A plant viral coat protein RNA binding consensus sequence contains a crucial arginine

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A defining feature of alfalfa mosaic virus (AMV) and ilarviruses [type virus: tobacco streak virus (TSV)] is that, in addition to genomic RNAs, viral coat protein is required to establish infection in plants. AMV and TSV coat proteins, which share little primary amino acid sequence identity, are functionally interchangeable in RNA binding and initiation of infection. The lysine-rich amino-terminal RNA binding domain of the AMV coat protein lacks previously identified RNA binding motifs. Here, the AMV coat protein RNA binding domain is shown to contain a single arginine whose specific side chain and position are crucial for RNA binding. In addition, the putative RNA binding domain of two ilarvirus coat proteins, TSV and citrus variegation virus, is identified and also shown to contain a crucial arginine. AMV and ilarvirus coat protein sequence alignment centering on the key arginine revealed a new RNA binding consensus sequence. This consensus may explain in part why heterologous viral RNA-coat protein mixtures are infectious.

Keywords: plant/replication/RNA binding consensus/RNA-protein interactions/RNA virus

Introduction

RNA binding proteins are important regulators of transcriptional, post-transcriptional and developmental processes (reviewed in Bandziulis *et al.*, 1989; Cullen, 1993; Melefors and Hentze, 1993; Burd and Dreyfuss, 1994). Since there are few high resolution structural analyses of RNA-protein complexes, our understanding of how RNA and protein molecules associate to form specific ribonucleoprotein complexes is limited. While some RNA binding proteins have been grouped by binding domain sequence homology (recently reviewed by Burd and Dreyfuss, 1994), such motifs have not been identified in numerous RNA binding proteins, including virus coat proteins, which have important roles in the viral life cycle. The model system analyzed here is the specific binding

of alfalfa mosaic virus (AMV) and ilarvirus coat proteins to their viral RNAs.

AMV and ilarviruses [type virus: tobacco streak virus (TSV)] are positive strand RNA plant viruses composed of three genomic RNAs (RNAs 1–3) in addition to a subgenomic RNA (RNA 4), which encodes the viral coat protein. The RNAs are 5' capped, lack a poly(A) tail, but share a region of 3' nucleotide sequence and potential secondary structure homology. Genomic RNAs 1–3 and subgenomic RNA 4 are encapsidated separately by a single coat protein species into bacilliform-shaped virions (reviewed in Jaspars, 1985). A defining feature of these viruses is that coat protein is required to initiate early stages of virus replication (Jaspars, 1985; Rybicki, 1995). Heterologous AMV and ilarvirus RNA-coat protein mixtures are infectious (Gonsalves and Garnsey, 1975; van Vloten-Doting, 1975; Reusken *et al.*, 1995). For example, AMV and TSV coat proteins, which are serologically unrelated (Gonsalves and Garnsey, 1975; van Vloten-Doting, 1975) and show little primary sequence identity (van Vloten-Doting, 1975; Cornelissen *et al.*, 1984), are functionally interchangeable in RNA binding (Zuidema and Jaspars, 1984, 1985; Zuidema *et al.*, 1984) and initiation of infection (Gonsalves and Garnsey, 1975; van Vloten-Doting, 1975). Both AMV coat protein (reviewed by Jaspars, 1985; Baer *et al.*, 1994; Reusken *et al.*, 1994) and its amino-terminal peptides (Baer *et al.*, 1994) bind specifically to the 3'-untranslated region of AMV RNA 4 and activate AMV genomic RNA replication in protoplasts (Alblas and Bol, 1978; Baer *et al.*, 1994). Recently, a 3'-terminal 39 nucleotide RNA binding site (AMV_{843–881}) for AMV coat protein and amino-terminal peptides has been identified (Houser-Scott *et al.*, 1994). These specific RNA-protein interactions may contribute to the multiple functions of coat protein in the viral life cycle, including initiation of viral RNA synthesis (Houwing and Jaspars, 1978; Jaspars, 1985; Taschner *et al.*, 1991; Baer *et al.*, 1994; Yusibov and Loesch-Fries, 1995), virion assembly (van Vloten-Doting and Jaspars, 1972; Kaper, 1973; Jaspars, 1985), mRNA stability (van der Kuyl *et al.*, 1991; Houwing and Jaspars, 1993; Neeleman *et al.*, 1993), regulation of the ratio of positive to negative strand RNA synthesis (Nassuth and Bol, 1983; Houwing and Jaspars, 1986; Quadt *et al.*, 1991; van der Kuyl *et al.*, 1991; van der Vossen *et al.*, 1994; de Graaff *et al.*, 1995) and viral cell-to-cell movement (Dore *et al.*, 1991; van der Vossen *et al.*, 1994).

