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Evidence That a Viral Replicase Protein Is Involved in the Disassembly of Tobacco Mosaic Virus Particles *in Vivo*

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Tobacco mosaic virus (TMV) particles have been shown to undergo bidirectional disassembly when they are introduced into host cells. Approximately three-quarters of the genomic RNA (i.e., the 126-kDa and 183-kDa protein ORFs) is first uncoated in the 5′-to-3′ direction and the process is then completed by removal of coat protein molecules in the 3′-to-5′ direction. An effort was made to determine whether the 126-kDa protein or the 183-kDa protein, both of which are involved in replication of the viral RNA, is required for the second part of the disassembly reaction. It was shown that progeny negative-strand viral RNA begins to be produced in inoculated cells at about the same time that 3′-to-5′ disassembly is initiated thus suggesting that the two processes may be coupled. Particles containing mutant forms of the viral RNA in which large sections of the 126-kDa and 183-kDa protein ORFs were missing were not disassembled in the 3′-to-5′ direction when they were introduced into cells. However, they were disassembled when the inoculum contained purified TMV RNA from which, presumably, the two functional proteins could be translated. Particles containing mutants of the RNA from which a few codons had been deleted in or near conserved regions in the 126-kDa protein ORF also did not undergo 3′-to-5′ disassembly unless mixed with wild type viral RNA prior to inoculation. These results suggest that the 126-kDa and/or 183-kDa protein plays a role in the completion of disassembly of TMV particles at the onset of the infection process.

One of the most important events that occurs during the establishment of infection of an organism by a virus is the uncoating of the genomic constituent of the virus particle. During this process, the virus particle is disassembled and the genome is released in a state in which it can be expressed and replicated in the initially infected cell of the host. In an investigation in this laboratory of the uncoating of the genomic RNA of tobacco mosaic virus (TMV), the classic virus of rod-shaped morphology, evidence that the process occurs in a bidirectional manner has been obtained. Within the first 2 min after inoculation of protoplasts with purified virus particles, at least the first 4635 nucleotides from the 5′ terminus of the 6395-nucleotide viral RNA are uncoated, and it is assumed that the removal of subunits occurs in the 5′-to-3′ direction (Wu *et al.,* 1994). This process is followed by uncoating of the remainder of the RNA in the 3′-to-5′ direction with a region near the origin-of-assembly sequence (nucleotide residues 5444–5518) being the last to be uncoated (Wu and Shaw, 1996).

The initial, rapid, 5′-to-3′ uncoating of TMV particles *in vivo* results in the release of coat protein subunits from the part of the viral genome that encodes the 126- and 183-kDa proteins. [The 183-kDa protein is the product of readthrough of the amber codon that terminates translation of the 126-kDa protein ORF (Goelet *et al.,* 1982).] Since these proteins constitute the viral RNA-dependent RNA polymerase (Ishikawa *et al.,* 1986; Meshi *et al.,* 1987), it is conceivable that one or both may participate in the 3′-to-5′ uncoating of the remainder of the genome and that the process may involve a coreplicational-disassembly mechanism. Such a possibility was suggested many years ago by Wilson (1985), who demonstrated that purified TMV particles could undergo partial disassembly in *in vitro* protein synthesis reaction mixtures by a cotranslational mechanism (Wilson, 1984) and who speculated that a coreplicational disassembly process might be required to complete the uncoating of the particles. The experiments described here were designed to examine this hypothesis, and the results provide evidence that the 126-kDa protein and/or 183-kDa protein may play a role in the uncoating of TMV RNA *in vivo.*

METHODS AND MATERIALS

Preparation of mutant viral RNAs

A plasmid (pTMV210) containing a nucleotide sequence equivalent to that of TMV RNA was kindly provided by Drs. W. O. Dawson and D. J. Lewandowski (University of Florida). This plasmid was used for the construction of altered forms of the viral cDNA from which mutant RNAs could be synthesized in *in vitro* transcription reactions. Four mutant RNAs were pro-

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duced; in two of them, large segments of the ORFs that are involved in replication of the viral RNA were deleted. For mutant ShortA, a *Tth*111 I–*Nsi*I fragment [equivalent to nucleotides 113–4653 in the viral genome; numbering as in Goelet *et al.* (1982)] was excised from pTMV210. The deleted sequence (a 4541-nucleotide segment) represents most of the 126-kDa/183-kDa protein ORF of TMV RNA. T4 DNA polymerase was used to produce blunt ends which were ligated. A similar approach was used to prepare a construct from which mutant MalA RNA could be transcribed. Digestion of pTMV210 with *Stu*I and *Nhe*I resulted in excision of a 245-nucleotide segment (nucleotides 1678–1922 in the middle of the 126-kDa protein ORF).

Two other mutants were constructed by PCR mutagenesis of pTMV210 with Deep Vent DNA polymerase (New England Biolabs) and pairs of oligonucleotide primers selected to yield products in which small segments at specific sites in the cDNA were deleted. The blunt-ended PCR products were then ligated and used to transform *Escherichia coli* cells. For mutant MT, primers with nucleotide sequences ATATGCTCGTCCCTT and AACCTGGACGTTCG yielded a product in which 18 nucleotides (435–452) were deleted from a region in the 126-kDa protein ORF near that which encodes a conserved motif in a putative methyltransferase domain (Rozanov *et al.,* 1992). The other mutant, HelA, produced by reactions involving primers TTACAGCCCGGAACT and AGAAATTCTTTCCAGGGT, gave rise to a 9-nucleotide deletion (nucleotides 2580–2588) in the region of the 126-kDa protein ORF, which encodes a three-amino-acid sequence (GKT) in a conserved motif of the putative helicase domain (Gorbalenya *et al.,* 1988). Each of the sequence modifications used to construct the mutants was confirmed by DNA sequence analysis.

