Cell

Riboswitch Control of Aminoglycoside Antibiotic Resistance

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http://dx.doi.org/10.1016/j.cell.2012.12.019

SUMMARY

The majority of riboswitches are regulatory RNAs that regulate gene expression by binding small-molecule metabolites. Here we report the discovery of an aminoglycoside-binding riboswitch that is widely distributed among antibiotic-resistant bacterial pathogens. This riboswitch is present in the leader RNA of the resistance genes that encode the aminoglycoside acetyl transferase (AAC) and aminoglycoside adenyl transferase (AAD) enzymes that confer resistance to aminoglycoside antibiotics through modification of the drugs. We show that expression of the AAC and AAD resistance genes is regulated by aminoglycoside binding to a secondary structure in their 5′ leader RNA. Reporter gene expression, direct measurements of drug RNA binding, chemical probing, and UV crosslinking combined with mutational analysis demonstrate that the leader RNA functions as an aminoglycoside-sensing riboswitch in which drug binding to the leader RNA leads to the induction of aminoglycosides antibiotic resistance.

INTRODUCTION

The aminoglycoside antibiotics have played a historically important role in the treatment of serious bacterial infections. They bind to 16S rRNA in the decoding region of the 30S ribosomal subunit at the A site and cause mistranslation of mRNA and inhibit translocation (Davies and Davis, 1968; Fourmy et al., 1996; Carter et al., 2000). Originally isolated as natural products, they have been progressively developed to combat the spread of antibiotic-resistant infections through first and now second generation semisynthetic approaches (Armstrong and Miller, 2010). The cationic amine groups of the aminoglycosides give them the propensity to bind to negatively charged pockets in structured RNA (Hermann and Westhof, 1999), and additional binding sites have been found in rRNA (Carter et al., 2000; Borovinskaya et al., 2007). Binding sites have also been identified in the HIV *trans*-activating-region and Rev response element (Zapp et al., 1993) and in auto catalytic ribozymes (von Ahsen et al., 1991), and drug binding at such sites can induce conformational changes in the RNA (Davis et al., 2004; Murchie et al., 2004). Their potential to mold RNA structures has been exploited to generate RNA aptamers (Famulok and Hüttenhofer, 1996).

Resistance emerged shortly after their introduction and is associated with the mobile elements on plasmids or integrons responsible for transmissible multidrug resistance (Liebert et al., 1999). Integrons were originally discovered through the proliferation of antibiotic resistance (reviewed in Mazel, 2006). Aminoglycoside resistance is conferred most commonly through enzymatic modification of the drug or of the target rRNA through methylation or by the overexpression of efflux pumps (Nikaido, 2009). Enzymatic inactivation of the drugs is achieved through either N-acetylation (by acetyl transferases), O-adenylylation (by adenyl transferases), or O-phosphorylation (by phospho transferases) of amine or hydroxyl groups by specific enzymes (Figure 1A) (Mingeot-Leclercq et al., 1999). Induction of resistance genes by many classes of antibiotics has been studied for several decades (Lovett and Rogers, 1996) although the molecular details of the mechanism of induction are not yet completely understood. Resistance to the aminoglycosides is known to be inducible (Swiatlo and Kocka, 1987; Mingeot-Leclercq et al., 1999). The best characterized example of induction of ribosomal antibiotic resistance is the induction of the erythromycin resistance methyltransferase ERMC. A key feature of the *ermC* system is ribosomal stalling during the course of translation of a leader peptide through nascent leader peptide-ribosome interactions (Dubnau, 1984; Weisblum, 1995; Vazquez-Laslop et al., 2008).

Over the last decade small-molecule RNA interactions have been identified as a means of regulating gene expression. Riboswitches are regulatory RNAs that bind small-molecule metabolites and cofactors; they exploit specific interactions between low-molecular-weight metabolites and noncoding regions of messenger RNAs to regulate the biosynthetic pathway of the metabolite (Mandal et al., 2003; Nudler and Mironov, 2004;

Grundy and Henkin, 2006). They utilize a simple feedback mechanism whereby the interplay between two distinct structures in the mRNA controls the level of gene expression, in response to cellular conditions. The equilibrium between competing structures can be controlled by environmental conditions such as fluctuations in metal ion concentrations (Cromie et al., 2006; Dann et al., 2007), changes in pH (Nechooshtan et al., 2009), or temperature (Johansson et al., 2002). They show precise selectivity in controlling the expression of the biosynthetic enzymes of a range of metabolites that represent an assortment of chemical types ranging from relatively large molecules such as coenzyme-B12 (Nahvi et al., 2002) down to small amino acids like Lysine (Grundy et al., 2003; Sudarsan et al., 2003).

