involves a noncovalently bound captan–enzyme intermediate which precedes the formation of the irreversibly bound captan, but it cannot be proven with the present data. Due to solubility, captan concentration was limited to 1 mM, and, therefore, the enzyme cannot be saturated if K_D is large (>1 mM). Values obtained were reasonable for a mechanism which includes an enzyme–captan intermediate, in which the modifying reagent has a low affinity for the enzyme. The estimated modification rate ($k_3 = 0.1\ s^{-1}$) is faster than the pseudo-first-order rate of the reaction of captan with water ($1.8 \times 10^{-5}\ s^{-1}$; Wolf et al., 1976) but considerably less than one would have predicted if a group as reactive as hydroxide were in close proximity to the bound captan.

In this study, we have identified a different aspect of the polynucleotide binding site. Although DNA binding can block modification by captan, the modification does not prevent the binding of polynucleotide to DNA pol I. Therefore, we propose that the polynucleotide binding site may have two compartments, one of which serves the polymerase and 3′→5′ exonuclease activities and can be blocked by captan. The other compartment can still bind polynucleotide even after DNA pol I has been treated with captan.

Registry No. DNA polymerase, 9012-90-2; 5′→3′ exonuclease, 79121-99-6; 3′→5′ exonuclease, 79393-91-2; captan, 133-06-2.

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Determination of RNA–Protein Contacts Using Thiophosphate Substitutions†

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ABSTRACT: The binding of the bacteriophage R17 coat protein to its RNA binding site is an example of a specific RNA–protein interaction. Extensive analysis has revealed that the binding is dependent upon a unique hairpin structure that contains four essential single-stranded nucleotides. Additional specificity is thought to be due to four or five ionic contacts between the protein and phosphates on the RNA. Transcription of synthetic DNA with T7 RNA polymerase, using one of the nucleoside 5′-*O*-(1-thiotriphosphates) [NTP(αS)s], allows the synthesis of RNAs specifically substituted with thiophosphates. Eleven sequence variants of the R17 coat protein binding site were synthesized with different NTP(αS)s and tested for coat protein binding to deduce positions of thiophosphates that alter the binding affinity. Of the twenty-one phosphate positions in the molecule, two were found to decrease the K_a 3-fold when substituted with a thiophosphate, one position decreased the K_a 10-fold, and one position increased the K_a 10-fold. Substitution of any of the other 17 positions with thiophosphates does not alter the K_a. The four positions that alter the K_a are located in a uniquely structured region of the RNA, and it is postulated that these thiophosphates affect binding because they contact coat protein directly.

One of the best characterized RNA–protein interactions is the binding of the bacteriophage R17 coat protein to the translational initiation region of the replicase gene, resulting in the repression of translation (Barnardi & Spahr, 1972). This interaction has been extensively characterized by using synthetic bacteriophage RNA fragments that contain the −17 to +4 positions of the replicase gene (Figure 1). The changes in the affinity of the coat protein with more than 100 synthetic sequence variants have allowed the sequence and structural requirements of binding to be determined (Carey et al., 1983a; Romaniuk et al., 1987; Wu & Uhlenbeck, 1987). The coat

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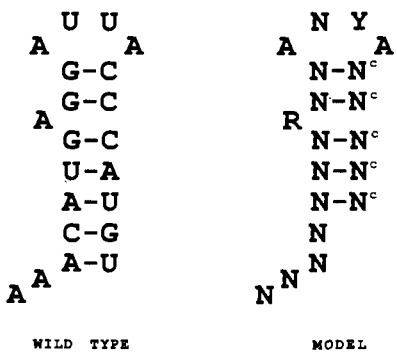

FIGURE 1: (Left) Secondary structure of the translational operator region of the R17 replicase gene. (Right) Model of the minimum length and sequence requirements for R17 coat protein binding.

protein recognizes a precise hairpin structure that contains four single-stranded residues whose identity and relative position must be maintained (Figure 1). It is not clear, however, whether the four essential residues interact directly with the protein or whether they are needed for the overall folding of the hairpin. Indeed, recent experiments with the bulged purine (Wu & Uhlenbeck, 1987) failed to clearly identify nucleotide functional groups that are involved in the interaction and instead suggested that the bulged purine may be needed for its ability to intercalate into the helix. It is therefore reasonable to consider that all of the specificity of the R17 coat protein–RNA interaction can be achieved through contacts between the protein and the ribose–phosphate backbone, whose shape is in turn determined by the RNA sequence.

In order to explore the interaction between the R17 coat protein and the ribose–phosphate backbone of RNA, various thiophosphate substitutions were introduced. The substitution of phosphates by thiophosphates has been used to study nucleotide binding proteins [reviewed in Eckstein (1985)] and DNA restriction enzymes (Connolly et al., 1984; Nakamaye & Eckstein, 1986). The substitution of a nonbridging oxygen by a sulfur is often found to alter the affinity of the protein for the nucleic acid. The S_p isomer of nucleoside 5′-*O*-(1-thiotriphosphates) [NTP(αS)] can be incorporated by T7 RNA polymerase with inversion of configuration, giving an RNA with thiophosphates of the R_p isomer (Griffiths et al., 1987). Since a large number of sequence variants that are active in R17 coat protein binding can be prepared by transcription using synthetic DNAs (Milligan et al., 1987; Milligan & Uhlenbeck, 1989), the effect of thiophosphate substitution at different positions in the coat protein binding site could be investigated. Data from such experiments can potentially allow the identification of phosphate positions that interact with the coat protein.