Viral RNA was generated from the mutant constructs or from pTMV210 in *in vitro* transcription reactions containing T3 RNA polymerase or T7 RNA polymerase (Gibco BRL) and performed according to the manufacturer's recommendations.

Infectivity assay of mutant RNAs

Nicotiana tabacum cv. Xanthi plants were inoculated with the products of the *in vitro* transcription reactions to determine whether the mutant RNAs were viable. The plants were maintained in a growth chamber until those that had been inoculated with RNA isolated by phenol–chloroform extraction from purified virus or with wild-type transcripts had developed systemic disease symptoms. Samples of tissue (1.2-cm diameter disks) were then collected from leaves above the inoculated leaves and homogenized in 100 μl 0.12 M Tris–HCl buffer (pH 6.8), 4% SDS, 10% β-mercaptoethanol, and 20% glycerol. The extracts were boiled and then subjected to Western immunoblot analysis.

The ability of the mutant RNAs to be replicated in protoplasts was also tested. Protoplasts were isolated from tobacco leaves and inoculated with transcript RNA by the procedures described by Luciano *et al.* (1987) and Klein *et al.* (1994). After incubation for 48 h, samples of the protoplasts were subjected to Western immunoblot analysis with an anti-TMV serum used as described by Klein *et al.* (1994). Other samples were subjected to Northern hybridization analysis with probes identical or complementary to a sequence (nucleotide residues 5080–6395) in the 3′-proximal part of the viral RNA.

Encapsidation of mutant RNAs

Two procedures were used for the assembly of particles containing the mutant transcript RNAs. With mutants MalA, MT, and HelA, transcription reaction products were subjected to DNase treatment and phenol–chloroform extraction and the RNA was precipitated with ethanol and resuspended in water. *In vitro* assembly of particles was performed by incubation of a 50-fold amount of TMV coat protein, isolated from purified virus particles by the procedure of Fraenkel-Conrat (1957), with transcript RNA under the conditions described by Butler (1984).

Particles containing ShortA RNA were obtained from tobacco plants that had been inoculated with a mixture of mutant transcripts and purified viral RNA (Raffo and Dawson, 1991). Virus particles were purified from extracts of systemically infected leaves and passed through a column of agarose beads (Wu *et al.,* 1994). Fractions containing shorter-than-full-length particles were collected and the particles concentrated by ultracentrifugation. To determine the nature of the short particles, RNA was isolated from them and subjected to Northern hybridization analysis with probes complementary to a region in the 3′ (nucleotides 5080–6395) or 5′ proximal part (nucleotides 999–3332) of the viral RNA.

Analysis of virus particle disassembly

Tobacco protoplasts were isolated and inoculated (by electroporation) with particles containing mutant or wild-type viral RNA (1 μg particles per million protoplasts) as previously described (Wu *et al.,* 1994). In some experiments, the particles were mixed with purified TMV RNA (0.1–0.5 μg per μg particles) prior to electroporation of the protoplasts. The collection of protoplast samples at various times (0–60 min) after inoculation and the subsequent handling of the samples were performed as described by Wu *et al.* (1994).

Analysis of disassembly of the mutant or wild-type virus particles in the 3′-to-5′ direction was conducted as previously described (Wu and Shaw, 1996). Briefly, the procedure involved extraction of the protoplasts in a medium in which the uncoated parts of the viral RNA were destroyed, isolation of the protected (still encapsi-

dated) fraction of the RNA molecule, and identification of the fragment by RT-PCR analysis. The RT and PCR primers and the reaction conditions were those used previously (Wu and Shaw, 1996). This procedure reveals the identity of the part of the viral RNA that has been uncoated *in vivo* at a particular time after inoculation of the protoplasts.

Detection of negative-strand viral RNA in inoculated protoplasts

Protoplasts were inoculated with purified TMV particles and samples were collected as described by Wu *et al.* (1994). Total RNA (instead of encapsidated viral RNA) was isolated from the protoplasts by extraction with TRI Reagent (Molecular Research Center) and alcohol precipitation. To eliminate the possibility that artifacts might arise as a result of the presence of positive-strand viral RNA in the extracts, the samples were subjected, prior to RT-PCR analysis, to periodate oxidation (Andrulis and Arfin, 1979) in order to prevent 3′-end priming by the positive strands.

Three separate RT reactions were performed with aliquots of each sample of total protoplast RNA. Each reaction mixture contained one of the following primers: primer J, 5′-GGCGAACCCCACGACTGCCG-3′ (equivalent to nucleotides 6011–6030 in TMV RNA); primer K, 5′-GTCTTACAGTATCACTACT-3′ (5714–5732); or primer L, 5′-GAGAGAAGATTACAAACGTG-3′ (5426–5445). A single pair of PCR primers was used to amplify products of each RT reaction. The nucleotide sequences of the PCR primers were 5′-ATTATGCATCTTGACTACCTC-3′ (nucleotides 6210–6190 in TMV cDNA) and 5′-TAGAGTAGACGACGCAACGGTGGCCATAA-3′ (equivalent to nucleotides 6050–6078 in TMV RNA). The RT and PCR amplifications were performed under the conditions described by Wu *et al.* (1994).

