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Interaction of the *trp* RNA-Binding Attenuation Protein (TRAP) of *Bacillus subtilis* with RNA: Effects of the Number of GAG Repeats, the Nucleotides Separating Adjacent Repeats, and RNA Secondary Structure

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The 11-subunit *trp* RNA-binding attenuation protein of *Bacillus subtilis*, TRAP, regulates transcription and translation by binding to several (G/U)AG repeats present in the *trp* leader and *trpG* transcripts. Filter binding assays were used to study interactions between L-tryptophan-activated TRAP and synthetic RNAs. RNAs that contained GAG and/or UAG repeats were tested while the length and sequence of the nucleotides separating adjacent trinucleotide repeats were altered. TRAP-RNA complexes formed with transcripts containing GAG repeats were more stable than those with transcripts containing UAG repeats or alternating GAG and UAG repeats. The stability of TRAP-RNA complexes also increased substantially when the number of GAG repeats was increased from five to six and from six to seven. A gradual increase in complex stability was observed when the number of GAG repeats was increased from 7 to 11. The optimal spacer between adjacent trinucleotide repeats was found to be 2 nucleotides, with A and U residues preferred over G and C residues. TRAP binding was specific for single-stranded RNA; TRAP could not bind to RNA containing GAG repeats base paired in a stable RNA duplex. Overall, our findings suggest that each L-tryptophan-activated TRAP subunit can bind one (G/U)AG repeat and that multiple TRAP subunit-RNA binding site interactions are required for stable TRAP-RNA association.

Expression of the tryptophan biosynthetic genes of *Bacillus subtilis* is regulated in response to changes in the intracellular concentration of L-tryptophan by transcriptional attenuation (4, 10, 12). When the *trp* RNA-binding attenuation protein of *B. subtilis*, TRAP, is activated by L-tryptophan, it can bind to a segment of the nascent *trpEDCFBA* operon leader transcript that includes a portion of an RNA secondary structure, the antiterminator (Fig. 1A) (5, 6, 13). TRAP binding prevents the formation of or disrupts the antiterminator such that formation of an overlapping rho-independent terminator is favored (Fig. 1A), resulting in transcription termination at a site approximately 65 nucleotides (nt) upstream of the *trpE* structural gene (6). In addition to regulating transcription, TRAP also regulates translation of *trpE* and *trpG*. TRAP can bind to *trp* operon transcripts that escape termination and indirectly inhibit *trpE* translation, presumably by promoting refolding of the *trp* leader transcript such that the *trpE* ribosome binding site is sequestered in a secondary structure (10, 11). The unlinked tryptophan biosynthetic gene, *trpG*, is located within a folate operon (14) and appears to be regulated at the level of translation by a TRAP binding site that overlaps the *trpG* ribosome binding site (Fig. 1B) (5, 14, 15).

The *trp* leader RNA-TRAP binding site is composed of 11 (G/U)AG repeats, separated by 2- or 3-nt spacers (Fig. 1A), while the *trpG* binding site contains 8 (G/U)AG repeats (Fig. 1B), with the length of the spacers separating adjacent repeats being more variable (Fig. 1B) (5). In both of the TRAP binding sites, the majority of the spacer nucleotides are A and U residues. The TRAP quaternary structure contains 11 identical subunits arranged in a single toroid ring (1–3). TRAP activation by L-tryptophan is cooperative and involves extensive hydrogen bonding between TRAP and L-tryptophan (1, 2, 7), with one molecule of L-tryptophan bound between adjacent subunits (2). The structure of TRAP suggests that each tryptophan-activated subunit interacts with one (G/U)AG repeat present in the *trp* leader and *trpG* transcripts.

Recent studies demonstrated that TRAP binds to transcripts composed of GAG or UAG repeats separated by 2 nt (AU) but not to transcripts in which GAG repeats were separated by 1, 3, or 4 nt (3). TRAP did not bind to transcripts composed of AAG, CAG, GCG, GGG, GUG, GAA, GAC, or GAU repeats (3). In the present study, we have further defined the nucleotide sequences that allow TRAP binding, using synthetic RNAs. We observed that the number of appropriately spaced GAG repeats dictates the overall stability of the TRAP-RNA complex. We also found GAG repeats contribute to the stability of TRAP-RNA complexes more than UAG repeats do, that the specific nucleotide sequence of the spacers separating adjacent repeats affects TRAP binding, and that TRAP cannot bind to a stable RNA duplex.

