RNA Structure Inhibits the TRAP (trp RNA-binding Attenuation Protein)-RNA Interaction*

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TRAP (trp RNA-binding attenuation protein) regulates expression of the tryptophan biosynthetic genes in response to tryptophan in Bacillus subtilis by binding to two sites containing a series of 9 or 11 (G/U)AG triplet repeats that are generally separated by two or three spacer nucleotides. Previous mutagenesis experiments have identified three TRAP residues, Lys-37, Lys-56, and Arg-58 that are essential for RNA binding. The precise nature of the structure of the residues on the TRAP oligomer supports the proposal that RNA binds TRAP by encircling the TRAP oligomer. In this work, we show that RNAs containing 11 GAG or UAG repeats separated by CC dinucleotide spacers (((G/U)AGCC)11) form stable structures that inhibit binding to TRAP. This conclusion is based on the effects of temperature and Mg2+ on the affinity of TRAP for RNAs with CC spacers combined with UV hyperchromicity and circular dichroism. Furthermore, introducing the base analogue 7-deazaguanosine in the (((G/U)AGCC)11) RNAs stabilized the TRAP-RNA interaction. This effect was associated with decreased stability of the RNA structure as measured by circular dichroism spectroscopy. The precise nature of the structure of the (((G/U)AGCC)11) RNAs is not known but evidence is presented that it involves noncanonical interactions. We also observed that substitution of Arg-58 with Lys further reduced the ability of TRAP to interact with structured RNAs. Since in vivo function of TRAP may involve binding to structured RNAs, we suggest a potential function for this residue, which is conserved in TRAP from three different bacilli.

The Bacillus subtilis TRAP protein (trp RNA-binding attenuation protein) negatively regulates expression of the tryptophan biosynthetic (trp) genes in response to tryptophan (1, 2). Upon binding tryptophan, TRAP associates with two specific RNA targets and regulates transcription of the trpEDCFBA operon (1–4) as well as translation of trpE (5) and trpG (6, 7). The RNA-binding sites for TRAP contain multiple GAG or UAG (rarely AAG) triplet repeats, generally separated by two or three spacer nucleotides (8, 9). The trp leader RNA contains 11 triplet repeats and the binding site in trpG contains 9 repeats.

Results from experiments using artificial RNA-binding sites have demonstrated that these trinucleotide repeats are crucial for TRAP binding (10–12). These studies also indicate that two nucleotide spacer regions are optimal. Lack of sequence conservation, as well as mutagenesis and footprinting experiments have suggested that the spacer nucleotides do not directly interact with the protein (8, 9).

The crystal structure of TRAP complexed with l-tryptophan reveals that TRAP is an oligomeric protein with 11 identical subunits arranged in a symmetrical ring (9, 13). The secondary structure of TRAP is entirely comprised of β-strands, β-turns, and random coils. Four β-strands from one subunit combine with 3 β-strands from the adjacent subunit, resulting in a novel quaternary structure consisting of 11 7-stranded antiparallel β-sheets.

Mutagenesis studies have identified three residues in TRAP, Lys-37, Lys-56, and Arg-58 that when substituted with alanine decrease the affinity of TRAP for trp leader RNA 600–800-fold without altering its ability to bind tryptophan (14). Structural analysis revealed that 11 clusters of this KKR motif, comprised of Lys-37 from one subunit and Lys-56 and Arg-58 from the adjacent subunit, are directly aligned and encircle the TRAP oligomer. This spatial arrangement suggests a model in which the trp leader RNA wraps around TRAP with the (G/U)AG repeats interacting with these 3 amino acid residues (14). This model implies that the RNA would have to be flexible in order to wrap around the protein. Consistent with this hypothesis, we found that the spacer nucleotides in RNA sequences selected for TRAP binding in vitro were predominantly pyrimidines, which stack less well than purines and allow greater flexibility in the RNA (12).

Arginine residues have been shown to be important in several other protein-RNA interactions (15, 16). Mutagenic analysis of Arg-58 in TRAP revealed that proteins with substitutions of alanine (R58A), glutamine (R58Q), and lysine (R58K) at this position display a broad range of activity (14). Whereas R58A bound trp leader RNA with greater than 600-fold lower affinity than wild-type TRAP, glutamine substitution was less deleterious and replacement with lysine had no effect on RNA binding. These studies suggested a role for hydrogen bonding interactions between this residue and the RNA in the TRAP-RNA complex. Furthermore, although lysine at this position allows TRAP to function similar to wild-type TRAP with regard to RNA binding and gene regulation, residue 58 is an arginine in TRAP proteins from three different bacilli, suggesting an important role for arginine at this position (17, 18).2

These studies led us to further examine the role of Arg-58 in the TRAP-RNA interaction. To test the role of this residue in specificity determination, we compared the sequences of RNAs that bind to wild-type TRAP to those that bind R58K TRAP.

