In Vitro Selection Analysis of Neomycin Binding RNAs with a Mutagenized Pool of Variants of the 16S rRNA Decoding Region†

Michael Famulok* and Alexander Hüttenhofer‡

Institut fur Biochemie der Ludwig-Maximilians-Universität München−Genzentrum, Würmtalstrasse 221, 81375 München, Germany

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ABSTRACT: An in vitro selection for neomycin B binding was carried out with an RNA pool containing a 47-nucleotide domain of the decoding region of 16S ribosomal RNA, mutated at 30% per base position. The degenerate region was comprised of an oligonucleotide analogue ("motif **A**") of the decoding region in 30S subunits which has previously been shown to interact with the aminoglycoside antibiotic neomycin B and tRNA ligands. After five cycles of selection/amplification, RNA sequences were isolated which specifically bound to neomycin B. Cloning and sequencing showed that none of the isolated clones shared primary sequence or secondary structure homology with the decoding region of 16S RNA. Instead, a new set of sequences was isolated which could be folded into a defined hairpin structure designated as motif **B**. We investigated the affinity of motif **A**, motif **B**, the unselected pool RNA, and the corresponding unmutagenized "parent" RNA to neomycin B at different Mg^{2+} concentrations. Under buffer conditions of low ionic strength all RNAs tested bound nonspecifically to neomycin B. However, motif **B** bound to neomycin B at Mg^{2+} concentrations at which binding of the other RNAs tested was significantly lower or not detectable. This is consistent with motif **B** exhibiting a higher affinity for neomycin B than motif **A** under these conditions. Motif **B** has previously been isolated from an in vitro selection to identify RNA sequences with affinity to neomycin B using a completely randomized RNA pool which shared no relationship to motif **A**. Our results indicate that motif **B** might represent a highly optimized RNA sequence for neomycin B binding; conversely, the A-site motif in 16S rRNA (motif **A**) might not be an optimal target for neomycin B recognition.

Many antibiotics interfere with protein synthesis by binding to the ribosome and perturbing distinct steps of translation. When bound to the A site in 16S ribosomal RNA, neomycin B and other aminoglycosides cause mistranslation, leading to misincorporation of amino acids into proteins (Noller, 1993). On the molecular level the function of aminoglycosides is poorly understood. The antibiotic may affect translation by interacting with the mRNA, the tRNA, or both. Alternatively, neomycin B could accomplish its effect by distortion of the ribosomal structure. Mutational analyses of drug-resistant strains (Cundliffe, 1990) and chemical modification analyses (Moazed & Noller, 1987; Woodcock et al., 1991) have identified the sites of interaction of antibiotics within the decoding region of 16S rRNA (Figure 1a). The putative function of the decoding region within the ribosome is to ensure selection of the correct aminoacyl-tRNA. Recently, it was shown by chemical probing analysis that a 49-mer oligonucleotide analogue (Figure 1a, "motif **A**") designed to mimic the decoding region of 16S rRNA is capable of interacting with antibiotics and tRNA anticodon stem−loop ligands in a similar way as within the context of the full-length 16S rRNA of the 30S ribosomal subunit [Purohit & Stern, 1994; for a comment see Schroeder (1994)].

The indication that large RNA molecules contain independent small functional domains prompted us to design a pool of highly mutagenized variants of this short oligonucleotide and to use it in an in vitro selection experiment [Tuerk & Gold, 1990; Robertson & Joyce, 1990; Ellington & Szostak, 1990; for reviews see Gold et al. (1995), Joyce (1994), Klug and Famulok (1994), and Bartel and Szostak (1994)] to obtain related neomycin B binding RNAs (Figure 1b). The objective of this study was to get an insight into the sequence constraints of the decoding region of 16S rRNA in binding to neomycin B as this region is highly conserved among various organisms and therefore phylogenetic variation is low (Gutell, 1993). A similar approach has previously been used to obtain an "artificial phylogeny" of an RNA domain within HIV-1 genomic RNA, the Rev responsive element (RRE), which interacts specifically with the HIV-1-Rev protein, and to use covariations among these sequences to propose a number of noncanonical base pairs which are important for Rev recognition (Bartel et al., 1991; Giver et al., 1993a,b; Leclerc et al., 1994; Williamson, 1994). Recently, this method has yielded considerable information about the secondary structure of an oligonucleotide ligase ribozyme (Ekland et al., 1995; Ekland & Bartel, 1995), isolated by in vitro selection (Bartel & Szostak, 1993). In the present study, we applied the same methodology to analyze the interaction of neomycin B with the decoding region.

