The 5′ Untranslated Region of Alfalfa Mosaic Virus RNA 1 Is Involved in Negative-Strand RNA Synthesis

A. Corina Vlot and John F. Bol*

Institute of Biology, Gorlaeus Laboratories, Leiden University, Leiden, The Netherlands

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The three genomic RNAs of alfalfa mosaic virus each contain a unique 5′ untranslated region (5′ UTR). Replacement of the 5′ UTR of RNA 1 by that of RNA 2 or 3 yielded infectious replicons. The sequence of a putative 5′ stem-loop structure in RNA 1 was found to be required for negative-strand RNA synthesis. A similar putative 5′ stem-loop structure is present in RNA 2 but not in RNA 3.

Alfalfa mosaic virus (AMV) is a positive-strand tripartite RNA virus belonging to the family Bromoviridae (reviewed in reference 2). RNAs 1 and 2 encode the replicase proteins P1 and P2, respectively. P1 contains a methyltransferase-like domain in its N-terminal half, as well as a helicase-like domain in its C-terminal half (6, 18). Residues in P1 that are highly conserved among alphaviral methyltransferase proteins or superfamily I helicases are required for AMV RNA replication (29, 30). P2 is the viral RNA-dependent RNA polymerase (RdRp) protein (16). RNA 3 encodes the movement protein (29, 30). P2 is the viral RNA-dependent RNA polymerase (RdRp) protein (16). RNA 3 encodes the movement protein (29, 30).

The 5′-terminal 11 nt of RNAs 1 and 2 are identical, and the 5′ UTRs of both RNAs contain a predicted 5′-terminal stem-loop structure consisting of a 12-bp stem and a 4-nt loop (M fold) (Fig. 1A and B). Interestingly, replication of RNA 3 with its 5′ UTR replaced with that of either RNA 1 or 2 was not supported in P12 plants (27).

Replication of AMV RNA 1 carrying the 5′ UTR of either RNA 2 or 3. In the present study, the 5′ UTR of RNA 1 (L1) was replaced with the 5′ UTR of RNA 2 (L2) or RNA 3 (L3). The mutations were introduced into pBSR1, which contains a DNA copy of RNA 1 cloned between a cauliflower mosaic virus 35S promoter and the terminator of the nopaline synthase gene (31). To that end, sequences containing L2 or L3 downstream of a 35S promoter were transferred to pBSR1 from pCA27T-Nco or pCA35T-Nco using KpnI and NcoI. pCA27T-Nco and pCa35T-Nco contain DNA copies of RNAs 2 and 3, respectively (26, 27). “Nco” refers to an NcoI site engineered into the cDNA across the position of the start codon of either P2 or P3. The resulting pBSR1 derivatives were cut with NcoI, after which the NcoI-NcoI fragment of pCA17T-Nco was inserted. In pCa17T-Nco, a mutation was introduced into cDNA 1 to create an NcoI site in the region containing the start codon of P1 (26). RNA 1 infectivity was not affected by the mutation introduced by the NcoI site (26).

RNA 1 derivatives carrying L2 or L3 instead of L1 are hereafter referred to as L2-R1 and L3-R1, respectively. L2-R1 contains the 54-nt-long RNA 2 5′ UTR fused directly to the start codon of RNA 1. L3-R1 contains the 345-nt-long RNA 3 5′ UTR fused directly to the start codon of RNA 1. 35S expression cassettes encoding L2-R1 or L3-R1 were transferred from the respective pBSR1 derivatives to pMOGR12 using KpnI and SsrI. pMOGR2 is a pMOG800 derivative carrying a 35S expression cassette containing cDNA 2, and pMOG800 is the transferred-DNA (T-DNA) vector used to express AMV RNAs in Nicotiana benthamiana by agroinfiltration (31). A derivative of pMOG800 carrying 35S expression cassettes of both RNAs 1 and 2 is called pMOGR12 (31). Infiltration of N. benthamiana leaves with a mixture of Agrobacterium tumefaciens clones containing pMOGR12 and pMOGR3 resulted in accumulation of viral RNA and protein at the level of a wt AMV infection (31). pMOGR3 carries a 35S expression cassette containing cDNA 3 in its T region (31).

