Translational Repression and Specific RNA Binding by the Coat Protein of the Pseudomonas Phage PP7*

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**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions**—The PP7 coat sequence cloned on a plasmid was kindly provided to us by Gordon Garde. We amplified the coat sequence using polymerase chain reaction and a 5′-primer (5′-GGGTCCTAGACGGTCGACCTCAGAGAAACGTAAG-3′) that introduced a XbaI site about 40 nucleotides upstream of the coat initiation codon, and a 3′-primer (5′-GGGGATCCATACACACGGGTACACCGGGTTCGGGTCGG-3′) that created a BamHI site a few nucleotides downstream of the stop codon. This and subsequent amplifications were conducted using Pfu DNA polymerase. After digestion with XhoI and BamHI, the fragment was cloned between the corresponding restriction sites within the polylinker of pUC119 (11), placing the coat sequence under control of the lac promoter. We call this plasmid pP7CT. Because of relatively low expression of the PP7 coat protein from pP7CT, we constructed another plasmid called pETP7CTNc. This was accomplished by PCR amplification of the coat sequence using the same BamHI site-containing 3′-primer described above, and a new 5′-primer (5′-GACCACGTTACACCGCAGGTTACACCGGGTTCGGGTCGG-3′), which introduced a NcoI site at the initiator AUG of the coat gene. After digestion with NcoI and BamHI, this fragment was inserted into pET3d (12), where it became attached to the T7 promoter and gene 10 ribosome binding site. Plasmid pETP7CTNc produces large amounts of PP7 coat and is a convenient source of the protein. However, it is unsuitable for translational repressor assays in our two-plasmid system. Therefore, we constructed pP7CTNcXb by transfer of the XhoI-BamHI fragment from pETP7CTNc to pUC119. This construction retains the fusion of coat to the T7 gene 10 ribosome binding site, but returns coat to lac promoter control in pUC119.

The plasmid we call pRZP7 is similar to the previously constructed pRZ5 (13). A synthetic EcoRI-BamHI fragment containing the putative PP7 translational operator replaces the corresponding MS2 operator sequence of pRZ5. This fuses the Escherichia coli lacZ gene to a synthetic version of sequences surrounding the PP7 replication start codon, which we assume contain the PP7 translational operator (14). This sequence is shown in Fig. 1. The object of this construction was to place the synthesis of β-galactosidase under translational control of PP7 coat protein. A plasmid we call pT7prop contains the same EcoRI-BamHI operator sequence under control of the T7 promoter in pT7-1 (from U.S. Biochemical Corp.).

**Protein Preparation and Reduction of Disulfide Bonds**—To determine whether coat protein contains interchain disulfide bonds, PP7 capsids produced in E. coli from either pETP7Nc or pP7CTNcXb were purified on Sepharose CL-4B by methods described previously (13), and incubated at a concentration of 1 mg/ml in 50 mM Tris, pH 8.5, with DTT at concentrations varying from 0 to 50 mM at 0 °C. After 60 min, reactions were terminated by addition of N-ethylmaleimide to a concentration of 200 mM. Samples were then subjected to electrophoresis under non-reducing conditions on 12.5% polyacrylamide gels containing SDS. Protein was visualized by staining with Coomassie Brilliant Blue.

Coat protein was prepared for RNA binding studies by incubation in 50 mM Tris, pH 8.5, 7 mM urea, 5 mM DTT at 0 °C for 60 min. followed by dialysis against 1 mM acetic acid (about pH 4). Renatured coat protein was stored at 4 °C, where it appears to be stable for several weeks. Measurement of Translational Repression and RNA Binding—Nitrocellulose filter binding was performed using a Schleicher & Schuell dot-blot

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apparatus and the method of Wong and Lohman (15). RNAs were labeled with \([\alpha-32P]ATP\) by in vitro runoff transcription of BamHI-cleaved pT7opP7, a plasmid that contains the P7 promoter sequence of Fig. 1 fused to the T7 promoter of pT7–1 (U.S. Biochemical Corp.). The quantity of RNA retained after passage of the samples through the nitrocellulose filter was determined using a Packard phosphorimager. To measure RNA binding, buffers in the pH range from 6.2 to 7.7 were made using 0.1 M MOPS (MoKM buffers). In the pH range from 7.0 to 8.5, buffers contained 0.1 M Tris (TMK buffers). To measure ionic strength dependence of binding, KCl was added to final concentrations ranging from 40 mM to 1.0 M. Magnesium ions were introduced into most reactions by the addition of magnesium acetate to a concentration of 10 mM. In our standard reactions, binding was allowed to come to equilibrium during an incubation period of 60 min on ice. In an effort to determine whether equilibrium was attained under these conditions, a series of protein-excess binding curves was produced after incubation for different time periods. Incubation times ranging from 30 min to 8 h gave identical results.

