The 5′-Proximal Hairpin of Turnip Yellow Mosaic Virus RNA: Its Role in Translation and Encapsidation

Hugo H. J. Bink,1 Jan Schirawski,2† Anne-Lise Haenni,2 and Cornelis W. A. Pleij1*

Leiden Institute of Chemistry, Leiden University, Leiden, The Netherlands,1 and Institut Jacques Monod, CNRS, Universités Paris VI et VII, Paris, France2

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The RNA genome of turnip yellow mosaic virus (TYMV) consists of more than 6,000 nucleotides. During a study of the roles of the two hairpins located in its 90-nucleotide 5′ untranslated region, it was observed that stabilization of the 5′-proximal hairpin leads to a delay in the development of symptoms on plants. This delay in symptom development for both locally and systemically infected leaves was found to be dependent on a change in the free energy of the hairpin caused by introduced mutations. A protoplast transfection assay revealed that the accumulation of plus-strand full-length RNA and subgenomic RNA, as well as protein expression levels, was affected by hairpin stability. Stabilization of this hairpin inhibited translation. A model is proposed in which a destabilized 5′-proximal hairpin allows maximal translation of the viral proteins. It is suggested that this hairpin may exist in close proximity to the 5′ cap as long as its stability is low enough to enable translation. However, at an acidic pH, the hairpin structure becomes more stable and is functionally transformed into the initiation signal for viral packaging. Slightly acidic conditions can be found in chloroplasts, where TYMV assembly is driven by low pH generated by active photosynthesis.

The 5′ untranslated region (5′ UTR) of single-stranded positive-sense RNA virus genomes often contains cis-acting elements involved in translation, replication, and/or encapsidation. Turnip yellow mosaic virus (TYMV) RNA has a 5′ leader of 90 nucleotides which may regulate all three processes by means of signals embedded within this short stretch of RNA. Translation of TYMV RNA has been assumed to take place according to general principles for eukaryotic mRNAs, since the genomic RNA and the subgenomic RNA (sgRNA) for the coat protein (CP) are capped at the 5′ end (10, 15). The cap would enable the binding of translation initiation factors, which subsequently would allow the docking of the 40S ribosomal subunit on the viral mRNA. By scanning the RNA, the ribosome reaches the start codons of the movement protein (MP) and the RNA-dependent RNA polymerase (RdRp). The AUG start codon of the latter open reading frame (ORF) overlaps that of the MP ORF and is situated only 4 nucleotides downstream of the first AUG (Fig. 1A). Translation of the RdRp gene was proposed to occur through leaky scanning (18, 34), although Weiland and Dreher reported that mutation of the first AUG did not affect translation starting at the downstream AUG (34), a finding which is at variance with leaky scanning. A number of exceptions to the eukaryotic scanning model of Kozak (18) exist, most of which come from the field of virology. Cap-independent initiation of translation was shown to occur through a strategy involving the use of an internal ribosomal entry site (IRES). The IRES-mediated translation initiation mechanism involves the binding of translation initiation factors and the ribosome to a complex RNA secondary structure, thereby placing the translation machinery in the start codon context (8, 19). For the translation of TYMV RNA, it was recently shown that in some way, internal initiation takes place as well. The 3′-terminal tRNA-like structure (TLS) of TYMV RNA appears to drive this internal initiation. However, whether an additional secondary structure is required or whether translation initiation factors are involved, in analogy to the IRES mechanism, is unknown (1). The signals involved in plus-strand synthesis of the sgRNA encoding the CP of TYMV are well defined. Initiation depends on the presence of a highly conserved sequence known as the tymobox (7, 29). Unfortunately, the signals involved in the replication of the TYMV full-length plus-strand RNA are less clear. It was suggested that the 5′ UTR is involved in a process of circularization, since complementary sequences were shown to be present in the 5′ UTR and the 3′ UTR (4, 32). On the other hand, one can speculate that the conserved m7GpppGUAAU motif at the extreme 5′ end of the tymovirus genomic RNA may have a function in viral plus-strand synthesis. The signals responsible for the initiation of synthesis of the full-length minus-strand RNA are located at the 3′ end of the TYMV RNA genome, in which a crucial role is played by the ACCA sequence at the 3′ end of the TLS (5, 6, 30).

