Use of Agarose Gel Electrophoresis to Monitor Conformational Changes of Some Small, Spherical Plant Viruses

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ABSTRACT

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Several small, spherical plant viruses undergo conformational changes (swell) under conditions that deprotonate carboxyl groups and/or remove divalent cations. In this study, swollen, ribonuclease A susceptible turnip crinkle carmovirus (TCV) and tomato bushy stunt tombusvirus (TBSV) particles migrated more slowly than contracted virions during agarose gel electrophoresis, and swollen TCV virions stained more intensely with ethidium bromide than did contracted virions. The swelling of TCV virions was prevented by the addition of 5.0 mM $CaCl_2$ to alkaline treatments, whereas the addition of 5.0 mM $MgCl_2$ did not prevent swelling. A portion of swollen TCV virions was contracted when the pH was shifted from 8.5 to 5.5. TBSV, which is more stable, expanded only in the presence of divalent cation chelators but not by alkaline treatments alone. Agarose gel electrophoresis is a quick, simple method that can be used to monitor pH- and chelator-induced conformational and permeability changes of some small, spherical plant viruses.

Several small, spherical plant viruses have been shown to undergo conformational changes (swell) under conditions that reduce carboxyl groups or remove divalent cations; they contract again when the cations are replaced but by a different pathway than swelling, as shown by a characteristic hysteresis in hydrogen ion titration (10). Divalent cations stabilize the particles of several plant viruses (20). Turnip crinkle carmovirus (TCV), southern bean mosaic sobemovirus (SBMV), brome mosaic bromovirus (BMV), and cowpea chlorotic mottle bromovirus (CCMV) swell when divalent cations, mainly calcium, are removed by chelation or by the shift of the pH from slightly acidic to slightly basic (1,12,19,23). Many plant viruses have binding sites for divalent cations (e.g., 7,8,20), and the ionic interactions and permeability of these viruses have been studied (10). In 1977, Durham et al (9) hypothesized that "there is one archetypal cation-binding site present in most, perhaps all, plant viruses." The same authors suggested that the evolutionary purpose of such sites is to bind calcium ions to ensure that plant viruses release their RNA only in the low calcium concentration of the host cytoplasm. Some plant viruses, for example alfalfa mosaic virus, are stabilized mainly by protein-RNA bonds and have been considered permanently swollen (21,28); others, like BMV, are stabilized by protein-RNA and protein-protein bonds (23,24). Many subunit-subunit bonds depend on the pH and the presence of divalent cations (21,35). The conformational changes known as swelling have been monitored by hydrogen ion titration, photon correlation spectroscopy, analytical ultracentrifugation, fluorescence techniques, X-ray crystallography, and small-angle X-ray and neutron scattering (4,22,31).

In addition to applications in nucleic acid analyses, agarose gel electrophoresis has been used to determine the sizes of multienzyme complexes (11), to quantify and model the sieving of spheres (14), to compare the sieving of rod-shaped virions with the sieving of spheres (15), to detect and characterize plant viruses at microgram (13,16,17,29,30,33) and nanogram levels (30), and to assay the capacity of TCV to form capsids in vivo (16). Two-dimensional agarose gel electrophoresis has been used to detect and characterize multimolecular cellular constituents (32), and polyacrylamide-agarose gels have been used to analyze tobacco mosaic virus disassembly intermediates (18). Until now, agarose gel electrophoresis has not been reported as an assay of the conformational states of spherical virions.

TCV, a member of the carmovirus group (for review see 27), is a 30-nm icosahedral plant virus with a 4,051-base, single-component, positive-sense RNA genome encapsidated by 180 copies of a 38-kDa capsid protein subunit. TCV virions swell at a slightly alkaline pH in the absence of added calcium ions (12). Tomato bushy stunt tombusvirus (TBSV), the type member of the tombusvirus group, is also a 30-nm icosahedral plant virus with properties similar to those of TCV (26). TBSV virions swell at a slightly alkaline pH, but only after cations have been removed (31).