RESULTS

Two approaches have been undertaken to examine the coreplicational disassembly hypothesis. First, it was reasoned that such a mechanism would result in the simultaneous removal of coat protein subunits from the end of the virus particle bearing the 3′-end of the RNA and the initiation of synthesis of progeny negative-strand viral RNA. Therefore, an attempt has been made to determine whether negative-strand TMV RNA could be detected in samples of inoculated protoplasts during the period when the virus particles were undergoing 3′-to-5′ disassembly. Second, some mutants of TMV RNA with alterations in the 126-kDa/183-kDa protein ORFs were prepared and packaged in coat protein subunits and the ability of the particles to be disassembled in the 3′-to-5′ direction was assessed. If disassembly could occur only when the viral RNA polymerase genes were unaltered, then the coreplicational disassembly hypothesis would gain some support.

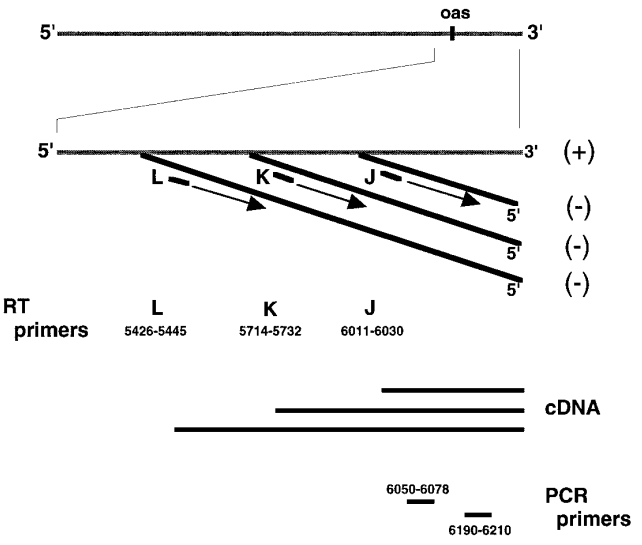

FIG. 1. Positions of RT and PCR primers used to detect the presence of negative-strand viral RNA in extracts of protoplasts inoculated with TMV particles. Faint horizontal lines represent viral RNA; boldface diagonal lines are negative-strand RNA; boldface horizontal lines are cDNA. Upper line shows the 6395-nucleotide TMV RNA with the origin-of-assembly sequence (nucleotides 5444–5518) indicated. J, K, and L (shown as boldface diagonal dashes) are RT primers (complementary to negative-sense TMV RNA); the nucleotide residue positions in TMV RNA to which they are equivalent are indicated. The three cDNAs are those that would be produced by reverse transcription of negative-strand TMV RNA in the presence of primers J, K, and L. The positions in the genome represented by the pair of PCR primers used to amplify a region in the cDNAs are indicated.

Detection of negative-sense viral RNA in TMV-inoculated protoplasts

Protoplasts were inoculated with purified TMV particles and samples were collected at various times thereafter. Extracts of the protoplasts were processed to yield their total RNA contents and separate aliquots were subjected to reverse transcription in the presence of primer J, K, or L. The sequences of these primers are equivalent to specific regions between the origin-of-assembly sequence and the 3′-terminus of TMV RNA (Fig. 1). RT products were amplified by PCR with a pair of primers representing a region nearer the 3′-terminus of the viral RNA.

As a test of the applicability of these procedures, purified TMV RNA or TMV particles were added to extracts of mock-inoculated protoplasts, which were then carried through the RNA isolation and RT-PCR steps. In no case was a PCR product detected when primer J, K, or L was present in the RT reaction (Fig. 2). (Only the samples in which RT primer J was used are shown in Fig. 2; reactions were also performed with primers K and L, and no PCR products were produced.) The expected PCR product was obtained when the sample to which

