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Transfer RNA-mediated antitermination *in vitro*

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ABSTRACT

The threonyl-tRNA synthetase gene (*thrS*) is a member of the T-box family of ~250 genes, found essentially in Gram-positive bacteria, regulated by a tRNA-dependent antitermination mechanism in response to starvation for the cognate amino acid. While interaction between uncharged tRNA and the untranslated leader region of these genes has been firmly established by genetic means, attempts to show this interaction or to reconstitute the antitermination mechanism *in vitro* using purified tRNAs have so far failed. In addition, a number of conserved sequences have been identified in the T-box leaders, for which no function has yet been assigned. This suggests that factors other than the tRNA are important for this type of control. Here we demonstrate tRNA-mediated antitermination for the first time *in vitro*, using the regulatory $tRNA^{Thr}$ isoacceptor isolated from *Bacillus subtilis* and a partially purified protein fraction. As predicted by the model, aminoacylation of $tRNA^{Thr(GGU)}$ with threonine completely abolishes its ability to act as an effector. The role of the partially purified protein fraction can be functionally substituted by high concentrations of spermidine. However, this polyamine does not play a significant role in the induction of *thrS* expression *in vivo*, suggesting that it is specific protein co-factors that promote T-box gene regulation in conjunction with uncharged tRNA.

INTRODUCTION

A large number (~250) of genes have been identified, primarily in Gram-positive bacteria, whose untranslated leader regions contain a conserved ~14 nt sequence element known as the T-box, just upstream of a transcription terminator (1–4). Most of these genes encode aminoacyl-tRNA synthetases, amino acid biosynthetic enzymes or enzymes involved in amino acid transport, all of which are likely to be induced upon starvation for their cognate amino acid. The leader regions of these genes are generally ~300 nt in length and are highly structured. A conserved secondary structure model of T-box leaders has been proposed (2,5) and has essentially been confirmed by experimental data (6). Uncharged cognate tRNA is thought to interact with the leader region in at least two places to stabilise an antiterminator structure at the expense of the terminator (Fig. 1). The specificity of the tRNA:leader interaction is achieved by binding of the anticodon of the tRNA to a 'specifier codon' bulged out of the first major RNA structure that forms in the leader, the specifier domain (2,7,8). The antiterminator structure is stabilised by Watson–Crick base pairing between the -NCCA 3′-end of the acceptor arm of all tRNAs and the -UGGN′- sequence in the central region of the T-box, located in a side-bulge of the antiterminator structure (7,9). Steric hindrance by the amino acid is thought to prevent charged tRNA from interacting with this sequence. Although an interaction between the full tRNA and the *thrS* leader was sought in structure probing experiments both *in vivo* and *in vitro*, it was never observed (6). Transfer RNA^{Tyr} was recently shown, however, to be capable of interacting with a model *tyrS* antiterminator domain, whose stability was significantly increased by mutation (10).

In addition to the T-box, a number of other small conserved sequences have been noted in the leaders of genes of this family. These include the so-called GNUG-box and AG-box in the specifier domain and a sequence known as the F-box further downstream (11). Mutations in any of these sequence elements have a dramatic negative effect on antitermination *in vivo* (12; our unpublished results); however, their function is as yet unknown. Furthermore, only 4 of the 14 nt of the T-box have been assigned a convincing role. Although some of these nucleotides are involved in base pairing interactions with nucleotides which are also used by the terminator, there is no particular reason why other nucleotide pairings would not be tolerated. The existence of the small conserved sequence elements and the fact that we have been unable to reconstitute the tRNA-dependent antitermination mechanism in *in vitro* transcription assays with the tRNA alone convinced us that protein co-factors are involved in promoting the tRNA:leader interaction.

An initial search for additional factors that might be involved in tRNA-mediated antitermination led to the discovery of an endoribonucleolytic cleavage in the loop of the *thrS* antiterminator structure (13). Cleavage at this site is RNase E-dependent in *Escherichia coli*, suggesting *Bacillus subtilis* has an analogue of this enzyme (14). *Bacillus subtilis* cleavage is much more efficient under threonine starvation conditions *in vivo* and results in the production of a shorter transcript, bounded by stable RNA hairpins, that is five times more stable than the full-length mRNA (13). Thus, the effect

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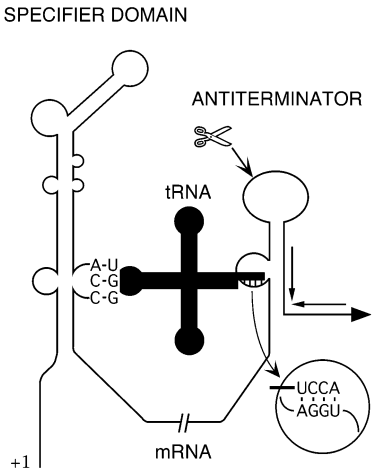

