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Functions of the 126- and 183-kDa Proteins of Tobacco Mosaic Virus

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Tobacco mosaic virus produces two proteins that contain domains similar to the methyltransferase (MT) and helicase (HEL)-like domains of the replicase-associated proteins of other RNA viruses. The more abundant 126-kDa protein contains only the MT and HEL-like domains, whereas the 183-kDa readthrough protein additionally contains the polymerase domain. We examined the functions of these proteins by constructing a bipartite system to express the 126- and 183-kDa proteins from separate RNAs. Mutants expressing the 183-kDa protein recognized promoters for negative- and positive-stranded RNA synthesis, transcribed subgenomic mRNAs, capped RNAs, synthesized proteins, moved cell to cell within the plant, and replicated defective RNAs (dRNAs). The principal function of the 126-kDa protein was to increase the rate of replication approximately tenfold. The 126-kDa protein appeared to function primarily *in cis,* and production of the 126-kDa protein *in trans* did not enhance replication of the helper virus. dRNAs producing a functional 126-kDa protein were replicated efficiently by helper viruses that produced only the 183-kDa protein but not by wild-type virus, suggesting that efficient replication required the 183-kDa protein to form a heterodimer with the 126-kDa protein already bound to the target dRNA.

INTRODUCTION

Tobacco mosaic tobamovirus (TMV) is a positive-stranded RNA virus and a member of the alphavirus-like superfamily that includes pathogens of plants and animals. Members of the superfamily have common replication strategies that include asymmetric synthesis of positive- and negative-stranded RNAs, production of subgenomic mRNAs (sgRNAs), and capping of viral RNAs (reviewed by Buck, 1996). Despite having evolved diverse genome organizations, members of the alphavirus-like superfamily produce replication-associated proteins with domains of high levels of sequence similarity (Gorbalenya *et al.,* 1989; Koonin, 1991; Rozaonv *et al.,* 1992). Capping-related guanylyltransferase and methyltransferase (MT) activities have been associated with the first domain (Mi and Stollar, 1991; Ahola and Kääriäinen, 1995; Kong *et al.,* 1999). The helicase (HEL)-like domain shares sequence similarity with a number of proteins with helicase activity (Gorbalenya *et al.,* 1989; Kadaré and Haenni, 1997), and the polymerase (POL) domain contains sequence motifs present in most RNA-dependent RNA polymerases (Koonin, 1991).

Viral replicase domains function in a range of combinations. Although expression strategies result in the coupling or separation of individual replicase domains, it is presumed that these domains are functionally equivalent. Alphaviruses produce polyproteins whose sequential cleavage regulates replication. The *Bromoviridae* have segmented genomes that divide the replicase-associated domains between two proteins, the MT and HEL-like domains in 1a and the POL domain in 2a. However, these proteins dimerize for replicase function (Kao *et al.,* 1992; O'Reilly *et al.,* 1995).

TMV places all three domains in one protein, but duplicates the MT and HEL-like domains in a second protein. The 126-kDa protein, which is terminated at the stop codon of open reading frame (ORF) 1, is the major translation product containing the MT and HEL-like domains. Approximately 10% of the time, the stop codon is suppressed, producing the overlapping 183-kDa protein, which contains all three domains. The 126-kDa protein has been shown to have guanylyltransferase (Dunigan and Zaitlin, 1990; Merits *et al.,* 1999) and MT activities *in vitro* (Merits *et al.,* 1999). Both proteins are present in partially purified replicase complexes (Young *et al.,* 1987; Osman and Buck, 1996) and can be isolated from infected cells as a heterodimer (Watanabe *et al.,* 1999). It is not understood why the MT and HEL-like domains are duplicated or why one protein is produced in such large excess. The specific functions of each protein in replication are not known.

It was previously shown that a tomato mosaic tobamovirus (ToMV) mutant could replicate poorly in protoplasts without the 130-kDa protein, but this virus was unstable in plants, quickly reverting to wild type (Ishikawa *et al.,* 1986, 1991). Indirectly, these results suggested a role for

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the 130-kDa protein because of its absence. However, defining separate functions for the tobamovirus 126- to 130-kDa and 180- to 183-kDa proteins have been difficult, primarily because they are produced from the same ORF. Mutations in either of the MT or HEL-like domains are expressed in both proteins.

We developed a bipartite system to independently manipulate and examine each protein. We found that the 183-kDa protein performed all of the known functions associated with TMV RNA synthesis, including recognition of promoters for synthesis of negative- and positive-stranded genomic RNAs and sgRNAs, polymerization, capping, protein synthesis, and cell-to-cell movement. Replication in the presence of the 126-kDa protein occurred approximately 10 times faster. The 183-kDa protein could function *in trans*, whereas the 126-kDa protein appeared to function preferentially *in cis*, enhancing replication of RNAs that could serve as its mRNA.