Mutations in the coat proteins of several plant viruses affect the symptoms expressed by various hosts (e.g., 17). Because of the ability to synthesize infectious plant viral RNA in vitro from cloned cDNA, many more mutations in coat protein genes can be constructed in the future. Whether specific coat protein mutations alter virion stability or the capacity to undergo conformational changes may, under certain circumstances, be an important question.

Like other small, spherical plant viruses, the swollen virions of TCV and TBSV were permeable to ribonuclease A, and the RNA genomes were, therefore, susceptible to degradation by the nuclease. Contracted, intact virions were not permeable to ribonuclease A, and their RNA genomes were protected against digestion. The ribonuclease A susceptible, and therefore swollen, particles of both viruses migrated more slowly than contracted particles during agarose gel electrophoresis. I report that, although it cannot replace more detailed and thorough analyses like X-ray crystallography and neutron scattering, agarose gel electrophoresis is a quick, simple method that can be used to monitor the conformational states of small, spherical plant viruses.

MATERIALS AND METHODS

Virus propagation and purification. TCV and TBSV were propagated in *Nicotiana benthamiana* (Domin.) in plant growth chambers at 22 C with a 16-h photoperiod of approximately 100 μmol $m^{-2}s^{-1}$. Both viruses were purified from fresh leaf tissue as described by Lommel et al for carnation mosaic carmvovirus (25). RNA was extracted from purified TCV virions as described by Carrington and Morris (5,6).

pH-induced conformational changes. Dissociated TCV was prepared as described by Wei et al (34). For various pH, cation, and chelator treatments, purified virions were suspended in 0.01 M sodium acetate, pH 5.5, at a virus concentration of approximately 5 mg/ml. Five micrograms of virions (1.0 μl) were brought to a final volume of 10 μl with 0.1 M Tris, pH 5.5, 7.5, or 8.5, depending on the experiment, and incubated at 4 C for 90 min. In some experiments, $CaCl_2$, $MgCl_2$, sodium (di) ethylenediaminetetraacetate (EDTA; Fisher Scientific, Pittsburgh, PA), or ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA; Sigma Chemical Company, St. Louis, MO) was added to a concentration of 5.0 mM. In other experiments, pH 8.5 was shifted to pH 5.5 (in the absence of added Ca^{++} or in the presence of 5.0 mM Ca^{++}) by the addition of 3 μl of 2 N hydrochloric acid. Incubation times were as noted in the Results section and in the figure legends.

Ribonuclease A protection assays. Virus particles were treated at pH 5.5, 8.5, or with EDTA as described above. After the 90-min treatment, 50 ng of ribonuclease A (Sigma Chemical Company) was added, and the reaction was incubated for an additional 30 min at 4 C. Sodium dodecyl sulfate (SDS; Fisher Scientific) was added to 1%, and RNA was extracted by the addition of 1 vol of Tris-buffered phenol, pH 8.0, with agitation. The mixture was spun in a microcentrifuge for 2 min. Five microliters of the upper aqueous phase was added to 2 μl of tracking dye (0.05% bromophenol blue, 0.05% xylene cyanol FF, and 3% Ficoll 400), and the samples were loaded into a 1% agarose-TBE gel (50.0 mM Tris, pH 8.0, 50.0 mM boric acid, 1.0 mM EDTA). Electrophoresis was at 10 V/cm. The RNA gel was stained for 10 min in distilled water that contained 0.5 mg/ml of ethidium bromide, destained for 10 min in distilled water, and photographed.

Agarose gel electrophoresis of virus particles. Approximately 1.25 μg of virus (2.5 μl of the pH treatment) was added to 3.5 μl of tracking dye. Samples were resolved at 7 V/cm in gels that contained 1% agarose, 38 mM glycine, and 5 mM Tris, pH 8.3. The electrophoresis buffer contained 38 mM glycine and 5 mM Tris, pH 8.3. Gels were stained for 10 min in 0.1 M Tris, pH 5.5, and 0.5 mg/ml of ethidium bromide and destained for 10 min in distilled water. In some cases, gels were photographed, restained in 0.1 M Tris, pH 8.5, and 0.5 mg/ml of ethidium bromide, destained, and photographed a second time.

