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Negamycin Restores Dystrophin Expression in Skeletal and Cardiac Muscles of *mdx* Mice

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The ability of aminoglycoside antibiotics to promote read-through of nonsense mutations has attracted interest in these drugs as potential therapeutic agents in genetic diseases. However, the toxicity of aminoglycoside antibiotics may result in severe side effects during long-term treatment. In this paper, we report that negamycin, a dipeptide antibiotic, also restores dystrophin expression in skeletal and cardiac muscles of the *mdx* mouse, an animal model of Duchenne muscular dystrophy (DMD) with a nonsense mutation in the dystrophin gene, and in cultured *mdx* myotubes. Dystrophin expression was confirmed by immunohistochemistry and immunoblotting. We also compared the toxicity of negamycin and gentamicin, and found negamycin to be less toxic. Furthermore, we demonstrate that negamycin binds to a partial sequence of the eukaryotic rRNA-decoding A-site. We conclude that negamycin is a promising new therapeutic candidate for DMD and other genetic diseases caused by nonsense mutations.

Key words: dystrophin, *mdx* mouse, muscular dystrophy, negamycin, nonsense mutation.

Aminoglycoside antibiotics are known to decrease translational fidelity and cause read-through of termination signals in eukaryotic cells (*1–4*). Considerable attention has therefore been focused on aminoglycoside antibiotics as potential therapeutic agents for genetic diseases. Recently, gentamicin has been used in clinical trials for the treatment of cystic fibrosis, Hurler's disease, infant neuronal ceroid and lipofuscinosis, diseases that are caused by nonsense mutations (*3–7*).

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder affecting 1 in 3,500 live-born males, and is characterized by a lack of the dystrophin protein in the sarcolemma. In 65% of patients with DMD, the gene exhibits gross rearrangement (predominantly deletion or duplication), while the remaining 35% have a dystrophin gene with either nonsense or other point mutations. The *mdx* mouse, an animal model of DMD, carries a nonsense mutation (CAA to TAA) at nucleotide position 3,185 in exon 23 of the dystrophin gene (*8*, *9*). This causes an absence of dystrophin and, thus, the dystrophin-associated glycoprotein complex in the sarcolemma. DMD patients are treated pharmacologically with corticosteroids and corticosteroid-sparing agents, however, the use of corticosteroids is accompanied by significant side effects and is of limited and short-lived benefit (*10*, *11*). Identifying clinically useful methods to suppress the nonsense mutation within the dystrophin gene would be of benefit to a significant number of patients with DMD.

In 1999, Barton-Davis *et al.* reported that gentamicin restores dystrophin function to the skeletal muscles of *mdx* mice (*12*). Gentamicin is currently in clinical trials for DMD patients. However, aminoglycoside antibiotics are associated with numerous side effects such as kidney disorders and hearing loss, and excessive use can lead to the emergence of drug-resistant bacteria.

The serious side effects of gentamicin led us to search for other possible drug candidates to suppress nonsense mutations. Uehara *et al.* reported that negamycin (δ-hydroxy-β-lysine linked with methylhydrazinoacetic acid), a dipeptide antibiotic discovered in 1970, also induces the read-through of stop codons in a prokaryotic translation system (*13*, *14*). We, therefore, injected negamycin subcutaneously into *mdx* mice over periods of 2 to 4 weeks. Immunofluorescence and immunoblotting analyses revealed that dystrophin protein expression was restored in skeletal and cardiac muscles of negamycin-treated *mdx* mice. The restoration of dystrophin was also found in immortalized *mdx* skeletal muscle cells (*mdx*SV40-T cells) prepared by introducing a temperature-sensitive SV40-T gene into *mdx* skeletal muscle cells using a retroviral vector.

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It has been reported that aminoglycoside antibiotics bind to the A-site of rRNA and change its conformation (*15–17*). In 1999, Griffey *et al.* showed the interaction between aminoglycoside antibiotics and the rRNA-decoding A-site of prokaryotes and eukaryotes using electrospray ionization mass spectroscopy (ESI-MS) (*18*). To understand the mechanism of the read-through activity induced by negamycin during protein synthesis, we show here the interaction between a partial sequence of rRNA and negamycin, as determined by time-of-flight mass spectroscopy (TOF-MS).

These results suggest that negamycin is a new therapeutic candidate for DMD and other genetic diseases caused by nonsense mutations.

MATERIALS AND METHODS

Antibiotic Treatment—The amount of negamycin administered was adjusted to be equivalent to the number of molecules of gentamicin administered in the study of Barton-Davis *et al.* (*12*). Negamycin (1.2×10^{-5} mol/kg body weight [BW]) was dissolved in PBS that had been sterilized by passage through membrane filters with a pore size of 0.22 μm (Millipore) just before injection each day. Male *mdx* mice (7–8 weeks old, n = 45, or 4 weeks old, n = 5) were injected subcutaneously with 0.2 ml of solution every day for 2 or 4 weeks. Untreated male *mdx* mice and normal male control C57BL10 mice (7–8 weeks old, n = 6) were injected subcutaneously with only 0.2 ml of PBS every day. Animals were sacrificed by an overdose of ether gas, and the tibialis anterior (TA), hindlimb, and cardiac muscles were removed and placed in tragacanthogum (Wako), frozen quickly in dry ice-cooled isopentane and stored at –80°C. Animal experiments were carried out according to the manual of the University of Tokyo.

Vital Staining with Evans Blue Dye—All animals were given an intraperitoneal injection of Evans Blue dye (EB: 2% EB in PBS, 0.1 ml/10 g, BW) 12 h prior to sacrifice. EB enables the visualization of degenerating muscle fibers that have permeable membranes, as described previously (*19*).

Cell culture of mdx mouse skeletal muscle cells (mdxSV40-T)—SV40-T cDNA was introduced into a retroviral vector. Muscle cells extracted from the leg skeletal muscle of an *mdx* mouse were infected with the SV40-T virus produced by Plat-E from packaging cell lines (*20*). At 32°C, infected cells (*mdx*SV40-T cells) grew rapidly, and cells that entered the cell cycle were chosen and cultures established. When the temperature was shifted to 38°C, the SV40-T protein was no longer effective, and the cells started to differentiate. The growth medium contained 20% FBS in DMEM including 50 U/ml penicillin, 50 μg/ml streptomycin, and 12.5 μg/ml amphotericin. The differentiation medium was 10% horse serum in Eagle's minimum essential medium (10% HS-MEM) without antibiotics.

