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# Design of 2'-*O*-Me RNA/ENA™ chimera oligonucleotides to induce exon skipping in dystrophin pre-mRNA

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## ABSTRACT

**2'-*O*-Me RNA/ENA chimera oligonucleotides complementary to exon 45 and 46 of the dystrophin gene induced exon 45 and 46 skipping of the dystrophin pre-mRNA, respectively. The induction of exon skipping by the most effective 2'-*O*-Me RNA/ENA chimeras led to the expression of dystrophin in dystrophin-deficient myocytes by correcting the translational reading frames. Also, in the process of 2'-*O*-Me RNA/ENA chimera optimization to induce exon skipping in several exons, it was found that the optimized target sequences of the chimeras included guanosine- or adenosine-rich sequences that might function as splicing enhancer sequences (SES).**

## INTRODUCTION

Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are both caused by a mutation in the dystrophin gene. However, the mutations in BMD do not disrupt the reading frame and thus, a partially functional dystrophin protein is still produced. On the other hand, DMD is a fatal myodegenerative condition that results from either a non-sense or frame-shifting mutation, leading to a deficiency of dystrophin protein in the muscle fibers. We have proposed a novel strategy for the treatment of DMD, that of changing a DMD-causing out-of-frame mutation into an in-frame mutation characteristic of BMD by inducing exon skipping. In previous studies, we have designed a 31-mer phosphorothioate oligodeoxynucleotide (S-oligo) complementary to the splicing enhancer sequence of exon 19 of the dystrophin gene and transfected it into DMD myocytes that contained a deletion of exon 20 (1). Successful transfection of the S-oligo induced exon 19 skipping and led to the production of an in-frame dystrophin mRNA with simultaneous deletion of exon 19 and 20. This exon skipping by the S-oligo led to the expression of dystrophin in DMD myocytes.

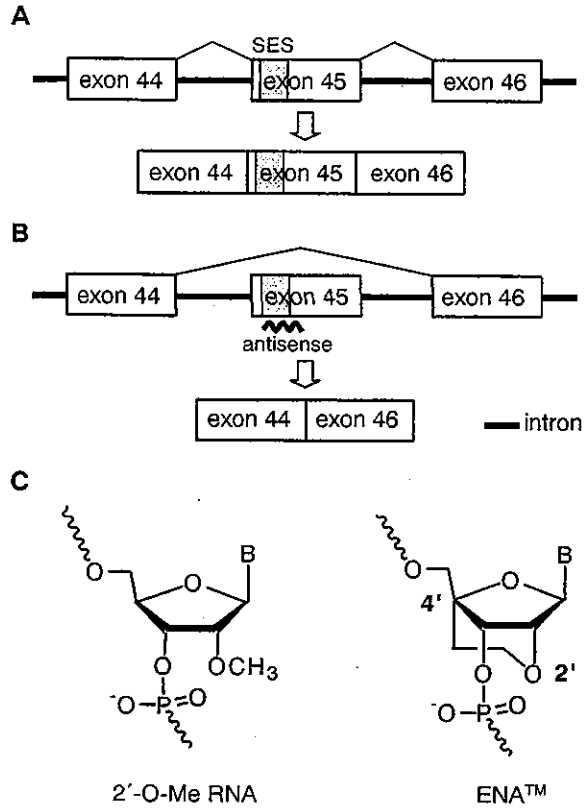


**Figure** (A), (B) Mechanism of exon-skipping of pre-mRNA using antisense molecules and (C) structures of 2'-O-Me RNA and ENA™

As more attractive oligonucleotides than S-oligos, we have reported 2'-*O*,4'-*C*-ethylene-bridged nucleic acid (ENA) oligonucleotides that have high affinity to

complementary RNA strands and high nuclease-resistance (2). Recently, we have reported that the exon 19 skipping activity of a 2'-*O*-Me RNA/ENA chimera oligonucleotide having the same sequence as the S-oligo was 40 times greater than that of the S-oligo (3). Here, we designed and evaluated 2'-*O*-Me RNA/ENA chimeras for the effective induction of exon 45 and 46 skipping.

## RESULTS AND DISCUSSION

### Exon skipping of dystrophin pre-mRNA by 2'-O-Me RNA/ENA chimera

Nucleotide sequences of exon 45 and 46 in the dystrophin gene were divided into 15- to 18-base fragments, and 2'-*O*-Me RNA/ENA chimeras containing sequences complementary to each fragment were chemically synthesized. Ten ENA residues were incorporated into 2'-*O*-Me RNA/ENA chimeras. A transfection reagent (Lipofectamine) and 200 nM of the chimera were added to a culture medium of myocytes, and after 2 days, the dystrophin mRNA expressed in the DMD myocytes was analyzed. Upon reverse transcriptase PCR (RT-PCR) amplification of the region spanning exon 44 to 48, two bands were visualized with the 2'-*O*-Me RNA/ENA chimera-treated myocytes. Sequence analysis of the amplified products revealed that the larger band corresponded to a normal, full-length fragment and the smaller band to exon 45 or 46 skipped fragment, as expected. In contrast, only normal product was observed when the myocytes were not transfected with the chimera. In the study of exon 45 skipping induction, an 18-mer 2'-*O*-Me RNA/ENA chimera, AO85, had the strongest activity, inducing exon 45 skipping in nearly 90% of the dystrophin mRNA. Among the ten 2'-*O*-Me RNA/ENA chimeras for exon 46, an 18-mer 2'-*O*-Me RNA/ENA chimera, AO27, had the strongest activity. When we synthesized an 18-mer 2'-*O*-Me RNA/ENA chimera, AO92, which had the same sequence and the same number of ENA residues, and in which the ENA residues were incorporated at different positions from the case of AO27, AO92 showed a much stronger activity than AO27.

### Induction of dystrophin expression by 2'-O-Me RNA/ENA chimera

After successful induction of exon 45 skipping in myocytes of patients, the dystrophin expression in these cells was examined. Immunohistochemical staining using an antibody recognizing the rod domain of dystrophin revealed that nearly 90% of the cultured myocytes transfected with the 18-mer 2'-*O*-Me RNA/ENA chimera, AO85, was shown to be dystrophin-positive. These results indicate that 2'-*O*-Me RNA/ENA chimeras may be applied clinically for the treatment of the out-of-frame mutation of the dystrophin gene.

### Guanosine- and adenosine-rich sequences to induce exon skipping

In previous and current studies, we found sequences of 2'-*O*-Me RNA/ENA chimera oligonucleotides that induced the skipping of some exons of the dystrophin pre-mRNA, such as exon 19, 41, 44, 45, 46, 50, 51, 53 and 55. These target sequences were generally located in the 5' region of the exons, and they included guanosine- and adenosine-rich sequences that were most likely to be sequences known as splicing enhancer sequences (4). These results suggest that analyzing the splicing enhancer sequences in other exons and other target genes may lead to presuming exon-skipping sequences and designing effective 2'-*O*-Me RNA/ENA chimeras that induce exon-skipping.