RESULTS

Replication of TMV in the absence of the 126-kDa protein

Previously it was shown that ToMV mutants producing only the 180-kDa protein could replicate poorly (Ishikawa *et al.,* 1991). However, the single nucleotide (nt) substitution was unstable and the Tyr codon rapidly mutated to a stop codon and revertants expressed both 130- and 180-kDa proteins (Ishikawa *et al.,* 1986). To examine the functions of the TMV 183-kDa protein in the absence of the 126-kDa protein, we needed to replace the amber

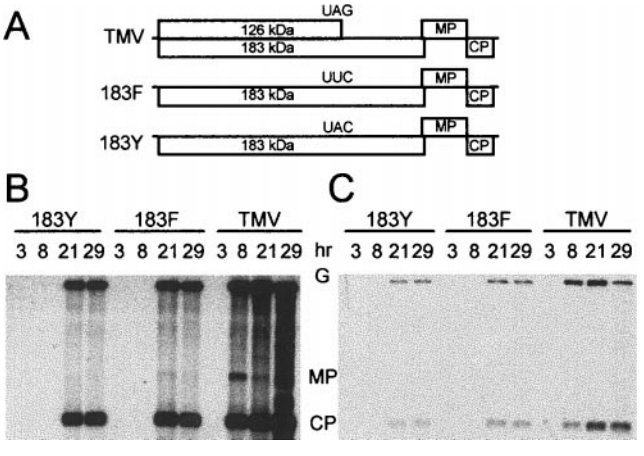

FIG. 1. Replication of TMV mutants deficient in 126-kDa protein expression. (A) Genome organization of TMV, 183F, and 183Y with stop codon replacements indicated. Time-course of positive- (B) and negative-stranded (C) RNA accumulation in tobacco protoplasts transfected with TMV, 183F, or 183Y transcripts. RNA was extracted at the times shown and analyzed by Northern blot hybridization with probes complementary to (B) or corresponding to (C) the 3′ untranslated region (Lewandowski and Dawson, 1998). Each lane contains RNA from ~2 × 10^3 protoplasts. Positions of genomic (G) RNA, movement protein (MP), and coat protein (CP) sgRNAs are indicated. Boxes = ORFs.

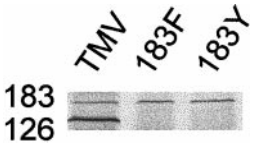

FIG. 2. Expression of replicase proteins in protoplasts. Western immunoblot of replicase proteins produced by TMV, 183F, and 183Y. Each lane contains total soluble protein from ~1 × 10^5 protoplasts. Blot was probed with polyclonal antisera against a common region of the 126- and 183-kDa proteins (Lehto *et al.,* 1990).

stop codon of the 126-kDa protein ORF with a codon for an amino acid. As it is unknown which amino acid is inserted as the amber stop codon is suppressed, we tested three different amino acids. 183Y contains a G to C substitution that changed the amber stop codon to a Tyr codon, the same amino acid used by Ishikawa *et al.* (1986). 183S has an AGC replacement of the amber stop codon that encodes Ser, also a polar residue, but with a smaller side group than that of Tyr. The amber stop codon in 183F was changed to UUC, encoding Phe, which like Tyr has a bulky side group.

Compared with wild-type TMV, 183Y and 183F accumulated reduced levels of positive- and negative-stranded RNAs at all time points (Fig. 1). SgRNAs for the movement protein and CP were detectable in protoplasts infected with 183F and 183Y, but at reduced levels compared with that of TMV (Fig. 1). 183S replicated much less than the other mutants, suggesting that insertion of Ser disrupted the function of the 183-kDa protein (data not shown), thus 183S was not examined further.

Synthesis of viral proteins was delayed and reduced without the 126-kDa protein. Neither 183Y nor 183F produced detectable levels of the 126-kDa protein (Fig. 2). However, 183Y and 183F produced levels of the 183-kDa protein similar to that of TMV, as determined by Western immunoblot analysis (Fig. 2). This is in contrast to what might be expected if the 183-kDa protein was produced without a leaky stop codon to a level approaching the tenfold higher level of translation products from both ORFs. This reduced protein synthesis probably was the result, at least in part, of the reduced rate of replication of the mutants.

Genomic RNA and CP sgRNA of the 183-kDa protein mutants were capped

Reduced replication of the 126-kDa protein-deficient mutants could have been the result of reduced translational efficiency. Wild-type TMV genomic RNA and CP sgRNA contain a 5′ 7-methyl guanosine cap (Guilley *et al.,* 1979; Zimmern, 1975). One possibility was that the 126-kDa protein is the capping enzyme and in its absence RNAs were not capped and thus were inefficiently translated. To examine whether viral RNAs were capped in the absence of the 126-kDa protein, total RNA was

