RNA Binding Site of R17 Coat Protein

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ABSTRACT: The specific interaction between R17 coat protein and its target of translational repression at the initiation site of the R17 replicase gene was studied by synthesizing variants of the RNA binding site and measuring their affinity to the coat protein by using a nitrocellulose filter binding assay. Substitution of two of the seven single-stranded residues by other nucleotides greatly reduced the K_a, indicating that they are essential for the RNA–protein interaction. In contrast, three other single-stranded residues can be substituted without altering the K_a. When several of the base-paired residues in the binding site are altered in such a way that pairing is maintained, little change in K_a is observed. However, when the base pairs are disrupted, coat protein does not bind. These data suggest that while the hairpin loop structure is essential for protein binding, the base-paired residues do not contact the protein directly. On the basis of these and previous data, a model for the structural requirements of the R17 coat protein binding site is proposed. The model was successfully tested by demonstrating that oligomers with sequences quite different from the replicase initiator were able to bind coat protein.

The coat protein of bacteriophage R17 represses translation of the R17 replicase gene by binding to a small hairpin loop containing the replicase initiation codon (Bernardi & Spahr, 1972). A synthetic 21-nucleotide fragment of RNA was found to bind coat protein with an affinity similar to that of intact R17 RNA, thereby defining a convenient system to study the details of a sequence-specific protein–RNA interaction (Krug et al., 1982; Carey & Uhlenbeck, 1983). By measurement of the affinity of coat protein to synthetic variants of the wild-type RNA fragment, the size of the binding site was established (Carey et al., 1983b). In addition, an intact secondary structure and at least two of the single-stranded residues of the RNA were found to be essential for protein binding.

In this paper we report the coat protein binding properties of 22 additional variants chosen to examine the importance of the remaining five single-stranded residues for binding, and we more thoroughly investigate the role of the base pairs in the interaction. From the combined information on all the variants, it is possible to deduce the structural elements in the RNA hairpin that are required for specific protein binding. This information allows the definition of other RNA sequences that should bind R17 coat protein. Several variants that differ substantially from the wild-type sequence were synthesized and tested for protein binding. In addition, a number of nucleic acid sequences were searched for potential coat protein binding sites.

Materials and Methods

Synthesis of RNA Fragments. The 24 RNA fragments shown in Figure 1 were synthesized enzymatically in a manner similar to that described by Krug et al. (1982) and Carey et al. (1983b). The control fragment **1** has the same sequence as R17 RNA, where +1 is the first nucleotide of the replicase gene. The synthetic scheme for each fragment is summarized in Table I. The general methods for the enzymatic synthesis of "quarter" molecules, the joining of quarter molecules to make "half" molecules, and the preparation of internally ^{32}P labeled binding fragments have been described in detail previously (Krug et al., 1982; Carey et al., 1983a). The synthesis of quarter molecules not previously described is given here.

Table I: Synthesis of Variant Binding Fragments[a]

[AAACAUG + AGGAU] + [NACCC + AUGU]	**1, 2**
[AAACAUG + AGGAN] + [UACCCAUGU]	**3–5**
[AAACAUG + AGGNU] + [UACCCAUGU]	**6, 7**
[AAACAUG + CGGAU] + [UACCCAUGU]	**8**
[AGACAUG + AGGAU] + [UACCCAUGU]	**9**
[GAACAUG + AGGAU] + [UACCCAUGU]	**10**
[AAACAUG + AGCAUU] + [AGCC + AUGU]	**11**
[AAACAUG + ACAUU] + [ACGC + AUGU]	**12**
[AAAAAAG + AGGAU] + [UACCC + UUUU]	**13**
[AAAAAAC + AGGAU] + [UACCG + UUUU]	**14**
[AAAAAAG + AGGAU] + [UACCCAUGU]	**15**
[AAACAUG + AGGAU] + [UACCC + UUUU]	**16**
[AAAAAAG + AGGAU] + [UACCG + UUUU]	**17**
[AAAAAAC + AGGAU] + [UACCC + UUUU]	**18**
[AAAAAAAAC + AAAC] + [AUG + UUUU]	**19**
[AAAAAAAAAAAAC] + [AUUUUUU]	**20**
[AAAAAAC + AGCAAN] + [AGC + GUUUU]	**21, 22**
[AAAAAAC + AGCAAN] + [AGC + GUUU]	**21A, 22A**
[AAAAAAC + AGCAAN] + [AGC + GUU]	**21B, 22B**
[AAAAAAU + AUAAAN] + [AUAA + UUUU]	**23, 24**

[a] Brackets indicate the two half-molecules used in the final ligation. N indicates positions where different nucleotides have been inserted (see Figure 1).