The initiation of encapsidation of TYMV RNA and the role of the 5′ UTR have been well characterized by a series of studies involving biochemical and biophysical experiments. Two hairpins (HP1 and HP2) (Fig. 1A) present in the 5′ UTR are strongly conserved among the members of the genus Tymovirus (13). Moreover, these hairpins contain internal symmetric loops consisting of C · C and/or C · A mismatches. Extensive mutagenesis of the internal loops of both HP1 and HP2 revealed that these non-Watson-Crick base pairs specifically are instrumental in the packaging of the viral genome (2, 14). The C · C and C · A mismatches become protonated under slightly acidic conditions, forming C+ · C and C+ · A base pairs.
which are suggested to share a proton with the carboxyl residues of the TYMV CP in the process of encapsidation. Binding experiments with the 5'-proximal hairpin (HP1) and empty capsids confirmed this interaction, which was found to be pH dependent and to require the presence of the polyamine spermidine (2). As part of our mutagenesis study of the internal loop of HP1 (Fig. 1), the question was raised as to whether the introduction of site-specific substitutions may have an effect on other features of the virus replication cycle. The possibility that changes in viral RNA or protein synthesis may affect the encapsidation process as well could not be excluded.

To further deepen the understanding of the possible roles of the 5'-proximal hairpin of TYMV RNA in translation and/or replication, HP1 mutants were analyzed in more detail. First, the development of symptoms on primary and secondary leaves of Chinese cabbage plants was monitored. Second, the translation of the MP and CP genes and the synthesis of full-length plus-strand RNA and the sgRNA were studied by using an improved protoplast transfection assay specifically developed for TYMV (28), allowing more precise determination of the influence of the introduced mutations on replication and translation efficiency. It was concluded that the behavior of the mutants during plant infection is strongly dependent on the stability of HP1. The introduction of hairpin-stabilizing substitutions decreases translation efficiency but has no major effect on replication. A model is proposed and discussed; in this model, stabilized HP1 is used as an encapsidation initiation signal, while destabilized HP1 leads to increased translation efficiency.

MATERIALS AND METHODS

Construction of cDNA mutants. To obtain mutant TYMV RNA, use was made of an infectious cDNA clone of the Blue Lake strain of TYMV, pBL16 (31). The introduction of the site-specific mutations was achieved by PCR mutagenesis. All oligonucleotides were synthesized by Eurogentec, Seraing, Belgium. The sequences of the oligonucleotides were described by Bink et al. (2). The resulting PCR products, containing the T7 promoter sequence as well as the 5' part of the viral genome, were digested with HindIII and NcoI and ligated in pUC21. Sequence analysis was performed by using a T7 sequencing kit (Pharmacia). These fragments were then cloned into pBL16 and sequenced again.

RNA preparation and plant inoculation. Plasmid DNAs of the various clones were linearized with NdeI. Transcription with T7 RNA polymerase (Pharmacia) and subsequent plant inoculation were performed as described by Hellendoorn et al. (14). However, instead of a sterile toothpick, a sterile glass spatula was used for applying the RNA to the leaves, leading to a higher proportion of plants showing symptoms. On average, 2 μg of capped TYMV RNA was applied per leaf. Symptom development was monitored daily for 4 weeks after inoculation. Unless otherwise stated, 15 plants were inoculated with each mutant.

Plants. Chinese cabbage (Brassica pekinensis) was grown under an 8- to 16-h light-dark regimen. Standard conditions in the growth chambers included a light intensity of 5,000 to 6,000 lx, a humidity of 70%, and a constant temperature of 23°C. Three-week-old plants were used for infection with RNA.

Protoplasts. An Arabidopsis thaliana cell culture suspension was used for the production of protoplasts (28). Transfection of protoplasts and analysis of the viral products formed were performed as described by Schiawski et al. (28). Protoplasts were harvested 48 h postinoculation. Quantitative analysis was performed by scanning the exposed X-ray films with a Bio-Rad GelDoc system including the Quantity One software package. Quantification of expression levels was based on the results of two or more independent experiments per mutant.

