VIROLOGY 160, 297–300 (1987)

The Location of Coat Protein and Viral RNAs of Alfalfa Mosaic Virus in Infected Tobacco Leaves and Protoplasts

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Received February 27, 1987; accepted March 20, 1987

The location of coat protein of alfalfa mosaic virus (AlMV) strain 425 was determined in protoplasts isolated from infected tobacco leaves and in *in vitro* inoculated tobacco protoplasts, using immunocytochemistry on ultrathin frozen sections labeled with colloidal gold. In infected tobacco leaves 5 days postinoculation (p.i.) coat protein is present in the cytoplasm and nucleus, especially around the nucleolus. In *in vitro* inoculated tobacco protoplasts coat protein was not present in the nucleus 6 hr p.i. These results indicate that the presence of coat protein in the nucleus is not necessary for viral replication. However, coat protein could be detected in the nucleus 48 hr p.i. Probably coat protein or virus particles accumulate in the nucleus late in infection. Minus-strand RNA, as part of the replication complex, could be detected in a 650 *g* pellet fraction of infected tobacco leaves but not in the nucleus, suggesting that replication of AlMV occurs outside the nucleus. © 1987 Academic Press, Inc.

The genome of alfalfa mosaic virus (AlMV) consists of three single-strand RNAs of the plus polarity which are separately encapsidated into virions. In addition, virion preparations contain a subgenomic RNA, the messenger for coat protein. The coat protein has at least two different functions in the multiplication of the genome: activation of the viral genome, probably by binding to the 3′-terminal homologous region of the RNAs (*1*) and encapsidation of the newly formed viral RNAs (*2*).

With standard electron microscopy techniques, AlMV particles have previously been visualized in the cytoplasm (*3*). No AlMV particles were seen in other organelles of cells infected with strain 425. Some nuclei of cells infected with AlMV (strain 15/64) contained groups of tubular bodies, reminiscent of virus particles (*4*). However, there is no evidence to show whether these tubular bodies are of viral origin. It has also been observed, using electron microscopic autoradiography, that leaf cells infected with AlMV and treated with actinomycin D to inhibit host-directed RNA synthesis incorporate ^{3}H-labeled uridine mainly into nuclei and cytoplasm (*5*). This suggests involvement of the nucleus in AlMV RNA synthesis. However, it is not clear whether labeling in the nucleus represented RNA synthesized there or the accumulation of RNA previously synthesized in the cytoplasm.

In order to obtain more information about the localization of coat protein and the replication complex of AlMV (strain 425), we investigated the location of this protein and viral RNA in protoplasts isolated from tobacco (*Nicotiana tabacum* L. Samsun NN) leaves 5 days after inoculation and in *in vitro* inoculated protoplasts early (6 hr p.i.) and late (48 hr p.i.) in infection. For the analysis of coat protein, we used a highly sensitive immunocytochemical technique in which ultrathin frozen sections of protoplasts are treated first with an antibody against coat protein and subsequently with a protein A/gold complex (*6, 7*). Using this method, complete virus particles and coat protein can be localized. Electron microscopic observation demonstrated gold particles in the cytoplasm and the nucleus, especially around the nucleolus, in protoplasts isolated from infected tobacco leaves (Figs. 1A–1C). Healthy protoplasts treated by the same procedure (Fig. 1D) as well as those from infected tissue incubated with preimmune serum (not shown) showed almost no label. No coat protein could be detected in the nucleus of tobacco protoplasts 6 hr p.i. (Fig. 1E), while viral minus-strand RNA was already detectable at this time point (*8*). These results indicate that the presence of coat protein in the nucleus is not necessary for activation of the viral genome. Coat protein could be detected in the nucleus in tobacco protoplasts 48 hr p.i. (Fig. 1F). Probably coat protein or virus particles accumulate in the nucleus late in infection.

