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Comparison of the Role of 5′ Terminal Sequences of Alfalfa Mosaic Virus

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The 5′ untranslated regions (UTRs) of the genomic RNAs 1, 2, and 3 of alfalfa mosaic virus (AMV) are 100, 54, and 345 nucleotides (nt) long, respectively, and lack extensive sequence similarity to each other. RNA 3 encodes the movement protein P3 and the coat protein and can be replicated in transgenic tobacco plants expressing the replicase proteins P1 and P2 (P12 plants). 5′ *Cis*-acting sequences involved in RNA 3 replication have been shown to be confined to the 5′ UTR. When the 5′ UTR of RNA 3 was replaced by the 5′ UTRs of RNAs 1 or 2, the recombinant RNA was not infectious to P12 plants. Also, when the P3 gene in RNA 3 was put under the control of a subgenomic promoter and the 5′ UTR of this RNA was replaced by 5′ terminal RNA 1 sequences of 103 to 860 nt long or RNA 2 sequences of 57 to 612 nt long, no accumulation of the hybrid RNAs was observed. Deletion of the 5′ 22 nucleotides of RNA 3 resulted in the accumulation of a major progeny that lacked the 5′ 79 nt. However, when the 5′ 22 nucleotides of RNA 3 were replaced by the complete 5′ UTR of RNA 1 or 5′ sequences of RNAs 1, 2, or 3 with a length of 5 to 15 nt, accumulation of the full-length mutant RNAs was observed. The effect of mutations in the 5′ viral sequences of 5 to 15 nt was analyzed. It is concluded that although elements within nucleotides 80–345 of the 5′ UTR of RNA 3 are sufficient for replication, a specific sequence of 3 to 5 nt is required to target the replicase to an initiation site corresponding to the 5′ end of the RNA.

INTRODUCTION

Alfalfa mosaic virus (AMV) is a member of the *Bromoviridae* family of plant viruses. The genome of this virus consists of three single-stranded RNA molecules of messenger-sense polarity, designated RNA 1, 2, and 3 in order of decreasing size. RNA 1 and 2 encode viral replicase proteins P1 and P2, respectively. RNA 3 is translated into the putative movement protein P3. The viral coat protein (CP) is translated from the subgenomic RNA 4, which is colinear with the 3′ terminal 881 nucleotides (nt) of RNA 3. Besides having a structural role in encapsidating the viral RNAs, the CP was shown to be involved in the initiation of infection, plus-strand RNA synthesis, and cell-to-cell transport (Bol *et al.,* 1971; de Graaff *et al.,* 1995; van der Vossen *et al.,* 1994). The 3′ terminal 145 nt of the three genomic RNAs are highly homologous and can form an almost identical secondary structure, containing a number of stem–loop structures flanked by AUGC-sequence motifs (Koper-Zwarthoff and Bol, 1980). These elements have been shown to serve as high-affinity binding sites for the CP and can also be found in the RNAs of the related ilarviruses (Houser-Scott *et al.,* 1994; Reusken *et al.,* 1994; Reusken and Bol, 1996).

In contrast to the presence of this homologous region at the 3′ terminus of the viral RNAs, sequence similarity at the 5′ end of the AMV RNAs of strain 425 (which is used in our experiments) is limited to 11 identical nucleotides at the 5′ end of RNAs 1 and 2. In RNAs 1, 2, and 3 of this AMV strain, the 5′ untranslated regions (UTRs) are 100, 54, and 345 nt long, respectively. For RNA 3, *cis*-acting sequences required for replication have been studied previously in transgenic tobacco plants expressing the viral replicase proteins P1 and P2 (P12-plants), or protoplasts thereof. Deletions in the P3 gene did not significantly reduce RNA 3 replication in P12 protoplasts as long as the subgenomic promoter for RNA 4 synthesis was not affected (van der Vossen *et al.,* 1995). Therefore, it was concluded that 5′ terminal *cis*-acting sequences involved in RNA 3 replication are limited to the leader sequence of this RNA, which contains several 27-nt repeats. Each of these repeats contains a sequence similar to the ICR2 motifs recognized in many plant viruses carrying tRNA-like structures at their 3′ terminus (Duggal *et al.,* 1994). A 5′ terminal sequence of 112 nt with one 27-nt repeat was sufficient for a 50-fold reduced accumulation of RNA 3 in P12 protoplasts but permitted near wild-type (wt) accumulation in P12 plants (van der Vossen *et al.,* 1993). The 27-nt repeat in this 112-nt sequence of RNA 3 is predicted to be located at

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the top of a stem–loop structure. Both the ICR2 motif and the secondary structure in this 112-nt sequence appeared to be important for RNA 3 accumulation (van der Vossen and Bol, 1996). On the other hand, an RNA 3 mutant with a deletion of nucleotides 1–79, including the 5′ most 27-nt repeat, also accumulated at wt levels in P12 plants (van der Vossen *et al.,* 1996). Possibly, when the 5′ most 27-nt repeat is deleted, its function is taken over by similar repeats located downstream. Although the ICR2 motif and the predicted element of secondary structure present in the 5′ terminal 112 nt of RNA 3 cannot be found at the 5′ terminus of AMV RNA 1 or 2, we investigated a possible functional equivalence between the 5′ termini of the three genomic AMV RNAs. The results demonstrated that replacement of the entire 345-nt leader sequence of RNA 3 by the leaders of RNA 1 or 2, or by much longer 5′ terminal sequences from these RNAs, resulted in a loss of infectivity of the RNA. However, when the 5′ terminal 22 nt of RNA 3 were replaced by full-length or partial leader sequences of RNAs 1 and 2, replication competent mutants were obtained. A mutational analysis indicated that the 5′ 3 to 5 nt of the AMV RNAs, or their equivalent in the corresponding minus-strand RNAs, play a crucial role in initiation of plus-strand RNA synthesis.