Figure 1. Model of the interaction of uncharged $tRNA^{Thr}$ with the *B.subtilis thrS* leader RNA. The schematic depicts the genetically identified points of interaction between the specifier codon and the tRNA anticodon and between the 3′-end of the acceptor arm of the tRNA and the side-bulge of the antiterminator structure. The scissors indicate a cleavage site upstream of the terminator believed to occur in many genes regulated by this mechanism. Inverted arrows represent the terminator structure.

of antitermination is amplified by cleavage and subsequent stabilisation of the coding portion of the *thrS* mRNA. The RNAs of several other genes of this family are cleaved in similar positions, suggesting that this phenomenon is conserved among T-box regulated genes.

In this study, we describe the reconstitution of the tRNA-mediated antitermination mechanism in *in vitro* transcription assays, using uncharged $tRNA^{Thr}$ and a partially purified protein extract. As predicted by the model, aminoacylation of this tRNA abolishes its ability to promote antitermination. We show that while high concentrations of spermidine can substitute for the protein fraction *in vitro*, a *B.subtilis* strain unable to produce spermidine *in vivo* has unaltered *thrS* expression following threonine starvation. These results provide strong evidence that specific protein co-factors are required for tRNA-mediated antitermination.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The *B.subtilis* strains used in this study are the prototrophic strain 168 (BGSC 1A2) and derivatives thereof. Strain SSB275 (*amyE*::*thrS′-lacZ*) contains a *thrS-lacZ* transcriptional fusion (plasmid pHMS69) integrated at the *amyE* locus. Strain SSB315 (*amyE*::*thrS′-lacZ*, Δ*speA*::*spc*) was constructed by transformation of strain SSB275 with chromosomal DNA isolated from strain BSIP7010 (15) and selection for spectinomycin resistance. The correct deletion of the *speA* gene was verified by Southern blot. Strain SSB1031 (*amyE*::*thrS′-lacZ*, *pnpA*::*spc*) was obtained by transforming strain SSB275 with chromosomal DNA from strain BD2411 (16).

Strain HP24 (*amyE*::*thrS′-lacZ*, pHMS11) contains the *thrS* overproducing plasmid pHMS3 (1). For the purification of the ThrS protein this strain was grown in LB medium supplemented with 0.5% glucose. For starvation experiments, cells were grown in SMS minimal salt medium (17) with 0.5% glucose. Threonine starvation was achieved by the addition of 650 μg/ml DL-threonine hydroxamate at $A_{600} = 0.4$. Cells were harvested 2 h after the addition of threonine hydroxamate. Antibiotics for selection of chromosomal plasmid integrants were added at 4 μg/ml for chloramphenicol, 100 μg/ml for spectinomycin and for the selection of the replicative plasmid pHMS3 at 20 μg/ml for tetracycline.

Plasmid constructs

pHMS17. A 0.5 kb *Dra*I–*Cla*I fragment of the *thrS* gene containing the promoter, the entire leader sequence and part of the *thrS* structural gene was inserted between the *Sma*I and *Cla*I sites of the plasmid Bluescript KS+.

pHMS69. The 0.44 kb *Dra*I–*Eco*RV fragment of the *thrS* gene containing the promoter, the entire leader sequence and part of the *thrS* structural gene was isolated as an *Eco*RI–*Bam*HI fragment and inserted between the *Eco*RI and *Bam*HI sites of the *lacZ* fusion plasmid pHM2 (18).

pHMS71. Plasmid pHMS71 is derived from plasmid pHMS17. The 65 nt between the *Eco*RV and *Cla*I sites of the *thrS* structural gene were replaced with the third leader region transcription terminator of the *thrZ* gene (1). The terminator-containing fragment was constructed by hybridising two complementary oligonucleotides carrying an *Eco*RV and a *Cla*I site, respectively, at either end.

Preparation of tRNAs

A 110 g frozen cell pellet harvested from an exponentially growing culture of strain BGSC 1A2 was added to 500 ml of a solution preheated to 50°C containing 250 ml of buffer I (10 mM Na–acetate pH 5, 10 mM $MgCl_2$, 0.5% SDS, 0.5% bentonite) and 250 ml phenol, pH 6. The contents were incubated at 50°C on a rotary shaker at 300 r.p.m. for 30 min. The phenol phase was separated by centrifugation for 15 min at 6000 *g* at 4°C. The aqueous phase was ethanol precipitated, dissolved in 30 ml water and phenolised once more (phenol pH 8). After ethanol precipitation 166 mg of RNA was obtained, consisting of >90% tRNA.

