is possible that these data represent degradation of the phosphoproteins during isolation and preparation, it is also possible that it reflects degradation during in vivo maturation (Dimuzio & Veis, 1978; Veis et al., 1981).

The present experiments, characterizing the phosphoproteins extracted from very young, fetal calf dentin and from mature, adult bovine dentin under identical conditions designed to eliminate or minimize proteolysis, strongly suggest that the single high molecular weight phosphoprotein of fetal calf dentin is degraded during the progressive mineralization of dentin during maturation. This may at least partially explain the earlier finding that the major phosphoprotein of young steer dentin was much smaller and had a much lower phosphorus content (4.8% vs. 8.0%).

Degradation of the phosphoproteins of mineralized tissues during maturation appears to be a general phenomenon. Direct evidence for the in vivo degradation of the enamel proteins has been obtained (Strawich & Glimcher, 1982), and there is highly suggestive evidence that the phosphoproteins of bone are also degraded during maturation (Lee & Glimcher, 1979, 1981).

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Sequence-Specific Interaction of R17 Coat Protein with Its Ribonucleic Acid Binding Site†

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ABSTRACT: The interaction between phage R17 coat protein and its RNA binding site for translational repression was studied as an example of a sequence-specific RNA–protein interaction. Nuclease protection and selection experiments define the binding site to about 20 contiguous nucleotides which form a hairpin. A nitrocellulose filter retention assay is used to show that the binding between the coat protein and a synthetic 21-nucleotide RNA fragment conforms to a simple bimolecular reaction. Unit stoichiometry and a K_d of about 1 nM are obtained at 2 °C in buffer containing 0.19 M salt. The interaction is highly sequence specific since a variety of RNAs failed to compete with the 21-nucleotide fragment for coat protein binding.

Efforts to understand the molecular basis of the specific interaction between an RNA sequence and a protein are often hampered by the complexity of the system. For example, in the case of multistep catalytic reactions such as the aminoacylation of tRNAs by synthetases, it may be difficult to provide a simple description of the reaction in terms of individual contacts on the surfaces of both RNA and protein molecules. This consideration prompted us to search for a sequence-specific RNA–protein interaction in which biological function is exerted in a single binding event. Such a system should permit description of the reaction as a simple bimolecular equilibrium amenable to detailed physical and biochemical characterization. The success of this approach in understanding sequence-specific DNA–protein interactions is exemplified by work on the *lac* repressor (Riggs et al., 1970; Record et al., 1977; Butler et al., 1977; Barkley et al., 1981; Winter & von Hippel, 1981).

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Good candidates for sequence-specific RNA binding proteins are the translational repressors. These proteins block the initiation of translation by binding to a specific initiation region on the mRNA. Although several examples of translational repressors have been described (Lemaire et al., 1978; Yates et al., 1980; Model et al., 1982), the best characterized case is the repression of the phage R17 replicase gene by R17 coat protein (Lodish & Zinder, 1966). Considerable genetic and biochemical evidence indicates that about 10 min after phage infection, the synthesis of replicase protein ceases due to the intracellular accumulation of coat protein (Lodish & Zinder, 1966). Physical evidence for a specific interaction between coat protein and a target site on the RNA is also available. Under conditions similar to those in which in vitro translation of the replicase cistron is specifically repressed by coat protein, a complex of R17 RNA and between one and six coat protein monomers can be demonstrated (Sugiyama et al., 1967; Spahr et al., 1969; Zagorska et al., 1975). Digestion of this complex with ribonuclease allowed the isolation of a 59-nucleotide protected fragment covering the region −53 → +6 where +1 is the first nucleotide of the replicase gene (Bernardi & Spahr, 1972). This purified 59-nucleotide fragment has been shown to rebind the coat protein with approximately the same relative affinity as intact R17 RNA (Berzin et al., 1978), suggesting that all the contacts necessary for the specific interaction reside in the protected fragment. The 59-mer contains two hairpin loops (Gralla et al., 1974). The smaller hairpin, −15 → +3, is most important for coat protein binding since its melting behavior is altered by the coat protein (Gralla et al., 1974) and because fragments containing only the smaller hairpin are also active in coat protein binding (Steitz, 1974; Jansone et al., 1979). Our laboratory has recently synthesized a 21-nucleotide RNA fragment containing the smaller hairpin (Krug et al., 1982). Since this molecule binds coat protein with the same affinity as the 59-mer, the binding site is contained in the region −17 → +4.

In this paper, several aspects of the interaction between R17 coat protein and its target site are investigated to determine whether it is a good example of a sequence-specific RNA–protein interaction. The secondary structure and size of the RNA binding site are examined in further detail. A nitrocellulose filter binding assay for the reaction is established, and the equilibrium constant and stoichiometry are determined. The specificity of the interaction is assessed by the extent to which other RNAs can compete in the binding reaction.

Materials and Methods

R17 Coat Protein. Phage R17 was propagated on *Escherichia coli* S26 and purified according to Kolakofsky (1971) as modified by R. Gesteland (personal communication). Coat protein was prepared by the method of Berzin et al. (1978) except that the phage stock was about 25 mg/mL and Sephadex G-10 replaced G-100 for desalting coat protein into 1 mM acetic acid in the last step. The G-10 column pool had an average protein concentration of 1.4 mg/mL and a pH of 4.0 and could be stored at 4 °C with 0.05% sodium azide for at least 3 months without any change in RNA binding properties. Incubation of coat protein with labeled RNA failed to reveal any nuclease activity. A molar extinction coefficient of 1.54×10^4 at 280 nm was used for the coat protein (Weber & Konigsberg, 1975).

RNA. R17 RNA was purified according to Kolakofsky (1971), and degradation was estimated to be less than 5% by electrophoresis in a 4% acrylamide–8M urea gel. The method of Gralla et al. (1974) for isolation of protected fragments was modified as follows. An incubation mixture was formed from 60 μL of 30 μM R17 RNA in water, 440 μL of autoclaved TMK buffer [0.1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.5 at 2 °C, 80 mM KCl, and 10 mM magnesium acetate], and 300 μL of 50 μM coat protein. After 10 min at 2 °C, the mixture was warmed to 22 °C, and 10 μL of 25 000 units/mL ribonuclease T_1 (Calbiochem) was added. After 30 min at 22 °C, the mixture was filtered through a stack of two presoaked 24-mm nitrocellulose filters (Millipore, HAWP) and washed twice with 2-mL aliquots of cold TMK. Protected fragments were recovered from the filter by extraction with 0.5 mL of 0.1 M Tris-HCl, pH 7.5, and 0.5 mL of neutralized phenol. After ether extraction of the aqueous layer, the fragments were concentrated by ethanol precipitation. The RNA fragments were purified by applying them to two wells of a 20% polyacrylamide–7.5 M urea gel (260 × 150 × 0.75 mm) and electrophoresing for 8 h at 800 V. When the gel was examined by ultraviolet shadowing, the major RNA bands observed migrated as a closely spaced doublet with an R_f of 0.56 with respect to the xylene cyanol dye. Each band of this doublet was excised and eluted from the crushed gel slice by soaking in 1.5 mL of 0.1 M Tris-HCl, pH 7.5, and 0.1 mM ethylenediaminetetraacetic acid (EDTA) at 4 °C for 8–12 h. The RNAs were recovered from the eluate by ethanol precipitation. About 5–10 μg of each band, corresponding to a 12–25% molar yield, was recovered by this procedure. The identities of the upper band as the 59-nucleotide coat protein binding fragment −53 → +6 and the lower band as the 56-nucleotide fragment −53 → +3 (Jansone et al., 1979) were confirmed by enzymatic RNA sequence analysis.