$(Ap)_6N$ (N = U, C, G), $(Ap)_8C$, and $(Ap)_{12}C$ were prepared by either RNase A or RNase T_1 digestion of the appropriate random copolymer as described by Uhlenbeck and Cameron (1979). ApUpUpU, $A(pU)_6$, $G(pU)_4$, and ApUpApA, and ApUpApApA were prepared from the appropriate dimer and nucleoside 5′-diphosphate by using polynucleotide phosphorylase as described by Krug et al. (1982).

The oligomers ApGpG, ApGpGpA, and CpGpGpA were chemically synthesized by the phosphotriester method (Everett et al., 1980) and were the gift of T. Neilson. ApGpCpC, ApCpGpC, ApGpGpC, and ApGpGpU were prepared by the addition of a 5′,3′-bisphosphate to the trinucleotide with RNA ligase followed by treatment with alkaline phosphatase. ApGpGpApN (N = C, A, G), ApGpGpNpU (N = U, C), UpApCpCpG, ApUpApApN (N = U, C), and CpGpGpApU were prepared from the corresponding tetramers in a similar

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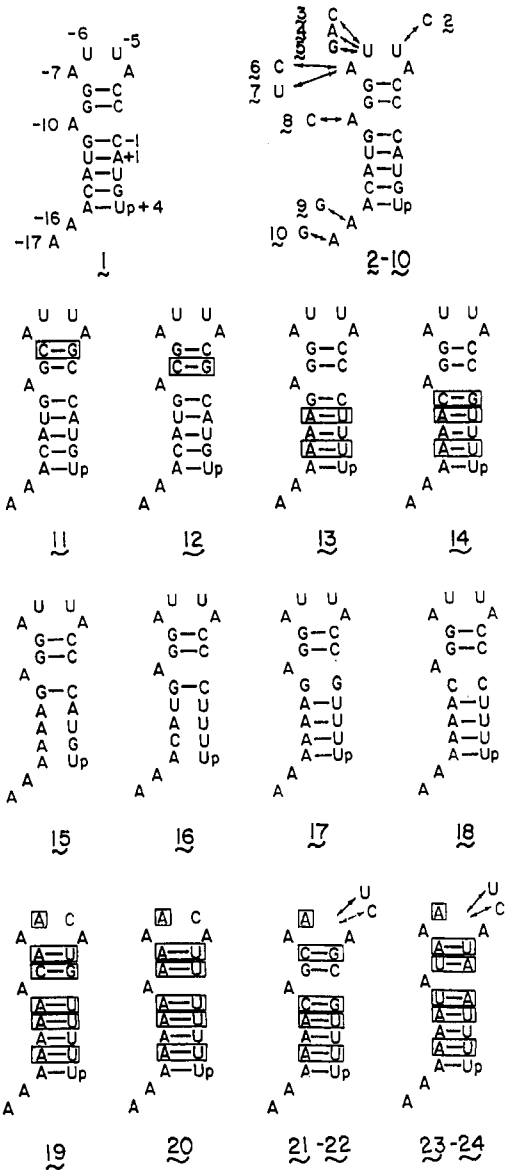

FIGURE 1: Sequences and possible secondary structures of coat protein binding fragments. Residue +1 in the wild-type fragment **1** is the first nucleotide in the R17 replicase gene. The nucleotides in fragments **11–14** and **19–24** that differ from **1** are boxed.

fashion. The reaction conditions for all 3′,5′-bisphosphate addition reactions were similar to those described for UpAp-CpCpU by Carey et al. (1983b). Trial reactions were performed to determine the optimal enzyme concentration for each donor and acceptor pair.

The heptamers AGACAUG and GAACAUG were prepared in three steps. AGA and GAA were made by the addition of A residues to the dimers, using polynucleotide phosphorylase. AGAC and GAAC were made by the addition of pCp to the trimers with RNA ligase followed by treatment with alkaline phosphatase. The addition of pAUGp to AGAC and GAAC with RNA ligase was carried out as described previously for the synthesis of A_3CAUG. The reaction conditions for all three steps were the same as described by Krug et al. (1982) and Carey et al. (1983b). In this case the heptamers were purified by ion pair high-performance liquid chromatography (HPLC) and desalted on BOND-ELUT columns (Beckett & Uhlenbeck, 1984).

