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Characterization of the Masked Strain of Tobacco Mosaic Virus: Identification of the Region Responsible for Symptom Attenuation by Analysis of an Infectious cDNA Clone

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A strain of tobacco mosaic virus (TMV) that produces mild (attenuated) symptoms on tobacco plants has been molecularly cloned to identify the region of the genome responsible for symptom attenuation. A full-length cDNA clone whose transcripts produce the parental disease phenotype on both systemic and hypersensitive host plants has been constructed. This infectious clone was sequenced, and 55 base changes relative to the published sequence of common TMV (strain U1) were identified. These changes resulted in 12 amino acid alterations in the open reading frames encoding the 126/183-kDa and 30-kDa movement proteins; two of these changes were determined not to be responsible for the attenuated phenotype. Exchange of restriction fragments between the infectious mild strain cDNA and an infectious U1 strain cDNA indicated that the determinants involved in symptom attenuation reside in the open reading frame encoding the 126/183-kDa proteins of TMV; these proteins are involved in viral replication.

Additional keywords: cDNA sequence, pathogenicity, sequence analysis.

Molecular biological techniques perfected in the last decade have made it possible to produce infectious cDNA clones from various animal (Mizutani and Colonno 1985; Dasmahapatra *et al.* 1986; Van der Werf *et al.* 1986; Rice *et al.* 1987) and plant (Ahlquist *et al.* 1984; Dawson *et al.* 1986; Meshi *et al.* 1986; Allison *et al.* 1988; Vos *et al.* 1988) RNA viruses. These clones can be used to identify sequences responsible for symptom development (see review by Daubert 1988), for example, by specific mutagenesis. A second strategy is to clone strains of viruses that produce symptoms which are either more or less severe than those produced by a closely related strain. By comparing the two sequences, changes in the viral genome can be identified as potentially responsible for the altered symptoms. This approach has been taken to identify sequences that could be responsible for symptom attenuation in poliovirus (Nomoto *et al.* 1982) and tomato mosaic virus (ToMV) (Nishiguchi *et al.* 1985). The results of the poliovirus work implicated, but did not prove, that mutations in the viral capsid protein were responsible for attenuation. In the case of the attenuated $L_{11}A$ strain of ToMV, 10 base substitutions were identified that distinguished it from a virulent strain. Three of the changes resulted in amino acid alterations in the open reading frame (ORF) encoding the 126- and 183-kDa proteins of ToMV; these proteins are involved in viral replication (see review by Quadt and Jaspars 1989). It was subsequently shown that this attenuated strain produced less movement protein (MP) than the more virulent strain, while synthesis of genomic RNA and viral coat protein (CP) was the same in both strains (Watanabe *et al.* 1987). This defect apparently is responsible for a reduced rate of cell-to-cell spread compared with the virulent strain (Nishiguchi and Oshima 1977). These experiments did not identify which particular base change(s) was linked to phenotype change(s), since specific mutagenesis or exchange of restriction fragments between infectious cDNA clones of the virulent and attenuated strains (Meshi *et al.* 1986) was not reported.

We have produced cDNA clones of an attenuated strain, the Holmes' masked (M) strain (Holmes 1934) of tobacco mosaic virus (TMV). This strain does not differ in most biological or physicochemical characteristics compared with the U1 (common) strain (Knight 1947; Siegel and Wildman 1954). However, in systemic *Nicotiana tabacum* L. hosts, the M strain produces at most a mild chlorosis on leaves compared with the light and dark green mosaic of the U1 strain; it is often symptomless (Holmes 1934). More recently Asselin *et al.* (1984) identified a significant alteration in the RNA fingerprint of the M strain compared with the U1 strain. The alteration was mapped to a fragment located in the MP ORF, and it was suggested that this change could be responsible for the diminution of symptoms.

