Mutational Analysis of the Pseudoknot Region in the 3' Noncoding Region of Tobacco Mosaic Virus RNA

NOBUHIKO TAKAMATSU,^{†*} YUICHIRO WATANABE, TETSUO MESHI, AND YOSHIMI OKADA

*Department of Biophysics and Biochemistry, Faculty of Science,
University of Tokyo, Hongo 113, Japan*

Received 12 March 1990/Accepted 6 May 1990

The approximately 200-nucleotide-long 3'-terminal noncoding region of tobacco mosaic virus (TMV) RNA contains a tRNA-like structure and, in its immediate upstream region, three consecutive pseudoknots, each of which is composed of two double-helical segments. To elucidate the biological functions of the pseudoknot region, we constructed several deletion mutant TMV-L (a tomato strain) RNAs by using an *in vitro* transcription system and tested their ability to multiply in both tobacco plants and protoplasts. When deletions were introduced just downstream of the termination codon of the coat protein gene in the 5'-to-3' direction progressively, five of six double-helical segments were dispensable for viral multiplication, indicating that the pseudoknot structures are not essential for multiplication. However, extension of the deletion into the central pseudoknot region resulted in reduction in viral multiplication, accompanied by loss of development of mosaic symptoms on systemic tobacco plants. Cessation of multiplication was observed when the sequence involved in formation of double-helical segment I just upstream of the tRNA-like structure was deleted irrespective of the start point and extent of deletion. Point mutations that destabilized double-helical segment I resulted in a loss or great reduction of viral multiplication, whereas the double mutants in which the double helix was restored by additional compensating base substitutions restored multiplication to nearly the wild-type level. Thus, double-helical segment I just upstream of the tRNA-like structure is a structural feature essential for viral multiplication.

Tobacco mosaic virus (TMV) is a plant virus with a messenger-sense, single-stranded RNA of about 6,400 nucleotides (24). The replication cycle of TMV RNA includes synthesis of the minus-strand RNA complementary to TMV RNA, and the 3'-terminal portion of TMV RNA is thought to play an important role in its synthesis.

Many single-stranded plant RNA viruses possess 3'-terminal tRNA-like structures that serve as substrates for tRNA-specific enzymes (7, 8). Despite many functional similarities between tRNAs and viral 3'-terminal tRNA-like structures, the predicted structures of the tRNA-like termini often deviate from the cloverleaf of canonical tRNA. However, structural resemblance of the viral 3'-terminal tRNA-like structures to tRNA can be improved when the contribution of pseudoknotting to the tertiary structure is taken into consideration (21). Moreover, in the case of TMV RNA, three consecutive pseudoknots exist in the region immediately upstream of the tRNA-like structure; they can be found in all of the tobamovirus RNAs whose sequences of the 3'-terminal portions have been determined, and their existence is supported by chemical and enzymatic structure mapping (6, 23). TMV-L (a tomato strain) chimeras that carry the 3' noncoding region replaced with the corresponding region of TMV-OM (a common strain), cucumber green mottle mosaic virus, or TMV-Cc (a cowpea strain) multiply in tobacco plants and protoplasts (11). Thus, assuming that the 3'-terminal portion of TMV RNA is recognized on initiation of minus-strand synthesis, its higher structural features rather than sequence may play an important role.

The development of an *in vitro* transcription system allows us to manipulate TMV RNA at the level of DNA (3, 16). To understand the biological functions of the well-conserved, highly structural pseudoknot region, we constructed mutant TMV RNAs and analyzed their multiplication in tobacco plants and protoplasts.

MATERIALS AND METHODS

Plasmid construction. All plasmids were constructed from pLFW3 (16) by standard recombinant techniques (15). pLFW3 is a plasmid carrying a full-length cDNA copy of TMV-L RNA of 6,384 nucleotides (19) immediately downstream of the *p_m* promoter (2). pLFW3 linearized at the unique *Mlu*I site, which is located just downstream of the TMV-derived sequence, was used as a template for *in vitro* runoff transcription of infectious TMV RNA. The TMV sequence is numbered from the G residue at the 5' end (19).