AGCAUUp, ACGAUUp, and AGCAAUp were prepared in three steps. Periodate oxidation of AU_3 was carried out in a 500-μL reaction containing 2 mM AU_3, 0.4 M cyclohexylamine hydrochloride, 0.1 M *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (Hepes), pH 7.5, and 10 mM freshly prepared $NaIO_4$. After incubation in the dark for 1 h at 25 °C, the reaction was terminated by the addition of one drop of dimethyl sulfide and further incubation for 30 min. The reaction was then evaporated to dryness, resuspended in 0.5 mL of water, and incubated at 37 °C for 2 h. AUUp was purified by paper chromatography. pAUUp and pAAUp were made in a 0.5-mL reaction containing 1 mM trimer, 2.5 mM ATP, 50 mM Hepes, pH 8.0, 20 mM $MgCl_2$, 3 mM dithiothreitol, and 15 units/mL *pseT 1* polynucleotide kinase. After incubation at 37 °C for 4 h, each reaction was terminated by heating to 90 °C for 3 min. Synthesis of the hexamers was carried out in a 1-mL reaction containing the above 0.5-mL kinase reaction, 0.8 mM trimer acceptor, 1 mM ATP, and 300 μg/mL RNA ligase in the same buffer. After incubation for 12 h at 14 °C, the products were purified on ion pair HPLC.

Each internally ^{32}P labeled fragment was shown to be homogeneous and have the expected length by analysis on denaturing polyacrylamide gels. In addition, each oligomer was digested with mixed nucleases to give nucleoside 3′-monophosphates or with P_1 nuclease to give nucleoside 5′-monophosphates, and these reactions were analyzed by two-dimensional thin-layer chromatography as described by Krug et al. (1982). This procedure verifies that the expected internucleotide linkage between donor and acceptor is formed in each case (Carey et al., 1983b).

Coat Protein Binding. The association constant between coat protein and each fragment was determined with a nitrocellulose filter retention assay described in detail by Carey et al. (1983a). A constant, low concentration of ^{32}P-labeled RNA was mixed with a series of coat protein concentrations between 0.1 nM and 1 μM in 10 mM magnesium acetate, 80 mM KCl, 80 μg/mL serum albumin, and 100 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.5. After incubation at 2 °C for 20 min, the mixture was filtered through a nitrocellulose filter and the amount of complex retained on the filter determined by liquid scintillation counting. For each experiment the data points were fit to a retention efficiency and a K_a value, assuming a bimolecular equilibrium (Carey et al., 1983a). The precision of determining K_a with this assay is within a factor of 3 for independent coat protein dilutions. Each reported K_a value is the average of two to five independent determinations.

Computer Search. Nucleic acid sequences were searched for putative R17 coat protein binding site by use of the program Search from the Delila system (Schneider et al., 1982). The program has been modified so that it can search for sites that have relationships between the bases. The four relationships that can be defined are identity, nonidentity, complementary, and noncomplementary. The bacteriophage and *Escherichia coli* sequences from GenBank release 29.0 were converted to Delila book format by use of DbPull and DbBk (Schneider et al., 1984).

RESULTS

Variants in Single-Stranded Residues. The association constants for R17 coat protein binding to fragments **3–10** are compared with that of fragment **1** in Table II. Five of the seven single-stranded residues in the fragment have been substituted with one or more different nucleotides. Fragments with changes in the remaining two singled-stranded positions were studied previously (Carey et al., 1983b).

The substitution of U_{-6} with other nucleotides (fragments **3–5**) has comparatively little effect on coat protein binding.

Table II: Coat Protein Binding to Variants in Single-Stranded Residues

fragment	nucleotide change	K_a (M^{-1})	fragment	nucleotide change	K_a (M^{-1})
1	none	3×10^8	**7**	$A_{-7} \rightarrow U$	$<10^5$
3	$U_{-6} \rightarrow C$	3×10^8	**8**	$A_{-10} \rightarrow C$	$<10^5$
4	$U_{-6} \rightarrow A$	1×10^8	**9**	$A_{-16} \rightarrow G$	3×10^8
5	$U_{-6} \rightarrow G$	1×10^8	**10**	$A_{-17} \rightarrow G$	3×10^8
6	$A_{-7} \rightarrow C$	3×10^6			

The C_{-6} variant binds just as well as **1**, whereas the introduction of either purine at this position results in a 3-fold lower K_a. These data contrast with those obtained in experiments substituting several other single-stranded residues where the effects on K_a are much greater [Carey et al. (1983b) and below]. For example, when the adjacent U_{-5} is changed to a C, the K_a increases 150-fold, and when it is changed to a purine, it decreases 10–100-fold. Thus the relatively small changes in K_a observed for −6 variants suggest that little protein–RNA stabilization occurs at this site.

