Engineering the rRNA decoding site of eukaryotic cytosolic ribosomes in bacteria

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ABSTRACT

Structural and genetic studies on prokaryotic ribosomes have provided important insights into fundamental aspects of protein synthesis and translational control and its interaction with ribosomal drugs. Comparable mechanistic studies in eukaryotes are mainly hampered by the absence of both high-resolution crystal structures and efficient genetic models. To study the interaction of aminoglycoside antibiotics with selected eukaryotic ribosomes, we replaced the bacterial drug binding site in 16S rRNA with its eukaryotic counterpart, resulting in bacterial hybrid ribosomes with a fully functional eukaryotic rRNA decoding site. Cell-free translation assays demonstrated that hybrid ribosomes carrying the rRNA decoding site of higher eukaryotes show pronounced resistance to aminoglycoside antibiotics, equivalent to that of rabbit reticulocyte ribosomes, while the decoding sites of parasitic protozoa show distinctive drug susceptibility. Our findings suggest that phylogenetically variable components of the ribosome, other than the rRNA-binding site, do not affect aminoglycoside susceptibility of the protein-synthesis machinery. The activities of the hybrid ribosomes indicate that helix 44 of the rRNA decoding site behaves as an autonomous domain, which can be exchanged between ribosomes of different phylogenetic domains for study of function.

INTRODUCTION

Accurate decoding of genetic information is a crucial step in protein synthesis. Genetic, biochemical and structural data provide evidence for a functional role of ribosomal RNA in mRNA decoding and tRNA selection (1–3). The functional relevance of rRNA residues in codon–anticodon recognition and stabilization is reflected in their universal conservation throughout the three phylogenetic domains of life. While this holds true for most nucleotides in helices 18, 34 and 44 of the small subunit rRNA that form the aminoacyl-tRNA acceptor site (A site), critical variations have evolved between different phylogenetic domains, most prominent in helix 44, the penultimate stem of 16S rRNA (4). These variations most likely account for a decoding region that has been highly optimized in the context of evolutionary differentiation.

Aminoglycosides are a class of structurally related antibiotics which interfere with decoding by binding to the A site of small subunit rRNA (5). These antibiotics preferentially target prokaryotic over eukaryotic ribosomes and affect protein synthesis by inducing codon misreading and inhibiting tRNA translocation (6–8). The binding site is located within a conserved loop of helix 44, which in part shows phylogenetic sequence variability, e.g. at position 1408 and at base pair 1409–1491 (9) (rRNA residues are numbered according to Escherichia coli nomenclature; see also Figure 2). Crystal structures of various bacterial 30S ribosomal particles in complex with different ligands, e.g. tRNA and antibiotics, have revealed the molecular mechanisms of the decoding step at atomic resolution (10,11). In the absence of high-resolution X-ray crystal structures of eukaryotic ribosomes, model oligonucleotides mimicking the ribosomal decoding site have been used for structural analysis of the A site in human cytosolic ribosomes (12).

Site-directed mutagenesis has been used in prokaryotes to study the functional relevance of individual drug-nucleotide contacts in aminoglycoside–ribosome interaction (13). A detailed analysis of the aminoglycoside target site in ribosomes of higher and lower eukaryotes is complicated by the complexity of eukaryotic rRNA genetics. Genetic manipulation of the rRNA component of eukaryotic ribosomes has proven exceedingly difficult.
due to the problem of the high copy number of corresponding operons. Higher eukaryotes have so far resisted any genetic manipulation of their ribosomal nucleic acids, virtually abolishing the possibility to test hypotheses by experimentation. Even in lower eukaryotic organisms, such as yeast, the presence of 100–200 tandemly repeated copies at the RDN locus, has made genetic studies of rRNA difficult (14–16).

The eukaryotic ribosome differs significantly from the prokaryotic one (17). It is impossible to predict the contribution of additional, phylogenetically diverse ribosomal elements and characteristics, as in the eukaryotic ribosome, to aminoglycoside susceptibility. It is, for instance, commonly assumed that translation in mammalian cells functions with higher fidelity than that of bacteria (18). To address this question experimentally, we have performed domain shuffling experiments in rRNA. Using genetic techniques, we successfully engineered a functionally critical domain in the ribosome by replacing the bacterial A-site rRNA of helix 44 with its counterpart from higher and lower eukaryotes. For functional characterization of these hybrid ribosomes, we studied their susceptibility to antibiotics targeting the ribosomal A site and which are known to affect bacterial versus eukaryotic ribosomes to different extents.