In vitro translation. Translation of TYMV RNA was performed by incorporation of [35S]methionine (ICN) with purified capped full-length plus-strand TYMV RNA in a wheat germ extract (L4380; Promega) or NdeI-linearized pBL16 DNA in a TNT T7-coupled wheat germ extract (L4140; Promega). Reactions were carried out according to the manufacturer's instructions. After translation, the protein products were precipitated with 10% trichloroacetic acid and filtered with glass microfiber filters (Whatman). Incorporation was determined by scintillation counting. Since [35S]methionine incorporation was found to be independent of the wheat germ system used, the results presented in the text are averages for each mutant without discrimination between the sources of the wheat germ extract.

Calculation of RNA secondary structure stability. The influence of the mutations on RNA secondary structure formation was calculated by using the efr/server running mFold version 3.0 (20). The thermodynamic parameters used were valid for pH 7.0 only. This means that the stability of hairpins containing C·A and C·C base pairs at a low pH could not be calculated.

RESULTS

Construction of mutants. The mutants used in this study were originally designed to investigate their influence on encapsidation initiation (2, 14). The mutations are restricted to site-specific substitutions within the internal loop region of HP1 (Fig. 1B). The formation of any unwanted alternative folding in the 5' UTR of the mutant RNAs was checked with the mFold program (20). No competitive RNA secondary structures were predicted as a result of the substitutions introduced. The predicted minimum free energy (ΔG) values of the various mutant hairpins ranged from −3.6 kcal mol⁻¹ (wild type, S1, S2, and S4) to −16.3 kcal mol⁻¹ (S6) (Fig. 1B).

Symptom development. The possible effects of the substitutions introduced were examined by monitoring the development of local and systemic symptoms on Chinese cabbage plants. For this purpose, capped T7 transcripts were synthe-
sized in vitro and used for plant infection. The time of the first appearance of symptoms on the primary and secondary leaves is summarized in Fig. 2. On average, the appearance of symptoms on primary leaves was observed 4 days after inoculation with wild-type TYMV RNA. The primary leaves exhibited lesions characterized by small bright, light yellow spots, which were not observed on mock-inoculated plants. Subsequent spreading of virus to the other leaves resulted in a systemic infection phenotype within 8 days. The systemic symptoms were, however, not uniform. In most cases, the typical mosaics patterns were observed, while in some cases, yellow and light green spots first became visible at the edges of the major veins of the leaves and only later migrated further into the leaves. Student’s t test analysis was used to calculate the significance of the delay in the appearance of symptoms caused by each mutation. The resulting probability factors are also indicated in Fig. 2. These calculations showed that mutants S2 and S4 and possibly also mutant S1 did not influence virus spreading, since the first appearance of local and systemic symptoms was identical to that seen with the wild type. All other mutants resulted in a statistically significant delay in TYMV infection of both primary and secondary leaves. An exception was mutant S8, which resulted in a significant delay in systemic symptom development, whereas the time of appearance of local symptoms was close to that seen with the wild type. Also, only for mutant S6 was it observed that not all plants became infected or exhibited symptoms. Of 44 plants inoculated with S6 transcripts, only 8 showed local symptoms, while 29 developed systemic symptoms. All other mutants generated symptoms on all of the plants tested.

Further analysis of the delay in symptom development yielded an unexpected result when the first appearance of local symptoms was plotted against that of systemic symptoms for each mutant (Fig. 3A). A linear relationship between the appearance of both types of symptoms was obtained. This result suggests that the mutations influenced another process besides encapsidation. Indeed, if only encapsidation initiation was affected, then a delay in the development or a complete absence of systemic symptoms would be expected, while the development of local symptoms would be expected to be similar to that seen with the wild type (3, 12). The different stabilities of the various HP1 mutants might explain this result, since the delay in symptom development was apparently linearly related to the calculated stability (Fig. 3B). Which process in the viral life cycle is influenced most strongly by these mutations cannot easily be determined from this plant infection assay only. In an attempt to answer this question, we made use of a protoplast transfection assay.