Minus-strand RNA, as part of the replication complex (*9*), and plus-strand RNA were analyzed in different

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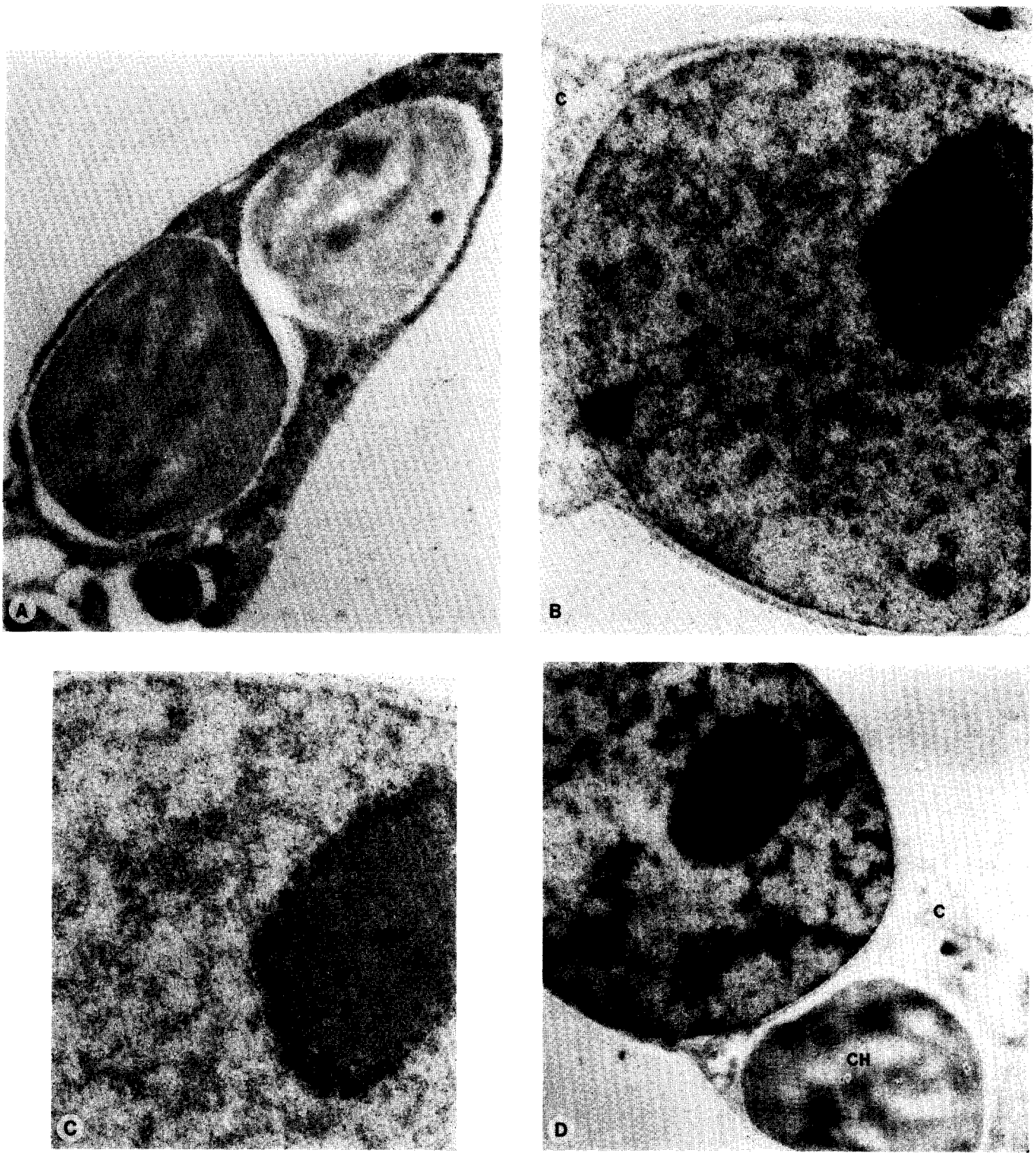

FIG. 1. Localization of coat protein in tobacco protoplasts isolated 5 days after inoculation of the leaves with AlMV and in tobacco protoplasts isolated, inoculated with AlMV, and incubated for 6 and 48 hr p.i. as described by Loesch-Fries *et al.* (*11*). The protoplasts were fixed 30 min in 0.5% glutaraldehyde in 0.1 *M* sodium phosphate buffer, pH 7.2, and embedded in gelatin. For electron microscopy, 100-nm cryosections were labeled with a serum against AlMV coat protein or preimmune serum and subsequently labeled with protein A complexed with 8-nm-diameter colloidal gold. The sections were stained with uranyl acetate. (A) Gold particles in the cytoplasm (C) of tobacco protoplasts isolated 5 days after inoculation of the leaves. Chloroplast (CH). Magnification ×52,000. (B) Nucleus (N) of protoplast isolated 5 days after inoculation of the leaves. Gold particles are seen around the nucleolus (NU). Magnification ×64,000. (C) Same nucleolus (NU) as in (B). Magnification ×88,000. (D) Cytoplasm (C) and nucleus with nucleolus (NU) of healthy tobacco protoplast. Chloroplast (CH). Magnification ×52,000. (E) Nucleus (N) of *in vitro* inoculated tobacco protoplast 6 hr p.i. Magnification ×54,000. (F) Cytoplasm (C) and nucleus (N) of *in vitro* inoculated tobacco protoplast 48 hr p.i. Magnification ×107,000.

