Translation of a nonpolyadenylated viral RNA is enhanced by binding of viral coat protein or polyadenylation of the RNA

Lyda Neeleman, René C. L. Olsthoorn, Huub J. M. Linthorst, and John F. Bol*

Institute of Molecular Plant Sciences, Gorlaeus Laboratories, Leiden University, PO Box 9502, 2300 RA, Leiden, The Netherlands

Communicated by Paul Ahlquist, University of Wisconsin, Madison, WI, October 12, 2001 (received for review August 27, 2001)

On entering a host cell, positive-strand RNA virus genomes have to serve as messenger for the translation of viral proteins. Efficient translation of cellular messengers requires interactions between initiation factors bound to the 5′-cap structure and the poly(A) binding protein bound to the 3′-poly(A) tail. Initiation of infection with the tripartite RNA genomes of alfalfa mosaic virus (AMV) and viruses from the genus *Ilarvirus* requires binding of a few molecules of coat protein (CP) to the 3′ end of the nonpolyadenylated viral RNAs. Moreover, infection with the genomic RNAs can be initiated by addition of the subgenomic messenger for CP, RNA 4. We report here that extension of the AMV RNAs with a poly(A) tail of 40 to 80 A-residues permitted initiation of infection independently of CP or RNA 4 in the inoculum. Specifically, polyadenylation of RNA 1 relieved an apparent bottleneck in the translation of the viral RNAs. Translation of RNA 4 in plant protoplasts was autocatalytically stimulated by its encoded CP. Mutations that interfered with CP binding to the 3′ end of viral RNAs reduced translation of RNA 4 to undetectable levels. Possibly, CP of AMV and ilarviruses stimulates translation of viral RNAs by acting as a functional analogue of poly(A) binding protein or other cellular proteins.

Circularization of the genome of RNA viruses plays an essential role in the replication and translation of viral RNAs. Formation of panhandle structures by base pairing between complementary sequences at the 5′ and 3′termini of several genera of minus-strand RNA viruses has been known already for some time (1). Recently, it became clear that cyclization by RNA–RNA interactions may also play an essential role in the replication of positive-strand RNA viruses such as flaviviruses (2, 3). However, for a growing number of positive-strand RNA viruses, circularization seems to be mediated by protein bridges between the 5′ and 3′ termini of the viral RNA. On entry into the host cell, the genome of positive-strand RNA viruses has to serve as messenger for the translation of viral proteins including replication factors. A number of viral RNAs resemble cellular mRNAs by having a 5′-cap structure (m^7GpppN) and 3′-poly(A) tail. In cellular mRNAs, these structures permit the formation of a protein bridge by interactions between eIF4G and eIF4E from the cap-binding complex of translation initiation factors and the poly(A) binding protein (PABP) associated with the poly(A) tail (4, 5). Picornaviruses do not have a cap structure, but the internal ribosome entry site in the 5′ untranslated region (UTR) of the RNA binds initiation factors and a poly(rC) binding protein that mediate the interaction with PABP. Circularization of *Poliovirus* RNA seems to play a role both in translation and replication of the viral RNA (6, 7).

The genomic RNAs of many viruses lack a poly(A) tail, and elements in the 5′ and/or 3′ UTR are believed to compensate for the absence of a poly(A) tail (8–12). Recently, the rotavirus (family *Reoviridae*) NSP3 protein was shown to interact with eIF-4G and with the 3′ UTR of the nonpolyadenylated viral mRNAs. Both interactions were required for efficient translation of the viral mRNA, and NSP3 was suggested to mimic the function of PABP (13). Here, we report evidence that the coat protein (CP) of alfalfa mosaic virus (AMV; genus *Alfamovirus*, family *Bromoviridae*) also may act as a functional equivalent of PABP.

RNAs 1 and 2 of the tripartite AMV genome encode the replicase proteins P1 and P2, respectively. P1 contains methyltransferase- and helicase-like motifs, whereas P2 contains polymerase-like motifs. RNA 3 encodes the movement protein and CP, which is translated from a subgenomic messenger, RNA 4. The RNAs contain a 5′-cap structure but the 3′ ends are not polyadenylated (14). At their 3′ termini, RNAs 1–4 contain a homologous sequence of 145 nucleotides that can be folded into two alternative conformations: a linear array of hairpins with flanking AUGC sequences that represents a strong CP binding site or a tRNA-like structure that represents the promoter for minus-strand RNA synthesis (15). A mixture of the three genomic RNAs becomes infectious only after addition of CP or its messenger, RNA 4, to the inoculum (16). Initiation of infection requires binding of a few molecules of CP to the 3′ termini of the genomic RNAs (14, 17). CP is required to initiate minus-strand RNA synthesis in protoplasts inoculated with T7 RNA polymerase transcripts corresponding to the genomic RNAs (18). However, CP is not required to initiate minus-strand RNA synthesis when the genomic RNAs are expressed from cDNAs flanked by the cauliflower mosaic virus 35S promoter and nopaline synthase terminator from nuclear transgenes in transgenic plants (19, 20), from a T-DNA vector in agroinfiltrated leaves (21), or from plasmids used to inoculate plants (22). We have suggested that in a natural infection, CP is required in a step before the initiation of AMV minus-strand RNA synthesis, probably the translation of the viral RNAs (15, 18). Moreover, we hypothesized that the CP-independent initiation of infection obtained with AMV 35S promoter-cDNA constructs was due to the poly(A) tail of the nuclear transcripts generated by pol II (18). In the present study, this hypothesis was tested by inoculation of plants and protoplasts with T7 RNA polymerase transcripts of AMV cDNAs extended with a 3′-terminal poly(A) tail. These poly(A) tails conferred infectivity to a mixture of the genomic RNAs, probably by enhancing translation efficiency of the RNAs. Moreover, we show that efficient translation of RNA 4 in plant cells depends on the ability of CP to bind to the 3′ end of its own messenger RNA in cis or in trans. We propose that binding of CP to the 3′ termini of AMV RNAs is functionally equivalent to the binding of PABP to the poly(A) tail of cellular mRNAs.

