JOURNAL OF BACTERIOLOGY, Apr. 1997, p. 2587–2594 Vol. 179, No. 8
0021-9193/97/$04.00+0

Specificity of tRNA-mRNA Interactions in *Bacillus subtilis tyrS* Antitermination

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Received 20 December 1996/Accepted 12 February 1997

The *Bacillus subtilis tyrS* gene, encoding tyrosyl-tRNA synthetase, is a member of the T-box family of genes, which are regulated by control of readthrough of a leader region transcriptional terminator. Readthrough is induced by interaction of the cognate uncharged tRNA with the leader; the system responds to decreased tRNA charging, caused by amino acid limitation or insufficient levels of the aminoacyl-tRNA synthetase. Recognition of the cognate tRNA is mediated by pairing of the anticodon of the tRNA with the specifier sequence of the leader, a codon specifying the appropriate amino acid; a second interaction between the acceptor end of the tRNA and an antiterminator structure is also important. Certain switches of the specifier sequence to a new codon result in a switch in the specificity of the amino acid response, while other switches do not. These effects may reflect additional sequence or structural requirements for the mRNA-tRNA interaction. This study includes investigation of the effects of a large number of specifier sequence switches in *tyrS* and analysis of structural differences between $tRNA^{Tyr}$ and tRNA species which interact inefficiently with the *tyrS* leader to promote antitermination.

Many aminoacyl-tRNA synthetase and amino acid biosynthesis operons in gram-positive bacteria are regulated by a common transcription antitermination system (11, 12, 16, 28). The genes in this group share a number of features in the 5′ region in their transcripts, including a set of conserved primary sequence and structural elements located upstream of the start of the coding sequence. The mRNA leader region structural elements include three large stem-loops, designated stems I, II, and III, preceding a factor-independent transcriptional terminator. The most prominent of the sequence elements, the 14-base T box, forms a portion of an antiterminator structure by pairing with conserved sequences on the 5′ side of the terminator. Expression of the genes in this group is therefore controlled at the level of readthrough of the leader region transcriptional terminator, by a switch between the antiterminator and terminator forms of the transcript.

Expression of several genes in this family has been shown to be induced in response to limitation for the cognate amino acid and not by general amino acid starvation (5, 6, 18, 27, 29). Examination of a number of the leader regions revealed a precisely located single codon, specifying the appropriate amino acid (11); the effector signalling amino acid limitation was therefore proposed to be tRNA, with the specificity of the amino acid response dictated by codon-anticodon pairing. The induction specificity of the *Bacillus subtilis tyrS* gene was switched from a response to tyrosine limitation to a response to phenylalanine limitation by a single base substitution in the UAC tyrosine codon of the *tyrS* leader to a UUC phenylalanine codon (11). These studies indicated that the leader region codon, termed the "specifier sequence," acts as a critical determinant of the specificity of the amino acid response. Similar specificity switches were subsequently carried out for *B. subtilis ilv-leu* (to phenylalanine, tyrosine, and arginine) and *pheS* (to threonine), while a similar experiment for *thrS* (to phenylalanine) failed to result in a switch in the amino acid response (21, 29).

A second interaction between the acceptor end of the tRNA and the antiterminator side-bulge was initially suggested by covariance at that position; mutational studies with *tyrS* indicated that pairing in this region also contributes to the specificity of the tRNA-mRNA interaction (13). Induction by uncharged tRNA is apparently blocked by the presence of charged tRNA matching the specifier sequence, suggesting that it is the ratio of uncharged to charged tRNA that controls readthrough (4, 13, 29).

Although certain specifier sequence mutations were shown to result in a switch in the amino acid response, other switches failed to work, even when accompanied by a secondary mutation in the antiterminator side-bulge to foster the interaction with the acceptor end of the tRNA (16, 29). Since uncharged tRNA is the effector recognizing these specificity determinants in the leader mRNA, the success of some but not all switches suggests that there are sequence or structural determinants important for mRNA-tRNA recognition in addition to codon-anticodon pairing at the specifier sequence and pairing of the acceptor end of the tRNA with the leader region antiterminator. The goal of this study is to further investigate these determinants.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Table 1. *B. subtilis* strains were routinely propagated on tryptose blood agar base medium (Difco). Minimal medium for amino acid limitation experiments has been described by Anagnostopoulos and Spizizen (1). *Escherichia coli* JM103 was used for propagation of phage M13 isolates, and DH5α was used for plasmid propagation. *E. coli* strains were generally grown in Luria-Bertani medium (23). Antibiotics were used at the following concentrations (in micrograms per milliliter): ampicillin, 50; chloramphenicol, 5; neomycin, 5; erythromycin, 1; lincomycin, 25; spectinomycin, 200.

