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A Functional Role for the Conserved Protonatable Hairpins in the 5′ Untranslated Region of Turnip Yellow Mosaic Virus RNA

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The 5′ untranslated region (UTR) of the RNA of several tymoviruses contains conserved hairpins with protonatable internal loops, consisting of C-C and C-A mismatches (K. Hellendoorn, P. J. A. Michiels, R. Buitenhuis, and C. W. A. Pleij, Nucleic Acids Res. 24, 4910–4917, 1996). Here, we present a functional analysis of the 5′ UTR of turnip yellow mosaic virus (TYMV) RNA, which contains two protonatable hairpins with nearly identical internal loops. Mutations were introduced in an infectious cDNA clone, and T7 RNA transcripts were used to infect Chinese cabbage plants. Different symptoms were observed for the various mutants, pointing to a functional role of the C-C and C-A mismatches in the hairpins of the 5′ UTR. The replication of the virus is influenced by the mutations made, while in vitro translation studies showed that the expression of the two overlapping reading frames of TYMV is not influenced by the secondary structure of the leader. Various mutants were propagated for up to five serial passages of infection, and the sequence of the 5′ UTR was determined. This resulted in virus RNA with new non-wild-type sequences that produced the wild-type phenotype in infected plants. Remarkably, in all cases C-C or C-A mismatches were introduced. The internal loop of the 5′-proximal hairpin seems to be more important for the viral life cycle than that of the second hairpin. A deletion of 75% of the leader, including the two hairpins, resulted in a virus that was deficient in viral spread. Since the ratio between filled and empty capsids was changed drastically by this mutation, a role of the 5′ UTR in viral packaging is proposed.

Tymoviruses are a group of positive-strand RNA plant viruses, which consist of a monopartite genome and a shell of 180 identical coat protein subunits, arranged with T=3 symmetry. The RNA genome of the tymoviruses has a remarkable base composition: it contains about 40% cytosines and less than 20% guanines. The excess of unpaired cytosines is thought to be important for RNA-protein interactions inside the virion, and a role for protonated cytosine residues in stabilizing the virions was suggested (reference 11 and references therein). In vitro reconstitution experiments with empty capsids of turnip yellow mosaic virus (TYMV), the type member of the tymoviruses, and various RNAs indicated that protonated cytosines in single-stranded regions are involved in RNA-protein interactions (1, 10). Laser-Raman measurements of TYMV (7) and belladonna mottle virus (18) and nuclear magnetic resonance spectroscopy experiments of both viruses (21) pointed to an interaction between protonated cytosines and acidic amino acid side chains of the coat protein. Furthermore, the coat protein gene of TYMV RNA was shown to contain long, C-rich single-stranded regions, which are therefore candidates for these protein-RNA interactions (8). Unfortunately, recent X-ray diffraction studies of the virion did not reveal any detail of the RNA within the virion (2), which means that the exact character of the RNA-protein interactions still remains unclear.

The genome of the tymoviruses contains three different genes, two of which are overlapping (16). The coat protein is expressed from a subgenomic mRNA. The 5′-proximal overlapping genes code for a transport protein (overlapping protein [OP]) and the replicase (RP) and are 1887 and 5535 nucleotides in length, respectively. The RP gene is in the +1 frame with respect to the OP gene, and there are 4 nucleotides between the two AUG start codons. The secondary structure of the 5′ untranslated region (UTR) of the RNA of TYMV (see Fig. 1A) and of several other tymoviruses has been proposed (9). The leaders all contain one or more hairpins with a cytosine-rich internal loop. It was shown by UV-melting experiments and nuclear magnetic resonance spectroscopy measurements that all these internal loops are protonatable at a slightly acidic pH. The finding of these conserved protonatable cytosines led us to the hypothesis that the 5′ UTR is important for binding to the coat protein and functions as the initiation site of RNA encapsidation. Binding experiments with the viral capsid of TYMV and these RNA hairpins, however, did not yet establish this putative high-affinity binding of the 5′ UTR (9).