Abbreviations: AMV, alfalfa mosaic virus; CP, coat protein; UTR, untranslated region; PABP, poly(A) binding protein; wt, wild type; BMV, brome mosaic virus.

*To whom reprint requests should be addressed at: Gorlaeus Laboratories, Leiden University, Einsteinweg 55, 2333 CC, Leiden, The Netherlands. E-mail j.bol@chem.leidenuniv.nl.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Materials and Methods

Plasmids and *in Vitro* RNA Synthesis. Nonpolyadenylated AMV RNAs 1–3 were transcribed with T7 RNA polymerase from plasmids pUT17A, pUT27A, and pAL3, respectively (18). Transcripts of *Sma*I-linearized pUT17A contained two nonviral C-residues at the 3′ end; transcripts of *Sal*I-linearized pUT27A and pAL3 contained 10 nonviral nucleotides at the 3′ end. To extend the viral cDNAs with 40 or 80 A-residues, 3′ fragments of the cDNAs were replaced by corresponding fragments with a poly(A) tail that were generated by PCR. The upstream primers corresponded to nucleotides 3315–3335 of RNA 1, 2285–2302 of RNA 2, 1952–1981 of RNA 3, and contained *Nco*I, *Bgl*II, and *Bst*XI restriction sites, respectively. The downstream primers consisted of 40 or 80 T-residues flanked by a 3′ sequence of 19 nucleotides complementary to the 3′ end of the viral RNAs and a 5′ sequence of three G-residues. The PCR fragments were inserted in pUT17A, pUT27A, and pAL3 that had been cleaved with *Nco*I, *Bgl*II, or *Bst*XI, respectively, in the viral cDNA, and with *Sma*I in the vector downstream of the cDNA. T7 RNA polymerase transcription of these clones was done after linearization with *Sma*I. Construction and transcription of clones of RNA 4 encoding wild-type (wt) or mutant CP was described (23). Linearization of pUT17A and pUT27A with *Pvu*II was done to obtain RNA 1 and 2 transcripts with a plasmid-derived 3′ extension of 110 nucleotides. Linearization of pAL3 with *Pvu*I resulted in RNA 3 transcripts with a plasmid-derived 3′ extension of 600 nucleotides.

Inoculation of Plants and Protoplasts. Inoculation of tobacco plants and protoplasts with T7 RNA polymerase transcripts was done as described (18, 23). AMV CP was prepared as described (18). When indicated, CP was added to the inoculum at a ratio of 40 CP molecules per RNA molecule.

RNA and Protein Analysis. Extraction of RNA and protein from plants and protoplasts was done as described (18, 23). Northern blots were hybridized with strand-specific probes detecting all AMV plus- or minus-strand RNAs, and Western blots were analyzed with an antiserum raised against AMV CP (18).

***In Vitro* Translation.** Translation of RNA 4 in a rabbit reticulocyte lysate (Promega) was done according the manufacturer's instructions.

Results

Initiation of AMV Infection with Polyadenylated RNAs. Tobacco plants were inoculated with capped T7 RNA polymerase transcripts corresponding to AMV RNAs 1–3 with modified 3′ termini. Inoculation was done plus or minus CP in the inoculum. No accumulation of viral RNA was detectable in leaves inoculated with wt RNAs (Fig. 1, lane 1), but the RNAs became highly infectious after addition of CP to the inoculum (Fig. 1, lane 5). Extension of the 3′ termini of the three wt RNAs with a poly(A) tail of 40 or 80 A-residues resulted in infection of the plants when no CP was present in the inoculum (Fig. 1, lanes 2 and 3). To see whether this infectivity was specifically mediated by the poly(A) tail, T7 transcripts were made from plasmids that were linearized 110 (RNAs 1 and 2) or 600 (RNA 3) bp downstream of the viral cDNA. AMV RNAs with plasmid-derived extensions of 110–600 nucleotides were not infectious to plants when no CP was added to the inoculum (Fig. 1, lane 4). In leaves inoculated with the polyadenylated RNAs, viral RNAs accumulated at levels that were about 5% of the level obtained in wt infected leaves. Note that the amount of RNA loaded in lanes 5–8 of Fig. 1 corresponded to 20-fold less leaf material than the RNA loaded in lanes 1–4. After addition of CP to the AMV RNAs with a 3′-poly(A) tail or plasmid-derived sequence, the RNAs were as

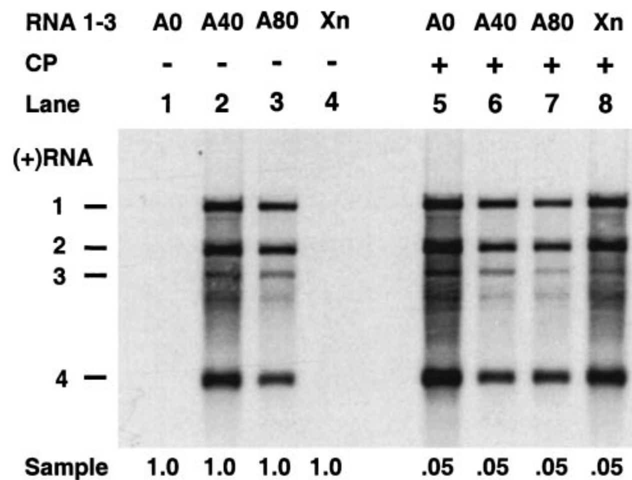

Fig. 1. Infection of tobacco plants with polyadenylated AMV RNAs. Plants were inoculated with RNAs 1–3 with modified 3′ termini as indicated. A0, no modification; A40 and A80, extension with 40 and 80 A-residues, respectively; Xn, extension with plasmid-derived transcripts of 110 (RNAs 1 and 2) or 600 (RNA 3) nucleotides. Inocula plus or minus CP were used as indicated. Northern blots were loaded with RNA extracted from 200 μg (lanes 1–4) or 10 μg (lanes 5–8) of leaf material and hybridized with a probe detecting plus-strand RNAs 1–4. Relative sample sizes are indicated at the bottom, and the positions of RNAs 1–4 are indicated at the left of the blot.

infectious as the wt RNAs (Fig. 1, lanes 6–8). Apparently, the 3′-terminal extensions did not interfere with the initiation of infection.

