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# Coat Protein Activation of Alfalfa Mosaic Virus Replication Is Concentration Dependent

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**Alfalfa mosaic virus (AMV) and ilarvirus RNAs are infectious only in the presence of the viral coat protein; therefore, an understanding of coat protein's function is important for defining viral replication mechanisms. Based on in vitro replication experiments, the conformational switch model states that AMV coat protein blocks minus-strand RNA synthesis (R. C. Olsthoorn, S. Mertens, F. T. Brederode, and J. F. Bol, *EMBO J.* 18:4856–4864, 1999), while another report states that coat protein present in an inoculum is required to permit minus-strand synthesis (L. Neeleman and J. F. Bol, *Virology* 254:324–333, 1999). Here, we report on experiments that address these contrasting results with a goal of defining coat protein's function in the earliest stages of AMV replication. To detect coat-protein-activated AMV RNA replication, we designed and characterized a subgenomic luciferase reporter construct. We demonstrate that activation of viral RNA replication by coat protein is concentration dependent; that is, replication was strongly stimulated at low coat protein concentrations but decreased progressively at higher concentrations. Genomic RNA3 mutations preventing coat protein mRNA translation or disrupting coat protein's RNA binding domain diminished replication. The data indicate that RNA binding and an ongoing supply of coat protein are required to initiate replication on progeny genomic RNA transcripts. The data do not support the conformational switch model's claim that coat protein inhibits the initial stages of viral RNA replication. Replication activation may correlate with low local coat protein concentrations and low coat protein occupancy on the multiple binding sites present in the 3' untranslated regions of the viral RNAs.**

RNA-protein interactions play important roles in a variety of biological processes and are of particular importance in the life cycle of positive-strand RNA viruses, where the genomic RNA serves as the template for protein synthesis and replication. Alfalfa mosaic virus (AMV) and ilarviruses provide an interesting model system in which to study RNA-protein interactions because the viral coat protein (CP) has multiple roles in the viral life cycle, not all of which are completely understood. In addition to virion assembly (21, 44) the viral CP has roles in cell-to-cell movement (12, 43), infectivity (6, 23), and translation (28, 29).

Most bromoviruses have a tRNA-like structure (TLS) on the 3' end of the RNA genome. The TLS has been found to be important for recruiting the viral replicase (8, 13). Conversely, AMV and ilarviruses lack a canonical CCA 3' end common to the TLS and further require the presence of their own viral CP to initiate replication (6). It has been shown that CP binds specifically to the 3' untranslated region found on all three AMV genomic RNAs as well as the subgenomic RNA4 (5, 22, 24). Genomic RNA1 and -2 encode proteins with replicase functions (33). RNA3 is dicistronic, encoding the viral movement protein (MP) and CP. Only the upstream open reading frame, encoding the viral MP, is translated from genomic RNA3. The viral CP is translated from a subgenomic RNA4, which is generated by the internal initiation of transcription from minus-strand RNA3. In vitro-transcribed RNA4 (CP

mRNA) can substitute for CP in the activation of replication when inoculated with the genomic RNAs into tobacco protoplasts (46).

It was first proposed by Houwing and Jaspars that an RNA conformational change accompanies CP binding to the 3' ends of the genomic RNAs and thereby presents a recognition signal for replicase binding (20). Results from circular dichroism analyses and native polyacrylamide gel electrophoresis experiments support the idea that the 3' untranslated region (UTR) of AMV RNA3 and -4 undergoes a conformational change upon binding amino-terminal CP peptides (3). Further investigation of this RNA-protein interaction revealed a 26-amino-acid RNA binding motif in the N terminus of the CP that is sufficient to activate replication in mesophyll protoplasts (3, 39). A minimal CP binding site was also identified at the terminal 39 nucleotides of the AMV 3' UTR (1, 17). While it is known that the genomic RNAs are not infectious in the absence of CP (6, 47), it is unclear how CP initiates infection or how it influences ongoing replication.

Several models have been proposed to explain the genome activation phenomenon. The interaction of CP with the genomic RNA 3' UTR may serve to protect the RNAs from degradation (30); however, AMV genomic RNAs have been shown to be stable for several hours when inoculated into protoplasts in the absence of CP or polymerase (19). Olsthoorn et al. (31) described the conformational switch model, wherein the protein-free 3' UTR assumes a pseudoknotted conformation that directs the synthesis of AMV minus strands. Upon binding the CP, the RNA switches to an extended structure that blocks minus-strand synthesis and promotes positive-strand synthesis. In the accompanying paper (32), we describe

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experiments that evaluate the conformational switch model. The data did not support the role of a pseudoknot in regulating viral RNA synthesis, and an alternate concept, the 3' organization model, was proposed as an improved fit to the available data. Here, we use a subgenomic reporter construct to directly test the role of viral CP in initiating replication, thereby testing a key element of the conformational switch model.

The conformational switch model states that minus-strand RNA synthesis occurs only on CP-free viral RNAs. However, upon entry into a wounded cell, each of the four encapsidated viral RNAs is surrounded by dozens of CP dimers (22, 23). Following the arguments of the conformational switch model (31), this abundance of CP molecules would create an unfavorable environment for minus-strand synthesis, implicitly requiring RNA and CP to be completely dissociated and sequestered in order to permit minus-strand synthesis. To further explore the implications of the conformational switch model, we performed experiments that examined the role of CP in viral RNA replication. We reasoned that, if CP blocks minus-strand synthesis as the conformational switch model suggests, then the earliest stages of viral RNA replication should be increasingly inhibited by adding progressively larger amounts of CP or CP mRNA to an inoculum of transcribed infectious RNAs. Alternatively, if CP binding facilitates an RNA conformational change that is correlated with generating replication-competent genomic RNAs, then increasing the amount of CP or CP RNA present in an inoculum of infectious RNAs would promote viral replication.

This paper describes the first analysis of AMV RNA replication as a function of inoculum CP concentration. The results demonstrate that replication is undetectable in the absence of CP, activated at low CP concentrations, and progressively inhibited as the CP dimer:RNA concentration ratio exceeds about 5:1. Mutations in the CP mRNA coding sequence that block CP translation were found to inhibit replication, suggesting that it is the translated CP and not viral RNA4 that is required for the stimulatory effect. In addition, mutations in the CP RNA binding domain that prevent CP binding to the RNA 3' UTR were also found to reduce replication levels, suggesting that CP binding to the 3' UTR is required to maintain AMV replication. These data are evidence that CP is not strictly inhibitory to AMV minus-strand synthesis as described in the conformational switch model (31). We propose that during the earliest stages of replication, CP concentrations are low, leading to low CP occupancy on the 3' termini of the viral RNAs and the activation of viral RNA replication.