RESULTS

Migration of virions relative to the migration of RNA. The migration of intact virions relative to viral RNA and the ribonucleoproteins of dissociated virions (34) was demonstrated in

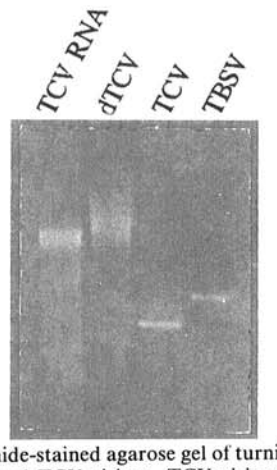

Fig. 1. Ethidium bromide-stained agarose gel of turnip crinkle carmovirus (TCV) RNA, dissociated TCV virions, TCV virions, and tomato bushy stunt (TBS) virions. TCV RNA extracted from virions (TCV RNA), the ribonucleoprotein complex (dTCV) of dissociated TCV, and intact TCV and TBSV particles were resolved in a 1% agarose-Tris glycine gel and stained with ethidium bromide.

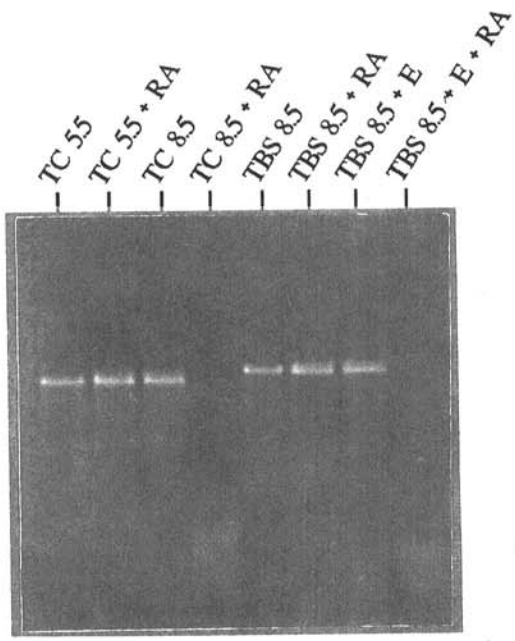

Fig. 2. Ethidium bromide-stained agarose gel of RNA extracted from treated virions. Turnip crinkle virus (TCV) and tomato bushy stunt virus (TBSV) particles were treated for 90 min at pH 5.5 or 8.5 with EDTA (E) or without EDTA (as indicated above each lane). Virions in lanes labeled RA were treated with ribonuclease A for an additional 30 min. RNA was extracted, resolved in a 1% agarose-TBE gel (50.0 mM Tris, pH 8.0, 50.0 mM boric acid, 1.0 mM EDTA), and stained with ethidium bromide.

the gel shown in Figure 1. TCV RNA and the ribonucleoproteins of dissociated virions nearly comigrated. The ribonucleoproteins migrated slightly more slowly than full-length RNA. Intact particles of both TCV and TBSV migrated as single bands, and both migrated faster than TCV RNA and the ribonucleoprotein of dissociated TCV. Intact TCV migrated faster than intact TBSV.

Ribonuclease A protection assays. Treated particles of TCV and TBSV were exposed to ribonuclease A. The RNA in TCV particles that had been treated at pH 5.5 was not degraded by ribonuclease A, whereas the RNA in TCV virions treated at pH 8.5 was degraded (Fig. 2). The RNA in TBSV particles was protected after treatment at pH 8.5; however, treatment with EDTA rendered TBSV RNA susceptible to ribonuclease A digestion. Swollen particles of TCV and TBSV that were exposed to ribonuclease A comigrated with virions that had not been exposed to the nuclease (data not shown).

