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REVIEW ARTICLE

Alfalfa mosaic virus and ilarviruses: involvement of coat protein in multiple steps of the replication cycle

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Introduction

The genera *Alfamovirus*, *Ilarvirus*, *Bromovirus* and *Cucumovirus* have been classified as members of the *Bromoviridae* family of plant RNA viruses (Rybicki, 1995). Recently, the proposal to include the genus *Oleavirus* in this family was ratified (Pringle, 1998). Viruses in the family *Bromoviridae* have a tripartite RNA genome of plus-strand polarity. RNAs 1 and 2 encode proteins involved in viral RNA replication, whereas RNA 3 encodes a protein involved in cell-to-cell movement and the coat protein (CP), which is translated from a subgenomic messenger, RNA 4. The four viral RNAs are separately encapsidated into bacilliform particles (alfamoviruses), spheroidal particles (ilarviruses), quasi-spherical to bacilliform particles (oleaviruses) or spherical particles (bromoviruses and cucumoviruses). In 1971 it was reported that a mixture of the three genomic RNAs of alfalfa mosaic virus (AMV), the type species of the alfamoviruses, was not infectious to plants unless a few molecules of CP were added per RNA molecule to the inoculum (Bol *et al.*, 1971). In this inoculum, the CP could be replaced by its subgenomic messenger, RNA 4. The early function of CP in the initiation of infection was termed 'genome activation'. In 1975 it was reported that, similar to AMV, a mixture of the three genomic RNAs of tobacco streak virus (TSV), the type species of the ilarviruses, required addition of CP to the inoculum to initiate infection. Interestingly, infection with the AMV genome was activated by the TSV CP and vice versa (van Vloten-Doting, 1975). Subsequently, data demonstrating a functional equivalence of CP in the initiation of infection were obtained for the ilarviruses citrus leaf rugose virus (CiLRV), citrus variegation virus (CVV), prunus necrotic ringspot virus (PNRSV) and prune dwarf virus (PDV) (Gonsalves & Garnsey, 1975; Gonsalves & Fulton, 1977; Halk & Fulton, 1978).

In 1972, it was observed that the AMV genome could be activated by addition of purified virus particles containing AMV RNA 1. After mixing RNAs and particles, the virions were disassembled and their CP molecules became distributed over the RNAs present in the inoculum (van Vloten-Doting & Jaspars, 1972). In 1978, binding sites with a high affinity for CP were localized at the 3′ end of AMV RNA 4 and subsequent studies revealed stem–loop structures involved in CP binding that were located in a 3′-terminal sequence of 145 nucleotides (nt) that is homologous in the four AMV RNAs (Houwing & Jaspars, 1978; reviewed in Jaspars, 1985). Similar structures were found at the 3′ termini of ilarvirus RNAs and it was shown that CP of AMV could bind to the 3′ termini of ilarvirus RNAs and vice versa. By the elegant use of a temperature-sensitive mutant CP, evidence was obtained that each of the three AMV genomic RNAs had to be complexed with a few molecules of CP to permit the initiation of infection (Smit *et al.*, 1981).

In contrast to AMV and ilarviruses, bromoviruses and cucumoviruses do not require CP or RNA 4 in the inoculum to initiate infection. The 3′ termini of the RNAs of these viruses contain a tRNA-like structure that is known to bind a number of host proteins, but has no particular affinity for CP (Giegé, 1996). Similar to bromoviruses and cucumoviruses, oleaviruses do not require the addition of CP to a mixture of the genomic RNAs for infectivity (Grieco *et al.*, 1995, 1996). In addition, the observation that the 3′ termini of oleavirus RNAs can be folded in a tRNA-like structure that closely resembles that of brome mosaic virus (R. C. L. Olsthoorn and J. F. Bol, unpublished) suggests that bromo-, cucumo- and oleaviruses may have a similar replication strategy. Since the initial discovery of the early function of CP of AMV and ilarviruses, it has been speculated that CP plays an essential role either in the translation or replication of the RNAs in the inoculum. In the following paragraphs, recent data on the various steps in the life-cycle of AMV and ilarviruses will be reviewed with particular emphasis on the role of CP in each of these.

Genome structure

The complete nucleotide sequence has been determined for the three genomic RNAs of AMV strain 425 (Leiden isolate) and the four ilarviruses TSV, CiLRV, elm mottle virus (EMoV) and spinach latent virus (SpLV) (Barker *et al.*, 1983; Cornelissen *et al.*, 1983*a*, *b*; Xin *et al.*, 1998 and references therein). In addition, the sequences of RNA 1 of PDV, and RNA 3 of PDV, PNRSV, CVV, apple mosaic virus (ApMV), lilac ring mottle virus (LRMV) and hydrangea mosaic virus (HdMV) (see Xin *et al.*, 1998) have been published. Incomplete

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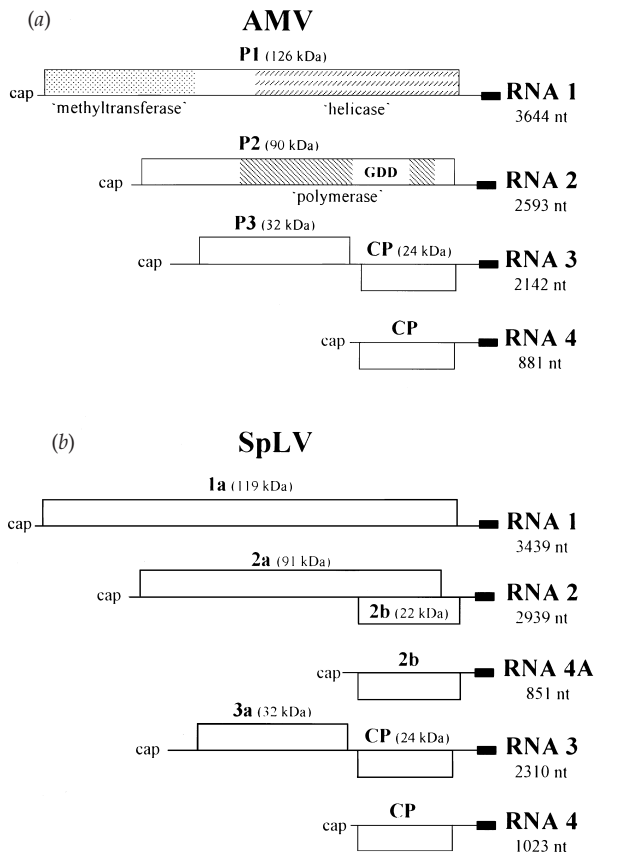

Fig. 1. Genome structure of AMV (*a*) and SpLV (*b*).

sequence data obtained for several AMV strains indicate an approximately 95% sequence similarity between different members of the *Alfamovirus* genus. Phylogenetic analysis of AMV and ilarvirus CPs reveals two major clusters, one containing AMV, PNRSV, ApMV and PDV, and the other containing TSV, CiLRV, CVV and LRMV (Sánchez-Navarro & Pallás, 1997). Within one cluster the sequence similarity is 45% or more whereas the similarity between clusters is 35–40%.