The sequence at positions −16 and −17 also does not appear to be important for coat protein binding. The substitution of either A_{-16} or A_{-17} with a G residue (fragments **9** and **10**) has no effect on the K_a. In addition, Cielers et al. (1982) have shown that the closely related bacteriophage fr translational operator (an A_{-6}, C_{-17} double variant) binds R17 coat protein tightly, so it appears that C_{-17} can be tolerated as well. Since it was previously established that deletion of A_{-17} eliminates protein binding, it is clear that a contact with the protein occurs at this position. The apparent absence of a sequence requirement suggests that the contact involves the sugar–phosphate backbone and not the nucleotide functional groups.

Substitution of A_{-7} with either a U or a C (fragments **6** and **7**) greatly reduces the affinity of the RNA to coat protein. In the case of the U_{-7} variant, very little complex could be detected at coat protein concentrations as high as 1 μM. Since higher protein concentrations result in the formation of empty viral capsids, the K_a of **6** could only be estimated to be less than 10^5 M^{-1}. The binding curve for the C_{-7} variant is nearly complete, so a K_a of 3×10^6 M^{-1} can be calculated. Similar very large decreases in the K_a were observed when the A at position −4 on the opposite side of the loop was substituted with other nucleotides (Carey et al., 1983b). Thus, both of the single-stranded A residues at the top of the stem are essential for coat protein binding. While the simplest interpretation is that one or more functional groups on the two adenosine rings make direct contacts with the coat protein, it is also possible that the two A residues interact with each other and orient the backbone in a conformation optimal for protein binding.

Another single-stranded nucleotide that appears essential for coat protein binding is the extrahelical A residue at position −10. Substitution of A_{-10} with a C reduces the K_a to below the detection limit of 10^5 M^{-1}. This result is consistent with the previous observation that deletion of A_{-10} abolishes protein binding (Carey et al., 1982). Since such "bulged" A residues appear in several different phage replicase translational operators (Weber, 1976; Inkuchi et al., 1986) and a number of putative ribosomal protein binding sites on *E. coli* 16S rRNA (Noller, 1984), it has been suggested that they constitute a specialized protein binding domain on RNA molecules (Peattie et al., 1981). While it is possible that the functional groups on A_{-10} interact with coat protein directly, recent NMR data on oligodeoxynucleotides suggest an alternate explanation. An extrahelical A residue in a short DNA helix appears to intercalate between the adjacent base pairs, resulting in a considerable distortion in the helical backbone (Patel et al., 1982). In contrast, an extrahelical C residue loops out and disrupts the helix much less (Morden et al., 1983). If a similar situation occurs in RNA, the deletion of A_{-10} or its substitution to a C residue may cause reduced protein binding by changing the relative distance between essential functional groups elsewhere in the RNA. Substitution of functional groups of A_{-10} that do not alter its ability to intercalate could distinguish between these two possibilities.

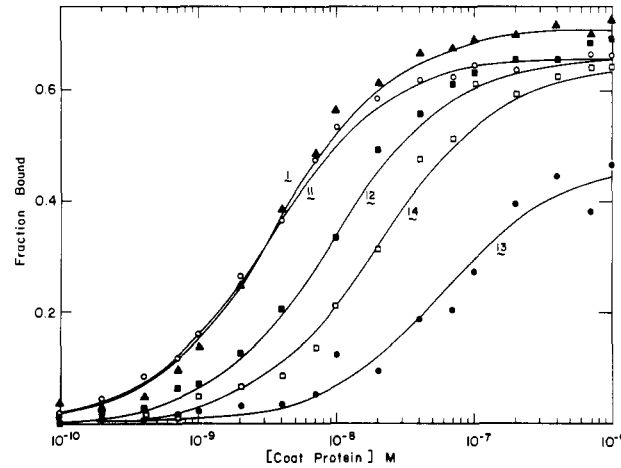

FIGURE 2: Coat protein binding to base-pair variants **11–14** compared with that to **1**. For each variant the solid lines are calculated for a bimolecular equilibrium, assuming a best fit retention efficiency and the K_a values given in Table II.