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The abbreviations used are: TRAP, trp RNA-binding attenuation protein; C7G, 7-deazaguanosine.

2 X. Chen, C. Baumann, and P. Gollnick, unpublished results.
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In vitro. The 25 nucleotides in the random region are capitalized. The trinucleotide ((G/U)AG) repeats involved in TRAP binding are underlined and bold, and the spacer nucleotides are italicized.

In Vitro Selection of RNAs That Bind to R58K TRAP—SELEX experiments with R58K TRAP were performed as described previously for wild-type TRAP (12). An oligonucleotide pool with 25 random positions, which can accommodate five trinucleotide repeats each separated by two nucleotide spacers was used. This pool size (425) was well represented by 2 nmol of synthetic oligonucleotide. RNAs that bound to TRAP were selected by filter binding and were eluted from the nitrocellulose filters as described previously (12). Eight rounds of SELEX were performed and selected RNAs were reverse transcribed, the cDNAs cloned in to pUC118 and individual clones sequenced using Sequenase (Amersham).

Thermal Denaturation of RNAs Measured by UV Hyperchromicity—Temperature-dependent UV absorption data of RNAs (5 μm in 250 mM KCl and 16 mM HEPES pH 8.0) were collected with a Beckman DU-640 spectrophotometer using a circulating water bath with a heating rates of either 1 °C or 0.25 °C per min. Both heating rates gave identical results. Data were normalized to the absorbance at 16 °C.

Circular Dichroism (CD)—CD spectra were obtained with a JASCO model J-500C spectropolarimeter using 1-mm path length cuvettes. CD spectra of RNAs (2.5 μm) were recorded between 25 and 80 °C in 50 mM potassium phosphate, pH 7.4. For studies of TRAP binding, 2.5 μm RNA was incubated with 5 μM wild-type TRAP and 50 μM l-tryptophan at 55 °C for 1 h. Neither TRAP nor tryptophan show significant dichroism in the range in which the data were recorded (230–300 nm) and therefore make no contribution to the spectra.

RESULTS

In Vitro Selection (SELEX) Experiments to Examine the Role of Arg-58 in RNA Binding—Previous studies identified Arg-58 as one of three residues important for RNA binding to TRAP (14). To further investigate the role of Arg-58 in the specificity of this interaction, we used SELEX to select from a pool of 1.2 × 1015 RNAs containing 25 random positions within the RNA that bound R58K TRAP with highest affinity (12). We then compared the sequence requirements for RNA binding to R58K TRAP to those for binding to wild-type TRAP (12), in that they all contained multiple GAG and UAG repeats (Fig. 1 and Table I).

The spacers separating the (G/U)AG repeats were also similar.
ilar to those obtained previously with wild-type TRAP (12), being predominately 2 nucleotides (90%), with 1 (8%) and 3 nucleotides (2%) spacers rarely observed (Table I). The composition of the spacers was also similar to that observed previously with wild-type TRAP, being predominately (>90%) pyrimidines with greater than 75% uridines. We suggested previously that this bias for pyrimidines reflects their lower stacking ability (as compared with purines) allowing greater flexibility for the RNA to wrap around the protein. However, this hypothesis does not explain the preponderance of U’s in the spacers since both C’s and U’s are predicted to stack similarly (25) and thus would be expected to be present with comparable frequencies if flexibility is the only consideration. Furthermore, few if any C’s occurred as CC dinucleotide spacers. In RNAs obtained with wild-type TRAP, 75% of C’s in the spacers were observed as CU, 7% as UC, and 6% each as AC, CA, or CC dinucleotides. With R58K TRAP, 88% of the C’s occurred as CU spacers, and 4% each as UC, CA, or single nucleotide C spacers. These results suggest that C’s in spacers are inhibitory to TRAP binding, particularly as CC spacers. These findings are consistent with previous studies of CU spacers in RNAs with 6 GAG repeats (11).

The only significant difference we observed between RNAs that bound R58K TRAP as compared with wild-type TRAP was the ratio of GAG to UAG repeats in the RNAs. While RNAs that bound wild-type TRAP contained more GAGs, RNAs obtained with R58K TRAP contained predominantly UAG repeats (Table I). These results indicate that amino acid substitution at position 58 in TRAP can alter the preference for the base in the first position of the trinucleotide repeat, suggesting that Arg-58 may interact directly with the first G or U in the triplet.

