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Altering the RNA Binding Specificity of a Translational Repressor*

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The coat proteins of RNA phages MS2 and GA are specific RNA-binding proteins which function to encapsidate viral RNA and to translationally repress synthesis of the viral replicase. The two proteins have highly homologous amino acid sequences, yet they show different RNA binding specificities, recognizing RNA stem-loop structures which differ primarily in the nucleotide sequences of their loops. We sought to convert MS2 coat protein to the RNA binding specificity of GA through the introduction of GA-like amino acid substitutions into the MS2 coat protein RNA-binding site. The effects of the mutations were determined by measuring the affinity of the coat protein variants for RNA *in vitro* and by measuring translational repression *in vivo*. We found five substitutions that affect RNA binding. One dramatically reduces binding of MS2 coat protein to both operators. Three others compensate for this defect by nonspecifically strengthening the interaction. Another substitution accounts for the ability to recognize the differences in the RNA loop sequence.

The structural motifs and the molecular interactions that allow proteins to recognize specific RNA molecules are still poorly understood. The RNA bacteriophage MS2 offers a convenient model system for the study of RNA-protein interactions, since its coat protein specifically interacts with a stem-loop structure in viral RNA to repress translation of the replicase cistron and to nucleate encapsidation of the RNA genome. Genetic and biochemical approaches have identified the RNA-binding site within the known three-dimensional structure of the MS2 coat protein (1). The RNA structural determinants of this interaction have also been determined (2). Thus, although we are now in possession of structural models for the coat protein RNA-binding site and for its RNA ligand, as yet we have no picture of how the two molecules interact in the RNA-protein complex.

MS2 and GA are related RNA phages. Their coat proteins are highly homologous, showing about 62% amino acid sequence identity (3). Despite their obvious relatedness, however, the two proteins have somewhat different RNA binding specificities. They bind RNA stem-loop structures which are highly similar, but differ by two nucleotide substitutions in the loop and by two substitutions in the stem (see Fig. 1). MS2 coat protein requires a pyrimidine at position −5 in the loop and thus binds the GA operator poorly, because it contains adenine at this position. GA coat protein, on the other hand, is relatively indifferent to this nucleotide substitution and binds both the GA and MS2 operators with similar affinities *in vitro* (see Ref. 4 and Table I). The stem substitutions create the potential for two interconvertible secondary structures in which the position of the bulged adenosine is shifted as shown in Fig. 1. It has been reported that GA coat protein is tolerant of this alternative bulge placement and that MS2 is not (4). These differences in specificity must be conferred by differences in amino acid sequence between the two proteins. Identification of amino acid substitutions that confer different RNA binding specificities may identify sites on coat protein which contact specific elements of RNA structure in the operator.

EXPERIMENTAL PROCEDURES

Bacteriophage GA was a gift of A. Hirashima (Keio University, Tokyo). The virus was grown and purified by standard methods (3), and RNA was extracted from the phage particle using phenol/chloroform. To clone the GA coat sequence, GA RNA was subjected to reverse transcription using a synthetic oligonucleotide primer complementary to a sequence downstream of the GA coat sequence. The coat portion of the cDNA product was amplified using polymerase chain reaction (5) with *Taq* DNA polymerase using primers that bounded the coat sequence and that introduced a *Kpn*I site about 45 nucleotides upstream of the coat initiation codon and a *Xba*I site about 20 nucleotides downstream of the termination codon. The GA sequence was cloned into pUC118 between its *Kpn*I and *Xba* I sites to produce the plasmid called pGACT. It directs the synthesis of GA coat protein under control of the *Lac* promoter.

The plasmid called pGM1 is shown in Fig. 4*A*. It contains both the GA and MS2 sequences with a unique *Xba*I site between them. Recombination between the MS2 and GA sequences was accomplished by digestion of pGM1 with *Xba*I followed by transformation of *Escherichia coli* strain CSH41F$^-$ with the linearized DNA. Individual transformants were picked into 1 ml of LB medium, grown overnight at 37 °C, and plasmids were prepared by the method of Birnboim and Doly (6). Restriction mapping identified the approximate locations of the recombination junctions, and these were later precisely identified by DNA sequence analysis.