Table III: Coat Protein Binding to Other Fragments

fragment	K_a (M^{-1})	fragment	K_a (M^{-1})
1	3×10^8	**19**	$<10^5$
2	5×10^{10}	**20**	$<10^5$
11	3×10^8	**21**	2.6×10^7
12	1.1×10^8	**21A**	2.6×10^7
13	2.2×10^7	**21B**	2.5×10^7
14	6×10^7	**22**	2.1×10^8
15	$<10^5$	**22A**	2×10^8
16	5×10^6	**22B**	2×10^8
17	$<10^5$	**23**	$<10^5$
18	$<10^5$	**24**	$<10^5$

Base-Pair Changes. Fragments **11–14** have changes in the base-paired portion of the binding site that maintain the secondary structure of the hairpin, while fragments **15–18** have sequences that prevent one or more base pairs from forming. The coat protein excess binding curves of **11–14** are compared with that of **1** in Figure 2. Although some differences in the K_a's are observed, it is clear that all four of these variants bind coat protein quite well. In contrast, **15–18** all bind coat protein poorly (Table III). Taken together, the data suggest that while the base pairs are important for protein binding, the type of base pair is not. Although a very limited set of base-pair variants have been tested, it is tempting to suggest that the coat protein does not interact with the nucleotide functional groups in the helical portion of the molecule. The protein may make contacts with the sugar–phosphate backbone of the helix. Alternatively, the sole purpose of the base pairs may be to maintain the correct relative orientation of the essential single-stranded residues identified in the previous section.

Model for the R17 Coat Protein Binding Site. By combining the data of Carey et al. (1983b) with those presented above, it is possible to propose a model for what is required in an RNA sequence to interact specifically with R17 coat protein. The RNA fragment must be at least 19 nucleotides in length, and additional 5′ or 3′ residues do not affect binding.

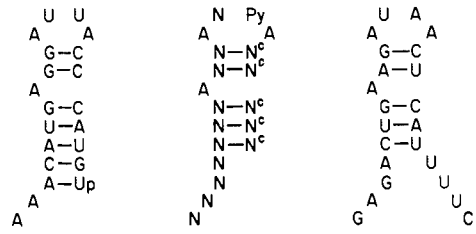

FIGURE 3: A comparison of **1** (left), a model for generalized R17 coat protein binding site (center), and a sequence from the MS2 A protein gene (right).

The sequence must be able to form the characteristic hairpin loop of four residues and have a single looped-out adenosine in the proper position. Four to five base pairs are needed to stabilize the structure although, to a first approximation, the sequence of the base pair is not important. The identities of the nucleotides at positions −6, −16, and −17 are not important although the nucleotides must be present. The nucleotides at positions −4, −7, and −10 must all be A residues while the residue at position −5 can vary, although the K_a depends upon the sequence. This model is summarized in Figure 3.

Fragments That Test the Model. Six oligonucleotides were synthesized (fragments **19**–**24**) that would appear to fit the general structural requirements for coat protein binding described above but have sequences substantially different from that of **1**. A shown in Table III, variants **19** and **20** have no detectable binding to R17 coat protein. The likely explanation for this result is that the structure shown for both variants **19** and **20** is not the thermodynamically most favorable one. Instead of looping out, the A residue at position −10 is more likely to pair with the opposing U residue. The remaining AU base pairs also will shift pairing partners, thereby forming an uninterrupted helix that is missing the essential bulged A_{-10}. Although the contribution of a bulged A residue to the stability of a helix has not been experimentally determined, it has been estimated to destabilize the helix by at least +3 kcal/mol (Tinoco et al., 1973). This difference in free energy would mean that >99% of the molecules would be in a conformation missing the bulged A residue. The shifting of pairing partners in the helix has also been shown to influence protein binding of other fragments (Carey et al., 1983b) and emphasizes the importance of considering the structure of the RNA when evaluating its activity.

Fragments **21** and **22** are much better tests of the binding site model since they do not appear to be able to form alternate conformational forms and differ from **1** and **2** at nine positions (four base pairs and one single-stranded residue). Fragment **21** binds with a K_a only 12-fold less than that for **1** (Table III). Some of the lower K_a of **21** can be attributed to the fact that it has an A residue at position −6 which reduces K_a 3-fold (fragment **4**). Fragment **22** is identical with **21** except that it has the stabilizing C at position −5, and this differs from **1** by 10 nucleotides. As expected, **22** binds coat protein better than **21**, although the K_a is only 10-fold greater instead of the 160-fold difference one sees comparing **2** and **1**. Perhaps the equilibrium of the transient covalent adduct thought to occur at position −5 (Romaniuk & Uhlenbeck, 1985) is altered slightly. In any case, the tight binding of fragments **23** and **24** substantiates the binding site model proposed in Figure 3.

