Translational Autoregulation of the *sgm* Gene from *Micromonospora zionensis*

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The sisomicin-gentamicin resistance methylase gene (*sgm*) from *Micromonospora zionensis* (the producer of antibiotic G-52 [6-*N*-methyl-sisomicin]) encodes an enzyme that modifies 16S rRNA and thereby confers resistance to 4,6-disubstituted deoxystreptamine aminoglycosides. Here, we report that this gene is regulated on the translational level. The *Escherichia coli lacZ* gene and operon fusion system was used, and it was shown that an extra copy of the *sgm* gene decreases the activity of the fusion protein. These results suggested that expression of the *sgm* gene is regulated by the translational autorepression because of binding of the methylase to its own mRNA. It was shown by computer analysis that the same hexanucleotide (CCGCCC) is present 14 bp before the ribosome-binding site and in the C-1400 region of 16S rRNA, i.e., the region in which most of the aminoglycosides act. A deletion that removes the hexanucleotide before the gene fusion is not prone to negative autoregulation. This mode of regulation of the *sgm* gene ensures that enough methylase molecules protect the cell from the action of its own antibiotic. On the other hand, if all of the ribosomes are modified, Sgm methylase binds to its own mRNA in an autorepressive manner.

Members of the order *Actinomycetales* are capable of producing a large number and wide variety of medically useful secondary metabolites. Among the actinomycetes, special attention has been paid to the genus *Streptomyces*, and the genetics and biochemistry of several antibiotic pathways have been extensively studied (for a list, see reference 6). On the other hand, the genetics of *Micromonospora* species has been far less studied. Thus far, cloning of several antibiotic resistance genes from *Micromonospora* species has been reported elsewhere (15, 21, 22, 25, 32, 33, 35). Concerning antibiotic biosynthetic genes, only the cloning of genes for fortimicin A biosynthesis has been reported (10, 11). Genetic analyses of antibiotic-producing organisms have revealed that, in most cases, the genes for resistance to antibiotics are clustered with their corresponding biosynthetic genes and, in some cases, with regulatory genes (for an example, see reference 20). This may indicate that expression of genes conferring resistance and biosynthetic genes are correlated, ensuring that resistance is always operational when the organism is exposed to its own toxic product.

The actinomycete *Micromonospora zionensis* produces antibiotic G-52 (6-*N*-methyl-sisomicin). This strain, like many other aminoglycoside-producing *Micromonospora* strains, protects itself against its own product by modification of the target site, i.e., ribosomes (29). An aminoglycoside resistance determinant (the *sgm* gene) from *M. zionensis* has been cloned in *Streptomyces lividans*, and it has been shown that the mechanism of resistance involves methylation of the 30S ribosomal subunit (25). Transcriptional analysis of the *sgm* gene in *S. lividans*, as well as in *M. zionensis*, revealed that this gene is transcribed from tandem promoters (24, 25). The promoter region of the *sgm* gene differs from that known for *Streptomyces* or *Micromonospora* (2, 36, 41). It has been shown that in *Micromonospora* species, tandem, temporally regulated pro-

moters are utilized by different RNA polymerase holoenzymes (28), as previously discovered in *Streptomyces* species (5, 45).

Concerning regulation of resistance genes in antibiotic-producing actinomycetes, three different models have been described (for a review, see reference 7). The first model describes a complex regulatory cascade for biosynthetic genes in streptomycin-producing *Streptomyces griseus*. The model includes the positive A-factor signal which is transferred to *strR*, a regulatory gene in the streptomycin biosynthetic cluster. The resistance gene *aphD* is transcribed mainly by read-through from the A-factor-dependent *strR* promoter (20). A second regulatory mechanism is based on translational attenuation of MLS (macrolide, lincosamide, and streptogramin) resistance genes, and it has been shown that it operates in various *Streptomyces* strains. However, it has recently been shown that the tylosin producer *Streptomyces fradiae* exerts regulation of the *tlrA* resistance gene via transcriptional attenuation (23). The third mechanism of regulation of antibiotic resistance genes in actinomycetes is demonstrated in *Streptomyces sphaeroides*, a producer of novobiocin. The resistance gene from this organism is driven by a promoter which responds to the superhelical density of DNA (42). In addition to these well-documented examples, it has been proposed that *kgmB* gene of *Streptomyces tenebrarius* might be down-regulated by translational autoregulation (19).