## MATERIALS AND METHODS

**Cloning of AMV genomic RNAs.** Infectious clones of AMV RNAs (Fig. 1A) were constructed as described elsewhere (26).

**Construction of RNA3 mutants.** To generate RNA3ΔMP (Fig. 1B), the MP open reading frame was deleted by digesting the RNA3 cDNA clone with XhoI and NdeI. The resulting termini were converted to blunt ends by digestion with mung bean nuclease and religated. Mutations of the RNA3ΔMP CP translational initiation codon (AUG to AGG) were introduced by PCR mutagenesis using the Quikchange site-directed mutagenesis kit (Stratagene). Arginine 17 within the CP RNA binding sequence (2) was mutated to alanine or lysine in both RNA3 and RNA3ΔMP.

**Construction of the RepLuc luciferase reporter.** The first step in constructing the luciferase reporter RepLuc was to introduce a single nucleotide deletion in RNA3ΔMP at position 1192 to create an NdeI site (Quikchange; Stratagene). The resulting plasmid was digested with NdeI and BstXI in order to remove the

CP coding sequence. The firefly luciferase coding sequence with complementary termini was amplified from pT3/T7 Luc (Promega) and ligated into the gel-purified plasmid DNA. Following luciferase DNA insertion, the NdeI site (CA TATG) was changed back to the wild-type sequence (CATCATG) and verified by nucleotide sequence analysis. Schematic representations of the constructs are presented in Fig. 1.

**RNA transcripts.** Capped runoff transcripts were transcribed from SmaI-linearized plasmids using the mMessage machine kit (Ambion, Inc, Austin, Tex.). Following incubation, RNAs were precipitated by the addition of lithium chloride according to the manufacturer's protocol.

**Labeled RNA transcripts.** Labeled transcripts were generated using the Ambion mMessage machine kit as described above with the inclusion of [ $\alpha$ - $^{32}$ P]UTP. The specific activities of all the transcripts were normalized prior to transfection into cells during viral replication assays.

**Viral replication assays.** Viral RNA replication was assayed as described previously (26). Briefly, in vitro-transcribed, capped AMV RNAs were transfected by electroporation into tobacco protoplasts prepared from *Nicotiana tabacum* NT-1 cells grown in liquid culture. Following digestion of the cell wall, 200  $\mu$ l of protoplasts ( $10^6$  cells) was mixed with 600  $\mu$ l of electroporation buffer (0.14 M NaCl, 3 mM KCl, 1.5 mM  $\text{KH}_2\text{PO}_4$ , 8 mM  $\text{NaH}_2\text{PO}_4$ , 0.4 M D-mannitol, pH 6.5) along with 3  $\mu$ g of each of the viral RNAs or variant RNA constructs. The protoplasts were electroporated at 300 V and 325  $\mu$ F and then allowed to recover on ice for 20 min. The cells were resuspended in 5 ml of protoplast medium (1 $\times$  Murashige and Skoog salt mixture, 88 mM sucrose, 0.6 mM *myo*-inositol, 1 mg of thiamine-HCl/liter, 0.2 mg of 2,4-dichloro-phenoxyacetic acid/liter, 0.4 M mannitol, pH 5.8) and incubated at 28°C for 24, 48, or 72 h.

Replication was activated by transfecting the genomic RNA1 through -3 along with RNA4-trUTR, a truncated RNA4, or purified virion CP. The reporter RepLuc RNA was added as indicated in the text and figure legends. RNA4-trUTR encodes the full-length CP but has a shortened 3' UTR (nucleotides 719 through 842 of the 3' UTR were deleted) that makes it distinguishable from newly transcribed subgenomic RNAs in Northern blot analyses (26). When virion CP was used to activate replication, the RNA transcripts and CP were prebound prior to electroporation under conditions previously identified for RNA-protein gel shift experiments (3, 17). For these experiments, the sum of genomic RNAs plus RepLuc reporter RNA totaled approximately 20 pmol. Therefore, 20 (1 $\times$ ), 100 (5 $\times$ ), 200 (10 $\times$ ), 400 (20 $\times$ ), or 2,000 (100 $\times$ ) pmol of CP dimer was added to the RNA in a 10- or 20- $\mu$ l binding reaction. The entire volume of the RNA protein solution was then electroporated into protoplast cells.

For protein analysis, 0.5 ml of protoplast suspension was centrifuged at 14,000  $\times$  g for 5 min for 24 to 72 h after transfection. After removal of the supernatant, 200  $\mu$ l of NT-1 extraction buffer (0.1 M  $\text{KH}_2\text{PO}_4$  [pH 7.8], 1 mM EDTA, 10 mM dithiothreitol, 5% glycerol) was added. The cells were then disrupted by sonication at 5 W for 3 s. The sonicated sample was then centrifuged for 10 min at 14,000  $\times$  g at 4°C to sediment cellular debris. An aliquot of the NT-1 supernatant was mixed with 5 $\times$  sodium dodecyl sulfate loading buffer, boiled, and analyzed by electrophoresis into a 12% polyacrylamide gel. Following transfer to a nitrocellulose membrane, the blot was probed for AMV CP by using a polyclonal rabbit anti-AMV CP antiserum.

Luciferase assays were performed using NT-1 cell supernatants prepared as described above from 0.5 ml of protoplast suspension. A total of 100  $\mu$ l of the NT-1 supernatant was mixed with 100  $\mu$ l of luciferase reagent (Promega) and read on a luminometer (Turner Designs).

For Northern blotting, protoplasts from 3 ml of suspension were collected at 72 h and sedimented at 14,000  $\times$  g for 5 min. Following removal of the supernatant, 1 ml of Trizol reagent (Ambion Inc.) was used according to the manufacturer's directions in order to isolate the RNA. Approximately 10  $\mu$ g of total RNA was run into each lane of a formaldehyde gel. Following transfer to a nitrocellulose membrane, the blot was probed with a double-stranded DNA probe corresponding to the CP coding region. The use of the double-stranded DNA probe provided the capability of detecting minus and plus strands of RNA3 along with the plus-strand RNA4.

**Virion CP.** CP prepared from AMV virions (25) was a gift from Ed Halk. Mass spectrometry analysis demonstrated the absence of contaminating proteins or nucleic acids in this preparation.

