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ANALYSIS OF SECONDARY STRUCTURE WITHIN *SGM* AND *KGMB* MRNA

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Abstract - Sgm methyltransferase from *Micromonospora zionensis* and KgmB methyltransferase from *Streptoalloteichus tenebrarius* are resistant to aminoglycoside antibiotics as a result of their ability to specifically methylate G1405 within the bacterial 16S rRNA A-site. The (C)CGCCC motif, assumed to be a regulatory sequence responsible for the autoregulation of the *sgm* gene, could most likely also be responsible for the autoregulation of the *kgmB* gene. This sequence, found within the 5' untranslated region of both *sgm* and *kgmB* mRNAs, as indicated by *in silico* prediction, may be involved in the formation of a specific stem-loop structure. Sgm and KgmB are mutually down-regulated and it is likely that they share the same *cis*-acting elements. Structure probing experiments confirmed the existence of a stable secondary structure within the 5' UTR of the *sgm* mRNA, while the analysis of *kgmB* mRNA failed to confirm the predicted structure.

Keywords: 16S rRNA methyltransferase, *kgmB*, *sgm*, secondary structure, 5' UTR

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INTRODUCTION

The great majority of mRNAs carry within their sequence information not only about the related protein product, but also about the efficiency of their synthesis and possible changes in the expression of the protein in response to various external signals. As transcription and translation are tightly coupled in prokaryotes, this information is usually contained in the structure of the 5' untranslated region (5' UTR).

Translation in prokaryotes is usually regulated by blocking access to the initiation site (Kaberdin and Bläsi, 2006). The structured 5' end of mRNA is significantly affected by temperature and sometimes functions as a thermoresponsive element that controls the initiation and efficiency of translation itself (Gualerzi et al., 2003). Alternatively, the binding of *trans*-acting factors like proteins, small molecules or antisense RNAs can allosterically control alternative structures influencing translation (Schlax and Worhunsky, 2003).

In order to regulate their own expression, some ribosomal proteins bind to the translational operators that are structurally similar to the regions in the ribosomal RNA with which these proteins interact during ribosome assembly (Boni et al., 2000, 2001; Rasmussen et al., 1993; Bénard et al. 1994, 1998). Similarly, the *thrS* gene encoding *Escherichia coli* threonyl-tRNA synthetase is autogenously controlled at the level of translation initiation (Romby and Springer, 2003). It was also shown that the phylogenetically conserved bacterial RNA chaperone Hfq binds to two sites in the 5' UTR of *hfq* mRNA and inhibits the formation of the translation initiation complex (Večerek et al., 2005). Furthermore, low molecular weight effectors such as amino acids, coenzymes or vitamins bind to the 5' UTR of many mRNAs to regulate their function. The structural elements responsible for effector binding and subsequent changes in the expression pattern of their cognate mRNAs are referred to as riboswitches (Tucker and Breaker, 2005; Winkler and Breaker, 2005). The rearrangements of the structure that occur either upon effector binding to or dissociation from its cognate

mRNA element can result in a stem–loop structure that sequesters the translation initiation region. Post-transcriptional mechanisms in bacteria often involve the action of *cis*- and *trans*-encoded antisense RNAs (Carpousis, 2003; Gottesman, 2005). Most of these small non-coding RNAs pair with the translation initiation region of their target mRNAs, resulting in the inhibition of translation.

