tRNA determinants for transcription antitermination of the *Bacillus subtilis* *tyrS* gene

FRANK J. GRUNDY, JENNIFER A. COLLINS, SEAN M. ROLLINS, and TINA M. HENKIN

Department of Microbiology, The Ohio State University, Columbus, Ohio 43210, USA

ABSTRACT

Transcriptional regulation of the T box family of aminoacyl-tRNA synthetase and amino acid biosynthesis genes in Gram-positive bacteria is mediated by a conserved transcription antitermination system, in which readthrough of a termination site in the leader region of the mRNA is directed by a specific interaction with the cognate uncharged tRNA. The specificity of this interaction is determined in part by pairing of the anticodon of the tRNA with a “specifier sequence” in the leader, a codon representing the appropriate amino acid, as well as by pairing of the acceptor end of the tRNA with an unpaired region of the antiterminator. Previous studies have indicated that although these interactions are necessary for antitermination, they are unlikely to be sufficient. In the current study, the effect of multiple mutations in tRNA^{Tyr} on readthrough of the *tyrS* leader region terminator, independent of other tRNA functions, was assessed using a system for in vivo expression of pools of tRNA variants; this system may be generally useful for in vivo expression of RNAs with defined end points. Although alterations in helical regions of tRNA^{Tyr} that did not perturb base pairing were generally permitted, substitutions affecting conserved features of tRNAs were not. The long variable arm of tRNA^{Tyr} could be replaced by either a short variable arm or a long insertion of a stable stem-loop structure. These results indicate that the tRNA–leader RNA interaction is highly constrained, and is likely to involve recognition of the overall tertiary structure of the tRNA.

Keywords: aminoacyl-tRNA synthetase; attenuation; gene expression; gene regulation; Gram-positive; termination

INTRODUCTION

The *Bacillus subtilis* *tyrS* gene, encoding tyrosyl-tRNA synthetase, is a member of the T box family of aminoacyl-tRNA synthetase and amino acid biosynthesis genes (Grundy & Henkin, 1993, 1994a; Henkin, 1994; Condon et al., 1996). Expression of the genes in this family is dependent on readthrough of a factor-independent transcriptional terminator located in the leader region of the mRNA, upstream of the start of the coding sequence. Readthrough of the terminator in each gene is stimulated by interaction of the cognate uncharged tRNA with the leader mRNA, resulting in formation of an antiterminator structure that prevents formation of the terminator.

Genetic studies have revealed two positions of base pairing between the tRNA and the leader RNA. The anticodon of the tRNA pairs with a single codon, designated the specifier sequence, and the acceptor end

of the tRNA pairs with a side bulge in the antiterminator (Fig. 1; Grundy & Henkin, 1993; Grundy et al., 1994). Experiments in which these determinants of the *tyrS* leader were replaced with those corresponding to a noncognate tRNA demonstrated that these elements are sufficient to direct interaction with certain other tRNAs, albeit at lower efficiency than the interaction with the cognate tRNA (Grundy & Henkin, 1993; Grundy et al., 1997). In other cases, substitutions at the specifier and antiterminator did not result in induction directed by another tRNA. Similar results have been observed for the *ilv-leu*, *thrS*, and *valS* genes (Putzer et al., 1995; Marta et al., 1996; Luo et al., 1997). These results indicate that elements of the tRNA in addition to the anticodon and acceptor end are probably required, and that additional features of the tRNA–leader interaction are likely to be important for efficient readthrough.

To facilitate analysis of large numbers of tRNA variants, a system for efficient in vivo tRNA^{Tyr} expression was generated. A normal cellular tRNA must direct its own processing, be specifically recognized by the cognate aminoacyl-tRNA synthetase, bind elongation factor Tu, enter the A-site of an elongating ribosome, and

Reprint requests to: Tina M. Henkin, Department of Microbiology, The Ohio State University, 484 W. 12th Avenue, Columbus, Ohio 43210, USA; e-mail: henkin.3@osu.edu.

FIGURE 1. Structural model of the stem I and antiterminator regions of the *tyrS* leader. The AMB-U222A *tyrS* variant is shown matched with tRNA^{Tyr}AMB-A73U. tRNA^{Tyr} is known to interact with the leader by pairing of the anticodon (CUA, outlined letters) with the specifier sequence (boxed UAG amber codon) and by pairing of the 3' acceptor end of the tRNA (UCCA, outlined letters) with the side bulge of the antiterminator (UGGA). The remainder of the *tyrS* leader is omitted for clarity (dashed lines). Numbering of the *tyrS* leader is relative to the start point of transcription (Henkin et al., 1992).

participate in peptide bond formation. In contrast, T box gene antitermination is mediated by uncharged tRNA, and tRNA^{Tyr} mutations that block interaction with tyrosyl-tRNA synthetase (and therefore all subsequent roles for charged tRNA) efficiently promote *tyrS* antitermination (Grundy et al., 1994). The requirement for self-processing can also be eliminated using an expression system that generates the mature 5' and 3' ends in vivo. In this study, we describe analysis of multiple tRNA^{Tyr} variants to identify features of the tRNA required for *tyrS* antitermination, independent of other roles of the tRNA in the cell. Possible effects on tRNA stability were assessed by Northern analysis. Mutations can affect tRNA folding rather than direct interactions with the leader; therefore, the most useful information arises from analysis of variants that successfully promoted antitermination, because these clearly show sequence variants that do not interfere with appropriate interaction with the leader RNA. We

find that although there is some flexibility in requirements for specific features of tRNA^{Tyr}, the general rules for tRNA structure are conserved for *tyrS* antitermination, indicating that the system recognizes the complete tertiary structure of the tRNA.