## RESULTS

**Development of a luciferase reporter system.** At the start of this work, we set out to design a reporter construct that would make it possible to follow AMV replication by using an assay distinct from CP or viral RNA accumulation. The accumula-

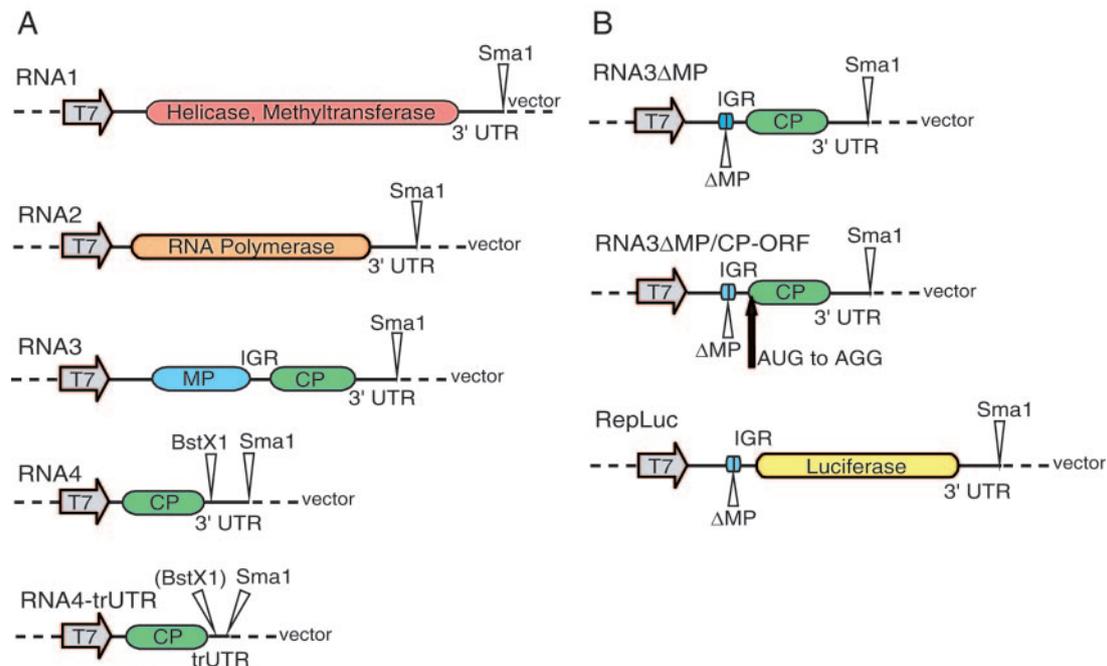


FIG. 1. Constructs used in in vivo protoplast replication assays. (A) cDNA clones of RNA1 to -4 are templates for the production of wild-type infectious RNAs by runoff transcription. RNA4-trUTR has a shortened 3' UTR wherein nucleotides 719 through 842 were deleted. (B) Luciferase reporter constructs are shown. In RNA3 $\Delta$ MP, the majority of the MP open reading frame was deleted. RNA3 $\Delta$ MP/CP-ORF is identical to RNA3 $\Delta$ MP with the exception that the translation initiation codon for the viral CP was disrupted by a point mutation changing the AUG to AGG. RepLuc was generated from RNA3 $\Delta$ MP and contained the luciferase coding sequence in place of CP. IGR, intergenic region.

tion of viral CP is a useful measure of viral RNA replication; however, quantifying AMV CP expression is complicated in experiments where CP rather than RNA4 (i.e., the CP mRNA) is added to activate replication. In other words, CP present in the inoculum cannot be distinguished without labeling techniques from newly synthesized CP generated through viral RNA replication and transcription. A reporter approach had the potential added benefit of providing greater sensitivity and range than CP detection by Western blotting, in addition to faster data collection. The reporter construct RepLuc was designed to report subgenomic RNA production. By this approach, luciferase protein is produced only as a result of viral RNA replication. To generate the reporter, most of the MP was deleted from RNA3 to create construct RNA3 $\Delta$ MP. RepLuc was then constructed by replacing the CP coding region of RNA3 $\Delta$ MP with the firefly luciferase coding region. In the RNA3 $\Delta$ MP and RepLuc constructs, the CP and luciferase coding regions, respectively, were preceded by a small region of the MP open reading frame that included the translational start and stop codons (Fig. 1B) immediately upstream of the intergenic region. For both constructs, ribosomes scanning from the 5' end of the input RNA are predicted to initiate on the MP AUG codon and to terminate on the in-frame MP translational stop codon, dissociating before reaching the downstream AUG preceding the CP or luciferase coding region. CP and luciferase protein were therefore translated only from transcribed subgenomic RNAs generated during replication and not as a result of ribosome scanning from the 5' end of the input RNA.

The RepLuc reporter RNA was tested by transfecting

RNA1, -2, and -3 transcripts along with RepLuc RNA and a modified RNA4 construct, RNA4-trUTR, wherein nucleotides 719 through 842 of the 3' UTR were deleted (26, 35). By using the truncated RNA4 to activate replication, the detection of subgenomic RNA4 by Northern blot analysis is clear evidence of viral RNA replication because the slower-migrating subgenomic RNA4 is produced only by transcribing the minus-strand RNA3 transcript. Viral CP is detected by Western blotting only if replication is ongoing; that is, CP translated from input RNA4 is not detected (26, 35) (see Fig. 4C and D, lanes 2). Routine use of the RNA4-trUTR RNA in translation and viral RNA replication experiments reveals that its activity is approximately 80% that of the full-length viral RNA4 (G. W. Martin, S.C. Low, and L. Gehrke, manuscript in preparation).

The combination of RNA1, -2, and -3; RNA4-trUTR; and RepLuc in the transfection mixture yielded viral RNA replication as evidenced by the production of viral CP (Fig. 2A, group 1). However, corresponding luciferase activity was not detected (Fig. 2B, group 1). Others have reported related technical problems when using AMV RNA3 with reporters (36); therefore, we asked if both CP and luciferase activities were detected if RNA3 $\Delta$ MP was used in place of transfected RNA3. The data (Fig. 2A and B, groups 2) showed that when RNA1 and -2, RNA3 $\Delta$ MP, RNA4-trUTR, and RepLuc were cotransfected, both CP and luciferase activity were readily detected. While the combination of RNA3 and the RepLuc reporter did not block viral RNA replication (Fig. 2C, group 1), the absence of luciferase activity (Fig. 2B, group 1) suggested a potential antagonism between RNA3 or MP and the luciferase coding region or luciferase protein.

