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In Vivo Localization of Ubiquitin in Tobacco Mosaic Virus Infected and Uninfected Tobacco Cells

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A small proportion of the coat protein subunits in virions of tobacco mosaic virus (TMV) have been shown to be ubiquitinated (D. D. Dunigan, R. G. Dietzgen, J. E. Schoelz, and M. Zaitlin, Virology 165:310-312, 1988). To determine if there was specific ubiquitination of virus-infected tissues, an analysis of both TMV-infected and uninfected tobacco tissues was made by an immunogold-labeling technique using polyclonal antiserum to human ubiquitin. Colloidal gold labeling, indicating the presence of ubiquitin and/or ubiquitin conjugates, was found in the cytoplasmic matrix, chloroplasts, nucleus, and nucleolus equivalently in both healthy leaf tissue and leaf tissue infected with TMV. Cell walls and vacuoles were not labeled. In virus-infected cells, the ribbonlike structures contained within viroplasms were tagged by the anti-ubiquitin antibodies. These ribbons contain the 126K TMV putative replicase protein (G. J. Hills, K. A. Plaskitt, N. D. Young, D. D. Dunigan, J. W. Watts, T. M. A. Wilson, and M. Zaitlin, Virology 161:488-496, 1987). TMV particle aggregates in tissues were not labeled by this technique, and neither were purified TMV virions.

Additional keywords: electron microscopy, H protein.

The virions of tobacco mosaic virus (TMV) have associated with them trace amounts of a protein originally termed "H protein" (Asselin and Zaitlin 1978), which was shown to be an unidentified host protein conjugated through an isopeptide linkage to a coat protein subunit (Collmer *et al.* 1983). Recently, the host-derived polypeptide moiety was identified as ubiquitin, most probably linked to the coat protein at lysine 53; ubiquitination of coat protein subunits was detected in five strains of TMV (Dunigan *et al.* 1988). Ubiquitin conjugates, interpreted as ubiquitinated coat protein, have recently been detected in five other plant viruses. Two plant viruses examined did not contain ubiquitinated conjugates in their virions (Hazelwood and Zaitlin, in press). Recently, other virus-associated ubiquitins have been discovered. The baculovirus *Autographa californica* nuclear polyhedrosis virus encodes a protein that has a 76% identity with animal ubiquitin (Guarino 1990), and a cytopathic strain of a togavirus, bovine viral diarrhoea virus, contained an insertion of a host-derived ubiquitin gene within the gene for a viral-encoded protein (Meyers *et al.* 1989).

Ubiquitin is a 76 amino acid protein present in all eucaryotes (Rechsteiner 1988; Vierstra 1987a, 1987b). It is present in the cells, both as free molecules and as conjugates to other proteins, normally coupled through its carboxy-terminus by an isopeptide bond to the epsilon NH_2 group of lysine residues (Finley and Varshavsky 1985). Ubiquitin seems to be involved in different cellular processes including ATP-dependent nonlysosomal protein degradation (Hershko and Ciechanover 1982), regulation of the cell cycle (Finley *et al.* 1984), cell-cell interactions (Siegelman *et al.* 1986), receptor functions (Yarden *et al.* 1986), and as a response to stress, such as heat shock (Parag *et al.* 1987; Christensen and Quail 1989). This study examines the localization of ubiquitin and/or ubiquitin conjugates in TMV-infected tissue with the immunogold-labeling technique for the electron microscope. Moreover, since the location of the H protein in the virion is unknown, that issue was also addressed by using the same technique.

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MATERIALS AND METHODS

Plant and virus. Tobacco plants (*Nicotiana tabacum* L. cv. Samsun) were inoculated mechanically with the common (U1) strain of TMV (0.1 mg/ml) suspended in 0.01 M KH_2PO_4, pH 7.0, buffer containing washed Celite at 0.05 g/ml. Uninoculated plants were used as controls.

Antiserum. The affinity-purified anti-human ubiquitin antiserum was a gift of Arthur L. Haas (Medical College of Wisconsin, Milwaukee). This antiserum recognizes ubiquitin conjugates and free ubiquitin (Haas 1988).