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# Cloning of cDNA Encoding a Regeneration-Associated Muscle Protease Whose Expression Is Attenuated in Cell Lines Derived from Duchenne Muscular Dystrophy Patients

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**In the dystrophin-mutant *mdx* mouse, an animal model for Duchenne muscular dystrophy (DMD), damaged skeletal muscles are efficiently regenerated and thus the animals thrive. The phenotypic differences between DMD patients and the *mdx* mice suggest the existence of factors that modulate the muscle wasting in the *mdx* mice. To identify these factors, we searched for mRNAs affected by the *mdx* mutation by using cDNA microarrays with newly established skeletal muscle cell lines from *mdx* and normal mice. We found that in the *mdx* muscle cell line, 12 genes, including L-arginine:glycine amidinotransferase and thymosin β4, are up-regulated, whereas 7 genes, including selenoprotein P and a novel regeneration-associated muscle protease (RAMP), are down-regulated. Northern blot analysis and *in situ* hybridization revealed that RAMP mRNA is predominantly expressed in normal skeletal muscle and brain, and its production is enhanced in the regenerating area of injured skeletal muscle in mice. RAMP expression was much lower in individual muscle cell lines derived from biopsies of six DMD patients compared to a normal muscle cell line. These results suggest that RAMP may play a role in the regeneration of skeletal muscle and that its down-regulation could be involved in the progression of DMD in humans. *(Am J Pathol 2004, 164:1773–1782)***

Point mutations or out-of-frame deletions in the dystrophin gene on the X-chromosome are known to cause Duchenne muscular dystrophy (DMD).[1] This disease occurs with a frequency of 1 of 3500 newborn males, which makes it the most common lethal myopathy. Dystrophin is a large membrane-associated protein that plays an important role in linking the intracellular cytoskeletal actin filaments to the sarcolemmal membrane.[2] In addition, it forms a multicomponent complex denoted as dystrophin-associated protein complex, which contains dystroglycans, sarcoglycans, syntrophins, and nitric oxide synthase.[3,4] Thus, dystrophin not only mechanically protects the sarcolemma from muscle contraction-induced tension,[5] it also affects intracellular signaling pathways, particularly in the $Ca^{2+}$-dependent enzymatic cascade.[6]

The *mdx* mutant mouse strain carries a nonsense mutation at position 3185 of the murine dystrophin gene.[7,8] However, despite the lack of subsarcolemmal dystrophin protein in these mice, their skeletal muscle degeneration is less severe than it is in DMD patients as after an initial period of skeletal muscle necrosis at 3 to 4 weeks of age, regenerative activity in the *mdx* mice gradually compensates for the muscle damage in the hindlimb.[9] As a result, the adult *mdx* mice show little functional disability. In contrast, in DMD patients, there is an imbalance between muscle degeneration and repair that leads to the loss of muscle fibers and increased fibrosis.[10] Consistent with these observations is that recent DNA microarray analyses revealed that mRNAs encoding proteins related to the muscle regeneration process are more abundantly

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expressed in the skeletal muscle of *mdx* mice than in the skeletal muscle of normal control mice.[10–13] Examples of these muscle-regenerating proteins are insulin-like growth factor-2, transforming growth factor $\beta$, procollagens, and osteopontin. The down-regulation of myostatin mRNA in the skeletal muscle of the *mdx* mouse is also related to its higher regeneration capacity.[10,12] Intriguingly, recent reports demonstrated that transgenic overexpression of insulin-like growth factor-1 in muscle[14] or administration of anti-myostatin neutralizing antibody[15] blocked the degeneration and fibrosis in the diaphragm in *mdx* mice, suggesting that the enhancement of muscle regenerative capacity may be a promising therapeutic approach for DMD.

Although the extensive gene profiling of DMD patient biopsies *versus* normal muscle samples has provided many clues about the secondary loss of or changes in DMD muscle,[16,17] it is difficult to be sure that net change observed in the gene expression of DMD muscle reflects an altered genetic program in the muscle cells because the necrotic DMD muscle areas are filled with many macrophages and other inflammatory immune cells. Intact muscle biopsies from young patients also contain many blood cells. Thus, it is difficult to be sure that a net change observed in the gene expression of DMD muscle reflects an altered genetic program in the muscle cells. To overcome this problem, we first immortalized skeletal muscle cells from *mdx* and control mice and compared their expression of several gene sets by using cDNA microarrays. We also established muscle cell lines from biopsies taken from DMD patients, Becker muscular dystrophy (BMD) patients, and an unaffected person to investigate the behavior of the genes whose expression patterns were found to be altered in the *mdx* muscle cell line. In this study, we report that, relative to the control murine muscle cell line, the transcription of 12 genes in the *mdx* muscle cell line is up-regulated while mRNA levels of 7 other genes is down-regulated. Among the down-regulated genes was a novel gene that we found encoded a secreted protease termed regeneration-associated muscle protease (RAMP). We found that RAMP mRNA levels are also often decreased in the muscle cell lines derived from the DMD and BMD patients.

## Materials and Methods

### Mice

Breeding pairs of C57BL/10 ScSn-Dmd$^{mdx}$ (*mdx*) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and propagated in a standard pathogen-free animal facility in the institute. C57BL/10 (B10) mice were purchased from Nihon SLC (Hamamatsu, Japan). All animal experiments were based on institutionally approved protocols.

### Primary Culture of Skeletal Muscle and Immortalization

*Ex vivo* culture of skeletal muscle was done according to the published protocol with a slight modification.[18] In brief, the hindlimb muscles were taken from 2-month-old B10 and *mdx* mice, thoroughly minced into a coarse slurry, and enzymatically dissociated with 5% trypsin (Difco, Detroit, MI) in phosphate-buffered saline (PBS) at 37°C for 30 minutes. The dissociated muscle tissues were resuspended in Dulbecco's modified Eagle's minimum essential medium-high glucose type (Sigma, St. Louis, MO) supplemented with 20% fetal calf serum and 0.5% penicillin-streptomycin (Sigma), triturated by using a 10-ml pipette, and passed through a 100-$\mu$m nylon mesh. The cells were cultured in Dulbecco's modified Eagle's medium containing 20% fetal calf serum (JRH Biosciences, Lenexa, KS), 4% Ultroser G (Biosepra, Cergy-Saint-Christophe, France) and 0.5% penicillin-streptomycin in gelatin-coated flasks for 24 hours. The nonadherent cells were then transferred to new flasks for the subculture of the primary myoblastic cells. Two days later, half of the cells were infected for 1 day with a recombinant retrovirus producing a temperature-sensitive form of the simian virus 40 large T antigen (SV40 tsT)[19] and then cultured at the permissive temperature of 32.5°C until continuously growing cells appeared. The retrovirus was produced as previously described by using the PLAT-E packaging cell line[20] and the pMESVts retrovirus vector[19] (a kind gift from Dr. Drinkwater, University of Wisconsin Medical School). After 1 month of culture, the Ultroser G was removed from the growth medium and myoblastic subclones were isolated by limiting dilution. For myotube formation, the culture medium was changed to Dulbecco's modified Eagle's medium supplemented with 5% horse serum (Invitrogen, Carlsbad, CA) followed by incubation at 39.5°C for 7 days.

### Immunocytochemistry

Cells grown in a 4-well culture dish (Nunc, Roskilde, Denmark) were treated with 0.1% Triton X-100 (Wako, Osaka, Japan) in PBS for 1 minute and fixed in cold methanol for 2 minutes. After rinsing with PBS, cells were blocked with 2% bovine serum albumin (Sigma) in PBS for 1 hour and incubated with rabbit anti-desmin antibody (Progen, Heidelberg, Germany) (1:100 dilution) for 1 hour. Samples were washed with PBS and incubated with anti-rabbit immunoglobulin conjugated with horseradish peroxidase (Jackson Immunoresearch Laboratories, West Grove, PA) (1:250 dilution) for 1 hour. After rinsing with PBS three times, the peroxidase activity was visualized by incubation with 0.25% diaminobenzidine (Sigma) in PBS containing 0.075% $H_2O_2$ and 3.36 mmol/L $NiCl_2$ (Nakalai, Kyoto, Japan).