The AMV genome structure is schematically shown in Fig. 1(*a*). RNAs 1 and 2 encode the replicase proteins P1 and P2, respectively. P1 contains an N-terminal methyltransferase motif and a C-terminal helicase motif; P2 contains a polymerase motif with the conserved GDD sequence. The 5′-proximal gene in RNA 3 encodes the P3 protein with a role in cell-to-cell movement and this gene is followed by the CP gene. The sequence of 145 nt in the 3′ untranslated region (UTR) that is 80% homologous in the viral RNAs is indicated. Fig. 1(*b*) shows the genome organization of SpLV as a representative of the ilarviruses. The AMV proteins P1, P2 and P3 are termed 1a, 2a and 3a in the ilarvirus nomenclature and the conserved sequence in the 3′ UTR of SpLV RNAs is 163 nt. A major difference between the genomes of AMV and the four ilarviruses sequenced to date is the presence in ilarviruses but not AMV of a conserved open reading frame (ORF) 2b that overlaps the 3′ end of ORF 2a. A subgenomic messenger corresponding to ORF 2b (RNA 4A) has been identified in infected plants and this RNA is translated *in vitro* into the expected 22 kDa protein (Xin *et al.*, 1998). The genome of cucumoviruses contains a 2b gene at a position similar to that of the ilarvirus 2b gene (Ding *et al.*, 1996). However, the ilarvirus 2b gene is almost twice as large as the cucumovirus 2b gene and there is no sequence similarity between the two types of gene whereas there is significant similarity between the four ilarvirus 2b genes. The cucumoviral 2b gene plays a role in systemic spread of the virus and virulence determination, possibly by suppressing a host RNA silencing mechanism (Ding *et al.*, 1996; Brigneti *et al.*, 1998). A possible role of the 2b gene in ilarvirus replication has not yet been analysed. Preliminary data on the sequences of RNA 2 of ApMV, PDV and *Humulus japonicus* virus indicate that a 2b gene is absent from the genome of these ilarviruses (P. Shiel & S. W. Scott, personal communication). It has been speculated that the 2b gene is either an ancestral gene of *Bromoviridae* that has been lost in bromoviruses, AMV and some ilarviruses, or an independent *de novo* creation in cucumoviruses and some ilarviruses (Xin *et al.*, 1998).

Virion structure

Preparations of AMV contain four major classes of particle called bottom component (B), middle component (M), top component b (Tb) and top component a (Ta). The bacilliform particles B, M and Tb contain the genomic RNAs 1, 2 and 3, encapsidated by 240, 186 and 150 CP subunits, respectively. Ta contains two molecules of RNA 4, encapsidated by 132 CP subunits, and can be subdivided into bacilliform Ta-b and spheroidal Ta-t particles (see Jaspars, 1985). The bacilliform particles are 19 nm wide and have lengths ranging between 30 nm (Ta-b) and 56 nm (B). In the absence of RNA, the isolated AMV CP reassembles under proper conditions into $T = 1$ icosahedral empty particles. Crystallization of these particles is facilitated through removal of the highly basic N-terminal 26 amino acids (aa) by mild trypsin digestion. The structure of CP in such $T = 1$ particles has been resolved by X-ray diffraction and cryoelectron microscopy at 4·0 Å resolution (Kumar *et al.*, 1997). The $T = 1$ particle is built from 60 subunits standing 'end on' on the viral surface with the body axis of CP almost parallel to the closest fivefold axis and the N- and C-terminal arms protruding inward. The CP has the canonical eight-stranded β-barrel fold which is common to the structure of most spherical viruses. In the CP–CP dimer interaction, the C-terminal arm of one CP subunit appears to be hooked around the N-terminal arm of the twofold related subunit and becomes sandwiched between this N-terminal arm and the body of the β-barrel of the partner. The dimer is further stabilized by interactions between the C-terminal arms at the icosahedral twofold axis (Kumar *et al.*, 1997).

The structure of ilarvirus particles has been studied in little detail. Virus preparations can be separated into four classes of

spheroidal particles with diameters of about 33, 30, 26 and 23 nm, containing RNAs 1, 2, 3 and 4, respectively. Studies with AMV mutants indicate that only a limited number of amino acid changes can influence the propensity of the CP to assemble into bacilliform or spheroidal structures (Roosien & Vloten-Doting, 1983; Thole *et al.*, 1998). Thus, a difference in particle morphology should not be given too much weight in classifying AMV and ilarviruses in separate genera.

CP–RNA interaction

AMV CP isolated from virions occurs as dimers in solution (Kruseman *et al.*, 1971). Initially, high-affinity binding sites for CP were localized to the 3′ end of AMV RNA 4 and complexes containing one, two or three dimers of CP per RNA molecule were identified (Houwing & Jaspars, 1978, 1980). An analysis of RNA fragments that were protected by CP against RNase T1 digestion revealed the binding of AMV CP to 3′-terminal sequences of AMV RNAs 1, 2, 3 and 4 and TSV RNA 3, as well as to a few internal sequences of these RNAs (Zuidema *et al.*, 1983, 1984; Zuidema & Jaspars, 1985; reviewed in Jaspars, 1985). The smallest fragment able to rebind CP corresponded to the 3′-terminal 36 nt of RNA 1; no binding was found for a 3′-terminal fragment of 28 nt. A model for the secondary structure of the 3′ UTRs of AMV RNAs 1, 2 and 3 as proposed by Koper-Zwarthoff & Bol (1980) and Houwing & Jaspars (1982) is shown in Fig. 2. The model is supported by data from enzymatic-structure probing (Quigley *et al.*, 1984). The 3′-terminal sequence of 145 nt that is 80% homologous in the three AMV RNAs can be folded into five stem–loop structures (labelled A–E in Fig. 2) which are flanked by single-stranded AUGC tetranucleotide sequences. Similar stem–loop structures and AUGC motifs are found at the 3′ termini of ilarvirus RNAs. Fig. 3 shows the predicted secondary structure of the 3′ termini of RNA 3 of AMV and a number of ilarviruses.

In the past 5 years, the interaction between CP and RNA of AMV and ilarviruses has been studied using RNA transcripts of wild-type (wt) and mutant cDNA clones, transcripts of synthetic deoxyribooligonucleotides and synthetic peptides. Band-shift assays with transcripts corresponding to the 3′ UTR of AMV RNA 3 done by Houser-Scott *et al.* (1994) revealed four RNA–CP complexes. Hydroxyl radical and RNase T1 footprinting showed that in these complexes five regions of the 3′ UTR interacted with CP: nt 9–18, 29–41, 63–72, 107–121 and 133–144 from the 3′ end. Binding studies with subfragments of the 3′ UTR showed that the 5′ half (nt 85–175 from the 3′ end) formed one complex with CP whereas the 3′ half (nt 1–86 from the 3′ end) formed three complexes. In agreement with the results of Zuidema *et al.* (1984), binding of CP to a sequence corresponding to the 3′-terminal 39 nt of RNA 3 was observed. This sequence contains hairpins A and B and AUGC motifs 1, 2 and 3, indicated in Fig. 2. Mutation of AUGC motif 2 to AAAA abolished binding of CP to the 39 nt fragment. Band-shift assays done by Reusken *et al.* (1994) also identified a minimum of two independent binding sites for CP in the 3′ UTR of AMV RNA 3, site I being located in the sequence of nt 1–127 and site II in nt 133–208 from the 3′ end. Mutation of AUGC motifs 1 and 2 to AGGC reduced the binding of CP to site I whereas such a mutation of AUGC motif 3 abolished CP binding to site I. Similarly, mutation of AUGC motifs 4 and 5 to AGGC reduced and abolished binding of CP to site II, respectively. The importance of AUGC motifs as determinants for CP binding was further supported by the application of the 'Selex' method starting from a pool of fragments corresponding to the 39 nt of AMV RNA 3 with random substitutions in the AUGC motifs. After six iterative rounds of *in vitro* genetic selection, 25% of the RNAs selected from the pool contained the wt AUGC motifs (Houser-Scott *et al.*, 1997). Mutation of AUGC motifs 1, 2 or 3 to AAAA abolished the binding of CP to the 39 nt fragment (Reusken & Bol, 1996). Also, mutation of AUGC motif 2 to AGGC, UAGC or UUGC abolished binding of CP to the 39 nt fragments but similar mutations in motifs 1 and 3 had little effect on CP binding. To explain why mutation of AUGC motif 2 to AAAA abolished CP binding to the 3′ 39 nt fragment but had little effect on CP binding to the 3′ 127 nt fragment, it was proposed that in the 127 nt fragment the UUGC sequence in between hairpins C and D (nt 68–71 in Fig. 2) also played a role in CP binding (Reusken & Bol, 1996). Mutations that disturbed the stem-structures of hairpins A and B interfered with CP binding to the 39 nt fragment but compensating mutations restored CP binding. From a mutational analysis it was concluded that the loops of hairpins A and B are not involved in sequence specific contacts with CP and it was suggested that these hairpins predominantly serve to bring AUGC motifs in the correct orientation and proximity for CP binding (Reusken & Bol, 1996). Possibly, the seven hairpin structures in the 3′ UTR of AMV RNA 3 arrange the five AUGC boxes and UUGC sequence in such a way that tandem binding sites are created that permit cooperative binding of CP dimers.

