

Activity of ribosomes and tmRNA of *Streptomyces aureofaciens* during development and stress conditions induced by changes in temperature and the presence of antibiotics

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Streptomyces are soil microorganisms exposed to various stresses that activate specialized responses whose coordinated action promotes growth under adverse conditions. Ribosomes having a highly cooperative structure are potential target for control mechanisms that generate signal and activate adaptive regulons or developmental programs. We examined how *Streptomyces aureofaciens* producing tetracycline responds to the presence of antibiotics and stresses induced by the changes in temperature. Tetracycline interacts with 16S RNA and decreases its thermodynamic stability. The drug also inhibits binding of ternary complex Phe-tRNA.EF-Tu.GTP to purified ribosomes. We have found that antibiotics that cause ribosome stall or pause could increase the requirement for tmRNA in the process *trans*-translation. Increase in tmRNA level was also demonstrated upon downshift in temperature.

Keywords: *Streptomyces aureofaciens*, ribosomes, translation, tmRNA, antibiotics

Introduction

The growing list of small non-coding RNA (sRNA) in bacteria and their expression patterns suggest that sRNA play a prominent role in regulation of cellular responses to environmental conditions. Regulation by sRNA is particularly advantageous during stress responses, when the energy cost must be managed with coordinated expression of multiple genes. Energy expenses for sRNA synthesis are several times lower than for synthesis of regulatory proteins. This type of regulation may represent ancestral form of control of gene functions probably before appearance of regulatory proteins. *Streptomyces* are soil microorganisms, exposed to various conditions inducing stresses that activate specialized responses including synthesis of antibiotic, hydrolytic enzymes and/or morphological differentiation from vegetative forms to aerial mycelium and spores. It has been shown that downshift in temperature is not sole inducer of cold shock response, because antibiotics that interfere with function of A-site of ribosome induce the response [1]. Bacterial cells evolved mechanisms to ensure quality control during protein biosynthesis. TmRNA is a versatile and highly conserved bacterial molecule endowed with the combined structural and functional properties of both a tRNA and a mRNA. The tmRNA was first discovered in *Escherichia coli* [2] and play a central role in releasing stalled ribosomes and targeting incomplete proteins and mRNAs in *trans*-translation system. When ribosome translates to the 3' end of an incomplete or stop-codon lacking mRNA, tmRNA is bound by a small protein SmpB that is required for structural integrity and interaction with ribosome [3]. TmRNA is charged with alanine by alanyl-tRNA synthetases and complex tmRNA.SmpB.EF-Tu.GTP enters the A-site of the ribosome. Nascent polypeptide is transferred to alanyl-tmRNA-SmpB and the complex is translocated to the P-site and translation switches from the original message to a short tmRNA open reading frame (ORF) that encodes a degradation tag. Translation of the ORF and normal termination releases the tagged polypeptide for degradation and permits disassembly and recycling of ribosomal subunits for new rounds of protein synthesis [4,5]. The final product is a chimeric polypeptide in which the specific tagged polypeptide is recognized and degraded by ATP-dependent proteases [6-8]. Experiments with *Synechocystis* showed

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that in the presence of subinhibitory concentration of protein synthesis inhibitors, this cyanobacterium requires tmRNA for growth [9]. Production of tmRNA tagged proteins in *Escherichia coli* has been observed to increase in the presence of both suppressor tRNA [10] and the miscoding drugs kanamycin and streptomycin [11] which cause translation read-through of stop codons. The presence of a functional *trans*-translation system is important to allow the survival of several species of bacteria under unfavorable conditions. In *Bacillus subtilis* the SsrA-SmpB system is important for growth in extreme temperature conditions. Strains without tmRNA activity are hypersensitive to high temperatures [12]. The absence of *trans*-translation in *B. subtilis* has significant impact on low-temperature growth. The recycling of stalled ribosomes makes an important contribution to initiation in cold stressed cells of *B. subtilis* [13]. In this study we investigated activity of ribosomes and abundance of tmRNA of tetracycline producer *Streptomyces aureofaciens* under temperature shift up and down and possible role of *trans*-translation system. Tetracycline inhibits protein synthesis by interfering with the binding of aminoacyl-tRNA to the A-site of ribosome [14]. There are at least two functionally important binding sites for tetracycline on the 30S subunit. One site is located between the head and body of small subunit near the acceptor site and the second binding site is positioned in the body of 30S subunit. Resistance mechanisms include proteins that are implicated in an energy-dependent exporting of tetracycline across the cell membrane or mimic the structure and function of the elongation factors (TetM, TetO, TetS and others) and are thus able to release tetracycline from the A-site of 30S subunits [15-17]. We have found that *trans*-translation system can contribute to survivance at the low temperature and at the beginning of tetracycline production when intracellular concentration of tetracycline is low.