### cDNA Microarray Analysis

Poly(A)$^+$ RNAs were isolated by using the FastTrack 2.0 (Invitrogen). According to the previously published method,[21,22] mouse microarrays carrying ~4000 different cDNAs derived from a mouse fetus at 17.5 days post coitum and an adult mouse brain were hybridized with Cy3- and Cy5-labled cDNA probes prepared from the mRNAs of the C57BL/10- and *mdx*-derived cell lines cultured in the

**Table 1.** Summary of the Expression Pattern and Primer Sequences of the Genes that Are Up-Regulated and Down-Regulated in *Mdx* Mice

| Name of gene/protein | Accession no. | Mdx/B10 cell line* | Mdx/B10 muscle† | Patient/normal human cell | Sense primer sequence (5′–3′) | Anti-sense primer sequence (5′–3′) | PCR band size (bp) |
|---|---|---|---|---|---|---|---|
| 1 SCHIP-1 | NM_01392 | ↑ | → | → | GTCTATCAGACAGAAGTTGGC | GAAGATCAGCGACGGGAGAC | 415 |
| 2 OX-2 | AF004023 | ↑ | N.T. | N.T. | | | |
| 3 Arg/Gly amidinotransferase | U07971 | ↑ | ↑ | ↑ | GGAAGTGATAGTGGGCAGAGC | CAGGATGTCTCGAGGCATTGC | 293 |
| 4 MAD/MEF2C | L08895 | ↑ | N.T. | N.T. | | | |
| | | | | | (m) CTCGAGATGAGCCACGGGAAGAGAAC | (m) GCGGCCGCCTAGCTGGAGACGGCCATCA | (m) 476 |
| 5 PC3 | M60921 | ↑ | ↑ | → | (h) ATGAGCCACGGGAAGGAAGGGAAC | (h) GGGTCAGCTCGCTGGGCAGC | (h) 304 |
| 6 mc7 | AJ278191 | ↑ | N.D. | → | GACGGCACGTGTGACGAGTG | CCTCGCTGTCTTCTTCACTC | 422 |
| 7 CGI 61 | AF151819 | ↑ | → | → | CCTCCTTAGCAAAGCTGAATG | TGCAGCATCCAAATCCAGTC | 393 |
| | | | | | (m) CCGGAATTCATGTCTGACAAACCCG | (m) GCTCTAGATTACGATTCGCCAGCTTG | (m) 135 |
| 8 Thymosin β4 | M34043 | ↑ | ↑ | → | (h) CAACCATGTCTCTGAGAAACCCG | (h) GATTCGCCTGCTTGCTTCTCC | (h) 135 |
| 9 EST-MNCb4008 Telomeric repeat binding | BF168890 | ↑ | → | ↑ | GGAGCAAAGAATGCATAAGC | CAGTGGGAAGAGAGGCCATG | |
| 10 Factor 2 | NM_02058 | ↑ | ↑ | → | CCTTTCCTGCCAACTCTTCCAC | GAGACTCTGGTTGGCCAGAG | 300 |
| 11 EST-MNCb1040 | AU035914 | ↑ | ↑ | N.T. | (m) ATGTGAGGCCGTTTGCGCGGA | (m) TTTTCTAATGGAATGCTTCCCC | (m) 540 |
| 12 mKIAA1039 protein | AK122424 | ↑ | ↑ | → | AGGTTTCCGAGGAGGCCTGG | CTTGTAGGATGGGGTCTCTG | |
| | | | | | (m) CCGGAATTCGAGAGCCAAGGCCAAAGC | (m) GCTCTAGATTAGTTTGAATGTCATTTC | (m) 1085 |
| 13 Selenoprotein P | X99807 | ↓ | ↑ | → | (h) CAATGTGGAGAAGCCTGGGG | (h) AGATGTGTGTATTTAATCG | (h) 312 |
| | | | | | (m) GCGAAGGCTCTGAAAGTGG | (m) GGGGTAACTCAGAATGCAGGGTTC | (m) 908 |
| 14 GARG16 *Phosphatidic acid* | Q64828 | ↓ | ↑ | → | (h) GACTGTGAGGAAGGATGGGC | (h) GGTCTGTGAGGACATGTTGG | (h) 330 |
| 15 Phosphatase type 2B | BC005558 NM_0320 03 | ↓ | ↓ | → | CCGCAGCCAGCGCCATGCAAA | GTAATAGATCCGGTAGAATTC | 344 |
| 16 Ectonucleotide pyrophosphatase | | ↓ | → | → | GATCACAAACCAGAGGGCAG | TCTGTGGAGTTCATGGCTTC | 684 |
| 17 Phosphodiesterase 5/amyloid beta (A4) precursor like protein 2 | NM_009691 | ↓ | → | → | GAGGCTCTTGCAGCCAATGC | CTGGAACTAGCAGGACATCAC | 337 |
| 18 Lysosomal membrane glycoprotein | J05287 | ↓ | → | → | TGTCTGCTGGCTACCATGGG | CTGCACTGCAGTCTTGAGCTG | 418 |
| 19 EST-MNCb1423 (RAMP) | XM_14918 | ↓ | ↑ | ↓ | CTGGGCAGCGCTGTGAAAATC | GTAATGGTGTCTCCCTTGAC | 366 |

The mRNA expression levels were determined by Northern blot analyses (*) or by semi-quantitative RT-PCR (†).
N.D., not detectable; N.T., not tested; M, mouse; H, human.

growth media at 32.5°C. The fluorescent signals were quantified by ScanArray 4000 (GSI Lumonics, Moorpark, CA) and the data were analyzed by QuantArray software (GSI Lumonics).

## *Construction of Human Microdystrophin cDNA and Transfection*

Construction of the human microdystrophin cDNA containing four rod repeats and three hinges was performed based on a published report.[23] By using cDNA derived from human skeletal muscle cells (Cambrex, Baltimore, MD) as the template, two polymerase chain reactions (PCRs) were independently performed with two sets of primers, namely, HD1 (5′-CTCGAGATGCTTTGGTGGGAAGAAGT-3′) and HD2(5′-TCTTTCAAGGGTATCCACAGTAATCTGCCTCTTC-3′) orHD3 (5′-ATTACTGTGGATACCCTTGAAAGACTC-CAGGAAC-3′) and HD4 (5′-GCGGCCGCCTACATTGTGTC-CTCTCTCAT-3′). Subsequently, a mixture of the two PCR products was reamplified with the HD1 and HD4 primers to obtain a long PCR band of 4.6 kb pairs that was subsequently cloned into a pPCR-Script Amp SK(+) vector (Stratagene, La Jolla, CA). A *XhoI*/*NotI* fragment was inserted into the reverse tet-regulated retrovirus vector pLRT-X[24] (a kind gift from Dr. Hagiwara, Tokyo Medical and Dental University, Tokyo, Japan). A subclone of the *mdx*-derived muscle cell line was transfected with the pLRT-microdystrophin vector by FuGENE 6 (Roche, Mannheim, Germany) and cultured in growth medium containing blasticidin S (Wako) at 5 μg/ml for 3 weeks to establish stable transfectants.

## *Western Blot Analysis*

Total cell lysates were separated by electrophoresis on a 5 to 10% sodium dodecyl sulfate-polyacrylamide gel, transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA), and incubated with anti-dystrophin DYS2 monoclonal antibody (1:100 dilution; Novocastra, Newcastle, UK). Immunoreacting bands were visualized by using the ECL-Plus detection reagent (Amersham Pharmacia Biotech, Piscataway, NJ).

