In Situ Localization of Plant Viral Gene Products

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ABSTRACT

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Methods for the in situ localization of both viral nucleic acids and gene products were developed. Well-established systemic infections of two taxonomically distinct viruses (cauliflower mosaic virus [CaMV] and tobacco mosaic virus [TMV]) were used to establish trends in gene product localization that transcend virus group characteristics. An analysis of variance of in situ labeling is combined with a least significance difference determination to quantify results. The in situ localization studies were augmented by in vitro analyses to compare the validity, specificity, and sensitivity of the methods used. In TMV-infected tissue, structural proteins, nonstructural proteins, and ubiquitin (a key element in a protein degradative process in plants) were localized in viral inclusions. Negative sense RNA, a critical component of the replication complex, was not detected in these inclusions. On the basis of these results we conclude that TMV inclusions function in sequestration and degradation of viral products in infected cells. The in situ hybridization technique used in this study detected statistically significant amounts of negative sense RNA between 24 and 72 h after inoculation in the cytoplasm only. This coincided roughly with the only times that the 130K and 180K proteins could be detected in the tissues by in vitro (western blot) techniques. A cytoplasmic site for TMV replication is, therefore, the only conclusion indicated by our studies. The in situ labeling results are discussed against the background of our knowledge of TMV replication and in relation to the in vitro data obtained here.

Inclusion bodies are common to the cytopathology of many virus-infected plant tissues (19) and specific to virus-host interactions. Notwithstanding the commonality of their occurrence, no function for these diverse structures has been described.

Inclusion bodies have been proposed to play a role in virus replication, translation, and assembly. More recently, the host protein ubiquitin, a key factor in a cellular proteolytic pathway, has been associated with inclusions formed in some plant virus infections (9).

The cauliflower mosaic virus (CaMV) inclusions have been extensively studied (4) and were therefore used in this study to determine the specificity and general efficacy of the methods that were developed for in situ localization of viral products. Some previously unreported details with regard to CaMV inclusions came to light and will be described. Furthermore, similarities in the localization patterns of viral products in inclusions of unrelated viruses such as CaMV and tobacco mosaic virus (TMV) may provide clues as to the functional similarities of inclusions in virus infections.

We report here on the temporal and spatial relationships of plant and TMV-engendered products found in the inclusion bodies in TMV-infected plants. Our results suggest that these inclusion bodies are not the sites of viral RNA replication but rather cellular regions for protein sequestration and eventual degradation by a ubiquitin-mediated pathway.

MATERIALS AND METHODS

Plants, viruses, and antisera. Seeds of *Brassica rapa* L. 'Just Right' (turnip) were obtained from Twilley Seed Company, Trevose, PA. Seeds of *Nicotiana tabacum* L. 'Xanthi' and 'Xanthi-nc' were provided by W. O. Dawson of the University of California-Riverside. In this study, Xanthi-nc is used only for lesion assays. Plants were grown under existing greenhouse conditions (22–28 C and with day length extended to 16 h with fluorescent lights) (135 μmol sec^{-1} m^{-2}, Li-Cor, data logger).

The CM1841 and Cabbage B strains of CaMV (symptoms produced by these strains described by Schoelz and Shepherd, 27) were a gift from R. J. Shepherd of the University of Kentucky-Lexington. CaMV inoculum was prepared by grinding approximately 0.5 g of dried systemically infected turnip leaves with 5 mg of Celite in 5 ml of a buffer containing 1 mM sodium sulfite and 50 mM sodium phosphate (pH 7.2). Tobacco plants were inoculated with the U_1 strain of TMV (30) by grinding approximately 0.5 g of dried systemically infected tobacco tissue with 5 mg of Celite in 5 ml of a 50 mM sodium phosphate-containing buffer (pH 7.2). Mock-inoculated control plants were rubbed with abrasive and buffer in the absence of virus. Inoculum was applied with a cotton swab to the cotyledons of turnip plants or the two lower leaves of tobacco plants.

A time course of TMV replication was established in tobacco plants. The fifth leaf (approximately 6–8 cm long) of each of a set of plants was mechanically inoculated with 10 mg/ml of TMV and 5 mg of Celite in 5 ml of a buffer containing 50 mM sodium phosphate (pH 7.2). Residual inoculum was removed after 10 min from the inoculated leaf by washing with buffer. A mechanically inoculated leaf was harvested for immunogold labeling, western blot analysis, local lesion assay, and northern blot analysis at 0, 12, 24, 36, 48, and 72 h or 5, 7, and 14 days after inoculation. The third leaf above the inoculated leaf also was harvested at these times to assay the time course of systemic infection. Tissues were prepared for electron microscopy immediately after harvest. Tissues for western and northern blot analyses were frozen in liquid nitrogen, lyophilized, and stored at −70 C until used. Tissues for local lesion assays (12) were excised (disks, #6 cork bore) and frozen at −70 C until all of the samples had been collected.

Antisera against CaMV-Cabbage B (coat protein [CP] antiserum), CaMV-CM1841 gene I product (gene I antiserum), and CaMV-CM1841 gene VI product (gene VI antiserum) were generously provided by R. J. Shepherd (37,38). Antisera against CaMV gene I and gene VI products were prepared from gene I and gene VI β-galactosidase fusion proteins expressed in *Escherichia coli* cells. Antiserum against TMV-U_1 (CP antiserum) and rabbit normal serum were from our stocks. Antisera against the common region of the 130K and 180K proteins of TMV and to the unique region of the 180K protein were generously provided (along with preimmune sera) by W. O. Dawson. Antiserum against oat ubiquitin (ubiquitin antiserum, 0.2 mg/ml) and preimmune immunoglobulins (0.2 mg/ml; 29) were provided by R. Vierstra at the University of Wisconsin-Madison. All antisera used were polyclonal sera.

Preparation for electron microscopy. Leaves of systemically CaMV-infected and mock-inoculated turnip plants were sampled

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6 wk postinoculation while systemically invaded leaves of TMV-infected and mock-inoculated tobacco plants were sampled 2 wk after inoculation. Leaves were diced in fixative (0.1% glutaraldehyde and 4% formaldehyde in a 50 mM sodium cacodylate [pH 7.2] buffer), gently vacuum infiltrated, and fixed at room temperature for 1.5 h. The tissues were washed four times (5 min each) with buffer and postfixed in 2% osmium tetroxide in buffer for 2 h. Tissues were washed four times (5 min each) with buffer and then dehydrated in a graded series of ethanol followed by infiltration with L. R. White resin (21). Ultra-thin sections were supported by 300-mesh nickel grids covered with Butvar film (10).