The enzymatic synthesis of the 21-nucleotide fragment −17 → +4 is described in Krug et al. (1982). The molar extinction coefficient of the fragment at 260 nm is assumed to be 2.1×10^5. That paper also describes the synthesis of the 21-mer with an internal ^{32}P label.

5′-^{32}P-Labeled 59-mer and 21-mer were prepared by incubating 1 μM [γ-^{32}P]ATP (2000 Ci/mmol), 1 μM RNA fragment, 25 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes), pH 7.5, 10 mM $MgCl_2$, 50 μg/mL bovine serum albumin (BSA), 10 mM dithiothreitol (DTT), and 20 units/mL *Pset* 1 polynucleotide kinase (Cameron et al., 1978) for 2 h at 37 °C. Dephosphorylated 59-mer was 3′ end labeled with [5′-^{32}P]pCp and RNA ligase (England & Uhlenbeck, 1978). Labeled RNAs were purified from 20% polyacrylamide–7.5 M urea gels as described above except that 200 μg/mL *E. coli* tRNA carrier was included in the gel elution buffer. As we show here, tRNA has no effect on the binding properties of the labeled RNA.

E. coli 5S, 16S, and 23S mixed ribosomal RNAs for competition assays were isolated by phenol extraction of purified 70S ribosomes; Qβ RNAs were gifts of P. Cole and K. Campbell; Brome mosaic virus RNA was a gift of P. Kaesberg.

Nuclease Mapping and Fragment Selection. Partial ribonuclease digestions of end-labeled 59-mer under native conditions (Vournakis et al., 1981) were performed in 10-μL reactions containing 20 nCi of 59-mer, 0.5 mg/mL tRNA carrier, 10 mM $MgCl_2$, and 50 mM sodium cacodylate, pH 6.0, with or without 10 μM coat protein. After 10 min at 25 °C, 1 or 2 μL of nuclease was added and incubation continued an additional 15 min at 25 °C. Stock nuclease concentrations were 1 unit/mL RNase A, 1000 units/mL RNase U_2, and 5000 units/mL RNase S_1.

Partial RNase T_1 digestions of the 5′-^{32}P-labeled 59-mer under denaturing conditions were performed as described by Donis-Keller et al. (1977). Limited alkaline hydrolysis of the

5′-^{32}P-labeled 59-mer was carried out in a 10-μL reaction by incubation in 50 mM sodium carbonate, pH 9.2, 1 mM EDTA, and 0.5 mg/mL tRNA carrier at 90 °C for 30 min. All partial digestion reactions were terminated by adding an equal volume of 0.3% xylene cyanol–0.6% bromophenol blue in 10 M urea and either applying directly to the gel or freezing.

For fragment selection experiments, 60 nCi of 5′ or 3′ end-labeled 59-mer was partially hydrolyzed with sodium carbonate as described above except that the reaction was terminated by the addition of 480 μL of TMK. Coat protein was then added to a final concentration of 3 or 0.3 μM, and the samples were incubated at 2 °C for 15 min. The samples were filtered through nitrocellulose filters and the bound fragments recovered and precipitated as described above.

End-labeled oligonucleotides were separated on 320 × 400 × 0.4 mm denaturing 20% polyacrylamide sequencing gels (Donis-Keller et al., 1977) and visualized by autoradiography.

Gel Chromatography. A Sephadex G-75 column (1 × 36 cm) was equilibrated at 15 mL/h in TMK at 4 °C and calibrated with lysozyme, bovine serum albumin, ovalbumin, carbonic anhydrase, and Blue Dextran 2000. For samples containing labeled RNA, 10 mg/mL *Torula* RNA (Sigma VI) was included in the column buffer to reduce nonspecific retention. Each 0.5-mL sample in TMK buffer contained 0.1 μCi of ^{32}P-labeled RNA and various concentrations of coat protein freshly diluted into 1 mM acetic acid. The radioactivity was located in 0.5-mL fractions by Cerenkov counting. Coat protein was detected by the method of Lowry et al. (1951).

Filter Binding Assays. Aliquots (490 μL) containing about 9 nCi (4.5 fmol) of labeled RNA fragment were prepared in TMK buffer. Coat protein was serially diluted to the desired concentrations in 1 mM acetic acid–20 μg/mL bovine serum albumin. A binding assay was initiated by adding 25 μL of the coat protein solution to the RNA solution. After 20 min at 2 °C, 450 μL of each reaction was applied to a 24-mm nitrocellulose (Millipore-HAWP) filter on a prechilled filtration apparatus. The filters were soaked for 30 min in cold TMK prior to use, and each was stripped of excess buffer by shaking before it was placed on the apparatus. House vacuum was turned on only after the sample was applied and resulted in a filtration time of about 3 s. Aliquots of labeled RNA were spotted on filters to determine the input. The filters were dried in scintillation vials at 100 °C and counted in 0.4% diphenyloxazole in toluene. Backgrounds obtained in the presence of diluent only were less than 10% of input and are subtracted in all cases. Note that this assay avoids both dilution of the complexes before filtration and washing the filter after filtration. The precision in determining K_d by using this assay is within a factor of 3 for independent replicates. Diluting and pipetting concentrated coat protein stocks were the largest sources of variability and were minimized by diluting into BSA-containing buffers and by not pipetting coat protein volumes less than 25 μL. The presence of serum albumin in the diluent had no effect on backgrounds or fragment binding properties.

Competition assays contained 50 nCi/mL internally ^{32}P labeled 21-mer equilibrated with coat protein in TMK buffer. Competitor RNA was diluted to the desired concentrations in water, and 5 μL was added to 500-μL aliquots of the equilibrated mixture. After 20 min at 2 °C, a 450-μL aliquot was filtered without washing. The competition data were analyzed according to eq 5 of Lin & Riggs (1972), which defines the parameter θ as the ratio of the radioactivity bound to the filter in the presence of competitor to the radioactivity bound in the absence of competitor.