In this paper, we present data that support the existence of an autoregulatory mechanism operating, at least in *Escherichia coli*, at the translational level of *sgm* gene expression.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Strain *E. coli* NM522 [*supE thi* Δ(*hsdMS-mcrB*) Δ(*lac-proAB*) F′ (*proAB*⁺ *lacI*q Δ*lac*ZM15)] was used (16). Luria broth was used as a rich medium and contained 15 g of agar liter⁻¹ when it was used as a solid medium (30). Antibiotics and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were added at standard concentrations for screening of various fusions. Cells were grown in MMA glucose medium (30) supplemented with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when they were assayed for β-galactosidase.

Recombinant DNA techniques. Bacterial transformations, plasmid preparations, ligations, restriction enzyme digestions, and gel electrophoresis were performed according to the method described by Sambrook et al. (37). The restriction enzymes were obtained from Bethesda Research Laboratories (Bethesda,

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TABLE 1. Plasmids constructed in this study

Plasmid	Relevant characteristic[a]
pSX1	*sgm* gene cloned at the *Xho*I site of pP$_L$tl7G
pF1	*sgm-lacZ* gene fusion constructed in the *Bgl*II site of pP$_L$tl7G
pF2	*sgm* gene cloned at the *Sal*I site of pF1
pF3	*sgm* gene inactivated in the *Xho*I site of pF2
pFK1	*kan* gene inserted in the *Eco*RI site of pF1
pFK2	*sgm* gene cloned at the *Xho*I site of pFK1
pFK3	*sgm* gene inactivated in the *Xho*I site of pFK2
pSOF1	*lacZ* gene cloned at the *Bgl*II site of the pSX1-operon fusion
pSOF2	*sgm* gene cloned at the *Xho*I site of pSOF1
pOF1	*lacZ* gene cloned at the *Bgl*II site of pP$_L$tl7G
pOF2	*sgm* gene cloned at the *Xho*I site of the pOF1-operon fusion
pOF3	*sgm* gene inactivated in the *Xho*I site of pOF2
pΔRK1	Deletion created in the *sgm-lacZ* gene fusion and constructed analogously to pFK1
pΔRK2	*sgm* gene cloned at the *Xho*I site of pΔRK1
pΔRK3	*sgm* gene inactivated in the *Xho*I site of pΔRK2
pTK1	*sgm-lacZ* gene fusion containing only 10 codons of the *sgm* gene
pTK2	*sgm* gene cloned at the *Xho*I site of pTK1
pTK3	*sgm* gene inactivated in the *Xho*I site of pTK2

[a] See text for details.

Md.) or Pharmacia (Uppsala, Sweden) and were used according to the manufacturers' directions. The nucleotide sequence was determined by the Sanger dideoxy-chain termination method (38), which was adapted for Sequenase version 2.0 (U.S. Biochemical Corp., Cleveland, Ohio). DNA fragments were separated by electrophoresis in 1% (wt/vol) low-melting-point agarose and extracted with the aid of cetyltrimethylammonium bromide (27).

Plasmid constructions. A list of plasmids constructed in this study is given in Table 1. All fusions were constructed on a multicopy plasmid, pP$_L$tl7G (26), which contains a strong IPTG-inducible hybrid P$_L$tl promoter. The *Sal*I-*Sal*I *sgm* fragment (1,122 bp) encoding the sisomicin-gentamicin resistance methylase (25) was inserted in the *Xho*I site of plasmid pP$_L$tl7G to yield pSX1. Expression of the *sgm* gene from this plasmid is completely dependent on the P$_L$tl promoter.

For construction of a plasmid encoding an *sgm-lacZ* gene fusion, the *Bam*HI-*Bam*HI fragment containing the *lacZ* gene was subcloned from plasmid pMC1871 (39). This fragment was inserted in pSX1 after digestion with the *Bgl*II enzyme. Both fragments, *Bam*HI carrying *lacZ* and *Bgl*II carrying most of the pSX1 plasmid, were treated with Klenow polymerase in order to obtain in-frame fusion. Blue colonies on X-Gal were analyzed, and from among them a strain containing plasmid pF1 was chosen for further analyses.