Genetic techniques. *B. subtilis* chromosomal DNA isolation and transformation were as previously described (17). Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs or Promega and were used according to the instructions of the manufacturer. Phage M13 derivatives were propagated as described by Messing (22). DNA sequencing was carried out with Sequenase DNA polymerase (United States Biochemicals) on single-stranded M13 or double-stranded plasmid templates. An asparagine auxotroph (strain SH101) was isolated by introduction of pooled DNA from a

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TABLE 1. Bacterial strains

Strain	Genotype	Source or reference
B. subtilis		
TH217	*tyrA1 trpC2 thr-5*	18
1A5	*glyB133 metC3 tre-12 trpC2*	BGSC[a]
1A9	*ald-1 aroG932 leuB8 trpC2*	BGSC
1A10	*hisA1 thr-5 trpC2*	BGSC
1A109	*ilvB2 trpC2*	BGSC
1A231	*ilvA2 trpC2*	BGSC
1A621	*serC82*::Tn*917 trpC2*	BGSC
1A622	*arg*(*GH*)*85*::Tn*917 trpC2*	BGSC
1A652	*pro*(*AB*)	BGSC
BR151MA	*lys-3 trpC2*	10
ZB307A	*SP*β*c2del2*::Tn*917*::pSK10Δ6	35
ZB449	*trpC2 pheA1 abrB703* (SPβ cured)	24
KS115	*cysA14 hisA1 leuA8 metC3 trpC2*	K. Sandman
SH101	*lys-3 trpC2 asn-1*::Tn*917lac*	This study
SH102	*lys-3 trpC2 asn-1*::Tn*917*::p917::Sp	This study
E. coli		
DH5α	ϕ80d*lacZ*Δ*M15 endA1 recA1 hsdR17*(r_K^- m_K^+) *thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)U169	Bethesda Research Laboratories
JM103	*endA1 supE44 sbcBC thi-1 rpsL* Δ(*lac-pro*)/F′*traD36 lacI*[q]ZΔM15 *proAB*	22
RZ1032	*thi-1 relA1 spoT1 dut-1 ung-1 zbd-279*::Tn*10 supE44* HfrKL16 Po/45	19

[a] BGSC, *Bacillus* Genetic Stock Center, Ohio State University.

Tn*917lac* library of transposon insertions (obtained from R. Yasbin) into strain BR151MA by selection for erythromycin and lincomycin resistance conferred by the *erm* gene of the transposon and screening for isolates which required asparagine for growth. Strain SH102 was generated by replacing the *erm* and *lac* portions of the transposon with a *spc* cassette by transformation with plasmid p917::Sp (31), selection for spectinomycin resistance, and screening for erythromycin sensitivity.

Site-directed mutagenesis of the *tyrS* specifier sequence. Oligonucleotide-directed mutagenesis was carried out as described by Kunkel (19), by using an M13 template containing the 0.8-kb *Hin*dIII-*Xba*I *tyrS* promoter-leader DNA fragment (18). The template DNA contained the TyrPHE mutation (11), since this variant contains an *Eco*RI restriction site spanning the specifier sequence; isolates containing new mutations could therefore be identified by screening for loss of the *Eco*RI site in the starting DNA. Mutagenic oligonucleotides were purchased from Cruachem. These oligonucleotides were designed to maintain the conserved C at the third position of the specifier sequence (11, 16) and to vary the first two positions. Mutants (Fig. 1) were identified by DNA sequencing. Two variants containing the ACC threonine specifier sequence were tested: TyrTHR2 contained the ACC at the normal UAC position, while TyrTHYR contained an extra base introduced after the normal UAC (UACC), so that the ACC triplet was at the usual specifier position, with the UAC displaced one position upstream of its normal location.

Construction of *tyrS-lacZ* fusions. The 0.7-kb *Bgl*II-*Xba*I *tyrS* fragment was subcloned from M13 into plasmid pFG328 (14) to generate a transcriptional fusion to a *lacZ* reporter gene. When necessary, the promoter-proximal *Bgl*II-*Bgl*I fragment was combined with a downstream *Bgl*I-*Xba*I fragment from a *tyrS* isolate containing the appropriate antiterminator side-bulge mutation, so that pairing at this region would be maintained for the tRNA matching the specifier sequence mutation. The *tyrS-lacZ* fusion constructs were introduced in single copy into the *B. subtilis* chromosome by recombination into the SPβ prophage carried in strain ZB307A and purified by passage of the phage through strain ZB449 (24, 35). The phage carrying the fusion was then introduced into a *B. subtilis* host strain carrying an appropriate amino acid auxotrophic mutation.

Amino acid starvation experiments. Cells were grown in minimal medium containing excess levels of all required amino acids (generally 50 μg/ml; 100 μg/ml for arginine and serine and 250 μg/ml for asparagine). Strains 1A621 (*serC82*::Tn*917*) and SH102 (*asn-1*::Tn*917*::*spc*) also required 0.02% Casamino Acids for good growth in Spizizen minimal medium. At mid-exponential growth phase, cells were collected by centrifugation and resuspended in media containing either excess amino acids or limiting levels (5 μg/ml) of a single amino acid. Growth was continued for 4 to 5 h, and samples were taken for β-galactosidase assays at 0.5- to 1-h intervals during the incubation period, as previously described (11). A range of growth conditions and different strain backgrounds were tested for isolates which failed to show a response, to ensure that the appropriate conditions were tested, and two independent constructs were tested for each specifier mutation. β-Galactosidase activity was measured in toluene-permeabilized cells as described by Miller (23).