The N terminus of CP was shown to be necessary and sufficient for binding of CP to the 3′ end of AMV and ilarvirus RNAs (Baer *et al.*, 1994). When the N-terminal 40 aa were deleted, no binding of CP to the 3′ UTR of AMV RNA 3 was observed in band-shift assays. However, binding was observed with a fusion protein consisting of the N-terminal 40 aa of AMV CP and α-globin, and with synthetic peptides corresponding to the sequence of the N-terminal 25 aa (CP25) or 38 aa (CP38) of AMV CP. Even a peptide corresponding to aa 13–26 of AMV CP conferred protection against RNase T2 digestion of the 3′-terminal nucleotides of the 39 nt RNA fragment (Ansel-McKinney *et al.*, 1996). Similar protection was obtained with peptides corresponding to the N termini of CP of the ilarviruses CVV (aa 25–47) and TSV (aa 39–57). The AMV CP25 peptide contains seven basic residues. Replacement of the lysine residues at positions 5, 6, 10, 13, 16 or 25 by alanine did not affect CP binding, but replacement of the arginine at position 17 by alanine or lysine abolished binding

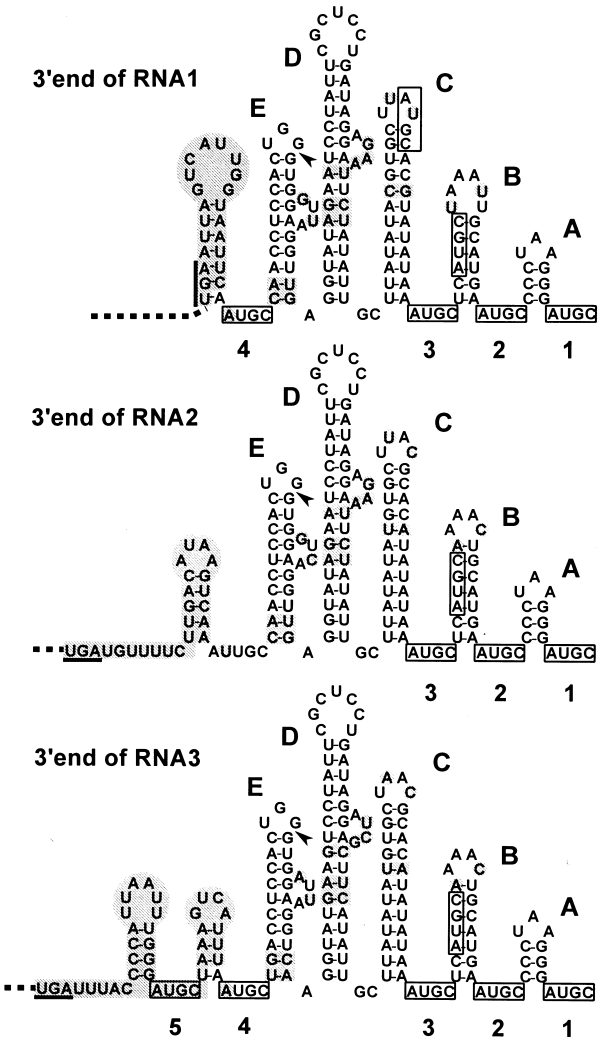

Fig. 2

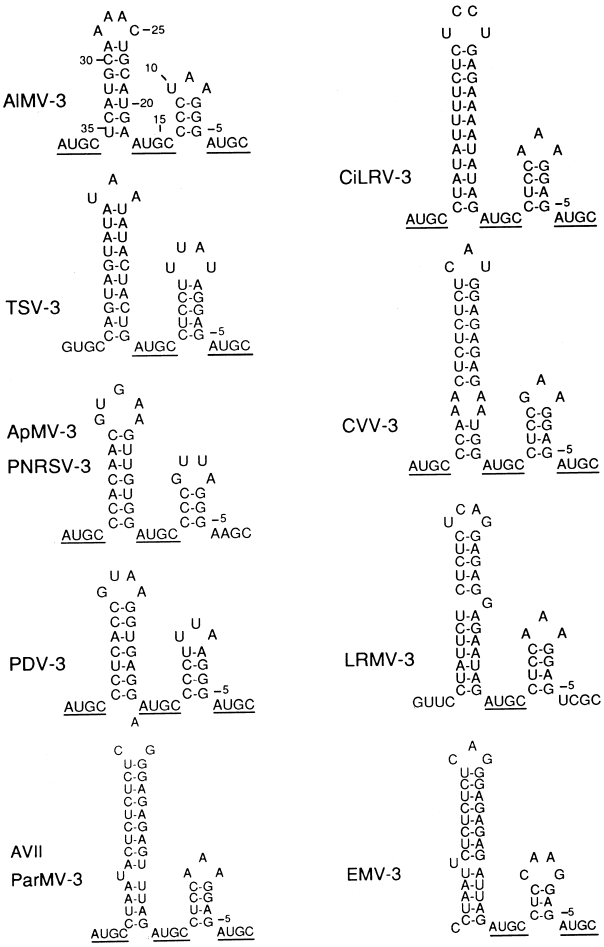

Fig. 3

Fig. 2. Schematic representation of the proposed secondary structure of the 3′ UTRs of the genomic RNAs of AMV. Bases that are not identical in the three RNAs are shaded. The stop codons of the P1, P2 and CP genes are indicated by black bars. AUGC sequence motifs are boxed. The arrowhead indicates a unique *Dra*III site in the viral cDNAs. Nucleotides of RNA 3 are numbered from the 3′ end. Reprinted from van Rossum *et al.* (1997*a*) with the permission of the American Society for Microbiology.

Fig. 3. Predicted secondary structure at the 3′ terminus of RNA 3 of AMV and several ilarviruses. AUGC sequence motifs are underlined. Reprinted from Reusken & Bol (1996) with the permission of Oxford University Press.

of CP to the 39 nt fragment. In CVV and TSV CP, arginines equivalent to the arginine at position 17 in AMV CP occur at positions 34 and 47, respectively, and alanine or lysine substitutions at these positions interfered with binding of the CVV and TSV peptides to the AMV 39 nt RNA fragment (Ansel-McKinney *et al.*, 1996). In AMV CP, the arginine at position 17 is also crucial for binding of the full-length CP to the 3′ UTR of AMV RNA 3 (Yusibov & Loesch-Fries, 1998). From an alignment of AMV and ilarvirus CP sequences, the consensus RNA binding sequence Q/K/R-P/N-T-X-R-S-R/Q-Q/N/S-W/F/Y-A was deduced (Ansel-McKinney *et al.*, 1996). Interestingly, the P/N-T-X-R-S motif of this consensus sequence is found in replicase proteins of several alphaviruses and turnip yellow mosaic virus, the parvovirus capsid protein VP1 and the hepatitis C virus core protein (Swanson *et al.*, 1998). Fig. 4 shows an alignment of the amino acid sequences of the AMV CP26 and CP10–26 peptides, the TSV CP39–75 peptide and the deduced consensus RNA binding sequence. In contrast to the lysine-rich AMV peptide, the TSV peptide is rich in arginine. The TSV CP39–75 peptide was found to bind specifically to RNA fragments corresponding to the 3′-terminal 39 nt of AMV RNA 3 and the 3′-terminal 50 nt of TSV RNA 3. Mutation of the arginine at position 47 to lysine or alanine disrupted the binding of the TSV peptide to both RNA

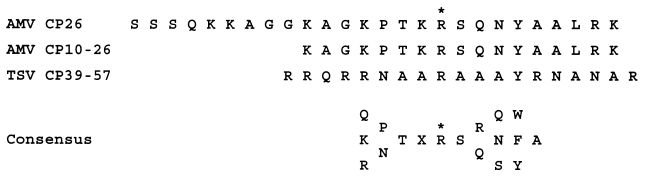

Fig. 4. Amino acid sequences of N-terminal peptides of the CPs of AMV and TSV aligned with the AMV and ilarvirus RNA binding consensus deduced by Ansel-McKinney *et al.* (1996). The arginine residue that is critical for binding of the peptides to viral RNA is indicated by an asterisk.