Here, we show that the expression of aminoglycoside antibiotic-resistance genes is controlled by a riboswitch. The aminoglycosides bind to the leader region of the aac/aad mRNA and cause a significant conformational change, leading to induction of a reporter gene. Aminoglycoside-RNA crosslinking and mutational analysis of the leader mRNA reveals the structural features that are important for antibiotic binding. We demonstrate a riboswitch mechanism of induction of aminoglycoside resistance genes in which antibiotic binding induces translation of the resistance gene.

RESULTS

A Conserved Sequence in the 5′ Leader RNA of Aminoglycoside Antibiotic-Resistance Genes

Resistance to the aminoglycoside antibiotics is most commonly achieved through modification of the antibiotic by specific enzymes (Figure 1A) (Mingeot-Leclercq et al., 1999) but can also be conferred by modification of the target site in rRNA or through excretion of the drug (Nikaido, 2009). We analyzed the 5′ leader RNA of a representative set of 50 aminoglycoside resistance genes from drug-resistant strains (Table S1A available online). Multiple sequence alignment (Larkin et al., 2007) of the 5′ leader RNA regions revealed that the leader RNA of two aminoglycoside acetyl transferase (AAC) and three aminoglycoside adenyl transferase (AAD) genes within this gene set show significant sequence identity (Figure 1B). The leader RNA has putative short open reading frames (ORFs) that encode leader peptides embedded upstream of the resistance gene, consisting of a ribosome-binding site (SD1) and start and stop codons for the leader peptides and a second ribosome-binding site (SD2) and start codon for the AAC/AAD coding sequences. The leader RNAs are predicted to adopt stable secondary structures. The sequence was found to be widely distributed upstream of aac and aad genes in the antibiotic-resistance (R) plasmids that confer multidrug resistance among a number of clinically important pathogens (Liebert et al., 1999; Hall et al., 2007; Nikaido, 2009). Leader RNA sequences from a range of organisms are shown in Figure 1C and Table S1B (the full sequence alignments are shown in Data S1). Further analysis of this RNA sequence revealed that nucleotides 1–39 were identical throughout the antibiotic-resistant strains, whereas the following nucleotides were more variable (Figure 1C). Significantly, the presence of identical nucleotides (1–39) upstream of two discrete classes of antibiotic-resistance genes, encoding an N-acetyl transferase and an O-adenyl transferase, respectively, for a number of bacterial strains suggests that there might be a common regulatory mechanism for expression of these enzymes.

The Aminoglycosides Induce Reporter Gene Expression through the 5′ Leader RNA of aac/aad