Here we examine protein determinants required for the specific binding of AMV and ilarvirus coat protein peptides to AMV_{843–881} RNA. Since basic side chains frequently contribute to specific non-covalent and/or electrostatic interactions with target RNAs, we sought to identify basic residues of the AMV amino-terminal RNA binding domain (RBD) important for viral RNA binding. AMV amino-

terminal peptides of varying lengths and variant peptides containing amino acid substitutions at basic residues were tested for specific AMV_{843–881} binding by RNase T2 protection and Fe(II)-EDTA footprinting assays. We found that the lysine-rich AMV RBD contains a single arginine whose position and specific side chain identity are crucial for RNA binding. In addition, the AMV RBD can be truncated to a 14 amino acid peptide containing the crucial arginine and three additional basic residues. We also identified the putative RBD of the TSV and citrus variegation virus (CVV) ilarvirus coat proteins which, like AMV, are shown to contain a crucial arginine. A new RNA binding consensus was revealed by centering the alignment of AMV and ilarvirus coat protein sequences on the key arginine. The data have significance for defining protein determinants involved in specific RNA binding and for elucidating the role of coat protein in the viral life cycle.

Results

Unlike the arginine-rich RNA binding proteins Tat (Weeks *et al.*, 1990; Calnan *et al.*, 1991a) and Rev (Kjems *et al.*, 1992), the AMV coat protein RBD is lysine rich (Baer *et al.*, 1994). To identify basic residues important for RNA binding, AMV amino-terminal peptides containing single alanine substitutions at basic residues were synthesized (Figure 1A) and RNA binding potentials were determined by RNase T2 protection (Figure 1B) and Fe(II)-EDTA footprint (Figure 1C) analyses. The single strand-specific RNase T2 cleaves AMV_{843–881} at 3'-terminal nucleotides 871–880 and hairpin loop nucleotides 853–856 (Figure 1B, lanes 5, 14, 22 and 26; Figure 1D). When AMV_{843–881} is incubated with AMV coat protein or peptides CP25 or CP38, which bind AMV_{843–881} specifically (Houser-Scott *et al.*, 1994), 3'-terminal nucleotides are protected from T2 cleavage while the loop nucleotides remain susceptible (Figure 1B, lanes 6, 27 and 28; Figure 1D). Peptides containing a single alanine substitution at basic residues K5, K6, K10, K13, K16 or R25 provided similar protection (Figure 1B, lanes 7–11 and 13). The diminished cleavage intensity is evidence of direct peptide–RNA interactions and/or a peptide-induced RNA conformational change that renders the RNA resistant to enzymatic attack. In contrast, the T2 cleavage pattern of AMV_{843–881} incubated with a peptide containing a single alanine substitution at residue R17 (R17A) matches that of free RNA (Figure 1B, compare lanes 12 and 14). This cleavage pattern (Figure 1B, lane 12) did not change significantly at R17A concentrations up to 25 μ M, which is $\sim$ 70-fold greater than the apparent dissociation constant (K_d) of wild-type peptide CP25 (Ansel-McKinney and Gehrke, unpublished results).

Fe(II)-EDTA footprint analysis was used as a high resolution assay for RNA–peptide interactions. In the presence of the Fe(II)-EDTA reagent, AMV_{843–881} RNA is cleaved, probably by hydroxyl radicals, at every ribose moiety, independently of primary sequence or secondary structure (Figure 1C, lanes 5, 14, 22 and 26) (Huber, 1993). AMV coat protein or peptides CP25 or CP38 partially protect the ribose groups of nucleotides U_{844–850}, A_{864–868} and A₈₇₈ from Fe(II)-EDTA-induced cleavage (Figure 1C, lanes 6, 27 and 28; Figure 1D). This same protection pattern is observed when AMV_{843–881} is incubated with peptides K5A, K6A, K10A, K13A, K16A

or R25A (Figure 1C, lanes 7–11 and 13). There was, however, no detectable protection provided by peptide R17A (Figure 1C, lane 12). The data presented in Figure 1B and C suggest that R17 is a critical determinant of RNA binding for peptide CP25. Single alanine substitutions at all other CP25 basic residues do not affect the T2 and Fe(II)-EDTA protection patterns obtained upon specific protein or peptide binding. These basic residues may contribute, however, to the affinity of the coat protein–RNA interaction.

