

Efficient translation of alfamovirus RNAs requires the binding of coat protein dimers to the 3' termini of the viral RNAs

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Summary

The coat protein (CP) of *Alfalfa mosaic virus* (AMV) is required to initiate infection by the viral tripartite RNA genome whereas infection by the tripartite *Brome mosaic virus* (BMV) genome is independent of CP. AMV CP stimulates translation of AMV RNA *in vivo* 50- to 100-fold. The 3' untranslated region (UTR) of the AMV subgenomic CP messenger RNA 4 contains at least two CP binding sites. A CP binding site in the 3' terminal 112 nucleotides of RNA 4 was found to be required for efficient translation of the RNA whereas an upstream binding site was not. Binding of CP to the AMV 3' UTR induces a conformational change of the RNA but this change alone was not sufficient to stimulate translation. CP mutant R17A is unable to bind to the 3' UTR and translation *in vivo* of RNA 4 encoding this mutant occurs at undetectable levels. Replacement of the 3' UTR of this mutant RNA 4 by the 3' UTR of BMV RNA 4 restored translation of R17A-CP to wild-type levels. Apparently, the BMV 3' UTR stimulates translation independently of CP. AMV CP mutant N199 is defective in the formation of CP dimers and did not stimulate translation of RNA 4 *in vivo* although the mutant CP did bind to the 3' UTR. The finding that N199-CP does not promote AMV infection corroborates the notion that the requirement of CP in the inoculum reflects its role in translation of the viral RNAs.

Introduction

Efficiency of translation of eukaryotic messenger RNAs is strongly enhanced by synergistic interactions of the 5' terminal cap structure and 3' terminal poly(A) tail. The cap structure is the target for binding of eIF4F, a complex of initiation factors including the cap-binding protein eIF4E and the multifunctional adapter protein eIF4G. The poly(A)-binding protein (PABP) binds to the poly(A) tail and interacts with eIF4G and eIF4B to convert the mRNA into a closed loop structure. These interactions stabilize the complex and enhance initiation of translation (Browning, 1996; Gallie, 1991; Imataka *et al.*, 1998; Le *et al.*, 1997, 2000; Tarun & Sachs, 1996; Wilkie *et al.*, 2003). The only known class of cellular mRNAs that do not contain a poly(A) tail is represented by the cell cycle-regulated histone mRNAs in animal cells. Recently, evidence was obtained that binding of the stem-loop-binding protein (SLBP) to a highly conserved 3' terminal stem-loop structure stimulates translation of histone mRNAs through a functional interaction of SLBP with eIF4G (Ling *et al.*, 2002).

The mRNAs of many animal and plant RNA viruses lack a cap structure, a poly(A) tail or both, but yet they efficiently compete with host mRNAs for the translational machinery. RNAs of animal picornaviruses and plant potyviruses lack a 5' cap structure and translation initiates at an internal ribosome entry site (IRES) in the 5' untranslated region (UTR). An interaction between eIF4G bound to the IRES and PABP bound to the poly(A) tail of picorna- and potyviruses appears to be required for efficient translation of the viral RNAs (Svitkin *et al.*, 2001; Gallie, 2001). Several genera of plant viruses contain a 3' terminal tRNA-like structure (TLS) instead of a poly(A) tail. The TLS of *Brome mosaic virus* (BMV, genus *Bromovirus*) and *Tobacco mosaic virus* (TMV, genus *Tobamovirus*) can be aminoacylated with tyrosine and histidine, respectively, and the 3' UTRs of these viruses stimulate translation of a reporter gene by mimicking the function

of a poly(A) tail (Gallie & Kobayashi, 1994). Plant viruses from the genus *Luteovirus* (family *Luteoviridae*) and *Necrovirus* (family *Tombusviridae*) contain neither a cap structure nor a poly(A) tail. Translation of the RNAs of the luteovirus *Barley yellow dwarf virus* requires base pairing between a stem-loop in the 5' UTR and a stem-loop in a 100 nucleotide (nt) translation element that is present in the 3' UTR, presumably to deliver translation factors and/or ribosomes to the 5' end (Guo *et al.*, 2001).

We have studied the translation strategy of *Alfalfa mosaic virus* (AMV, genus *Alfamovirus*). Within the family *Bromoviridae*, the tripartite plus-strand RNA genomes of viruses from the genera *Bromovirus* and *Cucumovirus* are infectious as such, whereas initiation of infection by viruses from the genera *Alfamovirus* and *Ilarvirus* requires addition of coat protein (CP) to a mixture of the genomic RNAs (reviewed in Bol, 1999, 2003; Jaspars, 1999). AMV RNAs 1 and 2 encode the replicase proteins P1 and P2; RNA 3 encodes the viral movement protein (P3) and CP, which is translated from a subgenomic messenger, RNA 4. This RNA 4 can replace CP in the inoculum to initiate infection. At their 3' termini, AMV RNAs contain a sequence of 145 nucleotides (nts) with a high level of sequence similarity. The 3' terminal 112 nts of this sequence can adopt two alternative conformations: a linear array of hairpins separated by the sequence AUGC with a high affinity for CP, or a structure that resembles the TLS of bromo- and cucumoviruses and is required for minus-strand promoter activity (Olsthoorn *et al.*, 1999). Binding of CP to AMV RNAs blocked minus-strand promoter activity *in vitro* and enhanced translation of viral RNA in tobacco protoplasts 50- to 100-fold (Olsthoorn *et al.*, 1999; Neeleman *et al.*, 2001). We have proposed that after infection of plants with AMV RNAs 1 to 4, initially RNA 4 is poorly translated into CP but then this CP stimulates translation of its own messenger and finally stimulates the translation of RNAs 1 and 2 into the replicase proteins. After targeting of the genomic RNAs to membrane bound replication complexes and dissociation of CP from the 3' end of the RNAs, the TLS

conformer can be formed and the replicase proteins initiate viral minus-strand RNA synthesis (Neeleman *et al.*, 2001; Bol, 2003).