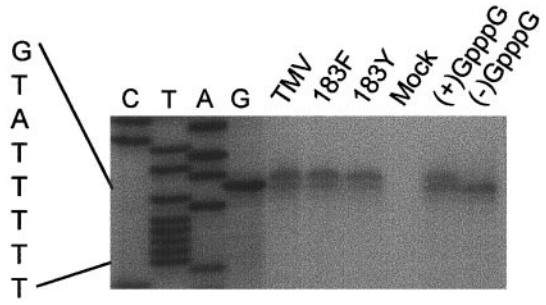

FIG. 3. Primer extension analysis of TMV, 183F, and 183Y genomic RNA. Products of primer extension using a primer complementary to nts 65–89 were resolved on a 6% sequencing gel. Total RNA was extracted from mock or TMV-, 183F-, or 183Y-infected protoplasts. Control primer extension reactions using *in vitro* transcripts of pTMV183Y synthesized in the presence (+) or absence (−) of cap analog. CTAG = DNA sequencing ladder of pTMV183Y.

extracted from protoplasts infected with 183F, 183Y, or TMV and analyzed by primer extension. *In vitro* transcripts of pTMV183Y synthesized with and without inclusion of the cap analog in the transcription reaction were used as positive and negative controls, respectively. Capped 183Y *in vitro* transcripts yielded two products (89 and 90 bases) from primer extension with a ^{32}P-end-labeled primer complementary to nts 65–89 (Fig. 3), characteristic of capped RNAs (Contreras *et al.,* 1982; Ahlquist and Janda, 1984), whereas the uncapped *in vitro* transcripts produced only the smaller DNA. Two products (89 and 90 bases) were also produced from primer extension reactions of RNAs from protoplasts infected with 183F, 183Y, or TMV, suggesting that the genomic RNAs were capped without the 126-kDa protein (Fig. 3).

To determine whether the 183-kDa protein could also cap the CP sgRNA, primer extension reactions were run with a ^{32}P-end-labeled primer complementary to nts 5746–5770. Two products (68 and 69 bases) were produced from the primer extension of RNA extracted from protoplasts infected with 183F, 183Y, or TMV (data not shown), suggesting that the CP sgRNAs were capped by the 183-kDa protein.

Cell-to-cell movement of TMV in plants in the absence of the 126-kDa protein

183Y and 183F replicated, transcribed the normal set of sgRNAs, appeared to cap RNAs, and expressed the proteins typical of a TMV infection, suggesting that the 126-kDa protein must have other functions. The mutation that substituted Tyr for the amber stop codon of ToMV, preventing the production of the 130-kDa protein, quickly reverted in plants (Ishikawa *et al.,* 1986), suggesting that the smaller protein is required in plants, perhaps for movement. The alternate Tyr codon (UAC) to that used for ToMV (Ishikawa *et al.,* 1986) was used to construct 183Y. However, 183Y was similarly unstable in plants, forming a range of sizes of local lesions in *N. tabacum* cv. Xanthi nc leaves (Fig. 4). Single lesion transfers from large lesions resulted in mostly large lesions characteristic of wild-type TMV in subsequently inoculated *N. tabacum* cv. Xanthi nc leaves, and single lesion transfers from small lesions produced a mixture of small and large lesions (data not shown), demonstrating instability of the mutation.

In contrast, 183F that contained a UUC codon had a more stable phenotype in plants than 183Y. 183F induced local lesions on *N. tabacum* cv. Xanthi nc that were small and uniform in size (Fig. 4) and that appeared late, 5 to 6 days postinoculation compared with 2 to 3 days for wild-type TMV. Single local lesion transfers of 183F resulted in a uniform population of small local lesions (data not shown), showing the stability of this mutant deficient in 126-kDa protein production. Importantly, these data show that TMV can move cell to cell without the 126-kDa protein, although at a reduced rate. The reduced movement of 183F in tobacco leaves compared to that of wild-type TMV likely was the result of reduced replication and delayed movement protein production observed in protoplasts.

The 183-kDa protein can function *in trans*

Despite having evolved monopartite genomes, tobamoviruses can replicate helper-dependent RNAs *in trans* including a satellite virus (Valverde and Dodds, 1987) and artificially created dRNAs (Ogawa *et al.,* 1991, 1992; Raffo and Dawson, 1991; Lewandowski and Dawson, 1998). However, these studies used helper viruses that expressed both overlapping replicase proteins. As 183F and 183Y replicated similarly in protoplasts, data from some experiments are presented for only one helper virus.

To examine whether the more abundant 126-kDa protein was required for replication of dRNAs, protoplasts were coinoculated with 183F plus ΔCla (Fig. 5A), a dRNA previously shown to be replicated efficiently by wild-type TMV (Lewandowski and Dawson, 1998). 183F efficiently replicated ΔCla (Fig. 5, lane 2), demonstrating that the 183-kDa protein formed replicase complexes that can amplify dRNAs *in trans.* Western immunoblot analyses

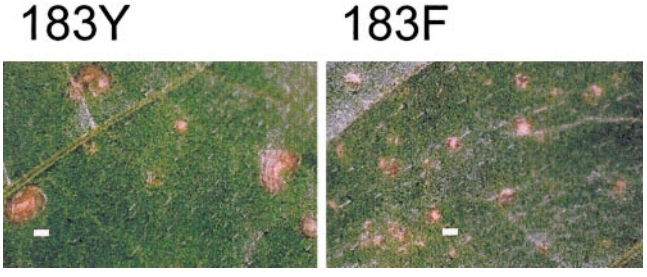

FIG. 4. Local lesion phenotypes on *Nicotiana tabacum* cv. Xanthi nc plants inoculated with the lysate of protoplasts infected with 183Y or 183F. Photographs were taken 7 days postinoculation. Bar = 1 mm.