**MATERIALS AND METHODS**

**Construction of the rrn plasmid exchange system**

Starting with *Mycobacterium smegmatis* mc²-155 SMR5 (19), a single-rRNA allelic strain bearing a deletion of ~5 kb in the chromosomal rrnA operon was constructed by gene replacement. The deletion strategy for chromosomal rRNA operons is shown in Supplementary Figure 1. Subsequently and following integration of the rrnB-replacement vector into the chromosomal rrnB locus, the strain was transformed with integration-proficient vector pMH-rrnB²⁰⁵₈G for rRNA complementation. pMH-rrnB²⁰⁵₈G is a derivative of plasmid pMV361-rrnB²⁰⁵₈G (20), in which the kanamycin resistance cassette has been replaced with a hygromycin resistance cassette. Counterselection and genetic screening for deletion of the chromosomal rrnB locus resulted in strain *M. smegmatis* ΔrrmA ΔrrnB attB::pMH-rrnB²⁰⁵₈G with homogenous populations of hybrid ribosomes.

Figure 1. Sequential strategy for the generation of a plasmid-rRNA exchange system. From top: Following deletion of chromosomal rrnA, a complementation vector pMIH-rrnA²⁰⁵₈G carrying a functional rrn operon was introduced to the chromosomal attB site. Subsequent deletion of rrnB resulted in *M. smegmatis* ΔrrmA ΔrrnB attB::pMIH-rrnB²⁰⁵₈G, in which ribosomal RNA is exclusively transcribed from the plasmid. From there, a plasmid-rRNA exchange system was established by replacing pMIH-rrnB²⁰⁵₈G with pMIG-rrnB-sacB. Transformation with hybrid rRNA genes pMIH-rrnB²⁰⁵₈G hybrid and selection on sucrose resulted in *M. smegmatis* ΔrrmA ΔrrnB attB::pMH-rrnB²⁰⁵₈G hybrid with homogenous populations of hybrid ribosomes.
and the sucrose sensitivity marker sacB. This vector was used to replace pMIH-rrnB<sup>2058G</sup> in strain <i>M. smegmatis</i> Δrrn pMIH-rrnB<sup>2058G</sup>, resulting in strain <i>M. smegmatis</i> Δrrn pMIH-rrnB-sacB. The latter was used as a plasmid exchange system in which the rrrB wild-type operon can be efficiently replaced with an rrnB mutant operon by transformation and selection on sucrose-containing media (Figure 1).

### Construction of mutant strains with hybrid ribosomes

Site-directed mutagenesis of rRNA genes was performed by PCR mutagenesis as described previously (21). In brief, hybrid rDNA oligonucleotides comprising the eukaryotic helix 44 decoding site sequence were used for gene amplification. Using restriction endonuclease recognition sites present in the operon, rRNA gene fragments in plasmid pMIH-rrnB were replaced to construct pMIH-rrnB<sup>hybrid</sup>, the plasmids coding for hybrid 16S rRNA genes. <i>Mycobacterium smegmatis</i> Δrrn pMIH-rrnB-sacB was transformed with the various pMIH-rrnB<sup>hybrid</sup> constructs and selected on hygromycin plates containing sucrose (Figure 1). Successful plasmid exchange was controlled by sequence analysis.

### Minimal inhibitory concentrations

Broth microdilution tests were performed in a microtitre plate format as described previously (22). In brief, bacterial strains were cultured on Luria–Bertani (LB) agar plates at 37°C. Freshly grown cultures were resuspended in LB broth supplemented with 0.05% of Tween 80, diluted to an absorbance at 600 nm of 0.025, and incubated in the presence of 2-fold serial dilutions of the following 2-deoxystreptamine aminoglycosides: paromomycin, neomycin, genetin (G418), gentamicin, netilmicin, tobramycin and kanamycin A (Sigma). After incubation at 37°C for 72 h, the minimal inhibitory concentration (MIC) was recorded as the lowest concentration of drug inhibiting visible growth.

