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The mechanism of RNA binding to TRAP: Initiation and cooperative interactions

MATTHEW B. ELLIOTT, PHILIP A. GOTTLIEB, and PAUL GOLLNICK
Department of Biological Sciences, State University of New York, Buffalo, New York 14260, USA

ABSTRACT

The *trp* RNA-binding Attenuation Protein (TRAP) from *Bacillus subtilis* is an 11-subunit protein that binds a series of 11 GAG and UAG repeats separated by two to three-spacer nucleosides in *trp* leader mRNA. The structure of TRAP bound to an RNA containing 11 GAG repeats shows that the RNA wraps around the outside of the protein ring with each GAG interacting with the protein in nearly identical fashion. The only direct hydrogen bond interactions between the protein and the RNA backbone are to the 2′-hydroxyl groups on the third G of each repeat. Replacing all 11 of these guanosines with deoxyriboguanosine eliminates measurable binding to TRAP. In contrast, a single riboguanosine in an otherwise entirely DNA oligonucleotide dramatically stabilizes TRAP binding, and facilitates the interaction of the remaining all-DNA portion with the protein. Studies of TRAP binding to RNAs with between 2 and 11 GAGs, UAGs, AAGs, or CAGs showed that the stability of a TRAP-RNA complex is not directly proportional to the number of repeats in the RNA. These studies also showed that the effect of the identity of the residue in the first position of the triplet, with regard to binding to TRAP, is dependent on the number of repeats in the RNA. Together these data support a model in which TRAP binds to RNA by first forming an initial complex with a small subset of the repeats followed by a cooperative interaction with the remaining triplets.

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

INTRODUCTION

The trp RNA-binding Attenuation Protein (TRAP) is an RNA-binding protein that negatively regulates expression of the tryptophan biosynthesis (*trp*) genes in *Bacillus subtilis* in response to intracellular levels of tryptophan (Gollnick, 1994; Babitzke, 1997). Six of the seven *trp* genes are clustered in the *trpEDCFBA* operon. Transcription of this operon is controlled by an attenuation mechanism involving two mutually exclusive RNA secondary structures that form in the leader region 5′ to the structural genes. One of these structures is an intrinsic transcription terminator and the other overlapping structure is an antiterminator. When activated by binding tryptophan, TRAP binds to a series of 11 triplet repeats composed of GAG or UAG that, in part, overlap a portion of the antiterminator. TRAP binding therefore prevents antiterminator formation, which leads to formation of the terminator, and transcription halts in the leader region. Under conditions of limiting tryptophan, TRAP does not bind RNA and the antiterminator structure is favored, allowing transcription of the operon. TRAP also regulates translation of several genes. By binding to the same site in the leader region of *trp* read-through mRNAs, TRAP induces formation of an RNA structure that sequesters the *trpE* ribosome binding site, thereby inhibiting translation initiation (Kuroda et al., 1988; Merino et al., 1995; Du & Babitzke, 1998). Translation of *trpG*, which is in the folate operon (Slock et al., 1990; Yang et al., 1995), and of *yhaG*, which is believed to encode a tryptophan transport protein (Sarsero et al., 2000a), is also regulated by TRAP. In these cases the TRAP binding site overlaps the ribosome binding site, and for *trpG,* Du et al. (1998) demonstrated that TRAP competes directly with ribosomes for binding to this mRNA.

The crystal structure of TRAP complexed with *L*-tryptophan shows that the protein consists of 11 identical subunits arranged in a symmetrical ring around a central hole (Antson et al., 1995; Chen et al., 1999). The TRAP oligomer is composed of 11 seven-stranded antiparallel β-sheets, each containing four β-strands from one subunit and three strands from the adjacent subunit. TRAP is activated to bind RNA by the cooperative binding of 11 molecules of *L*-tryptophan in pockets formed between adjacent subunits (Antson et al., 1995; Babitzke et al., 1995).

Reprint requests to: Paul Gollnick, Department of Biological Sciences, State University of New York, Buffalo, New York 14260, USA; e-mail: gollnick@ubunix.acsu.buffalo.edu.

Naturally occurring TRAP binding sites contain between 9 and 11 GAG and/or UAG (and rarely AAG) repeats (Babitzke et al., 1994; Antson et al., 1995; Yang et al., 1995; Chen et al., 1999; Sarsero et al., 2000a, 2000b). Mutagenesis and deletion studies have demonstrated the importance of the adenosines and guanosines in the second and third residues of the triplet repeats for binding to TRAP (Antson et al., 1995; Babitzke et al., 1995). We recently used nucleoside analog substitution studies to identify functional groups of these residues that are crucial for complex formation (Elliott et al., 1999). In contrast, numerous substitutions in the first residue of 11 RNA triplets had little effect on the stability of the complex. This result was surprising given the large bias (>90%) for G or U in the first residue of naturally occurring TRAP binding sites.

