Using Circular Permutation Analysis To Redefine the R17 Coat Protein Binding Site†

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ABSTRACT: The bacteriophage R17 coat protein binding site consists of an RNA hairpin with a single purine nucleotide bulge in the helical stem. Circular permutation analysis (CPA) was used to examine binding effects caused by a single break in the phosphodiester backbone. This method revealed that breakage of all but one phosphodiester bond within a well-defined binding site substantially reduced the binding affinity. This is probably due to destabilization of the hairpin structure upon breaking the ribose phosphates at these positions. One circularly permuted isomer with the 5′ and 3′ ends at the bulged nucleotide bound with wild-type affinity. However, extending the 5′ end of this CP isomer greatly reduces binding, making it unlikely that this circularly permuted binding site will be active when embedded in a larger RNA. CPA also locates the 5′ and 3′ boundaries of protein binding sites on the RNA. The 5′ boundary of the R17 coat protein site as defined by CPA was two nucleotides shorter (nucleotides −15 to +2) than the previously determined site (−17 to +2). The smaller binding site was verified by terminal truncation experiments. A minimal-binding fragment (−14 to +2) was synthesized and was found to bind tightly to the coat protein. The site size determined by 3-ethyl-1-nitrosourea-modification interference was larger at the 5′ end (−16 to +1), probably due, however, to steric effects of ethylation of phosphate oxygens. Thus, the apparent site size of a protein binding site is dependent upon the method used.

One of the best characterized RNA–protein interactions is the specific binding of the bacteriophage R17 coat protein to the translational initiation region of the replicase gene [reviewed in Witherell et al. (1991)]. The coat protein binds to a small RNA hairpin with a four-nucleotide loop and a stem interrupted by an unpaired purine. The size of the binding site has been determined previously from a series of RNA hairpins shortened progressively from either the 5′ or the 3′ end (Carey et al., 1983b). These experiments confined the binding site to within the −17 to +2 nucleotide positions of the replicase gene. In subsequent experiments, an RNA molecule comprising these 19 nucleotides was found to bind the R17 coat protein with high affinity (Carey et al., 1983b).

A circularly permuted RNA is produced by first connecting its normal termini and then cleaving its backbone at another site (Pan et al., 1991; Pan & Uhlenbeck, 1993). Recently, we have developed a method to determine the effects of circular permutation on the folding of tRNA (Pan et al., 1991). The method, termed circular permutation analysis (CPA),[1] is now modified to analyze RNA–protein interactions using the R17 coat protein binding site. Interestingly, CPA also appears to be well suited to map the boundaries of the protein binding site. The results from CPA are also compared to those obtained with 3-ethyl-1-nitrosourea-modification (ENU-modification) interference, which tests the effects of phosphate ethylation on coat protein binding (Bushman et al., 1985; Romby et al., 1985; Jessen et al., 1991; Kjems et al., 1992).

MATERIALS AND METHODS

Preparation of RNAs. RNAs were prepared by *in vitro* transcription from synthetic DNA templates using T7 RNA polymerase (Milligan et al., 1987). RNA transcripts were purified by electrophoresis on 20% polyacrylamide gels containing 7 M urea, visualized by UV shadowing, cut out, eluted overnight in 0.1 M Tris-HCl (pH 8)/1 mM EDTA, and ethanol precipitated in the presence of 0.4 M ammonium acetate. RNAs were treated with calf intestinal alkaline phosphatase and labeled at their 5′ ends using [γ-^{32}P]ATP and T4 polynucleotide kinase (Maniatis et al., 1982). 3′-End-labeled RNAs were prepared using [5′-^{32}P]pCp and RNA ligase (England et al., 1980).