Many aminoglycoside antibiotic-producing actinomycetes use 16S rRNA methylation to prevent self-intoxication (Cundliffe, 1992). These 16S rRNA resistance methyltransferases (MTases) act as nucleotides within their respective antibiotic binding site, adding a methyl group and thus sterically blocking antibiotic binding. Two distinct groups of 16S rRNA aminoglycoside resistance MTases have been distinguished based on their target nucleotides G1405 or A1408 (Savic et al., 2009). Both KgmB MTase from *Streptoalloteichus tenebrarius*, formerly *Streptomyces tenebrarius* (Tamura et al., 2008) and Sgm MTase from *Micromonospora zionensis* modify the N7 position of G1405 in helix 44 (C-1400 region) of 16S rRNA (Beauclerk and Cundliffe, 1987; Savic et al., 2009) and provide high-level resistance to 4,6-disubstituted 2-deoxystreptamines. As this high-level resistance is not usually related to gene dosage, it is assumed that relatively few enzyme molecules are sufficient to complete the modification of the target (i.e., 16S rRNA) (Cundlife, 1989). In light of this assumption, a model for the translational autoregulation of Sgm and KgmB MTases has been proposed explaining the maintenance of both constant and very low concentrations of these enzymes (Kojic et al., 1996; Vajic et al., 2004). The model is based on *in vivo* studies and suggests that Sgm and KgmB MTases recognize the same motif(s) within the 16S rRNA molecule and 5' untranslated regions (5' UTR) on their own mRNAs. According to the model, when a sufficient amount of the MTases is present in the cell to methylate all ribosomes and thus protect the cell from the action of its own antibiotic, the enzymes bind to their own mRNA to prevent further translation. Either primary or secondary structure, or even both, of the predicted motif(s) could be involved in the autoregulation. Computer modeling (Mfold; http://www.bioinfo.rpi.edu/applications/mfold) (Mathews et al., 1999) of 5' UTR of both *kgmB* and *sgm* mRNAs revealed the presence of a specific stem-loop structure.

The objective of this study was to probe the structure of the 5' UTR of *kgmB* and *sgm* mRNAs and compare computer-aided models with experimental data.

MATERIAL AND METHODS

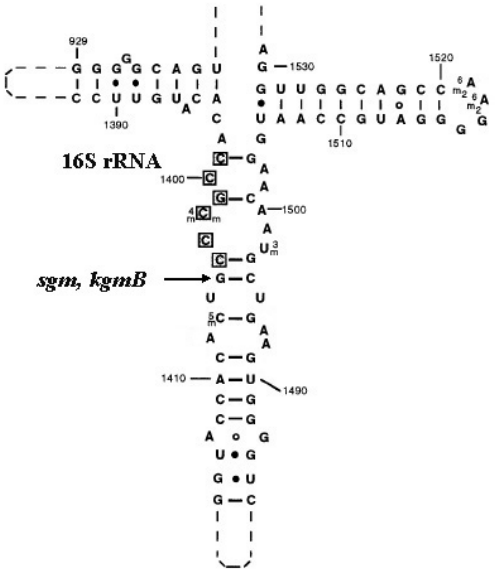
Bacterial strains and culture conditions

The strain *E. coli* NM522 (*supE, thi,* Δ(*hsdMS-mcrB*), Δ (*lac-proAB*), F' (*proAB*$^+$, *lacI*^{q}, Δ*lacZ*M15) was used (Gough and Murray,1983). A Luria-Bertani broth (LB – 10g tryptone, 5 g yeast extract and 5 g NaCl per 1l, pH 7.4) was used as a rich medium and contained 15 g l^{-1} agar when used as a solid medium (Miller, 1972). The antibiotic ampicillin was added at standard concentrations to the medium for bacteria harboring recombinant plasmids.

