Hearing loss is one of the most common human health problems, affecting 1 in 1,000 newborns (18, 19). Hearing loss can be caused by gene aberrations or environmental factors, including ototoxic drugs, such as aminoglycoside antibiotics (18). Aminoglycosides, such as gentamicin and tobramycin, are of great clinical importance for the treatment of bacterial infections. These antibiotics are known to exert their antibacterial effects by interacting with the bacterial ribosome (1, 2). Aminoglycosides bind directly to highly conserved sequences of bacterial 16S rRNA in the aminoacyl-tRNA site (A site) of the decoding region, where codon-anticodon recognition occurs (16, 24). These antibiotics interfere with protein synthesis by inducing codon misreading and inhibiting the translocation of the tRNA-mRNA from the A site to the peptidyl-tRNA site (P site) in the ribosome (2, 21). Exposure to aminoglycosides frequently causes toxicity, which involves the renal, auditory, and vestibular systems (6, 8). The renal impairment is usually reversible, whereas the auditory or vestibular failure is frequently irreversible. In familial cases of ototoxic hearing loss, aminoglycoside hypersensitivity is often maternally transmitted, suggesting that a mutation(s) in mitochondrial DNA (mtDNA) is the molecular basis for this susceptibility (6, 8).

Since mitochondrial ribosomes share more similarities with bacterial ribosomes than do their cytosolic counterparts, the mitochondrial small rRNA is proposed to be the primary targeting site for aminoglycosides (6, 8). Recently, the A1555G and C1494T mutations in human mitochondrial 12S rRNA have been associated with both aminoglycoside-induced and nonsyndromic hearing loss in many families worldwide (5, 14, 15, 22, 28).

The A1555G and C1494T mutations are located at the highly conserved A site of mitochondrial 12S rRNA (20, 21, 29). In wild-type Escherichia coli, aminoglycosides such as paromomycin and neomycin bind directly to the C1409–G1491 base pairing of the A site of 16S rRNA (7, 25). This C1409–G1491 base pair, which is adjacent to the A-site tRNA binding pocket (7). Binding of aminoglycosides to the rRNA induces a local conformational change in the A site of 16S rRNA, thereby affecting the efficiency and accuracy of codon-anticodon interaction (3, 4). Thus, E. coli strains carrying the C1409–G1491 base pairing of 16S rRNA exhibit sensitivity to aminoglycosides. In contrast, mutations that disrupt the C1409–G1491 base pairing of E. coli 16S rRNA confer aminoglycoside resistance (4). In human mitochondrial 12S rRNA, A1555 and C1494 (equivalent to positions 1491 and 1409 of E. coli 16S rRNA, respectively) are in apposition to each other but do not form a base-pair. The A1555G or C1494T mutation forms a new G-C or A-U base pair, thereby extending the adjacent stem by 1 nt and making the secondary structure of mitochondrial 12S rRNA more closely resemble the corresponding region of E. coli 16S rRNA (11, 22, 28) (see Fig. 1A). Thus, the new G-C or A-U pair is expected to create altered binding sites for aminoglycosides, such as paromomycin, neomycin, gentamicin, and kanamycin, as in the case of the C1409–G1491 base pairing of the E. coli 16S rRNA (16, 21, 24). In contrast, other antibiotics, such as streptomycin, interact with other segments of E. coli 16S rRNA (16, 21, 24) and should not, therefore,
interact with the 12S rRNA carrying these ototoxic mutations. It is also anticipated that the binding of aminoglycosides to the new G-C or A-U pair created by the A1555G or C1494U mutation causes conformational changes in the A site of 12S rRNA.

In this investigation, we employed an RNA-directed chemical modification approach to determine if the A1555G mutation increases the binding of aminoglycosides to 12S rRNA and if these bound drugs cause conformational changes in the A site of 12S rRNA with respect to its wild-type counterpart.

MATERIALS AND METHODS

Preparation of RNA oligonucleotide analogues. To prepare RNA oligonucleotide analogues carrying the A site of human mitochondrial 12S rRNA, a DNA template (65 nt), as shown in Fig. 1A and 2, was synthesized at a 250-mmol scale and purified on 10% (wt/vol) polyacrylamide gel electrophoresis (PAGE) (acylamidine-bisacylamidine, 19:1 [wt/wt]–7 M urea gels (Integrated DNA Technolo-
gies, Inc.). RNA oligonucleotide analogues (48 nt) consisted of wild-type or mutant human mtDNA sequences spanning the A site of 12S rRNA (bases 1488 to 1498 and 1551 to 1562 of mtDNA) (22, 28), two additional G-C base-pairs at the upper stem, and a tetraloop (UUCG) at the lower stem for stabilizing the structure, as well as an extended 17-base annealing site (5'-CCCAACGCGCC CGACC-GC3') for the primer extension detection of the chemical modification (23, 25). RNA analogues were synthesized by in vitro transcription with T7 RNA polymerase (New England Biolabs) in the presence of 300 nM T7 primer (18 nt) (as shown in Fig. 2) according to the manufacturer's protocol and were purified on 20% (wt/vol) PAGE (acylamidine-bisacylamidine, 19:1 [wt/wt]–7 M urea gels as detailed elsewhere (23, 29). Gel blocks with RNA fragments were eluted with an Elutrap electroelution apparatus (Schleicher & Schuell) and dialyzed with dialysis tubing (molecular weight cutoff, 1,000; Spectrum). Finally, RNA analogues were lyophilized and then dissolved in deionized water.