Immunogold labeling. Sections were etched for 10 min by floating grids, section side down, on drops of 0.42 M sodium meta-per-Iodate (1,5). Sections then were washed by floating grids on three drops of glass-distilled water for 5 min each. Grids were pretreated by floating on drops of TBST + 1% BSA (10 mM Tris-HCl [pH 7.2], 0.5 M NaCl, 0.3% Tween 20, and 1% bovine serum albumin [BSA]) to block nonspecific binding of antibody or protein A to the sections (5). The grids were transferred to drops of antiserum or normal serum diluted 1:250 to 1:2,000 in TBST + 1% BSA and incubated for 1 h. Grids were washed three times (5 min per wash) by flotation on fresh drops of TBST + 1% BSA. Grids then were labeled on drops containing goat antirabbit/15 nm gold (1.75×10^{-3} A520 absorbance units; Janssen Life Sciences Products, Olen, Belgium) or protein A/10 nm gold (1.25×10^{-3} A520 absorbance units) diluted 1:50 with TBST + 1% BSA. Labeling was allowed to proceed for 30 min and grids then were washed as described above followed by three washes in glass-distilled water. The grids then were stained in an LKB Automat stainer with aqueous uranyl acetate for 15 min at 40 C, followed by lead citrate staining for 8 min at 20 C.

Electron microscopy, sampling design, and statistical tests. Samples were examined with either a JEM-7 or a Hitachi H600 electron microscope operating at 80 or 75 kV, respectively. The efficacy of various treatments for labeling sensitivity using tissues from CaMV-infected turnip plants and TMV-infected tobacco plants was determined. The number of gold particles per field of CaMV inclusion body or paracrystalline arrays of TMV virions (referred to hereafter as TMV crystal) was counted. A field is defined as the area within the engraved rectangle on the screen of the electron microscope that was operating at a magnification of 40,000. For each sample, the number of gold particles present in 10 fields ($\pm$ 4 μm^2 per field) was averaged. Differences in these averages were compared between the various immunogold labeling parameters (e.g., fixation, embedding, etching, etc.) tested. Results of these optimization studies have been previously described (25).

The specificity of labeling was examined by determining the average number of gold particles per square micrometer per cellular structure (organelles or viral inclusion body). Each of the cellular structures was photographed at an original magnification of 12,000–20,000 in three different cells. The number of gold particles was counted over each of the organelles or viral inclusion bodies. The cross-sectional area of a cellular structure on a photomicrograph was determined by digitizing and stereological estimation (36). Control experiments showed no statistical variation between these methods in determining cross-sectional areas.

Differences in the average number of gold particles per area of organelle from two samples were compared by the Student's *t* test (31). Immunogold labeling results, collectively referred to as immunogold labeling profiles, were compared by a two-way analysis of variance (ANOVA) and a least significant difference method (LSD; 31) to determine confidence levels of differences between treatments.

Construction of TMV strand-specific probes. General recombinant DNA techniques were used as described by Maniatis (18). Unless otherwise stated all enzymes were obtained from Bethesda Research Laboratory.

First- and second-strand cDNA were synthesized essentially as described by Okayama and Berg (23) from polyadenylated TMV RNA. A clone of the *Hind*III/*Pst*I fragment from the 3′ end of TMV RNA (base 5083 internal to the 3′ end and base 6400, three bases into the polylinker's *Pst*I) was then subcloned into the dual transcription vector pT7 T3-18 (Bethesda Research Laboratory). Transcription plasmids containing the TMV insert were purified by cesium chloride-ethidium bromide density gradient centrifugation.

Strand-specific probes were produced by in vitro transcription of the opposing promoters on the pT7 T3-TMV vector prepared above. Transcription reactions were as described by Bethesda Research Laboratory for the production of biotinylated transcripts.

In situ hybridization. In situ hybridization procedures were adapted from methods described by Brahic and Haase for light microscopy (2) and from methods used to label whole mount chromosomes for electron microscopy (13). Based on preliminary experiments (25), the following in situ hybridization protocol was adopted.

Sections on Butvar-coated copper grids were prehybridized in 45% formamide, 0.3 M NaCl, 20 mM Tris-HCl (pH 7.2), 5 mM EDTA, 0.02% BSA, 0.02% Ficoll, 0.02% polyvinylpyrollidone, 10% dextran sulfate, 0.5 mg/ml of yeast tRNA, 0.2 mg/ml of sheared denatured salmon sperm DNA, in 50-μl aliquots in "reactivials" at 37 C for 1 h. Biotinylated transcripts were added to a final concentration of 2 μg/ml and incubated at 37 C overnight. Sections were washed four times for 5 min each with 0.3 M NaCl and 0.03 M sodium citrate (2× SSC) at room temperature, and washed two times for 5 min each with 0.03 M NaCl and 3 mM sodium citrate (0.2× SSC) at 52 C. Sections then were washed two times in 2× SSC. Biotin containing RNA/RNA hybrids on sections were detected by incubation of the grids with streptavidin 15 nm gold (Janssen Life Sciences Products). Sections were first blocked with 5% BSA in TBST and then labeled with streptavidin gold diluted 1:100 with TBST + 1% BSA. Sections then were sequentially washed with TBST + 1% BSA and distilled H_2O and finally stained with uranyl acetate and lead citrate before electron microscopic examination.

RESULTS

The results will be presented in two parts, the first dealing with CaMV and TMV advanced systemically infected tissues and the second addressing the time course studies of TMV infections.

To localize plant viral proteins in situ, tissue fixation and immunogold labeling parameters were optimized with respect to the preservation of protein antigenicity and ultrastructural detail (25). These optimized fixation and labeling conditions were employed in the following experiments.

The criteria for good ultrastructural and antigen preservation were validated by experiments (dot immunobinding assays and/or western blot analysis, data not shown) to show the specificity of the antisera used in the experiments. Preimmune serum controls were run at all times for all labeling experiments. Furthermore, in vitro experimentation (infectivity assays, western blots, RNA extraction) was performed to validate results obtained in situ.

Localization of CaMV and TMV structural proteins. Immunogold labeling of tissues systemically infected with CaMV and TMV using the described methods was specific (Fig. 1A–C). Antiserum against CaMV coat protein primarily labeled inclusion bodies, with the bulk of this label appearing on virions within inclusion bodies (Fig. 1A and B). Some plant cell wall labeling also was observed (not shown). Antiserum against TMV coat protein (Fig. 1C and D) densely labeled TMV crystals, as well as inclusion bodies, cytoplasm, and vacuoles.