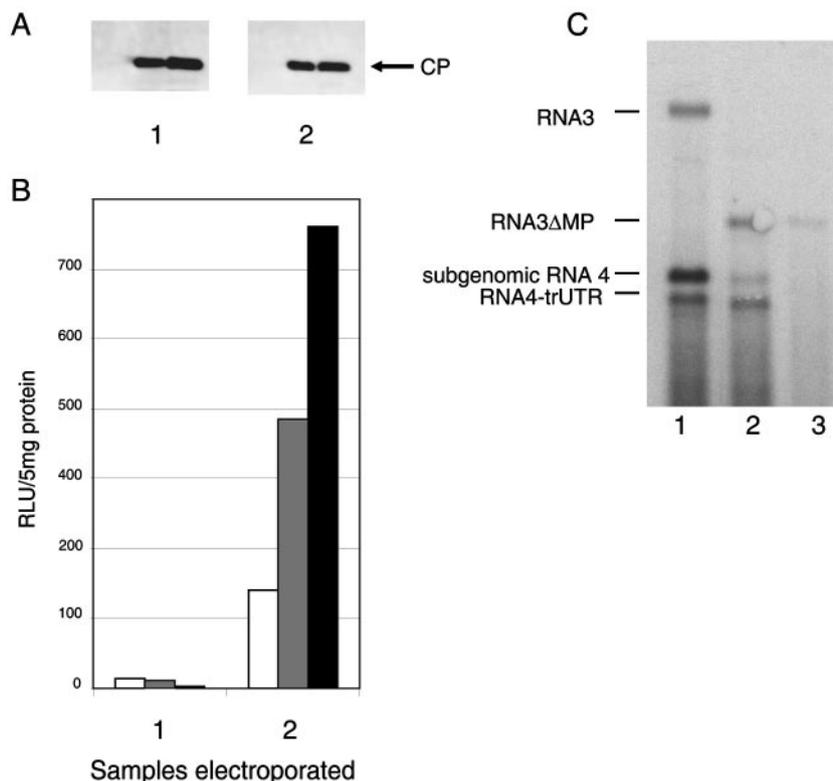


FIG. 2. Luciferase activity reflects CP expression and subgenomic RNA production. (A) Western blot analysis shows CP levels in transfected cells. Group 1, cells were transfected with a mixture of RNA1, -2, -3, RNA4-trUTR, and RepLuc RNA. The three lanes represent CP present in cells harvested at 24 h (left), 48 h (center), or 72 h (right). Group 2, same as group 1 except that RNA3 was replaced with RNA3ΔMP. (B) Luciferase activity is expressed as relative light units (RLU) per 5 μg of supernatant protein. Group 1, cells were transfected with genomic RNA1 through -3, RNA4-trUTR, and RepLuc reporter RNA. Group 2, cells were transfected with RNA1 and -2, RNA3ΔMP, RNA4-trUTR, and the RepLuc reporter. This experiment was repeated three times, and a representative experiment is shown. Time points were collected at 24 h (white bars), 48 h (grey bars), and 72 h (black bars). (C) Northern blot analysis shows RNA3ΔMP replication and subgenomic RNA expression. Lane 1, cells were transfected with genomic RNA1 through -3 plus RNA4-trUTR; lane 2, cells were transfected with RNA1 and -2, RNA3ΔMP, and RNA4-trUTR; lane 3, cells were transfected with RNA1 and -2 and RNA3ΔMP without added RNA4-trUTR.

Replication of the RNA3ΔMP construct was further characterized by Northern hybridization. Subgenomic RNA was detected when both genomic RNA3 (Fig. 2C, lane 1) and the RNA3ΔMP construct (Fig. 2C, lane 2) were coelectroporated with RNA1 and -2 and RNA4-trUTR. To demonstrate that viral RNA replication is dependent on CP, genomic RNA1 and -2 and RNA3ΔMP only were introduced into the cells. In this case, no evidence of subgenomic RNA expression was observed (Fig. 2C, lane 3). The RNA3ΔMP band in lane 3 represented input RNA. Taken together, the data (Fig. 2) demonstrated that luciferase expression from RepLuc mirrors the production of subgenomic RNA and viral CP when the inoculum includes RNA1 and -2, RNA3ΔMP, RNA4-trUTR, and RepLuc.

**Reporter activity and CP accumulation are stimulated by input CP or CP mRNA.** In order to examine the proposed inhibitory effects of CP on viral RNA replication (11, 18, 31), we incubated genomic RNA1 and -2 plus RNA3ΔMP and the RepLuc reporter RNAs with CP prior to transfection into tobacco protoplasts. For these experiments, 3 μg of each RNA was used, for a total of 12 μg or approximately 20 pmol of RNA per transfection. These RNAs were then incubated with increasing amounts of viral CP prior to electroporation into

protoplasts. We incubated the RNA and CP under the same conditions used for protein binding in electrophoretic mobility shift assays (26). The data presented in Fig. 3A indicate that luciferase activity does not exceed background levels in the absence of CP (group 1) and that the activity increases as the molar excess of CP to RNA in the inoculum is increased from equimolar to a fivefold molar excess (groups 2 and 3). At higher CP concentrations, this stimulation decreases (groups 4 and 5), and finally, when a 100-fold molar excess of CP is added, luciferase activity is inhibited and drops to background levels (group 6). Control experiments demonstrated that transfection of the RepLuc RNA alone yields the predicted low activity levels (group 7).

We considered the possibility that the diminished luciferase activities (Fig. 3A, groups 4 through 6) might result if the addition of CP decreased electroporation efficiencies. To test this possibility, we prepared radiolabeled RNA transcripts and transfected them into cells by electroporation. Cellular RNA was extracted, denatured, and analyzed by electrophoresis. The data (Fig. 3B) demonstrated that the amounts of intracellular viral RNA transcripts do not decrease as the CP levels rise; rather, there is an increase. To show that the RNAs were intracellular rather than adsorbed to the cell exterior, two