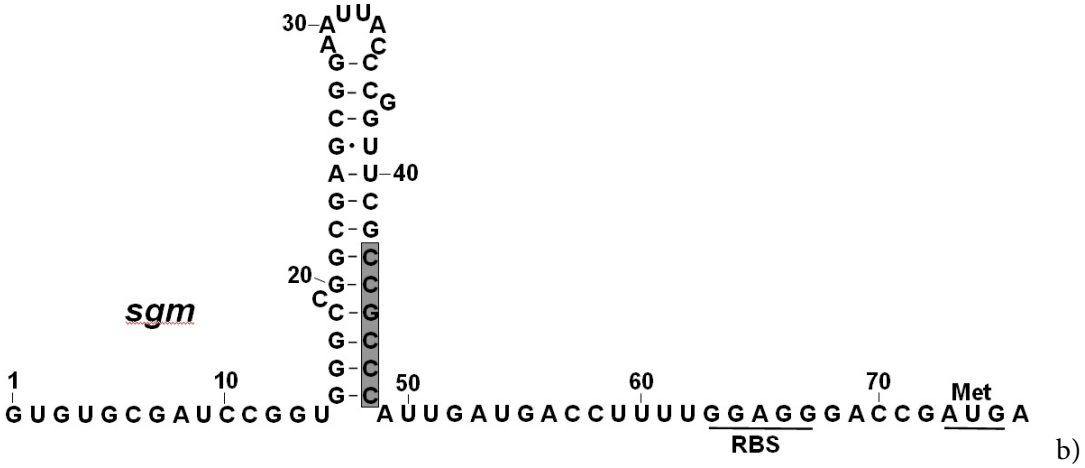
Recombinant DNA techniques

All routine DNA manipulation techniques, including plasmid preparation, restriction enzyme digestions, bacterial transformations, ligations and gel electrophoresis were performed according to standard protocols (Sambrook et al., 1989). The restriction enzymes were obtained from Fermentas (Vilnius, Lithuania) and were used according to the manufacturer's instructions. DNA templates for *in vitro* transcriptions were generated by PCR using Fkgm100RNA

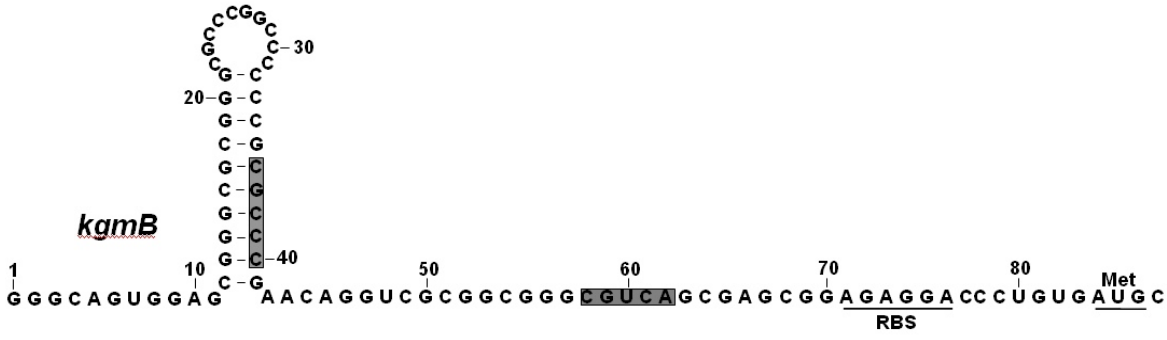
(5′ GGAATTCTAATACGACTCACTATAGGGAG AAGCCTCGCTAGGCTGG 3′) and Bg1IIkgm (5′ GGAGGGCGTCGTACTTGGG 3′) primers in the case of the *kgmB* gene and ForP1sgm (5′ CGGAATTCTAATACGACTCACTATAGGGAG AGTGTGCGATCCGGTG-3′) and Rev+80sgm (5' CGGCTAGCGCCACCGTCTGGTAACG 3') for the *sgm* gene. The underlined nucleotides cor-


16S rRNA
sgm, kgmB

a)


sgm
GUGUGCGAUCCGGU AUUGAUGACCUUUUGGAGGGACCGAUGA
RBS
Met

b)


kgmB
GGGCAGUGGAG AACAGGUCGCGGCGGGCGUCAGCGAGCGGAGAGGACCCUGUGAUGC
RBS
Met

c)

Fig. 1

respond to the T7 RNA polymerase promoter. The 376 bp-long PCR product of the *kgmB* gene bearing the 5'untranslated region and part of the coding sequence was purified from the agarose gel, digested with *Eco*RI and *Bgl*II, ligated into the *Eco*RI/*Bam*HI sites of pUC19 (Yanish-Perron et al., 1984) and the resulting plasmid was named pUKT7. The 191 bp-long PCR product of the *sgm* gene bearing the 5'untranslated region and part of the coding sequence was purified from the agarose gel, digested with *Eco*RI and *Nhe*I, ligated into the *Eco*RI/*Nhe*I sites of p3'HDV (Walker et al., 2003) and the resulting plasmid was named pHDVP1sgm.