By placing our full-length cDNA clones of the attenuated M strain behind the bacteriophage T7 promoter, we produced *in vitro* transcripts that induced disease symptoms in plants identical to those caused by the parental virus. This clone was fully sequenced to identify base changes that may be involved in symptom development. In addition, exchange of corresponding cDNA restriction fragments between the infectious M strain clone and an infectious U1 strain cDNA clone further delineated the nucleotide changes responsible for symptom attenuation. From these

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experiments, it was determined that the region responsible for symptom attenuation in the M strain was within the 126/183-kDa ORF where several base changes result in significant amino acid changes.

MATERIALS AND METHODS

Virus isolation. The M strain of TMV was obtained from the American Type Culture Collection, Rockville, MD (ATCC PV42), and the U1 strain of TMV was obtained from M. Zaitlin, Cornell University, Ithaca, NY. Both were serially transferred several times through a systemic host (*N. tabacum* cv. Xanthi) before inoculation onto a hypersensitive host (*N. tabacum* cv. Xanthi-nc). Single lesions were isolated and inoculated onto systemic hosts. After visually verifying the symptoms of each virus, leaves were harvested and the virus purified as described by Asselin and Zaitlin (1978).

cDNA cloning. M strain viral RNA was obtained from purified virus as described by Bruening *et al.* (1976). cDNA cloning was performed as described by Meshi *et al.* (1986) with the following modifications: first strand synthesis with a 3′ terminal oligodeoxynucleotide primer utilized AMV reverse transcriptase (Seikagaku America, St. Petersburg, FL) in a buffer described by Ahlquist *et al.* (1984). First strand cDNA was fractionated on a 2.5% polyacrylamide/8.3 M urea gel run overnight at 250 V, and high molecular weight cDNA was isolated. Second strand synthesis with a 3′ terminal oligodeoxynucleotide primer utilized the Klenow fragment of DNA polymerase I (Promega, Madison, WI) followed by AMV reverse transcriptase. *Pst*I linkers (New England Biolabs, Beverly, MA) were added by ligation and digested with *Pst*I, and the double-stranded cDNA was size fractionated on an A-150m (Bio-Rad, Richmond, CA) column to collect the largest cDNAs. The cDNA was then restricted with *Bam*HI (unique site at nucleotide 3332 of U1; Goelet *et al.* 1982) and cloned into plasmid pSP73 (Promega). cDNAs representing partial genomes of the M strain of TMV were ligated to a plasmid containing a bacteriophage T7 promoter designed to transcribe nucleotides 1–256 from a cDNA clone of the U1 strain of TMV (Holt and Beachy, in press). A cDNA clone representing the 5′ half of the M strain (nucleotides 257–3332) was ligated to this transcription cassette following a *Sma*I-*Bam*HI digestion. The U1 strain and M strain sequences from nucleotides 1–256 (the *Sma*I site) are identical except for a change at position 209, which did not alter the amino acid sequence specified by the ORF. A clone of the 3′ half of the M strain (nucleotides 3333–6395) was then inserted to create a full-length cDNA clone from which infectious RNA transcripts were produced *in vitro* by standard techniques (Nielsen and Shapiro 1986).

Sequencing. Viral RNA was sequenced by the procedure of Larson and Wilson (1989) utilizing dideoxynucleotides and AMV reverse transcriptase. A commercial kit (Pharmacia-LKB Biotechnology, Piscataway, NJ) using dideoxynucleotides and T7 DNA polymerase was used to sequence cDNA clones from double-stranded templates. Deaza analogues of dGTP (Mizusawa *et al.* 1986) and dITP as well as opposite strand primers were utilized where necessary to resolve difficult areas in the sequence.

Genome exchange. Corresponding segments of the infectious M strain cDNA and an infectious U1 strain cDNA (Holt and Beachy, in press) were exchanged using standard techniques (Maniatis *et al.* 1982). Enzymes were from Promega and New England Biolabs. Constructions were confirmed by restriction mapping and nucleotide sequencing.

Protein sequence alignments. Protein sequence alignments were conducted using the Clustal program (Higgins and Sharp 1988). Fixed and varied gap penalities were set at 10. Residues with a score of eight or greater in a Dayhoff PAM matrix of amino acid similarity (Dayhoff 1978) were considered conservative changes.