Isolation of tRNA^{Thr}. The gene encoding tRNA^{Thr} (GGU anticodon to match the ACC threonine specifier which is present in *B. subtilis thrS*) is located in the *trnI* operon (33). PCR primers (5′, ACCTGCGAAGCTTTGGTTCCATAGCT CAG[T/C]AGGTAGA; 3′ reverse complement, GGTAATAGTCGACTTAAG GTTCCAAGCGG[G/A]CTCGAAC) flanking this gene were designed based on the published sequence, with the inclusion of the C2G and G71C substitutions (Fig. 2), which were shown for *E. coli* tRNA^{Thr} to block charging by threonyl-tRNA synthetase (15). Chromosomal DNA of *B. subtilis* BR151MA was used as the template for PCR. The primers also included *Hin*dIII and *Sal*I restriction enzyme sites, to permit insertion of the tRNA gene into plasmid pDG148 (32), so that expression would be under the control of the P_{spac} promoter, as previously described for *B. subtilis* tRNA^{Tyr} (13). Isolates of tRNA^{Thr} with mutations at positions 16 and 60 were generated by incorporating a mixture of wild-type and mutant nucleotides at the corresponding positions of the PCR primers. Anticodon and position 32 mutations were generated by oligonucleotide-directed mutagenesis. All mutants were introduced into pDG148, and the resulting constructs, which contained identical segments of tRNA^{Thr} DNA, were verified by DNA sequencing. Plasmids were introduced into *B. subtilis* containing appropriate *tyrS-lacZ* fusions, by selection for the plasmid-borne neomycin resistance determinant. Expression of the tRNA was induced by addition of IPTG (isopropyl-β-D-thiogalactopyranoside) (1 mM), and samples were assayed for β-galactosidase as previously described (13).

RESULTS

Specifier sequence switches in the *B. subtilis tyrS* leader. Alteration of the UAC tyrosine codon in the *B. subtilis tyrS* leader to a UUC phenylalanine codon was previously shown to result in loss of response to tyrosine limitation and a switch to induction in response to limitation for phenylalanine, indicating that tRNA^{Phe} is capable of directing *tyrS* antitermination (11). To expand our understanding of the abilities of different tRNAs to interact with the *tyrS* leader, we introduced a variety of codons at the specifier sequence, with the goal of identification of tRNA features common to tRNAs which successfully interact with the *tyrS* leader. Since C is strongly conserved at the third position of the specifier (11, 16), and alteration of the UAC tyrosine codon of *tyrS* to a UAU tyrosine codon reduces readthrough 54-fold under inducing conditions (30), we chose to maintain a C at the third position and vary the first two nucleotides (Fig. 1). Pairing between the discriminator base of tRNA and the variable position of the antiterminator side-bulge has also been shown to contribute to the tRNA-mRNA interaction (13). We therefore combined the specifier sequence mutations with the appropriate antiterminator mutations (U222A or U222C) in those cases where the discriminator base (position 73) of the tRNA matching the

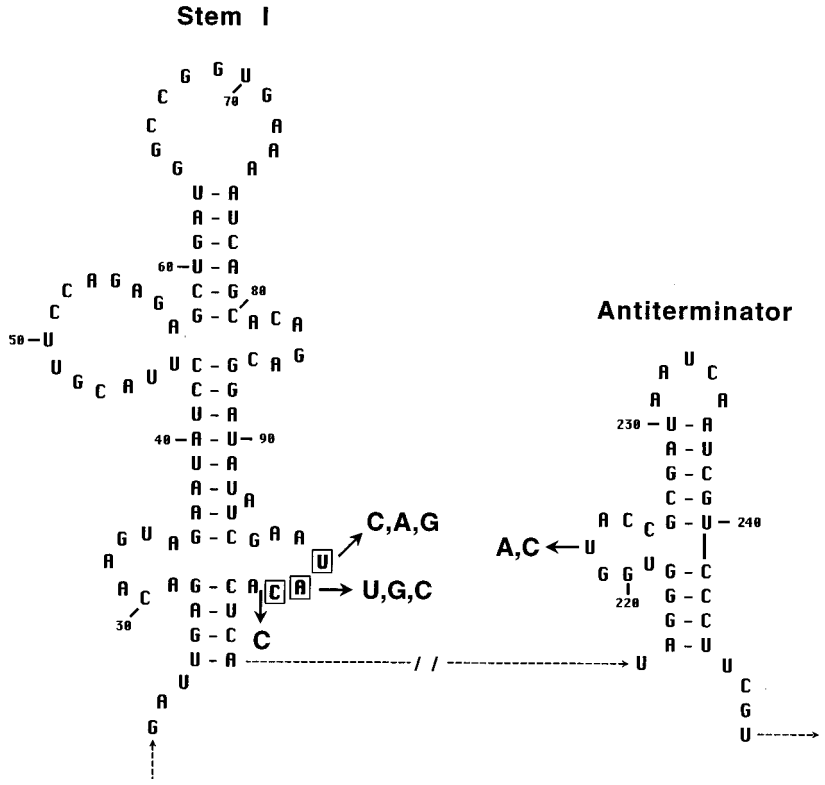

FIG. 1. Structural model of the *tyrS* leader RNA. Stems II and III are omitted for clarity, and only the antiterminator form, which interacts with tRNA, is shown. Positions relative to the start point of transcription are indicated. The UAC tyrosine specifier sequence is boxed, and arrows indicate mutational alterations.

mutant specifier sequence varied from the A found in tRNA^{Tyr}. *tyrS-lacZ* fusions containing the resulting mutant leaders were introduced into the appropriate auxotrophic strain background to test for induction in response to limitation for the amino acid specified by the new specifier sequence.

