Probing the TRAP–RNA interaction with nucleoside analogs

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ABSTRACT

The *trp* RNA-binding Attenuation Protein (TRAP) from *Bacillus subtilis* binds a series of GAG and UAG repeats separated by 2–3 nonconserved spacer nucleotides in *trp* leader mRNA. To identify chemical groups on the RNA required for stability of the TRAP–RNA complex, we introduced several different nucleoside analogs into each pentamer of the RNA sequence 5'-(UAGCC)-3' repeated 11 times and measured their effect on the TRAP–RNA interaction. Deoxyribonucleoside substitutions revealed that a 2'-hydroxyl group (2'-OH) is required only on the guanosine occupying the third residue of the RNA triplets for high-affinity binding to TRAP. The remaining hydroxyl groups are dispensable. Base analog substitutions identified all of the exocyclic functional groups and N1 nitrogens of adenine and guanine in the second and third nucleotides, respectively, of the triplets as being involved in binding TRAP. In contrast, none of the substitutions made in the first residue caused any detectable changes in affinity, indicating that elements of these bases are not necessary for complex formation and stability. Studies using abasic nucleotides in the first residue of the triplets and in the two spacer residues confirmed that the majority of the specificity and stability of the TRAP–RNA complex is provided by the AG dinucleotide of the triplet repeats. In addition to direct effects on binding, we demonstrate that the N7-nitrogen of adenosine and guanosine in UAG triplet and the 2'-OHs of (UAGCC)₁₁ RNA are involved in the formation of an as yet undetermined structure that interferes with TRAP binding.

Keywords: 2'-OH; base analogs; gene regulation; RNA–protein interactions; trinucleotide repeats; tryptophan

INTRODUCTION

trp RNA-binding Attenuation Protein (TRAP) is an RNA-binding protein that negatively regulates expression of the tryptophan biosynthetic (*trp*) genes in *Bacillus subtilis* in response to intracellular levels of tryptophan (Gollnick, 1994; Babitzke, 1997). Transcription of the *trpEDCFBA* operon is regulated by an attenuation mechanism involving formation of mutually exclusive RNA stem loop structures in the 203-nt 5' leader region of the transcript upstream of *trpE* (Fig. 1; Kuroda & Yanofsky, 1984; Shimotsu et al., 1986; Kuroda et al., 1988). In the presence of *L*-tryptophan, TRAP is activated and binds to the nascent *trp* leader RNA inducing formation of a transcription terminator stem loop, halting expression of the *trp* operon. Under conditions of limiting tryptophan, TRAP does not bind RNA and an alternative antiterminator stem loop structure forms, leading to read-through of the operon and expression of the *trp* genes. TRAP also controls translation of several *trp* genes.

TRAP binding to *trp* read-through mRNA transcripts that have escaped attenuation results in formation of another RNA secondary structure that sequesters the *trpE* ribosome-binding site (rbs) thus inhibiting translation of *trpE* (Kuroda et al., 1988; Merino et al., 1995; Du & Babitzke, 1998). Translation of *trpG*, the only *trp* gene that is not in the *trp* operon (Slock et al., 1990), is also regulated by TRAP (Yang et al., 1995). In this case TRAP competes directly with the ribosome by binding to a site surrounding the *trpG* ribosome binding site (Yang et al., 1995; Du et al., 1997).

The three-dimensional crystal structure of TRAP complexed with *L*-tryptophan shows that the protein contains 11 identical subunits arranged in a symmetric ring (Antson et al., 1995). The protein's secondary structure consists entirely of β -strands, β -turns, and random coils. The tertiary structure of TRAP is composed of 11 seven-stranded antiparallel β -sheets, each containing four strands from one subunit and three from the adjacent subunit. Alanine scanning mutagenesis studies identified three residues in each TRAP subunit (Lys-37, Lys-56, and Arg-58) that are important for RNA binding (Yang et al., 1997). The crystal structure shows that these residues are directly aligned on the outer edge of

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FIGURE 1. Schematic representation of transcription attenuation of the *B. subtilis* *trp* operon. A portion of the 203-nt leader region that precedes the structural genes is shown, with numbering corresponding to the start of transcription. The nucleotide sequences of the TRAP-binding site and of the terminator and antiterminator regions are given with the GAG and UAG repeats in bold. The arrows above the DNA sequence indicate the regions encoding the antiterminator and terminator. Nucleotides 108–111 that overlap between the antiterminator and terminator are boxed in the two structures. The 11-subunit TRAP protein is shown with β -strands indicated as arrows and the bound L-tryptophan molecules as van der Waals spheres. The bound RNA is indicated as a line wrapping around the protein.

the protein, suggesting that RNA binds to TRAP by wrapping around the protein ring (Antson et al., 1995; Yang et al., 1997).

TRAP has been shown to recognize RNAs containing multiple GAG and UAG (and rarely AAG) triplet repeats (Babitzke et al., 1994, 1995; Antson et al., 1995; Yang et al., 1997; Chen et al., 1999). The two characterized TRAP-binding sites in *B. subtilis* contain either nine (*trpG*) (Babitzke et al., 1994; Yang et al., 1995) or 11 triplet repeats (*trp* operon) (Babitzke et al., 1994; Antson et al., 1995). Furthermore, in vitro selection studies (SELEX) have confirmed the importance of these G/UAG triplet repeats for binding to TRAP (Baumann et al., 1997; Xirasagar et al., 1998). Appropriate spacing of the G/UAG repeats is also important for TRAP binding, with two “spacer” nucleotides being optimal (Babitzke et al., 1996). Several studies indicate that these spacer residues do not directly contact TRAP (Babitzke et al., 1994, 1996); however they can influence TRAP binding indirectly by affecting RNA structure (Baumann et al., 1997; Xirasagar et al., 1998). In contrast to most other characterized RNA–protein interactions (Nagai, 1996), the presence of secondary structure inhibits RNAs from binding to TRAP (Xirasagar et al., 1998).

Mutagenesis and substitution studies have demonstrated the importance of the adenosine and guanosine residues in the second and third positions, respectively, of the G/UAG triplet repeats in TRAP binding (Antson et al., 1995; Babitzke et al., 1995). Replacing either of these positions with other nucleosides drastically interferes with TRAP binding, suggesting that contacts occur between these residues in the RNA and TRAP during complex formation. The role of the residues in the first position of the triplets is less clear. Guanosine and uridine occupy the vast majority (70% and 25%, respectively) of the first positions in the triplets of naturally occurring TRAP-binding sites (Chen et al., 1999), and RNAs selected in vitro from pools containing 25 random positions reflect a similar bias in the first position (Baumann et al., 1997). Moreover, studies of RNAs containing six triplet repeats showed that those with GAG or UAGs bound TRAP with higher affinity than RNAs with AAGs or CAGs (Babitzke et al., 1995). Together these studies suggest that if specific contacts occur between TRAP and the first nucleotide of the triplet repeat, they either occur to functional groups that are similarly arranged in both guanosine and uridine or to different groups of each base that each facilitate binding of GAG or UAG repeats.