Nucleotide substitutions were introduced into the MS2 coat sequence using the method of Kunkel *et al.* (7). The template was single-stranded DNA derived from pCT119 (1, 8). Mismatched oligonucleotide primers were synthesized on the Applied Biosystems model 390 DNA synthesizer.

To determine their RNA binding properties *in vitro*, MS2 and GA coat proteins were produced from pCT119 and pGACT and purified as described previously (8). The plasmid pROP5 contains a cloned copy of the wild-type MS2 operator sequence positioned downstream of a promoter for T7 RNA polymerase. Digestion of the plasmid with *Bam*HI creates a template for production of run-off transcripts 45 nucleotides in length. Plasmid pROP6 is identical to pROP5 except for two nucleotide substitutions in the translational operator which convert it to the GA RNA loop sequence (see Fig. 1). These plasmids were the sources of ^{32}P-labeled operator RNA produced by run-off transcription *in vitro* (9) for use in filter binding analyses performed as described by Carey *et al.* (10).

In order to test the effects of alternative placement of the bulged A duplex, DNA representing the variant operator sequence shown in Fig. 1 was synthesized (ROPG). The corresponding RNA should fold so as to unambiguously move the bulged A to the position characteristic of the alternative conformation of the GA operator. This synthetic operator

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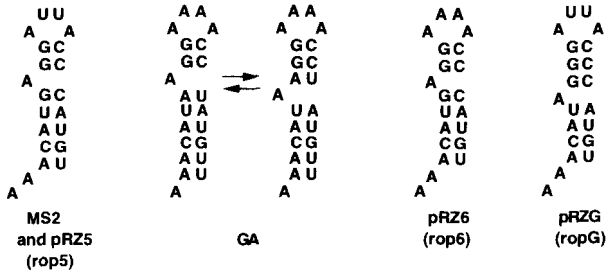

FIG. 1. **The sequences and predicted secondary structures of the various translational operators used in this study.**

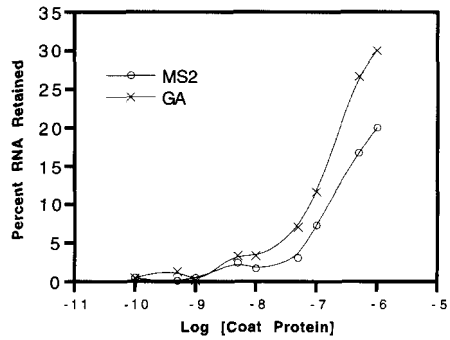

FIG. 2. **Binding of the wild-type MS2 and GA coat proteins to ROPG RNA.**

sequence was used in the construction of pRZG for translational repression measurements *in vivo* and in pROPG for production of operator RNA by transcription *in vitro*.

Assays of β-galactosidase activity were performed as described by Miller (11) using strain CSH41F⁻ containing pRZ5 or pRZ6 and the appropriate pCT119 mutant. Repressor efficiency is expressed as the fold difference between the repressed and unrepressed enzyme levels. The unrepressed state was represented by CSH41F⁻ containing either pRZ5 or pRZ6 and pUCter3 (8), a plasmid which expresses no coat protein. The values shown in Table II are the averages of three measurements.

RESULTS

The Effects of Alternative Placements of the Bulged A on Binding by MS2 and GA Coat Proteins—The GA operator seems capable of adopting two alternative conformations which differ in the position of the bulged A residue (see Fig. 1). It was reported previously that GA coat protein is able to bind both forms of the RNA (4) whereas MS2 coat protein is not. We set out to determine the protein structural differences between GA and MS2 coat proteins that account for this apparent difference in RNA binding specificity. To do so we synthesized a duplex oligonucleotide encoding the operator shown in Fig. 1 and constructed pRZG for measurements of translational repression *in vivo* and pROPG for synthesis of the operator RNA by transcription *in vitro*. The results are shown in Fig. 2. Neither coat protein was capable of binding this operator with high affinity. The dissociation constants could not be determined exactly, since over the range of protein concentrations used in this experiment saturation of the RNA was not achieved. However, it is clear that neither dissociation constant is less than about 10^{-6} to 10^{-7} M. Consistent with the *in vitro* result, neither MS2 nor GA coat protein repressed translation from pRZG *in vivo* (results not shown). We do not know how to explain this apparent discrepancy between our results and those reported previously (4). We point out, however, that the operators used in the previous study incorporated additional nucleotide substitutions, raising the possibility that other differences in RNA structure may account for this disagreement. Since the dissociation constants are at least 100-fold higher than for the interactions of either protein with their homologous operators, however, we did not investigate further the bindng of the coat proteins to this operator variant.