The whole RNA preparation was loaded on a MonoQ HR10/10 (Pharmacia) anion exchange column equilibrated with buffer A (20 mM K-PO_4, pH 7). After an initial wash step with 350 mM NaCl the tRNAs were fractionated with a 350–750 mM NaCl gradient. Fractions containing threonine accepting tRNA species (identified by aminoacylation assays using purified threonyl-tRNA synthetase) were pooled and $(NH_4)_2SO_4$ was added to a final concentration of 1.8 M. The tRNA preparation was then loaded on a Phenylsuperose HR10/10 (Pharmacia) column equilibrated with buffer B [1.8 M $(NH_4)_2SO_4$ in buffer A]. The two threonine isoaccepting tRNA species were resolved by a 1.8–0.4 M $(NH_4)_2SO_4$ gradient. The separation of the $tRNA^{Thr(GGU)}$ and $tRNA^{Thr(UGU)}$ isoacceptors was confirmed by dot-blot analysis (Fig. 2) using labelled oligonucleotides specific for $tRNA^{Thr(UGU)}$ (5′-CCCCCAACCTACTGATTACAAGTCAGT-3′) and $tRNA^{Thr(GGU)}$ (5′-CGCTGACCTCTTCCTTACCATGGA-3′). For each isoacceptor several fractions were pooled and the concentration of the specific tRNA was determined in aminoacylation assays with purified threonyl-tRNA

synthetase. The $tRNA^{Thr(UGU)}$ pool used here contained 1.6 pmol of the isoacceptor/μg tRNA; the concentration of $tRNA^{Thr(GGU)}$ was 2.2 pmol/μg tRNA.

Preparation of protein fractions

The cell pellet from a 100 ml culture of threonine-starved strain SSB1031 was sonicated in 2 ml buffer C (20 mM Tris–HCl pH 7.5, 4 mM $MgCl_2$, 10% glycerol, 10 mM β-mercaptoethanol) supplemented with 2 mM PMSF. An S30 supernatant containing 15 mg of total protein was loaded on a Mono Q HR5/5 (Pharmacia) anion exchange column equilibrated with buffer C. Proteins were fractionated by a 0–1 M KCl gradient. Samples of the fractions were combined in four separate pools and then tested in all possible combinations for their capacity to stimulate tRNA-dependent antitermination *in vitro*. The major activity was found in fractions eluting at 500 mM KCl. One of these fractions was used in the *in vitro* transcription assays described in the text.

Purification of the *B.subtilis* threonyl-tRNA synthetase ThrS

A 24 g cell pellet of strain HP24 overproducing the ThrS enzyme 10-fold was washed in 100 ml of buffer A (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 20% sucrose), centrifuged (10 000 *g* × 30 min) and resuspended in 50 ml buffer A. The suspension was heated to 37°C before addition of 6 mg of lysozyme and incubated for 20 min with gentle agitation. All subsequent steps were carried out at 4°C. The protoplasts were centrifuged (12 500 *g* × 30 min) and resuspended in 50 ml of buffer B (10 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$, 10% glycerol, 20 mM β-mercaptoethanol, 1 mM PMSF). After passage through a French Press (20 000 psi) the lysate was centrifuged (18 000 *g* × 45 min) and an S100 supernatant was obtained by ultracentrifugation (100 000 *g* × 4 h). Proteins were precipitated with 80% $(NH_4)_2SO_4$, the pellet dissolved in a minimum of buffer C (10 mM Tris–HCl pH 7.8, 10 mM $MgCl_2$, 10% glycerol, 10 mM β-mercaptoethanol, 0.1 mM PMSF) and dialysed against buffer C. Half of the preparation was loaded on a MonoS HR10/10 (Pharmacia) column and fractionated by a 0–200 mM KCl gradient in buffer C. ThrS eluted at 115 mM KCl. ThrS-containing fractions were directly loaded on a DEAE AP-1 8HR (Waters) column and fractionated by a 0–300 mM KCl gradient in buffer C. ThrS eluted at 200 mM KCl and was >95% pure. The pH of all buffers was adjusted at room temperature.

In vitro transcription assays

RNA polymerase was isolated from the *B.subtilis* wild-type strain BGSC 1A2 as described by Moran (19). The enzyme was stored at –20°C in storage buffer (10 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$, 0.1 mM EDTA, 100 mM KCl, 50% glycerol).