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* Corresponding author. Tel: +49-89-74017-410. Fax: +49-89-74017-448. e-mail: Famulok@lmb.uni-muenchen.de.

‡ Present address: Institut für Genetik und Mikrobiologie, Maria Ward Strasse 1a, 80638 München, Germany.

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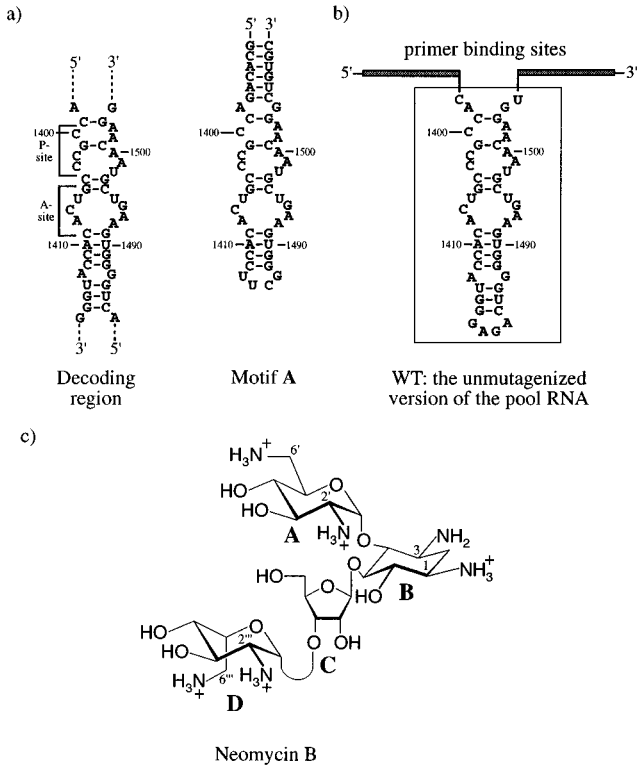

FIGURE 1: (a) Decoding region within 16S rRNA and motif **A** which has been shown to be sufficient for neomycin B binding under low salt conditions in buffer A. The numbers refer to the numbering of base positions in 16S rRNA. The location of the A and P site is indicated. (b) Construction of the mutant 47-mer pool used in the selection. The boxed region was randomized at a 30% degeneracy per base position. The primer binding sites are 5′-GGA ATT GAC GGG GAG ACT TGT ACA and 5′-AAC GGA AGT TGG ATC ACC TCC TTA GTA ATA AGG AGG AAA TAA AAA TGA AAA. (c) Structure of neomycin at pH 7.3. The mode of protonation of amino groups was determined previously by ^{15}N-NMR spectroscopic analysis (Botto & Coxon, 1983).

Aminoglycoside antibiotics have been shown to be inhibitory to several other biological functions involving RNA. Besides their effect on ribosomal RNAs, they have been found to inhibit self-splicing in catalytic RNAs such as the group I intron (Von Ahsen et al., 1992; Von Ahsen & Noller, 1993) or the hammerhead ribozyme (Stage et al., 1995). In addition, binding of neomycin to the Rev resposive element in HIV-1 RNA blocks the interaction of the Rev protein to its RNA target, resulting in a significant decrease of viral replication (Zapp et al., 1993). Although aminoglycosides have long been known to have an intrinsic nonspecific affinity to RNA (Dahlberg et al., 1978), these studies show that, depending on the applied salt and buffer conditions, aminoglycoside antibiotics can exert their effects in a highly specific manner. To test the dependence of neomycin recognition by RNA on the buffer conditions, we compared the binding of selected clones and the decoding site comprising RNA oligonucleotides to neomycin at different salt and bivalent cation concentrations.