RESULTS

Phenotype of parental virus and infectious cDNA clone. The attenuated M strain of TMV (Holmes 1934) induces local lesions equal in size to those produced by the U1 strain, and little or no symptoms compared with U1 in systemic hosts. To compare and contrast the genome sequences of both strains, full-length cloned cDNA of the M strain was prepared as described in the previous section. Transcripts produced *in vitro* from the infectious M strain cDNA clone caused identical visible disease symptoms on hypersensitive and systemic hosts compared with the parental M strain virus (not shown). As predicted, the necrotic local lesions produced by the infectious M strain clone were identical to those produced by the U1 virus (not shown). The use of the first 256 bases from a U1 cDNA clone in constructing the M strain infectious clone did not cause an alteration in the attenuated phenotype.

Sequence analysis. The two half-genome cDNA clones of the M strain that were combined to produce the full-length infectious clone were sequenced using primers which were, in most cases, complementary to (+) strand viral RNA. Areas that proved difficult to sequence were further analyzed using primers complementary to the opposite strand of the cDNA or deoxynucleotide analogues. There were 55 base changes compared with the published U1 strain sequence of Goelet *et al.* (1982) (Table 1). There was an uneven distribution of sequence alterations throughout the molecule, with no changes in the 5′ untranslated region, a slight underrepresentation of changes in the CP coding region, and a slightly greater percentage of changes in the 126/183-kDa ORF. There was no bias in the type of base that was changed, but there was a strong bias toward transitions (83% of the total) as opposed to transversions.

Twelve amino acid changes were predicted to result from the nucleotide changes, all of which were in either the 126/183-kDa proteins or the MP (Table 1). Potentially significant changes include those at amino acid residues 367 (Lys to Glu), 587 (Pro to Ala), and 601 (Glu to Lys) of the 126/183-kDa proteins. M strain viral RNA and the RNA from the U1 strain of TMV were also sequenced to verify the changes in the cDNA relative to the parental viral RNA (Table 1). Only one amino acid change, at residue 128 of the MP, was found in the M strain cDNA clone but not in the M strain viral RNA. This change had no apparent effect on the disease phenotype, since transcripts from the infectious cDNA clone caused attenuated symptoms identical to those induced by the M strain virus. In addition,

an amino acid change in the M strain cDNA clone relative to the published sequence (Goelet *et al.* 1982) was observed in both U1 strain and M strain viral RNAs (amino acid residue 185 of the 126/183-kDa proteins). Thus, these amino acid changes are not likely to be involved in the production of the attenuated phenotype.

Genome exchange. Experiments in which portions of an infectious U1 cDNA clone were exchanged with the corresponding portions of the infectious M strain clone were conducted to further delineate the region responsible for the attenuated phenotype (described in Fig. 1). RNAs transcribed *in vitro* from the chimeric genomes were inoculated onto the systemic Xanthi tobacco host, and the types of symptoms produced were compared with those induced by strains M and U1. As summarized in Figure 1, these experiments showed that the determinants responsible for the mild phenotype resided solely in the ORF encoding the 126/183-kDa proteins. More refined exchange experiments, in which quarter portions of the genomes from the two full-length clones were exchanged, indicated that a change or changes in the region 5′ to the *Stu*I site (nucleotide 1675) in addition to a change or changes in the region from the *Stu*I site to the *Bam*HI site were responsible for the attenuated phenotype. Clones that contained either of these regions from U1 produced an intermediate phenotype (Fig. 1B). The region comprising nucleotides 1–1675 of the attenuated strain contains amino acid changes at residues 325, 360, 367, and 416, while the segment including nucleotides 1676–3332 contains amino acid changes at residues 587, 601, 668, and 747 (Table 1).