Plasmid pF1 was used for construction of pF2 (the *Sal*I-*Sal*I fragment containing the *sgm* gene was inserted in the *Sal*I site of pF1) and pFK1 (the *Eco*RI fragment of pUC4K [44] carrying the *kan* gene was inserted in the *Eco*RI site of partially digested pF1). Furthermore, the *sgm* gene was inserted in the *Xho*I site within the *kan* gene of pFK1. In this way, the *sgm* gene was expressed from the promoter of the *kan* gene (plasmid pFK2). As a control in all experiments, the *sgm* gene was inactivated by filling in the *Xho*I site, resulting in gentamicin-sensitive transformants (plasmids named pF3 and pFK3, respectively).

Plasmids containing *sgm-lacZ* operon fusions were constructed by two different strategies. Plasmid pSOF1 was constructed by insertion of the 5′ end of the *lacZ* gene from pCON4 (the *Bam*HI-*Eco*RV fragment containing the ribosome-binding site [RBS] and N-terminal region) and the 3′ end of the *lacZ* gene from pMC1871 (12) (the *Eco*RV-*Bam*HI fragment containing the C-terminal region) in *Bgl*II-digested pSX1. In this way, the fragment of the *sgm* gene that was present in operon fusion was present in the gene fusion on plasmid pF1. On the other hand, plasmid pOF1 was obtained by using the same fragments of *lacZ* gene from pCON4 and pMC1871 and inserting them into the *Bgl*II site of pP$_L$tl7G. In both cases, the *sgm* gene was inserted in the *Xho*I site, resulting in plasmids pSOF2 and pOF2, respectively. As a control in experiments with pOF2, additional construct was prepared by inactivating the *sgm* gene in the *Xho*I site, and it was designated pOF3.

Three additional constructs which lack sequences upstream of the RBS of the *sgm* gene were made. First, a *Sal*I-*Bgl*II fragment from the *sgm* gene was cloned in pUC19 and an *Mnl*I fragment was isolated from such constructs. The *Mnl*I cleavage site is positioned six nucleotides upstream of the predicted RBS, as indicated in Fig. 4B. From this construct, a *Sal*I-*Sma*I fragment containing the RBS and 61 codons from the *sgm* gene was isolated and inserted together with the *lacZ* gene (the *Sma*I-*Sal*I fragment isolated from pMC1871) into the *Xho*I site of pP$_L$tl7G. In the resulting plasmid, the *kan* gene from pUC4K was inserted
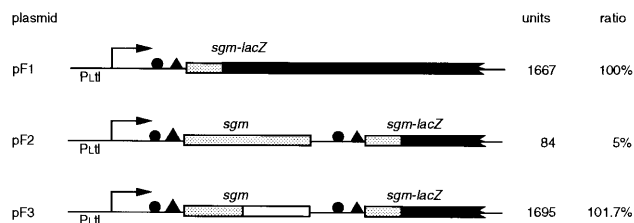

FIG. 1. The effect of an extra copy of the *sgm* gene on the *sgm-lacZ* gene fusion. Shaded boxes, coding regions of the *sgm* gene; empty box, inactivated gene; black boxes, *lacZ*; arrows, directions of the transcripts; triangles and circles, RBS and sequence proposed to be involved in autoregulation, respectively. β-Galactosidase activities are given in Miller units. The β-galactosidase activity of the strain carrying plasmid pF1 was taken as 100%. Only relevant regions of the plasmids are shown.

in an *Eco*RI site, yielding pΔRK1. Again, the *sgm* gene was inserted in the *Xho*I site of the *kan* gene (plasmid pΔRK2), after which the *sgm* gene in the *Xho*I site (plasmid pΔRK3) was inactivated.

β-Galactosidase assays. The levels of β-galactosidase in different strains were measured as described by Miller (30). Values are the averages of at least 10 experiments.

RESULTS

The Sgm protein is not detectable in *E. coli* cells. The *sgm* gene (from *M. zionensis*) and the *grm* gene (from *Micromonospora purpurea*) (22) show significant similarity in their coding regions, while the upstream nontranslated parts of the two genes differ in length and sequence. We tried to express both genes in an *E. coli* minicell system, in which both genes are expressed from the *lacZ* promoter. Surprisingly, while the Grm protein was detected on sodium dodecyl sulfate-polyacrylamide gels, the Sgm methylase was not detectable (data not shown), despite the fact that *E. coli* cells containing the construct were gentamicin resistant. Since both genes are expressed from the same promoter, we assumed that variance in upstream, nontranslated regions is responsible for differential expression of these genes. Our inability to detect the Sgm protein in gentamicin-resistant *E. coli* cells was possibly due to posttranscriptional regulation, which also indicated that a relatively low concentration of methylase molecules is sufficient for establishment of the gentamicin-resistance phenotype.