Translational repression was assessed by the ability of coat proteins expressed from pCT119, p7CTNxB, and pQC7 to inhibit the synthesis of \(\beta\)-galactosidase from pRZ5, pRZ7, and pRZQ5 (10, 13). Assays of \(\beta\)-galactosidase were performed using the method described by Miller (16).

Mapping the RNA Binding Site—Using site-directed mutagenesis (17) of single-stranded p7CTNxB, we constructed libraries of mutations directed to specific codons for amino acids potentially involved in RNA binding. We used degenerate oligonucleotide primers which converted target codons to NNNG/T, thus resulting in the possible introduction of all 20 amino acids and one stop codon. DNA from mutagenesis reactions was introduced by transformation into strain CSH41F-pSH141F (pRZP7) and plated at a density of about 500 colonies/plate on solid LB medium containing 5-bromo-4-chloro-3-indolyl-\(\beta\)-galactoside (X-gal). After 24 h of growth at 37 °C, individual blue colonies were used to inoculate 1-ml cultures in LB medium, which were grown to saturation overnight. To determine whether capsids were present, cells were harvested by centrifugation, resuspended in 0.25 ml of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and disrupted by sonication. After removal of cellular debris by centrifugation, glycerol and bromphenol blue were added and the samples were applied to a 1% agarose gel containing 50 mM sodium phosphate, pH 7.4, 1 mM EDTA. Electrophoresis was conducted until the dye had migrated 10 cm. The gel was stained with ethidium bromide, and capsids were visualized by UV illumination. Mutants that passed this capsid assembly test were subjected to DNA sequence analysis.

RESULTS

Construction of Plasmids for Overexpression of Coat Protein and in Vivo Assay of Its Translational Repressor Activity—The expectation that P7 coat protein would repress the translation of replicase was only an extrapolation from our understanding of the behaviors of better characterized RNA coliphages like MS2 and Q\(\beta\). Therefore, our first task was to determine whether P7 coat protein is indeed a translational repressor and to identify its RNA binding target. We had previously constructed two-plasmid systems for MS2, GA, and Q\(\beta\) in which coat protein expressed from one plasmid represses translation of a replicase-\(\beta\)-galactosidase fusion protein expressed from a second plasmid (9, 10, 13). Inspection of the sequence of P7 RNA reveals the potential RNA hairpin structure shown in Fig. 1. It contains the translational start of the replicase gene. By analogy to the other phages, we felt this was the best candidate for the P7 translational operator.

To test for translational repression in vivo, we constructed a two-plasmid system for P7. The first plasmid, analogous to the MS2 coat protein producer pCT119 (8, 13), expresses P7 coat protein from the lac promoter on a plasmid that confers ampicillin resistance. We PCR-amplified the coat sequence using primers that introduced an XhoI site about 40 nucleotides upstream of the coat protein start codon and a BamHI site just downstream of its stop codon. The resulting XhoI-BamHI frag-

tment was inserted into pUC119, resulting in the plasmid we call pP7CT.

A second plasmid, pRZP7, was constructed by the fusion of a synthetic sequence containing the putative P7 translational operator to a deletion mutant of the \(\beta\)-galactosidase gene, which lacks its own initiator AUG. This plasmid, from a different incompatibility group than pP7CT, expresses \(\beta\)-galactosidase from the lac promoter and confers chloramphenical resistance. If the sought-after RNA-protein interaction exists, these manipulations should place \(\beta\)-galactosidase under translational control of P7 coat protein in E. coli cells harboring both plasmids. Repression would be revealed by a failure to produce the blue color typical of \(\beta\)-galactosidase-producing colonies on X-gal plates.