The template plasmids pHMS71 and pHMS17 were purified using Nucleobond AX columns (Macherey-Nagel), as described by the manufacturer. They contain the *thrS* promoter, leader and leader region terminator, and the beginning of the structural gene. Plasmid pHMS71 also contains the third terminator of the *thrZ* leader (1) cloned immediately upstream of the *Cla*I cleavage site used to linearise the template DNA.

Transcription reactions (50 μl) contained 20 mM HEPES–KOH pH 8.0, 4 mM $MgCl_2$, 1 mM DTT, 40 mM KCl, 2 μg bovine serum albumin, 12 U RNasin (Promega), CTP, GTP and UTP at 10 μM, ATP at 400 μM, [α-^{32}P]UTP (0.5 μCi/reaction), template DNA at 4 nM and RNA polymerase at 0.5 nM. Pools of tRNA containing either $tRNA^{Thr(UGU)}$ or $tRNA^{Thr(GGU)}$ were added to 4 μM; this corresponds to 160 nM of $tRNA^{Thr(UGU)}$ and 220 nM of $tRNA^{Thr(GGU)}$. Charged tRNA was prepared by incubating the uncharged tRNA for 5 min at 30°C in a 20 μl reaction containing 20 mM HEPES–KOH pH 8.0, 4 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 1 mM threonine and 0.3 μg of purified *B.subtilis* threonyl-tRNA synthetase ThrS. Threonine was replaced by 1 mM valine as a negative control. The entire aminoacylation reaction was then added to the *in vitro* transcription reaction. Where indicated, 10 μg of a protein fraction from a total *B.subtilis* extract prepared as described above were added. Spermidine was added as indicated at various concentrations between 0.5 and 4 mM.

In vitro transcription reactions were incubated at 30°C for 20 min, and were then were stopped by addition of 5 μl of 3 M Na–acetate pH 5, phenolised and precipitated with 3 vol ethanol. The pellet was dissolved in 0.3× gel loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), heated to 95°C for 2 min and loaded on a 5% sequencing gel.

β-Galactosidase assay

The β-galactosidase activity of *lacZ* fusions was measured as described previously (1). Each experiment was performed three times.

RESULTS

The experimental system

In order to study tRNA-mediated transcription antitermination *in vitro*, we were careful to use a system which reproduced conditions *in vivo* as closely as possible, using native RNA polymerase and tRNAs purified from *B.subtilis*. As template, we used the leader region of the *B.subtilis* threonyl-tRNA synthetase gene (*thrS*), cloned on a plasmid and linearised at a *Cla*I site downstream of the leader transcription terminator. *ThrS* is a member of the T-box family of genes whose regulation has been extensively studied *in vivo* (1,7). The secondary structure of the entire *thrS* leader RNA has also been experimentally determined both *in vitro* and *in vivo* (6). Transcription of the *thrS* leader with *B.subtilis* RNA polymerase under classical *in vitro* transcription conditions, i.e. 100–200 μM NTPs, results in quite efficient read-through of the terminator (50% and more), in the absence of additional factors. This significantly reduces the sensitivity of the *in vitro* assay, as the maximum increase in terminator read-through that can be measured under these conditions is 2-fold. By lowering the NTP concentration in the assay to 10 μM NTP, only 5–10% of all transcripts extend beyond the terminator to produce a run-off (read-through) band at the site of template linearisation. The low NTP concentration is thought to reduce the elongation rate of RNA polymerase, thereby increasing the efficiency of the terminator (20). Under these conditions, 10–20-fold effects on antitermination can be measured.

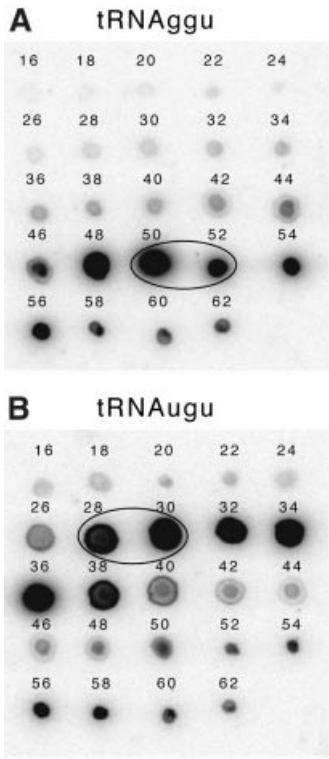

Figure 2. Dot-blot analysis of $tRNA^{Thr}$ isoacceptors following fractionation on a phenylsuperose column. (**A**) Aliquots of tRNA fractions were spotted on a membrane and hybridised to an oligonucleotide probe specific for $tRNA^{Thr(GGU)}$. (**B**) The same fractions as in (A) were hybridised to an oligonucleotide probe specific for $tRNA^{Thr(UGU)}$. Numbers correspond to the fractions as they eluted from the column. Encircled spots indicate the fractions that were combined to create the pools of $tRNA^{Thr(GGU)}$ and $tRNA^{Thr(UGU)}$ used in the *in vitro* transcription assays.

