
Mutations that increase the affinity of a translational repressor for RNA

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Received May 23, 1994; Revised and Accepted July 14, 1994

ABSTRACT

The coat protein of the RNA bacteriophage MS2 is a specific RNA binding protein that represses translation of the viral replicase gene during the infection cycle. As an approach to characterizing the RNA-binding site of coat protein we have isolated a series of coat mutants that suppress the effects of a mutation in the translational operator. Each of the mutants exhibits a super-repressor phenotype, more tightly repressing both the mutant and wild-type operators than does the wild-type protein. The variant coat proteins were purified and subjected to filter binding assays to determine their affinities for the mutant and wild-type operators. Each protein binds the operators from 3 to 7.5-fold more tightly than normal coat protein. The amino acid substitutions seem to extend the normal binding site by introducing new interactions with RNA.

INTRODUCTION

The coat protein of the bacteriophage MS2 is bifunctional. It serves both as the major structural protein of the virus particle and as the translational repressor that controls synthesis of the viral replicase. In performing these functions it binds a RNA stem-loop structure (the translational operator) which contains the replicase ribosome binding sequence, and which seems also to serve as the genomic packaging signal. The specific interaction of coat protein with RNA is a useful model for RNA-protein interactions in general. As such it has been the object of studies which have defined the RNA and protein structural requirements for binding to coat protein (1, 2). The identification of structural features of coat protein that participate in RNA binding has been aided by the development of a two-plasmid system in which coat protein expressed from one plasmid represses synthesis of a replicase- β -galactosidase fusion protein expressed from a second plasmid (3). This system permits the ready identification of coat mutants with altered repressor activities. We have employed it in the identification of amino acid residues that serve as components of the RNA-binding site (2).

We also isolated coat mutants which suppress the effects of a mutation in the translational operator (4). Most suppressors of this operator-constitutive (σ^c) mutation were also defective for

capsid assembly, and therefore accumulated higher levels of coat protein dimers. Each of these mutants had a super-repressor phenotype, repressing the wild-type and a variety of mutant operators better than the wild-type protein. This assembly-defective, super-repressor phenotype was also conferred when we engineered a deletion of 13 residues (called dIFG) from the 15 residue loop which connects the F and G β -strands and which the X-ray structure of MS2 shows to be important for interactions between dimers in the virus particle (5). These observations led us to propose that the assembly defects themselves, and not tighter RNA binding, were responsible for the super-repressor phenotypes of these mutants, and that they exert their effects by elevating the concentration of the repressor form (i.e. dimers) of coat protein. Only one of the amino acid substitutions we isolated in that study (V29I) resulted in super-repression without affecting virus assembly. We assumed this to be the result of tighter RNA binding.

In the present study we have isolated and characterized additional suppressors of the σ^c mutation. This time we used dIFG as the starting point for further mutagenesis so as to avoid isolating more assembly-defective mutations. As before, each of the mutants isolated by this approach is a super-repressor *in vivo*. We also show that each of the mutant proteins binds the wild-type and mutant translational operator RNAs more tightly *in vitro* than does the wild-type protein. The positions of the amino acid substitutions in the structure of coat protein are consistent with the idea that they extend the usual RNA-binding site by introducing new interactions with RNA.

MATERIALS AND METHODS

Mutagenesis of the dIFG coat sequence

The details of the two-plasmid genetic system used in screening for translational repressor mutants have been elaborated before (3). To allow for mutagenesis of both DNA strands, the dIFG mutant (4) was cloned as a *Hind*III-*Kpn*I fragment in both pUC118 and in pUC119 and single-stranded DNA was prepared after superinfection with M13KO7 (6). Random chemical mutagenesis was accomplished by treatment of single-stranded DNA with nitrous acid, formic acid, and hydrazine as described by Myers (7). After complementary strand synthesis using AMV

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reverse transcriptase and the universal M13 sequencing primer, the coat sequence was excised using *Hind*III and *Kpn*I and recloned into pUC119. The resulting library of mutations was introduced by transformation into strain DH5 α containing pRZ6 and plated on LB medium containing X-gal. Plasmid pRZ6 contains a translational operator that has been rendered defective for repression by the introduction of two nucleotide substitutions in the operator loop. Thus, it is an operator-constitutive (o^c) mutant. After about 24 h growth at 37°C white or pale blue colonies were picked for further analysis. Each mutant was subjected to DNA sequence analysis throughout the entire coding region to identify the nucleotide and predicted amino acid substitutions. Each of the resulting mutations was later introduced into the full-length (i.e. non-dIFG) coat sequence using site-directed mutagenesis performed by the method of Kunkel *et al.* (6).