Comparison of M strain 126/183-kDa sequence with other plant viral replicases. In an attempt to further identify amino acid changes that could cause an attenuated phenotype, amino acid sequences for putative RNA-dependent RNA polymerases (RdRps) from various related viruses were aligned (Fig. 2). The alignment showed that none of the eight amino acid changes between the attenuated M strain and the U1 strain were in highly conserved regions of the sequence. In addition, the changes at amino acid residues 325 (Ser to Gly) and 668 (Lys to Arg) were also found in ToMV (L strain) RdRp. The L strain of ToMV, although less severe than U1 in phenotype, does cause a green mosaic disease in tobacco; thus, it is unlikely that these amino acid changes result in the attenuated phenotype

Table 1. Nucleotide and predicted amino acid changes in an infectious cDNA clone of the M strain of tobacco mosaic virus and partial sequence analysis of M strain and U1 strain RNAs[a]

Region where change occurred	Nucleotide	Base change	Amino acid change and position	RNA sequence confirmation, Virus strain: M	RNA sequence confirmation, Virus strain: U1
126/183 kDa	209	G→A	Silent	+[b]	
126/183 kDa	482	C→T	Silent	+	
126/183 kDa	536	G→A	Silent	+	
126/183 kDa	623[c]	Deletion of A	Met→Cys^{185}	+	+
126/183 kDa	627[c]	Insertion of A		+	+
126/183 kDa	644	G→A	Silent	+	−
126/183 kDa	695	A→C	Silent	+	−
126/183 kDa	698	C→T	Silent		
126/183 kDa	713	G→A	Silent		
126/183 kDa	798	T→C	Silent		
126/183 kDa	878	T→C	Silent		
126/183 kDa	974	A→G	Silent		
126/183 kDa	1044	A→G	Ser→Gly^{325}	+	−
126/183 kDa	1149	T→A	Ser→Thr^{360}	+	−
126/183 kDa	1170	A→G	Lys→Glu^{367}	+	−
126/183 kDa	1301	G→A	Silent	+	−
126/183 kDa	1318	C→T	Ala→Val^{416}	+	−
126/183 kDa	1334	T→C	Silent		−
126/183 kDa	1535	G→A	Silent		
126/183 kDa	1614	T→C	Silent		
126/183 kDa	1656	A→C	Silent		
126/183 kDa	1830	C→G	Pro→Ala^{587}	+	−
126/183 kDa	1872	G→A	Glu→Lys^{601}	+	−
126/183 kDa	1943	G→A	Silent	+	
126/183 kDa	2074	A→G	Lys→Arg^{668}	+	−
126/183 kDa	2310	G→A	Ala→Thr^{747}	+	−
126/183 kDa	2330	C→T	Silent	+	−
126/183 kDa	2400	T→C	Silent		
126/183 kDa	2582	G→A	Silent		
126/183 kDa	2651	C→T	Silent		
126/183 kDa	2654	G→T	Silent		
126/183 kDa	3231	T→C	Silent		
126/183 kDa	3272	A→G	Silent		
183 kDa	3464	T→G	Silent		
183 kDa	3809	T→C	Silent		
183 kDa	3977	T→C	Silent		
183 kDa	4055	A→G	Silent		
183 kDa	4283	C→T	Silent		
183 kDa	4373	A→C	Silent		
183 kDa	4403	C→T	Silent		
183 kDa	4418	G→A	Silent		
183 kDa	4499	C→T	Silent		
183 kDa	4523	T→C	Silent		
183 kDa	4694	T→C	Silent		
183 kDa	4784	C→T	Silent		
MP[d]	5004	T→C	Silent		
MP	5288	C→G	Thr→Ser^{128}	−	−
MP	5329	A→G	Ile→Val^{142}	+	−
MP/OAS[e]	5416	T→C	Silent		
MP/OAS	5514	G→A	Silent	+	−
MP	5565	C→T	Silent	+	−
MP	5585	A→G	Asn→Ser^{227}	+	−
CP[f]	5738	T→C	Silent	+	−
3′ Untranslated	6208	A→G		+	−
3′ Untranslated	6326	T→G		+	−

[a] Sequence comparisons based on the results of Goelet *et al.* (1982). Superscripts after the amino acid residues refer to the position within the amino acid sequence exclusive of the initial methionine residue for each open reading frame.