Fig. 1A shows the structure of the 3' UTR of AMV RNAs 3 and 4 with the CP binding conformer at the 3' end. The sequence contains at least two CP binding sites: CPB1 in the sequence that RNAs 1, 2 and 3 have in common, and CPB2 which is unique to RNA 3 (Houser-Scott *et al.*, 1994; Reusken *et al.*, 1994). The TLS conformer is generated by a pseudoknot interaction between nts 5 to 8 and 90 to 93 from the 3' end (Olsthoorn *et al.*, 1999). In this study we addressed the following questions: (i) is CPB1 and/or CPB2 involved in stimulation of translation, (ii) is translation affected when the pseudoknot interaction is disrupted by mutations in the RNA rather than by CP binding, and (iii) is translation stimulated by the binding of CP monomers or dimers?

Methods

cDNA constructs. Full-length RNAs 1, 2 and 3 were transcribed from plasmids pUT17A, pUT27A and pAL3, respectively (Neeleman *et al.*, 1991; Neeleman & Bol, 1999). Wild-type (WT) RNA 4 was transcribed from plasmid p4kWT. This plasmid is a derivative of plasmid pT72-42 (Langereis *et al.*, 1986; Neeleman *et al.*, 2001) with a *KpnI* site introduced immediately downstream of the stopcodon of the CP gene (van der Vossen *et al.*, 1994). This plasmid contains a *SstI* site in the CP coding sequence and *PstI* and *SalI* sites immediately downstream of the cDNA 4 insert. The construction of cDNA 4 clones for expression of mutants R17A (pCPR17A; Tenllado & Bol, 2000), Δ N10, N63, N85, N199, AC3 and 2A (pCP Δ N10, pCPN63, pCPN85, pCPN199; pCPAC3; pCP2A; van der Vossen *et al.*, 1994) has been described previously. pCPWT- Δ T was made by deletion of the 3' UTR sequence between the *KpnI* site in cDNA 4 and

the *Sall* site in the poly linker sequence. To obtain pCPWT-BMV and pCPR17A-BMV a *KpnI-PstI* fragment with the 3' UTR sequence in p4kWT and pCPR17A (with a *KpnI* site introduced downstream of the CP gene), respectively, was replaced by a *NciI-PstI* fragment from the full-length BMV cDNA3 clone pBBMV3 (van der Vossen *et al.*, 1994). This *NciI-PstI* fragment contains the 3' UTR of BMV RNA 3 (nts 1807-2117). pCPWT- Δ L was made by replacing a fragment from p4kWT from a *HindIII* site (present 16 base pairs downstream of the T7 RNA polymerase start site) to an *EcoRI* site (in the polylinker downstream of cDNA 4) by a *NcoI-EcoRI* fragment from plasmid pT72-42NcoCP (van der Vossen *et al.*, 1994). This latter plasmid contains a cDNA 4 insert with a *NcoI* site over the start codon of the CP gene. Plasmid pCP Δ FG was made by replacing the *SstI-SmaI* fragment of pT72-42 by the *SstI-SmaI* fragment of plasmid p3 Δ KK (van Rossum *et al.*, 1997). Plasmids pCP- Δ E, - Δ D, - Δ C, - Δ B, - Δ A and - Δ AC were made by PCR amplification of mutant 3' UTR fragments flanked by *KpnI* and *PstI* sites as described by Olsthoorn & Bol (2002). The *KpnI-PstI* fragment from p4kWT was replaced by the mutant PCR fragments. To construct pCPWT- Δ PK and pCPR17A- Δ PK, the *SstI-PstI* fragments of p4kWT and pCPR17A, respectively, were replaced by the *SstI-PstI* fragment from plasmid p3kG3 which contains the relevant mutation (Olsthoorn *et al.*, 1999). Transcripts corresponding to the 3' terminal 208 nts of RNA 4 (3' UTR transcripts) were transcribed from plasmid pTE208 (van Rossum *et al.*, 1997).

Transcription with T7 RNA polymerase and inoculation of protoplasts.

Transcription with T7 RNA polymerase was done as described previously (Neeleman *et al.*, 2001). Samples of 200,000 tobacco protoplasts were inoculated with 10 μ g of transcripts of cDNA 4 derivatives, or with a mixture of transcripts of cDNAs 1, 2, 3 and 4 (2 μ g of each transcript) and incubated for 18 h as described (Neeleman & Bol, 1999).

In vitro translation and protein analysis. Translation of RNA 4 in a rabbit reticulocyte lysate (Promega) was done according to the manufacturer's instructions. Proteins synthesized *in vitro* or *in vivo* were analyzed by Western blotting (Towbin *et al.*, 1979) using antiserum against AMV CP or P3. The protein extracted from 100,000 protoplasts was loaded per slot (Neeleman & Bol, 1999). The relative accumulation of CP in protoplasts transfected with RNA 4 derivatives was estimated, taking the amount of CP in controls transfected with WT RNA 4 as 100%. To this goal, the sample from the control protoplasts was mixed with extracts from non-transfected protoplasts to obtain 50%, 30%, 20%, 10% and 5% dilutions and these mixtures were loaded on the blots for comparison.

Analysis of CP-RNA interactions. A rabbit reticulocyte lysate was programmed with RNA 4 transcripts (20 µg/ml lysate) in the absence or presence of 3' UTR transcripts (100 µg/ml lysate). After incubation of the lysate for 90 min at 25⁰, 1 µl was withdrawn for protein analysis by Western blotting and 1 µl was withdrawn for RNA analysis by Northern blotting. To analyze RNA/protein complexes formed in the lysate, 4 µl of the lysate was mixed with 200 µl IP buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA), and 5 µg carrier RNA (total RNA extracted from tobacco) and 0.5 µl antiserum against CP were added. After incubation for 60 min at 4⁰, 50 µl of a 50% suspension in IP buffer of protein A-Sepharose CL-4B (Amersham Pharmacia Biotech) was added. After incubation for another 60 min at 4⁰ under continuous rotation, the beads were washed four times with 1 ml of IP buffer. RNA bound to the beads was extracted with Trizol (Invitrogen) in the presence of 1 µg carrier RNA. DIG-labeled minus-strand RNA 3 was

used as probe to detect RNA 4 and 3' UTR transcripts on Northern blots (Neeleman & Bol, 1999).