(i) **Deletion constructions.** pLFW3 was digested with *Nsi*I at residue 6183 just downstream of the termination codon of the coat protein gene and treated with *Bal*31 nuclease. After digestion with *Bam*HI downstream of the TMV sequence, the *Bal*31-generated 3'-terminal noncoding fragments were cloned into pUC18 between the *Bam*HI and *Hinc*II sites to construct pL3NC plasmids. Deletion endpoints were characterized by restriction mapping and dideoxy sequencing (14), which revealed that the junction sequences derived from the *Hinc*II recognition sequence of pUC18 were heterogeneous, probably because of contaminated exonuclease (see later). The *Bal*31-generated 3'-terminal fragments were recovered from the selected pL3NC plasmids by digestion with *Pst*I in the polylinker sequence of pUC18 and *Mlu*I. pLD3N-series plasmids were constructed by replacing the *Nsi*I-*Mlu*I fragment of pLFW3 with the *Pst*I-*Mlu*I fragments of pL3NC plasmids. Thus, pLD3N-series plasmids should

* Corresponding author.

[†] Present address: Laboratory of Molecular Biology, School of Hygienic Sciences, Kitasato University, 1-15-1 Kitasato, Sagami-hara, Kanagawa 228, Japan.

have an additional GGTC sequence derived from the poly-linker sequence of pUC18 between the *HincII* and *PstI* sites, but this was not the case for all clones. The additional sequences were G for pLD3N-6207, -6209, and -6233, GG for pLD3N-6189, -6246, -6253, and -6263, GGT for pLD3N-6257, and GGTC for pLD3N-6204 and -6239.

To construct pLD3S-series plasmids, the filled-out *PstI*-*MluI* fragments of the pL3NC plasmids were used to replace the *SnaBI* (residue 6231)-*MluI* fragment of pLFW3. In the pLD3S-series plasmids, the junction sequences derived from the *SnaBI* recognition sequence, TAC, were not uniform, probably because of contaminated exonuclease: AC was lost for pLD3S-6239, -6253, and -6263, and TAC was lost for pLD3S-6275.

To introduce deletions confined to the 3' pseudoknot region, a fragment between the *NsiI* site (residue 6183) and residue 6249 was synthesized essentially according to Hong (9). The *Sau3AI*-*EcoRI* fragment of pLFW3 (residues 5934 to 6354) was first cloned into M13mp9 between the *BamHI* and *EcoRI* sites, and then the single-stranded DNA containing the TMV sequence in the plus polarity was prepared. The single-stranded DNA was annealed with the 15-mer synthetic DNA complementary to the TMV sequence between residues 6235 and 6249, and the first strand was synthesized with the large fragment of *Escherichia coli* DNA polymerase I. After synthesis of the second strand by using the M13 reverse primer, the resulting double-stranded DNA was digested with *NsiI*, and then the relevant fragment was ligated with the filled-out *PstI*-*MluI* fragment of pL3NC plasmids, the *KpnI*-*NsiI* fragment (residues 4390 to 6183), and the larger *KpnI*-*MluI* fragment of pLFW3 to construct pLD3P-series plasmids. Junction sequences were confirmed by the dideoxy method (14).

(ii) **Point mutations (pLNP-series plasmids).** To introduce point mutations in double-helical segment I in the 3' pseudoknot region, the *PvuII* (residue 6016)-*BamHI* (downstream of TMV cDNA) fragment of pLFW3 was cloned between the *EcoRV* and *BamHI* sites of pBluescript SK-M13(+) (pMTMV3). Mutagenesis was carried out on uracil-containing single-stranded DNA from pMTMV3 as described by Kunkel et al. (12), using a commercial kit (Takara Shuzo Co.). Mutants were identified by dideoxy sequencing (14). From the mutated plasmids, the *NsiI*-*MluI* fragments were purified and ligated with the *KpnI*-*NsiI* fragment (residues 4390 to 6183) and the larger *KpnI*-*MluI* fragment of pLFW3 to construct pLNP-series plasmids.

Transcription, reconstitution, and inoculation. pLFW3 and its derivatives were linearized at the unique *MluI* site and used to prepare in vitro transcripts (16). These transcripts were reconstituted with the coat protein of TMV-OM (a common strain) in vitro and inoculated into tobacco leaves according to Meshi et al. (16). *Nicotiana tabacum* L. cv. Xanthi nc and Samsun were used as a local lesion host and a systemic host, respectively. For inoculation into the systemic host, the reconstituted in vitro transcripts were diluted to a concentration at which the inoculum (20 to 60 μ l) gave 50 to 100 local lesions. Progeny viruses were extracted from the inoculated leaves of *N. tabacum* L. cv. Samsun and concentrated essentially as described previously (20). Viral spread in the systemic host plants was examined by back-inoculation: the upper, uninoculated leaves of the inoculated plants at 2 to 3 weeks postinoculation were ground in 10 mM phosphate buffer (pH 7.0), and an infectivity assay was carried out by inoculation into the local lesion host plants, using the extract as inoculum.