Nitrocellulose Filter Binding. R17 coat protein was isolated from purified phage by the method described in Beckett and Uhlenbeck (1988). The association constant between coat protein and each RNA fragment was determined with a nitrocellulose filter retention assay (Carey et al., 1983a). A constant, low concentration of ^{32}P-labeled RNA was mixed in 500-μL reaction mixtures with a series of coat protein concentrations between 0.1 nM and 1 μM in 10 mM $Mg(OAc)_2$, 80 mM KCl, 80 μg/mL BSA, and 100 mM Tris-HCl (pH 8.5 at 4 °C). After incubation at 4 °C for 20 min, the mixture was filtered with a water aspirator through a nitrocellulose filter (Nitro ME, 0.45 μm, from Micron Separations Inc., Westboro, MA) presoaked in the binding buffer. The amount of complex retained on the filter was quantitated by liquid scintillation counting, and a K_d was calculated according to Carey et al. (1983a). All K_d values were measured in duplicate, and the values differed by less than 20%.

Circular Permutation Analysis. RNA was synthesized by *in vitro* trancription using T7 RNA polymerase including a

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; CP, circularly permuted; CPA, circular permutation analysis; EDTA, (ethylenedinitrilo)-tetraacetic acid; ENU, 3-ethyl-1-nitrosourea; GMP, guanosine 5′-monophosphate; GTP, guanosine 5′-triphosphate; K_d, binding constant.

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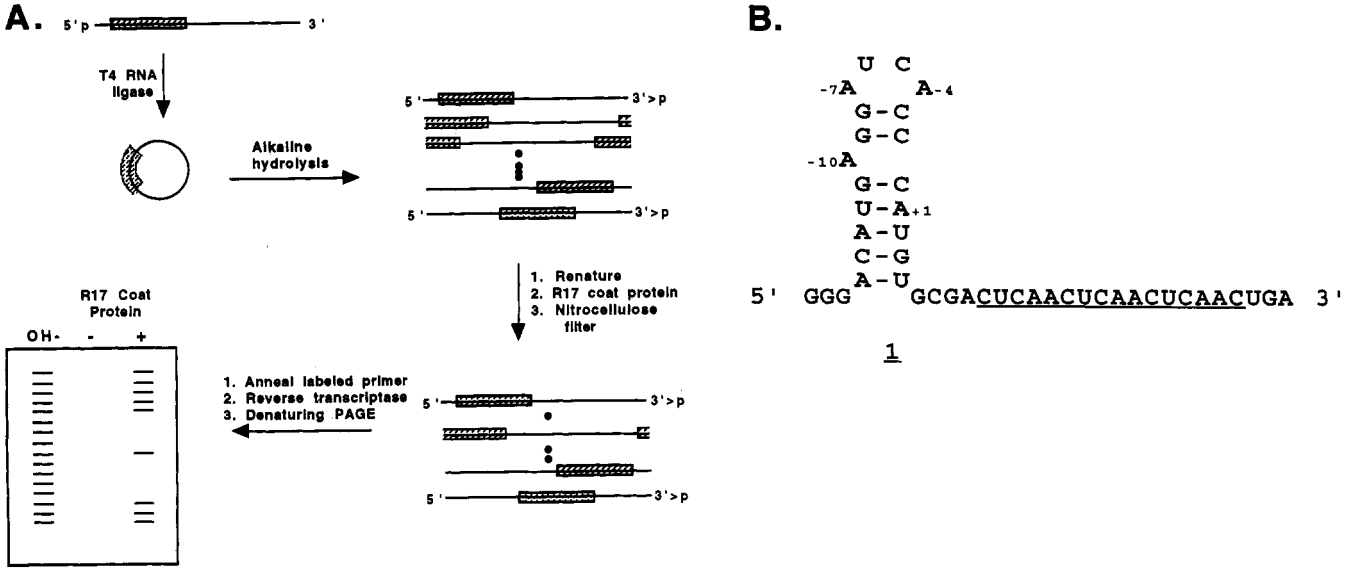

FIGURE 1: (A) Experimental protocol for circular permutation analysis (CPA). The hatched boxes represent the coat protein binding site. (B) Sequence and possible secondary structure of variant **1**. The primer binding site is underlined. The numbering of the nucleotides corresponds to that of the translational initiation region of the R17 replicase gene.