### Ribosome purification

Approximately 8 g of wet bacterial cell mass were resuspended in 25 ml of homogenization buffer (HB; 20 mM Tris–HCl pH 7.4, 100 mM NH₄Cl, 10.5 mM MgCl₂, 0.5 mM EDTA and 3 mM 2-mercaptoethanol) and lyzed with a French Pressure Cell (American Instrument Company, Maryland) at 16 000 psi, as described previously (23). The lysate was supplemented with DNase (RQI RNase-free, Promega, 2 U per gram cell mass), incubated on ice for 10 min, and adjusted with HB to a volume of 70 ml. Pre-cooled alumina (1.5 g per gram cell mass) was added to the cell lysate, stirred on ice for 20 min, and removed by slow-speed centrifugation (10 min at 2000g; all centrifugations were performed at 4°C). Cell debris was sedimented by centrifugation for 30 min at 10 000g and the lysate was passed through a tea filter. Centrifugation of the lysate for 60 min at 32 500g resulted in the S-30 supernatant. Sixteen milliliter portions of the supernatant were layered on 9 ml sucrose cushions (SC; 20 mM Tris–HCl pH 7.4, 350 mM NH₄Cl, 10.5 mM MgCl₂, 0.5 mM EDTA, 1.1 M sucrose and 3 mM 2-mercaptoethanol) and centrifuged for 15 h at 110 000g (Beckman Coulter Optima™ L-80 XP Ultracentrifuge) to separate ribosomal particles from the S-100 supernatant. The S-100 fraction was dialyzed for 24 h (6000–8000 MWCO, Spectra/Por, Serva) with three intermittent changes of buffer A (50 mM Tris–HCl pH 7.5, 70 mM NH₄Cl, 30 mM KCl and 7 mM MgCl₂), concentrated (Viva spin, 10 000 MWCO, Viva Science), and stored at −80°C. Ribosome pellets from the S-100 preparation were resuspended in washing buffer (WB; 20 mM Tris–HCl pH 7.4, 350 mM NH₄Cl, 10.5 mM MgCl₂, 0.5 mM EDTA and 7 mM 2-mercaptoethanol) and passed twice through sucrose cushions (6 h at 180 000g and 16 h at 83 000g). The final ribosome pellets were resuspended in buffer A, incubated for 30 min at 4°C, dispensed into aliquots, and stored at −80°C after shock freezing in liquid nitrogen. 70S ribosome concentrations were determined by absorption measurements on the basis of 23 pmol per A<sub>260</sub> unit.

### Qualitative evaluation of ribosome preparations

Integrity of 70S ribosomes was determined by analytical ultracentrifugation (14 h at 70 000g) through a 10–40% sucrose gradient in both association buffer (buffer A) and dissociation buffer (50 mM Tris–HCl pH 7.5, 70 mM NH₄Cl and 30 mM KCl). 70S, 50S and 30S ribosome fractions in different gradient layers were detected and quantified by absorption at 254 nm. Functional activity of purified ribosomes was determined by assessing their capacity to form initiation complexes. 70S ribosomes (0.5 M) were incubated in buffer A with m02 SD-MFTI-mRNA at 1.5 μM (5′-GGCAAGAGGAGUAAUAUAUG UUCACGAUC-3′; obtained from Dharmacon), initiation factors (IF) 1 and 3 from <i>M. smegmatis</i>, IF-2 from <i>E. coli</i> (1 μM each), GTP (1 mM), and [3H]-fMet-tRNA<sup>Fes</sup> in 1 μM for 60 min at 37°C; purified tRNA and IF2 from <i>E. coli</i> were a kind gift from Marina Rodnina. The initiation complexes formed were bound to nitrocellulose filters (Sartorius, pore size 0.45 μm), washed with 15 μl cold buffer A, dissolved in 10 ml scintillation cocktail (Filtersafe, Zinsser Analytic) and quantified in a Liquid Scintillation Analyzer (Tri-Carb 2900 TR, PerkinElmer).

