Modifications of the Tobacco Mosaic Virus Coat Protein Gene Affecting Replication, Movement, and Symptomatology

W. O. Dawson, P. Bubrick, and G. L. Grantham

Department of Plant Pathology, University of California, Riverside, CA 92521.
This work was supported in part by grants from USDA (85-CRCR-1-1795), NSF (DMB-8607638), and US-Israel BARD (I-882-85).
Accepted for publication 5 January 1988 (submitted for electronic processing).

ABSTRACT

Dawson, W. O., Bubrick, P., and Grantham, G. L. 1988. Modifications of the tobacco mosaic virus coat protein gene affecting replication, movement, and symptomatology. Phytopathology 78:783-789.

A series of tobacco mosaic virus (TMV) mutants with insertions and/or deletions in the coat protein gene were made by modification of the cDNA of the genome (pTMV204) followed by in vitro transcription. All mutants multiplied as free-RNA viruses and moved from cell to cell in inoculated leaves of Xanthi tobacco, although the mutants established systemic infections less efficiently than wild-type TMV. This included mutants that had the entire coat protein gene removed, deletions that extended 28 nucleotides into the 3'-nontranslated region, as well as a mutant containing the bacterial chloramphenicol acetyltransferase gene substituted for the coat protein gene. Most of the mutants produced coat protein-related polypeptides in vivo, although the size and amount of protein found in inoculated leaves varied considerably among different mutants. In general, polypeptides that contained the normal coat protein carboxyl terminus tended to accumulate in greater amounts. Three types of responses were observed in mutant-infected Xanthi tobacco: no symptoms, yellowing, and necrosis. Most mutants that produced no or low amounts of coat protein failed to induce symptoms. The mutants that retained the carboxyl terminus of the coat protein induced yellowing symptoms. All mutants including those completely lacking the coat protein gene induced local lesions in Xanthi-nc. Two mutants also induced necrotic local lesions in Xanthi tobacco as well as almost all host plants tested. These results demonstrate that the TMV coat protein gene has a multifunctional role during infection that includes encapsidation, symptom expression, and differential elicitation of resistant genes.

Additional keywords: construction of virus mutants, hypersensitive reaction, modification of genome of TMV, recombinant viral genetics.

Tobacco mosaic virus (TMV) has a genome consisting of one RNA molecule of 6,395 nucleotides that contains three open reading frames producing four proteins. The 126- and 183-kDa proteins presumably are involved in replication of RNA, the 30-kDa protein is involved in cell-to-cell movement, and the 17.5-kDa protein is structural. However, there are many more virus-associated functions and phenotypes than there are proteins produced. Replication has been subdivided into several functions, including production of double-stranded RNA (8), production of single-stranded RNA (7), and production of mRNA (4). Production of subgenomic mRNAs, regulation of the ratios of plus to minus RNAs, and shutdown of replication when a specific level of virus is produced probably are virus-encoded functions. Phenotypes also associated with the viral genome include control of the host range, symptoms induced in each host, ability to induce the hypersensitive reaction (HR) in certain hosts, ability to move from cell to cell, and ability to move long distances throughout the plant. Therefore, it is evident that some sequences of the TMV genome serve multiple functions.

We have begun a systematic examination of function-sequence relationships of TMV, beginning with the coat protein gene. Through recombinant DNA techniques we have made a series of insertions and deletions in the coat protein gene in order to address several questions.

First, is the coat protein gene required for replication? Although assembly defective mutants were described (23), it is possible that these coat proteins that were defective in assembly remained functional for some other aspect of replication or regulation. A mutant of TMV has been described that does not accumulate detectable amounts of coat protein (22). However, small amounts of coat protein or the RNA of the coat protein gene may be required for nonstructural functions.

Second, will the modified virus spread in whole plants? Deletions in the coat protein gene of brome mosaic virus (BMV) resulted in virus that could replicate in barley protoplasts but apparently could not spread from cell to cell in intact plants (10). Assembly-deficient mutants of TMV can move from cell to cell and, to a limited extent, can move long distances in whole plants (23). However, it is possible that these mutants were selected to have defective proteins that retain ability to effect spread of the viral infection. Deletions in the coat protein gene of TMV may likewise prevent cell-to-cell or long-distance movement.

Third, to what extent can the TMV genome be manipulated and still retain efficient replication? How much can the length of TMV RNA be increased or decreased? Does removal of coat protein gene sequences require replacement with other sequences for efficient replication?

Fourth, can we identify other functions associated with the coat protein gene? Is the coat protein gene associated with biological functions such as symptomatology and host-range?

In this paper, we report that insertions or deletions in the coat protein gene destroyed the assembly function but allowed replication, cell-to-cell movement, and limited systemic spread to continue. We also show that alteration of the coat protein greatly affected symptom production. A preliminary report was made earlier (6).

MATERIALS AND METHODS

Virus and plants. TMV 204 is a virus derived from plants infected with in vitro transcripts from pTMV204, which is a cDNA clone from TMV strain U1 (5). The virus was propagated systemically in Xanthi tobacco (*Nicotiana tabacum* L.), and infectivity was assayed in Xanthi-nc tobacco (25). Plants were grown in a greenhouse and maintained in plant growth chambers at 25 C with a 16-hr photoperiod of approximately 2,000 lx after inoculation unless stated otherwise.

Construction of coat protein mutants. Insertion and deletion mutants were all derived from pTMV204. The 3' half of pTMV204 cDNA (nucleotides 3332–6395) was subcloned into pUC18 to give a unique *Acc*I restriction site, which occurred in the coat protein gene. Two insertion mutants were made by addition of a *Xho*I linker (CCUCGAGG, from Pharmacia) into the *Acc*I site (Table 1). Mutant cp 5 was constructed by adding the linker after filling in the single-stranded overhang with T4 DNA polymerase, and mutant cp +2 was made by addition of the linker after removal of the overhang by digestion with Exonuclease VII. Deletion mutants

© 1988 The American Phytopathological Society

(Table 1) were made by *Bal*31 nuclease digestion (18) after *Acc*I nuclease digestion. Single-stranded regions produced by the *Bal*31 digestion were removed with T4 DNA polymerase and *Xho*I linkers were added. The modified 3′ halves of the TMV genome were removed from pUC18 and ligated to the 5′ half of pTMV204 and pBR322 as described previously (5). The dsDNA of each construct was sequenced through the modified region (27).