5-fold molar excess of 5′-GMP over GTP in the reaction to obtain RNA with a 5′-monophosphate. To form an intramolecular circle, 8.5 μM linear RNA was incubated in 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.2 mM ATP, 0.1 mg/mL BSA, 15% DMSO, and 1 units/μL T4 RNA ligase for 2 hours at 37 °C. Alkaline hydrolysis was carried out with 0.5 μM purified circular RNA in 1 mM glycine and 0.4 mM $MgSO_4$, pH 9.5, by boiling for 1 min 10 s. The hydrolysis mixture was neutralized by addition of 2.5 vol of 15 mM Tris-HCl (pH 7.5)/1 mM EDTA, diluted 15-fold in water, renatured by heating for 2 min at 90 °C, and cooled quickly on ice. This population of RNA molecules was bound to coat protein and then filtered through nitrocellulose filters. To elute the RNAs, the filters were cut into small pieces and soaked for 1 h in 400 μL of phenol saturated with 10 mM Tris (pH 7.5)/1 mM EDTA plus 200 μL of 7 M urea. One hundred microliters of water was then added, and the sample was centrifuged for 10 min at 12000*g* at room temperature. The aqueous phase was then extracted with chloroform/isoamyl alcohol (24:1), and the eluted RNA was ethanol precipitated in the presence of tRNA carrier and 0.3 M sodium acetate.

To determine the 5′ ends of the circularly permuted RNAs, the eluted RNA was annealed with a molar excess of 5′-^{32}P-labeled primer of the sequence 5′-GTTGAGTTGAGTTGAG-3′ by heating at 90 °C for 2 min followed by incubation at ambient temperature for 5 min. Reverse transcription was carried out in 50 mM Tris-HCl, pH 8.3, 6 mM $MgCl_2$, 40 mM KCl, and 2 units of avian myeloblastosis virus reverse transcriptase (U.S. Biochemicals) for 10 min at 37 °C. The reaction was stopped by addition of an equal volume of 9 M urea/50 mM EDTA, and the reaction mixture was analyzed on 15% polyacrylamide gels containing 7 M urea.

Terminal Truncation Experiments. End-labeled RNAs at 1 nM were partially hydrolyzed in 50 mM $NaHCO_3$ (pH 9.2)/1 mM EDTA/0.5 μg of unfractionated tRNAs by incubating at 90 °C for 30 min. Hydrolysis products were diluted directly into the binding buffer, bound to the coat protein, and retained on nitrocellulose filters. Bound RNA was eluted as described above and analyzed on 20% polyacrylamide gels. The radioactive bands were quantitated on a PhosphorImager. After each band was normalized to the total counts in the lane, the fraction of a given band bound to protein was calculated by dividing by the normalized amount of that band in the original hydrolysis mixture. The overall error in fraction bound for a given band was about ±20%.

ENU Modification and Cleavage. The procedure of Krol and Carbon (1989) was used with minor modifications. 5′- or 3′-^{32}P-labeled RNAs in water were heated to 90 °C for 3 min and cooled. After addition of buffer, a saturated solution in ethanol (~750 mM) of 3-ethyl-1-nitrosourea (Sigma) or ethanol alone was added to give final concentrations of 12 nM ^{32}P-labeled RNA, 200 mM HEPES, pH 8.0, 1 mM EDTA, 25% ethanol, and 188 mM ENU. Samples were incubated for 2 min at 90 °C and quickly cooled on ice, and the RNAs were ethanol precipitated twice in the presence of 0.15 mg/mL tRNA carrier and 0.3 M sodium acetate. RNAs were resuspended in water and renatured by heating for 5 min at 37 °C. Binding experiments were carried out in the presence of 0.6 μM coat protein, and the complex was retained on nitrocellulose filters. After elution, the RNAs bound by coat protein were resuspended in 100 mM Tris base (pH 9.5) and heated at 50 °C for 15 min to cleave the triester bond at the site of modification; they were then mixed with an equal volume of 7 M urea/TBE and electrophoresed on 20% polyacrylamide/7 M urea gels. Individual RNA bands on denaturing gels were quantitated using a Molecular Dynamics PhosphorImaging system. Since minor differences in the extent of ENU modification of different phosphates were observed, background values from the control lane without ENU were subtracted from each band and the lanes were normalized using the full-length, unmodified RNA product, which should be all bound to coat protein under these conditions.