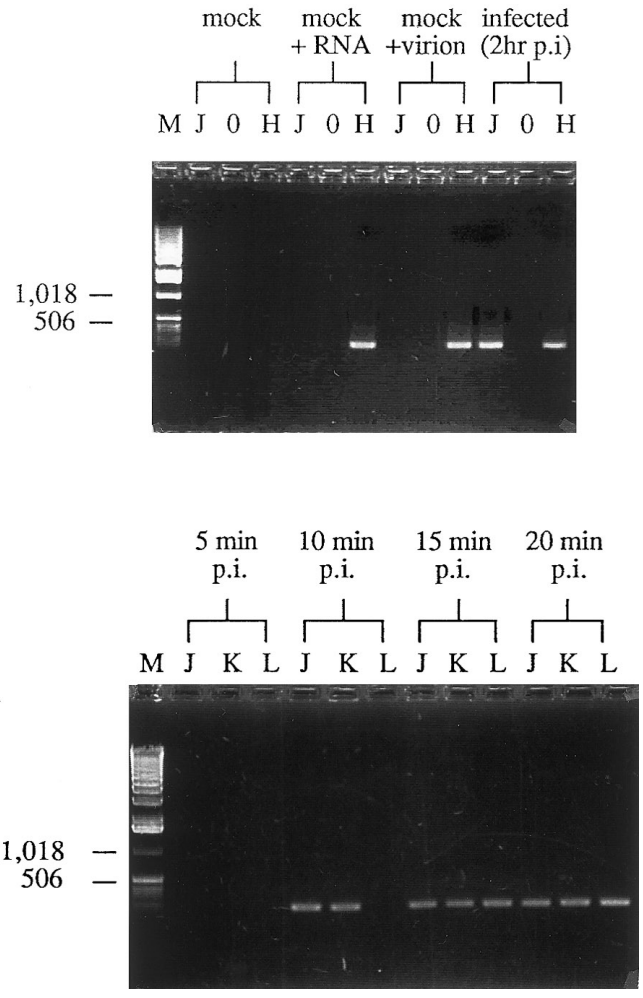

FIG. 2. Detection by RT-PCR of negative-strand viral RNA in protoplasts inoculated with TMV particles. (Top) Control to determine efficacy of RT-PCR procedure. Samples were extracts of mock-inoculated protoplasts to which were added nothing (mock), 20 ng purified TMV RNA per million protoplasts (mock + RNA) or 1 μg purified TMV particles per million protoplasts (mock + virion). A fourth sample [infected (2 h p.i.)] was an extract of protoplasts inoculated with TMV particles (1 μg per million protoplasts) that were collected 2 h after inoculation. Total RNA extracts of each sample of protoplasts were subjected to reverse transcription with primer J (Fig. 1), with primer H [an RT primer that anneals to a 20-nucleotide residue sequence at the 3′-terminus of TMV RNA (Wu and Shaw, 1996)], or with no primer (0). RT products were amplified by PCR performed with the primer pair shown in Fig. 1 (Bottom) Products detected by RT-PCR analysis of inoculated protoplasts. Times noted above lanes are the times after electroporation that the protoplast samples were frozen in liquid nitrogen. To lanes J, K, and L were applied the products of PCR amplification of cDNAs synthesized in reactions containing the designated RT primers. Lane M contains a standard DNA mixture with the sizes of some bands (in bp) indicated.

purified virus particles had been added was used with an RT primer (H) designed to detect positive-strand viral RNA (Wu and Shaw, 1996). Another control consisted of protoplasts that were incubated for 2 h after inoculation and then processed. These protoplasts were expected to contain positive-strand and newly synthesized negative-strand viral RNAs, and PCR products were obtained in reactions with either RT primer J or H (Fig. 2). These controls demonstrated the efficacy of the procedures for specific detection of negative-strand TMV RNA in extracts of inoculated protoplasts.

The results obtained with samples of protoplasts collected within the first 20 min after inoculation are shown in Fig. 2. In the 5-min samples, no PCR products were obtained. However, in samples collected 10 min after inoculation, RT reactions with primers J and K gave rise to products that could be amplified by PCR, and in 15- and 20-min samples, all three PCR products were produced. These results indicated the presence of the 5′-terminal region of the negative strand of TMV RNA within the first 10 min of inoculation and the appearance of more distal sequences in negative-strand RNA molecules within the next few minutes. Other experiments, in which an RT primer equivalent to a region near the 5′-terminus of the genomic RNA and the PCR primer pair shown in Fig. 1 were used, indicated the presence of near full-length negative-strand RNA in extracts of protoplasts collected 20–120 min after inoculation (data not shown).

3′-to-5′ uncoating of mutant TMV RNAs

A more direct test of the coreplicational disassembly hypothesis involved the preparation of TMV particles containing replication-defective genomes and the determination of whether such particles were able to undergo uncoating of the RNA in the 3′-to-5′ direction. Four mutants were constructed and cloned in plasmids from which transcript RNAs could be synthesized *in vitro* (Fig. 3). Mutant ShortA lacked most of the 126- and 183-kDa protein ORFs and mutant MalA lacked a segment of 245 nucleotides from the middle of the 126-kDa protein ORF. Smaller deletions were made in the 126-kDa protein ORF of two other mutants. In mutant MT, an 18-nucleotide residue segment near one of the conserved motifs of the putative methyltransferase domain was deleted and, in mutant HelA, a 9-nucleotide segment in a conserved motif of the putative helicase domain was missing. pTMV210 was used to produce wild-type TMV RNA transcripts.

Tobacco plants and protoplasts were inoculated with each of the preparations of transcript RNAs to determine whether any of the mutants was infectious. None of the plants inoculated with any of the mutants developed disease symptoms and no viral coat protein could be detected by Western immunoblot analysis of extracts of any of the leaves of the inoculated plants (data not shown). No viral RNA and no coat protein could be detected by Northern hybridization (with probes specific for either positive- or negative-strand TMV RNA) and Western immunoblot analyses of extracts of protoplasts that had been inoculated with any of the mutants and incubated for 48 h (data not shown). Plants inoculated