It was previously shown that the two 3′-terminal nucleotides of fragment **1** could be removed without altering protein binding (Carey et al., 1983b). In order to test whether this was also the case for fragment **21**, two fragments were synthesized (**21A** and **21B**) that were one and two nucleotides shorter, respectively, than **21** at the 3′ terminus. A similar pair of truncated variants of **22** were prepared. In each case the shorter molecules bound coat protein just as well as the corresponding 21-nucleotide fragment (Table III). Thus, the same size of the coat protein binding site could be deduced with sequences that differ substantially from the wild-type sequence.

Fragments **23** and **24** were made in an attempt to construct a coat protein binding site composed entirely of adenosine and uridine residues and unable to form alternate conformational forms. As shown in Table III, neither fragment binds coat protein. Fragments **23** and **24** only differ from the tightly binding **21** and **22** in that the upper three base pairs are AU pairs instead of GC pairs. Since it was previously concluded that the protein is unlikely to form direct contacts with these base pairs, the most likely explanation for the lack of binding of **23** and **24** is that the AU pairs are simply not stable enough to maintain the correct orientation of the essential A residues at positions −4, −7, and −10. Indeed, a calculation based on the available thermodynamic data (Tinoco et al., 1973) suggests that fragments **23** and **24** are not stable at 0 °C (ΔG = +1.6 kcal/mol).

Other Coat Protein Binding Sites. The definition of a minimal binding site for R17 coat protein makes it possible to search other nucleic acid sequences for potential binding sites. The set of sequences used to search for coat protein binding sites was $N_1N_2AN_3N_4AX_1X_2AN_4^CN_3^CN_2^CN_1^C$, where X_n is any nucleotide and N_n^C is any nucleotide complementary to N_n. These sequences will fold into a structure identical with the upper part of the binding site model in Figure 3. Since the essential upstream residues (positions −13 to −17) can have any sequence or secondary structure, they were not included in the search sequence. Only four base pairs are included in the search sequence since if the structure were stable, some protein binding might occur. The probability that a random 13-nucleotide sequence of RNA would be one of the search sequences is 1 in 16000.

A search of approximately 265 kilobases of *E. coli* DNA sequence in a recent GenBank listing revealed 15 potential coat protein binding sites, which is one less than would be expected at random. It is unlikely that coat protein acts as a translational repressor of any of the genes present in the sequence library since none of the potential binding sites are near the translational initiation sites of the genes. In particular, it is interesting that the *Tuf A*, *Tuf B*, *Tsf*, and *Rps A* genes do not contain potential coat protein binding sites although their gene products (elongation factors T_u and T_s and ribosomal protein S1) combine with the product of the phage replicase gene to form the functional replicase. It is unlikely that many *E. coli* genes are regulated by R17 coat protein since analysis of *E. coli* proteins on two-dimensional gels reveals no changes when high concentrations of coat protein are induced (Parker & Holtz, 1984; J. Parker, personal communication).

Potential coat protein binding sites were found in three of the *E. coli* rRNA operons. The *rrnE* and *rrnX* matches are located between the P1 and P2 promoters of the genes, and the *rrnB* match is 20 bases upstream of the mature 5′ end of 23S rRNA. The significance of these matches is unknown. Since none of the matches are present in the mature domains of 16S or 23S rRNA, the observation is consistent with the fact that an excess of 16S or 23S rRNA was unable to compete with 21-mer for coat protein binding (Carey & Uhlenbeck, 1983).

A search of the available *E. coli* bacteriophage sequences for potential R17 coat protein binding sites revealed several interesting results. No binding sites were found in the Qβ

RNA genome or in the minus strand of the MS2 RNA genome. However, *two* potential R17 coat protein binding sites were found in the plus strand of the MS2 RNA sequence. One site is the replicase initiator ($X_1 = U_{1748}$), which has the same sequence as R17 and interacts with the nearly identical MS2 coat protein in a similar fashion. The second site is located near the 5′ end of the A protein gene ($X_1 = U_{216}$), centering about the 27th codon of the protein (Figure 3). While it is not certain that this site is present in the closely related R17 RNA sequence, it should be able to bind MS2 coat protein. Since this second site has an A residue at position −5, it will probably have an affinity for coat proteins of at least 10-fold less than the replicase initiator. This fact may explain why a fragment corresponding to this sequence was not found in coat protein protection experiments (Bernardi & Spahr, 1972).