Negamycin treatment of mdxSV40-T cells—Cells (2.0×10^5 cells/35-mm culture dish) were grown at 32°C for 7 days in gelatin-coated culture dishes, after which the growth medium was replaced with differentiation medium and the cells were cultured at 38°C for an additional 5 days. After myotube formation, negamycin (4.0×10^{-7} mol/ml, 100 μg/ml, sterilized through a 0.22-μm pore size filter and diluted in differentiation medium) was added to the cultures. The cells were treated with negamycin each day for 7 days.

Staining of Dystrophin in Mouse Tissues by Immunohistochemistry—Immunofluorescent staining was carried out on 7–10-μm transverse cryosections of the TA muscle and ventricular muscle from experimental mice. After pre-incubation in blocking solution (10% HS-PBS) for 15 min at room temperature, the cryosections were washed with PBS three times for 10 min. The sections were then incubated with a rabbit polyclonal antibody against the 40 mer amino acid sequence of C-terminal region of dystrophin, diluted 1:200 in PBS containing 2% BSA for 1 h at room temperature. After being washed with PBS, the sections were labeled with a 1:100 dilution of FITC-conjugated anti–rabbit IgG antibody (Amersham Pharmacia Biotech) for 1 h at room temperature. Specimens were washed with PBS and then sealed with 20% glycerol-PBS, examined under a fluorescence microscope (ZEISS Axioplan), and photographed on color slide films (Fuji film, PROVIA400). All photographs were taken under identical conditions with the same exposure time.

Dystrophin Staining of Cultured Skeletal Muscle Cells—Cells were rinsed twice with PBS, fixed with methanol at –20°C for 10 min and then rinsed three times for 5 min with PBS. The cells were treated with 10% HS-PBS for 30 min at room temperature and then incubated overnight at 4°C with rat monoclonal antibody against the 40 mer amino acid sequence of C-terminal region of dystrophin, diluted 1:100, or for 1 h at room temperature with culture supernatant of mouse monoclonal anti-sarcomeric myosin heavy chain antibody (MF20: Developmental Studies Hybridoma Bank, University of Iowa). After the reaction, the cells were rinsed three times with PBS for 10 min. Then, the cells were incubated with secondary antibodies, Alexa Fluor 488–conjugated goat anti-mouse IgG (H+L) and Alexa Fluor 594–conjugated goat anti-rat IgG (H+L) (both from Molecular Probes) for 30 min at room temperature. The secondary antibodies were diluted 1:200 with 2% BSA-PBS. Cells were washed three times with PBS and photographed using a Polaroid PDMC Ie/OL. Photographs were taken under identical conditions with the same exposure time.

Partial Enrichment of the Dystrophin/Dystrophin-Associated Glycoprotein Complex Using the Lectin Wheat Germ Agglutinin (WGA)—According to the methods described by Yoshida *et al.* (*21*) and Campbell *et al.* (*22*), tissues were homogenized with a Teflon homogenizer in 15 ml of homogenizing solution (pyrophosphate mixture, 20 mM $Na_4P_2O_7$, 20 mM NaH_2PO_4, and 1 mM $MgCl_2$, pH 7.1) including 10% sucrose and 0.5 mM EDTA (pH 7.2). Muscle tissues of the left hindlimb (600 mg) and heart (100 mg) isolated from *mdx* or B10 mice were homogenized at 4°C for 1 min in 15 ml of homogenizing solution. The homogenates were centrifuged at 10,000 ×*g* for 15 min, and the supernatants were then centrifuged at 15,000 ×*g* for 30 min to obtain the microsome fractions. The pellet was resuspended in 1ml of 1% digitonin solution (0.5 M NaCl, 0.5 M sucrose, 0.1 mM PMSF, 50 mM Tris-HCl, 1U/ml aprotinin, pH 7.2). Sixty microliters of WGA-Sepharose CL-6B (Sigma, 1.5 mg/ml) were added to the solu-

tions, and the samples were incubated overnight at 4°C. After centrifugation at 15,000 ×*g*, the beads were collected and washed three times with 0.2% Nonidet P-40 (NP-40) in PBS. Then, 20 μl of SDS sample buffer (10% SDS, 10 mM EDTA, 70 mM Tris-HCl, pH 6.7, 5% β-mercaptoethanol) was added and the beads were heated at 90°C for 4 min.

Immunoprecipitation of the Dystrophin Protein from Skeletal Muscle Cell Culture—Immunoprecipitation was performed using a modification of the methods described by Abe *et al*. (*23*) and Arakawa *et al.* (*24*). The cells were washed twice with PBS, and then scraped in 400 μl of homogenizing solution (20 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 100 μg/ml DNase, 1 mM PMSF, 1 μg/ml *N*-tocyl-L-phenylalanyl chloromethyl ketone, 1 μg/ml *N*-tocyl-L-lysyl chloromethyl ketone, 200 U/ml aprotinin, and 5 mM EDTA) in a cold room at 4°C. The cells were collected and homogenized in a glass homogenizer. Homogenates were collected in microfuge tubes and centrifuged at 15,000 ×*g*. Supernatants were pre-incubated with protein A-Sepharose CL-4B (Sigma) for 2 h at 4°C to remove non-specific binding. After incubation, the supernatants were collected and incubated with rabbit polyclonal anti-dystrophin antibody (3 μl) for 30 min at room temperature. Protein A-Sepharose CL-4B (25 μl) was added to the solutions, and the samples were incubated overnight at 4°C. After incubation, the beads were collected and incubated in SDS sample buffer at 90°C for 4 min.