The 5′ UTR of TYMV RNA also functions in the initiation of translation. Unlike the Ω-leader of tobacco mosaic virus RNA, it probably does not strongly enhance translation (5). A correlation between the structure of the leader and the choice of the AUG start sites for the two alternate reading frames cannot be excluded.

Here we present a first analysis of the possible functional roles of the protonatable hairpins in the 5′ UTR of TYMV RNA by in vivo experiments, in which mutations were introduced in an infectious cDNA clone of TYMV. Most of these mutations led to a changed phenotype of the virus. Some of the mutant viruses were propagated for up to five passages, after which the 5′ UTR was sequenced. Several revertants were obtained with sequences differing from the wild type, which confirm the importance of the C-C and C-A mismatches in the 5′ proximal hairpin of the 5′ UTR of TYMV RNA. Also, the ratio of filled and empty viral capsids in a virus preparation was determined. Changes in this ratio indicated that in some of the mutants, the encapsidation was less efficient than for the wild-type virus.

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MATERIALS AND METHODS

Construction of cDNA mutants. To obtain the RNA of a set of mutants of TYMV, we used an infectious clone coding for the Blue Lake strain of TYMV, pBL16 (19). The 679-bp *Hin*dIII-*Bsr*GI fragment of the plasmid, containing the T7 promoter and the 5′ part of the viral genome, was cloned in M13mp18 cut with *Hin*dIII and *Acc*65I, leading to a construct named M13TYHB1. The same fragment was also ligated in a pUC19 plasmid cut with *Hin*dIII and *Acc*65I, leading to plasmid pTYBLHB1.

Mutations in hairpin 2 of the 5′ UTR of TYMV RNA were made by cutting the pTYBLHB1 construct with *Mun*I, filling in with T4 DNA polymerase in the presence of deoxynucleoside triphosphates or removing the unpaired nucleotides with nuclease S1, and religating the plasmid. These new constructs are pTYBLHB2 and pTYBLHB3. The *Hin*dIII-*Nco*I fragments of these plasmids were ligated into pBL16, which was cut with these enzymes, leading to the mutants pBL16a and pBL16b, respectively.

A *Bsi*WI restriction site was introduced in M13TYHB1 by Kunkel mutagenesis (14) just upstream of the region containing TYMV hp2 (see Fig. 1A), leading to the A61G substitution in the TYMV RNA. This construct, M13TYHB2, was cut with *Bsi*WI and *Mun*I, and two oligonucleotides with the sequences 5′GTAC ACTTGCAAGT3′ and 5′AATTACTTGCAAGT3′ were hybridized and ligated. This resulted in M13TYHB3, in which the internal loop of hp2 of TYMV RNA is removed. To delete hp2 completely, M13TYHB2 was cut with *Mun*I and *Bsi*WI, the ends were made blunt with T4 DNA polymerase in the presence of deoxynucleoside triphosphates, and the construct was religated, resulting in M13TYHB4. The *Hin*dIII-*Nco*I fragments of the last two new constructs were ligated into pBL16, leading to pBL16c and pBL16d, respectively.

Substitution mutants with mutations in hp1 of TYMV were made by Kunkel mutagenesis, introducing C33G and A35G and leading to a closure of the internal loop of hp1. This mutagenesis was performed on both M13TYHB1 and M13TYHB3. After the mutagenesis reaction, two other clones were picked up as well: A35G in M13TYHB1 and C33G/A35C in M13TYHB3. The *Hin*dIII-*Nco*I fragments of these four new constructs were ligated into pBL16, leading to pBL16e, pBL16f, pBL16g, and pBL16h, respectively. An additional *Mun*I restriction site was introduced just upstream of hp1 in the construct M13TYHB1 by Kunkel mutagenesis. This construct was cut with *Mun*I and religated, leading to a deletion of 66 nucleotides in the 5′ UTR of TYMV RNA. The *Hin*dIII-*Nco*I fragment of this construct was ligated into pBL16, leading to pBL16i.