## *Northern Blot Analysis and Reverse Transcriptase (RT)-PCR*

Poly(A)$^+$ RNAs (2 μg) from various tissues of adult (12 to 16 weeks old) C57BL/6 mice were separated by electrophoresis on a 1% formaldehyde-agarose gel, transferred to a Hybond-N$^+$ nylon membrane (Amersham Pharmacia Biotech), and hybridized with [α-$^{32}$P] dCTP-labeled probes prepared from each cDNA at 42°C overnight. To verify the amount of RNA loaded in each lane, the blot was rehybridized with a β-tubulin probe. After washing under the most stringent of conditions, the membrane was subjected to autoradiography. For RT-PCR analysis, total RNAs were prepared from skeletal muscle tissues and various muscle

cell lines using TRIzol (Invitrogen). Five $\mu$g of the RNA from each sample was reverse-transcribed by using the superscript preamplification system for first strand cDNA synthesis and an oligo(dT) primer (Invitrogen). Part (1/125) of the cDNA mixture was subjected to a PCR reaction using 56°C as an annealing temperature, ExTaq DNA polymerase (Takara, Tokyo, Japan), and the specific primer sets listed in Table 1.

### *Muscle Injury Model and* in Situ *Hybridization Analysis*

A crush-injury was given by puncturing the gastrocnemius muscle of 8-week-old male C57BL/10 mice with a 23-gauge needle. At different time points (5 hours to 14 days) after the injury, the gastrocnemius muscles were isolated and frozen in liquid nitrogen for RNA extraction. For *in situ* hybridization analysis, the tibialis anterior muscle samples from C57BL/10 and *mdx* mice were dissected on the day 6 after the crush injury or the injection of 100 $\mu$l of 10 $\mu$mol/L cardiotoxin (Wako), and they were frozen in isopentane precooled in liquid nitrogen. Ten-$\mu$m cryostat longitudinal sections were prepared and fixed in 4% paraformaldehyde in PBS (pH 7.4) and treated with 1 $\mu$g/ml of proteinase K (Wako) in PBS at room temperature for 7 minutes. After being acetylated with acetic anhydride in triethanolamine, the sections were hybridized with a digoxigenin-labeled anti-sense or sense RNA probe at 65°C for 18 hours and subjected to the colorimetric detection of signals as previously described.[25]

### *Establishment of Myoblastic Cell Lines from Patient Biopsy Samples*

Skeletal muscle biopsy samples were provided from a normal donor (52 years of age), two BMD patients (1 to 6 years of age), and six DMD patients (1 to 12 years of age) after obtaining the informed consent of donors or their parents. The enzymatically dissociated human cells were subjected to primary culture in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum and 4% Ultroser G or 5% of chicken embryo extract. Fibroblastic cells were removed by a 1-hour attachment in the tissue culture plate. Remaining myoblastic cells were subcultured for 1 to 2 weeks at 37°C, and infected with the SV40 tsT retrovirus for 2 days followed by a continuous culture at 32.5°C. The amphotropic retrovirus was produced by using the PLAT-A packaging line (SM and TK, unpublished). The above-described experimental protocols were approved by the ethical committees of the institute and associated universities.

## *Results*

### *Establishment of Skeletal Muscle-Derived Cell Lines from Mdx and B10 Mice*

To assess whether the gene expression program of myogenic cells in skeletal muscle is affected by the *mdx* mutation, we immortalized the myoblastic cells isolated from the skeletal muscle of *mdx* and B10 mice. The immortalization was achieved by introducing SV40 tsT by retrovirus-mediated gene transfer into the primary myoblast culture. The newly established skeletal muscle cell lines derived from the *mdx* mice were termed mdx-sm whereas those from the B10 control mice were termed B10-sm. In the absence of viral challenge, the primary cells ceased growing after the third to fourth passage. In contrast, the mdx-sm and B10-sm cell lines have proliferated for more than a year when grown at 32.5°C. Their doubling times are comparable (data not shown). When these cells are cultured at 39.5°C, all stop proliferating and myotube formation is initiated. This indicates that the cells have been successfully immortalized in a temperature-dependent manner. In terms of their morphology, both cell lines are myoblastic in general but some of the mdx-sm cells spontaneously differentiate into myotube-like structures in the presence of horse serum even at the permissive temperature of 32.5°C. Concordantly, the frequency of desmin-positive myotubes in the mdx-sm cell line is much higher than that in the B10-sm cell line (Figure 1A). This phenotypic difference was also observed when six subclones of the mdx-sm cell line (mdx-sm1 to mdx-sm6) and two subclones of the B10-sm cell line (B10-sm1, B10-sm2) were compared. Thus, it appears that the higher differentiation capacity of mdx-sm cells is not because of the mixed cell populations in the founding lines, rather it results from the altered genetic program in the *mdx* myoblasts. For the following studies, we used the mdx-sm2 and B10-sm1 subclones as representative cell lines.

### *Identification of Differentially Expressed Genes in Mdx-sm Cells by cDNA Microarray Analysis*

DNA tip analysis has been shown to be a powerful way to compare the overall gene expression profiles of test cells with those of control cells. We used mouse cDNA microarrays holding ~4000 distinct mouse cDNA fragments (>0.5 kb) originating from a mouse fetus and an adult mouse brain. After hybridization with fluorescently labeled cDNAs from the mdx-sm and B10-sm cells, we selected those genes in which the ratio of mdx-sm fluorescence to that of B10-sm was either higher than 1.5 or lower than 0.62 when both fluorescent color combinations were used (data not shown). In this way, we identified 20 up-regulated and 21 down-regulated genes in the mdx-sm cells.

To confirm the differential expression of these candidate genes, we performed Northern blot analyses with mRNAs from mdx-sm and B10-sm cells. As shown in Figure 1B, mRNAs for schwannomin-interacting protein 1 (SCHIP-1),[26] mc7 (dystrophin-interacting protein),[27] thymosin $\beta$4 (G-actin sequestering protein),[28] and L-arginine:glycine (Arg/Gly) amidinotransferase[29] are more abundantly expressed in mdx-sm cells than in B10-sm cells, whereas the mRNA levels of GARG16 (glucocorticoid-attenuated response gene),[30] selenoprotein P (anti-