Materials and Methods

Enzymes. T7 RNA polymerase was purified from *Escherichia coli* strain BL21, containing the plasmid pAR1219, by the method of Davanloo et al. (1984). RNA ligase was purified from T4-infected *E. coli* (Moseman-McCoy et al., 1979). Polynucleotide kinase lacking the 3′-phosphatase activity was purified from pseT1 T4 infected *E. coli* (Soltis & Uhlenbeck, 1982). Ribonuclease T_1 was purchased from Calbiochem-Behring. Ribonucleases U_2 and Phy.M. were purchased from P-L Biochemicals.

Nucleoside Triphosphates. NTPs were purchased as sodium salts from Sigma and were stored in solution at pH 8.1. Nucleoside 5′-*O*-(1-thiotriphosphates) were a gift from Dr. F. Eckstein and were stored in solution in the presence of 50 mM dithiothreitol. [α-^{32}P]NTPs and [γ-^{32}P]ATP (5000 Ci/mmol) were purchased from Amersham in aqueous form. [γ-^{32}P]GTP was prepared from [^{32}P]phosphoric acid (ICN, carrier free) by the method of Johnson and Walseth (1979).

Oligoribonucleotide Synthesis. Variants **1**–**9** and **11** were synthesized by use of T7 RNA polymerase and synthetic DNA templates (Milligan et al., 1987; Milligan & Uhlenbeck, 1989). DNA strands were synthesized on an Applied Biosystems 380B DNA synthesizer and purified by 20% polyacrylamide gel electrophoresis. Active transcription templates were made by annealing a template strand to a 17-nucleotide strand containing the promoter region (see Figure 4). To assure proper annealing, the two DNAs are heated to 90 °C for 3 minutes and then cooled on ice. Transcription reactions contained 100 nM DNA template, 1 mM in each NTP, 40 mM Tris-HCl (pH 8.1), 1 mM spermidine, 5 mM dithiothreitol, 6 mM $MgCl_2$, 50 μg/mL bovine serum albumin (BSA, fraction V, Sigma), and 30 units/μL T7 RNA polymerase and were incubated for 1–2 h at 37 °C. If the reactions are internally labeled with [α-^{32}P]UTP or [α-^{32}P]CTP, then the concentration of the corresponding nucleotide is lowered to 0.1 mM, which increases the specific activity of the transcript with only a small decrease in final yield (Milligan & Uhlenbeck, 1989).

Variant **10** was prepared by using RNA ligase to join two half-molecules, one of which was prepared by a modified transcription protocol. The acceptor half-molecule pppGGAGAGAGGAU was prepared by transcription of a template that normally yields pppGGAGAGAGGAUCACCCUCUCU by omitting CTP from the transcription reaction. This molecule is a good acceptor for RNA ligase but was inactive as a donor due to the presence of the 5′-triphosphate. The donor molecule UACCCUUUUp was made by using RNA ligase (Beckett & Uhlenbeck, 1984).

The donor and acceptor molecules were then ligated together to create variant **10** (see Figure 4). The donor molecule was first 5′-^{32}P-labeled in a reaction that contained 10 μM donor RNA, 50 μCi of [γ-^{32}P]ATP (5000 Ci/mmol), 50 mM HEPES (pH 8.3), 20 mM $MgCl_2$, 5 mM dithiothreitol, and 50 units/mL pseT1 polynucleotide kinase and was incubated for 1 h at 37 °C. The two molecules were then ligated by adding this reaction to 10 μM acceptor RNA, 1 mM ATP, and 250 units/mL RNA ligase and incubated 12–15 h at 14 °C.

Isolation of RNA. Oligoribonucleotides were isolated by denaturing 20% polyacrylamide gel electrophoresis and visualized by autoradiography. After isolation by ordinary crush and soak procedures, thiophosphorylated RNAs were often inactive with respect to coat protein binding. To avoid this inactivation, several important modifications were added to the isolation procedures. Ultrapure acrylamide (Bio-Rad) and urea (Schwartz/Mann, ICN) were used for making the gel stock solutions. The acrylamide/urea solution was thoroughly degassed under vacuum prior to polymerization.

Since the presence of one or more of the gel components appeared to cause the inactivation, it was important to remove the RNA from the gel as quickly as possible. The gel slice was thoroughly crushed with a Teflon stirring rod. Three volumes of elution buffer [0.5 M NaCl, 0.1 M Tris-HCl, 10 mM EDTA, and 50 mM dithiothreitol (pH 8.0)] was added and the sample vortexed vigorously for 30 s. The mixture was allowed to stand at room temperature for 5 min, and then 2 volumes of water-saturated phenol was added and vortexed vigorously for an additional 30 s. After 5 min at room temperature, the sample was centrifuged to separate the phases. The aqueous phase was removed and the phenol phase extracted a second time (Rubin, 1975). The pooled aqueous

phases were precipitated by 3 volumes of ethanol at −70 °C. The RNA was then stored in 10 mM dithiothreitol to prevent further inactivation. Despite these precautions, most thiophosphorylated RNAs inactivated slowly and after 5 days were often completely inactive.