Mutations of the UAC tyrosine codon in *tyrS* to phenylalanine (UUC), leucine (CUC), isoleucine (AUC), or valine (GUC) codons all resulted in efficient induction in response to limitation for the appropriate amino acid (Table 2). Glycine (GGC) or cysteine (UGC) specifiers also resulted in successful specificity switches, when combined with the U222A antiterminator mutation. These results indicate that the tRNAs matching these codons are capable of efficient interaction with the *tyrS* leader. The induction ratios for these mutants varied from 3.4- to 6.1-fold, in comparison to the 15-fold induction of the wild-type *tyrS-lacZ* fusion by limitation for tyrosine, suggesting that features in addition to the anticodon and discriminator base contribute to the tRNA-mRNA interaction. Alternatively, the amino acid limitation conditions may not result in as great an effect on the tRNA charging ratio, for unknown reasons. Changing the specifier to two different serine codons, UCC and AGC, also provided a successful switch, albeit at lower efficiency, when combined with the U222C mutation. A fusion with a CCC proline codon could be only weakly induced (1.5-fold), suggesting that tRNA^{Pro} differs from tRNA^{Tyr} and the tRNAs which interact efficiently with *tyrS* in some important feature(s); no tRNA^{Pro} with the appropriate anticodon to match a CCC specifier has been reported (9), although tRNAs capable of reading this codon in the context of translation, via wobble, do exist.

Three specifier sequence mutations, asparagine, arginine, and histidine, failed to result in induction in response to limitation for the amino acid specified by the mutant codon (Table 2). An AAC asparagine codon, in conjunction with the U222C antiterminator mutation which matches the discriminator position of tRNA^{Asn}, gave no response to limitation for asparagine. tRNA^{Asn} is unusual in that it contains a U at position 26, whereas nearly all tRNAs contain a purine at that position (Fig. 3); one variant of tRNA^{Gly} contains a C at this position, but this is not the species of tRNA^{Gly} which matches the GGC glycine specifier. tRNA^{Asn} and tRNA^{Gly} are nearly identical in unpaired regions, except for this position (33). Another unusual feature of tRNA^{Asn} is the U1-A72 pairing at the top of the acceptor arm; most tRNAs, including all known to function in *tyrS* antitermination, contain a G1-C72 pairing.

A CGC arginine codon, in combination with the U222C antiterminator mutation, gave no response to arginine limitation. Switches of the *ilv-leu* leucine specifier to arginine, tyrosine, and phenylalanine (21) and of *tyrS* to leucine and phenylalanine were successful, suggesting that the tRNA recognition properties of the *ilv-leu* and *tyrS* leaders are similar; however, the failure of the arginine switch to work for *tyrS* indicates that tRNA-mRNA recognition is likely to be complex. Although no tRNA^{Arg} with an anticodon to match CGC has been reported (9), the success of the arginine specifier switch in *ilv-leu* indicates that the cell must contain a tRNA capable of interacting with this specifier, at least in the context of the *ilv-leu* leader.

A CAC histidine specifier mutant also failed to give a switch to histidine specificity. One explanation for this result is that tRNA^{His} is unique in that base 73 in the acceptor stem is paired, i.e., there is no unpaired discriminator base (Fig. 3). Since pairing of base 73 of the tRNA with the antiterminator side-bulge was shown to be important for *tyrS* antitermination