MATERIALS AND METHODS

Materials. Neomycin sulfate (90−95% neomycin B, balance neomycin C, a stereoisomer at the C1 position of the B ring), DMS (dimethyl sulfate), and CMCT [1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate] were purchased from Sigma, KE (kethoxal) was from ICN, epoxy-activated agarose, Sephadex G-50, and dideoxy sequencing kits were from Pharmacia, and radiolabeled nucleotides were from Amersham. All enzymes, NTPs, dNTPs, glycogen, and vectors used were obtained from Boehringer Mannheim, except for RNasin (Promega), Superscript reverse transcriptase (Gibco-BRL), Taq-polymerase (Stratagene), PNKinase (New England Biolabs), T7 RNA polymerase [purified as described by Zawadzki and Gross (1991)], and nuclease T1 (Sigma). PCR primers were synthesized on a Millipore Expedite oligonucleotide synthesizer using standard phosphoramidite chemistry. Oligonucleotide purification was carried out as described previously (Famulok, 1994).

Degenerate Pool Synthesis. An unmutagenized 18-mer (sequence 5′-aac gga agt tgg atc acc-3′) was synthesized before coupling was continued for the 47 nucleotides homologous to bases 1397−1418 and 1482−1506 of the A/P site of 16S rRNA in Figure 1a. During coupling of these A/P-site domain nucleotides phosphoramidite mixtures were used to introduce point mutations at a rate of 30% per base position. The final 83-mer was obtained by synthesizing the constant 5′-primer binding site (sequence 5′-acg ggg gag act tgt aca-3′). The synthetic single-stranded 83-mer was purified on a 6% denaturing polyacrylamide gel [yield: 346 μg (12 nmol) of pure ssDNA] and PCR amplified for five cycles in a volume of 150 mL, using the oligonucleotide primers 44.AS (5′-tc**t aat acg act cac tat a**gg aat tga cgg ggg aga ctt gta ca-3′; 5′-primer, the T7 promotor is shown in bold face) and 51.AS (5′-ttt tca ttt tta ttt cct cct tat tac taa gga ggt gat cca act tcc gtt-3′; 3′-primer) to yield a double-stranded 142-mer DNA pool (pool 0) with the T7 polymerase promotor (boldface). The pool complexity was estimated as follows: of the 12 nmol of ssDNA 7% were found to be amplifiable by PCR under the PCR conditions used; 12 nmol of synthetic random DNA equals 7.2×10^{15} individual molecules; 7% of this equals 5×10^{14} full-length dsDNA molecules. Pool DNA was in vitro transcribed and purified as described previously (Famulok, 1994).

Pool Selection and Amplification. Neomycin-derivatized agarose was prepared as described previously (Wallis et al., 1995). In vitro selection cycles were carried out with 0.5 mL of derivatized agarose with *n*-propylamine-derivatized agarose used for negative selection. In the first cycle, five pool equivalents of pool 0 RNA (350 μg) were loaded onto the column, incubated for 10 min at 23 °C, washed with five column volumes (2.5 mL) of selection buffer [buffer A (80 mM K-Hepes, pH 7.6; 50 mM NH_4Cl, 5% poly(ethylene glycol)) adjusted to 5 mM $MgCl_2$ and 300 mM NaCl], and eluted with 3 column volumes (1.5 mL) of 5 mM neomycin B in selection buffer. The eluted RNA was precipitated in the presence of glycogen and subjected to reverse transcription and PCR amplification, followed by in vitro transcription. After five cycles of selection/amplification the pool 5 DNA was digested with *Eco*RI and *Bam*HI, cloned into the plasmid pvcBM20 (Boehringer Mannheim), and sequenced.

Binding Assays. Tests for metal ion dependence of binding of different RNA motifs to neomycin B were performed as described previously (Wallis et al., 1995) with the modification that 0.5 mL of neomycin agarose were used, and no affinity elution was carried out. RNAs were equilibrated in buffer A including the respective metal ions prior to being loaded onto the equally equilibrated neomycin column.