Materials and Methods

Streptomyces aureofaciens MBÚ was obtained from the collection of microorganisms of the Institute of Microbiology of the Czech Academy of Sciences. Aerial spores were used for inoculation of MJ complex medium (g/L): (NH₄)₂SO₄ 2.0, MgSO₄·7H₂O 0.2, yeast extract 2.0, soya extract 10 ml, casamino acids 10.0, glycerol 10.0 and K₂HPO₄ 1.0, pH 7.2. Cells were harvested by centrifugation and washed with the standard buffer: 20 mM Tris-HCl, pH 7.6, 40 mM NH₄Cl, 10 mM MgCl₂, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF). Cultures were grown at 28°C on reciprocal shaker at 135 rpm.

Isolation of RNA

Vegetative cells (0.6 g wet material) were mixed with RNA Blue containing guanidium thiocyanate, sodium citrate, 2-mercaptoethanol, Na₃VO₄ (Top-Bio) and disrupted with glass beads in FastPrep homogenizer (Qbiogene Inc). Homogenate (1 ml) was mixed with 50 µl of 10 % sarkosyl and incubated for 5 min at room temperature. The sample was shaken with 0.2 ml of chloroform, and then centrifuged at 14 500 x g for 10 min at 4° C. The water phase containing RNA was taken off and precipitated by 0.1 vol. 3 M CH₃COONa, pH 5.5 / 1 vol. isopropanol at 20° C. RNA was sedimented at 14 500 x g for 10 min and 4° C and washed with 1 ml of 75 % ethanol. The final sediment was solubilized in a sterile 10 mM Tris-HCl, pH 8.0. RNA was analyzed in 7 % polyacrylamide-6M urea sequencing gels and visualized with ethidium bromide.

Analysis of tmRNA by Northern hybridization

Aliquots of the total RNA were separated by electrophoresis in a 7 % polyacrylamide-6M urea gels. A nylon membrane was blotted with separated RNAs using a vacuum blotter Hybaid. Dig-11UTP-labeled *ssrA* probe was added for hybridization at 68° C for 16 h. Blot was washed with 0.5 x SSC and visualized by colorimetric substrate. The relative amounts of tmRNA were quantified using an Aida Image analyser.

Binding of formyl-Met-tRNA to ribosomes of *S. aureofaciens*

The binding of [³H]fMet-tRNA to the 30S subunits was assayed in a reaction mixture (100 µl) containing 50 mM Tris-HCl, pH 7.5, 4 mM MgCl₂, 80 mM NH₄Cl, 10 mM 2-mercaptoethanol, 30 pmol 30S subunits, 5 µg poly(ApUpG), 42 pmol [³H]fMet-tRNA (110 000 cpm), 0.2 mM GTP, 0.2 mM UTP and initiation factors (40 pmol of each). Initiation factors were isolated as described [18]. The reactions were preincubated for 5 min at 32° C and initiated by the addition of [³H]fMet-tRNA. The mixtures were incubated for 15 min at 32° C and finished by the dilution with cold wash buffer (20 mM Tris-HCl pH 7.5, 8 mM MgCl₂, 50mM NH₄Cl). Radioactive material retained on nitrocellulose filters was washed and radioactivity determined in a liquid scintillation counter.

Binding of ternary complex L-[¹⁴C]Phe-tRNA.EF-Tu.GTP to ribosomes

The complex EF-Tu.GTP was prepared in the reaction mixture (100 µl) containing 50 mM Tris-HCl, pH 7.5, 10 mM magnesium acetate, 60 mM NH₄Cl, 5 mM GTP, 120 mM EF-Ts, 40 mM phosphoenol pyruvate, 10 µg pyruvate kinase and 600 pmol EF-Tu from *S. aureofaciens* [27]. The incubation was carried out at 30° C for 10 min. Aliquots (100 µl) containing 50 mM Tris-HCl, pH 7.6, 8 mM magnesium acetate, 80 µg poly(U), 25 pmol ribosomes were preincubated with 50 pmol of deacylated tRNA^{Phe} for 30 min at 30° C. Then 120 pmol of EF-Tu.GTP and 200 pmol [¹⁴C]Phe-tRNA (1500 pmol/A₂₆₀ U) were added and incubation continued at 30° C for 10 min. Reactions were terminated by addition of 3 ml of cold buffer (5 mM Tris-HCl, pH 7.6, 100 mM NH₄Cl, 8 mM magnesium acetate). Radioactivity of bound Phe-tRNA to the A-site of ribosomes was determined by membrane filter assay.