MATERIALS AND METHODS

Plasmids. Plasmid pTZ18U, containing a T7 RNA polymerase promoter (U.S. Biochemical Corp.), was used to construct all of the plasmids described in this work. Plasmid pPB77 containing the region of DNA corresponding to the wild-type *trp* operon leader was described previously (5). Transcripts derived from plasmids pPB90 containing six GAG repeats with AU spacers, pPB100 containing six UAG repeats with AU spacers, and pPB202 containing six GAG repeats with AUU spacers were described previously (3). With the exception of pPB300, the plasmids used to generate specific templates for in vitro transcription were constructed by ligating double-stranded oligonucleotide cassettes flanked by *Eco*RI- and *Kpn*I-cohesive ends into the pTZ18U polylinker. The oligonucleotides used were designed so that the insert DNA would contain the desired

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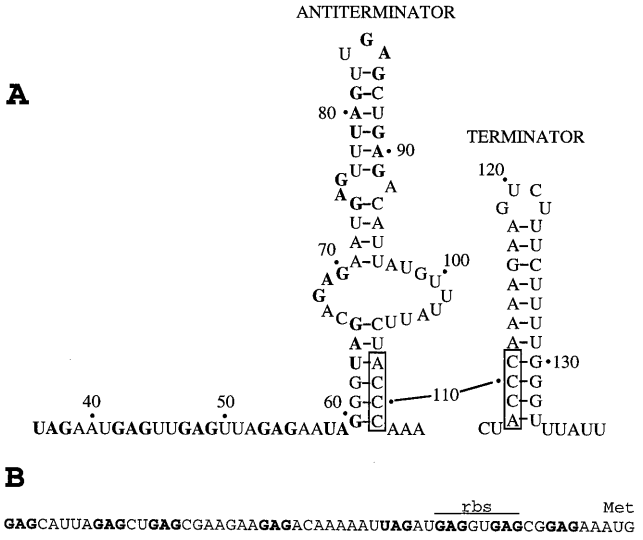

FIG. 1. *trp* leader and *trpG* TRAP binding sites. (A) Sequence of the *B. subtilis trp* operon leader transcript showing the presumed mutually exclusive antiterminator and terminator structures. Boxed nucleotides are overlapping segments of the competing secondary structures. GAG and UAG repeats involved in TRAP binding are indicated in boldface type. Secondary structure predictions were performed by using the MFOLD program of the Genetics Computer Group sequence analysis software package based on that of Zuker and Steigler (16). (B) Sequence of the *B. subtilis trpG* TRAP binding site. Positions of the *trpG* ribosome binding site (rbs) and the start of translation (Met) are indicated. GAG and UAG repeat sequences are indicated in boldface type.

number of trinucleotide repeats separated by the appropriate spacer nucleotides. Plasmid pPB300 was constructed by ligating a double-stranded oligonucleotide cassette flanked by *Kpn*I- and *Bam*HI-cohesive ends into pPB90 such that transcripts derived from this template would form a stable secondary structure containing six GAG repeats in the 5′ half of the RNA duplex ($\Delta G = -48.0$ kcal/mol [1 kcal = 4.184 kJ]). The sequences of all pTZ18U derived templates were confirmed by sequencing. Transformation and DNA isolation were performed as described previously (4).

RNA synthesis. Labeled runoff transcripts were synthesized in vitro with T7 RNA polymerase (New England Biolabs) according to the manufacturer's protocol from DNA templates linearized with the appropriate restriction enzyme. The labeling nucleotide was [5,6-^{3}H]UTP (approximately 6 Ci/mmol). Unlabeled runoff transcripts were synthesized in vitro with the Ambion MEGAscript transcription kit. Labeled and unlabeled transcripts were gel purified on 10% polyacrylamide gels containing 7 M urea.