RESULTS AND DISCUSSION

Expression of *Escherichia coli* tRNA^{Tyr} in *B. subtilis*

As a first step in determining the flexibility of the structural requirements for tRNA^{Tyr} for *tyrS* antitermination, *E. coli* tRNA^{Tyr} was introduced into *B. subtilis*. *E. coli* tRNA^{Tyr} contains multiple differences at the primary sequence level from its *B. subtilis* homolog (Fig. 2). These differences include some of the key tertiary interaction positions (Giege et al., 1993); although the 15·48 *trans* interaction is maintained, the 26·44 *cis* interaction (G·U)

is replaced by A·C, the (47·45)(10·25) interaction [(U·C)(G·C)] is replaced by (U·G)(C·G), and the (13·22)·47 interaction [(A·A)·U] is replaced by (G·A)·U in *E. coli* tRNA^{Tyr}. The *supF* amber suppressor allele of tRNA^{Tyr}, containing a CUA anticodon to match UAG amber codons (Kirsebom & Svard, 1992), was modified by introduction of an A73U mutation at the discriminator position of the tRNA to reduce possible interference by charged tRNA. This substitution blocks tRNA^{Tyr} charging by TyrRS (Bedouelle et al., 1993), and it was previously demonstrated that this mutation in *B. subtilis* tRNA^{Tyr}AMB, a *supF* analog, reduced amber suppression in vivo and permitted induction of a *tyrS-lacZ* fusion with a corresponding U222A substitution at the antiterminator and UAG specifier sequence during growth in rich medium (Grundy et al., 1994). The mutant tRNA was inserted into plasmid pDG148 (Stragier et al., 1988) so that expression would be directed by the IPTG-inducible *Pspac* promoter. This construct was introduced into *B. subtilis* tester strains containing single-copy *tyrS-lacZ* fusions integrated into an SP β prophage.

Expression of the *tyrS-lacZ* AMB-U222A fusion in the absence of plasmid-directed tRNA synthesis was low, and IPTG-induced synthesis of tRNAs lacking a match at the specifier (pWT) or at the U222A position (pAMB) failed to significantly increase expression of the fusion (Table 1). In contrast, IPTG-induced synthesis of tRNA^{Tyr}AMB-A73U resulted in an 18-fold increase in *tyrS-lacZ* expression. *E. coli* tRNA^{Tyr}SupF-A73U was twofold more efficient than the corresponding variant of *B. subtilis* tRNA^{Tyr} in inducing the *tyrS-lacZ* AMB-U222A fusion. This variant of *E. coli* tRNA^{Tyr} exhibited a more severe reduction of nonsense suppression activity than the equivalent *B. subtilis* tRNA^{Tyr}AMB-A73U, using an *rpsD*^{Am}-*lacZ* translational fusion, which contains an amber mutation in the ribosomal protein S4 coding sequence fused to a *lacZ* reporter (Table 1); this fusion was previously used to

assay translational suppression (Grundy & Henkin, 1994b). The difference in nonsense suppression activity is likely to reflect a difference in charging efficiency, which may be responsible for the small increase in effectiveness of *E. coli* tRNA^{Tyr} in inducing *tyrS* read-through. It therefore appears that the sequence differences between these tRNAs do not dramatically affect interaction with the *tyrS* leader, that is, that at least some alterations in *B. subtilis* tRNA^{Tyr} can be tolerated.

tRNA^{Tyr} expression cassette

An expression system that eliminated the requirement for the tRNA to provide the normal processing determinants was developed to facilitate generation of large numbers of variants of tRNA^{Tyr}, some of which could be expected to alter these determinants. The expression cassette (Fig. 3) placed the first position of the tRNA (using an *Xma*I restriction site) at the transcription start point of the very strong *B. subtilis* *rpsD* promoter (Grundy & Henkin, 1990), so that the primary transcript would contain the mature 5' end of the tRNA (with a 5' triphosphate, rather than the monophosphate normally generated by RNaseP cleavage of a longer precursor tRNA). The 3' terminal A of the tRNA was placed immediately upstream of the 5' position of a variant of tRNA^{Gln}, so that RNaseP cleavage at the 5' end of tRNA^{Gln}, directed by tRNA^{Gln}, would simultaneously liberate the mature 3' end of tRNA^{Tyr}. The placement of the 3' terminus was expedited by the use of an *Nco*I restriction site (CCATGG) that contained the 3' CCA common to all tRNAs adjacent to the TGG found at the 5' end of tRNA^{Gln}. Additional restriction sites were also introduced into the tRNA^{Tyr} coding sequence, within helical regions, to facilitate replacement of the central portions or 5' and 3' terminal portions of the tRNA with oligonucleotide cassettes containing nucleotide substitutions, deletions or insertions. All constructs contained the G34C anticodon and A73U discriminator base mutations to match the *tyrS-lacZ* AMB-U222A leader fusion.

The tRNA^{Tyr} expression cassette was generated via intermediate constructs deleted for either the D stem and loop (MINI + TV) or the T stem and loop and variable arm (MINI + D), or both (MINI; Fig. 3). These constructs resulted in essentially no increase in *tyrS-lacZ* expression (data not shown), but interpretation of these results is complicated by the fact that the major perturbations of tRNA structure introduced could result in misfolding or instability of the tRNA product, although minihelices of this type have been shown to be substrates for aminoacylation (Francklyn & Schimmel, 1989). More subtle alterations of the tRNA were therefore tested.

The composite (COMP) tRNA^{Tyr} construct generated from the intermediate constructs contained all of the features of a normal tRNA but had several primary

TABLE 1. Effect of tRNA^{Tyr} variants on *tyrS-lacZ* expression and nonsense suppression.

Plasmid-borne tRNA	β -galactosidase activity ^a					
	Fusion: <i>tyrS-lacZ</i> AMB-U222A			<i>rpsD</i> ^{Am} - <i>lacZ</i>		
	-IPTG	+IPTG	Induction ratio	-IPTG	+IPTG	Induction ratio
None	1.0	1.0	1.0	0.27	0.41	1.5
Wild-type	1.2	0.92	0.77	0.65	0.68	1.0
AMB	1.9	3.6	1.9	4.8	300	63
AMB-A73U	1.6	28	18	0.30	13	44
SupF-A73U	1.7	60	35	0.25	3.0	12

^atRNA^{Tyr} expression was under *Pspac* control, and was induced by the addition of IPTG (1 mM). β -galactosidase activities are in Miller units (Miller, 1972). Induction ratio is the ratio of expression in the presence versus absence of IPTG.