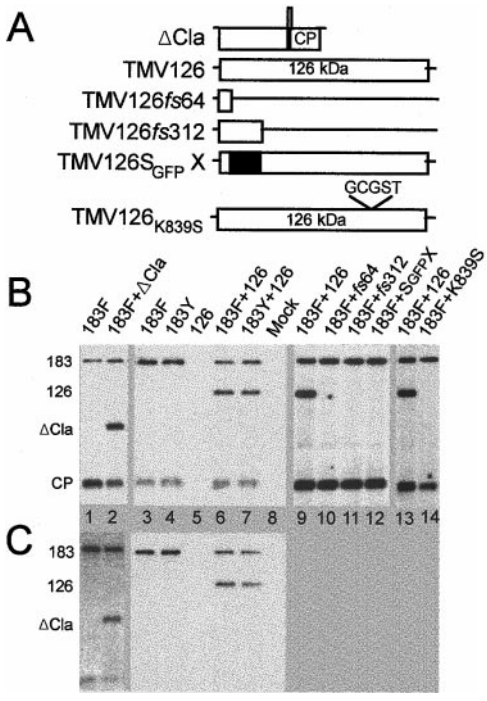

FIG. 5. Replication of dRNAs *in trans* by 126-kDa protein-deficient mutants. (A) Schematic diagram of dRNAs. Positive- (B) and negative-stranded (C) RNA accumulation in protoplasts transfected with 183F, 183Y, or dRNA transcripts or cotransfected with helper virus plus dRNA transcripts. Representatiive data are from at least four experiments; gaps signify separate experiments. RNA extracted at 20 h postinoculation was analyzed as described in Fig. 1. Helper virus (183), TMV126 (126), and ΔCla genomic-length RNAs and coat protein (CP) sgRNA are indicated. Open boxes = ORFs; black box = GFP ORF.

showed that no detectable 126-kDa protein was produced in these protoplasts (data not shown). Other dRNAs supported by TMV (Lewandowski and Dawson, 1998) were also replicated by 183F or 183Y (data not shown).

Development of a bipartite TMV genome to express the 126- and 183-kDa proteins separately

Previously we showed that if specific regions of the TMV genomic RNA were deleted, the resulting RNA could be replicated *in trans* (Lewandowski and Dawson, 1998). The minimal deletion required to produce *trans*-replicating dRNAs was the region encoding the POL domain. Precise deletion of this region allowed production of a dRNA containing the complete 126-kDa protein ORF. TMV126 lacks sequences encoding the POL domain, and the movement protein and CP ORFs (Fig. 5A). TMV126 did not replicate in the absence of a replicating helper virus, indicating that the 126-kDa protein is not the replicase (Fig. 5, lane 5). However, coinoculation of TMV126 plus either 183Y or 183F enabled us to examine uncoupled expression of the 126- and 183-kDa protein genes. Both 183F and 183Y supported the replication of TMV126 *in trans* and helper virus and TMV126 RNAs accumulated to similar levels (Fig. 5, lanes 6 and 7).

Production of the 126-kDa protein *in trans* did not augment the replication of the helper virus. 183F and 183Y replicated to similar levels in the presence or absence of TMV126 (Fig. 5). To compare the proteins produced in singly and doubly infected protoplasts, total soluble protein was analyzed by Western immunoblotting. Protoplasts inoculated with TMV126 did not accumulate detectable levels of 126-kDa protein (Fig. 6A). Protoplasts coinoculated with TMV126 plus either 183F or 183Y accumulated both 126- and 183-kDa proteins (Fig. 6A). However, the levels of 183-kDa protein were similar in the presence or absence of TMV126 (Fig. 6A). The levels of 126-kDa protein were similar to the level of 183-kDa protein, not tenfold higher as occurred in wild-type infections. Production of 126-kDa protein *in trans* also did not appreciably affect the levels of CP (Fig. 6B).

The 126-kDa protein is needed *in cis* for efficient replication of TMV126

To examine whether production of the 126-kDa protein was required for replication of the TMV126 RNA, the 126-kDa ORF was mutated. Frameshift mutations truncated the 126-kDa protein after 64 or 312 residues (Fig. 5A). Neither frameshift derivative produced detectable 126-kDa protein (Fig. 6C) nor was supported by 183F (Fig. 5, lanes 10 and 11) or 183Y (data not shown), suggesting that the 126-kDa protein might be required for replication of TMV126. To examine whether the dRNAs containing a truncated 126-kDa protein ORF could be rescued *in trans* by a functional 126-kDa protein, each TMV126 frameshift derivative was coinoculated with wild-type TMV. TMV126

