Functional recognition of fragmented operator sites by R17/MS2 coat protein, a translational repressor

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ABSTRACT

The R17/MS2 coat protein serves as a translational repressor of replicate by binding to a 19 nt RNA hairpin containing the Shine–Dalgarno sequence and the initiation codon of the replicase gene. We have explored the structural features of the RNA operator site that are necessary for efficient translational repression by the R17/MS2 coat protein in vivo. The R17/MS2 coat protein efficiently directs lysogen formation for P22 R17, a bacteriophage P22 derivative that carries the R17/MS2 coat protein in vivo. The R17/MS2 coat protein is necessary for efficient translational repression by binding to a 19 nt RNA hairpin at a distance of 9 nt from the Shine–Dalgarno sequence, implying that a discrete region of biological repression is defined by the coat protein–RNA hairpin interaction. The assembly of RNA species into capsid structures is not an efficient means whereby the coat protein achieves translational repression of target mRNA transcripts. The R17/MS2 coat protein exerts translational regulation that extends considerably beyond the natural biological RNA operator site structure; however, the coat protein still mediates repression in these constructs by preventing ribosome access to linear sequence determinants of the translational initiation region by the formation of a stable RNA secondary structure. An efficient translational regulatory mechanism in bacteria appears to reside in the ability of proteins to regulate RNA folding states for host cell and phage mRNAs.

INTRODUCTION

RNA-binding proteins can initiate translational repression by a variety of mechanisms. Most examples of translational regulation in prokaryotes focus on perturbing the formation of a functional initiation complex (1). The R17/MS2 coat protein causes translational repression by binding to an RNA secondary structure within the translational initiation region of the coliphage replicase gene (2). The most widely accepted model for translational repression by the coat protein suggests that the prevention of binary complex formation occurs by sequestering the Shine–Dalgarno sequence and initiation codon in secondary structure (1,3,4). Coat protein binding to the RNA stabilizes the secondary structure and prevents ribosomal access to the Shine–Dalgarno sequence (3). Another model proposes that the coat protein occludes access to the RNA by physically masking the Shine–Dalgarno sequence and the initiation codon (1). The latter model does not invoke a specific conformation that the RNA target sequence must adopt for translational regulation. These models are not mutually exclusive and are difficult to differentiate because sequestration and occlusion occur simultaneously in the natural R17/MS2 replicase operator site.

We have applied the RNA challenge phage system as a genetic tool (5) to evaluate different types of R17/MS2 coat protein binding site structures as translational operator sites. Our objective with these studies was to evaluate whether sequestration or occlusion represents the predominant mechanism for early translational regulation of an artificial operator site by the R17/MS2 coat protein. Our findings reveal that the coat protein can exert translational regulation well outside the confines of its RNA binding site.

Coat proteins may also direct translational repression late in the infection by drawing RNA transcripts into capsid assemblies. This mode of translational regulation has not been studied extensively in the context of the native R17/MS2 phage life cycle, but it would be amenable to investigation using heterologous reporter systems (6,7). Further insights into the capsid’s role in translational repression were obtained through our comparative studies of the effectiveness in which coat proteins encoding different types of defects in capsid assembly can functionally recognize artificial operator sites.

MATERIALS AND METHODS

Standard reagents

Biochemical reagents were of the highest grade obtainable from various manufacturers. Sterile water was initially deionized using a Millipore Milli-Q Plus water purification system. The
oligonucleotides were purchased from Operon Technologies, Inc. The [γ-32P]ATP (6000 Ci mmol) was obtained from DuPont-New England Nuclear. Horseradish peroxidase coupled goat anti-rabbit IgG antibody was purchased from Zymed Laboratories, Inc. The chemiluminescence reagents were obtained from Amersham, Inc. and DuPont-NEN. Enzymes were obtained from New England Biolabs.