Tissue preparation. Small pieces (2 × 0.5 mm) from young systemically infected or uninfected leaves were fixed in 1% glutaraldehyde in 0.05 M sodium cacodylate, pH 7.2, buffer for 16–18 hr at 4° C. They were washed three times for 30 min each and for 20 hr with the same buffer and then dehydrated in a graded series of ethanol starting with 30% ethanol at 4° C, followed with 50, 70, and 100% at −20° C. The infiltration with LR White acrylic resin (London Resin Co., Basingstoke, Hampshire, U.K.; obtained from Ernest Fullam, Inc. Latham, NY) was made following a schedule described by Hills *et al.* (1987) except

that the accelerator benzoin methyl ether was not used. The infiltrated tissue was embedded in polyethylene capsules and polymerized at 55° C for 48 hr.

Immunogold labeling of tissue sections. Thin sections of tissues were cut using a diamond knife and mounted on Formvar-coated nickel grids. The sections containing grids were floated on a drop of PBS-T (0.01 M phosphate buffer, pH 7.2, 0.15 M NaCl, 0.01% Tween 20) containing 1% bovine serum albumin (BSA) for 15 min at room temperature to block nonspecific reactions, and then incubated overnight at 4° C with the anti-ubiquitin antiserum (20 μg/ml) in PBS-T containing 0.01% BSA. (In some experiments antiserum was used at 4 μg/ml with the same qualitative results). After washing on three drops of PBS-T containing 0.01% BSA (10 min each drop), the grids were incubated for 1 hr at room temperature on drops of the protein A-gold complex (Bioclinical Services, Cardiff, U.K.; obtained from Probetech, Perkasie, PA) diluted 1:50 with PBS-T containing 0.01% BSA. After washing with buffer as above and with distilled water in the same way,