Filter binding assays. TRAP was purified by a previously published procedure (6). Filter binding assays were modified from established procedures (3, 5) and were used to determine if TRAP could bind to various test transcripts (standard filter binding reaction). Transcripts that allowed appreciable TRAP binding were also used to examine the rate of TRAP-RNA association and the relative stabilities of the resulting TRAP-RNA complexes. Standard filter binding reaction mixtures (0.1 ml) contained 0.25 pmol of [^{3}H]RNA, 0.5 pmol of TRAP 11-mer, 10 U of RNasin (Promega), 1 mM L-tryptophan, and 1 mM dithiothreitol in TKM buffer (40 mM Tris-HCl [pH 8.0], 250 mM KCl, 4 mM $MgCl_2$) (5). TRAP dilution buffer was 50 mM Tris-HCl (pH 8.0)–50 mM KCl–15% glycerol (6). Mixtures were incubated for 20 min at 37°C and filtered as described previously (5). The filters were dried, and counts were determined in a liquid scintillation counter. Amounts of label retained on the filter (in counts per minute) were corrected for the number of U residues present in each transcript, since UTP was used as the labeling nucleotide. Background counts from a 20-min incubation at 37°C in the absence of L-tryptophan, approximately 200 to 600 cpm depending on the transcript used, were subtracted from each value. Time course experiments used to determine the stabilities of various TRAP-RNA complexes were modified from a published procedure (5). Reaction mixtures (0.6 ml) contained 1.5 pmol of [^{3}H]RNA, 3.0 pmol of TRAP 11-mer, 60 U of RNasin, 1 mM L-tryptophan, and 1 mM dithiothreitol in TKM buffer. Mixtures were incubated 20 min at 37°C to allow equilibrium TRAP-RNA complex formation, at which time 15 pmol of unlabeled wild-type *trp* leader transcript was added. Aliquots (0.1 ml) were removed at various times and filtered as described above. Background counts from a 20-min incubation at 37°C in the absence of L-tryptophan and unlabeled RNA, approximately 200 to 600 cpm depending on the transcript used, were subtracted from each value. The method to determine the rate of TRAP-RNA association was modified from a previously published procedure (5). Reaction mixtures (0.6 ml) contained 3.0 pmol of TRAP 11-mer, 60 U of RNasin, 1 mM L-tryptophan, and 1 mM dithiothreitol in TKM buffer. Mixtures were incubated 10 min at 37°C, at which time 1.5 pmol of [^{3}H]RNA was added. Aliquots (0.1 ml) were removed at various times and filtered. Background counts in the absence of L-tryptophan, approximately 200 to 500 cpm depending on the transcript used, were subtracted from each value.

RESULTS

TRAP-RNA complex stability is determined by the number of GAG repeats present in the RNA. Previous binding studies with *trp* leader deletion transcripts suggested that the stability of a TRAP-RNA complex correlated with the number of (G/U)AG repeats present in the RNA; however, the transcripts previously tested also differed in the size and sequence of the spacer nucleotides separating the adjacent repeats as well as in the respective number of GAG and UAG repeats (5). To establish that the number of trinucleotide repeats affects the ability of TRAP to bind to RNA, we performed filter binding experiments with RNA targets that have defined sequences. We designed a series of plasmids that would serve as templates for in vitro synthesis of short, defined transcripts. The transcripts contained from 2 to 11 GAG repeats, each separated by AU. These RNAs were then examined in filter binding experiments with L-tryptophan-activated TRAP. Our findings show that the binding of test transcripts increased as the number of GAG repeats was increased (Table 1). These results indicated

TABLE 1. TRAP binding to synthetic RNA targets containing different numbers of GAG repeats

Plasmid	No. of GAG repeats	cpm[a]	Relative cpm[b]	Dissociation constant[c]	Complex half-life (min)
pPB97	2	0	0	ND[d]	ND
pPB96	3	34 ± 28	1	ND	ND
pPB94	4	234 ± 114	4	ND	ND
pPB220	5	2,280 ± 34	39	8.7×10^{-2}	8.0
pPB90	6	4,371 ± 112	74	2.8×10^{-2}	25
pPB221	7	4,162 ± 170	70	9.5×10^{-3}	73
pPB223	8	4,843 ± 146	82	3.5×10^{-3}	199
pPB224	9	5,736 ± 86	97	1.7×10^{-3}	418
pPB222	11	5,904 ± 152	100	—[e]	—

[a] Counts per minute retained on the filter were corrected for the number of U residues present in each transcript since UTP was used as the labeling nucleotide. The corrected value of input radioactivity for each sample was approximately 11,500 cpm.
[b] Counts per minute were normalized to the value obtained with the transcript containing 11 GAG repeats (pPB222), which was arbitrarily set to 100.
[c] Dissociation constants were calculated by the preferred method of Johnson and Faunt (8).
[d] ND, not determined.
[e] —, stable (i.e., no detectable dissociation during the 20-min time course).

