JOURNAL OF VIROLOGY, Feb. 1991, p. 861–868
0022-538X/91/020861-08$02.00/0

Vol. 65, No. 2

Specific Cessation of Minus-Strand RNA Accumulation at an Early Stage of Tobacco Mosaic Virus Infection

MASAYUKI ISHIKAWA,[1,2]* TETSUO MESHI,[1] TAKESHI OHNO,[2] AND YOSHIMI OKADA[1]†

Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Hongo, Tokyo 113,[1] Department of Agricultural Chemistry, Faculty of Agriculture, Hokkaido University, Sapporo 060,[2] Japan

Received 4 September 1990/Accepted 14 November 1990

The time course of accumulation of viral plus-strand RNAs (genomic RNA and subgenomic mRNA for the coat protein) and minus-strand RNA in tobacco protoplasts synchronously infected with tobacco mosaic virus (TMV) RNA was examined. In protoplasts infected with the wild-type TMV L RNA, the plus and minus strands accumulated differently not only in quantity but also in the outline of kinetics. The time courses of accumulation of the genomic RNA and coat protein mRNA were similar: they became detectable at 2 or 4 h postinoculation (p.i.), and their accumulation increased until 14 to 18 h p.i. The accumulation rate reached the maximum at about 4 h p.i. and then gradually decreased. In contrast, accumulation of the minus-strand RNA ceased at 6 to 8 h p.i., at which time the plus-strand accumulation was already about 100 times greater and still continued vigorously. This specific halt of minus-strand accumulation was not caused exclusively by encapsidation of the genomic RNA, because a similar halt was observed upon infection with a deletion mutant that lacks the 30K and coat protein genes. Upon infection with a mutant that could not produce the 130K protein (one of the two proteins that are thought to be involved in viral RNA replication), the accumulation levels of both plus and minus strands were lower than that of the parental wild-type virus. Given these observations, possible mechanisms of TMV replication are discussed.

The genome of tobacco mosaic virus (TMV) is composed of a single-stranded, messenger-sense 6.4-kb RNA with the cap structure at the 5′ end and a tRNA-like structure at the 3′ end (30). The TMV genome encodes at least three nonstructural proteins (130K, 180K, and 30K proteins) and the coat protein (5, 19). The 130K and 180K proteins are translated from the genomic RNA, and the latter is synthesized by suppression of the amber termination codon of the 130K protein gene, incorporating tyrosine (4). The 30K and coat proteins are translated from their respective subgenomic mRNAs generated during replication (8, 28, 30). These two proteins have been shown to be dispensable for replication (15, 29).

The 180K protein contains the Gly-Asp-Asp motif characteristic of a number of virus-encoded proteins shown or suspected to be RNA-dependent RNA polymerases (11). Furthermore, the 130K and 180K proteins have considerable amino acid sequence similarities with nonstructual proteins of several positive-strand RNA viruses, such as brome mosaic virus (BMV), alfalfa mosaic virus (AlMV), and Sindbis virus (an animal alphavirus), that are thought to be involved in replication (1, 7). This fact may imply that these viruses replicate by fundamentally similar strategies. However, the modes of expression of these nonstructural proteins are rather different: in the case of BMV and AlMV, two polypeptides are synthesized from their respective genomic RNAs; in the case of Sindbis virus, two overlapping polypeptides are first synthesized and subsequently cleaved proteolytically into four parts.

Previously, we showed an impairment in multiplication of several TMV mutants constructed in vitro that produced either of the 130K and 180K proteins or a modified 180K protein (9). No infectivity was detected in either plants or protoplasts inoculated with mutants that could not produce the 180K protein. Thus, production of the 180K protein is essential for viability (9; unpublished data). Furthermore, we constructed another mutant that carries a single base substitution at the amber termination codon of the 130K protein gene, resulting in its change to a tyrosine codon; thus, this mutant is expected to produce the authentic 180K protein but little if any 130K protein (9). In tobacco plants, this mutant retained infectivity, although its multiplication was greatly impaired compared with that of the wild type (9). From these data, we speculated that the 130K protein would be unnecessary for replication; however, for normal efficient replication, balanced synthesis of both 130K and 180K proteins would be necessary (9). In Sindbis virus, Li and Rice changed the leaky opal termination codon between nonstructural protein P3 (nsP3) and nsP4 to a serine, tryptophan, or arginine codon and revealed that RNA synthesis of these mutants was inhibited early in infection compared with that of the parental virus (12). Thus, also in the case of Sindbis virus, unbalanced production of viral nonstructural proteins (overproduction of nsP34 and decreased production of nsP3) was thought to result in poor viral RNA synthesis (12).

In TMV-infected cells, replicative form (RF) and replicative intermediate (RI) which contain minus-strand RNA are detected. Therefore, TMV RNA is thought to replicate via the minus-strand RNA complementary to the genomic RNA. However, precise analyses of plus- and minus-strand RNA syntheses have not been done yet. To understand the replication mechanism of TMV RNA and also the function of the 130K and 180K proteins in replication, we examined the time course of accumulation of viral plus- and minus-strand RNAs in protoplasts infected with the wild-type TMV as well as with two kinds of mutants: one lacking the 30K and coat protein genes and the other with the amber-to-tyrosine mutation resulting in deficiency of the 130K protein. During

* Corresponding author.
† Present address: Department of Biosciences, School of Science and Engineering, Teikyo University, Utsunomiya, Tochigi 320, Japan.

these experiments, it was found that the plus- and minus-strand RNAs accumulate differently.

MATERIALS AND METHODS

Plasmids. pLFW3 is the standard plasmid that has been used to obtain infectious TMV (L strain) RNA by in vitro transcription with *Escherichia coli* RNA polymerase (14). pLQDN, a derivative of pLFW3, lacks the 30K and coat protein genes (15). pLFR2 (9) and pLDR2, derivatives of pLFW3 and pLQDN, respectively, have a G-to-U substitution at the amber termination codon of the 130K protein gene, resulting in a change from the amber codon to a tyrosine codon (Fig. 1).

pLP1M and pLP7P were constructed by inserting the *Hin*cII (nucleotides 138 to 795 from the 5′ end of TMV L RNA [19]) and *Sau*3AI (nucleotides 809 to 1361) fragments of pLFW3 at the *Hin*cII and *Bam*HI sites of pSP64 (13), respectively. pLP2P and pLP2M were constructed by inserting the filled-in *Ava*II fragment (nucleotides 5557 to 6160) of pLFW3 at the *Hin*cII site of pSP64. Transcripts produced from pLP2P and pLP7P by SP6 polymerase had the sequences with plus polarity, while those from pLP2M and pLP1M had the sequences with minus polarity.