Protoplast transfection assay. Protoplasts derived from A. thaliana cell suspension cultures were transfected with T7 transcripts of wild-type or mutated TYMV cDNA constructs. Figure 4 shows a Western blot analysis of MP and CP (Fig. 4A and B, respectively). Northern blot analysis was used to investigate the synthesis of full-length genomic RNA and CP sgRNA (Fig. 4C). The expression of MP is believed to be dependent on canonical scanning of the ribosome after binding to the 5’ cap structure. This ribosome therefore has to pass the two conserved hairpins, HP1 and HP2, before reaching the first AUG, which corresponds to the start of the MP ORF (Fig. 1A). How the expression of the downstream RdRp ORF is regulated is not exactly known. Two possible mechanisms are proposed to explain the initiation of translation of the RdRp gene. First, leaky scanning has been proposed (18); this mechanism implies a certain fixed ratio of translation of the MP and RdRp ORFs and suggests that the levels of translation of MP may be indicative of the expression of RdRp. A second mechanism suggests cap-independent internal initiation of the ribosome on the RdRp ORF which is dependent on the TLS at the 3’ end of the TYMV RNA (1); however, the model does not indicate whether leaky scanning is excluded.
Anti-MP antibodies revealed the presence of two products migrating in very close proximity to each other, as observed earlier for TYMV MP by Bozarth et al. (3). Whether this finding is the result of posttranslational modifications, such as proteolytic cleavage or phosphorylation, is unclear. The production of MP clearly differed for the various mutants. The levels of expression of MP visualized were low, especially when compared with the amounts of CP synthesized, although the low levels might also reflect differences in the qualities of the antibody preparations. CP was visualized as a clear band migrating between 19 and 25 kDa (Fig. 4B).

Remarkably, the results of the Northern blotting analysis showed that a similar dependence on the type of mutation exists for the accumulation of full-length genomic RNA as for that of sgRNA (Fig. 4C). This finding was especially clear for mutant S6, where a strong decrease in CP production was paralleled not only by a low level of sgRNA accumulation but also by low levels of full-length RNA and MP production.

The amounts of RNA and protein synthesized were quantified and are presented in Fig. 5 as a function of hairpin stability. The expression level for each mutant hairpin was normalized to that of the wild-type hairpin. It is clear that an increase in hairpin stability resulted in decreased synthesis of both full-length plus-strand RNA and sgRNA as well as decreased MP and CP translation (Fig. 5A to D, respectively). The levels of repression of the synthesis of full-length plus-strand RNA and sgRNA were identical. Although the synthesis of both full-length genomic RNA and sgRNA uses the full-length minus-strand RNA as a template, a mutation in a presumed signal functional in full-length plus-strand synthesis might be expected not to be involved in the initiation of sgRNA synthesis, which is dependent on the tymobox sequence. Furthermore, the deletion of both HP1 and HP2 resulted in local symptom development comparable to that seen with the wild type, suggesting that these hairpins are not functional in replication (14). In view of these arguments, we interpret the results of Fig. 5A to D to indicate that the stability of the 5'-proximal hairpin may influence translation rather than replication. The observed effect on MP ORF translation likely reflects the level of RdRp synthesis, since there exists a presumed fixed ratio of translation of these two ORFs. A smaller amount of replicase could explain the lower yield of RNA synthesis.

**In vitro translation.** To study the direct influence of hairpin stability on translatability, a wheat germ in vitro translation assay was performed. The incorporation of [35S]methionine was monitored for each mutant and plotted as a function of hairpin stability. Analysis of the translation products by sodium dodecyl sulfate gel electrophoresis was performed. However, quantification was very difficult because of the multitude of
bands and the much lower efficiency of translation of T7 transcripts than of native TYMV RNA (14). When the amount of trichloroacetic acid-precipitable material was normalized to that of the wild-type construct, the translatability of the mutant RNAs decreased with increasing stability of HP1 (Fig. 5F). In this experiment, the same amounts of RNAs were used for the mutants. Therefore, decreased production of protein does not reflect a smaller amount of available RNA but is a direct measure of the suitability of the RNA for translation. Thus, this experiment shows independently that an increase in HP1 stability leads to a decreased efficiency of in vitro translation, corroborating our interpretation of the in vivo analysis with the protoplast transfection assay (Fig. 5E).

### DISCUSSION

In this study, evidence is presented that mutations stabilizing the 5'-proximal hairpin (HP1) in TYMV RNA result in a delay in the development of both local and systemic symptoms. A combination of plant infection assay, protoplast transfection assay, and in vitro translation experiments allowed us to establish which process in the life cycle of the virus is most strongly influenced by the mutations introduced. The mutants, which were designed originally to study encapsidation initiation, had an inhibitory effect on translation as a function of hairpin stability.