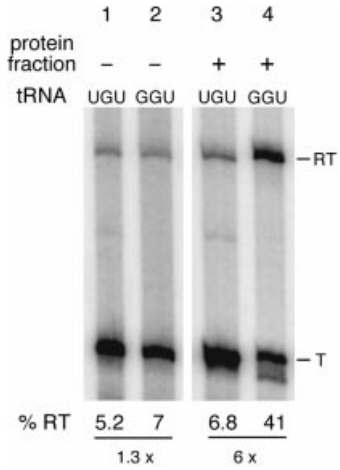

Figure 3. *In vitro* transcription of the *thrS* leader region. All reactions contained linearised pHMS71 plasmid DNA as template (4 nM) and *B.subtilis* RNA polymerase (0.5 nM). Pools of uncharged tRNA containing either $tRNA^{Thr(GGU)}$ (220 nM) or $tRNA^{Thr(UGU)}$ (160 nM) were added as indicated. Lanes 3 and 4, addition of 10 µg of a protein fraction from a total extract eluting at 500 mM KCl on a MonoQ (Pharmacia) column (see Materials and Methods). T and RT indicate products terminated at the *thrS* leader region terminator and read-through transcripts, respectively. % RT represents the percentage of read-through transcripts; the terminated and read-through transcripts taken together as 100%. The fold increase in antitermination is shown at the bottom of the figure.

The effector of the T-box regulatory system is the cognate uncharged tRNA. *Bacillus subtilis* has two $tRNA^{Thr}$ isoacceptors, with UGU and GGU anticodons. We have shown previously that only $tRNA^{Thr(GGU)}$ is able to interact productively with the *thrS* leader *in vivo* (7). To study the effect of $tRNA^{Thr}$ on *thrS* transcription antitermination *in vitro*, while protecting against the possibility that modified bases were necessary for the tRNA:leader interactions, we decided to use tRNAs purified from standard *B.subtilis* cultures, rather than *in vitro* synthesised tRNAs or tRNAs overproduced *in vivo*. Total tRNA was fractionated using HPLC columns creating two tRNA pools containing either the $tRNA^{Thr(UGU)}$ or the $tRNA^{Thr(GGU)}$ isoacceptor. Each was present in very similar quantities as measured by hybridisation assays with probes specific for each tRNA and by aminoacylation assays using purified threonyl-tRNA synthetase. The complete separation of the two isoacceptors is illustrated by dot-blot analysis of phenylsuperose column fractions (Fig. 2). We studied the effect of addition of $tRNA^{Thr(GGU)}$ on *thrS* transcription antitermination *in vitro*, using the $tRNA^{Thr(UGU)}$-containing fraction as a negative control.

tRNA-dependent antitermination *in vitro*

The addition of the $tRNA^{Thr(GGU)}$ isoacceptor to our *in vitro* assay was not sufficient to promote antitermination (see below). Thus, either our *in vitro* reaction conditions were not optimal or other factors, present *in vivo*, are required for tRNA-dependent terminator read-through *in vitro*. We decided to search for proteins capable of promoting antitermination in concert with, and dependent on, uncharged $tRNA^{Thr(GGU)}$. A *pnp* mutant strain was used for the preparation of *B.subtilis* cell extracts to help limit RNA degradation of the read-through transcript in particular during *in vitro* transcription. For these assays, we also used a template (pHMS71) where the 3′-end of the run-off transcript could fold back into a stable RNA hairpin in an attempt to further protect it from RNA degradation. A complete extract (S30) from a threonine-starved culture (*thrS* inducing conditions) was fractionated by anion exchange chromatography (see Materials and Methods) and fractions were tested for their ability to promote antitermination. Several fractions eluting at 500 mM salt clearly increased antitermination in the presence of uncharged $tRNA^{Thr(GGU)}$ but not $tRNA^{Thr(UGU)}$ isoacceptor. The effect of the addition of one of these fractions is shown in Figure 3. Addition of the $tRNA^{Thr(GGU)}$ isoacceptor alone to the *in vitro* transcription assay does not increase antitermination compared with $tRNA^{Thr(UGU)}$ (Fig. 3, lanes 1 and 2). In contrast, further addition of the partially purified protein fraction specifically stimulated read-through of the *thrS* terminator 6-fold in the presence of $tRNA^{Thr(GGU)}$ relative to that seen with the $tRNA^{Thr(UGU)}$ isoacceptor (Fig. 3, lanes 3 and 4). It is noteworthy that *E.coli* RNA polymerase can quite effectively replace the *B.subtilis* enzyme under these conditions (data not shown).