MATERIALS AND METHODS

Construction of recombinant DNAs

In this study, hybrids were used that were derived from constructs containing the AMV cDNAs flanked by the cauliflower mosaic virus 35S promoter and nopaline synthase (nos) terminator (referred to as 35S/cDNAs; Neeleman *et al.,* 1993). In order to exchange 5′ UTRs between the different genome segments, mutant 35S/cDNAs were used in which an *Nco*I restriction site was engineered at the start of the P1, P2, or P3 open reading frame. These constructs are referred to as 1-Nco, 2-Nco, and 3-Nco, respectively (van Rossum *et al.,* 1996). Mutants 3/L1 and 3/L2 (Fig. 1A) were constructed by digesting these three plasmids with the restriction enzymes *Nco*I and *Kpn*I and ligating the relevant DNA fragments. Mutant 3SG (Figs. 1B and 1C) was constructed by amplifying a DNA fragment corresponding to nucleotides 1126 to 1280 from RNA 3 in a PCR reaction using Vent DNA polymerase (New England Biolabs) and oligonucleotides 5′ ACCACTATAAATGGTATGGCACC 3′ and 5′ GAAAATTAAAAATAAAAACGGCC 3′ as primers. The amplified fragment contains the subgenomic promoter. This fragment was treated with T4 DNA-kinase (BRL) and ligated into construct 3-Nco after this was digested with *Xho*I and treated with T4 DNA-polymerase (BRL) to generate blunt ends. The resulting plasmid 3SG corresponds to construct SGP3 (van der Vossen *et al.,* 1995), but is derived from the infectious 35S/cDNA 3 construct rather than the clone containing a T7 promoter (Neeleman *et al.,* 1993). Furthermore, this construct contains an *Nco*I restriction site at the position of the original P3 startcodon. Mutant 3SG was subsequently used to construct hybrids in which the 5′ terminal 345 nt were replaced by 5′ terminal fragments of RNA 1 or 2 (Figs. 1B and 1C). For this purpose, plasmid 3SG was digested with *Nco*I. The DNA was subsequently digested with *Kpn*I, which cuts the plasmid just in front of the CaMV 35S promoter sequence. The large fragment was isolated and ligated to *Kpn*I–*Nco*I fragments from mutants 1-Nco and 2-Nco (van Rossum *et al.,* 1996), respectively, to yield mutants 3SG/L1-103 and 3SG/L2-57. Mutants 3SG/L1-204, 3SG/L1-361, and 3SG/L1-860 were constructed by ligating *Kpn*I–*Pst*I, *Kpn*I–*Sal*I, and *Kpn*I–*Bgl*II restriction fragments from wild-type 35S/cDNA 1 (in which the *Pst*I, *Sal*I, and *Bgl*II sites were treated with T4 DNA-polymerase to yield blunt ends) to a fragment from mutant 3SG. This fragment was obtained by digesting the plasmid with *Nco*I, generating blunt ends with T4 DNA-polymerase, and digesting the DNA subsequently with *Kpn*I. In a similar way, mutants 3SG/L2-266, 3SG/L2-407, and 3SG/L2-612 were constructed by inserting *Kpn*I–*Xho*I, *Kpn*I–*Eco*NI, and *Kpn*I–*Ssp*I fragments from wild-type 35S/cDNA 2 (in which *Xho*I and *Eco*NI sites were converted to blunt ends using T4 DNA-polymerase) into the fragment from mutant 3SG described above.

Mutant 3S/L1-103 was constructed by digesting mutant 1-Nco with *Nco*I, generating blunt ends with T4 DNA-polymerase, and digesting subsequently with *Kpn*I. The fragment containing the sequence corresponding to the 5′ terminal 103 nt of RNA 1 was isolated and ligated to the *Ssp*I–*Kpn*I fragment of the wild-type 35S/cDNA 3 construct. The same *Ssp*I–*Kpn*I fragment was ligated to the *Kpn*I–*Nru*I fragment from mutant 2-Nco to create 3S/L2-15 (Fig. 3). Mutants 3S/L2-10, 3S/L2-5, 3Δ6-22, 3Δ6-11, 3Δ1-71, 3Δ1-79, M1, M2, and M3 were constructed by PCR amplification of modified 5′ terminal sequences using Vent DNA-polymerase and the following oligonucleotide primers: 5′ GTTTTTATCTATTCCAATTCAACTCAATTAACGC 3′ (3S/L2–10), 5′ GTTTTATTCCAATTCAACTCAATTAACGC 3′ (3S/L2–5), 5′ GTATTATTCCAATTCAACTCAATTAACGC 3′ (3Δ6–22), 5′ GTATTATTTTCAAAATATTCCAATTCAACTC 3′ (3Δ6–11), 5′ GTAAGTATGTTTCTGTAAAAGCG 3′ (3Δ71), 5′ GTTTCTGTAAAAGCGTTTCTTG 3′ (3Δ79), 5′ GCCCCATTCCAATTCAACTCAATTAACGC 3′ (M1), 5′ GTCCCATTCCAATTCAACTCAATTAACGC 3′ (M2), and 5′ GCCCAGGACGTGCTCATTCCAATTCAACTCAATTAACGC 3′ (M3). In all cases a primer complementary to nt 471 to 490 in RNA 3 was used as downstream primer. PCR products were phosphorylated using T4 DNA-kinase and digested with *Xho*I. The relevant DNA fragments were isolated from agarose gels and ligated to the *Xho*I–*Stu*I fragment from the plasmid pCa(+11)3T (van der Vossen *et al.,* 1996). All muta-

tions were confirmed by restriction mapping and DNA sequence analysis.