FIGURE 2. (Legend on facing page.)

FIGURE 3. In vivo tRNA cassette expression system. **A:** Expression construct. The 5' position of tRNA^{Tyr} was placed at the +1 position of the *rpsD* promoter using an *XmaI* restriction site; the 3' position of tRNA^{Tyr} was placed immediately upstream of the 5' position of a variant of tRNA^{Gln} using an *NcoI* site, so that RNase P cleavage directed by the downstream tRNA^{Gln} would simultaneously release the mature 3' end of tRNA^{Tyr}. The *NcoI* site contains the CCA that is common to all tRNA 3' ends. In addition to the *XmaI* and *NcoI* sites, Site 1 and Site 2 represent unique restriction sites placed at various positions of the tRNA, permitting replacement of the region between the sites by oligonucleotide cassettes containing nucleotide substitutions at defined positions. **B:** MINI: contains the acceptor and anticodon arms. **C:** MINI + D: contains the acceptor, anticodon, and D arms. **D:** MINI + TV: contains the acceptor, anticodon, T, and variable arms.

sequence differences relative to tRNA^{Tyr} (Fig. 2C); none of these changes are predicted to disrupt known tertiary interactions (Giege et al., 1993). Expression of tRNA^{Tyr}COMP yielded 76 units of β -galactosidase expression from the *tyrS-lacZ*AMB-U222A fusion, in comparison to 1.5 units of expression in the absence of an appropriate tRNA^{Tyr} variant (Table 2). This level of *tyrS* readthrough was identical to that obtained with an equivalent construct containing tRNA^{Tyr}AMB-A73U, indicating that the stem region substitutions introduced did not negatively affect readthrough. A second variant

(BGII; Fig. 2D) was generated that contained an additional restriction endonuclease cleavage site in the D stem and consequently an extra base pair in the D stem (C13–G22), at a position that also participates in an interaction with U47. The BGII variant also contained a substitution of the 9·23·12 interaction of normal tRNA^{Tyr} (A·C·G) with A·U·U. In addition, position 11, which is a pyrimidine in nearly all bacterial tRNAs except tRNA^{fmet} (Sprinzl et al., 1998), was replaced with a purine. This variant resulted in a reduction of *tyrS-lacZ* fusion expression to 20 units, suggesting that

FIGURE 2. Variants of tRNA^{Tyr}AMB-A73U. **A:** *B. subtilis* tRNA^{Tyr}AMB-A73U. **B:** *E. coli* tRNA^{Tyr}SupF-A73U. **C:** COMP, contains intact tRNA^{Tyr}. **D:** BGII, derived from COMP, with additional substitutions in the D stem. **E:** Δ Var, derived from BGII, with a short variable arm. **F:** Δ Var-TAR, derived from BGII, with the variable arm replaced by the HIV TAR site. Numbering is according to Sprinzl et al. (1998). Numbered residues are discussed in the text. Asterisks indicate differences from *B. subtilis* tRNA^{Tyr}AMB-A73U. Restriction sites are labeled. The *Bgl*II site is lost in mutants with changes at position 26. Arrows indicate positions targeted for mutagenesis.

TABLE 2. Cassette expression of mutants of tRNA^{Tyr}.

Plasmid-borne tRNA ^{Tyr}	β -galactosidase activity ^a	
	<i>tyrS-lacZ</i> AMB–U222A	<i>rpsD</i> ^{Am} – <i>lacZ</i>
None	1.5	1.0
AMB–A73U	77	25
COMP	76	75
BGII	20	0.5
Δ Var–U44A	40	0.3
BGII–G26U	4.0	0.4
Δ Var–G26U	1.6	NT ^b
Δ Var–G26U,U44C	1.2	0.3
Δ Var–TAR	9.2	NT
69–72,AGCA	1.0	NT
69–72,UCCA	2.0	NT
5'EXT	22	NT

^a β -galactosidase activities are in Miller units (Miller, 1972).^bNT: not tested.

the D stem sequence can influence the system, although activity was still high.

Nonsense suppression by the tRNA variants was assessed as an indicator of charging efficiency (Table 2). The wild-type tRNA^{Tyr}AMB–A73U construct conferred 25 units of β -galactosidase expression using the *rpsD*^{Am}–*lacZ* translational fusion, somewhat higher than the 13 units obtained with *Pspac*-dependent expression (Table 1); this increase is presumably due to the increased level of tRNA expression directed by the stronger *rpsD* promoter (F. Grundy & T. Henkin, unpubl. results). The tRNA^{Tyr}COMP variant gave a threefold increase in amber suppression relative to wild-type tRNA^{Tyr}AMB–A73U, suggesting that the stem sequence alterations may enhance charging efficiency; because TyrRS does not recognize tRNAs with the A73U substitution (Bedouelle et al., 1993), another aminoacyl-tRNA synthetase may be responsible for this activity. In contrast, the D stem changes of the BGII construct, while resulting in a 3.8-fold decrease in *tyrS-lacZ* expression, dropped amber suppression to background levels (150-fold reduction relative to the COMP variant), indicating a severe defect in tRNA charging. This construct was therefore used for most further mutagenesis experiments, to eliminate possible tRNA charging variability.

Variable arm mutations

The variable arm of tRNA varies in both sequence and size, and position 44, the first position of the variable arm, also interacts with the nucleotide at position 26 (Giege et al., 1993). tRNA^{Tyr}, tRNA^{Leu}, and tRNA^{Ser} all contain long variable arm stem-loops, whereas other tRNAs contain short loops of 4–5 nt. It was previously noted (Grundy et al., 1997) that both classes of tRNAs could substitute for tRNA^{Tyr} in directing expression of a *tyrS-lacZ* fusion containing the appropriate specifier and

antiterminator sequences, albeit with reduced efficiency, suggesting that the variable arm is not a major determinant for interaction with the *tyrS* leader. This was tested directly by replacing the tRNA^{Tyr} variable arm with the sequence common to tRNA^{Ile} and tRNA^{Gly} (which function well in *tyrS* antitermination) and tRNA^{Asn} (which functions poorly). The influence of the 26–44 interaction was assessed by providing a mixture of G, A, C, and U at position 44. Constructs containing the short variable arm (Δ Var; Fig. 2E) were twofold more efficient in directing *tyrS* expression than the parental BGII construct (Table 2), but only in conjunction with an A at position 44, rather than the U normally found in tRNA^{Tyr}. This suggests that the length of the variable arm is not a determinant of *tyrS* leader interaction, as predicted, but that the context of this region is important, such that the identity of the nucleotide at position 44 is constrained by the size and/or sequence of the variable arm.