RNA preparation and inoculation. Plasmid DNA of the various clones was purified on a CsCl gradient and linearized with *Nde*I. Transcription with T7 RNA polymerase (purchased from Pharmacia or isolated by the method of Van Belkum et al., [20]) in the presence of ^{m7}GpppG was performed as described previously (19). About 20 μl of the transcription reaction mixture, containing about 2 μg of RNA, was put on the first two true leaves of 2.5- to 3-week-old Chinese cabbage (*Brassica pekinensis* cv. Granaat) plants that had been slightly dusted with carborundum powder and was rubbed on the surface with a sterile toothpick. The plants were grown in a climate-controlled room (16 h of daylight at 4,000 lux from fluorescent lamps, with day and night temperatures of 26 and 22°C, respectively).

Analysis of the viral RNA. Virus was isolated from plants, at least 14 days after inoculation, by homogenizing part of a well-infected leaf in an Eppendorf tube followed by centrifugation. The supernatant was used directly for inoculation of new plants. For further analysis of the virions, isolation with bentonite was performed (3). The virus was concentrated and purified by dialysis on a microsep microconcentrator (Filtron) with a cutoff mass of 300 kDa. RNA was isolated by phenol-chloroform extraction. Reverse transcription-PCR was performed to the 5′-terminal part of the viral RNA of several isolates to screen for possible revertants.

Analysis of the viral capsid. About 10 μg of purified virus was put on a 1% agarose minigel. After electrophoresis in TAE buffer (Tris-acetate-EDTA [pH 8.0]) for 1 h at 90 V, the gel was stained with Coomassie brilliant blue. In this way, differences in the ratio between filled and empty capsids can be observed, since empty capsids have a slightly lower migration rate than genuine virions on agarose (7a).

In vitro translations. Use was made of the TnT-coupled reticulocyte lysate system (Promega) to express the genes of the various viral mutants. The standard protocol of Promega was followed. In each reaction, 1 μg of DNA was used, and the reactions were performed at 30°C for 2 h. The products were analyzed on a denaturing 6.5% acrylamide gel (25 mM Tris-HCl, 250 mM glycine [pH 8.3], 0.1% sodium dodecyl sulfate) which was run for 3 h at 200 V.

RESULTS

Mutants and symptoms. The secondary structure of the 5′ UTR of TYMV RNA has been studied previously by structure probing (9) and appears to be conserved among tymoviruses. The finding of conserved hairpins with internal loops that are protonatable due to C-C and C-A mismatches led us to perform a functional analysis of this region, by studying the in vivo effects of mutations in the 5′ UTR of TYMV RNA. A set of nine different mutants of TYMV RNA were constructed with deletions, substitutions, or insertions in the 5′ UTR (Fig. 1A). The internal loop of the second hairpin (hp2) was modified (mutants a and b) or deleted (mutant c), or the entire hairpin was deleted (mutant d). In other mutants, the first hairpin (hp1) was modified (mutants e and f), or hp1 and hp2 were modified simultaneously (mutants g and h). In one case (mutant i) 75% of the 5′ UTR was deleted, leaving a leader of only 21 nucleotides, which is probably non-base-paired. In all mutants, the sequence preceding the first AUG start codon, GCAA, is identical to that in the wild type. The secondary structures of the leaders of the mutant RNAs were verified by computer predictions with the Mfold program (23) and are shown in Fig. 1A.

The wild-type cDNA clone and the various mutants were transcribed in vitro, and the RNA was used to inoculate plants. All constructs were transcribed with the same efficiency (data not shown). The development of viral symptoms on both the inoculated and the younger leaves was monitored for up to 4 weeks. The symptoms that were observed for the various mutants were highly reproducible. The number of plants that were inoculated and the time of appearance of the first symptoms of the various mutants both on the inoculated leaves and on younger, expanding leaves, are summarized in Fig. 1B. Most mutants caused symptoms that differed from those due to the wild type, varying from almost wild type to no lesions at all.

When the second hairpin of TYMV RNA was mutated by an insertion or a deletion, the plants developed symptoms on both the primary and the secondary leaves, although the symptoms were much less severe than those due to wild-type virus. When the internal loop of this hairpin was deleted (mutant c), there were clearly more severe symptoms than when there was a bigger hairpin loop or internal loop (mutants a and b). When the entire hairpin was deleted (mutant d), only half of the plants developed symptoms, which were similar to the symptoms of plants infected with mutants a and b.