From the beginning, translational repression was evident from the blueness (or, better, the relative lack of blueness) of colonies containing the two plasmids on X-gal plates. However, compared with the MS2 and Q\(\beta\) two-plasmid systems, repression was weak. Attempts to purify coat protein from cells bearing pP7CT revealed the probable cause of poor repression; very little coat protein was being produced (results not shown). Inspection of the P7P7 sequence suggested that the coat protein gene of this Pseudomonas phage possesses a ribosome binding sequence that may be poorly adapted for translation initiation in E. coli. Therefore, we attached the P7 coat protein coding sequence to a strong E. coli ribosome binding sequence as follows. We PCR-amplified the coat sequence using a 5′ primer that introduced an NcoI site at the initiator AUG. The 3′ primer was the same as described above and introduced a BamHI site downstream of the coat termination codon. The insertion of this NcoI-BamHI fragment into pET3d (12) attached the P7 coat protein sequence to the T7 promoter and gene 10 ribosome binding site. The resulting plasmid, which we call pETP7CTNc, was introduced into E. coli strain BL21(DE3)/plysS where, after induction with isopropyl-1-thio-\(\beta\)-d-galactopyranoside, the P7 coat protein was copiously produced (results not shown). We note, parenthetically, that the NcoI mutation changes the identity of the second amino acid in the coat sequence from serine to alanine. This change is predicted to result in the retention of the N-terminal methionine, which is normally removed by methionine aminopeptidase (18). However, as shown by the results that follow, these changes do not seem to compromise the ability of the protein to carry out translational repression or to assemble into a virus-like particle.

The plasmid pETP7CTNc provides a convenient source of P7 coat protein, which can be purified by methods described in this paper and elsewhere (13), but it is unsuitable for the translational repression assays we usually perform in strain CSH41F–, a strain that contains none of the apparatus for T7-based overexpression. Therefore, we excised the P7 coat sequence, together with the upstream, gene 10-derived ribo-
increased amounts of PP7 coat protein compared with pP7CT and pUC119, creating pP7CTNcXb. This plasmid produces in-

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virus-like particles by chromatography on Sepharose CL4B

pared the coat proteins of the various phages by purifying

(repression is specific since other coat proteins (MS2 and Qβ) do not efficiently repress pRZP7, and because PP7 coat does not efficiently repress the operators of these other phages (although it partially represses the Qb operator).

Disaggregation of Capsids and Refolding of PP7 Coat Protein for RNA Binding Studies in Vitro—We have previously pre-
pared the coat proteins of the various phages by purifying virus-like particles by chromatography on Sepharose CL4B (13). These were then subjected to denaturation in 50% acetic acid, 1 mM DTT. RNA-excess binding curves show that this treatment generally result in disaggregation of capsids and subsequent renaturation of coat protein dimers without reas-
semble into capsids. This procedure is necessary because the RNA binding site of the RNA phage coat proteins is inaccessible on the interior surface of intact virus-like particles. Unfor-

mately, when we applied these methods to PP7, they resulted in a coat protein preparation that RNA-excess titrations indi-
cated was no more than 0.15% active (results not shown). Thus, we sought an alternative disaggregation/renaturation protocol.

We noted the presence of two cysteine residues in the FG

loop of PP7 coat protein and wondered whether they were involved in the formation of interchain disulfide bonds in a manner similar to Qβ coat protein (3). The FG loops of different coat protein dimers converge at the 3-fold (quasi-6-fold) and 5-fold symmetry axes in the virus particle, where they participate in interactions that stabilize the capsid. If disulfides are present, their reduction could be important to the recovery of RNA binding activity during the renaturation step. To deter-
mine the presence of interchain disulfides and to establish conditions for their reduction, we conducted an experiment in which virus-like particles, prepared by chromatography on Sepharose CL4B, were exposed for 60 min to various concen-
trations of DTT at pH 8.5. The reaction was terminated by the addition of excess N-ethylmaleimide and applied to a polyacryl-

amidе gel containing SDS under non-reducing conditions. Fig.

2 shows that unreduced coat protein runs as two bands, which probably correspond to cyclic pentamers and hexamers. This interpretation is consistent with the formation of disulfide bonds between polypeptide chains at the 5-fold and 3-fold (qua-

si-6-fold) axes of the T = 3 icosahedral virus particle. These species presumably take the form of 5-membered and 6-mem-
bered coat protein rings because of the arrangement of coat proteins around the symmetry axes. This pattern of disulfide bonding was observed previously in Qb and, while this work was in progress, in the structure of the PP7 phage itself (5–7).