The molecular details of relatively few protein–RNA interactions are thoroughly understood, particularly for proteins that bind mRNA (Nagai, 1996). Moreover, the TRAP–RNA interaction is somewhat unusual in that TRAP is one of few characterized proteins that bind unstructured single-stranded RNA (Bernstein & Ross, 1989; Babitzke et al., 1996; Handa et al., 1999). We have therefore undertaken this study to identify the molecular components of the G/UAG triplets that confer specificity for binding to TRAP by performing a series of nucleotide analog substitution experiments. Our results show that a ribose sugar is only required in the third residue of the G/UAG triplet repeats for high-affinity binding to TRAP. We have also identified several molecular components of the bases occupying the second and third residues of the triplet repeats that are important in TRAP–RNA complex formation. In contrast we were unable to identify any specific requirements in the first residue of the triplets. Our studies further support the hypothesis that the bases in the spacer nucleotides do not specifically interact with TRAP.

RESULTS AND DISCUSSION

The work presented here involved making RNA modifications by the introduction of nucleoside analogs. By assessing the effect of these substitutions on the thermodynamic stability of the TRAP–RNA complex, we were able to gauge the importance of each group in the interaction. All the RNAs studied consisted of 11 identical pentameric repeats (triplet recognition sequences and 2 nt spacers). In all cases the analog substitutions at a given residue were made in all 11 repeats of the RNA. Given that TRAP contains 11 identical subunits arranged symmetrically (Antson et al., 1995), and that alanine-scanning mutagenesis identified 11 symmetrically arranged KKR patches specifically involved in RNA binding (Yang et al., 1997), we assume that all 11 identical repeats in our synthetic RNA interact with TRAP in a similar manner and contribute similarly to the thermodynamic stability of the TRAP–RNA complex. For example, if an analog eliminated one hydrogen bond between the RNA and the protein per repeated element, then a total of 11 hydrogen bonds would be disrupted in the entire complex, yielding a predicted $\Delta\Delta G^\circ$ of approximately 11–22 kcal/mol (Fersht, 1988; Lesser et al., 1990; Mazzarelli et al., 1992; Zhang & Gottlieb, 1993). Potentially, the resulting complex would be so destabilized that the forces that constitute the original bimolecular reaction would be unavailable. At this predicted level of disruption, the protein–RNA complex may assemble or adapt to an alternative interaction, partially compensating for the large positive change in free energy. Consequently we simply asked whether each chemical group tested is involved in binding TRAP under the given binding conditions, but did not assign individual energetic contributions to a predicted hydrogen bond.

Indeed we have observed in our experiments that adaptation to an alternate form of interaction may exist. For example, several analogs in our study fostered the formation of the TRAP–RNA complex in the absence of *L*-tryptophan, although not to the extent observed for specific interactions with *L*-tryptophan. Direct binding experiments of substituted RNA, in some instances, do not distinguish between specific binding, which is tryptophan dependent, and nonspecific binding, which is tryptophan independent. A true measure of specific binding is revealed only by a competition assay (see Materials and Methods) in which both the modified and unmodified nucleic acids compete for available TRAP-binding sites in the presence of tryptophan. It is important to note that nucleic acids that could be studied by direct binding were also tested by competition assay, and that both methods produced similar results.

Recently (after the conclusion of the experiments presented here), we have obtained the crystal structure of TRAP from *Bacillus stearothermophilus* complexed with a 53-base RNA containing 11 GAG repeats separated by AU dinucleotide spacers (A. Antson et al., 1999). The *B. stearothermophilus* and *B. subtilis* TRAP protein sequences are 77% identical (Chen et al., 1999), and the structure of the *B. stearothermophilus* TRAP–RNA complex is, in general, in very good agreement with the results presented in this article. Although not presented here, we will refer to the cocrystal structure at times to further support our findings, and conversely to verify the thermodynamic contribution of the intermolecular contacts seen in the cocrystal structure.

Deoxyribonucleoside substitution studies

TRAP binds RNAs containing GAG and/or UAG repeats, but does not bind similar sequences of deoxyribonucleotides (Otridge & Gollnick, 1993). To explore the role of the 2'-hydroxyl (2'-OH) groups of the ribose sugars in the TRAP–RNA interaction, we made a series of deoxyribonucleoside substitutions into (UAGCC)₁₁, an RNA containing 11 UAG repeats, each separated by CC dinucleotide spacers. This RNA binds TRAP with an apparent equilibrium dissociation constant (K_d) of 3.5 ± 1.2 nM. The advantage of this RNA sequence is that it contains a different nucleotide at each position of the triplet repeats, which in turn are distinct from the spacer regions. Consequently, we were able to make deoxyribonucleoside substitutions in each position of the triplet repeats or in the spacers by in vitro transcription using a mutant T7 RNA polymerase capable of incorporating deoxyribonucleotides (Sousa & Padilla, 1995). For simplicity, we use a nomenclature that designates the RNA portion of the oligonucleotides with upper case letters and the deoxyribonucleotides with lower case. To reduce formation of RNA structure, which inhibits complex formation with TRAP (Babitzke et al., 1996; Baumann et al., 1997; Xirasagar et al., 1998),

binding was conducted at 55 °C. TRAP has been shown to be highly thermostable and active for RNA binding up to 67 °C (Baumann et al., 1997).

Substituting deoxyribonucleosides in the first (deoxyribothymidine (data not shown) or deoxyriouridine) or second (deoxyriboadenosine) residues of the UAG triplet did not significantly change the affinity of the substituted RNAs (D/RNA chimeras) for TRAP as compared to (UAGCC)₁₁ RNA (Fig. 2A). Less than a twofold change in apparent binding constant was measured for each of these D/RNAs. Similarly, incorporating deoxyribocytidines into the spacer residues also had little effect on TRAP binding. Thus the various attributes of deoxyribonucleosides not in common with ribonucleosides, specifically the lack of a 2'-OH and the presence of the C5-methyl group of thymidine, have no effect on the TRAP-RNA interaction in these portions of the RNA. In contrast, substituting deoxyriboadenosine in the third position of all 11 triplet repeats drastically reduced the affinity of (UAGCC)₁₁ for TRAP such that binding was barely detectable up to 30 μM protein. This effect represents at least an 8,000-fold reduction in affinity as compared to (UAGCC)₁₁. These data indicate that the only 2'-OHs that are essential for formation of the TRAP-RNA complex are on the guanosine nucleotides of the UAG repeats. To test this proposal, we synthesized an oligonucleotide with deoxyribonucleosides in all residues except the third nucleotide of the 11 triplet repeats. This nucleic acid, (taGcc)₁₁, binds TRAP with a slightly higher affinity (K_d : 0.5 ± 0.4 nM) than (UAGCC)₁₁ RNA (K_d : 3.5 ± 1.2 nM) (Fig. 2B), confirming that 2'-OH groups are not required other than on the third nucleotide of the 11 triplet repeats.

