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Accumulation of Alfalfa Mosaic Virus RNAs 1 and 2 Requires the Encoded Proteins in *cis*

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RNAs 1 and 2 of the tripartite genome of alfalfa mosaic virus (AlMV) encode the replicase proteins P1 and P2, respectively. P1 expressed in transgenic plants (P1 plants) can be used in *trans* to support replication of AlMV RNAs 2 and 3, and P2 expressed in transgenic plants (P2 plants) can be used in *trans* to support replication of AlMV RNAs 1 and 3. Wild-type RNA 1 was able to coreplicate with RNAs 2 and 3 in P1 plants, but this ability was abolished by frameshifts or deletions in the P1 gene of RNA 1. Similarly, wild-type RNA 2 coreplicated with RNAs 1 and 3 in P2 plants, but frameshifts or deletions in the P2 gene of RNA 2 interfered with this replication. Apparently, the P1 and P2 genes are required in *cis* for the accumulation of RNAs 1 and 2, respectively. Point mutations in the GDD motif of the P2 gene in RNA 2 interfered with accumulation of RNA 2 in P2 plants, indicating that replication of RNA 2 is linked to its translation into a functional protein. Plants transformed with both the P1 and P2 genes (P12 plants) accumulate replicase activity that is able to replicate RNA 3 in *trans*. An analysis of the time course of the accumulation of RNAs 1, 2, and 3 in protoplasts of P12 plants supported the conclusion that translation and replication are tightly coupled for AlMV RNAs 1 and 2 but not for RNA 3.

The genome of alfalfa mosaic virus (AlMV) consists of three plus-strand RNAs. RNAs 1 and 2 encode the replicase proteins P1 and P2, respectively, whereas RNA 3 encodes the putative viral movement protein P3 and the coat protein (CP). RNA 4 is a subgenomic messenger for CP and is colinear with the 3′-terminal 881 nucleotides of RNA 3. A mixture of the three genomic RNAs of AlMV and ilarviruses is not infectious unless a few molecules of CP or RNA 4 are added per genomic RNA molecule (1, 10). This early function of CP has been termed "genome activation." The P1 and P2 proteins have been identified as subunits of the purified viral RNA-dependent RNA polymerase (replicase) (16). The P1 protein contains domains with homology to methyl transferases and helicases, and the P2 protein contains the GDD motif that is believed to be part of the catalytic center of the viral polymerase (11). Tobacco plants transformed with a DNA copy of the P1 gene (P1 plants) are able to support replication of AlMV RNAs 2 and 3, and tobacco plants transformed with the P2 gene (P2 plants) are able to support replication of RNAs 1 and 3 (17, 26). Tobacco plants transformed with both the P1 and P2 genes (P12 plants) support the replication of RNA 3 when inoculated with RNA 3 only (17). As for nontransgenic plants, infection of P1 and P2 plants required the presence of CP in the inoculum, but the early function of CP was found not to be required for the infection of P12 plants with RNA 3 (17). Several hypotheses to explain this phenomenon have been put forward (9, 14).

The ability of P1 and P2 proteins expressed in P12 plants to replicate AlMV RNA 3 in *trans* has been extensively used to map *cis*- and *trans*-acting functions involved in replication of this genome segment in vivo (20–22, 24, 25). Moreover, a virus-specific replicase isolated from healthy P12 plants permitted an investigation of the function of CP in viral RNA synthesis in vitro (5). The observation that wild-type (wt) RNA 1 was able to coreplicate with RNAs 2 and 3 in P1 plants and that wt RNA 2 was able to coreplicate with RNAs 1 and 3 in P2 plants suggested that these transgenic plants could be used to study *cis*-acting functions involved in replication in vivo of RNAs 1 and 2, respectively (17). However, we demonstrate here that any deletion or frameshift in the P1 gene in RNA 1 interfered with the ability of this genome segment to replicate in P1 plants, suggesting that the P1 protein produced by the plant could be used in *trans* for replication of RNAs 2 and 3 but not for replication of RNA 1. Similarly, deletions or frameshifts in the P2 gene of RNA 2 abolished the replication of this RNA in P2 plants. This phenomenon was further analyzed by engineering mutations in the GDD motif in RNA 2. In addition, we investigated the replication of AlMV RNAs 1, 2, and 3 in protoplasts from P12 plants to see whether RNAs 1 and 2 could be replicated in *trans* by the replicase activity expressed from the transgenes of the plant. The results indicate that replication of RNAs 1 and 2, but not RNA 3, is linked to translation of the RNAs in *cis* into functional proteins.

MATERIALS AND METHODS

Infection of plants and protoplasts. Nontransgenic *Nicotiana tabacum* cv. Samsun NN and transgenic P1, P2, and P12 plants were grown and inoculated with infectious cDNA clones as described previously (14, 17). Each sample was inoculated onto two plants, specifically onto one half of each of three leaves per plant. Isolation and inoculation of tobacco protoplasts with virus particles were done essentially as described previously (12, 26). For each protoplast experiment 1.5×10^6 protoplasts were inoculated with virus particles at a concentration of 5 μg/ml. The suspension of infected protoplasts was divided into samples of 3×10^5 protoplasts, which were incubated for different periods of time before total RNA extraction.