Specificity of labeling was examined by determining the average number of gold particles per square micrometer per organelle (defined as the immunogold labeling profile). To determine an immunogold labeling profile, the numbers of gold particles per square micrometer per organelle in three different leaf mesophyll cells were averaged. Because variation increased with increasing averages of gold per square micrometer per organelle, a square root transformation was used to stabilize this variation. The square root-transformed gold labeling densities of each of the organelles

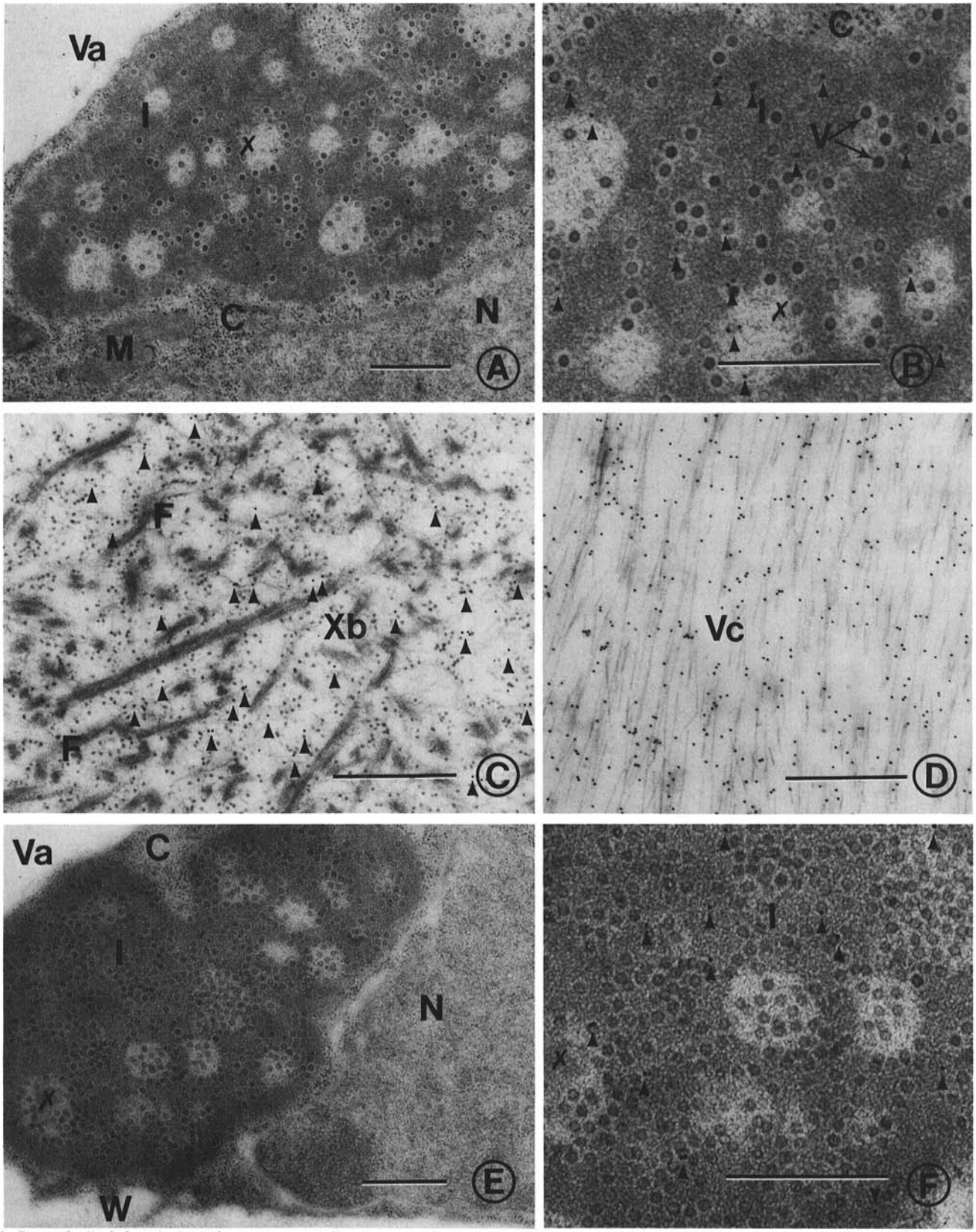

Fig. 1. Systemically infected plant tissues prepared for electron microscopy and immunogold labeling. Infected leaves were fixed, dehydrated, and embedded in L. R. White resin. Sections were etched with 0.42 M sodium periodate and immunogold labeled with 1:1,000 dilution of antisera followed by protein A/10 nm gold treatment. **A,** Turnip leaf cell infected with cauliflower mosaic virus (CaMV) strain CM1841 and labeled with CaMV coat protein (CP) antibodies. Note the preservation of the cellular detail of the inclusion body. **B,** A close-up of the area of the inclusion body indicated with the X mark in **A.** Note the localization of the gold label (arrowheads). Arrows point to virus particles. **C,** A mesophyll cell infected with tobacco mosaic virus (TMV) and containing an X body labeled with TMV-CP antibodies. Gold particles were mainly associated with the cytoplasm in these structures (arrowheads). **D,** A TMV crystal in an infected mesophyll cell labeled with TMV-CP antibodies. Gold particles are evident. **E,** A turnip mesophyll cell infected with CaMV-CM1841. Antibodies to the nonstructural protein product of gene VI were used in labeling. **F,** A close-up of the area of **E** indicated by an X mark. Gold particles are localized specifically in the inclusion (arrowheads). See Tables 1 and 2 for quantification and analysis. Bars = 0.5 μm. C = cytoplasm, F = fibril, I = inclusion, M = mitochondrion, N = nucleus, V = virus, Va = vacuole, Vc = virus crystal, W = wall, Xb = X body.

for each of the treatments were compared by two-way ANOVA (Minitab). Immunogold labeling data (shown in Fig. 1) for CaMV- and TMV-infected plants, labeled with their respective CP antisera, were quantified in this manner (Tables 1 and 2). This method of analysis for CP labeling of CaMV-infected plants revealed that all cellular structures, with the exception of the nucleus and mitochondria, contained CP (Table 1). Although inclusion bodies were densely labeled by CaMV-CP antiserum, other cellular structures also were labeled (Table 1). Western blot analysis revealed several additional protein bands that were detected by this antiserum (data not shown). A similar CP-labeling analysis of TMV-infected plants demonstrated specific labeling of TMV crystals, inclusion bodies, cytoplasm, and vacuoles by this method (Table 2). Furthermore, western blot analyses confirmed the specificity of the antiserum for TMV coat protein. In control experiments involving both CaMV and TMV-CP antisera, the labeling profile of mock-inoculated plants showed label densities below 0.2 gold particles per square micrometer over all cellular structures considered.

Localization of CaMV and TMV nonstructural proteins in systemically infected plants. To localize the intracellular site of CaMV nonstructural proteins, systemically infected turnip plants were immunogold labeled with antisera against gene I and gene VI nonstructural proteins and with their particular preimmune sera. Gene VI protein-specific antiserum from strain CM1841 labeled inclusion bodies in turnip plants infected with either strains MC1841 or Cabbage B (CM1841 illustrated in Fig. 1D and E and Table 1). Although antiserum against CaMV gene I protein also labeled inclusion bodies (not shown), the level of labeling was low and was not statistically significant (Table 1).