Analysis of RNA and protein syntheses in protoplasts.

Protoplasts were isolated from suspension-cultured cells of *N. tabacum* L. cv. BY-2 (26). In vitro transcripts were inoculated into tobacco protoplasts by the electroporation method as described by Watanabe et al. (26) except that DNase I treatment after the in vitro transcription was omitted. Two to five replicate experiments were done with each clone. For analysis of production of TMV-specific RNAs and proteins, protoplasts were labeled by adding [3 H] uridine or [3 S]methionine to the medium for 2 h as described by Watanabe et al. (25) except that dactinomycin was not added. Analysis of RNAs and proteins was done by fluorography after 1.0% agarose gel electrophoresis (13) and sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (25), respectively. For Northern (RNA) hybridization, total RNA was extracted from inoculated protoplasts at 24 h postinoculation and treated with DNase I. After normalization for amounts of rRNAs, Northern blot analysis of total RNA was performed as described previously (11), using a nick-translated full-length cDNA plasmid as probe. The amount of the genomic RNA accumulated at 24 h postinoculation was quantitated by densitometry of autoradiographs of several exposure times.

RESULTS

Construction of deletion mutant TMV RNAs. Of the 202-nucleotide-long 3' noncoding region of TMV-L RNA (19), the 3'-terminal portion of 105 nucleotides (residues 6280 to 6384) can be folded into a tRNA-like structure (22); its upstream sequence of 75 nucleotides (residues 6203 to 6277) contains three pseudoknot structures, each of which is composed of two double-helical segments (23) (Fig. 1A). The pseudoknots of residues 6203 to 6225, 6226 to 6247, and 6248 to 6277 are hereafter referred to as the 5', central, and 3' pseudoknots, respectively (Fig. 1A).

pLFW3 (16) carries the full-length cDNA of TMV-L RNA downstream of the modified lambda *p_R* promoter (2) and has been used as the standard template for in vitro transcription of infectious TMV-L RNA (10). On the basis of pLFW3, we constructed three types of mutant plasmids carrying deletions in the 3' noncoding region (Fig. 1B); the deletions of the respective series plasmids toward the 3'-end start from residue 6188 in the region immediately downstream of the termination codon of the coat protein gene (pLD3N-series plasmids), from residue 6234 in the central pseudoknot region (pLD3S-series plasmids), and from residue 6250 in the 3' pseudoknot region (pLD3P-series plasmids). In vitro transcripts derived from the respective series plasmids are designated N-, S- and P-X, where X stands for the number of the endpoint of the deletion. The wild-type transcript derived from pLFW3 is designated W3.

Deletions extending from downstream of the termination codon of the coat protein gene (N-series RNAs). To test the ability of the N-series RNAs to multiply, in vitro transcripts derived from pLD3N-series plasmids were reconstituted with the coat protein, followed by inoculation into *N. tabacum* L. cv. Xanthi nc (a local lesion host). RNAs for all N-series plasmids except N-6263 and -6275 produced local lesions (Fig. 1B). However, local lesions produced by N-6253 were a little smaller than those produced by TMV-L, W3, and the other infectious N-series RNAs.

When inoculated into a systemic host, *N. tabacum* L. cv. Samsun, N-6209, and the mutant RNAs with shorter deletions showed typical systemic mosaic symptoms in the upper, uninoculated leaves at about 1 week postinoculation, as did W3 (Fig. 1B) (see Materials and Methods). As for

FIG. 1. Deletion analysis of the pseudoknot region. (A) Model of the three-dimensional folding of the 3' noncoding region of TMV-L RNA (above) and a model showing the interactions (dashed lines) involved in the formation of the pseudoknots (below). They are depicted by analogy to TMV RNA (vulgare strain) (23). Roman numerals indicate the six double-helical segments involved. (B) Schematic representation of individual deletions and the results of an inoculation assay on a local lesion host (L) and a systemic host (S). The sequence is numbered from the 5' end of the genomic RNA (19) and by arrowheads above the sequence; nucleotides are numbered from residue 6190 every 10 nucleotides. CP, Coat protein gene. Residues underlined or overlined are those forming base pairs (23). The 5', central (C), and 3' pseudoknots, indicated at the bottom, consist of double-helical segments VI and V, IV and III, and II and I, respectively. The extent of deleted sequences for each mutant is represented by a solid bar. At the right are shown the results of an inoculation assay; +, production of local lesions (in column L) or development of systemic mosaic symptoms at 2 weeks postinoculation (in column S).