Chemical modification of RNA. To determine if the A1555G mutation increases the binding of aminoglycosides to 12S rRNA and if these bound drugs cause conformational changes in the A site of 12S rRNA with respect to its wild-type counterpart, RNA was chemically modified by a method detailed elsewhere (17, 23, 25). Briefly, chemical modification reactions (100 μl) were performed in 80 mM K-HEPES (pH 7.0) with 10 pmol RNA oligonucleotide, antibiotics (Sigma-Aldrich) were added and then incubated at room temperature for 10 min. Modification reactions were quenched by ethanol precipitation (dimethyl sulfate (DMS) (1:6 in ethanol), followed by incubation at room temperature for 5 min. RNA modifications were conducted by addition of 2 μl 25 mg/ml kanamycin (as shown in Fig. 2) according to the manufacturer's protocol and were purified on 20% (wt/vol) PAGE (acylamidine-bisacylamidine, 19:1 [wt/wt]–7 M urea gels as detailed elsewhere (23, 29). Gel blocks with RNA fragments were eluted with an Elutrap electroelution apparatus (Schleicher & Schuell) and dialyzed with dialysis tubing (molecular weight cutoff, 1,000; Spectrum). Finally, RNA analogues were lyophilized and then dissolved in deionized water.

RESULTS AND DISCUSSION

We employed an RNA-directed chemical-modification approach to determine if the A1555G mutation increases the binding of aminoglycosides to 12S rRNA and whether these bound drugs cause conformational changes in the A site of 12S rRNA with respect to its wild-type counterpart. As shown in Fig. 1A, we designed RNA oligonucleotide analogues corresponding to the A site with wild-type 12S rRNA sequences and to a mutated version harboring the A1555G mutation as well as two additional G-C base pairs at the upper stem and a UUCG tetraloop at the lower stem to stabilize the structure (24). This RNA oligonucleotide model system, which mimics both the affinity and the specificity of the interaction of aminoglycosides with the small subunit of the ribosome, has been extensively used to investigate aminoglycoside-ribosome interaction in E. coli (24, 25). Aminoglycosides bind to the oligonucleotides encompassing the A site of 16S rRNA specifically and similarly to the way that they bind to the ribosome with the same affinity in E. coli. The RNAs, either alone or complexed with aminoglycosides, were incubated under suitable conditions with the chemical probe DMS, which modifies RNA at the guanine N-7, adenine N-1, and cytosine N-3 positions (26). If an antibiotic(s) binds RNA by stable interactions with specific nucleotide bases, it should be possible to identify such sites by diminished or reduced reactivity with DMS probes. These RNA analogues, as shown in Fig. 2, were first synthesized by in vitro transcription with T7 RNA polymerase using a DNA template. The binding of aminoglycosides to these RNA oligonucleotides was assayed by chemical probing with DMS, either alone, as naked RNA, or in the presence of increasing concentrations of various aminoglycosides. In particular, 150 ng of RNA oligonucleotides carrying the wild-type sequence or RNA oligonucleotides carrying the A1555G mutation in the presence of DMS was incubated with 0.1, 1, 10, or 100 μM paromomycin, neomycin, tobramycin, gentamicin, kanamycin, or streptomycin. Positions of chemical modification were determined by primer extension using synthetic DNA primers. Reverse transcriptase stops or pauses at modified bases, thus producing prematurely terminated cDNA chains. Sequencing reactions utilizing dideoxynucleotides and untreated RNA were performed in parallel, and transcripts were analyzed on a DNA sequencing gel, which was autoradiographed. The cDNA binding patterns produced by primer extension of probes uncomplexed RNA and probes complexed RNA were compared. In every case, the bases in the RNA was denatured at 90°C for 1 min and cooled on ice immediately; then it was loaded onto 20% PAGE (acylamidine-bisacylamidine, 19:1–7 M urea gels. After electrophoresis, the gels were transferred to Whatman 3MM paper, dried, and autoradiographed 12 to 24 h at room temperature with Kodak XAr-5 film. Modification data were quantified by exposing the gel to a PhosphorImage screen (GE Healthcare) and were analyzed by Molecular Dynamics ImageQuant software.