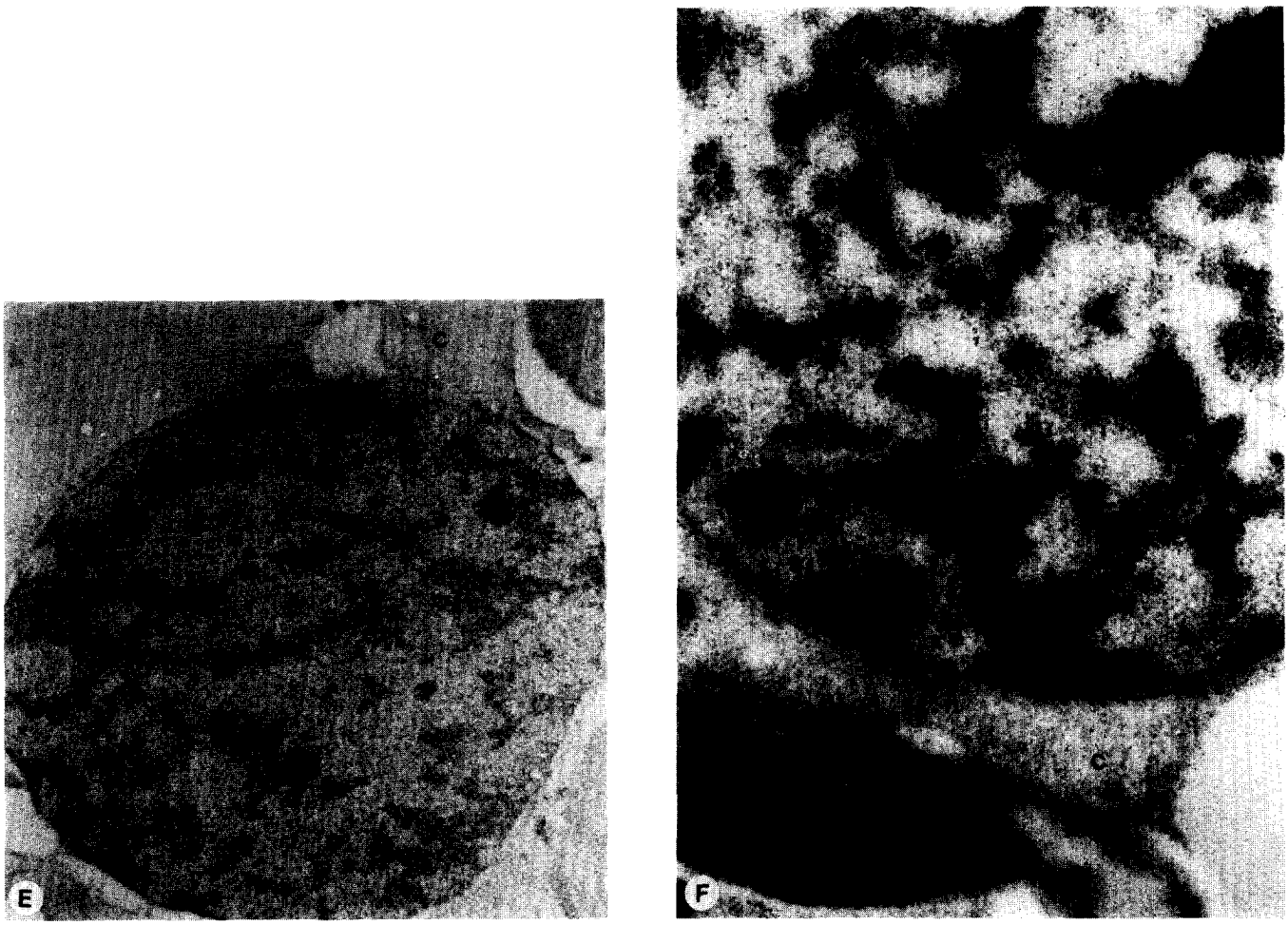

FIG. 1—*Continued.*

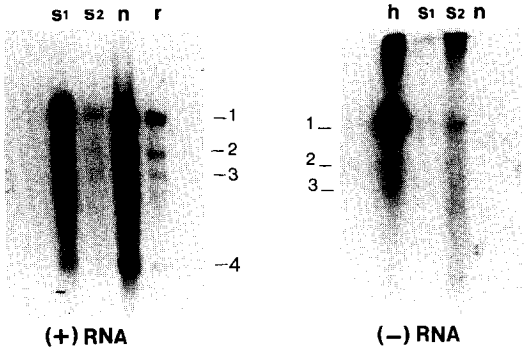

FIG. 2. Detection of viral plus- and minus-strand RNA in tobacco leaves 5 days after inoculation with AlMV strain 425. Leaves were homogenized at 4° in 2.5% Ficoll, 5% Dextran T-40, 0.25 *M* sucrose, 2 m*M* $MgCl_2$, 5 m*M* 2-mercaptoethanol, 25 m*M* Tris–HCl (pH 7.8). The brei was sieved through a set of four filters of pore sizes 0.25, 0.16, 0.045, and 0.010 mm to yield a leaf homogenate (h) which was centrifuged 10 min at 650 *g*. The supernatant was designated S_1 fraction. The 650 *g* pellet was suspended in 40% glycerol, 0.6 *M* sucrose, 20 m*M* KCl, 20 m*M* $MgCl_2$, 10 m*M* 2-mercaptoethanol, and 25 m*M* MES–NaOH, pH 6.0 (buffer A), containing 1.2% Nonidet-P40 and centrifuged 30 min at 2500 *g* on a cushion of 1.2 *M* sucrose. The supernatant was designated S_2. The pellet was resuspended in buffer A, loaded on a discontinuous sucrose gradient of 75 and 60% (w/v) sucrose, and centrifuged 30 min at 7000 rpm in a Beckman SW 27 rotor. The nuclei (n) were collected from the top of the 75% sucrose layer (*12*). The RNAs from the fractions h, S_1, S_2, and n were extracted with phenol, denaturated with glyoxal, electrophoresed on a 1.5% agarose gel, and transferred to GeneScreen (*10*). The blot was hybridized with ^{32}P-labeled AlMV cDNA (left panel) or ^{32}P-labeled viral RNA (right panel). Lane r was loaded with RNA extracted from purified AlMV.

