Viral Coat Protein Peptides with Limited Sequence Homology Bind Similar Domains of Alfalfa Mosaic Virus and Tobacco Streak Virus RNAs

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An unusual and distinguishing feature of alfalfa mosaic virus (AMV) and ilarviruses such as tobacco streak virus (TSV) is that the viral coat protein is required to activate the early stages of viral RNA replication, a phenomenon known as genome activation. AMV-TSV coat protein homology is limited; however, they are functionally interchangeable in activating virus replication. For example, TSV coat protein will activate AMV RNA replication and vice versa. Although AMV and TSV coat proteins have little obvious amino acid homology, we recently reported that they share an N-terminal RNA binding consensus sequence (Ansel-McKinney et al., EMBO J. 15:5077–5084, 1996). Here, we biochemically compare the binding of chemically synthesized peptides that include the consensus RNA binding sequence and lysine-rich (AMV) or arginine-rich (TSV) environment to 3′-terminal TSV and AMV RNA fragments. The arginine-rich TSV coat protein peptide binds viral RNA with lower affinity than the lysine-rich AMV coat protein peptides; however, the ribose moieties protected from hydroxyl radical attack by the two different peptides are localized in the same area of the predicted RNA structures. When included in an infectious inoculum, both AMV and TSV 3′-terminal RNA fragments inhibited AMV RNA replication, while variant RNAs unable to bind coat protein did not affect replication significantly. The data suggest that RNA binding and genome activation functions may reside in the consensus RNA binding sequence that is apparently unique to AMV and ilarvirus coat proteins.

The coat proteins of alfalfa mosaic virus (AMV) and the ilarviruses (type member, tobacco streak virus) bind to the 3′ termini of the genomic viral RNAs (23, 28, 29). The 3′-terminal coat protein binding sites of these positive-sense single-stranded viral RNAs are related both by predicted secondary structure and by the presence of tetranucleotide AUGC repeats (28, 57, 58). A defining feature of AMV and ilarviruses is that the genomic RNAs are not infectious; rather, infection is dependent upon a combination of genomic RNAs plus a few molecules of coat protein to initiate the early stages of virus replication (9, 50). The molecular basis for the coat protein requirement in viral RNA replication has not been elucidated. Although AMV and ilarvirus coat proteins specifically bind the 3′ end of the viral RNAs, it is not clear if genome activation directly involves a viral RNA-coat protein complex or if the RNA binding and genome activation functions are separable. Removal of the N-terminal RNA binding domain of AMV coat protein by mild trypsin treatment inactivates both RNA binding in vitro and functional activity in initiating virus replication (8, 56). Furthermore, by introducing amino acid substitutions into the RNA binding domain of full-length AMV coat protein, Yusibov and Loesch-Fries showed that disrupting RNA binding correlates with diminished viral RNA replication (55). These data are consistent with a hypothesis stating that a viral RNA-coat protein complex is associated with initiation of viral RNA replication (23); however, other interpretations have not been ruled out.

The unusual structure of the AMV and ilarvirus RNA 3′ termini help explain why coat protein is required for replication. Many plant viral RNAs have 3′-terminal pseudoknots (35) and/or tRNA-like structures that can be aminoacylated, and roles in replication have been proposed for both (16, 17, 20). Maizels and Weiner argue that tRNA evolved in the RNA world not for a role in protein synthesis but to tag genomic RNAs for replication and to function as telomeres, preventing loss of 3′-terminal nucleotides during successive replication rounds (32, 54). Although most plant viral RNAs in the family Bromoviridae have the 3′ tRNA-like structure and pseudoknots, AMV and ilarvirus RNAs are again distinguished because they lack both (20). At issue, therefore, is how the viral RNA-dependent RNA polymerase (replicase) recognizes the RNA 3′ termini and how the ends of the RNA are maintained during replication in the absence of an RNA substrate for CCA-nucleotidyltransferase (36). We recently proposed that a 3′-terminal coat protein-RNA complex may represent a functional tRNA equivalent, possibly explaining the unusual requirement for coat protein to activate viral RNA replication of full-length genomic RNAs (21).