Appropriate spacing of the triplet repeats is also important for TRAP binding (Babitzke et al., 1994; Antson et al., 1995), with two spacer nucleotides being optimal (Babitzke et al., 1996; Xirasagar et al., 1998). However the sequence of the spacer nucleotides is highly variable in naturally occurring binding sites, and several studies have shown that the spacer nucleotides do not directly interact with TRAP (Babitzke et al., 1994, 1996; Antson et al., 1999; Elliott et al., 1999). Spacer nucleotides do however, indirectly influence TRAP binding by affecting RNA structure. In contrast to most other RNA–protein interactions, the presence of stable secondary structure in the RNA is inhibitory to the TRAP–RNA interaction (Babitzke et al., 1996; Xirasagar et al., 1998; Elliott et al., 1999).

Antson et al. (1999) recently solved the crystal structure of TRAP in complex with an RNA containing 11 GAG repeats separated by AU dinucleotides. The bound RNA wraps around the outer perimeter of the TRAP ring. The phosphodiester backbone is on the outside of the ring and the bases point inward and interact with the protein. As predicted by our analog studies (Elliott et al., 1999), the majority of the specific interactions occur with the second and third nucleosides of each GAG and nearly all the contacts involve functional groups of the bases. There are no direct hydrogen bonds between TRAP and the spacer nucleotides between the GAG repeats. The only direct hydrogen bonds to the phosphodiester backbone are to the 2′-OHs on the ribose of the third G of each triplet. This contact, which was predicted by deoxynucleoside substitution studies (Elliott et al., 1999), explains how TRAP distinguishes RNA from DNA.

Our previous analog studies involved investigating the effects of making substitutions in all 11 repeats of TRAP binding RNAs (Elliott et al., 1999). This approach together with the structure of a TRAP–RNA complex (Antson et al., 1999) identified functional groups of both the RNA and the protein necessary for complex formation. However, these studies revealed little about the mechanism by which the RNA associates with TRAP and becomes wrapped around the protein ring. In this article, we further explore the role of the first residue in the triplet repeats in TRAP binding. We also extend our nucleoside analog studies to investigate the mechanism by which TRAP associates with its RNA targets by making substitutions into subsets (1 to 10) of the 11 triplet repeats of a TRAP binding site. The results of these studies provide evidence that TRAP forms an initial complex with as few as one or two G/UAG repeats in the RNA. We also provide evidence that a form of cooperativity is involved in RNA binding to TRAP. From these results we propose a model for RNA binding to TRAP that involves formation of an initial "nucleation" complex followed by cooperative interaction with the remaining triplet repeats in the RNA target.

RESULTS

Deoxyguanosine and deoxyinosine substitution studies

Our previous studies showed that only the third residue (guanosine) in each pentanucleotide repeat (triplet recognition sequence and two spacer residues) requires a ribose sugar for high-affinity binding to TRAP (Elliott et al., 1999). The remaining residues can be substituted with deoxynucleosides such that the DNA/RNA chimera[1] $(taGcc)_{11}$ binds TRAP with equal affinity and specificity as $(UAGCC)_{11}$ RNA. In contrast, substituting the 11 riboguanosines with deoxyguanosines eliminates measurable affinity of $(tagcc)_{11}$ or $(UAgCC)_{11}$ for TRAP (Table 1; see also Elliott et al., 1999). The importance of these ribose sugars is clarified by the TRAP–RNA crystal structure, which shows that the only direct hydrogen bond between the sugar–phosphate backbone of the bound RNA and the protein is made between the 2′-OH group of the third guanosine of each repeat and the main-chain NH group of Phe32 (Antson et al., 1999). To investigate the mechanism by which RNA binds to TRAP, we examined the effects of substituting deoxyguanosine (g) into subsets (between 6 and 10) of the taGcc repeats of $(taGcc)_{11}$. Substituting over half (6) of the 11 riboGs had a relatively small effect (threefold; $\Delta\Delta G^\circ = 0.76$ kcal/mol) on the stability of the complex with TRAP (Table 1). As more substitutions were made, the effects increased moderately and a chimera with only two riboGs, $(taGcc)_2(tagcc)_9$, displayed a dissociation constant (K_d) of 14 nM. The most surprising observation is that the presence of only one riboG in a DNA/RNA chimera such as $taGcc(tagcc)_{10}$ dramatically stabilizes the interaction with TRAP ($K_d =$ 90 nM) as compared to the DNA oligonucleotide $(tagcc)_{11}$, to which we were unable to measure any

[1] DNA/RNA chimeras are described using upper case letters (G, A, U, C) to designate ribonucleosides and deoxynucleosides are represented in lower case (g, a, t, c).

TABLE 1. Effect of deoxyguanosine substitutions on $(taGcc)_{11}$ affinity for TRAP

D/RNA[a]	K_d (nM)
$(taGcc)_{11}$[b]	0.5 ± 0.4
$(taGcc)_5(tagcc)_6$	1.5 ± 0.6
$(taGcc)_4(tagcc)_7$	5.1 ± 0.7
$(taGcc)_3(tagcc)_8$	10 ± 1.5
$(taGcc)_2(tagcc)_9$	14 ± 4.0
$taGcc(tagcc)_{10}$	90 ± 8.0
$(tagcc)_{10}(taGcc)$	75 ± 6.5
$(tagcc)_5taGcc(tagcc)_5$	70 ± 11
$(tagcc)_{11}$[b]	NB[c]