FIG. 2. N-series RNAs: detection of TMV-specific RNAs by Northern hybridization. Tobacco protoplasts were inoculated with W3 (lane 1), N-6209 (lane 2), N-6239 (lane 3), N-6246 (lane 4), N-6253 (lane 5), or N-6263 (lane 6) or were mock inoculated (lane M). Total RNAs were extracted at 24 h postinoculation, electrophoresed on a 1.0% agarose gel after DNase I treatment, and processed for hybridization, using a nick-translated full-length cDNA plasmid as probe. Positions of the genomic RNA and the subgenomic RNA for the coat protein (CP mRNA) are indicated on the right. The amounts of genomic RNA accumulation of the mutant RNAs at 24 h postinoculation relative to that of W3 were 0.7 (lane 2), 0.3 (lane 3), 0.2 (lane 4), and 0.1 (lane 5).

N-6233, -6239, and -6246, systemic mosaic symptoms did not develop (Fig. 1B), but infectivity that was one-fifth or less that of W3 was recovered from the upper, uninoculated leaves, indicating the systemic spread of these mutants. In the case of N-6253, infectivity was not recovered from the upper, uninoculated leaves by the back-inoculation tests (see Materials and Methods).

Multiplication of the N-series RNAs was further examined by inoculating N-6209, -6239, -6246, -6253, and -6263 into tobacco protoplasts. Accumulation of TMV-specific RNAs was analyzed by Northern hybridization of total RNAs extracted from the inoculated protoplasts at 24 h postinoculation (Fig. 2). The genomic RNA accumulation of N-6209 was a little lower than that of W3 (Fig. 2, lanes 1 and 2). In the cases of N-6239 and -6246, which are devoid of both the 5' and central pseudoknots, further deletion extending into the central pseudoknot region resulted in decreased levels of genomic RNA accumulation that were about one-fourth that of W3 (Fig. 2, lanes 3 and 4). N-6253 showed only 1/10 the genomic RNA accumulation of W3 (Fig. 2, lane 5). Since N-6253 lacks the sequence of residues 6248 to 6251, which is involved in formation of double-helical segment II (Fig. 1A), no pseudoknot structure is considered to be formed, and the observation thus indicates that the pseudoknot structures are not essential for viral multiplication. For N-6263, in which the sequence of residues 6257 to 6261, which are involved in formation of double-helical segment I, is eliminated (Fig. 1A), no TMV-specific RNA could be detected by Northern hybridization (Fig. 2, lane 6) even upon longer exposure.

TMV-specific RNA and protein syntheses were concurrently examined by pulse-labeling with [3 H]uridine and [35 S]methionine, respectively, for 2 h from 6 or 22 h postinoculation, followed by electrophoresis and fluorography. The results were consistent with those of Northern analysis described above (data not shown).

Deletions extending from the central pseudoknot region (S-series RNAs). The analysis of the N-series RNAs revealed that with expansion of the deletion extending from the region

FIG. 3. S- and P-series RNAs: detection of TMV-specific RNAs by Northern hybridization. Tobacco protoplasts were inoculated with W3 (lane 1), S-6239 (lane 2), S-6246 (lane 3), S-6253 (lane 4), S-6263 (lane 5), S-6275 (lane 6), P-6253 (lane 7), P-6263 (lane 8), or P-6275 (lane 9) or were mock inoculated (lane M). Northern hybridization of total RNAs extracted at 24 h postinoculation was carried out as for Fig. 2. The amounts of genomic RNA accumulation of the mutant RNAs relative to that of W3 were 0.1 (lane 2), 0.5 (lane 3), 0.9 (lane 4), and 0.3 (lane 7).

immediately downstream of the termination codon of the coat protein gene, the mutant RNAs showed reduction in ability to multiply and that the deletion reaching residue 6263 abolished detectable multiplication. To determine whether the loss of ability to multiply for N-6263 is due to lack of some sequence essential for multiplication or simply due to the large extent of deletion, and also to search for correlation between the lack of sequence in the central and 3' pseudoknot regions and the lack of the systemic mosaic symptom development, we constructed S-series RNAs (Fig. 1B).