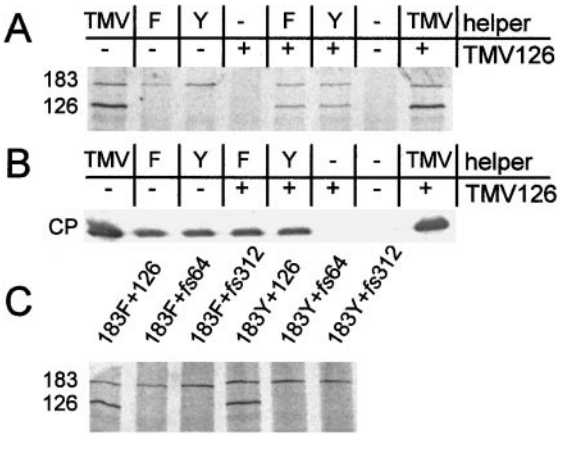

FIG. 6. Expression of TMV proteins in protoplasts coinoculated with bipartite TMV. Western immunoblots of 126- and 183-kDa proteins (A) and coat protein (B) from protoplasts transfected with *in vitro* transcripts of wild-type TMV, 183F, or 183Y without (−), or with (+) TMV126. (C) Western immunoblot of replicase proteins produced by TMV126 frameshift derivatives cotransfected with 183F or 183Y *in vitro* transcripts. Each lane contains total soluble protein from ~1 × 10^5 protoplasts isolated at 20 h postinoculation. (A) and (C) were probed as described in Fig. 2. (B) was probed with a monoclonal antibody against the CP (Schillberg *et al.,* 1999).

frameshift derivatives were not replicated by wild-type helper (data not shown), indicating that the defect was not simply the lack of 126-kDa protein, but that expression of a functional 126-kDa protein might be required *in cis*. Alternatively, it could suggest that translation or movement of ribosomes through the entire ORF was required for replication of the dRNA, perhaps to stabilize the RNA or to expose a binding site.

To test whether translation to the C-terminus of the 126-kDa protein could overcome the replication-negative phenotype, the ORF for the jellyfish green fluorescent protein (GFP) was substituted in frame within the 126-kDa ORF between the *Sma*I (256) and *Xba*I (999) sites. Protoplasts were coinoculated with TMV126ΔS_{GFP}X plus either 183F or wild-type TMV. TMV126ΔS_{GFP}X was not replicated *in trans* by either helper virus (Fig. 5B, lane 12, and data not shown), suggesting that ribosomal movement alone through the ORF was insufficient to enable replication of this dRNA. The fact that wild-type 126-kDa protein did not rescue TMV126ΔS_{GFP}X further indicates that a functional 126-kDa protein was required *in cis*.

In an attempt to disrupt a critical function of the 126-kDa protein with a less severe modification, a single amino acid change was made and tested for its effects on replication of TMV126. We mutated a conserved NTP-binding motif (Gorbalenya *et al.,* 1989) in the HEL-like domain of the wild-type 126-kDa protein. The conserved GxGKT NTP-binding motif is at residues 836–840 within the 126- and 183-kDa proteins. Mutagenesis of the conserved Lys residue within this NTP-binding motif has been lethal to or greatly impairs replication of several positive-stranded RNA viruses (Weiland and Dreher, 1993; Rikkonen, 1996; Davenport and Baulcombe, 1997). If the K839S substitution (Fig. 5A) disrupted a function of the 126-kDa protein essential for replication, replication of $TMV126_{K839S}$ might be altered. However, if function of the 126-kDa protein is *cis*-preferential, effects on the autonomously replicating 183F would be minor. $TMV126_{K839S}$ was not replicated by 183F and replication of the helper virus was unaffected (Fig. 5B; compare lanes 13 and 14). This single amino acid substitution apparently destroyed the function of the 126-kDa protein, further suggesting that functional 126-kDa protein is required *in cis* for replication of this dRNA.

TMV mutants lacking the ability to produce the 126-kDa protein replicated dRNAs. However, some striking differences between 126-kDa protein-deficient and wild-type helper viruses were observed. Figure 7 compares the relative levels of replication of ΔCla and TMV126. Although TMV replicated more efficiently, both helper viruses synthesized levels of ΔCla positive- and negative-stranded RNA nearly equivalent to the level of the helper virus (Fig. 7). However, 183F replicated TMV126 to levels exceeding helper virus positive- and negative-stranded RNA levels (Fig. 7). In sharp contrast, TMV126 positive- and negative-stranded RNA levels were approximately 1/10th and 1/20th, respectively, of the corresponding levels of the wild-type helper virus RNAs (Fig. 7). Moreover, although 183F replicated much less than TMV, 183F synthesized approximately fivefold more TMV126 than did TMV.