Immunoblot Analysis—The protein concentrations of SDS-treated samples were determined by the microbeuret method (*25*). The amount of protein in each SDS-treated sample was adjusted to 20 or 100 μg/20 μl with SDS sample buffer, and the samples were subjected to electrophoresis on 4–8% gradient SDS polyacrylamide gels (D.R.E. Co, Tokyo). The proteins were transferred to a PVDF membrane (Millipore) and treated with a blocking buffer (5% skim milk in PBS) overnight at 4°C. After blocking, the membrane was treated with monoclonal anti-dystrophin C-terminal antibody (DYS2: Novocastra) diluted 1:100 with 5% skim milk in PBS for 1 h at room temperature. After brief washing with 5% skim milk in PBS, the membrane was washed three times with 5% skim milk/0.05% Tween 20-PBS (PBS-T) for 20 min, and incubated with horseradish peroxidase (HRP)–conjugated anti–mouse IgG1 antibody (ZYMED) diluted 1:5,000 with 5% skim milk/PBS-T. Then, the membrane was washed with PBS-T three times for 30 min, and treated with an Enzyme Chemiluminescence (ECL) solution (Amersham Pharmacia Biotech). The pattern was visualized by exposing the membrane to X-ray film (Hyperfilm ECL, Amersham Pharmacia Biotech) for periods of 5 to 60 min at room temperature.

Interaction between Negamycin and rRNA (A-Site)—HPLC-purified partial eukaryotic rRNA sequences (A-site: 5′-GGCGUCGCUACUUCGGAAAAAGUCGCC-3′: 27 mer) were synthesized by the Takara Biotechnology Co. A reaction mixture containing 5.0×10^{-8} mol of negamycin and 5.0×10^{-9} mol of the rRNA sequence was prepared in distilled water to a total volume of 1ml. The formation of a negamycin:rRNA complex was examined by the Orthogonal Acceleration TOF-MS (AccuTOF, JEOL). Measurements were performed under conditions produced by a 50 μl/min syringe pump at temperatures under 100°C. To prepare a modified A-site, the bold nucleotides were changed in the partial 1406–1494 loop region of the A-site (modified A-site: 5′-GGCG**AC**U**C**U-**U**CUUCGG**AA**G**A**GUCGCC-3′, 26 mer).

Changes in Body Weights of mdx Mice Treated with Negamycin—Subcutaneous injection of male *mdx* mice (7 weeks old, male, $n = 4$ per dose) with negamycin was performed at various doses: 1.2×10^{-5} mol/kg (1×), 1.2×10^{-4} mol/kg (10×), 6.0×10^{-4} mol/kg (50×), and 1.2×10^{-3} mol/kg (100×) each day for 2 weeks. Gentamicin sulfate (1.2×10^{-3} mol/kg) was also injected into different male *mdx* mice (7 weeks old, n = 2). As a control, PBS was injected into *mdx* mice every day. Body weights were measured every day.

Ototoxicity—Male *mdx* mice (7 weeks old, n = 2) were treated with 1.2×10^{-4} mol/kg (10×) negamycin or gentamicin for 7 days. *Mdx* mice treated with PBS (0.1 ml/10 g BW) were used as controls. Hearing ability was deter-

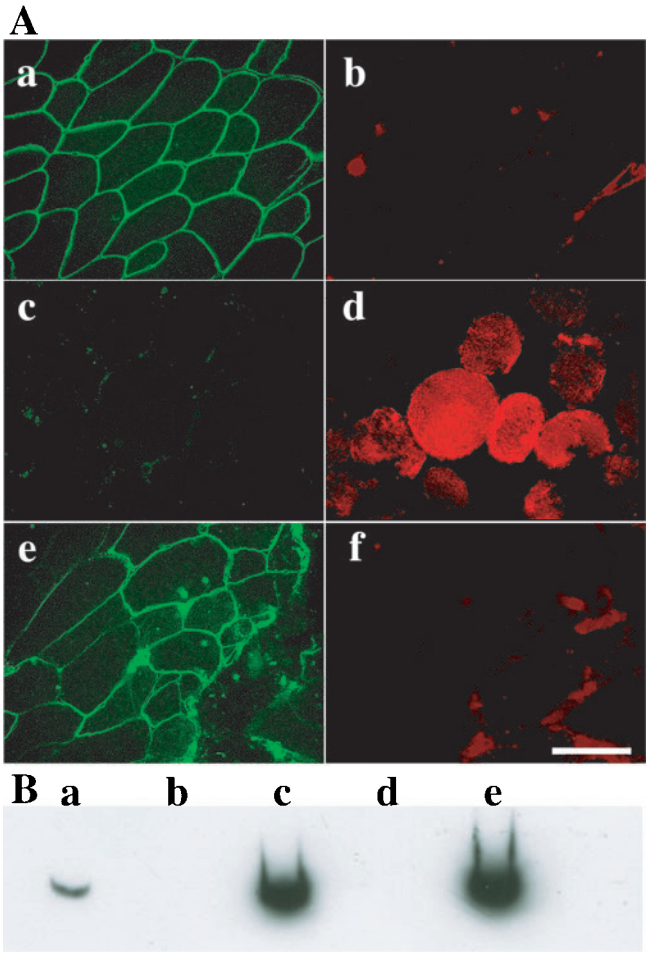

Fig. 1. **Dystrophin expression in skeletal muscles of negamycin-treated and untreated *mdx* mice.** A: Immunofluorescent staining of dystrophin (a, c, and e) and EB staining (b, d, and f) of degenerating fibers. a and b, normal B10 mouse; c and d, untreated *mdx* mouse; e and f, negamycin-treated *mdx* mouse. Bar = 100 μm. B: Immunoblot analysis of full-length dystrophin proteins from the left hindlimb muscles of *mdx* and control mice. a, negamycin-treated *mdx* mouse; b, sample buffer; c, negamycin-treated *mdx* mouse (ten times the amount of protein used in lane "a" was loaded); d, untreated *mdx* mouse; e, B10 control mouse. Dystrophin was visualized by ECL. Only the dystrophin area of the blot is shown.