The T-box regulation model predicts that only uncharged tRNA can interact productively with the leader mRNA to promote antitermination. It is assumed that base pairing between the side-bulge of the antiterminator (-UGGN′-) and

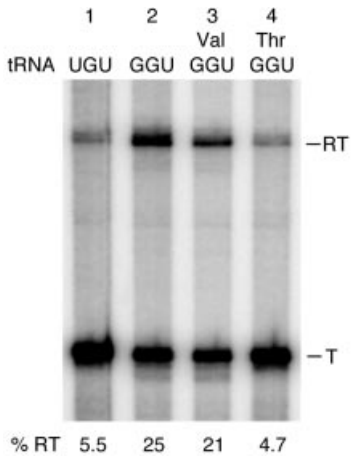

Figure 4. Effect of tRNA aminoacylation on protein-mediated *thrS* antitermination *in vitro*. Reaction conditions are as described in the legend to Figure 3. Val and Thr indicate that the $tRNA^{Thr(GGU)}$ preparation was incubated in the presence of valine or threonine, respectively, and purified threonyl-tRNA synthetase, prior to addition to the *in vitro* transcription assay. T and RT indicate products terminated at the *thrS* leader region terminator and read-through transcripts, respectively. % RT represents the percentage of read-through transcripts; the terminated and read-through transcripts taken together as 100%.

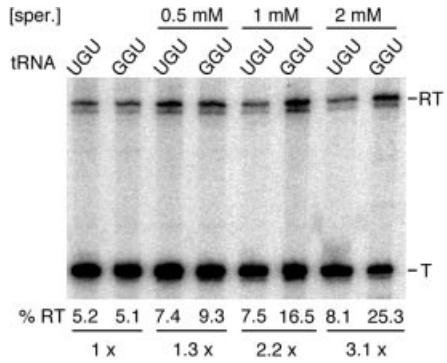

Figure 5. Effect of spermidine on *thrS* antitermination *in vitro*. Reaction conditions are as described in the legend to Figure 3, except that template pHMS17 was used. Spermidine [sper.] was added to the reactions at the indicated concentrations. T and RT indicate products terminated at the *thrS* leader region terminator and read-through (run-off) transcripts, respectively. % RT represents the percentage of read-through transcripts; the terminated and read-through transcripts taken together as 100%. The fold-increase in antitermination is shown at the bottom of the figure.

the -NCCA 3′-end of the cognate tRNA, which stabilises the antiterminator, is sterically hindered if the tRNA is aminoacylated. A prediction of this hypothesis is that charging of the tRNA should abolish $tRNA^{Thr(GGU)}$-dependent antitermination in our *in vitro* transcription assay. This is exactly what we observe (Fig. 4). In the presence of the active protein fraction, stimulation of antitermination is $tRNA^{Thr(GGU)}$-dependent as above (Fig. 4, lanes 1 and 2). When the $tRNA^{Thr(GGU)}$ was aminoacylated with its cognate amino acid (threonine) by purified threonyl-tRNA synthetase prior to its addition to the *in vitro* system it was completely unable to stimulate antitermination (Fig. 4, lane 4). In a key control experiment, where threonine was replaced by valine in the aminoacylation reaction, $tRNA^{Thr(GGU)}$ was still able to promote antitermination, as it remains uncharged (Fig. 4, lane 3). Our data clearly indicate that *B.subtilis* contains proteins which are capable of promoting tRNA-mediated antitermination *in vitro* and presumably necessary for this type of control *in vivo*. The protein fraction used here still contains a relatively large number of proteins (data not shown) and attempts to identify the individual proteins involved in T-box gene regulation are underway.

tRNA-dependent antitermination in the absence of added proteins

Previous studies from our laboratory aimed at the determination of the structure of the *B.subtilis thrS* leader revealed that the specifier domain (Fig. 1) is significantly more stable *in vivo* than *in vitro* (6). The high degree of thermodynamic instability of this domain probably explains why the tRNA:leader interaction could not be demonstrated *in vitro* (6), as under these circumstances it is unlikely that the specifier codon is correctly bulged out of the secondary structure and available for interaction with the anticodon of the tRNA. We believe that one of the roles of the protein fraction we have identified above is to stabilise the specifier domain and promote the tRNA:leader interaction necessary for this type of control.