Protoplast inoculation. In vitro transcripts were prepared as described previously (15). Hereafter, infectious in vitro transcripts and their replicates are referred to by the names of their originating plasmids without the prefix "p"; i.e., LFW3 is equivalent to the wild-type TMV L. All of the wild-type TMV L used in this work was purified from *Nicotiana tabacum* L. cv. Samsun leaves inoculated with LFW3 transcript and harvested at 7 days postinoculation (p.i.). Protoplast inoculation was carried out essentially as described previously (32). Briefly, protoplasts prepared from suspension-cultured cells (BY-2) derived from *N. tabacum* L. cv. Bright Yellow-2 were washed several times with 0.4 M mannitol and suspended in 0.3 M mannitol–70 mM KCl–5 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) (pH 5.8) at a concentration of 10^6 protoplasts per 600 μl. Then 600 μl of the protoplast suspension was mixed with in vitro transcripts (derived from 5 μg of the template DNA) or with the wild-type TMV L RNA (2 μg unless otherwise specified) in 200 μl of the same buffer and electroporated. After electroporation, the protoplasts were incubated at 0°C for 30 min and at 30°C for 5 min, collected by centrifugation, and suspended in protoplast medium (34) at a concentration of 10^5 protoplasts per ml. Aliquots of 1 ml were separately cultured at 28°C in the presence of 25 μg of dactinomycin (actinomycin D [AMD]) per ml in the dark. AMD did not significantly influence the outline of the kinetics of either plus- or minus-strand accumulation. However, in the absence of AMD the accumulation rate of the genomic RNA and coat protein mRNA (CP mRNA) declined from an earlier stage, and AMD raised the accumulation levels of all TMV-related RNAs at 24 h p.i. In addition, the molar ratio of CP mRNA to genomic RNA was about twofold higher in the absence of AMD than in its presence. Time zero is defined as the time when the electroporated protoplasts were transferred to 30°C.

Quantitation of TMV-related RNAs in protoplasts. Extraction of total nucleic acids from protoplasts and subsequent removal of DNA with DNase I were performed as described previously (10, 31). TMV-related RNAs were quantitated by the RNase protection method (13). Riboprobes were prepared with linearlized pLP plasmids as templates for SP6 RNA polymerase as described by Melton et al. (13), using [α-^{32}P]UTP. For quantitation of plus-strand RNAs, DNase I-treated RNAs derived from 1×10^3 to 2×10^4 protoplasts (RNAs from 2×10^4 protoplasts were used when the accumulation of plus strands was very small) were annealed with 0.2 pmol of ^{32}P-labeled riboprobe (referred to as the P1M and P2M probes, derived from pLP1M and pLP2M, respectively) in 20 μl of 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES)–0.4 M NaCl–1 mM EDTA–80% formamide (pH 6.4) by incubation at 100°C for 5 min and then at 45°C for 20 min. The single-stranded RNAs in the mixture were removed by digestion with 20 μg of RNase A (type 1-A; Sigma) in 200 μl of buffer A (0.2 M NaCl, 0.1 M LiCl, 10 mM Tris hydrochloride, 1 mM EDTA [pH 7.5]) (17) at 30°C for 30 min. RNase digestion was terminated by addition of 30 μg of proteinase K (Boehringer Mannheim) and sodium dodecyl sulfate (SDS) (final concentration, 0.4%) and incubation at 37°C for 10 min. The protected double-stranded RNAs (dsRNAs) were recovered by phenol extraction and ethanol precipitation, separated on a 3% polyacrylamide gel containing 8 M urea, and processed for autoradiography. Autoradiograms with appropriate exposures were scanned with a Shimadzu dual-wavelength scanner (model CS930) to quantitate the RNase-resistant dsRNA species.

For quantitation of minus-strand RNA, the following alterations were made. DNase I-treated RNAs from 2×10^4 protoplasts were first annealed with an excess amount (0.3 μg) of TMV L RNA in 30 μl of 0.3 M KCl–50 mM Tris hydrochloride–1 mM EDTA (pH 8.0) by sequential incubation at 80°C for 5 min, at 60°C for 10 min, and at 37°C for 10 min and treated with RNase A (final concentration, 1 μg/ml) at 30°C for 30 min. The undigested dsRNAs were recovered by treatment with proteinase K, extraction with phenol, and precipitation with ethanol. The dsRNAs derived from 2×10^4 protoplasts were then mixed with 0.07 pmol of the ^{32}P-labeled riboprobe (referred to as the P7P and P2P probes, derived from pLP7P and pLP2P, respectively) in 20 μl of buffer A. The mixture was heated at 100°C for 5 min to denature the dsRNAs and incubated at 45°C for 1 h to anneal the minus-strand RNA with the probe. Subsequent treatments were identical to those for plus-strand quantitation.

Relative molar amounts of TMV-related RNAs were calculated from band intensities, considering the specific activities of the probes and the numbers of U residues within the protected regions of the probes.

RESULTS

Detection and quantitation of TMV plus- and minus-strand RNAs. The RNase protection method (13) was used to detect and quantitate the plus- and minus-strand RNAs of TMV. Portions on the genomic RNA or its complement to which probes hybridize are shown in Fig. 1. Hybridization for quantitation of plus-strand RNAs (see Materials and Methods) was carried out under conditions such that the standard annealing mixture contained a sufficient amount of the probe and all of the plus-strand RNA molecules contained in the mixture could hybridize to the probe. These conditions were confirmed by the observation that when nucleic acids from 2×10^3 protoplasts inoculated with the wild-type TMV L RNA (7 h p.i.) were analyzed, the signal strengths of protected bands were unchanged even when the concentration of the probe was decreased to one-fifth. In addition, the signal strength of the protected band representing the genomic RNA was proportional to the amount of TMV L RNA in the standard annealing mixture at least within the range