Results

Secondary Structure of the 59-Nucleotide Fragment. The 59-nucleotide fragment of R17 RNA isolated by coat protein protection was 5′-^{32}P labeled and subjected to partial nuclease digestion with ribonucleases S_1, A, and U_2 under native conditions. The data for ribonuclease S_1 are shown in Figure 1. The S_1 nuclease cleavage sites shown in lanes b and c can be located on the sequence of the molecule by comparing them with the G residues identified by partial ribonuclease T_1 digestion shown in lane f and with the partial base hydrolysis sites in lane a. The nuclease S_1 sensitive sites are indicated on the RNA sequence in Figure 2. Results of ribonuclease A and U_2 digestion under the same reaction conditions are summarized in Figure 2 as well. Since the nucleotides at −4 to −7 and at −35 to −38 are sensitive to nucleases while the surrounding residues are not, the results are consistent with the two hairpin structures proposed by Gralla et al. (1974) and depicted in Figure 2. Direct evidence for two helical regions in the 59-nucleotide fragment has been obtained by using temperature-jump relaxation kinetics (Gralla et al., 1974) and NMR (Hilbers et al., 1974). The absence of an S_1 or U_2 cleavage site at the unpaired A at −10 is due to the relative resistance of single bulged residues to nuclease digestion (Vournakis et al., 1981). The reduced accessibility of this site to nucleases could result because the nucleotide ring may be stacked into the helix (Patel et al., 1982).

The remaining nucleotides in the molecule are relatively resistant to nuclease digestion despite their apparent single-stranded character. No S_1 or U_2 cleavages and only 4 of the 10 possible RNase A cleavages are observed in the three regions of the molecule which are not part of the hairpins. Berzin et al. (1978) also noted the surprising resistance of the region −16 to −22 to RNase S_1 digestion. Although few complementary base pairs can be formed between bases of the nuclease-resistant regions, it is possible that a tertiary structure involving non-Watson–Crick base pairs could prevent attack by nucleases. Alternatively, since the observed nuclease cleavage sites are obtained in partial digestion experiments, the extreme sensitivity of some cleavage sites could obscure the detection of less sensitive single-stranded sites. The residues in the small hairpin loops are likely to be strained and therefore quite sensitive to nuclease digestion. Thus, although the partial nuclease digestion data are consistent with the secondary structure in Figure 2, they do not totally confirm it.

Coat Protein Protection. Similar partial nuclease digestion experiments were performed in the presence of 10 μM coat protein to determine which cleavage sites are protected in the RNA–protein complex. As shown in lanes e and f of Figure 1, the nuclease S_1 cleavages between positions −4 and −7 are entirely eliminated by the presence of the protein while none of the other cleavages are significantly altered. Similar data were obtained with ribonucleases U_2 and A. The experiments indicate that the coat protein interacts with the upper part of the smaller hairpin but not with the single-stranded regions on either side of it. These results are in agreement with the observation that the kinetics of the helix–coil transition of the smaller helix were perturbed by the binding of the coat protein (Gralla et al., 1974). The larger helix is isolated as part of the coat protein protected fragment because it does not have any single-stranded G residues sensitive to the RNase T_1 used in the isolation.

Coat Protein Selection. In order to more precisely define the size of the protein binding site on the 59-nucleotide fragment, we performed coat protein selection experiments.

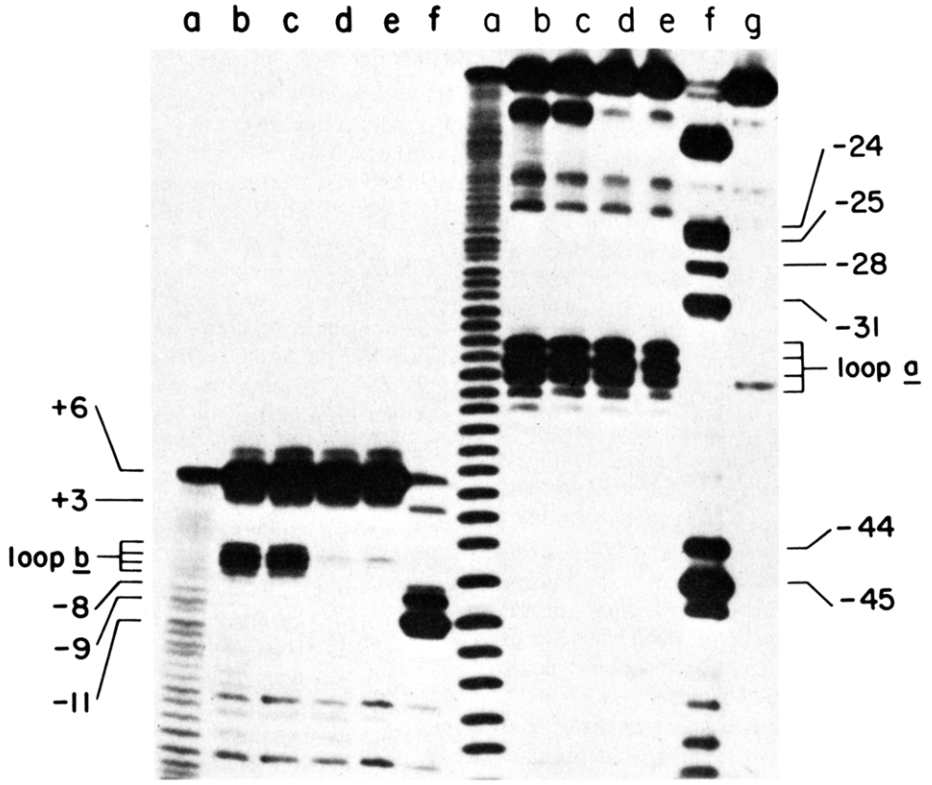

FIGURE 1: Partial ribonuclease S_1 cleavage of the 5′-^{32}P-labeled 59-mer with and without coat protein. Lane a, base hydrolysis; lanes b and c, no coat protein, 2 and 1 μL of S_1; lanes d and e, 10 μM coat protein, 2 and 1 μL of S_1; lane f, partial T_1 hydrolysis under denaturing conditions; lane g, untreated 59-mer. Note that some cleavages in regions of interest are present in the starting material. T_1 cleavage sites are numbered with respect to the first nucleotide in the replicase gene. Electrophoresis was for 24 h (left) or 10 h (right) at 800 V.

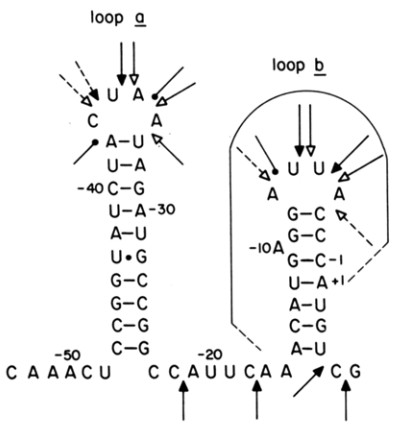

FIGURE 2: Summary of nuclease sensitivity and selection experiments on the coat protein binding site of R17 RNA. Arrows indicate positions of strong (—) or weak (---) cleavage by ribonuclease S_1 (Δ), A ($\blacktriangle$), or U_2 ($\bullet$). The nuclease cleavages within the region outlined are not made in the presence of coat protein. The limits of the outlined region were determined by the selection experiments shown in Figure 3. The nucleotides are numbered with respect to the first nucleotide in the replicase gene.