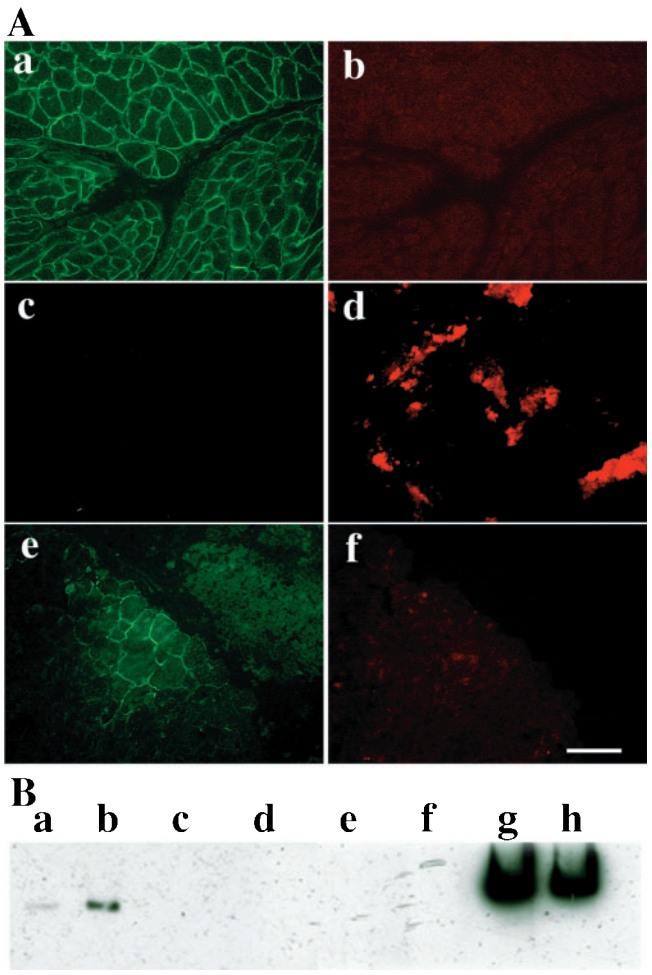

Fig. 2. **Dystrophin expression in cardiac muscles of negamycin-treated and untreated *mdx* mice.** A: Immunofluorescent staining of dystrophin (a, c, and e) and EB staining (b, d, and f) of degenerating fibers. a and b, B10 control mouse; c and d, untreated *mdx* mouse; e and f, negamycin-treated *mdx* mouse. Bar = 40 μm. B: Immunoblot analysis of full-length dystrophin protein from cardiac muscles. a and b, two individual, negamycin-treated *mdx* mice; c and f, empty lanes; d and e, two individual untreated *mdx* mice; g and h, two individual normal B10 mice. Dystrophin was visualized by ECL. Only the dystrophin area of the blot is shown.

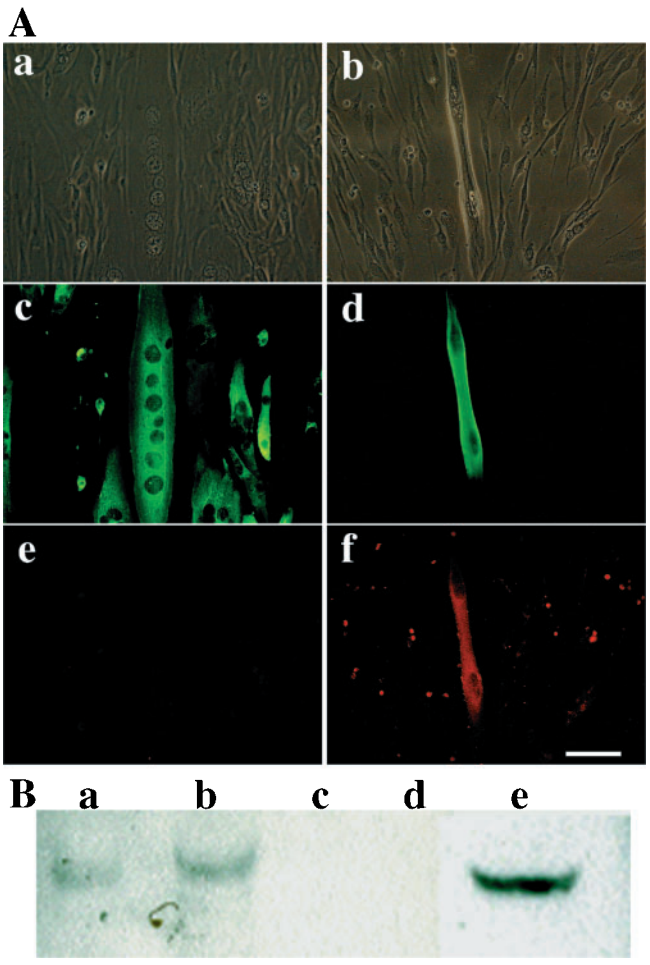

Fig. 3. **Dystrophin expression in cultured *mdx* skeletal muscle cells.** A: Untreated (a, c, and e) and negamycin-treated (100 μg/ml) (b, d, and f) cultured *mdx* myotubes. Phase contrast microscopy of cultured *mdx* myotubes (a and b), immunofluorescent staining of myosin heavy chain (c and d) and dystrophin (e and f). Bar = 40 μm. B: Immunoblot of dystrophin in cultured *mdx*SV40-T or C2C12 cells. a and b, two separate cultures of myotubes treated with negamycin for 1 week; c untreated *mdx* myotubes; d, empty lane; e, C2C12 myotubes.

mined by the auditory brainstem response, according to the method of Shapiro *et al.* (*26*)

RESULTS

Detection of Dystrophin in Negamycin-Treated mdx Muscles—Immunostaining of dystrophin and vital staining with EB were performed on TA muscles harvested from *mdx* mice that had received daily injections of negamycin (1.2×10^{-5} mol/kg BW) for 2 weeks (Fig. 1A). The number of dystrophin-positive fibers was greater in *mdx* TA muscle compared with controls. An average of 6% of 350–550 total TA muscle fibers in six negamycin-treated *mdx* mice were dystrophin-positive. Furthermore, the number of EB-positive fibers in the *mdx* mice decreased from 17% in untreated mice to 5% with negamycin treatment. Dystrophin accumulation was confirmed by immunoblots of partially enriched, WGA-purified dystrophin/dystrophin-associated glycoprotein complex (Fig. 1B). We estimate that the dystrophin level in the skeletal muscle of negamycin-treated *mdx* mice is approximately 10% that in normal skeletal muscles. Dystrophin-positive fibers were not observed in cardiac muscle of *mdx* mice treated with negamycin for 2 weeks (data not shown). The number of dystrophin-positive fibers in *mdx* cardiac muscle also increased after prolonged (4 weeks) negamycin treatment (Fig. 2A). There were no EB-positive fibers in the dystrophin-positive area. The full-length dystrophin protein was also detected by immunoblot analysis in the cardiac muscles of negamycin-treated *mdx* mice (Fig. 2B). The dystrophin level in the cardiac muscle was also estimated to be less than 10%.