Results

Role of 3' UTR in translation of RNA 4

The 3' UTR of RNAs 3 and 4 is 179 nts long and can form seven hairpins designated hpA to hpG in the CP binding conformer. The hairpins are interspersed by AUGC sequences 1 to 5 (Fig. 1A). Binding *in vitro* of CP to CBP1 requires hpA, hpB and AUGC-motifs 1, 2 and 3, whereas binding of CP to CPB2 requires hpF, hpG and AUGC-motifs 4 and 5 (Reusken *et al.*, 1994; Reusken & Bol, 1996; Houser-Scott *et al.*, 1997). The RNA 4 transcript referred to as "WT" in this study is schematically shown in Fig. 1B and contains a vector-derived 5' sequence of 16 nt (GGGAGACCCAAGCTCC) followed by the RNA 4 sequence consisting of a 5' UTR of 36 nts, a coding sequence of 660 nts and a 3' UTR of 179 nts. After transfection of tobacco protoplasts with this transcript the CP synthesis shown in Fig. 2A (lane 2) is observed. In mutant WT- Δ T, the 3' UTR of RNA 4 is replaced by a vector-derived sequence of 110 nts (Fig. 1B). Compared to mock-transfected protoplasts (Fig. 2A, lane 1) this mutant RNA 4 is not translated at a detectable level (Fig. 2A, lane 3). However, replacement of the AMV 3' UTR by the 3' UTR sequence of BMV RNA 4 permits the synthesis of WT levels of CP in transfected protoplasts (Fig. 2A, lane 4). As shown previously (Neeleman *et al.*, 2001), RNA 4 encoding CP with Arg-17 replaced by Ala (mutant R17A) is not translated at a detectable level in protoplasts (Fig. 2A, lane 5). The R17A-CP is unable to bind to the 3' UTR of AMV RNA and has lost its ability to initiate AMV infection (Ansel-McKinney & Gehrke, 1998; Yusibov & Loesch-Fries, 1995). In mutant R17A-BMV the AMV 3' UTR is replaced

by the BMV 3' UTR and this replacement fully complements translation of R17A-CP in *cis* (Fig. 2A, lane 6). As shown previously (Neeleman *et al.*, 2001), mutant R17A with the AMV 3' UTR can be complemented in *trans* by expression of CP with an N-terminal truncation of 10 amino acids from transcript Δ N10 (Fig. 2A, lane 7). This Δ N10-CP behaves like WT in all steps of the AMV replication cycle and is able to bind to the 3' UTR (Tenllado & Bol, 2000). As a control, it is shown that all RNA 4 mutants were translated with WT efficiency in a cell-free reticulocyte system (Fig. 2B). Together, the data show that the 3' UTR of AMV RNA 4 stimulates translation *in vivo* by a mechanism that is dependent on functional AMV CP whereas the BMV 3' UTR stimulates translation independently of CP.

Role of 5' UTR in translation of RNA 4

To analyze a possible role of the 5' UTR of RNA 4 in translation, this UTR was deleted in mutant WT- Δ L (Fig. 1B). Compared to protoplasts transfected with the WT transcript (Fig. 3, lane 2), translation *in vivo* of WT- Δ L was reduced by 80% (Fig. 3, lane 3). This defect in translation could not be complemented in *trans* by co-expression of the Δ N10-CP (Fig. 3, lane 4). These data indicate that in addition to the 3' UTR, also the 5' UTR is required for efficient translation of RNA 4.

Role of CPB1 and CPB2 in translation of RNA 4

The region of sequence similarity at the 3' termini of AMV genomic RNAs extends from the 3' end up to AUGC-motif 4 and contains CP binding site CPB1 (Fig. 1A). To analyze a possible role of CPB2 in the translation of RNA 4, hairpins hpF and hpG were deleted in mutant Δ FG (Fig. 1A). This deletion abolishes binding of CP to CPB2 *in vitro* (Reusken *et al.*, 1994). The finding that mutant Δ FG is translated with WT efficiency in

transfected protoplasts indicates that CPB2 is dispensable for translation (Fig. 4A, lane 2). Also, deletion of hpE did not affect translation (Fig. 4A, lane 3). Single hairpin deletions of hpD, hpC, hpB or hpA (shown in Fig. 1A) all reduced translation of RNA 4 to a 20 to 25% level (Fig. 4A, lanes 4 to 7). However, deletion of both hpA and hpC reduced translation to undetectable levels (Fig. 4A, lane 8). Together, the results demonstrate that elements in the 3'-terminal 112 nts are required for efficient translation of RNA 4.

Mutations in AUGC-motifs 1, 2 and 3 affected CP binding *in vitro* to the 3' 39 nt fragment of RNA 4 to various levels (Reusken & Bol, 1996). Here, we studied RNA 4 transcripts with mutations in AUGC-3 (mutant AC3), AUGC-2 (mutant 2A) or all three 3' AUGC-motifs (mutant 123A) as shown in Fig. 1C. Similar to the single hairpin deletions, these mutations reduced translation of RNA 4 in protoplasts to 20% of the WT level (Fig. 4B, lanes 2, 3 and 4).