In vitro trans-translation using the stalled complex of ribosomes

The composition of the reaction mixture was similar as described previously [19-21]. Polyphenylalanine was synthesized in 100 µl reaction mixtures containing 50 mM Tris-HCl, pH 7.6, 10 mM magnesium acetate, 80 mM NH₄Cl, 1.0 mM DTT, 5 mM phosphoenol pyruvate, 1 mM ATP, 0.5 mM GTP, and 15 pmol L-phenylalanine, 2A₂₆₀ Units of S30 fraction and 100 µg poly(U). In parallel experiment, time dependent incorporation of L-[³H]-phenylalanine (15 pmol) into polyphenylalanine was examined. After 15 min incubation at 32° C phenylalanine incorporation was saturated. To the stalled complex of ribosomes was added 20 µM each of L-U[¹⁴C]-alanine, 20 µM L-U[¹⁴C]-arginine, 20 µM L-U[¹⁴C]-leucine, 20 µM L-U[¹⁴C]-lysine and 50 µM each of remaining unlabeled amino acids. After incubation at 32° C for 15 min the reaction was terminated by trichloroacetic acid (TCA) to final concentration of 5 %. Material insoluble in hot TCA was filtered over Whatman GF/C filters and assayed for radioactivity.

Results

The development of *S. aureofaciens* is characterized by rapid rate of protein synthesis that reaches its maxima at 24 h of cultivation, thereafter the rate decreases and inorganic phosphate is exhausted. The decline in the rate of protein synthesis at 96 h cultivation was about 80 %. The synthesis of tetracycline started from 16 h cultivation and after 120 h cultivation reached 1.8 mg/ml (Fig. 1A).

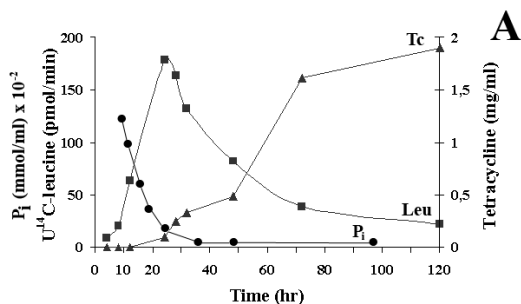


Fig. 1A. Kinetics of protein synthesis (Leu), utilization of inorganic phosphate (P_i) and production of tetracycline (Tc) in *S. aureofaciens*.

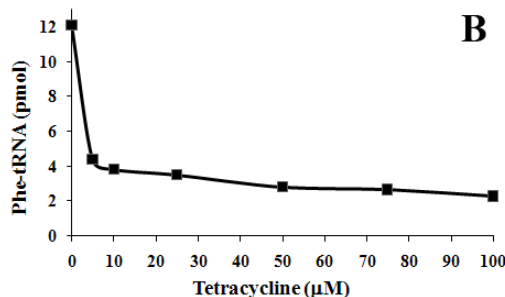


Fig. 1B. Inhibitory effect of tetracycline on binding of ternary complex $[^{14}C]Phe$ -tRNA.EF-Tu.GTP to purified ribosomes of *S. aureofaciens*.