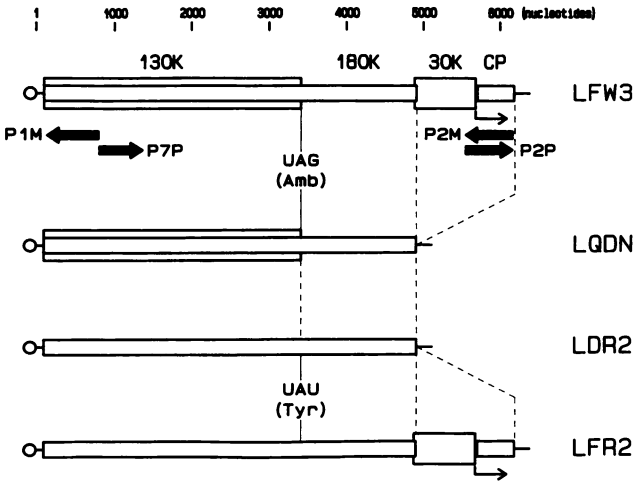


FIG. 1. Schematic representation of the genomic structures of the wild-type TMV L (LFW3) and its derivatives. Coding regions for the 130K, 180K, and 30K proteins and the coat protein (CP) are shown by open boxes. Open circles at the left indicate cap structures. Bold arrows below LFW3 denote the regions and directions that riboprobes carry: the P1M and P2M probes have the sequences with minus polarity and were used to detect plus-strand RNAs; the P2P and P7P probes have those with plus polarity and were used to detect minus-strand RNA. Bent arrows indicate the starting position of the CP mRNA.

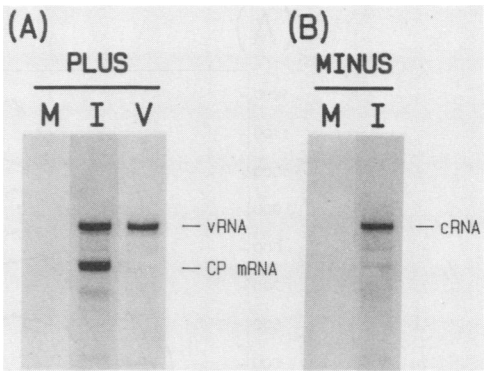


FIG. 2. Detection of TMV-related RNAs by the RNase protection method. Tobacco protoplasts (10^6) were mock inoculated (lane M) or inoculated with 40 ng of the wild-type TMV L RNA (lane I). Total nucleic acids were extracted at 14 h p.i. and treated to detect the plus- and minus-strand RNAs as described in Materials and Methods. The P2M and P2P probes were used to detect plus-strand (A) and minus-strand (B) RNAs, respectively. As a control, TMV L RNA (8 ng) was treated in the same manner (lane V; the band for the CP mRNA cannot be seen). Protected RNAs were separated by 8 M urea–3% polyacrylamide gel electrophoresis. Positions of the protected bands representing vRNA (0.60 kbp), CP mRNA (0.47 kbp), and cRNA (0.60 kbp) are indicated at the right.

from 2.5 to 100 ng. Preliminary experiments showed that the amount of the genomic RNA was 50 ng at most in 2×10^3 infected protoplasts. Thus, this range covers from the maximal amount to 1/20 of the genomic RNA in the standard annealing mixture. Under our conditions, 0.1 ng of TMV L RNA was readily detectable, although the signal strength of the protected band was underestimated below 2.5 ng.

It was a problem that a large amount of plus-strand RNAs over minus-strand RNA competitively inhibited the annealing between minus-strand RNA and the probe. To overcome this inhibition, minus-strand RNA was converted to a double-stranded form by annealing with an excess amount of TMV L RNA; subsequently, the single-stranded RNAs that remained were removed by RNase A digestion in a high-salt condition. The minus-strand RNA, thus protected as a double-stranded form, was then quantitated as was plus-strand detection except for a few modifications described below. Probes prepared for minus-strand detection contained a small amount of transcripts with plus polarity, which caused background signals. It was necessary to decrease background signals to quantitate a small amount of minus-strand RNA. Thus, the concentration of the probe was decreased to one-third that for plus-strand detection, and the hybridization period was three times longer. When total RNAs from 2×10^3 protoplasts inoculated with the wild-type TMV L RNA (10 h p.i.) were mixed with those from 1.8×10^4 mock-inoculated protoplasts, the signal strength for the minus strand became 1/10 that from the total RNAs from 2×10^4 inoculated protoplasts.

When the P2M probe was used, two major bands were detected (Fig. 2A). The higher-molecular-weight band represents the accumulation level of the plus-strand RNAs containing the sequence of nucleotides 5557 to 6160. Hereafter, the RNA molecules represented by this band, and which must include mainly the genomic RNA, will be called vRNA. However, vRNA probed with P2M included the subgenomic mRNA for the 30K protein (the amount of this subgenomic mRNA was quite small compared with that of the genomic RNA) and other minor TMV-related RNAs with plus polarity. The other, lower-molecular-weight band represents the subgenomic mRNA for the coat protein (called CP mRNA), the 5′ end of which is at nucleotide 5694 (6) (Fig. 1).

The P2P probe detected only one band with a strong intensity, which corresponded to the minus strand of the genomic RNA (hereafter called cRNA). The band corresponding to the minus strand of the CP mRNA was quite weak or almost undetectable (Fig. 2B; also see Discussion). In some experiments (e.g., Fig. 4), the P1M and P7P probes were used to quantitate the genomic RNA and its minus strand, respectively. In these cases also, the terms vRNA and cRNA will be used. vRNA probed with P1M did not include the 30K protein mRNA. Almost identical time courses of accumulation of vRNA were obtained with the P1M and P2M probes (see below).