Plasmid DNAs and construction of mutants. The level of infectivity of RNAs transcribed in vitro from available AlMV cDNA 1 and 2 clones fused to the T7 promoter was too low to be detectable in protoplasts (26a). Therefore, mutations in AlMV RNAs 1 and 2 were engineered in infectious cDNAs flanked by the cauliflower mosaic virus 35S promoter and *nos* terminator (14) (referred to hereafter as 35S/cDNA), and these clones were used to inoculate plants. By using different restriction enzymes and T4 DNA polymerase to generate blunt ends, deletions and frameshifts were made as indicated in Fig. 1. In the following list of mutants the nucleotides affected by the mutations are indicated in parentheses. Deletion mutants in 35S/cDNA 1 are 1-ΔPS (nucleotides [nt] 205 to 357; in frame [IF]), 1-ΔPAp (nt 205 to 1107; IF), 1-ΔHEn (nt 1118 to 1424; out of frame [OF]), 1-ΔEnBs (nt 1425 to 2059; OF), 1-ΔBsXb (nt 2064 to 2464; OF), and

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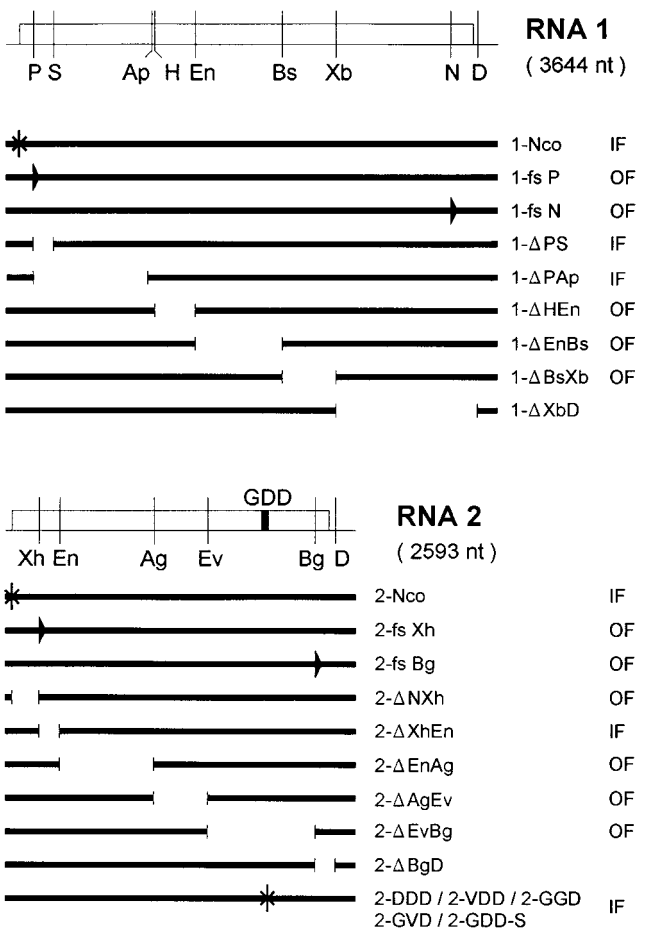

FIG. 1. Schematic representation of mutations engineered in cDNA clones corresponding to AlMV RNAs 1 and 2. The following restriction sites in the cDNAs are indicated: *Pst*I (P), *Sal*I (S), *Apa*LI (Ap), *Hpa*I (H), *Eco*NI (En), *Bsp*EI (Bs), *Xba*I (Xb), *Nco*I (N), *Dra*III (D), *Xho*I (Xh), *Age*I (Ag), *Eco*RV (Ev), and *Bgl*II (Bg). RNA 1 and RNA 2 mutants are indicated by the prefix 1 and 2, respectively. In addition, the names of the mutants specify the restriction sites that were used to make frameshifts (fs) or deletions (Δ). In mutants 1-Nco and 2-Nco the context of the ATG initiation codon was changed into that of an *Nco*I site. In mutants 2-DDD, 2-VDD, 2-GGD, and 2-GVD, the GDD motif in the RNA 2-encoded protein was changed as indicated. In mutant 2-GDD-S two translationally silent mutations were introduced into the sequence coding for this GDD motif. For mutations in the ORFs in RNAs 1 and 2, IF and OF mutations are indicated.