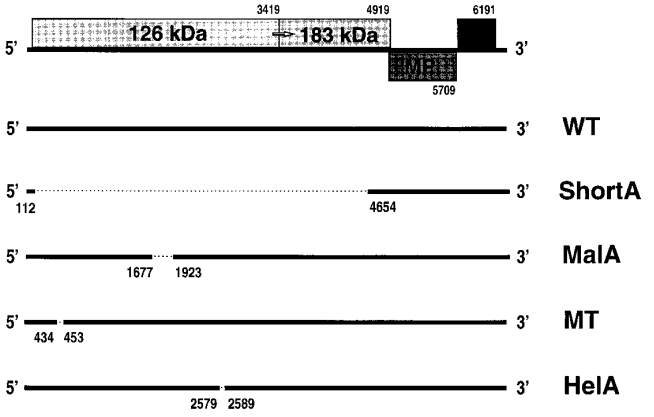

FIG. 3. Diagrammatic representation of mutants of TMV RNA used to investigate 3′-to-5′ disassembly of TMV particles in inoculated protoplasts. Gene map of TMV RNA with rectangular boxes denoting ORFs is shown at the top; numbers above and below map are the third nucleotides in the termination codon of each ORF. The ORFs marked 126 kDa and 183 kDa encode the viral RNA polymerase proteins [the 183-kDa protein is produced by readthrough of the termination codon (small arrow) of the 126-kDa protein ORF]; the MP and CP ORFs encode the 30-kDa movement protein and 17.5-kDa coat protein, respectively. Boldface horizontal lines represent the wild-type (WT) and mutant RNAs. The regions indicated by dotted lines have been deleted and the numbers below the lines are the nucleotides immediately before and after the deleted regions.

with wild-type transcripts developed typical symptoms and contained viral coat protein; viral coat protein and progeny viral RNA accumulated in protoplasts inoculated with wild-type transcripts (data not shown).

Transcripts containing mutant MalA, MT, or HelA RNA and wild-type transcript RNAs were packaged in TMV coat protein in *in vitro* assembly reactions. Particles containing transcripts of one mutant, ShortA, were produced in and isolated from plants co-inoculated with ShortA transcripts and TMV RNA. Northern blot hybridization analysis of the RNA isolated from the purified ShortA particles revealed that most of the particles contained 3′-proximal but not 5′-proximal sequences; even if present, short particles containing the latter would not be a complicating factor because they would lack sequences that could be detected by the RT and PCR primers used in the disassembly experiments.

Each type of particle was used in an analysis of 3′-to-5′ disassembly in inoculated protoplasts by the procedures described in Wu and Shaw (1996). The positions in TMV RNA represented by each of the RT and PCR primers used in the analyses are shown in Fig. 4 (top). With protoplasts inoculated with virus particles containing wild-type transcript RNA (Fig. 4, bottom), the same pattern of RT-PCR products was produced as had been observed in experiments with native TMV particles (Wu and Shaw, 1996). [Figure 4 does not include samples collected more than 25 min after inoculation; such samples produced results identical to those shown in Fig. 4 in Wu and Shaw (1996).] Samples collected 2 min after inoculation showed no evidence of disassembly at the end of the particles containing the 3′-terminus of the viral RNA, i.e., each of the four RT reactions resulted in cDNA products that could be amplified by PCR. However, with samples collected 5 min after inoculation, RT reactions with primer H did not yield an amplifiable product. In 10-min samples, only RT reactions involving primers E and F gave rise to PCR products, and in 20-min samples, a PCR product could only be obtained from RT reactions with primer E. This pattern of disassembly of TMV particles was taken as evidence of 3′-to-5′ uncoating of the viral RNA *in vivo* (Wu and Shaw, 1996).

There was no evidence of 3′-to-5′ disassembly of particles containing ShortA or MalA RNA in inoculated protoplasts (Fig. 5). With all samples, each of the RT reactions gave rise to products that could be amplified by PCR. (Only the analyses of protoplast samples col-