Discussion

The molecular basis of the highly specific interaction between R17 coat protein and its translational operator was studied by measuring the binding of RNA fragments that differ from the wild-type sequence by one or more nucleotides. Variants with reduced K_a's for binding to coat protein were assumed to either be missing one of the structural elements necessary for binding or have a secondary structure that prevents the essential RNA–protein contacts from forming. Variants that bind with a similar K_a were assumed to have all the necessary contacts for protein binding. This approach resembles a study of the interaction of synthetic operator variants with *lac* repressor (Caruthers, 1980; Mossing & Record, 1985) and can be considered the biochemical analogue to saturating a regulatory site with mutations. It is noteworthy that more than 40 variants were tested before a tentative model for the general features of the coat protein binding site could be proposed. Many additional variants will be needed to confirm and refine the model. To a certain degree, this comparatively exhaustive analysis was necessary because the regulatory site was composed of RNA with a defined secondary structure instead of DNA with a linear sequence of nucleotides. A simple saturation of the site by single nucleotide changes was insufficient to fully understand the nature of the regulatory site. Indeed, some of the most useful information was obtained from variants that had multiple nucleotide changes but showed normal binding. This emphasizes the utility of a synthetic approach in understanding RNA recognition sites.

It is clear that the replicase translational operator is defined by a subtle combination of RNA sequence and structure. Specific nucleotides are required at only four positions in the operator sequence. However, the remaining nucleotides must be specified such that the RNA can fold into its characteristic hairpin loop secondary structure. The four "specific" residues are all single stranded and probably must be maintained in the correct spatial orientation. The protein then interacts with the entire hairpin, forming a number of precise contacts at the interface of the two macromolecules. An important future goal is to learn the molecular details of these contacts.

The apparent absence of a specific sequence requirement for the helical residues suggests that the available functional groups of the base-paired nucleotides do not interact with coat protein directly. This situation is quite common for protein binding sites on RNA molecules. Several cases have been found where an interruption of a base pair causes a mutant phenotype that can be reverted by a second site change that forms a different base pair (Smith et al., 1970; Johnson & Roth, 1981). It is possible that the structure of the A-type RNA helix makes the base-paired residues less accessible to protein contact than is the case with proteins binding to B-type DNA.

It seems likely that the nucleotide functional groups of the four "specific" single-stranded residues each interact directly with amino acid side chains of the protein. Indeed, in another paper (Romaniuk & Uhlenbeck, 1985) we discuss the possibility that a transient covalent adduct forms between U_{-5} and a cysteine on the protein. However, given the clear RNA structural requirement for tight binding, it is important to consider the extent that the single-strand nucleotides determine the overall folding of the RNA chain in a sequence-specific fashion. As discussed above, A_{-7} could interact with A_{-4} and constrain the sugar–phosphate backbone of the hairpin loop while A_{-10} could intercalate into the helix and alter the phosphate–phosphate distance of adjacent base pairs. Thus, it remains possible that the protein only contacts the sugar–phosphate backbone of the hairpin loop. Binding experiments using RNAs containing nucleotide functional group modifications at defined positions may help determine how the specificity is achieved, although a complete structural determination of the complex will ultimately be required to resolve the issue.

The biological significance of an additional coat protein binding site near the 5′ end of the A protein gene is unclear. Pulse-labeling experiments indicate that late in infection (30–40 min) the amount of A protein synthesis of the closely related f2 phage drops substantially with respect to coat protein synthesis (Robertson, 1975), thereby suggesting that the A gene is regulated. Since the coat protein concentration is expected to be quite high late in infection, it is possible that the weaker A gene binding site could become saturated by coat protein and repress translation of the A gene. However, in vitro translation experiments using either f2 RNA or f2 replicative intermediate as message showed that added coat protein had no effect on the amount of A protein synthesized while translational repression of the replicase protein was clearly evident (Robertson, 1975). Although it is possible that the concentration of active coat protein in these experiments was not high enough to saturate the weak A protein binding site, no evidence for a translational repression mechanism can be claimed.

Another possible function of the A gene site could be in the phage assembly pathway. We have recently acquired evidence that the replicase translational operator is an initiation site of phage assembly (Beckett and Uhlenbeck, unpublished experiments). Perhaps the A site is a member of a group of weaker binding sites that ensure correct attachment of the genomic RNA inside the capsid. The phenotype of mutants in the A gene binding site that either strengthened or weakened the affinity of coat protein without changing the sequence of A protein would be very interesting.