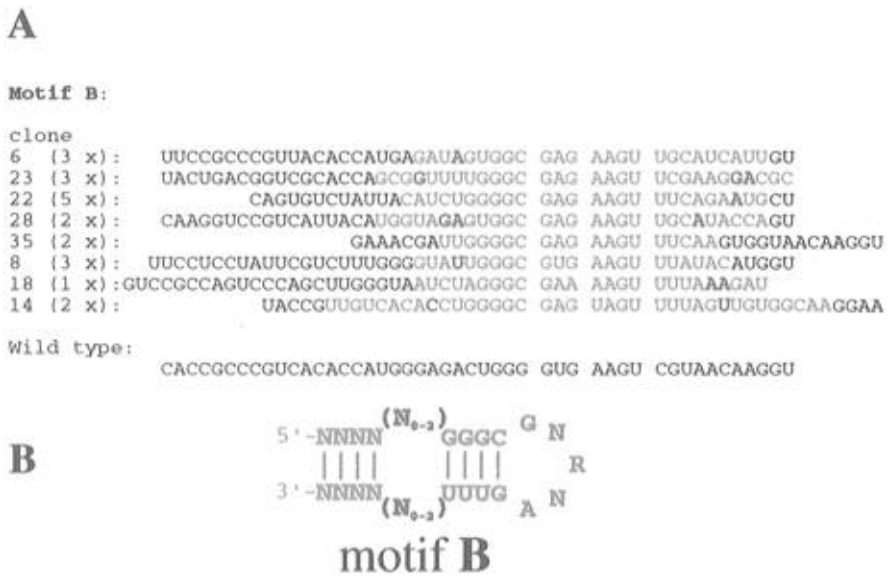

FIGURE 2: (A) Sequences of 23 selected neomycin binding clones. The bases which are relevant for forming motif **B** are shown in color. The color scheme is reflected in the secondary structure scheme of motif **B** shown below the aligned sequences. For comparison the wild-type sequence corresponding to the mutagenized insert is also shown. (B) Secondary structure scheme of motif **B**.

Partial Alkaline Hydrolysis and Nuclease Probing of RNA Secondary Structure. 5′ and 3′ end labeling and partial alkaline hydrolysis of RNA were carried out as described previously (Wallis et al., 1995). Selection of partially hydrolyzed RNA was performed under in vitro selection conditions in buffer A adjusted to 5 mM $MgCl_2$ and 300 mM NaCl. The fraction of RNA that was still capable of specific neomycin recognition was treated in the same way as described previously (Wallis et al., 1995). Probing of 5′-end-labeled RNA with nuclease T1 and nuclease S1 was performed as described elsewhere (Donis-Keller et al., 1977).

RESULTS

We carried out a selection using a pool which contained a 47-mer domain of the A and P site of 16S ribosomal RNA, mutated at a 30% degeneracy per base position (Figure 1b). With 70% of the positions in a given variant being wild type, the probability of synthesizing an oligonucleotide with the wild-type sequence is $0.7^{47} = 5.2 \times 10^{-8}$. The pool complexity of the starting pool was 5×10^{14} molecules so that the unselected pool contained an average of 2.5×10^{7} molecules of wild-type sequence. The mutated region of this pool is flanked by constant primer binding sites which are related to sequences in the analoguous domain within 16S rRNA (see Materials and Methods). We planned to carry out the selection under the same low salt and magnesium-free buffer conditions (buffer A: 80 mM K-Hepes, pH 7.4, 50 mM NH_4Cl, 5% PEG) which had previously been used by Purohit and Stern to demonstrate that motif **A** (Figure 1a) is sufficient for neomycin B binding. However, under these conditions nearly 100% of the unselected degenerate pool bound to the neomycin column. It must be considered highly unlikely that this binding is due to sequence-specific recognition of neomycin B by all of the 5×10^{14} different sequences; therefore, we consider binding under these conditions as nonspecific. After adjustment of buffer A to 300 mM NaCl and 5 mM $MgCl_2$, less than 0.5% of the unselected pool was retained on the column, indicative of nonspecific binding being abolished. Therefore, selection was performed using buffer A adjusted to 300 mM NaCl and 5 mM $MgCl_2$. Assuming that a particular array of positive charges provided by the ammonium side chains of neomycin B is required for specific interaction with the target RNA, we chose an immobilization protocol for neomycin which guarantees that the primary amino groups can still be protonated when coupled to the matrix. When immobilized on epoxy-activated agarose, the primary amino group is changed to a secondary alkylamine which retains the potential of the immobilized amino group for carrying a positive charge. The concentration of neomycin on the epoxy-activated agarose was between 1 and 2 mM.