Data analysis. The intensity of each band in the presence and absence of aminoglycosides was quantified with Kodak 1D Image Analysis software, version 3.6. To subtract the background variation, the average relative value of each band was normalized to the average relative value of bands located in the tetraloop area in each lane. The relative reactivities to DMS for each band in the RNA analogue carrying the A1555G mutation or in the wild-type 12S rRNA sequence in the presence of aminoglycosides were expressed as percentages of the average values for the corresponding band in the absence of drugs in each gel.
analogues were shielded from chemical modification in the presence of bound drugs. Thus, protection was observed as decreased intensities of the relevant gel bands.

The patterns of chemical modifications by DMS in the RNA oligonucleotides carrying the A1555G mutation, as shown in Fig. 3, were distinct from those of wild-type RNA oligonucleotides in the presence and absence of paromomycin. Interestingly, bases A1492, C1493, and C1494 in the RNA analogue carrying the G1555 base migrated faster than the corresponding bases in the RNA analogue carrying the A1555 base.

Higher reactivities to DMS in the absence or presence of paromomycin were observed for bases A1492, C1493, C1494, and A1557 in the RNA analogue carrying the G1555 base than those for the corresponding bases in the RNA analogue carrying the A1555 base. On the other hand, reduced reactivity to DMS in the presence of paromomycin was observed for bases G1555 and A1553 in the RNA analogue carrying the A1555G mutation. As an internal control, we also performed chemical modifications of the RNA oligonucleotides carrying the C1494U mutation in the presence of paromomycin. As shown

FIG. 1. Decoding-region analogues of human mitochondrial 12S rRNA with nucleotides either protected from DMS modification or exhibiting enhanced reactivity with DMS in the presence of paromomycin, neomycin, gentamicin, kanamycin, tobramycin, or streptomycin. (A) Secondary structure of E. coli 16S rRNA (with the A site boxed) and corresponding regions of oligoribonucleotides with portions of wild-type (WT) human mitochondrial 12S rRNA sequence or human mitochondrial 12S rRNA sequence containing the A1555G or the C1494U mutation. The analogue contains 12S rRNA decoding-region sequences from base 1488 to 1498 on the proximal side and base 1551 to 1562 on the distal side. (B) Protection (filled diamonds) and enhanced reactivity (filled circles) are indicated. Small or large symbols represent weak or strong reactivity, respectively.
in Fig. 3, reactivities to DMS in the presence of 0.1, 1, and 10 μM paromomycin were higher for bases A1492, C1493, U1494, and A1557 in the RNA analogue carrying the U1494 base than for the corresponding bases in the RNA analogue with the wild-type sequence. However, lower reactivities to DMS were observed in the presence of 100 μM paromomycin for bases A1492, C1493, U1494, A1555, C1556, and A1557 in the RNA analogue carrying the U1494 base than for the corresponding bases in the RNA analogue with the wild-type sequence. These results indicated that the A1555G and C1494T mutations in-

FIG. 2. Experimental schema for chemical modification.

FIG. 3. Autoradiographs of DMS probing reactions with human mitochondrial 12S rRNA oligonucleotides with the wild-type (WT) sequence or human mitochondrial 12S rRNA oligonucleotides carrying the A1555G or C1494T mutation. (A) Lanes a, b, c, and d show U, C, G, and A dideoxy sequencing reactions. (B) Lanes e and g, primer extension reactions using unmodified RNA analogues carrying base A1555 (WT) or G1555; lanes f and h, primer extension reactions using modified RNA analogues carrying base A1555 (WT) or G1555. (C) DMS probing reactions with RNA analogues in the presence of 0.1, 1, 10, and 100 μM paromomycin are shown in lanes 1 to 4 (WT), lanes 5 to 8 (A1555G mutation), and lanes 9 to 12 (C1494U mutation), respectively. The positions of bands corresponding to nucleotides of 12S rRNA are indicated to the right and left of the gels. An arrow indicates the nucleotide at position 1555.
creased the binding of paromomycin to the A site of 12S rRNA. These data also suggested that the A1555G mutation altered the local conformational changes of the A site of 12S rRNA and that such alterations were enhanced by the binding of aminoglycosides to the RNA analogue.