It is known that tetracycline blocks binding of ternary complex to A-site by sterically interfering with accommodation of the aminoacyl-tRNA [22]. To demonstrate whether ribosomes of *S. aureofaciens* are resistant to tetracycline, we examined binding of $[^{14}C]Phe$ -tRNA.EFTu.GTP to ribosomes. These experiments were performed with purified ribosomes (washed with 1M NH_4Cl to remove associated proteins) and 70S tight coupled ribosomes were isolated from sucrose density gradients. Results presented in Fig. 1B show that tetracycline at concentration of 5 μM inhibits the binding of the ternary complex by about 65 %. A further increase of the tetracycline concentration from 50 to 100 μM results only in an additional 2 % decrease of the binding. These results demonstrate that the purified ribosomes of *S. aureofaciens* are sensitive to tetracycline similarly as ribosomes of sensitive bacteria. Tetracycline binds primarily to the 30S ribosomal subunit [23] and inhibits polypeptide synthesis by blocking the binding of ternary complex to the A-site. The decoding of genetic information is not affected since ribosome dependent GTP hydrolysis by EF-Tu is not affected by tetracycline [24]. We have found that the formation of the 30S and 70S initiation complex in *S. aureofaciens* differ from that in *Escherichia coli* and *Bacillus stearothermophilus* [25] with respect to the requirement for nucleotide triphosphates for maximum activity. At physiological Mg^{2+} concentrations (5-6 mM) the 30S initiation complex formation depends on the presence of initiation factors, GTP and mRNA or ApUpG. We examined concentration dependence of GTP and mixtures of GTP and ATP, or UTP on MS2 RNA dependent binding of fMet-tRNA to 30S subunits (Fig. 2A). These experiments were performed at optimum concentrations of initiation factors. When only GTP was used in the reaction, the maximum binding was obtained at about 0.2 mM and increasing concentrations of GTP had no additional stimulatory effect. Experiments in the presence of 0.2 mM GTP and increasing concentration of ATP, CTP or UTP show that ATP and UTP have significant effect on the binding of initiator tRNA to the ribosomes. At the presence of 0.2 mM GTP and 0.3 mM UTP the fMet-tRNA binding was about 3.5 times higher than in the presence of GTP alone. The data also demonstrate that UTP alone cannot fulfil the requirement for GTP in the binding reaction. In additional experiments we wanted to find out whether the stimulatory effect of UTP is GTP dependent or whether GTP can be replaced with the nonhydrolyzable analogue guanylylimidophosphate (GDPNP). As shown in Fig. 2B,

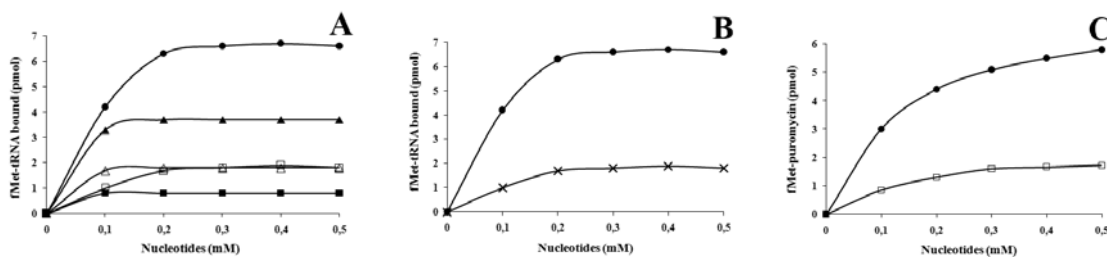


Fig. 2. The effect of nucleotide triphosphates on the binding of fMet-tRNA to 30S ribosomal subunits of *S. aureofaciens*. (A)-mRNA dependent binding in the presence of ATP (■), GTP (□), GTP+CTP (Δ), GTP+ATP (▲) or GTP+UTP (●). (B)-Effect of nonhydrolyzable analogue guanylylimidodiphosphate (GDPNP) on the stimulation of the binding of fMet-tRNA by UTP. Binding in the presence of GDPNP+UTP (×) and with GTP+UTP (●). (C)-initiator factor dependent binding of fMet-tRNA in the presence of GTP (□) and GTP+UTP (●) is reactive with puromycin.