Insertion of the HIV TAR site into tRNA^{Tyr}

The tolerance for deletion of the tRNA^{Tyr} long variable arm suggested that this region was not important for interaction with the *tyrS* leader. This was further tested by replacement of the variable arm of the Δ Var–U44A construct with a 39-bp stem-loop from the HIV TAR site, which is known to be a stable element and acts as the binding site for the Tat protein (Wang et al., 1998). This construct (Fig. 2F) directed *tyrS* antitermination fairly efficiently (Table 2), supporting the prediction that the variable arm does not interact directly with the leader, and must not interfere with the required regions of the tRNA–mRNA complex.

Position 26

As noted above, tRNA^{Asn} was previously shown to be unable to direct expression of a *tyrS-lacZ* fusion containing a matching specifier and antiterminator (Grundy et al., 1997). Comparison of tRNA^{Asn}, which failed to direct expression, and tRNA^{Gly}, which did direct *tyrS* expression, revealed few differences. The most unusual feature of tRNA^{Asn} is the presence of a U at position 26; this position is a purine in nearly all tRNAs (Sprinzl et al., 1998). A G26U mutation of tRNA^{Tyr} was therefore tested, and resulted in a fivefold reduction of expression in the context of the BGII construct (Table 2). Because position 26 normally interacts with position 44, and tRNA^{Asn} contains a short variable loop, the G26U allele was also generated in the context of the Δ Var construct, which contains a variable loop identical to that of tRNA^{Asn}, and with simultaneous variation of position 44. No variants in the Δ Var context containing the G26U mutation were able to direct *tyrS* expression; an isolate containing the U26–C44 pairing normally found in tRNA^{Asn} was among the mutants tested, and

this variant resulted in lower expression than that of the BGII–G26U allele (Table 2). This result suggests that U26 of tRNA^{Asn} may play an important role in the failure of that tRNA to interact properly with the *tyrS* leader, although it is also possible that this substitution in the context of tRNA^{Tyr} perturbs tRNA folding in a nonspecific manner.

Acceptor stem

The structure of the acceptor stem can be an important determinant for recognition by the cognate aminoacyl-tRNA synthetase (McClain et al., 1999). Of the 7 bp in this stem in *B. subtilis* tRNA^{Tyr}, the only position that differs in *E. coli* tRNA^{Tyr} is A3–U70 (to U3–A70; Fig. 2); the G4–C69 and G6–C67 pairings were modified to C4–G69 and C6–G67 in the expression constructs without apparent effect. To test if base-pair substitutions predicted to destabilize the acceptor stem would affect the ability to direct *tyrS* readthrough, positions 69–72 were varied in the context of the Δ Var–U44A construct. A pool of oligonucleotide cassettes containing all possible substitutions at these positions was used to generate multiple independently derived mutant pools. After subcloning of the pooled mutants into the tRNA expression cassette, ability to direct expression of a *tyrS-lacZ* AMB–A222U fusion in *B. subtilis* was tested. Only the wild-type sequence was identified in the isolates that effectively promoted *tyrS* expression. Several negative isolates were characterized to demonstrate that the appropriate mutants were contained in the pool (Table 2). These results indicate that the structure of the acceptor stem is important for tRNA^{Tyr}-directed antitermination.

D loop mutations

Garrity and Zahler (1994) identified mutations in the D loop of tRNA^{Leu}GAG that resulted in constitutive expression of the *ilv-leu* operon, a member of the T box family for which tRNA^{Leu}GAG is predicted to be the effector (Grundy & Henkin, 1994a; Marta et al., 1996). These mutations, which included G-to-A substitutions at the very highly conserved G18 and G19 positions, were postulated to reduce charging efficiency, thereby resulting in elevated *ilv-leu* expression during growth in the presence of leucine. Because substitutions at these positions were tolerated in tRNA^{Leu}, all possible substitutions at these positions of tRNA^{Tyr}BGII were tested using an oligonucleotide cassette with a mixture of all 4 nt at these positions, and the pooled mutants were screened for expression of the *tyrS-lacZ* AMB–A222U fusion. Thirteen blue isolates from nine independently derived pools were obtained, and DNA sequencing revealed that only the wild-type GG combination was represented. A number of white isolates were also characterized to ensure that the mutagenesis was success-

ful; 9 of the possible 15 variants were identified in a random sampling of 24 white isolates, indicating that the pools contained the expected sequence variants. In addition, variants in which the GG sequence was replaced with a single C or by three bases (UCA) were obtained, possibly as the consequence of errors in oligonucleotide synthesis, or misrepair during plasmid propagation. The G18A allele, which corresponds to tRNA^{Leu} mutant *leuG5*, was specifically tested in both the BGII and Δ Var contexts, and no increase in *tyrS-lacZ* expression was observed (Table 3).

The G18/G19 mutagenesis was repeated in the context of the COMP variant of tRNA^{Tyr}, to test whether the D stem structure could influence the effect of D loop sequence variation. Again, the only variants conferring increased *tyrS-lacZ* expression retained the wild-type GG sequence. A sample white isolate containing the G18U substitution conferred only background expression (Table 3); this allele also drastically reduced amber suppression (data not shown). The failure to obtain efficient *tyrS-lacZ* expression with mutants of the G18/G19 sequence indicates that these residues are critical in tRNA^{Tyr} for antitermination, although it is unknown whether this is due to specific interactions with the leader, or more general perturbations in tRNA structure (see below). Because A at these positions is apparently tolerated in tRNA^{Leu} induction of *ilv-leu* expression, there appear to be differences in the requirements for tRNA–leader RNA interaction in the two systems, possibly reflecting the complex folding of the leader RNAs and recognition of overall tRNA structure.

