December 13, 1996

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 50, Issue of December 13, pp. 31839–31845, 1996
Printed in U.S.A.

The RNA-binding Site of Bacteriophage Qβ Coat Protein*

(Received for publication, May 30, 1996, and in revised form, August 20, 1996)

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The coat proteins of the RNA bacteriophages Qβ and MS2 are specific RNA binding proteins. Although they possess common tertiary structures, they bind different RNA stem loops and thus provide useful models of specific protein-RNA recognition. Although the RNA-binding site of MS2 coat protein has been extensively characterized previously, little is known about Qβ. Here we describe the isolation of mutants that define the RNA-binding site of Qβ coat protein, showing that, as with MS2, it resides on the surface of a large β-sheet. Mutations are also described that convert Qβ coat protein to the RNA binding specificity of MS2. The results of these and other studies indicate that, although they bind different RNAs, the binding sites of the two coat proteins are sufficiently similar that each is easily converted by mutation to the RNA binding specificity of the other.

The coat proteins of the RNA bacteriophages play dual roles in the viral life cycle. In addition to serving as the major structural proteins of the virus particles, they act as translational repressors of viral replicase synthesis. This latter function is the result of coat protein interaction with an RNA stem loop which contains the replicase ribosome-binding site. The coat protein of bacteriophage MS2 is the most intensively studied of the RNA phage coat proteins. Its binding target on viral RNA has been thoroughly characterized (1), coat protein itself has been subjected to detailed genetic analysis of its RNA binding function (2–6), and x-ray structures of the coat protein in both the free and RNA-bound forms are available (7–9). The coat proteins of related phages are less well characterized, but, since some bind different RNAs, they provide opportunities to understand the basis of RNA binding specificity. The RNA binding targets of the coat proteins of MS2 and Qβ are shown in Fig. 1. The two coat proteins are about 25% identical in amino acid sequence and possess highly similar tertiary structures. Thus they utilize a common structural framework to bind structurally distinct RNAs.

We previously reported genetic analyses of the MS2 coat protein RNA-binding site utilizing a two-plasmid system in which coat protein expressed from one plasmid (pCT119) translationally represses synthesis of a replicase-β-galactosidase fusion protein from the second plasmid (pRZ5). We constructed an equivalent two-plasmid system for Qβ coat protein in order to similarly dissect its RNA-binding site. Here we describe this system and its application in identifying amino acid residues important for the interaction of Qβ coat protein with its RNA. We also describe the isolation and characterization of specificity mutations that confer to Qβ coat protein the ability to bind the MS2 translational operator.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—A two-plasmid system suitable for the isolation of MS2 coat mutants with altered translational repressor activities has been previously described in detail (2). In that system coat protein expressed from pCT119 represses the synthesis of a replicase-β-galactosidase fusion protein from pRZ5. For this study we constructed an analogous genetic system for Qβ. A cDNA of the Qβ viral genome had been previously cloned in plasmid pQβ7 (10). We excised the the Qβ coat sequence from pQβ7 as a *Bgl*II-*Bam*HI fragment and inserted it into the *Bam*HI site of pUC119 (11). The resulting plasmid, pQCT119, places the coat sequence under transcriptional control of the *lac* promoter of *Escherichia coli* on a plasmid which contains a ColEI replication origin and confers resistance to ampicillin. The plasmids pRZQ and pRZQ5 contain Qβ replicase-*lac*Z fusions and were constructed in a manner analogous to the previously described pRZ5 simply by inserting synthetic oligonucleotides containing different versions of the Qβ translational operator sequence (see Fig. 1) in place of the MS2 operator of pRZ5. The resulting plasmids use the *lac* promoter to drive transcription of the replicase-*lac*Z fusion. In addition each possesses a P15A origin of replication and confers resistance to chloramphenicol. The synthetic translational operator sequences were also inserted into pT7–2 (U. S. Biochemical Corp.) to produce pT7ropQ and pT7ropQ5. We also cloned an operator variant lacking the bulged A into pT7-2, creating pT7ropQ-NB (see Fig. 1).

Construction of Mutational Libraries of the Qβ Coat Sequence—Mutations were introduced into the Qβ coat sequence using error-prone polymerase chain reaction with primers that flanked the coat gene of pQCT119. Three different mutagenesis protocols were used. 1) For some experiments we followed the method of Cadwell and Joyce (12) without modification. The PCR[1] reaction conditions were as follows: 20 fmol of DNA, 30 pmol of each primer, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 7 mM $MgCl_2$, 0.5 mM $MnCl_2$, 1 mM dTTP, 1 mM dCTP, 0.2 mM dGTP, 0.2 mM dATP. After addition of 5 units of *Taq* DNA polymerase the reaction was subjected to 30 cycles of 94 °C for 1 min, 45 °C for 1 min, and 72 °C for 1 min. The mutant library produced by this method yielded about 50% repressor-defective clones, and sequence analysis showed that the mutations were highly random. However, the high mutation rate resulted in a predominance of multiple hit mutations. 2) To lower the mutagenesis frequency so as to favor single-hit mutations, conditions were altered to give 1.5 mM $MgCl_2$, 0.065 mM $MnCl_2$, and 1 mM each dNTP. These conditions resulted in libraries in which 10% of clones had the repressor-defective phenotype. However, DNA sequence analysis of the mutants showed that nucleotide substitutions were not as random as desired; a preponderance of transition mutations was obtained. 3) To reduce the mutagenesis frequency while retaining high randomness we made other modifications. First we altered the conditions of the mutagenesis reaction to include 7 mM $MgCl_2$, 0.25 mM $MnCl_2$, and 1 mM each dNTP. We also effectively reduced the size of the mutagenesis target. Taking advantage of the existence of internal *Nde*I and *Alw*NI sites we fragmented the PCR product into three pieces of approximately equal size, which were then used to replace the corresponding fragments in pQCT119. This resulted in three mutant libraries, each derived from mutagenesis directed to a different one-third of the coat sequence. About 10% of the clones obtained in this way produced defective translational repressors. Since it yielded the largest

* This work was supported by a grant from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: PCR, polymerase chain reaction; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; MES, 4-morpholineethanesulfonic acid.