### Cell-free (UUU)₁₂ translation assays

Cell-free translation reactions in buffer A (pH 7.5) were prepared on ice and contained <i>M. smegmatis</i> tRNA<sup>bulk</sup> (0.5 mg/ml), amino acids mixture (30 μM each) lacking phenylalanine and/or leucine, 10% (v/v) S100 extract, energy mix [DTT (1 mM), GTP (1 mM), ATP (4 mM), PEP (5 mM)], pyruvate kinase (0.1 mg/ml), and polyamines [spermidine (2 mM) and putrescine (8 mM)]. The reaction mixture was preincubated with radiolabeled phenylalanine and/or leucine (30 μM; obtained from Amersham) at 37°C for 15 min. The translation reaction was started by addition of ribosomes to a final concentration of 0.25 μM and (UUU)<sub>12</sub>-mRNA (5′-GCAGGCCAA GGAGGUAAGUG (UUU)<sub>12</sub> UAA GCAGG-3′, obtained from Dharmacon) to 1 μM; serially diluted aminoglycoside antibiotics were added simultaneously. Following incubation at 37°C for 60 min, the reaction was
stopped by addition of KOH to 0.5 M and subsequent hydrolysis at 37°C for 30 min. Synthesized polypeptides were precipitated with 200 µl of 5% tri-chloro-acetic acid (TCA) for 10 min on ice and bound to nitrocellulose filters (Sartorius, pore size 0.45 µm). Filter-bound polypeptides were washed with cold 30% 2-propanol and quantified in 10 ml of scintillation cocktail. Data analysis was performed with Prism (GraphPad Software Inc.). Best-fit nonlinear regression was used to define the 100% value of polypeptide synthesis and to calculate the aminoglycoside IC50 values of dose-dependent inhibition (Figure 3 and Table 2).

Cell-free luciferase translation assays

Purified 70S hybrid ribosomes were used in a coupled transcription–translation reaction of firefly luciferase (plasmid pBESTluc, Promega). A typical reaction (15 µl volume) contained 0.25 µM 70S ribosomes, 300 ng DNA, 40% (v/v) of M. smegmatis S100 extract, 100 µM amino acid mixture, and RNasin (40 U, Promega). rNTPs, tRNAs and energy were supplied by addition of commercial S30 Premix Without Amino Acids (Promega). Serially diluted aminoglycosides were added and the reaction mixture was incubated at 37°C for 60 min. The reaction was stopped on ice, 100 µl of luciferase assay substrate (Promega) was added, and the bioluminescence was measured in a luminometer (Bio-Tek instruments, FLx800). Data analysis was performed with Prism (GraphPad Software Inc.). Best-fit nonlinear regression was used to define the 100% value of luciferase synthesis and to calculate the aminoglycoside IC50 values of dose-dependent inhibition (Figure 4 and Table 3).

Rabbit reticulocyte translation assays

Cytosolic ribosomes present in commercially available rabbit reticulocyte lysate (Promega) were used for in vitro translation of a firefly luciferase control mRNA (Promega). A standard 30 µl reaction contained 20 µl reticulocyte lysate, 500 ng of luciferase mRNA, an amino acid mixture (100 µM each), and RNasin. Serially diluted aminoglycosides were added and the reaction mixture was incubated at 30°C for 90 min. After incubation, 100 µl of luciferase assay substrate (Promega) was added and luciferase activity was determined.