Role of the pseudoknot in translation of RNA 4

Under physiological conditions the TLS conformer of the 3' 112 nts of AMV RNAs is predicted to be more stable than the CP binding conformer (Olsthoorn *et al.*, 1999). Interaction of the 3' termini of the RNAs with CP disrupts the pseudoknot interaction between nts 5 to 8 and 93 to 90 to allow the formation of hpA (Olsthoorn *et al.*, 1999). To see whether disruption of the pseudoknot interaction is sufficient to enhance translation of RNA 4, the sequence of nts 93 to 90 (5'UCCU3') was mutated to 5'UGGG3' in WT RNA 4 (mutant WT- Δ PK) and in RNA 4 with the R17A mutation (mutant R17A- Δ PK). The same mutation has been shown to block minus-strand promoter activity *in vitro* (Olsthoorn *et al.*, 1999). Disruption of the pseudoknot interaction neither affected translation of RNA 4 encoding WT CP (Fig. 5, lane 2) nor did it stimulate translation of the R17A mutant to detectable levels (Fig. 5, lane 4). This indicates that merely

changing the structure of the 3' 112 nts of RNA 4 from the TLS conformer to the CP binding conformer is not sufficient to enhance translation efficiency.

Role of CP in the translation of RNA 3

With available antisera raised against P1 and P2 we were unable to detect translation products of RNAs 1 or 2 after transfection of protoplasts with mixtures of RNAs 1 and 4 or RNAs 2 and 4 (results not shown). However, with an antiserum raised against the C-terminus of the movement protein P3 we were able to detect P3 in protoplasts transfected with RNA 3. After transfection with an RNA 3 transcript a faint band of P3 was detectable on a Western blot (Fig. 6, lane 2). Translation of this RNA 3 was significantly stimulated by co-transfection of the protoplasts with an *in vitro* synthesized RNA 4 transcript (Fig. 6, lane 3) and even further stimulated by co-transfection with native RNA 4, purified from virions (Fig. 6, lane 4). Transfection of protoplasts with this native RNA 4 alone did not yield any P3 (Fig. 6, lane 5) demonstrating that the RNA 4 preparation was not contaminated with detectable amounts of RNA 3. The finding that native RNA 4 was more effective in stimulating the translation of RNA 3 than *in vitro* transcribed RNA 4 may be due to the 5' vector derived sequence of 16 nts or incomplete capping of the transcript.

Dimers of CP stimulate translation of RNA 4

The N-terminal sequence of AMV CP is involved in binding of the protein to viral RNA whereas the C-terminus is involved in CP dimer formation (Baer *et al.*, 1994; Choi & Loesch-Fries, 1999; Choi *et al.*, 2003; Tenllado & Bol, 2000). We have shown that CP with the C-terminal 21 amino acids replaced by four nonviral residues (mutant N199) is defective in dimer formation (Tenllado & Bol, 2000). Here, we tested the ability of this mutant to stimulate translation of RNA 4. In addition, we analyzed translation of RNA 4

transcripts encoding the N-terminal 85 or 63 amino acids of CP (mutants N85 and N63). *In vitro*, mutants N199 and N85 are translated into polypeptides of the expected size but the N63 peptide could not be visualized in the gel system used (results not shown). In transfected protoplasts, no translation of RNA 4 encoding the N85 or N199 mutant was detectable (Fig. 7A, lanes 3 and 5). However, co-transfection of mutant N199 with WT RNA 4 resulted in the efficient synthesis of both the WT and C-terminally truncated CP (Fig. 7A, lane 4). This shows that translation of mutant N199 can be complemented by WT CP and that the mutation does not affect the stability of the mutant CP in protoplasts. Complementation of mutant N85 resulted in a faint band of the N85 polypeptide that did not very well reproduce in Fig. 7A (lane 2).

Previously, we have used local lesion assays on bean plants and infection of tobacco plants to demonstrate that RNA 4 with mutations N63, N85 or N199 is largely defective in initiating infection by the three genomic RNAs (van der Vossen *et al.*, 1994; Tenllado & Bol, 2000). Here, we analyzed initiation of infection by these RNA 4 mutants in the tobacco protoplast system. Although a mixture of the genomic RNAs has a low intrinsic infectivity, virus accumulation in plants or protoplasts is stimulated approximately 1000-fold by the addition of WT RNA 4 to the inoculum (Houwing & Jaspars, 2000; Neeleman *et al.*, 2001). The accumulation of CP in protoplasts inoculated with mixtures of RNAs 1, 2 and 3 minus or plus WT RNA 4 is shown in lanes 2 and 6, respectively, of Fig. 7B. Addition of RNA 4 mutants N63, N85 or N199 to an inoculum with RNAs 1, 2 and 3 induced little or no increase in the accumulation of CP over the negative control (Fig. 7B, lanes 3, 4 and 5). Particularly the results with mutant N199 indicate that CP dimer formation is required for both translation of viral RNA and initiation of infection.

C-terminally truncated CP binds to viral RNA as a monomer

The defect of mutant N199 to stimulate translation and to initiate infection could be due to a defect in binding of the truncated CP to the viral RNA. Alternatively, the binding of CP monomers to viral RNA could be ineffective in performing these functions. A novel binding assay was developed to measure binding of CP to the 3' UTR of AMV RNAs. WT or mutant RNA 4 was translated in a rabbit reticulocyte lysate in the presence of a transcript corresponding to the 3' UTR of RNA 4. Subsequently, CP-antiserum and protein A-Sepharose beads were added to the translation mixture and CP/RNA complexes bound to the beads were analyzed. Fig. 8A shows an analysis of RNA 4 and 3' UTR transcripts present in a total RNA preparation extracted from the translation mixture whereas Fig. 8B shows an analysis of RNAs that were bound to the Sepharose beads. In the cell-free system, WT and mutant RNA 4 transcripts were translated into CP with similar efficiencies (Fig. 8C). When RNA 4 was omitted from the translation mixture, no binding of 3' UTR transcripts to the beads was observed (Fig. 8B, lane 8). Translation of RNA 4 into WT CP resulted in binding of both RNA 4 and 3' UTR transcripts to the beads (Fig. 8B, lane 2). Translation of WT CP from RNA 4 with its 3' UTR replaced by a plasmid-derived sequence (mutant WT- Δ T) resulted in a similar binding of 3' UTR transcripts to the beads but binding of mutant RNA 4 was significantly reduced (Fig. 8B, lane 7). The residual binding of mutant RNA 4 may reflect the interaction of CP with binding sites in the CP gene (see Discussion). CP with the R17A or Δ N16 mutation did not bind to the 3' UTR in this assay (Fig. 8B, lanes 4 and 6), in agreement with the results of other types of binding assays (Ansel-McKinney and Gehrke, 1998; Tenllado & Bol., 2000). However, CP with the N199 or Δ N10 mutations did bind the 3' UTR transcripts as efficiently as the WT CP did (Fig. 8B, lanes 3 and 5). From these results we conclude that CP-N199 binds as monomers to the 3' UTR of AMV RNAs. Apparently, this binding is insufficient to stimulate translation and to initiate infection.