1-ΔXbD (nt 2469 to 3517; OF). Frameshift mutants in cDNA 1 are 1-fsP (deletion of nt 205 to 208; OF) and 1-fsN (insertion of CATG between nt 3331 and 3332; OF). For mutants 1-Nco (IF) and 2-Nco (IF), *Nco*I restriction sites were created around the start codon of the P1 and P2 proteins, respectively, with a transformer site-directed mutagenesis kit (Clontech) and oligonucleotides 5′-GATTTCAC***C***ATG***G***ATGCTGACGC-3′ and 5′-AATCTTTTCA***CC***ATG***G***TC ACTCTTTTG-3′ as the mutagenesis primers, respectively. Deletion mutants in cDNA 2 are 2-ΔNXh (nt 57 to 262; OF), 2-ΔXhEn (nt 266 to 406; IF), 2-ΔEnAg (nt 408 to 1105; OF), 2-ΔAgEv (nt 1110 to 1488; OF), 2-ΔEvBg (nt 1489 to 2297; OF), and 2-ΔBgD (nt 2302 to 2466; OF). Frameshift mutants in cDNA 2 are 2-fsXh (insertion of TCGA between nt 262 and 263) and 2-fsBg (insertion of GATC between nt 2297 and 2298). Previously, we have generated mutations in cDNA 2 changing the sequence encoding the GDD motif in the P2 protein from GGT.GAT.GAT (GDD) into G***A***T.GAT.GAT (DDD), G***T***T.GAT.GAT (VDD), GGT.G***G***T.GAT (GGD) or GGT.G***T***T.GAT (GVD) (2). These mutations (indicated by bold italics) were transferred to the infectious cDNA2 clone as a 809-bp *Eco*RV-*Bgl*II fragment. As an additional control, a mutant (2-GDD-S) was constructed bearing two translationally silent mutations in this GDD motif (sequence GG***A***.GA***C***.GAT). This mutant was constructed by using the transformer site-directed mutagenesis kit (Clontech) with the oligonucleotide primer 5′-GCTTCCGG***A***GA***C***GATTCATTG-3′. The presence of all mutations was confirmed by sequence analysis.

Isolation of virus preparations. Isolation of virus from infected leaf tissue was done as described previously (1). Nontransgenic tobacco plants were used to isolate virus preparations containing all four AlMV RNAs; P1 plants were used to isolate preparations containing RNAs 2, 3, and 4; and P2 plants were used to isolate preparations containing RNAs 1, 3, and 4 (17).

RNA extraction and Northern blot hybridization. Five days after inoculation the inoculated leaves of infected plants were harvested and divided into two parts. One part was used to isolate total RNA (22); the other part was used to isolate virus particles. Total RNA and RNA extracted from purified virus particles were analyzed by Northern (RNA) blot hybridization. The amount of RNA loaded per slot corresponded to 5 mg of leaf material. As identical results were obtained with the two types of samples from the same plant, only the results with the total RNA extracts are shown. Total RNA was extracted from protoplasts with Trizol reagent (Gibco BRL). In this case the RNA from 10^5 protoplasts was analyzed by Northern blotting. Random-primed cDNA 1, 2, or 3 was used as the probe either separately or as a mixture (7). To confirm that the deletions did not affect the efficiency of the detection of mutant RNAs, the blots were loaded with T7 RNA polymerase transcripts of the deletion mutants.

RESULTS

Mutations engineered in AlMV RNAs 1 and 2. Figure 1 shows a schematic representation of the RNA 1 and RNA 2 mutants used in this study. The mutations were made in infectious cDNA clones of RNAs 1 and 2 that are flanked by the CaMV 35S promoter and *nos* terminator (35S/cDNAs). Previously, we have shown that tobacco plants can be infected by inocula consisting of wt AlMV 35S/cDNAs 1 to 4 (14). Restriction sites in the P1 and P2 genes were used to make deletions, and two restriction sites in each gene were used to make frameshifts early and late in the P1 and P2 open reading frames (ORFs). By sequence determination it was confirmed that the mutations in the P1 and P2 genes were IF or OF. In addition, mutants with changes in the context of the initiation codons of the P1 gene (ctATGa) and P2 gene (atATGt) to that of an *Nco*I restriction site (ccATGg) were made. This mutation changed the N-terminal amino acid of P1 from Asn to Asp (mutant 1-Nco) and the N-terminal amino acid of P2 from Phe to Val (mutant 2-Nco). Four point mutations made in the GDD motif of the P2 protein (Gly-Asp-Asp, amino acids 624 to 626) changed this sequence into DDD, VDD, GGD, or GVD. Finally, in mutant 2-GDD-S, two silent point mutations were introduced in the nucleotide sequence encoding the GDD motif.