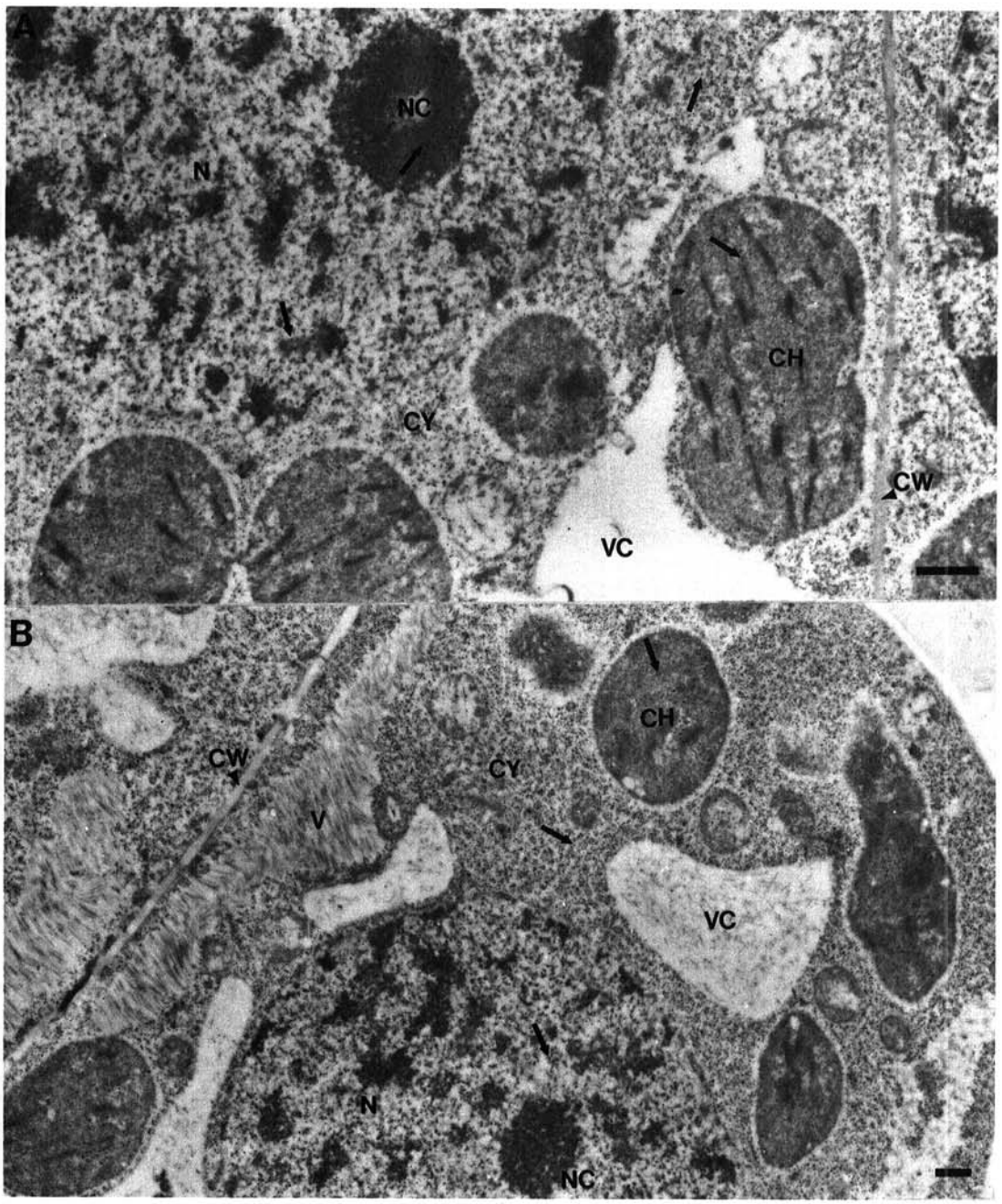

Fig. 1. Leaf sections probed with anti-ubiquitin antiserum and labeled with colloidal gold. Some gold particles are indicated by arrows. **A**, Uninfected cell. Labeling is seen over the cytoplasmic matrix (CY), chloroplasts (CH), nucleus (N), and nucleolus (NC), but not in the vacuole (VC) or cell wall (CW). In the nucleus the electron dense heterochromatin as well as euchromatin are gold-labeled. **B**, Cell infected with tobacco mosaic virus. The cytoplasmic matrix, chloroplasts, nucleus, and nucleolus are gold-labeled as indicated for panel **A**. There is no labeling over virus particles (V). Scale bars = 500 nm.

the grids were dried and stained for 1–2 min with 4% aqueous uranyl acetate. Sections were observed in a Phillips EM-200 electron microscope at 60 kV.

Immunogold labeling of TMV rods. Purified TMV particles in 0.01 M phosphate buffer, pH 7.0, were adsorbed to Formvar-coated nickel grids, and the grids were floated on drops of PBS-T containing 1% BSA for 15 min at room temperature. The grids were then incubated for 2 hr at room temperature with the anti-ubiquitin antiserum (20 μg/ml) diluted with PBS-T containing 0.01% BSA. After rinsing with the same buffer, the grids were floated for 1 hr on a solution of protein A conjugated with 10-nm-diameter gold particles diluted 1:50 with PBS-T containing 0.01% BSA. After washing with buffer and water, the grids were dried and stained for 30 sec with 2% aqueous uranyl acetate. In some experiments the virus particles were exposed to different treatments before the antibody reaction. These treatments included incubation with buffer at a high pH (Dore *et al.* 1988), fixation with glutaraldehyde, and incubation with methanol at −20° C (Eaton *et al.* 1988).

RESULTS AND DISCUSSION

Detection of ubiquitin in sections of tobacco tissue, uninfected and infected with TMV. Figure 1A shows a representative example of ubiquitin detection in uninfected tobacco leaf sections probed with anti-ubiquitin antiserum and labeled with 10-nm-diameter gold particles. Ubiquitin was localized in the cytoplasm and nucleus. In the cytoplasm, the matrix and the chloroplasts were the most abundantly labeled structures (Table 1). In the chloroplasts, gold particles were associated with the grana as well as stroma. In the nucleus, the heterochromatin and euchromatin were labeled but the nucleolus was the most highly labeled component. There was no label over the cell wall or vacuoles.

When leaf sections infected with TMV were probed with the same antiserum, the gold-labeling distribution (Fig. 1B; Table 1) was similar to that seen in uninfected leaf tissues. However, the X-bodies (a viroplasm typically induced by some strains of TMV) were also labeled (Fig. 2A), and the ropelike structures (Hills *et al.* 1987), a component of the viroplasm, were principally tagged. As seen in Figure 2, panels A and B, there was no significant labeling of TMV particles.

Sections of healthy (data not shown) and TMV-infected tissues incubated with nonhomologous antiserum or buffer instead of anti-ubiquitin antiserum as a control showed virtually no cellular gold labeling (Fig. 3). In the controls, serum directed against the 126K protein coded by TMV, as expected, labeled only the viroplasms in infected tissue and did not react with uninfected tissues.