RESULTS

Circular Permutation Analysis. The experimental protocol for CPA applied to protein binding is illustrated in Figure 1A. The RNA (Figure 1B) was circularized using T4 RNA ligase and subjected to partial alkaline hydrolysis to generate all circularly permuted (CP) isomers. This mixture of CP isomers was then renatured, incubated with R17 coat protein, and filtered through nitrocellulose filters to separate bound from unbound RNAs. To determine the 5′ end of the CP isomers retained on the filters, RNAs were eluted and annealed to a

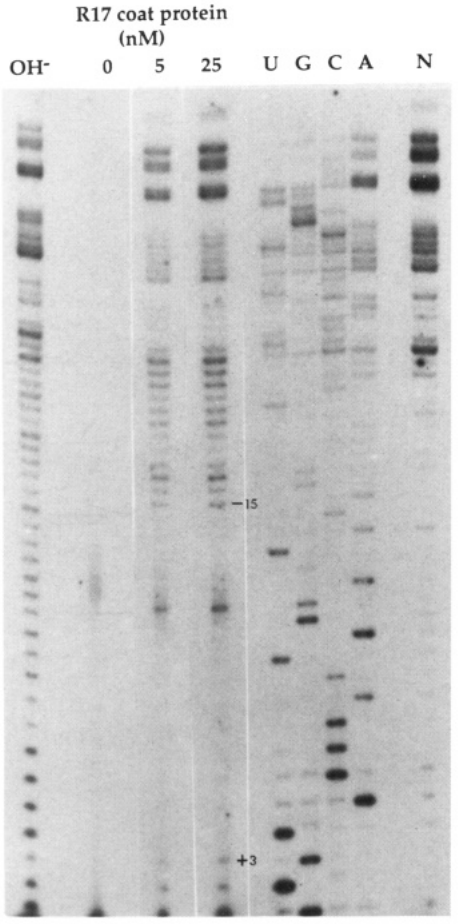

FIGURE 2: CPA of the R17 coat protein binding site. The 5′ ends of CP isomers are determined by runoff reverse transcription. OH^-: Alkaline hydrolysis of the circular form of **1**. R17 coat protein (0, 5, 25 nM): A trace amount of a mixture of CP isomers was incubated with coat protein at the indicated concentrations and retained on nitrocellulose filters, and the bound RNAs were analyzed by reverse transcription. The smear in lane 0 was a DNA contaminant of the primer. U, G, C, and A: Reverse transcriptase sequencing of the circular form of **1** using the same primer. N: Reverse transcription of the circular **1**.

5′-^{32}P-labeled DNA primer complementary to a region outside the coat protein binding site. The 5′ end of the CP isomers was identified by comparing the runoff bands generated by reverse transcription to sequencing reactions using the circular RNA as the template.

The starting 45-mer RNA (variant **1**) contains a consensus R17 coat protein binding site with a 3′ extension to serve as the primer binding site. This RNA binds to the coat protein with the same affinity as the hairpin without the extra nucleotides at the 3′ end (Witherell et al., 1990). At a concentration of 0.6 μM coat protein, all CP isomers were bound (data not shown), indicating that none of the backbone breaks within this molecule abolished binding completely. However, when lower coat protein concentrations were used, only a subset of the CP isomers was bound (Figure 2). These tightly bound RNAs include all RNAs having breaks outside the region containing nucleotides −15 to +2. In addition, one CP isomer with the ends within the hairpin structure bound with high affinity. This molecule has its 3′ end following the bulged A residue at −10 and its 5′ end at nucleotide −9 (Figure 2).