**Oligonucleotides and site-directed mutagenesis**

The following molecules were used in this study: L12: 5′-GGCTTCGGTGTTGCTAGTCTGCTTCCATGAGGAAACAATGAAATGATATAG-3′; L14: 5′-GGCTTGGTTGCTAGTCTGCTTCCATGAGGAAACAATGAAATGATATAG-3′; L18: 5′-GGCTTCGGTGTTGCTAGTCTGCTTCCATGAGGAAACAATGAAATGATATAG-3′; L19: 5′-GGCTTGGTTGCTAGTCTGCTTCCATGAGGAAACAATGAAATGATATAG-3′; L23: 5′-GGGCTTGGTTGACTACAGCACTATGAGGAAACAATGAAATGATATAG-3′; L28: 5′-GGCCGCTTGGTCATCTACAGCACTATGAGGAAACAATGAAATGATATAG-3′.

**PCR–RFLP analysis**

Bacteriophage or plasmid DNA served as the template for amplification reactions. PCR assays were done with phage suspension (~10^8 p.f.u.) or plasmid DNA (~10^8 molecules) in the presence of 0.4 µM each of Anti-Ωntm and Intra-Ant primers, 0.5 Unit Vent™ DNA Polymerase, 0.1 mM dNTPs in 10 mM KCl, 20 mM Tris–HCl (pH 8.8 at 25°C), 10 mM (NH₄)₂SO₄, 2 mM MgSO₄ and 0.1% Triton X-100. The PCR products (10 µl of a 100 µl PCR reaction) were subsequently digested with restriction endonucleases (0.25–1 U/µl) for 1 h according to the manufacturer’s instructions.

**Analysis of PCR products by dideoxynucleotide cycle sequencing**

The buffer components were separated from the amplified DNA products using dialfiltration in a 30 000 NMWL Ultra Free-MC ultrafiltration unit (Millipore, Inc.) according to the manufacturer’s instructions. The retentate containing the DNA was exchanged into TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) and adjusted to a final volume of 20 µl. Chain-termination sequencing reactions were performed with the PCR products (3 µl) using 5′-32P-labeled Intra-Ant and Arc-Ant primers and the cycle sequencing procedure as originally described (10).

**Bacteriological reagents and methods**

The genotypes of the bacteriological reagents used in this work are illustrated in Table 1. The P22 bacteriophages that encode a consensus operator site or a fragmented operator site in the 5′-end of the ant gene were constructed by homologous recombination in *Salmonella typhimurium* strain MS1883 as summarized in Figure 1c. In the original construction method (5), the DNA sequence encoding the desired RNA operator site was introduced into the P22 imm1 (arc+) region of pMMW20 using site-directed mutagenesis. An MS1883 transformant containing the resultant plasmid derivative was then infected with P22mnt::Ks9Arc(Am) to allow for homologous recombination between the resident plasmid and the infecting bacteriophage. Dilutions of the resultant phage lysate were plated on a lawn of MS1582 cells to permit the identification of turbid plaques that contain the recombinant arc allele. The arc⁺ phages that encode the RNA hairpin were identified by PCR–RFLP and direct sequence analyses. The arc(Am) allele was re-introduced into the resultant P22 phage derivative by performing a mixed infection with the phage encoding the RNA hairpin and a replication-defective helper phage derivative containing the arc(Am) allele [P22mnt::Kn9Arc(Am)] in MS1883. Phage lysates produced from the infection were plated on a lawn of MS1883 cells to permit identification of the clear phage plaques that contain the arc(Am) allele. The presence of the arc(Am) allele and the integrity of the encoded RNA hairpin in the final phage derivative were confirmed using PCR/RFLP and DNA sequence analyses.