Accumulation of RNA 1 and RNA 2 mutants in nontransgenic plants. To see whether the mutations in the P1 and P2 genes affected the biological activity of the clones, the mutated 35S/cDNA 1 clones were mixed with wt 35S/cDNAs 2, 3, and 4, and the mutated 35S/cDNA 2 clones were mixed with wt 35S/cDNAs 1, 3, and 4. These mixtures were used to inoculate nontransgenic tobacco plants, and the accumulation of viral RNA in the plants was analyzed by Northern blot hybridization. The results obtained with a number of these inocula are shown in Fig. 2. In addition to the wt inoculum (Fig. 2, lanes 2 and 13) only the RNA 1 mutant 1-Nco (lane 3) and the RNA 2 mutant 2-GDD-S (lane 15) accumulated in the plants. None of the other RNA 1 or RNA 2 mutants was found to be infectious (Fig. 2 and results not shown). It was expected that OF deletions and frameshifts early in the ORF (mutants 1-fsP and 2-fsXh) would affect the functions of P1 and P2 in RNA replication. The frameshift in mutant 1-fsN results in the replacement of the C-terminal 48 amino acids of P1 by 13 nonviral amino acids, and in mutant 2-fsBg the C-terminal 41 amino acids are replaced by 4 nonviral amino acids. Apparently, these mutations also are lethal. Previously, we have shown that a change of the context of the initiation codons of the P3 and CP genes to that of an *Nco*I site did not interfere with the infectivity of the virus (18, 24). Also, such a mutation of the P1 initiation codon did not affect the infectivity of RNA

FIG. 2. Accumulation of RNA 1 and RNA 2 mutants in nontransgenic tobacco plants. Lanes: 1, mock-inoculated plants; 2 and 13, plants inoculated with wt 35S/cDNAs 1 to 4; 3 to 5, plants inoculated with wt 35S/cDNAs 2 to 4 and mutant 1-Nco (lane 3), 1-fsP (lane 4) or 1-fsN (lane 5); 6 to 12, 14, and 15, plants inoculated with wt 35S/cDNAs 1, 3, and 4 and mutant 2-Nco (lane 6), 2-fsXh (lane 7), 2-fsBg (lane 8), 2-DDD (lane 9), 2-VDD (lane 10), 2-GGD (lanes 11 and 14), 2-GVD (lane 12), or 2-GDD-S (lane 15). Five days after inoculation, total RNA was extracted from the plants and analyzed by Northern blot hybridization. The blot was probed with a mixture of labeled cDNAs 1 to 3. The positions of RNAs 1 to 4 are indicated on the left. Samples loaded in lanes 1 to 12 and 13 to 15 are from two different experiments.

1 (mutant 1-Nco), but a mutation in the context of the P2 initiation codon abolished infectivity of RNA 2 (mutant 2-Nco). Similarly, amino acid substitutions in the GDD sequence interfered with the functioning of P2 as none of the GDD mutants was infectious. Previously, we had observed that transformation of tobacco with mutants 2-DDD, 2-GGD, and 2-GVD resulted in a number of virus-resistant lines whereas transformation with 2-VDD did not (2). The results reported here indicate that the Gly-to-Val change in mutant 2-VDD inactivates the polymerase domain of P2 to the same extent that the changes in the other GDD mutants do. The infectivity of mutant 2-GDD-S confirmed that the silent mutations in the GDD motif did not affect possible *cis*-acting sequences in RNA 2.

Accumulation of RNA 1 mutants in P1 plants. Inoculation of P1 plants with AlMV 35S/cDNAs 2 and 3 results in the accumulation of virus particles containing RNAs 2, 3, and 4, provided that 35S/cDNA 4 or CP is present in the inoculum (14). To see if the P1 protein expressed from the nuclear transgene in the P1 plants could complement in *trans* the replication of RNA 1 mutants with defective P1 genes, the mutant 35S/cDNA 1 constructs were mixed with wt 35S/cDNAs 2, 3, and 4. P1 plants were inoculated with these mixtures, and Fig. 3 shows the results of Northern blot analysis of the viral RNAs accumulating in the plants. As a control, P1 plants were inoculated with wt 35S/cDNAs 2, 3, and 4 (Fig. 3, lane 2). When wt 35S/cDNA 1 was added to this inoculum, RNA 1 coreplicated with RNAs 2 and 3 (Fig. 3, lane 3). Because P1 is expressed in the P1 plants in limiting amounts (14), this coreplication of RNA 1 permitted a slight increase in the accumulation of

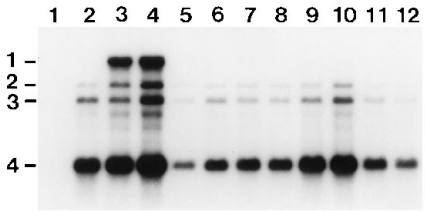

FIG. 3. Accumulation of RNA 1 mutants in transgenic P1 tobacco plants. Lanes: 1, mock-inoculated plants; 2, plants inoculated with wt 35S/cDNAs 2 to 4; 3 to 12, plants inoculated with wt 35S/cDNAs 2 to 4 and wt 35S/cDNA 1 (lane 3), mutant 1-Nco (lane 4), 1-fsP (lane 5), 1-fsN (lane 6), 1-ΔPS (lane 7), 1-ΔPAp (lane 8), 1-ΔHEn (lane 9), 1-ΔEnBs (lane 10), 1-ΔBsXb (lane 11), or 1-ΔXbD (lane 12). Five days after inoculation, total RNA was extracted from the plants and analyzed by Northern blot hybridization. The blot was probed with a mixture of labeled cDNAs 1 to 3. The positions of RNAs 1 to 4 are indicated on the left.