To further examine the importance of the 2'-OH of ribose on the third nucleoside of the triplets, we substituted the riboguanosines in (taGcc)₁₁ with 2'-O methyl riboguanosine (2'-Ome G), which contains a methoxy group (OCH₃) at C2' that presumably is incapable of hydrogen bonding. Moreover, in contrast to deoxyriboadenosine, which has a C2'-*endo* sugar pucker in DNA, 2'-Ome G prefers the C3'-*endo* pucker found in RNA, and is a useful probe for testing the role of sugar pucker for binding. The 2'-Ome G substitution resulted in nearly complete disruption of detectable binding of (taG_{2'-Ome}cc)₁₁ to TRAP (Fig. 2B), confirming the importance of the 2'-OH, and indicating that the change in sugar pucker was not likely responsible for the decrease in affinity of TRAP for (UAGCC)₁₁.

Remarkably, in the cocrystal structure of *B. steartophilus* TRAP bound to (GAGAU)₁₀GAG RNA, the only 2'-OHs close enough to interact directly with the protein are on the guanosines in the third residue of each repeat, which hydrogen bond with the main-chain amide of Phe-32 (Antson et al., 1999). Such H-bonds between RNA-binding proteins and 2'-OHs of their target RNAs have been implied in several other cases, including RNA polymerases (Szafranski et al., 1985;

FIGURE 2. Role of 2'-OHs of ribose sugars in TRAP binding. Equilibrium binding curves for RNA and D/RNA chimeras binding to TRAP. **A:** Effects of deoxyribonucleoside substitutions at individual positions in the UAG triplets or in the CC spacers. **B:** Effects of substitutions with multiple deoxyribonucleoside or with 2'-Ome G. Symbols for each RNA or D/RNA along with their apparent dissociation constant (K_d) for binding TRAP are as follows: (UAGCC)₁₁ (□), 3.5 ± 1.2 nM; (uAGCC)₁₁ (▼), 5.0 ± 1.0 nM; (UaGCC)₁₁ (○), 3.5 ± 0.2 nM; (UAgCC)₁₁ (▲), >30 μM; (UAGcc)₁₁ (◇), 9.5 ± 1.5 nM; (taGcc)₁₁ (◆), 0.5 ± 0.4 nM; (taG_{2'-Ome}cc)₁₁ (■), >30 μM. Upper case letters represent ribonucleosides and lower case letters designate deoxyribonucleosides. The curves shown are the best fit of the filter binding data using a nonlinear least-squares fitting algorithm. Background retention in the absence of TRAP was subtracted from the binding data and was usually less than 2% of the input counts. The y axis values are normalized to the total counts retained on the filter at saturation, which was greater than 50% of the input counts. Data are the average of at least four individual experiments.

Fedoroff et al., 1997) and ribonucleases (Loria & Pan, 1997). The cocrystal structure also shows a water-mediated hydrogen bond from the 2'-OH of adenosine in each GAG repeat to the main-chain carbonyl oxygen of Thr-30. However, we found that substituting deoxyriboadenosine in these positions had no effect on the stability of the complex in solution. This may simply reflect that these water-mediated H-bonds do not contribute significantly to the overall stability of this complex, even though similar interactions have been shown to be important in other protein-nucleic acid interactions, such as the *trp* repressor-operator complex in

Escherichia coli (Luisi & Sigler, 1990). Additionally it may be possible that organized water molecules surrounding the deoxyriboadenosine are sufficient to compensate for the loss of the 2'-OH at this residue.

Our studies have shown that the TRAP-RNA complex is not sensitive to changes in ionic strength, indicating that ionic interactions do not play an important role in stabilizing this complex (Baumann et al., 1997). This conclusion suggests that there are no salt links between TRAP and the phosphates of the bound RNA. Preliminary experiments using phosphorothioates, analogs that replace one of the nonlinking oxygens of the backbone phosphate with a sulfur atom, revealed little or no effect on TRAP-binding affinity for unstructured RNA, but large deleterious effects on structured RNA (data not shown). Consequently interpretation of these data is not straightforward, as we do not know the effect of phosphorothioate substitution on RNA structure (Scott & Uhlenbeck, 1999), which is known to inhibit TRAP binding (Babitzke et al., 1996; Xirasagar et al., 1998; see below). Together our studies suggest that the 2'-OHs of the third G in the triplet repeats are the only direct contacts between the RNA sugar-phosphate backbone and the protein. This conclusion is borne out in the cocrystal structure of *B. stearothermophilus* TRAP bound to RNA, which shows no evidence for any direct contacts to the phosphates.

Importance of the spacer bases for TRAP binding

The sequences of the spacer nucleotides separating the G/UAG repeats in naturally occurring TRAP-binding sites are highly variable (Babitzke et al., 1994; 1995; Antson et al., 1995; Chen et al., 1999). Moreover, mutagenesis (Babitzke et al., 1996; Xirasagar et al., 1998) and RNA footprinting studies (Babitzke et al., 1994) have suggested that TRAP does not directly interact with these nucleotides when binding RNA. To further investigate the role of the spacer nucleotides in the TRAP-RNA complex we created a chimeric molecule with 11 taG repeats each separated by two abasic 2'-deoxyribose residues lacking a base, (dSpacer, Glen Research), designated as "-". This chimera, (taG--)₁₁, bound to TRAP with virtually the same affinity as (taGcc)₁₁ (Table 1), further supporting the conclusion that TRAP does not interact with the bases of the nucleotides between the triplet repeats when binding RNA. These results are consistent with the cocrystal structure, which shows no direct contacts between the spacer nucleotides and TRAP.

Together these data imply that the only function of these nucleotides is to properly space the triplets to interact with the binding sites on the TRAP oligomer. Consequently, we are able to directly compare any RNAs containing appropriately spaced G/UAG repeats regardless of the identity of the residues between the

TABLE 1. Effect of abasic residue substitutions on D/RNA binding to TRAP.

D/RNA	K_d (nM) ^a
(taGcc) ₁₁ ^b	0.5 ± 0.4
(taG--) ₁₁ ^c	0.7 ± 0.1
(gaGat) ₁₁	1.0 ± 0.4
(-aGat) ₁₁ [*]	5.0 ± 1.0
(-aG--) ₁₁	6.1 ± 0.3

^a Apparent K_d s were determined by direct or competition (*) filter binding. Reactions were incubated at 55 °C in 250 mM K-glutamate and 16 mM HEPES, pH 8.0. Data are the average of at least four individual experiments.

^b Triplet repeat recognition sequences are underlined. Upper case letters represent ribonucleotides and deoxyribonucleotides are in lower case.

^c Each - indicates an abasic site.

triplets, provided that these studies are performed under conditions that do not favor RNA structure (55 °C, -Mg²⁺), as some spacer-nucleotide sequences lead to the formation of structure, which inhibits the TRAP-RNA interaction (Babitzke et al., 1996; Xirasagar et al., 1998). For this reason, all sequences tested in the following sections were first evaluated using the RNA *mfold* program (Mathews et al., 1999; Zuker et al., 1999) and are not predicted to form stable ($\Delta G > -10$ kcal/mol) base-paired structures.