[b] +, present in the RNA sequence; −, not present in the RNA sequence; no symbol, not checked.

[c] Base 623 of the sequence of Goelet *et al.* (1982) was found to be deleted, and an A residue was found inserted between bases 627 and 628 of Goelet *et al.* (1982).

[d] MP, movement protein.

[e] OAS, origin-of-assembly sequence.

[f] CP, coat protein.

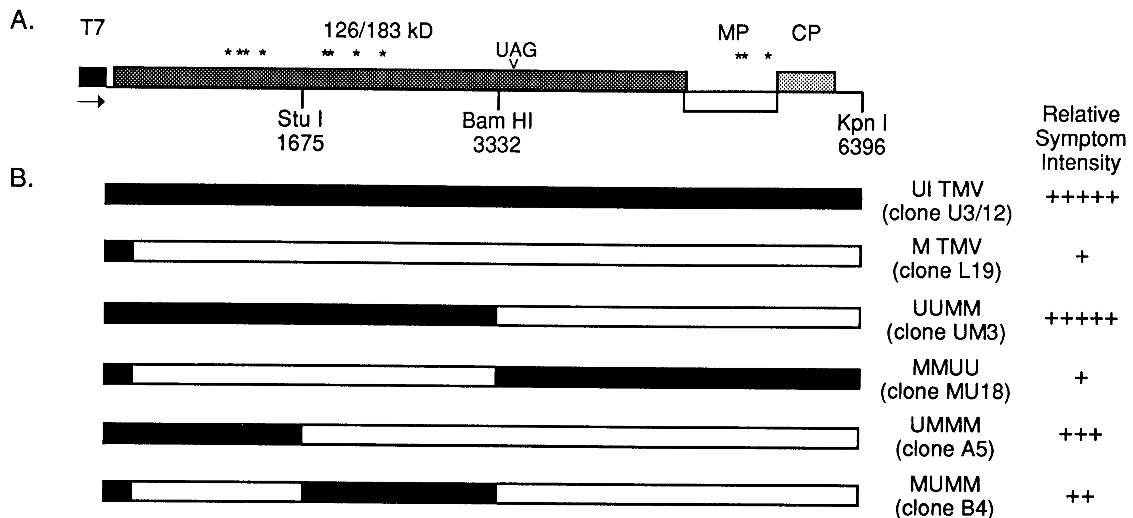

Fig. 1. A, Genome organization and partial restriction map of infectious cDNA clones. Open reading frames are represented by shaded or open bars: 126/183-kDa indicates the putative RNA-dependent RNA polymerase; MP, movement protein; and CP, coat protein. T7 designates the bacteriophage T7 promoter for production of *in vitro* RNA transcripts. Asterisks (*) indicate positions of amino acid changes in the M strain clone compared with the published U1 strain sequence (Goelet *et al.* 1982). UAG designates the leaky amber terminator (nucleotides 3417–3419). **B**, Summary of genome exchange experiments. Open bars indicate restriction fragments obtained from M strain cDNAs; closed bars indicate those from U1 strain cDNAs (Holt and Beachy, in press). *In vitro* transcripts from recombinant clones were inoculated onto Xanthi tobacco plants, and plants were visually scored for intensity of disease symptoms at 10 days after inoculation. +++++, severe light and dark green mosaic; +, mild chlorosis; and +++ and ++, intermediate symptoms.