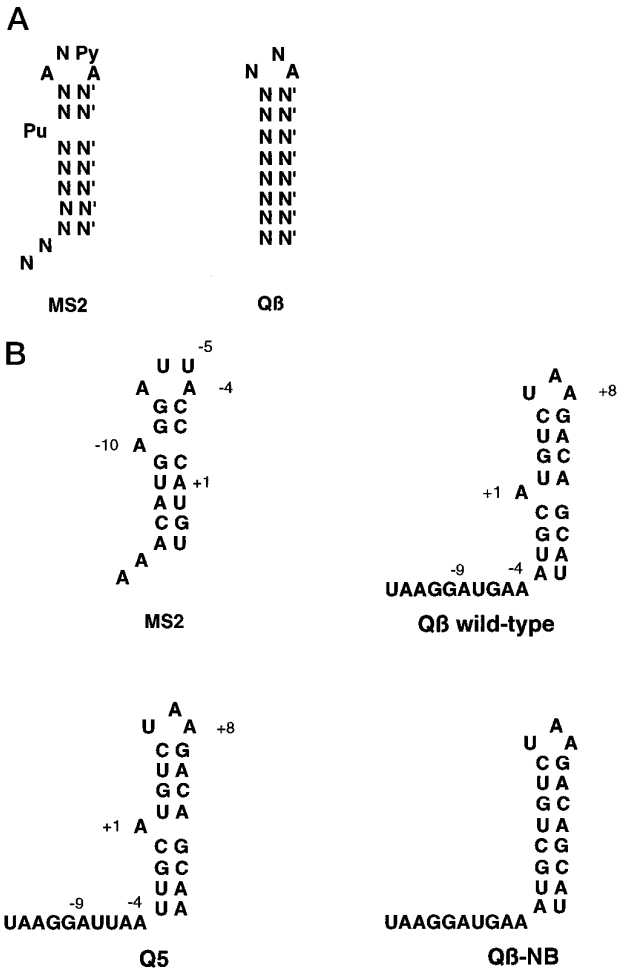

FIG. 1. *A*, structural features of the MS2 and Qβ translational operators required for interaction with their respective coat proteins. *B*, actual sequences and secondary structures of the Qβ translational operators used in this study for the construction of the various pRZ and pT7rop plasmids. The MS2 operator present in pRZ5 is also shown for comparison.

number of repressor-defective assembly competent mutants, most of the mutants described below were isolated by this last procedure.

Isolation and Characterization of Repressor-defective Mutants—Mutated DNA was introduced into strain CSH41F$^-$(pRZQ5). After about 24 h of growth on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), blue colonies were picked, transferred with sterile toothpicks into 1 ml of 2 × YT medium, and grown to saturation. Cells were then collected by centrifugation, resuspended in 0.5 ml of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and disrupted by sonication. After removing cellular debris by centrifugation, bromphenol blue and glycerol were added to the lysed samples. These were then applied to a 1% agarose gel (SeaKem Gold Agarose from FMC Corp. BioProducts) containing 50 mM sodium phosphate (pH 7.4), 1 mM EDTA, and electrophoresed until the dye had migrated 10 cm. Taking advantage of the presence of RNA in the capsids, they were visualized by staining the gel with ethidium bromide followed by UV illumination. Gels were also stained with Coomassie Brilliant Blue. Mutants that passed this capsid assembly screen were subjected to nucleotide sequence analysis and their protein products were purified (2). Filter binding analyses of their RNA binding functions were performed as described before (13) using ^{32}P-labeled RNA produced by run-off transcription of pT7rop plasmids cleaved with *Bam*HI (14). The dissociation constants of the various proteins were determined in a protein-excess filter-binding assay (15) in which a low concentration of radioactive RNA (about 10 pM) was mixed with varying concentrations of coat protein (1×10^{-10} M to 1×10^{-6} M) in MMK buffer (0.1 M MES, pH 6, at 0 °C, 80 mM KOAc, 10 mM $Mg(OAc)_2$, and 0.01 mg/mL bovine serum albumin). Each protein preparation was also subjected to titration with excess ^{32}P-labeled RNA in a filter-binding experiment in order to determine the concentration of active protein in each preparation. Individual protein preparations

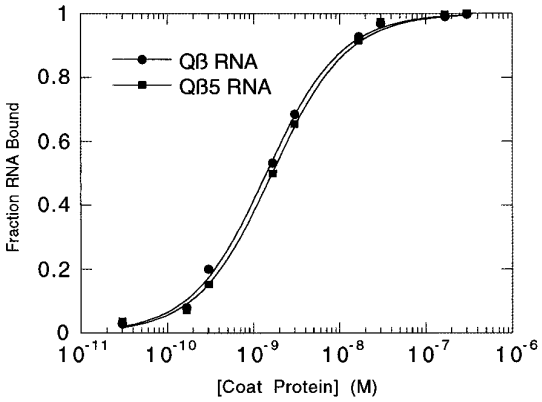

FIG. 2. **Protein-excess binding curves of the Qβ coat protein with the wild-type Qβ and Qβ5 operators.**

varied from 25 to 45% in their content of active protein. The binding data reported in Figs. 2–4 and in Table II are adjusted accordingly.

