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# The Minor Proteins in Tomato Bushy Stunt and Turnip Crinkle Viruses

A. ZIEGLER,[1] S. C. HARRISON,[2] AND R. LEBERMAN

*Max-Planck-Institut für medizinische Forschung, 69 Heidelberg, West Germany*

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Tomato bushy stunt virus (TBSV) and turnip crinkle virus (TCV) each contain two minor polypeptides in addition to the major coat protein. We have used quantitative estimation of band strengths after SDS-gel electrophoresis to show that the larger of these species (MW 87,000 in TBSV, 84,000 in TCV) is always present at a level of about one chain per particle. The smaller polypeptide (MW 28,000), present in variable amounts, is shown to be a cleavage product of the major coat protein (MW 41,000).

## INTRODUCTION

Tomato bushy stunt virus (TBSV) and turnip crinkle virus (TCV) have been the objects of detailed structure analyses. TBSV is the most suitable of the crystalline viruses for X-ray diffraction studies (Harrison, 1971), and a three-dimensional reconstruction from electron micrographs is also available (Crowther and Amos, 1971). The virus exhibits dimer clustering of subunits in a T = 3 surface lattice, with some additional "filling" of positions on the 5-fold axes. X-ray diffraction and electron microscopy have further shown that TCV and TBSV have essentially identical structures despite striking differences in amino acid composition (cf. Finch *et al.*, 1970). Both viruses have a principal subunit of molecular weight 41,000, and minor components have been detected by Butler (1970) and Hill and Shepherd (1972) using SDS polyacrylamide gel electrophoresis. Butler (1970) has proposed that one of these—a species migrating at 28,000—is responsible for the "filled" appearance of the 5-fold positions. His estimate of approximately 12 chains of this component per 180 chains of the major subunit is consistent with such a role. He has further suggested that the other—migrating near 80,000 is the viral RNA polymerase. We have used SDS-polyacrylamide gel electrophoresis to estimate the proportion of minor components in TBSV and TCV. We find that the smaller species is a breakdown product of the major protein, but that one chain of the larger component is apparently associated with each virus particle.

## MATERIALS AND METHODS

*Source of viruses.* TBSV was propagated in *Datura stramonium*. The virus was harvested about 1 month after infection. Purification was achieved by differential centrifugation. The virus was crystallized from ammonium sulfate by the method of Bawden and Pirie (1938). TCV was grown in Chinese cabbage (var. Pe-tsai) for 4–6 weeks after inoculation and isolated by differential centrifugation and/or precipitation with PEG/NaCl (Leberman, 1966). Some preparations were kindly donated by Dr. P. J. G. Butler. Virus concentrations were measured spectrophotometrically by using $OD^{1\%}_{260} = 52$ (TBSV) or 60 (TCV) (Markham, 1959).

*Reagents and standards.* All reagents were of analytical grade. Tobacco mosaic virus (TMV) was prepared by Ms. Ute Gallwitz in

[1] Present address: Basel Institute for Immunology, Grenzacherstrasse 487, 4058 Basel, Switzerland.

[2] Present address: Gibbs Laboratory, Harvard University, 12 Oxford St., Cambridge, Massachusetts 02138.

this laboratory and other calibration proteins were products of Boehringer Mannheim.

*Gel electrophoresis.* Electrophoresis was carried out in gels containing 7% (w/v) acrylamide cross-linked with 0.2% methylenbisacrylamide and polymerized with 0.075% ammonium persulfate in the presence of 0.075% (v/v) *N,N,N′,N′*-tetramethylethylenediamine. The electrode buffer was 0.1 *M* Tris-Bicine[3] pH 8.3 and 0.1% SDS ($I = 0.05$). The equimolar Tris-Bicine buffer, which has previously been used for cellulose-acetate electrophoresis (King and Leberman, 1973), contains no appreciable concentration of fast-moving small ions (the $Na^+$ concentration in 0.1% SDS is less than 0.01 *M*). Each gel was run at a current density of 25 mA/cm² for approximately 75 min. Samples were prepared by heating at 100° for 1 min in 5 m*M* Tris-Bicine pH 8.3, 0.07 *M* β-mercaptoethanol, 5% glycerol, 0.01% bromophenol blue, with SDS present in 3- to 5-fold weight excess over viral protein; 20–50 μl aliquots were applied to the gels. After electrophoresis, gels were fixed in 10% trichloroacetic acid, stained for 3 hr in a solution of 0.25% Coomassie Brilliant Blue in 5:5:1 methanol:water:glacial acetic acid, and destained with 7.5% acetic acid/5% methanol.