Inoculation of plants and isolation of virus particles

Transgenic *Nicotiana tabacum* cv. Samsun NN plants, expressing AMV replicase proteins P1 and P2 (P12 plants), were grown and inoculated with 35S/cDNA constructs as described previously (Neeleman *et al.,* 1993; Taschner *et al.,* 1991). Each sample was inoculated on two plants using three half-leaves per plant (van Dun *et al.,* 1988).

Analysis of viral RNA

Virus particles were isolated from inoculated leaves 5 days after inoculation as described previously (van Vloten-Doting and Jaspars, 1972). RNA isolated from these virus particles was analyzed by Northern blot hybridization. The amount of RNA loaded per slot corresponded to 5 mg of leaf material. Random primed ^{32}P-labeled cDNA 3 was used as a probe for the blot shown in Fig. 2 (Feinberg and Vogelstein, 1984). In the case of Figs. 4 and 7, viral RNAs were visualized using the DIG nonradioactive nucleic acid labeling and detection system (Boehringer Mannheim), also using the complete cDNA 3 as a probe. To examine the exact length of the progeny RNA, primer extension on the viral RNA was performed essentially as described (van der Vossen *et al.,* 1995), using reverse transcriptase and a primer complementary to nt 176–195 in AMV RNA 3.

RESULTS

Replacement of the 5′ UTR of RNA 3 by 5′ terminal sequences from RNA 1 or 2

In constructs 3/L1 and 3/L2 the 5′ UTR of RNA 3 is replaced by the full-length 5′ UTRs of RNA 1 and 2, respectively (Fig. 1A). As is shown in Fig. 2, inoculation of P12-plants with these constructs did not result in accumulation of RNA 3 and 4 (lanes 3 and 4), although inoculation with wt DNA 3 did (lane 2). Several explanations for this result are possible: First, the 5′ UTRs from RNA 1 and 2 may not contain all the necessary information required to promote plus-strand RNA synthesis. It is possible that a sequence extending into the P1 or P2 open reading frame is essential. Second, the expression of the P3 protein could be affected in these constructs. Since these experiments were performed in intact plants, translation of the P3 movement protein from the mutants will be required for accumulation of viral RNA. Finally, the possibility remains that the 5′ terminal sequences from the different AMV RNAs cannot be freely exchanged due to more complex interactions; e.g., with other parts of the viral RNAs.

To rule out the first two possibilities, we tested a series

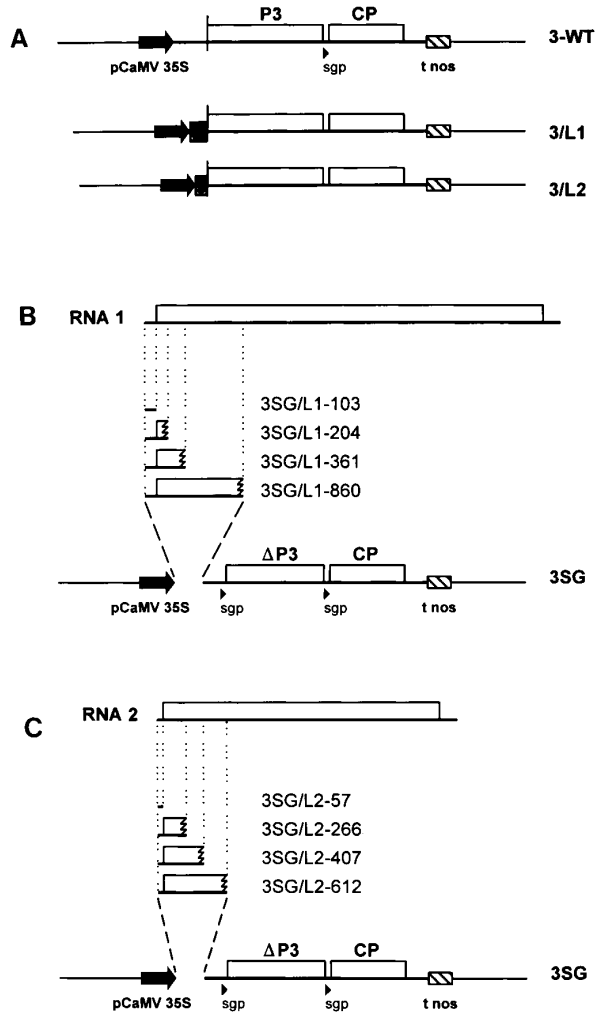

FIG. 1. Structure of RNA 3 leader mutants. A shows a schematic representation of the wild-type 35S/cDNA 3 construct (3-WT) and mutants 3/L1 and 3/L2. In mutants 3/L1 and 3/L2 the sequence corresponding to the 5′ UTR of RNA 3 (345 nt) was replaced by the leader sequences of RNA 1 (100 nt) and RNA 2 (54 nt), respectively. The dark arrow represents the CaMV 35S promoter and the hatched box represents the nopaline synthase terminator. The position of the subgenomic promoter (sgp) present on RNA 3 is indicated by an arrowhead. B and C show mutants derived from construct 3SG. In this construct a copy of the subgenomic promoter was inserted into the *Xho*I restriction site in cDNA 3. This results in the expression of a truncated P3 protein (ΔP3) from a novel subgenomic RNA (van der Vossen *et al.,* 1995). In the constructs shown here, the 5′ UTR of RNA 3 was replaced by 5′ terminal fragments from RNA 1 of 103, 204, 361, or 860 nt (B) or 5′ terminal fragments from RNA 2 of 57, 266, 407 or 612 nt (C).