Mutant cp S3-28 was constructed to have the entire coat protein gene removed. A synthetic dsDNA, which replaces the sequence 5′ of the coat protein gene and changes the AUG start codon to ACG, was ligated to the 3′ end of the cDNA of mutant cp 28, which has the 3′ end of the coat protein gene precisely excised with *Bal*31 exonuclease. The 3′-half of pTMVcp28 (nucleotides 3333 to the 3′-end of TMV) was subcloned in pUC18 and digested with *Cla*I (nucleotide 5664) and *Xho*I (nucleotide 6192). The resulting 5,708-bp fragment that contains two portions of TMV cDNA attached to pUC18 was ligated to the *Cla*I (nucleotide 5664) to *Aha*III (nucleotide 5708) fragment (44 bp) from pTMV204 and the annealed synthetic DNA (5′ AAATACGC 3′ and 5′ TCGAGCGTATTT 3′). This TMV cDNA was excised from pUC18 and ligated to the 5′ half of pTMV204 and pBR322 as described previously (5).

A mutant (S3-CAT-28) containing a substitution of the chloramphenicol acetyltransferase (CAT) gene for the coat protein was constructed as follows. The CAT gene was removed from pCM1 (Pharmacia) with *Sal*I and ligated into *Xho*I-cleaved pTMVS3-28. This construction produced pTMVS3-CAT-28 from which the mutant cp S3-CAT-28 was transcribed. Correct sequence and orientation were confirmed by sequencing (27).

Infectious RNA was produced by transcription of the DNA using the PM promoter (provided by P. Ahlquist: 1) and RNA polymerase of *Escherchia coli* (5). Infectivity was assayed by grinding leaves in cold 1% sodium pyrophosphate buffer, pH 9.0, plus 1% bentonite and 1% Celite with immediate inoculation, or from phenol extracted RNA in the same buffer (3).

Protein extraction, SDS-PAGE, and Western immunoblotting. Leaf tissue was pulverized in a mortar containing liquid nitrogen, transferred to SDS-PAGE sample buffer (final concentrations: 125 mM Tris, pH 6.8, 10% [w/v] glycerol, 2.5% [w/v] dithiothreitol [DTT], 2.0% [w/v] sodium dodecylsulfate [SDS], 0.01% [w/v] bromophenol blue), and incubated at 100 C for 4 min. Alternatively, tissue disks were immersed directly into sample buffer, ground, then incubated at 67 C for 20 min. SDS-PAGE was done as described by Laemmli (16) using acrylamide concentrations of 4.8 and 15% for stacking and separation gels, respectively. Proteins were stained with silver (2) or transferred to nitrocellulose (9) at 0.4 amps for 0.5 hr and 1.0–1.2 amps for 1 hr.

Coat protein polypeptides were detected using anti-coat protein antibody. All blockings and incubations were done in phosphate-buffered saline (PBS) skim milk (10%, w/v). Anti-coat protein antibody (1:1,000 dilution) and alkaline phosphate-conjugated goat anti-rabbit antibody (1:2,000 dilution) were incubated sequentially with blots for 2 hr at 37 C; enzyme was detected using BCIP-NBT (1-2 μg/gel) as described by Knecht and Dimond (14).

Polyclonal antibodies to TMV coat protein were raised using preparative SDS-PAGE purified antigen (11). Approximately 100 μg of protein (in 0.5 ml of PBS) was emulsified with incomplete Freund's adjuvant (0.5 ml) and injected intramuscularly into New Zealand White rabbits on a biweekly schedule; a total of five injections were administered. One week after the final booster injection, blood was collected, and the resulting serum was used for the detection of mutant coat proteins.

RESULTS

Mutants constructed. Modifications of the coat protein gene were made at a unique *Acc*I restriction site (nucleotide 6056 of pTMV204), which affects the coat protein RNA binding region, amino acids 90-123 that are encoded by nucleotides 5985–6083 (17,20,24). (This is not to be confused with the origin for assembly region for the RNA.) Two insertion mutants were made by ligating a *Xho*I linker into the cDNA at the *Acc*I site (Table 1). Mutant cp 5 had a net increase of 10 nucleotides, which caused a translation frame shift and premature termination of coat protein. This mutant should synthesize the normal coat protein through amino acid 114 and terminate with a carboxyl terminus of 15 non-TMV amino acids (Fig. 1). Mutant cp +2 had a net increase of six nucleotides, which maintained the correct reading frame. This mutant should synthesize a modified coat protein with two extra amino acids (leucine and glutamic acid) between residue 114 (valine) and residue 115 (aspartic acid).

A series of deletion mutants was made by *Bal*31 nuclease treatment after *Acc*I excision followed by addition of *Xho*I linkers. Six of these mutants representing a variety of sizes were chosen for examination (Table 1, Fig. 1). Mutants cp 10 and cp 25 and mutants cp 4 and cp 27 were chosen as pairs that had similar sized deletions but in opposite directions. Mutant cp 4 had a 134-nucleotide deletion consisting of 37 nucleotides 5′ and 97 nucleotides 3′ of the *Acc*I site. Mutant cp 27 had a deletion of 131 nucleotides resulting from removal of 108 nucleotides 5′ and 23 nucleotides 3′ of the *Acc*I site. Similarly, mutants cp 10 and cp 25 had 40 and 53 nucleotide deletions, respectively, but most of the cp 10 deletion is 3′, whereas most of the cp 25 deletion is 5′ of the original *Acc*I restriction site. Mutant cp 28 had the 3′ two-thirds of