The natural promoter and transcription start site of aac in *Pseudomonas fluorescens* has been identified (Jacquier et al., 2009). There are 126 nucleotides (nt) from the transcription start site to the coding sequence of the resistance protein and 75 nt from the first SD1 to the coding sequence. To investigate whether the 5′ leader RNA of aac/aad has a regulatory role, we constructed reporter plasmids pGEX-leaderRNAaac/aad-lacZa in which the leader RNA (126 or 75 nt) was under the control of the IPTG-inducible tac promoter (Ptac) and positioned upstream of a b-galactosidase (b-gal) reporter gene. This construct does not include the AAC/AAD protein. The controllable Ptac promoter enables careful analysis of leader RNA function (Bailey et al., 2008). The reporter plasmid was transformed into *E. coli* strain JM109 and b-gal activity was examined in the presence of aminoglycoside antibiotics by agar diffusion assays. Strains containing the aac genes are typically resistant to the 4,6 deoxystreptamine aminoglycosides; kanamycin B (KanB), sisomycin, tobramycin, netilmycin, gentamycin, amikacin (Mingeot-Leclercq et al., 1999). We therefore used these drugs for agar diffusion assays. The 4,5 deoxystreptamine derivatives ribostamycin and paromomycin, and neamine, a fragment molecule were used as controls. Initially, we performed agar diffusion assays using the reporter plasmid containing the 126 or 75 nt leader RNA in the presence of IPTG and antibiotics (KanB, sisomycin, ribostamycin, and neamine). For constructs containing the 126 or 75 nt leader RNA, we observe induction of reporter gene expression with KanB and sisomycin but not with ribostamycin or neamine (Figures 2A and 2B). A more detailed investigation with all controls was therefore carried out using the reporter plasmid containing the 75 nt leader RNA. On titration of the 4,6 deoxystreptamine antibiotics a blue-green circular zone of induction is visible around the filter for KanB and sisomycin (Figure 2D), and also for tobramycin, netilmycin, gentamycin, and amikacin (Figure S1A) but not for the control molecules ribostamycin, neamine (Figure 2D), or paromomycin (Figure S1C). No induction by KanB was observed in cells transformed with the reporter plasmid without IPTG (under conditions in which the Ptac promoter is inactive) (Figure 2C) and no induction was observed on plates without KanB (Figure 2C) or the other aminoglycosides (data not shown). To verify that the induction of the reporter is specific for the leader RNA, we performed analogous experiments on a control plasmid pGEX-leader RNA-cat-86-lacZa in which the leader RNA of the cat-86 (encoding chloramphenicol acetyltransferase) (Duvall et al., 1984) gene replaced that of aac/aad gene and found this control construct to be unresponsive to addition of KanB (Figure 2C). To further confirm and quantify the agar diffusion assay, we also measured b-gal activity in solution (Zhang and Bremer, 1995) for KanB, sisomycin, ribostamycin, neamine, and paromomycin. The solution measurements of b-gal activity are in good agreement with the plate based agar diffusion assay (Figures 2E, S1B, and S1D). Significantly, these results show that the 4,6