Translational control of Sgm synthesis. These results suggested that regulation of the *sgm* gene was dependent on *cis*-acting sequences present only in the noncoding region of this gene. This prompted us to construct gene and operon fusions using *lacZ* as the reporter gene. The *sgm-lacZ* gene fusion on pF1 is expressed from the strong inducible promoter P$_L$tl. This fusion contains 395 nucleotides of the *sgm* gene, i.e., the first 61 codons, which are preceded by the RBS and upstream sequences of the *sgm* gene and which were joined with the eighth codon of the *lacZ* gene. In plasmid pF1, the entire *sgm* gene was inserted such that both Sgm methylase and a fusion protein were driven by the same P$_L$tl promoter (plasmid pF2). The third construct, plasmid pF3, was made by inactivation of the *sgm* gene in plasmid pF2, giving rise to gentamicin-sensitive transformants. Expression of gene fusion in all of these constructs was analyzed by measuring β-galactosidase activities in cell extracts (Fig. 1). It is evident that the amount of fusion protein dramatically decreases when the active methylase is present, i.e., only 5% of β-galactosidase activity is retained. On the other hand, there is no difference in β-galactosidase activities between cells that harbor the pF1 plasmid and those that carry plasmid with fusion plus the inactivated *sgm* gene. There-
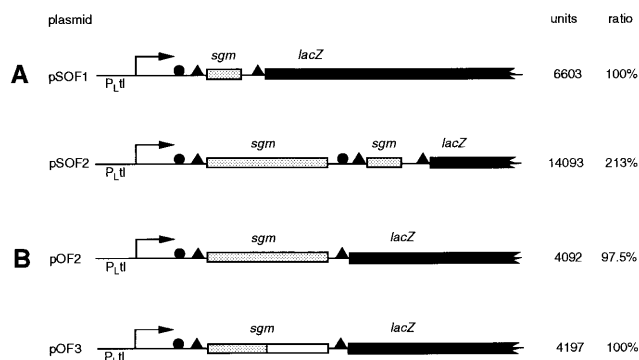
FIG. 2. Analysis of *sgm-lacZ* operon fusions. (A) The β-galactosidase activity of the strain carrying plasmid pSOF1 was taken as 100%. (B) The operon fusion on plasmid pOF2 was compared to the operon fusion carrying an inactivated copy of the *sgm* gene. Symbols used are as described in the legend to Fig. 1.

fore, we conclude that active Sgm methylase somehow controls expression of the *sgm* gene.

The operon fusion included the same part of the *sgm* gene as that in a gene fusion; however, it expressed a full-length *lacZ* gene with its own translational initiation signals (plasmid pSOF1). The expression of β-galactosidase from pSOF1 was compared with the expression of this enzyme from plasmid pSOF2, which contained an additional copy of the *sgm* gene driven by the same $P_L$tl promoter. Surprisingly, β-galactosidase activity was twofold higher when it was expressed from a plasmid that contained the *sgm* gene before *lacZ* (Fig. 2A). This could be explained by higher mRNA stability. To avoid this problem, two additional plasmids, pOF1 and pOF2, were constructed. Plasmid pOF1 contained the complete *lacZ* gene expressed from the $P_L$tl promoter, while in plasmid pOF2, the operon fusion included the entire *sgm* gene as well as the *lacZ* gene which contained its own RBS and translational start codon (Fig. 2B). As a control in this experiment, plasmid pOF3 was used. In this plasmid, the *sgm* gene was inactivated, and it could be used as a control since it had been shown previously that the inactivated gene has no effect on protein fusion. As shown in Fig. 2B, there is no difference in the β-galactosidase activities in extracts from those two cell types, indicating that expression of *lacZ* from the operon fusion was unaffected by Sgm methylase. Thus, we conclude that Sgm methylase specifically represses the translation of its own message.