To ensure that the RNA is in the proper hairpin conformation, unsubstituted RNAs that were stored frozen were heated to at least 65 °C for 2 min and then cooled on ice prior to the experiments. However, thiophosphorylated RNAs inactivated much more quickly when heated, so they were stored at 4 °C and used without further heating.

Product Identification. Transcripts were sequenced by one of two methods to confirm the correct sequence and the presence of the thiophosphates. Transcripts were 5′-end-labeled by transcribing in the presence of [γ-^{32}P]GTP. The transcripts containing normal nucleotides were sequenced by partial enzymatic hydrolysis using RNAses T_1, U_2, and Phy.M. (Donis-Keller et al., 1977). Transcripts containing thiophosphates were sequenced chemically by using 2-iodoethanol to alkylate the sulfurs, creating a thiophosphotriester which cleaves rapidly at high temperature (Gish & Eckstein, 1988). These sequencing reactions contained 7–10 nCi of 5′-end-labeled transcript, 5% 2-iodoethanol (v/v), 1 mM EDTA, 50% formamide, and loading dyes. The mixture was incubated at 95 °C for 4 min and then loaded directly on the gel. This technique confirmed the presence of the thiophosphates at the expected positions.

Coat Protein Binding. R17 coat protein was prepared from phage by the method of Beckett and Uhlenbeck (1989). The association constant (K_a) was determined by nitrocellulose filter retention assays (Carey et al., 1983b). For each variant 5–7 nCi (15 Ci/mmol) of RNA was mixed with 10 different concentrations of coat protein ranging from 0.2 nM to 0.6 μM in 100 mM Tris-HCl (pH 8.5), 80 mM KCl, 10 mM Mg(OAc)$_2$, and 80 μg/mL BSA (TMK buffer). These mixtures were incubated 2 h at 1 °C and filtered through nitrocellulose, and the amount of RNA–protein complex retained on the filter was determined by liquid scintillation counting. For each experiment the data were fit to a retention efficiency and then a K_a value was determined assuming a bimolecular equilibrium. Each reported K_a is the average of two to five independent determinations.

Many of the tighter binding variants cannot be accurately determined by using protein dilution assays due to the dissociation of the coat protein dimer into inactive monomers at concentrations below 1 nM (Beckett & Uhlenbeck, 1989). The K_a was therefore obtained from the half-life of the RNA–protein complex. The complex is formed by incubating 70 nCi of RNA with 20 nM coat protein in 7 mL of TMK buffer on ice for 2 h. Nonradiolabeled competitor RNA is added to 30 nM and the decay of the complex monitored by filtering 0.45-mL aliquots as a function of time. By assuming the same association rate (K_{on}) of 9.4×10^5 M^{-1} s^{-1}, as determined previously (Carey & Uhlenbeck, 1983), the K_a can be calculated from the half-life ($t_{1/2}$) of the complex since $K_a = K_{on}(t_{1/2})$.

Results

Thiophosphate Substitutions Cause Changes in the K_a. Binding of the R17 coat protein to unsubstituted variant **1** (Figure 2) and to the same RNA substituted with each of the four NTP(αS)s is shown in Figure 3. It is clear that the introduction of thiophosphates at different positions has a substantial effect upon the K_a. By comparing the K_a's to that of the unsubstituted RNA (Table I), we see that incorporation of GTP(αS) reduces the K_a 25-fold, while incorporation of