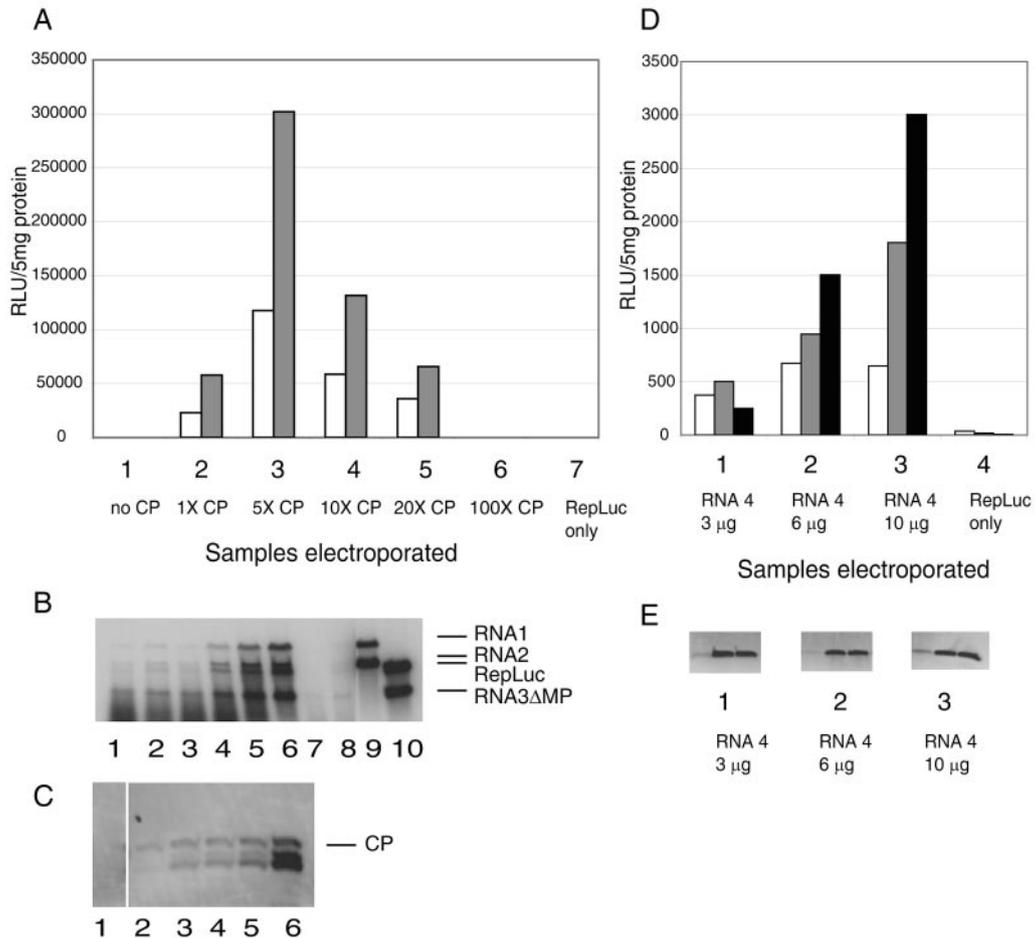


FIG. 3. CP activation of replication is concentration dependent. (A) Protoplasts were electroporated with infectious transcripts of AMV viral RNA1 and -2, RNA3ΔMP, and RepLuc. For those samples that included CP, RNA and CP were incubated prior to electroporation as described in Materials and Methods. Group 7 received only RepLuc RNA. Further additions of CP to the inoculum are indicated on the abscissa. Aliquots were removed for determination of luciferase activity at 24 h (white bars) and 48 h (gray bars). This experiment was repeated three times, and a representative experiment is shown. RLU, relative light units. (B) Protoplasts were electroporated with radiolabeled RNAs. Lanes 1 through 6 received RNA1 and -2, RNA3ΔMP, and RepLuc and increasing amounts of CP from 0× to 100× as indicated in groups 1 through 6 of panel A. Lanes 7 and 8 are identical to lanes 1 and 3 except that these samples were mock-electroporated. Lanes 9 and 10 are the transcribed RNAs, used here as mobility markers. (C) Western blot analysis shows intracellular CP levels in transfected protoplasts. The protocol for these samples was identical to that shown in panel A for groups 1 through 6 except that aliquots of the cells were removed at 24 h and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting using an anti-CP antibody. The numbering of the lanes is the same as for groups 1 through 6 of panel A. The position of CP is indicated. The smaller bands likely resulted from proteolysis at the trypsin-sensitive site. As an indication of the amounts of CP in the cells, the volume of cell extract analyzed was about 1/20 the amount analyzed in panel E. (D) Replication is activated by RNA4 in place of viral CP. Protoplasts in groups 1 through 3 were electroporated with infectious transcripts of AMV viral RNA1 and -2, RNA3ΔMP, and RepLuc, along with increasing amounts of RNA4, as indicated on the abscissa. Group 4 cells received the RepLuc reporter only. This experiment was repeated three times, and a representative experiment is shown. Luciferase activity was evaluated at three time points, 24 h (white bars), 48 h (gray bars), and 72 h (black bars) posttransfection. (E) Western blot for CP is shown. Parallel samples from panel C, above, were analyzed by Western blotting with anti-CP antibody.

samples were mock electroporated. The data (Fig. 3B, lanes 7 and 8) show that the amounts of RNA entering the cells without electroporation were nearly undetectable. As a further test, we also used immunoblotting to analyze the amounts of CP that entered the cells. The results of this experiment (Fig. 3C) were consistent with the radiolabeled RNA data (Fig. 3B) in demonstrating that intracellular CP levels also increase. These results strongly suggest that the diminished luciferase activity cannot be explained by a CP-mediated decrease in electroporation efficiency. The data were consistent with the interpreta-

tion that CP stimulates replication at low concentrations while inhibiting replication at high concentrations.

To further compare input RNA4 and input CP as initiators of replication, we performed experiments using increasing amounts of RNA4. For these experiments, 3, 6, or 10 µg of RNA4 was added to the RNA inoculum prior to protoplast electroporation (Fig. 3D). RepLuc RNA was electroporated alone as a control (group 4), yielding background levels of luciferase activity. Western blots showing the accumulation of viral CP during infection are shown in Fig. 3E. Although the

Western blot data for the 48- and 72-h time points appeared visually similar (Fig. 3E), the luciferase data (Fig. 3D) provided a rapid assay of replication that revealed quantitative differences. The data presented in Fig. 3D and E indicated that increasing amounts of input CP mRNA (RNA4) correlate with increased levels of CP and luciferase enzyme expression. The largest amount of RNA4 used in these experiments, 10  $\mu$ g, represented an approximately 3.5-fold molar excess over the sum of the other RNAs. The data presented in Fig. 3A demonstrated that viral RNA replication is stimulated by the addition of increasing amounts of viral CP up to a fivefold molar excess, while higher concentrations of CP are increasingly inhibitory. Increasing amounts of transfected RNA4 stimulated the accumulation of CP and luciferase activity (Fig. 3D and E). Technical and practical limitations of *in vitro* RNA synthesis (e.g., transcribing and electroporating hundreds of micrograms) prevented testing a broader range of added RNA4. Although published *in vitro* replication experiments suggest that CP inhibits minus-strand synthesis (11, 18), the data presented in Fig. 3 suggest that the important parameter to consider is the concentration of CP relative to that of AMV RNA.