In the revised construction method (8), the DNA sequence encoding the desired RNA hairpin was introduced into the P22 imm1 (arc(Am)) region of p9Gen1 using site-directed mutagenesis. An MS1883 transformant containing the resultant plasmid derivative was then infected with P22mnt::Ks9 to allow for homologous recombination between the resident plasmid and the infecting bacteriophage. Dilutions of the resultant phage lysate were plated on a lawn of MS1582 cells to permit the identification of clear phage plaques that contain the recombinant arc(Am) allele. The presence of the arc(Am) allele and integrity of the DNA sequence encoding the RNA hairpin was verified by PCR/RFLP analyses of the imm1 region and DNA sequence analyses of the 5′-end of the ant gene using the cycle sequencing procedure with Vent (exo–) DNA polymerase (10).

The plasmid pR17coat(+) [N55K] and pR17coat(+) [AID/N55K] were identified from a library of coat gene mutants that could promote lysogen formation for P22KAT(A–10)U using a genetic selection scheme described previously (11). The coding sequence that corresponds to the R17/MS2 coat protein mutant [AID] was originally isolated from a pR17coat(+)As plasmid library. The plasmid pR17coat(+) [AID] was constructed by removing the coat gene from pR17coat(+) [AID] using XhoI and HindIII restriction endonucleases and inserting the gene into pR17coat(+)-1.13 vector. Plasmid DNAs and bacteriophages were introduced into the *S.typhimurium* strains as described previously (5).

**RNA challenge plaque assays**

Briefly, MS1868 transformants containing one of the R17/MS2 coat protein expression plasmids were cultured to a cell density of ~5 × 10^6 cells/ml in LB media supplemented with ampicillin (100 µg/ml). MS1868 recipients (5 × 10^7 cells) were inoculated with the appropriate P22 challenge phage at a multiplicity of infection of 10–20. Following phage adsorption at 20°C for 20 min, the infected
cells were plated onto LB-agar plates containing the appropriate antibiotics. The number of lysogens that formed was determined by plating an appropriate serial dilution of infected cells on LB plates containing ampicillin (100 µg/ml) and kanamycin (50 µg/ml). Viable cell counts were determined in a similar fashion by plating uninfected cells on LB agar plates supplemented with ampicillin (100 µg/ml). The frequency of lysogenization (expressed as % lysogeny) was calculated as the number of colonies obtained on the LB plates containing ampicillin and kanamycin divided by the number of viable colonies obtained on the LB plates containing ampicillin, multiplied by 100.

Biochemical and immunological methods

The SDS–polyacrylamide gel electrophoresis experiments (SDS–PAGE) were carried out as described (11). The gels were fixed and stained with either silver nitrate or FastStain (Zoion, Inc.) according to the manufacturer’s instructions. Immunoblots were prepared by electrophoretic transfer of proteins from SDS–PAGE gels onto Immobilon PVDF membranes (Millipore Corp.). Membrane-bound coat proteins were detected with a rabbit anti-R17 coat protein polyclonal antiserum and visualized using a horseradish peroxidase linked goat anti-rabbit IgG (Zymed Laboratories, Inc.) with either an ECL Western detection kit (Amersham, Inc.) or the 3,3′-diaminobenzidine/H2O2/NiCl2 detection system (11).

MS1868 cultures (3 ml) that express a given coat protein were grown at 37°C to an OD600 of ~0.6, and the coat protein expression was induced by adding IPTG to a final concentration of 1 mM for 1 h. The cells were harvested by centrifugation at 12 000 g at 4°C for 3 min. Each cell pellet was resuspended in 0.3 ml of NGE buffer (0.05 M NaH2PO4, pH 7.0, 0.001 M MgCl2) and sonicated on ice. The cell lysates were clarified by centrifugation at 12 000 g at 4°C for 10 min. An aliquot of each lysate was adjusted to contain 1× loading buffer (5% glycerol, 0.04% xylene cyanole and 0.04% bromophenol blue) and subjected to electrophoresis (0.8 V/cm) in 0.9% agarose gels with circulating NGE buffer at room temperature until the bromophenol blue migrated to the bottom of the gel. The proteins were then transferred to a nitrocellulose membrane (MSI) by capillary blotting using NGE buffer before immunoblot analysis.