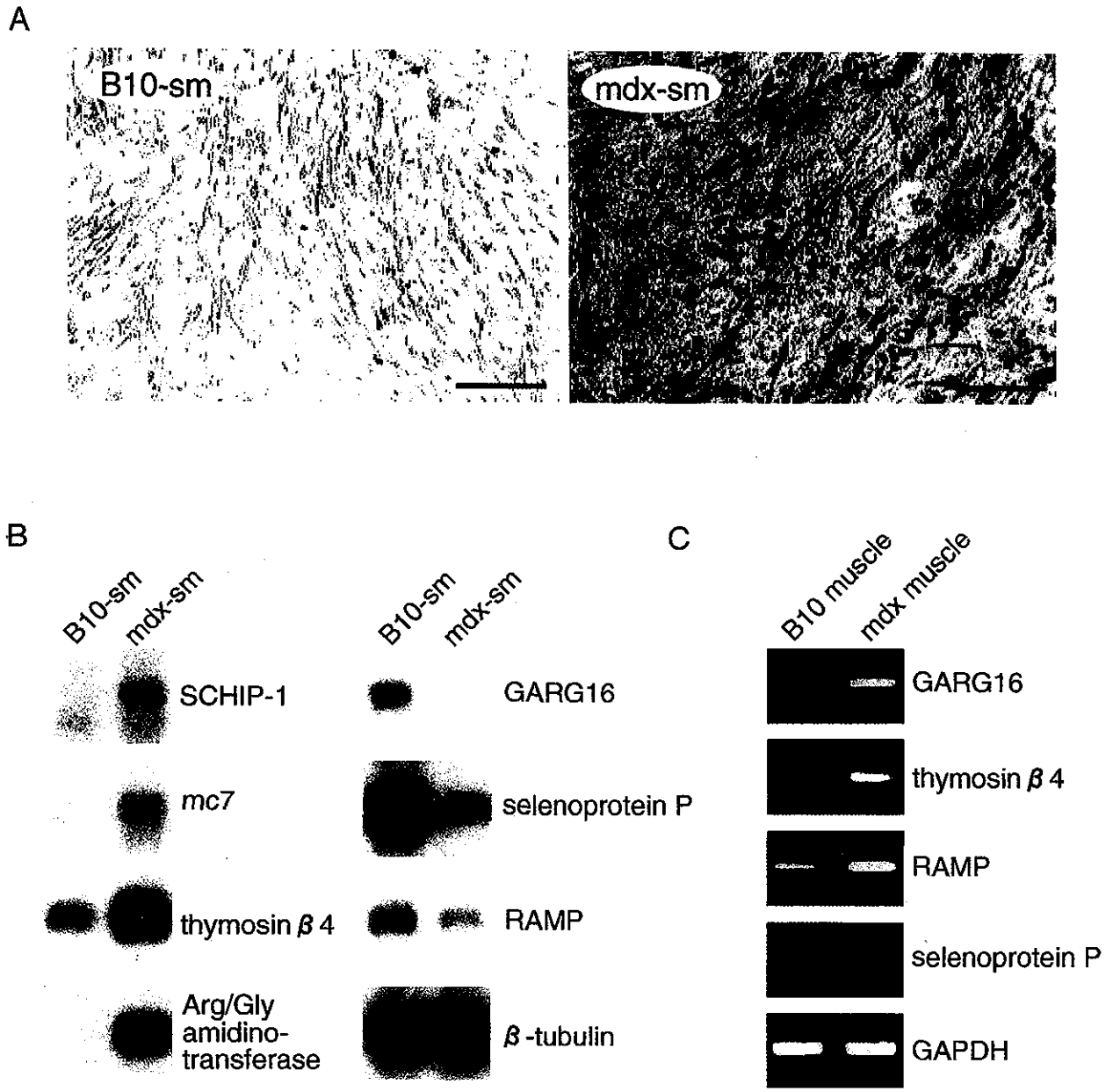


**Figure 1.** Identification of up- and down-regulated genes in the *mdx*-derived skeletal muscle cell line. **A:** Extent of spontaneous myogenic differentiation in the B10-sm and mdx-sm myoblastic cell lines derived from B10 and *mdx* mice, respectively. Cells cultured in differentiation-inducing conditions were immunostained with anti-desmin antibody. **B:** Northern blot analysis of various *mdx* up- and down-regulated genes in mdx-sm and B10-sm cell lines. The integrity and amount of loaded RNAs were assessed by probing with β-tubulin cDNA. **C:** RT-PCR analysis of various *mdx* up- and down-regulated genes in the intact skeletal muscle of B10 and *mdx* mice. Total RNAs were converted to cDNA by random hexamers and subjected to 20 cycles of amplification with specific primer sets for each gene as indicated. DNA bands after ethidium bromide staining are shown with GAPDH, which acts as the control that ensures equal amount of template cDNA were used.

-oxidant protein),[31] and EST-MNCb1423 (accession number, XM 149185; designated as RAMP in this study) are lower in mdx-sm cells. In addition, eight other genes including myogenic transcription factor MEFC[32] were found to be up-regulated in mdx-sm cells while four other genes were down-regulated (data not shown, Table 1). The expression patterns of these genes were similar in all of the mdx-sm subclones (data not shown).

Of the 19 differentially expressed genes, 15 encode proteins with known function and 4 are registered in the public database only as expressed sequenced tags (Table 1). When we examined the mRNA expression of these genes in the intact skeletal muscle of *mdx* and B10 mice by RT-PCR, five genes, including thymosin β4 and Arg/Gly amidinotransferase showed the same patterns revealed by the cell lines (Figure 1C, Table 1). In contrast, the mRNAs for GARG16, selenoprotein P, and RAMP were increased in the intact muscle of *mdx* mice (Figure 1C, Table 1). Although the exact reason for these discrepancies remains to be determined, our results demonstrate that the genetic programs of the growing myoblastic cells and the intact muscle fibers in *mdx* mice are not identical.

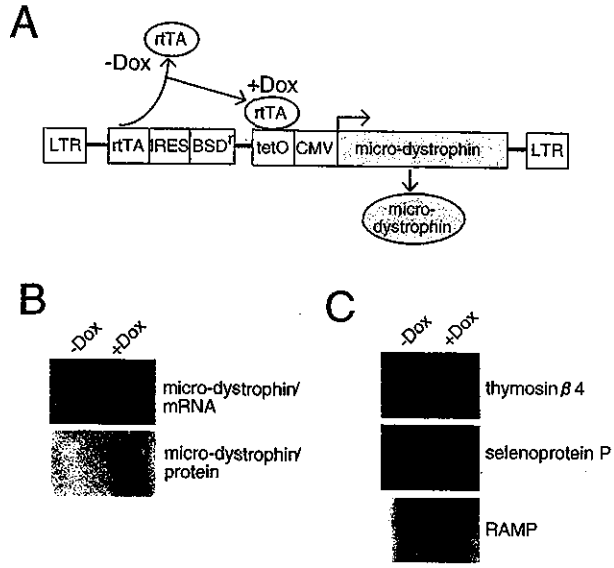


**Figure 2.** *Effect of enforced expression of microdystrophin protein in mdx-sm cell line.* **A:** Structure of the pLRT vector carrying a mouse microdystrophin gene under the Dox-inducible promoter. **B:** Dox-inducible expression of microdystrophin mRNA and protein in mdx-sm cells transfected with pLRT-microdystrophin. **C:** Effect of introducing microdystrophin into mdx-sm cells on the expression of some of the *mdx* up- and down-regulated genes. The levels of the mRNAs specific for thymosin β4, selenoprotein P, and RAMP before and after adding Dox were verified by Northern blot analyses.

## *Effect of Introducing Dystrophin on the Gene Expression in Mdx-sm Cells*

Dystrophin is known to be expressed in myotubes and mature muscle fibers but not in proliferating myoblasts. To exclude the possibility that the altered gene expression in mdx-sm cells is directly linked to the mutation in the dystrophin gene or to the lack of dystrophin mRNA or protein, we established a stable transfectant of mdx-sm cells that expresses the microdystrophin cDNA under the doxycyclin (Dox)-inducible promoter (Figure 2A). As shown in Figure 2B, 48 hours after the addition of Dox to the culture medium, microdystrophin mRNA and protein were produced in the mdx-sm transfectant cells. However, the expression levels of the genes that show disparate expression patterns in *mdx* mice, including thymosin β4, selenoprotein P, and RAMP, were not altered by the addition of Dox (Figure 2C, data not shown). These results suggest that the differential gene expression between mdx-sm and B10-sm cells is independent of dystrophin levels.