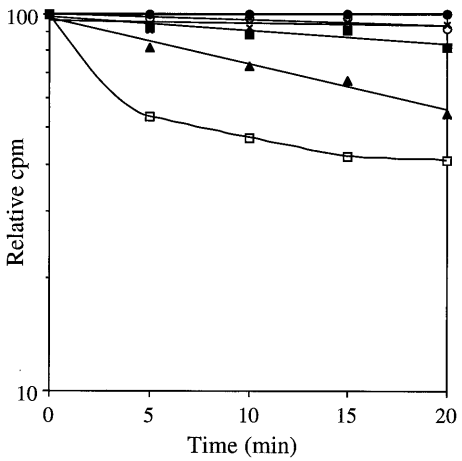

FIG. 2. Stability of TRAP-RNA complexes with RNA targets containing different numbers of GAG repeats. ^{3}H-labeled transcripts were preincubated with TRAP prior to the addition of unlabeled *trp* leader RNA. Aliquots were removed at the times indicated following the addition of unlabeled RNA and then were filtered. The value obtained following a 20-min incubation with each labeled RNA with TRAP and L-tryptophan (no unlabeled RNA) was arbitrarily set to 100. Values are averaged from at least four experiments. See Materials and Methods for experimental details. Symbols used to represent transcripts containing the indicated numbers of GAG repeats are as follows: ●, 11 repeats; ×, 9 repeats; ○, 8 repeats; ■, 7 repeats; ▲, 6 repeats; □, 5 repeats.

that, under our test conditions, a minimum of four GAG repeats was required for appreciable TRAP binding (Table 1). We also observed a significant increase in the binding of test transcripts when the number of repeats was increased from 4 to 5 and from 5 to 6, while a more gradual increase was observed as the number of repeats was increased one at a time from 6 to 11 (Table 1). TRAP-RNA association experiments established that the binding reactions were complete well within the 20-min incubation time of this assay.

To determine the relative stabilities of TRAP-synthetic RNA complexes, we compared complexes formed with RNAs that contained from 5 to 11 GAG repeats. Under the experimental conditions used, the amount of labeled test transcript retained in the complex with TRAP would be a function of the rate of dissociation of the TRAP-labeled RNA complex and the ability of free labeled RNA to compete with a 10-fold molar excess of unlabeled *trp* leader RNA. It was shown previously that *trp* leader RNA forms a stable complex with TRAP (3, 5). The results of the present analysis indicated that the transcript containing 11 GAG repeats formed a stable complex with TRAP; dissociation was not detected during the 20-min time course (Fig. 2). A gradual reduction in complex stability was observed as the number of GAG repeats was decreased from 11 to 9, from 9 to 8, and from 8 to 7. A more pronounced decrease in complex stability was observed as the number of repeats was reduced from seven to six and from six to five (Fig. 2; Table 1).

Filter binding experiments were also performed to determine if the number of GAG repeats present in the RNA targets influenced the rate of TRAP-RNA association. Results from these experiments demonstrated that association of TRAP with transcripts containing 11 or 9 repeats was complete by the earliest 30-s time point. In general, as the number of GAG repeats was decreased one at a time from nine to five, the rate of association was slightly reduced, although TRAP-RNA complex formation was essentially complete within 2 min for each of these transcripts (data not shown).