**Translation versus encapsidation.** In a study of encapsidation efficiency, some of the mutants generated reversions in the sequence of HP1 upon infection of Chinese cabbage (2). Mutant S6 is of special interest, since the original mutation was never recovered from systemically infected leaves. Moreover, this mutant gave rise to a lower yield of plants showing symptoms. The strong stabilization of HP1 ($\Delta G = -16.3 \text{ kcal} \cdot \text{mol}^{-1}$) probably is responsible for the low viability of this particular mutant. However, one has to assume that some low level of replication must take place to allow the introduction of reversions in the progeny isolated from the systemically infected leaves. Therefore, an HP1-destabilizing revertant might be superior in fitness, since its translational activity would be increased. For instance, for mutants S7 and S8, reversions leading to less stable variants of HP1 were observed (2). However, reversion of S7 and S8 appeared not to be an absolute requirement for these mutants to be fully infectious. Other mutants, such as S1, S2, and S4, showed no reversions.

From these results, we derived a schematic diagram in which the need for reversion of the mutated HP1 sequence is summarized. Based on the sequencing data, different modes of the requirement for reversion can be distinguished (Fig. 6A). Certain minimal and maximal stabilities for HP1 appear to be preferred, the limits being approximately $-8$ to $-10 \text{ kcal} \cdot \text{mol}^{-1}$ and $-12$ to $-16 \text{ kcal} \cdot \text{mol}^{-1}$, respectively. The most striking example is the gradual shift from mutant S6 to S6.1 and S6.2 over several rounds of infection; this shift crosses both thresholds. Figure 6B shows how after initial inoculation with mutant S6, the reversions introduce a gradual relaxation of the stability of HP1 over several rounds of infection. After four rounds of infection, only S6.2 and wild-type sequences were recovered (2).

Reversion requirement observed for mutant forms of the 5'-proximal hairpin of TYMV RNA. (A) Dependence of the reversion requirement on the stability of HP1. Three types of reversions were distinguished: required (+), preferred (+/−), and not required (−). Arrows indicate the change in stability resulting from an observed reversion in the progeny over multiple rounds of infection (2). When the original mutation could not be recovered in the virus progeny, a reversion was required. Mutants in the preferred reversion layer did not necessarily revert in the first or subsequent rounds of infection, whereas mutants in the layer that was not required (−) never introduced reversions during the four rounds of infection that were monitored. The areas of gray shading mark the apparent thresholds between the three types of reversion requirements. WT, wild type. (B) The reversion pathway of mutant S6 exhibited a turnover from S6 to S6.1, S6.2, and the WT over several rounds of infection (2). Initially, the infection assay started with the inoculation of mutant S6 ($\Delta G = -16.3 \text{ kcal} \cdot \text{mol}^{-1}$). In the following rounds of infection, the predominant (pseudo) revertants that were observed belonged to one of three genotypes: S6.1 ($\Delta G = -10.1 \text{ kcal} \cdot \text{mol}^{-1}$), S6.2 ($\Delta G = -8.1 \text{ kcal} \cdot \text{mol}^{-1}$), or WT ($\Delta G = -3.6 \text{ kcal} \cdot \text{mol}^{-1}$).

**Repression of translation efficiency.** Major features of virus infection, such as symptom development and the overall production of viral products, were negatively influenced by the mutations, possibly reflecting inefficient translation of the viral RNA. Functional coupling of hairpin stability and translatability can reflect (i) altered ribosomal scanning of the 5' leader sequence for the first AUG or (ii) altered binding of the 43S preinitiation complex, containing initiation factors and the 40S preinitiation complex, containing initiation factors and the 40S.
ribosomal subunit, to the leader. However, it has been established that a hairpin stability represented by a \( \Delta G \) value of \(-30\) kcal \( \cdot \) mol\(^{-1}\) does not interfere with ribosomal scanning of the messenger (16). Inhibition was observed only for a hairpin with a \( \Delta G \) value of \(-60\) kcal \( \cdot \) mol\(^{-1}\) (16). Inefficient scanning is therefore an unlikely explanation, since the most stable hairpin (S6) in this study has a \( \Delta G \) of \(-16.3\) kcal \( \cdot \) mol\(^{-1}\), while the level of repression of translation is 70 to 80\% (Fig. 5E).