[a]Upper case letters represent ribonucleosides and deoxynucleosides are in lower case.
[b]Data published previously (Elliott et al., 1999) and shown here for comparison.
[c]NB: no specific binding observed with up to 30 μM TRAP.

specific binding using up to 30 μM TRAP. The location of the single riboguanosine-containing triplet within the 11 repeat binding site made little difference (Table 1, compare $taGcc(tagcc)_{10}$ to $(tagcc)_5taGcc(tagcc)_5$ and $(tagcc)_{10}taG$). Preliminary crystallographic studies have shown that $(taGcc)_{11}$ forms a complex with TRAP that is similar to that seen with $(GAGAU)_{10}GAG$ (A. Antson and P. Gollnick, unpubl.). If $taGcc(tagcc)_{10}$ also forms a similar complex, then the chimera with a single riboguanosine would be capable of forming only one more hydrogen bond with TRAP than the all-deoxynucleotide $(tagcc)_{11}$. However, the energetics due to the difference of a single hydrogen bond cannot account for the large change in affinity for TRAP between $(tagcc)_{11}$ and $taGcc(tagcc)_{10}$ (Fersht, 1988; Lesser et al., 1990; Mazzarelli et al., 1992; Zhang & Gottlieb, 1993). Therefore we concluded that the presence of the single riboguanosine facilitates additional interactions between $taGcc(tagcc)_{10}$ and TRAP that $(tagcc)_{11}$ cannot form.

We hypothesized that the single riboG-containing taGcc repeat could allow the bases in the deoxyG-containing tagcc repeats to interact with TRAP, likely via similar contacts as seen for RNA repeats (Antson et al., 1999; Elliott et al., 1999). To test this hypothesis, we asked whether the affinity of $taGcc(tagcc)_{10}$ for TRAP is affected when modifications were made in the tagcc repeats while maintaining the single riboG-containing taGcc repeat. To do so, we substituted deoxyG residues in the tagcc repeats with deoxyinosine, which lacks the 2-amino group of guanosine (Fig. 1A). This functional group hydrogen bonds to Glu36 in the TRAP–RNA complex (Antson et al., 1999), and our previous studies showed that substituting inosine in all 11 repeats prevents complex formation (Elliott et al., 1999). Substituting deoxyinosine into various numbers of the tagcc repeats in $taGcc(tagcc)_{10}$ reduced the affinity for TRAP (Fig. 1B). The effects were proportional to the number of substitutions in the chimera, ranging from 5-fold for a single substitution to 34-fold when five deoxyinosines were present. A similar effect was also observed when deoxyadenosine in the second residue of the tagcc repeats was replaced with deoxycytidine (data not shown). Together these data demonstrate that the tagcc repeats contribute to the affinity of $taGcc(tagcc)_{10}$ for TRAP. Hence the presence of a single riboG in this D/RNA chimera facilitates the interaction of the remaining all-DNA portion [$(tagcc)_{10}$] with the protein. Consistent with this proposal, DNase I protection studies have shown that the tagcc repeats of such a chimera are protected by bound TRAP (P. Li and P. Gollnick, unpubl.).

Effects of the first residue in the triplet repeats

In the naturally occurring TRAP binding sites characterized to date, over 90% of the triplets repeats contain G or U in the first residue (Babitzke et al., 1994; Antson et al., 1995; Yang et al., 1995; Chen et al., 1999; Sarsero et al., 2000a, 2000b). Several studies have shown that for RNAs containing six or fewer repeats, those with GAGs or UAGs bind TRAP with higher affinity than do those containing AAGs or CAGs (Babitzke et al., 1995, 1996; Baumann et al., 1997). However, this difference is not seen for RNAs with 11 identical repeats, in which case the identity of the first residue of the triplets had little effect (Elliott et al., 1999). The structure of TRAP bound to $(GAGAU)_{10}GAG$ does not explain why G and U would be preferred over A and C (Antson et al., 1999). The structure suggests a distorted hydrogen bond between the NH_2 of the first G of each repeat and Asp39. No similar interaction is possible with U, and, moreover, mutagenesis (Yang et al., 1997) and nucleoside analog studies (Elliott et al., 1999) indicate this apparent hydrogen bond does not contribute to the stability of the complex. Hence our understanding of the G/U bias in the first residue of the repeats remains unclear.

To further investigate the role of the first nucleoside of the triplets, we compared the affinity of TRAP for RNAs containing various numbers of NAG repeats (N = G, A, U, or C). The triplets were separated by either AU or UU spacers, chosen to minimize secondary structure in the RNAs (Mathews et al., 1999; Zuker et al., 1999), which has been shown to interfere with binding to TRAP (Babitzke et al., 1996; Xirasagar et al., 1998). Furthermore, these binding studies were conducted at 55 °C in the absence of Mg^{2+} because TRAP is fully functional under these conditions, which minimize the effects of RNA structure (Xirasagar et al., 1998). As we reported previously (Elliott et al., 1999), for RNAs that contain 11 identical repeats, the identity of the first nucleoside of the triplets had little influence on the affinity for TRAP (Table 2). In contrast, when there were nine or fewer triplets per RNA, the effect of the first residue was more significant. For RNAs containing intermediate numbers of repeats (seven to nine) the base pref-