The pMOGR12 derivatives encoding L2-R1 and L3-R1...
were transformed to *A. tumefaciens* strain LBA4404 by electroporation. Subsequently, suspensions of transformants were infiltrated as described previously into *N. benthamiana* leaves together with bacteria containing pMOGR3 (30, 31). Prior to infiltration, the bacterial suspensions were mixed in a 1:1 ratio based on their optical densities at 600 nm. As a negative control, leaves were infiltrated with a mixture of bacteria containing pMOGR3 and the empty T-DNA vector pMOG800. As a positive control, leaves were infiltrated with a mixture of bacteria containing the wt constructs pMOGR12 and pMOGR3. Total RNA was isolated from the leaves 5 days after infiltration and analyzed by Northern blot hybridization as described previously (31). For the detection of negative-strand RNAs 1, 2, and 3 with digoxigenin-labeled probes, Northern blots were analyzed. The band of negative-strand RNA migrating between nt 268 to 290 of RNA 1 [Table 1]. Subsequently, the 5' UTR sequences of the respective RNAs were determined (Baseclear, Leiden, The Netherlands). Sequencing of the product of the 5' RACE-PCR performed on L2-R1 progeny RNA confirmed that the chimeric RNA 1 contained the 5' UTR of RNA 2. The largest product of the 5' RACE-PCR performed on L2-R1 progeny RNA had the expected size estimated from agarose gel electrophoresis, and sequencing of the DNA showed that L3 was fused to the coding sequence of RNA 1. However, sequencing of the entire PCR product was hampered by a highly thymidine-rich region complementary to the sequence $A_9G_1A_{14}G_5A_9C_8G_9A_9$, which occurs in L3 between nt 261 and 301. Thus, we cannot rule out the possibility that progeny RNA 1 of the L3-R1 infection acquired one or more point mutations in the 5' terminal 260 nt. Sequencing of a smaller 5'-RACE product generated with the L3-R1 progeny revealed an RNA 1 chimera with the 3' terminal 44 nt of L3 fused to the P1 gene. Probably, this RNA corresponds to the
lower band of the RNA 1 doublet seen in Fig. 1D, lane 2. Our data demonstrate that replacement of the 5' UTR of RNA 1 by that of RNA 2 or 3 yields viable replicons.

**Sequences in the 5' UTR of RNA 1 affect negative-strand RNA synthesis.** The putative stem-loop structure near the 5' ends of RNAs 1 and 2 (Fig. 1A and B) is absent in the 5' UTR of RNA 3 (24). To analyze a possible role of this stem-loop in RNA replication, a mutation was introduced into RNA 1, disrupting base pairing in the upper half of the stem of the predicted hairpin (Fig. 2A, mutation r3). Disrupting base pairing in the upper half of the stem (Fig. 3A, mutations 5'H11032 C1G2C) of RNA 1 by introducing a translationally silent mutation that disrupted sequences were transferred as and 35S expression cassettes containing the mutant cDNA 1 produced into pBSR1 by PCR-mediated mutagenesis (Table 1), and 35S expression cassettes containing the mutant cDNA 1 sequences were transferred as KpnI-SstI fragments to pMOGR2, resulting in pMOGR12 derivatives. These derivatives contained a translationally silent mutation that disrupted an AluI site at position 203 in cDNA 1. Bacteria containing pMOGR12 with the same modification (pMOGR12-Alu) in addition, the top 3 bp of the stem were reversed (Fig. 3A, d3). In d3 and 3, 3' loops of RNAs 1 and 2 in agroinfiltrated leaves as efficiently as bacteria containing wt pMOGR12 (results not shown).

The R12-Alu derivatives were transformed to *A. tumefaciens*, and leaves were infiltrated with mixtures of bacteria containing these derivatives and R3 constructs. Total RNA was isolated from the leaves 5 days after infiltration, and accumulation of viral negative-strand RNA (Fig. 2B) and positive-strand RNA (Fig. 2C) was analyzed by Northern blot hybridization. Expression of each of the two mutant RNAs (L1-3'd5 and L1-LC) induced the synthesis of negative-strand RNAs 2 and 3 (Fig. 2B, lanes 2 and 3), indicating that both RNA 1 species were translated into the P1 protein and that active RdRp complexes were assembled. However, no negative-strand synthesis of the two RNA 1 mutants was observed (Fig. 2B, lanes 2 and 3). Therefore, the accumulated positive-strand RNA 1 corresponding to the two mutants that was observed in Fig. 2C, lanes 2 and 3, was probably not synthesized by the viral RdRp but was transcribed from the T-DNAs by cellular polymerase II. The synthesis of subgenomic RNA 4 in the presence of each mutant demonstrates that at least RNA 3 was replicated by viral RdRp (Fig. 2C, lanes 2 and 3). Previously, it was shown that expression of CP from RNA 3 strongly enhances accumulation of viral T-DNA transcripts, probably by protecting them from degradation (31). As replication-defective AMV T-DNA transcripts accumulate in agroinfiltrated leaves at levels that are 20 to 50% of the levels of the RNAs produced during a wt virus infection (31) (Fig. 2C), the replication-defective transcripts would serve as an ample source of templates for negative-strand RNA synthesis. Thus, we conclude that mutants L1-3'd5 and L1-LC are defective in negative-strand RNA 1 synthesis.