After five cycles of in vitro selection more than 30% of the input RNA bound to the column and could specifically be eluted with neomycin B in binding buffer. The RNAs from the final cycle were converted to DNA by reverse transcription; the cDNA was PCR amplified, cloned, and sequenced.

Most abundant among the binding RNAs was a set of sequences which contained a conserved region, GGG CGN RNA GUU U, and exhibited a significant degree of variation compared to the wild-type sequence. None of the selected sequences could fold into the putative wild-type secondary structure motif, which is based on phylogenetic (Gutell, 1993) and mutational analyses (Cunningham et al., 1992, 1993). The sequences containing the consensus sequence fold into the hairpin loop secondary structure designated as motif **B** (Figure 2B). The same motif has been isolated in a previous study in which neomycin binding aptamers were selected from a *completely* randomized pool *which had no relationship to the decoding region* (Wallis et al., 1995). The secondary structure model of this motif was based on the covariations in the sequences and chemical probing analysis which confirmed the proposed folding and showed that neomycin B protects bases in the loop and bulge regions from modification. It might be argued that the repeated isolation of a particular motif could be due to possible contamination with those sequences isolated in the previous in vitro selection in the same laboratory (Wallis et al., 1995). Since the degenerate pool used in the present study was PCR amplified with a completely unrelated set of primers, this

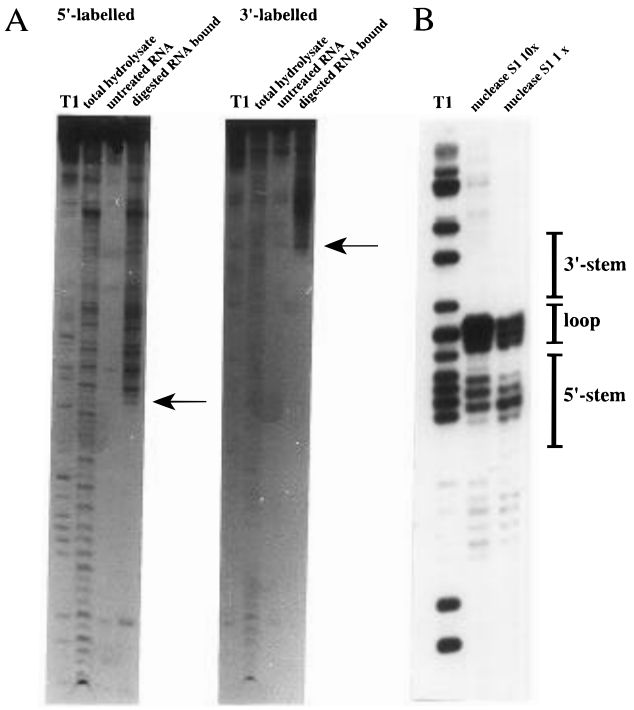

FIGURE 3: Partial alkaline hydrolysis damage selection and secondary structure probing of the most abundant motif **B** comprising clone 22. (A) Determination of the length requirement for motif **B**. The autoradiographs show 5′- and 3′-labeled clone 22. Lane labels: T1, clone 22 RNA digested with nuclease T1 under denaturing conditions (7.5 M urea); total hydrolysate, OH^--digested RNA prior to the selection; untreated RNA, unhydrolyzed and unselected labeled RNA; digested RNA bound, OH^--digested RNA that retained its ability to bind to neomycin B. (B) Secondary structure probing of clone 22 RNA with different concentrations of nuclease S1. Loop and stem regions are indicated by black bars.

would be quite unlikely to happen. Nevertheless, in order to exclude this possibility with certainty, we performed 30 cycles of PCR of the pool DNA described by Wallis et al. (1995) using the primers for the doped A-site pool and did not detect any amplification product.