*Estimation of band strengths and protein molecular weight on stained gels.* To facilitate quantitative estimation of band strength, a known amount of dissociated virus was applied along with a known amount of a standard protein (usually TMV). For bands of similar width, the eye can estimate *relative* strength quite accurately. By running a series of gels with different amounts of standard, it is possible with some experience to estimate by inspection the strength of an "unknown" band to within about 20%. For example, in a typical experiment 50 μg of dissociated TBSV is applied to each of five gels along with 0.1, 0.2, 0.5, 1, and 2 μg of TMV protein, respectively. It is quite easy to locate the intensity of a minor band in the standard series. This method assumes that all of each protein enters the gel and remains there during staining and that all proteins bind Coomassie brilliant blue to a similar extent. With fixed gels in a given run, we find that identical quantities of β-galactosidase, bovine serum albumin, aldolase, TMV protein, pancreatic ribonuclease, and the TBSV and TCV major proteins stain with identical intensities. Lowey and Risby (1971) have demonstrated similar results in establishing the stoichiometry of myosin light chains. Moreover, using TMV protein and controlled staining conditions, we have obtained a linear relation between the area of the stained band (measured photometrically) and the amount of protein applied, in the range 0.1–2 μg; however, the method of internal comparison described above makes it unnecessary to rely on this linearity.

Molecular weights were estimated in the usual way by plotting the logarithm of MW versus relative mobility (Weber and Osborn, 1969).

*Electron microscopy.* Electron micrographs of TBSV, negatively stained with 1% uranyl acetate, were taken on a Siemens Elmiskop 1A, fitted with a liquid nitrogen trap and used with an accelerating voltage of 60 kV.

## RESULTS

Figure 1 shows the results of SDS-polyacrylamide-gel electrophoresis of several preparations of TBSV. In accord with Butler (1970), we find minor bands in the gel patterns of this virus in addition to the major band at 41,000. These bands are at positions corresponding to molecular weights of about 87,000 and about 28,000. A band often appears at about 95,000, but it is significantly weaker than the 87,000 band and variable in its intensity from preparation to preparation. Samples of TBSV denatured at 100° for more than 1 min showed additional bands, the most prominent appearing as a doublet near 20,000. Their strength increased with the time during which the sample was kept at 100°. Since the 28,000 and 87,000 bands do not decrease correspondingly, the new bands are probably cleavage products of the coat protein. The sum of the molecular weights of the two components in the doublet is approximately equal to 41,000.

The amount of protein in the 87,000 and 28,000 bands, relative to the total viral pro-

[3] Tris = tris(hydroxymethyl)aminomethane; Bicine = *N,N*-bis(hydroxyethyl)glycine.