Purification of proteins

Wild-type coat protein and mutants not disrupted for assembly were purified as described previously (3). The assembly-defective mutants (i.e. dIFG and mutants derived from it) were purified as follows. Cells were grown to saturation in 0.5 l of LB medium at 37°C, collected by centrifugation, and frozen at -70°C. After thawing, the cells were resuspended in 0.1 M NaCl, 0.05 M Tris-HCl (pH 8.5), 0.01 M EDTA, 0.001 M DTT, 0.5 mM PMSF, and disrupted by treatment with lysozyme at 0.2 mg/ml for 30 min at 0°C. Sodium deoxycholate was then added to a final concentration of 0.05% and the cell suspension was incubated for another 60 min at 0°C. This was then sonicated for three 1 min intervals. Nucleic acids were removed by precipitation with polyethylenimine (PEI) added to a final concentration of 0.2%. After 1 h on ice the precipitate was removed by centrifugation at 12000 rpm. Proteins in the supernatant were precipitated by addition of ammonium sulfate to 50% of saturation. After centrifugation the pellet was dissolved in 0.1 M NaCl, 0.01 M Tris-HCl (pH 7.5), 0.1 mM MgSO₄, 0.01 mM EDTA, and applied to a 1.5×45 cm column of Sephadex G-75. Fractions were analyzed by electrophoresis in polyacrylamide gels containing SDS (8). Fractions containing coat protein were pooled and the buffer replaced with 0.02 M sodium phosphate (pH 5.8) by diafiltration (Macrosep concentrator; FILTRON, Inc.), and applied to a 1.5×25 cm column of S-Sepharose and eluted with 0.02 M sodium phosphate (pH 6.5), 0.4 M NaCl. Coat protein-containing fractions were pooled and the buffer replaced with 10 mM acetic acid. Protein quantitation was accomplished by Bradford assays (9) using chicken lysozyme as a standard.

RNA binding assays

The use of nitrocellulose filter-binding assays in the characterization of the coat protein-RNA interaction has been described previously (10). ³²P-labeled RNA was produced by transcription *in vitro* (11) of operator sequences cloned in pT7-2 (US Biochemicals). Dissociation constants were determined in a protein-excess filter binding assay in which a low concentration of radiolabeled RNA (about 10 pM) was mixed with varying concentrations of coat protein in TMKG buffer (100 mM Tris-HCl, pH 8.5, 80 mM KCl, 10 mM magnesium acetate, 10% glycerol, 10 μg/ml BSA). After incubation at 0°C for 1 h samples were passed through a nitrocellulose filter and the amount of retained complex was determined by liquid scintillation counting of the dried filter. We were surprised to find that under

the conditions previously used for filter binding of coat protein (i.e. TMK buffer without glycerol; see ref. 10) dIFG showed a peculiar biphasic binding curve compared to wild-type (results not shown). We don't know the explanation for this behavior, but suspect it may be related to the altered aggregation properties of the dIFG protein compared to wild-type. This could result in poorer retention of the protein on nitrocellulose filters at low protein concentrations. In any case, the problem was corrected by performing the experiment in TMK containing 10% glycerol (TMKG). Control experiments show that glycerol at this concentration has no effect on RNA binding by wild-type protein. Moreover, each of the mutations was introduced into the full length (non-dIFG) sequence and the binding behavior of the resulting proteins was determined.