The *sgm-lacZ* fusion can be repressed by Sgm methylase. To test whether the *sgm* gene product represses translation, we used plasmid pFK2, which contained the *sgm* gene expressed from the *kan* promoter (Fig. 3A). In this way, the *sgm-lacZ* fusion and the *sgm* gene, both of which were present on the same plasmid, were in the same copy number. Measurement of β-galactosidase activities confirmed that Sgm methylase reduces the amount of fusion protein. The residual activity of the fusion protein is approximately 28%, which is a much higher level than the level when the *sgm* gene was expressed from the same promoter as the *sgm-lacZ* fusion (5%). However, this level of repression is high enough to conclude that the product of the *sgm* gene is responsible for autoregulation.
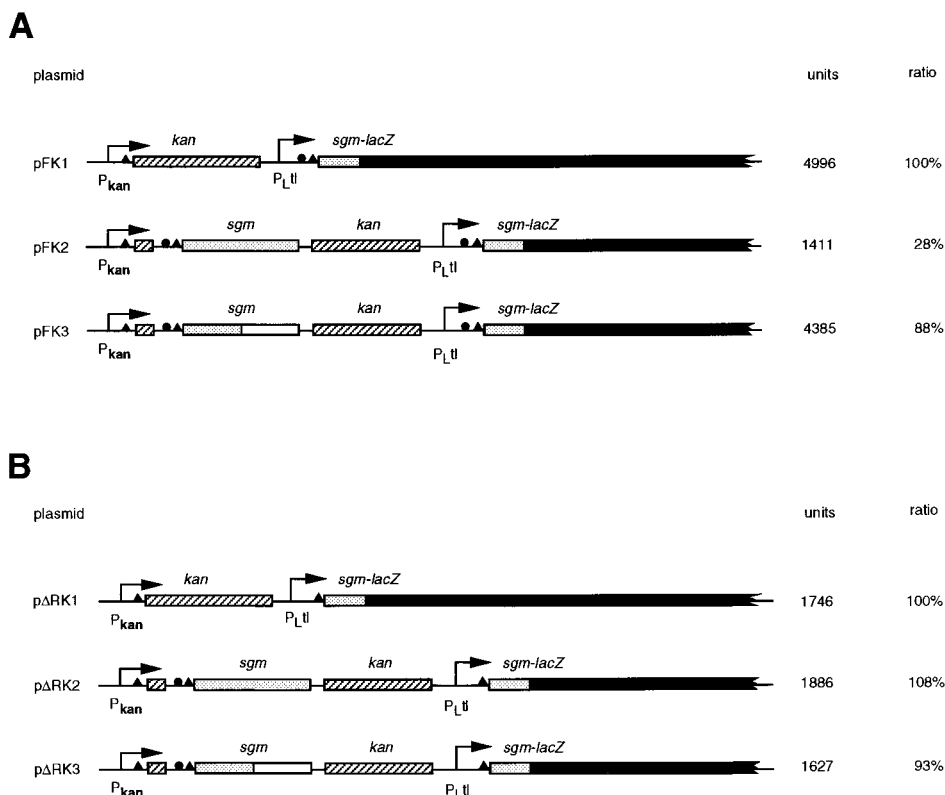