of constructs derived from mutant 3SG, in which a copy of the subgenomic promoter (sgp) was inserted in the *Xho*I restriction site early in the P3 open reading frame. It has been shown by van der Vossen *et al.* (1995) that the truncated ΔP3 protein expressed from this sgp is functional in cell-to-cell movement. An *Nco*I restriction site, present at the position of the original P3 startcodon in mutant 3SG, was used to replace the 5′ terminal 345

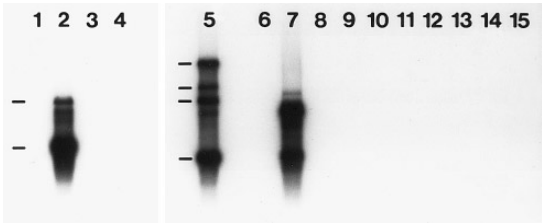

FIG. 2. Northern blot showing the accumulation of viral RNAs in P12 plants inoculated with wild-type and hybrid 35S/cDNA constructs. The results from two separate experiments (lanes 1–4 and lane 6–15) are combined in this figure. The following inocula were used : mock-inoculation (lanes 1 and 6), 3-WT (lane 2), 3/L1 (lane 3), 3/L2 (lane 4), RNAs isolated from AMV particles (lane 5), 3SG (lane 7), 3SG/L1-103 (lane 8), 3SG/L1-204 (lane 9), 3SG/L1-361 (lane 10), 3SG/L1-860 (lane 11), 3SG/L2-57 (lane 12), 3SG/L2-266 (lane 13), 3SG/L2-407 (lane 14), and 3SG/L2-612 (lane 15). To the left of lane 1 the positions of RNA 3 and 4 (top to bottom) are indicated. The marks to the left of lane 5 indicate the positions of RNAs 1, 2, 3, and 4 (top to bottom).

nt of RNA 3 with sequences derived from AMV RNA 1 or RNA 2. The resulting constructs are shown in Figs. 1B and 1C. In these constructs, the 5′ terminal sequences of the mutants will not affect translation of the ΔP3 protein as this will be translated from a subgenomic messenger. Lanes 7 to 15 in Fig. 2 show the accumulation of viral RNAs in tobacco plants inoculated with mutant 3SG and the hybrid constructs derived from it. Upon inoculation with mutant 3SG, a full-length product can be detected, as well as the subgenomic messenger RNAs for the expression of ΔP3 and CP (lane 7). However, if the 5′ terminal 345 nt from mutant 3SG were replaced by 5′ terminal fragments of 103, 204, 361, or 860 nt from RNA 1 (lanes 8–11) or the 5′ terminal 57, 266, 407, or 612 nt from RNA 2 (lanes 12–15), no accumulation of viral RNAs was observed.

Replacement of the 5′ terminal 22 nt of RNA 3 with sequences from RNA 1 or 2

Although overall sequence similarity between the leaders of AMV RNAs is limited, some similarity is found at the 5′ termini, particularly those of RNAs 1 and 2. To see if a small 5′ terminal part of the RNA 3 leader sequence could be replaced by 5′ terminal sequences from RNA 1 or 2, the constructs shown in Fig. 3 were made. By using an *Ssp*I restriction site in the leader of RNA 3, the 5′ terminal 22 nt of this RNA were replaced by the complete 5′ UTR of RNA 1 (construct 3S/L1–103), the 5′ terminal 15 nt of RNA 2 (3S/L2–15), and the 5′ terminal sequences of 10 or 5 nt that are identical in RNAs 1 and 2 (3S/L2–10 and 3S/L2–5). Previously, we reported that the RNA 3 mutant with a deletion of nucleotides 1–22 (3Δ1–22) was infectious to plants but showed an altered dependency of the infection for CP in the inoculum (van der Vossen *et al.,* 1996). In contrast to the inoculation of nontransgenic plants with AMV RNAs 1, 2, and 3, no CP is required to initiate infection of P12 plants with wt RNA 3 or 35S/cDNA 3 (Taschner *et al.,* 1991; Neeleman *et al.,* 1993) (Fig. 4, lanes 1 and 2). When the P12 plants are inoculated with mutant 3Δ1–22 the infection becomes largely dependent on the presence of CP or 35S/cDNA 4 in the inoculum (van der Vossen *et al.,* 1996) (Fig. 4, lanes 3 and 4). It was shown that the major progeny RNA resulting from infection with mutant 3Δ1–22 is an RNA 3 molecule with the 5′ terminal 79 nt deleted, and it was suggested that CP in the inoculum was required to permit internal initiation by the viral replicase at position 80 in the minus-strand RNA of mutant 3Δ1–22 (van der Vossen *et al.,* 1996). Infection of P12 plants with RNA 3 mutants with 5′ sequences derived from RNAs 1 or 2 was independent of the presence of 35S/cDNA 4 in the inoculum (Fig. 4, lanes 9–16). This could indicate that in contrast to 3Δ1–22, these chimeric constructs produced full-length progeny RNAs corresponding in size to the mutant RNAs expressed from the inoculum cDNAs.

To investigate this possibility, the nature of the progeny RNAs resulting from some of these inoculations was examined in the primer-extension experiment shown in Fig. 5. In lane 6, the product from plants inoculated with the wild-type 35S/cDNA 3 construct is seen. As expected, the length of the extension product indicates correct initiation of the produced plus-strand RNAs at the 5′ terminal G-residue (G1). As seen in lane 5, inoculation with mutant 3Δ1–22 resulted in three distinct extension products, the smallest of which is identical to the product described previously, starting at the G-residue at position 80 (van der Vossen *et al.,* 1996). The length of the other two extension products indicated the presence of RNA molecules probably resulting from initiation at G-residues at positions 56 and 72. As the experiments were done with RNAs from purified virus particles, all truncated RNAs are apparently encapsidated. The progeny RNAs with 5′ termini starting at positions 56, 72, and 80 were all found in leaves inoculated with mutant 3Δ1–22, but only the progeny starting at position 80 was found in systemically infected leaves of these plants (result not shown). The products seen in lanes 1–4 indicate that full-length products are produced upon inoculation of P12 plants with constructs 3S/L2-5, 3S/L2-10, 3S/L2-15, and 3S/L1-103.