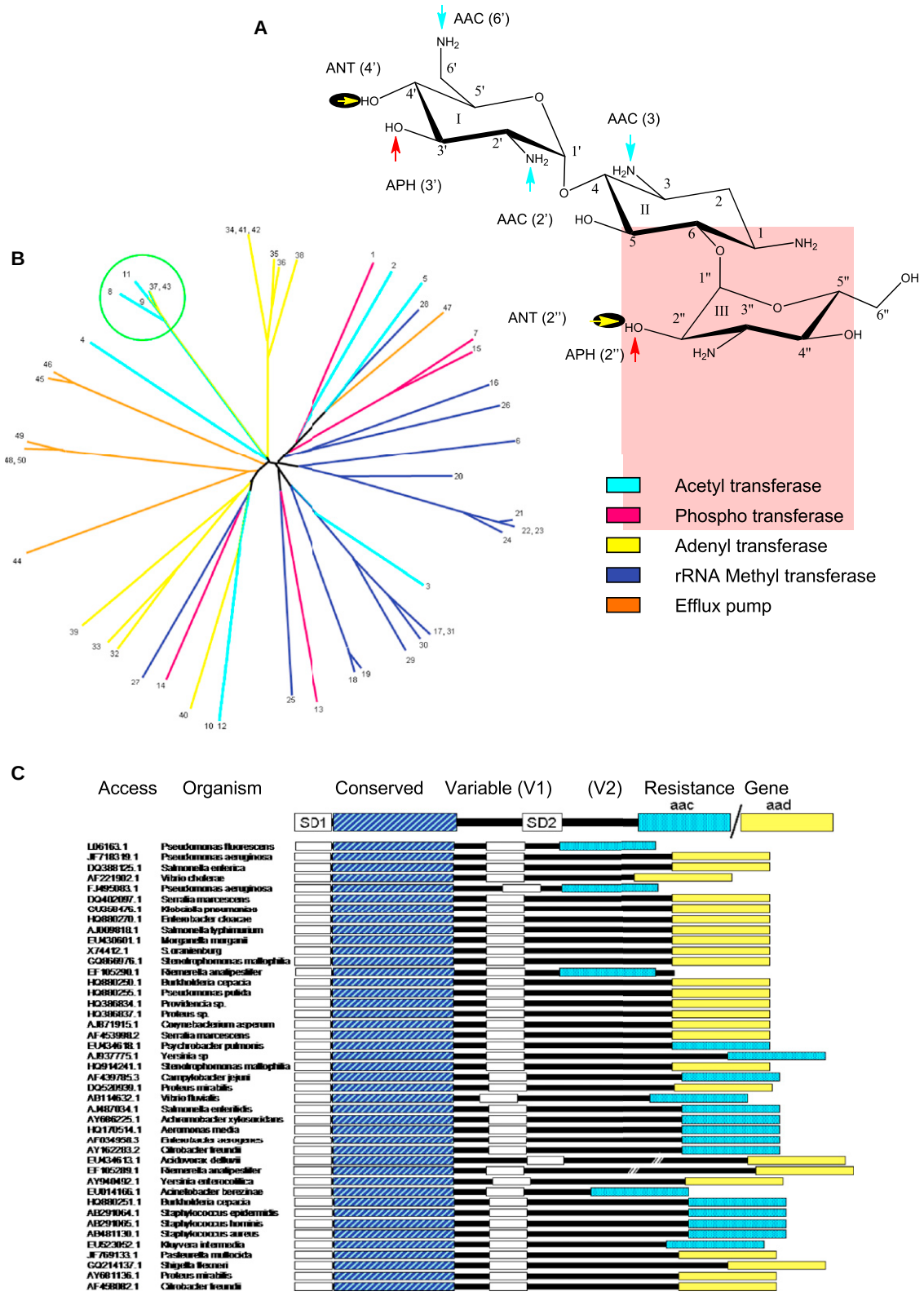

Figure 1. A Highly Conserved Sequence in the 5′ Leader RNA of Aminoglycoside Resistance Genes

(A) Structure of KanB (KanB), arrows indicate the positions that resistance acetyl transferase (AAC) (cyan), phospho transferase (APH) (red) and adenyl transferases (AAD) (yellow) modify.

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deoxystreptamine aminoglycosides (such as KanB or sisomycin) can induce expression of the reporter gene and that the leader RNA is required for induction. However, none of the 4,5 deoxystreptamine aminoglycosides ribostamycin, neamine, or paromomycin induced the reporter gene (Figures 2D, S1C, and S1D). Thus induction of reporter gene expression requires the presence of specific aminoglycosides and the leader mRNA, suggesting that the interaction of the specific aminoglycosides with the leader RNA may have a role in the induction of the resistance protein.

The Aminoglycosides Induce a Change in the Leader RNA Structure

To investigate the secondary structure of the leader RNA, we performed in-line probing, DMS probing and selective 2′-hydroxyl acylation analyzed by primer extension (SHAPE) on the 75 nt RNA in the absence of KanB by capillary electrophoresis with fluorescence detection. The data from the three independent probing methods are shown in Figure 3B and in combination with computational folding a potential RNA secondary structure was predicted (Figure 3A). To investigate the effect of drug binding on the leader RNA structure, in-line probing was further carried out on the 75 nt RNA on titration of KanB (Figure 3C). In response to KanB titration, some nucleotides show progressively increased or decreased cleavage and nucleotide scission is generally increased at the 3′ end (where SD2 and AUG is located) and decreased at the 5′ end (SD1) (Figures 3A and 3D), suggesting that KanB induces a structural transition. In particular, on titration of KanB fragmentation at SD2 is increased, implying that KanB causes changes in the RNA structure such that SD2 may become more accessible (Figures 3C and 3D). Similar results are observed by in-line probing of the 75 nt RNA on titration of the inducing antibiotics such as sisomycin and amikacin (Figures S2B and S2C). In contrast, the pattern of fragmentation remains unchanged with control antibiotics with no increase in cleavage at SD2 (Figures 3C and S2A). DMS probing of the 75 nt RNA with KanB is also consistent with the in-line probing data (Figure S3C). In-line probing of the full-length 126 nt RNA on titration with KanB show that the fragmentation patterns between SD1 and the resistance protein start site (that correspond to the shorter 75 nt RNA) are similar to those of the 75 nt RNA, suggesting that the folding and function of the 75 nt RNA is independent of the upstream sequence (Figures S3A and S3B). In reporter assays, KanB induces reporter gene expression from both the 75 and 126 nt RNAs. The 75 nt RNA can therefore be considered a minimal functional RNA. From the in-line probing data of the 126 nt RNA, we also observed increased accessibility at SD2 (Figures S3A and S3B). These data together suggest that aminoglycoside binding to the leader RNA causes a change in the RNA structure so that SD2 may become more accessible to the ribosome.