RESULTS

Mutagenesis and isolation of suppressors of an o^c mutation

A two-plasmid system for the genetic dissection of the RNA-binding function of coat protein has been described (3). In this system coat protein expressed from one plasmid (pCT119) represses translation of a replicase- β -galactosidase fusion protein expressed from a second plasmid (e.g. pRZ5 or pRZ6). Plasmid pRZ5 contains a wild-type MS2 operator and synthesis of the fusion protein is typically repressed about 50-fold. Plasmid pRZ6 contains a mutant operator which differs from the wild-type by the two nucleotide substitutions shown in Figure 1. This RNA sequence was modeled after the translational operator of the related RNA phage, GA. Coat protein expressed from pCT119 represses synthesis of replicase- β -galactosidase from pRZ6 about 5-fold. *In vitro* the mutant operator is bound about 100-fold less tightly than wild-type (Table I). We previously described the isolation of coat mutants that acquired the ability to repress translation from this mutant operator (4). Each of these was a super-repressor, meaning that they repressed translation from both wild-type and mutant operators more efficiently than did the wild-type protein. Three of the four mutants we isolated in that study as suppressors of the operator-constitutive mutation acquired their super-repressor phenotypes as secondary consequences of a capsid assembly defect. In those cases super-repression was apparently accomplished by an elevation in the intracellular level of repressor dimers at the expense of virus-like capsids. This interpretation of our results was supported by the super-repressor phenotype of a mutant deliberately engineered

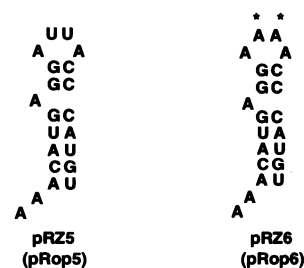


Figure 1. The sequences and secondary structures of the translational operators of pRZ5 and pRop5 (the wild-type MS2 operator) and pRZ6 and pRop6 (the mutant operator). The sites of nucleotide substitutions in the mutant are denoted by asterisks.

to result in an assembly defect. The three-dimensional structure of the MS2 virus particle shows that the so-called FG loop was involved in inter-dimer contacts permitting assembly of dimers into virus particles. When we deleted most of the FG loop (in the mutant called dIFG) the assembly-defective/super-repressor phenotype was obtained.

On the other hand, one of the four mutants, V29I, was a super-repressor without exhibiting any obvious assembly defect. We proposed that V29I bound RNA more tightly and set out to isolate more mutants of this class. In order to prevent the isolation of additional assembly-defective super-repressors we used the dIFG mutant as the starting point for mutagenesis and screened again for acquisition of the ability to repress synthesis from pRZ6. Since dIFG is itself a super-repressor and produces white colonies with

pRZ6 on X-gal plates, it was necessary to modify our system so as to produce blue colonies. This turned out to be a simple matter of using the *E. coli* strain DH5 α instead of our usual CSH41F⁻. Our two-plasmid translational repression system is obviously functioning in DH5 α , but for some reason colonies are generally bluer than with the other strain. Consequently, a library of mutants was constructed with dIFG by the method of Myers *et al.* (7). This was introduced by transformation into DH5 α cells containing pRZ6 and plated on solid medium containing X-gal. After sufficient time for growth and color development, colonies were picked that displayed a white or pale blue phenotype. DNA sequence analysis of the entire coat protein coding region identified the predicted amino acid substitutions shown in Table I. One mutation, V29I, had been isolated before (4). In this study it was isolated independently at least twice as the result of mutagenesis of different DNA strands. The V29I substitution results in the production of white colonies with pRZ6 on X-gal plates, indicating efficient repression. The other mutations resulted in new amino acid substitutions, K43R and K66R. Each of these produced colonies that were pale blue, suggesting slightly less efficient repression than V29I, but better than wild-type.

RNA binding properties of the mutants

We previously showed that the mutant dIFG has a super-repressor phenotype *in vivo* that is apparently the result of a failure to assemble coat protein dimers into stable capsids (4). If the assembly defect is responsible for the super-repressor activity of dIFG, the isolated protein should possess a similar affinity for RNA as the wild-type protein. This was tested by purifying the dIFG and wild-type coat proteins as described in Materials and