Polyadenylation of RNA 1 Is Sufficient for Poly(A)-Mediated Infection. To determine whether polyadenylation of all three genomic RNAs is required for poly(A)-mediated initiation of infection, tobacco protoplasts were inoculated with mixtures of transcripts in which only one or two of the genome segments were extended with 80 A-residues. An inoculum of nonpolyadenylated wt RNAs without CP induced the accumulation of relatively low amounts of minus-strand RNA (Fig. 2*B*, lane 1). The plus-strand RNAs detectable in these protoplasts represent mainly inoculum RNAs as no RNA 4 accumulation is detectable (Fig. 2*A*, lane 1). Polyadenylation of all three genomic RNAs in the inoculum resulted in accumulation of plus- and minus-strand RNAs (Fig. 2, lane 8) at levels that were 5–10% of the infection obtained by addition of CP (Fig. 2, lane 9; note the difference in loading of lane 9 and lanes 1–8). A similar level of RNA accumulation was observed when only RNA 1 in the inoculum was polyadenylated (Fig. 2, lane 2). Compared with the control (Fig. 2, lane 1), no increase in plus- or minus-strand RNA accumulation was observed when RNA 2, RNA 3, or both RNA 2 and 3 in the inoculum were polyadenylated (Fig. 2, lanes 3, 4, and 7). Moreover, polyadenylation of both RNAs 1 and 2 or RNAs 1 and 3 (Fig. 2, lanes 5 and 6) did not result in a higher level of RNA accumulation than was obtained with polyadenylation of RNA 1 only. Similar results were obtained when poly(A) tails of 40 instead of 80 A-residues were used (results not shown). The data indicate that initiation of infection by the three genomic RNAs is prevented primarily by a bottleneck in the translation of RNA 1, which can be partially relieved by polyadenylation of the RNA.

Previously, we have shown that CP-mediated initiation of infection requires the capping of RNAs 1 and 2 but not RNA 3 (18). A similar result was obtained for poly(A)-mediated initiation of infection (data not shown).

Role of 5′-Leader Sequences in AMV RNA Translation and Replication. Initiation of AMV infection can be obtained by adding either CP or RNA 4 to the genomic RNAs. If CP would be required to

BIOCHEMISTRY

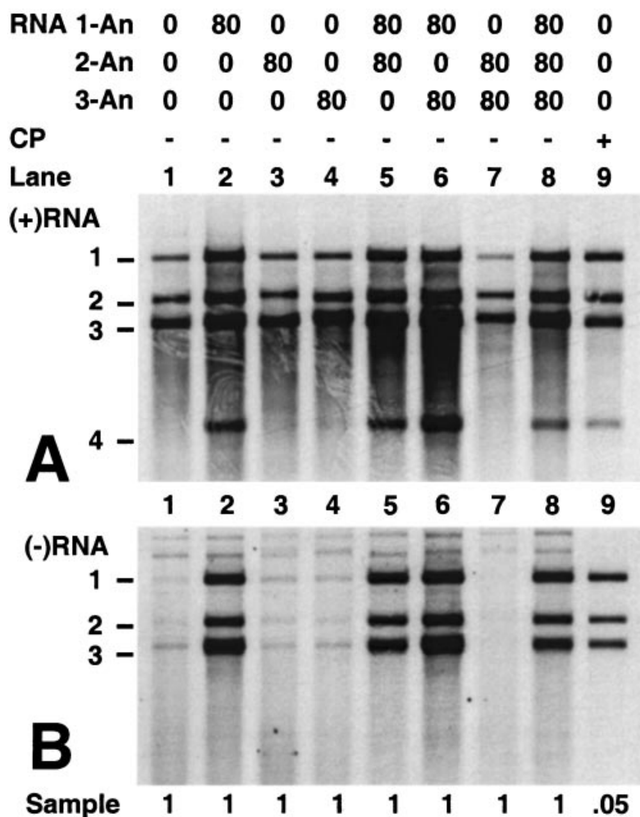

Fig. 2. Infection of tobacco protoplasts with mixtures of polyadenylated and nonpolyadenylated AMV RNAs. Protoplasts were inoculated with RNAs 1–3 with modified 3′ termini as indicated. 0, no modification; 80, extension with 80 A-residues. Inocula plus or minus CP were used as indicated. Northern blots were loaded with RNA extracted from 10,000 (lanes 1–8) or 500 (lane 9) protoplasts and hybridized with probes detecting plus-strand RNAs 1–4 (*A*) or minus-strand RNAs 1–3 (*B*). Relative sample sizes are indicated at the bottom, and the positions of plus-strand RNAs 1–4 and minus-strand RNAs 1–3 are indicated at the left of the blots.