The Aminoglycosides Bind to Specific Regions of the 5′ Leader RNA

To examine aminoglycoside-leader RNA binding directly, we used surface plasmon resonance spectroscopy (SPR) (Hendrix et al., 1997). The minimal leader RNA was prepared by in vitro transcription using T7 RNA polymerase. Biotinylated leader RNA was immobilized on an SA-biosensor chip and the binding of each aminoglycoside was measured by flowing them over the immobilized RNA. The same set of molecules was used as before. Titration of the antibiotics led to an increase in the measured response that was consistent with the formation of an aminoglycoside-RNA complex. Figure 4 shows the dissociation constants (k_D) for aminoglycoside- RNA complex formation measured by SPR (Figures 3E and S4). Note that tobramycin, KanB, and sisomycin have the highest affinity for the leader RNA at 2.19, 2.78, and 6.8 mM, respectively, and under these conditions display noncooperative-binding behavior (with Hill constants n ~1) consistent with the formation of a 1:1 complex (Figures 3E and S4). In contrast, ribostamycin, neamine and paromomycin bind with lower affinities at 589 mM, 47 mM, and 12 mM respectively and ribostamycin and paromomycin may exploit a different binding mode (n ~ 0.6) (Figure S4). Thus, we find that the aminoglycosides that induce reporter gene expression in the reporter assays induce a conformational change to the RNA upon binding also display the highest affinity for the leader RNA in SPR measurements. In contrast, the control antibiotics display a different pattern of fragmentation in in-line probing and have the lower affinity for the leader RNA.

Sisomycin is a 4, 6 deoxystreptamine aminoglycoside that has an unsaturated double bond between the 4′ and 5′positions of ring one (Figure 4) and that is suitable for UV crosslinking to RNA. Drug-RNA UV crosslinks can be mapped by primer extension (Porse et al., 1999). Sisomycin induces reporter gene expression (Figures 2A, 2B, 2D, and 2E), binds to the leader RNA in the mM range by SPR (Figures S4 and 4) and induces an altered RNA structure (Figure S2B). To identify the region of the leader RNA that the aminoglycosides bind, we performed UV crosslinking experiments in the presence of 100 mM sisomycin; RNA was reverse transcribed using a fluorescent primer and sequenced directly. The sites of specific crosslinks were detected by the position and incidence of abortive reverse transcripts when compared to UV-treated RNA in the absence of the drug (Porse et al., 1999), and a crosslink was identified between A18 and sisomycin, suggesting that A18 may be involved in aminoglycoside binding (Figures 3F, 3G, and 3A).

(B) Multiple sequence alignment of the leader RNA sequence; unrooted dendrogram of 50 aminoglycoside resistance genes including genes encoding AAC (cyan), APH (red), AAD (yellow), rRNA methyl transferases (blue) or efflux pumps (orange) for key see table S1. Two highly conserved AAC and three AAD genes are circled.

(C) Organization of the leader RNA of aac/aad in antibiotic-resistance strains; the highly conserved sequence and relative positions of ribosome-binding sites (SD1 and SD2), variable regions and resistance genes are marked for key see Table S2.

See also Data S1.