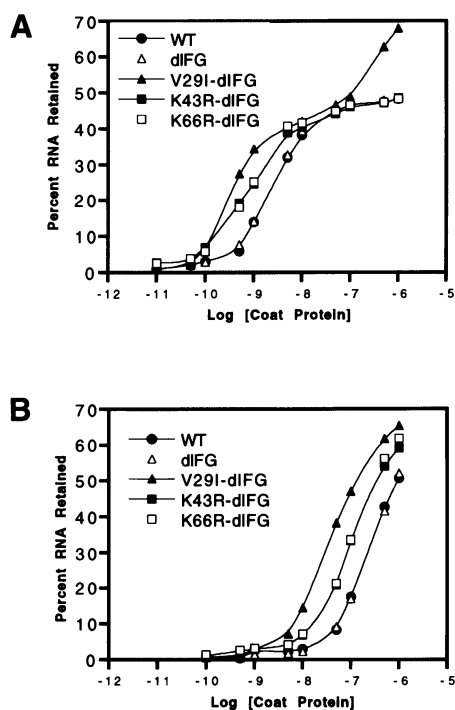


Figure 2. (A) Protein-excess binding curves of the wild-type and mutant coat proteins for the wild-type MS2 operator RNA. (B) Binding of the mutant operator by the same proteins.

Table I. Values of K_d of the various coat protein variants for the rop5 and rop6 RNAs as estimated from the protein-excess binding curves shown in Figures 2 and 3

Repressor	K_d (M)	
	rop5	rop6
MS2-WT	3×10^{-9}	3×10^{-7}
dIFG	3×10^{-9}	3×10^{-7}
V29I-dIFG	4×10^{-10}	4×10^{-8}
K43R-dIFG	1×10^{-9}	1×10^{-7}
K66R-dIFG	1×10^{-9}	1×10^{-7}
V29I	4×10^{-10}	4×10^{-8}
K43R	1×10^{-9}	1×10^{-7}
K66R	3×10^{-9}	3×10^{-7}

Note that we use the one letter amino acid code and a number to indicate the site and type of amino acid substitution. For example, the designation V29I means that valine 29 of the wild-type sequence has been substituted with isoleucine.

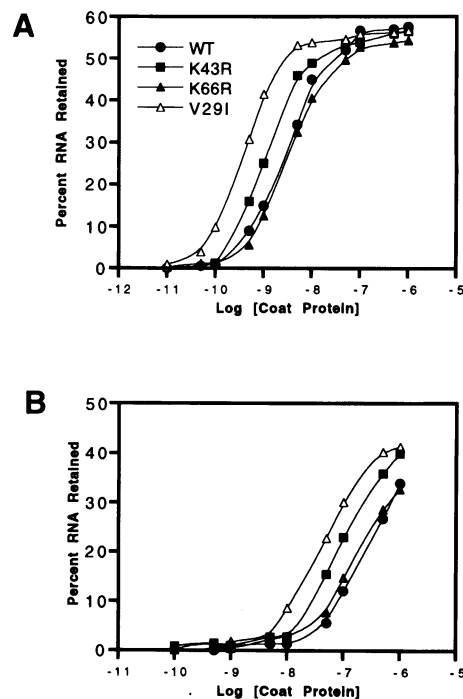


Figure 3. Effects of the presence of the FG loop on the RNA binding properties of the coat protein mutants described in the text and in Figure 2. Panels (A) and (B) show binding of these proteins to the wild-type and mutant operators respectively.

Methods and subjected them to tests of their ability to bind the wild-type (pROP5) and mutant (pROP6) operators. Figure 1 shows the protein-excess binding curves and Table I summarizes our estimates of the K_d values of these proteins for binding of the two operator RNAs shown in Figure 1. The binding behavior of dIFG is not distinguished from that of the wild-type protein. Each binds the wild-type operator (pROP5) with $K_d = 3 \times 10^{-9}$ M and the mutant operator (pROP6) with $K_d = 3 \times 10^{-7}$ M. This is consistent with our previous assertion that the assembly defect itself is responsible for the super-repressor phenotype of this class of mutants (4).

We also subjected each of the mutants to filter binding analyses. Consistent with their super-repressor colony-color phenotypes, each of them binds both RNAs more tightly than wild-type. V29I-dIFG binds most tightly. We estimate its K_d for the wild-type operator (pROP5) to be 4×10^{-10} M. It binds the mutant operator ROP6 with $K_d = 4 \times 10^{-8}$ M. This represents a 7.5-fold increase in binding of both RNAs. Unlike the other proteins, V29I-dIFG shows additional binding of RNA at protein concentrations above 10^{-7} M. We are unsure how to explain this behavior. However, we note that it is commonly observed that only about 50% of operator RNA is capable of being bound by coat protein *in vitro*. We suspect that this is the result of half the RNA assuming inappropriate conformations, and wonder whether V29I has an increased non-specific affinity for RNA so that it is able to bind this additional RNA.