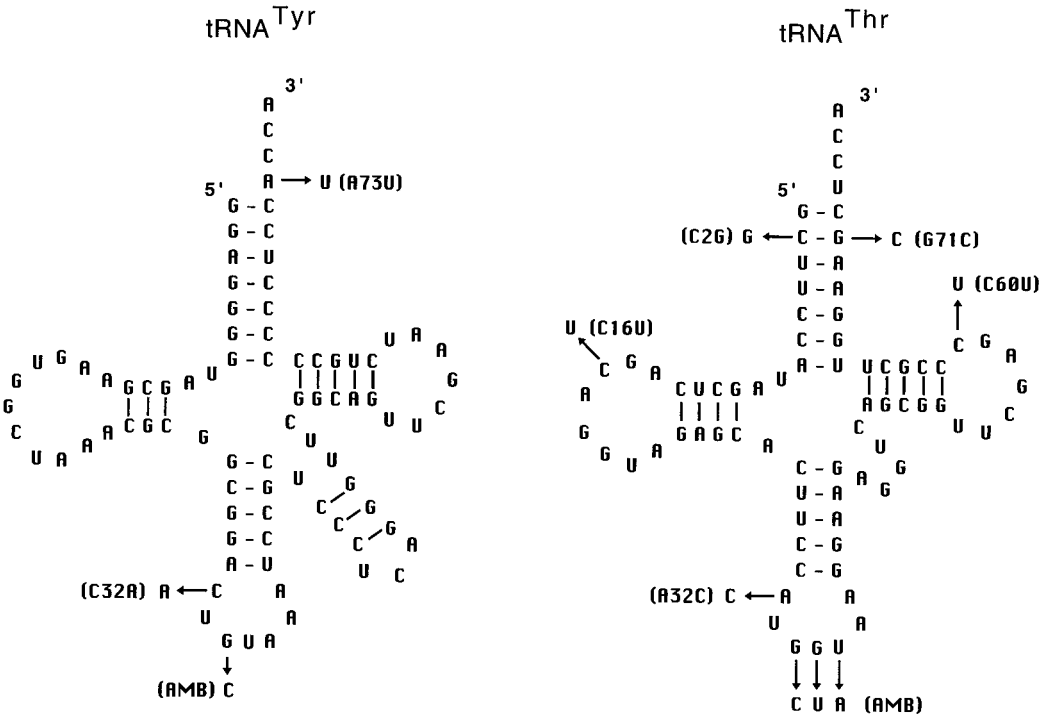

FIG. 2. Structure of $tRNA^{Tyr}$ and $tRNA^{Thr}$. Arrows indicate mutational alterations.

(13), the unavailability of this position in $tRNA^{His}$ may preclude pairing with the *tyrS* leader. The *Lactococcus lactis his* operon is apparently a member of the T-box family (12); other determinants in the *his* operon leader may compensate for the absence of pairing at this position, assuming that $tRNA^{His}$ in *L. lactis* also has this generally conserved feature of $tRNA^{His}$.

Two different variants containing ACC threonine codons responded weakly to threonine limitation (Table 3). The first (THYR) contained an extra C introduced after the UAC tyrosine codon, so that ACC was now located in an appropriate position in the specifier side-bulge. This mutation resulted in a threefold drop in the response to tyrosine but no response to threonine (Table 3); introduction of the U222A antiterminator mutation resulted in a drop in the response to tyrosine but only a very small response to limitation for threonine. Since the phenotype of this mutant could have been due to the altered placement of the specifier sequence, the THR2 mutant, in which the UAC codon was replaced by ACC in the normal specifier sequence position, was also tested. The THR2 mutant exhibited no response to tyrosine, as expected since the UAC tyrosine codon was now absent, and only a weak response to threonine even in combination with the U222A mutation. These variant leaders match the corresponding tRNAs at both known positions of mRNA-tRNA pairing, suggesting that

TABLE 2. Effects of specifier sequence mutations on expression of *tyrS-lacZ* fusions[a]

Specifier sequence	Amino acid	Antiterminator	Strain	β-Galactosidase activity (Miller units)		
				Uninduced	Induced	Ratio
UAC	Tyrosine	UGGU	TH217	17	260	15
UGC	Cysteine	UGGA	KS115	31	190	6.1
UUC	Phenylalanine	UGGU	ZB449	1.5	8.5	5.7
GUC	Valine	UGGU	1A109	20	92	4.6
GGC	Glycine	UGGA	1A5	23	99	4.3
AUC	Isoleucine	UGGU	1A231	18	73	4.1
CUC	Leucine	UGGU	1A9	16	54	3.4
AGC	Serine	UGGC	1A621	41	110	2.7
UCC	Serine	UGGC	1A621	47	96	2.0
ACC	Threonine	UGGA	1A10	5.9	14	2.4
CCC	Proline	UGGU	1A652	25	37	1.5
CGC	Arginine	UGGC	1A622	24	21	0.88
CAC	Histidine	UGGU	KS115	1.1	0.76	0.69
AAC	Asparagine	UGGC	SH102	78	62	0.80

[a] Cells were grown in Spizizen minimal medium containing excess levels of all required amino acids and then were split and grown in excess (uninduced) or limiting (induced) levels of the amino acid corresponding to the specifier sequence. Growth was continued for 4 h, and samples were harvested at intervals. Data for the time points resulting in maximal induction values for β-galactosidase activity are shown.