Partial reduction causes the appearance of two new bands, which migrate slightly more slowly than the completely unre-
duced cyclic pentamer/hexamer species. These probably repre-
sent the linearization of the cyclic pentameric and hexameric coat species by reduction of a single disulfide bond. Further reduction results in the appearance of species whose molecular sizes correspond to monomer, dimer, trimer, and tetramer. A DTT concentration of 10 mM results in nearly complete reduc-
tion during the 60-min time course of the reaction.

We tried several disaggregation protocols, including one in

which we employed the minimum concentration of acetic acid (20%) that leads to PP7 disassembly as assessed by agarose gel electrophoresis of virus-like particles. However, none of these resulted in a high yield of active protein upon dialysis against 1 mM acetic acid (as assessed by RNA-excess titrations), even when disulfide bonds were reduced (results not shown). The method we eventually settled on uses denaturation in 7 M urea in the presence of 10 mM DTT at pH 8.5 followed by dialysis against 1 mM acetic acid, 1 mM DTT. RNA-excess binding curves show that this procedure results in recovery of about 25–50% active protein,

| pUCter3 | pCT119 | pQCT | pP7CTNcXb |
|---------|--------|------|-----------|
| 100     | 87     | 100  | 7         |
| 100     | 31     | 3    | 19        |
| 100     | 2      | 74   | 72        |

Table 1

Relative β-galactosidase production by bacteria containing the indicated plasmids

The plasmids listed on the left, pCT119, pQCT, and pP7CTNcXb, produce MS2, Qb, and PP7 coat proteins, respectively. The plasmids called pRZP7, pRZQ5, and pRZ5 contain the PP7, Qb, and MS2 operators fused to lacZ. The values shown are percentages of the activities found in the non-repressing (pUCter3) controls.
assuming the stoichiometry of one RNA binding site per coat protein dimer typical of the other coat proteins characterized so far. The RNA binding activity of these preparations is stable for at least a few weeks when stored at 4 °C.

**RNA Binding**—The concentration of active protein in a given preparation was determined by RNA-excess titrations in TMK buffer at pH 8.5 (19). To determine appropriate conditions for binding reactions, protein-excess titrations were then performed with RNA held at about 10 pm under a variety of conditions. Specifically, pH was altered over a range from 6.2 to 8.5 using MoMK and TMK buffers (see “Experimental Procedures”). KCl concentration was also varied from 40 mM to 1.0 M, and Mg$^{2+}$ was omitted from some reactions. None of these alterations had much effect on binding, and most of the conditions we used gave a dissociation constant close to 1 nM (Figs. 3 and 4). Binding curves generated in the absence of Mg$^{2+}$ were indistinguishable from those produced in the presence of 10 mM magnesium acetate (data not shown). The interaction was also essentially independent of pH over the range we tested. Relatively large changes in KCl concentration also had only modest effects on binding; $K_d$ varied hardly at all between 0 and 0.6 M. Even 1 M KCl resulted in only a 4-fold elevation of $K_d$.

That RNA binding by PP7 coat protein *in vitro* is specific for PP7 RNA was indicated by the failure of MS2 RNA to be tightly bound (Fig. 5).