Table 1. Biological reagents used

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RESULTS

Previously, we described the use of a temperate DNA bacteriophage P22 derivative to evaluate RNA–protein interactions within a biological context (5). Two sets of genes normally determine the developmental fate of bacteriophage P22 in an infected *S. typhimurium* host cell (Fig. 1a). These genes reside in two genetically distinct loci within the bacteriophage genome termed the immunity regions, *immC* and *immI*. The expression of the c2 gene product, which resides in *immC*, causes the bacteriophage to establish and maintain a dormant, lysogenic state in the infected host cell. The c2 protein represses lytic gene functions by binding to two DNA operator regions (12). The *immI* region contains two additional repressor proteins termed Mnt and Arc, and an antirepressor protein called Ant. Expression of these genes occurs from two divergently transcribed operons. One transcription unit consists of P*mnt* and the *mnt* gene. The second unit consists of P*ant* and the *arc* and *ant* genes (12). The Mnt and Arc proteins selectively repress *ant* gene expression by binding to two different DNA operator sequences overlapping P*ant* (13–15). During the early phase of infection, P*ant* is utilized and a burst of Arc and Ant synthesis results. Arc represses transcription from P*ant* by binding to an overlapping operator site, O*ant*. Although Arc also represses its own expression through binding O*arc*, transcription of P*arc* is stimulated, leading to the synthesis of Mnt protein. Mnt binds to O*ant* to prevent further expression from P*ant*, and a stable lysogen can form. In addition, Mnt binding to O*ant* activates transcription from P*arc* (16).

Repression of *ant* gene expression by Mnt is critical for maintaining the lysogenic state because Ant is able to inactivate c2 repressor function by binding non-covalently to the protein (17). Without functional c2 repressor, lytic gene functions would be fully expressed, and the lytic growth phase would ensue. Although *ant* gene expression does not influence the developmental fate in the wild-type P22 context during normal infection (12), phages that contain an *arc(Am)* mutation dramatically overproduce Ant after infection (13), which can lead to lytic gene functions being expressed. The *arc(Am)*-bacteriophage can form lysogens, however, if transcription of *ant* is prevented. The relative amounts of c2 and Ant proteins synthesized in an infected cell will dictate whether an *arc(Am)*-bacteriophage becomes lysogenic or lytic.
RNA challenge phages are modified versions of P22 in which post-transcriptional regulatory events controlling the expression of ant determine the developmental fate of the phage. The modified phage encodes a disruption of mnt with a kanamycin resistance gene cassette and an amber mutation within arc (Fig. 1b). Sequence-specific RNA-binding activities can be detected using derivatives of P22 that have RNA target sequences substituted for the ant 5′-untranslated leader sequence region. The bacteriophage P22R17 is a derivative of P22 in which the chosen developmental pathway is regulated by the R17/MS2 coat protein interacting with its RNA target site located in the ant mRNA (5). Lysogenic development of the phage relies upon R17/MS2 coat protein expression in the susceptible host cell and the availability of a suitable coat protein binding site encoded by the phage genome. The system was used successfully to identify novel RNA ligands that display reduced affinity for the R17/MS2 coat protein (3) and to select for suppressor coat proteins that recognize mutant RNA ligands (11).

Biological repression specified by generic R17/MS2 RNA operator sites

Recognition of RNA by the R17/MS2 coat protein is specified by a small secondary structure element as shown in Figure 2a. Individual pairs of nucleosides within the helical regions of the structure are not uniquely required for coat protein’s RNA-binding activity (Fig. 2b; ref. 18). We assessed whether specific representatives of the consensus RNA ligand structure for coat protein recognition could serve as biological operator sites. RNA challenge phage derivatives were constructed in which the protein recognition could serve as biological operator sites. RNA hairpin in ant mRNAs generated from P22R17[L19], but is separated from the RNA hairpin in ant mRNA transcripts produced from P22R17[L23] and P22R17[L28] (Fig. 2c). The coat protein causes lysogen formation of P22R17[L19] at a frequency of ~40%, nearly twice that observed for P22R17 (Fig. 2c). The coat protein induces lysogens at an ~20-fold lower frequency following infection of recipient strains with P22R17[L23] (~2% lysogeny). No lysogens were observed above background levels following infection of recipient cells expressing the coat protein with P22R17[L28] (~10−6% lysogeny). The frequencies of lysogeny obtained with P22R17[L28] were comparable to phage lacking a translational operator (~10−5% lysogeny with P22mnt::KanBar(An)) (Fig. 3).