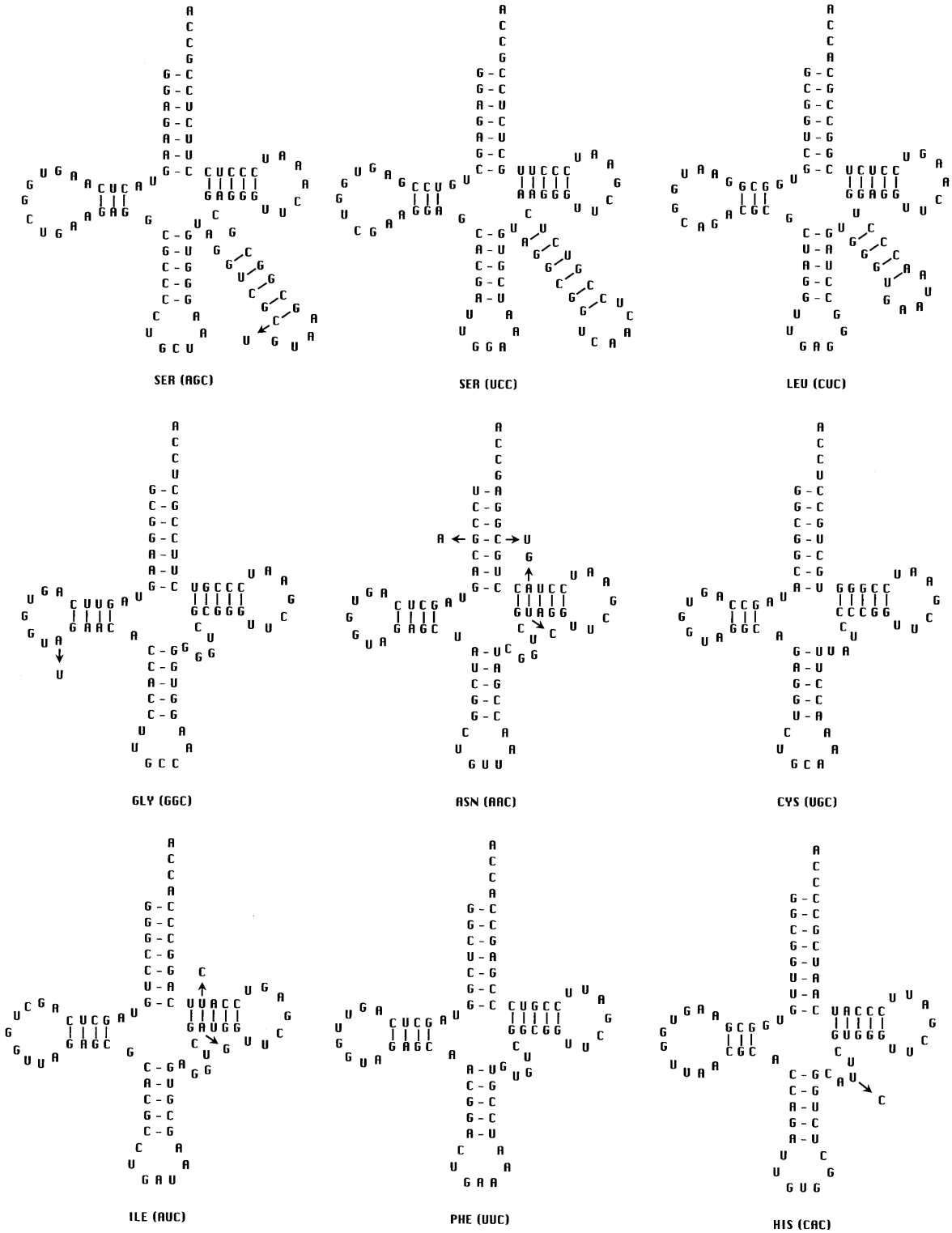

FIG. 3. Structure of tRNA species matching the specifier sequence mutations. No variants of $tRNA^{Val}$, $tRNA^{Arg}$, or $tRNA^{Pro}$ with the appropriate anticodon have been identified. Arrows indicate positions of sequence variation in tRNA variants with the same anticodon. tRNA sequences are from published data (3, 7, 8, 25, 33, 34).

there are additional sequence or structural determinants for mRNA-tRNA recognition.

Mutants of $tRNA^{Thr}$ with improved induction of *tyrS*. To confirm that $tRNA^{Thr}$ is unable to efficiently induce antitermination in a *tyrS* variant containing an ACC threonine specifier and U222A antiterminator mutations, unchargeable variants of $tRNA^{Thr}$ were tested. It has previously been shown that the presence in the cell of a chargeable tRNA capable of interaction with the leader region specifier sequence can interfere with induction (4, 13). We therefore tested a variant of

TABLE 3. Responses of *tyrS-lacZ* fusions containing threonine specifier sequences to limitation for threonine or tyrosine[a]

Host strain (limiting amino acid) and *tyrS-lacZ* fusion	β-Galactosidase activity		Induction ratio
	Uninduced	Induced	
TH217 (tyrosine)			
Wild type	17	260	15
THYR	1.4	7.4	5.3
THYR-KpA	1.2	1.8	1.5
THR2	1.8	2.3	1.3
THR2-KpA	2.9	3.4	1.2
1A10 (threonine)			
Wild type	36	26	0.72
THYR	5.3	5.8	1.1
THYR-KpA	3.5	5.9	1.7
THR2	4.6	6.0	1.3
THR2-KpA	5.9	14	2.4

[a] Cells were grown in minimal medium containing excess levels of all required amino acids and then were split and grown in medium containing excess or limiting levels of a single amino acid. Cell samples were harvested at 30-min intervals and were assayed for β-galactosidase activity (Miller units); data from the time points giving maximal induction values are shown.