Effects of the Operator Loop Sequence on the RNA Binding and Translational Repressor Properties of GA and MS2 Coat Proteins—Fig. 1 summarizes the differences between the MS2 and GA translational operators. In order to restrict our analysis to interactions of coat protein with the RNA loop, we synthesized two operators. Both have the MS2 stem sequence, but differ in the sequences of their loops. We inserted the operator containing the MS2 loop sequence into the plasmids called pRZ5 and pROP5. The operator with the GA loop sequence was inserted into plasmids pRZ6 and pROP6. The plasmids pRZ5 and pRZ6 were described previously (8) and link their respective translational operators to the *E. coli Lac* Z gene, making it possible to measure translational repression of β-galactosidase synthesis by coat protein *in vivo*. In plasmids pROP5 and pROP6, these same operators have been linked to the phage T7 RNA polymerase promoter in order to produce radiolabeled operator RNA by run-off transcription *in vitro* (9).

RNA binding affinities of the MS2 and GA coat proteins were measured by determining their abilities to retain ^{32}P-labeled operator RNA (10) on nitrocellulose filters (11). The results shown in Fig. 3 and summarized in Table I confirm the previous report that GA coat protein has relaxed specificity relative to MS2 *in vitro* (4). It binds both the pROP5 and pROP6 operators with affinities that differ by only severalfold ($K_d = 6.3 \times 10^{-9}$ M and $K_d = 2.3 \times 10^{-8}$ M, respectively). MS2 coat protein, on the other hand, binds its homologous pROP5 operator 100-fold more tightly than the GA-like pROP6 operator ($K_d = 3 \times 10^{-9}$ M *versus* 3×10^{-7} M).

Measurements of the *in vivo* translational repression efficiencies show that each coat protein prefers to repress its homologous operator. In other words, *in vivo* the two coat proteins show reciprocal specificities. Clearly, the conditions of our *in vitro* experiments do not mimic exactly the intracellular conditions under which RNA binding and translational repression naturally occur. However, the *in vivo* and *in vitro* results agree in the sense that in both assays the coat proteins show specificity based on the operator loop sequence.

Localizing the Determinants of Loop Binding Specificity Using GA-MS2 Recombinant Coat Proteins—We took advantage of the approach shown in Fig. 4*A* to produce GA-MS2 recombinant coat sequences. The aim was to construct hybrid coat proteins with either MS2 or GA RNA binding specificity, thus localizing the determinants of specificity. The method we used has been described before and promotes efficient recombination between homologous sequences (12). It relies on the fact that linearized plasmids transform *E. coli* inefficiently and must somehow circularize in order to replicate. The relatively small numbers of transformants obtained in this way are frequently the results of recombination at regions of homology. The plasmid pGM1 contains a copy of the GA coat protein sequence upstream of the MS2 coat protein coding sequence. A unique *Xba*I site is situated between them. After digestion with *Xba*I, pGM1 was introduced into strain CSH41F⁻ (11). Restriction analysis of the resulting recombinants revealed that in many clones recombination had produced hybrid GA-MS2 sequences. Subsequent nucleotide sequence analysis showed that recombination had occurred predominantly at two locations. Apparently recombination was not random, but occurred at hot spots, since only these two types were found among the half-dozen we sequenced. One type of recombination event occurred in such a way that the resulting sequence corresponded almost entirely to MS2 coat protein. Naturally these recombinants showed the translational repression properties of MS2. The second class of recombinants resulted in hybrid molecules in which sequences

