Failure of Translational Repression in the Phage f2 op3 Mutant Is Not Due to an Altered Coat Protein-RNA Interaction*

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A secondary phenotype of the op3 mutant of RNA bacteriophage f2 is the absence of translational repression of the phage replicase gene by the phage coat protein. We have synthesized RNA fragments corresponding to the site of translational repression for both the wild type and the op3 mutant. Using a quantitative assay, we show that the affinity of the closely related R17 coat protein for the mutant and wild type RNA fragments is the same. In addition, we find that the op3 and R17 coat proteins bind to the wild type RNA fragment with essentially identical dissociation constants. Thus, the altered regulation of replicase protein synthesis in the op3 mutant does not appear to be due simply to a reduced affinity of the translational repressor for its target site.

The op3 mutation of bacteriophage f2 creates a UGA termination codon from the CGA arginine codon at position 30 in the lysis protein gene (Fig. 1) (1-3). Premature termination of the lysis protein in a nonsuppressing host results in the accumulation of infectious phage without cell lysis (1). In addition, altered expression of the gene for the phage-encoded replicase subunit is observed in op3 infected cells. With wild type f2, replicase synthesis begins soon after infection and continues for about 20 min until it is repressed by the binding of coat protein to the replicase initiator (4). In an op3 phage infection, the synthesis of replicase does not start until 20 min after infection and continues unabated for at least 90 min (1). It is unclear whether this altered expression of the replicase gene is a result of incomplete translation of the lysis gene or due to the fact that the op3 mutation lies within the replicase initiation region. As shown in Fig. 1B, the C → U change in op3 at position +5 of the replicase gene (2) results in an additional base pair in a postulated secondary structure of the region (5). This change in the sequence and structure of the replicase initiator could lead to an alteration in ribosome or coat protein binding and thereby affect replicase expression. However, ribosome protection of op3 RNA showed no difference in the amount of replicase initiator recovered compared to wild type (2), and repression of in vitro replicase synthesis by coat protein appeared to be normal with op3 mRNA (1).

Our laboratory has been studying the binding of R17 coat protein with the replicase initiation region as an example of a sequence-specific RNA-protein interaction. We have synthesized a 21-nucleotide fragment (1) which contains residues -17 to +4 of the replicase gene (6) and appears to contain all the elements of the coat protein binding site (6, 7). In addition, we have shown that a filter retention assay can be used to determine the dissociation constant for the protein-RNA complex (7) and have provided evidence (7, 8) that the interaction of 1 with coat protein accurately reflects the interaction between the intact phage RNA and coat protein that is responsible for translational repression. It is likely that the protein-RNA interactions are identical in bacteriophages R17 and f2 since the two coat proteins differ by only a single amino acid (9) and the replicase initiation sequences are identical.

In this communication, we re-examine the interaction of coat protein with the op3 RNA sequence in hope of explaining the failure of translational repression. Because it is possible that a comparatively small change in the dissociation constant is responsible for the absence of repression of the replicase gene in an op3 infection, the large excess of coat protein used in the in vitro repression assay (1) might obscure such a difference. By using the more quantitative filter assay, a difference between the op3 and wild type interaction may be revealed.

MATERIALS AND METHODS

The enzymatic synthesis of the 21-nucleotide fragment of R17 RNA corresponding to -17 to +4 of the replicase gene (Fig. 1B) is described in Krug et al. (6). The fragment was dephosphorylated in a reaction containing 2 μM RNA and 80 μg/ml of bacterial alkaline phosphatase in ligase buffer (20 mM MgCl₂, 3 mM dithiothreitol, 50 mM Tris-HCl, pH 8.0) for 10 min at 65°C.

\[ \text{A} \]
\[ \text{B} \]

**Fig. 1. Sequence of the op3 mutant of phage f2.** A, the nucleotide sequence in the region of the mutation is shown with the position of the C → U change in boldface. The amino acid sequence of the prematurely terminated op3 lysis protein is indicated above the nucleotide sequence. The amino acid sequence of the replicase protein subunit is given below the nucleotide sequence. The nucleotide sequence is numbered with respect to the first nucleotide of the replicase gene. B, sequence 1 is a synthetic 21-nucleotide RNA fragment comprising residues -17 to +4 of the replicase gene shown in A. The sequence is drawn in a secondary structure that has been postulated (5) for the region. Addition of C to the 5' end of 1 creates 2, extending the wild type sequence, while addition of U to 1 results in 3, the op3 sequence, and addition of UU to 1 gives 4.
Translational Repression in the op3 Mutant of Phage f2

Preparation of [5'-32P]pCp, [5'-32P]pUp, and [5'-32P]pUpU from Cp, Up, or UpU was performed as described in England et al. (10). Additions to the 3' end of the 21-nucleotide fragment were accomplished in 10-μl reactions containing 9.94 μM RNA, 0.2 μM ATP, 3 mM dithiothreitol, 15% dimethyl sulfoxide, pH 8.3, ligase buffer, 100 μg of RNA ligase, and 0.2 μM of either [5'-32P]pCp (16 Ci/mmol), [5'-32P]pUp (5 Ci/mmol), or [5'-32P]pUpU (2.3 Ci/mmol). Incubation was at 0°C for 5 h. Preparation of [5'-32P]-labeled 21-nucleotide fragment is described in Krug et al. (6). Labeled RNA fragments were purified on a 20% polyacrylamide, 7 M urea gel and eluted without carrier RNA as described in Carey et al. (7).