**5' primary structure of RNA 1 is of primary importance.** To further investigate the role of the 5'-terminal sequence of L1 in negative-strand RNA synthesis, a series of mutations was introduced into RNA 1. Base pairing in the predicted 5' stem-loop structure was affected by mutation of the top 3 nt on the 5' or 3' side of the stem (Fig. 3A, mutations 5'd3 and 3'd3). In addition, the top 3 bp of the stem were reversed (Fig. 3A, mutation r3). The importance of the loop sequence 5'C5G3C3U was analyzed by mutating the 4 nt in the loop separately (Fig. 3A, mutations ClG, G2C, C3G, and U4A). Mutations were introduced into pMOGR12-Alu as described above, and the resulting derivatives were transformed to *A. tumefaciens* in agroinfiltrated leaves, wt RNA 2 and mutant RNA 1 are expressed from these derivatives. Expression of RNA 3 was omitted because RNA 3 is not required for the synthesis of negative-strand RNAs 1 and 2 (31). However, to circumvent possible effects of the mutations on the translation of RNA 1, the pMOGR12-Alu derivatives were coexpressed with pMOGR1Δ/Δ. The R1Δ/Δ construct encodes 3'-terminally truncated replication-defective RNAs 1 and 2, which serve as messengers for the synthesis of wt P1 and P2 proteins (31).

Leaves were infiltrated with mixtures of bacteria containing the R1Δ/Δ construct and the R12-Alu constructs encoding the mutations in RNA 1 shown in Fig. 3A. Two days after infiltration, RNA was extracted from the leaves and accumulation of
negative-strand RNAs was analyzed by Northern blot hybridization (Fig. 3B). wt RNA 2 expressed from all mutant R12-Alu derivatives served as a template for negative-strand RNA synthesis by RdRp expressed either from the R12-Alu construct or from the R1/H9004/2/H9004 construct (Fig. 3B, lanes 2 to 10). Mutant L1-LC (Fig. 2A) was included as a control. As before (Fig. 2B, lane 3), expression of the mutant RNA did not induce synthesis of negative-strand RNA 1 (Fig. 3B, lane 5), and synthesis of negative-strand RNA 2 was reduced compared to that of the wt control (Fig. 3B, lane 10). Similar to the disruption of the base pairing of the top 5 bp of the stem in mutant L1-3’d5 (Fig. 2B, lane 2), disruption of the base pairing of the top 3 bp in mutants L1-5’d3 and L1-3’d3 blocked the synthesis of negative-strand RNA 1 (Fig. 3B, lanes 2 and 3). However, reversion of these 3 bp in mutant L1-r3 also blocked the synthesis of negative-strand RNA 1 (Fig. 3B, lane 4). One-by-one mutation of the four loop nucleotides showed that mutation of the C3 residue into a G residue (mutant L1-C3G) did not affect the synthesis of negative-strand RNA 1 (Fig. 3B, lane 8). However, mutation of each of the three other loop nucleotides abolished the synthesis of negative-strand RNA 1 (Fig. 3B, lanes 6, 7, and 9). It should be noted that the nucleotides at positions 1, 2, and 4 from the 5’ ends of the loops of the predicted 5’-terminal hairpins in RNAs 1 and 2 are identical, whereas variation at position 3 is observed (Fig. 1A and B).

Replacement of the 5’ UTR of RNA 1 with that of subgenomic RNA 4 abolished the synthesis of negative-strand RNA 1 in protoplasts (13). Here, we showed that replacement of this UTR with that of RNA 2 or RNA 3 permitted stable replication of the RNA in plants. The 5’ UTRs of the genomic RNAs of AMV may contain sequences involved in positive-strand RNA promoter activity. As these 5’ UTRs vary in length

FIG. 2. Sequences in the 5’ UTR of RNA 1 affect negative-strand RNA synthesis. (A) The predicted 5’-terminal stem-loop structure in RNA 1, with mutated residues in the stem (solid line) and in the loop (dotted line) boxed. The sequences in L1 mutants 3’d5 and LC are indicated by arrows. (B and C) Leaves were infiltrated with mixtures of bacteria containing pMOGR3 and either the empty vector pMOG800 (lanes 1) or a pMOGR12-Alu derivative. The R3 construct expressed wt RNA 3 (lanes 1 to 4), and the R12-Alu construct expressed wt RNA 2 (lanes 2, 3, and 4) and either RNA 1-Alu (lanes 4), L1-3’d5 (lanes 2), or L1-LC (lanes 3), as indicated above the lanes. Total RNA was extracted from the leaves 5 days after infiltration and analyzed by Northern blot hybridization using digoxigenin-labeled probes specific for minus-strand (-) RNAs 1, 2, and 3 (B) or plus-strand (+) RNAs 1 to 4 (C). The positions of RNAs 1, 2, 3, and 4 are indicated on the left.