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                          G                                T      E
TMV   (315)FL---LYK--GVAHKSVDSEQFYTAM(335)..(350)SERILLEDSSSVNYWFPKMRDMVIVPLF(377)
ToMV  (315)FL---LYK--GVAHKGVDSEQFYKAM(335)..(350)SERILLEDSSSVNYWFPKMRDMVIVPLF(377)
TRV   (325)FS---IYRIAGVPRRSLSSQEYYRRI(347)..(349)-----------ISRW----ENMVVVPIF(361)
BSMV  (338)-A---MYH---MKYRGMKRDETFK--(354)..(357)P---LLKNSS------------VVVPLF(369)
BMV   (257)FFTESVHCIDGTTYLLEREMLKCNIM(283)..(294)CPRETLRHCV----WFEDISKYVGVSIP(317)
CMV   (288)FMTDQTYSFNGMTYGIERCVIYAGVM(313)..(324)CPPELIRHCI----WFPSMKDYVGLKIP(347)
AMV   (276)YLTYNAVDLGHAAYRIERKQHFGGVM(301)..(316)-PHSNGRSCA----WYNRVKGQMVVHTV(338)
                                                                       : :

                   V                                  A            K
TMV   (406)RTYQAKALTYANVLSFVESIR(426)..(577)SQMCQSLEVDPMTAAKVIVAVMSNESGLTLTFERP(611)
ToMV  (406)RTYQAKALTYSNVLSFVESIR(426)..(577)SQMCQSLEVDPMTAAKVIVAVMSNESGLTLTFEQP(611)
TRV   (390)ARLSDQQLTISNVKSYLSSNN(410)..(624)SAKFSNYSGSSVRTSPPSV-VGSSRSGLGLLLEDS(657)
BSMV  (396)MKNKEKDLNVAVLKNYLSAVN(416)..(573)-TECEK-DLGPLVQPIKEILVQLVMPNLVRALCRP(605)
BMV   (347)KESKEWTENMKAVASILSAKS(367)..(497)----------EVQTAKTKRSKKKAKVPPAAEIPQE(521)
CMV   (377)NDKKNWMDLFKIILGVLSSKS(397)..(517)----------AVADGTILAEEKAKKLADRLAVPVE(541)
AMV   (374)RPNADAHSAIQSIATMLSSST(394)..(596)AEWHQGNAIDASNYARTLLDDIRKQKEESLKAKAK(630)
              :     : : :::

                     R                                 T
TMV   (658)AGDHPESS--YSKNEEIESLEQF(678)..(737)T-----RQKFGVLDVASRKWLIKPTA(757)
ToMV  (658)SGDVPESS--YTRSEEIESLEQF(678)..(737)T-----RQKFGVLDVASKRWLVKPSA(757)
TRV   (723)ENVLPAVKPLVSKGKTVKRVDYF(745)..(800)TGGQNYPHGLGVWDVEMKNWCIRP--(823)
BSMV  (662)DEDMSRRTGMPPRPKVTSS---Y(681)..(727)A-------RFYSFQSLRPGWVFKTPS(745)
BMV        ---------------------       ..(599)----EIANKSIFETYHRIDDMVNVHL(620)
CMV   (579)--------------------PIA--(581)..(621)RNSLESNLKFVFDTYFSVDALVNVHF(646)
AMV   (681)--------------------PLPEF(685)..(740)WAGDDKRRAFIPKKNTWVGPTARS-Y(764)
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Fig. 2. Position of M strain amino acid changes in reference to aligned sequences from portions of various plant virus RNA-dependent RNA polymerases. Letters above the tobacco mosaic virus (TMV) sequence indicate changes in the M strain sequence compared with the U1 strain sequence. Numbers before and following amino acid residues represent the positions of those amino acid residues within the RNA-dependent RNA polymerase sequence. : designates strongly conserved amino acid residues with a score of eight or better in a Dayhoff PAM matrix of amino acid similarity. The Clustal program (Higgins and Sharp 1988) aligned the invariant GXXGXGKS/T consensus NTP-binding motif (Gorbalenya and Koonin 1989) of each sequence. TMV (translated from the nucleotide sequence of variant 1 of Goelet *et al.* [1982]); ToMV, tomato mosaic virus (Ohno *et al.* 1984); TRV, tobacco rattle virus (Hamilton *et al.* 1987); BSMV, barley stripe mosaic virus (Gustafson *et al.* 1989); BMV, brome mosaic virus (Ahlquist *et al.* 1984); CMV, cucumber mosaic virus (Rezaian *et al.* 1985); and AMV, alfalfa mosaic virus (Cornelissen *et al.* 1983).

of the M strain. A third amino acid change at position 416 (Ala to Val) is not conserved between U1 (Ala) and ToMV (Ser) and, therefore, is not likely to be involved in symptom attenuation. Thus, unless changes in RNA sequence that are silent in regard to amino acid sequence affect symptom development, the changes at amino acid residues 360, 367, 587, 601, and 747 are the most likely to be involved in symptom attenuation.