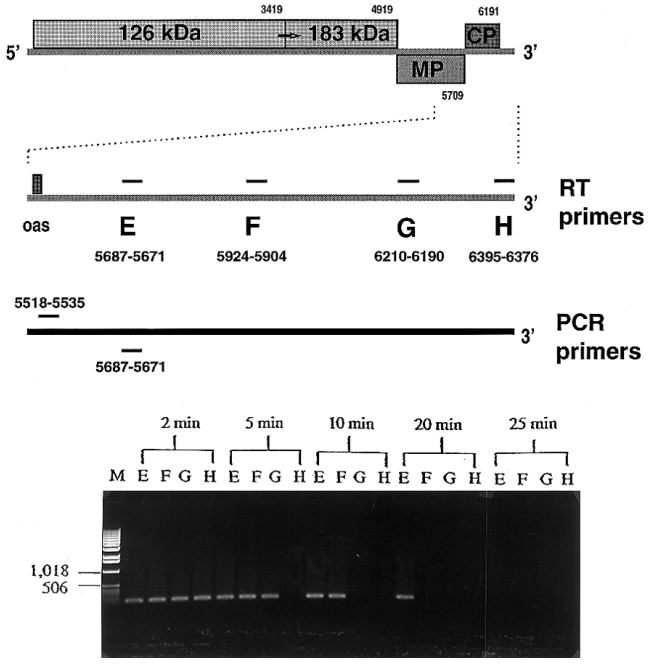

FIG. 4. Analysis of 3′-to-5′ uncoating of viral RNA after inoculation of protoplasts with TMV particles. (Top) Positions of RT and PCR primers. The gene map of TMV RNA (described in Fig. 3 legend) is shown at the top; below it is the region in TMV RNA from the origin-of-assembly sequence (oas) to the 3′-end showing the positions of primers E, F, G, and H used to reverse transcribe fragments of viral RNA that were protected by viral coat protein in extracts of inoculated protoplasts. The positions in TMV RNA of the PCR primers used for amplification of cDNAs are also indicated. The numbers represent the first and last nucleotide residues in the primers; the nucleotide sequences of the RT and PCR primers are presented in the text. (Bottom) RT-PCR analyses of protected viral RNA fragments in extracts of protoplasts inoculated with TMV particles. The virus particles were produced by *in vitro* assembly of TMV coat protein and RNA transcripts (wild type) of pTMV210. Times above the lanes are the period between electroporation and freezing of the protoplasts. With each sample, the protected (still-coated) viral RNA fragments were isolated and four separate RT reactions, with primers E, F, G, or H, were conducted. Electrophoretic analysis of the products of PCR amplification of the RT reaction products is shown. Lane M contained a standard DNA mixture; the sizes of some of the bands (in bp) is shown.

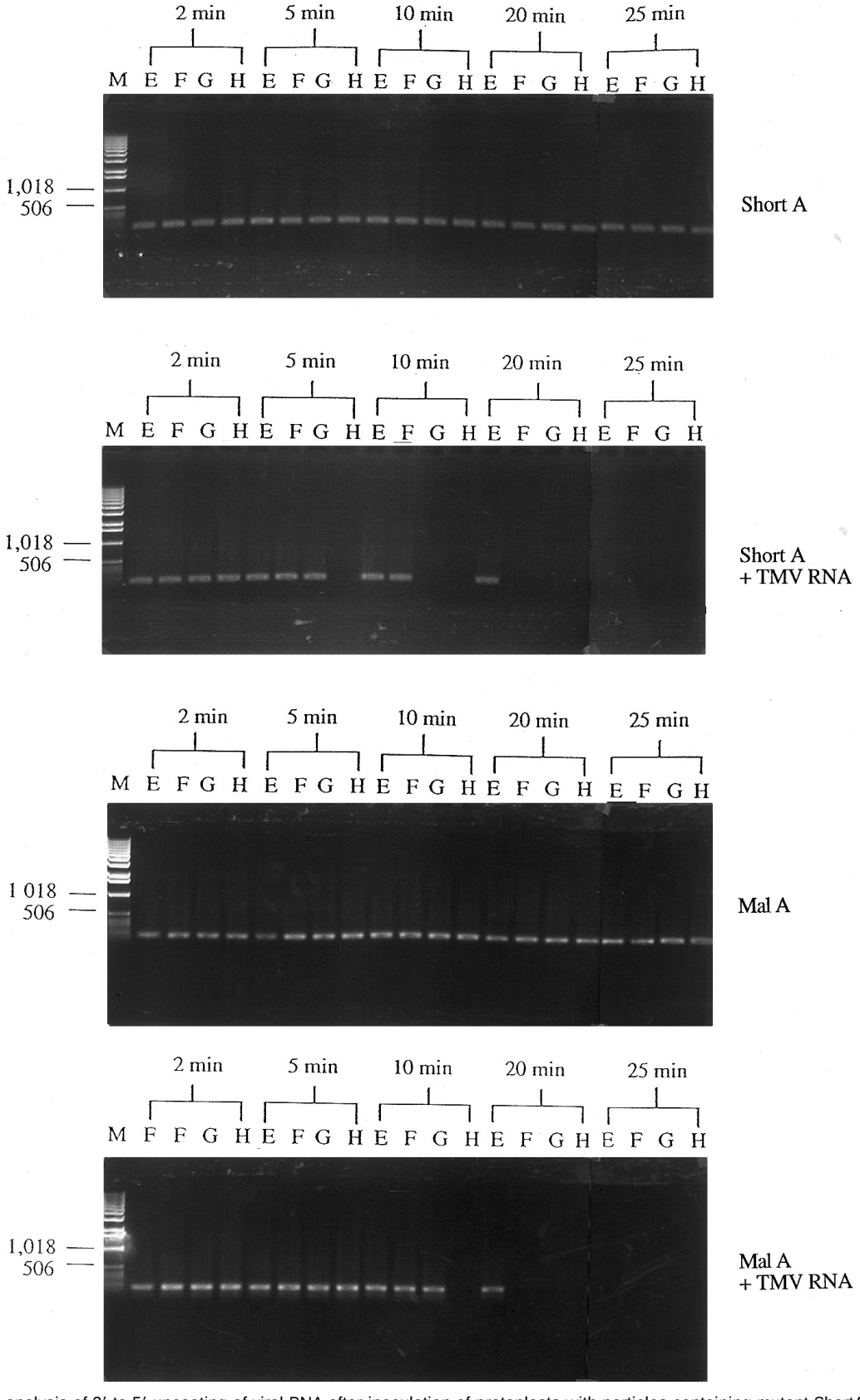

FIG. 5. RT-PCR analysis of 3′-to-5′ uncoating of viral RNA after inoculation of protoplasts with particles containing mutant ShortA or mutant MalA RNA. Protoplasts that had been inoculated with mixtures of purified TMV RNA and particles containing ShortA or MalA RNA were also analyzed. Analyses were conducted in the same manner as those of the samples shown in Fig. 4.

lected at times up to 25 min after inoculation are shown in Fig. 5; the results obtained with 30- to 60-min samples were the same as those with the 25-min samples.) When purified TMV RNA was included in inocula with ShortA RNA-containing particles, disassembly of the particles in the 3′-to-5′ direction occurred in the same manner as with particles containing wild-type transcripts (Fig. 4) or native viral RNA (Wu and Shaw, 1996). Mixture of viral RNA and particles containing MalA RNA resulted in disassembly of the particles although the timing of the process was slightly different than that obtained with particles bearing wild-type RNA.