$tRNA^{Thr}$ in which the anticodon had been changed to match a UAG amber nonsense specifier sequence, since in a wild-type cell background there should be no tRNA to match. Expression of these tRNAs was conditional and independent of starvation for threonine. Mutations at positions 2 and 71, in the acceptor stem, were also introduced since these mutations, in addition to anticodon mutations, were shown in *E. coli* to block charging by threonyl-tRNA synthetase (15).

IPTG-induced expression of $tRNA^{Tyr}$ (AMB/A73U) resulted in a 20-fold induction of a fusion containing a UAG amber specifier and the U222A antiterminator mutation (Table 4). In contrast, expression of $tRNA^{Thr}$ (with an amber anticodon) resulted in 13-fold induction. A $tRNA^{Thr}$ construct with these changes as well as an anti-amber anticodon failed to suppress an amber mutation in an *rpsD-lacZ* translational fusion, indicating that the tRNA is unchargeable (30a). This result suggests that there are important structural differences between $tRNA^{Tyr}$ and $tRNA^{Thr}$ in addition to the anticodon and discriminator positions.

TABLE 4. Induction of the AMB-U222A *tyrS-lacZ* fusion by expression of variants of $tRNA^{Tyr}$ and $tRNA^{Thr}$[a]

Plasmid tRNA and mutation	β-Galactosidase activity		Induction ratio
	− IPTG	+ IPTG	
None	1.4	1.4	1.0
Tyrosyl			
AMB/A73U	1.9	38	20
AMB/A73U/C32A	1.0	21	21
Threonyl			
AMB	1.8	24	13
AMB/A32C	1.8	48	27
AMB/C16U/C60U	1.4	20	14
AMB/C16U/C60U/C32A	1.4	43	31

[a] Cells were grown in 2XYT medium (23) until mid-exponential growth phase, diluted fourfold, and split, and IPTG (1 mM) was added to one culture to induce expression of the plasmid-borne tRNA. Cells were harvested after 2.5 h and assayed for β-galactosidase activity (Miller units). AMB, amber.

Comparison of $tRNA^{Thr}$ and $tRNA^{Tyr}$ revealed several structural differences (Fig. 2). $tRNA^{Tyr}$ contains a long variable arm; however, other tRNAs with short variable arms (like $tRNA^{Phe}$) interacted efficiently with the *tyrS* leader, indicating that this difference was unlikely to be important. Another difference between $tRNA^{Thr}$ and $tRNA^{Tyr}$ (and other tRNAs which interact with *tyrS*) is the presence of C residues at positions 16 and 60 in $tRNA^{Thr}$, instead of U residues in the other tRNAs; $tRNA^{Ile}$ also contains a C at position 16. An unusual feature of $tRNA^{Thr}$ is the presence of an A at position 32 in the anticodon loop; this position is a C or U in all of the tRNAs which direct *tyrS* antitermination and is highly conserved as a pyrimidine in tRNAs in general. Mutations of $tRNA^{Thr}$ in which bases 16, 60, and 32 were changed to the corresponding residues of $tRNA^{Tyr}$ were therefore tested to determine whether these alterations could increase the efficiency of $tRNA^{Thr}$-directed *tyrS* antitermination.

Introduction of the mutations at positions 16 and 60 had no effect on $tRNA^{Thr}$ induction of *tyrS-lacZ* expression, while the base 32 mutation gave a twofold increase in induction, either alone or in combination with the mutations at positions 16 and 60. The base 32 mutation had the same relative effect in the context of an ACC threonine specifier, although the induction efficiency was much lower for the ACC specifier constructs (30a). Poor induction of ACC specifier constructs could be due to sequence requirements for codon-anticodon pairing in the context of the *tyrS* leader, because of specific structural constraints in this region, or secondary effects of the anticodon sequence on tRNA structure or modifications (2); alternatively, interference by cellular $tRNA^{Thr}$ could play a role. Alteration of the C at position 32 in $tRNA^{Tyr}$ to an A, as found in $tRNA^{Thr}$, had no effect (Table 4), indicating that the base at this position does not act as a strong identity determinant in the context of the otherwise perfectly matched cognate tRNA.

DISCUSSION

The anticodon and discriminator base of tRNA have previously been demonstrated to be critical determinants of the specificity of tRNA-directed induction of antitermination in T-box family genes (11, 13, 21, 29). The goal of this study was to determine if these are the only elements responsible for the specificity of the tRNA-mRNA interaction. One approach to this question was to alter the specifier sequence of the *tyrS* gene to a variety of different codons in order to determine if the tRNAs matching those codons were capable of directing *tyrS* antitermination. These experiments revealed that many specifier sequence switches were successful, although to different degrees. The second approach was to directly compare $tRNA^{Tyr}$ with tRNAs which apparently interact inefficiently with the *tyrS* leader, to attempt to identify critical differences, and to test the importance of these sequence elements by mutagenesis.

Twelve new specifier sequence mutations were tested in this study, in addition to the normal UAC tyrosine codon and the UUC phenylalanine mutation previously reported (11). Mutants with aspartate and alanine specifier codons were also obtained but could not be tested because the corresponding auxotrophic mutant strains were not available. A new asparagine auxotroph was isolated in the course of this study, so that the effect of this specifier sequence switch could be determined.