## *Primary Sequence and Expression of RAMP*

Because myoblasts play a central role in muscle regeneration, we hypothesized that the 13 differentially expressed genes in mdx-sm cells might play a role in the progression of muscle wasting seen in DMD patients and/or the milder myopathy in *mdx* mice. We especially focused on the RAMP gene because we found its mRNA expression was enhanced in regenerating muscle fibers (see Figure 4). Mouse RAMP cDNA in the database is 3085 bp in length and its predicted open reading frame codes for 720 amino acid

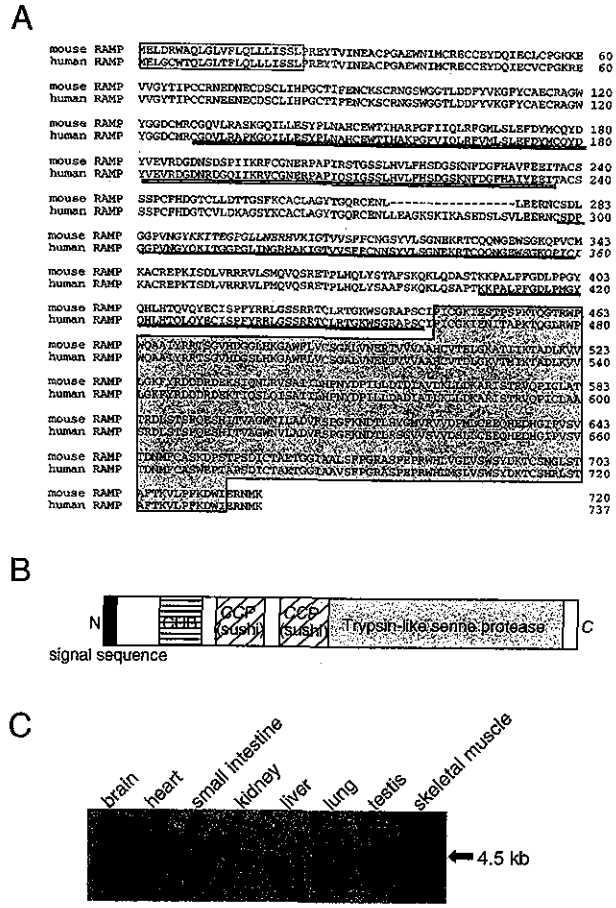


**Figure 3.** Sequence and tissue distribution of RAMP. **A:** Sequences of human and mouse RAMP proteins. Predicted signal sequences are **boxed**. CUB and CCP/Sushi domains are indicated by a **double line** and a **solid line**, respectively. The trypsin-like serine protease domain is shown by a **light gray box**. **B:** Schematic representation of the structural motifs in RAMP. **C:** Predominant expression of RAMP mRNA in skeletal muscle and brain. Two μg of poly(A)$^+$ RNA prepared from various organs of adult C57BL/6 mouse were electrophoresed, blotted, and hybridized with $^{32}$P-labeled mouse RAMP cDNA. The band size of the detected transcript is shown.

residues (Figure 3A). A BLAST search of the database revealed that mouse RAMP protein shows 88% identity at the amino acid level with the uncharacterized human protein DKFZP586H2131 (accession number, MN 015430), which we refer to as human RAMP in this study. As shown schematically in Figure 3B, mouse RAMP protein contains a putative signal peptide at its N-terminal region (1 to 22),[33] the CUB domain (122 to 236),[34] two complement control protein (CCP) modules (also known as Sushi domains) (280 to 342 and 389 to 442),[35] and a trypsin-like serine protease domain (444 to 715).[36] CUB and CCP/Sushi domains are often found in developmentally regulated proteins and cell adhesion molecules, respectively. These subdomain structures are conserved in human RAMP as well. The calculated molecular mass of mouse RAMP was 82.1 kd with an isoelectric point of 7.3. In the normal adult mouse, RAMP mRNA is only detectable in the brain and skeletal muscle as a single band at the position of 4.5 kb (Figure 3C). Thus, it appears that RAMP is a novel secreted protease that potentially plays some functions in skeletal muscle.

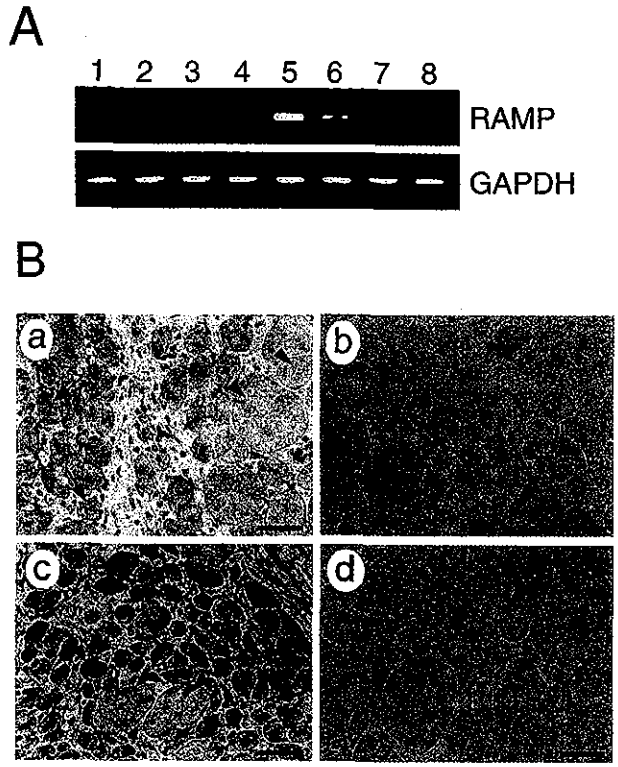


**Figure 4.** Up-regulation of RAMP mRNA expression in skeletal muscle after injury. **A:** Gastrocnemius muscle of mice was dissected 5 hours (**lane 1**) or 1 (**lane 2**), 2 (**lane 3**), 3 (**lane 4**), 4 (**lane 5**), 7 (**lane 6**), 10 (**lane 7**), or 14 (**lane 8**) days after injuring the muscle with a needle. Total RNA from each sample was extracted, reverse-transcribed, and amplified by 30 cycles of PCR using RAMP-specific primers. RT-PCR for GAPDH cDNA was performed to verify that equal amounts of template cDNA were used. **B:** Detection of RAMP mRNA in regenerating muscle fibers by *in situ* hybridization. Transverse cryosections of the tibialis anterior muscle of C57BL/10 mice harvested 6 days after crush injury were hybridized with digoxigenin-labeled anti-sense (**a**) or sense (**b**) cRNA probes for RAMP. Tibialis anterior muscle sections prepared from *mdx* mice after cardiotoxin injection were also hybridized with digoxigenin-labeled anti-sense (**c**) or sense (**d**) cRNA probes for RAMP. Specific signals for RAMP mRNA were detected as blue (**a**) or brown paints (**c**) in the centrally nucleated muscle fibers (**arrows** in **a** and **c**), but not in mature fibers (**arrowheads** in **a** and **c**). Scale bars, 50 μm.

## *Induction of RAMP mRNA in Regenerating Skeletal Muscle Fiber*

We next examined whether the expression of RAMP mRNA is changed in regenerating skeletal muscle fibers. A crush injury of gastrocnemius muscle was induced in normal mice and the expression of RAMP mRNA was measured. RAMP expression gradually increased throughout the days after the injury, reaching the highest levels 4 days after the injury, and then being reduced to the baseline within 1 to 2 weeks (Figure 4A). Consistent with the transient up-regulation of RAMP mRNA in the muscle injury model, *in situ* hybridization assays revealed that RAMP mRNA was specifically detected in centrally nucleated regenerating muscle fibers (shown by arrows), but not in unaffected fibers (arrowheads) (Figure 4B, a). In the tibialis anterior muscle of *mdx* mice after cardiotoxin injection, expression of RAMP was again specifically observed in regenerating muscle fibers (arrows) (Figure 4B, c). Thus, the RAMP gene is induced by the injury of skeletal muscle.