To further understand coat protein’s role in viral RNA replication, we have focused on defining nucleotide and amino acid determinants that are required for specific coat protein-RNA interactions (4, 6, 21, 22). In this paper, we report the results of experiments aimed at understanding coat protein binding to the RNA of the type member of the ilarviruses, tobacco streak virus (TSV). Despite their lack of amino acid similarity, AMV and TSV coat proteins contain an RNA bind-
Concentrations of RNA were determined by amino acid analysis. RNAs were radioactively labeled and purified as described previously (4).

EMSA. Details concerning the electrophoretic mobility shift assay (EMSA) were published previously (21, 22). Briefly, prior to use, the RNA was heated to 65°C for 2 min in REN buffer (10 mM Tris-HCl [pH 7.5], 50 mM NaCl, 10 mM MgCl₂, 1 mM EDTA) to dissociate aggregates and was then cooled slowly to room temperature over 15 to 30 min to allow renaturation. 32P-end-labeled, gel-purified RNAs (4) were incubated with or without peptide in a total volume of 10 μl of EMSA binding buffer (10 mM Tris-HCl [pH 7.5], 50 mM NaCl, 0.1 mM EDTA, 55 pmol of tRNA) at room temperature for 30 min. Peptide concentrations in the binding reactions are indicated in the figure legends. RNA-peptide mixtures were analyzed by electrophoresis in a native 10% polyacrylamide gel (acylamidobis ratio, 46:1) in 0.5× Tris-borate-EDTA (TBE) at 4 W constant power at room temperature. Gels were prerun at the same power for 30 min prior to loading of the samples. Dried gels were exposed to X-ray film (Kodak).

Hydroxyl radical footprinting. Details of the hydroxyl radical footprinting method have been published elsewhere (3, 4). A protocol based on molecular oxygen was used. 5'-labeled RNAs were renatured prior to treatment by heating at 65°C in footprinting REN buffer (10 mM NaPO₄ [pH 6.5], 3 mM MgCl₂, 50 mM NaCl, 0.1 mM EDTA) for 2 min and were then cooled slowly to room temperature over 15 to 30 min. Renatured 5'-labeled RNAs with or without peptide were incubated in 8 μl of footprinting binding buffer (10 mM NaPO₄ [pH 6.5], 50 mM NaCl, 0.1 mM EDTA, 55 pmol of tRNA) at room temperature for 30 min. One microliter of a freshly prepared solution of Fe(III) EDTA (40 mM ammonium iron(II) sulfate hexahydrate [Aldrich], 40 mM EDTA) and 1 μl of 200 mM dithiothreitol were added, and the RNA was incubated for 1 h at room temperature. The reaction was stopped by adding 1 μl of 100 mM thiourea and was mixed with 11 μl of 10 M urea. RNAs were analyzed by electrophoresis in a 20% polyacrylamide, 0.15-mm-thick gel containing 8 M urea in TBE buffer with an ammonium acetate gradient in the running buffer to minimize the separation between small RNA fragments running near the bottom of the gel (0.75 M ammonium acetate in the bottom reservoir).

Other RNA digestions. RNase T1 and RNase ΦM ladders were generated by digesting 5'-end-labeled RNA under partial denaturing conditions (3.5 M urea, 16 mM sodium citrate [pH 5.0], 0.8 mM EDTA, 55 pmol of tRNA) for 12 min at 55°C (27). Formamide ladders were generated by digesting 6.25 pmol of labeled RNA and 22.5 μg of unlabeled tRNA in a total of 25 μl of formamide at 100°C for 25 min. Samples were then mixed with an equal volume of 10 M urea–2 M TBE and were loaded onto the gel.