To identify the intracellular location of TMV nonstructural proteins, tobacco plants systemically infected with TMV were immunogold labeled with antiserum (130K) against the common region of the 130/180K readthrough protein, with antiserum (180K) against the unique region of 180K TMV readthrough protein, and with preimmune sera. Antiserum against the common region of the 130K and 180K labeled viral inclusion bodies (Table 3). Within the TMV inclusion body (X body) the broad helical fibrils were preferentially labeled (Fig. 2A). Because the level of gold labeling by these antisera was much lower than that of TMV coat protein, immunogold labeling data from these antisera and their preimmune sera were analyzed as a group (Table 3). Antiserum against the unique region of the 180K protein was demonstrated to label inclusion bodies and plant cell walls; however, the level of labeling was not statistically significant (Table 3). Cell walls from mock-inoculated tobacco plants also were labeled by the 130K and 180K antisera (1.23 and 1.45 gold particles per square micrometer, respectively). Because neither 130K nor 180K preimmune serum significantly labeled any systemically infected cell components, it appeared that cell wall labeling was a specific feature of the 130K and 180K antisera and possibly artifactual as it seemed to occur when antisera prepared with complete adjuvant were used.

Localization of ubiquitin in systemically infected plants. Because inclusion body formation in virus-infected tissues could be a response to foreign or aberrant proteins, antiserum against ubiquitin, a host protein involved in cellular protein degradation (8,34) was used to immunogold label systemically infected plant tissues. In CaMV-infected plants both the matrix and virions of CaMV inclusions were labeled by ubiquitin antiserum (Fig. 2B). None of the other cellular structures was significantly labeled by ubiquitin antiserum (Table 1). Mock-inoculated control plants were not significantly labeled by the antiserum (data not shown). Thus, our data taken together showed that the host protein, ubiquitin, as well as CaMV coat and gene VI proteins, were local-

TABLE 1. Immunogold labeling profile of turnip plants infected with cauliflower mosaic virus (CaMV)

Cellular structure	Antisera specific for the viral gene products shown[a,b]				
	CP	VI	I	Ub	PI
Nucleus	0.83	0..49	0.26	0.71	0.21
Chloroplast	1.10	0.30	0.23	0.10	0.00
Mitochondrion	0.00	0.00	0.00	0.00	0.00
Inclusion body	6.15*	3.34*	0.83*	2.18*	0.00
Cytoplasm	1.19	0.00	0.06	0.68	0.00
Vacuole	1.13	0.51	0.31	0.00	0.10
Cell wall	3.61*	0.52	0.70	0.57	0.00
Intercellular space	1.41	0.42	0.00	0.00	0.33

[a] Turnip plants were mechanically inoculated with CaMV. Six weeks after inoculation, tissue sections of systemically infected leaves were immunogold labeled with antiserum against CaMV coat protein (CP), gene VI (VI), gene I (I), ubiquitin (Ub), and the preimmune serum control of the ubiquitin antiserum (PI), as described in the text.

[b] The square roots of the number of gold particles per area of organelles in three different cells were averaged and the values were analyzed by two-way analysis of variance (ANOVA) and least significant difference (LSD) methods. Values in the labeling profiles that differ from each other by more than the LSD are significant at the 95% confidence level (see *). LSD = 0.601.

TABLE 2. Immunogold labeling profile of tobacco plants infected with tobacco mosaic virus (TMV)

Cellular structure	Antisera specific for the viral gene product shown[a,b]		
	CP	Ub	PI
Nucleus	1.09	1.19	0.24
Chloroplast	1.03	1.47	0.17
Mitochondrion	0.86	0.43	0.59
Inclusion body	4.29*	2.59*	0.09
TMV crystal	14.37*	0.93	0.09
Cytoplasm	5.03*	2.01*	0.23
Vacuole	7.51*	0.00	0.19
Cell wall	0.80	0.00	0.22
Intercellular space	1.12	0.59	0.00

[a] Tobacco plants were mechanically inoculated with TMV. Fourteen days after inoculation, tissue sections of systemically infected leaves were immunogold labeled with antiserum against TMV coat protein (CP), ubiquitin (Ub), and the preimmune serum control of the ubiquitin antiserum (PI), as described in the text.

[b] The square roots of the number of gold particles per area of organelles in three different cells were averaged and the values were analyzed by two-way analysis of variance (ANOVA) and least significant difference (LSD) methods. Values that differ from each other by more than the LSD are significant at the 95% confidence level (see *). LSD = 1.442.

TABLE 3. Immunogold labeling profile of nonstructural viral proteins in tobacco plants infected with tobacco mosaic virus (TMV)

Cellular structure	Antisera specific for the viral gene products shown[a,b]			
	130K	130K PI	180K	180K PI
Nucleus	0.31	0.50	0.81	0.95
Chloroplast	0.39	0.00	.078	0.00
Mitochondrion	0.00	0.00	0.00	0.00
Inclusion	3.18*	0.25	1.42*	0.75
TMV crystal	0.78	0.26	0.00	0.79
Cytoplasm	0.00	0.31	0.81	0.20
Vacuole	0.00	0.00	0.34	0.00
Cell wall[c]	1.23	0.00	1.23	0.21

[a] Tobacco plants were mechanically inoculated with TMV. Fourteen days after inoculation, tissue sections of systemically infected leaves were immunogold labeled with antiserum against the common region of the 130K and 180K proteins, the unique region of the 180K protein, and their respective preimmune sera (PI), as described in the text.

[b] The square roots of the number of gold particles per area of organelles in three different cells were averaged and the values were analyzed by two-way analysis of variance (ANOVA) and least significant difference (LSD) methods. Values that differ from each other by more than the LSD are significant at the 95% confidence level (see *). LSD = 0.504.

[c] 130K As and 180K As also labeled cell walls of mock-inoculated plants (1.23 and 1.45, respectively).

ized in the viral inclusion body in tissues systemically infected with CaMV.

For TMV-infected plants, immunogold labeling by ubiquitin antiserum also was primarily localized in the TMV inclusion body (Fig. 2C). Nearly 80% of the gold particles observed on TMV inclusion bodies were further localized to the 60–70 nm fibrils within the inclusion bodies (Fig. 2C). Although ubiquitin antiserum also labeled other cellular structures, including TMV crystals, the level of labeling observed was not statistically significant (Table 2). Ubiquitin labeling also was not detected in mock-inoculated control plants (less than 0.20 gold particles per square micrometer per organelle). Thus, these data suggest that both the TMV 130K protein and host ubiquitin were specifically localized to the 60–70 nm fibrils of the TMV inclusion body.

Localization of TMV RNA in systemically infected plants. To further characterize the possible functional role(s) of inclusion bodies in TMV-infected tissues, biotinylated strand-specific TMV RNA probes were prepared and used for in situ hybridization and gold labeling. In initial in situ hybridization labeling experiments, positive strand-specific probes preferentially labeled TMV crystals. The conditions for in situ hybridization then were optimized by evaluating the effects of different labeling conditions on the extent of labeling of these crystals. Among the parameters evaluated were prehybridization treatments, probe concentration, temperatures of hybridization and washing, and posthybridization treatment (25). Based on the results of these experiments, sections were hybridized with full-length probes at a concentration of 2 μg/ml at 37 C and washed at 52 C before the addition of streptavidin gold.