TABLE 1. Description of coat protein deletion mutants

	Deletion[a]							Symptoms on Xanthi	
	5′ ←	3′ →	Reading frame[b]	Expected protein (kDa)	Infectivity of transcripts[c]	Extractable infectivity[d]	Systemic movement	Local	Systemic
cp 5	+1	+1	−	14.3	10	37	+	none	none
cp +2	−1	−1	+	17.7	15	43	+	yellow spots	yellowing
cp 10	−14	−26	−	13.1	38	45	−[e]	necrosis	···
cp 4	−37	−97	+	12.9	45	7	+	yellowing	yellowing
cp 25	−38	−15	+	15.7	15	51	+	yellow spots	yellowing
cp 27	−108	−23	+	13.0	15	46	−[e]	necrosis	···
cp 28	−164	−135		7.1	44	16	+	yellow spots	none
cp 35	−216	−163		5.4	27	18	+	none	none
cp 35-5	−216	+1	+	9.7	50	4	+	yellow areas	yellowing
cp 5-35	+1	−163	−	14.3	15	3	+	none	none
cp S3-28	−345	−135		0	11	4	+	none	none
cp S3-CAT-28	−345	−135		···	17	6	+	none	none
TMV 204	0	0	+	17.5	16	>200	+	none	mosaic

[a] Number of nucleotides deleted or added 5′ or 3′ of *Acc*I restriction site (6056) on TMV cDNA. Each construction has a *Xho*I linker (CCUCGAGG) inserted between the DNA remaining after the described deletion of TMV nucleotides.

[b] Reading frame beyond deletion and linker insertion is same as normal reading frame at 3′ end of gene (+) or different reading frame (−). No symbol means that all of the 3′ end of the coat protein gene is deleted.

[c] In vitro transcripts of cDNA were assayed on Xanthi-nc. Results of one representative experiment (average number of local lesions per lear).

[d] Phenol extracts of infected Xanthi were assayed on Xanthi-nc (average number of local lesions per leaf).

[e] Mutants cp 10 and cp 27 usually were restricted to local lesions on inoculated leaves, but occasionally moved systemically.

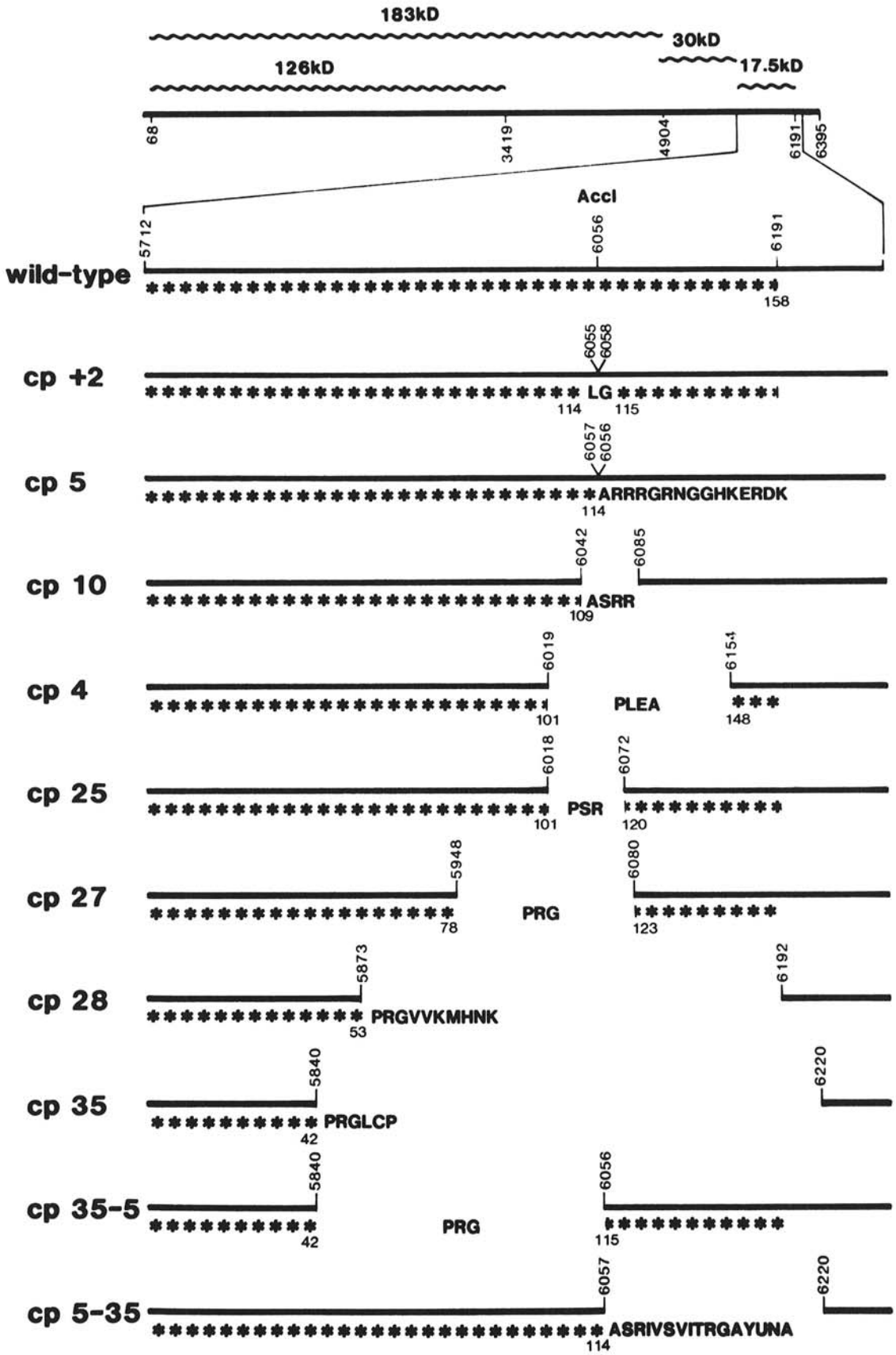

Fig. 1. Comparison of the coat protein produced and the insertions and/or deletions in the coat protein gene of TMV predicted from the sequence of the cDNA construct. The RNA is diagrammed above the protein. Numbers above the RNA denote last normal nucleotide 5′ of the deletion and the first normal nucleotide 3′ of the deletion. A *Xho*I linker (CCUCGAGG) is inserted between the TMV nucleotides designated. Numbers below the protein designate the last normal coat protein amino acid produced before deletions and, when the correct reading frame is continued, the first normal coat protein amino acid after the deletion. Letters denote amino acids incorporated into the modified coat protein either by the inserted linker and/or from an incorrect reading frame.

the coat protein gene removed with the deletion ending precisely after the stop codon but leaving the 3'-nontranslated region of the viral RNA unaltered. Mutant cp 35 had a larger deletion that continued for 28 nucleotides into the 3' nontranslated region.