The K43R-dIFG and K66R-dIFG mutants also bind RNA more tightly than the wild-type protein. The binding curves for K43R-dIFG and K66R-dIFG are indistinguishable from one another, each binding the ROP5 operator with K_d of 1×10^{-9} M and the mutant ROP6 operator with $K_d = 1 \times 10^{-7}$ M. These values differ by only a few-fold from wild-type, but were reproducibly obtained in several repetitions of these experiments and with different preparations of the mutant proteins.

Each of the mutants described above is, of course, a double mutant, since in addition to a nucleotide substitution each has suffered a deletion of residues 68–80 in the FG loop. We wondered whether the deletion might affect the RNA-binding properties of any of the mutants. In particular, we imagined that the properties of K66R might be altered by the dIFG deletion, since this amino acid substitution is situated so close to the beginning of the deletion and because inspection of the X-ray structure of the wild-type coat protein (5) reveals that the side chain of residue 66 normally projects from the wrong side of the β -sheet to be obviously implicated in RNA binding. Consequently, we introduced each of the V29I, K43R, and K66R substitutions into the full-length sequence and repeated the binding studies with the wild-type and mutant operators. The results show that the behaviors of V29I and K43R are identical to those of V29I-dIFG and K43R-dIFG with respect to their abilities to bind either of the two operator RNAs (Figure 3 and Table I). K66R, however, binds wild-type RNA like the wild-type protein. The enhancement of binding of the mutant operator is also eliminated when the FG loop is present. In other words, the effect of the K66R substitution virtually disappears if the FG loop is left intact.

DISCUSSION

Mutants of bacteriophage MS2 coat protein are readily isolated that interact more tightly with RNA than does the wild-type protein. We had hoped initially to isolate specificity mutants that would prefer to bind the mutant operator (i.e. allele-specific suppressors). No such mutants were found, but we now believe we know why. Recently we produced a mutant MS2 coat protein with the desired change in specificity (12). Its construction required the introduction of at least two amino acid substitutions. Single nucleotide substitutions predominated in the mutant library from which the super-repressors described in this paper were