FIG. 3. 5’ primary structure of RNA 1 is of primary importance. (A) Predicted 5’-terminal stem-loop structure in RNA 1 with mutated residues in the stem (boxed) and in the loop. Mutant sequences in the stem (mutations 5’d3, 3’d3, and r3) are indicated by solid arrows, and mutant residues in the loop (mutations C1G, G2C, C3G, and U4A) are indicated by dotted arrows. (B) Leaves were infiltrated with mixtures of bacteria containing pMOGR1/H9004/2/H9004 and either the empty vector pMOG800 (lane 1) or a pMOGR12-Alu derivative. The R1/H9004/2/H9004 construct expressed wt P1 and P2 (lanes 1 to 10), and the pMOGR12-Alu construct expressed wt RNA 2 (lanes 2 to 10) and either RNA 1-Alu (lane 10) or RNA 1-Alu with mutations in L1 (lanes 2 to 9), as indicated above the lanes. Total RNA was extracted from the leaves 2 days after infiltration and analyzed by Northern blot hybridization using digoxigenin-labeled probes specific for minus-strand RNAs 1 and 2. The positions of RNAs 1 and 2 are indicated on the left.
from 54 to 345 nt and show little sequence similarity, it is difficult to distinguish possible promotor elements. A common element in the 5′ UTRs of RNAs 1 and 2 is the predicted 5′ stem-loop structure. Our data indicate that the putative stem-loop structure in RNA 1 is required for negative-strand RNA synthesis, but an additional role of the structure in positive-strand RNA synthesis cannot be ruled out. The 5′ regions of BMV RNAs 1 and 2 contain stem-loop structures with loop sequences resembling box B elements that are homologous to the TΨC stem-loop of TRNAs. These elements mediate recruitment of the RNAs to replication complexes and are thus required for negative-strand RNA synthesis (3, 19, 20). The box B consensus sequence in bromovirus and cucumovirus RNAs is GGUUCAANNCC with N being any possible nucleotide (3), but no such sequence is found in the 5′ UTRs of AMV RNAs 1 and 2. Moreover, our mutational analysis indicates that the primary sequence rather than the secondary structure of the putative stem-loop near the 5′ end of AMV RNA 1 is important for negative-strand RNA synthesis. Thus, there is no evidence supporting a role of this stem-loop in template recruitment.

For an increasing number of viruses, it is becoming clear that circularization of plus-strand genomic RNAs is involved in the regulation of translation, replication, and subgenomic-RNA synthesis. Long-distance RNA-RNA interactions between 5′- and 3′-terminal sequences are involved in the translation of barley yellow dwarf virus RNA and in the replication of several flavivirus RNAs (4, 7, 10, 33, 34). In the case of the flavivirus dengue virus, this long-distance interaction is thought to be required for negative-strand RNA synthesis (33, 34). In addition, structural features in the 5′ UTRs of bovine viral diarrhea virus and tomato bushy stunt virus may be involved in negative-strand RNA synthesis (32, 35). Moreover, secondary-structure elements in the 5′ UTRs of Sindbis virus and poliovirus RNAs bind viral or host factors involved in replication and are required for negative-strand RNA synthesis (1, 5, 8, 9, 11). Formation of a protein-protein bridge between the 5′-terminal cloverleaf structure and the 3′-terminal poly(A) tail in poliovirus RNA and between the 5′ UTR and the 3′-terminal poly(A) tail in Sindbis virus RNA has been implicated as an essential step in the initiation of negative-strand RNA synthesis (1, 8, 9). It has been proposed that a protein-protein bridge between initiation factors bound to the cap structure and CP bound to the 3′ end of AMV RNAs converts these RNAs into a closed-loop structure that is essential for translation of the RNAs (13). However, CP is dispensable for negative-strand RNA synthesis (12, 31). So far, we have been unable to identify 3′-terminal sequences in AMV RNAs that could potentially base pair with 5′ sequences involved in negative-strand RNA synthesis.

REFERENCES