RNA preparation for in vitro studies

The 198 nucleotides (nt)-long *kgmB* mRNA and 159 nt-long *sgm* mRNA were synthesized from linearized plasmids pUKT7 and pHDVP1sgm, respectively, using a T7 Transcription Kit (MBI Fermentas). They were then gel-purified following the standard procedure according to Ambion protocols. The transcripts were dephosphorylated with FastAP Thermosensitive Alkaline Phosphatase (MBI Fermentas), 5'-end-labeled with [γ-^{32}P]ATP (PerkinElmer) and gel-purified on 6% polyacrylamide-8M urea gels following standard procedures. The RNA concentration was determined by measuring the A_{260}.

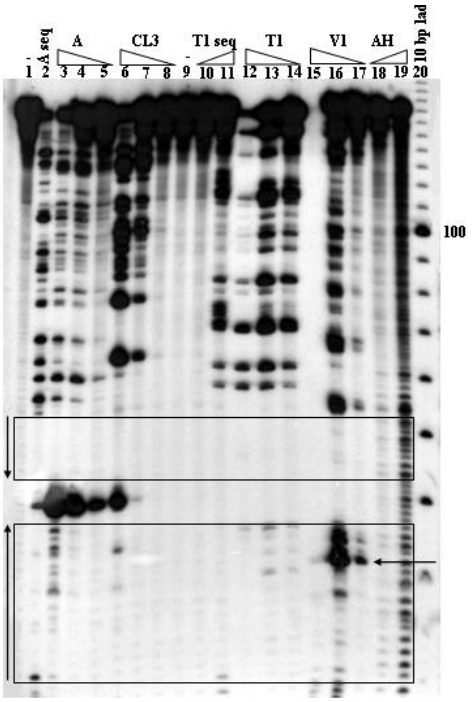
RNA secondary structure determination

RNA secondary structure determination with partial digestions of *in vitro* synthesized 5'-end-labeled both *kgmB* and *sgm* mRNAs with RNAse V1 (Ambion), RNAse T1 (Fermentas), RNase A (Fermentas) and RNAse CL3 (Gibco BRL) has been described in detail elsewhere (Moine et al., 1998; G. Knapp, 1989). In brief, approximately 2μg aliquots of *kgmB* and *sgm* mRNA were incubated in the presence of declining concentrations of T1, V1, CL3 and A RNases in a 10 μl final volume starting with 10^{-1} U of RNAse V1, 10^{-3} U of RNAse T1, 10^{-4} U of RNase A and 4 U of RNAse CL3 for 15 min at room temperature. An enzyme-free aliquot was processed together and used as a control. The cleavage products were recovered by ethanol precipitation, separated on a 8% polyacrylamide gel containing 8 M urea, and detected by autoradiography. As RNA size markers, RNase T1 and RNase A ladders prepared by digestion of labeled RNAs in an appropriate buffer for 15 min at 50°C were used as well as the alkaline-hydrolysis ladder (AH) obtained by digestion of labeled RNAs in an appropriate buffer for 2 or 5 min at 95°C. The 10 bp DNA ladder (Invitrogen) was run in parallel with RNA size markers. *In silico* secondary structure predictions were performed using the Mfold (http://www.bioinfo.rpi.edu/applications/mfold) (Mathews et al., 1999)