The nucleotide sequence separating adjacent (G/U)AG repeats influences TRAP-RNA recognition. The spacer nucleotides that separate the 11 (G/U)AG repeats in the *trp* leader RNA are primarily A and U residues. Of the 23 spacer nucleotides there are only two C and two G residues (Fig. 1A). This underrepresentation of C and G spacer nucleotides could indicate that these residues are inhibitory to TRAP binding. To test this possibility, we performed filter binding experiments with synthetic RNAs that contained six GAG repeats separated by AA, AU, CU, GU, or UU residues. TRAP bound with comparable effectiveness to transcripts in which the GAG repeats were separated by AA, AU, or UU residues (Table 2). However, TRAP binding was greatly diminished when the spacers consisted of CU or GU residues (Table 2).

TRAP-RNA complex stability experiments were carried out with the transcripts containing AA, AU, or UU spacers. Complexes between TRAP and the RNA containing UU spacers were twice as stable as complexes formed with AU spacer transcripts. Complexes formed with transcripts containing AA spacers were considerably less stable than complexes formed with UU or AU spacer transcripts (Fig. 3; Table 2). The rates

TABLE 2. TRAP binding to various synthetic RNA targets

Plasmid	RNA repeat sequence	Spacer sequence	cpm[a]	Relative cpm[b]	Dissociation constant[c]	Complex half-life (min)
pPB90	GAG	AU	4,422 ± 163	100	3.0×10^{-2}	23
pPB100	UAG	AU	1,386 ± 142	31	1.2×10^{-1}	6.0
pPB210	GAG-UAG[d]	AU	4,240 ± 64	96	9.2×10^{-2}	7.6
pPB202	GAG	AUU	0	0	ND[e]	ND
pPB230	GAG	AU-AUU[f]	2,282 ± 93	52	2.2×10^{-1}	3.2
pDC4	GAG	AA	4,567 ± 157	103	1.2×10^{-1}	6.0
pDC2	GAG	UU	4,687 ± 357	106	1.6×10^{-2}	42
pDC1	GAG	GU	767 ± 130	17	ND	ND
pDC3	GAG	CU	577 ± 117	13	ND	ND
pPB300	GAG[g]	AU	0	0	ND	ND

[a] Counts per minute retained on the filter were corrected for the number of U residues present in each transcript since UTP was used as the labeling nucleotide. The corrected value of input radioactivity for each sample was approximately 11,500 cpm.
[b] Counts per minute were normalized to the value obtained with the transcript containing six GAG repeats and AU spacers (pPB90), which was arbitrarily set to 100.
[c] Dissociation constants were calculated by the preferred method of Johnson and Faunt (8).
[d] This transcript alternated between GAG and UAG repeats, with GAG being the first repeat (three GAG and three UAG repeats).
[e] ND, not determined.
[f] The spacers separating adjacent repeats in this transcript alternated between AU and AUU, with AU being first (three AU spacers and two AUU spacers).
[g] These GAG repeats are present within a stable RNA secondary structure.

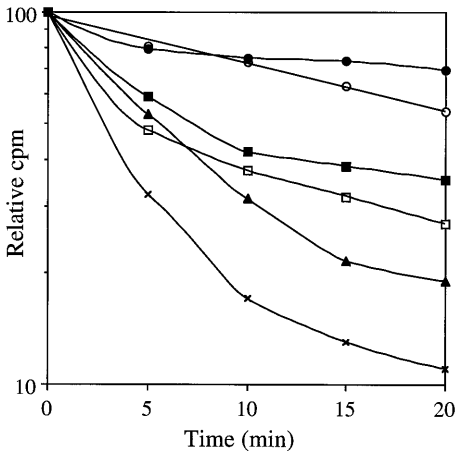

FIG. 3. Stability of TRAP-RNA complexes with various RNA targets. ^{3}H-labeled transcripts were preincubated with TRAP prior to the addition of unlabeled *trp* leader RNA. Aliquots were removed at the times indicated following the addition of unlabeled RNA and then were filtered. The values obtained following a 20-min incubation with each labeled RNA with TRAP and L-tryptophan (no unlabeled RNA) was arbitrarily set to 100. Values are averaged from at least four experiments. See Materials and Methods for experimental details. Symbols used to represent the various transcripts are as follows: ●, transcript with six GAG repeats (UU spacers); ○, transcript with six GAG repeats (AU spacers); ■, transcript with six repeats alternating between GAG and UAG (AU spacers); □, transcript with six UAG repeats (AU spacers); ▲, transcript with six GAG repeats (AA spacers); ×, transcript with six GAG repeats (alternating AU and AUU spacers).

of association of TRAP with each of these transcripts were similar, and binding was complete within 2 min (data not shown).