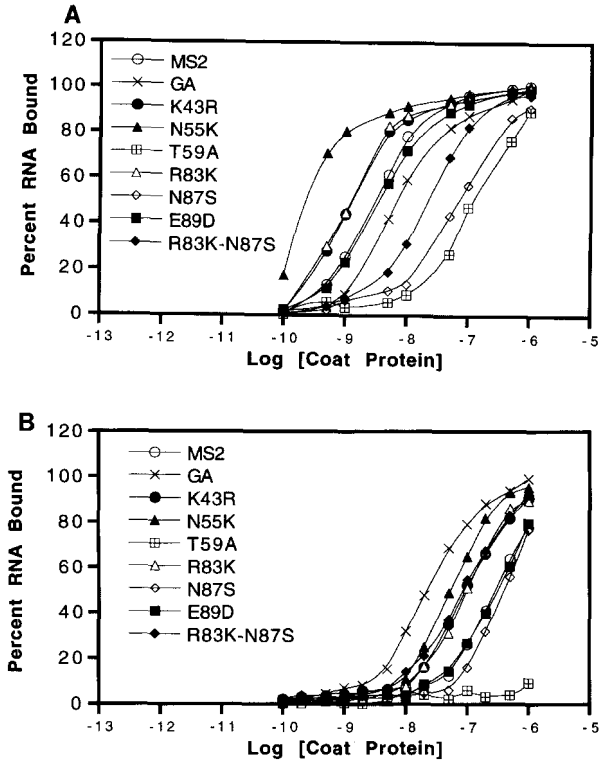

FIG. 3. *A*, binding of ROP5 RNA (*i.e.* the wild-type MS2 translational operator) to MS2 and GA coat proteins and to the various MS2 mutants described in the text. *B*, binding to ROP6 of the same proteins as in *A*.

TABLE I

K_d values for the interaction of the various coat proteins with the rop5 (wild-type MS2) and rop6 (GA-like loop) operators shown in Fig. 1

Also shown are the ratios of the K_d values of individual proteins for the two RNAs.

Repressor	K_d		
	rop5	rop6	rop6/rop5
		M	
MS2	3.0×10^{-9}	3.0×10^{-7}	100
GA	6.3×10^{-9}	2.3×10^{-8}	3.7
K43R	1.0×10^{-9}	1.0×10^{-7}	100
N55K	2.0×10^{-10}	5.0×10^{-8}	250
T59A	1.2×10^{-7}	$>10^{-6}$	ND[a]
R83K	1.0×10^{-9}	1.0×10^{-7}	100
N87S	6.3×10^{-8}	4.0×10^{-7}	6.4
E89D	3.0×10^{-9}	3.0×10^{-7}	100
R83K-N87S	2.0×10^{-8}	8.5×10^{-8}	4.3

[a] ND, not determined.

upstream of amino acid 83 were derived from GA, whereas those downstream of this site were from MS2. One of these recombinants, pGXM10, was tested for its RNA binding specificity by assessing its ability to repress translation of β-galactosidase synthesis from pRZ5 (MS2 operator) and pRZ6 (GA-like operator). The results are shown in Table II in the form of fold repression values. The pGXM10 protein shows a repressor specificity very similar to that of MS2 coat protein, even though most of its sequence is derived from GA. Inspection of the structure of MS2 coat protein (13) shows that in the pGxM10 hybrid protein most of the RNA-binding site is contributed by GA, with only β-strand G coming from MS2 (see Fig. 4*C*).