To extend the study, we designed experiments to differentiate between an initial, one-time requirement for CP to activate initial replication and an ongoing requirement for CP expression throughout replication. To test the requirements for CP at the initiation of replication, we designed an RNA3 $\Delta$ MP variant, RNA3 $\Delta$ MP/CP-ORF (Fig. 1B), where the initiation codon of the CP open reading frame was changed from AUG to AGG. Lacking a functional initiation codon, subgenomic RNA4 transcribed from this construct was not translatable to generate CP, leaving input CP as the sole source for the infection. For these experiments, RNA4-trUTR was included in the inoculum to activate replication. The subgenomic RNA4-trUTR does not replicate but provides an initial, limited source of translated CP, thereby allowing us to test whether this initial amount was sufficient to maintain viral replication over the course of the 72-h incubation. *In vitro*-transcribed genomic RNAs were synthesized and transfected into protoplast cells by electroporation in order to assay viral RNA replication.

The controls for the replication experiment (Fig. 4A and B, lanes 1) showed that both subgenomic RNA4 and viral CP were detected when the cells received genomic RNA1 and -2 in addition to RNA3 $\Delta$ MP and RNA4-trUTR. However, neither subgenomic RNA nor CP synthesis was observed when cells were transfected with RNA3 $\Delta$ MP/CP-ORF (Fig. 4A and B, lanes 2). These results suggested that the input CP RNA alone is insufficient to maintain replication in the absence of a CP translated from viral RNAs generated during replication. This experiment also strongly suggested that the viral CP, and not the CP RNA, is required to promote ongoing replication. These results support a model in which an ongoing source of CP is needed to sustain viral RNA replication. In a related study, van der Vossen et al. (43) reported previously that a mutation in the CP initiation codon significantly reduces RNA3 replication.

In a further experiment, we asked if this requirement for CP requires an interaction between the CP and the viral RNA. CP binds specifically to the 3' untranslated region found on all three genomic RNAs as well as the subgenomic RNA4 (24). Previously, we described an RNA binding motif (PTxR<sub>17</sub>SxxY)

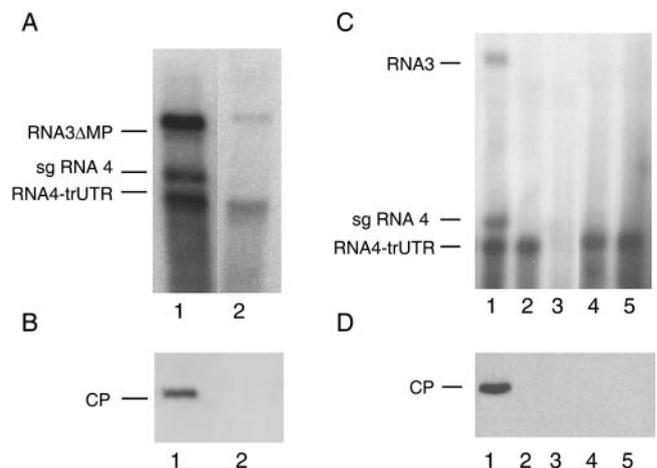


FIG. 4. Mutation of the CP open reading frame or the CP RNA binding domain decreases replication. (A) Northern blot analysis showing the accumulation of RNA3 and -4, sg, subgenomic. Lane 1, cells were transfected with RNA1 and -2, RNA3 $\Delta$ MP, and RNA4-trUTR; lane 2, cells were transfected with RNA1 and -2, RNA3 $\Delta$ MP/CP-ORF, and RNA4-trUTR. (B) Western blot corresponding to the Northern blot in panel A showing the accumulation of CP. (C) Northern blot showing the accumulation of RNA3 and -4. Lane 1, cells were transfected with wild-type RNA1, -2, and -3 and RNA4-trUTR; lane 2, cells were transfected with RNA4-trUTR only; lane 3, cells were transfected with RNA1 through -3 only; lane 4, cells were transfected with RNA1 and -2, RNA4-trUTR, and RNA3 containing the R<sub>17</sub>-to-lysine mutation in the CP open reading frame (RNA3 $\Delta$ MP/CP-R17K); lane 5, cells were transfected with RNA1 and -2, RNA4-trUTR, and RNA3 containing the R<sub>17</sub>-to-alanine mutation (RNA3 $\Delta$ MP/CP-R17A). (D) Western blot corresponding to the Northern blot in panel C showing the accumulation of CP.

within the N terminus of the viral CP (2). The central role of this arginine for RNA binding has been demonstrated both biochemically (2) and structurally (15). Substituting lysine or alanine for R<sub>17</sub> prevents RNA-peptide binding interactions (2). To determine if mutations at R<sub>17</sub> affected CP and RNA3 expression in our system, we substituted alanine or lysine codon sequences for R<sub>17</sub> in the context of genomic RNA3. These construct RNAs were transfected into tobacco protoplasts with genomic RNA1 and -2 and RNA4-trUTR. The Northern blot and corresponding Western blot demonstrated that viral RNA replication is not detected when the R17A (Fig. 4C and D, lanes 4) or R17K (Fig. 4C and D, lanes 5) substitutions are present in the CP open reading frame of genomic RNA3. The controls for this experiment revealed that subgenomic RNA4 and viral CP were detected when an inoculum of wild-type genomic RNA1 through -3 plus RNA4-trUTR was transfected into protoplasts (Fig. 4C and D, lanes 1) but not when the cells received RNA4-trUTR only (Fig. 4C and D, lanes 2) or genomic RNA1 through -3 only (Fig. 4C and D, lanes 3). The absence of the RNA3 band in Fig. 4C, lane 3, suggests that without viral RNA replication, the input RNA3 degrades by 48 h after transfection, while the input RNA4-trUTR exhibits greater stability (Fig. 4C, lane 2). These data suggest that CP molecules with an intact RNA binding domain must be present in the inoculum and also expressed in an ongoing manner during replication in order to maintain viral RNA replication.