The results obtained with P22R17[L28] were so surprising that we suspected defects in P22R17[L28], such as a defective c2 gene product, might account for the observed low frequency of lysogeny. To test this idea, two additional independent clones of P22R17[L28] were constructed and evaluated in lysogen assays using MS1868[pR17coat(+)] as the recipient strain. The results obtained with these two phage clones were identical to the data obtained with the original P22R17[L28] (data not shown). Furthermore, the frequency of lysogeny of P22R17[L28] was unchanged following phage infection of recipient cells in which coat protein expression was increased by IPTG addition (data not shown). These data strongly imply that the coat protein exerts regulatory control over a discrete portion of the ant translational initiation region.

Other bacteriophages employed in these studies yielded unexpected results. The bacteriophage P22R17[L18] contains 1 nt of the Shine–Dalgarno sequence within the coat protein binding site; consequently, we anticipated that the coat protein would direct lysogen formation at a frequency between ~1 and ~40%. These values correspond to lysogen frequencies obtained following infection of MS1868[pR17coat(+)] recipient cells with P22R17[L14] and P22R17[L19], respectively (Fig. 2c). A frequency of only ~2 × 10−5% lysogeny was obtained following infection of recipient cells with P22R17[L18] (Fig. 2c). This result could be explained by the fact that the RNA hairpin from P22R17[L18] is predicted to be the least stable of the RNA secondary structures described in this study (19). A bacteriophage derivative that encodes a hybrid RNA hairpin (P22R17[L18/19]) was created to yield a more thermodynamically stable secondary structure in which the upper 3 bp of the helix from the RNA hairpin encoded by P22R17[L18] were substituted with the corresponding nucleotides from P22R17[L19] (Fig. 2c). Lysogen formation occurred at a frequency of 2.1% following infection of recipient cells with the resultant phage P22R17[L18/19] (Fig. 2c). This suggests that the failure of coat protein to effectively lysogenize P22R17[L18] is attributed to the protein’s altered ability to recognize a thermodynamically unstable RNA hairpin rather than to the position of the Shine–Dalgarno sequence within the ant mRNA transcript.

Translational regulation of fragmented RNA operator sites

The consensus coat protein binding site does not require the specific nucleotide pairs within the RNA secondary structure (18). We investigated whether the Shine–Dalgarno sequence could be positioned outside the RNA hairpin bound by the coat protein. The bacteriophages P22R17[L19], P22R17[L23] and P22R17[L28] encode fragmented RNA operator sites, as the coat protein binding determinants within the RNA hairpin do not overlap with the canonical sequences necessary for translation initiation of the Ant open reading frame. These phage derivatives contain the same binding site structure and Shine–Dalgarno sequence 5′ to the ant coding sequence. The Shine–Dalgarno sequence lies adjacent to the RNA hairpin in ant mRNAs generated from P22R17[L19], but is separated from the RNA hairpin in ant mRNA transcripts produced from P22R17[L23] and P22R17[L28] (Fig. 2c). The coat protein causes lysogen formation of P22R17[L19] at a frequency of ~40%, nearly twice that observed for P22R17 (Fig. 2c). The coat protein induces lysogens at an ~20-fold lower frequency following infection of recipient strains with P22R17[L23] (~2% lysogeny). No lysogens were observed above background levels following infection of recipient cells expressing the coat protein with P22R17[L28] (~10−6% lysogeny). The frequencies of lysogeny obtained with P22R17[L28] were comparable to phage lacking a translational operator (~10−5% lysogeny with P22mnt::KanBar(An)) (Fig. 3).
RNA packaging via encapsidation is a poor means of translational regulation by the R17/MS2 coat protein