Mode of accumulation of virus-related RNAs in protoplasts infected with the wild-type TMV RNA. The time course of accumulation of vRNA, CP mRNA, and cRNA in tobacco protoplasts inoculated with the wild-type TMV L RNA was first examined. We determined the time course of accumulation of TMV-related RNAs per total inoculated protoplasts (Fig. 3A and B) or per infected protoplasts calculated on the basis of the percentages of infected protoplasts (Fig. 3C and D). The amount of TMV L RNA contained in the inoculum did not significantly influence the outline of the time course of accumulation (Fig. 3C and D), although it affected the percentages of infected protoplasts and consequently the final level of accumulation (Fig. 3A and B). The plus strands (vRNA and CP mRNA) became detectable at 2 to 4 h p.i., and their amounts increased until 14 to 18 h p.i. (Fig. 3A and C). The accumulation rate reached the maximum at about 4 h p.i. and then gradually decreased. After 14 or 18 h p.i., the accumulation level decreased, indicating that degradation somewhat exceeded de novo synthesis. This decrease also

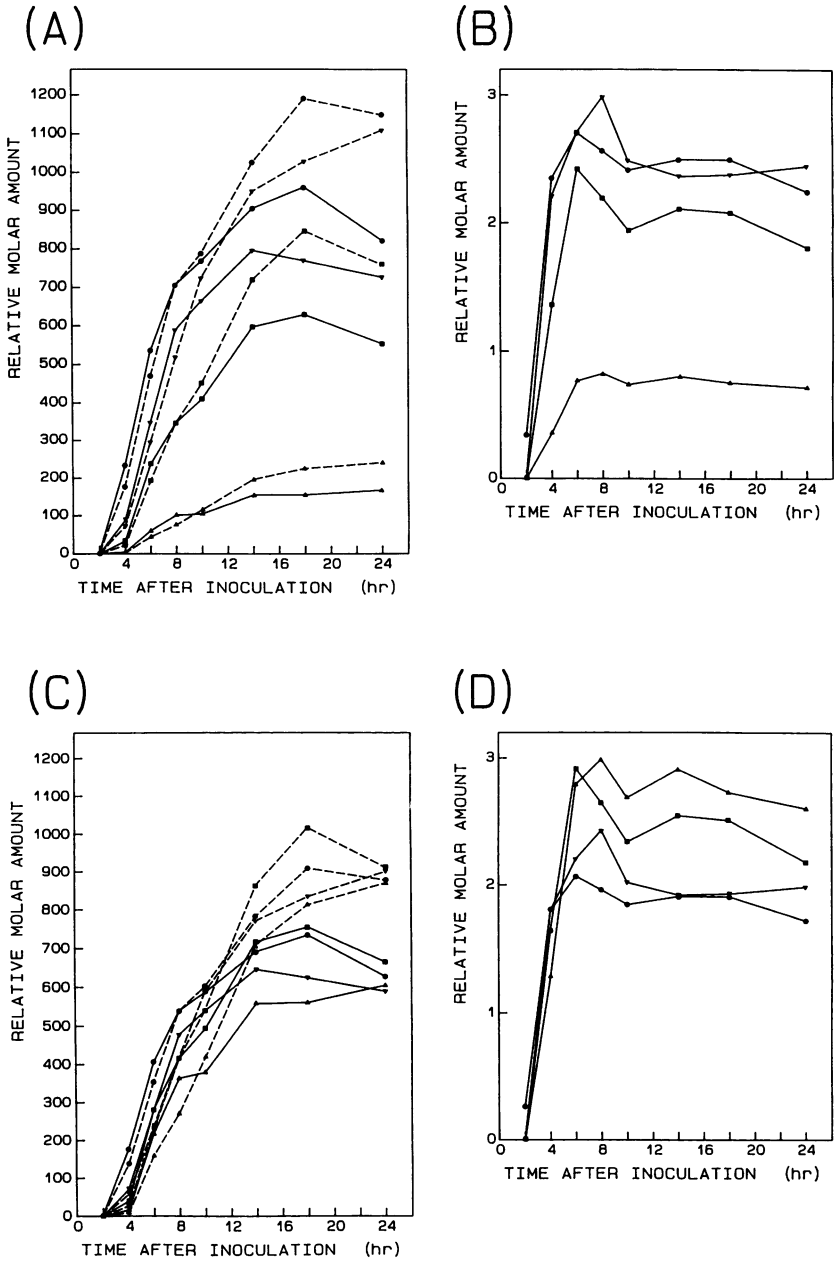
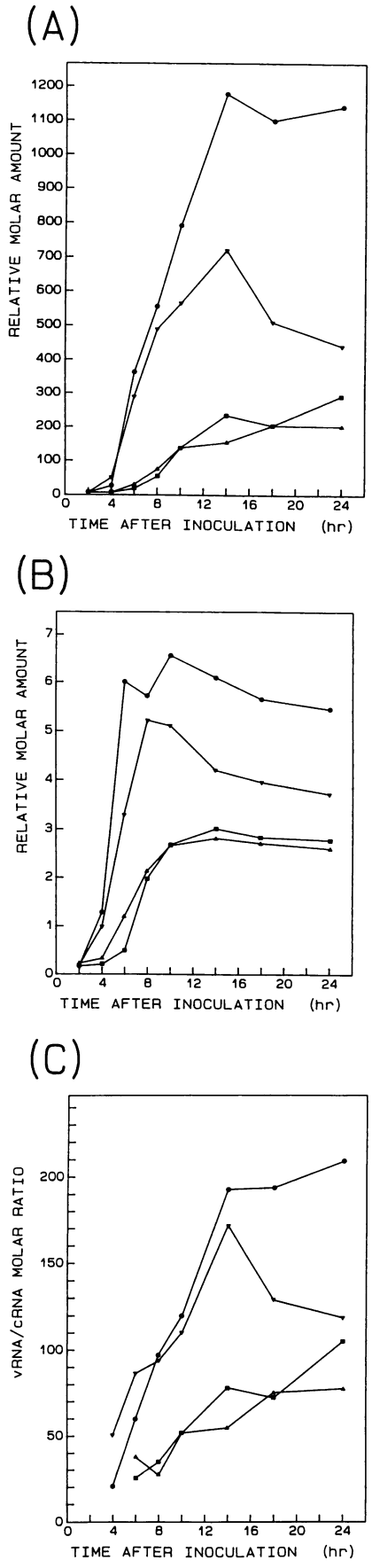


FIG. 3. Time course of accumulation of TMV plus-strand RNAs (vRNA and CP mRNA; A and C) and minus-strand RNA (cRNA; B and D) in tobacco protoplasts inoculated with various amounts of the wild-type TMV L RNA. Amounts of inoculum RNA: 5 μg (●), 1 μg (▼), 0.2 μg (■), and 0.04 μg (▲). The RNase protection assay was performed by using the P2M (A and C) and P2P (B and D) probes. In panels A and B, relative molar amounts per total inoculated protoplasts are shown, and the relative molar amount of 8.2×10^2 is defined as the vRNA accumulation in protoplasts inoculated with 5 μg of TMV L RNA at 24 h p.i. In panels C and D, relative molar amounts per infected protoplasts calculated on the basis of the percentages of infected protoplasts are shown, and the relative molar amount of 6.7×10^2 is defined as the vRNA accumulation in protoplasts inoculated with 5 μg of TMV L RNA at 24 h p.i. Percentages of protoplasts infected were 85, 80, 54, and 18 when 5, 1, 0.2, and 0.04 μg, respectively, of TMV L RNA were used as inocula (determined by staining with anti-TMV fluorescent antibody). In panels A and C, accumulation levels of vRNA and CP mRNA are shown by solid and broken lines, respectively. Relative molar amounts of TMV-related RNAs were calculated as described in Materials and Methods.

resulted partly from the damage of infected cells in the later stages of infection. The kinetics of accumulation of vRNA and CP mRNA were similar during the time course except that the accumulation rate and the final accumulation level (molar amount) were slightly larger for CP mRNA than for vRNA (Fig. 3A and C).