Base analog substitution studies

To examine the importance of the nucleobase functional groups for binding to TRAP, base analogs were inserted in one of the following sequences: (UAGCC)₁₁, (taGcc)₁₁, or (gaGat)₁₁. These nucleic acids all bind TRAP with similar affinities (see footnote of Table 2). (UAGCC)₁₁ was used to test ribonucleotide analogs that were incorporated into RNA by in vitro transcription. The chimera (taGcc)₁₁, which is the D/RNA homolog of (UAGCC)₁₁, was used in cases involving chemical synthesis using deoxyribonucleotide and ribonucleotide analog phosphoramidites. We used (gaGat)₁₁ to study purine analog phosphoramidites in the first residue of the triplets. Each analog has one or more changes to either the exocyclic functional groups or the heterocyclic rings of the base as shown in Figure 3.

Several analogs were used to examine the importance of the functional groups on the guanosine of the UAG triplets. Substituting inosine, which lacks the 2-amino of guanosine (Fig. 3), in the third nucleotide of all 11 triplets resulted in the loss of detectable binding of (UAICC)₁₁ to TRAP (Table 2). This effect suggests that the NH₂ of guanosine in the UAG triplet interacts with the TRAP protein. Similar results were obtained when guanosine in (taGcc)₁₁ was replaced with 2-amino-purine (2-AP), which lacks O6 and the N1-proton

TABLE 2. Effect of analog substitutions in the G/UAG triplet repeats on TRAP binding.

Analog substitution ^c	K_d (nM) ^a		
	Position in triplets ^b		
	First	Second	Third
Inosine	3.0 ± 0.5*	NB ^d *	NB
Nebularine	4.0 ± 1.8*	150 ± 80*	— ^e
2-aminopurine	—	—	NB*
7-deazaadenosine	—	1.8 ± 0.1	—
7-deazaguanosine	—	—	0.4 ± 0.2

^aApparent K_d values were determined by either direct or competition (*) filter binding by incubating each binding reaction at 55 °C in 250 mM K-glutamate and 16 mM HEPES, pH 8.0. K_d values of unsubstituted nucleic acids under these conditions are as follows: (taGcc)₁₁: 0.5 ± 0.4 nM, (UAGCC)₁₁: 3.5 ± 1.2 nM, (gaGat)₁₁: 1.0 ± 0.4 nM. Data are the average of at least four individual experiments.

^bTriplets refer to the first 3 nt of each pentameric repeat of (taGcc)₁₁, (UAGCC)₁₁, or (gaGat)₁₁.

^cAll substitutions were made into (taGcc)₁₁ with the exception of inosine in the first and third positions, which were made into (gaGat)₁₁ and (UAGCC)₁₁, respectively. Additionally, both of the 7-deaza purine substitutions were made into (UAGCC)₁₁.

^dNB, no detectable binding observed up to 1 μM TRAP (direct binding assay) or 1 μM competitor D/RNA (competition assay).

^e—: this analog was not substituted at this position.

(Figs. 3 and 4 and Table 2). We conclude from these results that O6 and/or the N1-nitrogen of guanosine in the third residue of the triplets also participate in the stability of TRAP–RNA complex. The analog 7-deazaguanosine (^{C7}G) has the N7 ring nitrogen replaced with carbon. Substituting this analog for guanosine did not decrease affinity for TRAP but, as we have shown previously (Xirasagar et al., 1998), resulted in a slight (≈8-fold) increase in affinity (K_d : 0.4 ± 0.2 nM) (Table 2) as compared to unsubstituted (UAGCC)₁₁ (K_d : 3.5 ± 1.2 nM), indicating that N7 of guanosine does not interact directly with TRAP.

Every functional group on the third guanosine of each triplet that we identified as being important for complex formation interacts with *B. stearotherophilus* TRAP in the cocrystal structure. Both N1 (NH) and N2 (NH₂) donate hydrogen bonds to the side-chain carbonyl of Glu-36, and O6 accepts H-bonds from the amino groups of Lys-56 and Arg-58. Moreover, as predicted, N7 does not interact with the protein.

We also used this approach to identify several molecular components of the adenosine in the UAG triplets that are involved in the TRAP–RNA complex. Substituting adenosine with nebularine, a deoxyribonucleotide analog with only the base purine ring (lacking the 6-amino group), reduced the affinity for TRAP 300-fold as compared to unsubstituted (taGcc)₁₁ (Table 2 and Fig. 4). These results indicate a role for the NH₂ of adenosine in formation of the TRAP–RNA complex. Inosine differs from adenosine in that the 6-amino group is replaced with an oxygen and a proton is added to N1 (Fig. 3). Substituting inosine in the second nucleotide

of the 11 triplets resulted in the loss of detectable binding of (tiGcc)₁₁ to TRAP (Table 2 and Fig. 4). These results suggest that, in addition to the 6-amino group, the N1-nitrogen of adenosine may also interact with TRAP, as replacing adenosine with inosine results in a greater decrease in affinity than nebularine substitution. The 6-amino is altered in both analogs, but in inosine the N1-nitrogen is also changed from a hydrogen-bond acceptor to a donor (Fig. 3). Similar to the results seen with ^{C7}G, replacing the adenosines of the UAG triplets with 7-deazaadenosine (^{C7}A) (containing a carbon at N7) had little effect on the affinity for TRAP as compared to (UAGCC)₁₁ (Table 2).

The cocrystal structure also correlates very well with our analog substitution studies in the second position of the triplet. Both the 6-amino and N1-nitrogen form H-bonds with the backbone carbonyl and amide groups, respectively, of Lys-37 in each TRAP subunit. Again, the N7-nitrogen does not contact the protein.

The nucleotide requirements for TRAP recognition at the first residue of the triplet repeats are less clear than for the second and third residues. Analysis of natural TRAP-binding sites (Babitzke et al., 1994), artificial TRAP-binding RNAs (Antson et al., 1995; Babitzke et al., 1995), and in vitro selected RNAs (Baumann et al., 1997; Xirasagar et al., 1998) all indicate a strong preference for guanosine or uridine rather than adenosine or cytidine, with guanosine being the most favored for TRAP binding. To examine the role of several functional groups of guanosine in the first position, we tested two purine analogs. Interestingly, replacement with either inosine or nebularine had very little effect on the affinity for TRAP as compared to (gaGat)₁₁ (Table 2 and Fig. 4), indicating that the 2-amino, the N1-nitrogen, and the O6 of these guanosines are not essential in the TRAP–RNA complex. Previous studies have also indicated that N7 of this residue does not directly interact with the protein (Xirasagar et al., 1998). Therefore we were unable to identify any functional elements of guanosine in the first residue of the repeats that are essential for TRAP–RNA complex formation.