Because CPA was carried out using a mixture of RNA molecules, it was necessary to confirm the tight binding of the CP isomer with ends at the bulge by synthesizing it individually and analyzing its binding to the coat protein (variants **2**–**5**,

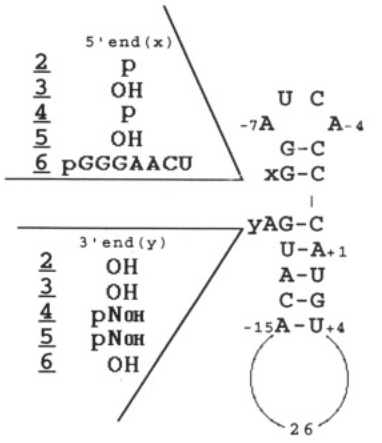

FIGURE 3: Binding of RNA variants **2**–**6** to the R17 coat protein. The 5′ (x) and 3′ (y) groups for each variant are shown. The loop sequence is 5′-GCGACUCAACUCAACUCAACUGAGGG-3′.

Table I: Binding Constants of RNA Variants

variant	K_d (10^{-9} M)	variant	K_d (10^{-9} M)
1	0.7	**6**	>10 000
2	200	**7**	0.4
3	2.1	**8**	20
4	>1000	**9**	80
5	6.0	**10**	36

Figure 3; Table I). Initially, the RNAs were primed with 5′-GMP so that they contained primarily 5′-monophosphates. Variant **2** did not bind R17 coat protein well, having a binding constant (K_d) almost 300-fold higher than the original hairpin (**1**). Since the 5′ end of the CP isomers in the CPA experiment was a hydroxyl group, not a phosphate, the 5′-phosphate of variant **2** was then removed by alkaline phosphatase to yield variant **3**. Consistent with the data in Figure 2, **3** had a K_d of 2 nM, approximately 100-fold lower than the same RNA containing a 5′-phosphate. A similar result was seen with an RNA extended at the 3′ end by one nucleotide (variants **4** and **5**). The 3′ extension increased the K_d in each case, but only 3–6 fold, while the presence or absence of a 5′-phosphate had a drastic effect on coat protein binding. Finally, consistent with binding of variants **2** and **4**, addition of several nucleotides at the 5′ end of **2** completely eliminated binding (variant **6**, Table I).

Redefining the Boundaries of the Binding Site. Since all CP isomers with ends outside the region from −15 to +2 are considered to bind tightly, the boundaries of the binding site appear to be at nucleotide −15 at the 5′ end and nucleotide +2 at the 3′ end. Compared to the site size determined by Carey et al. (1983b), CPA results show an identical 3′-end limit, but give a shorter 5′-end limit by 2 nucleotides. The experiments by Carey et al. (1983b) were done using RNA variants with one nucleotide deleted at a time from the 5′ end. We performed a similar experiment by truncating a 3′-^{32}P-labeled molecule (Figure 4A, variant **7**) in alkaline solution, binding the mixture with the coat protein, and isolating the bound RNA truncates by retention on nitrocellulose filters. These experiments were done at high protein concentrations (0.6 μM) to ensure complete binding of the full-length molecule ($K_d \sim 0.4$ nM) and partial binding for molecules with higher K_d values. Under these conditions, an RNA bound at 50% of saturation will have a K_d of 600 nM. We have arbitrarily chosen this reduction in K_d as defining the boundaries of the binding site. The resulting 5′-end limit was located at nucleotide −13, even shorter than that determined by CPA which was done at much lower protein concentrations (≤25