Symptom formation by RNA 3 leader mutants

Infection of P12 plants with wt RNA 3 results in high levels of virus accumulation without detectable symptom formation. After inoculation with mutant 3Δ1–22, necrotic ringspot-like symptoms developed systemically, which were attributed to the progeny of this mutant lacking nucleotides 1–79 of RNA 3 (van der Vossen *et al.,* 1996). As summarized in the legend to Fig. 3, similar systemic necrosis developed in plants inoculated with mutants 3S/L2-15, 3S/L2-10, 3S/L2-5, but no necrosis

Construct	5' terminal sequence	Infectivity -CP	Infectivity +CP	Progeny FL/TR	Necrosis - / +
3S/L1-103	GUUUUUAUCUUACACACG ··· 85 ····· AUUCCAAUUCAACUCAAU ·····	+	+	FL	-
3S/L2-15	GUUUUUAUCUUUUCG ——— AUUCCAAUUCAACUCAAU ·····	+	+	FL	+
3S/L2-10	GUUUUUAUCU ——— AUUCCAAUUCAACUCAAU ·····	+	+	FL	+
3S/L2-5	GUUUU ——— AUUCCAAUUCAACUCAAU ·····	+	+	FL	+
3WT	GUAUUAAUACCAUUUUCAAAUAUUCCAAUUCAACUCAAU ····· (*Ssp* I)	+	+	FL	-
3Δ1-22	AUUCCAAUUCAACUCAAU ·····	-	+	TR	+
3Δ6-22	GUAUU ——— AUUCCAAUUCAACUCAAU ·····	+	+	FL	+
3Δ6-11	GUAUU ——— AUUUUCAAAAUAUUCCAAUUCAACUCAAU ·····	+	+	FL	-
3S/M1	GCCCC ——— AUUCCAAUUCAACUCAAU ·····	-	+	TR	+
3S/M2	GUCCC ——— AUUCCAAUUCAACUCAAU ·····	-	+	TR	+
3S/M3	GCCCAGGACGUGCUC ——— AUUCCAAUUCAACUCAAU ·····	-	+	TR	+

FIG. 3. Schematic representation of the RNAs corresponding to mutant 35S/cDNA constructs, in which the 5′ terminal 22 nt of AMV RNA 3 were replaced by the 5′ terminal 103 nt of RNA 1 (3S/L1-103, RNA 1 sequence drawn partially), the 5′ terminal 15 nt of RNA 2 (3S/L2-15), or the 5′ terminal 10 or 5 nt present in both RNA 1 and RNA 2 (3S/L2-10 and 3S/L2-5, respectively). In addition, the 5′ terminal sequences are shown of wild-type RNA 3 (3WT) and RNA 3 mutants in which nucleotides 1 to 22, 6 to 22, or 6 to 11 were deleted (designated 3Δ1–22, 3Δ6–22, and 3Δ6–11, respectively). In mutants M1, M2, and M3, the 5′ sequences differ from those of RNAs 1, 2, or 3 as indicated. For each construct, data on infectivity to P12 plants, nature of the progeny RNA, and symptoms on P12 plants are summarized. Infectivity was assayed in the presence or absence of CP in the inoculum and is indicated by a plus sign (infectious) or a minus sign (noninfectious). Primer extension was used to determine whether the progeny RNA was full-length (FL) or 5′ terminally truncated (TR) compared to the inoculum RNA. Symptoms are represented by a plus sign (necrotic symptoms) or a minus sign (no detectable symptoms).

was detectable in plants inoculated with 3S/L1-103. To map the determinant for symptom formation in more detail, RNA 3 constructs were made with a deletion of nucleotides 6–22 (3Δ6–22) or 6–11 (3Δ6–11) (see Fig. 3). In the sequence of the 5′ terminal five nt, RNA 3 differs only in the third position from RNAs 1 and 2. Therefore, mutant 3Δ6–22 differs only in the third position from mutant 3S/L2–5 and both mutants induced systemic necrosis in P12 plants. As summarized in the legend to Fig. 3, mutant 3Δ6–11 did not induce detectable symptoms. Figure 4 shows that mutants 3Δ6–22 (lanes 5 and 6) and 3Δ6–11 (lanes 7 and 8) accumulated at wt levels in P12 plants whether or not 35S/cDNA 4 was present in the inoculum. The minor difference between lanes 5 and 6 in Fig. 4 was not observed in other experiments.

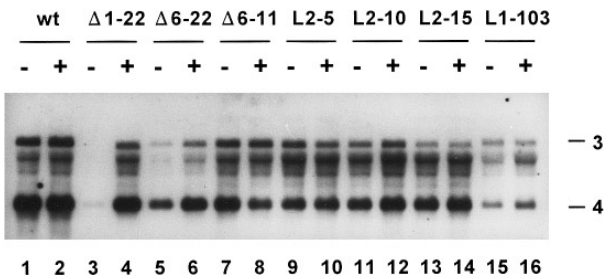

FIG. 4. Northern blot showing the accumulation of encapsidated viral RNAs in P12 plants 5 days after inoculation with wild-type and mutant 35S/cDNA 3 constructs in the presence or absence of 35S/cDNA 4 (indicated by + and − signs). The positions of RNAs 3 and 4 are indicated in the right-hand margin. P12 plants were inoculated with wt RNA 3 (lanes 1, 2) and mutants 3Δ1–22 (lanes 3, 4), 3Δ6–22 (lanes 5, 6), 3Δ6–11 (lanes 7, 8), 3S/L2–5 (lanes 9, 10), 3S/L2–10 (lanes 11, 12), 3S/L2–15 (lanes 13, 14), and 3S/L1–103 (lanes 15, 16). Viral RNAs on the blot were detected using the DIG nonradioactive nucleic acid labeling and detection system (Boehringer Mannheim) with the complete cDNA 3 as a probe.