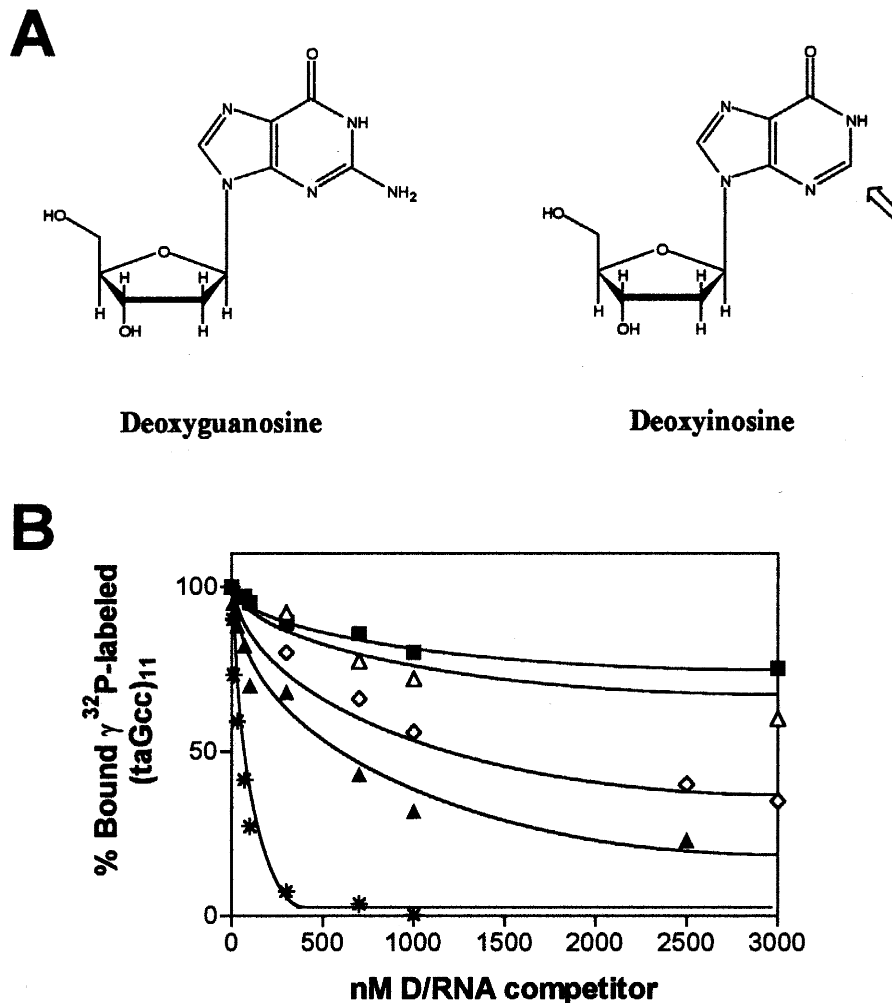

FIGURE 1. Competition assays comparing analog substituted and unsubstituted D/RNAs. **A**: Chemical structures of deoxyguanosine and deoxyinosine. The arrow indicates that deoxyinosine lacks the 2-amino group found in deoxyguanosine. **B**: Effects of deoxyinosine (i) substitutions on TRAP binding. Symbols for each RNA or D/RNA along with their apparent dissociation constant (K_d) for binding TRAP are as follows: taGcc(tagcc)$_{10}$ (*), 90 ± 8.0; taGcctaicc(tagcc)$_9$ (▲), 450 ± 9.0 nM; taGcc(taicc)$_2$(tagcc)$_8$ (◇), 1,050 ± 14 nM; taGcc(taicc)$_3$(tagcc)$_7$ (△), 2,600 ± 32 nM; taGcc(taicc)$_5$(tagcc)$_5$ (■), 3,020 ± 25 nM. Upper case letters represent ribonucleosides and lower case letters designate deoxynucleosides. Studies were performed at 55 °C in 250 mM K-glutamate, 16 mM HEPES, pH 8.0. Curves are the best fit of the competition 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 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 each D/RNA was carried out to 10 μM competitor; however, all curves are presented to 3 μM to show the detail of the early portion of the curves.

erence was G > U > C > A in the first residue of the triplets. With six or fewer repeats, AAGs had higher affinity than CAGs. As the number of triplets in each RNA decreased, the magnitude of the effects of the identity of the first residue on TRAP binding affinity increased. For example, the highest affinity eight-repeat RNA (GAG-containing) bound to TRAP with 16-fold greater affinity than that with the lowest affinity (AAGs), whereas for five repeats per RNA, there was over a 200-fold difference between GAGs (K_d = 14 nM) and CAGs (K_d = 3,000 nM).