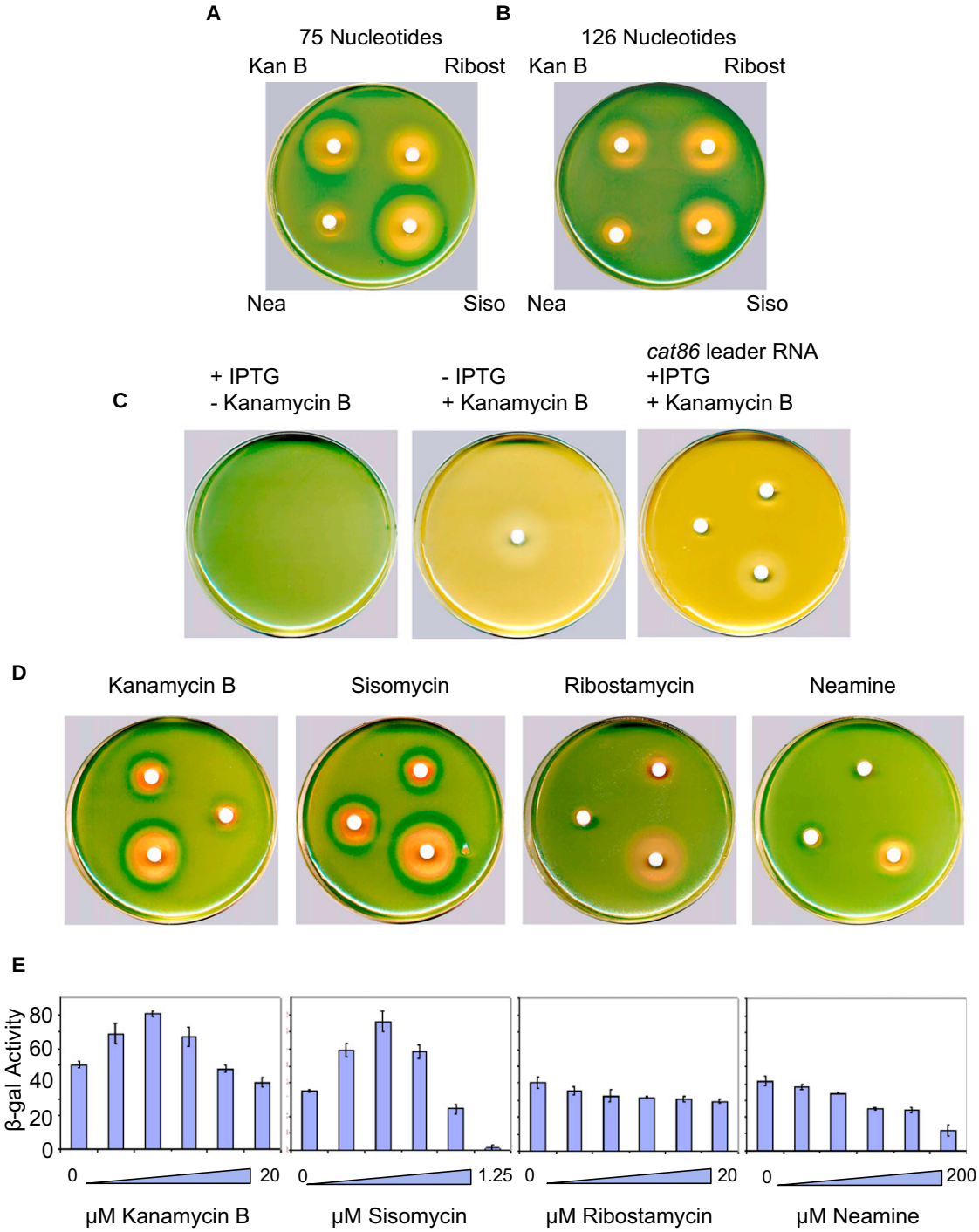

Figure 2. The Aminoglycosides Induce Reporter Gene Expression through the 5′ Leader RNA of aac/aad

(A and B) Agar diffusion assays of cells transformed with the reporter plasmid containing the 75 or 126 nt RNA grown on plates with IPTG; each filter disc was spotted with 1 ml of 100 mM KanB (Kan), sisomycin (Siso), ribostamycin (Ribost), or neamine (Nea).

(C) Control plates for (D); cells transformed with the reporter plasmid containing the 75 nt RNA grown on plate without KanB in the presence of IPTG, cells with the reporter plasmid grown on plate with 1 ml of 100 mM KanB in the absence of IPTG under conditions in which the Ptac promoter is inactive, cells transformed with plasmid pGEX-leaderRNAcat-86-lacZa that have the cat-86 leader RNA in place of aac/aad, with 1 ml of 100 mM (bottom filter [B]), 10 mM (top [T]) and 3 mM KanB (left [L]), and IPTG.

(D) Agar diffusion assay of cells transformed with the reporter plasmid containing the 75 nt RNA grown on plates in the presence of IPTG and titration of aminoglycosides. Filters were spotted with 1 ml of 100 mM (B), 10 mM (T) and 3 mM KanB (Right (R), 1 ml of 100 mM (B), 10 mM (L) and 3 mM (T) sisomycin, 1 ml of 100 mM (B), 10 mM (T) and 3 mM (L) ribostamycin, and 1 ml of 100 mM (B), 10 mM (L) and 3 mM (T) neamine.