In this study, immunogold labeling of healthy tissue and tissue infected by TMV with a polyclonal antiserum immunoreactive with ubiquitin and its conjugates revealed that ubiquitin (and/or conjugates) is located in the cytoplasmic matrix, chloroplasts, nucleus, and nucleolus. The significance of ubiquitin in higher plants is not known, but it is likely that ubiquitin has similar functions in plants and animals (Vierstra 1987a). If so, the ubiquitin distribution that we found in tobacco is quite expected because in animal cells ubiquitin is associated primarily with cytoplasmic and nuclear functions and has been found in subcellular fractions (Carlson and Rechsteiner 1987). In higher plants, it has been shown that the protein phytochrome is ubiquitinated *in vivo* after phototransformation to the far-red light absorbing form, indicating that it is degraded via ubiquitin intermediates (Shanklin *et al.* 1987). This observation is supported by the demonstration that higher plants contain ATP-dependent ubiquitin-conjugating activities (Vierstra 1987b).

The explanation for the presence of ubiquitin in the chloroplasts is yet to be established, but a role in the turnover of the chloroplasts' proteins cannot be ruled out. There is no evidence to suggest that chloroplasts have a ubiquitin conjugating system, although free ubiquitin has been found in several organelles in hepatoma cells by immunoelectron microscopic localization (Schwartz *et al.* 1988). With chloroplasts it is conceivable that proteins with transit sequences targeting them to the chloroplasts could be ubiquitinated before transport into the chloroplast.

TMV coat protein has been found in chloroplasts of tissues infected with TMV (Reinero and Beachy 1986), and a hypothesis could be made that the coat protein in chloroplasts is ubiquitinated. If this hypothesis were true, higher levels of ubiquitin labeling would be expected in chloroplasts of cells infected with TMV in comparison with

Table 1. Distribution of gold-tagged anti-ubiquitin antibodies on cell components of cells uninfected and infected with tobacco mosaic virus (TMV)

Cell component	Uninfected						Infected with TMV					
	μm^2 examined		Total gold particles observed		Gold particles per μm^2		μm^2 examined		Total gold particles observed		Gold particles per μm^2	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Cytoplasmic matrix	12.9	11.1	257	200	20	18	13.3	12.3	186	197	14	16
Chloroplasts	20.05	17.8	441	374	22	21	21.1	19.6	484	431	23	22
Vacuoles	7.3	5.2	4	2	<1	<1	6.8	4.9	9	4	1	<1
Nuclei (not including nucleoli)	16.7	17.2	484	447	29	26	18.6	16.3	315	344	17	21
Nucleoli	8.4	6.2	503	335	60	54	7.8	8.7	403	443	52	51
Viroplasms	...	...	...	...	...	...	11.4	10.7	171	182	15	17
Virus particle aggregates	...	...	...	...	...	...	22.7	18.0	21	11	<1	<1

those of uninfected cells. However, it seems not to be the case, and our results (Table 1) indicate that we did not detect increased levels of ubiquitin in the chloroplasts of infected tissues using the immunogold-labeling technique. Ubiquitin coat protein conjugates were also not detected in isolated chloroplasts of TMV-infected tissues in western transfers (data not shown). However, in the same blots, other ubiquitin conjugates were detected.

An interesting finding is the ubiquitin label associated with the ropelike structures of the viroplasms. A similar observation was made by K. Reinke and G. A. de Zoeten (personal communication). The viroplasms are composed, at least in part, of the TMV-coded 126K protein (Hills *et al.* 1987; Wijdeveld *et al.* 1989), and it has been suggested that they could represent the site of TMV replication (Saito *et al.* 1987). However, Hills *et al.* (1987) suggested that TMV replication occurs at the edges of the viroplasms rather than within them. Within the viroplasm, the 126K protein (Hills *et al.* 1987) and the 183K protein (our unpublished data) are mostly associated with the ropelike structures. A possible explanation is that the proteins in the viroplasms are tagged to be degraded in a ubiquitin-