T loop

The T loop region of tRNA contains certain highly conserved positions, whereas other positions are variable.

TABLE 3. D loop and T loop mutants of tRNA^{Tyr}.

Plasmid-borne tRNA ^{Tyr}	<i>tyrS-lacZ</i> AMB–U222A fusion expression ^a
None	1.5
BGII	20
BGII–G18A	0.9
BGII–G18C	0.7
BGII–G18U	0.7
COMP–G18U	1.8
Δ Var–U44A	37
Δ Var–G18A	1.3
Δ Var–G57A	16
Δ Var–A59U	10
Δ Var–A59G	47
Δ Var–G57A,A59U	8.0
Δ Var–G18A,C56G	1.1
Δ Var–G18C,C56G	1.5

^a β -galactosidase activities are in Miller units (Miller, 1972).

The consensus sequence for this region is UUCRANY; the first U (position 54) pairs with the A at position 58, and the second U (position 55) is modified to a pseudouridine which, together with the C at position 56, forms a tertiary interaction with the conserved G18/G19 of the D loop (Giege et al., 1993). Because analysis of other regions of the tRNA indicated a strong requirement for maintenance of conserved positions, mutations of the T loop at the more variable positions 57, 59, and 60 were tested, in the context of the Δ Var-U44A construct. Pools containing all possible combinations at the three positions were screened. As shown in Table 3, a number of variants were identified which retained the ability to direct *tyrS-lacZ* expression. In the tRNA variants, only G and A were found at position 57, consistent with conservation of a purine at this position. A, U, and G were found at position 59, normally not a conserved position, whereas only the U normally found in tRNA^{Tyr} was found at position 60, although tRNAs in general contain either U or C at this position, and C is found in 15 of the 86 *B. subtilis* tRNA genes (Sprinzl et al., 1998).

Only 5 of the 64 possible combinations at bases 57, 59, and 60 of the T loop were found among isolates exhibiting detectable *tyrS* expression. Nine combinations exist among the 86 tRNA genes in *B. subtilis*; 4 of the 5 functional combinations (including that found in wild-type tRNA^{Tyr}) were included among those found naturally in *B. subtilis*. The fifth functional combination (A57,U59,U60) is also absent from *E. coli* (which has 85 tRNA genes in 7 categories, all of which are found in *B. subtilis*), but is found in some other bacterial tRNAs (Sprinzl et al., 1998). The limited number of functional combinations in the T loop demonstrate restrictions on tRNA sequence and/or structure for antitermination. Other sequence combinations may be functional in a different context. At least three other *B. subtilis* tRNAs known to be active in antitermination exhibit sequence combinations in this region different from those found to be functional in the tRNA^{Tyr} context: tRNA^{Leu}GAG (A57,G59,U60); tRNA^{Val}GAC (G57,G99,C60); and tRNA^{Thr}GGU (G57,G99,C60). Although tRNA^{Thr}GGU is the effector for the *B. subtilis* *thrS* gene, it functions inefficiently for the *tyrS* leader with the appropriate specifier and antiterminator changes (Grundy et al., 1997). A C60U mutation in tRNA^{Thr}, which confers a T loop sequence represented within the functional classes in tRNA^{Tyr}, did not increase tRNA^{Thr}-directed antitermination of the *tyrS* leader, indicating that this is not the critical difference between the tRNAs (Grundy et al., 1997). The unique class found in this mutant pool but not within the normal pool of tRNAs in either *B. subtilis* or *E. coli* may indicate differences in requirements for antitermination *versus* other cellular activities of tRNA, because the mutant isolation system separated antitermination from other functions.

Covariation of T loop and D loop sequences

Positions 18 and 19 of the D loop (conserved G residues) form a tertiary interaction with the pseudouridine and C at positions 55 and 56 in the T loop (Giege et al., 1993). Because variations at the G18/G19 positions were tolerated in tRNA^{Leu} but not in tRNA^{Tyr}, we tested the possibility that these variations might be permitted in tRNA^{Tyr} in the context of covariation at the corresponding positions of the T loop. We therefore simultaneously varied all four positions in the context of both the Δ Var-U44A and BGII constructs. Only variants with the wild-type sequence at positions 18, 19, 55, and 56 were identified among isolates with significant levels of *lacZ* expression. Several isolates with low activity were sequenced to demonstrate that the pool contained the expected variants (Table 3). These results contrast with the analysis by Sampson et al. (1990) of yeast tRNA^{Phe}, where a combination of G19C and C56G substitutions allowed efficient aminoacylation by PheRS; in that case, the G18-U55 interaction was found to be essential. It therefore appears that these positions play an important role in *tyrS* antitermination beyond base pairing, and that requirements for aminoacylation may in some cases be less stringent than requirements for antitermination.

RNaseP-dependent generation of the tRNA 5' end

The effect of introduction of a requirement for RNase P processing was tested by insertion of an additional 10 bp between the transcription start point of the *rpsD* promoter and the +1 position of tRNA^{Tyr} Δ Var-U44A. This construct (5'EXT) resulted in a twofold decrease in expression (Table 2), suggesting that the tRNA from the Δ Var-U44A construct, the product of which is identical in sequence to that of construct 5'EXT, is in fact generated by transcription initiation and that RNase P processing is a limiting factor in expression of the tRNA from 5'EXT. Transcription initiation leaves a triphosphate on the 5' end of the tRNA product, rather than the normal RNase P-generated monophosphate, at a position that abuts the antiterminator in the tRNA-mRNA complex (Fig. 1). This triphosphate apparently does not interfere with interaction with the *tyrS* leader.