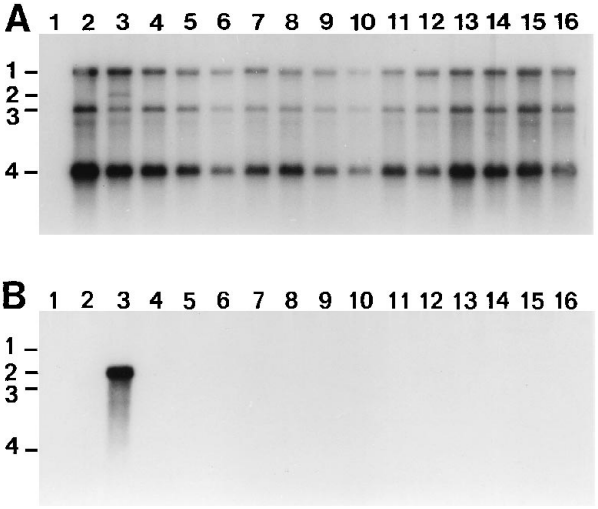

FIG. 4. Accumulation of RNA 2 mutants in transgenic P2 tobacco plants. The RNA samples were hybridized with a probe detecting all AlMV RNAs (A) or RNA 2 only (B). Lanes: 1, mock-inoculated plants; 2 to 16, plants inoculated with wt 35S/cDNAs 1, 3, and 4 and wt 35S/cDNA2 (lane 3), mutant 2-Nco (lane 4), 2-fsXh (lane 5), 2-fsBg (lane 6), 2-ΔNXh (lane 7), 2-ΔXhEn (lane 8), 2-ΔEnAg (lane 9), 2-ΔAgEv (lane 10), 2-ΔEvBg (lane 11), 2-ΔBgD (12), 2-DDD (lane 13), 2-VDD (lane 14), 2-GGD (lane 15), or 2-GVD (lane 16). Five days after inoculation, RNA was extracted from the plants and analyzed by Northern blot hybridization. The positions of RNAs 1 to 4 are indicated on the left.

RNAs 2, 3, and 4. Also, the RNA 1 mutant 1-Nco coreplicated with RNAs 2 and 3 (Fig. 3, lane 4) but none of the other RNA 1 mutants did (Fig. 3, lanes 5 to 12). To rule out the possibility that some deletion mutants were overlooked because of their comigration with RNA 2, the blot was hybridized with an RNA 1-specific probe (results not shown). This confirmed that only wt RNA 1 and mutant 1-Nco were able to coreplicate with RNAs 2 and 3. It is difficult to envisage that all deletions or frameshifts made in the P1 gene of RNA 1 would affect *cis*-acting sequences involved in recognition of RNA 1 by the enzyme activity that replicates RNAs 2 and 3. Particularly, the results with the frameshift mutants 1-fsP and 1-fsN indicate that translation of the P1 reading frame in RNA 1 is required in *cis* for replication of this genome segment. Mutant 1-Nco encodes a protein that is functional in nontransgenic plants (Fig. 2), and in P1 plants this mutant behaves like the wt.

Accumulation of RNA 2 mutants in P2 plants. P2 plants support the accumulation of AlMV RNAs 1, 3, and 4 when they are inoculated with 35S/cDNAs 1 and 3 plus either 35S/cDNA 4 or CP (14). Figure 4 shows that when wt 35S/cDNA 2 is added to this inoculum, RNA 2 coreplicates with RNAs 1 and 3 (Fig. 4A, lane 3). The mutant with silent mutations in the sequence encoding the GDD motif (2-GDD-S) was fully infectious to nontransgenic plants (Fig. 2) and accumulated at wt levels in P2 plants (result not shown). However, none of the other mutant RNA 2 molecules could be replicated by the enzyme activity that replicates RNAs 1 and 3 (Fig. 4A, lanes 4 to 16). To increase the sensitivity of the detection of RNA 2 mutants, the same samples that were analyzed in the blot shown in Fig. 4A were reanalyzed with a probe consisting of cDNA 2 only (Fig. 4B). No accumulation of mutant RNA 2 was detectable in Fig. 4B, even after longer exposure of the autoradiogram. As was observed for RNA 1, the reading frame in RNA 2 appears to be required in *cis* for replication of this genome segment. The mutation of the N-terminal amino acid (mutant 2-Nco) or the point mutations in the GDD motif (mutants 2-DDD, 2-VDD, 2-GGD, and 2-GVD) do not affect