RNA binding by additional peptides was examined to evaluate the importance of the side chain identity and the relative position of R17. RNase T2 protection and Fe(II)-EDTA footprint patterns obtained when AMV_{843–881} was incubated with wild-type peptide CP26 (Figure 1A) were indistinguishable from those of CP25, CP38 or AMV coat protein (Figure 1B and C; compare lane 15 with 6, 27 and 28). However, peptides containing alanine (26/R17A) or lysine (26/R17K) substitutions at position R17 in CP26 failed to significantly protect AMV_{843–881} from T2- or Fe(II)-EDTA-induced cleavage (Figure 1B and C; lanes 19 and 20). Similarly, AMV_{843–881} protection is reduced markedly when the lysine at position 16 and the arginine at position 17 of CP26 are inverted (26/R16K17) (Figure 1B and C; lane 21). Even in the presence of 30 μ M 26/R17A, 26/R17K or 26/R16K17 peptides ($\sim$ 600 times the apparent K_d of wild-type CP26 binding), AMV_{843–881} protection is diminished (Ansel-McKinney and Gehrke, unpublished results). Neither an arginine-rich human immunodeficiency virus (HIV) Tat-like peptide nor a plant viral coat protein (CCMV) with a basic amino-terminal domain (Dasgupta and Kaesberg, 1982) binds specifically to AMV_{843–881} RNA, at concentrations of 1 μ M and 10 μ M, respectively (Figure 1B and C; lanes 29 and 32). These data strongly indicate that RNA binding requires arginine, not just a basic charge, at position 17 of CP26. Furthermore, the R16K17 inversion data suggest that the position of the arginine in relation to one or more of the surrounding CP26 residues is crucial for binding.

To examine the minimal length of an RBD, a series of amino-terminal truncated peptides (Figure 1A) were tested and found to protect AMV_{843–881} from T2- and Fe(II)-EDTA induced cleavage (Figure 1B and C, lanes 15–18). The shortest peptide, CP13–26, contains R17 and three additional basic residues, suggesting four positive charges are sufficient for specific RNA binding.

Initiation of AMV and ilarvirus infection by heterologous RNA–coat protein mixtures (Gonsalves and Garnsey, 1975; van Vloten-Doting, 1975; Reusken *et al.*, 1995) suggests functionally equivalent RBDs. Using AMV R17 as a reference, we aligned AMV and ilarvirus coat protein sequences and identified the RNA binding consensus Q/K/R·P/N·T·X·R·S·R/Q·Q/N/S·W/F/Y·A (Figure 2). This sequence alignment predicts the RNA binding domain of other ilarvirus coat proteins, and two approaches were used to test the binding potential of additional peptides. First, by probing a series of peptides synthesized on polypropylene membranes, we found that CVV peptide 25–47 (Figure 2A) (Scott and Ge, 1995a) binds AMV_{843–881} specifically (compare Figure 3A and B, position 1), but substituting alanine for CVV R34 (which aligns with AMV R17) abolishes the interaction (compare Figure 3A and B, top row, position 5). All other CVV basic residues in peptide

Fig. 1. (A) Alfalfa mosaic virus (AMV) and tobacco streak virus (TSV) peptides. Basic residues are underlined; amino acid substitutions are in bold and boxed. AMV peptides were synthesized with an amino-acetyl (ac) group since virion coat protein contains acetyl-Ser at its amino-terminus (van Beynum *et al.*, 1977). TSV peptides were numbered with reference to the TSV coat protein translational initiation site identified by Reusken *et al.* (1995). (B) RNase T2 protection analysis of AMV₈₄₃₋₈₈₁ binding to AMV and TSV wild-type and variant peptides. Prior to T2 cleavage, AMV₈₄₃₋₈₈₁ RNA was incubated without peptide (lanes 5, 14, 22 and 26), with 5 μ M peptide (lanes 6–13, 15–21, 27 and 32) or 30 μ M peptide (lanes 23–25). (C) Fe(II)-EDTA footprint analysis of AMV₈₄₃₋₈₈₁ binding to AMV and TSV wild-type and mutant peptides. Prior to Fe(II)-EDTA-induced cleavage, AMV₈₄₃₋₈₈₁ was incubated without peptide (lanes 5, 14, 22 and 26), with 10 μ M peptide (lanes 6–13, 15–21 and 32) or 20 μ M peptide (lanes 23–25). Numbers in the margin indicate nucleotides whose ribose groups are partially protected by peptides. (B and C) Lane 1, RNA only; lanes 2 and 31, alkaline hydrolysis ladder (Knapp, 1989); lane 3, RNase Φ M cleavage (adenosine and uracil) (Knapp, 1989); lanes 4 and 30, RNase T1 cleavage (guanosine) (Knapp, 1989); lanes 28 and 29 contain 1 μ M AMV coat protein or cowpea chlorotic mottle virus (CCMV) coat protein (Dasgupta and Kaesberg, 1982) respectively; lane 32 contains an arginine-rich, Tat-like peptide with the sequence YGKKRRQRRRP. The specificity of RNA-peptide binding as assayed by T2 and Fe(II)-EDTA protection was confirmed using the wild-type AMV₈₄₃₋₈₈₁ RNA and a non-binding variant RNA where UGC₈₆₆₋₈₆₈ was changed to AAA as described previously (Baer *et al.*, 1994; Houser-Scott *et al.*, 1994). (D) T2 protection and Fe(II)-EDTA footprinting patterns observed upon specific peptide or protein binding to AMV₈₄₃₋₈₈₁. Outlined letters (A U G C) represent nucleotides (U₈₄₄–U₈₅₀, A₈₆₄–C₈₆₈ and A₈₇₈) whose ribose moieties are protected from Fe(II)-EDTA-induced cleavage upon specific binding. Arrows (filled or open) mark sites of AMV₈₄₃₋₈₈₁ cleavage by RNase T2: upon specific binding, nucleotides remaining susceptible to cleavage are represented by filled arrows while nucleotides protected from cleavage are shown as open arrows. Enzymatic structure mapping data indicate that the extreme 3' terminus of AMV RNA 4 assumes multiple conformations and is susceptible to single strand-specific nucleases (Quigley *et al.*, 1984; Ansel-McKinney and Gehrke, unpublished results). Putative base pairing is indicated by dotted lines.