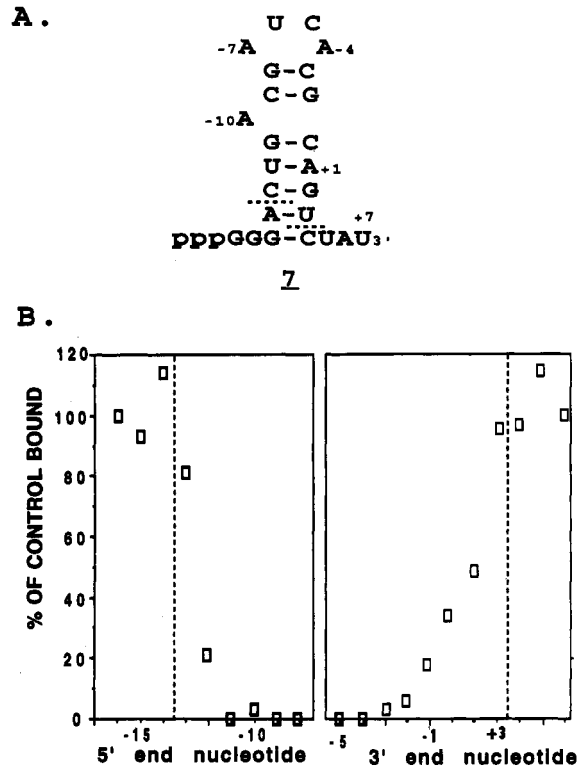

FIGURE 4: (A) Sequence and secondary structure of variant 7. The dashed lines include the binding site determined by terminal truncation experiments. (B) Terminal truncation experiment of 7 to determine the 5′- and 3′-end limits of the binding site. The dashed lines include the nucleotides of the binding site.

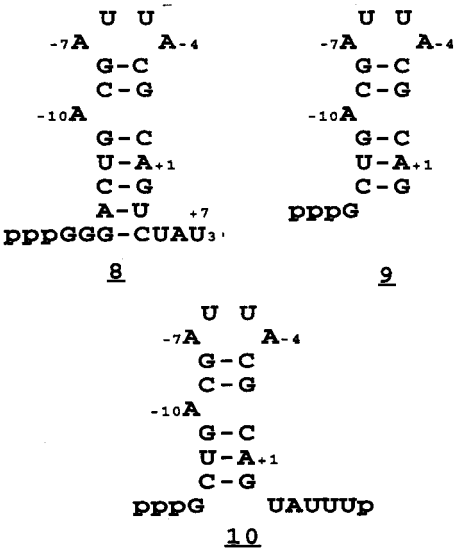

FIGURE 5: Sequences and secondary structures of variants **8**, **9**, and **10**.

nM). Thus, the more realistic 5′ end lies between −13 and −15. Truncation using the 5′-^{32}P-labeled variant **7** gave the 3′-end limit at +3 (Figure 4B).

On the basis of these results, three variants were made to verify the minimal-binding motif of the R17 coat protein (Figure 5). Variant **8** has a sequence identical to that of variant **7** except for a U at position −5. Although U^{-5} represents the wild-type sequence in the phage R17 genome, RNA variants with C^{-5} bind with 50-fold higher affinity than those with U^{-5} (Romaniuk et al., 1987; Lowary & Uhlenbeck, 1987). Variant **9** is a putative minimal-binding motif and has its 5′ end at −14 and its 3′ end at +2. Variant **10** has five nucleotides

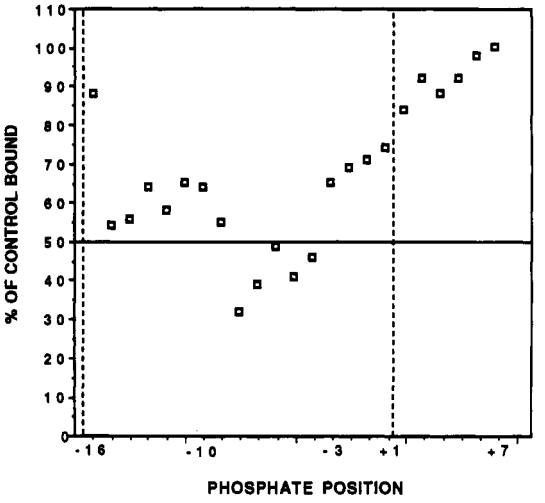

FIGURE 6: Effect of ENU modification on R17 coat protein binding. The results are presented as the percent of RNA in the protein-bound fraction divided by the same RNA band in the unselected RNA lane for variant **7**. The dashed lines include the region which we interpret as the binding site as determined by ENU-modification interference.

added to the 3′ end of variant **9**. Compared to the control molecule with ends at −17 and +7 (**8**), binding of the putative minimal motif (**9**) was decreased in K_d by only 4-fold (Table I). However, this loss in binding energy could be partially compensated by the addition of 5 nucleotides to the 3′ end of the minimal-binding motif (**10**).