**Mapping the RNA Binding Site of PP7 Coat Protein**—We previously mapped amino acid residues contributing to the RNA binding sites of MS2 and Qβ coat proteins by random mutagenesis, followed by selection of mutants that failed to repress β-galactosidase synthesis in the two-plasmid systems already described (9, 10, 13). A further screen for capsid assembly eliminated mutants whose repressor defects were consequences of failure to properly fold, and DNA sequence analysis identified the affected amino acid residues. This procedure resulted in the maps of the MS2 and Qβ sites shown in Fig. 6. They use overlapping but non-identical sets of amino acids, and in each case the binding site resides on the surface of the coat protein β-sheet. Because of the likelihood that the RNA binding site of PP7 also resides on the β-sheet, we adopted a site-directed mutational strategy to identify amino acids involved in RNA binding. At the outset our only guide to PP7 coat protein structure was its sequence homology with other coat proteins (14). On the basis of amino acid sequence alignments with proteins of known three-dimensional structure, we were able to identify amino acids likely to reside within each of the β-strands. In βE and βG, the alignments were sufficiently clear to allow us also to predict with reasonable confidence those amino acids that reside on the surface of the sheet and that therefore are potential RNA-contacting residues. We targeted the codons for each of these amino acids for randomization using the procedure given under “Experimental Procedures.” In βF, however, ambiguities in the sequence alignments made the assignment of surface residues uncertain. Since the side chains of neighboring amino acids project from opposite sides of the sheet, improper assignment of the register of the β-strand could result in mistaken assignment of the inside/outside arrangement of the amino acid side chains for the entire strand. Therefore, in βF we randomized the codons for every residue, reasoning that because substitutions oriented toward the hydrophobic core are unlikely to exert any direct effect on RNA binding, mutations that destroy translational repression by substituting such residues probably exert their effects by disrupting protein folding or stability. Mutants of this class should yield blue colonies and will not produce virus-like particles. On the other hand, residues whose substitution results in an RNA binding defect with retention of proper folding (*i.e.* blue colonies with production of capsids) must reside on the solvent-exposed surface of the β-sheet where they can directly alter contacts with RNA. The method we used for determining the presence of
virus-like particles in cell extracts is described under "Experimental Procedures," and the results are shown in Fig. 7. The results of mutational analysis, described in detail below, are summarized in Table II and correlated with coat protein structure in Fig. 6.

In βE we mutagenized the codons for residues Arg39, Thr41, Ser43, and Arg45. We found that Arg39 and Arg45 yielded substitutions resulting in the repressor-defective, assembly-competent phenotype, thus implicating these amino acids in the RNA binding site. The two other βE surface residues, Thr41 and Ser43, never gave mutants in this class despite repeated efforts to isolate them. All repressor-defective Thr41 and Ser43 mutants were also defective for assembly. However, sequence analysis of some randomly chosen repressor-competent clones taken from Thr41 and Ser43 mutagenesis experiments revealed that Thr41 can be replaced by arginine while Ser43 tolerates substitution by alanine. Thus, the identities of these residues are not crucial for RNA binding.

In βG residues, Val83, Ser85, and Thr89 are identified as contributors to the RNA binding site because substitution of these amino acids can result in the repressor-defective, assembly-competent phenotype (although we should note that no Ser85 substitutions gave normal capsid yields). No repressor-defective mutants that satisfied the capsid assembly criterion were found for Thr81 and Asp87. However, sequence analysis of several repressor-competent clones randomly selected from the mutant library shows that RNA binding tolerates replacement of Thr81 by serine, while Asp87 can be replaced by asparagine, serine, or cysteine. Because of the close similarity of threonine and serine, we cannot state with confidence that Thr85 is not a constituent of the binding site.

TABLE II

| Translational repression by the PP7 repressor-defective mutants of β-galactosidase synthesis from pRZP7 |
|---------------------------------------------------------------|
| The values shown are the amounts (percent) of enzyme activity compared to a non-repressing control (pUCter3). |

| Mutant     | Relative Activity |
|------------|------------------|
| pUCter3    | 100              |
| PP7 WT     | 7                |
| R39L       | 17               |
| R45V       | 18               |
| R45P       | 30               |
| R45S       | 17               |
| A52L       | 20               |
| A53P       | 74               |
| R54H       | 44               |
| R54M       | 100              |
| R54L       | 82               |
| R54T       | 45               |
| R54F       | 86               |
| R54E       | 80               |
| K58N       | 100              |
| K58N       | 52               |
| K58H       | 27               |
| D60Y       | 19               |
| V83A       | 25               |
| V83Y       | 58               |
| V83L       | 29               |
| V83H       | 27               |
| S85A       | 48               |
| S85T       | 29               |
| S85Q       | 37               |
| T89A       | 64               |
| T89W       | 100              |
| T89F       | 89               |
| T89V       | 62               |