The S-series RNAs were first assessed for infectivity by inoculation into local lesion host plants (Fig. 1B). S-6239, -6246, and -6253 produced local lesions, whereas neither S-6263 nor -6275 did so (Fig. 1B). When inoculated into systemic host plants, none of the five S-series RNAs showed mosaic symptoms (Fig. 1B).

The S-series RNAs were further analyzed by inoculation into tobacco protoplasts (Fig. 3). In the cases of S-6263 and -6275, no TMV-specific RNA could be detected (Fig. 3, lanes 5 and 6). Thus, it is likely that the deletion passing over residue 6263 was responsible for the loss of viral multiplication irrespective of the extent of deletion. Among the other three viable S-series RNAs, S-6239, whose deletion is shortest, showed the smallest amount of genomic RNA accumulation, and S-6253, with the largest deletion, showed the largest accumulation (Fig. 3, lanes 2 to 4).

Deletions introduced in the 3' pseudoknot region (P-series RNAs). To define the sequence essential for viral multiplication in the pseudoknot region, three P-series RNAs that had deletions of 4 (P-6253), 14 (P-6263), and 26 (P-6275) nucleotides confined to the 3' pseudoknot region were tested for multiplication. Only P-6253 produced local lesions (Fig. 1B). No mutant RNA showed systemic mosaic symptoms (Fig. 1B). By protoplast inoculation analysis, TMV-specific RNAs were detected only for P-6253 (Fig. 3, lanes 7 to 9). Results for the P-series RNAs confirmed that abolishment of multiplication of the deletion mutants was due not to the extent of deletion but rather to deletion of the sequence essential for viral multiplication. The results for P-6253 and -6263 suggest that the sequence between residues 6254 and 6263 contains information essential for viral multiplication.

Point mutations in double-helical segment I (LNP-series RNAs). The 5'-proximal five double-helical segments (II to VI) are dispensable for multiplication, and all of the inviable deletion mutants lack the ability to form double-helical segment I. To clarify whether the stem-loop structure formed by double-helical segment I is important for viral multiplication and whether the sequence identity between residues 6254 and 6263 plays an important role, we created mutant TMV RNAs by introducing point mutations into double-helical segment I (LNP-1 to -9) or the unpaired loop region (LNP-10 to -12) (Fig. 4A) and examined their ability to multiply in protoplasts. These LNP-series RNAs are derivatives of W3 and should retain intact double-helical segments II to VI.

When one or two nucleotides in either strand of the helix were changed to their Watson-Crick complements, LNP-1, -2, -4, and -6 (Fig. 4A), genomic RNA accumulation could not be detected at 24 h postinoculation (Fig. 4B) except that in the case of LNP-4, genomic RNA less than 1/1,000 that of W3 was detected in one experiment (data not shown). Next analyzed were LNP-3, -5, and -7, in which the secondary point mutations were introduced to compensate for the mutations of LNP-1, -2, -4, or -6 to restore the helix (Fig. 4A). These double mutants multiplied to a level comparable to that of W3 (Fig. 4B). In contrast, two mutants, LNP-8 and -9 (Fig. 4A), multiplied to a level about one-fourth that of W3 (Fig. 4B), although their mutations are located in the center of the helix and should destabilize the helix. These observations indicate not only the existence of double-helical segment I but also its importance for viral multiplication. Thus, it is likely that the inviability of N-, S-, and P-6263 resulted from destruction of double-helical segment I. The results also imply that the sequence identity at least at the mutated positions (residues 6257 to 6260 and 6274 to 6277) is not important. Since the U-to-A point mutation at residue 6260 in LNP-8 did not abolish multiplication, the U · A base pair between residues 6260 and 6274 is not essential; this is also likely to be the case with the C · G base pair between residues 6261 and 6272 because this base pair does not seem to occur in LNP-9. The result for LNP-9 indicates that two C · G base pairs between residues 6257 and 6277 and residues 6258 and 6276 seem sufficient for the functioning of double-helical segment I. Although LNP-6 has mutations at the same positions as those of LNP-9, the inviability of LNP-6 may be ascribed to an alternate, incorrect secondary structure (see Discussion).