Accumulation of the minus-strand RNA (cRNA) became detectable at 2 or 4 h p.i. and increased only until 6 h p.i. (in some experiments, until 8 h p.i.) (Fig. 3B and D). It is clear that minus-strand accumulation ceased at a time when the plus strands were still vigorously accumulating even if encapsidation of a part of vRNA is considered. This obvious



difference in the mode of accumulation of plus and minus strands implies that distinct molecular interactions and mechanisms underlie the plus- and minus-strand RNA syntheses of TMV (see Discussion). The ratio of the amount of vRNA to cRNA (vRNA/cRNA ratio) increased almost linearly from 4 h p.i. to 14 to 18 h p.i. (see the line for LFW3 in Fig. 4C), and no obvious change was observed in the vRNA/cRNA ratio around the time of cessation of cRNA accumulation. The results of another independent experiment using a different preparation of protoplasts and 2 μg of TMV L RNA as the inoculum were consistent with those shown in Fig. 3, confirming the results presented above.

Accumulation of LQDN, a deletion mutant lacking the 30K and coat protein genes. To determine reason for the obvious halt of cRNA accumulation at an early stage of infection, we first tested the possibility that the encapsidation of the genomic RNA was the cause, because it is likely that the encapsidated genomic RNA no longer works as a template for minus-strand RNA synthesis. For this purpose, we compared the modes of accumulation of vRNA and cRNA in protoplasts inoculated with LFW3 and LQDN (Fig. 1). LQDN is a replication-competent deletion mutant lacking the coat protein and 30K protein genes whose sequence includes the assembly origin; thus, this mutant produces no coat protein and virus particles.

The time courses of accumulation of both vRNA and cRNA were similar between LQDN and LFW3 except for the vRNA level in later stages (Fig. 4A and B). The halt of cRNA accumulation was also observed in LQDN infection; moreover, the time point of the halt was similar to that of LFW3 (usually around 6 h p.i. [Fig. 4B] but in some experiments slightly later). The vRNA/cRNA ratios for LFW3 and LQDN infections were similar until 14 h p.i. (Fig. 4C). These results suggest not only that no *cis*-acting element for viral RNA replication exists in the region where LQDN is lacking, i.e., between nucleotides 4933 and 6183, but also that the halt of minus-strand accumulation did not result from encapsidation of the genomic RNA. In addition, the halt of cRNA accumulation did not result from the action of the 30K or coat protein.

After 8 h p.i., the accumulation rate of LQDN vRNA declined more than that of LFW3 vRNA, and the accumulation thereafter was considerably lower than that of LFW3 vRNA. This result may be explained by the instability of unencapsidated RNA. Next, we determined the fraction of the genomic RNA (as represented by vRNA) that had been encapsidated. When probed with P2M, about 7 and 30% of LFW3 vRNA was resistant to micrococcal nuclease treatment at 8 and 21 h p.i., respectively. The conditions of the micrococcal nuclease treatment used ensured degradation of the CP mRNA, which is not encapsidated (8, 28), to a background level. These results suggest that the difference in

FIG. 4. Time course of accumulation of plus-strand RNAs (vRNA; A) and minus-strand RNA (cRNA; B) and time course of the molar ratio of plus-strand versus minus-strand RNAs (vRNA/cRNA ratio; C) in tobacco protoplasts inoculated with the wild-type LFW3 (●), LQDN (▼), LFR2 (■), and LDR2 (▲). The RNase protection assay was performed by using the P1M (A) and P7P (B) probes. In panels A and B, the relative molar amount of 1.1×10^3 is defined as the vRNA accumulation in protoplasts inoculated with LFW3 transcript at 24 h p.i. The calculation was performed as described in Materials and Methods. For each construct (LFW3, LQDN, LFR2, and LDR2), we performed at least one more independent experiment and obtained results consistent with those shown.

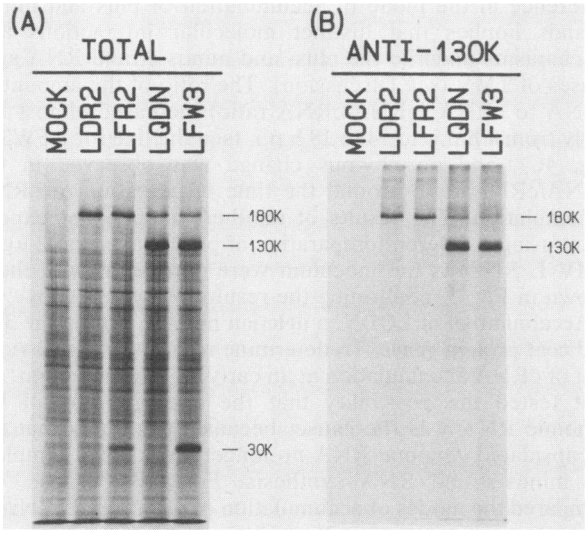


FIG. 5. Synthesis of the 180K and 130K proteins of TMV in tobacco protoplasts inoculated with in vitro transcripts of LFW3, LQDN, LFR2, and LDR2. Inoculated tobacco protoplasts were labeled with [^{35}S]methionine (40 μCi/ml) for 2 h from 11 h p.i. Total proteins (A) or proteins immunoprecipitated with anti-130K protein antibody (22) (B) were separated on an SDS–8% polyacrylamide gel and processed for fluorography by the dimethyl sulfoxide–2,5-diphenyloxazole method. Positions of the 180K, 130K, and 30K proteins are indicated in the margins.

vRNA accumulation between LFW3 and LQDN results at least partly from the difference in the ability to produce virus particles. However, it may be necessary to postulate some additional cause(s) to explain the large difference in vRNA accumulation after 14 h p.i. These results also agree with the idea that encapsidation of the genomic RNA is not the crucial cause for the halt of cRNA accumulation, because the portion of the encapsidated RNA was quite small at the time (8 h p.i.) when cRNA accumulation reached its plateau.