RESULTS AND DISCUSSION

The two chromosomal rrn operons in M. smegmatis were inactivated to produce a strain in which ribosomal RNA is exclusively transcribed from plasmid-encoded rRNA genes. Operon inactivation was achieved by means of unmarked deletion mutagenesis spanning the complete rRNA gene sequences and part of the promoter region (Supplementary Figure 1). For complementation, a fully functional wild-type rrnB operon encompassing promoter and termination sequences was cloned into the integration-proficient plasmid pMIG-sacB. In M. smegmatis Δrrn pMIG-sacB-rrnB, the wild-type rRNA operon carried on pMIG-sacB is efficiently replaced with a mutant rRNA operon by plasmid exchange following transformation with integration-proficient plasmid pMIG-rrnBmut (for details, see the Materials and Methods section and Figure 1). The rRNA plasmid exchange system was used to replace the bacterial 16S rRNA helix 44 within the decoding region with its homologous structures from parasitic kinetoplastid protozoa. The kinetoplastid genera Leishmania and Trypanosoma comprise species that are responsible for leishmaniasis and trypanosomiasis, diseases that are widespread in many tropical and subtropical countries. The insect trypanosome Blastocrithidia is characterized by an rRNA decoding site with an adenine at the position homologous to 16S rRNA residue 1408, and therefore represents a very rare trait within the eukaryotic domain. We also constructed human–bacterial hybrid ribosomes by introducing the human helix 44 rRNA homolog into functional ribosomes of strain M. smegmatis ΔrrnB by a method described previously (13). Both approaches resulted in bacterial mutant strains with homogenous populations of hybrid ribosomes in which a 34 nucleotide portion of helix 44 was substituted with its counterpart of eukaryotic small-subunit rRNA (Figure 2). Together with A-site residues in helices 18, 34 and 44 that are universally conserved throughout the phylogenetic domains, the engineered decoding site resembles that of eukaryotic cytosolic ribosomes. Engineering the eukaryotic decoding sites into bacterial ribosomes had relatively little effect on cell growth, with only slightly increased generation times as compared to wild-type cells (generation times: wild-type 3.7 ± 0.3 h; Leishmania hybrids 4.8 ± 0.5 h; Blastocrithidia hybrids 4.2 ± 0.1 h; H. sapiens hybrids 5.1 ± 0.6 h).

A bacterial oligoribonucleotide analog of 16S rRNA helix 44 has been shown to interact with both antibiotic and RNA ligands of the 30S subunit in a manner that correlates with normal subunit function (24). Since the decoding-site rRNA in the hybrid ribosomes is identical to that of eukaryotic ribosomes, the hybrid ribosomes may serve as a model to investigate specific features associated with the eukaryotic A-site rRNA structure. Towards this end, we investigated the susceptibility of the hybrid ribosomes to the 2-deoxystreptamine antibiotics paromomycin, neomycin, gentamicin (G418), gentamicin, netilmicin, tobramycin and kanamycin A (see Supplementary Figure 2 for chemical structures). In MIC assays, which determine growth inhibition at the whole cell level, M. smegmatis mutants carrying human–bacterial hybrid ribosomes were highly resistant to all aminoglycoside antibiotics tested except for gentamicin (Table 1). Relative resistance of the human hybrids to gentamicin, calculated as the ratio of mutant to wild-type MIC, was 8 to 16 as compared to ≥1024 for paromomycin, neomycin, gentamicin, netilmicin, tobramycin, and kanamycin. The Leishmania hybrids showed high-level resistance to neomycin, gentamicin, netilmicin, tobramycin and kanamycin, with relative resistance levels of ≥1024. However, the Leishmania hybrids showed distinct susceptibility to paromomycin and gentamicin, i.e. aminoglycosides with a hydroxyl group at position 6’ of ring I. Mycobacterium smegmatis hybrids with a Blastocrithidia decoding region were susceptible to all aminoglycoside antibiotics; the minimal inhibitory concentrations of the 4,6- and
The relative amount of $^{14}$C-phenylalanine incorporated by 5 pmol of purified 70S ribosomes after 60 min. incubation in 16S rRNA.

Figure 2. 16S rRNA sequence within helix 44 of M. smegmatis wild-type and hybrid ribosomes after transplanting the A-site rRNA of eukaryotic ribosomes. (A) Mycobacterium smegmatis. (B) Human–bacterial hybrid ribosomes. (C) Hybrid ribosomal RNA containing the decoding-site rRNA of the protozoan Leishmania, which is also identical to Trypanosoma; and (D) Blastocrithidia. Base substitutions rendering the bacterial 16S rRNA eukaryotic are depicted in blue; the transplanted region is boxed. rRNA residues are numbered according to the nucleotide numbering used in E. coli 16S rRNA.