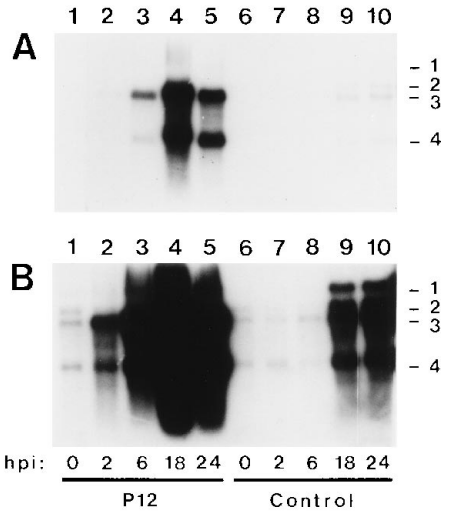

FIG. 5. Time course of accumulation of AlMV RNAs 1 to 4 in protoplasts from nontransgenic and P12 tobacco plants. Protoplasts from P12 plants (lanes 1 to 5) and nontransgenic control plants (lanes 6 to 10) were inoculated with AlMV particles containing RNAs 1 to 4. At the times hpi indicated at the bottom of the figure, RNA was extracted from the protoplasts and analyzed by Northern blot hybridization. The blots were probed with a mixture of labeled cDNAs 1 to 3 and the autoradiograms were exposed for 10 min (A) or 16 h (B). The positions of RNAs 1 to 4 are indicated on the right.

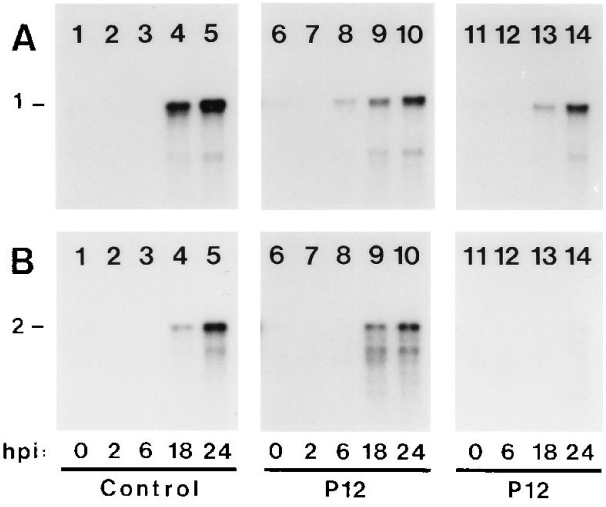

FIG. 6. Time course of accumulation of AlMV RNAs 1 and 2 in protoplasts from nontransgenic and P12 tobacco plants. Protoplasts were isolated from nontransgenic control plants (lanes 1 to 5) and P12 plants (lanes 6 to 14). The protoplasts were inoculated with AlMV particles containing RNAs 1 to 4 (A and B, lanes 1 to 10), particles containing RNAs 1, 3, and 4 (A, lanes 11 to 14), or particles containing RNAs 2, 3, and 4 (B, lanes 11 to 14). At the times indicated at the bottom of the figure, RNA was extracted from the protoplasts and analyzed by Northern blot hybridization. The blots were probed with labeled cDNA 1 (A) or cDNA 2 (B). The positions of RNAs 1 and 2 are indicated on the left.

the reading frame but cannot be complemented in *trans* by P2 produced by the plant. This result indicates that translation of RNA 2 into a functional P2 protein is required in *cis* for replication of this genome segment.

No evidence for recombination between RNA 1 or RNA 2 mutants. When P12 plants were inoculated with a mixture of two RNA 3 mutants, a rapid recombination to wt RNA 3 was observed (22). When nontransgenic plants or P1 plants were inoculated with a mixture of wt 35S/cDNAs 2, 3, and 4 plus 35S/cDNAs of the RNA 1 frameshift mutants 1-fsP and 1-fsN, no accumulation of any RNA was observed in the nontransgenic plants and only accumulation of RNAs 2, 3, and 4 was observed in the P1 plants. No recombination of the mutants to wt RNA 1 was detectable (results not shown). A similar experiment was done by inoculating nontransgenic plants and P2 plants with a mixture of the RNA 2 mutants 2-fsXh and 2-fsBg, supplemented with wt 35S/cDNAs 1, 3, and 4. Also, no recombination of the mutants to wt RNA 2 was observed (results not shown).