Northern analysis

It has previously been found that tRNA variants may exhibit reduced stability, and these effects are often unpredictable (McClain et al., 1988; Ramesh et al., 1997). Northern analysis was therefore used to measure the steady-state levels of mutant tRNAs, to assess whether effects on *tyrS* readthrough were due to differences in tRNA abundance rather than the efficiency of the tRNA-leader interaction. As shown in Fig-

ure 4, mutant tRNAs that failed to efficiently direct *tyrS* readthrough (e.g., Δ Var-G26U,U44C, BGII-G18A, Δ Var-G18A,C56G) were present at levels similar to those of variants that were efficient in antitermination (Δ Var-U44A, 5'EXT). Other mutant tRNAs gave similar results (data not shown). The 5'EXT tRNA, which includes a 10-bp extension at the 5' end of the primary transcript so that RNase P processing is required to generate the mature 5' end, was the only tRNA that appeared to be at lower abundance; this construct exhibited a small decrease in antitermination efficiency as compared to the same tRNA expressed without the 5' extension (Table 2). The larger band apparent in all lanes except 5'EXT presumably represents the tRNA^{Tyr}-tRNA^{Gln} precursor. The absence of the precursor band in the 5'EXT RNA may reflect a difference in RNase P processing kinetics when the transcript contains two RNase P cleavage sites. The control lane contains RNA from cells containing the expression plasmid with tRNA^{Gln} but lacking tRNA^{Tyr}; no hybridization was detected with the tRNA^{Tyr}-specific probe, which contained sequences maintained in all of the test tRNA constructs, but is apparently unable to hybridize under these conditions to chromosomally encoded tRNA^{Tyr}. It therefore appears that the differences in antitermination promoted by the different tRNA variants are not due to differences in tRNA abundance in the cell.

FIGURE 4. Northern analysis of tRNA levels in vivo. Total RNA was isolated from exponential growth phase cells containing a tRNA expression plasmid. RNA samples (7.5 μ g) were electrophoresed in an 8-M urea, 6% polyacrylamide gel, electroblotted to a membrane and probed using an antisense RNA probe complementary to the Δ Var-U26G variant; this probe did not hybridize to chromosomally encoded tRNA^{Tyr}. Lane 1: 5'EXT; lane 2: Δ Var-U26G; lane 3: Δ Var-G18A,C56G; lane 4: Δ Var-A44C; lane 5: BGII-U26G,G18A; lane 6: pDGS4P-tRNQ (expression plasmid with no tRNA^{Tyr}). Arrow A: tRNA^{Tyr}-tRNA^{Gln} precursor; arrow B: tRNA^{Tyr}. The increased size of the BGII-G18A tRNA is due to the presence in that variant of the wild-type long variable arm (8 additional nt).

CONCLUSIONS

The sequence and structural requirements for tRNA^{Tyr}-directed antitermination of the *B. subtilis* *tyrS* leader were analyzed using a system in which tRNA expression in vivo was separated from other tRNA functions. This study revealed that although many substitutions were tolerated, especially within helical regions of the tRNA, virtually all of the elements normally conserved in tRNA were required for productive interaction with the *tyrS* leader. Substitutions in sequence elements known to be important for formation of the tRNA tertiary structure resulted in loss of *tyrS* antitermination, indicating that the interaction between the leader and the tRNA is likely to involve recognition of the overall tertiary structure of the tRNA rather than only base-pairing interactions. In all positions of the tRNA where certain residues are conserved in all tRNA species, only the normal residues were permitted; in some cases, such as the T loop, the constraints for tRNA^{Tyr} function in antitermination appeared to exceed the stringency of the rules as established by sequence conservation.

It is likely that different members of the T box system may vary in the details of the tRNA-leader RNA interaction, because each leader has probably evolved to permit efficient interaction with its cognate tRNA and discrimination against noncognate tRNA species; these constraints are analogous to those required for specific tRNA recognition by aminoacyl-tRNA synthetases. Recognition of tRNAs by aminoacyl-tRNA synthetases in many cases involves both sequence and structural elements (Sampson et al., 1990; Bedouelle et al., 1993; Giege et al., 1993; Puglisi et al., 1993; McClain et al., 1999). It is therefore not surprising that the tRNA-leader interaction is similarly constrained, although the requirements for aminoacylation and antitermination are different. Although much of the specificity for antitermination is derived from the specifier-anticodon pairing, as well as the pairing of the variable position of the antiterminator with the discriminator position of the tRNA, it is apparent from both leader and tRNA mutant analyses that other elements participate in this recognition process.

MATERIALS AND METHODS

Bacterial strains and growth conditions

E. coli strain DH5 α (ϕ 80d*lacZ* Δ M15 *endA1 recA1 hsdR17* (*r_K⁻ m_K⁺*) *thi-1 gyrA96 relA1* Δ (*lacZYA-argF*)U169) was used for plasmid propagation, and MC1000 (*leu araD139* Δ (*ara-leu*)7697 Δ *lacX74 galE galK rpsL*) was used for generation of multimeric plasmid DNA. *B. subtilis* TH217::SP β AMB-U222A and BR151MA::SP β *rpsD*^{AM}-*lacZ* were described previously (Grundy et al., 1994). *E. coli* strains were propagated in LB medium, and *B. subtilis* strains were grown in 2XYT broth or on tryptose blood agar base plates (TBAB, Difco). Antibiotics were used at the following concentrations: ampi-

cillin: 50 $\mu\text{g}/\text{mL}$; neomycin: 5 $\mu\text{g}/\text{mL}$; chloramphenicol: 5 $\mu\text{g}/\text{mL}$ for selection, 0.1 $\mu\text{g}/\text{mL}$ for induction. IPTG (isopropyl- β -D-thiogalactopyranoside) was used at 1 mM to induce expression of the *Pspac* promoter. Xgal was used at 40 $\mu\text{g}/\text{mL}$ as an indicator of β -galactosidase activity.

Genetic techniques

Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs and were used as described by the manufacturer. DNA sequencing was carried out on plasmid DNA purified using Wizard Prep columns (Promega), and used Sequenase 2.0 (US Biochemicals). PCR was carried out using Taq DNA polymerase (Amersham) and a Perkin-Elmer 480 thermocycler. Oligonucleotide primers were purchased from Retrogen. Transformation of *E. coli* was by standard techniques. Transformation of *B. subtilis* was carried out as previously described (Henkin & Chambliss, 1984).