In the systemically infected leaf, positive strand-specific probes primarily detected TMV RNA in the TMV crystals (Fig. 2D). Positive strand TMV RNA also was detected in the inclusion body, TMV crystal, cytoplasm, and vacuole (Table 4). The vacuole was labeled only when virus particles also were present (not

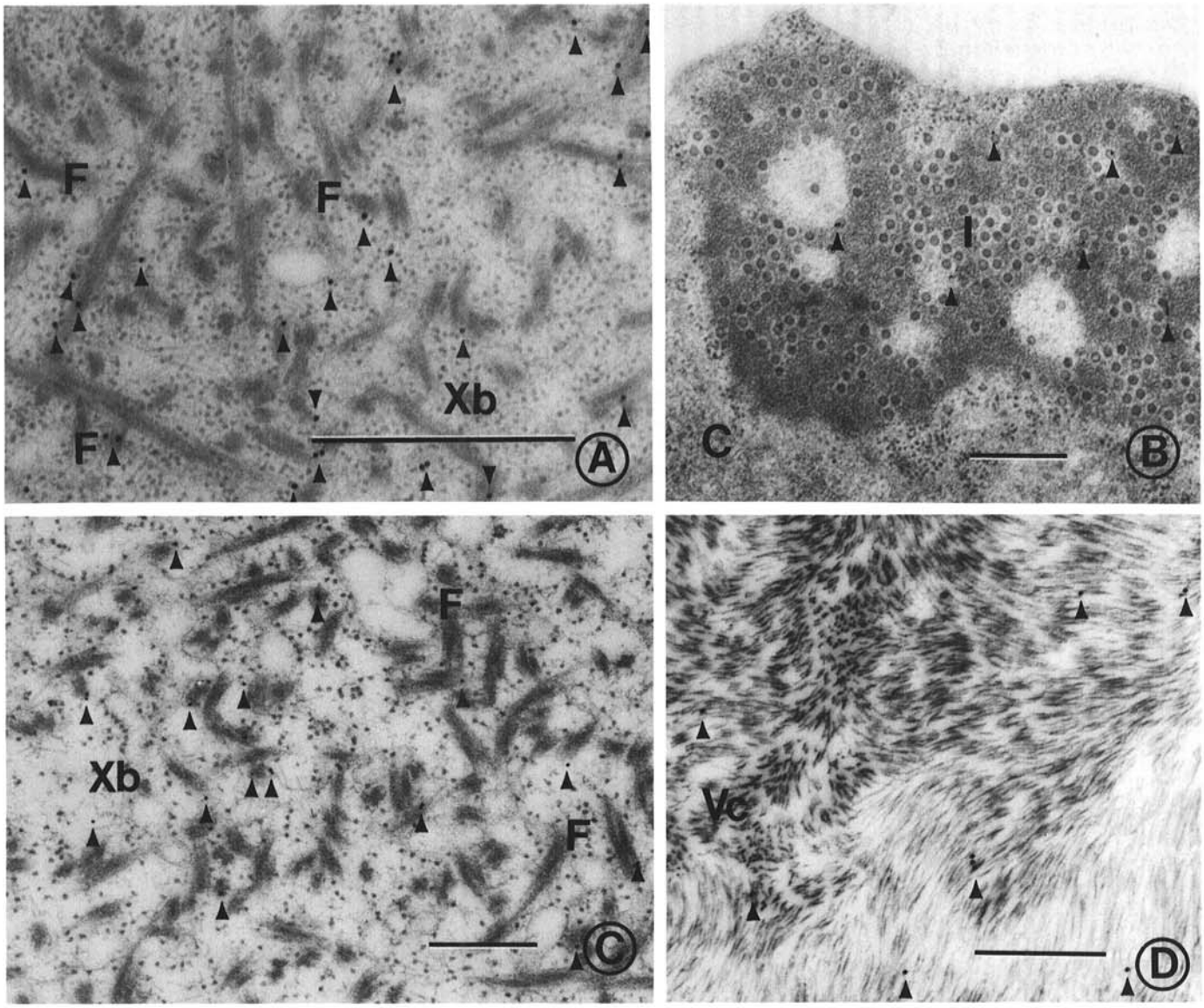

Fig. 2. Systemically infected plant tissues prepared for electron microscopy and immunogold labeling as described for Figure 1. Sections except for those of **D** were etched and immunogold labeled. **A,** An X body in a tobacco mesophyll cell systemically infected with tobacco mosaic virus (TMV) and labeled with a polyclonal antiserum against the 130K protein, followed by visualization of specific antibody binding by means of goat antirabbit γ-G conjugated with 15-nm gold particles. The fibrils are preferentially labeled with gold (arrowheads). **B,** Immunogold localization of ubiquitin in mesophyll cells infected with cauliflower mosaic virus (CaMV) strain CM1841. Sections were treated with antibodies raised against oat ubiquitin followed by localization of specific binding by means of protein A/10 nm gold. Both particles and matrix are labeled with gold (arrowheads). **C,** An X body in a TMV-infected mesophyll cell treated as the sections in **B.** The 60–70 nm fibrils are preferentially labeled with gold (arrowheads). **D,** In situ hybridization of TMV RNA. Tobacco plants were systemically TMV infected (14 days). Sections were hybridized overnight at 37 C with a solution containing 45% formamide, 0.3 M NaCl, and 2 μg/ml of biotinylated probe. Sections were washed with 0.03 M NaCl at 52 C. Biotin label was detected with streptavidin gold (arrowheads). The probe is specific for (+) TMV RNA. See Tables 1–3 for quantification and analysis. Bars = 0.5 μm. C = cytoplasm, F = fibril, I = inclusion, Vc = virus crystal, Xb = X body.

shown). Negative strand-specific probes, however, did not label systemically infected (14 days after infection) plant tissues (Table 4). Mock-inoculated tissues and unlabeled probe (biotin 11-UTP left out of the transcription reaction) also resulted in no significant gold labeling (Table 4).

Time course studies of TMV-infected tobacco plants. To further elucidate what role, if any, inclusion bodies had in TMV plant infections, time-course studies of the appearance and accumulation of viral RNAs, viral proteins, and assembled virus particles were performed. Data from this time-course study are shown in Table 5. The individual parts of this experiment will be briefly described in the following paragraphs.

Immediately after mechanical inoculation, there was an eclipse period in which no viral-specific components were localized. Because TMV RNA, coat protein, and infectivity were not detected at this early time point (Table 5), the initial inoculum or its degradation products were not detected by these methods.

TMV infectivity was detected by a local lesion bioassay 12 h after inoculation, and infectivity increased throughout the sampling period (not shown). Infectivity was not recovered from the systemically infected leaf (third leaf above a mechanically inoculated leaf) until 5 days postinoculation. Because samples were not infectious immediately postinoculation (0 h), the effects of initial inoculum were not observed. The local lesion assay demonstrated TMV infectivity at the same time point (12 h postinoculation) that western blot analysis detected coat protein.