Figure 3. Kanamycin-induced inhibition of polypeptide synthesis using (UUU)$_{12}$-directed phenylalanine incorporation. (A) Homo sapiens cytosolic hybrid ribosomes (closed circles) versus M. smegmatis wild-type ribosomes (open circles). (B) Leishmania (closed squares) versus Blastocrithidia (open squares) hybrid ribosomes. The relative amount of $^{14}$C-phenylalanine incorporated by 5 pmol of purified 70S ribosomes after 60 min. incubation in the presence of varying concentrations of kanamycin A is shown. SEs are indicated. The corresponding IC$_{50}$ values for kanamycin A and selected aminoglycoside antibiotics are given in Table 2.

4,5-disubstituted deoxystreptamines against Blastocrithidia hybrids were similar to those observed for the wild-type M. smegmatis strain (Table 1).

To analyze the antibiotic effects on translation in more detail, we determined the IC$_{50}$ values for selected aminoglycosides in (UUU)$_{12}$-mRNA-directed phenylalanine incorporation in cell-free translation assays. In vitro translation assays were established with purified 70S ribosomes of wild-type and mutant M. smegmatis strains. A polyphenylalanine mRNA with a ribosomal-binding site, twelve consecutive (UUU) triplets coding for phenylalanine, and a stop codon was used to assess aminoglycoside-induced inhibition of $^{14}$C-phenylalanine incorporation in translating wild-type and hybrid ribosomes. Bacterial wild-type ribosomes were highly susceptible to all aminoglycosides tested, with IC$_{50}$ values for poly-Phe synthesis below 3 µM (Figure 3 and Table 2). Hybrid ribosomes with a Blastocrithidia decoding region were likewise susceptible to low aminoglycoside concentrations. In contrast, hybrid ribosomes with the human decoding site were resistant, exhibiting IC$_{50}$ values that are more than 500-fold higher than those observed in bacterial wild-type ribosomes. Leishmania hybrid ribosomes were resistant to all aminoglycosides tested except paromomycin. In order to assess aminoglycoside-stimulated misreading (25) in hybrid ribosomes, paromomycin was used...
to measure drug-induced incorporation of leucine in a cell-free (UUU)\textsubscript{12} translation assay. In line with the other assays used to determine ribosomal drug susceptibility, the \textit{Leishmania} hybrids were significantly more susceptible to paromomycin-induced mistranslation than the human hybrids (data not shown).

We next wished to validate the hybrid ribosome approach by comparing the human–bacterial hybrid ribosomes to rabbit reticulocyte ribosomes (the decoding-site rRNA of human cytosolic ribosomes is identical to that of most vertebrates, including rabbit). In addition, with a view to define the selectivity of aminoglycoside compounds for protozoan versus human ribosomes more accurately, we wished to use an assay which is not limited by its sensitivity. Note that both the whole-cell determination of minimal inhibitory concentrations (MIC; Table 1) and the (UUU)\textsubscript{12} assay using purified ribosomes (Table 2) are limited by the amount of drug which can be applied. Towards this end we analyzed synthesis of luciferase based upon a coupled transcription–translation assay. Figure 4 shows the effect of paromomycin on protein synthesis in bacterial versus human–bacterial hybrid ribosomes and compared to rabbit reticulocyte ribosomes translating a luciferase mRNA. The paromomycin concentration required to inhibit synthesis of active luciferase in human–bacterial ribosomes to 50\% (IC\textsubscript{50}) closely resembled the IC\textsubscript{50}

![Figure 4. Paromomycin-induced inhibition of protein synthesis measured as luciferase activity in cell-free translation assays of firefly luciferase mRNA. Rabbit reticulocyte (closed triangles) versus human–bacterial hybrid (closed circles) and wild-type \textit{M. smegmatis} (open circles) ribosomes; error bars represent the SEM \((n = 3)\). The corresponding IC\textsubscript{50} values for paromomycin and the aminoglycosides tested are given in Table 3.](image)