To further explore the base requirements of the first nucleotide of the repeats in the TRAP–RNA interaction, we examined the binding of several chimeras containing other nucleotides in this location. Surprisingly, we found that D/RNAs containing any nucleotide in the first residue of the 11 triplet repeats all bind TRAP with similar affinities (Table 3, top). These results contrast previous findings by Babitzke et al. (1995), who reported that RNAs containing six AAG or CAG repeats bound TRAP with lower affinity than GAG- or UAG-containing RNAs. One possible explanation for this discrepancy is that the chimeric D/RNA nucleic acids used in our studies lack some characteristic that makes AAG or CAG RNA triplets unfavorable for TRAP–RNA complex formation. To test this hypothesis, we compared the binding of TRAP to RNAs containing 11 AAGs or

Position in triplet	Normal structure	Nucleoside analogues tested and effect on TRAP affinity		
3rd	Guanosine	Inosine >1000-fold decrease	2-aminopurine >1000-fold decrease	7-deazaguanosine 8-fold increase
	Adenosine	Inosine >1000-fold decrease	Nebularine 300-fold decrease	7-deazaadenosine <2-fold decrease
	Guanosine Uridine	Inosine <2-fold decrease	Nebularine 8-fold increase	(Abasic) dSpacer 10-fold decrease

FIGURE 3. Chemical structures of the nucleobases used in this work and their effect on TRAP binding. Arrows indicate sites of modification. Inosine lacks the 2-amino group found in guanosine, and differs from adenosine in that the 6-amino is replaced with a keto group and the N1-nitrogen is protonated. 2-aminopurine differs from guanosine in that the carbonyl group and N1-proton are absent. Nebularine lacks the 6-amino found in adenosine, and differs from guanosine in that the O6 group and N1 proton are absent. Both the 7-deaza purine analogs contain a carbon at position 7 in place of the nitrogen present in adenosine and guanosine. The sugar component of each nucleoside is represented with an R. Fold decrease or increase indicates the difference in binding affinity of the analog substituted as compared to the unsubstituted nucleic acids at 55 °C.

CAGs to those with 11 GAGs or UAGs, and again found no significant differences in affinity under our standard binding conditions (55 °C, $-Mg^{2+}$) (Table 3, bottom).

To rule out the possibility that the formation of RNA structure at 37 °C in the presence of magnesium provides the selective pressure against AAG and CAG repeats described above, we examined the affinity of the D/RNA chimeras and RNAs in Table 3 for TRAP under conditions that favor structure in RNA (37 °C, 4 mM Mg^{2+}), and compared these results to those obtained previously. None of the D/RNA chimeras or RNA homopolymers in Table 3 showed a significant decrease in affinity for TRAP under these conditions as compared to 55 °C, no Mg^{2+} , indicating that these nucleic acids are not sufficiently structured at 37 °C, $+Mg^{2+}$ so as to inhibit binding. Although the RNAs (CAGUU)₁₁

and (AAGAU)₁₁ bound TRAP with 8–10-fold lower affinity at 37 °C, $+Mg^{2+}$ than at 55 °C, $-Mg^{2+}$, these data do not seem sufficient to explain the low frequency of these triplets in naturally occurring TRAP-binding sequences. The 8–10-fold effects on TRAP affinity observed here are the result of having 11 A/CAG repeats per RNA, and it is unusual to find even one of these triplets in naturally occurring TRAP-binding sites (Babitzke et al., 1994; Chen et al., 1999). We did not test (AAGUU)₁₁ because it is predicted to form a stable hairpin structure via Watson–Crick base pairing.

It is likely that the differences between our results and those of Babitzke et al. (1995) and Baumann et al. (1997) reflect the different numbers of repeats (11 vs. 5–6) in the RNAs used in these studies. Perhaps having more repeats in some way compensates for an

FIGURE 4. Competition assays comparing analog substituted and unsubstituted D/RNAs. Symbols for each RNA or D/RNA along with their apparent dissociation constant (K_d) for binding TRAP are as follows: (taGcc)₁₁ (◆), 0.5 ± 0.4 nM; (iaGat)₁₁ (+), 3.0 ± 0.5 nM; (tnGcc)₁₁ (△), 150 ± 80 nM; (tiGcc)₁₁ (*), $>1,000$ nM; (ta2-APcc)₁₁ (×), $>1,000$ nM; (naGcc)₁₁ (◇), 4.0 ± 1.8 nM. Upper case letters represent ribonucleosides and lower case letters designate deoxyribonucleosides. Studies were performed at 55°C in 250 mM K-glutamate, 16 mM HEPES. Curves shown are the best fit of the filter binding data using a nonlinear regression, one binding site competition algorithm. Background retention in the absence of TRAP was subtracted from the binding data and was usually less than 2% of the input counts. The y axis values are normalized to the total counts retained on the filter at saturation, which was greater than 50% of the input counts. Data are the average of at least four individual experiments. The competition curve for (tnGcc)₁₁ was carried out to 500 nM competitor; however, all curves are presented to 20 nM to show the detail of the early portion of the curves.

intrinsically weaker association with individual AAG or CAG repeats as observed by Babitzke et al. (1995) with six repeat RNAs. A detailed study of the interaction of TRAP with RNAs containing variable numbers of each type of triplet may be required to fully understand this specificity.

Our data show that under some circumstances any nucleoside can occupy the first residue of the triplet repeats with equal affinity for TRAP, suggesting that the protein does not make specific contacts to the base at this location. To test this hypothesis, we created an abasic site in the first position of all 11 triplets. This nucleic acid, (-aGat)₁₁, bound TRAP with only 5–10-fold lower affinity than (gaGat)₁₁ or (taGcc)₁₁ (Table 1), confirming that under these conditions, a base is not required at the first residue of the triplets. These effects ($\Delta\Delta G = 1\text{--}2$ kcal/mol), while significant, are likely too weak to represent elimination of 11 hydrogen bonds in the interaction (Fersht, 1988; Lesser et al., 1990). However, it is possible that weaker forces, such as van der Waals interactions, intermolecular stacking, or hydrophobic effects, are being disrupted.

The only significant discrepancies between the crystal structure and our analog studies involve the residue in the first position of each triplet repeat. First, the crystal structure shows that the 2-amino group of the first G of each GAG repeat is in proximity of the carbonyl

TABLE 3. Effect of Mg^{2+} and temperature on RNA or D/RNA binding to TRAP.

Nucleic acid	K_d (nM) ^a	
	55 °C, $-Mg^{2+}$	37 °C, $+Mg^{2+}$
D/RNA chimeras		
(taGcc) ₁₁ ^b	0.5 ± 0.4	1.0 ± 0.3
(gaGat) ₁₁	1.0 ± 0.4	3.0 ± 0.5
(aaGcc) ₁₁	3.0 ± 1.0	0.3 ± 0.1
(caGcc) ₁₁	1.5 ± 0.2	0.3 ± 0.05
RNA homopolymers		
(UAGUU) ₁₁	0.3 ± 0.1	0.3 ± 0.1
(GAGUU) ₁₁	1.2 ± 0.6	2.7 ± 0.5
(AAGAU) ₁₁	4.0 ± 2.0	40 ± 5.0
(CAGUU) ₁₁	3.5 ± 1.5	27 ± 3.0
(GAGAU) ₁₁	2.5 ± 1.0	8.0 ± 2.5

^a Apparent K_d values were determined by competition (D/RNA chimeras) or direct (RNA homopolymers) filter binding. Reactions were incubated at the indicated temperature (± 4 mM $MgCl_2$) in 250 mM K-glutamate and 16 mM HEPES, pH. 8.0. Data are the average of at least four individual experiments.