Electrophoresis of swollen and contracted virions. Particles of TCV and TBSV were incubated at pH 5.5, 7.5, or 8.5 for 90 min before electrophoresis. TCV particles that were treated (swollen) at pH 7.5 or 8.5 (12) migrated more slowly than TCV treated at pH 5.5 (contracted) (Fig. 3). In some experiments, virions treated at pH 7.5 migrated as two bands: one that comigrated with virions treated at pH 5.5 and one that comigrated with virions treated at pH 8.5 (e.g., Fig. 4, lane 2). The RNA of swollen virions stained more intensely with ethidium bromide than did the RNA of contracted virions (e.g., upper panels of Figs. 3,4; Fig. 5). Particles of TBSV treated at pH 5.5, 7.5, or 8.5 comigrated and stained with equal intensity (Fig. 3). When gels were restained with ethidium bromide at pH 8.5, all bands were slightly more intense, but the increase in intensity was greater for TCV that had been treated at pH 5.5 (Fig. 1, lane 1; Fig. 4, lanes 1,4).

When 5.0 mM $CaCl_2$ was included in the pH 8.5 treatment, TCV virions comigrated with those that had been treated at pH 5.5 (Fig. 4). Virions treated with 5.0 mM $MgCl_2$ at pH 8.5 comigrated with those treated at pH 8.5 with no added cation. In all cases, the RNA of slower migrating, swollen TCV particles stained more intensely than the RNA of faster migrating, contracted TCV particles (Fig. 4, upper panel). When particles of TBSV were treated with EDTA at pH 8.5, they migrated more slowly than those treated at the same pH without EDTA (Fig. 5). The more slowly migrating TBSV particles did not stain more intensely than the faster migrating ones, but there was a more slowly migrating smear that probably represented partially dissociated virions.

When TCV was treated at pH 8.5 and then shifted to pH 5.5 in the presence or absence of Ca^{++}, the virions migrated as two bands; one band comigrated with contracted virions (those treated at pH 5.5), and the other comigrated with swollen virions (those

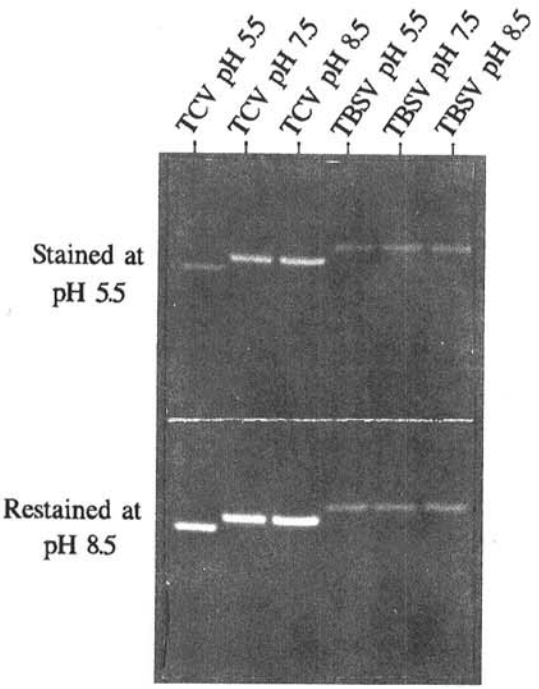

Fig. 3. Ethidium bromide-stained agarose gel of turnip crinkle virus (TCV) and tomato bushy stunt virus (TBSV) particles treated at pH 5.5, 7.5, and 8.5. TCV and TBSV particles were treated for 90 min at pH 5.5, 7.5, or 8.5 and resolved in a 1% agarose-Tris glycine gel. The gel was stained first at pH 5.5 and was restained at pH 8.5.