### Table 1. Activity of aminoglycoside antibiotics against cells carrying hybrid ribosomes

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC (µg/ml)</th>
<th>\textit{Mycobacterium smegmatis}</th>
<th>\textit{Homo sapiens}</th>
<th>\textit{Leishmania}</th>
<th>\textit{Trypanosoma}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paromomycin</td>
<td>1</td>
<td>&gt;1024</td>
<td>128</td>
<td>4</td>
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<tr>
<td>Neomycin</td>
<td>0.5</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
<td>1</td>
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<tr>
<td>Gentamicin</td>
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<td>128</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td>1</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Netilmicin</td>
<td>2</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
<td>8</td>
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<tr>
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<td>1024</td>
<td>1024</td>
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<td>&gt;1024</td>
<td>&gt;1024</td>
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\(^{a}\)Aminoglycoside concentrations required to inhibit \(^{14}\text{C}\)-phenylalanine incorporation to 50\% (IC\textsubscript{50}). Inhibition kinetics are exemplified by the graphs for kanamycin A presented in Figure 3. Best-fit nonlinear regression was used to define the 100\% value and to calculate the IC\textsubscript{50}. An antibiotic concentration of 1 µM corresponds to approximately four aminoglycoside molecules per ribosome.

### Table 2. Aminoglycoside-induced inhibition of (UUU)\textsubscript{12}-directed phenylalanine incorporation

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC\textsubscript{50} (µM)(^{a})</th>
<th>\textit{Mycobacterium smegmatis}</th>
<th>\textit{Homo sapiens}</th>
<th>\textit{Leishmania}</th>
<th>\textit{Trypanosoma}</th>
<th>\textit{Blastocrithidia}</th>
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<td>Paromomycin</td>
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<td>Gentamicin</td>
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<td>&gt;500</td>
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<td>7.5</td>
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\(^{a}\)Aminoglycoside concentrations required to inhibit \(^{14}\text{C}\)-phenylalanine incorporation to 50\% (IC\textsubscript{50}). Inhibition kinetics are exemplified by the graphs for paromomycin presented in Figure 4. Best-fit nonlinear regression was used to define the 100\% value and to calculate the IC\textsubscript{50}.

\(^{b}\)The rRNA decoding region of rabbit reticulocyte ribosomes is identical to that of human cytosolic ribosomes shown in Figure 2B.

### Table 3. Aminoglycoside-induced inhibition of luciferase synthesis

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC\textsubscript{50} (µM)(^{a})</th>
<th>\textit{Mycobacterium smegmatis}</th>
<th>\textit{Homo sapiens}</th>
<th>\textit{Leishmania}</th>
<th>\textit{Trypanosoma}</th>
<th>\textit{Blastocrithidia}</th>
<th>\textit{Rabbit reticulocyte lysate}(^{b})</th>
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<tr>
<td>Paromomycin</td>
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<td>116</td>
<td>204</td>
<td>0.03</td>
<td>67</td>
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</table>

\(^{a}\)Aminoglycoside concentrations required to inhibit synthesis of active luciferase to 50\% (IC\textsubscript{50}). Inhibition kinetics are exemplified by the graphs for paromomycin presented in Figure 4. Best-fit nonlinear regression was used to define the 100\% value and to calculate the IC\textsubscript{50}.
in rabbit reticulocyte lysate. In fact, the IC$_{50}$ values of the human hybrid ribosomes are virtually identical to the IC$_{50}$ values determined for rabbit cytosolic ribosomes for all aminoglycosides tested in this study (Table 3). In contrast, the IC$_{50}$ values in bacterial ribosomes were two to three orders of magnitude lower. Thus, transplanting helix 44 of the eukaryotic decoding region into bacterial ribosomes testified to be a valid approach for studying the specificity of aminoglycoside antibiotics, compounds that selectively affect prokaryotic versus eukaryotic ribosomes.

Analysis of aminoglycoside-induced inhibition of luciferase synthesis in *Leishmania* hybrid ribosomes and comparison to human hybrid ribosomes also allowed quantifying the differences in drug susceptibility. *Leishmania* hybrid ribosomes are ~20- to 30-fold more susceptible to the 4,5-disubstituted aminoglycoside paromomycin than human hybrid ribosomes. In line with the results from MIC determinations (Table 1) and aminoglycoside-induced inhibition of (UUU)$_{12}$-driven phenylalanine incorporation (Table 2), there is a significant difference in drug susceptibility between *Leishmania* hybrid and human hybrid ribosomes for aminoglycosides with a 6'-hydroxy group. This is in contrast to aminoglycosides with a 6'-amino group where no such difference is present.