Peptide and RNA numbering. The coat proteins of AMV and ilarviruses, including tobacco streak virus, are translated from subgenomic RNA (RNA 4) during infection. Genomic RNA 3, which contains nucleotide sequences for both the 32-kDa virus movement protein and the viral coat protein, is first copied into a negative strand, which is followed by the synthesis of RNA 4 by internal initiation of transcription on RNA 3. Coat protein is translated only from RNA 4. The 5' end of TSV RNA 4 has not been mapped precisely; however, Reusken et al. recently reported (39) that the coat protein translational initiation site is located at the AUG codon at nucleotides 1246 to 1248 in RNA 3 (14). Here, we have numbered the TSV coat protein amino acids starting from the methionine encoded at nucleotides 1246 to 1248 relative to the 5' end of RNA 3. To maintain consistency, we have also presented the nucleotide numbering of both TSV and AMV RNAs in terms of genomic RNA 3 (Fig. 1). We note, however, that the AMV RNA presented as AMV1999-2037 RNA in Fig. 1 is identical to AMV843-881 and AMV RNAs in terms of genomic RNA 3 (Fig. 1). We note, however, that the AMV RNA presented as AMV1999-2037 RNA in Fig. 1 is identical to AMV843-881.

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**RESULTS**

The predicted secondary structure folding patterns for the 3' termini of AMV RNA 3 and 4 and TSV RNA 3 and 4 are shown in Fig. 1A and B, respectively, along with the corresponding sequences of the AMV and TSV peptides (Fig. 1C) used in the RNA binding experiments. The RNA binding consensus sequence that we reported previously (4) is also shown in Fig. 1C. Peptide sequences are aligned relative to arginine 17 of AMV coat protein and arginine 47 of TSV coat protein, which were...
found to be crucial for specific AMV RNA binding (4). As shown in Fig. 1A and B, the 3′-terminal AMV and TSV RNA sequences are related by their potential to fold into two hairpin structures that are flanked by single-stranded AUGC or UAGC tetranucleotide repeats (22).

EMSA was used to analyze TSV peptide 39-57 binding to a 50-nucleotide RNA transcript representing the 3′ terminus of TSV RNA 3 and 4 (Fig. 1B and 2A, TSV 2156-2205 RNA). At a concentration of 0.25 μM TSV peptide 39-57, a shifted band representing an RNA-peptide complex is observed (Fig. 2A, lane 3), and more than half of the RNA is shifted into the complex at a 4 μM concentration. When the peptide concentration was increased to 16 μM, essentially all of the RNA was bound to peptide (Fig. 2A, lane 7). At higher peptide concentrations, an additional RNA-peptide complex formed at 8 μM and in the presence of 16 μM TSV 39-57 peptide. Nonspecific interactions. We demonstrated previously that comparable amino acid substitutions in the AMV and TSV coat proteins significantly diminished RNA binding potential for AMV RNAs (4). The results in Fig. 4A support the amino acid alignment (Fig. 1C) by providing evidence that R47 of the TSV coat protein is an important determinant of TSV coat protein binding.

The results of binding lysine-rich AMV coat protein peptides, we discovered that the affinity of the lysine-rich AMV peptides for TSV RNA is significantly higher than that of the arginine-rich TSV peptides for cognate TSV RNA; moreover, amino acid substitutions in arginine 47 of TSV coat protein disrupt TSV RNA binding. First, EMSA was used to assess peptide binding to TSV 2156-2205 RNA. The data presented in Fig. 4A illustrate the effect of substituting lysine or alanine for arginine 47 (Fig. 1C) in the TSV peptide 39-57. In lanes 1 to 8, labeled TSV 2156-2205 RNA was incubated with increasing concentrations of TSV peptide 39-57, and the results suggest that the apparent binding constant for the interaction is 0.5 to 1 μM (Fig. 4A, lanes 3 and 4). RNA binding by peptides containing single amino acid substitutions of R47 to lysine (R47K) or R47 to alanine (R47A) are shown in Fig. 4A, lanes 9 to 19. Al- though more than one half of the input TSV 2156-2205 RNA is bound at 1 μM wild-type peptide (lane 4), there is no observable complex formed at 8 μM concentrations of the two variant peptides (Fig. 4A, compare lane 7 with lanes 11 and 16). At higher peptide concentrations (Fig. 4A, lanes 12, 13, 17, and 18), the RNA shifted into diffuse complexes characteristic of nonspecific interactions. We demonstrated previously that comparable amino acid substitutions in the AMV and TSV peptides significantly diminished RNA binding potential for AMV RNAs (4). The results in Fig. 4A support the amino acid alignment (Fig. 1C) by providing evidence that R47 of the TSV coat protein is an important determinant of TSV coat protein-RNA interactions.