GDPNP can substitute GTP in the binding of fMet-tRNA to ribosomes, but increasing concentrations of UTP had no stimulation effect. To investigate whether the stimulatory effect of UTP possibly influences the correct position of fMet-tRNA on ribosome, the transfer of fMet-tRNA from 70S ribosomes to puromycin was assayed (Fig. 2C). The initiator factor dependent binding of fMet-tRNA to 70S ribosomes at 0.2 mM GTP is remarkably stimulated by the presence of UTP and initiator tRNA is reactive with puromycin. These results indicate that the fMet-tRNA is correctly positioned at the P-site of ribosome. Since only attachment of formylated tRNA to 70S ribosomes was stimulated by the initiation factors [26] we obtained additional evidence that UTP really stimulates the fMet-tRNA binding. Our data suggest that initiation complexes in streptomycetes might use a novel pathway. We have shown previously that ribosomes isolated from late phases of growth (from 48 to 96 h) lost activity and formed aggregates [27]. Tetracycline binds to the head of the 30S subunits and six tetracycline binding sites were identified [28]. The Tet-1 site interferes with location where the aminoacyl-tRNA is bound to A-site of ribosome. The presence of five additional sites and their functional relevance is not yet known. We examined the effect of Mg^{2+} and tetracycline on thermal denaturation of 16S RNA. Optical melting studies monitor transition of duplex regions to single strand by recording the absorbance of the mixture at 260 nm during slow increase of temperature. The midpoint of the transition (melting temperature) T_m corresponds to thermodynamic stability of the duplex regions of nucleic acids. Characteristic melting curve of 16S RNA in the presence or absence of 1 mM $MgCl_2$ and after addition of tetracycline is shown in Fig. 3. It is evident that electrostatic interaction of 1 mM Mg^{2+} with backbone phosphates stabilizes the RNA. In the presence of 1 mM Mg^{2+} T_m of RNA increased from 42° C to 57° C. Tetracycline (0.1 mM) in absence or presence Mg^{2+} caused decrease of thermodynamic stability of the 16S RNA. These data support existence of several binding sites of tetracycline on 16S RNA which are dependent or independent on the presence of Mg^{2+} .

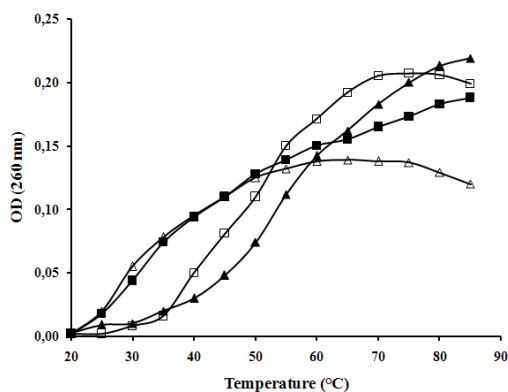


Fig. 3. Thermal denaturation of 16S RNA in absence (■) or presence (▲) of 1 mM Mg^{2+} . Reaction contained 0.1mM tetracycline in absence (Δ) or presence (□) of 1 mM Mg^{2+} .

During environmental stress, microorganisms limit protein synthesis. Translation pausing can lead to cleavage of the A-site codon and facilitate conscription of the quality control mechanism to suffering ribosomes. The *ssrA* gene codes for tmRNA, which functions both as tRNA and mRNA to rescue ribosomes stalled at 3' termini of truncated mRNA and directs degradation of their aberrant

products. Antibiotics that cause ribosome stall or pause could increase the requirement for tmRNA in the process called *trans*-translation. We examined effect of tetracycline, kirromycin, chloramphenicol and streptomycin on the level of tmRNA in exponential growing cultures (Fig. 4A). In the presence of tetracycline the level of tmRNA grows up after 10 min incubation and then remains unchanged for the next 180 min. The significant effect on rise in tmRNA level also exhibits chloramphenicol. The most pronounced effect on increase of tmRNA has streptomycin. This aminoglycoside antibiotic binds to 30S subunits and induces conformation changes resulting in translational read-through of stop codons. Kirromycin had little effect on tmRNA abundance. The results of these experiments show that induction of tmRNA expression under conditions where tetracycline has been added that is naturally produced is instantaneous and remains constant. We conclude that tmRNA system contributes to resistance to drug produced during the transition from exponential phase of growth to the antibiotic producing phase, before the more efficient resistant mechanisms such as active transport (efflux) are expressed, and/or in the presence of low levels of antibiotics synthesized by other streptomycetes.