Isolation and Characterization of Specificity Mutants—Mutant library DNA was introduced into CSH41F$^-$(pRZ5) and plated on solid medium containing X-gal. After 24 h of growth at 37 °C, colonies displaying white to pale blue colony color phenotypes were picked into 1 ml of LB medium and grown to saturation. Since suppression of the operator-constitutive phenotype may sometimes be the result of an assembly defect rather than changes in RNA binding activity (5), the products of the mutant coat genes were assayed for capsid formation by agarose gel electrophoresis as described above. Those mutants passing the capsid-assembly test were selected for DNA sequence analysis. Solution assays of β-galactosidase were performed by the method of Miller (16). The wild-type and mutant coat proteins were purified by methods described elsewhere (2). Operator RNAs labeled with ^{32}P were produced by transcription *in vitro* of *Bam*HI-cleaved pT7rop5 (3) or pT7ropQ5 (see above) as described by Draper *et al.* (14). Protein-excess nitrocellulose filter-binding assays of RNA binding activity were conducted as described by Witherell and Uhlenbeck (15).

RESULTS

Construction of a Two-plasmid Genetic System for Qβ—The details of the two-plasmid genetic system used in the screening for MS2 translational repressor mutants have been elaborated before (2). For the present study we constructed an analogous system for Qβ coat protein. Initially we constructed pRZQ, which contains a synthetic version of the wild-type Qβ operator fused to *lac*Z. Unfortunately, this version of the operator was utilized poorly for translational repression by Qβ coat protein. Inspection of the operator sequence revealed the presence of two AUG triplets (at −9 and −4) in addition to the true replicase start site at +1 (Fig. 1*B*). We wondered whether the existence of these potential translational start sites might increase ribosome binding at the expense of coat binding. Therefore, we constructed pRZQ5. It contains a mutant version of the Qβ operator which eliminates the extra AUGs while retaining the elements required for recognition by coat protein (see Fig. 1*A*). Filter binding assays revealed that coat protein binds both operators *in vitro* with a K_d of about 1×10^{-9} M (Fig. 2). Cells containing both pQCT119 and pRZQ5 plasmids resulted in white colonies, and β-galactosidase activity was repressed 44-fold. Therefore, these modifications resulted in efficient translational repression *in vivo* while leaving the *in vitro* binding characteristics of the operator unaltered.

The Significance of the Bulged A Nucleotide in the Qβ Operator—The sequence and structural requirements of the Qβ operator have been previously defined (15). Those studies showed that binding activity for coat protein required the presence of an 8-base pair stem and a 3-nucleotide loop. Analysis of the binding properties of hairpin variants showed that the exact sequence of the stem was unimportant as long as its secondary structure was preserved. Moreover, the identity of

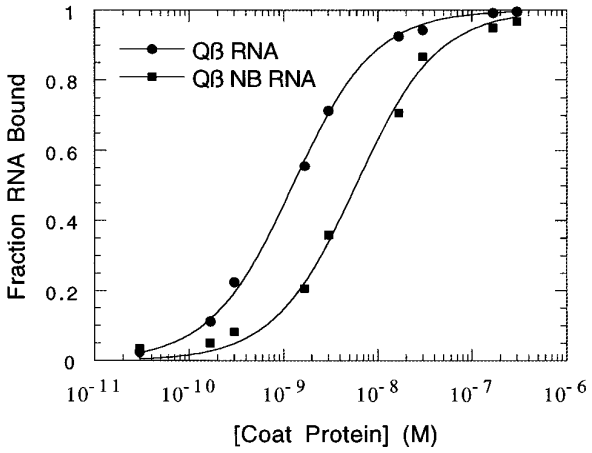

FIG. 3. **Protein-excess binding curves of the Qβ coat protein with the wild-type Qβ translational operator and the QβNB (bulge-less) variant.**

only one of the three loop nucleotides was essential (the A at +8). It was also found that the bulged A nucleotide could be deleted without substantially affecting coat protein binding. We found this surprising, since the activity of the MS2 operator is highly dependent on the presence and identity of the nucleotide at the bulged position (17), and because a bulged A residue is a conserved feature in the translational operators of a number of related RNA phages (18). We constructed pT7ropQβNB (no bulge) to test the role of the bulged A. Transcription *in vitro* produced the bulge-less operator variant shown in Fig. 1*B*. The results of filter binding studies are given in Fig. 3, where it is seen that the wild-type operator, in our hands, gives a K_d of 1×10^{-9} M, whereas the bulge-less variant has a K_d of about 5×10^{-9} M. This represents a 5-fold loss of binding affinity. Although there is a slight disagreement with Witherell and Uhlenbeck (15) over the exact magnitude of the effect of the bulge deletion (they found a 1.6-fold difference), our results confirm that the effects are much smaller than for the similar mutation of the MS2 operator (1). The consequences of bulge deletion on translational repression *in vivo* could not be tested, because the deletion of the extrahelical A destroys the replicase translational start site.