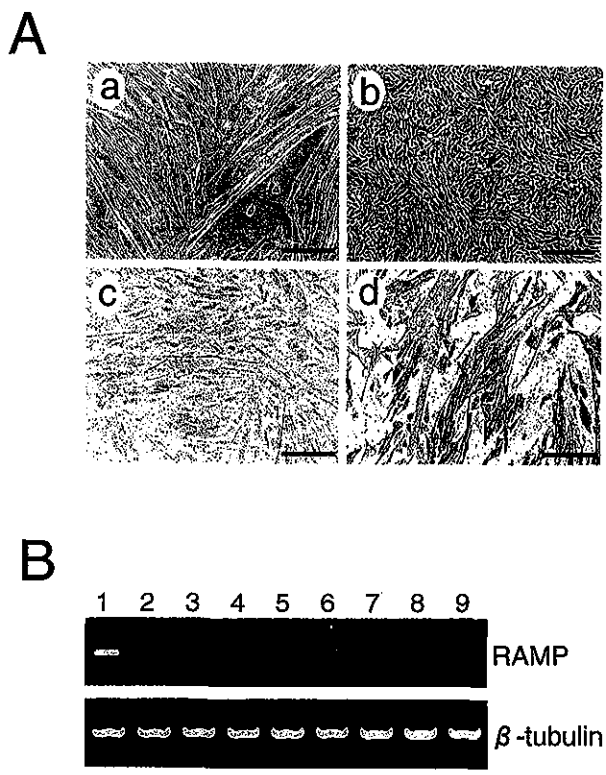


**Figure 5.** Expression of RAMP mRNA in cell lines derived from human BMD and DMD patients. **A:** Myotube differentiation capacity of BMD-sm1, a representative cell line derived from a BMD patient's skeletal muscle. The morphology is shown by phase contrast microscopy of the BMD-sm1 cell line cultured at 39.5°C (**a**) or 32.5°C (**b**). BMD-sm1 cells cultured at 39.5°C were immunostained with (**d**) or without (**c**) anti-desmin antibody. **B:** RT-PCR analysis of RAMP mRNA expression in nine human cell lines derived from skeletal muscle biopsies of patients. Total RNAs were extracted from the control (**lane 1**), two BMD (**lanes 2** and **3**), and six DMD (**lanes 4** to **9**) human muscle cell lines, reverse-transcribed, and amplified by 30 cycles of PCR using specific primers for human RAMP cDNA. Ethidium bromide-staining patterns of DNA bands are shown with β-tubulin, which shows that equal amounts of template cDNA were loaded. Scale bars, 150 μm.

## *Expression of RAMP mRNA in Skeletal Muscle Cell Lines Derived from BMD and DMD Patients*

To evaluate the importance of RAMP in DMD pathology, we established skeletal muscle cell lines derived from biopsies of an unaffected donor and BMD/DMD patients. The strategy used to achieve this was the same as that used to establish the mouse muscle cell lines except that an amphotropic retrovirus was used and the cells were exposed to the virus for a longer period. Continuously growing cells at 32.5°C were obtained from one normal, two BMD, and six DMD biopsies within 1 to 2 months after introducing SV40 tsT. In contrast, uninfected parallel control cultures exhibited senescence. Similar to the mdx-sm cell line, the human BMD/DMD-derived muscle cell lines differentiated into myotubes when cultured at 39.5°C. Figure 5 shows a representative BMD cell line, BMD-sm1, during its undifferentiated growth (b) and after it has been placed in differentiation-inducing conditions (a). Immunohistochemical analysis demonstrated that there was a high frequency of desmin-positive myotubes in the differentiated BMD-sm1 cell line (Figure 5A, d). The other eight human patient-derived cell lines proliferated similarly and

also possessed myogenic properties (data not shown), although their growth rates were very low compared to the mouse cell line. By designing specific sets of primers for human orthologs to the *mdx*-up and -down genes, we examined the mRNA expression of these genes in the nine human muscle cell lines by RT-PCR. We found that relative to the unaffected donor-derived cell line, mRNAs for Arg/Gly amidinotransferase and EST-MNCb4008 were always increased in the BMD/DMD patient-derived cell lines (data not shown, Table 1). In contrast, all of the BMD/DMD patient-derived cell lines showed lower RAMP mRNA expression levels relative to the control line (Figure 5B). That both the mdx-sm- and the BMD/DMD-derived cell lines show an attenuation of RAMP expression suggests that RAMP could be involved in the pathogenesis of DMD.

## Discussion

In this study, we identified 19 genes that are differentially expressed by newly established skeletal muscle cell lines from *mdx* and control B10 mice. We also showed that the expression of the novel gene RAMP, which is down-regulated in the *mdx* line, was frequently impaired in the muscle cell lines derived from six DMD and two BMD patients.

Gene expression studies using *mdx* mice or DMD biopsies have been published before[10–13,16,17] but unlike these studies, we used immortalized skeletal muscle cell lines as the initial materials for the cDNA microarray and Northern blot analyses. The immortalization was achieved by conditional transformation of the primary cells by infecting them with the SV40 tsT-bearing retrovirus vector. This method has been used successfully to expand target cell types before.[19] It has been reported that myogenic cell lines established by the SV40 large T antigen retain their differentiation capacity *in vitro*.[37,38] Supporting this is that the myoblastic cell lines established in this study possess myogenic differentiation activity. It is also known that rodent cells can be fully immortalized by the SV40 T antigen.[39] However, as the life span of human primary cells *in vitro* is dependent on their telomerase activity, human cells carrying the SV40 large T antigen will eventually undergo senescence and crisis because of telomere shortening. Nevertheless, our approach yielded sufficient numbers of progeny human myoblastic cells from single biopsies from DMD or BMD patients.

The skeletal muscle cell line that was established from *mdx* mice (mdx-sm) differentiated more frequently into a myotube-like structure *in vitro* than the line derived from the control C57BL/10 mice (B10-sm) (Figure 1A). That MEF2C expression in mdx-sm cells is enhanced (Table 1, data not shown) confirms that the myogenic differentiation program in these cells is engaged. This property could reflect the status of the myoblastic precursor cells in the *mdx* mouse muscle at the time of virus infection. In adult mice, skeletal muscle stem cells (satellite cells) that reside beneath the basal lamina of muscle fibers are mitotically quiescent and are only activated in response to mechanical stimuli such as exercise and injury.[40] In the process of muscle regeneration, satellite cells are believed to generate myoblasts that proliferate and differentiate to make myotubes before fusing with existing myofibers. In *mdx* mice, the destruction of muscle fibers caused by the lack of dystrophin and compensatory muscle regeneration are thought to occur continuously.[9] As is consistent with a previous report, the injury-response signaling pathway mediated by JNK1 MAP kinase may thus be activated in the majority of myoblasts in *mdx* mice.[40] All of the subclonal cell lines from the original bulk culture of mdx-sm produced desmin$^+$ myotube-like structures at a higher rate (data not shown). Because retrovirus infection occurs only in dividing cells in culture, the mdx-sm line may represent a proliferating subpopulation of myogenic precursor cells in *mdx* skeletal muscle. This may also be true for the human patient-derived muscle cell lines. Thus, the genetic programs in these cell lines are likely to represent those that are present in most, if not all, myoblasts in intact skeletal muscle.

The comparison of the mdx-sm and B10-sm cell lines revealed that 12 genes are up-regulated and 7 genes are down-regulated in the mdx-sm line (Figure 1B, Table 1). Dystrophin is expressed in muscle fibers and mature myotubes but not in proliferating myoblasts. To verify that the differential gene expression in mdx-sm cells is not because of the absence of dystrophin, we introduced a microdystrophin gene lacking the 4th to 23rd rod domains into mdx-sm cells and induced these cells to express the truncated dystrophin protein. Microdystrophin has been proven to be fully functional in restoring myopathy in *mdx* mice.[23,41] However, the presence of this protein in mdx-sm cells did not affect the expression of the 19 genes whose expression patterns are altered in mdx-sm cells (Figure 2B, data not shown). Thus, the altered gene expression pattern in mdx-sm cells is the result of a dystrophin-independent event in the myoblasts of *mdx* mice.