Affinities of Wild-type and R58K TRAP for RNAs with GAG or UAG Repeats—One mechanism by which Arg-58 could interact with the first guanosine of a GAG repeat would be by forming two hydrogen bonds with the O-6 and N-7 positions of the base. Such bidentate interactions between arginines and guanosines have been demonstrated to be important in other protein-nucleic acid complexes (15, 16). This model makes several predictions about the interaction of TRAP with RNA. First, wild-type TRAP should have a much higher affinity for GAG- than for UAG-containing RNAs because arginine can form two hydrogen bonds with guanosine but only one with uracil. This would result in a total of 11 additional hydrogen bonds in a complex between wild-type TRAP and an RNA with 11 GAG repeats as compared with one with 11 UAG repeats ($\Delta G^a = 11$–22 kcal/mol) (26–28). Conversely, R58K TRAP should have similar affinities for both GAG and UAG repeat-containing RNAs since lysine can only form one hydrogen bond with either guanosine or uridine.

We therefore examined the affinities of wild-type and R58K TRAP for RNAs with 11 GAG repeats ((GAGUU)$_{11}$) or 11 UAG repeats ((UAGUU)$_{11}$) (Table II). In contrast to our predictions, at 37 °C in the presence of Mg$^{2+}$, wild-type TRAP bound (GAGUU)$_{11}$ and (UAGUU)$_{11}$ with equal affinities ($K_d = 0.3$ nM). In addition, both wild-type and R58K TRAP bound similarly to both RNAs, although R58K displayed a slightly lower affinity for (GAGUU)$_{11}$ ($K_d = 0.8$ nm) than for (UAGUU)$_{11}$ ($K_d = 0.3$ nm). Moreover, at higher temperature (55 °C), both R58K and wild-type TRAP behaved identically when binding to these RNAs (Table II). The results of these binding studies are consistent with our SELEX data, however, they do not support the model of bidentate hydrogen bonding between Arg-58 and guanosine in the first position of the triplet repeats.

Effect of 7-Deazaguanosine (C7G) Substitution on the TRAP-RNA Interaction—Although the binding data did not support the model for two hydrogen bonds between the first guanosine of a GAG repeat and Arg-58, they did not rule out a role for N-7 of guanosines in interacting with TRAP. We tested whether the N-7 position of guanosines are involved in binding to TRAP using RNAs substituted with 7-deazaguanosine (C7G), an analogue of guanosine, in which the nitrogen at position 7 is replaced by carbon (Fig. 2). If the N-7 of guanosine interacts with TRAP via hydrogen bonding, substitution with C7G will eliminate this interaction and therefore lower the affinity of the substituted RNA for TRAP. We were particularly interested in the first position of the triplet repeat, since our studies suggested a possible interaction between Arg-58 and guanosine in this position. However, since it is not possible to specifically replace only the first guanosine of each GAG repeat by in vitro transcription, we first tested the effect of C7G in the third position of each UAG repeat in the RNA (UAGCC)$_{11}$. We then compared this effect with that of substituting the analogue at both the first and the third positions in each GAG repeat, using RNAs (GAGCC)$_{11}$ and (GAGUU)$_{11}$.

Surprisingly, we found that introduction of C7G in (UA$^{C7G}$GCC)$_{11}$ did not reduce the affinity of this RNA for either wild-type or R58K TRAP but instead resulted in increased affinity for both proteins as compared with the unsubstituted RNA (Table III). With wild-type TRAP we observed a 30-fold effect, and R58K TRAP bound (UA$^{C7G}$GCC)$_{11}$ with an apparent $K_d$ of 7 nM but showed no detectable binding to the unsubstituted (UAGCC)$_{11}$ (Table III). Introduction of C7G in both positions of each triplet repeat in (C7GA$^{C7G}$GUU)$_{11}$ had little effect on the affinity of this RNA for wild-type or R58K TRAP (Table III). The observation that incorporation of C7G into either GAG- or UAG-containing RNAs never reduced the affinity of these RNAs for TRAP, and in several cases significantly increased their affinities, suggests that the N-7 of guanosine at neither the first nor the third position of the triplet repeats interacts directly with the protein.

The C7G-induced enhancement in affinity for TRAP is inde-
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### Table II

| RNA                  | TRAP WT | R58K |
|----------------------|---------|------|
| (UAGCC)$_{11}$      | 1       | 1    |
| (GAGUU)$_{11}$      | 1       | 1    |

* Effect of Mg$^{2+}$ and temperature on RNA binding to wild-type and mutant TRAP

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**FIG. 2.** Structure of the nucleotide analogue 7-deazaguanosine. The arrow indicates the site of modification where nitrogen is replaced by a carbon atom.