In the time course study of TMV infection the 130K and 180K antisera labeled plant cell walls in the mechanically inoculated leaves within the first 72 h postinoculation. Vacuoles also were labeled during the first 24 h (Table 6) after inoculation. The cytoplasm of infected cells, however, was specifically labeled by CP antiserum at 48 h postinoculation. Isolated virions were observed in mechanically inoculated tissues by 72 h and small virus crystals were present by 5 days postinoculation. Finally, TMV inclusion bodies were first observed 7 days postinoculation. In systemically infected tissues (the third leaf above the mechanically inoculated leaf), TMV crystals were first observed at 5 days and inclusion bodies 14 days postinoculation. At every time point when inclusion bodies were present in the infected cell, they were specifically labeled by 130K antiserum. Although virus progeny was detected by 72 h postinoculation, 130K protein was not detected until 7 days postinoculation, by which time 130K protein was associated with the viral inclusion body.

Coat protein was detected by western blot analysis 12 h postinoculation and CP concentration increased throughout the sampling period (Fig. 3 shown as an example of our western blot controls). Also, double bands representing 130K and 180K proteins were detected by western blot analysis with 130K antiserum in leaves 36, 48, and 72 h after mechanical inoculation (data not shown). No bands were detected when similar blots were labeled with 180K specific antiserum. In contrast to the in situ labeling data above, the 130K and 180K proteins were detected in infected tobacco tissues only between 36 and 72 h after inoculation. This result also was unlike the coat protein analysis that revealed increasing amounts of coat protein during the infection time course.

During the time course of infection, positive strand TMV RNA was detected 72 h postinoculation (Table 7). Negative strand RNA, however, was detected between 24 and 72 h postinoculation (Table 7), although the level of labeling at 48 h was not statistically significant. Cell walls were not labeled by either probe in these in situ hybridization experiments. By 7 days postinoculation the cytoplasm, inclusion body, and TMV crystal were labeled by positive strand-specific probes (1.79, 1.19, and 2.76 gold particles per square micrometer of organelle, respectively). At 5 days postinoculation the cytoplasm was specifically labeled by negative strand probes, but by 7 days postinoculation no organelles were specifically labeled. Thus, these experiments show that positive and negative strand TMV RNAs appear at distinct times in the cytoplasm of infected cells.

TABLE 4. In situ hybridization labeling profile of tobacco plants systemically infected with tobacco mosaic virus (TMV)

	RNA strand detected[a,b]		
Cellular structure	Positive	Negative	Control[c]
Nucleus	0.00	0.00	0.00
Chloroplast	0.25	0.13	0.00
Mitochondrion	0.00	0.00	0.00
Inclusion	0.98*	0.00	0.00
TMV crystal	4.65*	0.41	0.15
Cytoplasm	2.73*	0.00	0.00
Vacuole	1.14*	0.00	0.00
Cell wall	0.00	0.00	0.00

[a] Tobacco plants were mechanically inoculated with TMV. Fourteen days after inoculation, tissue sections of systemically infected leaves were hybridized in situ and labeled with streptavidin 15 nm gold.
[b] The values represent the averages of the square roots of the number of gold particles of organelles in three different cells analyzed by two-way analysis of variance (ANOVA) and least significant difference (LSD) methods. Values that differ from each other by more than the LSD are significant at the 95% confidence level (see *). LSD = 0.608.
[c] These values were obtained from hybridization experiments performed with unlabeled probe.

TABLE 5. Summary of the time course of events in tissues infected with tobacco mosaic virus (TMV)

	Time postinoculation[a,b]								
Observed in tissues	0 h	12 h	24 h	36 h	48 h	72 h	5 days	7 days	14 days
Virions	−	−	−	−	−	Cyt	Cyt	Cyt	Cyt
TMV crystals	−	−	−	−	−	−	Cyt	Cyt	Cyt
Inclusions	−	−	−	−	−	−	−	Cyt	Cyt
In situ localization									
130K/180K	−		−		−	−	−	Inc	Inc
180K unique	−		−		−	−	−	−	−
Coat protein[c]	−		−		Cyt	Cyt	Cyt	Cyt	Cyt
dsRNA	−		−		−	−	−	−	−
+ TMV RNA	−		−			Cyt	Cyt	Cyt	Cyt
− TMV RNA	−		Cyt		Cyt	Cyt	Cyt	−	−
In vitro detection									
130K/180K	−	−	−	+	+	+	−	−	−
180K unique	−	−	−	−	−	−	−	−	−
Coat protein	−	+	+	+	+	+	+	+	+
+ TMV RNA	−	−	−	−	+	+	+	+	+
− TMV RNA	−	−	−	−	−	−	−	−	−
Infectivity	−	+	+	+	+	+	+	+	+

[a] Summary of the data from the time course of infection study of the inoculated tobacco leaf.
[b] The organelle in which a particular detail was localized is abbreviated as follows: Cyt = cytoplasm; Inc = TMV inclusion body.
[c] Coat protein also was detected in the TMV crystal, inclusion body, and vacuole.

By northern blot analysis, TMV positive strand RNA was detected at 48 h postinoculation and throughout the sampling period (data not shown). While positive strand TMV was detected earlier by northern blot analysis than by in situ hybridization, northern blot analysis failed to detect negative strand TMV RNA from either mechanically inoculated leaves or systemically infected tissue preserved by lyophilization after freezing in liquid nitrogen. The low concentration of negative strand RNA in relation to positive strand RNA (virus particles), RNA degradation during tissue preparation before extraction and the fact that we used the same biotinylated probe (suboptimal compared with ^{32}P-labeled probes) for northern analysis and in in situ hybridization may account for the failure to detect the negative sense RNA in this manner.

TABLE 6. Immunogold labeling profile of tobacco mosaic virus (TMV) 130K, 180K, and coat protein (CP) after mechanical inoculation

Cellular structure	Time postinoculation (h)[a,b,c] 0	24	48	72
130K antiserum				
Nucleus	0.18	0.27	0.22	0.41
Chloroplast	0.47	0.21	0.27	0.40
Mitochondrion	0.00	0.20	0.45	0.00
Cytoplasm	0.50	0.45	0.55	0.48
Vacuole	0.65	0.76	0.26	0.17
Cell wall	1.52	1.51	1.70	0.92
180K antiserum				
Nucleus	0.00	0.22	0.13	0.21
Chloroplast	0.00	0.26	0.00	0.00
Mitochondrion	0.00	0.00	0.00	0.00
Cytoplasm	0.00	0.27	0.64	0.64
Vacuole	0.57	0.12	0.00	0.00
Cell wall	1.28	2.07	1.43	0.78
CP antiserum				
Nucleus	0.13	0.00	0.22	0.36
Chloroplast	0.10	0.00	0.00	0.18
Mitochondrion	0.00	0.00	0.00	0.00
Cytoplasm	0.00	0.30	0.78	1.04
Vacuole	0.00	0.25	0.27	0.19
Cell wall	0.00	0.33	0.00	0.00

[a] Tobacco plants were inoculated with TMV as described in the text. At the indicated times postinoculation, tissue samples were sectioned and immunogold labeled as described in the text.