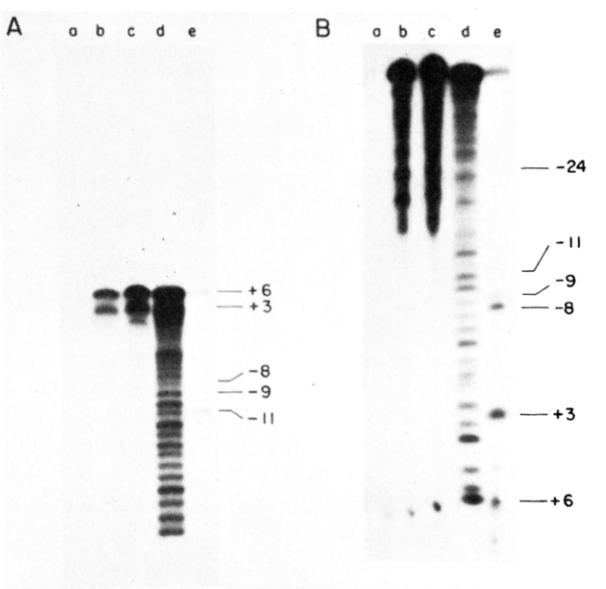

FIGURE 3: Selection by coat protein of truncated fragments of the 59-mer labeled at the 5′ (panel A) or 3′ (panel B) terminus. The partial base hydrolysate (lane d) was neutralized, incubated with either 0 (lane a), 0.3 (lane b), or 3 μM (lane c) coat protein, and filtered. The RNA fragments recovered from the filters are shown. Lane e shows the products of partial RNase T_1 digestion under denaturing conditions, and the residues are numbered as in Figure 1.

5′-^{32}P-Labeled 59-mer was partially hydrolyzed with base to produce a nested set of 5′ end-labeled oligomers from 1 to 59 nucleotides long. A similar nested set of 3′ end-labeled oligomers was made by hydrolyzing the 3′ end-labeled 59-mer. Each set of oligomers was incubated with two different concentrations of coat protein (3 and 0.3 μM), both of which were sufficient to saturate binding of the 59-mer. A nitrocellulose filter was used to retain those fragments which formed stable complexes with the coat protein. The bound labeled oligomers were eluted from the filter and separated on a polyacrylamide–urea gel. Only those fragments retaining the minimum length of RNA required for coat protein binding appear on the gel autoradiogram. Their lengths can be determined by comparing them with a sample of the original base hydrolysate as well as with the partial T_1 hydrolysate obtained under denaturing conditions. The results of these experiments using 5′ end-labeled 59-mer are shown in Figure 3A. Only the fragments from about 54 to 59 nucleotides long are visible on the autoradiogram after selection (lanes b and c). Thus, although the resolution of the gel was only about ±2 nucleotides, it is clear that 5 or 6 nucleotides can be removed from

the 3′ end of the 59-nucleotide fragment before coat protein binding is abolished. Although the distribution of oligomers in the original hydrolysate is not uniform, there seems to be a fairly abrupt decrease in protein binding when a nucleotide in the vicinity of position +1 is removed.

The results of similar experiments with 3′ end-labeled 59-mer are shown in Figure 3B. Fragments from 59 to as short as about 21 nucleotides are visible on the autoradiogram after selection (lanes b and c). Although the accuracy of this localization is also only about ±2 nucleotides, it is clear that all of the larger hairpin and most of the interloop region can be removed without abolishing coat protein binding. A fairly abrupt decrease in protein binding occurs when a nucleotide in the vicinity of position −15 is removed. These experiments support the data of Krug et al. (1982), who found that a synthetic 21-nucleotide fragment comprising −17 → +4 has an affinity constant equal to that of the 59-mer. These results also agree with those of Steitz (1974), who showed that the replicase initiation fragment derived by ribosome protection covering −13 → +11 is also able to bind coat protein, and of Jansone et al. (1979), who found that a nuclease S_1 subfragment of the 59-mer, −17 → +6, retains coat protein binding activity.

The conclusions from the two coat protein selection experiments are represented by the dashed line in Figure 2 and agree quite well with the coat protein protection experiments. The region of the 59-mer essential for protein binding extends from about −15 → +1 and encompasses the four residues in the hairpin loop, the upper part of the stem region, and the bulged A residue at −10. The lower part of the stem region may be less important since nucleotides +2 to +6 can be removed without loss of coat protein binding, although the protein will not bind in the absence of the complementary nucleotides −12 to −14. Thus, the binding site appears to be quite large and asymmetrically located about the hairpin, extending further in the 5′ than in the 3′ direction.

Equilibrium Constants and Stoichiometry. The interaction between the coat protein and the 21- and 59-nucleotide RNA binding fragments was investigated by using a nitrocellulose filter binding assay. A constant, low concentration (~10 pM) of labeled RNA was incubated at 2 °C in 0.1 M Tris-HCl, pH 8.5, 80 mM KCl, and 10 mM magnesium acetate with varying concentrations of coat protein for 20 min. The reactions were filtered without dilution or subsequent washing. Proteins and nucleic acid–protein complexes are retained by the filter matrix while uncomplexed RNA passes through. As seen in Figure 4, the formation of the RNA–protein complex is detectable at 0.1 nM coat protein, has a midpoint at about 3.0 nM, and reaches a plateau above 10 nM with about 70–80% of the input RNA bound. Little difference is seen between the synthetic 21-mer and the 59-mer isolated from R17 RNA, confirming the result of Krug et al. (1982) that the two RNA fragments interact similarly with the protein.

Several important points must be considered in order to interpret the data in Figure 4 in terms of a simple binding equilibrium between RNA and protein and thus allow the calculation of an equilibrium constant. First, since the assay involves separation of the protein–RNA complex from the free RNA, it is important to demonstrate that the complex does not dissociate during the filtration process. This possibility was a concern since preliminary experiments in which the complex was formed in a small volume and diluted prior to filtration indicated that the complex dissociated quite rapidly, with a half-life of less than 1 min. To test whether the RNA could dissociate from the complex and leach off the filter

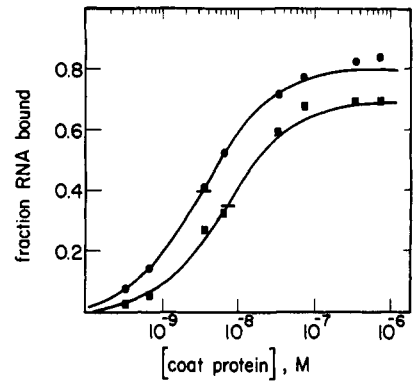

FIGURE 4: Coat protein excess binding curves for 5′-^{32}P-labeled 21-mer (●) or 5′-^{32}P-labeled 59-mer (■) in TMK buffer at 2 °C. The hash marks indicate half-saturation at 3.3 nM coat protein monomer for the 21-mer and 6.7 nM for the 59-mer. Using these values for K_d and a retention efficiency of 80% for the 21-mer and 70% for the 59-mer, we calculated the solid lines for a bimolecular equilibrium by assuming that the concentration of complex is negligible compared to the total protein concentration.

during the filtration process, we examined the effect of washing the filters with additional aliquots of buffer. Each reaction in a series similar to that in Figure 4 was first filtered as usual, the vacuum released, the wash buffer applied to the filter, and the vacuum reapplied. Up to two 2-mL washes had no effect on the binding curve although the background was reduced. Thus, RNA is not leached out of the complexes during filtration by the standard protocol. Since the multiple washings took several minutes, this result appears to be in conflict with the relatively rapid off rate. The two results can be reconciled by assuming that the filter-bound complexes dissociate more slowly than complexes in solution. This behavior has been observed for synthetase–tRNA complexes on nitrocellulose filters (Yarus & Berg, 1967).