Converting the MS2 RNA-binding Site to GA-like Specificity by Site-directed Mutagenesis—We assume that the MS2 and GA coat proteins are structurally homologous. This is almost certainly the case, since they have 62% identical amino acid sequences. Similarity of tertiary structure is also suggested by their abilities to co-assemble into a hybrid virus-like particle

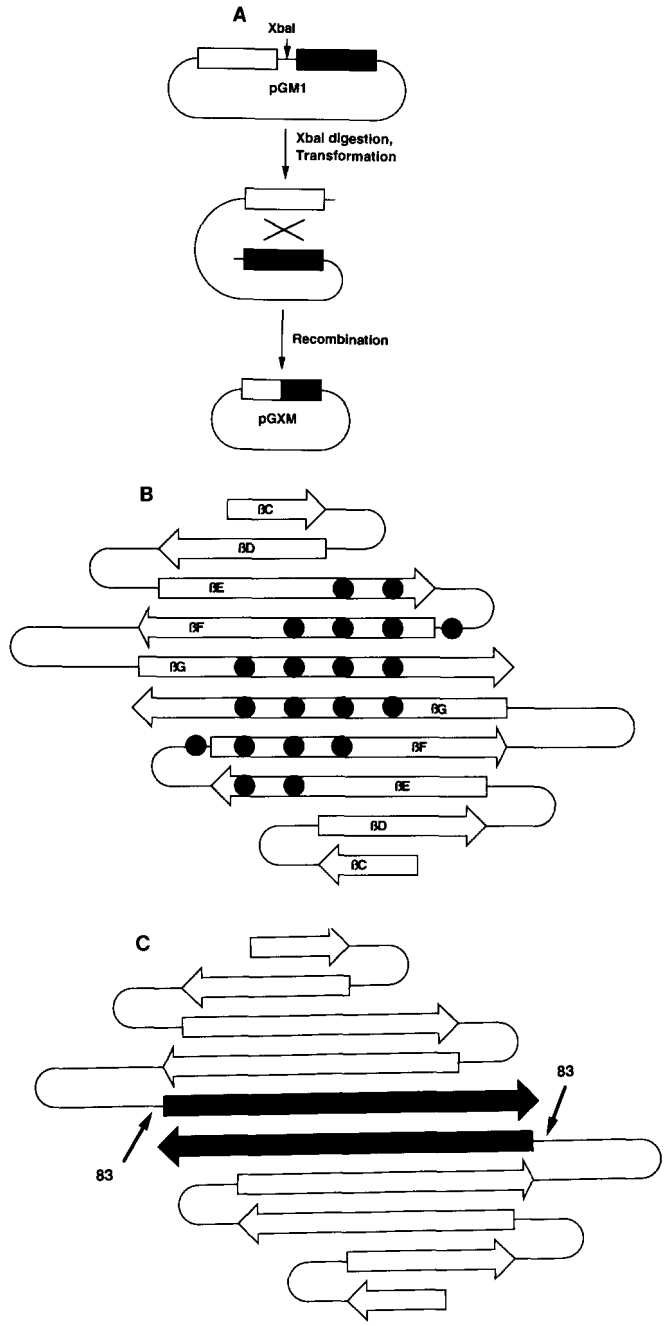

FIG. 4. *A*, the structure of pGM1 and the procedure used for the production of recombinant GA/MS2 coat sequences. *B*, a schematic drawing of the β-sheet of the symmetric MS2 coat protein dimer. Residues making up the RNA-binding site reside on three adjacent β-strands of each monomer. Their positions are indicated here by the *filled circles*. *C*, in the recombinant encoded by pGxM10, a hybrid coat protein is produced. It is comprised of GA sequences, shown in *white*, and MS2 sequences, shown in *black*. The recombination junction is at amino acid residue 83.

when expressed together in the same cell from pGM1.[1] We also assume that as with MS2 the amino acid residues involved in RNA binding reside on the surface of the GA coat protein β-sheet. Only 6 amino acid substitutions are found when comparison of the sequences of the two coat proteins is restricted to residues whose side chains reside on the solvent-exposed surface of the three β-strands that make up the binding site. These are listed in Table III. Presumably, changing some or all of

[1] D. S. Peabody, unpublished results.

TABLE II

Repression of β-galactosidase synthesis caused by the various repressor variants binding the translational operator of pRZ5 or pRZ6

-Fold repression values were calculated as the amount of β-galactosidase activity produced in the unrepressed state divided by that produced when repressed by the repressors listed on the left. β-Galactosidase levels were also assessed as relative blueness of colonies on 5-bromo-4-chloro-3-indoyl β-D-galactoside (X-gal) plates.