The *Xho*I restriction sites placed between the *Bal*31 deletions allowed combinations of different 5' and 3' deletions. Thus, mutant cp 35-5 contains only the 5' deletion of cp 35, while mutant cp 5-35 contains only the 3' deletion of mutant cp 35. Mutants cp 5 and cp 5-35 are designed to produce essentially the same truncated coat protein but to have different RNA sequences (Fig. 1).

Mutant cp S3-28 was designed so that all of the coat protein gene was removed. Because other workers have demonstrated that RNA plant viruses can express foreign genes (10,26), mutant cp S3-CAT-28, which contains the CAT gene from *E. coli* substituted for the coat protein gene, was made to examine the effects of a foreign gene on the biology of the virus-host interaction. Each construction was confirmed by sequencing the dsDNA.

Ability of the mutants to replicate. To determine whether these in vitro constructed mutants could replicate and spread in tobacco plants, RNA from in vitro transcriptions was used to inoculate two varieties of tobacco. The cultivar Xanthi becomes systemically infected by wild-type TMV resulting in mosaic symptoms, whereas Xanthi-nc restricts wild-type TMV to necrotic local lesions. Transcripts of all in vitro constructed mutants produced local lesions on Xanthi-nc of approximately the same size and number as produced by the parental wild-type virus, TMV 204. Table 1 shows the results of one representative experiment. These data are not stringently quantitative because infectivity from different transcriptions varies depending on the quality of the DNA transcribed and susceptibility of the hosts. However, the lesion numbers demonstrate that transcripts of all mutants had similar levels of infectivity.

To determine whether infectious RNA could be found in the inoculated Xanthi leaves, we extracted RNA 7 days after inoculation and assayed on Xanthi-nc. All mutants produced infectious RNA in vivo. Again, Table 1 shows the result of one experiment. Data from several different experiments indicate that all of the mutants produced approximately the same amount of infectivity. Variation depended on the level of the initial infection of leaves from which the RNA was extracted (data not presented). The greater amount of infectivity found in leaves infected with TMV 204 probably was due to the greater stability of virions in cells. The progeny RNA of all mutants produced local lesions on leaves of Xanthi-nc similar in size to those of TMV 204. Thus, all of the mutants were able to replicate effectively in tobacco leaves.

All mutants could be propagated serially from plant to plant or infectious RNA could be stored after phenol extraction. Storage of RNA extracts obviates the requirement for further in vitro transcription of the cDNA of TMV.

We next examined whether any of the mutants produced virions. The simplest procedure was to determine whether infectious RNA was protected from degradation when incubated in crude sap. Virions also were assayed by examining leaf dips and thin sections by electron microscopy and purification by differential centrifugation. These procedures failed to detect virions in plants infected with any of the mutants.

Ability of the mutants to move in plants. The ability of the mutants to spread from cell to cell was estimated by infection of Xanthi-nc leaves with each mutant and with TMV 204 and incubation at 32 C for 3 days followed by a temperature shift to 25 C. The size of necrotic lesions should be a measurement of spread from cell to cell during the incubation at 32 C. All of the mutants produced lesions similar in size to those produced by wild type (data not presented), suggesting that the localized movement of all mutants in inoculated leaves was similar to that of TMV 204.

Long distance movement in the plant was monitored by assaying upper, uninoculated leaves for infectious RNA. All mutants were able to move from inoculated leaves to upper leaves (Table 1). However, long distance spread throughout the plant by the mutants was less efficient than that of wild type. Systemic movement of all mutants was erratic and was similar to that initially reported for other free-RNA mutants (23). Movement was monitored more easily in plants infected by mutants that induce distinct symptoms where extractable infectivity was associated with symptomatic areas. However, infectivity was recovered from some nonsymptomatic leaves of plants infected by the other mutants. Often, plants contained some leaves where infectivity was distributed throughout the leaf, while only localized areas of other leaves became infected, and some upper leaves failed to become infected.

Mutant coat protein synthesis. All of the mutants except cp S3-28 and cp S3-CAT-28, both of which completely lack the coat protein gene, were expected to produce coat protein related polypeptides in vivo (Table 1). When total soluble protein from infected leaves was analyzed for TMV coat protein by SDS-PAGE and Western blotting followed by immunodetection, coat-protein-related polypeptides were detected from several mutants (Fig. 2). Different mutants accumulated different amounts of coat protein. Some mutants, such as cp +2 and cp 35-5, accumulated nearly as much as wild type. However, most mutants accumulated less protein than wild-type TMV in mechanically inoculated Xanthi leaves. In spite of the fact that the mutants multiplied and accumulated infectious RNA in infected leaves, we have not yet definitively detected coat protein in leaves infected with mutants cp 35, cp 28, cp 5, and cp 5-35. Mutants cp 35 and cp 28 should produce small polypeptides that may be difficult to resolve by standard SDS-PAGE analysis. Mutants cp 5 and cp 5-35, however, should produce larger polypeptides (14.3 kDa), which should easily be resolved.