Accumulation of wt RNAs 1 and 2 in P12 protoplasts. In healthy P12 plants the majority of the transgenic P1 and P2 proteins are associated with a membrane structure from which a replicase can be solubilized in vitro (5). When P12 plants or protoplasts thereof are inoculated with RNA 3, an efficient accumulation of RNAs 3 and 4 is observed (17). Apparently, RNA 3 can be replicated in *trans* by the P1- and P2-containing enzyme complex that is present in the transgenic plants. If replication of RNAs 1 and 2 requires the encoded replicase proteins in *cis*, this requirement could interfere with the possibility of these RNAs to use in *trans* the preassembled transgenic replicase from P12 plants. To investigate this possibility, we inoculated P12 protoplasts with purified virus particles containing AlMV RNAs 1 to 4. Accumulation of viral RNAs was analyzed by Northern blot hybridization at several times after inoculation. In Fig. 5 short and long exposures of the same blot are shown. In Fig. 5A RNAs 3 and 4 accumulate much more rapidly and to higher levels in P12 protoplasts (lanes 1 to 5) than in nontransgenic protoplasts (lanes 6 to 10). This difference in the time course of accumulation of RNAs 3 and 4 in P12 and nontransgenic protoplasts was observed in four independent experiments. In Fig. 5A, the accumulation of RNAs 1 and 2 is hardly visible, but a longer exposure confirmed that RNAs 1 and 2 are both accumulating in the P12 protoplasts and nontransgenic protoplasts (Fig. 5B) (see also below). This longer exposure time permitted the detection of the inoculum RNAs at the time zero (Fig. 5B, lanes 1 and 6) and showed that in P12 protoplasts the accumulation of RNAs 3 and 4 started within 2 h postinfection (Fig. 5B, lane 2) whereas in nontransgenic protoplasts this accumulation started after 6 h postinfection (Fig. 5B, lane 8). From another experiment, which included a 12-h time point, it was concluded that in nontransgenic protoplasts the accumulation of RNAs 3 and 4 started between 6 and 12 h postinfection (results not shown).

Because the signal of RNA 3 obscured the detection of the accumulation of RNAs 1 and 2 in P12 protoplasts in Fig. 5B, similar blots were hybridized to an RNA 1-specific probe (Fig. 6A) or an RNA 2-specific probe (Fig. 6B). These results confirmed that RNAs 1 and 2 accumulated with similar kinetics in nontransgenic protoplasts (Fig. 6, lanes 1 to 5) and P12 protoplasts (Fig. 6, lanes 6 to 10) when the protoplasts were inoculated with the complete AlMV genome. In addition, Fig. 6 includes the results of experiments in which either RNA 1 or RNA 2 was omitted from the inoculum. When P12 protoplasts were inoculated with virus particles containing AlMV RNAs 1, 3, and 4 (Fig. 6A, lanes 11 to 14), the time course of RNA 1 accumulation resembled the accumulation of RNA 1 in P12 protoplasts or nontransgenic protoplasts inoculated with the complete genome. However, when P12 protoplasts were inoculated with virus particles containing AlMV RNAs 2, 3, and 4 (Fig. 6B, lanes 11 to 14), virtually no accumulation of RNA 2 was detectable. That the omission of RNA 1 or 2 from the inoculum had no effect on the accumulation of RNAs 3 and 4 was checked (results not shown). A possible explanation for these results is discussed below.

DISCUSSION

A coupling between translation and replication of viral RNA has been reported for poliovirus (3, 8, 15), the coronavirus mouse hepatitis virus defective interfering RNAs (6, 23), defective RNAs of the potexvirus clover yellow mosaic virus (28), the tymovirus turnip yellow mosaic virus (27), and both RNAs of the bipartite comovirus cowpea mosaic virus (19). AlMV is the first virus with a tripartite RNA genome for which *cis*-preferential replication was investigated. Here, we present evidence that replication of AlMV RNAs 1 and 2 is tightly linked to translation of these RNAs whereas the replication of RNA 3 is not. RNA 3 is bicistronic and serves as a messenger for the 5′ terminally encoded P3 protein. Deletions or frameshifts in the P3 gene do not affect replication of RNA 3 in protoplasts from P12 plants (20, 21). The 3′ terminally encoded CP is believed not to be expressed from RNA 3 but to be translated exclusively from the subgenomic RNA 4. On the other hand, the results presented in Fig. 3 indicate that any frameshift or deletion in the P1 ORF in RNA 1 interfered with the coreplication of this RNA with RNAs 2 and 3 in P1 plants. Apparently, replication of RNA 1 cannot make use of the P1 protein expressed from the plant genome but requires translation of the P1 ORF in *cis*. Similarly, the P2 protein expressed in P2 plants can be used in *trans* for the replication of RNAs 1 and 3 but not for RNA 2 (Fig. 4). This conclusion is most convincingly supported by the results with the GDD mutants. Experiments with transgenic plants have shown that these mutants are correctly expressed from the 35S promoter (2). The point mutations in these mutants do not affect the reading frame in RNA 2. The results with the silent mutations in the GDD motif indicate that this sequence does not represent a *cis*-acting element required for RNA 2 replication. The observation that amino acid substitutions in the GDD sequence interfere with the accumulation of RNA 2 in the presence of transgenic wt P2 suggests that replication of RNA 2 requires translation of this RNA into a functional protein rather than translation per se. If mutant replicase proteins did inhibit the replication of mutant RNAs, such inhibition occurred only in *cis*, as none of the mutants affected the replication of wt inoculum RNAs.