Role of nucleotides 1 to 5 in RNA 3 replication

Comparison of the results with mutants 3S/L2–5 and 3Δ6–22 with those obtained with mutant 3Δ1–22 suggested that a 5′ sequence of 5 nt in the first two mutants was sufficient to permit initiation of plus-strand RNA synthesis with a G-residue corresponding to the 5′ nucleotide of the mutant. Possibly, the replicase is unable to start plus-strand synthesis with the 5′ A-residue of mutant 3Δ1–22 and selects G-residues at positions 56, 72, and 80 as alternative initiation sites. To investigate whether the 5′ sequences of 5 nt of mutants 3S/L2–5 and 3Δ6–22 contain determinants involved in intiation of plus-strand RNA synthesis other than the 5′ terminal G-residue, mutants M1, M2, and M3 were constructed (see Fig. 3). Mutants M1 and M2 contain the 5′ sequences GCCCC and GUCCC, respectively, which differ in four or three positions from the 5′ termini of mutants 3S/L2–5 (GUUUU) or 3Δ6–22

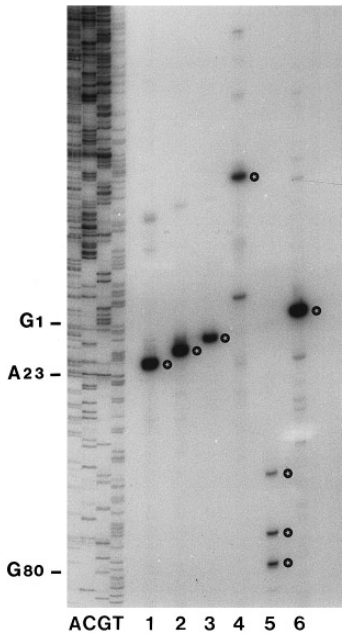

FIG. 5. Analysis of 5′ termini of viral RNAs by primer extension. A primer complementary to nt 176 to 195 in RNA 3 was annealed to the RNA and extended using reverse transcriptase. The sequence ladder at the left is the result of a sequencing reaction incorporating ^{35}S-dATP on wild-type cDNA 3, using the same primer. The products were analyzed on a 6% acrylamide gel. The primer-extension products were hybridized to ^{32}P-dCTP labeled cDNA 3. Extension products discussed in the text are indicated by a dot. The RNAs analyzed were purified from virus particles isolated from P12 plants infected with mutants 3S/L2–5 (lane 1), 3S/L2–10 (lane 2), 3S/L2–15 (lane 3), 3S/L1–103 (lane 4), 3Δ1–22 (lane 5), and wt RNA 3 (3WT, lane 6). To the left of the sequence ladder the positions of nucleotides 1, 23, and 80 in the wild-type RNA 3 sequence are indicated.

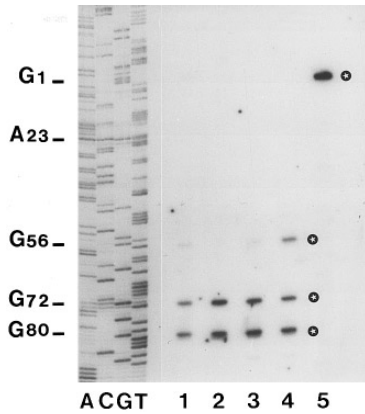

FIG. 6. Analysis of 5′ termini of viral RNAs by primer-extension. The experiment was done as described in the legend of Fig. 5. The RNAs analyzed were purified from virus particles isolated from P12 plants infected with mutants M1 (lane 1), M2 (lane 2), M3 (lane 3), 3Δ1–22 (lane 4), and wt RNA 3 (lane 5). Extension products discussed in the text are indicated by a dot.

(GUAUU). Mutant M3 contains a randomized 5′ sequence of 15 nt fused to the A-residue at position 23 of RNA 3. Mutants M1, M2, and M3 accumulated in P12 plants at levels similar to those of mutant 3Δ1–22 (data not shown) and the 5′ termini of the progeny RNAs of these mutants were analyzed by primer extension. Figure 6 shows that the progeny of mutants M1 (lane 1), M2 (lane 2), and M3 (lane 3) is similar to that of mutant 3Δ1–22 (lane 4). Only truncated RNAs are produced with 5′ termini mainly corresponding to G-residues G-72 and G-80 and no full-length mutant RNAs are detectable. This demonstrates that the 5′ G-residue of mutants M1, M2, and M3 is not sufficient to permit initiation of plus-strand RNA synthesis at the 5′ end of the mutant RNAs. Similar to mutant 3Δ1–22, mutants M1, M2, and M3 caused necrotic symptoms on P12 plants and the initiation of infection was strongly stimulated by addition of 35S/cDNA 4 to the inoculum (data summarized in Fig. 3).