The AAAUCGAA sequence (residues 6267 to 6274) that spans the loop and helix regions (Fig. 4A) is conserved among the tobamovirus RNAs except TMV-U2, whose sequence is AAAUAUAA (6, 23). To determine whether the conserved sequence in the loop region of the stem-loop structure formed by double-helical segment I is important for viral multiplication, we analyzed three mutants, LNP-10, -11, and -12 (Fig. 4A). All three mutants multiplied (Fig. 4B). The amount of genomic RNA accumulated at 24 h postinoculation was comparable to that of W3 for LNP-12 and reduced to about one-fourth and one-half for LNP-10 and -11, respectively (Fig. 4B). Thus, the conserved sequence is not essential for viral multiplication, but the reduction in genomic RNA accumulation for LNP-10 and -11 suggests the importance of this sequence in effective replication.

DISCUSSION

TMV RNA carries three consecutive pseudoknot structures in the region immediately upstream of the 3'-terminal

tRNA-like structure, and their existence is supported by nuclease digestion experiments and sequence comparison of tobamovirus RNAs (6, 23). Analyses of the deletion mutant RNAs described above have shown that the 5'-proximal five double-helical segments (II to VI) out of six in the pseudoknot region are dispensable for viral multiplication. Thus, the pseudoknot structures are not essential. In contrast, it is also shown that the pseudoknot region is necessary for productive multiplication to the wild-type level. Presumably, the 3'-terminal region plays an important role in the initiation of minus-strand synthesis during replication. The pseudoknot structures might be required for efficient recognition of the 3'-terminal tRNA-like structure by TMV replicase. It is still possible that the pseudoknot structures contribute to stability of the genomic and 3'-coterminal subgenomic RNAs or influence translation.

Point mutational analyses have further revealed that double-helical segment I in the pseudoknot region that is located immediate upstream of the tRNA-like structure is a structural feature essential for viral multiplication. The result for LNP-9 suggests that the two base pairs at the bottom of double-helical segment I are likely to be sufficient for functioning of the helix. However, this does not apply simply to inviable LNP-6, whose mutations are at the same positions as those of viable LNP-9. It is plausible that the base substitutions in LNP-6 result in an incorrect secondary structure. In fact, a stable stem-loop structure can be formed in LNP-6 (Fig. 5), in which double-helical segment II remains intact. If formation of the alternate structure causes inviability of LNP-6, the result for LNP-6, together with the results for S- and P-6275, implies that the existence of a stem-loop structure upstream of the tRNA-like structure alone would not be sufficient for viral multiplication. The essential structural feature might be deduced from the differences between the two stem-loop structures in wild-type and LNP-6. The two structures differ in at least two respects: one is the distance between the 3' pseudoknot structure and the tRNA-like structure, and the other is the sequence of the loop region.

The AAAUCGAA sequence in the loop region (residues 6267 to 6274) is conserved among the tobamovirus RNAs except TMV-U2, whose sequence is AAAUAUAA (6, 23). The reduction in the genomic RNA accumulation of LNP-10 and -11 suggests that the sequence in the loop region satisfies some sequence requirement. Considering the possible role of the 3'-terminal portion in the minus-strand synthesis during replication, a *trans*-acting factor might directly recognize the sequence in the loop context of the stem-loop structure formed by double-helical segment I. Alternatively, the loop region might interact with the sequence in another region to form a higher-order structure essential for replication. In the case of brome mosaic virus (BMV), coinoculation experiments with BMV RNA1, RNA2, and deletion mutant RNA3 have shown that not only the tRNA-like structure but also the upstream sequence are required for normal accumulation of BMV RNA3 in vivo (5). In this upstream sequence of the tRNA-like structure of BMV RNA, stem-loop structures can be formed (1). Thus, for TMV and BMV, the tRNA-like structure may function in combination with its upstream stem-loop structure(s), although it remains unclear whether there are direct interactions between the two structures.