The effect of the first residue of the triplets is particularly evident with regard to the minimum number of each type of NAG repeat that must be present in an RNA to be able to measure a stable complex with TRAP. We could measure a K_d (800 nM) with an RNA containing as few as three GAGs; however we observed virtually no binding to fewer than four UAGs, five AAGs or five CAGs (Table 2). There were also several cases where very large changes in the stability of the complex were observed when the number of repeats was changed by only one. For example, an RNA with four UAGs bound TRAP with a K_d of 80 nM, but there was virtually no detectable binding to an RNA with three UAGs. Likewise an RNA with seven CAGs bound with a K_d of 40 nM, whereas six CAGs showed almost no

TABLE 2. Equilibrium dissociation constants of triplet repeat containing RNAs and TRAP

	K_d (nM)			
No. of repeats	GAG	UAG	AAG	CAG
11	1.1 ± 0.4	0.5 ± 0.1	4.0 ± 2.0	3.5 ± 1.5
9	1.4 ± 0.3	2.0 ± 0.3	35 ± 3.1	15 ± 2.0
8	3.0 ± 0.5	6.5 ± 1	50 ± 7.0	18 ± 0.7
7	5.2 ± 0.4	20 ± 0.8	250 ± 18	40 ± 1.0
6	5.5 ± 0.1	25 ± 2.0	375 ± 24	IND[a]
5	14 ± 1.0	50 ± 5.5	550 ± 20	3,000 ± 45.5
4	92 ± 5.0	80 ± 3.0	IND	NB[b]
3	800 ± 25	IND		
2	IND			

[a]IND (indeterminate): significant binding was detected but a dissociation constant was not determined due to the absence of binding saturation with up to 30 μM TRAP.
[b]NB: no binding observed with up to 30 μM TRAP.

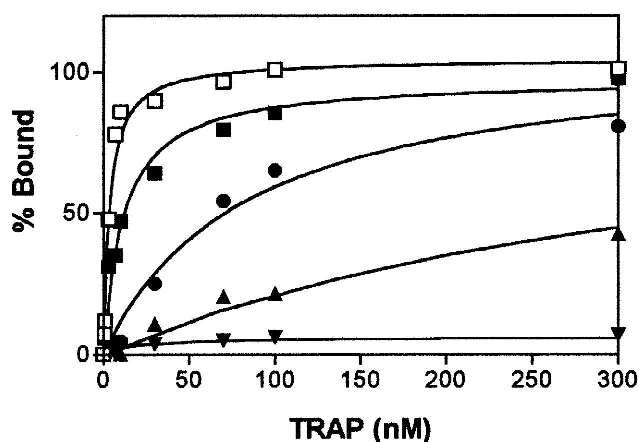

FIGURE 2. Effects of varying the first residue of each NAG repeat in RNAs or D/RNAs containing six repeats. Symbols for each RNA or D/RNA along with their apparent dissociation constant (K_d) for binding TRAP are as follows: $(GAGAU)_6$ (□), 5.6 ± 0.1 nM; $(UAGUU)_6$ (■), 25 ± 2.0 nM; $(-aGat)_6$ (●), 60 ± 4.0 nM; $(AAGAU)_6$ (▲), 375 ± 24; $(CAGAU)_6$ (▼), unable to determine K_d. Upper case letters represent ribonucleosides and lower case letters designate deoxynucleosides. Studies were performed at 55 °C in 250 mM K-glutamate, 16 mM HEPES. The curves shown are the best fit of the direct 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.

binding to TRAP. There is only one case where an RNA with fewer repeats (five CAGs) bound with higher affinity than an RNA with more of the same type of repeat (six CAGs) (Table 2). The explanation for this observation is not clear.

Guanosine in the first residue enhances binding whereas cytidine destabilizes the TRAP–RNA interaction

The higher affinity of TRAP for short RNAs (less than nine repeats) containing G or U in the first residue of the triplets may derive from favorable interactions between these nucleosides and the protein that A and C are not able to forge. Alternatively, A and C may act negatively to inhibit complex formation. To attempt to distinguish between these two possibilities, we created a six-repeat DNA/RNA chimera containing an abasic 2′-deoxyribose residue lacking a base (dSpacer, Glen Research; designated as “−”) in the first position of each repeat. We then compared the affinity of $(-aGat)_6$ to RNAs with six NAG repeats (N = G, U, A, or C) for TRAP. As shown in Figure 2, $(-aGat)_6$ binds to TRAP with lower affinity than $(GAGAU)_6$ or $(UAGUU)_6$, but with higher affinity than $(AAGAU)_6$ or $(CAGAU)_6$. These data suggest that elements of both models described above contribute to the higher affinity of G/U in the first position compared to A/C.

DISCUSSION

The TRAP–RNA interaction has several unusual features. First, the complex involves an entirely single-stranded RNA that is wrapped around the protein to form a circle. Second, the complex is composed of multiple small repeated recognition sites in the RNA interacting with 11 identical binding sites on the symmetric protein (Antson et al., 1999). In this work, we used two lines of experimentation to investigate the mechanism by which RNA binds to TRAP. Our studies of the effects of deoxyG substitutions into the third residue of various numbers of repeats indicate that TRAP forms an initial complex with a small subset of the RNA target. Secondly, our studies of TRAP binding to RNAs with different numbers of NAG repeats suggest that a form of cooperativity is involved in RNA binding to TRAP. Together these results suggest a model for RNA binding to TRAP in which an initial “nucleation” complex is formed followed by cooperative interaction with the remaining repeats in the RNA. Below we will discuss the evidence for each feature of this model and its potential implications regarding TRAP-mediated gene regulation.