Plasmid constructions

Plasmid pSu3 (*supF*) was obtained from L. Kirsebom (Kirsebom & Svard, 1992). Plasmid pDG148 (Stragier et al., 1988) was obtained from W. Nicholson. All tRNA expression constructs were first generated and characterized in pGEM7Z(+) (Promega), and all constructs were confirmed by DNA sequencing. pGEM7-S4P-tRNQ was generated by joining a 185-bp fragment of the *rpsD* promoter region, isolated by PCR using primers that placed *SphI* and *HindIII* sites upstream of the promoter and an *XmaI* site at the +1 position of the promoter, with a 120-bp fragment containing the tRNA^{Gln} gene, isolated by PCR using primers that placed an *XmaI* and an *NcoI* site upstream of the tRNA, and *PstI* and *SalI* sites downstream of the tRNA; these fragments were inserted into pGEM7Z(+) digested with *SphI* and *NsiI*. tRNA^{Gln} was chosen because fusion of the 5' end of this tRNA (TGG) to the 3' CCA of a tRNA gene inserted upstream yields an *NcoI* restriction site, to facilitate tRNA cassette insertion. All isolates obtained after propagation in *E. coli* contained a U8A substitution in the tRNA^{Gln}; because this is a highly conserved position, it is likely that this mutation, which is presumed to cause a defect in charging, arose because of negative effects of overproduction of functional tRNA^{Gln} in *E. coli*.

The initial MINI tRNA segment was generated as a complementary pair of oligonucleotides designed to have an *XmaI* overhang on the 5' end of the tRNA gene and an *NcoI* overhang on the 3' end, to permit insertion into the *XmaI* and *NcoI* sites of pGEM7-S4P-tRNQ. Oligonucleotides (20 pmol, in 70 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 5 mM DTT, 50 μM rATP) were heated to 72 °C for 5 min, mixed, heated to 95 °C for 5 min, and allowed to cool at room temperature for 90 min. This mixture was added to linearized plasmid DNA, followed by incubation at 45 °C for 5 min, cooling at room temperature for 45 min, addition of T4 DNA ligase (800 U), and overnight incubation at 16 °C. The resulting DNA was introduced into *E. coli* strain MC1000, and transformants were selected on media containing ampicillin. The MINI tRNA segment (Fig. 3B) corresponded to the acceptor and anticodon arms of tRNA^{Tyr}, with *MluI*, *BspEI*, and *BssHII* sites introduced into the central region. MINI + D (Fig. 3C) was generated by insertion of a similar complementary pair of oligonucleotides inserted into

the MINI construct digested with *MluI* and *BspEI*, whereas MINI + TV (Fig. 3D) was generated in a similar way using MINI digested with *BspEI* and *BssHII*. The COMP (Fig. 2C) construct was generated from MINI + TV digested with *MluI* and *BspEI*. Additional variants were constructed in a similar manner. The 5'EXT tRNA was generated by PCR amplification of the $\Delta\text{Var-U26G}$ tRNA using a 5' primer that contained an additional 10 nt (GGGTTAATGT) between the *XmaI* site and the first position of the tRNA. All tRNA constructs, flanked by the *rpsD* promoter and tRNA^{Gln}, were subsequently moved as a *HindIII-SalI* fragment into the shuttle vector pDG148 for expression in *B. subtilis*. Ampicillin was used for selection for pDG148 in *E. coli*, and neomycin was used for selection in *B. subtilis*.

tRNA cassette mutagenesis

The mutagenesis protocol followed the general scheme for generation of tRNA variants described above, except that the complementary oligonucleotides used contained specific substitutions, mixed nucleotides at certain positions, insertions or deletions. Pairs of oligonucleotides were used to target specific regions in each mutagenesis experiment. Multiple samples of plasmid DNA generated after the ligation step were used in separate transformations so that multiple independent pools (at least 1,000 transformants total) could be screened. Plasmid DNA was isolated from the pooled transformants, and the tRNA expression cassette was isolated by digestion with *HindIII* and *SalI* and introduced into plasmid pDG148. The resulting DNA, containing a pool of insert molecules, was amplified by propagation in *E. coli* MC1000, and introduced into *B. subtilis* strain TH217::SP β AMB-U222A. Transformants (at least 500, from multiple pools of plasmid DNA) were screened for blue color on media containing Xgal as an indicator of β -galactosidase activity. Colonies with detectable color were purified and assayed for activity. Plasmid DNA was isolated and transferred into MC1000, and the DNA sequence of the insert region was determined. After sequencing, the mutant tRNA plasmids were reintroduced into *B. subtilis* and β -galactosidase activity was measured to confirm the observed phenotype.

β -galactosidase assays

Cells were grown in 2XYT medium containing neomycin and chloramphenicol until early exponential phase. Samples of the culture were collected by centrifugation, and β -galactosidase activity was measured as described by Miller (1972) using toluene permeabilization of the cells. All assays were carried out in duplicate, and growth of each mutant was performed at least twice; variation was less than 10%. For tRNA variants expressed under the control of the *Pspac* promoter, cultures were grown to early exponential phase and split, and IPTG (1 mM) was added to one of the cultures. Samples were taken from each culture 3 h after addition of IPTG.

Northern analysis

RNA was isolated from exponential growing cells as previously described (Wu et al., 1989). RNA samples (7.5 μg)

were loaded onto a 6% polyacrylamide gel containing 8 M urea. After electrophoresis, RNA was transferred to an Ambion BrightStar-Plus positively charged membrane using a BioRad Trans-Blot SD semi-dry transfer cell. The membrane was baked at 80 °C for 30 min, then probed using a Northern Max Kit (Ambion). The antisense RNA probe was generated by *in vitro* transcription of plasmid 5'EXT, linearized at a *Hind*III site at the 5' end of the insert region, using SP6 RNA polymerase and α -³²P-UTP. Hybridization and washing were carried out at 65 °C. RNA load and transfer efficiency were verified using a tRNA^{Gln} probe.