If coat protein molecules bound to the filter can bind free RNA passing through the filter, an erroneously high determination of the association constant could result. This possibility was tested by first filtering a set of reactions similar to that in Figure 4 without any RNA present and then filtering an additional 0.45-mL aliquot of labeled RNA onto the same filters. At less than 100 mM coat protein, no binding of RNA to filters containing prebound coat protein was observed, while at coat protein concentrations greater than 100 nM some binding of RNA to prebound protein was observed. Since only 20% of the RNA was bound at 100 nM coat protein, the effect is small enough to conclude that binding of additional free RNA to filter-bound protein does not add to the amount of complex detected in the concentration range from which the K_d is determined.

Another aspect of Figure 4 that merits consideration is that, even at the highest protein concentration, only 80% of the input RNA is bound to the filter. If the binding is governed by a simple equilibrium which the filter assay accurately reflects, one would expect that above a coat protein concentration of 100 nM virtually all the RNA would be retained on the filter as complexes. Although incomplete retention of protein–acid complexes on nitrocellulose filters is generally found (Strauss et al., 1980; de Haseth & Uhlenbeck, 1980; Giacomoni, 1981), it is important to demonstrate that this observation does not contradict the simple equilibrium model. Although RNA does not leach out of the complexes during filtration, it is possible that the complexes are not completely retained by the filter. However, either refiltering the filtrate on a fresh filter or placing a second filter underneath the first does not retain more RNA. In addition, the complete retention of coat protein by

the filter was demonstrated by trichloroacetic acid precipitation of the filtrate with 20 μg/mL ovalbumin carrier and analysis for coat protein on a sodium dodecyl sulfate ($NaDodSO_4$) microgel (Matsudaira & Burgess, 1978) using the sensitive silver stain of Oakley et al. (1980). Even at input coat protein concentrations of 5–10 μM, less than 0.01% of the coat protein appeared in the filtrate. Therefore, since the coat protein and presumably also the complex are quantitatively retained on filters up to quite high concentration, the fractional retention efficiency does not appear to be due to incomplete retention of coat protein by the filters.

A remaining possibility is that the RNA which is not retained by the filter is altered and thus cannot bind protein. This possibility was tested by incubating 0.1–1 μM coat protein with the RNA in the filtrate and refiltering the mixture onto a fresh filter. The fraction of RNA retained upon refiltering was much less than the fraction originally retained at the same concentrations of protein. Analysis of the RNA in the filtrate on polyacrylamide–urea gels, however, showed that it was intact. Therefore, the presence of an altered form of the RNA, which cannot bind protein, may partially account for the fractional retention at the plateau in Figure 4.

We have observed fractional retention efficiences ranging from about 40 to 90%. The fraction of RNA bound at the plateau was always very similar for RNAs labeled in parallel but was not constant among different labeled preparations. It is thus likely that some subtle aspect of purification of labeled fragments accounts for the observed fractional retention. The fraction of RNA retained at the plateau was unaffected by a wide range of solution conditions (Carey & Uhlenbeck, 1983) and was constant for RNAs of quite different sequences and lengths when labeled in parallel (J. Carey, P. T. Lowary, and O. C. Uhlenbeck, unpublished experiments). We thus conclude that incomplete retention of RNA at the plateau is not inconsistent with the simple bimolecular equilibrium model for the interaction. We therefore follow the approach of others (de Haseth & Uhlenbeck, 1980; Giacomoni, 1981) and assume that the percent of RNA bound at the plateau represents complete binding of active RNA. This assumption allows the calculation of a retention efficiency, defined as the fraction of RNA bound at the apparent plateau, which is used to correct the rest of the data to give the fractional saturation at each protein concentration.

When a retention efficiency of 80% for the 21-mer and 70% for the 59-mer is used, the data in Figure 4 accurately fit a simple bimolecular equilibrium relation calculated by considering the RNA concentration to be negligible compared to the total protein concentration throughout the binding curve. The interaction does not appear to be cooperative since all the experimental points fit this equilibrium. Kinetic experiments which further support the simple bimolecular model are described in Carey & Uhlenbeck (1983). The dissociation constant for the interaction between the 21-mer and a coat protein monomer is 3.3 nM, and for the 59-mer, K_d = 6.7 nM. These values are identical within the error of the filter assay, which can be up to a factor of 2–3 in K_d for RNAs prepared separately. The finding that both the 59-mer and 21-mer bind similarly to the protein is in agreement with Krug et al. (1982), who found K_d = 50 nM for the two RNAs. The K_d values obtained here are higher due to the pH (8.5 here vs. 7.5) and temperature (2 °C here vs. 20 °C) at which the assays were performed.

The stoichiometry of the coat protein–21-mer interaction was determined with the filter retention assay under similar conditions by using a coat protein concentration near the K_d

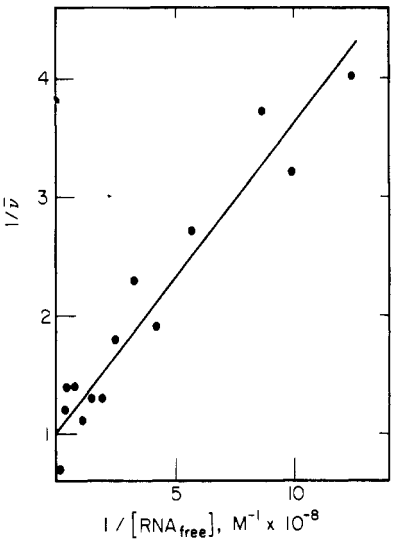

FIGURE 5: Double-reciprocal plot of coat protein–21-mer binding data. The filter assay was used with 3 nM coat protein and 0.2–20 nM 21-mer in TMK buffer at 2 °C. The fraction of complex, $\bar{\nu}$, was calculated by using a retention efficiency determined in a separate experiment to be 50%. The slope of the line is 1.3 nM, and the intercept is 1.0.

and varying the concentration of the 21-mer over 2 orders of magnitude spanning the K_d. These data are analyzed by a double-reciprocal plot in Figure 5. The intercept indicates one RNA binding site per protein monomer. The intercept can be combined with the slope to provide an independent measure of K_d and yields a value of 1 nM, in excellent agreement with the K_d obtained in Figure 4. These results imply that all protein monomers are active in binding RNA, in agreement with the unit stoichiometry observed by Gralla et al. (1974) for the 59-mer–coat protein complex in sedimentation equilibrium experiments.