enhance translation of RNA 1 and possibly RNA 2 into levels of P1 and P2 that are sufficient to initiate minus-strand RNA synthesis, we must assume that translation of RNA 4 does not require CP in the inoculum. The unstructured 5′-leader sequence of AMV RNA 4 has been shown to act as a translational enhancer *in vitro* (24) and *in vivo* (25). To see whether this leader sequence could enhance translation of P1 and P2, we replaced the 5′-leader sequences of RNAs 1 and 2 by the 5′-leader sequence of RNA 4, and the chimeric RNAs were used to inoculate protoplasts. After inoculation with wt RNAs 1 and 2 or wt RNAs 1–3, the inoculum RNAs were detectable but no minus-strand RNA synthesis was observed (Fig. 3, lanes 1 and 2). Addition of CP to inoculum RNAs 1 and 2 permitted minus-strand RNA synthesis (Fig. 3*B*, lane 7) and addition of CP to RNAs 1–3 permitted a wt infection (Fig. 3, lanes 8 and 13). (Note that lanes 8 and 10 in Fig. 3 were loaded with 20-fold reduced amounts of the same samples loaded in lanes 13 and 14, respectively.) Addition of a poly(A) tail to inoculum RNAs 1 and 2 did not permit a level of translation of P1 and P2 that was sufficient for a detectable synthesis of minus-strand RNAs 1 and 2 (Fig. 3*B*, lane 3). However, addition of wt RNA 3 to this inoculum permitted plus- and minus-strand RNA synthesis at a level that was about 5% of the wt infection (compare lane 4 of Fig. 3 with lanes 10 and 14). From the results shown in lanes 2–4 of Fig. 3, we conclude that polyadenylation of RNAs 1 and 2 enhanced translation of the RNAs to a level that is sufficient to initiate replication of RNA 3 in the inoculum. CP expressed from this RNA 3 probably further enhanced translation of RNAs 1 and 2 into replicase proteins that permitted RNA accumulation at 5% of the wt level.

Replacement of the 5′-leader sequence of RNAs 1 and 2 by the 5′ leader of RNA 4 did not permit detectable levels of minus-strand synthesis in the absence or presence of CP in the inoculum (Fig. 3*B*, lanes 5 and 11). Apparently, the 5′-leader sequences of RNAs 1 and 2 are required for the minus-strand RNA synthesis observed in Fig. 3*B* (lane 7). Inoculation of protoplasts with the chimeric RNAs 1 and 2 and wt RNA 3 did not permit a detectable level of RNA 3 replication (Fig. 3, lane 6). Apparently, in contrast to polyadenylation of RNAs 1 and 2, replacement of the 5′-leader sequences by the 5′ leader of RNA 4 did not enhance translation of P1 and P2 at detectable levels. However, addition of CP to this inoculum did permit a low level of RNA 3 replication (Fig. 3, lane 12). This observation demonstrates that CP could still stimulate translation of the chimeric RNAs 1 and 2.

CP Stimulates Translation of RNA 4. Another possibility to explain the evidence that RNA 4 in the inoculum can be translated into CP without a requirement for addition of CP to the inoculum is that translation of RNA 4 is autocatalytically stimulated by its own translation product. To investigate this possibility, we inoculated protoplasts with wt RNAs 1 and 2 and RNA 4 transcripts encoding wt or mutant CPs (Fig. 4 *A–C*). In addition, the RNA 4 transcripts were translated in a reticulocyte lysate (Fig. 4*D*). The six mutant CPs expressed in this experiment have been analyzed for their ability to function in several steps of the AMV replication cycle (23). Addition of capped wt RNA 4 or wt CP to RNAs 1 and 2 in the inoculum permitted the synthesis of minus-strand RNAs 1 and 2 to similar levels (Fig. 4*B*, lanes 3 and 10). *In vitro*, noncapped, and capped RNA 4 were translated with similar efficiency (Fig. 4*D*, lanes 2 and 3) but *in vivo*, translation of the noncapped RNA 4 was relatively poor (Fig. 4*C*, lanes 2 and 3). In some experiments, CP translated from noncapped RNA 4 was able to initiate minus-strand RNA synthesis (Fig. 4*B*, lane 2), whereas in other experiments it was not (data not shown). RNA 4 transcripts encoding three CP mutants were able to promote minus-strand RNA 1 and 2 synthesis: ΔN10, R25A ,and K13A (Fig. 4*B*, lanes 6, 7, and 9). However, mutants R17A, ΔN16, and R16K17 did not promote minus-strand RNA synthesis (Fig. 4*B*, lanes 4, 5, and 8). *In vitro*, all CP mutants translated with wt efficiency (Fig. 4*D*, lanes 4–9). However, *in vivo* translation was detectable only for those mutants that promoted minus-strand RNA synthesis (Fig. 4*C*, lanes 6, 7, and 9). The N-terminal 25 amino acids have been shown to be required and sufficient for binding of CP to the 3′ end of AMV RNAs (26). Lysines at positions 5, 6, 10, 13, 16, or 25 were not important for CP binding, but replacement of the arginine at position 17 by alanine (mutant R17A) or lysine abolished CP binding to the 3′ end of AMV RNAs (27). In addition, the R17A and R16K17 mutants (Lys-16 and Arg-17 reversed) were shown to be defective in initiation of infection (28). The data presented in Fig. 4 point to a strict correlation between the ability of CP to bind to the 3′ end of viral RNA, to promote translation of its own messenger, and to promote initiation of minus-strand RNA synthesis.

We propose that after inoculation of plants with AMV RNAs 1–4, CP translated from RNA 4 initially promotes translation of its own mRNA and subsequently enhances the translation of RNA 1 and possible other genomic RNAs by its ability to bind to the 3′ termini of the RNAs. Because P1 and P2 are not detectable in infected protoplasts with available antisera, we were unable to analyze the effect of CP on translation of RNAs 1 and 2 by Western blotting. However, we were able to analyze a trans-acting effect of CP on the translation of RNA 4 because mutants ΔN10 and ΔN16 (N-terminal deletions of 10 and 16