The natural *trp* leader binding site contains seven 2-nt and three 3-nt spacers, suggesting that 3-nt spacers are tolerated if they are in an appropriate context. To test this possibility we performed filter binding experiments with transcripts containing six GAG repeats such that each repeat was separated by either 2 nt (AU) or 3 nt (AUU) or with a transcript alternating between 2- and 3-nt spacers (three AU and two AUU spacers). As shown previously (3), TRAP did not bind to a transcript in which each repeat was separated by 3 nt (Table 2). However, TRAP did bind to the transcript with alternating 2- and 3-nt spacers, although not as effectively as to the transcript containing only 2-nt spacers (Table 2). When we tested the stabilities of these two TRAP-RNA complexes we found that the transcript containing only 2-nt spacers was far more stable than the transcript with alternating 2- and 3-nt spacers (Fig. 3; Table 2). The rates of association of TRAP with these two transcripts were similar, and binding was complete within 2 min (data not shown). These results indicate that 3-nt spacers are tolerated if they do not follow each other in the transcript.

TRAP-RNA complex stability is influenced by the relative number of GAG and UAG repeats present in the transcript. To determine the relative contribution of GAG or UAG repeats in TRAP binding, we performed filter binding experiments with transcripts containing six GAG repeats or six UAG repeats or with a transcript that had alternating GAG and UAG repeats (three GAG and three UAG repeats). The results presented in Table 2 show that TRAP binding to the transcript containing three GAG and three UAG repeats was similar to TRAP binding to the transcript consisting of six GAG repeats and that both of these RNAs were significantly better TRAP targets than the transcript with six UAG repeats. However, the stability of the TRAP-RNA complex formed with the GAG-UAG transcript was similar to that formed with the UAG repeat transcript, both of which were less stable than the complex formed between TRAP and the RNA that contained only GAG repeats (Fig. 3; Table 2). The rates of association of TRAP with each of these transcripts were similar, and binding was complete within 2 min (data not shown).

TRAP-RNA recognition is influenced by RNA secondary structure. Previously published results (5) demonstrated that TRAP could bind to transcripts that were predicted to be entirely single stranded or to transcripts that contained a partially double-stranded antiterminator structure ($\Delta G = -6.9$ kcal/mol). To determine if TRAP could bind to double-stranded RNA, filter binding experiments were performed with a transcript that contained six GAG repeats within a perfect duplex ($\Delta G = -48.0$ kcal/mol). TRAP was unable to bind to this transcript, indicating that TRAP is specific for single-stranded RNA (Table 2).

DISCUSSION

In the present study we have further defined the RNA sequences that affect TRAP binding. We found that the number of GAG repeats present in a test transcript has a dramatic effect on the ability of TRAP to bind to the RNA (Table 1) and on the stability of the resulting TRAP-RNA complexes (Fig. 2). In general, TRAP bound more tightly to transcripts as the number of GAG repeats was increased (Fig. 2). TRAP-RNA complexes formed with transcripts containing 11 repeats were stable. A gradual reduction in TRAP-RNA complex stability was observed when the number of GAG repeats was decreased from 11 to 9, from 9 to 8, and from 8 to 7. The stability of TRAP-RNA complexes decreased more rapidly when the number of GAG repeats was reduced one at a time from seven to five (Fig. 2; Table 1). The length and specific nucleotide sequence of the spacers separating adjacent GAG repeats also influence TRAP binding. We found that A and U residues were the preferred spacer nucleotides. The stabilities of TRAP-RNA complexes with GAG repeats separated by UU or AU residues were comparable, while complexes formed with similar transcripts containing AA spacers were considerably less stable (Fig. 3; Table 2). The presence of G or C residues in the spacers adversely affected TRAP binding (Table 2). TRAP bound to a transcript in which the spacers separating adjacent GAG repeats alternated between 2 and 3 nt (Table 2). However, the TRAP-RNA complex formed with this transcript was less stable than complexes in which the transcript contained only 2-nt spacers (Fig. 3; Table 2). TRAP did not bind to a GAG repeat transcript that contained only 3-nt spacers (Table 2). We also observed that the binding effectiveness of a transcript that alternated between GAG and UAG repeats was intermediate compared with those of similar transcripts containing only GAG or UAG repeats (Table 2; Fig. 3). SELEX experiments confirm the importance of (G/U)AG repeats, as well as that of UU and AU spacers, in TRAP-RNA recognition (7a). TRAP-RNA association experiments suggest that the number of GAG repeats in test transcripts affects the rate of complex formation (data not shown). However, TRAP-RNA complex formation was so rapid that an accurate determination of the association rates was not possible.