Since coat protein dimers bind to the RNA hairpin from P22R17[L19], the failure of the wild-type coat protein to effectively lysogenize recipient cells upon infection with P22R17[L28] may be due to the coat protein’s ability to efficiently assemble into capsid structures lacking ant mRNA transcripts. Previously, we obtained genetic and biochemical evidence suggesting that the coat protein forms capsids readily in S. typhimurium and at the expense of increased translational repressor capacity in vivo (5,11). The coat protein produced from pR17coat(+) is expressed under the control of an IPTG-inducible promoter. Pre-treatment of recipient cells containing this plasmid
toward both wild-type and mutant operator sites (11,20). We protein sequence (N55K), encodes a super-repressor activity such as lysine for asparagine at position 55 in the mature coat assembly properties and expanded RNA-binding activities (11). Recently we identified an example of an RNA secondary structure differing from the intracellular concentration of coat protein dimers above the equilibrium constant for capsid formation would result in newly synthesized coat protein being shunted along the pathway leading to capsid structures. The lysogenic behavior of coat protein mutants with well-defined characteristics was analyzed to determine the features of the coat protein that are responsible for translational regulation. Recently we described two classes of R17/MS2 coat proteins with altered capsid assembly properties and expanded RNA-binding activities (11). One class of these coat protein mutants, exemplified by substitutions in Figure 2. The standard deviations for the data were calculated from an average of at least three independent infection assays.

with IPTG results in a 15-fold increase in the steady-state levels of coat protein (11), yet there is no corresponding increase in the frequency of lysogeny following infection by P22 r17 (5). Our interpretation of these data is that coat protein expression occurs very efficiently from pr17coat(+) without IPTG treatment and that the frequency of lysogeny obtained with P22 r17 is limited by the intracellular concentration of coat protein dimers that would be available to bind to ant mRNA transcripts. An increase in the intracellular concentration of coat protein dimers above the equilibrium constant for capsid formation would result in newly synthesized coat protein being shunted along the pathway leading to capsid structures.

The lysogenic behavior of coat protein mutants with well-defined characteristics was analyzed to determine the features of the coat protein that are responsible for translational regulation. Recently we described two classes of R17/MS2 coat proteins with altered capsid assembly properties and expanded RNA-binding activities (11). One class of these coat protein mutants, exemplified by substitutions such as lysine for asparagine at position 55 in the mature coat protein sequence (N55K), encodes a super-repressor activity toward both wild-type and mutant operator sites (11,20). We tested whether the mutant coat protein [N55K] could direct lysogen formation for several phage derivatives in a fashion similar to that observed by the protein mutant with P22 r17 (A–10)U (Fig. 3; ref. 11). Recipient cells harboring pr17coat(+) [N55K] readily survived challenge to lytic infection by P22 r17 [L18] as well as by other phages (P22 r17 [L12], P22 r17 [L14] and P22 r17 [L18/19]; Fig. 3). The mutant protein was able to direct lysogen formation ∼3- to ∼103-fold more effectively than the wild-type coat protein with operator sites.

Ant protein translation occurs efficiently from mRNAs with operator sites

One concern raised by these studies is whether the frequencies of lysogeny displayed by the P22 phage derivatives might be attributed to RNA–RNA interactions or to RNA association with other cellular proteins besides the coat protein. We recently identified an example of an RNA secondary structure differing from the intracellular concentration of coat protein dimers above the equilibrium constant for capsid formation would result in newly synthesized coat protein being shunted along the pathway leading to capsid structures.