The structure of the TRAP–RNA complex shows that the ribose sugar of the guanosine in the third residue of each triplet repeat makes a single hydrogen bond to the protein (Antson et al., 1999). Hence each replacement of a riboG in $(taGcc)_{11}$ with deoxyG is predicted to disrupt one hydrogen bond. However, the changes in the stability of the resulting complexes between TRAP and these chimeras were not directly proportional to the number of deoxyG substitutions (Table 1). Most significantly, we found that the presence of just one riboG in chimeras such as $taGcc(tagcc)_{10}$ dramatically stabilizes the complex with TRAP as compared to $(tagcc)_{11}$ with no riboGs. Further analog substitution studies demonstrated that the all-DNA portion of $taGcc(tagcc)_{10}$ interacts with the protein (Table 1). Hence

we propose that the single riboG in chimeras such as $taGcc(tagcc)_{10}$, $(tagcc)_5taGcc(tagcc)_5$, or $(tagcc)_{10}taGcc$ allows formation of an initial complex with TRAP. We envision that the initial complex tethers the nucleic acid to the protein, thereby reducing its degrees of freedom, as well as placing the trinucleotide repeats in proper register such that the remaining tagcc repeats can interact with their binding sites on TRAP even though they lack one of the functional groups (the 2′OH) that hydrogen bonds to the protein. Apparently the initial complex cannot form with the all-DNA oligonucleotide entirely lacking riboG and hence we are unable to detect any binding of $(tagcc)_{11}$ to TRAP.

One of the surprising findings from these studies is the very limited requirement for ribose residues in the TRAP/RNA interaction. The only residue that needs to contain a ribose is the third residue (G) of each pentanucleotide repeat. Moreover, only a small subset of the 11 repeats need contain a ribose to form a complex with the protein (Table 1). There are several other examples of RNA-binding proteins with relaxed 2′-OH requirements including BIV RNA-dependent RNA polymerase (Siegel et al., 1998), MS2 coat protein (Baidya & Uhlenbeck, 1995) and *Escherichia coli* alanine-tRNA synthetase (Musier-Forsyth & Schimmel, 1992). However none of these proteins bind to their respective RNA targets when more than 90% of the ribonucleotides have been substituted with deoxynucleotides such as we see for TRAP binding to $taGcc(tagcc)_{10}$.

Another feature of our model for the interaction of RNA with TRAP is cooperative binding of the remaining triplet repeats to the protein after the formation of the initial complex. The structure of TRAP complexed with RNA (Antson et al., 1999), together with site-directed mutagenesis (Yang et al., 1997) and nucleoside analog studies (Elliott et al., 1999), have shown that each triplet repeat interacts with TRAP via six to seven direct hydrogen bonds, several water-mediated H bonds, as well as hydrophobic and stacking interactions. Furthermore, the crystal structure shows that, for an RNA containing 11 GAG repeats, each triplet interacts with TRAP in nearly identical fashion (Antson et al., 1999). Based on these structural and biochemical data, we expected that the stability of a TRAP–RNA complex would be directly proportional to the number of repeats in the RNA. However, we found that this is not true (Table 2). Within a series of RNAs containing the same triplets (GAG, UAG, AAG, or CAG), those with fewer repeats do have lower affinity for TRAP; however, the stabilities of the complexes are not directly proportional to the number of repeats. For example the $\Delta\Delta G°$ for comparing complexes with three versus six GAG repeats is 3.25 kcal/mol (Table 2 and Fig. 3). In contrast, the difference in TRAP binding affinity between complexes involving RNAs with 6 or 11 GAGs is only 1.0 kcal/mol, even though the six-repeat RNA lacks nearly half of the binding elements present in 11 repeats. Babitzke and

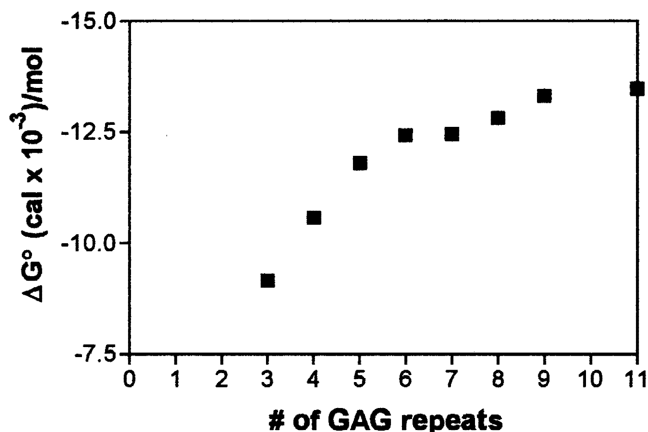

FIGURE 3. Stability of TRAP complexes with RNAs containing various numbers of GAG repeats. The $\Delta G°$ values in calories per mol for TRAP complexes with RNAs containing between 2 and 11 GAGAU repeats is plotted versus the number of repeat in the RNA. The data are from Table 2.

coworkers (1996) obtained similar results with RNAs containing various numbers of GAG repeats. We also found similar effects for RNAs with UAG or CAG repeats, although, for AAG-containing RNAs, the stabilities of the complexes with TRAP were more proportional to the number of repeats (Table 2).