DISCUSSION

The production of an infectious cDNA clone has made it possible to identify the molecular cause of symptom attenuation shown by the M strain of TMV. Through genome exchanges, the attenuated phenotype was mapped to base changes in the 126/183-kDa ORF (Fig. 1). Thus, an alteration in the MP coding sequence is not likely to be the cause of attenuation, as suggested by Asselin *et al.* (1984). The attenuated phenotype of several polioviruses has been mapped to either the 5′ noncoding region or the CP coding region; however, in some hepatitis viruses the nonstructural proteins may be important for attenuation (reviewed by Cohen 1989).

Apart from our sequence for the M strain of TMV, the L_{11}A strain of ToMV is the only other naturally derived attenuated plant virus that has been sequenced (Nishiguchi *et al.* 1985). L_{11}A, which shows attenuated symptoms on both tomato and tobacco (Goto and Nemoto 1971), has 10 base mutations compared with the more virulent L strain, three of which result in amino acid changes. All of the amino acid changes reside in the 126/183-kDa coding region (amino acid residues 348, 759, and 894). The authors suggested that the amino acid substitution at residue 348 contributed significantly to the attenuated symptoms. However, specific mutagenesis or genome exchange experiments between infectious cDNA clones of the L_{11}A and L strains (Meshi *et al.* 1986) were not reported, and thus the contribution of this amino acid change in attenuation has not been conclusively demonstrated. In addition, results of experiments with both the L_{11}A strain and M strain attenuated viruses do not address the possibility that mutations in the RNA sequence which are silent for amino acid changes may affect phenotype (Daubert 1988). It is also possible that alterations in the (+) strand may affect (−) strand RNA structure or proteins synthesized from the (−) strand, although in TMV only small ORFs are found in the (−) strand and no protein encoded by these ORFs has yet been detected.

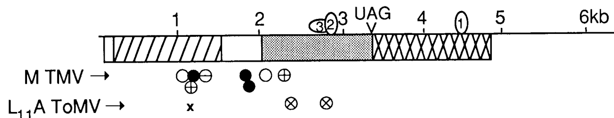

Fig. 3. Position of amino acid changes within the open reading frame of the putative RNA-dependent RNA polymerases (RdRps) of tobacco mosaic virus (TMV) and tomato mosaic virus (ToMV). The open reading frame of the 126/183-kDa RdRps is represented by a bar. UAG indicates the leaky amber terminator. The three internal regions that have sequence similarities with RdRps from other plant viruses are indicated by hatch marks or shading (adapted from Strauss and Strauss 1988, with permission from the Annual Review of Microbiology, Vol. 42, © 1988 by Annual Reviews Inc.). **Circled** numbers indicate the position of conserved sequence motifs within RdRps from this family: 1, the GDD motif (Kamer and Argos 1984); 2, the Hodgman motif (Hodgman 1986); and 3, the GXXGXGKS/T motif (Gorbalenya and Koonin 1989). Symbols below the bar indicate the position of amino acid changes from the virulent parental virus strains. M strain: ●, amino acid changes resulting in different charge or backbone properties and not seen in the virulent ToMV strain; circled +, amino acid changes not seen in the virulent ToMV strain; circled −, amino acid change not identical to the change observed in the virulent ToMV strain; and O, amino acid changes identical to that in the virulent ToMV strain. L_{11}A strain: X, amino acid change observed in both L_{11}A and a second attenuated ToMV strain, L_{11} (Oshima *et al.* 1965); and circled X, amino acid changes observed only in L_{11}A.