Interestingly, among the three viable S-series RNAs, S-6239, -6246, and -6253, the extent of deletion and the reduction of the ability to multiply did not parallel each other (Fig. 3, lanes 2 to 4). This observation would be a line of evidence supporting involvement of a higher structure of the

FIG. 4. Point mutational analysis of double-helical segment I. (A) Diagrams of the predicted base pairing within double-helical segment I of W3 and LNP-series RNAs. The conserved sequence (AAAUCGAA at residues 6267 to 6274) is boxed. Altered nucleotides are indicated by asterisks. (B) Detection of TMV-specific RNAs. Tobacco protoplasts were inoculated with W3 (lane W3) or LNP-series RNAs or were mock inoculated (lane M). Northern hybridization of total RNAs extracted at 24 h postinoculation was carried out as for Fig. 2.

pseudoknot region in viral multiplication. In S-6239 and -6246, the central pseudoknot structure was completely destroyed, and consequently the 5' and 3' pseudoknots were separated by 18 and 11 nucleotides, respectively. The difference in the distance between the two pseudoknots may

result in the difference in their ability to multiply. However, the central pseudoknot region contains three each of CGU and ACG sequences, all of which are responsible for formation of base pairing involved in the pseudoknot structure, and both mutant RNAs still retain one each, leaving the

FIG. 5. Possible RNA secondary structure for LNP-6. Altered nucleotides are indicated by asterisks.

possibility of formation of a stem-loop structure between the CGU and ACG sequences, thus, structural features in the residual sequence of the central pseudoknot region are also likely to have an effect on their multiplication. For S-6253, the central pseudoknot structure and double-helical segment II were destroyed, but if base pairs were formed between GUG of residues 6227 to 6229 and CAC of residues 6262 to 6264, a pseudoknot structure similar to the 3' pseudoknot could be reconstructed, separated from the 5' pseudoknot by only one nucleotide (Fig. 6). It would be possible that this highest ability for multiplication among the S-series RNAs resulted from the close location of the 5' and newly formed 3' pseudoknots.

Removal of the sequence in the central or 3' pseudoknot region resulted in the lack of the mosaic symptom development on *N. tabacum* L. cv. Samsun, the systemic host plants. Phytopathologically, the observation would suggest the difference in biological functions between the 5' and the other two pseudoknots. Considering the results of the inoculation assay of the S- and P-series mutant RNAs, the lack of mosaic symptom development was not dependent on the extent of the deletion but correlated with deletion in the central and 3' pseudoknot structures (Fig. 1B). As for N-6233, -6239, and -6246, S-6246 and -6253, and P-6253, although systemic mosaic symptoms did not develop, infectivity was recovered from the upper, uninoculated leaves of the inoculated systemic host plants, indicating the systemic spread of these mutants (data not shown). In the cases of N-6253 and S-6239, the back-inoculation experiments failed to detect the systemic spread (data not shown), but given their low ability to multiply, this observation would not necessarily rule out systemic spread. To determine whether the symptomless spread was due to the low concentration of the infectious transcript in the inoculum (see Materials and Methods), purified progeny virus of N-6239 was inoculated into *N. tabacum* L. cv. Samsun. Whereas the progeny virus of W3 caused systemic mosaic symptoms at 1 week postinoculation when inoculated at a concentration of 0.1 $\mu\text{g/ml}$,

FIG. 6. Schematic representation of possible interactions in the pseudoknot region of S-6253. The nucleotides added during plasmid construction are indicated by arrowheads. Residue numbers of the borders of the deletion are denoted.

the N-6239 progeny did not cause visible symptoms at 1 month postinoculation even on plants inoculated at a concentration of 10 $\mu\text{g/ml}$ (data not shown). Thus, the attenuated phenotype would be due to the reduced ability of the mutant RNA to multiply. TMV-L₁₁A, an attenuated strain derived from TMV-L, carries three amino acid substitutions in the 130K and 180K proteins (18), which are involved in viral RNA multiplication (10). In the case of TMV-L₁₁A, a reduced synthesis of the subgenomic RNA for the 30K protein, which is responsible for viral cell-to-cell movement (4, 17), has been observed in a protoplast system and is postulated to be the cause of the low virus yield in plants (27). Thus, in both cases, the low level of viral propagation in plants seems to result in the attenuated phenotype.

ACKNOWLEDGMENTS

We thank P. Ahlquist and Agrigenetics Research Associates for use of the p_m promoter and T. Shiba and K. Yoshioka for helpful discussion.

This work was supported by grants-in-aid from the Ministry of Education, Science and Culture and from the Ministry of Agriculture, Forestry and Fisheries, Japan.