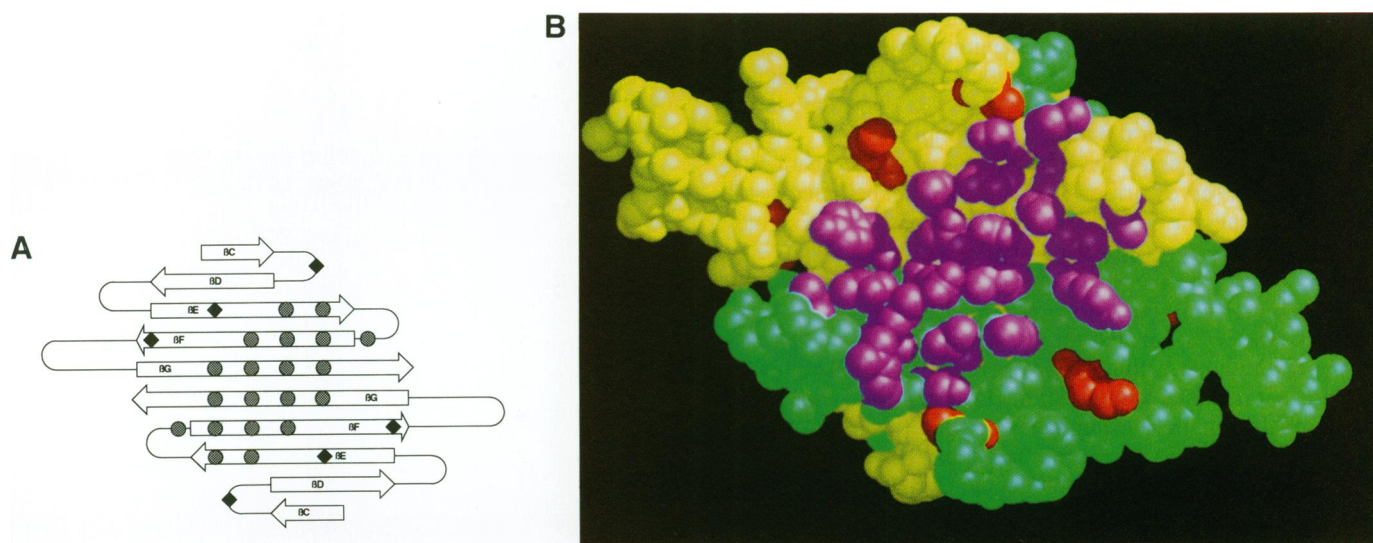


Figure 4. (A) A schematic representation of the RNA-binding site of coat protein. Amino acid residues whose substitution resulted in RNA binding defects are denoted by the shaded circles (see ref. 3). The filled diamonds indicate the positions of amino acid substitutions resulting in enhancement of RNA binding as described in the text. The arrows that signify β -strands point in the direction of the C-terminus. (B) The RNA-binding surface of coat protein is depicted here. The two subunits of the dimer are colored yellow and green. Residues whose substitution resulted in RNA-binding defects (2) are shown in magenta. The sites of the super-repressor substitutions described in the text are colored red. Note that residue 66 is barely visible since its side chain projects from the other side of the β -sheet.

isolated. We believe this to be the case for two reasons. First, none of the isolates we sequenced contained more than one nucleotide substitution. Second, the mutant library produced fewer than 10% blue colonies on X-gal plates when tested for translational repression with the wild-type operator. Therefore, appropriate double mutations must be extremely rare or even non-existent in this library.

The enhancement of binding of the wild-type operator by K66R is observed only when the substitution is present in the double mutant K66R-dIFG. The effect of the substitution disappears when the FG loop (residues 67–81) is intact. We have pointed out that in the wild-type protein residue 66 would be predicted not to participate in RNA binding since its side chain projects from the non-RNA-binding surface of the coat protein β -sheet. In the K66R-dIFG double mutant, however, the dramatic shortening of the FG loop to two residues (Gly–Ala) may cause a local distortion of the polypeptide chain, allowing residue 66 to participate in the binding site. Direct structural analysis will be required to test this speculation.

Inspection of the three-dimensional structure of coat protein and the locations of the relevant amino acid substitutions provide a possible explanation for the tight-binding behavior of the super-repressor mutants. Figure 4 shows the positions of residues previously identified as components of the RNA-binding site because their substitution results in defects in RNA binding and translational repression (2). That analysis revealed that the RNA-binding site is comprised of alternating amino acid residues making up the hydrophilic surface of the β -sheet of the protein. Residues on three adjacent β -strands in each monomer are involved. Each of the super-repressor mutations we have described results in an amino acid substitution at a site outside the previously identified binding site. We suspect that the tighter RNA binding properties of the mutant proteins are the results of additional contacts with RNA. In other words, we propose that the binding site has been expanded by these amino acid substitutions so that it interacts with operator RNA over a larger surface. This could be the direct effect of establishing new interactions between RNA and the substituted amino acid residues, or the indirect effect of changing the shape of the site so that new contacts are formed with unsubstituted residues. Alternatively, the mutations may result in the removal of unfavorable contacts in the wild-type complex.

It should also be noted that the magnitude of the changes in K_d which we observe correspond to relatively small changes in DG. For example the 7.5-fold decreased K_d of V29I, the tightest binding of these super-repressors, implies a DDG of about -1.1 kcal/mol. This indicates that super-repression is the result of relatively weak additional contacts with RNA.

Finally, we point out that the very existence of mutant coat proteins with higher affinities for operator RNA illustrates the principle that maximal strength of interaction is not synonymous with optimal strength. During the evolution of the RNA phages the coat protein–RNA interaction has apparently been tuned so that it is tight enough to ensure genome encapsidation without being so tight as to prematurely repress replicase synthesis.

ACKNOWLEDGEMENT

This work was supported by a grant from the National Institutes of Health.

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