Presuming that the amino acid changes in the 126/183-kDa ORF are significant, none of the changes in the attenuated M strain occur in any of the recognized sequence motifs identified in this region (Fig. 3) (Kamer and Argos 1984; Hodgman 1986; Gorbalenya *et al.* 1988a, 1988b; Gorbalenya and Koonin 1989; Habili and Symons 1989). Ahlquist *et al.* (1985) identified three domains of similarity in the RdRps of several plant viruses and an animal virus (Sindbis) (recently reviewed by Goldbach 1986; Strauss and Strauss 1988). The amino acid changes observed between the RdRps of virulent and attenuated strains of TMV and ToMV all reside in the first two conserved domains as well as in the less conserved region between these domains in TMV (Fig. 3). None of these changes in the M strain occur in strongly conserved positions when compared with sequences from other plant viruses within the Sindbis-like superfamily (indicated amino acid residues in Fig. 2). We are currently introducing point mutations in the M strain infectious clone to further define the base changes responsible for the attenuation.

Although the M and L_{11}A strains both have changes in the 126/183-kDa region relative to the more virulent U1 or L strains, respectively, their phenotypes on inoculated leaves are apparently different from each other. M strain viral RNA and transcripts from the infectious cDNA clone produce normal size necrotic lesions on Xanthi-nc tobacco and normal virus accumulation in the inoculated leaves of Xanthi tobacco when compared with the U1 strain of TMV (unpublished data). L_{11}A produces smaller necrotic lesions (Nishiguchi and Oshima 1977), and virus accumulation declined significantly in inoculated leaves 5 days after inoculation compared with the more virulent L strain of ToMV (Kiho and Nishiguchi 1984). Thus, although the lack of virulence of M and L_{11}A maps to the same region and both strains were selected under high-temperature regimes (Holmes 1934; Oshima *et al.* 1965), the phenotype is markedly different.

It has been shown that *cis*-acting alterations such as mutation or deletion of the viral CP (Dawson *et al.* 1988; Culver and Dawson 1989; Saito *et al.* 1989), the 3′ noncoding region (Ishikawa *et al.* 1988; Eggen *et al.* 1989), or intercistronic promoter regions (French and Ahlquist 1987; Marsh *et al.* 1988; Grakoui *et al.* 1989) can all modify symptom development. It is also possible that alterations in the RdRp can, through *trans*-acting functions, result in attenuation. Kroner *et al.* (1989) produced defined mutations in RNA 2 of an infectious brome mosaic virus cDNA clone. RNA 2 contains the third conserved domain found in RdRps from the Sindbis-like virus family (Strauss and Strauss 1988). Although several of their mutants did not replicate, some mutants differentially suppressed genomic

over subgenomic RNA accumulation. The authors suggested that the 2a protein may have a role in differential initiation of replication of specific viral RNA classes. Traynor and Ahlquist (1990), through the production of precise hybrids of brome mosaic virus and cowpea chlorotic mottle virus infectious cDNA clones, have obtained evidence suggesting that RNA 1, which contains conserved domains 1 and 2, may also play a role in differential responses to specific viral RNA templates. Similarly, Roossinck and Palukaitis (1990) prepared pseudorecombinants between the RNAs of mild and severe strains of cucumber mosaic virus, and they determined that RNA 1 is responsible for the severity and rate of symptom induction in zucchini squash.

At this time, we cannot eliminate the possibility that *cis*-acting phenomena, such as RNA stability or replication of the RdRp ORF, could cause the attenuated phenotype in our infectious M strain clone. However, the observation that infection causes normal size necrotic lesions on hypersensitive hosts and virus accumulation equal to U1 in systemic hosts as determined by ELISA (unpublished data) suggests that virus replication and movement from cell to cell are unimpaired in these infections. Thus, the RdRp and MP transcripts and their translation products do not appear to be limiting. We are currently investigating whether the mutations in the attenuated M strain could disrupt the ability of the RdRp to recognize the promoters in regions other than those for the MP or RdRp transcripts.

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