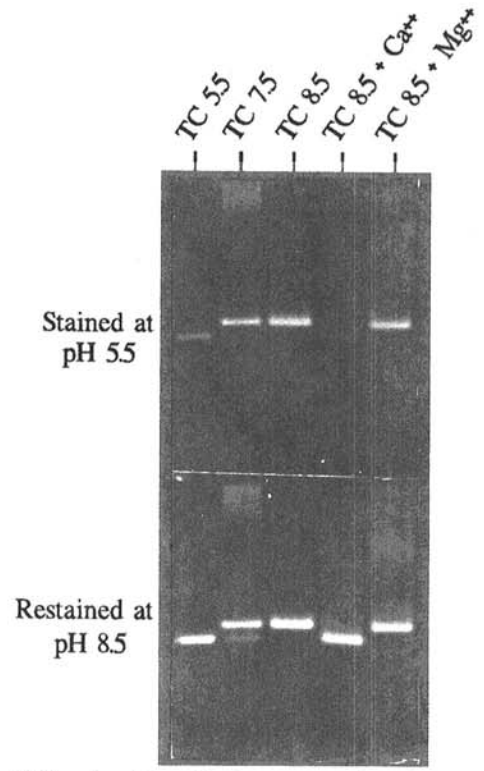

Fig. 4. Ethidium bromide-stained agarose gel of turnip crinkle virus (TCV) particles treated at pH 5.5, 7.5, and 8.5 with and without added $CaCl_2$ or $MgCl_2$. TCV (TC) particles were treated for 90 min at pH 5.5, 7.5, or 8.5. Five millimolar $CaCl_2$ (Ca^{++}) or $MgCl_2$ (Mg^{++}) was added to the treatment as indicated above each lane. Treated virions were resolved in a 1% agarose-Tris glycine gel, which was stained at pH 5.5. The same gel was restained at pH 8.5.

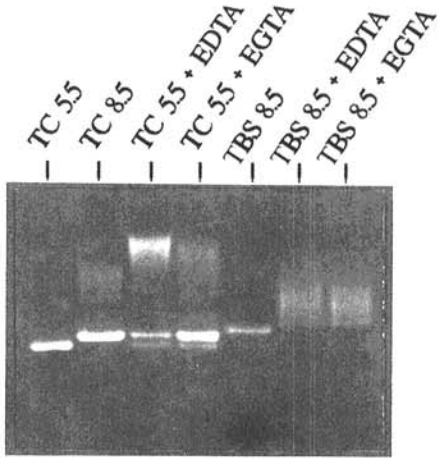

Fig. 5. Ethidium bromide-stained agarose gel of turnip crinkle virus (TCV) and tomato bushy stunt virus (TBSV) virions treated with EDTA or EGTA. TCV (TC) virions were treated for 90 min at pH 5.5 or 8.5, and at 5.5 with EDTA or EGTA as indicated above each lane. TBSV (TBS) particles were treated for 90 min at pH 8.5, and at 8.5 with EDTA or EGTA as indicated above each lane.

treated at pH 8.5; Fig. 6). Although the intensity of all bands increased slightly with restaining at pH 8.5, the increase in intensity was greater for the faster migrating bands (Fig. 6, lanes 3,5–7). In some experiments, TCV treated at pH 5.5 in the presence of EDTA or EGTA migrated as two bands, one swollen and the other not swollen (Fig. 5). In other experiments, all particles were swollen at pH 5.5 in the presence of EDTA (Fig. 6, lane 1).

DISCUSSION

Several small, spherical plant viruses swell when they are exposed to slightly alkaline conditions or to divalent cation chelators (e.g., 10). Swelling occurs when the divalent cations, mainly calcium, that maintain structural integrity are removed (1,12, 21,23). Swollen particles of several small, spherical plant viruses are capable of directing the synthesis of virus-specific polypeptides in in vitro translation systems (2,3,36,37). The conclusion drawn from these experiments is that the RNA genome is partially released from swollen virions and is, therefore, available for translation. It has been hypothesized that swelling, caused in vivo by differences in pH and calcium ion concentration between the external environment and the cytoplasm, is at least part of the mechanism by which virions disassemble to begin the replication cycle (9).

As in other studies, TCV became swollen when treated at pH 7.5 or 8.5, whereas the more stable TBSV was swollen only when chelators (EDTA or EGTA) were used to remove divalent cations (12,22,31). As shown in Figures 3–6, swollen particles of TCV migrated more slowly in 1% agarose gels than contracted virions. Kruse et al (22) used small-angle X-ray and neutron scattering to show that the TBSV radius swells by about 12% in elevated pH after removal of divalent cations. Their results were in agreement with those from the X-ray crystallography study of Robinson and Harrison (31), who reported that swelling is triggered by deprotonation of the aspartate residues in the calcium-binding sites (31). The local buildup of negative charges forces subunits apart, which results in swelling and an increase in the diameter of the virion by approximately 20%. The slower migration of swollen TCV and TBSV virions in this study must, therefore, have been due to the increased diameter.