FIG. 3. Repression of the *sgm-lacZ* gene fusion by Sgm methylase. (A) Influence of Sgm methylase expressed from different transcriptional units. The activity of the fusion represented on plasmid pFK1 was taken as a control. (B) Effect of the *sgm* gene on the *sgm-lacZ* gene fusion constructed without the supposed regulatory region (circle before the *sgm* gene). The β-galactosidase activity of pΔRK1 was used as a control. All symbols are the same as those described in the legend to Fig. 1. Hatched box, the *kan* gene.
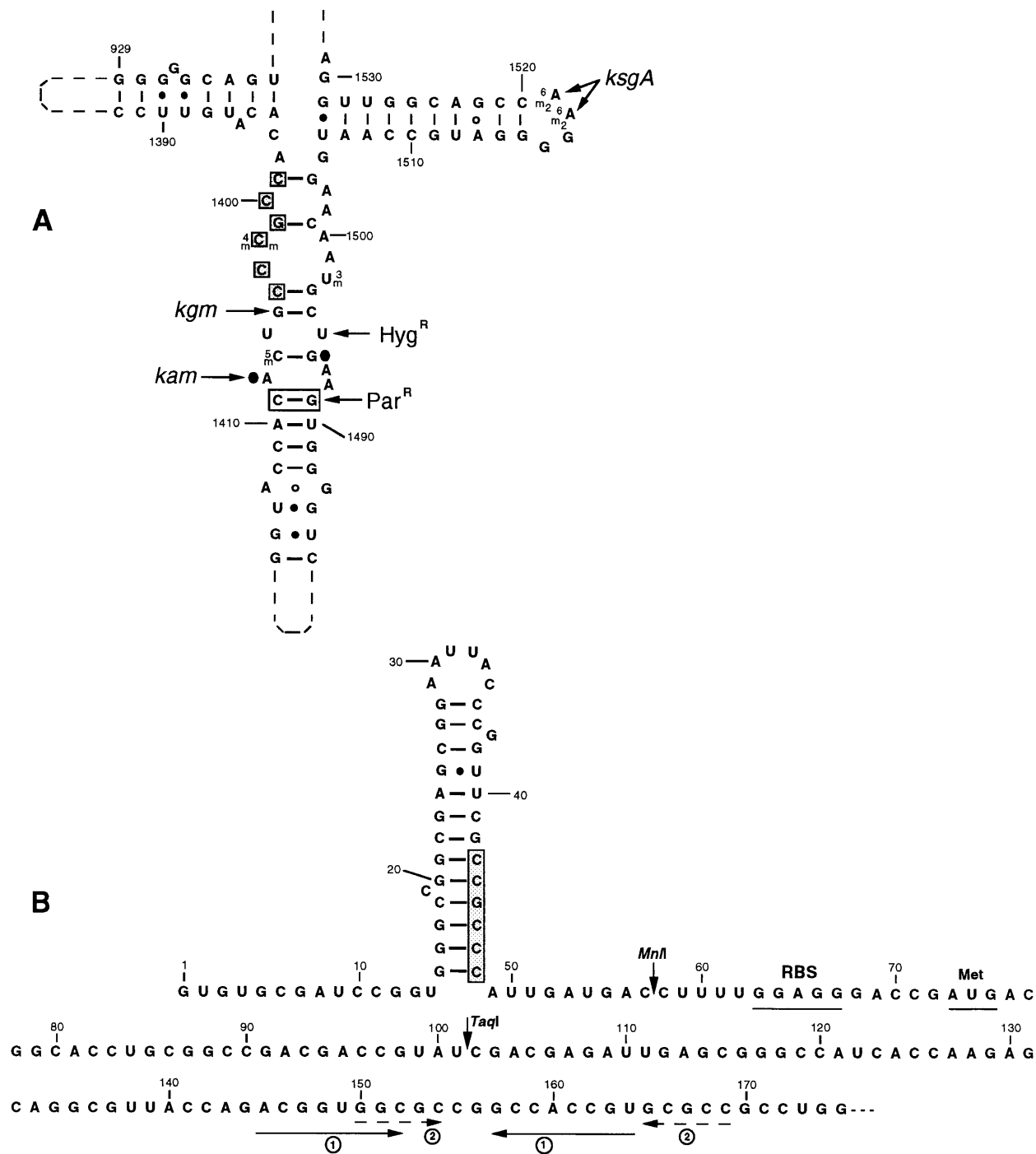
FIG. 4. Secondary-structure models of the 3′ end of 16S rRNA and the 5′ end of *sgm* mRNA. (A) Part of the recently refined secondary structure of the 3′ end of *E. coli* 16S rRNA with proposed tertiary interactions (9). The sites of action of antibiotic resistance methylase mutations giving resistance to antibiotics are from reference 8. Nucleotides identical to those found in the *sgm* mRNA are shaded. (B) Secondary structure of the 5′ end of the *sgm* mRNA derived from computer-aided RNA folding analysis. The hexanucleotide involved in autoregulation is shown in the shaded box. The RBS and translation initiation codon are underlined. Two possible secondary structures downstream of the RBS are indicated by arrows. The positions of *Mnl*I and *Taq*I cleavage sites in the corresponding DNA fragments are indicated. These sites were used for construction of different gene fusions.

Sequence required for translational autoregulation. To localize the *cis*-acting regulatory element(s) responsible for *sgm* autoregulation more precisely, two regions were taken into consideration. The first one includes sequences present within the *sgm* mRNA leader in which there exists a hexanucleotide sequence (CCGCCC) close to the putative RBS of the *sgm* gene (Fig. 4B). An identical sequence is also found in the 16S rRNA region in which most aminoglycoside resistance methylases act. A further *sgm-lacZ* fusion which contained the same 61 codons of the *sgm* gene preceded by its own RBS was

constructed; however, the CCGCCC hexanucleotide in the leader mRNA sequences was deleted (plasmid pΔRK1). The results of these experiments are outlined in Fig. 3B, and as can be seen, there is no significant difference in β-galactosidase activities between strains carrying only the mutated fusion and strains harboring an additional copy of the *sgm* gene (whether active or inactivated).