Mutating the first hairpin by two point mutations (mutant e), leading to a hairpin consisting of Watson-Crick base pairs only, resulted in symptoms in 7 of 23 inoculated plants. On the primary leaves of these plants, however, only a few or no symptoms were present. Analysis of the sequence of the RNA of virus isolated from these infected plants showed that these viruses were revertants (see below). When this mutation was combined with a deletion of the internal loop of hairpin 2 (mutant g), the RNA was no longer infectious. When only the C-A mismatch of the internal loop of hairpin 1 was changed into a C · G base pair (mutant f), the symptoms were indistinguishable from those due to the wild type. A mutant in which hairpin 2 lacked the internal loop and hairpin 1 contained a single, anomalous C-C mismatch (mutant h) caused symptoms in almost all plants, although the number of symptoms on secondary leaves was rather small and was restricted to the proximity of the veins. In another mutant, 75% of the viral leader was deleted (mutant i), so that no protonatable hairpins were present. On the inoculated leaves, a small number of lesions was visible, with only a few days delay in development compared to the symptoms due to the wild type. In most of the plants, however, no symptoms were observed on the other leaves, which means that systemic spread was hindered. Only in two cases did some minor veins of an older leaf clearly become yellow. When virus was isolated from the primary leaves and transferred to a new plant, similar phenomena were observed. The symptoms on the primary leaves now appeared after only 5 or 6 days. When large amounts of inoculum were used, viral spread through the entire plant was observed, although it was restricted to the immediate surroundings of the veins.

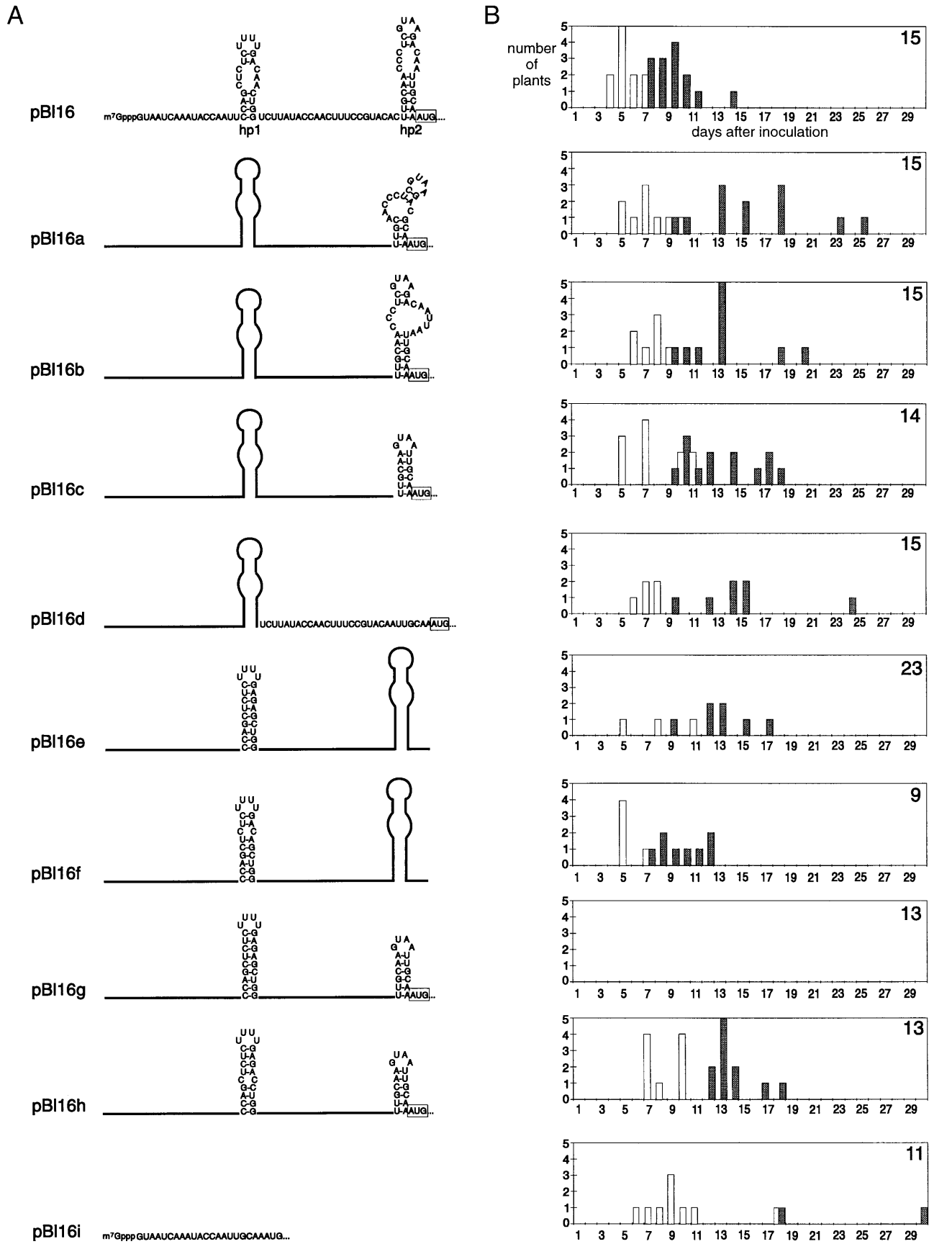
A
B
pBl16
pBl16a
pBl16b
pBl16c
pBl16d
pBl16e
pBl16f
pBl16g
pBl16h
pBl16i
hp1
hp2
number of plants
days after inoculation
15
15
15
14
15
23
9
13
13
11