Role of CP in the initiation of infection by RNA 3 leader mutants

As is summarized in Fig. 3, the initiation of infection in P12 plants by mutants that produce a full-length (FL) progeny is not dependent on CP in the inoculum but production of a truncated (TR) progeny by mutants 3Δ1–22, M1, M2, and M3 is accompanied by a dependency of the infection on CP. It has been suggested that formation of this truncated progeny occurs by internal initiation of the replicase on a minus-strand copy of the parental mutant RNA, and that CP is involved in this internal initiation (van der Vossen *et al.,* 1996). Once the 5′ sequence of the parental mutant RNA is lost, the replication of the truncated progeny will no longer involve internal initiation on the minus-strand template and the infection is expected to become independent of CP. To test this hypothesis, infectious cDNA clones were made corresponding to the progeny RNAs of mutant 3Δ1–22 that lacked the 5′ 71 or 79 nt of RNA 3. In Fig. 7, lanes 4–7 show that

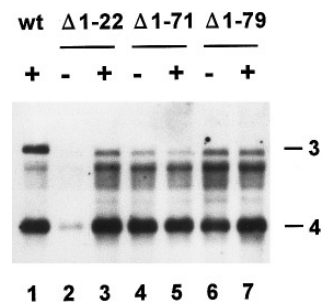

FIG. 7. Northern blot showing the accumulation of encapsidated viral RNAs 5 days after inoculation of P12 plants with wild-type and 5′-truncated 35S/cDNA 3 constructs. The presence or absence of 35S/cDNA 4 in the inoculum is indicated by + or − signs. P12 plants were inoculated with wild-type 35S/cDNA 3 (lane 1) and mutants 3Δ1–22 (lanes 2, 3), 3Δ1–71 (lanes 4, 5), and 3Δ1–79 (lanes 6, 7). The position of RNAs 3 and 4 is indicated in the right-hand margin. Viral RNAs on the blot were detected using the DIG nonradioactive nucleic acid labeling and detection system (Boehringer Mannheim), with cDNA 3 as a probe.

infection of P12 plants by these mutants is indeed independent of CP in the inoculum. As a control, the CP dependency of mutant 3Δ1–22 is shown in lanes 2 and 3 of Fig. 7. Apparently, CP from the inoculum is required only in a very early stage of infection by mutant 3Δ1–22 until the sequences from nucleotides 22–71 or 22–79 have been lost.

DISCUSSION

Cis-acting elements required for efficient amplification of a plus-strand RNA viral genome are generally expected to be found at or near the 5′ and 3′ termini, although additionally, internal sequence elements may be required as in the case of bacteriophage Qβ (Meyer *et al.,* 1981) or brome mosaic bromovirus (BMV) RNA 3 (French and Ahlquist, 1987). In a multipartite genome, a common element required for plus-strand RNA synthesis would be expected to be present in the 5′ termini of all viral RNAs. However, in many multipartite viruses, sequence similarity at the 5′ end of the RNAs was shown to be rather limited, especially when compared to the more extensive similarity at the 3′ termini of these RNAs. This is also the case for members of the *Bromoviridae* family of plant viruses. Homologous regions at the 3′ terminus of the RNAs of these viruses contain either tRNA-like structures in members of the genera bromovirus or cucumovirus (Duggal *et al.,* 1994), or CP binding sites in the case of AMV or ilarviruses (Jaspars, 1985). We have observed that replacement of the full-length 3′ UTR of AMV RNA 3 by the full-length 3′ UTRs of RNAs 1 or 2 did not affect replication of RNA 3 in P12 plants, indicating that the 3′ UTRs of the three AMV RNAs are functionally equivalent (van Rossum *et al.,* 1997).

For many members of the *Bromoviridae,* significant sequence similarity is found between the 5′ termini of RNAs 1 and 2, whereas similarity of these termini with the 5′ end of RNA 3 is limited. This has been found for the bromoviruses BMV (Ahlquist *et al.,* 1984), broad bean mottle virus (Dzianott and Bujarski, 1991; Romero *et al.,* 1992) and cowpea chlorotic mottle virus (Allison *et al.,* 1989; Dzianott and Bujarski, 1991), for the cucumovirus cucumber mosaic virus (Rezaian *et al.,* 1984, 1985; Davies and Symons, 1988), and for the ilarvirus citrus leaf rugose virus (Ge and Scott, 1994; Scott and Ge, 1995a and 1995b). In AMV strain 425, RNA 1 and RNA 2 share 11 identical nt at the 5′ end, whereas compared to RNA 3, only the first two nucleotides are identical.

Recently, we presented evidence that the AMV P1 and P2 proteins are required in *cis* for the replication of RNAs 1 and 2, respectively. This requirement interfered with a deletion analysis of *cis*-acting nucleotide sequences involved in replication of RNAs 1 and 2 in transgenic plants that expressed the P1 and P2 proteins *in trans* (van Rossum *et al.,* 1996). Therefore, hybrid constructs were studied in which the leader sequence of RNA 3 was replaced by 5′ terminal sequences of RNAs 1 and 2. For cowpea mosaic virus, a similar approach had shown that M-RNA, in which the 5′ UTR was replaced by that of B-RNA, could replicate in the presence of wild-type B-RNA (van Bokhoven *et al.,* 1993). When fused to wt AMV cDNAs 1 and 2, the T7 promoter is only active when transcripts with a 5′ nonviral G-residue are produced and this nonviral nucleotide largely interferes with infectivity of the transcripts (van Rossum *et al.,* 1996, and unpublished results). In the 35S/cDNA constructs used in this study the context of the 5′ terminus of the viral cDNA appears not to be critical for infectivity or 35S promoter activity. Extension of the 5′ end of cDNA 3 with nonviral sequences, modification of the 5′ end, or truncation by 22, 71, or 79 nt had little effect on the infectivity of 35S/cDNA 3 constructs (van der Vossen *et al.,* 1996, and this study). Moreover, point mutations and deletions in cDNAs 1 or 2 had no significant effect on 35S promoter driven transgene expression in plants (Brederode *et al.,* 1995). Therefore, it is highly unlikely that the noninfectious nature of the mutants analyzed in Fig. 2 is due to an effect of the mutations on the activity of the 35S promoter although this possibility cannot be completely excluded. The results presented in Fig. 2 indicate that the 5′ UTRs of the AMV RNAs are not functionally equivalent: Replacement of the 5′ UTR of RNA 3 by those of RNAs 1 or 2 rendered the RNA noninfectious to P12 plants even when expression of the movement protein gene in RNA 3 was put under the control of a subgenomic promoter to avoid an effect of the leader sequences on translation of this gene. Replacement of the 5′ UTR of RNA 3 by longer 5′ terminal RNA 1 or RNA 2 derived sequences up to 860 or 612 nt in length, respectively, did also not permit accumulation of the chimeric RNAs. Although it cannot be excluded that even longer fragments from RNA 1 and RNA 2 would be able to replace this region, it seems more likely that the 5′ UTR of RNA 3 contains signals necessary specifically for the replication of this RNA, which are absent in the other AMV RNAs. Possibly, such signals could have a dual role in the synthesis of genomic RNA 3 and subgenomic RNA 4. In potato virus X, sequences at the 5′ end of the genomic RNA were shown to be involved in subgenomic RNA synthesis (Kim and Hemenway, 1996).