Random Mutagenesis and Isolation of Repressor-defective Coat Mutants—The two-plasmid system facilitates the isolation of coat mutants with altered translational repressor activities. Wild-type coat protein expressed from the pQCT119 plasmid efficiently represses the synthesis of the replicase-β-galactosidase fusion protein encoded on the plasmid pRZQ5. This results in white colonies on X-gal plates. Mutations that give rise to defective repressors yield blue colonies. After transformation of CSH41F$^-$(pRZQ5) with a mutant library in pQCT119 and plating on LB medium containing X-gal, clones representing a spectrum of color-colony phenotypes were picked. In order to discard any mutants whose repressor defects might be secondary consequences of wholesale disruption of protein structure, each mutant coat protein was screened for the ability to assemble into virus-like particles using the electrophoretic assay described under "Experimental Procedures." Since capsid assembly requires multiple intersubunit contacts, we assume this to be a sensitive indicator of native folding. Capsids were easily visualized by ethidium bromide staining of host RNA within the particle (results not shown). We worried, however, that the repressor-defective mutants might fail to encapsidate RNA and would be unstainable with ethidium bromide. For this reason gels were also stained with Coomassie Blue. However, in no case did we find a mutant that stained with Coomassie Blue and not ethidium bromide.

TABLE I
The amino acid and nucleotide substitutions found in the repressor defective mutants of Qβ coat protein

The identities of structurally homologous amino acids of MS2 coat protein are shown in parentheses for comparison.

Amino acid substitution	Codon change	No. of isolates
V32A (Val^{29})	GTT to GCT	13
T49A (Thr^{45})	ACC to GCC	7
S56P (Ser^{52})	TCT to CCT	8
R59H	CGT to CAT	3
R59C	CGT to TGT	4
N61D (Asn^{55})	AAC to GAC	7
N61S (Asn^{55})	AAC to AGC	4
K63E (Lys^{57})	AAG to GAG	9
K63T (Lys^{57})	AAG to ACG	2
K63M (Lys^{57})	AAG to ATG	2
Y89H (Tyr^{85})	TAT to CAT	1
S95L (Tyr^{91})	TCG to TTG	5

Three different error-prone PCR mutagenesis methods were utilized in this study (see "Experimental Procedures" for details). The first used exactly the conditions of Cadwell and Joyce (12). This method is reported to introduce highly random mutations at a frequency of about 0.7%. A library of coat mutations produced in this way yielded about 50% blue colonies, but, of the 900 clones we screened, only 21 passed the capsid assembly test. Nucleotide sequence analysis showed that the high mutagenesis rate resulted in multiple mutations, increasing the likelihood that any given isolate would be defective for protein folding or stability. Consequently, we altered the conditions of PCR to lower the mutation rate to a point that favors single-hit mutations. However, we found that the resulting mutations were less random with regard to substitution type. We eventually found compromise conditions which lowered the mutation rate to an intermediate level while retaining high randomness. We further reduced the frequency of multiple hits by targeting mutations to three different restriction fragments of the gene. This strategy reduces the mutagenesis target size. Most of the mutants we characterized were from this library. To assess the spectrum of mutations present in this library, 21 repressor-defective mutants which also failed the capsid assembly test were subjected to nucleotide sequence analysis. The 32 nucleotide substitutions we identified in the 21 mutants were distributed throughout the coat coding sequence and were equally comprised of transition and transversion mutations. A disadvantage of targeting mutations to restriction fragments is that it precludes the isolation of mutations within the recognition sequences of the restriction endonucleases used to generate the fragments. The *Nde*I site contains parts of codons 88–90, and the *Alw*NI site contains portions of codons 40–43. Both sites contain sequences affecting potential binding site residues. However, this constraint does not apply to the 21 repressor-defective assembly-competent mutants derived from heavy mutagenesis of the whole gene (see above), and, indeed, one of these mutations alters codon 89 within the *Nde*I site. Nevertheless, mutations in these positions may be under-represented in our collection.

Characterization of the Mutant Repressors—Mutants that survived the screen for native structure were subjected to DNA sequence analysis. The nucleotide and amino acid substitutions we found are listed in Table I. Representative proteins were purified (2), their concentrations of active protein were determined (see "Experimental Procedures"), and each was tested for its ability to retain ^{32}P-labeled wild-type Qβ operator RNA on nitrocellulose filters (15). At some positions in the amino acid sequence more than one substitution was found to result in the repressor-defective phenotype. In such cases only one of the mutant proteins was subjected to RNA binding studies. The

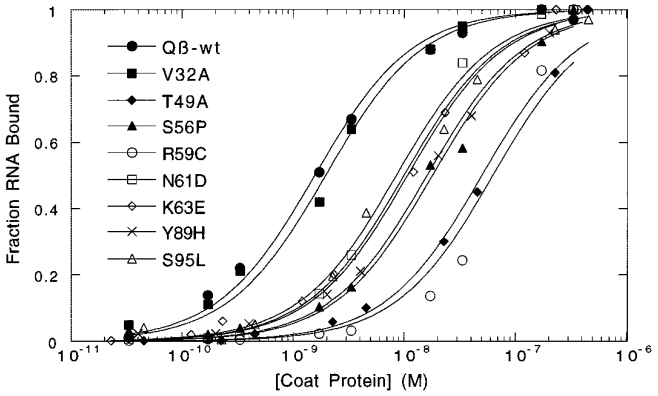

FIG. 4. **Protein-excess binding curves of the wild-type and repressor-defective coat proteins with the Qβ operator RNA.**

protein-excess binding curves are shown in Fig. 4, and the results are summarized in Table II. Each repressor-defective mutant with the possible exception of V32A corresponded to a defect in RNA binding. The locations of the affected amino acids in the structure of Qβ coat protein are shown in Fig. 5*A*. As was the case with MS2, the Qβ RNA-binding site resides on the surface of the coat protein β-sheet.