![FIG. 2. EMSA of TSV RNA-peptide complexes. Peptide concentrations in the binding reactions are indicated above the lanes. (A) A total of 20 nM of end-labeled TSV 2156-2205 RNA (diagrammed schematically at the left) was incubated with increasing amounts of TSV peptide 39-57. Lane 1, TSV 2156-2205 RNA only; lanes 2 to 7, TSV 2156-2205 RNA plus increasing concentrations of TSV peptide. (B) A total of 20 nM of end-labeled TSV 2156-2205 RNA (diagrammed schematically at the left) was incubated with increasing amounts of TSV peptide 39-57. Lane 1, TSV 2156-2205 RNA only; lanes 2 to 7, TSV 2156-2205 RNA plus increasing concentrations of TSV peptide.](http://jvi.asm.org/)

![FIG. 3. Competitive binding analysis. Radiolabeled TSV 2156-2205 RNA was incubated with TSV peptide 39-57 in the presence or absence of unlabeled competitor TSV 2156-2205 RNA or variant TSV 2185-2187AAA RNA. All reactions contained 20 nM of end-labeled TSV 2156-2205 RNA, and reactions analyzed in lanes 2 to 10 also contained 1 μM TSV 39-57 peptide. Concentrations of nonradioactive competitor RNAs included in the binding reactions are indicated above the lanes.](http://jvi.asm.org/)
tides to TSV2156-2205 RNA are shown in Fig. 4B. Two different peptides from AMV coat protein were tested, i.e., peptides representing amino acids 1 to 26 (CP26) and 5 to 26 (CP5-26) (Fig. 1C). In both cases, approximately half of the TSV RNA was shifted into RNA-peptide complexes at a peptide concentration of about 30 nM (Fig. 4B, lanes 3 and 11). These results can be compared to the relative affinity value of 0.5 to 1 μM with TSV peptide 39-57 (Fig. 4A, lanes 3 and 4). The TSV and AMV peptides used in these experiments are of similar sizes and charge densities. The lower comparative affinity of the TSV peptide may result from the absence of several amino acids that are conserved in AMV-like coat proteins and that may have roles in RNA binding (i.e., T15, S18, Q19, and N20 in AMV coat protein) (Fig. 1C and D) (4). A potential role for a zinc finger in the TSV coat protein is discussed below.

Heterologous binding of AMV peptide CP26 to TSV2156-2205 RNA was also analyzed by competition binding experiments (Fig. 4C). When unlabeled TSV2156-2205 RNA was present in the binding reaction, competition for peptide binding to labeled input RNA was complete at 0.5 to 1.25 μM competitor (Fig. 4C, lanes 1 to 6). Similar results were observed with AMV1999-2037 RNA competitor (Fig. 4C, lanes 7 to 11). Specificity was assessed by including the nonbinding TSV2185-2187AAA RNA in the binding reactions (Fig. 4C, lanes 12 to 18). The results indicate that 50 μM variant TSV2185-2187AAA RNA was required to yield the same competitive effect observed with 0.5 μM wild-type TSV2156-2205 RNA (Fig. 4C, compare lanes 16 and 4). By defining TSV2185-2187AAA RNA as a nonspecific substrate for AMV coat protein binding, then the ratio of differential binding to specific versus nonspecific substrates is approximately 100 (53).