FIG. 1. The mutations in the 5′ UTR of TYMV RNA that were used in this study and the time of appearance of symptoms on Chinese cabbage plants. (A) The putative secondary structure of the 5′ UTR of the mutants that were made. pBL16 is the infectious clone that was used to make the mutants (19). The black lines indicate regions that are identical to the wild-type sequence. The 5′-proximal start codon is boxed. (B) The time of appearance of the first symptoms of the various mutants on a set of plants, on inoculated leaves (open bars) and secondary leaves (solid bars). The graphs correspond to the mutants in panel A. The number of plants inoculated with RNA transcripts of the mutants is indicated in the right upper corner of each panel.

Mutants and revertants. To see whether the sequence of the various mutants was stable or if reversions or second-site mutations had taken place, most mutants were propagated for several cycles of infection. Virions of all viable mutants were isolated after one to five cycles of propagation. The 5′ end was multiplied by reverse transcription-PCR, and the sequence of the 5′ UTR was determined by dideoxy sequencing. The mutants with a modified or deleted hairpin 2 all turned out to be stable, since no reversions, insertions, or deletions were observed in the 5′ UTR of any of these mutants after five cycles of propagation.

The RNA sequences of viruses that were isolated after infection with mutant e were all different from those of the original construct (Fig. 2). Interestingly, in all eight isolates, C-C or C-A mismatches were regenerated in hp1. In five cases (revertant e1), the C-C mismatch of the wild-type virus was restored by the mutation G33C, leading to the sequence and structure of mutant f (Fig. 1A). The symptoms caused by these revertants were indistinguishable from those due to the wild-type virus. In two cases (revertant e3), the mutation G33A was found, resulting in a C-A mismatch at the position of the C-C mismatch in the wild type. Furthermore, one revertant (revertant e2) showed a combination of G33C and U23C, leading to a neighboring C-C and C-A mismatch. The RNA sequence of mutant f, which differs from that of the wild type by a single point mutation, was unchanged after the second cycle of propagation. However, after five cycles, the sequence was returned to wild type by the reversion G35A. Revertants e2 and e3 were propagated for only one cycle. Revertant e2 remained stable, as well as revertant e3. The sequence of mutant h, which contains only a single C-C mismatch in the 5′ UTR (Fig. 2) at a position where originally a C-A mismatch was present, turned out to be stable, since no reversions were observed in the 5′ UTR even after five cycles of propagation.