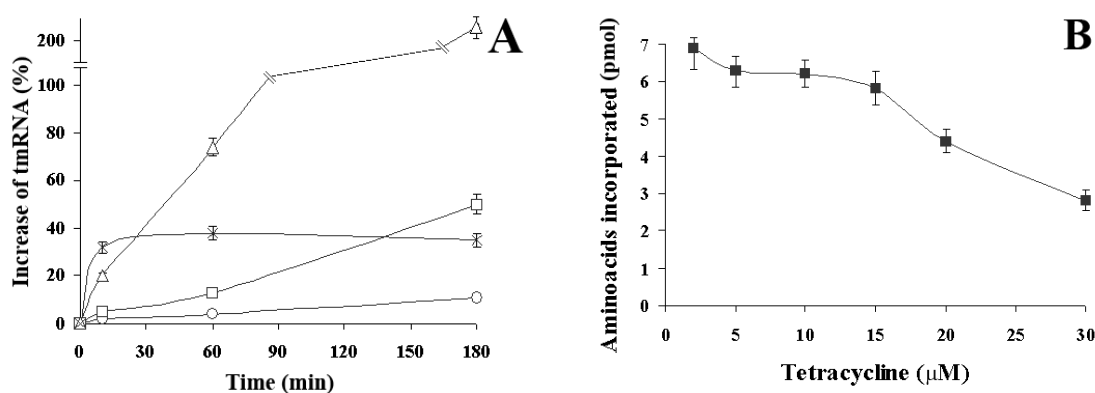


Fig. 4. (A)-The effect of antibiotics on the level of tmRNA in *S. aureofaciens*. To cultures from the exponential phase of growth (14 h) were added (○) kirromycin (5 µg/ml), (□) chloramphenicol (8 µg/ml), (Δ) streptomycin (5 µg/ml) or (×) tetracycline (50 µg/ml). In the time intervals, total RNA was isolated and aliquots (20 µg) were analyzed by electrophoresis in 7% acrylamide-6M urea gel. Northern blots were hybridized with Dig-11UTP-labeled *ssrA* probe and detected by anti-Dig-AP-conjugate and colorimetric substrate. The relative amounts of tmRNA were quantified using BioRad Molecular Imager FX. **(B)**-Sensitivity of *trans*-translation system to tetracycline.

To examine sensitivity of *trans*-translation system to tetracycline, S30 fraction from 24 h culture was used for preparation of stalled complex of ribosomes with poly(U) as previously described [19-21]. After 15 min incubation the polyphenylalanine incorporation was saturated and stalled complex of ribosomes was then mixed with ¹⁴C-labeled amino acids, which are constituents of the tag-peptide of *S. aureofaciens* tmRNA. The system was resistant to the presence of tetracycline up to 10 µM and higher concentrations of tetracycline inhibited incorporation of labeled amino acids to tag-peptides (Fig. 4B). These data indicate that *trans*-translation system can contribute to resistance at the initial phase of tetracycline production. In the next experiments the level of tmRNA upon stress induced by cold and heat shock was examined. Cells from middle of the exponential phase of growth at 28° C were transferred to 12° C or 37° C, respectively, and cultivated for 240 min. At time intervals samples were taken and RNA was used for the Northern hybridization with Dig-labeled probe (Fig. 5).

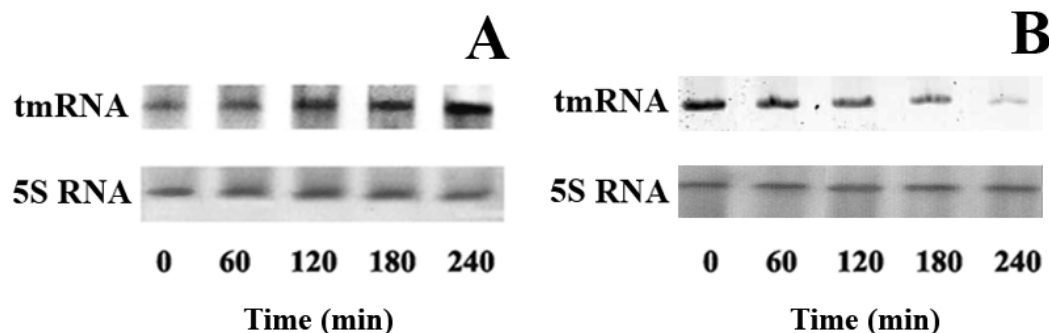


Fig. 5. TmRNA abundance upon cold (A) and heat shock (B). At time intervals RNA was isolated and Northern blots were hybridized with Dig-labeled probe and quantified. 5S RNA from control cells and cells exposed to temperature shifts were stained with ethidium bromide and quantified.

The increase in the tmRNA level was observed from 10 min after downshift in temperature. During downshift in temperature, amount of 5S RNA was unchanged. In contrast, transition from 28° C to 37° C caused decrease of tmRNA level and after 240 min about 80 % of tmRNA was degraded while the level of 5S RNA remained stable.

These data indicate that during cold shock, stalled ribosome requires recycling and tmRNA system thus regulates active pool of translating ribosomes.

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