^b Triplet repeat recognition sequences are underlined. Upper case letters represent ribonucleotides and deoxynucleotides are in lower case.

oxygen of Asp-39 such that an H-bond, although distorted, is predicted. This is somewhat surprising given that we saw little if any effect of substitutions in the first residue of the triplet repeats (Tables 1, 2, 3, and 5). In particular, replacing guanosine with inosine (Table 2 and Fig. 4) (lacking the 2-amino) or with an abasic residue (Table 1) resulted in only small decreases in affinity, suggesting that, at least with the *B. subtilis* protein under the conditions studied in this work, this putative H-bond is not important for stabilizing the overall complex. Furthermore, previous studies of the *B. subtilis* protein showed that replacing Asp-39 with Ala did not reduce the affinity of the mutant protein for *trp* leader RNA in vivo or in vitro (Yang et al., 1997).

The crystal structure also shows that this guanosine is in van der Waals contact with the aliphatic side chain of Lys-37. Furthermore, mutagenesis studies have indicated a role for the side chain of position 37 in RNA binding (Yang et al., 1997), and support the importance of hydrophobic interactions at this residue (A. Wendt & P. Gollnick, unpubl.). Nevertheless, we found that chimeras containing an abasic site at this position [(-aGat)₁₁] bound to TRAP with only slightly lower affinity than (GAGUU)₁₁ or (UAGCC)₁₁ (Tables 1, 2, and 5). These results seem contradictory and may suggest that the stabilizing effect that was lost due to disruption of hydrophobic interactions between Lys-37 and this guanosine in each repeat was in some way compensated for in the interaction with the abasic site containing chimeras. Perhaps the abasic backbone provides additional flexibility and loss of steric effects offsetting the requirement to unstack the bases in the first position for TRAP–RNA complex formation. Clearly more

studies of this putative interaction are necessary to understand this apparent discrepancy as well as the overall role of the first residue in the triplet repeats in the specificity and stability of this protein–RNA interaction.

Minimum TRAP-binding site

Our studies of D/RNA chimeras containing abasic sites demonstrated that TRAP does not require a base in either the first position of the triplet or in the spacer residues for high-affinity binding. Together these results suggest that 11 AG repeats appropriately separated should be sufficient for TRAP binding. To test this hypothesis, we created $(-aG-)_11$, a D/RNA consisting of 11 deoxyriboadenosine–riboguanosine dinucleotides, each separated by three abasic residues. This chimera bound TRAP with a K_d of 6.1 ± 0.3 nM at 55 °C, only 5–10-fold higher than $(gaGat)_11$ and $(taGcc)_11$ (Table 1 and Fig. 5). The small difference in the free energy of complex formation ($\Delta\Delta G^\circ = 1.2$ kcal/mol) suggests that under these conditions, the majority of the specificity and stability of the TRAP–RNA complex comes from contacts between the protein and the AG dinucleotides in the repeats. It should be pointed out that this conclusion assumes that the $(-aG-)_11$ molecule forms a similar complex with TRAP as 11 triplet-containing RNAs. Moreover, there may be differences in flexibility of $(-aG-)_11$ as compared to RNA, which may influence the interaction with TRAP (Baumann et al., 1997).

FIGURE 5. Equilibrium binding assays comparing affinity of TRAP for $(taGcc)_11$ versus $(-aG-)_11$. Symbols for each RNA or D/RNA along with their apparent dissociation constant (K_d) for binding TRAP are as follows: $(taGcc)_11$ (●), 0.5 ± 0.4 nM; $(-aG-)_11$ (▽), 6.1 ± 0.3 nM. Upper case letters represent ribonucleosides and lower case letters designate deoxyribonucleosides. Studies were performed at 55 °C in 250 mM K-glutamate, 16 mM HEPES. Curves shown are the best fit of the filter binding data using a nonlinear least-squares fitting algorithm. Background retention in the absence of TRAP was subtracted from the binding data and was usually less than 2% of the input counts. The y axis values are normalized to the total counts retained on the filter at saturation, which was greater than 50% of the input counts. Data are the average of at least four individual experiments.

Identification of molecular components involved the structure of $(UAGCC)_11$

RNA structure has been shown to play an inhibitory role in the formation of the TRAP–RNA complex (Babitzke et al., 1996; Baumann et al., 1997; Xirasagar et al., 1998). Studies by Xirasagar et al. (1998) found that RNAs with G/UAG repeats separated by CC dinucleotide spacers (but not UU spacers) adopt stable structures that inhibit binding to TRAP. Destabilization of this structure at elevated temperatures or by removing magnesium increases the affinity for TRAP. Additionally, substituting the guanosines in $(UAGCC)_11$ with 7-deazaguanosine (C^7G) (Fig. 3) also increases affinity for TRAP, suggesting that the N7-nitrogen is involved in stabilizing the RNA structure.

To determine if N7 of the adenosines in the UAG triplets are also involved in stabilizing the $(UAGCC)_11$ structure, we substituted the adenosines with 7-deaza-adenosine (C^7A), and examined the affinity for TRAP at various temperatures (Table 4). $(U^{C^7A}GCC)_11$ bound TRAP with higher affinity than unsubstituted $(UAGCC)_11$ at temperatures between 24 and 37 °C, suggesting that the N7 nitrogen of the adenosines in the UAG triplets are also involved in the stability of the structure of this RNA. To ascertain whether the effects of the 7-deaza substitutions at different residues in the triplet repeats are independent, we compared TRAP binding to doubly substituted $(U^{C^7A}C^7GCC)_11$, singly substituted $(U^{C^7A}GCC)_11$, or $(UA^{C^7}GCC)_11$ to unsubstituted $(UAGCC)_11$ at various temperatures (Table 4). Substitution with either C^7A or C^7G increased the affinity of the RNA for TRAP at 37 °C or lower, with C^7G always having a larger effect than C^7A . Moreover, these effects appear to be independent, as the doubly substituted RNA had the highest affinity for TRAP at each temperature tested. The effects of 7-deaza purine analog substitution were most pronounced at temperatures of 24 °C and lower, where binding was not detectable for the unsubstituted $(UAGCC)_11$. We conclude from these studies that the N7 of both adenosine and guanosine of the UAG triplets contribute to the stability of the structure of $(UAGCC)_11$, with the N7 of guanosine contributing more than the N7 of adenosine. These findings also imply that noncanonical interactions are involved in this structure, as the N7-nitrogens do not participate in Watson–Crick pairs.

None of the D/RNA chimeras shown in Table 3 displayed any temperature- and magnesium-dependent effects on TRAP binding, including those with CC dinucleotide spacers, which form stable structures in RNAs containing G/UAG triplets (Xirasagar et al., 1998; also see $(UAGCC)_11$ in Table 5). These data suggest that some or all of the 2'-OHs of the ribose sugars are also involved in stabilizing the structure of the $(G/UAGCC)_11$ RNAs. We further investigated the role of the 2'-OHs of the ribose sugars in each location of the

TABLE 4. Effect of 7-deaza (C^7 NTP) purine substitutions on (UAGCC)₁₁ RNA binding at various temperatures.