25–47 can be mutated individually to an alanine without loss of binding (compare Figure 3A and B, top row, positions 2–4 and 6–8). Second, TSV 39–57 (Figure 1A), a peptide containing basic sequences located within the proposed TSV coat protein RNA binding domain (Cornelissen *et al.*, 1984), provided protection against T2- and Fe(II)-EDTA-induced cleavage that was qualitatively

indistinguishable from the AMV peptides (Figure 1B and C; lane 23); however, changing the aligned arginine (TSV R47) to alanine (TSV/R47A) or lysine (TSV/R47K) reduced protection significantly (Figure 1B and C; lanes 24 and 25). Thus, the RBD of at least two ilarvirus coat proteins, CVV 25–47 and TSV 39–57, has been identified and shown to contain an arginine crucial for AMV₈₄₃₋₈₈₁

Fig. 2. Identification of an AMV/ilarvirus RNA binding consensus sequence. **(A)** AMV and AMV-like ilarvirus coat proteins. **(B)** TSV and TSV-like ilarvirus coat proteins. Basic residues are underlined, consensus sequence residues are boxed and the AMV/TSV arginine required for binding is marked with an * in the section labeled 'consensus sequence'. Conservative aromatic to hydrophobic residue changes are in dashed boxes. The numbers refer to the residue positions of each coat protein represented: alfalfa mosaic virus (AMV) (Barker *et al.*, 1983); prune dwarf virus (PDV) (Bachman *et al.*, 1994); lilac ring mottle virus (LRMV) (Scott and Ge, 1995b); citrus leaf rugose virus (CiLRV) and citrus variegation virus (CVV) (Scott and Ge, 1995a); elm mottle virus (EMoV), spinach latent virus (SpLV), asparagus virus 2 (AV-2) (AV-2 coat protein sequence was determined from EMBL accession no. X86352) and Parietaria mottle virus (PMoV) (Scott and Ge, unpublished data); tobacco streak virus (TSV) (Cornelissen *et al.*, 1984); hydrangea mosaic virus (HdMV) (Scott and Ge, unpublished data); apple mosaic virus (ApMV-G) (Guo *et al.*, 1995) or (ApMV) (Sanchez-Navarro and Pallas, 1994); and *Prunus* necrotic ringspot virus (PNRSV) (Guo *et al.*, 1995) or (PNRSV_#) (Hammond and Crosslin, 1995).

binding which aligns with AMV R17 at position 5 of the consensus (Figure 2).