Detection of Dystrophin in Negamycin-Treated mdx Skeletal Muscle Cells in Culture—In order to study the restoration of dystrophin in cultured *mdx* skeletal muscle cells, we first established *mdx*SV40-T cells. Muscle cell

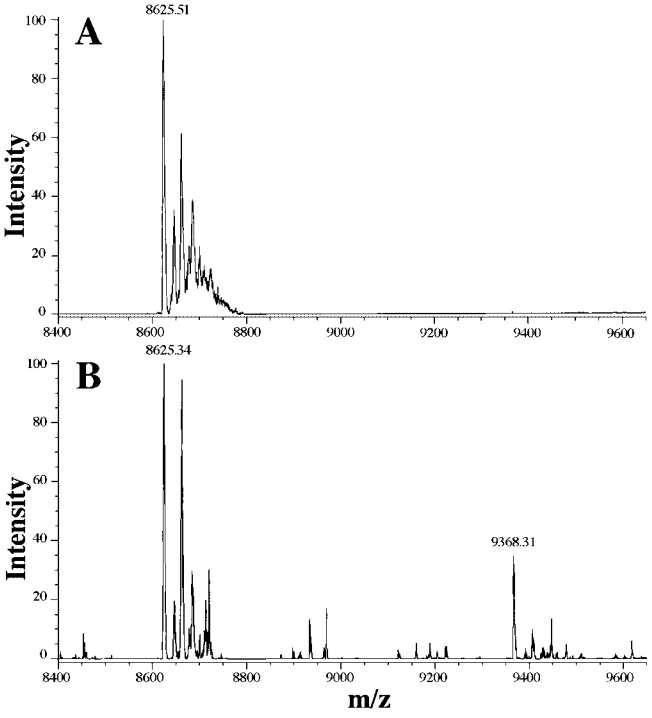

Fig. 4. **Interaction between negamycin and a eukaryotic partial rRNA A-site.** A: Deconvolution of a mass spectrum of the eukaryotic rRNA A-site (MN: 8625.51). B: Deconvolution of a mass spectrum of the negamycin/rRNA A-site complex (MN: 9368.31) corresponding to a binding ratio of negamycin to rRNA of 3:1 was observed by TOF-MS.

differentiation of *mdx*SV40-T cells was confirmed morphologically as well as immunohistochemically by the expression of the sarcomeric myosin heavy chain (Fig. 3A). After 1 week of negamycin treatment (4.0×10^{-7} mol/ml, 100 μg/ml), dystrophin was detected immunohistochemically in an average of 6% of a total of 340 sarcomeric myosin heavy chain-positive myotubes from 4 different experiments, and dystrophin accumulation was confirmed by immunoblot analysis (Fig. 3B). Dystrophin was not detected in untreated *mdx*SV40-T cells.

Interaction between Negamycin and the Eukaryotic rRNA A-Site—The ESI-MS was obtained from a mixture of 5 nmol partial eukaryotic rRNA A-site and 5.0×10^{-8} mol negamycin in distilled water. A partial eukaryotic rRNA A-site was detected at a mass number (MN) of 8625.51 (M+H) by TOF-MS (Fig. 4A). A negamycin/eukaryotic rRNA A-site complex (MN: 9368.31), corresponding to a 3:1 ratio of negamycin to eukaryotic rRNA, was detected (Fig. 4B).

To examine the affinity site more closely, binding of negamycin and a modified rRNA A-site (modified 5-nucleotides in the loop region as a match pair in the eukaryotic rRNA A-site sequence) was also performed under these same conditions. The modified rRNA A-site was also detected, however, no interaction between negamycin and the modified A-site was detected (data not shown). The eukaryotic rRNA A-site showed stoichiometric and stereospecific affinities that were significantly higher than those of the modified rRNA A-site.

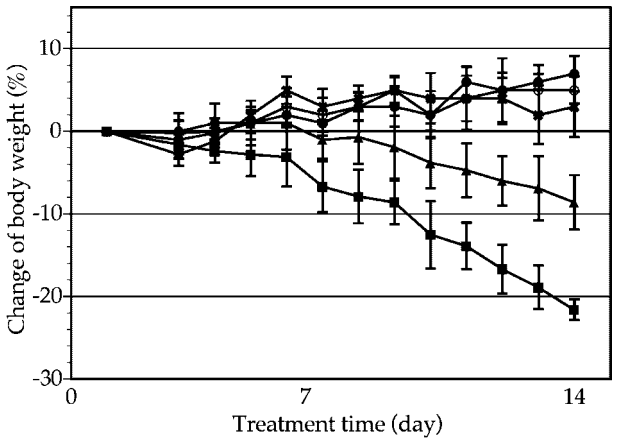

Fig. 5. **Changes in body weights of *mdx* mice during negamycin treatment for 2 weeks.** *Mdx* mice were treated with negamycin and weighed every day. *Mdx* mice treated with PBS (open circles), or with negamycin at 1.2×10^{-5} mol/kg (closed circles), 1.2×10^{-4} mol/kg (crosses), 6.0×10^{-4} mol/kg (closed triangles), or 1.2×10^{-3} mol/kg (closed squares).