Inoculation of protoplasts with particles containing mutant MT or mutant HelA RNA produced the same results as those obtained with mutants ShortA and MalA (Fig. 6). No 3′-to-5′ uncoating occurred when protoplasts were inoculated with the particles alone but, when viral RNA was included in either inoculum, the pattern of disassembly was the same as that obtained with particles containing wild-type RNA.

DISCUSSION

The investigation reported here was undertaken to determine whether the second stage of the bidirectional disassembly of TMV particles *in vivo* might be carried out by a mechanism in which virus-encoded RNA polymerase molecules simultaneously effect the release of coat protein subunits from the 3′-end of the viral RNA and the synthesis of progeny negative-strand RNA molecules. A coreplicational disassembly mechanism of this type would be of advantage to the virus since the 3′-end of the genomic RNA would be protected until it became engaged in the initiation of its replication.

As a first test of this hypothesis, an attempt was made to determine whether the start of the 3′-to-5′ disassembly process and the initiation of production of negative-strand RNA occur simultaneously in protoplasts inoculated with TMV particles. The results indicate that disassembly begins between 2 and 5 min after inoculation and that both disassembly and negative-strand RNA synthesis take place during the next 5 min and continue thereafter (Figs. 2 and 4). These observations suggest that the two processes may occur simultaneously, although closer examination of the data leaves the impression that, once started, the synthesis of negative-strand RNA proceeds more rapidly than the positive-strand template RNA is being uncoated. Such a situation should be impossible and, in fact, there is a reasonable explanation of the apparent discrepancy. It is likely that the degree of synchrony of events in the infection process declines sharply soon after virus particles have been introduced into the protoplasts. Thus, any sample of protoplasts should contain particles in varying stages of disassembly. Only the parts of the viral RNA that remain coated are detected in the RT-PCR analyses of 3′-to-5′ uncoating and, therefore, these procedures do not account for the particles that have undergone more rapid disassembly (i.e., the "faster" particles). However, the uncoated RNA molecules in the "faster" particles will be available to serve as templates for the synthesis of progeny negative-strand RNA sooner than those in the "slower" particles, and this probably accounts for the perception that transcription may precede uncoating. Thus, it seems reasonable to conclude that the timing of the two processes does not rule out the possibility that they may be coupled activities.

Experiments in which total RNA extracts were subjected to RT-PCR analysis with an RT primer specific for a region near the 5′-terminus of TMV RNA revealed the presence of full-length negative-strand viral RNA molecules in extracts of protoplasts collected 20–120 min after inoculation (data not shown). This raises the possibility that the seemingly slow rate of transcription of the 3′-end of the RNA seen in Fig. 2 is due to the requirement for concomitant removal of the remaining coat protein subunits from the RNA but that, once this has been accomplished, the part of the template RNA molecule that was previously uncoated during the 5′-to-3′ disassembly process can be transcribed much more rapidly.

A more direct test of the coreplicational disassembly hypothesis was provided by the experiments involving inoculation of protoplasts with particles containing altered genomes. With two of the mutant RNAs, ShortA and MalA, large parts of the 126- and 183-kDa protein ORFs were incapable of being translated. Most of the two ORFs had been deleted from the RNA contained in the ShortA particles. MalA particles lacked a 245-nucleotide sequence in the 126-kDa protein gene and contained newly introduced stop codons beginning 62 nucleotide residues downstream from the deleted segment. Neither of these types of particles was disassembled in the 3′-to-5′ direction when they were electroporated into protoplasts. If some physical features or biochemical materials in host cells are solely responsible for the disassembly of TMV particles, it might have been expected that coat protein subunits would be released from the 3′-ends of the ShortA or MalA particles. The results therefore suggested that the 126-kDa and/or 183-kDa proteins were required for uncoating. This possibility gained support from experiments in which the protoplasts were inoculated simultaneously with the ShortA or MalA particles and purified viral RNA. In these, disassembly of the particles in the 3′-to-5′ direction proceeded in the same manner as observed with particles containing wild-type RNA. The most obvious explanation of these observations is that the presence or participation of one or both of the polymerase proteins is required for 3′-to-5′ disassembly of TMV particles and that, when the mixed inocula were used, translation of the full-length viral RNA provided the functional protein(s) that could not be provided by translation of the mutant RNAs.