Discussion

A comparison of TSV/AMV coat protein amino acid homology and binding data suggests that specific RNA binding requires a basic environment including the proline/asparagine, critical arginine and the conserved aromatic (W/F/Y) while other consensus residues increase peptide binding affinity. Indeed, the apparent K_d for AMV₈₄₃₋₈₈₁ binding is $\sim 7 \mu\text{M}$ for TSV 39-57 compared with $\sim 50 \text{ nM}$ for AMV CP 10-26 and $25-50 \text{ nM}$ for CP26 (P. Ansel-McKinney and L. Gehrke, unpublished results). Although AMV peptides bind AMV RNA with affinities similar to that of the full-length coat protein (Baer *et al.*, 1994; Ansel-McKinney and Gehrke, unpublished results), the apparent K_d of full-length TSV coat protein for AMV₈₄₃₋₈₈₁ is not known. It is interesting to note, however, that all but one of the viral coat proteins shown in Figure 2B have a perfect (Sehnke *et al.*, 1989; Guo *et al.*, 1995; Hammond and Crosslin, 1995) or near perfect (Sanchez-Navarro and Pallas, 1994) zinc finger motif (Berg, 1986) amino-terminal to the proposed RBD. Zinc finger motifs are found in a number of nucleic acid binding proteins (Berg, 1986; Burd and Dreyfuss, 1994). For example, in the *Xenopus* TFIIA protein, a different subset of zinc fingers is responsible for TFIIA high affinity binding to 5S rRNA (fingers 4-7) (Clemens *et al.*, 1993) and DNA (fingers 1-3) (Theunissen *et al.*, 1992; Clemens *et al.*, 1993). Thus, the TSV coat protein may bind RNA with

Fig. 3. AMV₈₄₃₋₈₈₁ RNA binding to peptides synthesized on polypropylene membranes. Membranes containing peptides were probed with (A) radiolabeled AMV₈₄₃₋₈₈₁ to assay specific RNA binding or (B) a radiolabeled non-binding AMV₈₄₃₋₈₈₁ variant to assay non-specific binding. Hybridizations included equal counts and molar quantities of RNA and autoradiographs represent 1 h exposures. AMV peptides which specifically bind AMV₈₄₃₋₈₈₁ (Figure 1B and C, lanes 16–18) hybridize to AMV₈₄₃₋₈₈₁ probe (A, bottom row, positions 2–4) but not a non-binding AMV₈₄₃₋₈₈₁ variant probe (B, bottom row, positions 2–4). When AMV R17 or CVV R34 is changed to an alanine, specific binding is lost (compare A and B, bottom row, position 7 or top row, position 5). In (A) and (B) peptides were synthesized on polypropylene membranes at defined spots as follows. Top row: position 1, wild-type peptide containing CVV coat protein residues 25–47 (CVV 25–47); positions 2–8, single alanine substitutions in CVV 25–47 at basic residues R25, R28, R29, R34, R36, R46 and R47 respectively. Bottom row: position 2, AMV CP 5–26; position 3, AMV CP 10–26; position 4, AMV CP 13–26; positions 6–8, single alanine substitutions in AMV 5–26 at basic residues K16, R17 and R25 respectively.

greater affinity than TSV 39–57 peptide due to its putative zinc finger.

The properties of many of the amino acids of the coat protein consensus sequence are consistent with RNA binding roles. Arginine, glutamine and asparagine residues can form bidentate interactions with phosphates, sugars and/or bases of RNA targets (Rould *et al.*, 1991; Puglisi *et al.*, 1992, 1995; Cavarelli *et al.*, 1993; Biou *et al.*, 1994; Oubridge *et al.*, 1994; Valegard *et al.*, 1994) and have been implicated in sequence-specific base pair recognition (Seeman *et al.*, 1976). The guanidinium group of AMV R17 may hydrogen bond with the O6 and N7 of a guanosine in AMV₈₄₃₋₈₈₁, much like the U1A protein-U1 snRNP (Oubridge *et al.*, 1994) and proposed HIV (Puglisi *et al.*, 1992) and bovine immunodeficiency virus (BIV) (Puglisi *et al.*, 1995) Tat-TAR complexes. Indeed, AMV₈₄₃₋₈₈₁ contains two guanosines perfectly conserved among the ilarviruses, and modification of RNAs at these positions with the base-specific reagents diethylpyrocarbonate and dimethylsulfate interferes with CP26 binding (Ansel-McKinney and Gehrke, unpublished results). Serine, threonine and lysine residues may serve as hydrogen bond donors (Rould *et al.*, 1991; Cavarelli *et al.*, 1993; Biou *et al.*, 1994; Oubridge *et al.*, 1994; Valegard *et al.*, 1994; Puglisi *et al.*, 1995). The aromatic residue at consensus position 9 may stack with RNA bases (Cavarelli *et al.*, 1993; Oubridge *et al.*, 1994; Valegard *et al.*, 1994) while basic residues may stabilize the ribonucleoprotein complex through electrostatic interactions with the ribophosphate backbone (Cavarelli *et al.*, 1993; Tao and Frankel, 1993; Biou *et al.*, 1994; Valegard *et al.*, 1994).