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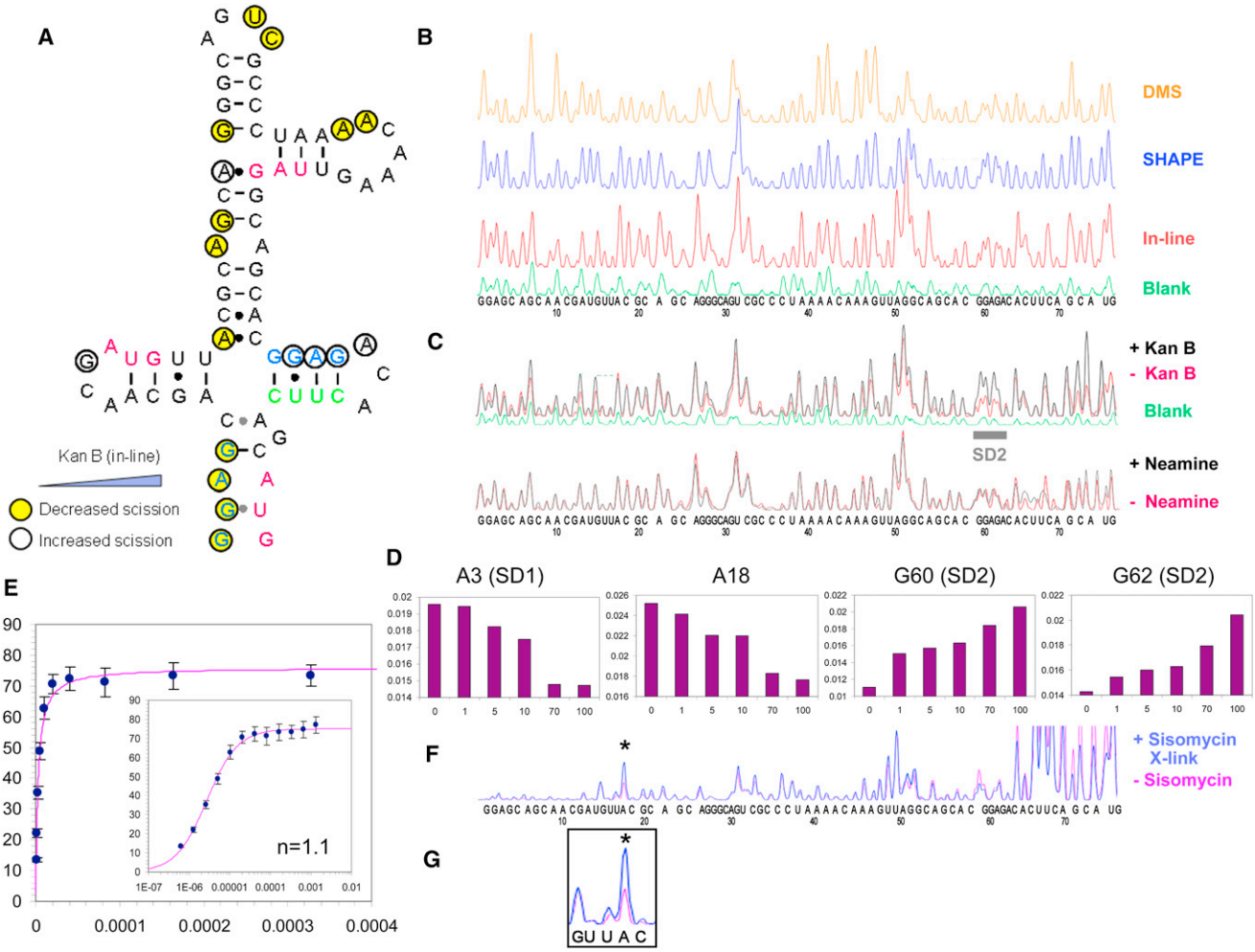

Figure 3. The Aminoglycosides Bind to Specific Regions of the 5′ Leader RNA of aac/aad and Induce a Change in the RNA Structure

(A) Predicted secondary structure of the 75 nt RNA by computational folding and structure probing analysis; the start and stop codon of the peptide and the start codon of the resistant protein are in red, SD1 and SD2 are in blue, the anti-SD is in green.

(B) DMS probing, SHAPE and In-line probing analysis of the 75 nt RNA in the absence of drug.

(C) In-line probing analysis of the 75 nt RNA ± 100 mM KanB and the control antibiotic neamine (100 mM).

(D) In-line probing analysis of position A3, A18, G60, and G62 on titration of KanB.

(E) Change in SPR signal in response units (RU) on KanB binding to immobilized aac/aad 5′ leader RNA, inset is a Hill plot of KanB binding and the Hill coefficient (n). Error bars are standard deviations of at least three independent experiments.