Coat Protein Aggregation. Under the conditions used in the RNA binding experiments, the 14 000-dalton R17 coat protein monomer can aggregate to form multimers which are presumptive intermediates in capsid formation. At a relatively high concentration (10 μM), the coat protein migrates on Sephadex G-75 in TMK buffer as a single fairly symmetrical peak with an apparent molecular weight of 37 000 (Figure 6a). Thus, the average size of the population of aggregates is between a dimer and a trimer at the highest protein concentration generally employed in the binding assays. Although the aggregation state at lower protein concentrations was not determined, Shafranski et al. (1975) observed monomers and dimers of ^{14}C-labeled MS2 coat protein on a Sephadex G-100 column in a buffer similar to TMK. Thus, if the single amino acid substitution between R17 and MS2 coat proteins does not alter the aggregation properties, the protein appears to be a monomer below about 10 nM. The relatively weak association between protein monomers means that the position of the protein peak in Figure 6a must be regarded as the average mobility resulting from a dynamic equilibrium among monomers, dimers, and higher order forms.

It is likely that protein aggregation is incompatible with RNA binding. Gralla et al. (1974) added up to 2.8 μM coat protein to 1 μM 59-mer in a buffer similar to TMK and found that the sedimentation constant of the complex reached a limiting value at 1 μM coat protein that was consistent with a bimolecular complex. Since free coat protein is expected to aggregate in their experiment, it apparently must disaggregate to bind to the RNA. Although it is possible that the protein aggregation equilibrium could alter the observed value

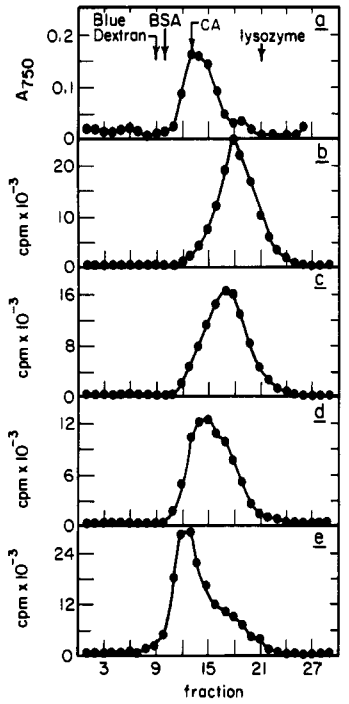

FIGURE 6: Mobility of coat protein and 21-mer on Sephadex G-75 in TMK buffer at 4 °C. (a) 10 μM coat protein applied to the column. The migration positions of bovine serum albumin (BSA) and carbonic anhydrase (CA) are indicated. In the lower panels, samples contained 5′-^{32}P-labeled 21-mer mixed with 0 (b), 30 nM (c), 300 nM (d), and 3 μM (e) coat protein.

of the binding constant between the protein and the RNA, this is not likely to be the case for the K_d determined from Figure 5, since in that experiment the coat protein concentration was low enough (3 nM) that aggregation is expected to be minimal.

In an attempt to directly determine whether coat protein aggregates could bind RNA, mixtures of 10 pM labeled 21-mer and varying concentrations of coat protein were chromatographed on Sephadex G-75. As shown in Figure 6, the migration position of the 21-mer changes from an apparent molecular weight of 23 000 (Figure 6b) to a single faster moving peak that increases in mobility as the coat protein concentration is increased from 30 nM to 3 μM (Figure 6c–e). A simple interpretation of these data is that the 21-mer combines with the protein to form a complex which is nearly fully formed in panel e and which migrates with an apparent molecular weight of about 40 000. This is approximately the mobility expected for a complex of a 14 000-dalton protein monomer and an RNA of apparent molecular weight 23 000. Since the resolution of the column is not high, separation of free RNA from the complex is not complete, resulting in broader peaks of intermediate mobility in panels c and d. If 10-fold dilution of the sample during chromatography is assumed, the profiles in panels c–e can be resolved into relative amounts of free and complexed RNA that are consistent with the binding curve in Figure 4. One would thus conclude that although coat protein can aggregate, only the monomer can bind RNA to form a complex.

This simple interpretation, however, rests on the assumption that the protein–RNA complex is relatively stable on the time scale of the Sephadex experiment. If this condition is not met, the position of the labeled RNA no longer reflects the size of the complex since labeled RNA will be released from the complex and rebind free protein many times during chromatography. It is indeed possible that the observed mobility shift of the labeled RNA in Figure 6c–e reflects simply the changing average size of the coat protein aggregates as a function of

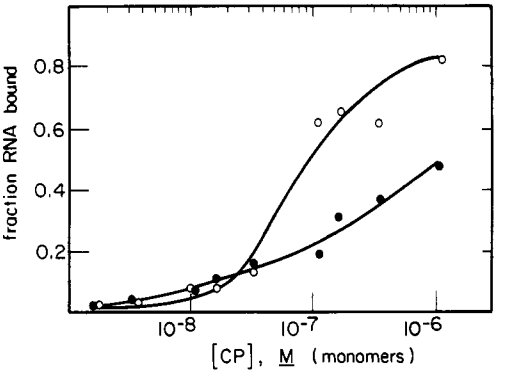

FIGURE 7: Binding of 5′-^{32}P-labeled 21-mer with coat protein (○) or capsids (●) in TMK buffer at 2 °C. For the formation of capsids, 25 μM coat protein (CP) was incubated in 1.2 M Tris–acetate, pH 6.8, for 12 h at 4 °C. The control was incubated in the same buffer at 25 °C, where capsids do not form.

concentration. The RNA may comigrate with the coat protein peak since this is the position where reassociation is most favored, regardless of which form of coat protein is responsible for RNA binding. If such rapid equilibration between protein–protein and protein–RNA complexes can occur on the column, the data in Figure 6 cannot eliminate the possibility that coat protein aggregates bind the 21-mer.

One defined aggregate of coat protein which does not dissociate rapidly is the empty phage capsid. Mafthews & Cole (1972) have shown that preformed capsids do not dissociate even when transferred to solution conditions where their formation is inhibited. Thus 21-mer binding to capsids was tested. Capsids were prepared by incubating 25 μM coat protein in 1.2 M Tris–acetate, pH 6.8, for 12 h at 4 °C. Under these conditions, capsid formation is about 80% complete (Matthews & Cole, 1972). As a control, an identical solution of coat protein was incubated at 25 °C, under which conditions capsids will not form (Matthews & Cole, 1972). Each sample was then diluted as usual into 1 mM acetic acid and 20 μg/mL BSA and assayed for RNA binding in TMK buffer. As shown in Figure 7, the capsids bind 21-mer considerably less well than the control coat protein. However, the control coat protein binds more poorly than untreated coat protein (compare Figure 4), possibly due to formation of noncapsid aggregates which are inactive in binding. In any case, these experiments support the view that coat protein aggregates are not able to bind RNA.