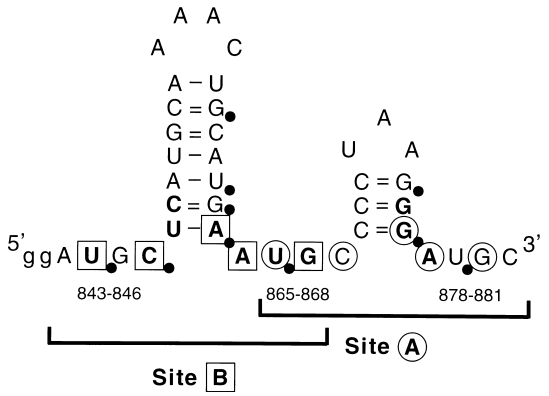

Fig. 5. Secondary structure of the 3′-terminal 39 nt of AMV RNA 3 with nucleotides interacting with N-terminal peptides of AMV CP indicated. Circled and boxed letters represent putative base-specific contacts for proposed peptide-binding sites A and B, respectively. Filled circles represent potential phosphate contacts. Reprinted from Ansel-McKinney & Gehrke (1998) with the permission of Academic Press.

fragments (Swanson *et al.*, 1998). A putative CCCH-type zinc-finger is present in aa 13–30 of TSV CP. The absence of this zinc-finger motif in TSV CP39–75 may explain the observation that the affinity of this peptide for its cognate RNA is lower than the affinity of the AMV peptides for the TSV RNA fragment (Swanson *et al.*, 1998). Mutation of AUGC motif 2 in the TSV RNA fragment to AAAA abolished peptide binding. Apparently, AUGC motifs 2 in AMV and TSV RNAs have equivalent functions in CP binding.

Recently, the sugar moieties, phosphates and bases of the 3′-terminal 39 nt fragment of AMV RNA 3 that interact with the AMV CP26 peptide were mapped in detail by hydroxyl radical footprinting, ethylation and chemical modification interference experiments (Ansel-McKinney & Gehrke, 1998). A summary of the results is shown in Fig. 5. Determination of the stoichiometry of the peptide–RNA complex provided evidence that two peptides are binding to one RNA fragment. The two binding sites are indicated as sites A and B in Fig. 5 and are believed to reflect the binding sites of the N termini of the two subunits of a native dimer of AMV CP. When the loops of the 5′ or 3′ hairpin in Fig. 5 were replaced by GNRA or UNCG tetraloops, which stabilize hairpin structures, no reduction of binding of the CP26 peptide was observed. This further substantiated the conclusion of Reusken & Bol (1996) that the loops are not involved in base-specific contacts with CP. Potential phosphate and base-specific contacts as well as ribose moieties protected upon peptide binding cluster in lower hairpin stems and in the flanking AUGC motifs 1, 2 and 3 of the 39 nt RNA fragment (Fig. 5). It was suggested that the base of the G-residue of AUGC motif 2 (G-867 in Fig. 5) may hydrogen bond with the crucial arginine at position 17 of CP (Ansel-McKinney & Gehrke, 1998).

Genome activation

There is ample evidence indicating that initiation of infection by AMV and ilarviruses requires the binding of CP to specific sites in the inoculum RNAs. Using a temperature-sensitive CP that activated the genome at the restrictive temperature only when bound to RNA, evidence was obtained that each of the AMV genomic RNAs has to be complexed with a few CP molecules to initiate infection (Smit *et al.*, 1981). When AMV RNAs 1, 2 and 3 in the inoculum are replaced by the corresponding cDNAs fused to the CaMV 35S promoter, the initiation of infection becomes largely independent of CP in the inoculum (Neeleman *et al.*, 1993). However, when one of the cDNAs in such an inoculum is replaced by the corresponding RNA, the infection becomes again fully dependent on the presence of CP in the inoculum. This supports the notion that each of the three genomic RNAs has to complexed with CP for genome activation. Apparently, a transient expression of 35S/cDNAs 1, 2 and 3 largely obviated the requirement of CP in the inoculum. Yet, infection by the 35S/cDNA inoculum was stimulated severalfold by addition of CP or 35S/cDNA 4. In contrast to RNA 4, 35S/cDNA 4 was not able to activate an inoculum consisting of AMV genomic RNAs. Probably, CP expressed from 35S/cDNA 4 is synthesized later than CP translated directly from RNA 4 in the inoculum, and it was concluded that CP is required in a narrow time-window after inoculation to permit activation of a mixture of AMV genomic RNAs (Neeleman *et al.*, 1993). When AMV 35S/cDNAs 1 and 2 are integrated in the genome of tobacco plants, the transgenic plants (P12 plants) can be infected with RNA 3 only, and the initiation of infection is fully independent of CP in the inoculum (Taschner *et al.*, 1991). Possible explanations for this phenomenon will be discussed later.

CP has been shown to bind both to the 3′ termini and to internal sequences of AMV and TSV RNAs (Zuidema & Jaspars, 1984). Circumstantial evidence indicates that binding of CP to 3′-terminal sequences rather than to internal sequences of AMV and ilarvirus RNAs is essential for genome activation. Peptides corresponding to the N-terminal 25, 26 or 38 aa of AMV CP specifically bind to the 3′-terminal 39 nt of AMV RNAs and are functional in genome activation (Baer *et al.*, 1994). Mutation of arginine-17 to alanine interferes with binding to the 3′-terminal 39 nt sequence (Ansel-McKinney *et al.*, 1996) and introduction of the same mutation in full-length CP interferes with binding of the mutant CP to an RNA

fragment corresponding to the 3′ 192 nt of AMV RNA 3 and with genome activation (Yusibov & Loesch-Fries, 1995, 1998). The observation that the AMV CP26 and the TSV CP39–57 peptides interact with similar nucleotides in the 3′-terminal AUGC sequences and hairpin structures of AMV and TSV RNAs was offered as an explanation why heterologous AMV and ilarvirus CP–RNA mixtures are infectious (Swanson *et al.*, 1998; Ansel-McKinney *et al.*, 1998). However, as the primary and secondary structures of several internal CP binding sites in AMV and TSV RNAs resemble that of the 3′-terminal binding sites (Zuidema & Jaspars, 1984), a possible role of the internal binding sites in genome activation cannot be ruled out.