Accumulation of LFR2, a 130K protein-defective mutant. We previously constructed a mutant, designated LFR2 (9), in which the amber termination codon (UAG) of the 130K protein gene was changed to a tyrosine codon (UAU) (Fig. 1). As a result, LFR2 produces only the 180K protein (9). LFR2 retains the ability to replicate in plants, but the efficiency of multiplication is much lower than that of LFW3 (9). Assuming that the function of the 130K protein in replication could be deduced by determining the direct effect of the 130K protein deficiency on inefficient replication, we compared the modes of accumulation of virus-related RNAs in protoplasts inoculated with LFR2 and LFW3. LDR2, a LQDN derivative carrying the amber-to-tyrosine change (Fig. 1), was analyzed concurrently.

We first evaluated the deficiency of the 130K protein in protoplasts infected with LFR2 or LDR2. No discrete band representing the 130K protein was detected on the fluorogram before and after immunoprecipitation with anti-130K protein antibody (Fig. 5). This result indicates that no or only a minute amount of the 130K protein was produced in either LFR2- or LDR2-infected protoplasts and that generation of revertants was negligible. Therefore, replication of LFR2 and LDR2 in protoplasts must essentially reflect the state of 130K protein deficiency.

Figure 4 shows the time courses of accumulation of vRNA and cRNA, quantitated with the P1M and P7P probes, in LFR2- and LDR2-infected protoplasts. The accumulation of both vRNA and cRNA, especially of vRNA, decreased compared with that in the wild-type infection (Fig. 4A). The fact that a considerable amount of vRNA was detected in the virtual absence of the 130K protein indicates that the 130K protein is nonessential for replication and suggests that the 180K protein can compensate for the functions of the 130K protein, although inefficiently, confirming the previous in planta results (9).

The difference in kinetics profile between vRNA and cRNA accumulation was not as clear in LFR2 or LDR2 infection as in the wild-type infection (Fig. 4A and B). Nevertheless, the cessation of cRNA accumulation was observed at 10 to 14 h p.i., and the maximal amount of cRNA accumulation was about half of that in LFW3 and LQDN infections (Fig. 4B). The vRNA/cRNA ratio in LFR2 and LDR2 infections increased with time and was smaller than that in LFW3 and LQDN infections at the same time p.i. (Fig. 4C). Given this comparison and provided that the difference in stability of each viral plus and minus strand is negligible, at least plus-strand synthesis seems to be impaired by the absence of the 130K protein. Since LFR2 and LDR2 replicate less actively than LFW3 and LQDN, an alternative comparison may be made at a similar stage in replication, i.e., when cRNA accumulation of the respective viruses reaches the maximum level. The vRNA/cRNA ratio in LFR2 (or LDR2) infection at 14 h p.i. was similar to or slightly smaller than that in LFW3 (or LQDN) infection at 7 h p.i. (Fig. 4C). On the basis of the latter comparison, it seems unlikely that the absence of the 130K protein impairs only plus-strand synthesis. However, we could not discriminate empirically whether the functions of the 130K protein in the normal replication process were in both plus- and minus-strand syntheses or in either of them.

The time courses of accumulation of vRNA and cRNA in LFR2 infection, quantitated by using the P2M and P2P probes, were almost the same as those shown in Fig. 4. The ratio of CP mRNA accumulation to vRNA accumulation, determined by using the P2M probe, was almost the same between LFW3 and LFR2 infections during the time course tested (data not shown). Thus, the loss of authentic 130K protein production affects both genomic and CP subgenomic mRNA syntheses to similar extents.

DISCUSSION

Previously, Aoki and Takebe (2) examined the kinetics of the synthesis of TMV-infection-specific RNA species (viral RNA, RF, and RI) in protoplasts by quantitating incorporation of ^{32}P-labeled precursor ($^{32}P_i$) into these RNA species. Although RF and RI contain the minus-strand RNA of TMV, incorporation of ^{32}P label into RF and RI does not reflect the synthesis of the minus-strand RNA directly. In this study, we examined the mode of accumulation of vRNA, CP mRNA, and cRNA in TMV-infected protoplasts by using the RNase protection method. The time courses of accumulation of vRNA and CP mRNA were quite similar, in contrast to the time course of cRNA accumulation. These results imply that the replication mechanisms for plus- and minus-strand syntheses may involve different kinds of factors or different modes of molecular interactions.

The accumulation of minus-strand RNA reached the maximum level after about 6 h p.i., at which time the accumulation of plus-strand RNAs still continued vigorously. The observation that the specific halt of minus-strand accumulation occurred even in the absence of the coat protein and the

assembly origin sequence on the genome excludes the possibility that all genomic RNA is encapsidated and thus cannot be used as the template for minus-strand synthesis, and as a result, minus-strand synthesis ceases. As for the cause of this specific halt of minus-strand accumulation, several possibilities can be considered: (i) a putative host factor required for minus-strand synthesis might not be utilized repeatedly, so that the amount of this factor would govern the minus-strand accumulation level; (ii) the number of sites at which the minus-strand RNA can stably exist and function might be limited; and (iii) the replication machinery might have a higher affinity for the minus-strand RNA than for the plus-strand RNA even if the same machinery is used for the synthesis of both RNAs. While the amount of the minus strand is smaller than that of the machinery, minus strand would be synthesized, but after the amount of the minus strand exceeds that of the machinery, almost all of the machinery would be bound to the minus strand, resulting in exclusive synthesis of plus-strand RNAs. Of course, it is possible that the machinery for minus-strand synthesis is highly sensitive to an intracellular concentration of substrates, such as ribonucleotide triphosphates, or to the overall energy level, which will decrease as replication proceeds.