fractions from tobacco leaves 5 days after inoculation. RNAs were extracted from a total leaf homogenate (h), a fraction containing mitochondria (S_1), a fraction containing disrupted plastids and cytoplasmic membranes from the 650 *g* pellet fraction (S_2), and a fraction containing purified nuclei (n). The RNAs were detected by the Northern blotting technique (10). The lanes of the gel (Fig. 2), except lane n, were loaded with RNA extracted from fractions isolated from 0.1 g of leaf tissue. Since there was a possible degradation of RNA during isolation of the nuclei, lane n was loaded with RNA extracted from the nuclear fraction isolated from 1 g of leaf tissue. No bands were detected in the lanes

loaded with RNA extracted from the healthy tissue fractions after hybridization with viral plus- and minus-strand RNA probes (not shown). As shown in Fig. 2 (left panel), plus-strand RNA appeared to be present in the purified nuclear fraction (n). However, electron microscopic observation revealed that this fraction was contaminated with virus particles (not shown). Therefore plus-strand RNA in this fraction may not be taken as evidence that plus-strand RNA is present in the nucleus. Viral minus-strand RNA was observed in the total leaf homogenate (h) and in the fraction S_2, which contained disrupted plastids and cytoplasmic membranes from the 650 *g* pellet fraction (Fig. 2, left panel). A faint band was visible in the fraction S_1. No bands were detectable in the purified nuclear fraction (n). Since the tobacco leaves 5 days p.i. probably contained cells in all stages of infection, the absence of minus-strand RNA as part of the replication complex in the purified nuclear fraction indicates that AlMV replication does not take place in the nucleus but that it is associated with structures in the 650 *g* pellet fraction.

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