Genome activation has been studied by addition to a mixture of AMV genomic RNAs of RNA 4 transcripts encoding truncated CP molecules. Transcripts encoding the N-terminal 37 aa of AMV CP did not activate the genome, possibly because of instability of the translation product, but transcripts encoding the N-terminal 51, 63, 85 or 199 aa of the 220 aa long CP did activate the genome (van der Vossen *et al.*, 1994). Deletion of aa 2–10 of the encoded CP did not interfere with genome activation but deletion of aa 2–18 abolished genome activation (Yusibov & Loesch-Fries, 1995). CP mutant 4P, in which aa 85 and 86 are replaced by six nonviral amino acids, was fully active in genome activation but defective in encapsidation of viral RNAs (van der Vossen *et al.*, 1994) and in CP–CP dimer formation as measured in the two-hybrid system (F. Tenllado & J. F. Bol, manuscript in preparation). This suggests that dimer formation of CP is not essential for genome activation.

Translation of viral RNAs

In cell-free extracts AMV RNAs are translated with different efficiencies. When the efficiency of translation of RNA 3 in wheat germ or reticulocyte systems is taken as 1, the relative efficiencies of translation of RNAs 1, 2 and 4 are 10, 60 and 150, respectively (Godefroy-Colburn *et al.*, 1985). Available evidence indicates that binding of CP to AMV RNAs does not affect their translation *in vitro* (van Vloten-Doting *et al.*, 1977; van Vloten-Doting & Neeleman, 1982; Gallie & Kobayashi, 1994). The 5′ UTR of AMV RNA 4 is principally single-stranded (Gehrke *et al.*, 1983). Uncapped and capped RNA 4 transcripts are translated efficiently *in vitro* (Loesch-Fries *et al.*, 1985; Langereis *et al.*, 1986) but enhancement of translation of RNA 4 by a cap structure has been reported (Fletcher *et al.*, 1990). However, capping of RNA 4 is essential for genome activation, indicating that the cap structure is essential for *in vivo* translation (Loesch-Fries *et al.*, 1985; Langereis *et al.*, 1986). Deletions in the 3′ UTR of AMV RNA 4 cause a lagged RNA translation in wheat germ extracts, suggesting a role for the 3′ UTR in translation initiation (Ryabova *et al.*, 1993). Initially, Loesch-Fries *et al.* (1985) concluded that the 3′ UTR of RNA 4 conferred stability to the mRNA in *Xenopus* oocytes. More recently, Hann *et al.* (1997) showed that the 3′ UTR was required for the efficient competition of RNA 4 with cellular messengers in oocytes and wheat germ extracts, rather than for mRNA stability. Mutation of the AUG codon in AUGC motif 5 (Fig. 1) abolished translation of RNA 4 in oocytes whereas a mutation of the AUG codon in AUGC motif 4 of the 3′ UTR did not affect this translation (Hann *et al.*, 1997). When the 3′ UTR of RNA 4 was fused to the *β*-glucuronidase coding sequence, no enhanced translation of GUS was observed *in vitro* whether or not CP was added (Gallie & Kobayashi, 1994). Possibly, the 5′ and 3′ UTR of AMV RNA 4 have to act in concert to promote efficient CP expression.

Replication of viral RNA: role of coding sequences

The synthesis of AMV RNAs has been studied *in vitro* and *in vivo*. Studies *in vitro* became possible as a result of the painstaking work of E. M. J. Jaspars and co-workers at Leiden University in identifying the critical conditions for plant growth and infection, permitting the isolation of a soluble template-dependent RNA-dependent RNA-polymerase (RdRp or replicase) from AMV-infected plants. Initial experiments were done with RdRp from bean plants infected with AMV strain YSMV (Houwing & Jaspars, 1986). Later, isolation of RdRp from *Nicotiana tabacum* or *N. benthamiana* infected with AMV strain 425 appeared to yield a more stable enzyme with better reproducibility (Quadt *et al.*, 1991). Available evidence indicates that in the infected cell, AMV replication complexes are associated with chloroplast membranes (de Graaff *et al.*, 1993). Solubilization *in vitro* of the RdRp with Nonidet P-40 under high salt conditions followed by purification of the extract in a glycerol gradient yields an enzyme that is strongly dependent for activity on exogenous AMV template RNA. The enzyme contains the viral proteins P1, P2 and CP and can be immunoprecipitated with antisera against these proteins (Quadt *et al.*, 1991). Studies *in vivo* have been mainly done in protoplasts from transgenic tobacco plants expressing the AMV P1 and P2 proteins (P12 plants). These plants and protoplasts can be infected with T7 transcripts of a cDNA clone of RNA 3 without the need for CP in the inoculum (Taschner *et al.*, 1991; see Fig. 6) and have been extensively used to analyse functions involved in RNA 3 replication. Only recently, infectious T7 transcripts corresponding to AMV RNAs 1 and 2 were obtained that can be used to study *cis*- and *trans*-acting functions in these RNAs (Neeleman & Bol, 1999).

Available evidence indicates that AMV CP has no role in the synthesis of viral minus-strand RNA. RdRp isolated from mock-infected P12 plants does not contain CP but specifically transcribed AMV plus-strand RNAs *in vitro* into minus-strand copies (de Graaff & Jaspars, 1995). Addition of CP to this assay mixture inhibited minus-strand RNA synthesis. When P12 protoplasts were inoculated with RNA 3 with a defective CP gene, accumulation of wt levels of minus-strand RNA 3 was

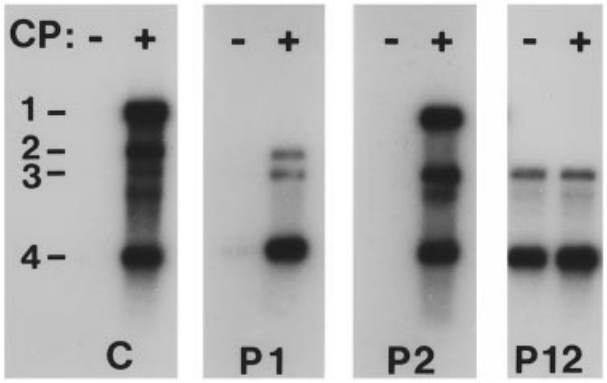

Fig. 6. Northern blot analysis of the accumulation of AMV RNAs in plants transformed with viral replicase genes. Nontransgenic plants (panel C) were inoculated with AMV RNAs 1, 2 and 3. Plants transformed with the P1 gene (panel P1) were inoculated with AMV RNAs 2 and 3. Plants transformed with the P2 gene (panel P2) were inoculated with AMV RNAs 1 and 3. Plants transformed with both the P1 and P2 genes (panel P12) were inoculated with AMV RNA 3. Inoculation was done plus or minus CP in the inoculum as indicated. The positions of AMV RNAs 1, 2, 3 and 4 are indicated.

observed although CP was neither present in the inoculum nor was it expressed from RNA 3 (van der Kuyl *et al*., 1991*a*; van der Vossen *et al*., 1994). These data demonstrate that CP is not required for minus-strand synthesis *in vitro* or *in vivo*. However, under conditions where CP was required in the inoculum to initiate RNA replication, minus-strand RNA synthesis was observed only when CP was added to the inoculum. It was proposed that this minus-strand RNA synthesis reflected a role of CP in protecting the plus-strand inoculum RNA from degradation rather than a role in the mechanism of minus-strand RNA synthesis per se (Neeleman & Bol, 1999).

Studies done *in vitro* and *in vivo* revealed an essential role of CP in AMV plus-strand RNA synthesis. In the absence of CP, the RdRp extracted from mock-infected P12 plants did not recognize AMV minus-strand RNA 3 as a template, but after addition of CP to the assay mixture plus-strand RNA 4 was synthesized (de Graaff & Jaspars, 1995). Also, when CP was added to membrane-bound AMV replication complexes that contain an endogenous minus-strand template, an increase in the accumulation of plus-strand RNA was observed. It was proposed that CP facilitates the release of plus-strand RNA from these replication complexes (Houwing & Jaspars, 1993; Houwing *et al*., 1998). *In vivo*, mutations that interfere with the expression of the CP gene in RNA 3 result in a 100-fold drop in the accumulation of plus-strand RNAs 3 and 4 in P12 protoplasts (van der Kuyl *et al*., 1991*a*; van der Vossen *et al*., 1994) and a similar reduction in the accumulation of plus-strand RNAs 1, 2 and 3 in nontransgenic protoplasts (Neeleman & Bol, 1999). Analysis of the phenotype of a number of CP mutants resulted in the conclusion that this drop in plus-strand RNA accumulation was due to a reduced synthesis of RNA rather than to enhanced degradation (van der Vossen *et al*., 1994).