RNA	K_d (nM) ^a				
	55 °C	42 °C	37 °C	24 °C	16 °C
(UAGCC) ₁₁ ^b	3.5 ± 0.1	14 ± 0.1	250 ± 32	NB ^c	NB
(U ^{C7} AGCC) ₁₁	1.8 ± 0.1	17 ± 0.7	46 ± 18	900 ± 75	NB
(UA ^{C7} GCC) ₁₁	0.4 ± 0.2	2.5 ± 0.3	7.2 ± 0.7	21 ± 7.0	120 ± 15
(U ^{C7} A ^{C7} GCC) ₁₁	0.05 ± 0.2	0.75 ± 0.3	1.1 ± 0.1	13 ± 1.1	20 ± 5.0

^aAll K_d values determined by direct filter binding by incubating each reaction at the indicated temperatures in 250 mM K-glutamate, 16 mM HEPES, pH 8.0, and 4 mM MgCl₂. Data are the average of at least four different experiments.

^bTriplet repeat recognition sequences are underlined.

^cNB: no binding detected up to 30 μM TRAP.

triplet repeat and the spacer nucleotides by examining the effect of deoxyribonucleotide substitutions on the temperature and Mg²⁺ dependence of the D/RNAs described in Figure 2A in binding to TRAP. Substitutions at either the first or second nucleotide of the UAG triplet or at the CC spacers all increased the affinity of (UAGCC)₁₁ for TRAP to an intermediate level between that of (UAGCC)₁₁ and (taGcc)₁₁ at 37 °C in the presence of Mg²⁺ (Table 5), suggesting that all the 2'-OHs play a role in stabilizing the structure of (UAGCC)₁₁.

The results from this work suggest a role for both the 2'-OH of each ribose sugar (except guanosine, which we were unable to assess in this role) and the N7-nitrogens of adenosine and guanosine in this structure. Both magnesium and 2'-OHs have been reported to be involved in stabilizing RNA structure (Laing et al., 1994; Allain & Varani, 1995; Wang & Kool, 1995; Agris, 1996; Li & Turner, 1997). Moreover, in several cases, Mg²⁺ interacts with 2'-OH groups (Sugimoto et al., 1989; Perreault et al., 1991; Smith & Pace, 1993). Perhaps this type of interaction is involved in the (UAGCC)₁₁ struc-

ture, which would explain why our deoxyribonucleotide substitutions destabilize this structure even in the presence of Mg²⁺ (Table 5). Another possible role for the 2'-OHs is involvement in water-mediated hydrogen bonds. Egli and coworkers (1996) showed that multiple water-mediated H-bonds between 2'-OHs in the minor groove of (C₄G₄) significantly increase the RNA-duplex stability. The N7-nitrogens have also been shown to coordinate divalent cations in some RNA structures (Scott et al., 1995; Correll et al., 1997), and to be involved in purine–purine interactions such as G-quartets and G-A mismatches (Wu & Turner, 1996). Since the structure of (UAGCC)₁₁ is Mg²⁺ dependent (Xirasagar et al., 1998; M.B. Elliott, P.A. Gottlieb, & P. Gollnick, unpubl.), this effect may also be a function of the N7 nitrogens. Confirmation of these hypotheses awaits determining the structure of (UAGCC)₁₁; however these results continue to demonstrate that, unlike most protein–RNA interactions, the presence of structure inhibits RNAs from binding to TRAP.

CONCLUSION

Through the use of nucleoside analogs, we have identified several functional elements of RNA that are essential for TRAP recognition and complex stability. These elements, the N1 and 6-amino groups of A, and the N1, 2-amino, O6, and 2'-OH groups of G, all reside on the second and third nucleosides of the G/UAG triplet repeats. All but one of these groups (the 2'-OH of guanosine) are contained on the nucleobases of adenine and guanine, respectively. Identification of these RNA elements as interacting with the protein explains why TRAP is specific for single-stranded RNA, because these groups all participate in Watson–Crick base pairing. Moreover, lysine and arginine residues of RNA-binding proteins (Tao & Frankel, 1992; Oubridge et al., 1994) and numerous DNA-binding proteins (Tucker-Kellogg et al., 1997; for a survey, see Mandel-Gutfreund et al., 1995) have been shown to participate in hydrogen bonding with adenines and guanines. Because of

TABLE 5. Effect of temperature and magnesium on the affinity of TRAP for (UAGCC)₁₁ ± deoxyribonucleoside substitutions.

Nucleic acid	K_d (nM) ^a	
	55 °C, −Mg ²⁺	37 °C, +Mg ²⁺
(UAGCC) ₁₁ ^b	3.5 ± 1.2	250 ± 17
(uAGCC) ₁₁ ^c	5.0 ± 1.0	15 ± 3.0
(tAGCC) ₁₁	10 ± 4.0	30 ± 10
(UaGCC) ₁₁	3.5 ± 0.2	18 ± 3.0
(UAgCC) ₁₁	NB ^d	NB
(UAGcc) ₁₁	9.5 ± 1.5	25 ± 7.0
(taGcc) ₁₁	0.5 ± 0.4	1.0 ± 0.3

^a K_d values were determined by direct filter binding. Reactions were incubated at the indicated temperature (± 4 mM MgCl₂) in 250 mM K-glutamate and 16 mM HEPES, pH 8.0. Data are the average of at least four individual experiments.

^bTriplet repeat recognition sequences are underlined.

^cUpper case letters represent ribonucleotides and lower case represent deoxyribonucleotides.

^dNB: no binding observed up to 30 μM TRAP.

the repetitive nature of both the TRAP oligomer and the RNA binding site, we assume that the chemical groups of each triplet contribute equally to the stability of the TRAP–RNA complex. Furthermore, our 7-deaza purine substitutions indicate that none of the N7 nitrogens of the purines interact with the protein, but appear to play a role along with the 2'-OHs in the formation of RNA structure in (UAGCC)₁₁ RNA. Our evidence indicates that the bases in the first residue of the triplets as well as in the spacer nucleotides do not play a specific role in the TRAP–RNA interaction. These data are, in general, in good agreement with previous studies of the specificity of the TRAP–RNA interaction, both in vivo (Antson et al., 1995) and in vitro (Babitzke et al., 1994, 1995). Moreover, these results confirm the thermodynamic importance of the intermolecular contacts seen in the cocrystal structure of *B. stearotheophilus* TRAP bound to an RNA containing 11 GAG repeats (Antson et al., 1999).