[b] The square roots of the number of gold particles per area of organelles in three different cells were averaged and the values were analyzed by two-way analysis of variance (ANOVA) and least significant difference (LSD) methods. Values that differ from each other by more than the LSD are significant at the 95% confidence level. LSD = 0.646.

[c] Half-leaves of these samples were analyzed by local lesion assay and western blot analysis.

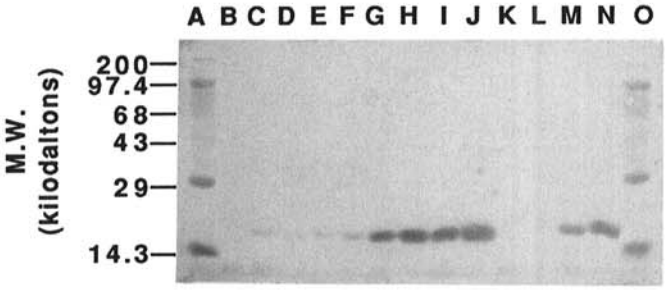

Fig. 3. Western blot analysis of tobacco mosaic virus (TMV) coat protein (CP) appearance with time after inoculation. Tobacco plants were mechanically inoculated with TMV. At the times indicated, tissues were harvested, lyophilized, and ground in 30 mM potassium phosphate (pH 7.4), 0.4 M NaCl, and 10 mM 2-mercaptoethanol. Cellular debris was pelleted and the supernatant fraction mixed with an equal volume of Laemmli buffer. Proteins were fractionated on a 13% polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked with 1% BSA, labeled with CP antiserum followed by goat antirabbit/alkaline phosphatase, and developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP and NBT). Lanes A and O are markers. Lanes B through J are protein extracts from tobacco leaves (B = 0 h, C = 12 h, D = 24 h, E = 36 h, F = 48 h, G = 72 h, H = 5 days, I = 7 days, J = 14 days) after mechanical inoculation. Lanes K through N are extracts from systemically infected leaves (K = 3 days, L = 5 days, M = 7 days, N = 14 days) after inoculation.

DISCUSSION

Cauliflower mosaic virus and its cytopathology provided the means to test both the specificity and the efficacy of the methods developed for in situ localization. Our experiments corroborated the in vitro work by Young et al (37,38), who reported that CaMV gene products are present in the inclusion body. Because western blot assays indicated the presence of antibodies in CP antiserum that labeled proteins in extracts of mock-inoculated plants, it was reassuring to see that this lack of specificity was reflected in the high background readings found in in situ studies (Fig. 1A and Table 1). Because the CP antiserum was raised against virions isolated from infected plants, antibodies to host contaminants may account for the high background labeling. In contrast, the use of antisera raised against both gene VI- and gene I-β-galactosidase fusion proteins expressed in *E. coli* resulted in lower levels of background labeling than did antisera prepared against CaMV-CP (Table 1). Using this approach we have provided unequivocal evidence that the gene VI product is a major component of the matrix of the CaMV inclusion. In contrast to Odell and Howell (22) and Covey and Hull (4), we used an antibody raised to an in vitro-produced gene VI product rather than an antibody made against the matrix protein isolated from plants.

The loss of gene I protein antigenicity or the loss of the gene I protein itself during specimen preparation may explain the fact that we could not localize gene I protein in significant amounts. Although immunogold labeling provides great spatial resolution of the antigens that can be successfully preserved, the sensitivity of this method is rather poor compared with conventional in vitro means of detection (16).

Studies with animal cells have shown ubiquitin associated with cytopathological inclusions (20) or abnormal proteins (17). One way eukaryotic cells specifically eliminate proteins from cellular metabolism is to covalently link them with a ubiquitin molecule that then serves as a reversible recognition signal for the selective degradation of the modified protein (8,34). Immunogold labeling studies of ubiquitin were initiated to determine whether plant

TABLE 7. In situ hybridization labeling profile of tobacco mosaic virus (TMV) RNAs after mechanical inoculation

Cellular structure	Time postinoculation (h)[a,b] 0	24	48	72
Positive strand specific				
Nucleus	0.00	0.11	0.37	0.00
Chloroplast	0.00	0.21	0.29	0.00
Mitochondrion	0.00	0.00	0.00	0.00
Cytoplasm	0.00	0.00	0.36	1.38
Vacuole	0.12	0.00	0.29	0.00
Cell wall	0.00	0.00	0.00	0.00
Negative strand specific				
Nucleus	0.00	0.24	0.13	0.00
Chloroplast	0.00	0.00	0.31	0.00
Mitochondrion	0.00	0.00	0.00	0.00
Cytoplasm	0.00	0.74	0.40	0.78
Vacuole	0.00	0.17	0.17	0.16
Cell wall	0.00	0.00	0.00	0.00

[a] Tobacco plants were inoculated with TMV. At the indicated times postinoculation, tissue samples were prepared for electron microscopy, sectioned, hybridized in situ, and labeled with streptavidin 15 nm gold as described in the text.

[b] The values represent the averages of the square roots of the number of gold particles per area of organelles in three different cells analyzed by two-way analysis of variance (ANOVA) and least significant difference (LSD) methods. Values that differ from each other by more than the LSD are significant at the 95% confidence level. LSD = 0.454.

viral inclusion bodies, like their mammalian counterparts, also are associated with ubiquitin. In the present study, ubiquitin was localized in both TMV and CaMV inclusion bodies. In plants, ubiquitin is associated with the specific degradation of phytochrome (32). Perhaps the formation of inclusion bodies and the association of ubiquitin with these inclusion bodies represents a common cellular defense strategy to isolate foreign proteins and remove them from the metabolism of the cell. Although inclusions may have some role in the viral replication cycle (e.g., in CaMV), our results suggest that they also serve as cellular centers of protein degradation.

Although some TMV coat protein subunits in virions have been demonstrated to be associated with ubiquitin (6), ubiquitin was not specifically associated with the TMV crystals in the present study. Our results showed that ubiquitin antiserum primarily labeled the 60–70 nm fibrils of the TMV inclusion body. These results have been corroborated by the work of Gaspar et al (9). Interestingly, these fibrils are the same structures that are preferentially labeled by antisera against the TMV 130K protein in the present study (see Fig. 7; 11). We have no evidence to suggest whether or not these antisera are labeling common determinants on the 60–70 nm fibrils. Indeed, the nature and composition of the fibrils remains unknown.