Complementation studies indicated that CP is required in *trans* for the synthesis of plus-strand RNAs 1 and 2, but is required in *cis* for the synthesis of plus-strand RNA 3. When protoplasts were coinfected with a mutant with a defective P3 movement protein gene and a mutant with a defective CP gene, the wt CP expressed by the P3 mutant neither increased the low level of accumulation of RNA 3 with the defective CP gene nor did it encapsidate this RNA 3 (van der Vossen *et al*., 1996*a*; Neeleman & Bol, 1999). These data indicate that in the infected cell the replication of RNA 3, synthesis of RNA 4 and CP and encapsidation of RNA 3 are tightly coupled.

Surprisingly, complementation studies with transgenic tobacco plants that express the AMV P1 protein (P1 plants) or P2 protein (P2 plants) indicated that P1 and P2 act in *cis* in the replication of RNAs 1 and 2, respectively. The transgenic P1 supports the replication of RNAs 2 and 3 (see Fig. 6) and does not interfere with the coreplication of wt RNA 1 with RNAs 2 and 3 (Taschner *et al*., 1991). However, the transgenic P1 does not support coreplication in this system of RNA 1 which contains lethal point mutations in the P1 reading frame (van Rossum *et al*., 1996; Neeleman & Bol, 1999). Similarly, the transgenic P2 supports replication of RNAs 1 and 3 in the P2 plants (see Fig. 6), does not interfere with coreplication of wt RNA 2 with RNAs 1 and 3, but is unable to support coreplication of RNA 2 which contains lethal mutations in the P2 reading frame (van Rossum *et al*., 1996). Possibly, the *cis*-acting functions of P1 and P2 are required to ensure the assembly of a replicase activity on the inoculum RNAs before these are targeted to the chloroplast membranes where replication complexes are formed. The *cis*-acting functions of P1 and P2 may explain the evidence that RNAs 1 and 2 are unable to make use of the transgenic replicase expressed in P12 plants. Replication of RNA 3 in P12 protoplasts does not require CP in the inoculum or the presence of RNAs 1 and 2, but RNAs 1 and 2 are dependent on each other for replication in P12 protoplasts and require CP in the inoculum to initiate their replication as is the case in nontransgenic protoplasts (van Rossum *et al*. 1996; Neeleman & Bol, 1999). When P12 protoplasts are inoculated with RNAs 1, 2 and 3, CP is expressed from RNA 3 within 2 h of inoculation but this CP is unable to fulfil the early function of CP in the inoculum required for initiation of replication of RNAs 1 and 2 (van Rossum *et al*., 1996; Neeleman & Bol, 1999).

Replication of viral RNAs: role of non-coding sequences

The 3′ UTRs of AMV RNAs 1, 2 and 3 consist of a 3′-terminal sequence of 145 nt with 80% similarity in the three RNAs and upstream sequences of 18–34 nt that are unique for each RNA molecule (Fig. 2). Replacement of the full-length 3′ UTR of RNA 3 by the corresponding UTRs of RNA 1 or 2 did not affect RNA 3 replication in P12 plants, indicating that the three UTRs are functionally equivalent. A 3′-terminal sequence

of 127 nt of RNAs 1, 2 or 3 was not sufficient for template activity in an *in vitro* RdRp assay or replication *in vivo* but a 3′-terminal sequence of 145 nt permitted relatively low levels of RNA synthesis in the *in vitro* RdRp assay and low levels of replication in plants. It was concluded that the 3′ homologous sequence in the three AMV RNAs represents a core promoter for a low level of minus-strand synthesis which requires upstream sequences that are unique in the three RNAs to enhance the activity to wt levels (van Rossum *et al*., 1997*a*, *b*). Mutation of AUGC motifs 1–5 in the 3′ UTR of RNA 3 to the sequence AGGC, or mutation of AUGC motif 2 to AAAA, did not affect template activity of RNA 3 in the *in vitro* RdRp assay. Probably, the AUGC motifs are involved in CP binding but not in recognition of the minus-strand promoter by the RdRp (van Rossum *et al*., 1997*b*; Reusken *et al*., 1997). Surprisingly, mutation of several of the AUGC motifs selectively affected RNA 4 synthesis in P12 protoplasts (van der Vossen *et al*., 1994; Reusken *et al*., 1997). Particularly, mutation of AUGC motif 2 of RNA 3 into the sequence AAAA had no significant effect on minus-strand or plus-strand RNA 3 synthesis in protoplasts but strongly reduced RNA 4 synthesis (Reusken *et al*., 1997). Similarly, disruption of the stems of hairpins A or B in Fig. 2 strongly reduced RNA 4 synthesis with little effect on RNA 3 synthesis. Moreover, disruption of hairpin A strongly reduced encapsidation of RNA 3. The mutations that affect RNA 4 synthesis all interfere with CP binding to RNA fragments corresponding to the 3′ 39 nt of RNA 3, suggesting that binding of CP to this site is somehow connected to the regulation of subgenomic RNA synthesis (Reusken *et al*., 1997).

Studies on *cis*-acting sequences at the 5′ termini of AMV RNAs have focussed on RNA 3. The 5′ UTR of RNA 3 of the Madison isolate of AMV strain 425 is 240 nt long and contains three repeats of 27 nt each harbouring a motif with similarity to internal control region 2 (ICR2) recognized by DNA-dependent RNA-polymerase III. In the 5′ UTRs of RNA 3 of the Strasbourg strain of AMV, the Leiden isolate of strain 425 and strain YSMV, sequences of 56, 75 and 149 nt, respectively, have been duplicated. Exchange experiments showed that the leader sequences controlled the specific ratios in which RNAs 3 and 4 are synthesized for each strain. A 5′ sequence of 112 nt containing one ICR2 motif was sufficient for a 20-fold reduced level of RNA 3 accumulation in protoplasts and a delayed accumulation in plants compared to wt (van der Vossen *et al*., 1993). A mutational analysis of this 112 nt sequence supported a role in RNA replication of the ICR2 motif and a stem–loop structure between nt 42 and 96 carrying this motif in the loop (van der Vossen & Bol, 1996). When P12 plants are infected with 35S/cDNA 3 constructs in which the 5′ end of RNA 3 is extended with a nonviral sequence, or in which the 5′ 22 nt of RNA 3 are deleted, the infection became dependent on CP in the inoculum and the progeny consisted predominantly of a mutant with a deletion of the 5′ 79 nt of RNA 3 (van der Vossen *et al*., 1996*b*; van Rossum *et al*., 1997*c*). As infection of P12 plants with this progeny virus was no longer dependent on CP in the inoculum, it was proposed that CP was required to permit deletion of a 5′ sequence from the parental RNAs by internal initiation of the RdRp on the corresponding minus-strand RNAs. Although a deletion of the 5′ 22 nt of RNA 3 resulted in the RdRp selecting the G-residue at position 80 to start plus-strand RNA synthesis, a deletion of nt 6–22 (mutant 3D6–22) permitted initiation at G-residue 1 of RNA 3. When the 5′ sequence GUAUU of mutant 3D6–22 was changed into GCCCC or GUCCC, the RdRp again selected G-80 to initiate plus-strand RNA 3 synthesis. It was concluded that the 5′-terminal 5 nt contain a *cis*-acting element that specifically directs the RdRp to initiation at the 3′ end of a minus-strand RNA 3 template (van Rossum *et al*., 1997*c*).