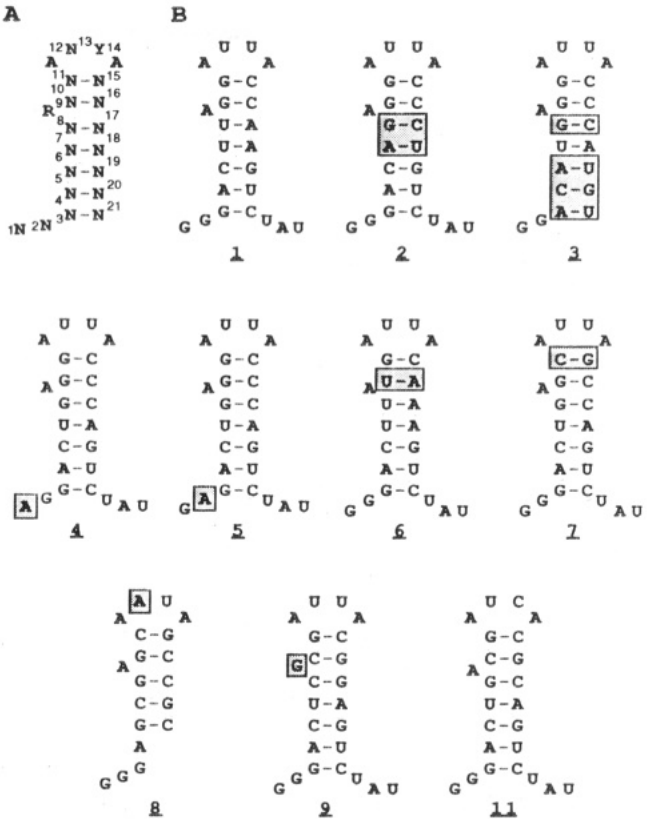

FIGURE 2: (A) Phosphate numbering system for thiophosphate substitutions. (B) Sequence variants used to deduce the thiophosphates that affect binding. Shaded areas highlight the changes made to test the adjacent thiophosphates. All variants have 5′-triphosphates.

Table I: Relative Binding Affinity of Thiophosphate-Substituted Variants[a]

variant	K_a	relative K_a ATP(αS)	GTP(αS)	CTP(αS)	UTP(αS)
1	2.0×10^8	10.5	0.040	0.38	1.0
2	3.3×10^8	11.0	0.031	0.27	1.1
3	3.3×10^8	9.2	0.035	0.35	1.0
4	3.3×10^8	9.0	0.030		
5	3.3×10^8	9.5	0.033		
6	1.5×10^8	11.4	0.11	0.29	0.29
7	5.0×10^8	8.7	0.09	0.11	1.0
8	2.0×10^8	12.5	0.083	0.083	1.0
9	1.5×10^8	11.4	0.11		
10	3.3×10^7	10.3[b]			
11	8.4×10^9	8.0	0.078		

[a] The association constants (K_a) are in M^{-1}. All constants are the average of two to five separate determinations. Relative K_a's are with respect to the unsubstituted variant. [b] Variant **10** was substituted with ATP(αS) only in the 5′ half of the molecule (see Figure 4).

CTP(αS) results in a 2.5-fold decrease. On the other hand, incorporation of UTP(αS) had no effect upon binding, and ATP(αS) substitution *increased* the K_a more than 10-fold. Since each of the substituted RNAs have thiophosphates at several positions in the molecule, the observed changes in the K_a could be due to substitution at more than one position. In addition, since thiophosphates can both increase and decrease the K_a, it is possible that offsetting effects could occur which result in no net change in the K_a, as might be seen for the UTP(αS)-substituted **1**.

Four variants were used to establish that none of the thiophosphates that alter binding of the coat protein are located in the lower part of the hairpin (positions 1–7 and 17–21 in Figure 2). The sequence of variant **2** differs from that of **1** by two base pair changes (Figure 2) but binds to the R17 coat

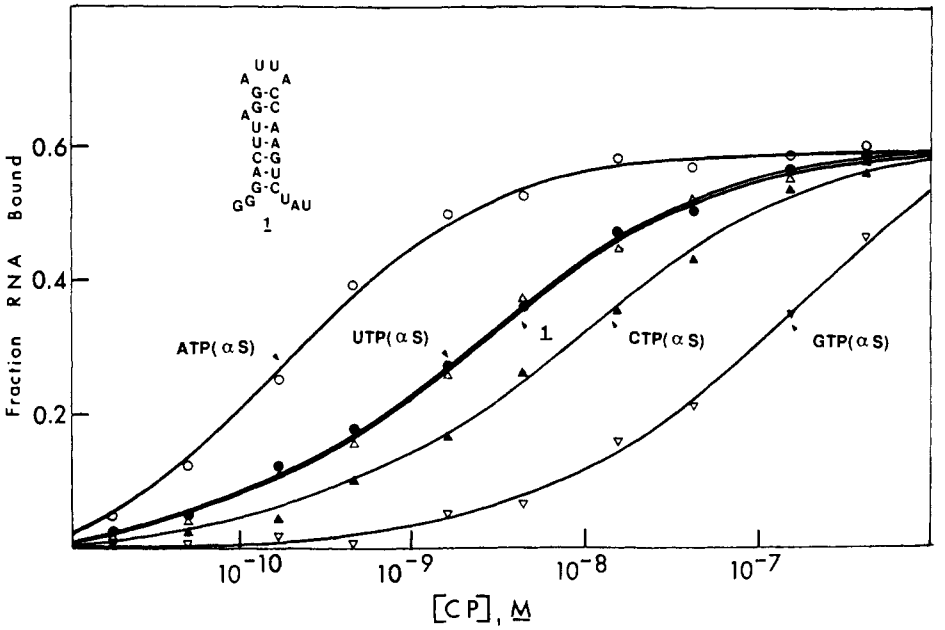

FIGURE 3: Protein excess binding curves of thiophosphate-substituted variant **1**. ^{32}P-Labeled RNAs were assayed in TMK buffer, pH 8.5, 1 °C. The solid line represents a theoretical binding curve, calculated by using a retention efficiency of 60% and determining the K_a from the point of half-saturation. The K_a's are given in Table I.

protein with a very similar K_a. When **2** was substituted with each of the NTP(αS)s, it was found that the effect on K_a was nearly the same as found with **1**. As shown in Table I, **2** synthesized with GTP(αS) decreases the K_a approximately 30-fold, CTP(αS) decreases the K_a more than 3-fold, ATP(αS) increases the K_a 11-fold, and UTP(αS) has no effect on the K_a. The simplest explanation for these data are that the four positons where **2** differs from **1** (positions 6, 7, 17, and 18) are not among those that cause a change in K_a when substituted with thiophosphates and therefore can be considered to be neutral positions with respect to coat protein binding.