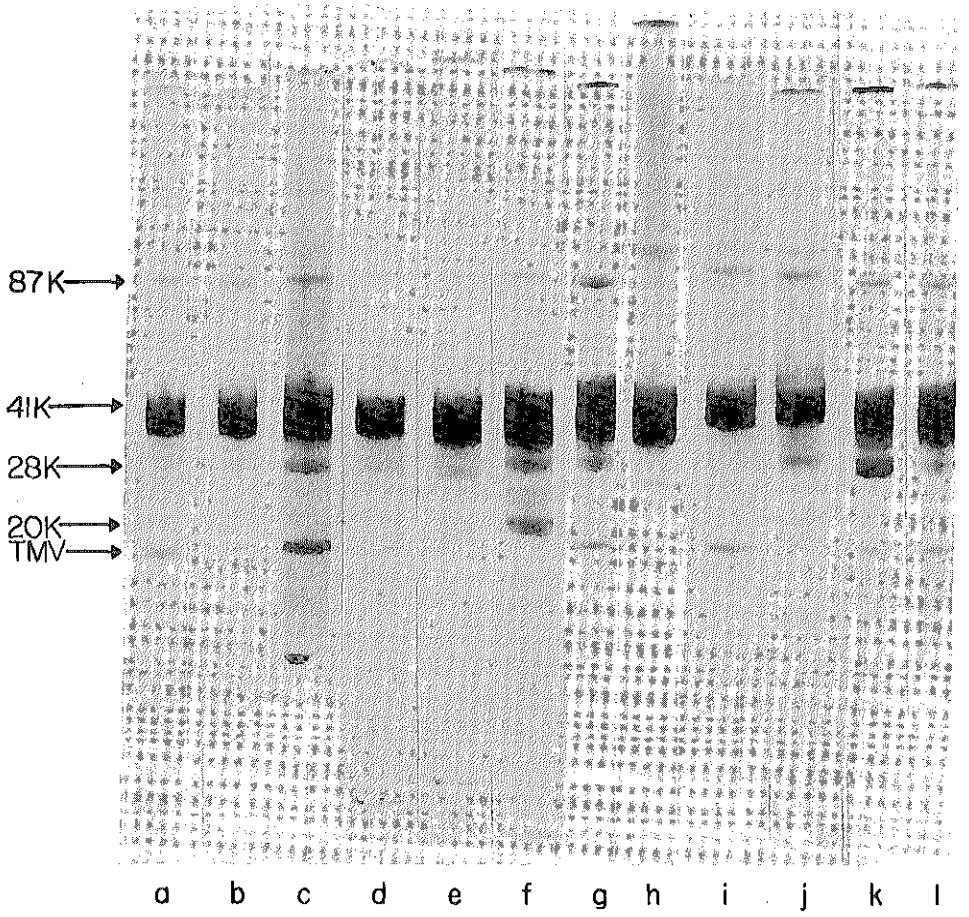


FIG. 1. SDS-Polyacrylamide gel electrophoresis of various preparations of TBSV. Varying amounts of TMV protein were added as intensity standards for the weak bands (see text: the photographic reproduction is not always faithful to the stained intensities). The molecular weights of the various bands are shown. The TBSV on gel a was the gift of Dr. C. A. Knight (Berkeley); TBSV I-V (gels b–h) were different preparations of the same strain, grown in Heidelberg. Unless otherwise stated, virus samples were incubated in sample buffer, at 100°C for 1 min prior to electrophoresis. (a) 40 μg TBSV (Berkeley), 0.5 μg TMV; (b) 40 μg TBSV-I, 0.1 μg TMV; (c) 50 μg TBSV-II, 0.5 μg TMV; (d) 40 μg TBSV-III; (e) 40 μg TBSV-III, incubated 100°C for 5 min; (f) 50 μg TBSV-III, incubated 100°C for 15 min; (g) 60 μg TBSV-IV, 0.25 μg TMV; (h) 50 μg TBSV-V; (i) 30 μg TBSV-I, crystals, 0.25 μg TMV; (j) 33 μg TBSV-I, crystal mother liquor, 0.1 μg TMV; (k) 60 μg TBSV-IV, supernatant after final centrifugation during purification, 0.25 μg TMV; (l) 48 μg TBSV-IV, pellet after final centrifugation, 0.1 μg TMV.

tein in the gel, is shown in Table 1. The 28,000 protein appears in variable quantity, but in no case are there more than 3 chains per 180 chains of the major species present. In some cases, there are clearly two weak bands migrating in this region. The 87,000 protein, by contrast, appears in all preparations as about 1 chain per particle. The one preparation tested was infectious in *Datura stramonium*. Various further experiments show that the strength of the 28,000 band can be reduced by crystallization, differential centrifugation or isopycnic centrifugation in CsCl; the results are included in Fig. 1 and Table 1. Crystals from TBSV preparation I were washed with 1 *M* ammonium sulfate and redissolved in water. The band at 28,000 has almost completely vanished. On the

TABLE 1

MINOR PROTEINS IN TBSV[a]

| | 87,000 Protein | 28,000 Protein | Gel in Fig. |
|---|---|---|---|
| TBSV-Berkeley | 1 | 1 | 1a |
| TBSV-I | 1 | 1–2 | 1b |
| -II | 1 | 2–3 | 1c |
| -III | 1 | Doublet, 2 chains each | 1d |
| -IV | 1 | Doublet, 1–2 chains each | 1g |
| -V | 1 | Doublet, <1 chain | 1h |
| TBSV-I, redissolved crystals | 1 | ≪1 | 1i |
| TBSV-I, crystal mother-liquor | 1 | 3 | 1j |
| TBSV-II, after isopycnic banding in CsCl | 1 | Doublet, <1 chain each | |
| TBSV-IV, pellet after third centrifugation during preparation (100,000 *g*, 1 hr) | 1 | Doublet, <1 chain each | 1l |
| TBSV-IV, supernatant after third centrifugation | 1 | Doublet, 3 chains each | 1k |
| TBSV-IV, pellet after five additional centrifugations | 1 | Not visible | |