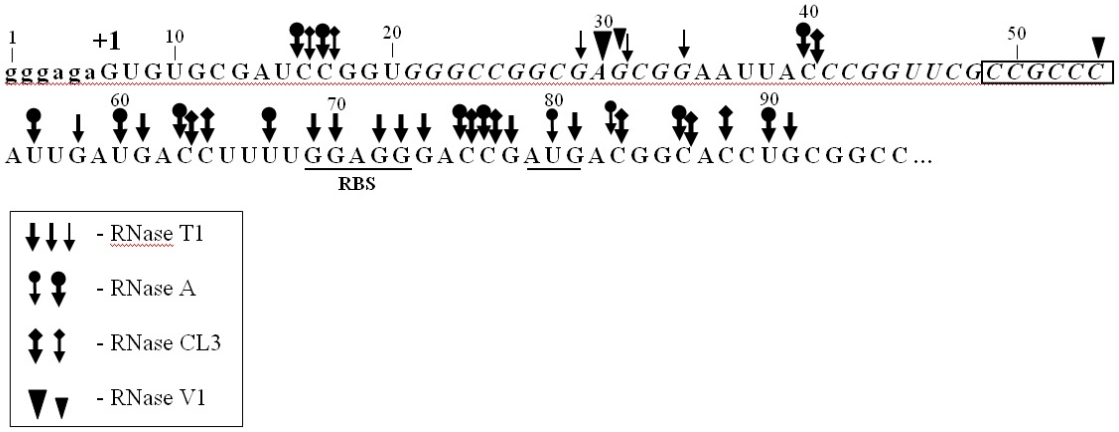
RESULTS AND DISCUSSION

In vivo analysis of *sgm* and *kgmB* gene expression revealed the existence of negative autoregulation at the translational level, while deletion analysis of *sgm* 5' UTR stressed the importance of its segment containing the CCGCCC hexanucleotide for autoregulation of the *sgm* gene (Kojic et al., 1996). A model of translational autoregulation was proposed for both *sgm* and *kgmB* genes (Kojic et al., 1996; Vajic et al., 2004). This is based on the suspected ability of these methyltransferases to recognize the same motif(s) on 16S rRNA and their own mRNA.

Sequence comparison of helix 44 (C-1400 region) of 16S rRNA (Fig. 1a) and the 5' UTRs of the *sgm* and *kgmB* MTases' mRNAs (Fig. 1b and 1c) revealed the presence of a (C)CGCCC motif, while a pentanucleotide CGUCA (that partially overlaps with the previous sequence), which includes the target nucleotide G1405 of 16S rRNA, is found 6 bp upstream of the *kgmB* ribosome binding site (RBS). The involvement of the 5'UTR of both genes in downregulation has been investigated, and mutual negative translational regulation has been shown between Sgm and KgmB MTases (Vajic et al., 2004). The proposed model of autoregulation suggests that both MTases recognize and bind to the same motif within the 16S rRNA molecule as well as motif(s) within the 5' UTRs on their own mRNAs and thereby prevent further translation when all ribosomes are methylated.



a)



b)

Fig. 2. Secondary structure probing of the *sgm* mRNA with different RNases. (a) Gel analysis of products obtained by limited cleavage with RNases. Lanes 1 and 9 correspond to *in vitro* transcribed control mRNA. Lane 2 corresponds to a RNase A ladder generated under denaturing conditions. Lanes 10 and 11 – RNase T1 ladder generated under denaturing conditions. Lanes 18 and 19 – alkaline-hydrolysis ladder (AH) with increasing incubation time. Lane 20 – a 10 bp DNA ladder. Lanes 3-8 and 12-17 correspond to products of limited cleavage with decreased concentrations of the indicated RNases. Vertical arrows and boxed parts of the autoradiogram indicate position of a stem. Horizontal arrow points to digestion with RNase V1. (b) Summary of secondary structure probing of the *sgm* mRNA with different RNases. The putative regulatory sequence (CCGCCC) is shown in the box. Transcription start site of the *sgm* gene in *M. zionensis* is indicated (+1). RBS and the translational start codon are underlined. Major and minor cuts are indicated by thicker and thinner symbols as listed in the separated box. Small letters represent nucleotides from T7 promoter sequence. Letters in italics represent nucleotides predicted to be in a stem.