.dependent of the position of substitution in the (G/U)AG repeats, since it occurred with (UAGCC)$_{11}$ and (GAGCC)$_{11}$ but not with (GAGUU)$_{11}$, again suggesting that this effect was not due to changing direct interactions with the protein. These observations suggested that C$^7$G may enhance the affinity of (GAGCC)$_{11}$ and (UAGCC)$_{11}$ for TRAP indirectly by affecting RNA structure. Previous work has suggested a role for RNA structure in binding to TRAP since an RNA in which the GAG repeats are predicted to be present in a stable duplex did not bind to TRAP (11). We therefore further investigated the role of RNA structure in (UAGCC)$_{11}$ and (GAGCC)$_{11}$ binding to TRAP.

**Effects of Temperature on the TRAP-RNA Interaction**—One prediction of the hypothesis that RNAs (UAGCC)$_{11}$ and (GAGCC)$_{11}$ are structured in a way that inhibits binding to TRAP is that increasing the temperature would enhance the affinity of these RNAs for TRAP by destabilizing their structure. Since TRAP is stable up to 70 °C (12), we were able to directly test this prediction. Consistent with this hypothesis, we observed increased affinity of (UAGCC)$_{11}$ for both wild-type (40-fold) and R58K TRAP upon raising the temperature from 37 to 55 °C (Table II). For (GAGCC)$_{11}$, increasing the temperature to 55 °C resulted in a slightly smaller (7–9-fold) increase in affinity for TRAP proteins (Table II). In contrast to our observations with RNAs containing CC spacers, increasing the temperature to 55 °C had little or no effect on the affinity of either (UAGUU)$_{11}$ or (GAGUU)$_{11}$ for either of these TRAP proteins (Table II). Rather, at 55 °C (UAGUU)$_{11}$ binds with slightly lower affinity to both proteins. These results further support the hypothesis that RNA structure has a negative influence on the binding of RNAs with CC spacers to TRAP.

**Effect of Mg$^{2+}$ on the TRAP-RNA Interaction**—Mg$^{2+}$ has been shown to stabilize secondary and tertiary structure in RNAs (29). Since the above results suggested an inhibitory role for RNA structure in the binding of RNAs with CC spacers to TRAP, we further investigated this possibility by testing whether Mg$^{2+}$ stabilized this RNA structure. If this were the case, we would predict that removing Mg$^{2+}$ would destabilize the structure leading to increased affinity for TRAP. Consistent with this prediction, we observed that at 37 °C wild-type TRAP bound to (UAGCC)$_{11}$ with 10-fold higher affinity in the absence of Mg$^{2+}$ than in the presence of 4 mM Mg$^{2+}$ (Table II). Increased affinity of this RNA for R58K TRAP was also observed in the absence of Mg$^{2+}$. (GAGCC)$_{11}$ also showed increased affinity for wild-type and R58K in the absence of magnesium (Table II). In contrast, Mg$^{2+}$ had no effect on the binding of (UAGUU)$_{11}$ to either TRAP protein. Furthermore, at 55 °C Mg$^{2+}$ had almost no effect on the affinity of wild-type TRAP for (UAGCC)$_{11}$, which bound with a $K_d$ of 5 nM in the presence of Mg$^{2+}$ and 4 nM in its absence. Thus the effects of Mg$^{2+}$ and temperature on binding of the (G/U)AGCC-repeat RNAs to TRAP suggest that both factors influence the same element of the TRAP-RNA interaction, RNA structure, which inhibits binding to TRAP.

**UV Hyperchromicity and Circular Dichroism Studies of Thermal Denaturation of RNAs**—To test whether the RNAs with CC spacers possess more stable structure than those with CC spacers, we examined the melting properties of these RNAs using UV hyperchromicity and CD spectroscopy. In the previous section, we found that temperature and Mg$^{2+}$ had the greatest effect on TRAP binding to (UAGCC)$_{11}$, suggesting that this RNA contains the most stable structure. We therefore compared the melting of this RNA to (UAGUU)$_{11}$, (GAGUU)$_{11}$, and (GAGCC)$_{11}$, which all seem less structured based on our binding studies.

RNA (UAGCC)$_{11}$ displayed a highly cooperative UV hyperchromic transition with an approximate melting temperature ($T_m$) of 75 °C (Fig. 3). In contrast, (UAGUU)$_{11}$ and (GAGUU)$_{11}$ showed less cooperative transitions with lower $T_m$ values of approximately 40 and 45 °C, respectively. These results further indicate that (UAGCC)$_{11}$ has a more stable structure than either (UAGUU)$_{11}$ or (GAGUU)$_{11}$. Surprisingly, thermal dena-
turation of the C7G substituted (UAC7GCC)11 was similar to the unsubstituted RNA, being cooperative with only slightly lower Tm of approximately 70 °C. The C7G-substituted (UAC7GCC)11 underwent a larger hyperchromatic shift than (UAGCC)11, suggesting that the C7G-substituted RNA undergoes a different temperature-induced structural change than the unsubstituted RNA. However, these studies did not explain the increased affinity of TRAP for the C7G-substituted RNA. Therefore we also examined the structure of these RNAs by CD spectroscopy.