The basic mechanisms of translation of mRNA to protein are conserved throughout all organisms (17,26). Experimental data suggest that the selectivity of aminoglycoside antibiotics for bacterial ribosomes is due to defined phylogenetic variations of ribosomal RNA in an otherwise highly conserved binding site (13). The results obtained with the hybrid ribosomes are in excellent agreement with the drug susceptibilities determined in rabbit reticulocyte lysate (Table 3) and with data reported in the literature on complete eukaryotic ribosomes (25,27), demonstrating that the model system adequately reflects the situation in eukaryotic cytosolic ribosomes. Drugs targeting protein synthesis are infrequently used for treatment of infections with lower eukaryotes such as protozoan parasites, helminths or fungi, although the potential for selective activity appears to exist (28–33). Using various assays of drug activity, we demonstrated that the 4,5-disubstituted aminoglycoside paromomycin exhibits a distinct activity towards hybrid ribosomes carrying the cytosolic decoding site of *Leishmania* and *Trypanosoma*. These results provide an explanation for the activity of paromomycin in treatment of leishmaniasis (34,35), by pointing to the cytosolic ribosome as a drug target. Little treatment options involving quite toxic drugs, such as the trivalent arsenic compound melarsoprol, exist for late stage *T. brucei rhodense* sleeping sickness (36). The availability of compounds with antitrypanosomal activity would be most significant, if only as second-line option for patients who experience relapse.

Defined point mutations have been introduced previously into the bacterial rRNA A site to study individual drug—nucleotide interactions of aminoglycosides (13). These studies demonstrated the role of nucleotides 1408, 1409 and 1491 in drug binding. A base substitution of adenine at position 1408 with a guanine interrupts the pseudo-base-pair formation of the adenine with ring I of the disubstituted 2-deoxystreptamines and alterations of the 1409–1491 bp interfere with the stacking interaction between 1491 and ring I. Our study complements this reductionistic approach by transferring the complete eukaryotic binding site into bacterial ribosomes. Given the complex structure of the ribosome and its various components that contribute to translational fidelity, e.g. ribosomal proteins S4, S5 and S12 (3), it is remarkable that a hybrid bacterial ribosome carrying the eukaryotic H44 shows a drug-susceptibility pattern identical to that of complete rabbit reticulocyte ribosomes. From this we conclude that additional phylogenetically variable ribosomal structures do not affect aminoglycoside susceptibility of the complete eukaryotic ribosome. In line with previous findings (13,37–39), a more general picture emerges, where ribosomes with a 1408 guanine in small subunit rRNA are resistant to aminoglycosides carrying an amino group at position 6' of ring I (neomycin, gentamicin, netilmicin, tobramycin, kanamycin), while A sites with a non-canonical base-pair interaction between residues 1409 and 1491 are predominantly resistant to geneticin and paromomycin, drugs which carry a hydroxyl group at the 6' position of ring I. Together, these results suggest that paromomycin and geneticin have a specific activity against ribosomes that are characterized by a canonical 1409–1491 bp; that neomycin, gentamicin, netilmicin, tobramycin and kanamycin are active against ribosomes with a 1408 adenine; and that metazoan cytosolic ribosomes are generally resistant to 2-deoxystreptamines.

Successful crystallizations of the ribosome and ribosome–drug complexes have initiated a rebirth of interest in the ribosome as drug target (40–43). Apparently, helix 44 of the rRNA decoding A site behaves as an autonomous domain, which can be dissected from the remaining part of the ribosome and shuffled between ribosomes of different phylogenetic domains *in vivo* while retaining its functional characteristics. It is likely that this possibility will help to study mechanisms of the various eukaryotic rRNA decoding sites in the future and to test hypotheses built upon X-ray structures. The construction of hybrid ribosomes, as demonstrated in this study, should also help characterizing the specificity of ribosomal inhibitors already available and of future compounds to come.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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