A potential concern with regard to interpreting the data in Table 2 is that these RNAs vary not only in the number of repeats they contain but also in overall length, and this feature may influence their ability to interact with the protein. However, we found that increasing the length of RNAs containing five or six NAG repeats with 30 residues of nonspecific RNA had little or no effect on the stability of their complexes with TRAP (results not shown). Another question is whether more than one RNA may be binding to a single TRAP 11mer, particularly for RNAs with five or fewer repeats. Several lines of evidence indicate that only one RNA is binding to each protein. First, the binding studies were conducted under conditions of excess TRAP (20–10,000-fold) over RNA, which would reduce the likelihood of multiple RNAs binding to a single protein, assuming no cooperativity of binding between two RNAs. Second, the data from these studies fit one-site binding curves (rectangular hyperbolae) very well (root mean square (r.m.s.) $>$ 0.98) and binding saturates at approximately 1:1 stoichiometry of TRAP to RNA. Finally, previous studies (Baumann et al., 1997) as well as unpublished mobility shift gel experiments from our lab (M.B. Elliott and P. Gollnick) have provided no evidence for multiple RNAs binding to TRAP even in the presence of excess RNA. The 1:1 stoichiometry observed, even for small RNAs, could be a consequence of the thermodynamics of binding. One interpretation of the data in Figure 3 is that binding of the first six trinucleotide repeats contributes -12.5 kcal/mol of free energy, and that the sub-

sequent five triplets contribute only 0.16 kcal/mol each (for a total of −13.5 kcal/mol). Hence, in the case of two RNAs, the majority of the binding energy would be contributed by the first bound and binding of a second RNA to TRAP might be weak and undetected.

In many characterized cooperative interactions between protein and ligands, cooperativity is generated by an allosteric conformational change in the protein induced by ligand binding. However, the structure of TRAP, particularly the backbone, changes very little (r.m.s. difference of 0.39 Å) upon RNA binding (Antson et al., 1999). Hence we propose that tethering the RNA to the protein through the initial complex generates cooperativity in this system by reducing the degrees of freedom of the remaining repeats in the RNA. In some ways this interaction is reminiscent of that which occurs between two complementary strands of DNA (or RNA). In either case, the limiting step is formation of an initial nucleation complex between the two molecules, a second-order process, followed by a first-order annealing of the two molecules. Our results in Table 1 indicate that nucleation can begin equally well from any of the triplet repeats. An interesting consequence of the circular symmetry of the TRAP protein is that a nucleation complex forming between any triplet on the RNA and any binding site on the protein can equally well initiate complex formation between the protein and an RNA with a full complement of 11 repeats.

As mentioned previously, the naturally occurring TRAP binding sites contain over 90% GAG and UAG repeats with AAGs and CAGs rarely present (Babitzke et al., 1994; Antson et al., 1995; Yang et al., 1995; Chen et al., 1999; Sarsero et al., 2000a, 2000b). From the results presented here as well as prior studies by Babitzke et al. (1995), it is clear that for RNAs with fewer than 11 NAG repeats, those with GAGs and UAGs have higher affinity for TRAP (Table 2). The effects of the identity of the first residue in the NAG repeats become more pronounced as the RNAs have fewer repeats. We also found that for RNAs with six repeats, the presence of G or U in the first residue of the repeats stabilizes the interaction with TRAP as compared to having no base ($(-\text{aGat})_6$; Fig. 2). In contrast, the presence of A or C in the first position of the repeats is detrimental to TRAP binding. In the crystal structure of TRAP complexed with an RNA containing 11 GAGs, the first guanosine base in each repeat packs against the aliphatic side chain of Lys37, and the NH_2 of the base appears to H bond to Asp39 (Antson et al., 1999). Mutagenesis (Yang et al., 1997) and analog studies (Elliott et al., 1999) do not support any role for the proposed H bond. In contrast, data from studies in which we substituted Lys37 with other nonpolar amino acids containing side chains of varying length indicate that hydrophobic interactions between the Lys37 side chain and the RNA contribute to the stability of the complex (A. Wendt and P. Gollnick, unpubl. observations). It is not clear that G and U would be better suited than A or C for making hydrophobic interaction with the lysine side chain. Clearly the abasic sugar–phosphate in $(-\text{aGat})_6$ is unable to make a similar hydrophobic interaction with Lys37; yet this chimera has higher affinity for TRAP than RNAs with A or C in the first position of the repeats (Fig. 2). It may be that for $(-\text{aGat})_6$, the sugar of the abasic residue forges hydrophobic interactions with the Lys37 side chain. It is also interesting to note that in the structure of TRAP complexed with $(\text{GAGAU})_{10}\text{GAG}$, The first G of each repeat has an unusual conformation including a C2′-*endo* sugar pucker and the nucleoside is twisted with respect to A_2. Together with the U of the preceding repeat, this G forms a dinucleotide with a left-handed twist (Antson et al., 1999). It is possible that increased flexibility in the backbone when this residue lacks a base makes up for the loss of hydrophobic interactions with Lys37. Perhaps the presence of A or C in this position reduces the ability of the RNA to adapt this conformation.