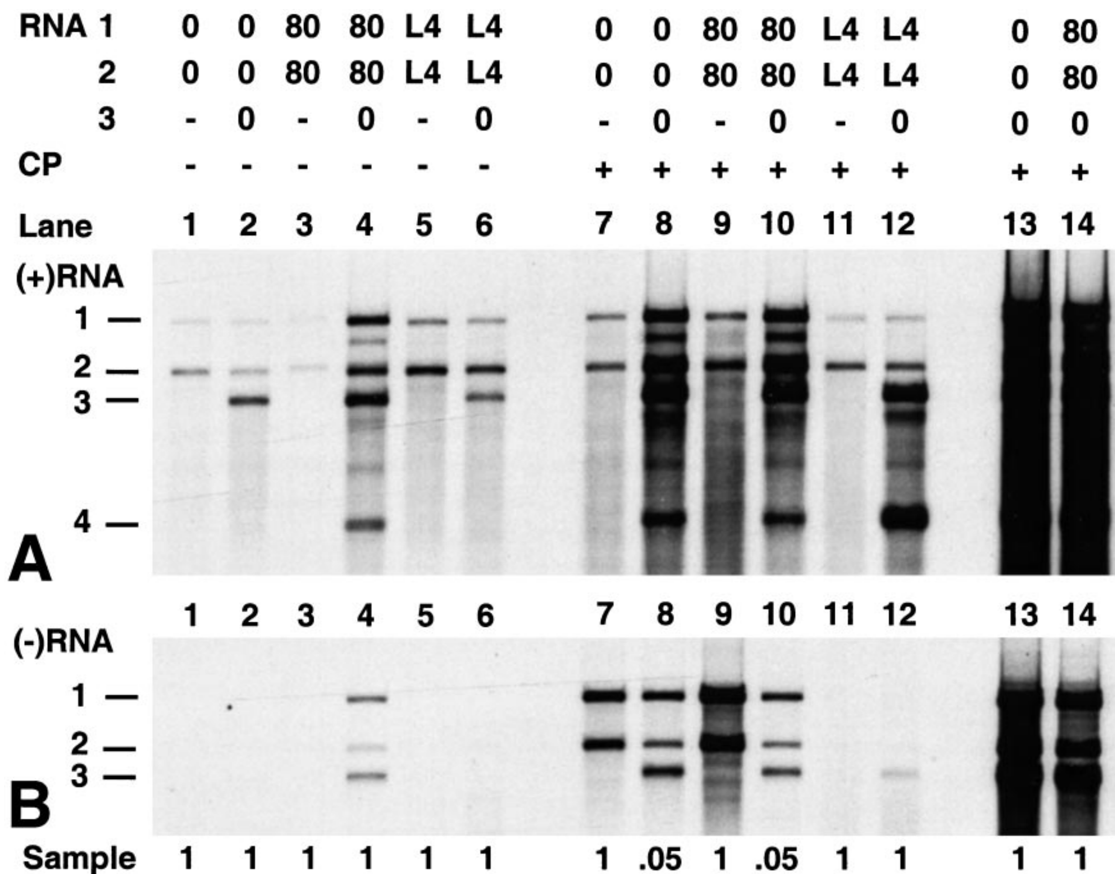

Fig. 3. Role of 5′-leader sequences and polyadenylation of RNAs 1 and 2 in the initiation of AMV RNA replication. Protoplasts were inoculated with RNAs 1–3 with modified 5′ or 3′ termini as indicated. 0, no modification; 80, 3′ extension with 80 A-residues; L4, 5′ UTR replaced by 5′ UTR of RNA 4; minus sign, no RNA 3 present in the inoculum. Inocula plus or minus CP were used as indicated. Northern blots were loaded with RNA extracted from 10,000 (lanes 1–7, 9, and 11–14) or 500 (lanes 8 and 10) protoplasts and hybridized with probes detecting plus-strand RNAs 1–4 (*A*) or minus-strand RNAs 1–3 (*B*). Relative sample sizes are indicated at the bottom, and the positions of plus-strand RNAs 1–4 and minus-strand RNAs 1–3 are indicated at the left of the blots.

amino acids, respectively) can be distinguished on Western blots from full-length CP. Fig. 5 shows that RNA 4 transcripts of mutants R17A and R16K17 are not translated when transfected to protoplasts alone (Fig. 5, lanes 3 and 4) but are translated at near wt levels when cotransfected with the translatable mutant ΔN10 (Fig. 5, lanes 7 and 8). Similarly, mutant ΔN16 is not translated when transfected to protoplasts alone (Fig. 5, lane 5) but is translated at near wt levels when cotransfected with wt RNA 4 (Fig. 5, lane 10). Thus, in addition to their own mRNAs, the wt and ΔN10 CPs stimulate translation of other RNA 4 molecules in trans.

Discussion

Addition of CP or RNA 4 to a mixture of AMV genomic RNAs increases the infectivity toward plants and protoplasts ≈1,000-fold and without this addition, infectivity is detectable only at extremely high inoculum concentrations (refs. 16 and 29; this study, Fig. 3). Addition of a poly(A) tail of 40 or 80 A-residues to the 3′ end of RNA 1 increased the infectivity of a mixture of genomic RNAs about 50-fold and resulted in a level of virus accumulation in plants and protoplasts that was 5% of the CP-mediated infection under our standard inoculation conditions. Addition of CP or polyadenylation of RNA 1 in the inoculum were both required to initiate minus-strand AMV RNA synthesis (Figs. 2 and 3). The observation that minus-strand RNA synthesis does not depend on CP when AMV RNAs are expressed from nuclear transgenes in transgenic plants (18, 20) or from a T-DNA vector in agroinfiltrated leaves (21) may be due to quantitative and qualitative differences between the transcripts made by the cellular polymerase II and wt inoculum RNAs. The transcripts produced in transgenic or agroinfiltrated leaves are detectable by Northern blot analysis, whereas inoculum RNAs in freshly inoculated leaves are not (30). In addition to this quantitative difference, the poly(A) tail generated by pol II transcription of AMV RNAs may affect the expression of viral proteins. Polyadenylation of RNA 1 is clearly responsible for the 50-fold increase in the synthesis of minus-strand RNAs 1–3 in inoculated protoplasts (Fig. 2, lanes 1 and 2). Quantitative differences in inoculum doses may explain why expression of polyadenylated RNAs 1 and 2 in agroinfiltrated leaves results in wt levels of minus-strand RNA synthesis (21), whereas inoculation of protoplasts with polyadenylated RNAs 1 and 2 does not (Fig. 3, lane 3). The observation that addition of RNA 3 to polyadenylated RNAs 1 and 2 results in detectable RNA replication in protoplasts, whereas addition of RNA 3 to wt RNAs 1 and 2 does not (Fig. 3, lanes 2 and 4), indicates that polyadenylation of the inoculum RNAs stimulates translation of the replicase proteins P1 and P2 to a level sufficient to initiate replication of RNA 3. Subsequently, CP expressed from RNA 3 may bind to the 3′ termini of the inoculum RNAs to further enhance translation. CP expressed from RNA 4 in the inoculum will be available at an earlier time point than CP expressed from RNA 3. It has been shown that initiation of infection by CP requires the presence of CP in a narrow time window early after inoculation (22, 29). This finding may explain why expression of CP from RNA 3 results in only 5% of wt minus-strand synthesis