In vitro translation. Since some of the mutants caused no symptoms at all (mutant g) or caused symptoms only after a spontaneous mutation had taken place (revertants e1 to e3), we wanted to know which step of the viral life cycle was affected. For this reason, in vitro translation of each viral mutant was performed, by using a coupled transcription-translation system, in which cDNA plasmids of the various mutants were transcribed by T7 RNA polymerase and translated by a reticulocyte lysate in a single-tube reaction. No qualitative differences in the protein patterns of the different mutants were observed (Fig. 3), showing that the structure of the viral leader probably does not influence the relative efficiencies of the translation of the two overlapping genes (OP and RP). The mutants with a strongly stabilized 5′-proximal hairpin (mutants e and g) clearly were translated less efficiently. The efficiency of the translation of mutant i, which has a much shorter leader, did not differ from that of the wild type.

Analysis of encapsidation. Virus preparations of TYMV contain a relatively large number of empty viral particles containing no RNA (natural top component) which can be separated from the infectious virions (B1 component) on a CsCl gradient (17). Empty viral particles migrate slightly more slowly than the filled particles when analyzed by agarose gel electrophoresis (7a), so that the ratio between filled and empty particles in a virus preparation can be analyzed easily by this technique. This ratio may give an indication of the efficiency of the process of encapsidation of the RNA. However, the results differ slightly for different preparations of the same mutant. In Fig. 4, the result of such an agarose gel electrophoresis experiment is shown, with virus preparations of several mutants and the wild-type virus. The ratio between filled and empty particles was not changed when hp2 was deleted completely (mutant d), although smaller mutations clearly led to less efficient packaging (mutants a and b). Mutations in hp1 had a more drastic effect. For mutants h and i, the virus preparation contained a large excess of empty viral shells, and even revertant e3 and mutant f, which caused wild-type symptoms, seem to encapsidate the RNA slightly less efficiently.

DISCUSSION

The 5′ UTR of TYMV RNA contains two hairpins, which have an internal loop that can be protonated due to the presence of C-C and C-A mismatches (9). This is a conserved feature of all tymoviruses sequenced so far, except for kennedya yellow mosaic virus, which contains only one protonatable hairpin. To study the functions of these hairpins, we created mutations in an infectious cDNA clone of TYMV. The two hairpins were mutated or deleted separately (mutants a to f) or together (mutants g to i) and Chinese cabbage plants were inoculated with RNA transcribed in vitro.

When the internal loop of hp1 of the 5′ UTR of TYMV RNA was replaced by Watson-Crick base pairs, the virus was not viable (mutant g), or reverted (mutant e). This may be ascribed in part to the reduced expression of the nonstructural genes, as was observed with the in vitro translation experiments. It is known that a stable hairpin that is positioned close

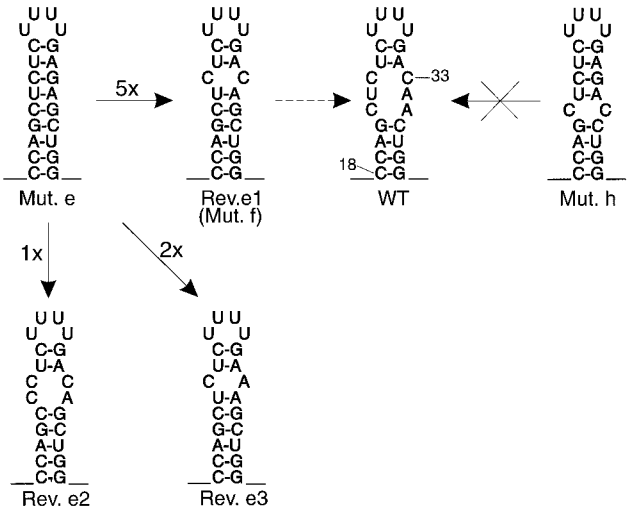

FIG. 2. Spontaneous mutations that were observed in hp1 of mutant e. The names of the original mutants and of the various revertants are indicated below the hairpins. The number of viral isolates in which a certain reversion was observed is indicated above the arrows. The dashed arrow indicates a reversion that was observed only after five cycles of propagation. The sequence of mutant e itself was never observed. Mutant h did not revert at all.