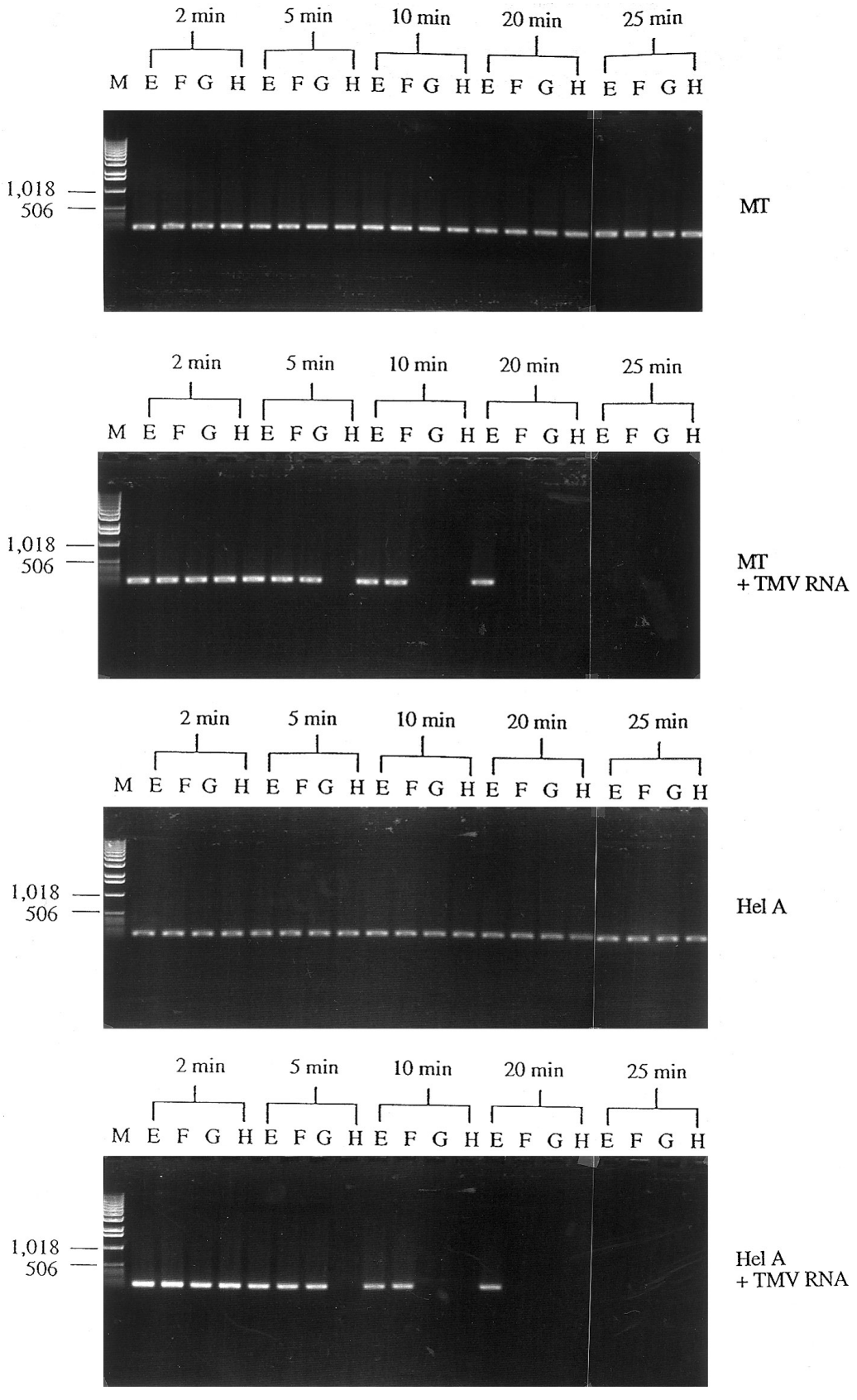

FIG. 6. RT-PCR analysis of 3′-to-5′ uncoating of viral RNA after inoculation of protoplasts with particles containing mutant MT or mutant HelA RNA. Protoplasts that had been inoculated with mixtures of purified TMV RNA and particles containing the mutant forms of RNA were also analyzed. Analyses were conducted in the same manner as those of the samples shown in Fig. 4.

From this interpretation, it would follow that the replicase proteins of TMV can operate *in trans*.

With mutants MT and HelA, much smaller deletions (six and three codons, respectively) were made and these did not result in the introduction of new stop codons in the 126- or 183-kDa protein ORFs. The mutations were in or near the conserved methyltransferase and helicase motifs in the 126-kDa protein ORF that are associated with replication of the viral RNA. As noted with the other mutants, neither the MT nor the HelA particles were able to undergo disassembly in the 3′-to-5′ direction when used alone to inoculate protoplasts but they were disassembled when the inoculum also contained purified viral RNA. These results provide additional evidence that the 126- or 183-kDa protein is involved in uncoating in the 3′-to-5′ direction. Since these mutations result in deletions in both proteins, it is not appropriate at this time to specifically assign the involvement to one or the other of the two proteins.

The purpose of the work reported here was to determine whether replication-associated proteins were involved in the completion of the disassembly process, not to attempt to identify a putative disassembly determinant. Such an endeavor will require a more extensive analysis of both the 126- and 183-kDa protein ORFs. Nevertheless, the results with the MT and HelA particles would seem to suggest that a fully functional 126- or 183-kDa protein rather than a specific sequence motif therein is required for the completion of particle disassembly. Whether the process occurs by means of a coreplicational disassembly mechanism, however, has not been determined. It is possible that the protein may only trigger the disassembly process, perhaps by dislodging the first few coat protein subunits, but that the subsequent removal of subunits occurs by some other mechanism not involving the polymerase proteins. It is also possible that the 126- or 183-kDa protein interacts with or recruits some host component that actually carries out the uncoating process. On the other hand, if a viral polymerase protein should be shown to be actively engaged in the initiation and continuation of disassembly as negative-strand RNA molecules are being synthesized, then the coreplicational disassembly hypothesis would gain favor.

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