Fig. 6. A schematic representation of the structural locations of amino acids substituted in repressor-defective, assembly-competent mutants of MS2 (A), Qβ (B), and PP7 (C).
In $\beta_F$, where we mutated every amino acid, we never found an assembly-competent repressor defect affecting any of the odd-numbered residues, 51, 53, 55, 57, 59, or 63. On the other hand, we identified a number of even-numbered residues in $\beta_F$ whose substitution resulted in the repressor-defective, assembly-competent phenotype. These results argue that the side chains of the odd-numbered amino acids of $\beta_F$ reside in the core of the protein where they cannot contact RNA, thus identifying the even-numbered amino acids as residing on the solvent-exposed side of the sheet. This assignment has now been verified in the x-ray structure of PP7 (5). Because they gave repressor-defective, assembly-competent mutants, residues Ala$^{52}$, Arg$^{54}$, Lys$^{58}$, and Asp$^{60}$ are identified as constituents of the RNA binding site. Residues Asn$^{56}$ and Ala$^{62}$, although they must reside on the RNA binding surface, did not yield repressor-defective, assembly-competent mutants. Furthermore, sequence analysis of randomly selected repressor-competent clones shows that Asn$^{56}$ tolerates substitution by lysine, and that Ala$^{62}$ tolerates threonine, arguing that they play no crucial role in RNA binding.

The results of our mutational analyses are illustrated in Fig. 6, where a map of residues whose substitution specifically affects RNA binding is shown superimposed on a schematic of the coat protein $\beta$-sheet.

**DISCUSSION**

Our experiments demonstrate that the RNA hairpin shown in Fig. 1 is indeed a target for binding by PP7 coat protein. It is a functional translational operator in vivo (Table I) and is bound with a $K_D$ of about 1 nM in vitro (Fig. 5). Thus, it seems that, like the other RNA phages, PP7 probably utilizes coat protein to translationally repress replica synthesis.

The structure of the PP7 operator differs significantly from those of other RNA phages characterized previously, and neither the MS2 nor Q$\beta$ coat proteins efficiently repressed translation from the PP7 operator. Moreover, PP7 coat protein showed little ability to repress the operators of these other phages. Thus, the interaction is specific and represents an example of the ability of the coat protein structural framework to adapt by mutation to the binding of different RNAs. Future studies will determine which features of RNA structure are necessary for interaction with PP7 coat protein.

The protein-RNA interaction shows little dependence on salt concentration over the range of 40 mM to 1 M, suggesting that electrostatic interactions are relatively unimportant in stabilizing the PP7 RNA-protein complex. This is a little surprising, given that the RNA binding surface of PP7 coat protein has four basic residues (three arginines and one lysine) and that translational repression is sensitive to substitution at these sites. Moreover, RNA binding by other coat proteins is sensitive to salt. For example, MS2 coat protein, which utilizes five ion pair contacts in its complex with RNA, shows a significant dependence on salt concentration. Its $K_D$ for operator RNA increases about 40-fold as the KCl concentration approaches 1 M (19). GA and Q$\beta$ coat proteins show similar dependences on salt concentration (20, 21).

Changing pH over the range 6.2–8.5 also had little effect, indicating that RNA binding is not substantially affected by protonation or deprotonation of any groups with $pK_a$ values in this range. Other coat proteins so far characterized exhibit a variety of behaviors with respect to pH. MS2 and GA have broad pH optima centered around pH 7.0 and show only small changes in binding affinity between pH 6.5 and pH 8.0 (19, 20).

Above pH 8.0 the binding affinities of MS2 and GA begin to fall, behavior that has been attributed, at least in the case of MS2, to the deprotonation of Tyr$^{85}$ (22). In the crystal structure of the MS2 RNA-protein complex, Tyr$^{85}$ is H-bonded through its phenolic hydroxyl to the phosphate of U-5. Substitution with phenylalanine abolishes this pH dependence. RNA binding by Q$\beta$ coat protein also exhibits a broad optimum, but it is centered around 6.0 and drops precipitously above pH 8 (21). Q$\beta$ coat protein has tyrosine at the position 85 equivalent, but the details of its interactions with RNA are as yet unknown. However, this tyrosine presumably participates in binding since substitution with histidine reduces its affinity for RNA about 5-fold (10). PP7 contains valine at the position 85 homologue.

Our mutational analyses provide a map of the PP7 coat protein RNA binding site (Fig. 6). However, it should be noted that this could be an incomplete map for reasons that include the following.