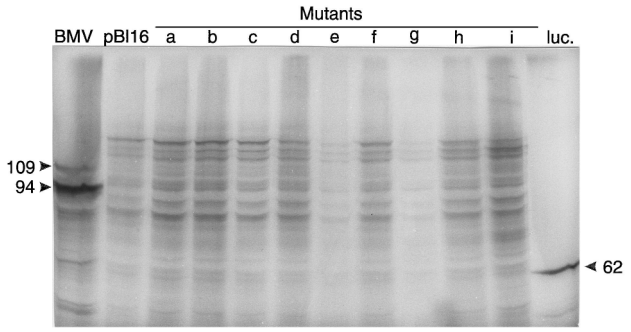

FIG. 3. In vitro translation of the RNA of TYMV and several mutants. Use was made of a coupled transcription-translation system to express the proteins of TYMV. The proteins were analyzed on an 8% polyacrylamide gel. Brome mosaic virus (BMV) RNA and luciferase (luc.) RNA were translated and used as size markers.

to the cap can hinder the binding of ribosomes to the mRNA (13). In mutants e and g, the calculated stability of hp1, which is located 17 nucleotides downstream of the cap, is −13.5 kcal/mol. Since even a hairpin with a stability of −30 kcal/mol does not hinder the ribosome once it has started scanning the messenger (12), it is more likely that in our case the stabilized hairpin interferes with the binding of the ribosome to the cap structure. A single point mutation in this closed hairpin 1 strongly reduces the stability, thereby restoring translation. In mutant f, for example, which is identical to revertant e1 (found in five cases), the hairpin has a stability of −7.1 kcal/mol and the efficiency of translation is restored. The same holds for revertant e3 (data not shown). The stability of the hairpin per se, however, is not the only prerequisite for a proper function of hp1. For both an A · U and a G · C pair, there are five point mutations possible that lead to a mismatch (G · U pairs not taken into account). If we do not take into account the base pairs at the extremities of the stem of hp1, for the remaining 7 bp there are 35 different point mutations that would strongly reduce the stability of the hairpin, 11 of which introduce a C-C or C-A mismatch. Interestingly, the eight revertants that we isolated, starting from mutant e, all contain a C-C or C-A mismatch at the position where the wild-type hairpin has a C-C mismatch. This strongly emphasizes the necessity of these protonatable mismatches in this hairpin. This is, to the best of our knowledge, the first example of a functionally important role of such a mismatch in viral RNA. The mismatch at the top of the internal loop appears to be the most important element, as shown by the sequence of the revertants and the virulence of mutant f. Revertant e2 has a phenotype indistinguishable from that of the wild-type virus, although in this case the C-C and C-A mismatches are adjacent. In this respect, revertant e2 is more similar to the hairpins found in the RNA of the tymoviruses eggplant mosaic virus and onionis yellow mosaic virus (9), where symmetric internal loops of four cytosines are present. It is remarkable that mutant h, which contains only a single C-C mismatch in the entire 5′ UTR, does not lead to revertants, even after five cycles of propagation, although this mutant causes less severe symptoms than the wild-type virus does. A reversion or another spontaneous mutation in a viral RNA will take place only if the mutant replicates at a certain level, allowing the replicase to make mistakes, and will only be observed if the new mutants are more viable than the original one. The fact that mutant g does not produce revertants may be ascribed to the first reason, and the absence of revertants of mutant h can be ascribed to the second reason. The fact that a protonatable mismatch at the bottom of the internal loop of hp1 also plays a functional role is confirmed by the observation that mutant f slowly reverts to the wild type.