Repressor	-Fold repression		Blueness on X-gal	
	pRZ5	pRZ6	pRZ5	pRZ6
MS2	92	12	+–	+++
GA	18	39	+	+–
GXM10	56	6	–	++
K43R	85	17	–	+
N55K	330	19	–	+–
T59A	8	1	++	+++
R83K	430	38	–	+–
N87S	8	12	++	++
E89D	56	5	+–	++
R83K-N87S	20	24	+	+–

TABLE III

Amino acid substitutions that distinguish the RNA binding sites of MS2 and GA coat proteins

Position	MS2	GA
43	Lys (K)	Arg (R)
55	Asn (N)	Lys (K)
59	Thr (T)	Ala (A)
83	Arg (R)	Lys (K)
87	Asn (N)	Ser (S)
89	Glu (E)	Asp (D)

these 6 amino acids in MS2 to their GA counterparts should confer GA RNA binding specificity. Moreover, the experiments described above with hybrid GA-MS2 coat proteins suggest that the specificity determinants may reside in β-strand G. Therefore, GA specificity might be conferred to MS2 coat protein by introducing no more than the 3 amino acid substitutions at residues 83, 87, and 89. Note that some of the GA substitutions are in sites we have shown previously to be important components of the RNA-binding site of MS2 coat protein (1).

Each of the single amino acid substitutions shown in Table III was introduced into the MS2 sequence. The abilities of the wild-type MS2 and GA proteins and the various MS2 mutants to repress translation were determined by measurements of β-galactosidase activity in strains containing either pRZ5 (MS2 operator) or pRZ6 (GA-like operator). The results are summarized in Table II. As we have already shown each of the wild-type proteins clearly prefers to repress its homologous operator. One of the substitutions, E89D, has little effect on repressor efficiency. This mutant is only slightly repressor-defective. T59A is clearly repressor-defective for both the pRZ5 and pRZ6 operators. Three of the substitutions, K43R, N55K, and R83K, result in a super-repressor phenotype, repressing both pRZ5 and pRZ6 better than the wild-type MS2 protein. (Note that in this particular experiment K43R is not obviously a better repressor that wild-type, but comparison of colony color on 5-bromo-4-chloro-3-indoyl β-D-galactoside plates indicates that this is so.) N87S is the one substitution that shows a clear operator-specific effect. This substitution renders the MS2 coat protein defective for repression of the MS2-like operator of pRZ5, but has little or no effect on its ability to repress the GA-like operator of pRZ6. In other words the N87S substitution confers on MS2 coat protein a tolerance for the nucleotide substitutions characteristic of the GA operator loop.

Bearing in mind the differences we have already noted between the *in vitro* and *in vivo* data for the two parental proteins the results of filter binding experiments correlate with the repression efficiencies. The binding curves are shown in Fig. 3, and the K_d values are listed in Table I. First, it is clear that the E89D substitution has little or no effect on the ability MS2 coat protein to bind either operator and that T59A is dramatically reduced relative to wild-type in its ability to bind these RNAs. Consistent with their super-repressor phenotypes, three substitutions, K43R, N55K, and R83K, result in the tighter binding of both operators. Again, N87S seems to be the key substitution in defining the specificity differences between the two proteins. Its K_d for the pROP5 operator is increased more than 20-fold compared with the wild-type MS2 protein (6.3×10^{-8} *versus* 3.0×10^{-9} M), but its K_d for the pROP6 operator is hardly increased at all (4.0×10^{-7} *versus* 3.0×10^{-7} M). Moreover, MS2 coat protein shows a 100-fold increased K_d for the pROP6 operator compared with pROP5 (3.0×10^{-7} *versus* 3.0×10^{-9} M), but the N87S mutant shows only a 6.7-fold increase (6.3×10^{-8} *versus* 4.0×10^{-7} M). These results are consistent with the idea that a contact with the loop is lost in N87S so that the identity of the nucleotide at position –5 becomes relatively unimportant for binding *in vitro*.