Discussion

Previously, we reported a strict correlation between the ability of CP mutants to bind to AMV RNAs and their ability to promote translation of RNA 4 (Neeleman *et al.*, 2001). In the present work we analyzed sequences in RNA 4 that are important for efficient translation and we addressed the role of CP dimer formation in this process. *In vitro*, CP binds to the 3' termini of the four AMV RNAs as well as to internal coding sequences (Zuidema & Jaspars, 1984). The internal binding sites may play a role in encapsidation of the RNAs as the 3' UTRs of RNAs 1 and 2 are not required in *cis* for virion formation (Vlot *et al.*, 2000). In RNA 4, specific CP binding sites have been identified in the CP gene between nts 425 to 474 and in the 3' UTR (Houwing & Jaspars, 1982; Baer *et al.*, 1994; Reusken *et al.*, 1994). The observation that CP mutants R17A and Δ N16 did not bind to 3' UTR transcripts but showed a low level of binding to full-length RNA 4 (Fig. 8) suggests that different domains of CP are involved in CP binding to internal and 3' terminal sites. Because RNA 4 with a plasmid-derived 3' UTR was untranslatable (Fig. 2), the internal CP binding site in RNA 4 is not sufficient to stimulate translation. Therefore, we focussed our studies on translation determinants in the 3' UTR of RNA 4. The results with the RNA 4 derivative WT- Δ L indicate that also the leader sequence of RNA 4 contributes to translation efficiency of the RNA *in vivo* (Fig. 2). *In vitro*, this leader sequence has been reported to stimulate translation of a reporter gene 35-fold (Jobling & Gehrke, 1987).

The TLS conformer and hpE are the key elements in the 3' UTR required for minus-strand promoter activity of AMV RNAs (Olsthoorn *et al.*, 1999; Olsthoorn & Bol, 2002). The results with pseudoknot mutant WT- Δ PK (Fig. 5) and mutant Δ E (Fig. 4A)

demonstrate that neither of these elements plays a role in translation of RNA 4. The sequence of CPB2 (Fig. 1A) is outside the 3' region of 145 nts that shows sequence similarity in RNAs 1, 2 and 3 and this sequence is not found in RNAs 1 and 2. Deletion Δ FG removes the major part of this CP binding site but the deletion does not affect translation of RNA 4 (Fig. 4A). Moreover, mutant Δ AC contains an intact CPB2 sequence but is untranslatable (Fig. 4A). Thus, CPB2 plays no role in translation of RNA 4 and its significance in the AMV replication cycle is presently unclear. In contrast to our data, studies by Hann *et al.* (1997) indicated that AUGC-motif 5 was part of a translation determinant.

The deletion analysis presented in Fig. 4A shows that elements in the 3' terminal 112 nts of RNA 4 govern translation efficiency of this RNA. Deletion of one of the four hairpins in this region (hairpins A, B, C or D) reduced translation 4- to 5-fold whereas deletion of two of these hairpins (hairpins A and C) reduced translation to undetectable levels. The region of the 3' 39 nts (hairpins A and B, and AUGC-motifs 1, 2 and 3) is the minimal sequence that is sufficient for binding of CP *in vitro* (Reusken & Bol, 1996; Houser-Scott *et al.*, 1997). This sequence is present in mutants Δ C and Δ D, which translate with an efficiency of about 20% (Fig. 4A). Apparently, the CPB1 sequence is not sufficient for WT levels of translation of RNA 4. AUGC-motifs 1, 2 and 3 are essential for binding of CP to CPB1 (Reusken *et al.*, 1996) and mutation of these motifs affected translation efficiency of RNA 4 (Fig. 4B). This supports the notion that the CP binding activity of CPB1 is required for translation. Possibly, sequences required for CP binding to AMV RNAs *in vivo* are longer than the minimal sequence of 39 nt required *in vitro* and extend into the 3' terminal 112 nts. Alternatively, efficient translation of RNA 4 may require a CP binding site of 39 nt and an upstream element of unknown function between nts 39 and 112 from the 3' end.

Baer *et al.* (1994) reported that a peptide consisting of the N-terminal 25 or 38 amino acids of AMV CP was sufficient to bind the 3' terminal binding site in AMV RNAs and to initiate virus replication in protoplasts. In later experiments, it became clear that N-terminal peptides were much less active in initiating infection than full-length CP (Ansel-McKinney *et al.*, 1996; unpublished data quoted in Choi *et al.*, 2003). Here, we showed that RNA 4 transcripts encoding peptides corresponding to the N-terminal 63, 85 or 199 amino acids of CP were untranslatable *in vivo* and were unable to initiate infection in protoplasts (Fig. 7). Similar to our results with mutant N199 (which lacks the C-terminal 21 amino acids of CP), Choi *et al.* (2003) recently reported that RNA 4 transcripts encoding CP with C-terminal deletions of 18 or 19 amino acids (mutants CP Δ C18 and CP Δ C19) were largely defective in initiating AMV replication in protoplasts. Mutant N199 was found to be defective in dimer formation by using the yeast two-hybrid system (Tenllado & Bol, 2000) whereas mutants CP Δ C18 and CP Δ C19 did not form dimers in an assay involving glutaraldehyde cross-linking (Choi & Loesch-Fries, 1999). Our results presented in Fig. 8B and band-shift assays done by Choi *et al.* (2003) demonstrate that the C-terminally truncated CPs interact with the 3' UTR as monomers. Apparently, binding of monomers of N199 to viral RNA neither stimulated translation nor did it stimulate initiation of infection at significant levels.