According to *in silico* analysis, the CCGCCC hexanucleotide in the 5' UTR of the *sgm* is located within double-stranded stem consisting of 13 paired nucleotides interrupted by two bulged-out bases and a 6 nt-long loop (Fig. 1b). Secondary structure prediction of the *kgmB* 5' UTR showed that the CGCCC sequence is engaged in specific stem-loop structure formation, as in the case of the *sgm*. This double-stranded stem comprises 10 paired nucleotides interrupted by a 10 nt-long loop (Fig. 1c). The same sequence is present and structured within helix 44 in 16S rRNA (Fig. 1a). The pentanucleotide motif CGUCA, present in both the 16S rRNA and 5' UTR of the *kgmB*, is engaged in the stem in the former, while unstructured in the latter.

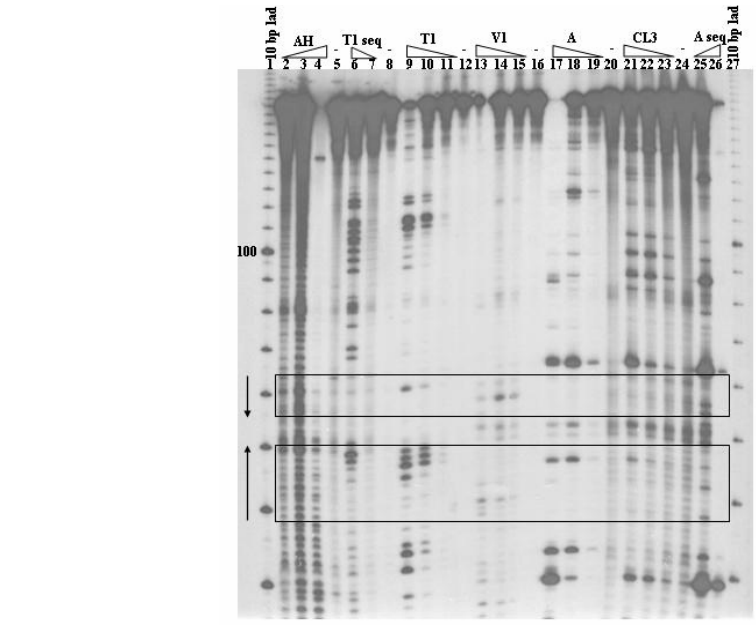
Since computer-predicted RNA structures often deviate from experimentally determined ones (Heidrich et al., 2007), *in vitro* experiments of limited digestions with structure-specific ribonucleases have been conducted to determine if the predicted secondary structure of the *sgm* and *kgmB* mRNA exists. The 159 nt-long *sgm* mRNA and 198 nt-long *kgmB* mRNA were 5'-end labeled, gel-purified and treated with RNase T1 (that cleaved the 3'-end of unpaired G residues), RNase A (that cleaved the 3'-end of unpaired C and U residues), RNase CL3 (that cleaved the 3'-end of unpaired C) and RNase V1 (that cleaved double-stranded or stacked regions). The results of limited digestions are shown in Fig. 2a and Fig. 3a.

Structure probing experiments of the *sgm* mRNA revealed that there are no cleavages with single-strand-specific RNases at nt 21 to 34 and nt 41 to 54, predicted to be in a double-stranded stem, while there is undeniable cleavage at nt 30 with double-strand-specific RNaseV1 (Fig. 2b). Two minor cuts with RNase V1 at nt 31 and 32 are also identified, although expected cleavages at the opposite side (nt 41 to 54) have not been detected. The absence of the bands could be explained as the consequence of the inherent characteristics of the method. Even though minor cuts with RNase T1 at nt 29, 31 and 34 were observed in the probed structure, it is presumed that this region is double-stranded albeit wobbly, possibly due to the presence of bulged nucleotide and G-U pairing. The obtained results for the *sgm* 5' UTR showed significant agreement with its computer prediction. Since deletion analysis of the *sgm* 5' UTR did not clarify if the secondary structure containing the CCGCCC hexanucleotide or the sequence itself is responsible for the autoregulation of the *sgm* gene, further study should consider the contribution of a secondary structure to this mechanism.