In a eukaryotic mRNA, such as the genomic RNA of TYMV, the primary binding site is the cap structure, which must be recognized by initiation factor eIF-4F (9). Unfortunately, no data are available on the exact size or sequence of the leader required by the 43S preinitiation complex to facilitate binding to the (unfolded) mRNA (22). However, the values for translation inhibition found in this study compare very well to the results obtained with yeast and rabbit cell extracts. Both in vivo and in vitro experiments showed pronounced inhibition of translation when stem-loop structures were introduced in close proximity to the cap structure (17, 23, 27, 33). The level of translation inhibition rose to 90\% for a hairpin with a \( \Delta G \) value of \(-15\) kcal \( \cdot \) mol\(^{-1}\) (27, 33). In TYMV RNA, HP1 is only 18 nucleotides downstream of the 5' cap and exhibits comparable levels of translation inhibition for the hairpin-stabilizing mutants. Moreover, the introduction of stem-loop structures with different free energy values into the 5' UTR of herpes simplex virus thymidine kinase mRNA at 11 nucleotides from the cap site resulted in reduced binding of eIF-4B in direct proportion to the amount of secondary structure introduced. In contrast, increased hairpin stability 38 nucleotides from the cap site had no effect on this binding (24). Apart from the effect of the stability of hairpin HP1, the optimal length of the leader between the hairpin and the cap in terms of translation efficiency remains to be determined. In addition, it was observed that deletion of the 3' TLS in TYMV RNA decreased RdRp synthesis in vitro, suggesting an interaction between the 3' UTR and the 5' UTR (1). However, the in vitro translation of an HP1-stabilizing mutant viral RNA (14) decreased RdRp synthesis as well. It is therefore proposed that the interaction of the 3' TLS and the 5' UTR is facilitated by eIFs.

**Biological consequences.** For TYMV, it is not exactly known how translation, replication, and encapsidation relate to one another in the infected cell. Translation of the viral RNA takes place in the cytoplasm, while replication has been shown to occur within characteristic vesicles formed by invaginations of the chloroplast membrane (11, 21, 25). Encapsidation has been suggested to proceed at the necks of the invaginations (21). Moreover, it is supposed that translation occurs under slightly alkaline conditions, whereas encapsidation is driven by a low pH (26). Although the environmental conditions in proximity of the replication machinery are not known, we assume that translation and encapsidation are uncoupled events due to different pH requirements. In the context of changing pH conditions, HP1 is believed to adopt at a neutral pH a relatively unstable or unfolded structure that does not interfere with translation initiation, whereas it is functional as a more stable hairpin during the initiation of encapsidation, which takes place at a lower pH (2) (Fig. 7).

As mentioned earlier, HP1 is in close proximity of the cap structure. Nevertheless, optimal translation of the MP and RdRp ORFs is reached. This potential effect is clearly demonstrated by the HP1-stabilizing mutants. The change in pH has dramatic consequences for the stability of HP1. Analysis of the UV melting curves obtained with a synthetic RNA fragment corresponding to wild-type HP1 showed that the \( \Delta G \) value is increased upon a decrease in the pH, in contrast to the behavior of HP1 of mutant S6 (2). Identical results were obtained for HP2 and its S6 variant (13). The \( \Delta G \) value for both the wild-type hairpins HP1 and HP2 and the hairpins containing all of the Watson-Crick base pairs reached 4.2 kcal \( \cdot \) mol\(^{-1}\) as the pH was decreased. The major contribution to stabilization comes from the protonation of cytosine and adenine residues, which results in the formation of C\(^+\) \cdot C and A\(^+\) \cdot C base pairs. Apparently, the composition and location of hairpins HP1 and HP2 observed in TYMV RNA as well as in all other
members of the genus *Tymovirus* generate a situation in which minimal interference with translation initiation is combined with optimal encapsidation ability of the viral RNA. Moreover, the positioning of the encapsidation initiation signal at the far 5′ end allows the packaging of the full-length plus-strand RNA immediately after the new strand appears from the replication machinery, in keeping with the TYMV encapsidation model presented by Matthews (21).

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