To provide further evidence for the proposed hairpin loop structure and to determine the minimal sequence requirements for antibiotic binding of the hairpin motif, we carried out a damage selection by partial alkaline hydrolysis of end-labeled RNA aptamers as well as structural analysis by enzymatic and chemical probing. Partial alkaline hydrolysis of the end-labeled RNAs and subsequent analysis of those RNA fragments still capable of binding to neomycin B revealed that the portion of the sequence which folds into motif **B** (shown in color in Figure 2A) is sufficient for neomycin binding (Figure 3A). Chemical probing with DMS, CMCT, and KE (data not shown) confirmed the proposed secondary structure. S1 nuclease cleavage showed bases in the putative loop region being susceptible to nuclease attack. Interestingly, G bases in the 5′-half of the proposed stem region which are involved in the formation of three consecutive G·U wobble base pairs are also moderately susceptible to nuclease attack (Figure 3B). This might be indicative of a higher order tertiary structure of this RNA motif where nucleotides in the 5′-half of the stem are more exposed to the solvent than in the 3′-half. Together, these data confirm that the sequences selected from our mutagenized pool of A-site variants are indeed identical to the

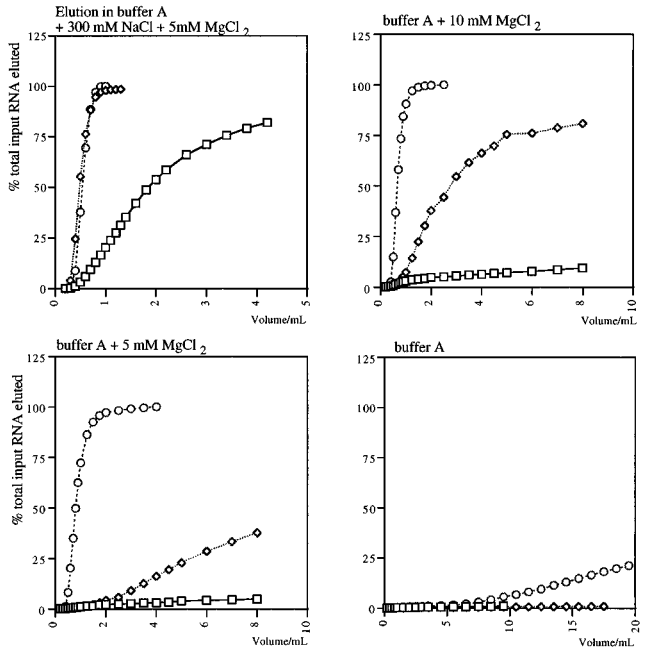

FIGURE 4: Elution of motif **A**, WT, and motif **B** (clone 22) RNA under selection conditions and different bivalent cation conditions. Cumulative output cpm are expressed as the percentage of the input cpm. Symbols: (□) sum % total eluted motif **B**; (◇) sum % total eluted WT; (○) sum % total eluted motif **A**.

motif obtained from the completely randomized pool (Wallis et al., 1995).

In order to get an insight into the binding properties of motif **B** in comparison with motif **A** as well as the unmutagenized "wild-type" 16S RNA analogue (WT, Figure 1B), we studied and quantified the binding of these RNAs to neomycin under different mono- and bivalent cation concentrations. The finding that the unselected pool, consisting of approximately 5×10^{14} different individual sequences, bound nonspecifically to the neomycin column in the absence of Mg^{2+} and monovalent cation concentrations below 100 mM suggested a strong dependence of aminoglycoside binding on the applied buffer conditions. Furthermore, in vitro selections for lividomycin and kanamycin which were carried out in the absence of magnesium at high monovalent cation concentrations (500 mM Na^+) yielded a large number of different tight-binding sequences without any obvious primary sequence homology to each other, indicating that aminoglycoside recognition can be achieved by many different RNAs but only under certain buffer and salt conditions (Lato et al., 1995).