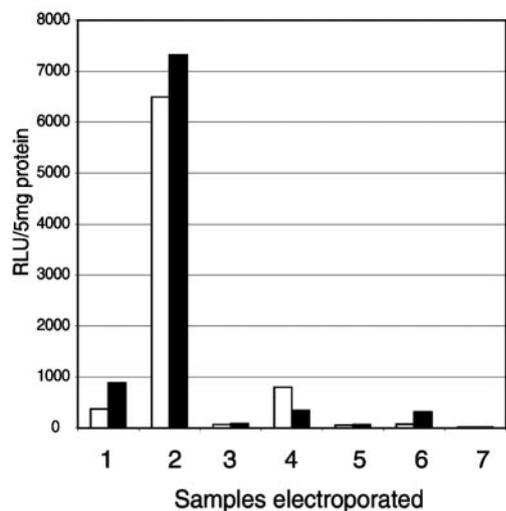


FIG. 5. Increased amounts of input RNA4 stimulate replication but do not rescue RNA3 mutants. Group 1 cells were electroporated with RNA1 and -2, RNA3 $\Delta$ MP, RepLuc, and 3  $\mu$ g of RNA4. Group 2 was identical to group 1 but included 10  $\mu$ g of input RNA4. Group 3 cells were electroporated with RNA1 and -2, RNA3 $\Delta$ MP/CP-ORF, RepLuc, and 3  $\mu$ g of RNA4. Group 4 was identical to group 3 but included 10  $\mu$ g of input RNA4. Group 5 cells were electroporated with RNA1 and -2, RNA3 $\Delta$ MP/CP-R17A, RepLuc, and 3  $\mu$ g of RNA4. Group 6 was identical to group 5 but included 10  $\mu$ g of input RNA4. Group 7 cells received the RepLuc reporter only. Cells were collected at 30 h (white bars) and 45 h (black bars) posttransfection for analysis of luciferase activity levels. RLU, relative light units.

We next asked if increasing the amount of transfected CP RNA4 would maintain replication in the absence of ongoing expression of functional CP. RNA1, -2, and -4 and RepLuc were electroporated along with either RNA3 $\Delta$ MP or variant RNA3 $\Delta$ MP constructs. Construct (RNA3 $\Delta$ MP/CP-ORF) produces subgenomic RNA4 that is defective in translating viral CP because the AUG initiation codon is changed to AGG, while construct (RNA3 $\Delta$ MP/CP-R17A) generates subgenomic RNA4 whose CP translation product is defective in RNA binding because the critical R<sub>17</sub> is changed to alanine. For these experiments, both 3 and 10  $\mu$ g of RNA4 were cotransfected with RNA1 and -2, RepLuc, and the RNA3 $\Delta$ MP constructs. Luciferase activity was measured at 30 and 45 h posttransfection. The control for this experiment (Fig. 5, groups 1 and 2) showed that when RNA1, -2, and -4 and RNA3 $\Delta$ MP were cotransfected, luciferase activity increased as a function of input RNA4 levels (Fig. 5, compare groups 1 and 2). However, when RNA3 $\Delta$ MP/CP-ORF was used in the place of RNA3 $\Delta$ MP (Fig. 5, groups 3 and 4), luciferase activity was low, irrespective of the amounts of input RNA4 (Fig. 5, groups 3 and 4). This result is consistent with the Northern and Western blot results presented in Fig. 4. Similarly, mutation of R<sub>17</sub> to alanine within the CP N terminus also resulted in greatly diminished luciferase activity; moreover, the increased amount of input RNA4 did not stimulate luciferase activity levels (Fig. 5, groups 5 and 6). We were able to detect a reproducible slight increase in luciferase activity at 30 h posttransfection when 10  $\mu$ g of RNA4 was cotransfected with RNA3 $\Delta$ MP/CP-ORF RNA (Fig. 5, group 4), but similar results were not observed when 10  $\mu$ g of RNA4 was included with the RNA3 $\Delta$ MP/CP-

R17A RNA (Fig. 5, group 6). One possible explanation for the lack of early stimulation in the latter experiment is that the defective R17A CP translated from newly transcribed subgenomic RNA acts as a dominant negative by forming inactive CP heterodimers with wild-type CP translated from the input RNA4. Taken together, the results (Fig. 5) strongly indicated that an ongoing supply of functional CP translated from transcribed subgenomic RNA4 is required to maintain AMV replication.

## DISCUSSION

The results presented here demonstrate that CP-mediated activation of AMV replication is concentration dependent. These data argue against the conclusion that CP binding to the viral RNAs is exclusively inhibitory to replication (31). Viral RNA replication, scored by the appearance of luciferase activity, was undetectable in the absence of CP, strongly stimulated at CP concentrations less than or equal to an approximately fivefold molar excess, and progressively inhibited as CP concentrations increased above a fivefold molar excess. The results also suggest that CP containing a functional RNA binding domain must be present to stimulate replication. Mutations of the critical R17 residue of the RNA binding domain or mutation of the CP translational initiation codon in genomic RNA3 severely reduced the levels of viral RNA and CP produced in protoplast transfection experiments (Fig. 4) (27). The ongoing expression of functional CP cannot be replaced by large amounts of a nonreplicating CP source (RNA4) in the inoculum (Fig. 5). The data indicate that genome activation is continuous during the infection and not restricted to the earliest phase.

The conformational switch model (31) states that minus-strand AMV RNA synthesis requires the viral RNAs to be free of CP and in a 3' pseudoknotted conformation. Then, upon CP binding, the RNA switches from the pseudoknot to an extended conformation that blocks minus-strand synthesis and favors positive-strand RNA synthesis. Our data are not consistent with this model for two reasons. First, the addition of CP to the inoculum, using concentrations less than a 10-fold molar excess under binding conditions known to convert the viral RNA to RNA-CP complexes, did not block viral RNA replication (Fig. 3). An alternate interpretation for the results would be that CP is somehow completely stripped from the RNAs either during the electroporation or after cellular entry, thereby generating CP-free pseudoknotted RNA for replication. We are not aware of evidence for sequestering the viral RNA from CP to permit minus-strand synthesis. Furthermore, if CP were removed or sequestered upon entry, it does not follow that increasing the amounts of CP in the inoculum up to a fivefold molar excess would correlate directly with enhanced replication, as demonstrated in our experiments. Second, by the criteria of the conformational switch model, the predicted outcome of the experiment shown in Fig. 4A would be the accumulation of minus-strand viral RNA, which was not observed. Here, the translational initiation codon for CP expression was ablated in genomic RNA3, preventing the expression of CP that would make the switch from minus- to plus-strand synthesis. Although RNA4 was added to the inoculum (providing a source of CP to stimulate RNA1 and RNA2 transla-

tion according to the model), hybridization with the double-stranded DNA probe did not reveal genomic RNA3 or subgenomic RNA4 expression (Fig. 4A, lane 2).