We cannot rule out the possibility that some RNA 1 or RNA 2 mutants did not accumulate because reduction of their reading frame interfered with stabilization of the RNA by the translation machinery. However, several deletion and frameshift mutants of RNAs 1 and 2 retained 50 to 90% of their reading frames. Moreover, we have observed that mutations that abolish translatability of RNA 3 had little effect on the accumulation and stability of this RNA in protoplasts (20, 21). To further analyze the stability of mutant RNAs, we inoculated P1 and P2 protoplasts with in vitro-capped T7 RNA polymerase transcripts of the RNA 1 and RNA 2 deletion mutants listed in Fig. 1 (except mutant 2-ΔNXh). Although the presence of a 5′-terminal nonviral G residue interfered with the infectivity of the wt transcripts and possible infectivity of mutant transcripts, time course experiments demonstrated that the half-lives of wt and mutant transcripts were the same in these protoplasts (26a). Previously, we have shown that wt RNAs 1 and 2, a 5′ truncated version of RNA 2 and the GDD mutants 2-DDD, 2-VDD, 2-GDD, and 2-GVD are not subjected to splicing when these RNAs are expressed from nuclear transgenes (2, 18, 26). Thus, the inability of the GDD mutants to accumulate in P2 plants is not due to a possible splicing of transcripts expressed from the mutant 35S/cDNAs.

Virus accumulation in plants requires both RNA replication and cell-to-cell spread, and P1 and P2 could be required in *cis* for either of these two processes. Because we have been unable to infect protoplasts with 35S/cDNAs 1 and 2 thus far, a *cis*-acting function of P1 and P2 in cell-to-cell transport of RNAs 1 and 2 cannot be ruled out. Although such a function would be of considerable interest, a *cis*-acting function of P1 and P2 in RNA replication is more likely in view of the well-documented role of these proteins in this process (5, 13, 17). Moreover, the absence of detectable recombination between RNA 1 mutants or between RNA 2 mutants indicates that these mutants do not replicate to high levels in primary infected cells of P1 or P2 plants, respectively. In contrast, RNA 3 mutants of AlMV recombine readily to wt RNA 3 (22).

Further evidence that P1 and P2 are required in *cis* for replication rather than for cell-to-cell transport stems from the experiments with P12 protoplasts. In these protoplasts, accumulation of RNAs 3 and 4 was initiated approximately 6 h earlier than in nontransgenic protoplasts and the RNAs accumulated at higher levels (Fig. 5). Previously, we have shown that replication of RNA 3 in P12 protoplasts is independent of the presence of RNAs 1 and 2 in the inoculum (17). Probably, in P12 protoplasts RNA 3 can directly associate with the replicase expressed from the nuclear transgenes whereas in nontransgenic protoplasts it has to wait until replicase activity has been expressed from the inoculum RNAs 1 and 2. In contrast, the time course of the accumulation of RNAs 1 and 2 is similar in P12 protoplasts and nontransgenic protoplasts infected with the complete AlMV genome (Fig. 6). This indicates that in P12 protoplasts RNAs 1 and 2 have to be translated in *cis* to enable their replication. Apparently, these RNAs do not make use of the transgenic replicase enzyme to initiate their replication. Omission of RNA 2 from the inoculum did not affect replication of RNA 1 (Fig. 6A). Probably, transgenic P1 and P2 are not expressed in P12 protoplasts in the same ratio as they are incorporated in the replicase complex. P1 translated from the inoculum RNA 1 can possibly associate with transgenic P2 that is expressed in excess of the transgenic P1. When RNA 1 was omitted from the inoculum, RNA 2 largely failed to replicate (Fig. 6B). Possibly, there is insufficient free P1 available in the P12 protoplast for association with P2 translated from inoculum RNA 2. Previously, we observed that RNA 1 alone was able to replicate in P12 protoplasts whereas RNA 2 was unable to replicate alone but could coreplicate with RNA 1 (17). This dependency of the replication of RNA 2 in P12 protoplasts on the presence of RNA 1 in the inoculum is in agreement with the results reported here.

Several models have been proposed to explain the coupling of translation and replication of viral RNAs (see reference 15). Weiland and Dreher proposed a model in which the *cis*-preferential replication of the turnip yellow mosaic virus genome is due to the interaction of newly synthesized p150 and p70 turnip yellow mosaic virus proteins preferentially with the RNA genome from which they have been made, resulting in the channeled formation of a replication initiation complex in *cis* (27). It has been shown that in the infected cell AlMV replication complexes are associated with the chloroplast outer membrane (4). At this time, the mechanism by which components of the viral replicase are targeted to this site is not known. One possibility is that P1 and/or P2 itself contains signals for sorting to the chloroplasts. We favor the idea that the *cis*-acting functions of P1 and P2 are required to ensure that viral replicase and template RNAs arrive together at the chloroplast membrane where replication complexes are established. In this model, inoculum RNAs 1 and 2 remain associated with a fraction of their translation products whereas another fraction of the translation products may be released to permit association to the heterologous template RNA. Initiation of replication of RNAs 1 and 2 may give rise to levels of P1 and P2

sufficient for association of these proteins with RNA 3, or P1 and P2 translated from the inoculum RNAs may initiate RNA 3 replication in *trans*. To further investigate this model we are analyzing putative binding sites in RNAs 1 and 2 for P1 and P2, respectively.

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