Results from a previous study (5) demonstrated that TRAP can bind to *trp* leader deletion transcripts in which the (G/U)AG repeats are predicted to be entirely single stranded or to transcripts that consist of a partially double-stranded antiterminator structure (Fig. 1A). However, TRAP was unable to bind to appropriately spaced GAG repeats when the repeats were present within a stable RNA duplex ($\Delta G = 48.0$ kcal/mol)

(Table 2), indicating that TRAP is specific for single-stranded RNA. The reduced binding that was observed with the CU spacer transcript (Table 2) could be due to RNA secondary structure, since this transcript is predicted to fold into a relatively stable hairpin ($\Delta G = -10.5$ kcal/mol). This might explain the high level of underrepresentation of C residues in the *trp* leader and *trpG* TRAP binding sites (Fig. 1).

A model in which a segment of *trp* leader or *trpG* RNA is wrapped around the perimeter of L-tryptophan-activated TRAP was previously proposed (2, 3, 5). The correlation of the TRAP subunit structure with the number of (G/U)AG repeats present in the *trp* leader and *trpG* transcripts suggests that each trinucleotide repeat interacts with one tryptophan-activated subunit or a combination of adjacent subunits (2). Once a critical number of repeats are encountered, the RNA would wrap around the TRAP complex. Our demonstration that the number of GAG repeats present in a transcript affects the stability of the resulting TRAP-RNA complex is consistent with this model.

The results presented here and those from previous studies (2, 3, 5) have indicated that the optimal TRAP binding site consists of single-stranded RNA containing 11 GAG repeats separated by 2 nt (UU or AU). However, the natural *trp* leader and *trpG* binding sites consist of a mixture of GAG and UAG repeats, spacer segments containing G and/or C residues, spacers containing more than 2 nt, and some secondary structure (Fig. 1). The function of TRAP binding to *trp* leader RNA is to prevent formation of or to disrupt the antiterminator structure. It was also proposed that disruption of the antiterminator structure by TRAP would be necessary to promote refolding of the *trp* leader transcript thought to be involved in regulating translation of *trpE* (11). Thus, the presence of trinucleotide repeats in a single-stranded RNA segment preceding the antiterminator structure may have evolved to facilitate TRAP binding prior to antiterminator formation, while those within the antiterminator structure probably exist to promote TRAP binding as well as antiterminator disruption. The two spacer G residues within the base of the antiterminator stem appear to be strategically placed such that the resulting G-C base pairs add stability to this portion of the antiterminator structure. This is especially significant since these G residues can base pair with residues necessary for terminator formation (Fig. 1A). Previous studies have demonstrated that TRAP can be titrated out in vivo if the strain contains a plasmid expressing *trp* leader RNA (2, 10, 13). Thus, it is possible that TRAP would be titrated out if the TRAP binding sites did not allow appreciable TRAP-RNA complex dissociation, as was observed for the optimal TRAP binding site in vitro (Fig. 2). The presence of UAG repeats, G and C residues in the spacers, and spacers containing more than 2 nt in the *trp* leader and *trpG* TRAP binding sites might be necessary to prevent titration of TRAP in vivo. *trpG* encodes an amidotransferase subunit involved in both folate and tryptophan biosynthesis (9). While the expression of *trpG* is negatively regulated by tryptophan (9, 15), it does not appear that the other genes in the folate operon are controlled by tryptophan (15). Thus, it is possible that the suboptimal TRAP binding site in the *trpG* mRNA, including the unusually long spacers, is used to allow some *trpG* translation in the presence of tryptophan-activated TRAP to maintain folate biosynthesis (15).

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