Base stacking interactions in RNA result in a strong maximum centered near 265 nm in the CD spectrum (30) with the intensity of the band proportional to the degree of stacking. The CD spectra of (UAGCC)11 as well as the C7G-substituted RNA showed strong intensities at 265 nm at 25 °C indicative of base stacking in both RNAs (Fig. 4, A and B). The intensity of this band in the spectra of the substituted (UA C7GCC)11 reduced as the temperature was increased from 25 to 55 °C, whereas negligible changes were observed in the spectra of (UAGCC)11 in the same temperature range (compare Fig. 4, A and B; see also Fig. 4C). This difference in temperature-induced spectral changes between these two RNAs suggests that the C7G containing RNA undergoes conformational changes unlike the unsubstituted (UAGCC)11 RNA in the temperature range where we observe differences in the binding of these RNAs to TRAP. This propensity for conformational change may better enable C7G-substituted RNA to bind to TRAP.

Previous studies with the trp leader RNA showed that the intensity of the band at 265 nm in the CD spectra of this RNA is greatly reduced upon binding to TRAP, indicating that the bases become unstacked (12). Similarly, the CD spectra of both the unsubstituted and C7G-substituted (UA C7GCC)11 RNAs showed a reduction in the intensity of this maximum in the presence of tryptophan-activated wild-type TRAP (Fig. 5). However, a greater TRAP-induced change was seen with the C7G-substituted (UA C7GCC)11 as compared with the unsubstituted RNA (Fig. 5). The maximum in the spectra of the substituted (UA C7GCC)11 underwent a larger red shift and also displayed a greater reduction in intensity. Furthermore, in the presence of TRAP, a negative band at around 240 nm was observed only with the substituted RNA. Once again, this observation is consistent with the hypothesis that C7G-substituted (UA C7GCC)11 is more capable of undergoing conformational changes than the unsubstituted RNA.

**DISCUSSION**

Our studies strongly suggest that the interaction of TRAP with RNA is inhibited when the RNA is sufficiently stably structured. In the current model for the TRAP-RNA interaction, the bound single-stranded RNA is wrapped around the TRAP protein ring (9, 14), and hence any RNAs that are incapable of unfolding under the binding conditions would be incapable of interacting with TRAP. It is therefore essential when analyzing the TRAP-RNA complex formation to distinguish between direct effects, meaning stabilization by intermolecular hydrogen bonds, salt links, or hydrophobic interaction, and indirect effects on RNA structure.

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**TABLE III**

| (UA C7GCC)11  | (UA C7GCC)11  | (UA C7GCC)11  | (UA C7GCC)11  | (UA C7GCC)11  | (UA C7GCC)11  |
|--------------|--------------|--------------|--------------|--------------|--------------|
| Kd (nM) 200  | Kd (nM) 7    | Kd (nM) 60   | Kd (nM) 7    | Kd (nM) 0.3  | Kd (nM) 0.2  |
| TRAP WT  R58K | TRAP WT  R58K | TRAP WT  R58K | TRAP WT  R58K | TRAP WT  R58K | TRAP WT  R58K |

a Apparent Kd values were determined by filter binding at 37 °C in the presence of 4 mM Mg2+. Values are the averages of at least two independent experiments with standard deviations being less than 10% of the mean.

b No binding detected up to 3 μM TRAP.