Results presented here support the key role of arginine in RNA-protein interactions, now extending from bacterio-

phages (Lazinski *et al.*, 1989) and animal viruses (Calnan *et al.*, 1991b; Puglisi *et al.*, 1992, 1995; Tao and Frankel, 1992; Tan *et al.*, 1993; Tan and Frankel, 1994) to include plant viruses. Bacteriophage N proteins (Lazinski *et al.*, 1989) as well as the HIV Tat (Weeks *et al.*, 1990; Calnan *et al.*, 1991a) and Rev (Kjems *et al.*, 1992) proteins contain an arginine-rich RNA binding motif. The HIV Tat peptide requires a single arginine residue for specific TAR binding (Calnan *et al.*, 1991b; Puglisi *et al.*, 1992; Tao and Frankel, 1992) while surrounding basic residues stabilize this interaction, presumably by electrostatic interactions with phosphates of the TAR backbone (Calnan *et al.*, 1991a; Delling *et al.*, 1991). Furthermore, wild-type RNA binding and transactivation activity depends on the position of this arginine in relation to the surrounding basic charges (Calnan *et al.*, 1991b; Tao and Frankel, 1993). Similarly, the AMV peptide CP26 requires an arginine whose side chain identity and position are crucial for AMV₈₄₃₋₈₈₁ binding. Furthermore, this arginine is centered in a lysine-rich environment. However, neither an arginine-rich Tat-like peptide nor the cowpea chlorotic mottle virus (CCMV) coat protein with its basic amino-terminus bind AMV₈₄₃₋₈₈₁ (Figure 1B and C; lanes 29 and 32), suggesting that, unlike HIV Tat, an arginine within a basic environment is not sufficient for specific, high affinity AMV RNA binding. In the same way that the HIV Rev peptide requires several sequence determinants (four arginines, a threonine and an asparagine) for specific RRE binding (Tan *et al.*, 1993; Tan and Frankel, 1994), it is unlikely that the AMV R17 is solely responsible for specific RNA recognition and/or binding.

This RNA binding consensus also contains residues that may have structural roles important for RNA binding. Although the AMV coat protein amino-terminus is unstructured (Kan *et al.*, 1982; Baer *et al.*, 1994), an α -helical structure may be stabilized upon binding as basic residues align on one face of the helix and interact with RNA phosphates (van Beynum *et al.*, 1977; Argos, 1981). Thus, the highly conserved proline or asparagine residue at position 2 may have a structural role as an α -helix breaker (Richardson and Richardson, 1988) while the alanine at position 10 may serve as an α -helix former (Scholtz and Baldwin, 1992). Finally, the protein sequence immediately amino-terminal to the RNA binding consensus of AMV and many ilarvirus coat proteins (Figure 1) is glycine rich. The presence of larger side chains at these glycine positions may interfere sterically with RNA binding, as suggested for two glycine residues important for the formation of the BIV Tat-TAR complex (Chen and Frankel, 1995; Puglisi *et al.*, 1995).

The ability to form an α -helical conformation upon binding is a property common to many but apparently not all arginine-rich RNA binding proteins (Tan and Frankel, 1995). In solution, the HIV Tat (Calnan *et al.*, 1991a) peptide, like AMV, is unstructured, while HIV Rev (Tan *et al.*, 1993) and bacteriophage N peptides (Tan and Frankel, 1995) tend to form α -helices. While bacteriophage N and HIV Rev peptides require an α -helical conformation for specific RNA binding, the HIV (Tan and Frankel, 1995) and BIV (Puglisi *et al.*, 1995) Tat peptides do not. Structural changes that occur upon AMV peptide-RNA binding are probably most similar to those reported for the CCMV coat protein peptides upon oligophosphate

binding. CD and NMR studies revealed that peptides containing the basic amino-terminal 25 residues of the CCMV coat protein (Dasgupta and Kaesberg, 1982) alternated between random coil (80%) and α -helical (20–21%) conformations in solution (van der Graaf and Hemminga, 1991; van der Graaf *et al.*, 1991, 1992). However, upon addition of oligophosphates, the α -helical structure is stabilized (van der Graaf and Hemminga, 1991; van der Graaf *et al.*, 1992), presumably by electrostatic interactions between basic side chains and phosphates (Argos, 1981; Vriend *et al.*, 1981, 1986).