The new bands found by SDS-PAGE analysis of protein from mutant-infected tobacco leaves were shown to be coat protein related polypeptides by immunoblotting with antibodies made against TMV coat protein (Fig. 2B). However, the data in Figure

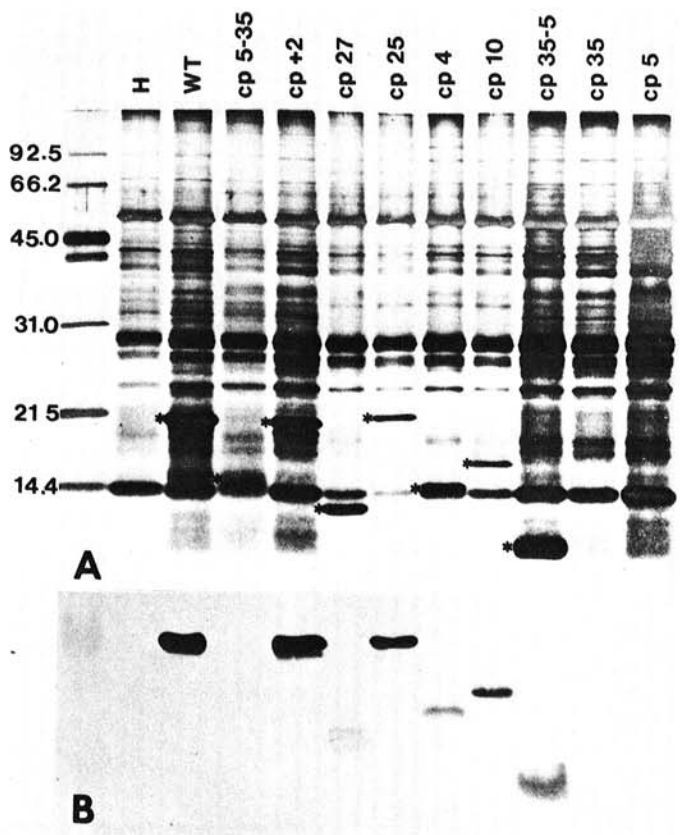

Fig. 2. A, SDS-PAGE and, **B,** immunoblot analysis of coat protein-related polypeptides produced by coat protein deletion mutants in inoculated leaves of Xanthi tobacco. H is total protein from uninfected tobacco and WT is wild-type TMV 204. Mutants are listed above individual lanes and suspected coat protein-related polypeptides have an asterisk on the left of the band. Numbers on the left indicate the molecular weights of the standards in kilodaltons. Lanes cp 5-35, cp 5, and cp 35 did not have an immunoreactive band.

2B are not quantitative since some of the mutant polypeptides bound the antibodies less efficiently than others, probably because some epitopes were deleted.

At this point, we do not know whether the reduced accumulation of coat protein by some of the mutants is due to a reduced rate of synthesis, to reduced stability of the proteins, or to combinations of both. However, during pulse-labeling with ^{3}H-leucine, the rate of coat protein synthesis by the mutants that accumulate reduced amounts of protein was proportionally less than that of wild type (data not presented).

Several of the proteins migrated anomalously during electrophoresis in SDS-polyacrylamide gels. For example, the proteins of mutants cp 4, cp 10, and cp 27 should be approximately the same size and, yet, clearly had different electrophoretic mobilities (Fig. 2). Also, the protein of mutant cp 25 migrates more slowly than expected. Electrophoresis in the presence of 8 M urea and SDS did not eliminate these mobility differences. Others have shown that identically sized coat proteins from different TMV strains can migrate differently during SDS-PAGE analysis (21).

Mutant cp S3-CAT-28 failed to produce a band during SDS-PAGE that could positively be identified as chloramphenicol acetyltransferase, but functional enzyme activity was detected in plant extracts that acetylated ^{14}C-chloramphenicol with acetyl CoA (data not presented).

Symptomatology of the coat protein mutants. A surprising feature of the coat protein mutants was the induction of a variety of different symptoms in the systemic host, Xanthi. Figure 3 shows some examples of symptoms on inoculated or systemically infected leaves. Mutants cp +2, cp 25, cp 35-5, and cp 28 produced distinct yellow spots on inoculated Xanthi leaves, while mutant cp 4 produced distinct yellow rings (Fig. 3B). All of these mutants produced some type of yellowing on systemically infected leaves except cp 28, which was symptomless in the infected upper leaves. Mutant cp +2 produced a general yellowing in all systemically infected areas. Mutant cp 25 often caused a herringbonelike symptom in systemically infected leaves (Fig. 3D). Mutant cp 35-5 produced the most dramatic yellowing, with bright yellow areas in inoculated and systemically infected leaves and often produced a yellow oakleaf pattern (Fig. 3C). In contrast, mutants cp 5, cp 5-35, cp 35, cp S3-28, and cp S3-CAT-28 produced no local or systemic symptoms in Xanthi. Table 1 and Figure 1 allow comparisons of symptom type with the predicted proteins produced by these mutants.

All mutants induced local lesions in Xanthi-nc, which has the N gene for resistance to TMV. This included the mutant with no coat protein gene and the mutant with the chloramphenicol acetyltransferase gene in place of the coat protein gene. This demonstrates that the coat protein gene is not required for induction of HR in plants with the N gene.

Remarkably, mutants cp 10 and cp 27 induced distinct necrotic lesions on inoculated leaves of Xanthi (Fig. 3E). Xanthi tobacco has the nn genotype and normally does not respond hypersensitively to TMV. The lesions produced on inoculated leaves of Xanthi were slightly larger and occurred later than lesions produced on Xanthi-nc. Lesions developed on Xanthi nc after 3 days and on Xanthi after 5 days at 25 C. When inoculated plants were maintained at 25 C, the virus usually was restricted to the necrotic areas and did not move systemically. Occasionally the virus would move systemically, resulting in systemic yellowed and necrotic areas.