Hydroxyl radical footprinting experiments were done to compare regions of the TSV RNA protected by AMV and TSV coat protein peptides. Hydroxyl radicals are small, readily diffusible reagents that cleave RNA without base or secondary structure specificity (3, 30, 48), and they are useful high-resolution probes for characterizing RNA-protein interactions (2, 52). The TSV2156-2205 RNA protection patterns observed with the AMV peptides CP26 and CP10-26 (Fig. 5, lanes 11 and 12) and TSV39-57 peptide (Fig. 5, lanes 6 and 9) are qualitatively similar.
C2161 are diminished in the autoradiograph, indicating that peptide protected by AMV peptides, but the protection is less obvious with the TSV 39-57 protected in the same way that we reported ribose A878 (A2034 by the AMV peptides. Conversely, the G2183 and C2187 ribose groups are protected by the AMV peptides, but the protection is less obvious with the TSV 39-57 peptide.

Consistent with the EMSA results presented in Fig. 2, the hydroxyl radical cleavage data suggest that amino acid substitutions at arginine 47 of the TSV 39-57 peptide diminish binding affinity and specificity (Fig. 5, lanes 7 and 8). The R47A substitution diminished specific protection more than the R47K change (Fig. 5, compare lanes 8 and 7), a fact that was also suggested when comparable substitutions were made in the AMV CP26 peptide (Fig. 5, compare lanes 14 and 15).

Although protected regions are clearly visible in the lanes containing TSV peptides (Fig. 5, lanes 6, 7, and 9), the results also indicate that the relative intensities of the bands in these lanes are reduced compared to those of reactions lacking added peptide (Fig. 5, lanes 5 and 10). Because of the lower-affinity TSV peptide-TSV RNA interaction, higher concentrations of peptide were required in the footprinting reactions containing TSV peptides (Fig. 5, lanes 6, 7, and 9).

The protection patterns observed with the AMV and TSV peptides are essentially identical, although subtle comparative differences are indicated in Fig. 6.

To extend the biochemical data and to gain further insight into the functional significance of coat protein-RNA complexes in viral RNA replication, we tested the effects of including the TSV and AMV RNA fragments (shown in Fig. 1A and B) in inocula applied to infect plant protoplasts. When genomic AMV or ilarivirus RNAs are inoculated into tobacco protoplasts in the presence of viral coat protein, the viral RNAs replicate, as evidenced by the accumulation of viral coat protein that can be detected by immunofluorescence (6, 31). The RNAs are not replicated if coat protein or coat protein mRNA is omitted from the inoculum (1, 50).

We reasoned that if a coat protein-RNA complex was required for initiating viral replication, then adding the 3′-terminal coat protein binding fragment of AMV or TSV RNAs (Fig. 1A and B) may inhibit replication by competitive binding. The results of this experiment (Table 1) indicate that infection was indeed affected by the RNA fragments. About 49% of the protoplasts inoculated with genomic RNA plus coat protein became infected, while less than 1% of those inoculated without coat protein became infected. The addition of wild-type TSV or AMV RNA fragment reduced infection by 73 or 95%, respectively, while TSV 2185-2187AAA RNA or AMV 2022-2024AAA RNA fragments that do not bind AMV coat protein did not affect infection. The results strongly indicate that viral RNA replication was inhibited significantly by adding the coat protein binding domains of AMV or TSV RNAs but not by their variant AUGC—AAAAA counterparts that fail to bind coat protein with high affinity. The decrease in replication that accompanies addition of the viral RNA fragments suggests that coat protein is prevented from forming essential interactions with protein or RNA molecules in the replication complex.

**FIG. 6.** Summary of hydroxyl radical footprint data for the TSV 2156-2205 RNA. The boxed regions represent nucleotides whose ribose groups were protected from hydroxyl radical-induced cleavage in the presence of TSV 39-57 peptide and AMV peptides. Subtle differences between the protection patterns with TSV and AMV peptides are illustrated by the dotted lines. The ribose of U2164 is protected somewhat by TSV peptide 39-57, without obvious protection by the AMV peptides. Conversely, the G2183 and C2187 ribose groups are protected by AMV peptides, but the protection is less obvious with the TSV 39-57 peptide.