Variant **3** differs from **1** at eight positions and is three nucleotides shorter (Figure 2), but also has a similar binding affinity. Again, the effect of substituting **3** with each of the four NTP(αS)s is the same as that for **1** and **2** (Table I). The base pair changes in **3** were selected so that no two combinations of base pairs in the lower part of the hairpin were the same in variants **1** and **3**. This means that any combination of effects which would not have been detected in **1** or **2** should have been revealed in **3**. Since no changes in the K_a were observed in **3**, it can be concluded that positions 3–5 and 19–21 are neutral. Additionally, **3** establishes that positions 22–24 are also neutral, which was anticipated since the 3′-terminal trinucleotide sequence present in **1** and **2** does not contribute to the K_a and thus is unlikely to interact with the protein (Carey et al., 1983a).

Variants **4** and **5** were used to test the 5′ single-stranded positions 1 and 2 (Figure 2). When substituted with ATP(αS), both **4** and **5** bind with nearly a 10-fold increase in K_a, while the GTP(αS)-substituted variants bind with about a 30-fold decrease in K_a. Since these effects are the same as those observed with **1**, it was concluded that both positions 1 and 2 are also neutral.

Thus far, the data establish that out of the 21 phosphate positions in the coat protein binding site, fourteen (1–7, 17–21) can be substituted with thiophosphates without altering the K_a to the coat protein.

Locating the Positions That Reduce the K_a. Since substitution of **1** with GTP(αS) lowers the K_a 25-fold and the four G residues in the lower part of the stem are adjacent to neutral positions, the thiophosphates at positions 9 and/or 10 must be responsible for the decrease in the binding affinity. Similarly, the 2.5-fold decrease in K_a caused by substitution of **1** with CTP(αS) must be due to thiophosphates at positions 15 and/or 16. The thiophosphate positions responsible for these effects can be determined by variant **6**, which changes a G·C pair to a U·A pair (Figure 2), thereby separating positions 9 and 16 from 10 and 15. Substitution of **6** with UTP(αS) causes about a 3-fold decrease in the K_a, whereas the UTP(αS)-substituted **1** had the same K_a as its unsubstituted counterpart (Table I). This establishes phosphate 9 as a position responsible for a 3-fold decrease in binding affinity. Substitution of **6** with ATP(αS) still shows an 11-fold increase in binding, indicating that position 16 must be neutral when substituted with a thiophosphate. Since position 16 is neutral, this suggests that thiophosphate substitution at position 15 must be responsible for the 2.5-fold decrease in the K_a of **1** when substituted with CTP(αS). This is confirmed by substitution of **6** with CTP(αS), which shows about a 3-fold decrease in K_a which must be due to position 15 since the remaining two positions (5 and 21) have already been shown to be neutral. Finally, substitution of **6** with GTP(αS) causes a 9-fold decrease in the K_a. This must be due to position 10 since the remaining substituted positions (1–3 and 19) are all neutral.

Thus, the substitution data of variant **6** establishes that the presence of thiophosphates at three different positions results in lower K_a values. Thiophosphates at positions 9 and 15 each lower the K_a about 3-fold and at position 10 lower the K_a about 10-fold. The 25-fold decrease in K_a observed when **1** is substituted with GTP(αS) is the result of two positions, the 3-fold decrease at position 9 and the 10-fold decrease at position 10, which exert a multiplicative effect upon the binding affinity, as would be expected if they acted independently.

Variant **7** was synthesized to test whether the above model could accurately predict K_a values of other thiophosphate substituted RNAs. Variant **7** is very similar to **2** except that the G·C pair at the top of the hairpin is changed to a C·G pair (Figure 2). On the basis of the model, GTP(αS)-substituted **7** should show a 9-fold total decrease in K_a as a result of thiophosphates at positions 9 and 15, each of which causes a 3-fold decrease. Substitution of **7** with CTP(αS) should result

in a 10-fold decrease in K_a as a result of the thiophosphate at position 10, since position 16 is neutral. The K_a's of the GTP(αS)- and the CTP(αS)-substituted **7** were both determined to be about 10-fold less than that of the unsubstituted molecule, almost exactly as was predicted (Table I). Thus, the data on variant **7** confirm the model.

Variant **8** was synthesized to test positions 12 and 13. Although variant **1** has no change in the K_a when substituted with UTP(αS) at both these positions, it is possible that positions 12 and 13 have opposite and complementary effects on K_a. Variant **8** is significantly different from **1** yet still binds the coat protein well. Variant **8** contains only one uridine, at the essential pyrimidine, and an adenosine at the nonessential loop nucleotide. When **8** is substituted with UTP(αS), it is found to have an identical K_a as its unsubstituted counterpart, indicating that position 13 is neutral. ATP(αS)-substituted **8** has a 12.5-fold increase in K_a compared with the unsubstituted **8**. Thus the increase is similar to that seen in other ATP(αS)-substituted variants, indicating that position 12 is also neutral.