The RNA binding consensus presented in Figure 2 may explain in part why heterologous AMV and ilarvirus RNA-coat protein mixtures are infectious. A coat protein-RNA complex may be involved directly in initiating replication (Houwing and Jaspars, 1978), and it was reported recently that a full-length AMV coat protein containing alanine substitutions at consensus residues K13, K16 and R17 failed to initiate AMV infection, suggesting that these residues were important for AMV RNA binding (Yusibov and Loesch-Fries, 1995). Database searches (Swanson and Gehrke, unpublished results) reveal that the PTxRS motif of the consensus sequence is found in RNA virus proteins with polymerase or helicase functions (i.e. Cornelissen *et al.*, 1983; Keese *et al.*, 1989; Dominguez *et al.*, 1990). For example, the PTxRS sequence is present in the nsP4 protein (Strauss *et al.*, 1988; Kinney *et al.*, 1989) (the putative RNA polymerase) of several alphaviruses which, along with AMV and ilarviruses, form the alphavirus superfamily (reviewed by Strauss and Strauss, 1994). While alphaviruses regulate replication by controlling their minus-strand RNA synthesis (reviewed by Strauss and Strauss, 1994), the presence of AMV coat protein is necessary for asymmetric AMV plus-strand RNA accumulation (Nassuth and Bol, 1983; Houwing and Jaspars, 1986; Quadt *et al.*, 1991; van der Kuyl *et al.*, 1991; van der Vossen *et al.*, 1994; de Graaff *et al.*, 1995). Therefore, the consensus domain may have dual roles in RNA binding and possibly in regulating the ratio of plus- to minus-strand RNA synthesis by interacting either with the template RNA (van der Vossen *et al.*, 1994) and/or protein components of the replication complex (Nassuth and Bol, 1983; de Graaff *et al.*, 1995).

Materials and methods

Peptides

Peptides were synthesized with an acetylated amino-terminus (ac) as described (Baer *et al.*, 1994; Houser-Scott *et al.*, 1994). Peptide concentrations were determined by amino acid analysis; peptide integrity and concentrations were confirmed by denaturing gel electrophoresis, spectrophotometric analysis and/or high pressure liquid chromatography.

In vitro transcription

AMV₈₄₃₋₈₈₁ RNA was transcribed *in vitro* from synthetic DNA templates (Milligan *et al.*, 1987; Milligan and Uhlenbeck, 1989) using T7 RNA polymerase purified from a plasmid-expressing bacterial strain essentially as described (Grodberg and Dunn, 1988). Transcription reactions contained 3.3 mM rNTPS, 40 mM Tris-HCl (pH 8.1), 18 mM MgCl₂, 25 mM NaCl, 10 mM dithiothreitol (DTT), 2 mM spermidine, 5 μ M template and ~0.045 μ g/ μ l T7 RNA polymerase. To label RNA internally, the rGTP concentration was changed to 1.1 mM and 0.33 μ M [α -³²P]GTP was added to the reaction. Transcription reactions were incubated at 37°C for 1–2 h, phenol-chloroform extracted and ethanol precipitated. RNA products (i.e. N, N+1, N-1) were denatured in 7 M urea/1 $\times$ TBE at 68°C for 5 min, separated on a 0.15 mM thick 20% polyacrylamide-

urea gel, located by UV light shadowing and buffer eluted overnight at 4°C (Calnan *et al.*, 1991a). Eluted RNA was passed through a 0.22 µm Millipore filter pre-equilibrated with STE (pH 7.5), phenol–chloroform extracted twice, chloroform extracted and ethanol precipitated. Purified RNA was resuspended in 0.1 mM EDTA (pH 7.8). Unlabeled RNAs were quantitated by absorption spectroscopy while labeled RNAs were quantitated by incorporation of [α - 32 P]GTP into the transcripts.

End labeling

AMV_{843–881} RNA was dephosphorylated with calf intestinal alkaline phosphatase (CIP) essentially according to the manufacturer's instructions (Boehringer Mannheim). AMV_{843–881} RNA (200 pmol) was incubated in a 200 µl volume of a 50 mM Tris–HCl (pH 8.5), 1 mM EDTA buffer with 20 U of CIP for 1 h at 50°C and then supplemented with 10 U of CIP for an additional 30 min. To inactivate the CIP, EGTA was added to a final 50 mM concentration and the reaction was heated to 68°C for 10–15 min, phenol–chloroform extracted twice and precipitated with 1/10 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol. Dephosphorylated RNA was 5' end-labeled with T4 polynucleotide kinase (NEB) and [γ - 32 P]ATP (NEN-NEG002z) essentially according to the manufacturer's instructions. Dephosphorylated RNA was incubated in a 200 µl reaction containing 70 mM Tris–HCl (pH 7.6), 10 mM MgCl₂, 5 mM DTT, ~400 µCi [γ - 32 P]ATP and 50 U of T4 polynucleotide kinase at 37°C for 60–90 min. The reaction was phenol–chloroform extracted and precipitated in 2.5 M ammonium acetate, 3 volumes of ethanol with ~4 µg of carrier tRNA. 5' End-labeled RNA was separated from unincorporated label on a 20% polyacrylamide–urea gel, located by autoradiography or UV light shadowing and eluted as described above.