In the pGXM10 recombinant, the introduction of β-strand G of MS2 coat protein confers MS2-like RNA binding specificity to a site that is otherwise derived from GA (see Fig. 4*C*). This being the case, we wondered whether the combined effects of the two β-strand G substitutions, R83K and N87S, might convert MS2 coat protein to GA operator binding specificity. In the *in vivo* translational repression assay, the double mutant displays some of the characteristics of GA coat protein, although it does not quite achieve the ability of GA to distinguish the two operators (Table II). *In vitro* the double mutant exhibits the GA-like indifference to the RNA loop substitutions and binds both RNAs more tightly than the N87S mutant, although it does not quite achieve the tightness of binding of GA coat protein.

DISCUSSION

We used two basic strategies to localize the determinants of MS2 and GA RNA binding specificity. The first involved the utilization of homologous recombination in the construction of hybrid GA/MS2 coat proteins. We found that the presence of MS2 sequences COOH-terminal to residue 82 was sufficient to confer MS2-like behavior to the recombinant protein. The significance of this observation is made clearer by inspection of the structure of the RNA-binding site of MS2 coat protein as it has been defined by x-ray crystallography (14) and by genetic and biochemical analysis (1). The binding site is shown diagrammatically in Fig. 4*B*. It resides on the surface of an extensive β-sheet and involves at least three β-strands from each of the two monomers. Substitution of amino acids at any of 10 sites on these adjacent β-strands can result in failure to bind RNA (1). Notice that residue 82 is the last amino acid before β-strand G, the most COOH-terminal of the β-strands present within the binding site. Since in pGxM10 all residues carboxyl-terminal to residue 82 are derived from the MS2 sequence, it appears that MS2 repression specificity has probably been conferred to an otherwise GA-like molecule by substitution of β-strand G (see Fig. 4*C*). Since the only differences in the pRZ5 and pRZ6 operators occur in the operator loop, residues in β-strand G may interact with nucleotides there.

The second strategy relied on site-directed mutagenesis to introduce specific GA-like substitutions into the MS2 sequence. The results of these experiments show that the RNA-binding site of GA coat protein has been extensively remodeled compared with that of MS2. Of the five substitutions that make a difference in binding properties, one of them (T59A) results in a generalized defect in RNA binding. Others (K43R, N55K, and R83K) bind both operator RNAs more tightly. Another (N87S) causes a specific defect for the MS2 operator, but little effect on

the already poor ability of MS2 coat protein to bind the GA-like operator. Thus the main determinant of the difference in GA and MS2 specificity seems to be the N87S substitution. It accounts for the relative indifference to the RNA loop substitutions that characterize the *in vitro* RNA binding behavior of GA coat protein. Of course the loss of this contact causes a reduced affinity for MS2 operator RNA, and, as with T59A, the negative effects of the substitution are apparently compensated by the super-repressor mutations K43R, N55K, and R83K.

We imagined that full GA specificity might be conferred to the MS2 molecule by the introduction of all six GA-like substitutions. When we introduced all six changes, however, the stability and/or folding properties of the protein were apparently perturbed, since this mutant failed to repress translation from either operator and was unable to assemble into virus-like particles (results not shown). Implicit in our original experimental design was the assumption that the GA and MS2 molecules achieve their respective RNA binding specificities by incorporating a few different surface amino acids on an otherwise identical structural framework. Indeed, we have so far ignored the potential roles of residues outside the solvent exposed portions of β-strands E, F, and G. Eventually the importance of other residues will have to be taken into account, including the possibility that amino acids within the hydrophobic core of the protein influence the shape of the binding site.

The results of our experiments suggest that Asn-87 may form an interaction with the RNA loop in the wild-type MS2 complex. This is the most straightforward interpretation of our results, but not the only one possible. For example, Asn-87 may contact a site in the RNA whose conformation is altered when loop nucleotides are substituted. In other words, the structural effects of nucleotide substitutions in the operator loop may not be confined to the actual sites of substitution. Alternatively, residue 87 may affect the conformation of the relevant RNA-contacting amino acid residue. The resolution of these questions awaits the results of further experimentation and eventual determination of the structure of the RNA-protein complex.

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