[a] The results, expressed as polypeptide chains per virus particle, were determined by visual estimates of band strengths on SDS-polyacrylamide gels relative to a TMV standard (see Materials and Methods) and calculated assuming equal stain uptake by TBSV proteins and by TMV protein and knowing the total amount of TBSV and TMV protein applied (cf. captions to Figs. 1, 3, and 4).

other hand, the supernatant of the crystal suspension contained virions with a higher proportion of the 28,000 band (3 chains compared to 1 chain/virion). Furthermore, some additional weak bands were apparent, which could not be found in the crystal suspension. Similarly, a comparison of gels of the supernatant and the pellet after the third cycle of centrifugation at 100,000 *g* for 1 hr in preparation IV showed about a 5-fold decrease of each of the components present in the 28,000 region. Both X-ray diffraction and electron microscopic evidence indicate that the particles in these preparations are identical in structure to those previously studied. Diffraction photographs from crystals of preparation I show the usual pattern, both in low and high concentrations of ammonium sulfate. Electron micrographs (Fig. 2) also reveal familiar images, including 2-fold views with evident filling of the 5-fold positions. A preliminary three-dimensional image reconstruction confirms this impression (J. T. Finch and R. A. Crowther, personal communication).

For comparison, we have examined five samples of TCV (Fig. 3). The proportion of 28,000 protein (Table 2) varies in these preparations from about 3 to 4 chains per particle in the fresher and apparently "cleaner" samples to about 20 chains in others. Differential centrifugation of the latter samples appears to give considerable purification (Table 2). A protein that migrates at 84,000 is also present, in more variable but similar proportion to the corresponding component in TBSV, at least in new preparations.

It is known that treatment of TCV with high salt at pH 9 results in dissociation of the particles (Leberman, 1968). A sample of TCV was incubated in a buffer containing 0.65 *M* NaCl, 0.05 *M* Tris·HCl pH 9, 0.005 *M* β-mercaptomethanol. After various times samples were withdrawn and subjected to polyacrylamide electrophoresis. The results (Fig. 4) clearly show that the coat protein is cleaved to yield two smaller proteins with molecular weights 28,000 and 9000–12,000, respectively. Addition of phenylmethylsulfonylfluoride (PMSF) at a concentration of $10^{-3}$ *M* to the incubation mixture results in a reduction of the rate of cleavage (Fig. 4).

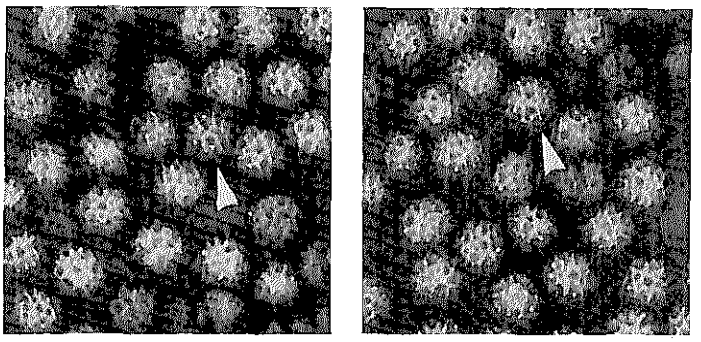

FIG. 2. Electron micrograph of TBSV-IV, negatively stained with uranyl acetate. Arrows indicate 2-fold views of the particle, showing "filled" 5-fold positions (to the left and right of the central "chiasma": cf. Finch *et al.*, 1970).