Isolation of Qβ Coat Mutants That Bind MS2 RNA—Given the fact that the MS2 and Qβ coat proteins share homologous tertiary structures, we wondered whether the Qβ coat protein could be modified to bind the MS2 RNA operator. Wild-type Qβ coat protein expressed from pQCT119 fails to repress synthesis of the MS2 replicase-β-galactosidase fusion enzyme encoded on pRZ5 and results in the formation of blue colonies on X-gal plates. Random mutations within the Qβ coat sequence were generated by the error-prone PCR method of Cadwell and Joyce (12), the mutant library was introduced into CSH41F$^-$(pRZ5) and plated on solid medium containing X-gal. About 4000 colonies were screened for the white or pale blue phenotype and seven were picked for further analysis. Four of these produced coat proteins competent for assembly into virus-like particles as judged by electrophoretic mobility in agarose gels (results not shown). Sequence analysis of the other three revealed that they possessed multiple nucleotide substitutions, and that each contained mutations affecting the so-called FG loop (8). We showed previously that mutations within the FG loop of MS2 coat protein blocked assembly of coat protein dimers into capsids (5). This resulted in an elevated intracellular concentration of the active repressor species and increased translational repression without any improvement of RNA binding affinity. We assumed that these three Qβ mutants were also in this category and did not characterize them further.

Table III shows the nucleotide and amino acid substitutions of the four specificity mutants that passed the capsid-assembly test. The mutants we call suM1 and suM2 have two and three amino acid substitutions respectively, but have the D91N substitution in common. This led us to suspect that the D91N mutation alone was responsible for the translational repressor phenotypes of these mutants. This is also supported by the fact that, of the affected amino acids, only Asp^{91} resides in a location in coat protein where it is likely to directly influence RNA binding. The D91N single mutant was constructed by isolating the *Eco*RI-*Nde*I fragment of the D91N,N22Y,T18S mutant and ligating it to pQCT119 cut with the same enzymes. For similar reasons we also suspected that the translational repressor phenotype of the suM4 mutant was conferred by the Q65H substitution. In this case, the appropriate single mutant had already been isolated (suM3). The translational repression efficiencies of the various mutants were determined and comparison of the

TABLE II
Dissociation constants of the Qβ wild-type and mutant proteins for binding of Qβ operator RNA determined by nitrocellulose filter-binding as described under "Experimental Procedures"

Repressor	K_d
	M
QB	1×10^{-9}
V32A	2×10^{-9}
T49A	5×10^{-8}
S56P	2×10^{-8}
R59C	1×10^{-7}
N61D	1×10^{-8}
K63E	1×10^{-8}
Y89H	2×10^{-8}
S95L	1×10^{-8}

β-galactosidase activities revealed that the two single mutants D91N and Q65H, repressed the Qβ and MS2 operators similarly to the multiple mutants (see Table IV).

The RNA Binding Properties of the Specificity Mutants—The coat proteins produced by the Q65H and D91N mutants were purified and subjected to nitrocellulose filter-binding assays. In these experiments no attempt was made to correct for the fraction of active protein, but the binding activities measured *in vitro* are consistent with the translational repressor activities determined *in vivo*. Q65H and D91N exhibited K_d values of 5×10^{-10} M and 2×10^{-9} M with the Qβ operator, respectively, whereas the wild-type protein displayed a K_d of 4×10^{-9} M (Fig. 6 and Table V). On the other hand, the Qβ coat protein interacted with MS2 RNA poorly, yielding a K_d of 1×10^{-7} M. As predicted from their activities as translational repressors *in vivo*, both mutants possessed stronger affinities for the MS2 RNA operator, with dissociation constants of 3×10^{-9} M for Q65H, and 5×10^{-9} M for D91N. A schematic diagram of the Qβ coat β-sheet structure depicts the positions of residues 65 and 91 within the RNA-binding site (Fig. 5*A*).

DISCUSSION

Our genetic approach to dissection of the Qβ RNA-binding site relies on translationally repressing the synthesis of a hybrid replicase-β-galactosidase protein. We initially constructed pRZQ for this purpose. It contains a synthetic version of the wild-type Qβ operator. Unfortunately, pRZQ was not susceptible to efficient translational repression by Qβ coat protein expressed from pQCT119. We did not know how to explain this behavior, but noticed the presence of two AUG triplets (at −9 and −4 in Fig. 1*B*) in addition to the replicase initiation codon (at +1). Since translational repression is the result of competition between coat protein and ribosomes for binding of the operator RNA, we speculated that the extra AUGs might somehow tip the balance in favor of ribosome binding. This was the reasoning behind the construction of pRZQ5, which contains nucleotide substitutions that preserve the required RNA secondary structure, but destroy the two extra AUGs (see Fig. 1). These changes confer the desired capacity for translational repression. This is not the result of any difference in affinity of the two operator RNAs for coat protein, since their binding behaviors *in vitro* are indistinguishable (Fig. 2).