Although these results indicated the involvement of the first region in autoregulation, we also tested a second possible regulatory region. This region includes downstream sequences (within the coding region of the *sgm* gene) and consists of two alternative hairpin structures (Fig. 4B). New gene fusions were constructed by exploiting the *Taq*I site such that the new fusion contained only 10 codons of the *sgm* gene before the β-galactosidase gene. With such constructs, the same level of repression was obtained as that with plasmid pF2 (data not shown). Therefore, it is most likely that the sequences within a coding region of the *sgm* gene are not involved in autoregulation. This result, in conjunction with the results obtained with the fusion lacking mRNA leader sequences, indicates that the CCGCCC hexanucleotide before the RBS sequence might be required for translational repression.

DISCUSSION

Our interest in regulation of the *sgm* gene derives from the fact that we have been unable to detect Sgm protein synthesis in *E. coli* gentamicin-resistant cells. In the case of ribosome-modifying enzymes, such as Sgm methylase, expression of the resistance gene should not necessarily be maximized to achieve a higher level of resistance. It is anticipated that relatively few enzyme molecules are sufficient for complete modification of the target (i.e., rRNA) (7). Therefore, it is clear that a negative autoregulatory system could be involved in maintenance of a constant and also very low concentration of the Sgm protein. This low concentration is evidently sufficient for establishment of the gentamicin-resistance phenotype. Although repression could in principle be accomplished at the level of transcription, we consider this very unlikely the mechanism by which the *sgm* gene is controlled in *E. coli*. Two independent facts support such a consideration. First, expression of the *sgm* gene in a heterologous system, such as *E. coli*, is dependent on the host transcriptional signals. Consequently, we consider it highly questionable that a transcriptional regulatory system that is capable of acting upon such evolutionarily diverse promoter sequences is present in two different bacteria. Second, the Sgm protein can repress the activity of β-galactosidase only in the gene fusions but cannot repress *lacZ* in the operon fusions.

Many components of protein synthesizing machinery are autoregulated at the translational level, such as S1 (34), S4 (17, 18), S10 (14), S15 (3), and L11 (1) ribosomal proteins and also aminoacyl tRNA synthetase (40). Among posttranscriptionally autoregulated genes are those coding for RNA methylases. The *ermC* gene confers resistance to erythromycin in gram-positive bacteria, and in addition to translational attenuation, this gene is autoregulated (4, 13). In addition, the *ksgA* gene, a 16S rRNA-modifying enzyme, is autoregulated at the level of translation (43). The autoregulatory mechanisms in these examples are based on similarity between the mRNA (in sequence or secondary structure) and the target site of the respective protein. Therefore, we searched the *sgm* mRNA sequence for regions that might display such similarity with 16S rRNA. Unfortunately, the precise site of action of the *sgm* gene product in 16S rRNA is not known; however, the search could be reduced to the region of 16S rRNA where many aminoglycoside antibiotics exert their action. It has been shown

that gentamicin strongly protects A-1408 and G-1494, while residues A-1394, A-1413, and G-1487 are protected only weakly against chemical attack in the presence of gentamicin (31). Computer analysis revealed that the same hexanucleotide (CCGCCC) is present 14 bp before the *sgm* RBS and in the C-1400 region of 16S rRNA. This hexanucleotide might be involved in the formation of a specific hairpin structure in the *sgm* mRNA. However, obvious structural similarities between 16S rRNA and the *sgm* mRNA leader sequences were not found (Fig. 4). This suggested that the Sgm methylase recognizes primarily the same sequence within 16S rRNA and its own mRNA. Currently, we are undertaking more-detailed studies to determine the role of sequence and hairpin structure (i.e., site-directed mutagenesis and changing of the distance between the regulatory sequence and the RBS).

It is interesting that the *grm* gene from the closely related *M. purpurea* has a completely different mechanism of regulation. Apart from lacking regulation at the level of translation, the *grm* gene is transcribed from only one promoter close to the putative translational start site, and it is thus devoid of longer mRNA leader sequence. On the other hand, the *sgm* gene is transcribed from two promoters with different strengths (24). In this respect, it is tempting to speculate that development of an additional level of regulation (i.e., autogenous repression at the translational level) was useful on energetic grounds. Since few copies of methylase can protect an antibiotic-producing organism, unnecessary translation of this protein is prevented by binding to its own mRNA especially under conditions in which a stronger promoter is active.

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