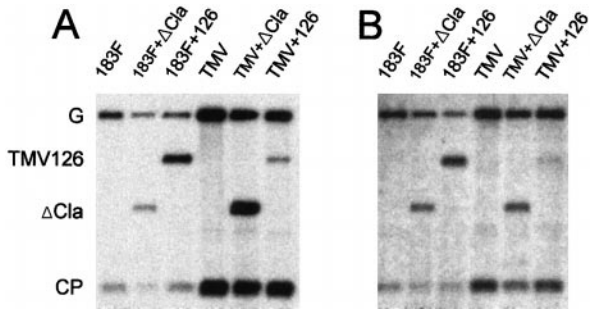

FIG. 7. Differential replication of dRNAs by wild-type and mutant helper viruses. Protoplasts were transfected with 183F or wild-type TMV transcripts alone or in combination with ΔCla or TMV126 transcripts. Positive-(A) and negative-stranded (B) RNA extracted at 20 h was analyzed as described in Fig. 1. Helper virus (G), TMV126, and ΔCla genomic-length RNAs and coat protein (CP) sgRNA are indicated.

DISCUSSION

Substantial evidence suggests that the viral components of tobamovirus replicase complexes consist of both the highly produced smaller protein containing the MT and HEL-like domains and the larger readthrough protein additionally containing the POL domain. It has been shown that both tobamovirus replicase-associated proteins are present in partially purified replicase preparations (Young *et al.,* 1987; Osman and Buck, 1996). Also, the TMV 126- and 183-kDa proteins coimmunoprecipitate with antisera specific to the POL domain at a 1:1 ratio (Watanabe *et al.,* 1999). However, the need for duplication of the MT and HEL-like domains in the replicase complex is not known.

Formation of replicase heterodimers may be a common theme among viruses. Determination of the structure of human immunodeficiency virus reverse transcriptase revealed the formation of an asymmetric heterodimer between the truncated p51 protein and its readthrough product p66 (Wang *et al.,* 1994). Furthermore, p66 assumes an open structure, while p51 remains closed in the heterodimeric state. Perhaps the TMV MT and HEL-like domains also assume different conformations in the 126- and 183-kDa proteins of the replicase complex. Although the brome mosaic virus replicase domains are expressed as two proteins, the domains appear to unite and overlap. Brome mosaic virus 1a and 2a proteins must join to be functional (O'Reilly *et al.,* 1995). Additionally, when the POL domain from 2a is covalently fused to the C-terminus of 1a to create a protein with an organization analogous to the TMV 183-kDa protein, free 1a is still required for replication (Smirnyagina *et al.,* 1996).

Dissecting functions of overlapping proteins involved in virus replication and gene expression is complicated by the fact that mutagenesis of one protein also introduces the same mutation(s) into the overlapping region of the second protein. Studying functions of each protein requires the development of systems to uncouple the production of the two proteins. Replicase ORFs of some positive-stranded RNA viruses coexpressed by a readthrough mechanism have been artificially divided between two RNAs (White *et al.,* 1995; Molinari *et al.,* 1998; Oster *et al.,* 1998). Similarly, we have uncoupled the production of the TMV 126- and 183-kDa proteins by genetically dividing these ORFs between two coreplicating RNAs. However, unlike TMV, when the carmo- (White *et al.,* 1995) and tombusvirus (Molinari *et al.,* 1998; Oster *et al.,* 1998) replicase ORFs are divided between two RNAs, both proteins are still required for replication.

Functions associated with the 183-kDa protein

In what would appear to conflict with the normal formation of heterodimers of the 126- and 183-kDa proteins, the tobamovirus readthrough protein alone can provide the same functions of replication (Ishikawa *et al.,* 1991; this study). Without the smaller protein, the TMV 183-kDa protein recognized the 3′ promoter and synthesized negative-stranded RNA, recognized the 5′ *cis*-acting elements and amplified positive-stranded RNA, recognized sgRNA promoters, and capped genomic RNA and CP sgRNA. The ratio of mutant positive- to negative-stranded RNAs was similar to that of the wild-type virus.

Interestingly, neither the ToMV (Ishikawa *et al.,* 1991) nor TMV mutants deficient in production of the 126- to 130-kDa protein produced tenfold more readthrough protein that might be expected from direct translation of the RNA without a stop codon, compared with levels resulting from infrequent suppression of the stop codon. However, determining whether the reduced protein level was a cause or effect of reduced replication was impossible.

Additionally, mutants deficient in production of 126-kDa protein could move cell to cell within plants. Previous studies showed that mutations that mapped to the common region of the 126- and 183-kDa proteins affected movement, but determining which of the mutant tobamovirus replicase-associated proteins affected movement was not possible (Nelson *et al.,* 1993; Derrick *et al.,* 1997). The formation of small local lesions in *N. tabacum* cv. Xanthi nc by 183F showed that the 126-kDa protein is not required for cell-to-cell movement, but it greatly affects the rate of movement, probably because of its effects on the rate of replication. However, it is possible that the 126- to 130-kDa protein normally functions in movement, but that the 180- to 183-kDa protein can perform these functions without the 126-kDa protein, as previously suggested (Ishikawa *et al.,* 1986).

Functions of the 126-kDa protein

The most obvious function of the 126-kDa protein is to enhance the rate of replication. Apparently the 126-kDa protein interacts with the 183-kDa protein to make replication proceed faster. No information is available concerning a specific function of the 126-kDa protein in this enhancement process (initiation, rate of polymerization, or other). However, the net contribution of the 126-kDa protein is that most measurable characteristics, from negative-stranded RNA synthesis to production of sgRNAs and proteins, and even cell-to-cell movement went approximately ten times faster.