Ribonuclease T2 protection analysis

AMV_{843–881} RNA was renatured in REN buffer (Baer *et al.*, 1994) by heating to 68°C for 5 min and slowly cooled for ~30 min to <35°C. For binding, 3×10^5 counts of RNA were incubated with coat protein (1 µM) or peptide (5 or 30 µM) in binding buffer [10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 5% glycerol, 0.15 µg/µl yeast tRNA] for 20 min at room temperature. For T2 digestions, 1 µl of a 1 U/µl T2 enzyme (GibcoBRL)–8 µg/µl *Escherichia coli* tRNA mixture was added to each 10 µl of binding reaction for 5 min at room temperature. To stop the reactions, the tubes were incubated on ice after adding 11 µl of loading buffer (10 M urea/2× TBE) to each reaction. Reactions were heated to 68°C for 5 min before loading ~ 10^5 counts/reaction onto a 15% polyacrylamide–urea gel to resolve the cleavage patterns. Autoradiography was performed on wet gels.

Fe(II)-EDTA footprinting

5' End-labeled AMV_{843–881} RNA was renatured in a 10 mM sodium phosphate (pH 6.5), 50 mM NaCl, 3 mM MgCl₂ and 0.1 mM EDTA buffer as described above. For binding, 1×10^6 counts of RNA were incubated with coat protein (1 µM) or peptide (10 or 20 µM) in Fe(II)-EDTA binding buffer [10 mM sodium phosphate (pH 6.5), 50 mM NaCl, 1 mM EDTA, 0.15 µg/µl yeast tRNA] for 30 min at room temperature. Fe(II)-EDTA cleavage was performed as described (Latham and Cech, 1989; Houser-Scott *et al.*, 1994) except that cleavage was at room temperature for 1 h in 2 mM Fe(II), 4 mM EDTA and 10 mM DTT. Briefly, 1 µl of a 20 mM ammonium iron (II) sulfate hexahydrate/40 mM EDTA solution and 1 µl of a 100 mM DTT solution, both freshly prepared, were pipetted carefully into the 8 µl binding reaction. To quench the reaction, 1 µl of a 100 mM thiourea solution was added for a 5 min incubation followed by the addition of 11 µl of 2× loading buffer (10 M urea, 0.25× TBE, dyes). Reactions were heated to 68°C for 5 min and 3.3×10^5 counts/reaction were loaded onto a 20% polyacrylamide–urea gel to resolve the cleavage products. Autoradiography was performed on wet gels.

RNA binding to peptides synthesized on polypropylene membranes

Peptides were synthesized using Fmoc chemistry at defined spots on polypropylene membranes (Research Genetics). To assay specific binding of AMV_{843–881} RNA to membrane-bound peptides, a protocol was adapted from the 'dot blot RNA binding assay' of Qian and Wilusz (1993). Blots were pre-wetted in 0.1% Tween 20 at room temperature for 1 h with shaking and rinsed twice for 10 min in 10 mM Tris–HCl (pH 7.8). To block non-specific binding, blots were placed in a sealable bag and incubated in 10 ml of blocking/binding buffer [10 mM Tris–HCl (pH 7.8), 50 mM NaCl, 1 mM EDTA, 0.15 µg/µl *E. coli* tRNA] for 30 min at room temperature with shaking. Next, to assay binding, 1.5×10^6 counts of [α - 32 P]GTP-labeled RNA were renatured in REN

buffer as described above, added to the blocking/binding buffer and incubated with shaking for 1 h at room temperature. Blots were washed in a 10 mM Tris (pH 7.8)/1 mM EDTA buffer containing 200 mM NaCl (2×) followed by 400 mM NaCl (1×) for 10 min at room temperature with shaking. Autoradiography was performed on wet blots. To assay non-specific binding, blots were first probed with a non-binding variant RNA where UGC_{866–868} is changed to AAA (Baer *et al.*, 1994; Houser-Scott *et al.*, 1994). Blots were then washed once in 10 mM Tris–HCl (pH 7.8), 1 mM EDTA, 800 mM NaCl for 15 min at room temperature while shaking, rinsed in H₂O and reprobed with binding wild-type AMV_{843–881} RNA.

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