Enhanced translation mediated by CP binding to the viral RNAs (29) may contribute to the stimulation of replication observed in our experiments; however, we argue that enhanced translation alone cannot explain CP's role in the initiation of early replication in transfected cells. If the CP or CP mRNAs supplied in our inocula augmented translation, the predicted outcome would be to stimulate CP mRNA translation, thereby producing additional CP molecules that would, according to the conformational switch model, inhibit minus-strand synthesis. The data presented here suggest that CP stimulates the early stages of viral RNA replication.

The literature detailing CP's role(s) in viral RNA replication describes a range of conclusions. The use of *in vitro* assays to analyze CP's role in replication is complicated by the presence of copurified CP in the replicase enzyme preparations (33). Replicase preparations that lack CP show only a few percentage points of the activity observed in the presence of CP (21), consistent with our finding that replication activity is stimulated in the presence of CP. However, other *in vitro* studies indicate that replication is inhibited by the presence of additional CP (11, 31). How can the two data sets be reconciled? One potential explanation is to consider that AMV RNAs have multiple CP binding sites (17, 34) and that CP occupancy of these sites may influence RNA function. A prediction of this concept is that replication might be favored on a viral RNA having only one or two CP binding sites occupied, while assembly is favored when additional sites are occupied. By band-shift analysis, a fivefold excess of CP converts all free RNA into RNA-protein complexes; however, the presence of multiple shifted bands (RNA-CP complexes) suggests that not all of the available binding sites are completely filled (17). The presence of higher levels of CP may saturate the available CP binding sites and promote particle assembly, leading to diminished replication potential. Another interpretation consistent with our results is that the presence of high concentrations of CP prevents disassembly of the viral RNA-CP complexes for translation and replication functions.

The work reported in this paper is the first to examine intracellular AMV replication as a function of CP concentrations. DeGraaff et al. performed *in vitro* experiments with purified replicase by using a 115-fold excess of CP over RNA (11). The conclusion of those experiments was that CP is inhibitory to viral RNA replication. The use of such a large excess of CP may create a competitive environment wherein RNA binding to free excess CP is favored over RNA binding to replicase-associated CP, thereby diminishing replication levels. DeGraaff et al. suggested that a CP titration experiment *in vitro* might reveal that CP is not inhibitory under all conditions; however, an experiment using lower concentrations of CP has not been reported prior to our work.

The advantages and disadvantages of using transgenic plants and their protoplasts should also be weighed. Transgenic plants expressing the AMV polymerase proteins P1 and P2 have been important tools for exploring AMV replication mechanisms (40). However, unlike the state of native AMV infections, CP is not required for replication of AMV RNA3 in transgenic (P12) plants and protoplasts that constitutively ex-

press the replicase proteins (40, 42). It seems, therefore, that the replication environment in the transgenic plants is distinct from that of native plants, and these differences may influence experimental results and their interpretations. Schwartz et al. (37) recently reported that constitutive expression of brome mosaic virus (BMV) replicase proteins causes significant rearrangements of cellular membranes, which are the sites of viral RNA replication. In related data, Smirnyagina et al. reported that N-terminal sequences of the BMV 2a polymerase protein are dispensable for BMV RNA replication when expressed from DNA plasmids (38). These results contrast with the findings from using native infections, wherein the N-terminal 120 amino acids of the 2a protein were found to be necessary and sufficient for localization to the endoplasmic reticulum membrane as mediated by interactions with the 1a protein. A significant difference in the environments for these two experiments is that, in a native infection, the replicase protein RNAs are introduced at low multiplicity per cell, leading to low initial concentrations of replicase proteins. Chen and Ahlquist noted that protein-protein interactions formed in the native state and that of high-level constitutive transgenic expression may differ, leading to altered functional outcomes (9). With respect to the subject of this paper, most of the prior published conclusions about CP's inhibitory effects on viral RNA replication are based on *in vitro* replication assays using replicase preparations that included copurified CP or on the use of transgenic plants or protoplasts that may not have recapitulated the native environment. The experiments reported here, based on RNA transfections into nontransgenic cells, suggest that CP does not, by definition, inhibit minus-strand synthesis.

The viral capsid protein of another positive-strand RNA virus, rubella virus, has been found to regulate viral RNA replication in a concentration-dependent manner. Chen et al. identified stably transformed cell lines that express the rubella capsid protein at high and low levels (10, 41). The replication data demonstrated that virus infectivity was stimulated substantially in cells expressing low concentrations of capsid protein while being inhibited in cells expressing higher capsid protein levels. We propose that at low concentrations, the capsid protein may stabilize the viral genome, participate in protein-protein interactions that enhance replication, or help localize the RNA for replication (10). Alternatively, at high concentrations, replication may be limited because the viral RNAs become sequestered in the assembly pathway (10). Taken together, effects of rubella virus capsid protein on viral RNA replication bear similarities to those reported here for AMV.

What is CP's early role in AMV RNA replication? We demonstrated that the 3' untranslated region of viral RNA3 or RNA4 undergoes a conformational change upon binding peptides representing the RNA binding domain of the viral CP (3, 15). This conformational change converts the RNA to a more compact form in a manner similar to that described for folding active catalytic RNAs (7, 45) or rRNA-protein complexes (4). It has been proposed that CP-free AMV RNAs fold into a TLS (31); however, AMV and ilarvirus RNAs lack key structural and functional determinants of TLS, including the canonical CCA 3' terminus, the ability to be charged by an aminoacyl synthetase, and activity as a substrate for nucleotidyltransferase (discussed in the accompanying paper [32] and refer-

ences 14, 16, and 31). The RNAs of a closely related bromovirus, BMV, have a tRNA-like 3' end, the functionality of which is evident in that BMV RNAs can be aminoacylated. In comparing AMV and BMV, we noted that BMV infectious RNAs do not require CP to replicate, while AMV and ilarviruses, on the other hand, lack key features of the tRNA-like end and also require the presence of CP to replicate. We propose, therefore, that a structural feature common to AMV and BMV replication is the requirement for an organized 3' RNA terminus. In the case of BMV, the organization is provided by the presence of the tRNA-like end. We propose that the organization of the AMV and ilarvirus 3' termini is accomplished by RNA-CP binding. Further evidence for the organization of the AMV RNAs is found in the co-crystal structure of a 39-nucleotide 3'-terminal AMV RNA bound to the N terminus of the viral CP (15).

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