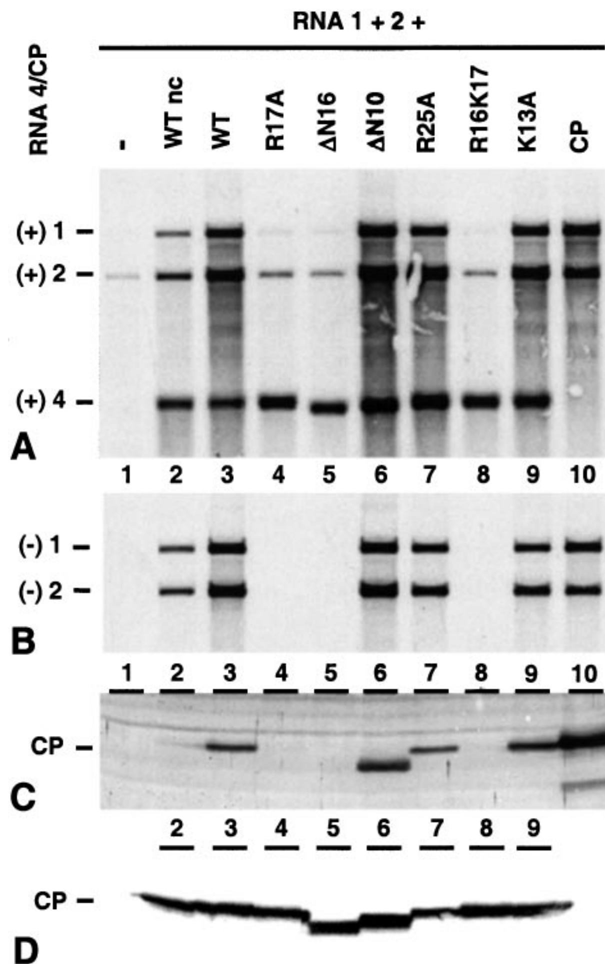

Fig. 4. Initiation of minus-strand RNA synthesis and enhancement of translation of RNA 4 in protoplasts by wt and mutant CP. (*A–C*) Protoplasts were inoculated with wt RNAs 1 and 2 (lane 1), wt RNAs 1 and 2 plus CP (lane 11), or wt RNAs 1 and 2 plus RNA 4 encoding wt CP (lanes 2 and 3), or the mutant CPs R17A, ΔN16, ΔN10, R25A, R16K17, and K13A (lanes 4–9, respectively). RNA 4 in the inoculum was capped (lanes 3–9) or not capped (lane 2). Northern blots were loaded with RNA extracted from 10,000 protoplasts and hybridized with probes detecting plus-strand RNAs 1–4 (*A*) or minus-strand RNAs 1–3 (*B*). The positions of plus-strand RNAs 1, 2, and 4 and minus-strand RNAs 1 and 2 are indicated at the left of the blots. (*C*) Analysis of CP translated from RNA 4 (lanes 2–9) or derived from the inoculum (lane 10). Protein was extracted from 10,000 protoplasts and analyzed by Western blotting with a CP antiserum. The position of wt CP is indicated at the left of the blot. (*D*) Translation in reticulocyte lysates of RNA 4 transcripts corresponding to the RNA 4 transcripts used in *A–C*. Synthesis of CP in the lysates was analyzed by Western blotting with a CP antiserum. The position of wt CP is indicated at the left of the blot.

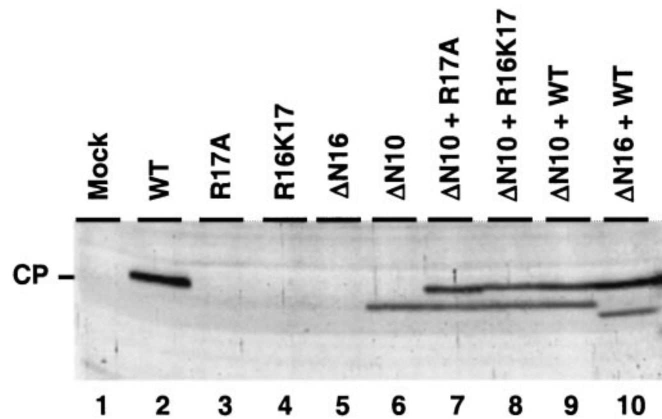

Fig. 5. CP stimulates translation of AMV RNAs in trans. Tobacco protoplasts were mock-inoculated (lane 1) or inoculated with RNA 4 transcripts encoding wt CP (lane 2), mutant CPs R17A (lane 3), R16K17 (lane 4), ΔN16 (lane 5), ΔN10 (lane 6), or mixtures of ΔN10 and R17A (lane 7), ΔN10 and R16K17 (lane 8), ΔN10 and wt CP (lane 9), and ΔN16 and wt CP (lane 10). Protein was extracted from 10,000 protoplasts and analyzed by Western blotting with a CP antiserum. The position of wt CP is indicated at the left of the blot.