LITERATURE CITED

- Ahlquist, P., R. Dasgupta, and P. Kaesberg. 1981. Near identity of 3' RNA secondary structure in bromoviruses and cucumber mosaic virus. *Cell* 23:183-189.
- Ahlquist, P., and M. Janda. 1984. cDNA cloning and in vitro transcription of the complete brome mosaic virus genome. *Mol. Cell. Biol.* 4:2876-2882.
- Dawson, W. O., D. L. Beck, D. A. Knorr, and G. L. Grantham. 1986. cDNA cloning of the complete genome of tobacco mosaic virus and production of infectious transcripts. *Proc. Natl. Acad. Sci. USA* 83:1832-1836.
- Deom, C. M., M. J. Oliver, and R. N. Beachy. 1987. The 30-kilodalton product of tobacco mosaic virus potentiates virus movement. *Science* 237:389-393.
- French, R., and P. Ahlquist. 1987. Intercistronic as well as terminal sequences are required for amplification of brome mosaic virus RNA3. *J. Virol.* 61:1457-1465.
- García-Arenal, F. 1988. Sequence and structure at the genome 3' end of the U2-strain of tobacco mosaic virus, a histidine-accepting tobamovirus. *Virology* 167:201-206.
- Haenni, A.-L., S. Joshi, and F. Chapeville. 1982. tRNA-like structure in genomes of RNA viruses. *Prog. Nucleic Acid Res. Mol. Biol.* 27:85-104.
- Hall, T. C. 1979. Transfer RNA-like structures in viral genomes. *Int. Rev. Cytol.* 60:1-26.
- Hong, G. F. 1981. A method for sequencing single-stranded cloned DNA in both directions. *Biosci. Rep.* 1:243-252.
- 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.
- Ishikawa, M., T. Meshi, Y. Watanabe, and Y. Okada. 1988. Replication of chimeric tobacco mosaic viruses which carry heterologous combinations of replicase genes and 3' noncoding regions. *Virology* 164:290-293.
- Kunkel, T. A., J. D. Roberts, and R. A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* 154:367-382.
- Laskey, R. A., A. D. Mills, and N. R. Morris. 1977. Assembly of SV40 chromatin in a cell-free system from *Xenopus* eggs. *Cell* 10:237-243.
- Luckow, B., R. Renkawitz, and G. Schutz. 1987. A new method for constructing linker scanning mutants. *Nucleic Acids Res.* 15:417-429.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- 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.
17. Meshi, T., Y. Watanabe, T. Saito, A. Sugimoto, T. Maeda, and Y. Okada. 1987. Function of the 30-kD protein of tobacco mosaic virus: involvement in cell-to-cell movement and dispensability for replication. *EMBO J.* **6**:2557–2563.
18. Nishiguchi, M., S. Kikuchi, Y. Kiho, T. Ohno, T. Meshi, and Y. Okada. 1985. Molecular basis of plant viral virulence; the complete nucleotide sequence of an attenuated strain of tobacco mosaic virus. *Nucleic Acids Res.* **13**:5585–5590.
19. Ohno, T., M. Aoyagi, Y. Yamanashi, H. Saito, S. Ikawa, T. Meshi, and Y. Okada. 1984. Nucleotide sequence of the tobacco mosaic virus (tomato strain) genome and comparison with the common strain genome. *J. Biochem.* **96**:1915–1923.
20. Otsuki, Y., I. Takebe, T. Ohno, M. Fukuda, and Y. Okada. 1977. Reconstitution of tobacco mosaic virus rods occurs bidirectionally from an internal initiation region: demonstration by electron microscopic serology. *Proc. Natl. Acad. Sci. USA* **74**:1913–1917.
21. Pleij, C. W. A., K. Rietveld, and L. Bosch. 1985. A new principle of RNA folding based on pseudoknotting. *Nucleic Acids Res.* **13**:1717–1731.
22. Rietveld, K., K. Linschooten, C. W. A. Pleij, and L. Bosch. 1984. The three-dimensional folding of the tRNA-like structure of tobacco mosaic virus RNA. *EMBO J.* **3**:2613–2619.
23. Van Belkum, A., J. P. Abrahams, C. W. A. Pleij, and L. Bosch. 1985. Five pseudoknots are present at the 204 nucleotides long 3' noncoding region of tobacco mosaic virus RNA. *Nucleic Acids Res.* **13**:7673–7686.
24. Van Regenmortel, M. H. V., and H. Fraenkel-Conrat. 1986. The plant viruses, vol. 2. The rod-shaped plant viruses. Plenum Publishing Corp., New York.
25. Watanabe, Y., Y. Emori, I. Oshika, 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.
26. 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.
27. 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.