**DISCUSSION**

Cross-activation of AMV and ilarivirus replication by heterologous RNA-coat protein mixtures was described more than 20 years ago (18, 19, 50), but the molecular mechanism has not been defined. One unanswered question is whether coat protein functions in replication as a free (unbound) form or if the replication function requires a coat protein-RNA complex. Although AMV and TSV coat proteins have little amino acid homology (14, 50) and are serologically unrelated (19, 50), we recently reported that they share an RNA binding consensus sequence containing a crucial arginine residue (4). This consensus RNA binding sequence, which seems to be unique to AMV and ilarviruses that require coat protein to initiate viral replication, was indeed affected by the RNA fragments. About 49% of the protoplasts in inocula applied to infect plant protoplasts contained replicating RNA 3′-terminal AMV or TSV RNA fragments (shown in Fig. 1A and B) in inocula applied to infect plant protoplasts.
RNA replication, may explain in part why the proteins are interchangeable in RNA binding and genome activation. In addition to common protein determinants, the cross-activation phenomenon also requires the coat proteins to specifically recognize different viral RNAs. For example, AMV coat protein must specifically recognize both AMV and TSV RNAs. To test for the presence of common RNA sequences or structures, we have compared the binding sites for AMV and TSV coat protein peptides on TSV RNA.

Coat protein binding domains in AMV and TSV RNAs were previously localized to the 3′ termini (28, 57). We have now used high-resolution hydroxyl radical footprinting to define protection sites, and the results are evidence that lysine-rich AMV coat protein peptides and the arginine-rich TSV peptide protect the same target RNA regions (Fig. 5). Although the 3′-terminal AMV and TSV RNAs are related by the (G/A)UGC repeats and by the potential to fold into similar secondary structures (Fig. 1) (23, 28), the predicted TSV RNA hairpins are longer and have a different primary sequence compared to that of the AMV RNA (Fig. 1). The hydroxyl radical-resistant domains identified with AMV and TSV peptides are essentially coincident and cluster at the base of the RNA hairpins and in the single-stranded AUGC nucleotides (Fig. 5 and 6) that are common in AMV and ilarvirus RNAs (22). Chemical modification interference data (3a) further indicate that the AMV peptide binding sites on AMV RNA are coincident with the protected regions shown in Fig. 6, again suggesting that the upper stem and loop nucleotides are not major contact points for coat protein or peptides.

Although the AMV and TSV peptides bind similar sites on TSV RNA, the EMSA data (Fig. 4) strongly suggest that peptide TSV 39-57 binds TSV RNA with significantly lower affinity than AMV peptide CP26 or CPS-26. Furthermore, we reported previously that the TSV peptide has a lower affinity for AMV RNA than the cognate AMV coat protein peptides (4). These results were not anticipated. van Vloten-Doting demonstrated that upon incubation of deproteinized AMV RNA with AMV virions, coat protein subunits were withdrawn from the viral particles to bind to the RNA (51). However, TSV protein subunits were not withdrawn from TSV virions by AMV or TSV RNA, suggesting that the TSV RNA-protein interaction is more stable (24, 50). In addition, Gonsalves and Garmsen noted that AMV and TSV coat proteins were essentially equivalent in activating replication (19), suggesting that the two coat proteins were not noticeably different in the functional activation experiments. Together, these results suggest that the TSV peptide used in these experiments may not reflect the RNA binding affinity of full-length TSV coat protein (discussed in reference 4). However, we have not determined a relative RNA binding value for full-length TSV coat protein. The TSV coat protein has a putative zinc-finger motif (7, 42) positioned 11 amino acids N terminal of the AMV-ilarvirus consensus sequence (Fig. 7), and it is conceivable that the zinc finger region interacts with RNA to increase RNA binding affinity of the full-length protein (4). Zinc fingers found in other nucleic acid binding proteins play important roles in DNA or RNA binding (12, 13, 46).