These experiments demonstrated that tRNAs specific for phenylalanine, cysteine, valine, isoleucine, leucine, and glycine could interact relatively efficiently with the *tyrS* leader, although less effectively than the cognate $tRNA^{Tyr}$. Two differ-

ent seryl-tRNAs, as well as $tRNA^{Thr}$ and $tRNA^{Pro}$, interacted inefficiently with *tyrS*, while $tRNA^{Arg}$, $tRNA^{Asn}$, and $tRNA^{His}$ apparently failed to interact at all to promote antitermination. A match between the discriminator base of the tRNA and the *tyrS* antiterminator side-bulge was maintained in all of these experiments, so that the efficiency of induction reflects the overall interaction of the tRNA with the leader, beyond codon-anticodon and acceptor end-antiterminator base pairing. The histidine specifier mutant is an exception to this rule, in that $tRNA^{His}$ lacks a free discriminator base for pairing with the antiterminator; this may explain the inability of this tRNA to direct *tyrS* antitermination.

Certain specifier mutations (phenylalanine, threonine, and histidine) resulted in a drop in basal expression. This could be due to variations in the ability of an unknown tRNA to interact with the leader to induce readthrough in the absence of amino acid limitation. Alternatively, the specifier sequence change could perturb leader structure in some way to reduce readthrough.

The absence of a tRNA containing the appropriate anticodon (e.g., proline or arginine) could be responsible for failure of the switch, if constraints for specifier-anticodon interaction are different from those for codon-anticodon interaction during translation. For example, a single species of $tRNA^{Tyr}$, with an anticodon to match the UAC *tyrS* specifier, has been identified; this tRNA must translate UAU tyrosine codons, by wobble pairing, yet a UAU specifier sequence mutation results in a severe decrease in *tyrS* readthrough (30). In contrast, no variant of $tRNA^{Val}$ with the appropriate anticodon has been reported, yet the switch to a valine specifier was successful. Failure of a switch could be due to inefficient wobble pairing between the specifier sequence and the anticodon or to structural differences from $tRNA^{Tyr}$ which prevent productive interaction with the *tyrS* leader. Structural differences from tRNAs which function in *tyrS* antitermination were most obvious for $tRNA^{His}$, $tRNA^{Asn}$, and $tRNA^{Thr}$.

The inefficiency of the interaction between $tRNA^{Thr}$ and the *tyrS* leader was chosen for further analysis, because of obvious structural differences between $tRNA^{Thr}$ and those tRNAs which could mediate antitermination and because of supporting data from the analysis of *B. subtilis thrS*, where a switch from the threonine specifier sequence to a phenylalanine specifier also failed to result in a switch in the amino acid limitation response (29). Three residues of $tRNA^{Thr}$ (C16, A32, and C60) were identified as possible candidates for modulating the tRNA-mRNA interaction. Positions 16 and 60 participate in formation of the variable pocket, which is a patch of the tRNA that at least for yeast $tRNA^{Phe}$ forms a distinct region on the surface of the molecule (20). Position 32 is nearly always a pyrimidine, while $tRNA^{Thr}$ contains an A. These positions of $tRNA^{Thr}$ were altered to match the sequence of $tRNA^{Tyr}$, and the effects of these modifications, alone and in combination, on a variant of *tyrS* containing a matching UAG amber specifier and the U222A antiterminator mutation were tested. The A32U mutation proved to have a modest influence on *tyrS* induction, while the other mutations had no effect.

The variability in the efficiency of induction of *tyrS* variants containing different specifier sequences could be due to differences in accumulation of uncharged tRNA under amino acid limitation conditions. A variety of conditions were tested, by time course assays, to avoid this problem. It is nevertheless possible that ideal conditions could not be identified for a given amino acid. The overall result, however, is that a number of different tRNAs could be directed to interact with the *tyrS* leader by changing the specifier sequence and the variable position of the antiterminator side-bulge, indicating that these are the major determinants of the specificity of the tRNA-mRNA interaction.

Extensive comparisons of tRNAs which function well in *tyrS* antitermination and tRNAs which function poorly did not reveal a simple pattern of identity determinants which correlate with antitermination efficiency. The failure to identify single base substitutions in $tRNA^{Thr}$ with a major effect on interaction with the *tyrS* leader suggests that there may be a complex array of structural determinants for the three-dimensional interaction between these RNA molecules. Lack of complete information about modified nucleotides in *B. subtilis* tRNA further complicates this analysis. Nucleotide modifications can influence the properties of tRNA in a number of ways, including interactions with aminoacyl-tRNA synthetases (26); it is likely that effects on interaction with the leader RNA could also occur. Biochemical studies of the tRNA-mRNA interaction and detailed structural information about the leader RNA structure will be necessary to further elucidate the molecular basis of this interaction. It should also be noted that this study measured tRNA-dependent antitermination and not tRNA-mRNA binding; it is possible that certain tRNAs can bind to the leader RNA but fail to cause antitermination because of unknown structural constraints.

ACKNOWLEDGMENT

This work was supported by NIH grant GM47823.

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