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**FIG. 4.** Thermal denaturation of RNAs as monitored by near UV circular dichroism (CD) spectroscopy. Spectral data from RNAs (2.5 μM) were collected between 230 and 300 nm as a function of temperature, every 5 °C between 25 and 80 °C. A shows representative spectra of RNA (UA C7GCC)11, and B shows that of C7G-substituted RNA (UA C7GCC)11, at 25, 55, and 80 °C. In panel C, each point represents the difference between the maximum value of the positive band determined at the given temperature and the value of the maximum at 80°C. ▲, RNA (UA C7GCC)11; ■, C7G-substituted RNA (UA C7GCC)11. Data are the average of two independent sets of measurements.
Introducing the analogue 7-deazaguanosine in (UA)GCC\textsubscript{11} and (CA)GCC\textsubscript{11} resulted in an enhancement in the affinity of these RNAs for TRAP. For several reasons, we believe that this effect results not from a direct interaction between C\textsuperscript{7}G and the protein, but rather by the ability of this analogue to interfere with formation of structures in these RNAs that are deleterious to TRAP binding. First, in the analogue, a carbon that is incapable of hydrogen bonding replaces the nitrogen at position 7 of the base, a potential hydrogen bond acceptor. Thus, this replacement seems unlikely to create new favorable interactions, or to eliminate unfavorable contacts between the RNA and TRAP. Insertion of C\textsuperscript{7}G at both the first and third positions in (GAGUU)\textsubscript{11} resulted in a smaller increase in TRAP affinity (ΔΔG = -1.3 kcal/mol) as compared with the single substitution made in the RNA of (GAGUU)\textsubscript{11} (ΔΔG = -2.1 kcal/mol). This could lead one to conclude that analogue substitution at the first position resulted in the loss of a direct interaction with TRAP at this site, which was partially nullified by a positive effect of substitution at the third position. However, assuming that the contribution of the third position is similar in each RNA used, the negative effect on affinity represents a total net increase of +0.8 kcal/mol for C\textsuperscript{7}G at position 1 of all 11 repeats. This amounts to approximately +0.07 kcal/mol per interaction (26–28), which is too small to indicate hydrogen bonding at this site, since current estimates ΔΔG for individual hydrogen bonding interactions are 1–2 kcal/mol (26–28). Second, there is no correlation between the position of C\textsuperscript{7}G in the repeat and its effect, because enhanced affinity for TRAP is observed with (GGA)GCC\textsubscript{11}, RNAs that contain C\textsuperscript{7}G but not with (GAGUU)\textsubscript{11} RNA. This effect of C\textsuperscript{7}G on the affinity of the TRAP-RNA complex, by perturbing the structure of the nucleic acid, is similar to observations with EcoRI (31) and the lac repressor (32). Incorporation of modified nucleotides in the target RNAs of these proteins also enhances their enzymatic and/or binding activities by affecting the structures of these DNAs.

Effects of temperature and Mg\textsuperscript{2+} on the binding of (G/U)-AGCC\textsubscript{11} RNAs to TRAP as well as thermal denaturation studies are all consistent with the proposal that increased structural stability of these RNAs (relative to similar RNAs with UU spacers) inhibits TRAP binding (Table II and Figs. 3 and 4). Previous work done by Babitzke et al. (11) has shown that TRAP does not bind to an RNA in which the (G/U)AG triplet repeats are present in a sequence predicted to form a canonical Watson-Crick base-paired duplex. One explanation for our results would be that the RNAs with CC spacers tend to form such duplexes. Computer predicted folding of these RNAs supports this possibility showing that both RNAs with CC spacers are predicted to have more stable structure (ΔG = -16 kcal/mol) than the RNAs with UU spacers ((GAGUU)\textsubscript{11} ΔG = -9.8 kcal/mol and (UAGUU)\textsubscript{11} ΔG = -6.9 kcal/mol). However, although at present we are unable to specify the type of structures in (UAGCC)\textsubscript{11} and (GAGCC)\textsubscript{11} that are inhibitory to TRAP binding, it is clear that they do not involve canonical Watson-Crick secondary structures. The effect of C\textsuperscript{7}G substitution on the affinity of these RNAs for TRAP clearly indicates involvement of the N-7 of guanosine in these structures, suggesting that non-canonical base pairings are involved. The effect of magnesium may indicate that the N-7 site coordinates divalent cations to stabilize this structure such as has been observed in other RNA structures (33, 34). Furthermore, recent studies have shown that removal of the 2-OH group of the adenosine ribose at the second position of each triplet repeat is sufficient to relax the inhibitory effects of both temperature and magnesium seen with (UAGCC)\textsubscript{11}, suggesting that this functional group is also involved in this structure. Additionally, the observation that this effect is seen in RNAs with CC but not UU spacers suggests a role for the N-3 and/or the N-4 groups of cytosine, although at this point it is not clear if both residues in the spacers are involved in this structure. Analysis of the structure of these RNAs may provide useful insights regarding the TRAP-RNA interaction.