The stimulation of translation of RNA 4 by its own translation product from undetectable to WT levels could be simply explained by the hypothesis that binding of CP stabilizes the RNA by protecting it from degradation. The results with mutant R17A and R17A-BMV (Fig. 2) showed that replacement of the 3' UTR of AMV RNA 4 by the 3' UTR of BMV resulted in efficient translation of the chimeric RNA independently of CP binding. Thus, the hypothesis would imply that the AMV 3' UTR stabilizes the RNA by binding of CP whereas the BMV 3' UTR stabilizes the RNA independently of CP,

possibly by the binding of host factors. However, there is evidence that the role of the 3' UTR of AMV and BMV in translation is more complex than mere protection of the RNAs against non-specific degradation. RNA 4 transfected into protoplasts survived an incubation period of 18 hours at similar levels whether or not the RNA was translated into CP (Neeleman *et al.*, 2001). By inoculation of protoplasts with AMV RNAs at different time points, Houwing and Jaspars (2000) showed that the messenger and replicon functions of these RNAs is preserved for several hours after inoculation. This argues against an exceptional sensitivity of these RNAs to degradation in the absence of CP. The observation that extension of the 3' termini of AMV genomic RNAs with a poly(A) tail obviated the requirement for CP in the inoculum to initiate infection made us to suggest that binding of CP to the 3' termini of AMV RNAs could mimic the function of the binding of PABP to the 3' poly(A) tail of cellular messengers (Neeleman *et al.*, 2001). Recently, it was shown that translation of the non-polyadenylated rotavirus mRNAs requires simultaneous interaction of the nonstructural protein NSP3 with initiation factor eIF4G and the mRNA 3' end (Vende *et al.*, 2000). By a similar mechanism, AMV CP could stabilize the complex of viral RNAs and initiation factors and promote the recruitment of 40S ribosomal subunits through the formation of a closed loop structure. Possibly, dimer formation is required for the putative interaction of CP with translation initiation factors. The 3' UTR of BMV has been proposed to act as a poly(A) mimic (Gallie & Kobayashi, 1994). If in the family *Bromoviridae* the 3' UTR of the RNAs of viruses from the genera *Bromovirus* and *Cucumovirus* act as a poly(A) mimic while CP of viruses from the genera *Alfamovirus* and *Ilarvirus* acts as a PABP mimic, this would explain the requirement of CP to initiate infection by the latter two genera.

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Figure Legends

Fig. 1. AMV RNA 4 mutants. (A) Secondary structure of the CP binding conformation of the 3' UTR of AMV RNA 4. The UGA-stopcodon of the CP gene is underlined and in italics. AUGC motifs 1 tot 5 are numbered and underlined with bold lines. Coat protein binding (CPB) sites 1 and 2 are underlined. Hairpins A to G are labeled and hairpin deletions discussed in the text are boxed. Nucleotide positions discussed in the text are indicated; numbering is from the 3' end of the RNA. (B) Schematic representation of T7 RNA polymerase transcripts corresponding to three RNA 4 derivatives. WT RNA 4 consists of a 5' sequence of 16 nts transcribed from the vector (P16), a 5' UTR of 36 nts (L36), the CP gene (CP) and a 3' UTR of 179 nts (T179). In transcript WT- Δ L the 5' UTR is deleted. In transcript WT- Δ T the 3' UTR is replaced by a vector-derived sequence of 110 nts (P110). (C) Mutations made in AUGC-motif 3 (mutant AC3), AUGC-motif 2 (mutant 2A) and AUGC-motifs 1, 2 and 3 (mutant 123A).

Fig. 2. Role of the 3' UTR in translation of AMV RNA 4. (A) Tobacco protoplasts were transfected with the indicated RNA 4 transcripts. Transcripts WT and WT- Δ T are shown in Fig. 1B. In transcript WT-BMV the 3' UTR of WT is replaced by the 3' UTR of BMV RNA 4. In transcript R17A the triplet in the CP gene encoding Arg-17 is replaced by an Ala-codon. In R17A-BMV the 3' UTR of R17A is replaced by the 3' UTR of BMV RNA 4. In lane 7, the protoplasts were co-transfected with transcript R17A and transcript Δ N10 in which the codons of the N-terminal 10 amino acids of CP are deleted. (B) The indicated transcripts were translated in a rabbit reticulocyte lysate. Accumulation of CP *in vivo* and *in vitro* was measured by Western blotting using a CP antiserum. The

position of CP is indicated in the left margin. The relative accumulation of CP in protoplasts is indicated below panel A (see Materials and Methods).

Fig. 3. Role of the 5' UTR in translation of AMV RNA 4. Tobacco protoplasts were transfected with the indicated RNA 4 transcripts. Transcripts WT and WT- Δ L are described in Fig. 1B; transcript Δ N10 is described in the legend of Fig. 2A. Accumulation of CP was measured by Western blotting using a CP antiserum. The position of CP is indicated in the left margin. The relative accumulation of CP is indicated below the panel.

Fig. 4. Role of hairpins A to G and AUGC-motifs 1, 2 and 3 in translation of AMV RNA 4. Tobacco protoplasts were transfected with the indicated RNA 4 transcripts. (A) Transcripts with hairpin deletions Δ A, Δ B, Δ C, Δ D, Δ E and Δ FG are shown in Fig. 1A. Transcript Δ AC contains a combination of deletions Δ A and Δ C. Transcript WT- Δ T is shown in Fig. 1B and was used as a negative control. (B) Transcripts contained mutations in AUGC-motifs 3 (AC3), 2 (2A) and 1, 2 and 3 (123A) as described in Fig. 1C. Transcript WT- Δ T (Fig. 1B) was included as a negative control. Accumulation of CP was measured by Western blotting using a CP antiserum. The position of CP is indicated in the left margin. The relative accumulation of CP is indicated below the panel.