In the present study we indeed find that neomycin B binding of both the selected RNA motif **B** and the A-site variant used by Purohit and Stern (1994) (motif **A**) is strongly affected by the concentration of Mg^{2+}. Adjustment of buffer A [the buffer which has been used to demonstrate the binding of neomycin B to motif **A** (Purohit & Stern, 1994)] to increasing concentrations of Mg^{2+}, however, abolished the binding of this motif to neomycin B; e.g., at 5 mM Mg^{2+}, no binding of motif **A** to neomycin B was observed. A similar decrease in neomycin affinity of motif **A** was measured when 5 mM Mg^{2+} was substituted by Ca^{2+} or Mn^{2+} (data not shown). In contrast, our selected sequences (comprising motif **B**) did bind tightly in buffer A including 5 mM Mg^{2+} (Figure 4).

Table 1: Relative Binding Affinities of Different RNAs[a]

RNA	buffer conditions	elution vol (mL)	app K_d (μM)
motif **A**	buffer A	46	1
	+1 mM $MgCl_2$	21	5
	+2 mM $MgCl_2$	3.3	34
	+5 mM $MgCl_2$	0.5	>1000
	+10 mM $MgCl_2$	0.5	>1000
	+5 mM $MgCl_2$ + 300 mM NaCl	0.5	1000
motif **B** (clone 22)	buffer A	nd[b]	
	+5 mM $MgCl_2$	197	0.5
	+10 mM $MgCl_2$	90	1
	+5 mM $MgCl_2$ + 300 mM NaCl	2.2	55
WT	buffer A	nd[b]	
	+5 mM $MgCl_2$	11	10
	+10 mM $MgCl_2$	2.5	48
	+5 mM MgCl + 300 mM $NaCl_2$	0.5	>1000

[a] Apparent dissociation constants were determined by analytical affinity chromatography as described (Ellington & Szostak, 1990). [b] nd = not determined.

The wild-type construct WT (i.e., the nonmutagenized sequence shown in Figure 1a plus the invariant sequences which served as primer binding sites; see Materials and Methods) bound in buffer A in the presence of 5 mM Mg^{2+}, although with a 20-fold weaker affinity than the selected sequences (Figure 4). In contrast, motif A does not show significant affinity to neomycin B under these conditions. The only difference between WT and motif A is the presence of the constant regions in the WT construct which served as primer binding sites. These constant regions are related in their primary sequence to regions within 16S rRNA which flank the decoding site (see Materials and Methods). When 300 mM NaCl was added, binding of the wild-type construct WT was not detectable. We quantified the binding studies by determining the apparent dissociation constants of some selected sequences under different buffer conditions (Table 1).

DISCUSSION

Our in vitro selection approach to search for variants of the 16S rRNA decoding region which are able to bind to neomycin B provides the first example in which a degenerate pool of a biologically relevant RNA sequence with known affinity to a low molecular weight ligand has been applied in a selection. It is an approach to test whether a small RNA motif which folds into a biologically significant conformation (Purohit & Stern, 1995) is particularly suited for neomycin recognition compared to other RNAs. It resulted in an RNA motif, designated as motif **B**, which significantly differed in its primary sequence as well as secondary structure from the original wild-type motif, designated as motif **A**. This small decoding site comprising RNA oligonucleotide has previously been shown by chemical probing analysis to interact with neomycin B (Purohit & Stern, 1994). Although the mutagenized pool of different RNA molecules which covered about 5×10^{14} different sequences contained an average of 2.5×10^7 molecules of the wild-type sequence resembling motif **A**, this motif was not isolated under our selection conditions.

Selection conditions contained 5 mM Mg^{2+} and 300 mM NaCl in addition to the same buffer which has previously been used for probing the interaction of motif **A** and neomycin B with chemical probes (Purohit & Stern, 1994). We show that omitting these mono- or bivalent cations results in nonspecific binding of the unselected pool RNA to neomycin. Under the selection conditions, however, neither motif **A** nor the wild-type sequence WT including 16S rRNA flanking regions to motif **A** bound to neomycin B, demonstrating that under increased ionic strength these RNA oligonucleotides exhibited only a very weak affinity to neomycin B. The observed differences in binding affinity between WT and motif **A** at 5 and 10 mM Mg^{2+}, respectively, are apparently due to motif **A** lacking the constant regions present in the WT construct. These regions, therefore, contribute to increased binding to neomycin, either ionic or nonionic in character, under these conditions.