ENU-Modification Interference. Another way to evaluate the binding site of R17 coat protein is to ethylate the nonbridging phosphate oxygens using 3-ethyl-1-nitrosourea (ENU). Variant **7** was modified to a low extent (less than 35% in our experiments) so that most ethylated molecules had only a single modified phosphate oxygen. The mixtures of modified RNAs were incubated with relatively high concentrations of coat protein (0.6 μM) to allow complete binding of the unmodified RNAs in the sample. The bound RNA was separated from the unbound RNA by retention on nitrocellulose filters. After elution of bound RNAs from the filters, the triester bonds of the modified phosphates were cleaved by mild alkaline treatment to determine the site of modification (Krol & Carbon, 1989). Under these conditions, modification of phosphate oxygens within a large region of the RNA hairpin (from −15 to +1) showed significant effects (Figure 6). Since an ethylated phosphodiester can give rise to two stereoisomers which may not affect coat protein binding equally, only values below 50% indicate that both isomers interfere with binding. Interpretation of values above 50% is more ambiguous; it may mean either that both isomers affect binding to a lesser extent or that modification of only one isomer affects binding. Nevertheless, interference within the 50–70% range still implies that phosphate ethylation at these positions generates a net effect on binding. By these criteria, modification of phosphate −15 interferes with binding, while modification of phosphate −16 does not. Since phosphate −15 is positioned between residues −15 and −16, the 5′-end limit should include nucleotide −16. Similar analysis for the 3′-end limit shows that phosphates up to +1 give modification interference, implying a 3′-end boundary at nucleotide +1 (Figure 6).

DISCUSSION

To examine the effects of single backbone breaks on RNA–protein interactions, circular permutation analysis was per-

formed with the R17 coat protein binding site. Outside of a well-defined region corresponding to the binding site, all CP isomers bind tightly to the coat protein. Thus, CPA should be useful in locating protein binding sites within other RNAs. At high protein concentrations (0.6 μM), all CP isomers with breaks within the binding site are bound. We interpret this result as an indication that an R17 hairpin with any single break still retains the intrinsic ability to form a structure similar to that of the intact RNA in the protein–RNA complex. Binding of all but one of these CP isomers, however, is weaker than the binding of intact RNA hairpin since almost no binding was observed at low protein concentrations (Figure 2). This result can be explained by assuming an equilibrium for these CP isomers between a structure resembling the normal hairpin and a different structure that does not bind to the protein. When the ribose phosphates are broken at these positions, a certain fraction of the binding energy is needed to shift the equilibrium to the correct RNA structure, thus reflecting a decrease in affinity. Because of the small size of the R17 hairpin, the integrity of most of the phosphodiester bonds is important to maintaining its normal structure. This result is different from CPA of yeast $tRNA^{Phe}$, where many phosphodiester bonds within the central core of the tRNA could be broken without affecting the tertiary folding of the RNA molecule (Pan et al., 1991).