In addition to *cis*-acting sequences at the 3′ and 5′ termini of AMV RNAs, sequences controlling subgenomic RNA 4 synthesis *in vitro* and *in vivo* have been analysed (van der Kuyl *et al*., 1990; van der Vossen *et al*., 1995). When the transcription start site of the subgenomic promoter is taken as $+1$, the sequence of nt $-26/+1$ was found to be sufficient for a basal level of promoter activity *in vivo*. Extension of this sequence to $-136/+12$ was required to obtain maximum levels of subgenomic promoter activity. Recently, we observed that protoplasts of P12 tobacco plants support replication of RNA 3 of the ilarvirus PNRSV (F. Aparicio, J. Sánchez-Navarro, V. Pallás & J. F. Bol, unpublished). Apparently, the AMV RdRp recognizes the promoters for minus-strand, plus-strand and subgenomic RNA synthesis of this ilarvirus.

Virus movement in plants

Mutations in the P3 gene and CP gene both affect cell-to-cell movement of AMV (van der Kuyl *et al*., 1991*b*; van der Vossen *et al*., 1994, 1995). By immunoelectron-microscopy, P3 could be localized in the middle lamella of the walls of those parenchymal or epidermal cells that had just been reached by the infection front (Stussi-Garaud *et al*., 1987). Expression of wt and mutant P3 in transgenic plants showed that a domain between aa 13 and 77 was necessary for association of P3 with cell walls (Erny *et al*., 1992*a*). Expression of the wt P3 resulted in an increase in the size-exclusion limit of transgenic plants although the increase was considerably smaller than the increase obtained with the tobacco mosaic virus movement protein (Poirson *et al*., 1993). Similar to other viral movement proteins, AMV P3 expressed in *E. coli* or yeast showed nonspecific RNA binding properties (Schoumacher *et al*., 1992*a*, *b*). A domain within aa 36–81 of P3 was shown to be necessary for RNA binding (Schoumacher *et al*., 1994). Cowpea protoplasts infected with AMV assembled P3 into long tubular structures at the surface of the protoplasts and these tubules appeared to be filled with virus particles (Kasteel *et al*., 1997). These findings suggested a mechanism of tubule-guided movement of AMV virions through modified plasmodesmata as has been described for comoviruses, nepoviruses, tospo-

viruses, caulimoviruses and geminiviruses. However, immunocytochemical and electron microscopical analysis of AMV-infected *N. benthamiana* have not revealed virion-containing tubules in plasmodesmata so far (van der Wel *et al.*, 1998). In contrast to the earlier work of Stussi-Garaud *et al.* (1987), no association of P3 with middle lamella of cell walls was observed but the movement protein could be localized in plasmodesmata at the infection front in a layer of three to four cells adjacent to uninfected cells. CP was also identified in plasmodesmata between cells in this infection zone but not in plasmodesmata from cells of fully infected tissue. In these latter cells, CP was predominantly localized in the cytoplasm. In the infection zone, two populations of plasmodesmata could be distinguished: one population with an average diameter of 19·5 nm similar to uninfected tissue, and one population with an average diameter of 36·1 nm specific to the infection zone (van der Wel *et al.*, 1998).

Deletion of the C-terminal 3 aa of P3 was reported to abolish cell-to-cell movement of AMV (van der Vossen *et al.*, 1995). A similar deletion interfered with tubule formation by a P3–GFP fusion protein transiently expressed in tobacco protoplasts (Zeng *et al.*, 1997). In addition, deletions of aa 1–77 and 84–142 were also found to affect tubule formation in this study. A mutational analysis of the CP gene revealed two CP mutants defective in cell-to-cell transport: mutant 4P (aa 85/86 of CP replaced by six nonviral amino acids) and mutant N199 (C-terminal 21 aa replaced by four nonviral amino acids). Both mutants accumulate viral RNA and CP in protoplasts but formation of stable virions was not detected. In plants, no accumulation of mutant 4P was detectable, but mutant N199 accumulated at a level 5% that of wt (van der Kuyl *et al.*, 1991*b*; van der Vossen *et al.*, 1994). Recently, it was found that deletion of the C-terminal 14 aa of CP resulted in a mutant that showed RNA 3 accumulation in plants at about 50% of the wt level but no stable virions could be isolated from the plants (F. Tenllado & J. F. Bol, unpublished). Possibly, these mutants move from cell to cell as nonvirion RNA–CP complexes or as unstable virions that are degraded upon homogenization of the cells.

Tobacco is not a host for the ilarvirus PNRSV and chimeras of AMV RNA 3 with P3 and/or CP replaced by the corresponding PNRSV genes accumulated poorly in P12 tobacco plants (Sánchez-Navarro *et al.*, 1997). However, PNRSV RNA 3 with its 5′ UTR replaced by the corresponding sequence of AMV RNA 3 was able to accumulate in P12 tobacco plants (F. Aparicio, J. Sánchez-Navarro, V. Pallás & J. F. Bol, unpublished), indicating that the PNRSV P3 and CP proteins are functional in cell-to-cell transport in this nonhost.

Models to explain early and late functions of CP

When the requirement of CP for the initiation of AMV infection was first reported, several hypotheses were put forward to explain a possible role of CP in either translation or replication of the inoculum RNAs (Bol *et al.*, 1971). The finding of specific binding sites for CP at the 3′ end of AMV RNAs led to the hypothesis that a conformational change at the 3′ ends of the genomic RNAs brought about by CP was required for recognition of the RNAs by the viral replicase (Houwing & Jaspars, 1978). However, synthesis of minus-strand AMV RNAs appeared to be possible *in vivo* and *in vitro* under conditions where no CP was present (van der Kuyl *et al.*, 1991*a*; van der Vossen *et al.*, 1994; de Graaff & Jaspars, 1995). To explain the ca. 100-fold drop in plus-strand AMV RNA synthesis observed in protoplasts when RNA 3 was omitted from the inoculum, it was proposed that binding of the RNA-3-encoded CP to the P1/P2-replicase would yield a P1/P2/CP–enzyme complex in which the recognition site for the plus-strand template would be masked, thereby directing the enzyme complex towards plus-strand synthesis (Nassuth & Bol, 1983). However, it was shown that *in vitro* CP stimulates synthesis of plus-strand RNA 4 on a minus-strand RNA 3 template in the absence of a competing plus-strand RNA template (de Graaff & Jaspars, 1995).

Although it is clear that CP has a positive regulatory role in plus-strand viral RNA synthesis, the question whether this role also explains the early function of CP in the initiation of infection remains unsolved. Recently, three new models have been proposed to explain the early function of CP in genome activation. Model 1, the 'replicase model', was proposed by Jaspars & Houwing (1990) and de Graaff & Jaspars (1995). In this model, binding of CP to each of the three genomic RNAs in the inoculum would ensure that CP is targeted to all membrane-bound replication complexes. In these replication complexes, CP would not be involved in transcription of inoculum RNAs into corresponding minus-strand RNAs but once minus-strand synthesis is complete CP would associate with the replicase to change the characteristics of the enzyme complex towards plus-strand RNA synthesis. Models 2 and 3 are both based on the assumption that CP binding sites at the 3′ termini of AMV and ilarvirus RNAs have functions similar to the tRNA-like structures at the 3′ termini of bromovirus and cucumovirus RNAs, and focus on putative functions of these tRNA-like structures in RNA replication and translation, respectively. Model 2, the 'telomere model', was proposed by Houser-Scott *et al.* (1997). These authors hypothesize that binding of CP to the 3′ end of plus-strand AMV RNAs stabilizes a conformation that positions the 3′-terminal nucleotide in the correct orientation for initiation of replication. The mechanism is analogous to the proposed role of EF-Tu and EF-Ts in immobilizing the extreme 3′ end of the Qβ genome for accurate replication. Binding of CP to AMV RNAs would serve to prevent the loss of 3′-terminal nucleotides during successive rounds of replication of the RNAs. Model 3, the 'translation model', was put forward by Neeleman *et al.* (1993). In this model it is proposed that binding of CP to the 3′ termini of AMV RNAs serves to prevent the loss of 3′-terminal

nucleotides during successive rounds of translation of the RNAs. Binding of host proteins to tRNA-like structures or poly(A) tails at the 3′ termini of other RNA viruses could confer a similar protection against 3′-terminal exonucleolytic degradation during translation of the viral RNAs.