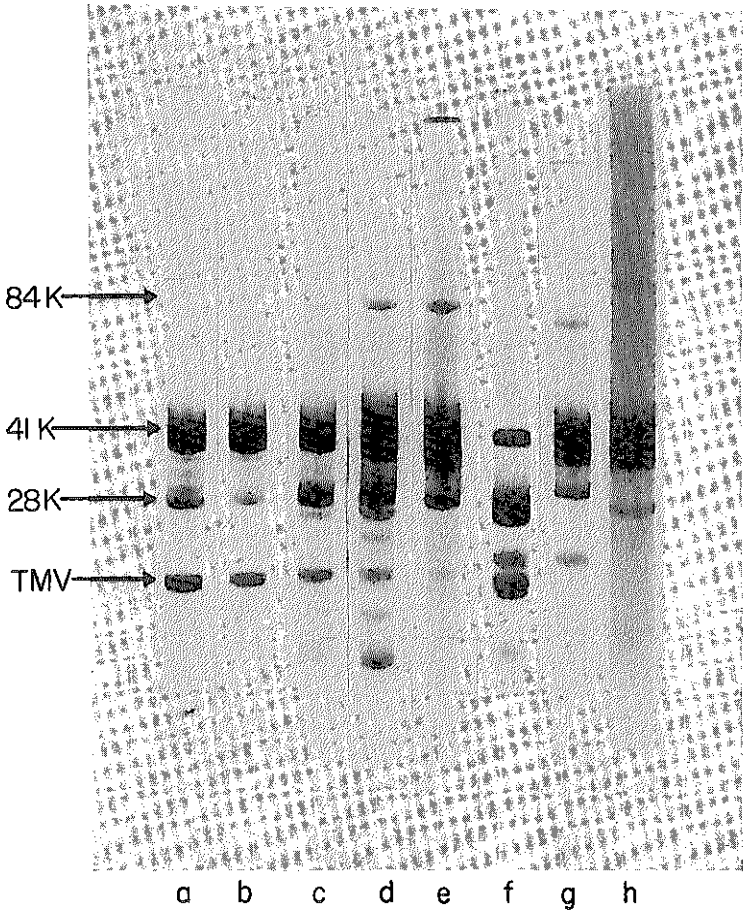


FIG. 3. SDS polyacrylamide gel electrophoresis of various samples of TCV. Gels a-d: TCV as received from Dr. P. J. G. Butler. Gels e and f: results of further purification. A number of minor bands appear in addition to the 28 K and 84 K species, but they are highly variable from preparation to preparation. (a) 27 μg TCV-I, 1.7 μg TMV; (b) 27 μg TCV-II, 0.83 μg TMV; (c) 27 μg TCV-III, 0.5 μg TMV; (d) 80 μg TCV-IV, 0.33 μg TMV; (e) 80 μg TCV-IV: pellet after centrifugation (100,000 *g*, 1 hr) 0.15 μg TMV; (f) TCV-IV: supernatant after centrifugation, 0.7 μg TMV; (g) 50 μg TCV-V (Heidelberg preparation), 0.67 μg TMV; (h) 50 μg TCV-VI (Heidelberg preparation).

TABLE 2
MINOR PROTEINS IN TCV

| Preparation | 84,000 Protein | 28,000 Protein | Gel in Fig. |
|---|---|---|---|
| TCV-I | 0.5 | 5–6 | 3a |
| -II | 1 | 3 | 3b |
| -III | 1 | 20 | 3c |
| -IV | 0.3 | 20–30 | 3d |
| TCV-IV, pellet after centrifugation (100,000 *g*, 1 hr.) | 0.3 | 7 | 3e |
| TCV-V | 1 | 4–5 | 3g |
| -VI | 1 | 3–4 | 3h |

[a] See Table 1 for experimental details; preparations I-IV were from Cambridge, V and VI from Heidelberg.

## DISCUSSION

In considerably purified samples of TBSV and TCV (Tables 1 and 2), two other protein components in addition to the coat protein of MW 41,000 are found; a species of MW 87,000 (TBSV) or 84,000 (TCV) and one of approximately 28,000. What are the origins and functions of these minor components?