Do these observations imply that the extra AUGs are bound by ribosomes? N-terminal sequence analysis of the replicase protein suggests that the AUG at +1 is the bona fide translation initiation codon (19). Although the AUG at −9 is in the replicase reading frame and could potentially lead to the synthesis of a protein three amino acids longer, we are unaware of any evidence for the existence of such an elongated product in Qβ-infected cells. Since pRZQ produces about 50% more β-ga-

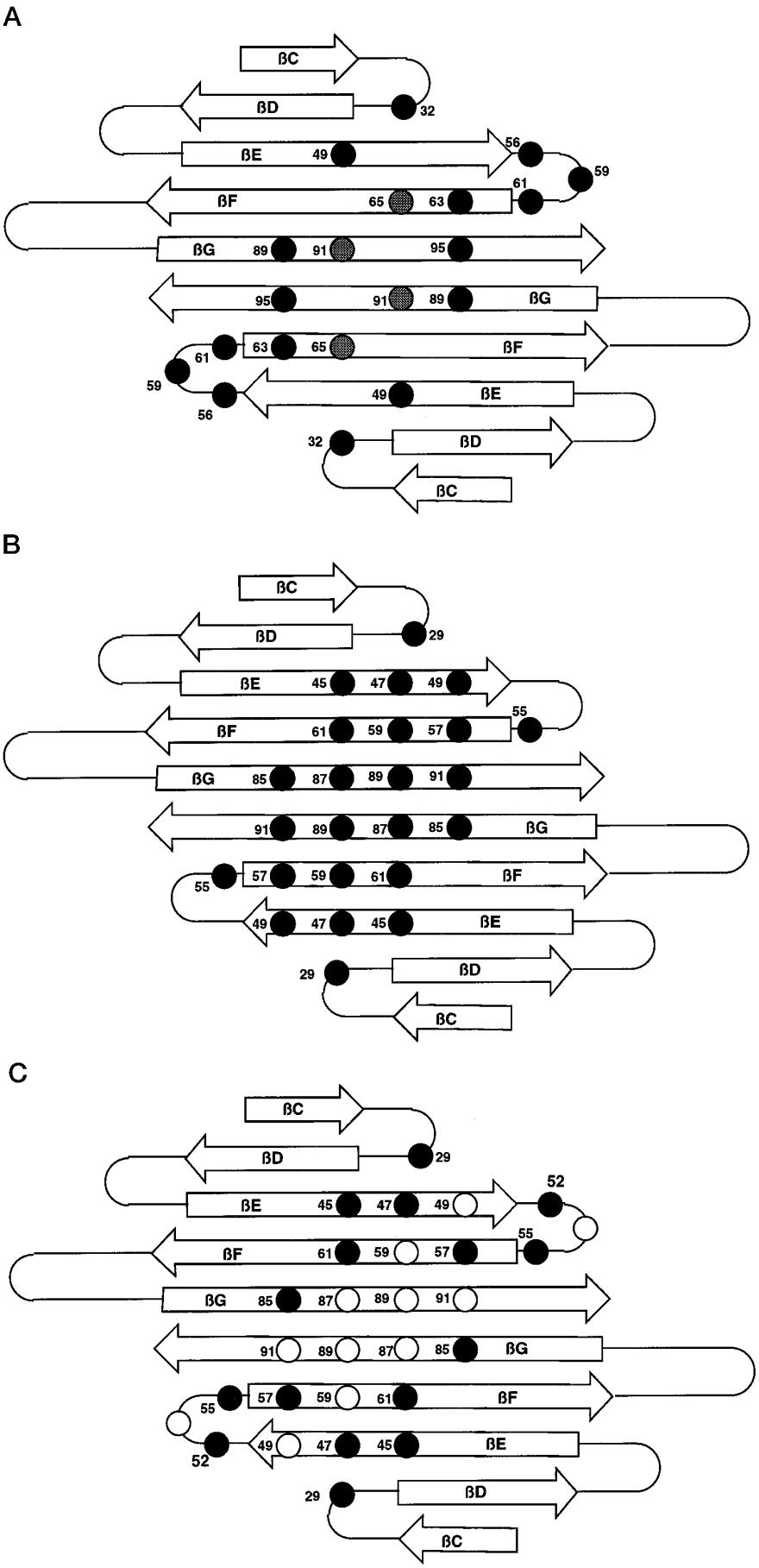

FIG. 5. **A schematic view of the *β*-sheet surface of coat protein where the RNA-binding site resides.** It is this surface of the protein which, in the assembled virus-particle, is oriented toward the interior of the capsid. Since the Q*β* and MS2 coat proteins are close structural homologues, we use the same basic illustration of *β*-strands connected by loops to represent them both. Shown in *A* are the locations of amino acids identified in this study whose substitution affects translational repression by Q*β* coat protein (Q*β* numbering). *Blackened circles* represent the repressor-defective mutations listed in Table I. *Shaded circles* correspond to the locations of the specificity mutations Q65H and D91N. For comparison *B* illustrates the locations of amino acids (MS2 numbering) in MS2 coat protein whose substitution resulted in the repressor-defective phenotype (3). In order to illustrate the extent of conservation of RNA-binding site residues in the Q*β* and MS2 coat proteins *C* shows a superposition of *A* and *B*. Positions which contain identical amino acids at structurally homologous positions are indicated as *filled circles*. Here the amino acids are identified by MS2 numbering.