The lysogenic behavior of coat protein mutants with well-defined characteristics was analyzed to determine the features of the coat protein that are responsible for translational regulation. Recently we described two classes of R17/MS2 coat proteins with altered capsid assembly properties and expanded RNA-binding activities (11). One class of these coat protein mutants, exemplified by substitutions such as lysine for asparagine at position 55 in the mature coat protein sequence (N55K), encodes a super-repressor activity such as lysine for asparagine at position 55 in the mature coat protein. We recently identified an example of an RNA secondary structure differing from the intracellular concentration of coat protein dimers above the equilibrium constant for capsid formation would result in newly synthesized coat protein being shunted along the pathway leading to capsid structures.
The primary sequence determinants necessary for coat protein binding have enabled assessment of substituted generic operator sites for the natural operator sequence. Our results with several phage derivatives indicate that the coat protein can regulate translation of an artificial RNA operator site that provides the native secondary structure of the RNA hairpin. Mutant coat proteins that possess enhanced RNA-binding activity can suppress some defects in the RNA hairpin structure such as those manifested in ant mRNA transcripts encoded by P22[ant][L18] and P22[ant][A(–10)U]. Several other RNA hairpins that are bound efficiently by the coat protein in vitro lack an adequate Shine–Dalgarno sequence (26); therefore, these RNA structures could not be evaluated in the RNA challenge phage system.

Translational regulation by the coat protein can be effectively accomplished for mRNA transcripts in which the Shine–Dalgarno sequence is removed entirely from the RNA secondary structure recognized by the protein. The extent to which the Shine–Dalgarno sequence can be positioned away from the RNA hairpin is limited. The wild-type coat protein behaves as a poor translational repressor of ant mRNA transcripts when the distance separating these two genetic elements is 4 or 9 nt. Coat protein mutants with a compromised ability to form native capsid structures recognize and repress several RNA operator sites that are not normally subject to translational regulation by the wild-type protein [e.g., A(–10)U; ref. 5]. The RNA-binding activity of these mutant proteins may be attributed to an increase in the intracellular concentration of dimer.

Figure 5. Lysogen frequencies for bacteriophages in MS1868 recipient cells. Cases in which the lysogen frequency was below the limits of detection for the dilution series analyzed in plating experiments are indicated by downward arrows. The standard deviations for the data were calculated from an average of at least three independent infection assays.
species or to subtle differences in the manner whereby the mutant protein dimer species interacts with the RNA hairpin.

Our data with the phages encoding the fragmented RNA operator sites allows us to expand our understanding of how a translational repressor functions and to discriminate between various models of translational repression. Appropriate regulation of translation is retained for RNA operators in which the Shine–Dalgarno sequence is placed entirely outside the coat protein binding site; therefore, we discount the importance of any model that stipulates that the Shine–Dalgarno sequence must be sequestered within a stable RNA secondary structure for translational repression. The occlusion model is difficult to reconcile when one considers all the data obtained with this system. The structural information gleaned from the crystallographic and solution data for the RNA–coat protein complex (25, 27) provide several constraints on the manner in which the coat protein interacts with the RNA hairpin. The coat protein dimer within the capsid establishes asymmetric contacts with its RNA ligand. Most of the RNA–protein contacts reside on the dimer within the capsid establishes asymmetric contacts with its coat protein interacts with the RNA hairpin. The coat protein nucleotide –20 lie 3′ determinants within the translational initiation region through sequence and the initiation codon (Fig. 6).

Our bacteriophage data can be rationalized with a model in which a coat protein-stabilized RNA secondary structure contributes to translational regulation. Stabilization energy is provided to the RNA secondary structure by the binding of the coat protein (28). The ribosome encounter site is experimentally defined as a region –17 to +16 on the mRNA transcript (29). Nucleotides at positions –16 and –17 lie within the RNA hairpin for ant mRNA transcripts encoded by P22R17[L23]. Sequence determinants within the translational initiation region through nucleotide –20 lie 3′ of the RNA hairpin for P22R17[L28], the only phage that was not lysogenized by recipient strains that express the coat protein. The coat protein exerts translational regulation by precluding access of ribosomal components on the mRNA transcript, including additional determinants within the stem region of the operator site other than the Shine–Dalgarno sequence and the initiation codon (Fig. 6).