Although the collective data suggest that the AMV and ilarvirus RNA and coat protein molecules share similar features that are important for specific RNA-protein interactions, there is no direct evidence that RNA-coat protein binding is critical for viral RNA replication. Indeed, others (15, 34) have proposed that coat protein, apparently free of RNA, may interact with viral replicase to regulate positive-strand viral RNA accumulation. Acknowledging that the mechanistic details of coat protein's role in activating replication are still sketchy, we argue that the available data suggest that RNA binding and genome activation functions colocalize in the AMV coat protein. First, we reported that amino-terminal AMV coat protein peptides (25 or 38 amino acids in length) encompassing the RNA binding consensus sequence (4) both bind viral RNA specifically and functionally substitute for full-length coat protein in activating viral RNA replication in tobacco protoplasts (6). AMV coat protein molecules lacking the basic N-terminal arm and RNA binding consensus sequence fail to activate viral RNA replication (8, 56). These data strongly suggest that RNA binding and genome activation functions reside in the same small protein domain. Second, substituting alanine for a key arginine in the AMV coat protein peptide prevents RNA binding (4) and also severely diminishes genome activation potential (5, 55, 55a). Finally, we report here that the RNA binding domains of AMV or TSV coat proteins significantly inhibit virus replication when included in an inoculum, while nonbinding RNA variants have no effect (Table 1). Collectively, RNA or protein mutations that interfere with the coat protein-RNA interaction correlate with changes in viral RNA replication efficiency. The small RNA fragments (Table 1) may inhibit replication by sequestering coat protein and preventing its interaction with the genomic RNAs; alternatively, protein-protein (possibly coat protein-replicase) interactions could be impeded if there are overlapping binding sites on coat protein for RNA and other proteins. To date, a coat protein variant that separates RNA binding and activation functions (i.e., a peptide that binds RNA but does not activate replication) has not been identified, although many amino acid substitutions in the RNA binding consensus sequence remain to be tested.

Although other plant virus coat proteins, including those of bromovirus mosaic virus (40, 43) and cowpea chlorotic mottle virus (47), have flexible amino-terminal basic arms, they do not bind AMV or ilarvirus RNAs specifically nor do they activate viral RNA replication (4, 6, 19, 50). Database search results suggest that the RNA binding consensus sequence identified in AMV and ilarvirus coat proteins (4) is unique to these viruses, and we suggest that this singular feature plays an important functional role in coat protein-dependent virus replication. The database searches also revealed a core PTXRS subdomain that is found not only in the AMV and ilarvirus RNA binding consensus but also in a number of other viral proteins that have
TABLE 2. Presence of the PTXRS subdomain in viral proteins

<table>
<thead>
<tr>
<th>Viral protein</th>
<th>Amino acid sequence</th>
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<tr>
<td>AMV and ilarvirus RNA binding consensus</td>
<td>Q P T V R S a v p a</td>
</tr>
<tr>
<td>SFV nsP4</td>
<td>Q P T V R S a v p a</td>
</tr>
<tr>
<td>VEE nsP4</td>
<td>Q P T V R S a v p a</td>
</tr>
<tr>
<td>ONN nsP4</td>
<td>Q P T V R S a v p a</td>
</tr>
<tr>
<td>Parvovirus VP-1</td>
<td>K P T E R S k p p p</td>
</tr>
<tr>
<td>TYMV polyprotein</td>
<td>S P T R S p H F p</td>
</tr>
<tr>
<td>HCV core</td>
<td>Q P T G R S g p g p</td>
</tr>
</tbody>
</table>

*PTXRS, a subdomain of the AMV and ilarvirus RNA binding consensus sequence (4), is found in a number of virus proteins that have RNA binding potential. Amino acids comprising the RNA binding consensus are enclosed in boxes; multiple amino acids in a single box reflect conservative substitutions among the AMV and ilarvirus coat proteins (4). The PTXRS subdomain is surrounded by a dotted line; X, any amino acid. The crucial arginine that is required for RNA binding (4) is indicated by the arrow. Abbreviations: SFV, Semliki Forest virus; VEE, Venezuelan equine encephalitis virus; ONN, o'nyong-nyong virus; nsP4, nonstructural protein 4, the putative polymerase; VP-1, coat protein; TYMV, turnip yellow mosaic virus; HCV, hepatitis C virus. Homology with the AMV and ilarvirus consensus sequence is indicated by uppercase letters.