The single CP isomer within the binding site which binds to the coat protein with high affinity has a 3′ terminus after the bulged A residue. It has been noted (Wu & Uhlenbeck, 1987; Witherell et al., 1991) that this bulged A is likely to intercalate into the double helix. One possible explanation of why this CP isomer binds far better than the other internal CP isomers is that it can maintain a stacked conformation that is quite similar to the correct hairpin structure. The other CP isomer at the bulge has the single-stranded A at the 5′ end of a short helix and may not stack as well as the 3′-terminal A. These differential stacking effects have been demonstrated in thermodynamic studies of similar RNA helices (Turner et al., 1988). To our surprise, the high affinity of protein binding to this CP isomer is lost upon addition of a single phosphate to its 5′ end. The phosphate at position −9 is one of the four phosphates that significantly affect coat protein binding upon substitution with a phosphorothioate (Milligan & Uhlenbeck, 1989). This suggests that a direct protein–RNA contact forms at this position. Compared to a 2′,3′-cyclic phosphate, a 5′-phosphate is more negatively charged. Thus, it is possible that the additional charge weakens the affinity of this contact. We cannot, however, rule out the possibility that addition of the 5′-phosphate destabilizes the structure of the CP isomer. The introduction of a 5′-terminal phosphate has been observed to destabilize model RNA helices, albeit only at low concentrations of monovalent ions (Freier et al., 1983).

In principle, circularly permuted protein binding sites can exist internally within large RNAs (Pan et al., 1991; Pan & Uhlenbeck, 1993). These CP sites will differ in primary sequence from the normal binding sites. A potential application of CPA is to provide an experimental means to locate the 5′ and 3′ ends of these new CP isomers. Since the RNA ends in the CPA experiment consist of 5′-hydroxyl and 2′,3′-cyclic phosphate groups, it is also essential to determine whether nucleotides can be added to these ends for internal CP sites. In the case of the R17 coat protein binding site, a CP isomer with its 5′ end at −9 and its 3′ end at −10 was found. Addition of one nucleotide at the 3′ end leads to an increase in K_d of only 3-fold, suggesting that extension at the 3′ end is possible. However, addition of even a single phosphate at the 5′ end increases the K_d by at least 2 orders of magnitude, indicating that the 5′ end cannot be extended without losing affinity for the coat protein. This result means that a circularly permuted binding site embedded within the R17 phage genome would not bind tightly to the coat protein. Nevertheless, CPA is expected to be useful in identifying circularly permuted binding sites in other RNA–protein complexes.

Another result of CPA indicates that the 5′-end limit of the binding site is smaller by two nucleotides than previously determined. Subsequent terminal truncation experiments as well as binding studies of a minimal-binding motif confirm that up to three nucleotides can be removed from the 5′ end (−17 to −15) without a significant loss of affinity. The quantitative effect of removing these three nucleotides is only about 4-fold in K_d ($\Delta\Delta G$ = 0.77 kcal/mol). This effect is significantly smaller than that observed by Carey et al. (1983b) in which removal of nucleotide −17 alone caused a decrease in binding by at least 100-fold ($\Delta\Delta G > 2.55$ kcal/mol). We believe that this difference may be due to the differential stabilities of the R17 hairpins used in both studies. Removal of nucleotides from the 5′ end may lead to a higher degree of destabilization of the helical structure in the RNA used by Carey et al. (1983b), which contained more A·U base pairs in the stem than all variants used here. It is interesting to note that the 4-fold loss in binding affinity can also be partially regained by addition of five nucleotides to the 3′ end. This 3′ extension presumably stabilizes the hairpin with a short 5′ end (Turner et al., 1988; Limmer et al., 1993). Alternatively, this result could be explained by assuming that a phosphate in the −17 to −15 region contributes to the overall affinity. When residues −17 to −15 are removed, a phosphate in the extended 3′ nucleotides makes this contribution.

The site size determined by ENU-modification interference is larger by one or two nucleotides at the 5′ end, and shorter by one nucleotide at the 3′ end, than the site size from either CPA or terminal truncation. A likely explanation for this discrepancy is that, unlike the other methods, ENU modification adds a bulky group to the RNA molecule that can interfere with protein binding. Thus, the larger site size is probably the result of unfavorable steric interference of ethylation, rather than disruption of favorable RNA–protein contacts. Since modification of every phosphate oxygen within a large region (−16 to +1) shows some effects on coat protein binding, the surface area of the RNA covered by the coat protein is probably quite large. Thus, the size of a protein binding site can vary depending on the method used.

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