Since crystalline samples of TBSV contain less than 1 molecule of the 28,000 MW component per particle, this protein does not appear to be an obligatory occupant of the 5-fold axes; indeed, the object of the crystallographic studies of TBSV must be a structure altogether lacking this component. It appears that in the case of TCV the 28,000 and 9000–12,000 MW polypeptides are cleavage products of the 41,000 coat protein. Incubation of the virus at pH 9.0 leads to the disappearance of the 41,000 protein and the coordinated appearance of bands at 28,000 and about 12,000. The cleavage may be due to the action of proteases of either plant or bacterial origin, as suggested by the partial inhibition of the degradation of the coat protein by PMSF, a serine-protease inhibitor (Fahrney and Gold, 1963) and by accumulation of the 9000–12,000 MW polypeptide in the supernatant after high-speed centrifugation during purification of the virus (Fig. 4g). Similar degradations of TMV coat protein have been observed by Durham (1972), and the high specificity of the cleavage site is reminiscent of the observations on methionyl-tRNA-synthetase (Cassio and Waller, 1971), on the DNA-dependent DNA-polymerase of *E. coli* (see Goulian, 1971) on hexokinase (see Steitz *et al.*, 1973), etc. The 28,000 MW material appears to remain as a component of some virions. These seem to be less stable than particles containing only uncleaved coat protein, since it is possible to reduce the amount of 28,000 MW component present in the virus by differential centrifugation; the supernatant material contains a relatively higher concentration of 28,000 MW component compared to the sedimented material. Moreover, crystals of TBSV con-

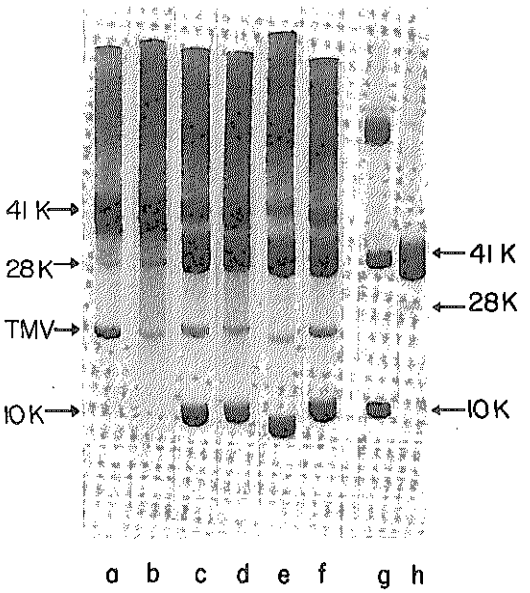


FIG. 4. SDS gel electrophoresis of TCV incubated at alkaline pH with and without PMSF. The incubation buffer contained 0.65 *M* NaCl, 0.05 *M* Tris·HCl, pH 9.0, and 0.005 *M* $\beta$-mercaptoethanol. (a) 60 $\mu$g TCV VI, no incubation, no PMSF (1.6 $\mu$g TMV); (b) 60 $\mu$g TCV VI, no incubation, $10^{-3}$ *M* PMSF (0.3 $\mu$g TMV); (c) 60 $\mu$g TCV VI, 64 hr incubation, no PMSF (0.8 $\mu$g TMV); (d) 60 $\mu$g TCV VI, 64 hr incubation, $10^{-3}$ *M* PMSF (0.4 $\mu$g TMV); (e) 60 $\mu$g TCV VI, 144 hr incubation, no PMSF (0.4 $\mu$g TMV); (f) 60 $\mu$g TCV VI, 144 hr incubation, $10^{-3}$ *M* PMSF (0.8 $\mu$g TMV); (g) TCV VI: supernatant after final differential centrifugation during purification; (h) TCV VI: pellet after final differential centrifugation (60 $\mu$g TCV). The last two gels were run for a different time than the first six.

tain a smaller proportion of the 28,000 polypeptide than their mother liquor.

We conclude that the 28,000 MW species found in preparations of TCV and TBSV are not responsible for the filled appearance of the 5-fold axes of these viruses and that they have no functional role. TCV and TBSV have different host ranges, but they have very similar surface structures and coat protein subunits of nearly identical molecular weight. The observation that preparations of both viruses contain a 28,000 MW degradation product, suggests that the coat proteins are folded into two compact portions, corresponding to ¾ and ¼ of the polypeptide chain, connected by a protease-susceptible bridge. The larger portion probably remains after cleavage as a "decapitated" subunit in its usual position in the icosahedral surface lattice.

The 87,000 (84,000) MW species must reside inside the virus particles, since the symmetry of crystals of TCV and TBSV implies a high symmetry for their surface interactions. The high basic amino acid content of this protein in TCV (Butler, 1970) suggests that it could be bound to the viral RNA. Butler (1970) has proposed that this large polypeptide represents a viral RNA-dependent RNA polymerase. Work is currently in progress to examine this suggestion.

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