In addition, variant **8** can be used to test the three thiophosphate positions that decrease the K_a. Like **7**, GTP(αS)-substituted **8** should show a 9-fold total decrease in K_a as a result thiophosphate substitutions at positions 9 and 15, each of which causes a 3-fold decrease. Substitution of **8** with CTP(αS) should result in a 10-fold decrease due to a thiophosphate at position 10. As predicted, the K_a's of GTP(αS)- and CTP(αS)-substituted **8** were determined to both be 12-fold less than that of the unsubstituted molecule (Table I). Thus variant **8** also confirms the model for the three positions that decrease K_a when substituted with a thiophosphate.

The Increase in the K_a Is Due to a Single Position. All variants tested thus far show approximately a 10-fold increase in K_a when substituted with ATP(αS), suggesting that positions 8, 11, and 14 are responsible either individually or in combination. Variant **9** was synthesized to test position 8 by replacing the bulged adenosine with a guanosine (Figure 2). While the molecule shows a slightly reduced K_a, it still binds the coat protein quite well (Wu & Uhlenbeck, 1987). When ATP(αS)-substituted **9** was tested, it was found to bind with an 11-fold higher K_a than the unsubstituted RNA. The GTP(αS)-substituted **9** bound with a 9-fold decrease in the K_a, as would be predicted for a molecule with a thiophosphate at position 10. Therefore, position 8 is neutral, and the positions responsible for the enhancement of binding must be 11 and 14, either individually or in combination.

Since positions 11 and 14 are adjacent to the two adenosines which cannot be substituted with other nucleotides without causing a tremendous decrease in binding (Uhlenbeck et al., 1983), variant **10** was created to test position 11 (Figure 4). A 12-nucleotide acceptor molecule made by transcription was joined to a 5′-^{32}P-labeled 9-nucleotide donor molecule by using RNA ligase (see Materials and Methods). By transcribing the acceptor with ATP(αS), a molecule that is substituted at position 11 but not position 14 can be created. While **10** binds to the coat protein rather poorly, perhaps due to the two stem G·U pairs, it is clear that the ATP(αS)-substituted molecule binds with a 10-fold increase in K_a similar to other ATP(αS)-substituted RNAs. This strongly suggests that position 11 is solely responsible for the increase in K_a, since positions 4, 6, and 8 have been shown to be neutral.

Double Substitution of Thiophosphates. RNAs containing thiophosphates introduced by two different NTP(αS)s were used to further test the multiplicative effect of the thiophosphates on the K_a. Variant **6** substituted with ATP(αS)

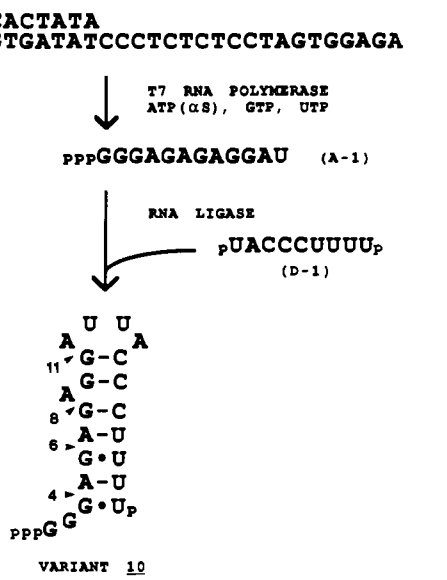

FIGURE 4: Synthesis scheme for variant **10**. The 5′ half of the molecule (A-1) was made by transcription. The 3′ half of the molecule (D-1) was made by using RNA ligase. When A-1 is made by using ATP(αS) and then ligated to D-1, the result is variant **10**, which contains thiophosphates only at positions 4, 6, 8, and 11.

Table II: Relative Binding Affinity of Double Thiophosphate-Substituted Variants

variant	relative K_a predicted	relative K_a exptl
6 substituted with ATP(αS) and UTP(αS)	3	3.8
6 substituted with ATP(αS) and GTP(αS)	1	1.2
6 substituted with ATP(αS) and CTP(αS)	3	8.4
3 substituted with ATP(αS) and GTP(αS)	0.3	0.5

and either UTP(αS), GTP(αS), or CTP(αS) was used to determine if the 10-fold increase in K_a due to the thiophosphate at position 11 could be offset by a thiophosphate at either position 9, 10, or 15 which decreases K_a. The predicted changes in the K_a and the experimental results are given in Table II. When **6** is substituted with ATP(αS) and UTP(αS), the K_a increases 3-fold, nearly as would be predicted for a 10-fold increase at position 11 combined with a 3-fold decrease at position 9. Similarly, when **6** is substituted with ATP(αS) and GTP(αS), the K_a remains unchanged, again as predicted for a 10-fold increase at position 11 offset by a 10-fold decrease at position 10. When **6** was substituted with ATP(αS) and CTP(αS), the K_a increased more than 8-fold, somewhat greater than that predicted from the combination of the 10-fold increase at position 11 and the 3-fold decrease at position 15. It appears that the thiophosphate at position 15 can only partially offset the increase at position 11.