Registry No. **1**, 82642-77-1; **2**, 106587-75-1; **3**, 106587-76-2; **4**, 106587-84-2; **5**, 106587-85-3; **6**, 106587-67-1; **7**, 106587-66-0; **8**, 106587-68-2; **9**, 106587-73-9; **10**, 106587-74-0; **11**, 106587-71-7; **12**, 106587-72-8; **13**, 106587-69-3; **14**, 106587-70-6; **15**, 106587-86-4; **16**, 106587-64-8; **17**, 106587-81-9; **18**, 106587-65-9; **19**, 106587-80-8; **20**, 106587-78-4; **21**, 106587-82-0; **22**, 106587-83-1; **23**, 106587-77-3; **24**, 106587-79-5; $(Ap)_6U$, 106470-72-8; $(Ap)_6C$, 33708-21-3; $(Ap)_6G$, 56078-77-4; $(Ap)_8C$, 106470-73-9; $(Ap)_{12}C$, 106587-63-7; ApUpUpU, 74713-41-0; $A(pU)_6$, 106470-74-0; $G(pU)_4$, 56931-06-7; ApUpApA, 5288-62-0; ApUpApApA, 3390-96-3; ApGpCpC, 55095-45-9; ApCpGpC, 106470-75-1; ApGpGpC, 56399-93-0; ApGpGpU, 56399-95-2; ApGpGpApC, 106470-76-2; ApGpGpApA, 106470-77-3; ApGpGpApG, 106470-78-4; ApGpGpUpU, 106470-79-5; ApGpGpCpU, 79507-40-7; UpApCpCpG, 106470-80-8; ApUpApApU, 106470-81-9; ApUpApApC, 52724-61-5; CpGpGpApU, 106470-82-0; ApGpApCpApUpG, 106470-83-1; GpApApCpApUpG,

106470-84-2; ApGpCpApUpUp, 106470-85-3; ApCpGpApUpUp, 106470-86-4; ApGpCpApApUp, 106470-87-5; $A(pU)_3$, 74713-41-0; ApUpUp, 6200-33-5; pApUpUp, 106470-88-6; pApApUp, 106470-89-7; pCp, 2922-94-3; ApGpA, 3393-24-6; GpApA, 3308-20-1; pApUpGp, 82604-39-5; ApG, 3352-23-6; GpA, 6554-00-3; ApGpApC, 79507-37-2; GpApApC, 106470-90-0.

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Mobilization of Hepatic Calcium Pools by Platelet Activating Factor†

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ABSTRACT: In the perfused rat liver, platelet activating factor, 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (AGEPC), infusion produces an extensive but transient glycogenolytic response which at low AGEPC concentrations (i.e., 10^{-11} M) is markedly dependent upon the perfusate calcium levels. The role of calcium in the glycogenolytic response of the liver to AGEPC was investigated by assessing the effect of AGEPC on various calcium pools in the intact liver. Livers from fed rats were equilibrated with $^{45}Ca^{2+}$, and the kinetics of $^{45}Ca^{2+}$ efflux were determined in control, AGEPC-stimulated, and phenylephrine-stimulated livers during steady-state washout of $^{45}Ca^{2+}$. AGEPC treatment had only a slight if any effect on the pattern of steady-state calcium efflux from the liver, as opposed to major perturbations in the pattern of calcium efflux effected by the α-adrenergic agonist phenylephrine. Infusion of short pulses of AGEPC during the washout of $^{45}Ca^{2+}$ from labeled livers caused a transient release of $^{45}Ca^{2+}$ which was not abolished at low calcium concentrations in the perfusate. Moreover, there occurred no appreciable increase in the total calcium content in the liver perfusate at either high or low concentrations of calcium in the perfusion fluid. Infusion of latex beads, which are removed by the reticuloendothelial cells, caused the release of hepatic $^{45}Ca^{2+}$ in a fashion similar to the case with AGEPC. Our findings indicate that AGEPC does not perturb a major pool of calcium within the liver as occurs upon α-adrenergic stimulation; it is likely that AGEPC mobilizes calcium from a smaller yet very important pool, very possibly from nonparenchymal cells in the liver.

1-*O*-Hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (AGEPC), a potent phospholipid mediator of biological inflammatory responses in various cell types, e.g., basophils (Hensen & Cochrane, 1971; Pinckard et al., 1979; Benveniste, 1974), mast cells (Camussi et al., 1977; Clark et al., 1980), platelets (Demopoulos et al., 1979; Hanahan et al., 1980), and neutrophils (Clark et al., 1980; O'Flaherty et al., 1981a,b), has been shown to exert several additional biological responses in tissues, such as guinea pig heart (Burke et al., 1982) and guinea pig ileum (Findlay et al., 1981). Recently, we have

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