Fig. 5. Role of the pseudoknot interaction in translation of AMV RNA 4. Tobacco protoplasts were transfected with the indicated RNA 4 transcripts. In transcript WT- Δ PK nucleotides 90 to 93 from the 3' end of RNA 4 are mutated to disrupt the pseudoknot interaction with nucleotides 5 to 8. In WT RNA 4 this interaction is disrupted by binding of CP. The mutation of nucleotides 90 to 93 was also introduced in transcript R17A described in the legend of Figure 2 (mutant R17A- Δ PK). Accumulation of CP was

measured by Western blotting using a CP antiserum. The position of CP is indicated in the left margin.

Fig. 6. Role of CP in the translation of AMV RNA 3. Tobacco protoplasts were mock-transfected (lane 1) or transfected with an RNA 3 transcript (lane 2), a mixture of RNA 3 and RNA 4 transcripts (lane 3), a mixture of an RNA 3 transcript and native RNA 4 purified from virions (lane 4) or native RNA 4 only (lane 5). Accumulation of the movement protein P3 was measured by Western blotting using a P3 antiserum. The position of P3 is indicated in the left margin. The asterisk indicates a plant protein detected by the antiserum.

Fig. 7. Role of CP dimers in the translation of AMV RNA 4. (A) Tobacco protoplasts were mock-transfected (lane 1) or transfected with a mixture of WT RNA 4 and RNA 4 encoding the N-terminal 85 amino acids of CP (N85) (lane 2), transcript N85 only (lane 3), a mixture of WT RNA 4 and RNA 4 encoding the N-terminal 199 amino acids of the 220 amino acids long CP (N199) (lane 4), or the N199 transcript only (lane 5). (B) Tobacco protoplasts were mock-inoculated (lane 1) or inoculated with transcripts corresponding to AMV RNAs 1, 2 and 3 plus buffer (lane 2), transcript N63 (lane 3), transcript N85 (lane 4), transcript N199 (lane 5) or WT RNA 4 (lane 6). RNA 4 corresponding to transcript N63 encodes the N-terminal 63 amino acids of CP. Accumulation of CP was measured by Western blotting using a CP antiserum. The position of CP (panels A and B) and the C-terminally truncated peptides N199 and N85 (panel A) is indicated in the left margin.

Fig. 8. Analysis of the binding of mutant CP to RNA 4 and to transcripts corresponding to the 3' UTR of RNA 4. A rabbit reticulocyte lysate was programmed with WT RNA 4

(lane 1), with mixtures of transcripts corresponding to the 3' UTR of RNA 4 (3' UTR) and the indicated RNA 4 transcripts (lanes 2 to 7), or with the 3' UTR transcript only (lane 8). After incubation of the lysates, samples were taken to analyze the RNAs in the lysate by Northern blotting (panel A) and to analyze CP translated from the RNA 4 transcripts by Western blotting (panel C). Subsequently, CP antiserum and Protein A-Sepharose beads were added to the lysate, the beads were washed several times and RNA in RNA/protein complexes that were bound to the beads were analyzed by Northern blotting (panel B). The Northern blots were hybridized to a probe corresponding to AMV RNA 3. The blot of panel A was exposed for 2 min., whereas the blot of panel B was exposed for 12 min.. The positions of RNA 4 and the 3' UTR transcript are indicated in the right margin. The Western blot was analyzed with a CP antiserum. The position of CP is indicated in the right margin.

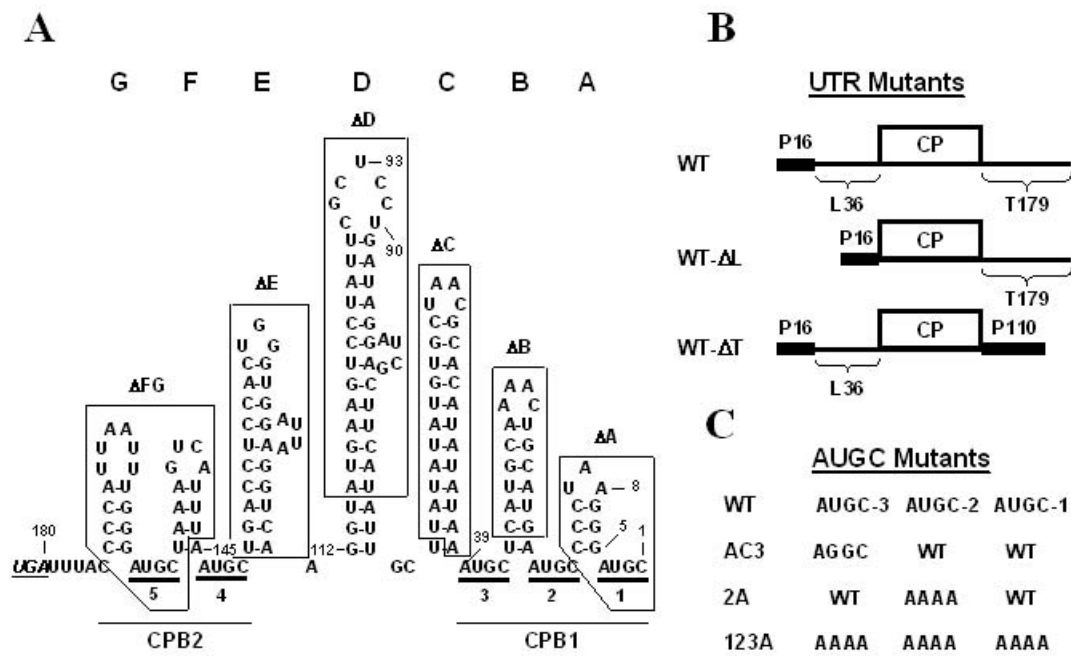


Fig. 1 Neeleman et al.