On the other hand, accumulation of the plus-strand RNAs was detectable at 2 or 4 h p.i. and reached the maximal level at 14 to 18 h p.i. The accumulation rate reached the maximum at about 4 h p.i. and then gradually decreased. Since the plus strands accumulated in a rather linear fashion between 2 h p.i. and 8 or 10 h p.i., some factor must determine the accumulation rate. It is unlikely that the rate-limiting factor for plus-strand synthesis is the amount of the 130K and 180K proteins, because these two proteins are synthesized and accumulated even after 24 h p.i. (31). Both host factors involved in plus-strand synthesis and the amount of the minus strand are candidates for this limiting factor. If the amount of the minus strand determines the accumulation rate of the plus strand, it would reach the maximum when accumulation of the minus strand reaches the maximum. This possibility is not inconsistent with our data, although precise evaluation is difficult. At the same time, however, putative changes in the amount of active machinery and templates and of their stability during time course might also influence the kinetics of accumulation of both the plus and minus strands. We could not quantitate the accumulation levels of TMV-related RNAs before 2 h p.i. because of their low amount and inhibition by the remainder of the inoculum. During this period, however, initial translation of the 130K and 180K proteins from the input genomic RNA, formation of an active replication complex, and a subsequent burst of both plus- and minus-strand accumulation must occur. Another distinct phase in replication might exist in this earliest time after infection.

Nucleotide sequence comparisons have revealed that the alphaviruses and several plant positive-strand RNA viruses, including TMV, BMV, and AlMV, share common features in the coding regions for proteins thought to be involved in replication. Therefore, these viruses may replicate in mechanistically similar manners. Shutoff of minus-strand synthesis has been reported for animal alphaviruses, such as Semliki Forest virus (SFV) and Sindbis virus (23, 25). In SFV-infected cells, minus-strand RNA synthesis ceases between 3 and 4 h p.i., whereas plus-strand RNA synthesis continues at a maximal rate to a later time p.i. (23). It has been suggested that in SFV infection, encapsidation of the 49S genomic RNA is not the cause of the shutoff of minus-strand synthesis (24). These observations are quite similar to our results for TMV infection. In SFV-infected cells, the synthesis of nonstructural proteins decreased after 3 to 4 h p.i. (23). However, it has been suggested that the cessation of minus-strand synthesis is not due to the failure of continuous synthesis of viral nonstructural proteins (27). Furthermore, it is unlikely that the rate of plus-strand synthesis is governed solely by the amount of nonstructural proteins (27) or minus-strand RNA (26). Nassuth and Bol reported that in AlMV infection, normal differential regulation of viral plus- and minus-strand RNA syntheses is largely abrogated when the inoculum omits RNA3, which encodes the coat protein and the AlMV analog of TMV 30K protein (18). This observation seems somewhat different from that for TMV infection.

Several mechanisms for cross-protection by plant viruses have been proposed (3, 20). In particular, the coat protein-mediated mechanism has been extensively investigated by using transgenic plants expressing the coat protein (21). However, from the observation that cross-protection occurred even when mutant TMV that encodes the coat protein incapable of encapsidating viral RNA was used as the first infecting virus, it has been suggested that mechanisms for cross-protection which does not include coat protein also exist (35). Our observation of the cessation of minus-strand accumulation at an early stage of infection may imply another possible mechanism of viral cross-protection: in cells in which a given TMV strain has already been infected and its replication has reached a stage at which further accumulation of the minus-strand RNA does not occur, de novo minus-strand synthesis of a challenge virus may be strongly repressed. Such a situation would be substantially similar to cross-protection.

None or only a small amount of the minus-strand RNA of the CP mRNA was produced during replication (Fig. 2B), confirming previous findings (e.g., reference 33). If the putative minus strand to the CP mRNA was not highly unstable in vivo compared with the minus-strand to the genomic RNA, these data suggest not only that the subgenomic RNAs are synthesized by internal initiation from the genome-size minus-strand RNA (16) but also that the CP mRNA is rarely used as a template for minus-strand RNA synthesis. This process would be explained by the postulation that a *cis* element necessary for initiation of minus-strand RNA synthesis is also located in the region on the genomic RNA that is not covered by the CP mRNA. Alternatively, a minus-strand RNA synthesis (e.g., formation of the active replication complex) might be coupled with the translation of replicase components encoded on the virus genome (namely, the 130K and 180K proteins), which would result in extremely preferential utilization of the genomic RNA (located near the translation machinery), as discussed previously (23).

ACKNOWLEDGMENTS

We thank P. Ahlquist and Agrigenetics Research Associates for the use of the p_M promoter and Y. Watanabe, T. Saito, and D. Takezawa for help and discussion.

This work was supported in part by a grant-in-aid from the Ministry of Education, Science and Culture, Japan.

REFERENCES

1. **Ahlquist, P., E. G. Strauss, C. M. Rice, J. H. Strauss, J. Haseloff, and D. Zimmern.** 1985. Sindbis virus proteins nsP1 and nsP2 contain homology to nonstructural proteins from several RNA plant viruses. J. Virol. **53**:536–542.