Specificity of the Coat Protein–21-mer Interaction. We have examined the interaction of R17 coat protein with a wide variety of other RNA molecules by a competition assay. Unlabeled competitor RNAs at concentrations up to 50 μM were added to an equilibrium mixture of ~10 pM labeled 21-mer and a saturating concentration of coat protein; after reequilibration, the reactions were filtered. Competition by the unlabeled RNA will result in reduction in the amount of labeled RNA retained on the filter as the concentration of competitor is increased. Representative competition data are shown in Figure 8. Although the precision of the data is low, unlabeled R17 RNA competes with labeled 21-mer about as well as expected from a theoretical competition curve calculated with the assumption that the K_d for R17 RNA is 3 nM and the K_d for the 21-mer is 6 nM (Figure 8A). A similar result is obtained when unlabeled 21-mer is used as competitor (not shown).

Figure 8B shows the data for three RNAs which do not compete significantly with the 21-mer for coat protein binding. The RNAs shown represent a total of over 17 000 nucleotides of sequence complexity, including the RNA from the closely

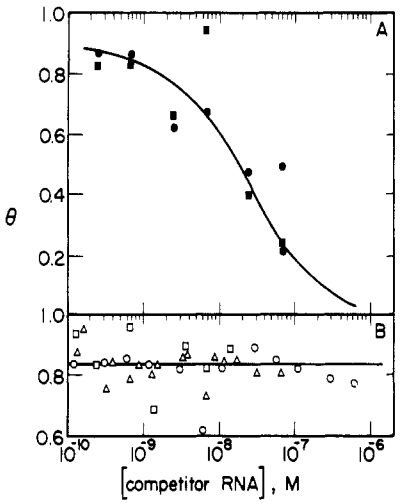

FIGURE 8: Competition for coat protein binding between ~10 pM labeled 21-mer and unlabeled RNAs. (A) R17 RNA, 38 nM coat protein; two independent experiments (●, ■). The solid line is the theoretical curve calculated for K_d = 3 nM for the R17 RNA and 6 nM for the 21-mer. (B) Heterologous RNAs, 20 nM coat protein: *E. coli* mixed ribosomal RNAs (○); Brome mosaic virus RNA (□); Qβ RNA (△). θ is defined as the ratio of radioactivity bound to the filter in the presence of competitor to the radioactivity bound in the absence of competitor.

related phage Qβ. Since no reduction in the amount of labeled 21-mer retained on the filter is observed at even the highest competitor concentrations, the K_d values for the competitors must be much weaker than the highest concentration of competitor tested. A number of other RNAs were also tested by competition for coat protein binding. These included *E. coli* 16S rRNA at concentrations up to 0.4 μM, *Drosophila melanoaster* 5S rRNA up to 0.1 μM, $tRNA^{Phe}_{yeast}$ up to 2.5 μM, *E. coli* mixed tRNA up to 50 μM, poly(rU) up to 1 mg/mL; poly(rA) up to 1 mg/mL, and $(A_p)_{10}A$ up to 8.7 μM. None of these RNAs showed detectable competition. Thus, the interaction of R17 coat protein with its binding site is highly specific.

Discussion

The selection experiments presented here suggest that the entire binding site for R17 coat protein repressor is localized to a 20-nucleotide segment of the 3569-nucleotide genome of R17 RNA. This conclusion is strengthened by the finding that the synthetic 21-nucleotide fragment can compete effectively with intact R17 RNA for coat protein. Because its binding site is a single fragment of RNA, the R17 coat protein is a particularly attractive RNA binding protein for study. Many other RNA binding proteins, including *E. coli* host factor (de Haseth & Uhlenbeck, 1980), several of the tRNA synthetases (Schimmel, 1979), and some of the ribosomal proteins [e.g., see Mackie & Zimmerman (1975)], bind noncontiguous regions of their target RNA molecules, thereby complicating the analysis of these interactions.

Some details about the RNA binding site for coat protein can be deduced from our results. The four single-stranded residues at the top of the hairpin helix are protected from nuclease digestion by the coat protein and are thus implicated as part of the binding site. In addition, the selection experiments suggest that the binding site is asymmetric with respect to the bottom of the helix. Several residues 5′ to the helix appear to be required for binding, but destroying the secondary structure of the lower part of the helix by removing residues from the 3′ side does not appear to alter binding. Gralla et al. (1974) found that, although the melting properties of the helix are altered by coat protein binding, the helix is not denatured. Thus, it seems likely that the structure of the RNA is an essential feature of the target site. The protein apparently forms several contacts at sites which, though widely dispersed on the RNA, are held in a particular orientation by the RNA secondary structure. An estimate of the distance between the contact sites on the 21-mer can be made from the crystallographic dimensions of an A-type RNA helix of 7 base pairs (Arnott et al., 1969). If it is assumed that the 21-mer does not fold into a more compact size, the distance from the contacts on the top of the loop at residue -6 to those on the 5′ terminus at -17 is about 30 Å. This is about the same as the diameter of 33 Å we calculate for the R17 coat protein monomer from its sedimentation constant (Matthews & Cole, 1972) by assuming a spherical shape and a partial specific volume of 0.75 mL/g.

We have employed a nitrocellulose filter binding assay to study the interaction of R17 coat protein and its RNA binding site. Even though separation of the equilibrium mixture occurs upon filtration, we have concluded on the basis of several control experiments that the filter-bound radioactivity accurately reflects the amount of complex present in solution at equilibrium. These experiments showed the following: (i) once free coat protein had bound to the filter, it did not bind additional RNA; (ii) the half-life of the complex is much slower than the filtration time; and (iii) the dissociation rate of the filter-bound complex is even slower. However, as is often the case with nitrocellulose filter binding assays, the binding curve reaches a plateau of less than 100%. Thus, a fractional retention efficiency was introduced to calculate the amount of complex from the filter-bound radioactivity. Since supplies of coat protein are plentiful and labeled RNA is more difficult to obtain, it is most convenient to determine the equilibrium constant of the interaction by varying the coat protein concentration at a very low RNA concentration. The K_d obtained in this manner agrees quite well with the value obtained by varying the RNA concentration at a constant coat protein concentration.

The aggregation of R17 coat protein at higher concentrations can potentially complicate the study of the RNA–protein interaction. Since several protein–protein binding domains are needed to form the capsid (Paranchych, 1975), several types of protein dimers and trimers may be possible. At 10 μM protein, the average size of the aggregate is a trimer. The protein concentration must be below about 10 nM before all the protein is in a monomer form (Shafranski et al., 1975). Thus, the equilbrium constant for protein aggregation is only slightly weaker than that of the protein–RNA interaction. Although coat protein monomers are clearly active in binding [Figure 5 and Gralla et al. (1974)], higher order aggregates may also be active (Shafranski et al., 1975; Zagorska et al., 1975). In the case of *E. coli* host factor, higher order aggregates of protein hexamers can bind RNA, and RNA binding increases aggregation (de Haseth & Uhlenbeck, 1980). This behavior is not observed with R17 coat protein.