(i) Any amino acids that might contribute to the binding site but not targeted for substitution are naturally omitted from this analysis. We directed mutations to amino acids whose structural locations made them likely candidates for RNA-contacting residues, and we cannot rule out the possibility that other amino acids, for example those in the loops that connect $\beta$-strands, or in $\beta_C$ or $\beta_D$, might play a role in RNA binding.

(ii) We also point out that substitution of a non-RNA-contacting residue might sometimes cause a failure to bind RNA. For example, a substitution may result in the introduction of a larger amino acid than wild type, thus interfering sterically with RNA binding. However, at only three positions (Ala$^{52}$, Asp$^{60}$, and Val$^{63}$) did we fail to isolate substitutions whose side chains are smaller than the wild-type residue. It is also possible that the substituted amino acid could alter the conformation of a true RNA-contacting residue, affecting RNA binding secondarily. These limitations are inherent in the approach utilized here and emphasize the importance of structural studies as a companion to genetics and biochemistry.

(iii) Since our method includes a screen for assembly of ethidium bromide stainable capsids, any amino acid whose substitution always results in a folding or stability defect, or which causes failure to encapsidate host RNAs, would be invisible to this analysis. However, none of the sites we targeted in the coat protein surface failed to yield at least one mutant that satisfied this requirement.

(iv) Any mutations that were to increase the nonspecific affinity of coat protein for RNA might produce a repressor-defective phenotype by increasing the competitive effects of host RNAs.

It is instructive to compare the binding site of PP7 with that of MS2, whose complex with RNA is understood in great detail and was dealt with in a recent review (23). Many of the amino acids that make up the two sites are located in homologous positions on their respective $\beta$-sheets. However, only 5 of the 15
amino acids on the surfaces of the E, F, and G $\beta$-strands of the two proteins are identical (Table III). Three of these are at positions 41, 43, and 58 (PP7 numbering), amino acids whose identities are highly conserved among coat proteins generally. In MS2 it is easy to understand their significance to the interaction with RNA; these amino acids are involved in important interactions with A-10 and A-4 in the translational operator. However, our results show that translational repression by PP7 is tolerant of substitution of two of these residues, Thr$^{41}$ and Ser$^{43}$. Given the striking divergence of their RNA binding site sequence requirements, and considering the obvious differences in their translational operators, it seems likely that MS2 and PP7 use somewhat different modes of RNA binding.

Of course it is important to remember that the maps shown here are, in actuality, composites of two sites. The 2-fold symmetry of the dimer means that the RNA ligand may bind in either of two orientations. The makeup of a single, asymmetric site has already been determined for MS2 on the basis of heterodimer complementation experiments and by direct structural analysis of the RNA-protein complex, but similar information is not yet available for PP7.

It is striking that the coat protein-operator RNA interaction is conserved across a broad evolutionary spectrum of RNA phages. Thus, the translational repressor and its RNA target represent members of a co-evolving pair; mutations in one are compensated for by changes in the other so as to preserve the interaction. Clearly, retention of this RNA-protein interaction is important to these phages generally. This is also indicated by the observation that, although the coat protein-operator RNA interaction apparently is not absolutely required for virus viability, MS2 mutants with operator-inactivating mutations suffer a growth disadvantage compared with wild-type (24, 25). Possible explanations for the importance of this interaction include the following. (i) Replicase overproduction might reduce virus burst size by unnecessarily taxing the protein synthetic apparatus of the cell. (ii) The interaction may aid in recognition of viral RNA for selective encapsidation. (iii) Translational repression might serve to clear the replicase cistron of ribosomes, thereby facilitating genome replication or packaging. Any or all of these explanations may apply, as well as others we have not yet imagined.

The RNA phage coat proteins provide a structural platform upon which a diversity of RNA structures can be bound. Although the translational operators of the various RNA phages share some common features (i.e. they are all stem-loop structures with bulged adenosines), the length of the stem, the size of the loop, and the position and importance of the bulge vary. For at least one coat protein-RNA interaction (Q$\beta$), deletion of the bulge has only a modest effect on binding, whereas, for others (MS2 and GA), the bulge is essential. Although at present it is unknown how far the RNA binding specificity of coat protein can be made to diverge from these natural examples, we suggest that the coat protein $\beta$-sheet presents a surface adaptable to the binding of many different RNAs, some of which could differ substantially from any of the natural RNA targets so far characterized. Work currently under way seeks to address this issue.

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