The presence of C\textsuperscript{7}G could affect RNA structure in two ways. First, the N-7 in guanosine is known to stabilize alternate RNA structures such as quartets via non-Watson-Crick base pairs (35, 36), and replacement with the analogue C\textsuperscript{7}G would destabilize such structures. CD studies have confirmed that RNAs which contain C\textsuperscript{7}G do not form these noncanonical structures (37). Destabilization of such structures in the RNA by C\textsuperscript{7}G could increase the concentration of unfolded RNAs, which then bind TRAP. Second, C\textsuperscript{7}G could affect the stacking interactions in RNA by reducing the strength or by changing the geometry of stacking. CD studies indicate that poly(C\textsuperscript{7}G)-poly(C) and poly(G\textsuperscript{7}G)-poly(C) form similar duplex structures (37), suggesting that the bases are oriented similarly in both cases. Seela and co-workers (37) compared the melting of poly(G) to that of poly(C\textsuperscript{7}G) and showed that poly(G) has a highly cooperative thermal denaturation profile while poly(C\textsuperscript{7}G) shows less cooperativity and a lower T\textsubscript{m} (37). Furthermore, calculation of the dipole moments, 7.2 D for G and 3.0 D for C\textsuperscript{7}G, suggests that stacking energies are different for these two bases (37). The implication is that stacking is significantly weakened in the presence of 7-deazaguanosine, which accounts for the differences in the melting profiles. The effect of C\textsuperscript{7}G on destabilization of noncanonical structures and stacking interactions are not mutually exclusive and could both contribute to enhancing the binding to TRAP.

Thermal denaturation of both (UAGCC)\textsubscript{11} and C\textsuperscript{7}G-substituted (UA\textsuperscript{7}GCC)\textsubscript{11} is highly cooperative and both had similar melting points of 75 and 70 °C, respectively (Fig. 3). Clearly, a single C\textsuperscript{7}G substitution per pentameric repeat is not disruptive to those elements that gave rise to these UV hyperchromatic shifts. However, our CD studies indicate that the C\textsuperscript{7}G-substituted RNA (UA\textsuperscript{7}GCC)\textsubscript{11} undergoes conformational changes unlike the unsubstituted RNA that affect base stacking in the temperature range where the substituted RNA has greater affinity for TRAP (Fig. 4). These changes may indicate local structural changes in the RNA, which may serve to facilitate binding of TRAP at temperatures well below the T\textsubscript{m} of the RNA.

Our results are in sharp contrast to those observed with several other RNA-binding proteins, which recognize and interact with specific RNA structures. A variety of RNA structures, including helices, hairpin loops, bulges, internal loops, helical junctions, and pseudoknots have been shown to be crucial for recognition by specific RNA-binding proteins (38). However,}

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\*M. Elliott, P. Gottlieb, and P. Gollnick unpublished results.
ever, in the TRAP-RNA interaction, an inverse relationship between stability of RNA structure and binding is observed. This difference in the role of RNA structure is also reflected in the effect of Mg$^{2+}$ on the TRAP-RNA interaction as compared with other protein-RNA interactions. Magnesium stabilizes RNA structure, which inhibits RNA binding to TRAP, whereas it enhances the interactions of several other RNA-protein complexes. For example, stabilization of the hairpin loop of U1A snRNA (39) and the helical region of loop E in 5 S rRNA (34) by Mg$^{2+}$ greatly enhances binding of the U1A and the L25 proteins, respectively. In contrast, results with TRAP are akin to that observed with single-stranded DNA-binding proteins such as the gene 5 protein from the phage fd, where Mg$^{2+}$ inhibits binding to DNA (40).

This work has further defined the role of the spacer regions in RNA binding to TRAP. Analysis of the composition of the nucleotides in the natural TRAP-binding sites including, the trp leader RNA (10), the binding site in trpG (6, 7), and the yhaf$^4$ gene reveals that while 50% of the spacer nucleotides are pyrimidines, only 7% of these are C residues. Furthermore, in the trp leader, the few C residues that are present are in the stem-loop region that is required for proper regulatory activity, suggesting that their function in these regions is to maintain the secondary structures by G-C base pairing. Among the in vitro selected RNAs, while greater than 90% of the spacer nucleotides are pyrimidines, a large bias against C residues was also observed (Table I), with the majority of the spacers being UU dinucleotides. Other studies have shown that an RNA containing 6 GAG repeats and CU spacers does not bind TRAP (11). We suggest that the low fraction of Cs observed in the spacers among both in vivo and in vitro selected sequences reflects the inhibitory role of CC dinucleotides in stabilizing RNA structure. Hence, although the spacer nucleotides do not appear to directly interact with TRAP, they affect the ability of TRAP to interact with the RNA through a secondary effect on RNA structure.