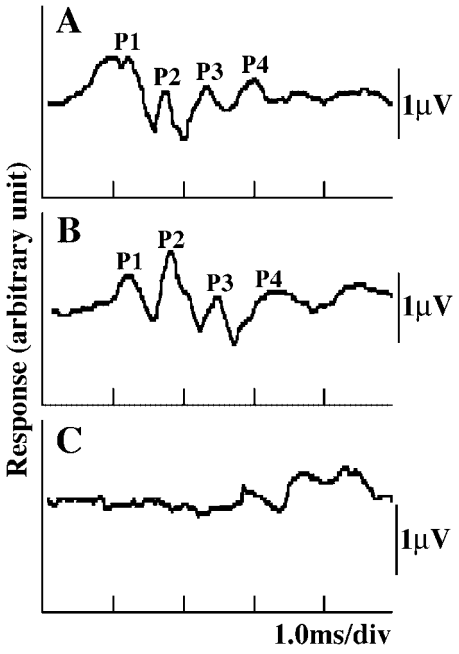

Fig. 6. **Changes in auditory brainstem response.** Audiometrc profiles according to the auditory brain stem response of antibiotic-treated mice are shown. A, control mouse, four positive peaks, P1, P2, P3, and P4, were recorded; B, negamycin-treated mouse, four positive peaks, similar to those in the control mouse, were recorded; C, gentamicin-treated mouse, the four positive peaks are absent.

Negamycin Is Less Toxic than Gentamicin in mdx Mice—It is well known that aminoglycoside antibiotics are associated with severe side effects. Although these antibiotics are used routinely for the treatment of bacterial infections, they often cause nephrotoxicity and ototoxicity. We examined negamycin toxicity by measuring changes in body weight and hearing acuity in drug-treated mice (Figs. 5 and 6). Male *mdx* mice (7 weeks old) were injected subcutaneously with varying doses of negamycin for 2 weeks. the body weights of mice treated with

lower doses of negamycin (1× and 10× the minimal effective dose) increased continuously (Fig. 5); however, the body weights of mice treated with higher doses of negamycin (50× and 100×) decreased. It is noteworthy that the highest dose of negamycin was not lethal. In contrast, a high dose of gentamicin (1.2×10^{-3} mol/kg/day BW, 100×) killed all mice tested within 4 h (data not shown). Furthermore, to investigate ototoxicity, we conducted otologic studies on untreated and antibiotic-treated mice. Auditory brainstem responses to sounds ranging from 95 to 25 decibels (dB) were recorded. In both untreated and negamycin-treated *mdx* mice, the normal P1, P2, P3, and P4 peaks of an auditory brainstem response were observed. On the other hand, gentamicin-treated mice lacked all waves of auditory brainstem response under 80 dB (Fig. 6). This study indicates that hearing loss under 80 dB was apparent only in the gentamicin-treated mice, and not in negamycin-treated mice.

DISCUSSION

Many human genetic diseases have a relatively high proportion of disease-causing nonsense mutations. These mutations are found in approximately 5–15% of patients with DMD (*12*). To overcome nonsense mutations, one can expect that the naturally occurring suppressor tRNA may play important roles. Especially in the case of selenoprotein synthesis, the selenocysteyl tRNA carries the anticodon for UGA, a stop codon (*27*, *28*). Unfortunately, however, the selenocysteyl tRNA can function in selenoprotein synthesis only in the presence of a selenocysteine insertion sequence (SECIS) in the 3′ untranslated region (UTR) of the mRNA, and SECIS is found only in the mRNAs encoding for selenoproteins. Except selenocysteyl-tRNA, the level of other suppressor tRNAs is not high enough to overcome common nonsense mutations. Therefore, it is reasonable to explore new drugs that induce read-through of premature stop codons.

The restoration of dystrophin was observed in the skeletal and cardiac muscles of *mdx* mice treated with negamycin. There was a difference between skeletal and cardiac muscles in the susceptibility to negamycin. In contrast to skeletal muscle, no dystrophin was detected in the cardiac muscle of *mdx* mice treated with negamycin for 2 weeks. However, dystrophin staining was observed after prolonged negamycin treatment of 4 weeks. Using WGA-conjugated affinity beads, we successfully enriched the dystrophin/dystrophin-associated protein complex from the muscles of negamycin-treated *mdx* mice. This study indicates that the accumulation of dystrophin-associated glycoprotein might be recovered by the restoration of dystrophin in the muscles of these animals. Furthermore EB-positive, degenerating fibers were not observed in the dystrophin-positive area. Moreover, with reference to Fig. 1, serum creatine kinase activity was decreased 35% in *mdx* mice treated with negamycin for 2 weeks (data not shown). Accordingly, it is reasonable to conclude that the physiological function of the muscle cell membrane is present not only in the TA muscle but also in other muscles in the whole body following negamycin treatment. However, muscle contraction profiles in negamycin-treated and untreated *mdx* mice remain to be compared in detail.

The mild Becker-type muscular dystrophy (BMD) is also caused by mutations in various portions of the dystrophin gene (*29*). Hoffman *et al.* reported that in several cases of BMD, the phenotype was improved by dystrophin expressed at a level greater than or equal to 20% of normal (*30*). Although we have no direct evidence as to whether 10% of the normal dystrophin level observed in this study would be sufficient to improve muscle performance *in vivo*, it is likely to be far better than a complete lack of dystrophin.

Danko *et al*. reported that dystrophin-positive fibers, called revertant fibers, were observed in 1% or fewer of the skeletal muscle fibers of 8-week-old *mdx* mice (*31*). These dystrophin-positive *mdx* muscle fibers presumably arose from alternative splicing and/or a second mutation that skipped the primary mutation in exon 23. The number of revertant fibers increases as *mdx* mice age (*32*). In the present study, the percentage of dystrophin-positive fibers in the skeletal muscle of *mdx* mice treated with negamycin was an average of 6%, which is significantly higher than the percentage of revertant fibers. In age-matched *mdx* skeletal and cardiac muscles, a truncated or shortened form of dystrophin was not detected in negamycin-treated *mdx* mice.

The read-through of termination codons during normal protein synthesis may also be considered a source of side effects of negamycin. However, according to the cDNA databases that include the dystrophin gene, two or more stop codons usually exist near the termination codon in the UTR (data not shown). Extensive read-through of termination signals during protein synthesis may not occur often. Both the type of stop codon and the nucleotide sequence surrounding the nonsense mutation have been reported to be critical for the read-through activity of gentamicin and other aminoglycoside antibiotics (*33*, *34*). Therefore, the type of nonsense mutation and surrounding nucleotide sequence affected by the read-through activity induced by negamycin should be investigated.