In addition to its role as a translational repressor of replicase, the coat protein packages phage genomic RNA. The RNA operator site was originally thought to be the principal site where the packaging reaction initiates during natural infection since one coat protein dimer would bind to this site on the RNA genome midway through the replication cycle (18). The P22R17[L28] phage did not form lysogens in any recipient strains that expressed either the wild-type coat protein or one of the super-repressor coat proteins. Ant protein synthesis can occur during infection because the coat protein dimer and ribosome components are presumed to bind to adjacent sites on the ant mRNA transcript from P22R17[L28]. Lysogen formation should result for all phages that contain an RNA operator site at any location within the ant mRNA transcript if RNA encapsidation occurs. The low frequency of lysogeny data obtained with P22R17[L28] indicates that RNA encapsidation is not a plausible means of translational regulation. The coat protein can efficiently package genomic RNA that lacks an adequate RNA operator site (7) and other large heterologous RNAs into capsids (7, 30), suggesting that the RNA operator is not an essential cis-acting determinant of RNA packaging. A key determinant of RNA packaging undoubtedly includes an RNA substrate devoid of ribosomes. The translationally repressive nature of RNA secondary structure alone would provide such an RNA substrate (31–35) and probably contributes significantly to the selectivity of genomic RNA packaging during a natural infection cycle for the R17/MS2 coliphage.

The occupancy of the translational initiation region by a repressor protein to prevent binary complex formation is not limited to the R17/MS2 coat protein; this mechanism represents a common mode of translational regulation in prokaryotic phage and host cell proteins. The bacteriophage T4 regA protein represses translation of a number of early T4 mRNAs (36). RegA binds specifically to a single-stranded RNA sequence located in the translational initiation region (1, 4). The protein occupies the region encompassing the Shine–Dalgarno sequence and part of the initiation codon for the gene 44 mRNA (37) and binds to the RNA region that includes the initiation codon for the rib gene mRNA (38). The T4 DNA polymerase binds to a stem–loop structure located 5′ to the Shine–Dalgarno sequence and occupies the region extending from the initial RNA-binding site to near the initiation codon (39). The T4 gene 32 protein initially binds to a pseudoknot structure located 5′ to the Shine–Dalgarno sequence, and then multimerizes along the RNA until it physically masks the

Figure 6. Protein-stabilized RNA folding prevents translation for an artificial operator site. The coat protein can exert regulatory influence on a translational initiation region as long as the linear sequence determinants necessary for ribosome recognition and binding are contained within the RNA secondary structure (denoted here as the overlapping region in the ant mRNA transcript encoded by P22R17[L23]).
Shine–Dalgarno sequence (40). The threonyl-tRNA synthetase binds to a tRNA-like clover-leaf structure located 5′ of the Shine–Dalgarno sequence for the gene encoding thrS, thereby controlling its own synthesis (41,42). These diverse biological operators function according to a common mechanism and one that is shared with the R17/MS2 coat protein. Similar flexibility in operator structure might also exist for the function of these translational regulatory systems.

The translational repressor system of the R17/MS2 coliphage provides an elegant example of the compromise struck in a natural RNA operator site in balancing the requirement of the RNA secondary structure for regulatory protein binding with the need for ribosome access to linear sequence determinants. The study of the artificial RNA operator sites described in this work not only reveals new insights about the manner in which a translational repressor exerts regulatory control over its mRNA substrate but highlights how RNA-binding proteins may regulate the folded structure of RNA and its function. In the example reported here, translational regulation was modulated by the folded state of an RNA ligand bound by a protein. The extensive region required for translational initiation in bacterial mRNA transcripts offers a rich playground in which numerous types of interacting RNA and protein molecules can affect translation. The full measure of the diversity of translational control remains to be explored.

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