The distribution of each of four of the TMV gene products was determined in systemically infected tobacco tissues, although each has separately been localized before (CP, 28; 130K/180K protein, 11,26; 30K protein, 33). We found that coat protein and 130K protein localized essentially as previously reported (11,26,28). Unlike Tomenius et al (33), we were unable to localize 30K protein in infected tissues, mainly because the 30K antiserum we had lacked the requisite specificity for in situ localizations. Notwithstanding, the fact that the 130K protein (putative replicase) could only be localized in situ in inclusion bodies 7 days after inoculation, one could infer a role for such inclusions and the 130K protein in the virus replicative process. However, other viral replicative intermediates such as dsRNA and negative sense complementary RNA should likewise be present in these inclusions. Antisera directed against dsRNA detected dsRNA in wound tumor virus (WTV) inclusions and in the cytoplasm of WTV-infected cells but failed to detect dsRNA in TMV inclusions as well as in inclusions associated with the replication of pea enation mosaic and cowpea mosaic virus (25). This may indicate that dsRNA per se is not a replicative intermediate in plus-sense RNA virus replication. In light of this, single-stranded negative sense RNA must be part of the replicative intermediate. We could not, however, find evidence for the presence of negative sense RNA in TMV inclusion bodies by in situ hybridization methods that can detect such RNAs in the cytoplasm of TMV-infected cells at earlier times. This indicates an absence of negative sense TMV RNA associated with the inclusions, negating the notion of an association of viral replication with inclusions.

Because the original inoculum did not produce residual infectivity, the presence of infectivity 12 h postinoculation suggests that the full cycle of events leading to progeny virion production must have occurred. Western blot analysis detected coat protein from plant extracts beginning at 12 h postinoculation, consistent with this cycle of events. However, most of the anticipated early products were not detected in infected cells with our in situ methods until later in infection. For example, 130K and 180K proteins were not detected in infected plants until 36 h postinoculation by western blot analysis and 7 days postinoculation by immunogold labeling (Table 6). By in situ hybridization, negative strand TMV RNAs were localized by 24 h postinoculation; however, positive strand TMV RNAs were not observed until 72 h postinoculation (Table 7). Therefore, immunogold labeling techniques do not appear to have the sensitivity of either the bioassay (local lesion assay) or western blot analysis in the time course analysis. Interestingly, however, results from northern blot analysis of RNAs were similar in sensitivity to in situ hybridization methods (using the same biotin containing probes). Neither method detected TMV RNAs until 24–48 h postinoculation (Tables 5 and 7).

By electron microscopy, TMV virions were regularly observed in infected tobacco cells 72 h postinoculation. By this time, both positive and negative strand TMV RNA were localized in infected cells by in situ hybridization, and the 130K and 180K proteins were detected by western blot analysis (Table 5). During this time period TMV replication was active, yet 130K and 180K proteins were not detected in situ in infected cells, and inclusion bodies were not observed. By 7 days postinoculation, however, inclusion bodies were observed in mechanically inoculated tissues (by electron microscopy) and antiserum against the 130K protein immunogold labeled the inclusion bodies. Similar results were previously obtained by Hills et al (11). Also, at early times in systemically infected leaves (7 days postinoculation), Saito et al (26) showed that the 130K label was concentrated on dense granular bodies in infected leaves. Dense granular inclusions were not observed in the present studies.

Several conclusions can be drawn from the work described here. First, TMV replication is not associated with the nucleus. In these studies, neither the 130K and 180K proteins nor negative strand TMV RNA was localized in the nucleus. Watanabe and Okada (35), however, demonstrated viral RNA-dependent RNA polymerase activity in cell fractions containing nuclear membranes. Perhaps during their fractionation procedures, viral RNA-dependent RNA polymerase activity became associated with a host component with which it is not normally associated. Alternatively, cytoplasmic membranes proposed to be associated with TMV replication (24) could not be differentiated from nuclear membranes and contaminated their preparations.

Second, the results of our in situ localization work do indicate a cytoplasmic site for TMV replication. The negative-stranded RNA of the replicative intermediate was localized in the cytoplasm of infected cells. Positive strand TMV RNA also was localized in the cytoplasm of infected cells before the accumulation of TMV virions in crystals. Although the 130K and 180K proteins, components of the viral-specific RNA-dependent RNA polymerase complex, were not localized in situ in cells early in infection (Table 6), these proteins must have been present based on the appearance of progeny virions detected by local lesion assay (Tables 5 and 6). Also, when viral replicative intermediates were found, they were located in the cytoplasm, not in chloroplasts or nuclei. Therefore the results presented here support a cytoplasmic site for TMV replication.

A third conclusion from these studies is that TMV replication has no demonstrable association with any particular membrane component. Neither components of the TMV RNA-dependent RNA polymerase (130K and/or 180K proteins) nor the replication intermediate (negative strand TMV RNA) was associated with host membranes. The 130K and/or 180K proteins were only localized in these studies to the viral inclusion bodies that appeared late within the infected cells. Although endoplasmic reticulum is a component of TMV inclusion bodies (7), the 130K and/or 180K proteins were localized primarily in the broad tubules of the TMV inclusion body and were not associated with the reticular components of these inclusions. Our results do not support the conclusion that the endoplasmic reticulum constitutes the cellular backbone for TMV replication.

Fourth, double-stranded RNA per se was not detected as an intermediate in TMV viral replication. Double-strand RNA antiserum did not detect double-stranded RNA in plant tissues infected with TMV. Although the replicative form in TMV viral replication may contain both positive and negative sense RNAs, the actual structure of the molecule in replicating cells may not be a classical RNA-RNA hybrid. There is evidence to support the proposition that classical double-stranded RNAs are not single-stranded RNA virus replication intermediates. For example, pulse chase labeling experiments by Jackson et al (14) showed that radioactivity could not be chased from double-stranded RNAs into progeny virions. Also, double-stranded RNAs do not function as templates for the replicase of RNA bacteriophage Q-β (15). Our in situ localization of negative strand RNA early in infections suggests, however, that negative strand TMV RNA (not a double-stranded RNA) is the template for

replication in TMV-infected cells.

Finally, we conclude that TMV inclusion bodies are not involved in the normal replication process of TMV because they appear only late in the virus infection process (7 days after inoculation). Furthermore, they do not contain negative-stranded RNA, a replicative intermediate, and therefore no link between the 130K protein (putative replicase) present in the inclusions and replicative intermediates of TMV infection could be made. Our methods would allow us to make such connections since the same or adjacent sections can be used to do the immunolabeling as well as the in situ hybridizations.

This conclusion, combined with the observation made here that ubiquitin, a marker for specific proteolysis in eukaryotic cells, is preferentially associated with the TMV inclusion bodies, suggests that inclusion bodies are the result of a general cellular defense mechanism and are involved in the degradation rather than the synthesis of viral materials. Related to the latter point, the seemingly anomolous observation that western blot assays detected the 130K and 180K proteins early in infections only (36–72 h postinoculation), while our in situ methods only detected these proteins later, now has a plausible explanation. We suggest that the 130K and/or 180K proteins have undergone partial proteolysis in the TMV inclusion body resulting in the exposure in situ of epitopes that react with 130K antibody. These epitopes somehow do not react in situ when the replicase is associated with RNA.

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