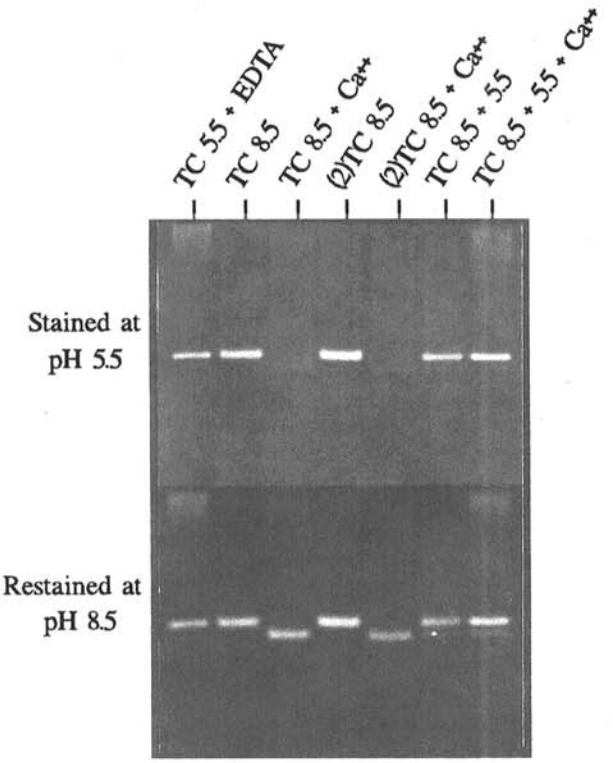

Fig. 6. Ethidium bromide-stained agarose gel of turnip crinkle virus (TCV) virions treated at pH 5.5 and at 8.5 with and without EDTA and added $CaCl_2$. TCV (TC) virions were treated for 90 min at pH 5.5 with EDTA, and at pH 8.5 with and without added $CaCl_2$, as indicated above each lane. Virions in lanes labeled (2)TC were treated for 180 min at pH 8.5 with and without added $CaCl_2$ (Ca^{++}), as indicated above each lane. Virions in lanes labeled 8.5 + 5.5 were treated for 90 min at pH 8.5, after which the pH was shifted to 5.5 with or without added $CaCl_2$, as indicated above each lane, for an additional 90 min. The virions were resolved in a 1% agarose-Tris glycine gel, which was stained at pH 5.5 (upper panel) and restained at pH 8.5 (lower panel).

In some experiments, but not in others, the induced expansion of TCV was incomplete (Fig. 4, lane 2; Fig. 5, lanes 3,4), and in most experiments the recontraction after the pH was shifted from basic to acidic was incomplete (Fig. 6, lanes 6,7). I cannot explain why expansion and recontraction were sometimes incomplete, but the ability to monitor the extent of the reaction is evidence that agarose gel electrophoresis is a quick, easy, inexpensive assay of the conformational states of TCV and TBSV. In all cases in which TCV migrated as two bands, there was never a smear between the bands. It seems, by this analysis and in agreement with Robinson and Harrison (31), that swelling is a cooperative phenomenon.

The major criterion used here to assay the conformational states of TCV and TBSV was mobility, but the intensities of ethidium bromide-stained bands were also indicative of the virions' conformational states. With the initial staining, swollen virions usually stained more intensely than contracted ones (Figs. 3–6). Although the intensity of most bands increased somewhat with restaining, differences were much more striking when virions were initially treated at pH 5.5 and swollen in situ by restaining at pH 8.5. Expansion at pH 8.5 caused the particles to become more porous and, therefore, the RNA in virions became more accessible to ethidium bromide. This is in agreement with Kruse et al (22) who used fluorescent methods, in addition to analytical ultracentrifugation and small-angle X-ray and neutron scattering, to analyze the conformational states of TBSV.

Because the migration of swollen and contracted virions was clearly resolvable in this system, agarose gel electrophoresis can be used to monitor pH- and chelator-induced conformational and permeability changes of these viruses. Agarose gel electrophoresis is not a replacement for more thorough analyses, but it is a quick, simple method that can be used to monitor the gross conformational changes of small, spherical plant viruses.

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