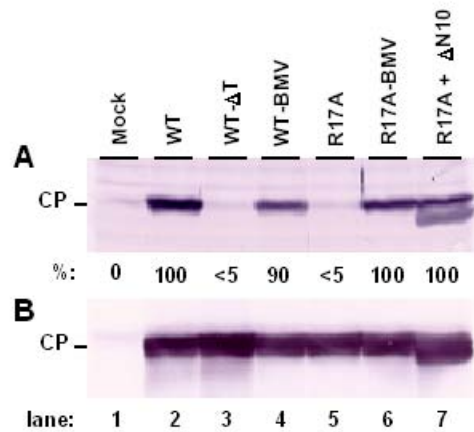


Fig. 2 Neeleman et al.

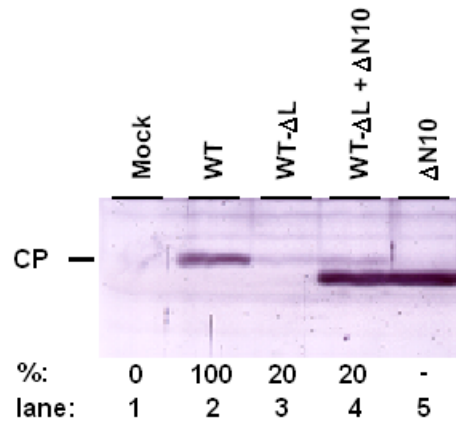


Fig. 3 Neeleman et al.

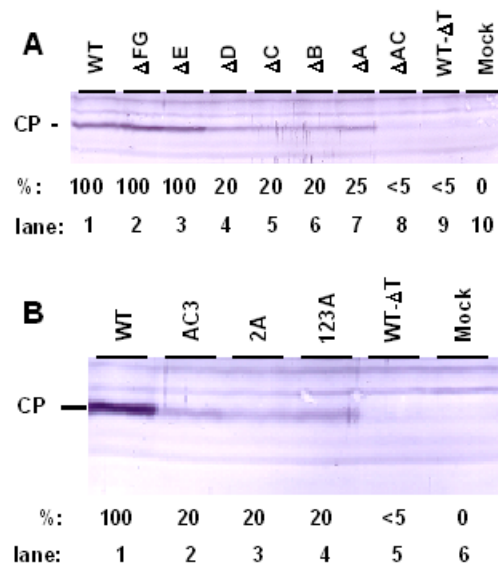


Fig. 4 Neeleman et al.

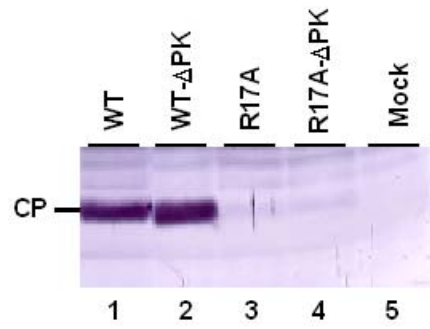


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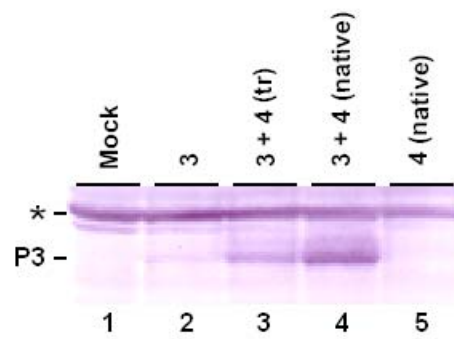


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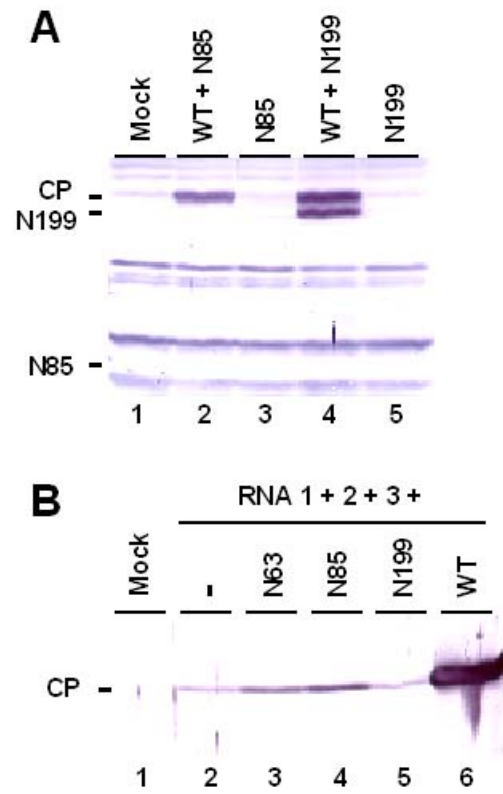


Fig. 7 Neeleman et al.

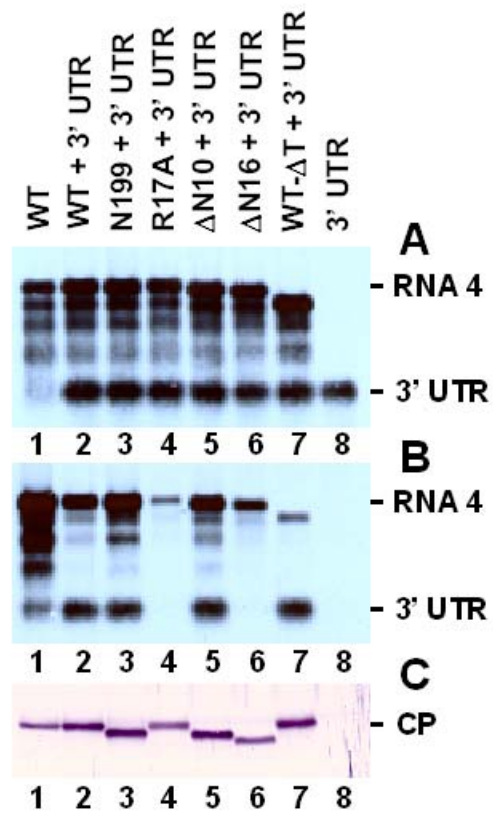


Fig. 8 Neeleman et al.