Nevertheless, we were curious to see whether it was possible to get the T-box system to function *in vitro* without proteins. In this respect, we turned our attention to polyamines, which, although dispensable under normal laboratory growth conditions, play an important role in the cell, in particular modulation of RNA structure and translational accuracy (21). Spermidine is the predominant polyamine (90–95%) in *B.subtilis* (22) and its capacity to mould RNA structure is becoming increasingly appreciated (21). To analyse a potential effect of spermidine on tRNA-dependent antitermination, we set up the same *in vitro* transcription assays used for testing protein fractions but where the proteins were replaced by varying concentrations of spermidine. In these assays, as no protection from RNA degradation is necessary, we used a template (pHMS17) where the 3′-end of the run-off transcript consists of native unstructured *thrS* sequence. This template results in two read-through bands, the reason for which is unclear. As shown in Figure 5, $tRNA^{Thr(GGU)}$ was unable to stimulate terminator read-through compared with the $tRNA^{Thr(UGU)}$ isoacceptor in *in vitro* transcription reactions performed in the absence of spermidine (Fig. 5, lanes 1 and 2). Addition of spermidine to concentrations of 0.5 mM or more specifically increased the relative abundance of read-through transcript in the presence of the $tRNA^{Thr(GGU)}$ isoacceptor compared with that of $tRNA^{Thr(UGU)}$. The stimulatory effect of spermidine was dose-dependent, ranging from 1.3-fold at 0.5 mM to almost 6-fold at 4 mM (Fig. 5, lanes 3–8 and Fig. 6, lanes 1 and 2). Moreover, just as in the case of the partially purified protein fraction, spermidine-stimulated antitermination was strictly dependent on the uncharged state of $tRNA^{Thr(GGU)}$. Prior charging of the $tRNA^{Thr(GGU)}$ isoacceptor with threonine completely abolished the spermidine effect, whereas the substitution of threonine with valine in the aminoacylation reaction left the tRNA competent for antitermination (Fig. 6, lanes 3 and 4).

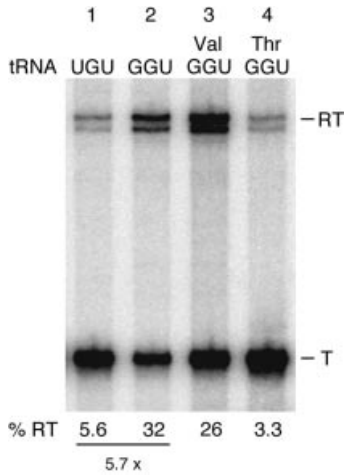

Figure 6. Effect of tRNA aminoacylation on spermidine-mediated *thrS* antitermination *in vitro*. Reaction conditions are as described in the legend to Figure 3, except that template pHMS17 was used. Val and Thr indicate that the $tRNA^{Thr(GGU)}$ preparation was incubated in the presence of valine or threonine, respectively, and purified threonyl-tRNA synthetase, prior to addition to the *in vitro* transcription assay. T and RT indicate products terminated at the *thrS* leader region terminator and read-through (run-off) transcripts, respectively. % RT represents the percentage of read-through transcripts; the terminated and read-through transcripts taken together as 100%.

Effect of a *speA* mutation on tRNA-mediated antitermination *in vivo*

Bacillus subtilis cells contain millimolar quantities of spermidine, most of it complexed with RNA (23). The concentration of free spermidine is only ~10 μM (24). The question thus arose as to what extent the rather high spermidine concentrations, required to observe tRNA-mediated antitermination *in vitro*, reflect the physiological context. Unlike in *E.coli*, spermidine is produced by a single pathway from arginine in *B.subtilis*, catalysed by the *speA*, *B* and *E* gene products (15). Danchin and co-workers (15) have thus shown that in a *speA* deletion strain no trace of spermidine or its precursor, putrescine, can be detected. To see whether spermidine plays a role in the regulation of *thrS* expression *in vivo*, we transferred this *speA* deletion construct (BSIP7010) into a strain containing a *thrS* leader-*lacZ* transcriptional fusion and compared β-galactosidase production from this strain to that of the wild-type. Under conditions of threonine starvation, both wild-type and *speA* strains showed very similar levels of *thrS* induction (Table 1). Thus, it appears that while spermidine may have an effect on tRNA-mediated antitermination *in vitro*, this role is played by proteins rather than spermidine *in vivo*.