The reaction of Xanthi to cp 10 and cp 27 was examined to determine whether the local lesion response was temperature sensitive. In Xanthi-nc, necrosis is induced by wild type only when infected plants are maintained at temperatures below approximately 30 C. As a control, Xanthi and Xanthi-nc plants were inoculated with TMV 204 and maintained at 32 C. As expected, necrosis occurred in neither plant. However, when Xanthi and Xanthi-nc were inoculated with cp 10 or cp 27 and incubated at 32 C, necrosis and yellowing occurred on inoculated leaves of both plants. When Xanthi and Xanthi-nc plants inoculated with cp 10 or cp 27 were incubated at 35 C, only yellowing occurred on inoculated leaves, and both mutants always moved systemically causing yellow areas in upper leaves (Fig. 3F). Thus, the hypersensitivity induced by these mutants appears to be temperature sensitive similar to the reaction of Xanthi-nc to wild type, except that the temperature required to prevent production of necrosis by cp 10 and cp 27 is about 5 C higher.

All mutants also were examined in *N. sylvestris* Speg. & Comes, which carries the N′ gene. Only mutants cp 10 and cp 27 produced necrosis in this plant. Additionally, mutants cp 10 and cp 27 were compared to wild type in a series of other hosts (Table 2). These mutants produced necrosis in almost all of the plants tested. In several of the plants, the mutants differed in ability to induce necrosis. In *N. glauca* Graham & Physalis *floridana* Rydb., cp 10 induced local lesions, while cp 27 only produced yellow spots. In Ace tomato, cp 27 induced necrosis in inoculated leaves, whereas cp 10 induced no symptoms. Only in *Zinnia elegans* Jacq. did the mutants fail to induce necrosis, although cp 27 produced yellow spots on inoculated leaves, and cp 10 caused the midrib of leaves to turn brown.

DISCUSSION

These results demonstrate that genetic manipulation of the coat protein gene did not greatly reduce the ability of TMV to infect and replicate in inoculated leaves of intact plants. All mutants with insertions or deletions in the coat protein gene were able to undergo localized cell-to-cell movement but compared with wild type were diminished in their ability to systemically invade plants. The entire coat protein gene (cp S3-28) or even 28 nucleotides beyond the coat protein gene into the 3′ nontranslated region could be removed (cp 35 and cp 5-35) without destroying the capability of replication. Similar results were obtained when the size of the genome was increased by substituting a larger foreign gene, chloramphenicol acetyltransferase (787 nucleotides), for the coat protein gene (478 nucleotides). All mutants had alterations in the RNA-binding region within the coat protein and, as expected, none of them formed virions. Thus, these mutants replicated and moved as free-RNAs that behaved similarly to the original assembly deficient mutants PM1 and PM2 (23).

Most of the mutants produced altered coat proteins of reduced size in vivo. However, the amounts of proteins found in cells varied among the different mutants. We do not yet know the reason for this variability. It is possible that the altered coat proteins are much more susceptible to degradation than protein subunits of virions, which are normally refractory to proteolytic digestion. We noted that proteins that retained the normal C-terminus tended to accumulate in higher amounts than proteins that had this region deleted, suggesting that the C-terminal amino acids may lend stability. However, there was large variation in amounts of accumulation of different mutant proteins that retain the normal C-terminus. Mutants cp 4, cp 25, and cp 27 produced much less protein than mutants cp 35-5 and cp +2. Also, in preliminary labeling experiments, the kinetics of precursor incorporation paralleled the amounts of protein accumulation. We have not yet quantified the amounts of subgenomic mRNA produced by the mutants. Although the promoter region of the coat protein subgenomic mRNA was not altered, other regions of the genome or the coat protein itself may affect transcription or efficiency of translation of the coat protein mRNA. For example, mutants cp 5 and cp +2 differ only by four nucleotides, and the subgenomic mRNAs would be expected to differ by only these four nucleotides, yet the amount of cp +2 coat protein produced was substantially higher than that of cp 5. This difference will be examined further.

The most surprising result of these experiments was the extent to which the coat protein gene could be involved in production of symptoms in plants. Most of the symptoms produced were distinctive, and individual mutants could be identified by their symptomatology. When the sizes or regions of the alterations of TMV RNA were compared to the symptoms induced, there were no immediately obvious correlations. However, there was a correlation between the protein structure and symptoms produced (Table 1 and Fig. 1). Several of the mutants produced proteins with the normal C terminal region deleted, either because the 3′ portion

of the gene was deleted (cp 28, cp 35, cp 5-35, cp S3-28, and cp S3-CAT-28) or because the reading frame was lost (cp 5 and cp 10). Mutants that produced proteins without the normal C terminus were usually symptomless. Exceptions were cp 28, which caused yellowing of inoculated leaves but, in contrast to the other yellowing mutants, caused no systemic symptoms, and cp 10, which induced necrosis. All of the mutants that produced the normal C terminal region of the coat protein induced some degree of yellowing. We do not know whether this is a direct effect of the normal C terminal region of the protein on chloroplasts, or whether some secondary effect like protein stability or concentration affects chloroplasts. The yellowing in inoculated leaves probably results from damage to mature chloroplasts, in contrast to the abnormal development of chloroplasts that culminates in mosaic symptoms in developing leaves (19).

There also was a correlation between the amount of altered coat protein that accumulated in cells and the amount of yellowing of leaves. The largest amount of protein accumulated in leaves infected with mutants cp 35-5 and cp +2, which also induced greater amounts of yellowing. The least amount of protein was found in leaves infected with mutants cp 5, cp 35, and cp 5-35, which induced no symptoms in plants other than the local lesion host Xanthi-nc. Mutants that did not produce any coat protein (S3-28) or produced a foreign protein (S3-CAT-28) also induced no symptoms similar to earlier reports (22,26). The proteins that were produced by the mutants were probably free protein in contrast to that in wild-type infected leaves, which is mostly associated with virions. It would be interesting to test whether the