Mutant i, which lacks 75% of the leader including the two hairpins, shows several interesting properties. This mutant virus does cause symptoms on the inoculated leaves, although the symptoms are less abundant than those due to the wild-type virus, and they develop later. This means that the first 17 nucleotides of TYMV RNA are sufficient for a certain level of replication. Moreover, the in vitro translation experiments showed no difference in the expression of the nonstructural genes for the wild-type RNA and mutant i. Although it has been shown that two 80S ribosomes can bind simultaneously to the leader of tobacco mosaic virus RNA and TYMV RNA (4), in TYMV a large reduction in the length of the leader does not lead to a lower translation efficiency in vitro. In vivo experiments showed that the leader of tobacco mosaic virus RNA functions as a translational enhancer, in contrast to the leader of TYMV RNA (5), although in those experiments with TYMV RNA the leader was probably base paired with the reporter gene. Also, the in vitro translation of the other mutants does not result in a change in the pattern of the proteins synthesized. Apparently, the secondary structure of the RNA of the 5′ UTR does not influence the relative expression levels of these two overlapping open reading frames with adjacent start codons, as was also observed in Sendai virus RNA (6).

The results of the determination of the ratio of filled to empty viral particles (Fig. 4) strongly suggest that the 5′ UTR has a function in the initiation of viral encapsidation. Since encapsidation is required for systemic spread (22), a deletion of the packaging signal will affect the systemic spread as well. We assume that the ratio of filled to empty viral particles is dependent on the efficiency of RNA encapsidation. Since the majority of the viral particles that were isolated from leaves infected with mutants i and h do not contain RNA, it is likely that the protonatable hairpins, or, in more detail, the C-C and C-A mismatches, function in coat protein binding. The first hairpin of the 5′ UTR in this respect seems more important than the second one, since a deletion of hp2 does not influence the ratio of filled and empty particles (mutant d). It is surprising that more subtle mutations in hp2 (mutants a and b) have an effect on the viral encapsidation. Possibly such a mutation in hp2 in some way hinders the binding of the coat protein to hp1 whereas a complete deletion of hp2 does not. However, if the coat protein also binds to hp2 of TYMV RNA, it may function as a translational repressor. This could explain the reduced virulence of the RNAs with mutations in this hairpin. In the 5′ UTR of the RNA of the tymovirus kennedya yellow mosaic

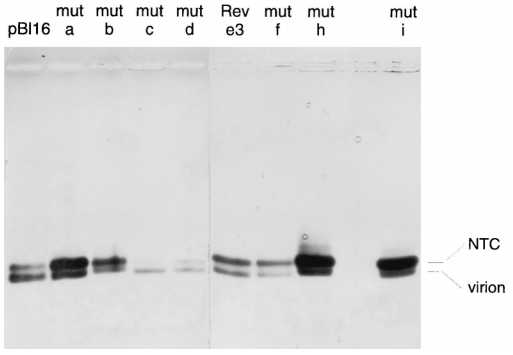

FIG. 4. Agarose gel electrophoresis of the virus preparations isolated from plants infected with the various mutants of TYMV RNA. The various mutants or revertants are indicated above the lanes. NTC, natural top component (empty virus shells).

virus, only hp1 has a protonatable internal loop (8); therefore, in that case one such hairpin is apparently sufficient.

The most important element of the hairpins of the 5′ UTR of TYMV RNA seems to be the C-C mismatch at the top of the internal loop of hp1, as was also concluded from the revertants. A role of C-C and C-A mismatches in the encapsidation is not unlikely, since protonated cytosines are important for RNA-protein interactions in TYMV (1, 7, 10, 18, 21). Furthermore, some electron microscopic observations of infected cells of Chinese cabbage plants show that packaging of plus strands into the virions has already started during their synthesis by the replicase (15). This makes it likely that the viral packaging signal is located in the 5′ part of the viral RNA.

It is clear now that there is an important functional role for the conserved protonatable hairpins in the 5′ UTR of tymoviral RNA, since C-C or C-A mismatches are required for a proper functioning. The hypothetical role in the initiation of encapsidation (9) has been strengthened by the results described here. More experiments are required, however, to elucidate the exact requirements of the hairpins in the 5′ UTR. Furthermore, a protoplast system may be helpful to further unravel the structural requirements for this region and will enable us to study the consequences of the mutations in hp1 on RNA replication and protein synthesis in a more quantitative way.

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