(Figs. 2 and 4). Our results indicate that addition of CP to AMV RNAs enhances translation much more efficiently than extension of the RNAs with a poly(A) tail. On entry in the host cell, the viral RNAs have to compete with about 300,000 cellular messengers for the translational machinery. Probably, the CP of AMV and ilarviruses provides the viral RNAs with a competitive advantage over the polyadenylated cellular mRNAs. The finding that polyadenylation of RNA 1 permits CP-independent infection at a 5% level might suggest that translation of this RNA is a major bottleneck in the initiation of infection, but it is quite possible that CP expressed from RNA 3 in this inoculum also stimulates translation of other AMV RNAs. In fact, our observation that translation of RNA 4 in protoplasts is strongly enhanced by wt or mutant CPs that are able to bind to the 3′ end of RNA 4 (Figs. 4 and 5) indicates that CP stimulates translation of all AMV RNAs. Recent results indicate that CP binding sites in the 3′ UTR of RNA 4 are required for the CP-mediated stimulation of translation (L.N., unpublished data). To our knowledge, the results shown in Figs. 4 and 5 are the first demonstration of the stimulation of translation of a viral RNA by its CP.

Within the family *Bromoviridae*, initiation of infection by the three genomic RNAs of bromo- and cucumoviruses is independent of CP or RNA 4 in the inoculum. Fusion of the 3′ UTR of brome mosaic virus (BMV; genus *Bromovirus*) to the 3′ end of a glucuronidase or luciferase reporter gene by Gallie and Kubayashi (8) enhanced translation of the reporter in carrot protoplasts 60- to 70-fold, but no enhancement was observed when the AMV 3′ UTR was fused to these reporters. On the other hand, Gehrke and coworkers (31) showed that deletion of the 3′ UTR of AMV RNA 4 abolished expression of CP in oocytes. Our data indicate that both the CP gene and 3′ UTR are required for *in vivo* translation of RNA 4. Differences between the 3′ UTRs of AMV and BMV probably correlate with differences in CP dependency of infection. Fusion of the 3′ UTR of tobacco mosaic virus (TMV; genus *Tobamovirus*) to a glucuronidase gene enhanced translation in protoplasts similar to the BMV 3′ UTR-mediated translation, and it was concluded that the BMV and TMV 3′ UTRs are functionally equivalent to the poly(A) tail of cellular messengers (8). Particularly, the pseudoknot domain upstream of the tRNA-like structure in the TMV 3′ UTR was responsible for increasing expression (32). The translational enhancer in the BMV 3′ UTR has not yet been identified.

The poly(A) tail of cellular messengers enhances translation synergistically with the cap structure by interactions of poly(A)-bound PABP with cap-binding initiation factors eIF-4F and eIF-4B (4, 5). eIF-4F consists of the cap-binding subunit eIF-4E, the helicase subunit eIF-4A, and the multiadapter subunit eIF-4G. Interactions of PABP with eIF-4B and eIF-iso4G from wheat germ have been demonstrated (33). The interaction of PABP with eIF-4B/eIF-4G results in increased affinity of PABP for the poly(A) tail and affinity of the initiation factors for the cap structure. Moreover, the interaction promotes recruitment of the 40S ribosomal subunit by interactions between eIF-4G and eIF-3 and confers protection against 5′-exonucleolytic degradation of the mRNA. In yeast, the poly(A) tail of mRNAs

undergoes progressive shortening by the PABP-dependent poly(A) nuclease Pan1p. Dissociation of PABP from the mRNA disrupts the interaction of PABP with initiation factors and renders the cap structure vulnerable to removal by the decapping enzyme Dcp1p. Decapping is quickly followed by degradation of the mRNA by the 5′->3′ RNA exoribonuclease Xrn1p (5, 34). The synergy between the cap and poly(A) tail is lost in an *xrn1* mutant (35).

Similar to the binding of PABP to cellular mRNAs or polyadenylated viral RNAs, the binding of CP to the 3′ ends of AMV RNAs could enhance translation by promoting the recruitment of 40S ribosomal subunits, by enhancing the stability of the viral RNAs, or both. In inoculated protoplasts, there was no clear difference in stability between RNA 4 transcripts encoding CP that was functional or nonfunctional in RNA binding (Fig. 4*A*). This observation might suggest that CP does not enhance translation by stabilizing the viral RNAs. BMV is able to replicate in yeast cells, and the yeast *LSM1* gene was found to allow the replication of BMV RNAs independently of a poly(A) tail. A defect in replication enhancer-dependent RNA 3 responsiveness to the BMV 1a protein in an *LSM1* mutant could be compensated by polyadenylation of BMV RNA 3 (36). *LSM1* is known to play a role in 5′ decapping of cellular mRNAs. AMV CP could have a function similar to that of the LSM1 protein in permitting poly(A)-independent translation and replication of the viral RNAs. Another possibility is that AMV CP promotes circularization of the viral RNAs analogous to the role of PABP in the circularization of cellular mRNAs. The CP might directly interact with initiation factors from the cap-binding complex as has been shown for the rotavirus nonstructural protein NSP3 (13). We have shown that binding of CP to the 3′ UTR of AMV RNA induces a conformational change of the RNA (15). Possibly, CP does not bind directly to initiation factors but induces an RNA structure that interacts with such factors as has been shown for the internal ribosome entry site in the 5′ UTR of picornavirus RNAs (37) and the translational enhancer in the 3′ UTR of the satellite RNA of tobacco necrosis virus (12, 38).