The specificity of the coat protein for the binding sequence appears to be quite high. A wide variety of RNAs of considerable sequence complexity was unable to compete with the 21-mer for protein binding. This finding supports the idea that a fairly complex binding site on the R17 RNA having a particular three-dimensional array of phosphates and nucleoside functional groups is needed for protein binding. We can calculate a lower limit for the K_d of the nonspecific interaction if we assume, as has been done for DNA (McGhee & von Hippel, 1974), that the number of nonspecific binding

sites on an RNA molecule equals the number of nucleotides in the RNA. With this assumption, our data yield a minimum estimate of 5 mM for the dissociation constant between R17 coat protein and nonspecific RNA binding sites. Thus a specificity ratio, defined as $K_{specific}/K_{nonspecific}$, of at least 10^6 is obtained. This ratio is in the range found for several sequence-specific DNA binding proteins which form nonspecific nucleic acid–protein complexes stabilized mostly by ionic forces (de Haseth et al., 1977, 1978; Newport et al., 1980; Strauss et al., 1980). In the case of the *lac* repressor, the nonspecific binding mode is thought to be an important mediator of the specific binding mode (Berg et al., 1981) since the intracellular concentration of nonspecific binding sites can approach the K_d of the nonspecific interaction. Because the R17 system has a similar specificity ratio, it was of interest to evaluate whether any physiological importance of the nonspecific binding mode is possible. Assuming a burst size of 10^3 phage, we calculate that the intracellular concentration of nonspecific binding sites on R17 RNA is about 6 mM at lysis; since repression occurs well before lysis when fewer phage RNA molecules are present, even fewer nonspecific sites are available. It is thus unlikely that a nonspecific binding mode facilitates the interaction between the coat protein and replicase initiator in vivo.

In conclusion, the interaction of R17 coat protein with its binding site is an excellent example of a sequence-specific RNA–protein interaction which merits additional study. The RNA binding site is on a single reasonably small fragment which has a secondary structure that is probably essential for its binding activity. The complete synthesis of the binding fragment has recently been achieved (Krug et al., 1982). The equilibrium constant is in a range that is readily accessible with the amounts of materials available. Finally, the specificity of the interaction is extremely high. In the following paper (Carey & Uhlenbeck, 1983), we investigate several solution properties of the RNA–protein interaction. In a later paper, we will examine the protein binding properties of 21-mers with defined sequence alterations.

Registry No. 21-mer, 82642-77-1.

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Kinetic and Thermodynamic Characterization of the R17 Coat Protein–Ribonucleic Acid Interaction†

Jannette Carey and Olke C. Uhlenbeck*

ABSTRACT: A filter retention assay is used to examine the kinetic and equilibrium properties of the interaction between phage R17 coat protein and its 21-nucleotide RNA binding site. The kinetics of the reaction are consistent with the equilibrium association constant and indicate a diffusion-controlled reaction. The temperature dependence of K_a gives $\Delta H = -19$ kcal/mol. This large favorable ΔH is partially offset by a $\Delta S = -30$ cal mol^{-1} deg^{-1} to give a $\Delta G = -11$ kcal/mol at 2 °C in 0.19 M salt. The binding reaction has a pH optimum centered around pH 8.5, but pH has no effect on ΔH. While the interaction is insensitive to the type of monovalent cation, the affinity decreases with the lyotropic series among monovalent anions. The ionic strength dependence of K_a reveals that ionic contacts contribute to the interaction. Most of the binding free energy, however, is a result of nonelectrostatic interactions.

The binding of regulatory proteins to nucleic acids is fundamental to the control of gene expression. It is of interest to understand the nature and relative importance of the elements which confer specificity on such interactions. One useful approach has been to reproduce the binding reaction with purified components, determine the association constant, and observe the effect of changing solution conditions on the association constant. When this approach has been applied to several DNA–protein binding systems, inferences have been made about the mechanism and molecular basis for these interactions (de Haseth et al., 1977; Strauss et al., 1980; Barkley et al., 1981; Winter et al., 1981). The previous paper (Carey et al., 1983) established that the interaction of R17 coat protein with a synthetic 21-nucleotide binding site is an excellent example of a simple, sequence-specific RNA–protein binding system. In the present paper, the association constant of the coat protein–21-mer[1] interaction is determined under a variety of solution conditions. The kinetics of the binding reaction are also presented. The results are interpreted in terms of the types of contacts made between the protein and nucleic acid and of the relative contributions of the various types of contacts to the free energy of the interaction. The results are compared with those of similar experiments on DNA–protein interactions to highlight certain differences in binding properties that can be attributed to the structural differences between the two nucleic acid targets.

Materials and Methods

The synthesis of the internally ^{32}P-labeled 21-nucleotide binding fragment is described in Krug et al. (1982). The specific activity averaged 2000 Ci/mmol. The isolation of R17 coat protein and the details of the nitrocellulose filter retention assay are described in Carey et al. (1983). For the various buffers used in this work, the pH was adjusted at the temperature used in the incubation. Standard TMK buffer is 0.1 M Tris-HCl, pH 8.5 at 2 °C, 10 mM magnesium acetate, and 80 mM KCl.

Results

Kinetics. The dissociation rate of the coat protein–RNA complex was measured by forming complexes between ~10 pM ^{32}P-labeled 21-mer and several different protein concentrations (5–60 nM). Two different methods, both based on the filter retention assay, were used to follow the dissociation kinetics. In the first method, dissociation of complexes formed with labeled 21-mer was observed by adding 50 nM unlabeled intact R17 RNA. Since R17 RNA competes effectively with the 21-mer (Carey et al., 1983) and is added in 10-fold molar excess over coat protein, dissociated 21-mer must compete with the unlabeled RNA to rebind to the protein, resulting in reduction of the amount of labeled 21-mer in the complexes with time until a new equilibrium is established. An example of this first type of dissociation experiment is shown in Figure 1A. A control experiment in which unfractionated *Escherichia coli* tRNA is added at zero time shows no decrease in the amount of bound 21-mer with time. Since tRNA does not compete with the 21-mer, this result indicates that once the labeled 21-mer has dissociated it can rebind the protein during the period of the experiment. In the second method, dissociation was initiated by diluting the reaction 5- or 10-fold with buffer, and samples were withdrawn and filtered at intervals until the new equilibrium was reached. Since it was previously shown that complexes trapped on the filter do not dissociate any further, the filtration procedure will accurately determine

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[1] Abbreviations: 21-mer, 21-nucleotide synthetic RNA fragment comprising residues −17 to +4 of the R17 RNA, where +1 is the first nucleotide of the replicase gene; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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