The mass measurement accuracy and resolution of MS easily provides this information in a rapid and sensitive manner. In the case of rRNA-aminoglycoside antibiotic and RNA-argininamide complex analysis, changes in the chemical shift of the rRNA complex were caused by binding to other small molecules (*17*, *18*, *35*, *36*). In the present study, to examine the functional relationship between negamycin and its read-through activity, we investigated interactions between negamycin and the eukaryotic rRNA-decoding A-site using TOF-MS. Our results indicate that an interaction occurs involving three molecules of negamycin and one molecule of rRNA. The negamycin/eukaryotic rRNA complex might also induce a local conformational change in the eukaryotic rRNA and cause the read-through of the nonsense mutation.

Barton-Davis *et al.* reported that gentamicin could restore functional dystrophin in *mdx* mice (*12*). However, Dunant *et al.* reported that gentamicin treatment failed to lead to a significant increase in the expression of the full-length dystrophin protein in *mdx* mice (*37*). In human trials, Politano *et al*. reported that one of four gentamicin-treated DMD patients exhibited increased dystrophin re-expression by both immunohistochemistry and Western blot analysis (*38*). In contrast, Wagner *et al*.

and Serrano *et al*. reported that the dystrophin protein was not detectable by immunohistochemistry or immunoblot analysis in clinical trials of DMD/BMD patients treated with gentamicin(*39*, *40*). These discrepancies in gentamicin clinical trials can probably be explained by the likely variations in the purity of commercially available gentamicin sulfate and in the ratios of its three distinct isomers.

Compared with gentamicin, negamycin treatment exhibited lower toxicity. While the antibacterial activity of negamycin resembles that of gentamicin, it is possible that negamycin may have different side effects. There is considerable interest in understanding the mechanisms underlying negamycin toxicity so that negamycin can be used to treat a variety of medical conditions. The side effects of negamycin treatment remain to be clarified.

We conclude that negamycin is a promising therapeutic candidate for DMD and many other genetic diseases caused by nonsense mutations. We emphasize that negamycin has not yet been approved for use in humans.

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REFERENCES

1. Wilhelm, J.M., Jessop, J.J., and Pettitt, S.E. (1978) Aminoglycoside antibiotics and eukaryotic protein synthesis: stimulation of errors in the translation of natural messengers in extracts of cultured human cells. *Biochemistry* **17**, 1149–1153
2. Burke, J.F. and Mogg, A.E. (1985) Suppression of a nonsense mutation in mammalian cells *in vivo* by the aminoglycoside antibiotics G-418 and paromomycin. *Nucleic Acids Res.* **13**, 6265–6272
3. Howard, M.T., Frizzell, R.A., and Bedwell, D.M. (1996) Aminoglycoside antibiotics restore CFTR function by overcoming premature stop mutations. *Nat. Med.* **2**, 467–469
4. Bedwell, D.M., Kaenjak, A., Benos, D.J., Bebok, Z., Bubien, J.K., Hong, J., Tousson, A., Clancy, J.P., and Sorscher, E.J. (1997) Suppression of a CFTR premature stop mutation in a bronchial epithelial cell line. *Nat. Med.* **3**, 1280–1284
5. Clancy, J.P., Bebok, Z., Ruiz, F., King, C., Jones, J., Walker, L., Greer, H., Hong, J., Wing, L., Macaluso, M., Lyrene, R., Sorscher, E.J., and Bedwell, D.M. (2001) Evidence that systemic gentamicin suppresses premature stop mutations in patients with cystic fibrosis. *Amer. J. Respir. Crit. Care Med.* **163**, 1683–1692
6. Keeling, K.M., Brooks, D.A., Hopwood, J.J., Li, P., Thompson, J.N., and Bedwell, D.M. (2001) Gentamicin-mediated suppression of Hurler syndrome stop mutations restores a low level of alpha-L-iduronidase activity and reduces lysosomal glycosaminoglycan accumulation. *Hum. Mol. Genet.* **10**, 291–296
7. Sleat, D.E., Sohar, I., Gin, R.M., and Lobel, P. (2001) Aminoglycoside-mediated suppression of nonsense mutations in late infantile neuronal ceroid lipofuscinosis. *Europ. J. Paedi. Neuro.* **5 (Suppl. A)**, 57–62
8. Bulfiled, G., Siller, W.G., Wight, P.A., and Moore, K.J. (1984) X chromosome-linked muscular dystrophy (*mdx*) in the mouse. *Proc. Natl Acad. Sci. USA* **81**, 1189–1192
9. Sicinski, P., Geng, Y, Ryder-Cook, A.S., Barnard, E.A. Darlison, M.G., and Barnard, P.J. (1989) The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. *Science* **244**, 1578–1582
10. Granchelli, J.A., Pollina, C., and Hudecki, M.S. (2000) Pre-clinical screening of drugs using the mdx mouse. *Neuromusc. Disord.* **10**, 235–239
11. Griggs, R.C., Moxley, R.T. 3rd., Mendell, J.R., Fenichel, G.M., Brooke, M.H., Pestronk, A., Miller, J.P, Cwik, V.A., Pandya, S., and Robinson, J. (1993) Duchenne dystrophy: randomized, controlled trial of prednisone (18 months) and azathioprine (12 months) *Neurology* **43**, 520–527
12. Barton-Davis, E.R., Cordiner, L., Shoturma, D.I., Leiland, S.E., and Sweeney, H.L. (1999) Aminoglycoside antibiotics restore dystrophin function to skeletal muscles of *mdx* mice. *J. Clin. Invest.* **104**, 375–381
13. Hamada, M., Takeuchi, T., Kondo, S., Ikeda, Y., Naganawa, H., Maeda, K., Okami, Y., and Umezawa, H. (1970) A new antibiotic, negamycin. *J. Antibiot.* **23**, 170–171
14. Uehara, Y., Hori, M., and Umezawa, H. (1974) Negamycin inhibits termination of protein synthesis directed by phage f2 RNA *in vitro*. *Biochim. Biophys. Acta* **374**, 82–95
15. Fourmy, D., Recht, M.I., Blanchard, S.C., and Puglisi, J.D. (1996) Structure of the A site of Escherichia coli 16S ribosomal RNA complexed with an aminoglycoside antibiotic. *Science* **274**, 1367–1371
16. Yoshizawa, S., Fourmy, D., Eason, R.G., and Puglisi, J.D. (2002) Sequence-specific recognition of the major groove of RNA by deoxystreptamine. *Biochemistry* **41**, 6263–6270
17. Loo, J.A., DeJohn, D.E., Du, P., Stevenson T.I., and Ogorzalek Loo, R.R. (1999) Application of mass spectrometry for target identification and characterization. *Med. Res. Rev.* **19**, 307–319
18. Griffey, R.H., Hofstadler, S.A., Sannes-Lowery, K.A., Ecker, D.J., and Crooke S.T. (1999) Determinants of aminoglycoside-binding specificity for rRNA by using mass spectrometry. *Proc. Natl Acad. Sci. USA* **96**, 10129–10133
19. Matsuda, R., Nshikawa, A., and Tanaka, H. (1995) Visualization of dystrophic muscle fibers in mdx mouse by vital staining with Evans blue: evidence of apoptosis in dystrophin-deficient muscle. *J. Biochem.* **118**, 959–964
20. Morita, S., Kojima, T., and Kitamura, T. (2000) Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther.* **7**, 1063–1066
21. Yoshida, M., Suzuki, A., Shimizu, T., and Ozawa, E. (1992) Proteinase-sensitive sites on isolated rabbit dystrophin *J. Biochem.* **112**, 433–439
22. Campbell, K.P. and Kahl, S.D. (1989) Association of dystrophin and an integral membrane glycoprotein. *Nature* **338**, 259–262
23. Abe, M., Saitoh, O., Nakata, H., Yoda, A., and Matsuda, R. (1996) Expression of neurofilament proteins in proliferating C2C12 mouse skeletal muscle cells. *Exp. Cell Res.* **229**, 48–59
24. Arakawa, M., Nakayama, Y., Hara, T., Shiozuka, M., Kondo, S., Kitamura, T., Takeda, S., and Matsuda, R. (2001) Negamycin can restore dystrophin in *mdx* skeletal muscle. *Acta. Myol.* **20**, 154–158
25. Itzhaki, R.F. and Gill, D.M. (1964) A micro-biuret method for estimating protein. *Anal. Biochem.* **9**, 401–410
26. Shapiro, S.M., Moller, A.R., and Shiu G.K. (1984) Brain-stem auditory evoked potentials in rats with high-dose pentobarbital. *Electroencephalogr. Clin. Neurophysiol.* **58**, 266–276
27. Berry, M.J., Banu, L., Harney, J.W., and Larsen, P.R. (1993) Functional characterization of the eukaryotic SECIS elements which direct selenocysteine insertion at UGA codons *EMBO J.* **12**, 3315–3322
28. Zavacki, A.M., Mansell, J.B., Chung, M., Klimovitsky, B., Harney, J.W., and Berry, M.L. (2003) Coupled tRNA(Sec)-dependent assembly of the selenocysteine decoding apparatus. *Mol. Cell* **11**, 773–781
29. Emery, A.E.H. (1993) *Duchenne Muscular Dystrophy*, 2nd ed., Oxford Monographs on Medical Genetics, Vol. **24**, Oxford Medical Publications, Oxford
30. Hoffman, E.P., Kunkel, L.M., Angelini, C., Clarke, A., Johnson, M., and Harris, J.B. (1989) Improved diagnosis of Becker mus-