TABLE III
Amino acid and nucleotide substitutions found in Qβ coat mutants that bind the MS2 translational operator

Mutant	Amino acid substitutions	Codon changes
suM1	D91N	GAC to AAC
	A114G	GCT to GGT
suM2	D91N	GAC to AAC
	N22Y	AAT to TAT
	T18S	ACT to TCT
suM3	Q65H	CAG to CAT
suM4	Q65H	CAG to CAT
	T29S	ACT to TCT

TABLE IV
The repressor activities of wild-type and specificity mutants of Qβ coat protein as assessed by their abilities to inhibit synthesis of a replicase-β-galactosidase fusion protein

Repressor	Repression		Blueness on X-gal	
	pRZQ5	pRZ5	pRZQ5	pRZ5
	-fold			
QB	44	4	−	+++
D91N	50	16	−	+−
D91N, A114G	40	12	−	+−
D91N, N22Y, T18S	52	14	−	+−
Q65H	58	11	−	+−
Q65H, T29S	27	13	+−	+−

lactosidase than pRZQ5[2] it is possible that the −9 AUG is utilized for translation initiation in this system. On the other hand, the AUG at −4 cannot produce an elongated protein, since it resides in the −1 reading frame and is followed, after two codons, by a nonsense triplet. Since pRZQ5 simultaneously inactivates both of the extra AUGs, we cannot distinguish their relative contributions to inhibition of repression. Moreover, we do not know whether the behavior of pRZQ means that the operator is poorly repressed in intact Qβ RNA in infected cells. Placement of the operator in the unusual context of pRZQ may have altered its function, perhaps somehow making the extra AUGs more susceptible to ribosome binding.

We previously reported the identification of amino acid residues making up the RNA binding site of MS2 coat protein. Their side chains reside on one surface of a large β-sheet. The location and make-up of the RNA-binding site was confirmed by the x-ray structure of the MS2 RNA-protein complex (7). In the current study we applied a similar genetic strategy to identify the amino acid constituents of the Qβ RNA-binding site. Given that MS2 and Qβ coat proteins are evolutionary relatives and possess homologous amino acid sequences, we assumed that Qβ coat protein would have high structural similarity to MS2, and would probably utilize a similar β-sheet surface for RNA binding. These expectations were confirmed by the mutational analyses reported here and by the x-ray structure of Qβ coat protein which Lars Liljas and colleagues kindly made available to us while this manuscript was in preparation. Table I shows the nucleotide and amino acid substitutions that resulted in the repressor-defective phenotype. Table II and Fig. 4 show that these substitutions also caused defects in RNA binding *in vitro*. As with MS2, the RNA-binding site mainly resides on the surface of the coat protein β-sheet, although some loop residues are also implicated. Most of the essential Qβ amino acids occupy positions that are structurally equivalent to important RNA-binding site residues of MS2 coat protein. The degree of conservation of these residues is shown in Table I which lists, in parentheses, the equivalent MS2 residues in

[2] F. Lim, M. Spingola, and D. S. Peabody, unpublished observations.

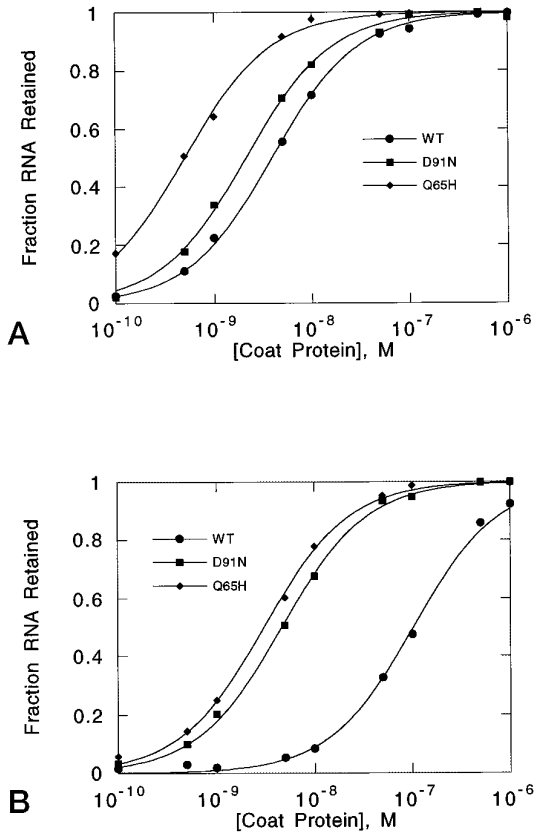

FIG. 6. *A*, protein-excess binding curves for wild-type and specificity mutants of Qβ coat protein with Qβ translational operator. *B*, binding curves of the same proteins with the MS2 operator.

TABLE V
Dissociation constants for the binding of Qβ and MS2 translational operators by the wild-type and D91N and Q65N mutants of Qβ coat protein

Repressor	K_d	
	Qβ RNA	MS2 RNA
	M	
Wild type	4×10^{-9}	1×10^{-7}
D91N	2×10^{-9}	5×10^{-9}
Q65H	5×10^{-10}	3×10^{-9}

those cases where a clear structural homologue is readily identified. Nearly all these amino acids are conserved between the two proteins. Fig. 5*B* shows the locations of the amino acid residues identified by genetic analysis as important for RNA binding by MS2 coat protein. A similar map of the Qβ-binding site, based on the studies reported here, is shown in Fig. 5*A*. Fig. 5*C* is a superposition of Fig. 5, *A* and *B*, and shows the degree of amino acid identity in residues required for RNA binding by the two proteins. The conserved aspects of the two sites may represent portions of coat protein structure required for binding of structurally similar parts of the operators.

It is possible, for several reasons, that the Qβ RNA-binding site includes residues not identified by this analysis. For example, some types of mutations could be absent in our mutational library. However, sequence analysis of 32 mutations in 21 repressor-defective assembly-defective mutants revealed a wide spectrum of different substitution types (not shown). Therefore, it seems unlikely that any amino acid residue whose identity is crucial for RNA binding is missing from this analysis, but, of course, we cannot rule out the possible existence of mutational cold spots which would lead to under-representa-

tion of certain mutants. It is also possible that certain residues play a dual role, functioning both in RNA binding and in protein folding or stability. Mutants with substitutions at these sites will not have passed our screen for capsid formation. Although we obtained multiple isolates of all but one of the repressor-defective mutants, it is also possible that the mutant library contains repressor defects not yet isolated. For these reasons the RNA-binding site could be more extensive than is indicated by the present set of repressor-defective mutations.