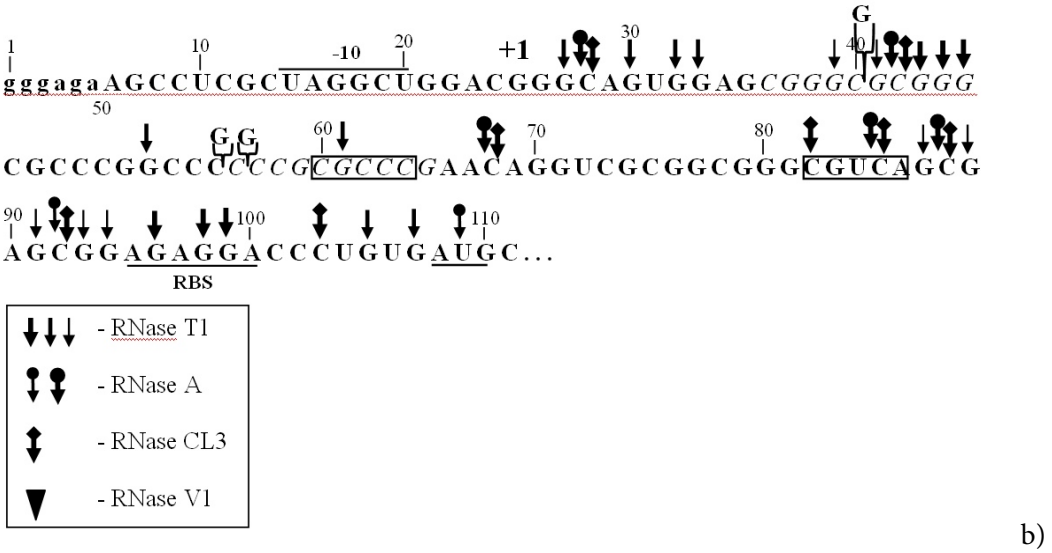
Many ribosomal proteins such as S1 (Boni et al., 2000, 2001; Rasmussen et al., 1993) or S15 (Bénard et al. 1994, 1998) are autoregulated at the translational level, and in most cases this mechanism is based on the similarity between the target site of the corresponding protein and its mRNA. The S1 protein, when synthesized in excess over the ribosomes, competes in binding to the translation initiation region with S1 coupled to 30S subunits (Boni et al., 2001; Tchufistova et al., 2003). On the other hand, the secondary structure, stabilized in the binding of the S15 protein to a pseudoknot-containing motif in the 5' UTR of its mRNA, prevents the mRNA from entering the ribosome channel and thereby causing the ribosomal machinery to stall at the preinitiation step (Ehresmann et al., 2004; Marzi et al., 2007).

The threonyl-tRNA synthetase binds to a specific site, the operator, positioned in the leader of its mRNA, and inhibits the initiation of translation by competing with the binding of the 30S ribosomal subunit (Romby and Springer, 2003). The operator is composed of two single-stranded domains and two stem-loop structures that resemble the anticodon loop of $tRNA^{Thr}$.

Structure probing of the *kgmB* 5' UTR has shown that the region predicted to be in a double-stranded stem (containing nt 39 as well as nt 41 to 45 (Fig. 3b)) is in fact single-stranded. There were no cleavages with double-strand-specific RNase V1 at the nucleotides predicted to be in a stem. Similarly, it has been shown that the region with the CGUCA sequence is also single-stranded. There are no unambiguous structure data regarding the



a)



b)