One of the goals of this work was to characterize the role of Arg-58 in the TRAP-RNA interaction. A comparison of the affinities of wild-type and R58K TRAP for RNAs with UU and CC spacers has allowed us to identify a possible role for Arg-58 in binding structured RNA. Both proteins bind ((G/U)AGUU)$_{11}$ with similar affinities. However, R58K TRAP bound (UAGCC)$_{11}$, which we have shown contains the most stable structure of the RNAs we studied (Fig. 3), with far lower affinity than wild-type TRAP under all conditions studied (Table I). The effects of temperature and Mg$^{2+}$ are also consistent with this role of Arg-58. Increasing the temperature to 55 °C in the presence of Mg$^{2+}$ destabilizes the structure of (UAGCC)$_{11}$ such that wild-type TRAP binds with 40-fold higher affinity than at 37 °C, however, R58K TRAP still bound with greater than 100-fold lower affinity than the wild-type protein. Removing Mg$^{2+}$ at 55 °C to further destabilize RNA structure had no effect on the affinity of wild-type TRAP for this RNA (K$_d$ = 4 nM), whereas a 30-fold increase in affinity was seen with the lysine-substituted protein (K$_d$ = 18 nM as compared with 600 nM in the presence of Mg$^{2+}$). These results suggest that at 55 °C the structure of (UAGCC)$_{11}$ has been destabilized enough to allow efficient binding to TRAP when position 58 is arginine but not lysine. Furthermore, incorporation of C7G into (UAGCC)$_{11}$ destabilizes the RNA structure in such a way as to allow both proteins to bind equally (Table III). These results suggest that the differences seen between these proteins with the unsubstituted RNA do not reflect differences in direct contacts to the RNA but reflect a reduced ability of R58K TRAP to interact with certain RNA structures. Moreover, a similar reduction in TRAP’s ability to bind structured RNAs was observed in TRAP with glutamine substitution at position 58 (data not shown).

The effect of substitutions at position 58 on TRAP’s ability to interact with structured RNAs suggests a possible reason why arginine is conserved at this position in three different strains of bacilli. Translational regulation of trpE by TRAP involves binding to the full-length trp leader RNA, which is predicted to adopt a secondary structure in which most of the repeats are proposed to be in a duplex (3). Hence, changes to the protein, even conservative substitutions, that alter the protein’s ability to interact with structured RNA would be disadvantageous to proper control of tryptophan biosynthesis. Consistent with this hypothesis, previous experiments have shown subtle differences in the in vivo regulatory abilities of wild-type and R58K TRAP (14). A kinetic analysis of the TRAP-RNA interaction of wild-type and mutant proteins with different RNAs would provide valuable insight into the regulation of the trp operon. In vitro selection (SELEX) studies to characterize the role of Arg-58 in determining specificity in the TRAP-RNA interaction showed that changing residue 58 from arginine to lysine altered the bias at the first position of the triplet repeats in the RNAs from G to U (Table I). To explain this change we proposed a direct interaction between Arg-58 and the first position in the (G/U)AG repeats, in which arginine would form two hydrogen bonds with G but Lys could only form one bond. However, further studies, particularly those involving C7G, indicated that this model is not correct. How then can we explain the SELEX results with R58K TRAP? Examination of Table II shows that the SELEX results are consistent with the measured affinities of this mutant protein for RNAs containing 11 GAG or 11 UAG repeats. UAGUU-binding sites are the most frequently observed sites (27% of all triplet/spacer combinations) in the in vitro selected RNAs, and R58K TRAP had the highest affinity for (UAGUU)$_{11}$ (Table II) with 3-fold lower affinity for (GAGUU)$_{11}$ and much lower affinity for either RNA with CC spacers. Since we have shown that R58K TRAP is less able to interact with structured RNAs than the wild-type protein, we propose that the difference in affinity of R58K for (UAGUU)$_{11}$ and (GAGUU)$_{11}$ is based on structural differences between these RNAs rather than on direct interactions with residue 58 in the protein. This hypothesis predicts that (GAGUU)$_{11}$ should have a more stable structure than (UAGUU)$_{11}$ and our thermal melting curves show that this prediction is true, with the T$_m$ of (GAGUU)$_{11}$ being about 5 °C higher than that of (UAGUU)$_{11}$ (Fig. 3). Additionally, at a higher temperature (55 °C), R58K TRAP behaves identically to the wild-type protein, binding with higher affinity to (GAGUU)$_{11}$ than to (UAGUU)$_{11}$.

Further support for our suggestion that Arg-58 does not directly interact with the G or U in the first position of the triplet repeat has recently been obtained. After the completion of the studies presented here, we have recently obtained a crystal structure of Bacillus stearothermophilus TRAP complexed with an RNA containing 11 GAG repeats separated by AU spacers. This structure shows that Arg-58 interacts with the G residue in the third position rather than the first position of the triplet repeat. Therefore the results presented in this paper clearly demonstrate how an indirect effect of RNA structure can influence the specificity of a protein-RNA interaction.

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$^4$ J. Sarsero, personal communication.

$^5$ A. Antson and P. Gollnick, unpublished data.
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