As an additional test, variant **3** was substituted with ATP(αS) and GTP(αS). This molecule was predicted to bind with about a 3-fold decrease in the K_a, due to a 10-fold increase at position 11, a 10-fold decrease at position 10, and a 3-fold decrease at position 9. Variant **3** substituted with ATP(αS) and GTP(αS) binds with a 2-fold decrease in the K_a, nearly that predicted. It is therefore apparent that the thiophosphate substitutions cause changes in the K_a's reflecting independent changes which, in almost all combinations, exert multiplicative effects upon binding.

Thiophosphate Substitution at Position 11 Acts Independently of the Essential Pyrimidine. The introduction of the thiophosphate at position 11 is only the second example of an RNA that binds R17 coat protein with a higher K_a. It has been previously demonstrated that a uridine to cytidine

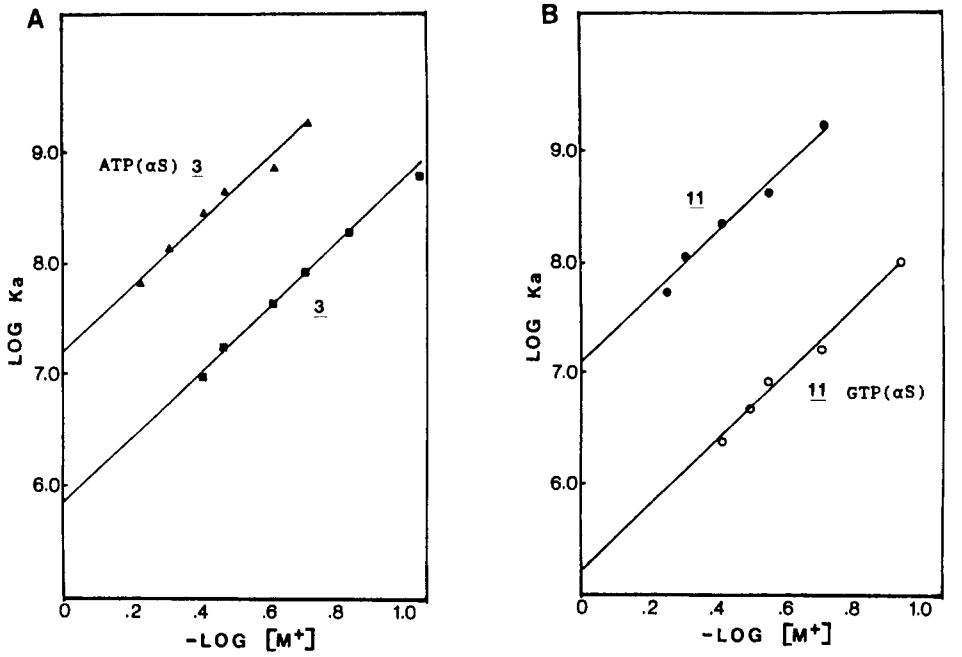

FIGURE 5: Salt dependence of K_a. (A) Comparison of **3** with ATP(αS)-substituted **3**. The slopes are 3.4 and 3.6, respectively. (B) Comparison of **11** with GTP(αS)-substituted **11**. The slopes are 3.5 and 3.3, respectively. For the ionic strength dependence the slope is equal to $Z\Psi$, where Z is the number of counterions displaced and Ψ is estimated to be 0.85 for these RNAs. Thus, for all RNAs, four ions are displaced upon coat protein binding.

substitution in the essential loop pyrimidine can cause an increase in K_a of nearly 100-fold (Lowary & Uhlenbeck, 1987). It is possible that the two phenomena are connected. For example, one could propose that the presence of the thiophosphate at position 11 alters the conformation of the uridine across the loop, thereby allowing a more favorable contact with the protein. Variant **11**, with a cytidine at the essential pyrimidine site, was used to test whether the two effects were interdependent. Since the binding is expected to increase dramatically, the change in binding was determined by changes in the half-life of the complex (see Materials and Methods). The half-life of the RNA–protein complex increases from 150 min, for unsubstituted **11**, to almost 20 h with ATP(αS)-substituted **11**. If the association rates are assumed to be the same, the introduction of the thiophosphate increases binding almost an additional 8-fold over the increase caused by the cytidine (Table I). Thus, the two effects appear to be independent, arguing against the model which proposes that the thiophosphate alters the conformation on the loop nucleotide.