RNA binding potential (Table 2). The GENPEPT database was searched for PTXRS with no mismatches, and of 393 total identities, 87 were virus sequences and 15 were from plant viruses. Six sequences representing viral RNA-dependent RNA polymerases or viral proteins thought to have an RNA binding function are presented in Table 2. Semliki Forest virus (45), Venezuelan equine encephalitis virus (26), and o'nyong-nyong virus (44) are all alphaviruses (single-stranded RNA viruses of positive polarity) that have PTXRS homology in their nsP4 protein, which contains a GDD motif and is likely the viral RNA-dependent RNA polymerase. Homology was also identified in the turnip yellow mosaic virus replicase protein (25). Parvoviruses are single-stranded DNA viruses; the PTXRS homology (37) is found in the capsid protein VP1. Hepatitis C virus is a positive-sense single-stranded RNA virus; the PTXRS homology is found in the core protein, which is presumed to bind nucleic acid (11, 49). Additional work is required to determine if these sequence homologies are functionally significant.

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REFERENCES

31. Loesch-Fries, L. S., and T. C. Hall. 1980. Synthesis, accumulation and en-
capsidation of individual brome mosaic virus RNA components in barley
the molecular fossil record that tRNA originated in replication, not
Oligoribonucleotide synthesis using T7 polymerase and synthetic DNA tem-
minus-strand RNAs induced by RNAs 1 and 2 of alfalfa mosaic virus in the
function of the tRNA-like structure of brome mosaic virus RNA. Proc. Natl.
37. Reed, A. P., E. V. Jones, and T. J. Miller. 1988. Nucleotide sequence and
region of alfalfa mosaic virus RNA 3 contains at least two independent
streak virus coat protein to substitute for late functions of alfalfa mosaic virus
arm of brome mosaic virus coat protein on RNA packaging and systemic
synthesis in alfalfa mosaic virus infected alfalfa protoplasts. Virology 131:
455–462.
1989. A “zinc-finger”-type binding domain in tobacco streak virus coat pro-
43. Sgro, J. V., B. Jacrot, and J. Chroboczek. 1986. Identification of regions of
brome mosaic virus coat protein chemically cross-linked in situ to viral RNA.
Nonstructural proteins nsP3 and nsP4 of Ross River and O’Nyong-nyong
viruses: sequence and comparison with those of other alphaviruses. Virology
45. Takkinen, K. 1986. Complete nucleotide sequence of the nonstructural pro-
and DNA binding zinc fingers in Xenopus TFIIIA. Cell 71:679–690.
47. Tremaine, J. H., H. O. Agrawal, and J. Chidlow. 1972. Partial sequence of the
N-terminal portion of the protein of cowpea chlorotic mottle virus.
49. van Doorn, L. J., G. E. M. Kleton, L. Stuyver, G. Maertens, J. T. Brouwer,
S. W. Schalm, R. A. Heijink, and W. G. V. Quint. 1995. Sequence analysis
of hepatitis C genotypes 1 to 5 reveals multiple novel subtypes in the Benelux
50. van Vloten-Doting, L. 1975. Coat protein is required for infectivity of tobacco
streak virus: biological equivalence of the coat proteins of tobacco streak and
application to pre-mRNA splicing complexes. Proc. Natl. Acad. Sci. USA
86:7795–7799.
53. Weeks, K. M., and D. M. Crothers. 1992. RNA binding assays for Tat-
54. Weiner, A. M., and N. Maizels. 1987. tRNA-like structures tag the 3’ ends of
genomic RNA molecules for replication: implications for the origin of pro-
alfalfa mosaic virus coat protein involved in the initiation of infection.
N-terminal amino acids of alfalfa mosaic virus. Virology, in press.
N-terminal part of alfalfa mosaic virus coat protein interferes with the
specific binding to RNA 1 and genome activation. Virology 129:255–
260.
coat protein binding sites of the genomic RNAs of alfalfa mosaic virus and
protein interaction in alfalfa mosaic virus and related viruses. Virology 140:
342–350.