An attractive feature of models 1 and 2 is that roles of CP in genome activation and plus-strand RNA synthesis are attributed to one single function of this protein. In model 1 (replicase model) we have to assume that there is a mechanism preventing CP in the inoculum from switching the specificity of the replicase towards plus-strand synthesis before a minus-strand template has been generated. Moreover, N-terminal peptides of 25 aa of the CP that are able to activate the genome and to bind specifically to the 3′ end of viral RNAs also should have binding specificity for the RdRp. Interestingly, trypsinized CP that lacks the N-terminal 25 aa was unable to stimulate plus-strand RNA 4 synthesis *in vitro* (de Graaff, 1995). To explain the CP-independency of infection of P12 plants by RNA 3, model 1 predicts that the relatively high level of transgenic replicase that is present at the time of inoculation results in the synthesis of abnormally high amounts of RNA 4 early in infection that obviate the need for CP in the inoculum (de Graaff & Jaspars, 1995). It has been shown that CP translated from this RNA 4 was unable to support the initiation of the replication of RNAs 1 and 2 in P12 protoplasts (Neeleman & Bol, 1999). In model 1 we have to assume that replication complexes containing RNAs 1 and 2 without CP became inactive soon after inoculation for reasons other than truncation of the 3′ termini of the RNAs predicted by models 2 and 3. The mechanism of replication of RNA 3 by the transgenic RdRp in P12 plants and by the RdRp translated from RNAs 1 and 2 in nontransgenic plants is probably the same. With model 2 (telomere model) it is difficult to explain why CP is required in the inoculum to prevent loss of 3′-terminal nucleotides during replication of RNA 3 in nontransgenic plants but not during replication in P12 plants. The presence of high levels of transgenic RdRp at the onset of RNA replication would not relieve a telomere problem. Available evidence indicates that replication of RNA 3 in P12 protoplasts starts immediately after inoculation (van Rossum *et al*., 1996). Apparently, the presence of the transgenic replicase circumvents the phase of translation of parental RNA. This was one of the reasons to suggest that in nontransgenic plants binding of CP to the 3′ termini of the viral RNAs was necessary to protect the RNAs during translation (model 3, translation model). To explain the observation that infection of nontransgenic plants by AMV cDNAs 1, 2 and 3 is only slightly dependent on CP in the inoculum, model 3 proposed that early transcripts of the transiently expressed viral cDNAs would serve as messengers for subunits of the RdRp whereas late transcripts with intact 3′ termini served as templates for this RdRp (Neeleman *et al*., 1993). However, this interpretation is difficult to reconcile with subsequent indications that P1 and P2 are required in *cis* for the replication of RNAs 1 and 2, respectively (van Rossum *et al*., 1996; Neeleman & Bol, 1999).

From a mutational analysis of the CP gene, van der Vossen *et al*. (1994) concluded that functions of CP in genome activation, plus-strand RNA synthesis, encapsidation of RNAs and cell-to-cell transport of virus could be mutated separately. The phenotype of mutant 4P showed that CP defective in virion formation and cell-to-cell transport was able to fulfil its functions in genome activation and plus-strand RNA synthesis. CP of mutant N199 was also defective in virion formation but permitted a low level of cell-to-cell movement. Recently, we obtained CP mutants defective in virion formation that spread efficiently in plants, and CP mutants that showed normal virion formation in protoplasts but did not move cell-to-cell or systemically in plants (F. Tenllado & J. F. Bol, unpublished). Together, these results indicate that different functions of CP are involved in virion formation, cell-to-cell movement and systemic spread of the virus. The conclusion that functions of CP in plus-strand RNA synthesis could be mutated separately was based on the observation that mutants 4P and N199 showed large differences in plus-strand RNA accumulation while CPs of both mutants were able to activate the genome (van der Vossen *et al*., 1994). It has been argued that the difference in phenotype of mutants 4P and N199 could reflect quantitative differences between the two mutant CPs in their function in RNA replication rather than different roles of CP in early and late steps of the replication cycle (de Graaff & Jaspars, 1995). Moreover, differences in plus-strand RNA synthesis between mutants 4P and N199 in nontransgenic protoplasts were less pronounced (Neeleman & Bol, 1999). Recently, we obtained several AMV CP mutants that showed wt levels of plus-strand RNA synthesis and virion formation in P12 protoplasts and wt levels of accumulation in P12 plants, but the CP of these mutants was unable to permit initiation of infection of nontransgenic plants with wt RNAs 1, 2 and 3 (F. Tenllado, L. Neeleman & J. F. Bol, unpublished). Probably due to a defect of CP in genome activation, the mutant RNA 3 could not coreplicate with RNAs 1 and 2 in nontransgenic plants whereas it was fully infectious to P12 plants which do not require CP for the initiation of infection. This supports the notion that different functions of CP are involved in genome activation and plus-strand RNA synthesis. The observation that several mutations in the CP binding site at the 3′ end of RNA 3 selectively affected RNA 4 synthesis may indicate that different functions of CP are involved in the synthesis of genomic and subgenomic RNAs (Reusken *et al*., 1997).

Concluding remarks

Studies on AMV and ilarviruses have revealed that the CP of these viruses is not just a structural protein but is involved in almost every step of the virus replication cycle. The observation that CP of AMV and ilarviruses can reciprocally activate the genomes of viruses within the two genera and that

protoplasts of transgenic P12 plants, which express the AMV RdRp, support replication of RNA 3 of the ilarvirus PNRSV, indicates that the replication strategy of viruses in the genera *Alfamovirus* and *Ilarvirus* is highly similar. A major difference separating the two genera is their mode of transmission in the field. AMV can be transmitted by aphids and by seed (Bol, 1999); in addition to transmission by seed, ilarviruses can be transmitted in association with thrips (Sdoodee & Teakle, 1987; Greber *et al.*, 1991*a*, *b*, 1992). Morphological differences between the bacilliform alfamoviruses and spheroidal ilarviruses may be a minor criterion to keep the two genera separate as spheroidal particles are present in AMV preparations and bacilliform particles can be found in preparations of several ilarviruses. The genome organization of the four ilarvirus genomes that have been completely sequenced to date indicates that the occurrence of a 2b open reading frame in RNA 2 may provide a new criterion for classification of viruses within the *Alfamovirus* and *Ilarvirus* genera.

Recently, chimeric constructs of AMV RNA 3 have been made that successfully express green fluorescent protein in plants (J. Sánchez-Navarro & J. F. Bol, unpublished results; V. Yusibov, personal communication). Such constructs will be of great help in analysing the mechanism of cell-to-cell movement and long distance movement of AMV. Moreover, AMV may offer a useful RNA vector system for the expression of heterologous proteins in plants. Experiments using tobacco mosaic virus-based expression vectors have shown the potential for use of AMV CP as a carrier of antigenic peptides (Yusibov *et al.*, 1997; Modelska *et al.*, 1998). Replication of AMV RNA 3-based expression vectors in transgenic P12 plants would reduce the risk of spread of the vector to other plants in the field. The use of CP mutants that replicate in P12 plants but not in nontransgenic plants would further contribute to the biological containment of AMV RNA 3-based expression vectors.

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