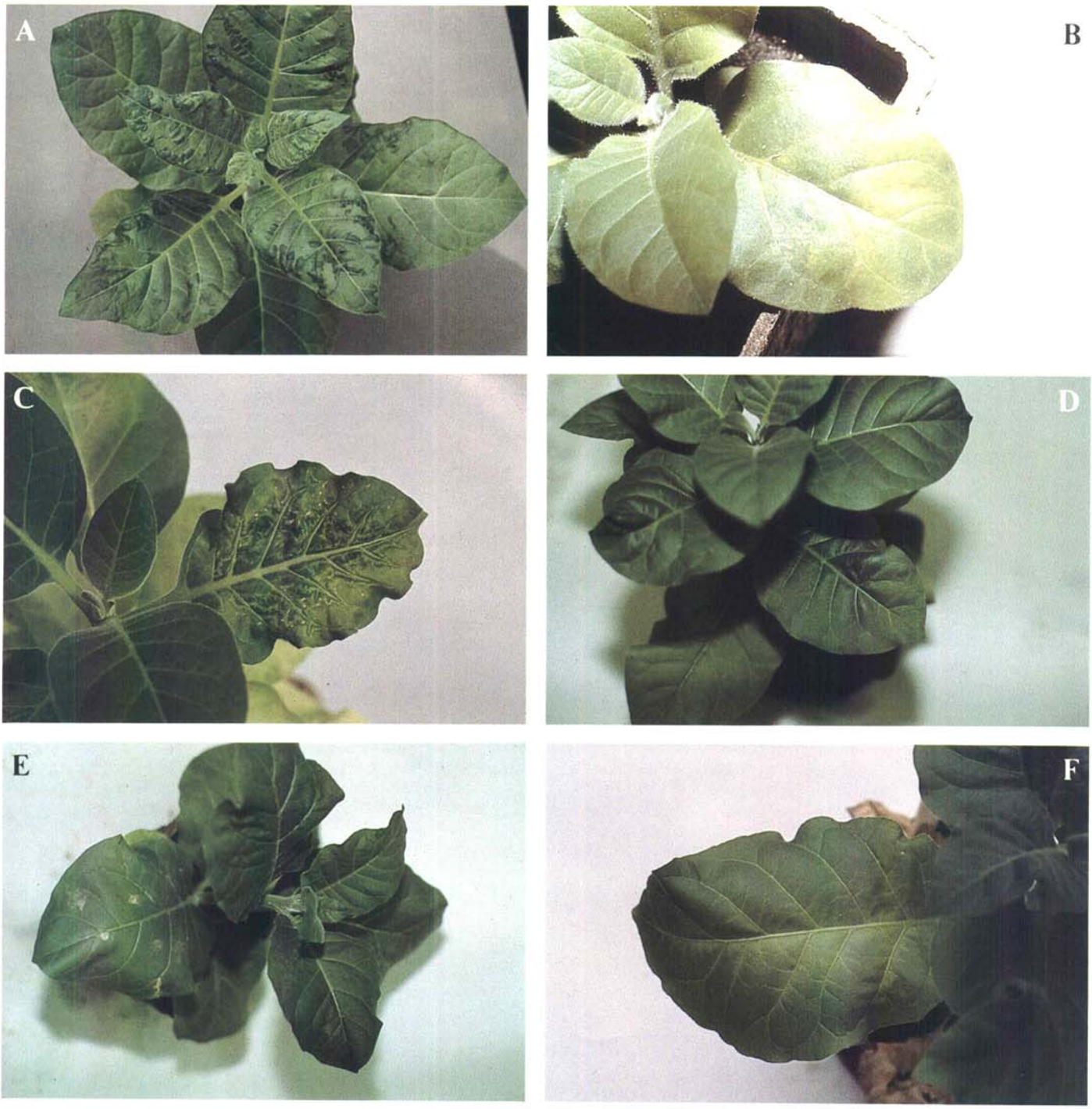

Fig. 3. Symptoms produced in Xanthi tobacco by coat protein deletion mutants. **A,** Wild type; **B,** cp 4 inoculated leaf; **C,** cp 35-5 systemic infection; **D,** cp 25 systemically infected leaf; **E,** cp 27 inoculated leaf; and, **F,** cp 27 inoculated leaf incubated at 35 C.

TABLE 2. Symptoms of tobacco mosaic virus (TMV) coat protein-deletion mutants cp 10 and cp 27 in different hosts

	Symptoms on inoculated leaves[a]		
	TMV 204	cp 27	cp 10
Nicotiana tabacum L. 'Xanthi'	L	N	N
Nicotiana tabacum L. 'Xanthi-nc'	N	N	N
Nicotiana sylvestris Speg. & Comes	L	N	N
Nicotiana benthamiana Domin	N	N	N
Nicotiana glauca Graham	L	Y	N
Phaseolus vulgaris L. 'Pinto'	N	N	N
Solanum nigrum L.	L	N	N
Lycopersicon esculentum Mill. 'Ace'	L	N	L
Zinnia elegans Jacq.	L	Y	B
Petunia hybrida Vilm.	L	N	N
Physalis floridana Rydb.	L	Y	N
Nicandra physalodes (L.) Gaertn.	L	N	

[a] L = Latent, N = necrotic local lesion, Y = yellow spots, B = browning of veins.

normal amount of wild-type protein existing as free protein instead of in virions would cause increased yellowing.

The coat protein gene of TMV also was shown to be involved in production of the hypersensitive reaction in plants in which wild-type TMV normally does not induce local lesions. Mutants cp 10 and cp 27 induced necrosis in almost all of the plants that were tested. These mutants have some similarities to natural mutants of TMV described earlier (12,13). Jensen's strain 14 induces local lesions on tobacco, *N. sylvestris*, and tomato and has reduced infectivity and resistance to incubation at high temperatures.

It is difficult to identify correlations between mutants cp 10 and cp 27 in relation to their ability to induce necrosis. Both mutants have deletions in the region where the coat protein binds to the RNA during assembly (amino acids 90–123). However, several of the other mutants have deletions in this area. Mutants cp 27 and cp 10 are deleted to similar extents in the region 3′ of the *Acc*I site (23 and 26 nucleotides, respectively), but mutant cp 27 retains the correct reading frame and maintains the normal C terminus, while cp 10 does not. This is being investigated further by making hybrid combinations between mutants that do and do not induce necrosis to better define nucleotide alterations required for induction of HR in various hosts.