Coat protein from the op3 mutant of phage f2 and from wild type phage R17 was prepared as described (7) without azide and used immediately. Mutant f2 op3 phage were a gift of P. Model (Rockefeller University). The concentration of op3 coat protein was determined by Lowry (11) assay with wild type R17 coat protein as a standard, since the extinction coefficient of the latter is known (9).

Protein excess binding curves were obtained in a buffer of 0.1 M Tris-HCl, pH 8.5, at 2°C, 80 mM KCl, 10 mM magnesium acetate (TMK). Labeled RNAs are diluted to ~10 μM in this buffer and coat protein is diluted in 1 mM acetic acid, pH 4, 20 μg/ml of bovine serum albumin. Protein is added to aliquots of RNA to give final protein concentrations of ~0.1 nM to ~1 μM. After 20 min, an aliquot of each reaction is filtered, without prior dilution or subsequent washing, on a nitrocellulose membrane (Millipore, HAWP). Because complexes and free protein are retained on the filter while unbound RNA passes through, scintillation counting of the filters yields the fraction of RNA bound as a function of protein concentration.

At high protein concentrations, a plateau of RNA binding is reached where less than 100% of the input RNA is bound. This plateau defines the retention efficiency, which is used to correct the rest of the data to give the fractional saturation at each protein concentration. The coat protein concentration at half saturation is equal to \( K_d \), the dissociation constant for the interaction. The values of \( K_d \) and retention efficiency obtained from the data are used to calculate theoretical curves for a binomial equilibrium of the form A + B ⇌ AB by considering the RNA concentration to be negligible compared to the total protein concentration throughout the binding curve.

RESULTS AND DISCUSSION

Three different variants of the RNA binding site shown in Fig. 1B were prepared by adding nucleotides to the 3' end of the 21-mer using RNA ligase. The addition of [5'-32P]pCp creates a 22-nucleotide fragment (2) with the wild type sequence while addition of [5'-32P]pUp creates the op3 sequence (3). Addition of [5'-32P]pUpU creates a 23-nucleotide fragment (4) with a new sequence that extends the helix by 2 base pairs compared to wild type. Thus, if the extra base pair in op3 affects the interaction with coat protein, the variant with two extra base pairs might display a more pronounced effect. The filter retention assay (7) was used to determine the dissociation constant between R17 coat protein and each of the variants; 5'-32P-labeled 21-mer (1) was included as a control.

As shown in Fig. 2, all four RNA fragments interact with R17 coat protein with an identical affinity. The \( K_d \) for each RNA fragment is 3 nM, which is the same as reported previously for 1 (7). The finding that the 21-mer (1) birds with the same \( K_d \) as the extended wild type sequence (2) was anticipated since 1 had been previously shown to bind as well as a 59-nucleotide fragment containing residues -53 to +6 (6, 7). Thus, residues +4 and +6 were not expected to be part of the coat protein binding site. However, since both the op3 (3) and the UpU (4) RNAs also bind as well as 1, we can conclude that base pairing of +5 to -16 and +6 to -17 does not alter coat protein binding. This result is significant since residues -16 and -17 have clearly been implicated as part of the coat protein binding site. Deletion of one or both of these residues greatly reduces coat protein binding (7, 12). Thus, although residues on the 5' side of the hairpin helix are important for coat protein binding, it does not matter whether they are base paired. Therefore, the op3 RNA sequence change does not cause an altered interaction with coat protein.

Although revertants of op3 by Escherichia coli normally and have a C at position +5, they form minute plaques and can revert further to fully wild type (1), suggesting that there may be a second mutation elsewhere in the op3 genome. Thus, there is a possibility that a lesion in the op3 coat protein gene could account for the failure of the repression of replicase by coat protein in op3-infected cells. We therefore determined the binding constant between 1 and coat protein derived from op3 phage. As shown in Fig. 3, the \( K_d \) measured for the op3 coat protein is about a factor two less than that for wild type R17 coat protein. Since this difference is comparable to the error of measuring \( K_d \) (7), we cannot detect any difference between the binding of wild type and op3 coat protein to the replicase initiator. A difference of only a factor of two in \( K_d \) is at any rate inadequate to account for the lack of repression between 20 and 90 min in an op3 infection, since the intracellular coat protein concentration more than doubles during this time (13).

Our findings suggest that the abnormal regulation of replicase in the op3 mutant cannot be accounted for by an altered interaction of the coat protein with the op3 replicase initiator. The molecular explanation of replicase expression in op3

1 The abbreviation used is: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
consequently awaits further study.

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