In contrast, the selected RNA motif **B** bound to neomycin with micromolar apparent dissociation constants even under conditions of the higher ionic strength applied during the selection. This is indicative of motif **B** exhibiting a generally higher binding affinity to neomycin than does the original wild-type sequence and motif **A**. In this sense, sequences containing motif **B** must be considered as being optimized for neomycin recognition because they exhibit micromolar apparent K_ds at high mono- and bivalent cation concentrations. The fitness of this motif to bind neomycin is also reflected by the outcome of the present selection: although it was much less abundant in the doped pool than variants of motifs which resemble the decoding region, it was nevertheless selected. On the other hand, motif **B** containing sequences were also the most abundant to be isolated from a completely randomized pool, which further underlines their neomycin binding ability. The isolated RNA motif presents a consensus sequence which folds into a hairpin secondary structure including a GNRNA loop and a stem which might feature a widened major groove, due to the presence of three lined-up G·U wobble base pairs identical to motif **B** isolated in the present study.

The binding affinity between neomycin B and all RNAs tested in this study is clearly a function of the Mg^{2+} concentration. However, increasing concentrations of Mg^{2+} seem to have a significantly stronger effect on binding affinity for motif **A** and WT than for motif **B** (Table 1). In this context it might be important to note a previous finding which showed that the modification pattern as well as the neomycin induced protection from chemical modification in motif **B** is identical at 0 and 5 mM Mg^{2+}, which indicated that the specific interaction of motif **B** with RNA is not of pure electrostatic nature (Wallis et al., 1995). The more dramatic effect of [Mg^{2+}] for neomycin B binding of motif

A and WT might be indicative of a direct competition between the antibiotic and Mg^{2+} for RNA binding sites to play a role for the binding affinity. In a similar study, Clouet-d'Orval et al. (1995) tested the neomycin B induced inhibition of RNA cleavage by the hammerhead ribozyme as a function of [Mg^{2+}]. Increasing [Mg^{2+}] led to significantly reduced inhibitory activity of neomycin B. This effect was also interpreted in favor of a competition between Mg^{2+} ions and neomycin B for binding to the RNA.

The fact that motif **B** has a considerably higher affinity to neomycin B than motif **A** implies that the 16S rRNA decoding region might not be an optimal target for neomycin B binding. So far, a variety of other natural sequences, such as the Rev responsive element, the group I intron, and hammerhead ribozymes have also been shown to exhibit specific sites for interaction with aminoglycosides. However, these RNA structures might not be optimally evolved for neomycin recognition as their evolutionary selection criterion was not to bind effectively to neomycin. This is supported by the observation that—taken together—none of the in vitro selection experiments carried out with aminoglycoside ligands (Wang & Rando, 1995; Lato et al., 1995) have revealed primary sequence or secondary structure homology to any of the known aminoglycoside-responsive RNAs. Aminoglycosides represent a class of compounds which possess a stereochemically well-defined array of hydrogen bond donors and acceptors as well as positive charges (Figure 1c) and should therefore be able to interact with a variety of different natural and "artificial" RNA structures in a specific manner (Hirao & Ellington, 1995).

On the other hand, binding to the 16S RNA decoding site might be neccessary but not sufficient for the biological activity of aminoglycosides. The A site in 16S rRNA evolved to optimize bacterial ribosome function and not to bind aminoglycosides. However, it seems plausible that the antibiotic warfare in aminoglycoside-producing microorganisms evolved in response not only to preexisting RNAs but also to the associated ribosomal proteins. Lastly, it is still unknown how aminoglycosides exert their antibiotic function on a molecular level. The fact that motif **A** was not obtained as a winning neomycin B binding sequence despite its high abundance in the randomized pool suggests that binding to the 16S rRNA decoding site and causing mistranslation might represent two independent functions of this compound. Accordingly, the binding affinity of aminoglycoside antibiotics to the decoding region might not be a sufficient measure for the antibiotic potential of the respective drugs.

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