The large bias for G or U in the first residue of the NAG repeats in naturally occurring TRAP binding sites with 9–11 repeats does not strongly reflect the differences in equilibrium binding affinity of RNAs with 9–11 identical NAG repeats (Table 2). Rather the bias more closely resembles the differences in affinity seen for RNAs with six or fewer repeats. This effect, coupled with our evidence for a mechanism for RNA binding to TRAP that involves an initial complex with a subset of the repeats, suggests that evolutionary pressure may have been based more on formation of the initial complex. The formation of this complex is likely rate limiting with regard to the kinetics of RNA binding, which may be particularly relevant to the mechanism of TRAP-mediated transcription attenuation (Gollnick, 1994; Babitzke, 1997). The timing of TRAP binding to *trp* leader RNA is very important for this mechanism to function properly in vivo. To cause attenuation, TRAP must bind and prevent formation of the antiterminator before RNA polymerase has advanced beyond the transcription terminator, which is only 42 nt beyond the 3′ end of the 11-repeat TRAP binding site. Hence the formation of the initial complex between TRAP and a small subset of its RNA target would be particularly crucial for this mechanism, and triplet repeats of GAG and UAG appear optimal for this role.

MATERIALS AND METHODS

Plasmids and strains

All plasmids were propagated in *E. coli* JM107 and all were stably maintained. The plasmids pTZ18UAGUU, pTZAAGAU, and pTZCAGUU have been described previously (Xirasagar et al., 1998; Elliott et al., 1999). Plasmids pPB96, pPB94, pPB220, pPB90, pPB110, pPB120, pPB221, pPB222, pPB223, and pPB224, containing various numbers of GAGAT

repeats following the T7 promoter, were graciously provided by Paul Babitzke and have been described previously (Babitzke et al., 1995, 1996). The plasmid pTZ18UAGUU9 has nine repeats of the sequence 5′-TAGTT-3′ cloned downstream of the T7 promoter in pTZ18U (US Biochemical). The insert was created by annealing oligonucleotides UAGUU-9 (5′-AGGGAATTC(TAGTT)$_8$TAGAAGCTTGGCACTGGCCG TCG-3′) and H3UNIV20MER (5′-CGACGGCCAGTGCCA AGCTT-3′). The 3′ ends of the resulting DNA fragments were extended using the Klenow fragment of DNA polymerase I and the resulting duplex DNA was digested with *Eco*RI and *Hin*dIII and ligated into similarly digested pTZ18U. The plasmids pTZUAGUU3, pTZAAGAU9, pTZAAGAU7, and pTZCAGUU6, containing the sequence 5′-AAGAT-3′ repeated nine times, 5′-AAGAT-3′ repeated seven times, and 5′-CAGTT-3′ repeated six times, respectively, were made by a similar approach, with the exception of the sequence of the oligonucleotides that were used to make the duplex DNA. The remaining DNA templates used for in vitro transcription in this study were made using the following procedure. For RNAs containing different numbers of UAG repeats, we used the oligo pTZT7PRIME with the sequence 5′-TACGACTCA CTATAGGGAATTC-3′ and the oligo UAGSLIDE, 5′-AGGG GATCCAACTAAACTAAACTAAACTA-3′, containing a *Bam*HI site and a stretch of 20 nt containing 4 ATCAA pentamers that anneal randomly to any contiguous group of 4 of the 11 TAGTT pentamers in pTZ18UAGUU (Xirasagar et al., 1998). Using PCR we generated various products containing between 4 and 11 TAGTT repeats flanked by *Eco*RI and *Bam*HI restriction sites. The PCR products were then digested with *Eco*RI and *Bam*HI and cloned downstream of the T7 promoter in similarly digested pTZ18U vector. Oligonucleotides used as templates for in vitro transcription of RNAs containing variable numbers of AAG and CAG were made by a similar approach, except for the sequences of the randomly annealing oligonucleotides and either pTZ18AAGAU or pTZ18CAGUU were used as template.

RNA and D/RNA synthesis

In vitro transcription reactions to prepare ^{32}P-labeled samples were performed with T7 RNA polymerase and [α-^{32}P]-ATP, or [α-^{32}P]-UTP (NEN) as described previously (Baumann et al., 1996; Xirasagar et al., 1998; Elliott et al., 1999) using plasmids linearized with *Bam*HI or *Hin*dIII as templates. All D/RNA chimeras were chemically synthesized as described previously (Elliott et al., 1999) by the CAMBI Nucleic Acid Facility, State University of New York, Buffalo, New York, and 5′-end labeled using [γ-^{32}P]-ATP and polynucleotide kinase (Sambrook et al., 1989).

TRAP purification

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

Filter binding assays

Quantitative direct and competition filter binding assays were performed as described previously (Elliott et al., 1999) 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. For direct filter binding assays, the concentration of TRAP was varied from 0.07 nM–30 μM, and the RNA concentration was 3 pM. The data were analyzed using nonlinear regression algorithms (Prism 3.0, GraphPad Software Incorporated, San Diego, California). Data are the average of at least four individual experiments.

Competition assays were performed in filter binding buffer with 2.3 fmol of γ-^{32}P-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 K_d 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 the equation below (Duggan et al., 1995):

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

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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