In the past decades, several hypotheses have been proposed to explain the early function of CP in AMV infection (14, 17). The results reported here indicate that CP in the inoculum has a major role in enhancing the translation of viral RNAs. In addition, we have shown that binding of CP to the 3′ end of AMV RNAs disrupts a conformation of the 3′ UTR that is required for minus-strand promoter activity *in vitro* (15). In the initiation of infection, CP derived from parental virions or translated from RNA 4 in the inoculum may have a dual role in promoting translation of the viral RNAs and preventing premature initiation of minus-strand RNA synthesis by newly synthesized replicase proteins. Later in the infection cycle, *de novo* synthesis of CP will result in increased protein levels that may lead to encapsidation of viral RNAs into progeny virus particles.

This research was supported by The Netherlands Organization for Scientific Research (NWO), Earth and Life Sciences division (ALW).

1. Hsu, M. T., Parvin, J. D., Gupta, S., Krystal, M. & Palese, P. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 8140–8144.
2. Khromykh, A. A., Meka, H., Guyatt, K. J. & Westaway, E. G. (2001) *J. Virol.* **75,** 6719–6728.
3. You, S., Falgout, B., Markoff, L. & Padmanabhan, R. (2001) *J. Biol. Chem.* **276,** 15581–15591.
4. Gallie, D. R. (1991) *Genes Dev.* **5,** 2108–2116.
5. Gallie, D. R. (1998) *Gene* **216,** 1–11.
6. Gamarnik, A. V. & Andino, R. (2000) *J. Virol.* **74,** 2219–2226.
7. Herold, J. & Andino, R. (2001) *Mol. Cell* **7,** 581–591.
8. Gallie, D. R. & Kobayashi, M. (1994) *Gene* **142,** 159–165.
9. Wells, D. R., Tanguay, R. L., Le, H. & Gallie, D. R. (1998) *Genes Dev.* **12,** 3236–3251.
10. Qu, F. & Morris, T. J. (2000) *J. Virol.* **74,** 1085–1093.
11. Timmer, R. T., Benkowski, L. A., Schodin, D., Lax, S. R., Metz, A. M., Ravel, J. M. & Browning, K. S. (1993) *J. Biol. Chem.* **268,** 9504–9510.
12. Lipzig, R., van Montagu, M., Cornelissen, M. & Meulewaeter, F. (2001) *Nucleic Acids Res.* **29,** 1080–1086.
13. Vende, P., Piron, M., Castagné, N. & Poncet, D. (2000) *J. Virol.* **74,** 7064–7071.
14. Bol, J. F. (1999) *J. Gen. Virol.* **80,** 1089–1102.
15. Olsthoorn, R. C. L., Mertens, S., Brederode, F. T. & Bol, J. F. (1999) *EMBO J.* **18,** 4856–4864.
16. Bol, J. F., van Vloten-Doting, L. & Jaspars, E. M. J. (1971) *Virology* **46,** 73–85.
17. Jaspars, E. M. J. (1999) *Arch. Virol.* **144,** 843–863.
18. Neeleman, L. & Bol, J. F. (1999) *Virology* **254,** 324–333.
19. Taschner, P. E. M., van der Kuyl, A. C., Neeleman, L. & Bol, J. F. (1991) *Virology* **181,** 445–450.
20. Thole, V., Garcia, M., van Rossum, C. M. A., Neeleman, L., Brederode, F. T., Linthorst, H. J. M. & Bol, J. F. (2001) *J. Gen. Virol.* **82,** 25–28.
21. Vlot, A. C., Neeleman, L., Linthorst, H. J. M. & Bol, J. F. (2001) *J. Virol.* **75,** 6440–6449.
22. Neeleman L., van der Vossen, E. A. G. & Bol, J. F. (1993) *Virology* **196,** 883–887.
23. Tenllado, F. & Bol, J. F. (2000) *Virology* **268,** 29–40.
24. Jobling, S. A. & Gehrke, L. (1987) *Nature (London)* **325,** 622–625.
25. Gallie, D. R., Sleat, D. E., Watts, J. W., Turner, P. C. & Wilson, T. M. A. (1987) *Nucleic Acids Res.* **15,** 8693–8711.
26. Baer, M., Houser, F., Loesch-Fries, L. S. & Gehrke, L. (1994) *EMBO J.* **13,** 727–735.
27. Ansel-McKinney, P. & Gehrke, L. (1998) *J. Mol. Biol.* **278,** 767–785.
28. Yusibov, V. & Loesch-Fries, L. S. (1995) *Virology* **208,** 405–407.
29. Houwing, C. J. & Jaspars, E. M. J. (2000) *Arch. Virol.* **145,** 13–35.
30. van der Vossen, E. A. G., Neeleman, L. & Bol, J. F. (1994) *Virology* **202,** 891–903.
31. Hann, L. E., Webb, A. C., Cai, J.-M., Gehrke, L. (1997) *Mol. Cell. Biol.* **17,** 2005–2013.
32. Gallie, D. R. & Walbot, V. (1990) *Genes Dev.* **4,** 1149–1157.
33. Le, H., Tanguay, R. L., Balasta, M. L., Wei, C.-C., Browning, K. S., Metz, A. M., Goss, D. J. & Gallie, D. R. (1997) *J. Biol. Chem.* **272,** 16247–16255.
34. Caponigro, G. & Parker, R. (1995) *Genes Dev.* **9,** 2421–2432.
35. Niepel, M., Ling, J. & Gallie, D. R. (1999) *FEBS Lett.* **462,** 79–84.
36. Diez, J., Ishikawa, M., Kaido, M. & Ahlquist, P. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 3913–3918. (First Published April 4, 2000; 10.1073/pnas.080072997)
37. Ohlmann, T., Rau, M., Pain, V. M. & Morley, S. J. (1996) *EMBO J.* **15,** 1371–1382.
38. Bailey-Serres, J., Rochaix, J.-D., Wassenegger, M. & Fillipowicz, W. (1999) *EMBO J.* **18,** 5153–5158.