Although the TMV 126-kDa protein is not required for function of the readthrough protein, it appeared to be required for replication of its template RNA. Frameshift mutations in the 126-kDa protein ORF destroyed the ability of the dRNA to be replicated. However, the ability of dRNAs to be replicated is often dependent on ribosomal travel through large parts of the RNA (reviewed by Graves *et al.,* 1996, and White and Morris, 1999). Yet, some dRNAs with in-frame deletions (Lewandowski and Dawson, 1998) or the in-frame insertion of the GFP ORF were not replicated. A more compelling argument for the need for a functional 126-kDa protein was the demonstration that substitution of a single residue in the 126-kDa protein in an NTP-binding motif prevented amplification of the dRNA.

A remaining enigma is that TMV produces a great excess of 126-kDa protein (tenfold the level of the 183-kDa protein). Watanabe *et al.* (1999) showed that the TMV replicase complex consists of a heterodimer of 126- and 183-kDa proteins in a 1:1 ratio. What are the functions of the other 90% of the 126-kDa proteins? Does this mean that the excess 126-kDa protein has a function for something other than replication?

In cis versus *in trans* functions of the replicase proteins

The 183-kDa protein can provide some of its functions both *in cis* and *in trans.* Replicase complexes from a pool formed during TMV replication are thought to be reused *in trans* to amplify positive-stranded genomic and subgenomic RNAs from the negative-stranded templates. We previously presented evidence that replication of TMV negative-stranded RNA is *cis*-preferential, based on observations that full-length RNAs were not replicated *in trans,* suggesting that only RNAs producing functional replicase proteins were replicated (Lewandowski and Dawson, 1998). The observation that TMV mutants unable to produce the 126-kDa protein replicated dRNAs demonstrated that the 183-kDa protein could synthesize negative-stranded RNA *in trans*. Thus, the 183-kDa protein can function without the 126-kDa protein both *in cis* and *in trans*. However, the observation that 126-kDa protein-deficient mutants efficiently replicated TMV126 *in*

trans suggested that the 183-kDa protein (perhaps with 126-kDa protein from TMV126) formed the replicase complexes that synthesized TMV126 negative-stranded RNA.

In contrast to the readthrough protein, it appears that the 126-kDa protein primarily functions *in cis*, that is, the 126-kDa protein enhances the synthesis of the RNA that was its mRNA (TMV126 or wild-type TMV genomic RNA). Addition of 126-kDa protein into infected protoplasts via the TMV126 dRNA did not detectably enhance replication of the 126-kDa protein-deficient helper virus. Thus, the 126-kDa protein preferentially enhanced replication of its template RNA.

One remarkable observation was that although TMV126 was efficiently replicated by 126-kDa protein-deficient helper viruses, to levels exceeding that of the helper virus, it was barely replicated by helper viruses that produced both replicase proteins. This suggests that a replicase complex containing both proteins cannot interact and initiate replication of the TMV126 dRNA, whereas replicase complexes consisting of only the 183-kDa protein can work efficiently. A possible explanation is that the nascent 126-kDa protein binds to its mRNA, perhaps near the 3′ promoter for negative-stranded RNA synthesis, and targets that RNA for replication. The 183-kDa replicase complex might be able to interact with the 126-kDa protein already bound to the RNA and initiate replication. However, replication complexes containing 126/183-kDa protein heterodimers might initiate negative-stranded RNA synthesis inefficiently *in trans* because of strong interactions between the 126-kDa protein and the dRNA or between the helper virus-derived 126- and 183-kDa proteins. On the other hand, the small dRNA with only the MT domain (ΔCIa) was equally replicated by wild-type and 126-kDa protein-deficient replicase complexes. An explanation could be that the MT domain might target dRNAs for replication, but could be displaced and the dRNA replicated by existing replication complexes.

Based on the available data, we propose a model for TMV replication. Following translation of TMV genomic RNA, nascent 126-kDa protein binds to the 3′ region of the RNA, which would target the RNA for replication. As the 183-kDa protein is produced, it would bind to the 126-kDa protein already attached to the promoter for initiation of negative-stranded RNA synthesis. TMV sgRNAs, which do not produce the 126-kDa protein, would not be targeted for negative-stranded RNA synthesis, consistent with the general lack of subgenomic dsRNAs of TMV (Dawson and Dodds, 1982). Following completion of negative-stranded RNA synthesis, the replicase complexes would constitute a pool available for positive-stranded genomic RNA or sgRNA synthesis. In an infection by a 126-kDa protein-deficient mutant, the 183-kDa protein would bind *in cis* to its genomic RNA, but probably less effectively than the 126-kDa protein.