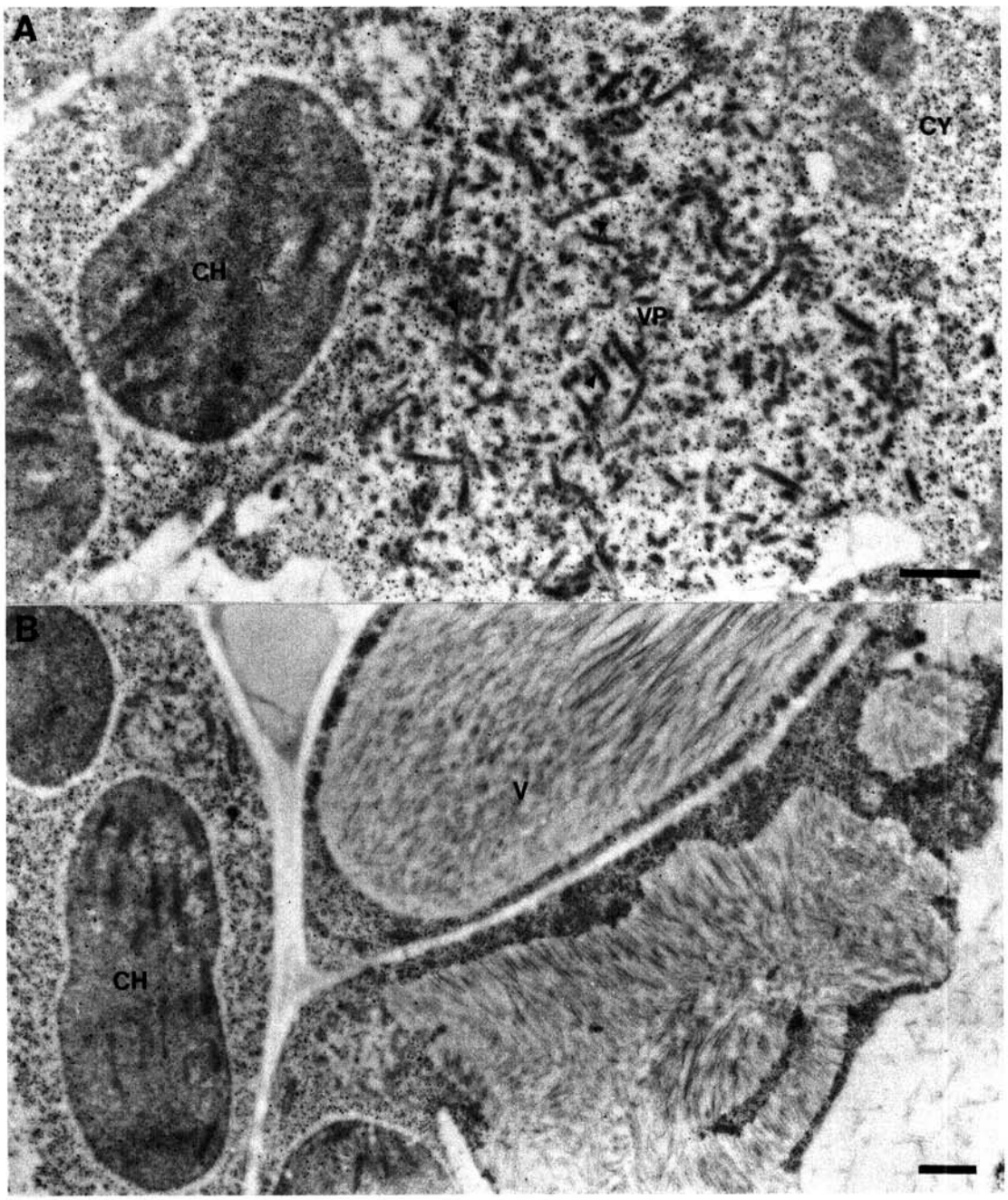

Fig. 2. Sections of a cell infected with tobacco mosaic virus probed with anti-ubiquitin antiserum and gold labeled. **A**, Cytoplasmic matrix (CY), chloroplasts (CH), and the viroplasm (VP) are labeled. Note that the ropelike structures (indicated by arrows) within the viroplasm are strongly gold-labeled. **B**, Chloroplasts are labeled, but virus particle aggregates (V) are not. Scale bars = 500 nm.

dependent proteolytic system. However, a more exciting possibility is that ubiquitination of the viroplasmic proteins is involved in virus replication.

The possibility that the 126K and 183K proteins themselves could be ubiquitinated was checked in western transfers. When the blots were incubated with the anti-ubiquitin antiserum, no reaction was found with the 126K and/or 183K bands or with upper bands. However, the presence of these two proteins in the blots was confirmed by the specific reaction with the respective antisera (data not shown). It is possible that the concentration of ubiquitin, associated with these two TMV-coded proteins, is below the level of detection with the western blotting technique. Also, there is a possibility that, besides the 126K and 183K proteins, the viroplasm ribbons contain a different ubiquitinated protein. Purification and analysis of the viroplasms induced by TMV would be needed to support this hypothesis.