(F) UV crosslinking of sisomycin and the leader RNA * indicates the position of the crosslink.

(G) Enlargement of crosslink site.

See also Figures S2, S3, S4 and Table S3.

Mutational Analysis of the Leader RNA

To investigate the importance and function of the structural elements of the leader RNA a series of deletions and mutations were introduced into the RNA (Figure 5A and 5B). The effect of these mutations on reporter gene induction in the presence of KanB was determined by reporter assays. The minimal leader RNA comprises three possible loops. For Loop1, a point mutation in Loop1 (M3) and a mutation that disrupts the stem (M5) result in a loss of reporter gene induction by KanB. For Loop2, deletion of Loop2 (M6) eliminates induction of the reporter gene. The formation of stem 2 is confirmed by the disruptive (M7) and restorative (M8) mutations in the reporter assay (Loop 2 and stem 2 are confirmed by the chemical probing data (Figures 3A and 3B). Loop 3 and stem 3 are also consistent with the chemical

(E) b-gal activity (Miller units) of the reporter gene on titration of aminoglycosides; cells were grown in the presence of 0, 2.5, 5, 10, 15, and 20 mM KanB, 0, 0.08, 0.16, 0.31, 0.63, and 1.25 mM sisomycin, 0, 0.31, 1.25, 2.5, 5, and 20 mM ribostamycin, 0, 5, 10, 50, 100, and 200 mM neamine. Error bars are standard deviations of at least three independent experiments.

See also Figures S1 and S7, and Table S2.

	Ring I				Ring II			Ring III									
R1	R2	R3	R4	R5	R1	R2	6	R1	R2	R3	R4	R5	Antibiotic	Affinity	(μM) (+/-)	Charge	Induction	In-line
H	NH2	OH	H	NH2	OH	NH2		OH	NH2	H	CH2OH	OH	**Tobramycin**	2.19	(0.12)	+ 5	+	+
H	NH2	OH	OH	NH2	OH	NH2		OH	NH2	H	OH	CH2OH	**Kanamycin B**	2.78	(0.26)	+ 5	+	+
(		¥		)	OH	NH2		OH	NHMe	Me	NH2	OH	**Sisomycin**	6.				

probing data (Figures 3A and 3B). A compensatory mutation to stem 3 (M20) shows near wild-type levels of reporter gene induction and deletions to Loop 3 and stem 3 (M9, M14−16, M19) show reduced levels of induction of the reporter gene. A18 was identified as a possible binding site for the drug (Figures 3A and 3 F) by UV crosslinking, and the point mutation, M4, at A18 causes a significant reduction in induction of the reporter gene. Consistent with this, the mispaired nucleotides A18:C58 and C19:A57 appear to be critical to the function of the leader RNA; the mutations M21 and M22 that introduce Watson-Crick base pairs at these positions eliminate induction of the reporter gene. These mutational data are supported by the chemical probing data and the crosslinking data. Taken together these data show the structural and functional importance of stem loops 1−3 and specific structural features for antibiotic binding.

We observe that the ribosome-binding site SD2 (GGAG nucleotides 59−62) becomes more accessible toward chemical probes with the drugs that induce reporter gene expression. In contrast, accessibility of SD2 shows no change on addition of drugs that cannot induce reporter gene expression (Figures 3C and S2A). Furthermore, the nucleotides 66−69 (CUUC) are complementary to both SD2 and SD1 (GGAG nucleotides 1−4). We therefore speculate that in the absence of the antibiotic SD2 is sequestered by the anti-SD2 sequence blocking ribosomal access. Specific antibiotic binding induces a structural transition that allows anti-SD2 to pair with SD1 and consequently frees SD2 for ribosome binding (Figure 6). To assess this proposed model, we made some mutations to SD1 or SD2 and anti-SD2 sequence. Predictably M11 and M13, mutations in the ribosome-binding site SD2, show a significant reduction in the reporter gene expression, suggesting that function of the leader RNA is dependent on ribosome recognition and binding. The mutation M18 to the anti-SD2 that abolishes base-paring between SD2 and anti-SD2 exhibits a similar level of reporter gene expression to wild-type leader RNA with the drug. In contrast, M12, a point mutation to the anti-SD2 sequence that strengthens the base-paring between SD2 and anti-SD2 shows a reduced level of reporter gene. The point mutation M1, in SD1,