In the bipartite system in which the 126- and 183-kDa proteins are produced from different RNAs, the 126-kDa protein binds preferentially to its mRNA, which would target that RNA for replication. Replication of the dRNA with bound 126-kDa protein would require the 183-kDa protein produced by the helper virus RNA. If the source of 183-kDa protein was a mutant that did not produce 126-kDa protein, free 183-kDa protein would be available to form a heterodimer with the 126-kDa protein and replicate the dRNA. Thus, the 183-kDa protein would function both *in cis* and *in trans* and provide all of the basic functions of replication. However, if wild-type TMV is the source of the 183-kDa protein, 183-kDa protein would form heterodimers with wild-type 126-kDa protein, thus rendering it unavailable for interacting with dRNA-derived and bound 126-kDa protein. The 126-kDa protein would function primarily *in cis* to target RNAs for replication and greatly increase the rate of replication of those RNAs. In contrast, defective 126-kDa protein could bind, but would form inactive heterodimers that would prevent replication of the dRNA.

MATERIALS AND METHODS

Plasmid construction

Constructs were built using standard recombinant DNA techniques (Ausubel *et al.,* 1987) and are derivatives of an infectious wild-type TMV cDNA clone (Dawson *et al.,* 1986). Clones giving rise to wild-type TMV (pTMV004), 183-kDa protein mutants with Tyr (pTMV183Y), or Phe (pTMV183F) substitutions of the stop codon for the 126-kDa protein ORF, and the defective RNAs (dRNAs) pTMVΔCIa (Δ1343–5664) and pTMVΔ3420–6191, have been described (Lewandowski and Dawson, 1998). pTMVΔ3420–6191 was renamed pTMV126 to indicate that this construct produces a dRNA that produces wild-type 126-kDa protein. pTMV183S contains a Ser substitution for the amber stop codon resulting from a UAG to AGC substitution at nts 3417–3419. Viruses derived from pTMV183F, pTMV183Y, and pTMV183S are designated 183F, 183Y, and 183S, respectively.

Frameshift mutations were introduced after 64 (pTMV126*fs*64) or 312 (pTMV126*fs*312) amino acid residues by end-filling the unique *Ava*I or *Xba*I sites, respectively, of pTMV126 with T4 DNA polymerase and religating. The GFP ORF was inserted between the *Sma*I (256) and *Xba*I (999) sites of the 126-kDa protein ORF by ligating the *Xba*I/*Kpn*I fragment from pTMV126 into *Xba*I/*Kpn*I-digested pD4GFP (derivative of pTMVΔCIa containing the GFP ORF inserted between the *Sma*I and *Xba*I sites) (V. Grdzelishvili, unpublished observations) to create pTMV126ΔS_{GFP}X.

To alter the conserved GxGKT NTP-binding motif within the 126-kDa protein ORF, pTMV126 was mutated with the U. S. E. Mutagenesis Kit (Amersham) using a mutagenic primer 5′ GGAGTTCCGGGCTGTGGA**TCC**AC-

CAAAGAAATTCTTTCCAGG to create the K839S codon substitution (bold) creating pTMV126$_{K839S}$.

Protoplast inoculation and analysis of viral RNA and protein

T7 *in vitro* transcription, tobacco suspension cell protoplast preparation, and inoculation and analysis of progeny RNAs were as described (Lewandowski and Dawson, 1998), except that a subculture of the cell line was adapted to grow at 34°C and was maintained at 34°C with weekly subcultures. Infected protoplasts were incubated at 26°C.

To determine whether TMV RNAs were capped, total RNA extracted from tobacco protoplasts infected with TMV, 183F, or 183Y *in vitro* transcripts was subjected to primer extension analysis. As controls, *in vitro* transcripts of pTMV183Y were synthesized in the presence or absence of G(5′)ppp(5′G) (Pharmacia). Primers complementary to TMV nts 65–89 and nts 5746–5770 were used for primer extension analysis of genomic RNA and coat protein (CP) sgRNA, respectively. 0.3 pmol of primer ($\sim 10^5$ dpm) end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase were annealed to RNA extracted from 1–5 $\times$ 10^4 protoplasts or 1 to 2 μg of *in vitro* transcripts. Primer extension reactions using AMV reverse transcriptase (US Biochemicals) were incubated at 42°C for 30 min, denatured, and resolved on 6% sequencing gels.

Total soluble protein was separated on 10% SDS–PAGE and analyzed by Western immunoblotting using polyclonal antisera generated against a common internal fragment of the 126- and 183-kDa proteins (Lehto *et al.,* 1990) or with a monoclonal antibody (mAb29) that recognizes TMV CP (Schillberg *et al.,* 1999).

Plant inoculations

Nicotiana tabacum cv. Xanthi nc plants with three fully expanded leaves were inoculated with the lysate of infected protoplasts. Protoplast pellets were disrupted in 50 μl 25 mM sodium phosphate, pH 7.0, mixed with an equal volume of inoculation buffer (Dawson *et al.,* 1986) and ground in a mortar and pestle. Plants were maintained in a growth room with 14 h light and photographed 7 days postinoculation. Single lesions were ground in a minimal volume of inoculation buffer and inoculated to *N. tabacum* cv. Xanthi nc plants.

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