Fig. 3. Secondary structure probing of the *kgmB* mRNA with different RNases. (a) Gel analysis of products obtained by limited cleavage with RNases. Lanes 1 and 27 correspond to the 10 bp DNA ladder. Lanes 2-4 corresponds to an alkaline-hydrolysis ladder (AH) with increasing incubation time. Lanes 5, 8, 12, 16, 20 and 24 – *in vitro* transcribed mRNA control. Lanes 6 and 7 – RNase T1 ladder generated under denaturing conditions. Lanes 25 and 26 corresponds to a RNase A ladder generated under denaturing conditions. Lanes 9-11, 13-15, 17-19 and 21-23 correspond to products of limited cleavage with decreasing concentrations of the indicated RNases. Vertical arrows and parts in boxes of the autoradiogram indicate the position of the predicted stem. (b) Summary of secondary structure probing of the *kgmB* mRNA with different RNases. The putative regulatory sequences (CGCCC and CGUCA) are shown in boxes. The transcription start site of the *kgmB* gene and -10 region of the natural transcript in *St. tenebrarius* are indicated as (+1) and the underlined region, respectively. The RBS and translational start codon are underlined. Major and minor cuts are indicated by thicker and thinner symbols as listed in the separate box. Small letters represent nucleotides from the T7 promoter sequence. Letters in italics represent nucleotides predicted to be in the stem. Positions of three additional G nucleotides within *kgmB* 5' UTR are shown in curly brackets.

region of the *kgmB* 5' UTR between two putative regulatory sequences (CGCCC and CGUCA). This region was not cleaved by either single-strand- or double-strand-specific RNases. The obtained results for the *kgmB* 5' UTR did not show the presence of an explicit *in silico* predicted secondary structure but they permit speculation about the existence of other secondary structures in the region of the *kgmB* 5' UTR, since the region containing nt 46 to 81 is rarely cut by either single-strand- or double-strand-specific RNases.

It is necessary to emphasize that repeated sequencing of the *kgmB* 5' UTR showed the existence of three additional G nucleotides, in comparison to the sequence deposited in GenBank (acc. no. S60108). A correct sequence of 5' UTR includes those G nucleotides between the 16th and 17th, 31st and 32nd, and 32nd and 33rd nucleotides (numbered from the start of transcription (Fig. 1c)), with respect to the sequence deposited in the database. As the nucleotides labeled from the 12th to 41st in the originally submitted sequence are predicted to be in a hairpin structure, the presence of three additional nucleotides raises a question as to whether the prediction is correct. *In silico* analysis of the corrected sequence (Fig. 3b) with Mfold software failed to predict a secondary structure similar to the one previously published, and this finding is in accordance with the obtained experimental data.

Structure probing data indicated that both *sgm* and *kgmB* minimal translational initiation regions (RBS and ATG start codon) are single-stranded.

In conclusion, our current data strongly support the computer prediction of secondary structure within the *sgm* 5' UTR. Although the *in silico*-predicted secondary structure within the *kgmB* 5' UTR was not documented in secondary structure probing experiments, further exploration of negative autoregulation should be focused not only on primary structure involvement but also on investigation as to whether other *in vitro* synthesized regions of the *kgmB* mRNA contain stable secondary structures. In spite of the differences between the predicted and observed secondary structures in the *sgm* and *kgmB* 5' UTRs, the fact that Sgm and KgmB are mutually down-regulated still stands, implying that they share the same *cis*-acting elements.

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АНАЛИЗА СЕКУНДАРНЕ СТРУКТУРЕ У ОКВИРУ *SGM* И *KGMB* MRNA

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Sgm метилтрансфераза из соја *Micromonospora zionensis* и KgmB метилтрансфераза из соја *Streptoalloteichus tenebrarius* остварују резистенцију на аминогликозидне антибиотике метилацијом нуклеотида на позицији G1405 у оквиру A места на 16S рРНК. Сматра се да је за ауторегулацију *sgm* гена одговоран (C)CCGCCC мотив. Највероватније је иста секвенца одговорна и за ауторегулацију *kgmB* гена. По компјутерској предикцији, овај мотив, лоциран у 5' нетранслатирајућем региону иРНК молекула оба гена, би могао учествовати у формирању секундарне структуре типа укоснице. Како Sgm и KgmB метилтрансферазе једна другу ауторегулишу, могуће је да препознају исте *cis* елементе у иРНК молекулима. Експерименти испитивања структуре су, с једне стране потврдили присуство стабилне секундарне структуре у оквиру 5' нетранслатирајућег региона иРНК молекула *sgm* гена, а са друге, нису доказали постојање моделоване секундарне структуре у иРНК молекулу *kgmB* гена.