ACKNOWLEDGMENTS

We thank Leif Kirsebom for providing plasmid pSu3, Wayne Nicholson for providing plasmid pDG148, and Wade Winkler for generating the pSupF-A73U construct. This work was supported by National Institutes of Health Grant GM47823.

Received September 24, 1999; returned for revision October 22, 1999; revised manuscript received May 11, 2000

REFERENCES

- Bedouelle H, Guez-Ivanier V, Nageotte R. 1993. Discrimination between transfer RNAs by tyrosyl-tRNA synthetase. *Biochimie* 75:1099–1108.
- Condon C, Grunberg-Manago M, Putzer H. 1996. Aminoacyl-tRNA synthetase gene regulation in *Bacillus subtilis*. *Biochimie* 78:381–389.
- Francklyn C, Schimmel P. 1989. Aminoacylation of RNA minihelices with alanine. *Nature* 337:478–481.
- Garrity DB, Zahler SA. 1994. Mutations in the gene for a tRNA that functions as a regulator of a transcriptional attenuator in *Bacillus subtilis*. *Genetics* 137:627–636.
- Giege R, Puglisi JD, Florentz C. 1993. tRNA structure and aminoacylation efficiency. *Prog Nucl Acid Res* 45:129–206.
- Grundy FJ, Henkin TM. 1990. Cloning and analysis of the *Bacillus subtilis rpsD* gene, encoding ribosomal protein S4. *J Bacteriol* 172:6372–6379.
- Grundy FJ, Henkin TM. 1993. tRNA as a positive regulator of transcription antitermination in *B. subtilis*. *Cell* 74:475–482.
- Grundy FJ, Henkin TM. 1994a. Conservation of a transcription antitermination mechanism in aminoacyl-tRNA synthetase and amino acid biosynthesis genes in Gram-positive bacteria. *J Mol Biol* 235:798–804.
- Grundy FJ, Henkin TM. 1994b. Inducible amber suppressor for *Bacillus subtilis*. *J Bacteriol* 176:2108–2110.
- Grundy FJ, Hodil SE, Rollins SM, Henkin TM. 1997. Specificity of tRNA-mRNA interactions in *Bacillus subtilis tyrS* antitermination. *J Bacteriol* 179:2587–2594.
- Grundy FJ, Rollins SM, Henkin TM. 1994. Interaction between the acceptor end of tRNA and the T box stimulates antitermination in the *Bacillus subtilis tyrS* gene: A new role for the discriminator base. *J Bacteriol* 176:4518–4526.
- Henkin TM. 1994. tRNA-directed transcription antitermination. *Mol Microbiol* 13:381–387.
- Henkin TM, Chambliss GH. 1984. Genetic mapping of a mutation causing an alteration in *Bacillus subtilis* ribosomal protein S4. *Mol Gen Genet* 193:364–369.
- Henkin TM, Glass BL, Grundy FJ. 1992. Analysis of the *Bacillus subtilis tyrS* gene: Conservation of a regulatory sequence in multiple tRNA synthetase genes. *J Bacteriol* 174:1299–1306.
- Kirsebom LA, Svard SG. 1992. The kinetics and specificity of cleavage by RNase P is mainly dependent on the structure of the amino acid acceptor stem. *Nucleic Acids Res* 20:425–432.
- Luo D, Leautey J, Grunberg-Manago M, Putzer H. 1997. Structure and regulation of expression of the *Bacillus subtilis* valyl-tRNA synthetase gene. *J Bacteriol* 179:2472–2478.
- Marta PT, Ladner RD, Grandoni JA. 1996. A CUC triplet confers leucine-dependent regulation of the *Bacillus subtilis* *ilv-leu* operon. *J Bacteriol* 178:2150–2153.
- McClain WH, Gabriel K, Bhattacharya S, Jou Y-Y. 1999. Functional compensation by particular nucleotide substitutions of a critical G·U wobble base-pair during aminoacylation of transfer RNA. *J Mol Biol* 286:1025–1032.
- McClain WH, Wilson JH, Seidman JG. 1988. Genetic analysis of structure and function in phage T4 tRNA^{Ser}. *J Mol Biol* 203:549–553.
- Miller J. 1972. *Experiments in molecular genetics*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
- Puglisi JD, Putz J, Florentz C, Giege R. 1993. Influence of tRNA tertiary structure and stability on aminoacylation by yeast aspartyl-tRNA synthetase. *Nucleic Acids Res* 21:41–49.
- Putzer H, Laalami S, Brakhage AA, Condon C, Grunberg-Manago M. 1995. Aminoacyl-tRNA synthetase gene regulation in *Bacillus subtilis*: Induction, repression, and growth-rate regulation. *Mol Microbiol* 16:709–718.
- Ramesh V, Varshney U, RajBhandary UL. 1997. Intragenic suppression in tRNA: Evidence for crosstalk between the D and T stems. *RNA* 3:1220–1232.
- Sampson JR, DiRenzy AB, Behlen LS, Uhlenbeck OC. 1990. Role of the tertiary nucleotides in the interaction of yeast phenylalanine tRNA with its cognate synthetase. *Biochemistry* 29:2523–2532.
- Sprinzi M, Horn C, Brown M, Ioudovitch A, Steinberg S. 1998. Compilation of tRNA sequences and sequences of tRNA genes. *Nucleic Acids Res* 26:148–153.
- Stragier P, Bonamy C, Karamazyn-Campelli C. 1988. Processing of a sporulation sigma factor in *Bacillus subtilis*: How morphological structure could control gene expression. *Cell* 52:697–704.
- Wang S, Huber PW, Cui M, Czarnik AW, Mei H-Y. 1998. Binding of neomycin to the TAR element of HIV-1 RNA induces dissociation of Tat protein by an allosteric mechanism. *Biochemistry* 37:5549–5557.
- Wu JJ, Howard MG, Piggot PJ. 1989. Regulation of transcription of the *Bacillus subtilis* *spoIIA* locus. *J Bacteriol* 171:692–697.