Figure 4 shows the binding of paromomycin, neomycin, tobramycin, gentamicin, kanamycin, and streptomycin to RNA oligonucleotides with the wild-type sequence or those carrying the A1555G mutation, assayed by chemical modifications and monitored by primer extension. In agreement with the results for paromomycin, bases A1492, C1493, and C1494 in the RNA analogue carrying the G1555 base migrated faster than the corresponding bases in the RNA analogue carrying the A1555 base in the presence of the other five aminoglycosides. Various degrees of chemical modifications by DMS were detected in the RNA oligonucleotides carrying the A1555G mutation in the presence of different aminoglycosides. In particular, increasing reactivities to DMS in the absence or presence of aminoglycosides were observed for bases A1492, C1493, C1494, and A1557 in the RNA analogue carrying the A1555G mutation. In contrast, reduced reactivity to DMS was observed for bases G1555, A1556, and A1553 in the presence of 0.1 μM, 1 μM, 10 μM, and 100 μM paromomycin, neomycin, gentamicin, kanamycin, and tobramycin, as well as streptomycin. We have quantified these reactivities to DMS in the bases at positions 1555, 1556, and 1553 of RNA analogues in the presence of aminoglycosides by using Kodak 1D Image Analysis software, version 3.6. The relative values for each nucleotide in the RNA analogue carrying the A1555G mutation or wild-type 12S RNA sequence in the presence of aminoglycosides were expressed as percentages of the average values for the corresponding band in the corresponding RNA analogue in the absence of drugs in each gel. Table 1 summarizes the relative reactivities to DMS for base G1555 in the presence of aminoglycosides at 0.1 μM, 1 μM, 10 μM, and 100 μM (expressed as percentages of the reactivities in the absence of aminoglycosides) ranged from 74.5% to 59.1% with paromomycin, from 56.4% to 51.6% with neomycin, from 62.2% to 55.9% with gentamicin, from 77.7% to 53.6% with tobramycin, from 65% to 52.8% with kanamycin, and from 69.8% to 52.3% with streptomycin. In particular, base G1555 exhibited marked but similar levels of protection in the presence of 0.1 μM to 100 μM neomycin, gentamicin, and kanamycin. In contrast, the levels of protection for base G1555 appeared to be correlated with the concentrations of paromomycin, tobramycin, and streptomycin. Apparently, these relative reactivities to DMS for base G1555 in the presence of aminoglycosides were lower than those for base A1555. These observations strongly indicated that the A1555G mutation increased the binding of aminoglycosides to base G1555 in the RNA analogues.

As shown in Table 1, the reactivities to DMS for bases A1553 and C1556 of RNA analogues carrying the A1555G mutation in the presence of aminoglycosides were reduced to various degrees, compared with those for the corresponding bases in the absence of aminoglycosides. As shown in Fig. 1A, the nucleotides at positions A1492, C1494, and A1557 of human mitochondrial 12S rRNA correspond to positions A1408, C1409, and A1493 of E. coli 16S rRNA, respectively. In E. coli, A1408 and A1493 are critical nucleotides for aminoglycoside binding to the A site of 16S rRNA (16, 24). In fact, these antibiotics bind in the major groove of the bacterial 16S RNA with a pocket created by the A1408–A1493 base pair, a single bulged adenine (A1492), and the C1409–G1491 base pair. In particular, the C1409–G1491 base pair provides the floor for the antibiotic binding pocket (7, 13, 27). Here, the new G1555–C1494 base-pair created by the A1555G mutation altered the local conformation of the A site of mitochondrial 12S rRNA. Like its bacterial counterpart, the G1555–C1494 base-pair may produce a binding pocket of 12S rRNA for aminoglycosides. Hence, aminoglycosides bind to the A site of 12S rRNA carrying the A1555G mutation.
Figure 1B summarizes the protection of nucleotides in human mitochondrial 12S rRNA analogues from DMS modification or their enhanced reactivities to DMS in the presence of various aminoglycosides. These data reveal how aminoglycosides interact with human mitochondrial 12S rRNA carrying the A1555G mutation. Our previous work has shown that the A1555G mutation led to a mild mitochondrial dysfunction but was insufficient to produce a deafness phenotype (9, 10). Exposure to aminoglycosides, which are concentrated in the perilymph and endolymph of the inner ear but are rapidly cleared from other tissues or organs (12), worsened mitochondrial dysfunctions caused by the A1555G mutation, leading to cell dysfunction or death in auditory systems (8). Therefore, exposure to aminoglycosides induces or worsens hearing loss in individuals carrying these mtDNA mutations. These data deepen our understanding of the pathogenesis of aminoglycoside ototoxicity and have a significant clinical impact.

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REFERENCES


### TABLE 1. Quantification of reactivity to DMS in RNA analogues in the presence of aminoglycosides

<table>
<thead>
<tr>
<th>Aminoglycoside</th>
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<th>RNA oligonucleotide carrying 1555G</th>
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<tr>
<td></td>
<td>0.1 µM</td>
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* The relative reactivities to DMS for each nucleotide in RNA analogues carrying the A1555G mutation or wild-type 12S rRNA sequence in the presence of aminoglycosides were expressed as percentages of the average values for the corresponding band in the corresponding RNA analogue in the absence of drugs in each gel. Quantifications of the relative reactivities were based on three independent experiments.