2. **Aoki, S., and I. Takebe.** 1975. Replication of tobacco mosaic virus RNA in tobacco mesophyll protoplasts inoculated in vitro. Virology **65**:343–354.
3. **Beachy, R. N.** 1988. Virus cross-protection in transgenic plants, p. 313–331. *In* D. P. S. Verma and R. B. Goldberg (ed.), Temporal and spatial regulation of plant genes. Springer-Verlag, New York.
4. **Beier, H., M. Barciszewska, G. Krupp, R. Mitnacht, and H. J. Gross.** 1984. UAG readthrough during TMV RNA translation: isolation and sequence of two $tRNAs^{Tyr}$ with suppressor activity from tobacco plants. EMBO J. **3**:351–356.
5. **Goelet, P., G. P. Lomonossoff, P. J. G. Butler, M. E. Akam, M. J. Gait, and J. Karn.** 1982. Nucleotide sequence of tobacco mosaic virus RNA. Proc. Natl. Acad. Sci. USA **79**:5818–5822.
6. **Guilley, H., G. Jonard, B. Kukla, and K. E. Richards.** 1979. Sequence of 1000 nucleotides at the 3′ end of tobacco mosaic virus RNA. Nucleic Acids Res. **6**:1287–1308.
7. **Haseloff, J., P. Goelet, D. Zimmern, P. Ahlquist, R. Dasgupta, and P. Kaesberg.** 1984. Striking similarities in amino acid sequence among nonstructural proteins encoded by RNA viruses that have dissimilar genomic organization. Proc. Natl. Acad. Sci. USA **81**:4358–4362.
8. **Hunter, T. R., T. Hunt, J. Knowland, and D. Zimmern.** 1976. Messenger RNA for the coat protein of tobacco mosaic virus. Nature (London) **260**:759–764.
9. **Ishikawa, M., T. Meshi, F. Motoyoshi, N. Takamatsu, and Y. Okada.** 1986. In vitro mutagenesis of the putative replicase genes of tobacco mosaic virus. Nucleic Acids Res. **14**:8291–8305.
10. **Ishikawa, M., T. Meshi, T. Ohno, Y. Okada, T. Sano, I. Ueda, and E. Shikata.** 1984. A revised replication cycle for viroids: the role of longer than unit length RNA in viroid replication. Mol. Gen. Genet. **196**:421–428.
11. **Kamer, G., and P. Argos.** 1984. Primary structural comparison of RNA-dependent RNA polymerases from plant, animal and bacterial viruses. Nucleic Acids Res. **12**:7269–7282.
12. **Li, G., and C. M. Rice.** 1989. Mutagenesis of the in-frame opal termination codon preceding nsP4 of Sindbis virus: studies of translational readthrough and its effect on virus replication. J. Virol. **63**:1326–1337.
13. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.
14. **Meshi, T., M. Ishikawa, F. Motoyoshi, K. Semba, and Y. Okada.** 1986. In vitro transcription of infectious RNAs from full-length cDNAs of tobacco mosaic virus. Proc. Natl. Acad. Sci. USA **83**:5043–5047.
15. **Meshi, T., Y. Watanabe, T. Saito, A. Sugimoto, T. Maeda, and Y. Okada.** 1987. Function of the 30kd protein of tobacco mosaic virus: involvement in cell-to-cell movement and dispensability for replication. EMBO J. **9**:2557–2563.
16. **Miller, W. A., T. W. Dreher, and T. C. Hall.** 1985. Synthesis of brome mosaic virus subgenomic RNA in vitro by internal initiation on (−)-sense genomic RNA. Nature (London) **313**:68–70.
17. **Myers, R. M., Z. Larin, and T. Maniatis.** 1985. Detection of single base substitutions by ribonuclease cleavage at mismatches in RNA:DNA duplexes. Science **230**:1242–1246.
18. **Nassuth, A., and J. F. Bol.** 1983. Altered balance of the synthesis of plus- and minus-strand RNAs induced by RNAs 1 and 2 of alfalfa mosaic virus in the absence of RNA 3. Virology **124**:75–85.
19. **Ohno, T., M. Aoyagi, Y. Yamanashi, H. Saito, S. Ikawa, T. Meshi, and Y. Okada.** 1984. Nucleotide sequence of tobacco mosaic virus (tomato strain) genome and comparison with the common strain genome. J. Biochem. **96**:1915–1923.
20. **Palukaitis, P., and M. Zaitlin.** 1984. A model to explain the "cross-protection" phenomenon shown by plant viruses and viroids, p. 420–429. *In* T. Kosuge and E. W. Nester (ed.), Plant-microbe interactions, molecular and genetic perspectives, vol. 1. Macmillan Publishing Co., New York.
21. **Powell-Abel, P., R. S. Nelson, B. De, N. N. Hoffman, S. G. Rogers, R. T. Fraley, and R. N. Beachy.** 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science **232**:738–743.
22. **Saito, T., Y. Watanabe, T. Meshi, and Y. Okada.** 1986. Preparation of antibodies that react with the large non-structural proteins of tobacco mosaic virus by using *Escherichia coli* expressed fragments. Mol. Gen. Genet. **205**:82–89.
23. **Sawicki, D. L., and S. G. Sawicki.** 1980. Short-lived minus-strand polymerase for Semliki Forest virus. J. Virol. **34**:108–118.
24. **Sawicki, D. L., and S. G. Sawicki.** 1987. Alphavirus plus strand and minus strand RNA synthesis, p. 251–259. *In* M. A. Brinton and R. R. Rueckert (ed.), Positive strand RNA viruses. Alan R. Liss, Inc., New York.
25. **Sawicki, D. L., S. G. Sawicki, S. Keränen, and L. Kääriäinen.** 1981. Specific Sindbis virus-coded function for minus-strand RNA synthesis. J. Virol. **39**:348–358.
26. **Sawicki, S. G., and D. L. Sawicki.** 1986. The effect of loss of regulation of minus-strand RNA synthesis on Sindbis virus replication. Virology **151**:339–349.
27. **Sawicki, S. G., and D. L. Sawicki.** 1986. The effect of overproduction of nonstructural proteins on alphavirus plus-strand and minus-strand RNA synthesis. Virology **152**:507–512.
28. **Siegel, A., V. Hari, I. Montgomery, and K. Kolacz.** 1976. A messenger RNA for capsid protein isolated from tobacco mosaic virus-infected tissue. Virology **73**:363–371.
29. **Takamatsu, N., M. Ishikawa, T. Meshi, and Y. Okada.** 1987. Expression of bacterial chloramphenicol acetyltransferase gene in tobacco plants mediated by TMV-RNA. EMBO J. **6**:307–311.
30. **van Regenmortel, M. H. V., and H. Frankel-Conrat.** 1986. The plant viruses 2. The rod-shaped plant viruses. Plenum Press, New York.
31. **Watanabe, Y., Y. Emori, I. Ooshika, T. Meshi, T. Ohno, and Y. Okada.** 1984. Synthesis of TMV-specific RNAs and proteins at the early stage of infection in tobacco protoplasts: transient expression of the 30K protein and its mRNA. Virology **133**:18–24.
32. **Watanabe, Y., T. Meshi, and Y. Okada.** 1987. Infection of tobacco protoplasts with in vitro transcribed tobacco mosaic virus RNA using an improved electroporation method. FEBS Lett. **219**:65–69.
33. **Watanabe, Y., N. Morita, M. Nishiguchi, and Y. Okada.** 1987. Attenuated strains of tobacco mosaic virus. Reduced synthesis of a viral protein with a cell-to-cell movement function. J. Mol. Biol. **194**:699–704.
34. **Watanabe, Y., T. Ohno, and Y. Okada.** 1982. Virus multiplication in tobacco protoplasts inoculated with tobacco mosaic virus RNA encapsulated in large unilamellar vesicle liposomes. Virology **120**:478–480.
35. **Zaitlin, M.** 1976. Viral cross-protection: more understanding is needed. Phytopathology **66**:382–383.