Unconserved amino acids within the two binding sites presumably account for their differing RNA binding specificities. We isolated four specificity mutants of Qβ coat protein based on their abilities to repress the MS2 operator. Three of them contained multiple amino acid substitutions. This was a consequence of the high mutation rate of the error-prone PCR method we used in generating the mutant library. We restricted our analyses to residues 91 and 65. The D91N,A114G and T18S,N22Y,D91N mutants had only the D91N substitution in common, and, of the affected amino acids, Asp^{91} was the only one present on the surface of the β-sheet where the RNA-binding site resides. The single mutant D91N was constructed to test this assertion, and we found that the translational repressor activity of both of the original mutants was conferred by the D91N substitution alone (Table IV). Similarly, Q65H possessed the translational repressor properties of the T29S,Q65H double mutant. For these reasons only the D91N and Q65H single substitutions were characterized further. Both mutants possess slightly increased affinities for the normal binding target of Qβ coat protein, the Qβ translational operator. This is evident both in the translational repression data presented in Table IV and in the *in vitro* binding affinities of the proteins for Qβ operator RNA (Fig. 6 and Table V). Thus, D91N binds the Qβ operator 2-fold more tightly than does wild-type Qβ coat protein. Meanwhile, Q65H shows an 8-fold improvement in binding of this RNA. The most dramatic effects, however, are found in the increased affinities for the MS2 operator. D91N and Q65H, respectively, bind the MS2 operator RNA 20-fold and 33-fold more tightly than does wild-type Qβ coat protein.

The effects of the D91N substitution are easily rationalized. Asp^{91} of Qβ coat protein occupies a position which is structurally homologous to Asn^{87} of MS2 coat protein. Genetic and structural studies identified Asn^{87} of MS2 as an important site of interaction with RNA (6, 7). It forms a hydrogen bond with the −5 uridine residue in the MS2 operator. On the other hand, Asp^{91} of Qβ is apparently not required for binding of the Qβ operator, since no substitutions at this site were found in our extensive collection of repressor-defective Qβ coat mutants, and because the D91N substitution itself clearly does not impair binding to Qβ RNA. It is striking that converting Asp^{91} to its MS2 counterpart dramatically improves activity for the MS2 operator. In fact, Qβ-D91N binds MS2 RNA nearly as well as does MS2 coat protein itself. Clearly, the RNA binding surface of Qβ coat protein is sufficiently similar to that of MS2 that it readily accommodates MS2 RNA, and a single amino acid substitution converts Qβ coat protein to a good repressor of MS2.

The effects of the Q65H substitution are not as easily understood. Gln^{65} in Qβ coat protein occupies a position which is structurally homologous to Thr^{59} of MS2. Substitutions like T59S or T59A result in repressor defects in MS2 coat protein (3, 6). However, the MS2 mutant T59Q is not repressor-defective for the MS2 operator.[2] Although it is clear from the crystal structure of the MS2 coat protein-RNA complex that Thr^{59} makes contact with RNA, the contact apparently does not involve the side chain (7). The effects of the Q65H substitution on RNA binding by Qβ coat protein suggest that histidine in this position establishes new contacts with the two RNAs, substantially increasing affinity for both the Qβ and MS2 operators.

We have shown that Qβ coat protein easily acquires binding activity for MS2 RNA by mutation. In work being reported elsewhere we show that MS2 coat protein acquires the ability to bind the Qβ operator with similar ease. Thus, the MS2 and Qβ coat proteins are sufficiently similar that their RNA-binding specificities are readily interconverted. Perhaps this is not unexpected given the similarities in the amino acid sequences of their RNA-binding sites. However, some may find it surprising that these similar proteins bind operators with the large apparent structural differences illustrated in Fig. 1. Compared to MS2, the Qβ operator requires a longer base paired stem, a smaller loop, and is relatively indifferent to deletion of the bulged A. In MS2 the critical nature of the bulge is easily reconciled with the structure of the protein-RNA complex where quasi-symmetric interactions between the two halves of the dimer and the As at −4 and −10 are observed. The amino acid residues (Val^{29}, Thr^{45}, Ser^{47}, and Lys^{61}) of MS2 coat protein that form the binding sites for these two As are conserved in Qβ, thus satisfying one of the important requirements for interaction with MS2 RNA. However, given the relative dispensability of the Qβ bulged A and the apparent difference in its spatial relationship to the loop A, the quasi-symmetry of these interactions is likely abolished in the Qβ coat protein-RNA complex. We suspect that the interaction of coat protein with the loop A is conserved between the two phages, but the interaction with the bulged A probably is not. Other contacts must be formed to compensate for this loss. The differences in makeup of the RNA-binding site of the two coat proteins presumably reflect this fact. On the other hand, the similarities of the binding sites might not reflect any significant similarities in the precise modes of interaction of the two RNAs with their respective coat proteins. Experiments currently in progress should determine whether the nature of the interaction with the loop A is a conserved feature in the RNA-protein complexes of MS2 and Qβ.

Acknowledgment—We thank Lars Liljas for providing the x-ray structure of Qβ coat protein in advance of publication.

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J. Biol. Chem. 1996, 271:31839-31845.
doi: 10.1074/jbc.271.50.31839