Ionic Strength Dependence of Binding. It has been previously predicted from the change of K_a with increasing ionic strength that four to five ions are displaced from the RNA during coat protein binding (Carey & Uhlenbeck, 1983). Since the thiophosphate substitutions also indicate four phosphate positions that affect binding, the ionic strength dependence of binding was determined to see whether the increase and decrease in binding upon thiophosphate substitution is due to a change in the total number of ions displaced or a difference in the energy of the interaction between the counterions and the thiophosphates. Protein excess binding curves were used to determine the K_a at various KCl concentrations in the presence of 100 mM Tris-HCl (pH 8.5 at 1 °C) and 10 mM $Mg(OAc)_2$. Variant **3** substituted with ATP(αS) was compared with its unsubstituted counterpart as an example of a 10-fold increase in K_a (Figure 5A). For an example of a 10-fold decrease in K_a, variant **11** substituted with GTP(αS) was compared to its unsubstituted counterpart (Figure 5B). The tight binding variant **11** was chosen so that decreases in binding with increasing ionic strength can more easily be determined. Differences in the effect of ionic strength are determined by plotting log K_a vs the negative log of the total cation concentration. This is a linear function whose slope is directly proportional to the number of ions displaced during coat protein binding (Lohman et al., 1980). The data are shown in Figure 5. The slopes are nearly identical for all four molecules, indicating that the number of ions displaced during coat protein binding are the same. This implies that the increase or decrease in K_a observed with thiophosphate substitution does not reflect a change in the number of ions displaced.

Discussion

Incorporation of NTP(αS)s into oligoribonucleotides has been used to determine the phosphate positions of an RNA hairpin that interact with the R17 coat protein. Since in vitro transcripts contain thiophosphates at many positions in the RNA molecule, it was necessary to be able to make many sequence variants in order to be able to independently evaluate each phosphate position. The interaction between the R17 coat protein and its operator is ideally suited for such studies since the sequence requirements for binding have been clearly established. The result of the analysis indicates that substitution with thiophosphates at four of the twenty-one phosphate positions affects the affinity of the coat protein. However, the effect is not the same at each of the four positions. Thiophosphates at positions 9 and 15 each reduce K_a 3-fold, whereas a thiophosphate at position 10 reduces K_a 10-fold and one at position 11 increases K_a 10-fold.

The substitution of phosphates by thiophosphates could potentially alter an RNA–protein interaction in a number of different ways. While sulfur has only a slightly larger van der Waals radius than oxygen and the length of the sulfur–phosphorous bond is nearly the same as the oxygen–phosphorous bond, the net negative charge in a thiophosphate resides almost exclusively on the sulfur instead of being evenly distributed on both oxygens of a phosphate (Frey & Sammons, 1985). In addition, since the thiophosphate anion is a more polarizable Lewis base than the oxyanion, its spectrum of

affinities to different counterions is also quite different (Pecoraro et al., 1984). Since the thiophosphate is a chiral center and only the R_p isomer is present, either the sulfur or oxygen could potentially interact with a given amino acid side chain with quite different consequences. If one also considers that phosphates are capable of forming both ionic contacts with lysine and arginine and hydrogen bonds with seven other amino acids, it is not surprising that substituting phosphates by thiophosphates can affect the K_a differently at different positions.

We measured the ionic strength dependence of K_a for several thiophosphorylated RNAs to the R17 coat protein in an attempt to determine whether the observed increase or decrese in K_a upon thiophosphate substitution was a result of a difference in the number of counterions released. This possibility was attractive because it had previously been estimated from the slope of log K_a vs $-\log [M^+]$ that four to five counterions were released upon coat protein binding and these studies have identified four phosphates sensitive to thiophosphate substitution. However, the ionic strength dependences of K_a for two different thiophosphorylated RNAs were identical with those of their unsubstituted counterparts. Since no information is available on the affinity of potassium ions to thiophosphates, one cannot calculate the number of ions released with complete confidence. However, the simplest interpretation of the data is that potassium ions bind thiophosphates with an affinity similar to that for phosphates and the number of ions released is the same for both RNAs. While it is then tempting to conclude that the differences in K_a between phosphorylated and thiophosphorylated RNA are entirely due to nonionic interactions, it may be possible that lysine or arginine may not resemble potassium and show a differential affinity to a thiophosphate, much like divalent metal ions. Thus it is not possible to relate the four ions released with the four positions identified by thiophosphate substitution in any defined manner. As few as four or as many as eight phosphate–protein contacts may be forming. If the former is true, the different ionic side chains are interacting with thiophosphates with differing affinities. If the latter is true, the four phosphates identified in this work are forming nonionic contacts and an additional four phosphates are forming ionic contacts.

We cannot rule out the possibility that the substitution of thiophosphates for phosphates may also cause small perturbations in the structure of the RNA that could in turn affect the binding affinity. However, the fact that thiophosphate substitutions affect K_a independently in multiple substituted molecules argues against this possibility.

In conclusion, we favor the interpretation that the elemental substitution of oxygen by sulfur in certain positions of the R17 coat protein binding site changes the binding energy as a result of altering specific protein–phosphate contacts. The four interactions we have identified all lie in the upper part of the hairpin and adjacent to the four nucleotide-specific sites identified previously (Romaniuk et al., 1987). Such extensive contact between the protein and the phosphate backbone of the RNA would be expected to contribute substantially to the specificity of the interaction since the structure of the RNA would be expected to constrain the spatial position of the phosphates considerably.

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