cular dystrophy by dystrophin testing. *Neurology* **39**, 1011–1017
31. Danko, I., Chapman, V., and Wolff, J.A. (1992) The frequency of revertants in *mdx* mouse genetic models for Duchenne muscular dystrophy. *Pediat. Res.* **32**, 128–131
32. Lu, Q.L., Morris, G.E., Wilton, S.D., Ly, T., Artem'yeva, O.V., Strong, P., and Partridge, T.A. (2000) Massive idiosyncratic exon skipping corrects the nonsense mutation in dystrophic mouse muscle and produces functional revertant fibers by clonal expansion. *J. Cell Biol.* **148**, 985–995
33. Manuvakhova, M., Keeling, K., and Bedwell, D.M. (2000) Aminoglycoside antibiotics mediate context-dependent suppression of termination codons in a mammalian translation system. *RNA* **6**, 1044–1055
34. Howard, M.T., Shirts, B.H., Petros, L.M., Flanigan, K.M., Gesteland, R.F., and Atkins, J.F. (2000) Sequence specificity of aminoglycoside-induced stop condon readthrough: potential implications for treatment of Duchenne muscular dystrophy. *Ann. Neurol.* **48**, 164–169
35. Chow, C.S. and Bogdan, F.M. (1997) A Structural Basis for RNAminus signLigand Interactions. *Chem. Rev.* **97**, 1489–1513
36. Puglisi, J.D., Tan, R., Calnan, B.J., Frankel, A.D., and Williamson, J.R. (1992) Conformation of the TAR RNA-arginine complex by NMR spectroscopy. *Science* **57**, 76–80
37. Dunant, P., Walter, M.C., Karpati, G., and Lochmuller, H. (2003) Gentamicin fails to increase dystrophin expression in dystrophin-deficient muscle. *Muscle Nerve* **27**, 624–627
38. Politano, L., Nigro, G., Nigro, V., Piluso, G., Papparella, S., Paciello, O., and Comi, L.I. (2003) Gentamicin administration in Duchenne pacients with premature stop codon. Preliminary results. *Acta Myol.* **22**, 15–21
39. Wagner, K.R., Hamed, S., Hadley, D.W., Gropman, A.L., Burstein, A.H., Escolar, D.M., Hoffmann, E.P., and Fischbeck, K .H. (2001) Gentamicin treatment of Duchenne and Becker muscular dystrophy due to nonsense mutations. *Ann. Neurol.* **49**, 706–711
40. Serrano, C., Wall, C., Moore, S.A., King, W., Barresi, R, , Campbell, K.P, , Noticewala, P., Brown, R.H., Nagarja, H.N., and Mendell, J.R. (2001) Gentamicin treatment for muscular dystrophy patients with stop codon mutations. *Neurology* **56 (Suppl)**, A79