The production of HR in n and N′ plants is associated with the coat protein gene. In a separate report, we examined the sequence alteration of a natural mutant of TMV that induces HR in *N. sylvestris* (N′ gene). The alteration of the phenotype of this mutant that continues to produce normal virions is due to a single nucleotide change (nucleotide 6157) that changes coat protein amino acid 148 from serine to phenylalanine (15). Here, we found that mutants with alterations in different areas of the coat protein gene could induce HR in plants with either the N′ or n gene. However, the coat protein gene appears not to be involved in invoking HR in plants with the N gene. Mutants with the entire coat protein gene deleted or the addition of a foreign gene retained the ability to induce HR in Xanthi-nc.

Our experiments demonstrate that the coat protein gene of TMV is multifunctional. Not only is this gene responsible for the structural features and long distance movement of the virus, but it also appears to be intimately involved with the physiological interactions between the virus and its host. The range of functions associated with alterations in the coat protein gene appears to depend on how the plant interprets changes in viral RNA or protein. In this regard, the present experiments have revealed new information concerning host resistance to TMV. The fact that mutants cp 10 and cp 27 induced necrosis in the normally susceptible Xanthi and almost all other plants tested demonstrates that plants that normally exhibit no resistance to TMV do have the genetic capacity to exhibit a hypersensitive response to aberrant forms of the virus. Learning how to manipulate this phenomenon could lead to the development of new methods to control virus diseases.

LITERATURE CITED

1. Ahlquist, P., and Janda, M. 1984. cDNA cloning and in vitro transcription of the complete brome mosaic virus genome. Mol. Cell Biol. 4:2876-2882.
2. Blum, H., Beier, H., and Gross, H. J. 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8:93-99.
3. Dawson, W. O. 1976. Synthesis of TMV RNA at restrictive high temperatures. Virology 73:319-326.
4. Dawson, W. O. 1983. Tobacco mosaic virus protein synthesis is correlated with double-stranded RNA synthesis and not single-stranded RNA synthesis. Virology 125:314-323.
5. Dawson, W. O., Beck, D. L., Knorr, D. A., and Grantham, G. L. 1986. cDNA cloning of the complete genome of tobacco mosaic virus and production of infectious transcripts. Proc. Natl. Acad. Sci. USA 83:1832-1836.
6. Dawson, W. O., and Grantham, G. L. 1986. Characterization of mutants of TMV with insertions or deletions in the coat protein gene. Phytopathology 76:1095.
7. Dawson, W. O., and White, J. L. 1978. Characterization of a temperature-sensitive mutant of tobacco mosaic virus deficient in synthesis of all RNA species. Virology 90:209-213.
8. Dawson, W. O., and White, J. L. 1979. A temperature-sensitive mutant of tobacco mosaic virus deficient in synthesis of single-stranded RNA. Virology 93:104-110.
9. Dunn, S. D. 1986. Effects of the modification of transfer buffer, composition and the renaturation of proteins in gels on the recognition of proteins on western blots by monoclonal antibodies. Anal. Biochem. 157:144-153.
10. French, R., Janda, M., and Ahlquist, P. 1986. Bacterial gene inserted in an engineered RNA virus: Efficient expression in monocotyledonus plant cells. Science 231:1294-1297.
11. Higgins, R. C., and Dahmus, M. E. 1979. Rapid visualization of protein bands in preparative SDS-polyacrylamide gels. Anal. Biochem. 93:257-260.
12. Holmes, F. O. 1952. Use of primary lesions to determine relative susceptibility of individual plants. Phytopathology 42:113.
13. Jensen, J. H. 1937. Studies on representative strains of tobacco-mosaic virus. Phytopathology 27:69-84.
14. Knecht, D., and Dimond, R. L. 1984. Visualization of antigenic proteins on western blots. Anal. Biochem. 136:180-184.
15. Knorr, D. A., and Dawson, W. O. 1988. A point mutation in the tobacco mosaic virus capsid protein gene induces hypersensitivity in *Nicotiana sylvestris* Proc. Natl. Acad. Sci. USA 85:170-174.
16. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.
17. Mandelkow, E., Stubbs, G., and Warren, S. 1981. Structures of the helical aggregates of tobacco mosaic virus protein. J. Mol. Biol. 152:375-386.
18. Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Pages 135-139 in: Molecular Cloning. Cold Spring Harbor Laboratory, Cold Spring, NY.
19. Matthews, R. E. F. 1973. Induction of disease by viruses, with special reference to turnip yellow mosaic virus. Ann. Rev. Phytopathol. 11:147-170.
20. Namba, K., and Stubbs, G. 1986. Structure of tobacco mosaic virus at 3.6 A resolution: Implications for assembly. Science 231:1401-1406.
21. Sano, Y., Nozu, Y., and Inoue, H. 1978. Anomalous mobility of cucumber green mottle virus protein in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biochem. (Tokyo) 84:1041-1049.
22. Sarkar, S., and Smitamana, P. 1981. A truly coat-protein-free mutant of tobacco mosaic virus. Naturwissenschaften 68:145-147.
23. Siegel, A., Zaitlin, M., and Sehgal, O. P. 1962. The isolation of defective tobacco mosaic virus strains. Proc. Natl. Acad. Sci. USA 48:1845-1851.
24. Stubbs, G., Warren, S., and Holmes, K. 1977. Structure of RNA and RNA binding site in tobacco mosaic virus from 4-A map calculated from X-ray fibre diagrams. Nature (London) 267:216-221.
25. Takahashi, W. N. 1956. Increasing the sensitivity of the local-lesion method of virus assay. Phytopathology 46:654-656.
26. Takamatsu, N., Ishikawa, M., Meshi, T., and Okada, Y. 1987. Expression of bacterial chloramphenicol acetyltransferase gene in tobacco plants mediated by TMV-RNA. EMBO J. 6:307-311.
27. Zagursky, R. J., Baumeister, K., Lomax, N., and Berman, M. L. 1985. Rapid and easy sequencing of large linear double-stranded DNA and supercoiled plasmid DNA. Gene Anal. Technol. 2:89-94.