Detection of ubiquitin in virions. Previous studies have indicated that there is an average of one ubiquitinated coat protein (H protein) per TMV particle (Asselin and Zaitlin 1978; Collmer *et al.* 1983), but the location on the virion is not known. Our early conclusion was that it was distributed at random in the virion (Asselin and Zaitlin 1978), but more recently we postulated that it was restricted to the end of the rod with the 3′ end of the RNA (Dunigan *et al.* 1988). In an attempt to resolve this question and to visualize ubiquitin in TMV virions, we incubated the virus particles adsorbed to Formvar-coated grids with the anti-ubiquitin antiserum and labeled the antibodies with protein A-gold particles. We never detected labeling of the rods with this technique; however, the ubiquitin coat protein conjugate is readily detected from disrupted purified virions in western transfers (Hazelwood and Zaitlin, in press). In some experiments we induced structural modifications in the virions by incubating them at pH 9.6 (Dore *et al.* 1988) or in methanol at −20° C (Eaton *et al.* 1988) in an attempt to expose ubiquitin conjugates that might be buried within the virion. No labeling was observed, however. It is probable that the technique, at the conditions used, is not sensitive enough to detect one ubiquitin molecule per virion. Alternatively, the ubiquitin might be contained within the virion, and it is not accessible to the antibodies, even after the treatments used. Support for this hypothesis comes from the observation that the H protein in the virion is not sensitive to degradation by proteolytic enzymes (Asselin and Zaitlin 1978). It is also conceivable that the antiserum used does not react with the H protein at native conformation. The latter possibility is more probable because the antiserum used is made to ubiquitin denatured by sodium dodecyl sulfate and, in western blotting where we can detect the ubiquitin in disrupted virions, we autoclave the membranes after electrotransfer to increase the sensitivity of the detection of ubiquitin by denaturing the protein on the membrane (Swerdlow *et al.* 1986).

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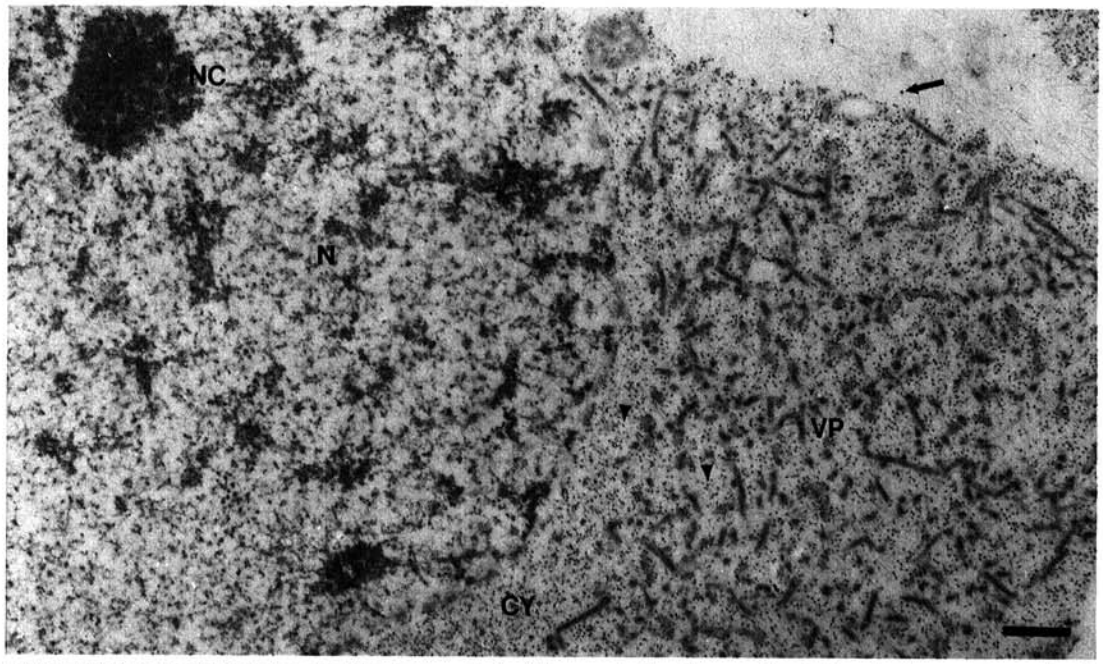

Fig. 3. Section of a cell infected with tobacco mosaic virus probed with a control antiserum directed against cowpea mosaic virus coat protein and then gold labeled. There is almost no labeling (arrow). Nucleus (N), nucleolus (NC), viroplasm (VP), cytoplasmic matrix (CY), and ribosomes (arrowheads). Scale bars = 500 nm.

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