The observation that in the Strasbourg strain of AMV the 5′ terminal 22 nt of RNA 3 are apparently replaced by the sequence of the 5′ terminal 38 nt of RNA 1 suggests that chimeric leader sequences may confer a stable genotype (Ravelonandro *et al.,* 1984). The presence of RNA 1-derived sequences at the 5′ end of RNA 3 was also reported for a temperature-sensitive mutant of AMV strain 425 (Huisman *et al.,* 1989). Our results on the replication of RNA 3 molecules with chimeric leader sequences indicate that different elements in the 5′ UTR

are involved in plus-strand promoter activity and selection of the initiation site for plus-strand RNA synthesis. The stem–loop structure with an ICR2 motif that occurs four times in the leader sequence of RNA 3 (strain 425) may be important for promoter activity (van der Vossen and Bol, 1996). However, a 5′ most sequence appears to be required for correct initiation of plus-strand RNA synthesis. The results with mutants 3S/L2–5 and 3Δ6–22 demonstrate that a sequence of 5 nt is sufficient to target the replicase to an initiation site corresponding to the 5′ end of the RNA. Apparently, the distance between this initiation site and promoter elements in the leader sequence of RNA 3 is not critical. In mutant 3S/L1–103 the leader sequence of RNA 1 may act as a 98-nt spacer between a 5′ initiation site of 5 nt and promoter elements in the truncated RNA 3 leader sequence. When the 5′ initiation site is deleted in mutant 3Δ1–22, the replicase selects alternative initiation sites starting with G-80 and less efficiently with G-72 or G-56. The 5′ terminal sequence of RNAs 1, 2, 4, and the mutant progeny starting at G-80 is GUUUU, whereas RNA 3 starts with GUAUU. In the mutants starting with G-56 and G-72 the 5′ sequence is more degenerate, being GUAAU and GUAAG, respectively. In leaves inoculated with mutant 3Δ1–22, the accumulation of progeny starting with G-56, G-72, and G-80 is detectable but only the G-80 progeny is detectable in systemically infected leaves. Possibly, the G-56 and G-72 mutants cannot compete with the G-80 mutant or convert into the G-80 mutant. In mutants M1, M2, and M3 the 5′ sequences GCCCC, GUCCC, and GCCCA are ignored by the replicase and initiation occurs predominantly at G-72 and G-80. This demonstrates that a 5′ G-residue or GU sequence is insufficient for 5′ initiation. The finding that the 5′ initiation site consists of 3 to 5 nt may explain why G-residues at positions 45, 54, 63, and 76 in the leader of RNA 3 are not selected to initiate plus-strand RNA synthesis upon infection with mutant 3Δ1–22. In addition, the predicted secondary structure of the RNA 3 leader sequence or complementary minus-strand sequence may affect selection of internal initiation sites (van der Vossen and Bol, 1996).

The observation that infection of P12 plants with mutant 3Δ1–22 is dependent on CP, whereas infection by the truncated progeny of this mutant is not, supports the hypothesis that CP plays a role in internal initiation by the replicase on minus-strand RNA of mutant 3Δ1–22 to generate mutants such as 3Δ1–71 and 3Δ1–79 (van der Vossen *et al.,* 1996). This role may mimic the stimulatory function of CP in the internal initiation of RNA 4 synthesis on a minus-strand RNA 3 template that was observed in *in vitro* assays (de Graaff *et al.,* 1995). We have not yet analyzed the stability of the cloned mutant 3Δ1–71 or its ability to systemically invade the plant.

Previously, we showed that deletion of the 5′ 79 nt from AMV RNA 3 changed the undetectable symptoms of the wt in P12 plants into severe necrosis (van der Vossen *et al.,* 1996). Apparently, replacement of the 5′ 22 nt of RNA 3 by 5′ sequences of 5 to 15 nt from RNAs 1, 2, or 3 resulted in similar necrotic symptoms (Fig. 3). The observation that mutant 3Δ6–22 induced necrosis, whereas mutant 3Δ6–11 did not, demonstrates that the presence of nucleotides 12–22 of RNA 3 caused a suppression of the hypersensitive response. Also, when nucleotides 1–22 of RNA 3 were replaced by the full-length 5′ UTR of RNA 1, no necrotic symptoms were observed. In a mixed infection, the truncated progeny RNA lacking the 5′ terminal 79 nt was unable to compete with wt RNA 3, indicating that the sequence of nucleotides 1–79 enhances the fitness of RNA 3 in P12 plants (van der Vossen *et al.,* 1996). Possibly, the 5′ termini of the wt RNAs contribute to the fitness of the virus in tobacco by avoiding the induction of defense responses of the plants.

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