MATERIALS AND METHODS

Plasmids and strains

All plasmids were propagated in *E. coli* JM107. Plasmids pTZ18UAGCC, pTZ18GAGUU, pTZ18UAGUU, pSP64GAGCC, and pUC118GAGAU have been described previously (Baumann et al., 1996; Xirasagar et al., 1998). The plasmid pTZ18CAGUU has ten repeats of the sequence 5'-CAGTT-3' followed by 5'-CAG-3' cloned downstream of the T7 promoter in pTZ18U (U.S. Biochemical). The insert was created by annealing oligonucleotides CAGUU-11 (5'-AGGGAATTC(CAGTT)₁₀CAGAAGCTTGGCACTGGCCGTCG-3') and H3UNIV20MER (5'-CGACGGCCAGTGCCAAGCTT-3'). The 3' ends were extended using the Klenow fragment of DNA polymerase I and the resulting duplex DNA was digested with *Eco*RI and *Hind*III and ligated into similarly digested pTZ18U. The plasmid pTZ18AAGAU, containing 10 repeats of the sequence 5'-AAGAT-3' followed by AAG, was made by a similar approach, with the exception of the sequence of the oligonucleotides that were used to make the duplex DNA. This plasmid was made using oligonucleotides AAGAU-11 (5'-AGGGAATTC(AAGAU)₁₀AAGAAGCTTGGCACTGGCCGTCG-3') and H3UNIV20MER (see above).

RNA and D/RNA synthesis

Deoxyribonucleoside and nucleotide analog substitutions were made in either (UAGCC)₁₁ RNA, or the D/RNA chimeras (taGcc)₁₁ and (gaGat)₁₁. These nucleic acids all bind TRAP with similar apparent *K_d*s of 3.5 ± 1.2 nM, 0.5 ± 0.4 nM, and 1.0 ± 0.4 nM, respectively. We used *Hind*III-linearized pTZ18UAGCC as a template for in vitro transcription reactions to make the RNA sequence 5'-(UAGCC)-3' repeated 11 times ((UAGCC)₁₁). This template was also used for the deoxyribonucleoside substitutions and for incorporation of the nucleotide analogs inosine triphosphate (ITP), 7-deazaadenosine triphosphate (^{C7}ATP), and/or 7-deazaguanosine triphosphate (^{C7}GTP). The chimera (taGcc)₁₁, which is the

D/RNA homolog of (UAGCC)₁₁, was used for some constructs, because it allowed substitution of commercially available deoxyribonucleotide and ribonucleotide analog phosphoramidites. Substitutions into (gaGat)₁₁ were made to test inosine (a deoxyriboguanosine derivative) in the first residue of the triplet ((iaGat)₁₁) and the abasic (-) substituted (-aGat)₁₁.

In vitro transcription reactions of ³²P-labeled RNA with T7 RNA polymerase were performed as described previously (Baumann et al., 1996; Xirasagar et al., 1998), using plasmids linearized with *Hind*III or *Bsp*120I as templates. RNAs used in this study that were synthesized by in vitro transcription were labeled with either [α -³²P]UTP, [α -³²P]ATP, or [α -³²P]CTP (NEN). For in vitro transcriptions to make (UAICC)₁₁, GTP was replaced with 6 mM ITP, 3 μ g of pTZ18UAGCC were used, and 0.1 mM ApG was added to the reaction to initiate transcription. In vitro transcriptions of 7-deaza purine- (^{C7}NTP) substituted RNAs contained 2 mM ^{C7}GTP and/or ^{C7}ATP substituted for GTP and/or ATP, respectively, with 2.5 mM GMP to initiate transcription. All RNAs and D/RNAs were gel purified as described previously (Baumann et al., 1996). For in vitro transcription reactions to make chimeric D/RNAs containing deoxyribonucleoside substitutions, a mutant T7 RNA polymerase in which Tyr-639 is substituted with Phe was used (Sousa & Padilla, 1995). This enzyme incorporates both rNTPs and dNTPs. Additionally, one rNTP was replaced with the corresponding dNTP (2.5 mM) and the concentration of dithiothreitol (DTT) was increased to 50 mM.

D/RNA chimeras that were not made by in vitro transcription were chemically synthesized using a 381A DNA synthesizer (Applied Biosystems Instruments). All synthesis reagents, supports, and CE phosphoramidites (dA, Ac-dC, dmf-dG, dT, dmf-G, dl, 2'-O-Me-G, 2'-DeoxyNebularine, and dSpacer), were purchased from Glen Research, Sterling, Virginia, or synthesized as described below. D/RNAs produced by chemical synthesis were 5'-end labeled with [γ -³²P]ATP using 1 pmol of D/RNA, 30 μ Ci of γ -³²P-ATP (6,000 Ci/mmol), T4 PNK buffer (50 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 10 mM 2-Mercaptoethanol) (USB) in a final reaction volume of 0.02 mL. These nucleic acids were purified as described previously (Baumann et al., 1996).

Synthesis of 7-deaza GTP, 7-deaza ATP, and 2-aminopurine

The nucleotide 7-deaza GTP was synthesized as described previously (Xirasagar et al., 1998). 7-deaza ATP was synthesized as described (Wieczorek et al., 1994). The phosphoramidite N2-isobutryl-2-aminopurine ribonucleoside was synthesized as described (Tuschl et al., 1993). NMR chemical shifts were identical to those previously described.

TRAP purification

Wild-type *B. subtilis* TRAP was purified by overexpressing *E. coli* as described previously (Antson et al., 1994).

Direct filter binding assay

Quantitative filter binding assays were performed as described previously (Xirasagar et al., 1998) in filter binding

buffer (250 mM potassium glutamate and 16 mM HEPES, pH 8.0) with standard conditions including incubation at 55 °C for 1 h, as described previously (Xirasagar et al., 1998). In some reactions, MgCl₂ was added to a final concentration of 4 mM. The data were analyzed using a nonlinear regression algorithm (GraphPad Prism 1.00, GraphPad Software Incorporated, San Diego, California). Data are the average of at least four individual experiments with a variation in K_d not exceeding 40%.

Competition filter binding assay

Competition assays were performed in filter binding buffer with 2.3 fmols of γ -³²P-labeled (taGcc)₁₁ or (gaGat)₁₁ (3×10^4 dpm), depending on whether a purine or pyrimidine analog was being substituted in the first position, and various concentrations of unlabeled competitor D/RNA. TRAP was then added to the reaction mixture to a final concentration of 1 nM (the dissociation constant of the labeled D/RNA). After 5 min, *L*-tryptophan was added to a final concentration of 1 mM. The final reaction volume was 0.1 mL. Other reaction conditions, filtration, and data acquisition were performed as described for the direct filter binding assay. The data was analyzed using a nonlinear regression, one binding site competition algorithm (GraphPad Prism 1.00, Graphpad Software Incorporated, San Diego, California) using Equation 1 (Dugan et al., 1995):

$$RO = \frac{1}{2}[K_{RO} + (K_{RO}/K_{RC})C_t + R_t + O_t - \sqrt{(K_{RO} + (K_{RO}/K_{RC})C_t + R_t + O_t)^2 - 4O_tR_t}] \quad (1)$$

where R_t , K_{RO} , K_{RC} , C_t , and O_t are the total protein, equilibrium dissociation constant for labeled D/RNA, equilibrium dissociation constant for competitor, total competitor D/RNA, and total labeled D/RNA, respectively. Data are the average of at least four individual experiments, with no more than 40% variation.

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