DISCUSSION

In recent years several important aspects of the tRNA-mediated antitermination mechanism have been revealed, mostly through *in vivo* experiments. Two points of interaction between the cognate uncharged tRNA and the leader mRNA have been clearly defined by mutational studies on several genes of the T-box family: the specifier codon:anticodon interaction, which confers the specificity of induction, and the base pairing between 3′-end of the uncharged tRNA and the central region of the T-box, believed to transiently stabilise the antiterminator (reviewed in 4,25,26). The experimentally determined structure of the *thrS* leader is in good agreement with this model (6), but no specific interaction between the leader transcript and the tRNA could be observed in these experiments. Nevertheless, on a smaller scale mini-helices corresponding to the acceptor arm of $tRNA^{Tyr}$ can interact with the bulge of a model antiterminator RNA in the absence of additional factors (10). For some time now, we and others (3) have attempted to reconstitute the T-box regulatory system *in vitro* by addition of uncharged cognate tRNA, but without success. This strongly suggested that additional factors present *in vivo* must be required for the tRNA:mRNA interaction to occur in a productive fashion. This is also supported by the fact that, besides the T-box, several conserved sequence elements have been identified within the leaders of genes of this family for which no function has yet been assigned.

At the outset of this study, we optimised our *in vitro* transcription assay to improve the chances of detecting antitermination activity in protein extracts. This was done by first lowering the NTP concentration in order to obtain termination levels >90%, a condition *sine qua non* if one wants to achieve the sensitivity required to detect small changes in termination efficiency. Secondly, we used native non-overproduced tRNAs rather than *in vitro* synthesised tRNA transcripts to circumvent the possibility that modified tRNA nucleotides play an important role in leader recognition. Thirdly, we took precautions against degradation of the read-through transcript in particular, upon addition of complex protein mixtures, by preparing our extracts from a *pnp* mutant strain and by cloning a stable hairpin at the 3′-end of the run-off transcript. Lastly, we used an all-inclusive S30 cell extract (rather than an S100 supernatant) from a threonine-starved culture as a starting point to look for potential antitermination

Table 1. Effect of a *speA* deletion on the induction of *thrS-lacZ* expression

Strain	Genotype	Threonine hydroxamate	β-Galactosidase specific activity (U/mg)	Factor of induction
SSB275	*amyE*::(*thrS′-lacZ*)	–	276	6.6×
		+	1818	
SSB315	*amyE*::(*thrS′-lacZ*) Δ*speA::spc*	–	208	6.7×
		+	1409	

factors. While we are not sure what contribution any of these individual precautions provided to the identification of a partially purified protein fraction capable of tRNA-dependent antitermination *in vitro*, undoubtedly their combination has led to success, where other attempts have failed.

The lack of thermodynamic stability of the specifier domain *in vitro* prompted us to examine the role of potential stabilisers of RNA structure in the tRNA-mediated antitermination mechanism. At high concentrations, the polyamine spermidine could essentially substitute for the partially purified protein fraction in promoting antitermination by uncharged $tRNA^{Thr(GGU)}$. The effect of spermidine on the *in vitro* antitermination assay was intellectually unsatisfying from two points of view. First, the concentrations of spermidine required for the antitermination effect are much higher than its free concentration *in vivo*. Secondly, the conservation of small sequence elements in the T-box leaders could not be explained by a relatively non-specific co-effector such as spermidine. The fact that induction of a *thrS-lacZ* fusion following threonine starvation was not affected by deletion of the *speA* gene provided evidence that this intuition was correct. Spermidine is by far the dominant polyamine in *B.subtilis* but no traces of it nor of putrescine, the sole precursor of polyamine synthesis, are detected in a *speA* mutant (15). As spermidine is itself the precursor for another well known polyamine, spermine, it seems unlikely that other polyamines can replace spermidine function *in vivo*. Thus, while the effect of spermidine on tRNA-mediated antitermination *in vitro* may provide an interesting clue as to how the protein antitermination co-factors function, we do not believe spermidine is involved in the T-box control mechanism *in vivo*.

The threonine-starved S30 extract, still containing ribosomes, was fractionated on an anion exchange column, with the proteins eluting at 500 mM KCl containing the tRNA-dependent antitermination activity. However, the elution profile does not necessarily reflect the binding characteristics of the individual proteins to the column matrix. As ribosomes bind very strongly to this type of column, we cannot exclude the possibility that the antitermination activity was eluted from the ribosome rather than directly from the column matrix. Indeed, preliminary experiments suggest that an essential component of the antitermination activity is actually associated with the ribosome (data not shown). Further fractionation steps lead to complete loss of antitermination activity, suggesting that several proteins are involved. This would also explain the difficulty we have encountered in selecting mutants defective in tRNA-mediated control by genetic means. Although laborious, we believe the pooling of inactive fractions from subsequent chromatography steps, followed by the purification of the individual components one by one, provides the best chance of identifying the proteins involved in the control of T-box regulated genes.

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