Among the 12 up-regulated genes in the mdx-sm cell line, Arg/Gly amidinotransferase is of particular interest because it is a first and rate-limiting enzyme for the creatine biosynthesis that is a major energy source in skeletal muscle.[42] A previous study has shown that Arg/Gly amidinotransferase mRNA is rapidly induced in kidney *in vivo* by adding creatine and growth hormone.[29] It is reasonable to suppose that skeletal muscle cells of *mdx* mice also produce this enzyme more abundantly in response to the creatine and creatine kinase that is released by disrupted muscle fibers. We found that all DMD- and BMD-derived muscle cell lines also produce a higher level of Arg/Gly amidinotransferase (Table 1, data not shown). This suggests that the measurement of mRNA for this enzyme in muscle cells as well as detection of higher creatine kinase levels in serum may be useful in the diagnosis of DMD and other myopathies.

Increased expression of thymosin $\beta$4 in the *mdx* mouse muscle has been reported in previous studies.[12] We found that its expression was up-regulated in mdx-sm cells and *mdx* muscle tissue, but not in the DMD patient-derived cell lines. Thymosin $\beta$4 is a bifunctional protein that sequesters G-actin in the cytoplasm and stimulates the migration of endothelial cells and monocytes once secreted outside of cells.[43,44] The acetylated tetrapeptide acSDKP that is proteolytically released from the N-

terminus of thymosin β4 inhibits proliferation of hematopoietic progenitor cells and enhances angiogenesis.[45] Presumably, thymosin β4 may play some roles in the regenerating muscle area by acting on inflammation-associated cells. A study is underway to determine whether higher amounts of thymosin β4 could ameliorate DMD pathogenesis.

The gene products of SCHIP-1 and mc7 are structural proteins in the muscle and brain. It has been suggested that SCHIP-1 links membrane proteins to the cytoskeleton[26] and that mc7 directly binds to the C-terminal region of dystrophin (GenBank data base), although clear evidence for these functions is not yet available. The relevance of the up-regulation of these two genes in the mdx-sm cell line remains uncertain. However, it is tempting to speculate that the expression of sarcolemmal membrane-associated proteins is induced in the absence of dystrophin by a compensatory mechanism. The expression of utrophin in *mdx* mice is the most famous example of this. Recent gene profiling studies of *mdx* mice and DMD biopsies also revealed such tendencies.[10,17]

The selenoprotein P gene is also down-regulated in the mdx-sm cell line. This down-regulation could affect the anti-oxidant defense of cells. Selenoproteins possess thioredoxin reductase activity that neutralizes the cytotoxic response of cells to the oxidant stress mediated by thioredoxin.[46] Recently, 10 families with congenital muscular dystrophy were shown to carry mutations in the selenoprotein N gene.[46] Moreover, there are reports showing that selenium deficiency is associated with muscular dystrophy in animals and cardiomyopathy in humans.[47,48] However, we found that the overall mRNA level of selenoprotein P in *mdx* mice was higher than that in control mice, as has also been shown in a previous report.[10] The presence of increased amounts of selenoprotein P as well as RAMP in adult *mdx* mice may be related to the milder skeletal myopathic phenotype observed in this model.

Of the seven down-regulated genes in mdx-sm cells, RAMP was the only one whose expression was always lower in muscle cell lines from six DMD and two BMD patients compared to the expression in a line from an unaffected control. Although the normal human myoblastic cell line used in this study was derived from a relatively aged donor, this difference appears to be significant because most of the other genes that showed differential expression in the mdx-sm line had a similar pattern of expression between the BMD/DMD patient-derived cell lines and the unaffected control cell line (Table 1). RAMP is a novel secreted protease that carries three major molecular signatures (Figure 3B). One of these is the CUB domain, which is often found in the extracellular domain of developmentally regulated proteins. Examples of these are bone morphogenic protein 1 (a metalloendopeptidase that induces cartilage and bone formation) and neuropillin (a calcium-independent cell adhesion molecule that functions during the formation of neuronal and vascular circuits). Another molecular signature is the CCP modules/Sushi domain. These exist in a wide variety of complement and adhesion proteins. The third significant molecular motif of RAMP is its serine protease domain belonging to the trypsin family at its C-terminus. In normal unchallenged mice, RAMP mRNA is only detectable in the skeletal muscle and brain. Previous studies showed that genes encoding cathepsin S and cathepsin H proteases are up-regulated in *mdx* muscle,[11,12] but RAMP-related proteases have not been described previously. The reason why the RAMP gene has not been identified in the microarray studies in the past by using *mdx* mice is probably because its expression level is low and the amount of mRNA in the intact skeletal muscle of *mdx* and normal mice do not differ significantly. A unique finding of this study is that RAMP mRNA is specifically induced in regenerating muscle fibers found after skeletal muscle injury. As described above, during muscle regeneration, myoblasts differentiate into myotubes and the cells then fuse with existing muscle fiber. Accumulating evidence from previous publications suggests that an elevated calcium concentration in regenerating muscle activates calcineurin, which dephosphorylates nuclear factors of activated T cells (NF-ATs).[49,50] Specific NF-AT isoforms in skeletal muscle play crucial roles in the myogenic differentiation and myoblast fusion events. As with the interleukin-4 induction that triggers myotube fusion in regenerating muscle areas,[51] RAMP mRNA is induced in small centrally nucleated myofibers (Figure 4B). Thus, RAMP could be an important regulator of the muscle regeneration process. Further analysis of its enzymatic targets and biological activities in skeletal muscle would shed light on this novel protease and enhance our understanding of the processes involved in muscle regeneration and the malignant progression of DMD.

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# Chimeric RNA and 2′-*O*, 4′-*C*-Ethylene-Bridged Nucleic Acids Have Stronger Activity Than Phosphorothioate Oligodeoxynucleotides in Induction of Exon 19 Skipping in Dystrophin mRNA

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## ABSTRACT

**Antisense phosphorothioate oligodeoxynucleotides against exon 19 of the dystrophin gene have been shown to induce exon 19 skipping and promote the expression of internally deleted dystrophin by correcting the translational reading frame. Because phosphorothioate oligonucleotides are associated with a variety of toxic nonantisense effects, several modifications of nucleic acid have been introduced to alleviate this toxicity. Recently, a 2′-*O*, 4′-*C*-ethylene-bridged nucleic acid (ENA™, Sankyo Lifetech Co., Ltd., Tokyo, Japan) was reported to have high affinity to complementary RNA strands and be resistant to nuclease digestion. Here, we examined the ability of this modified nucleic acid to induce exon skipping. Oligonucleotides having the same sequence as the phosphorothioate oligonucleotides but with some stretches of modified backbone (2′-O-methyl RNA with an ENA5-mer at the 5′-end and 3′-end) (RNA/ENA chimera) were transfected into myocytes, and the expressed dystrophin mRNA was analyzed. The RNA/ENA chimera induced exon 19 skipping in a dose-dependent and time-dependent manner. Remarkably, the exon 19-skipping activity of the RNA/ENA chimera was more than 40 times stronger than that of the corresponding conventional phosphorothioate oligodeoxynucleotide. This is the first report of such strong activity of an RNA/ENA chimera in the induction of exon skipping in the dystrophin gene. This new technology will allow the development of less toxic antisense drugs, making long-term therapy possible.**

## INTRODUCTION

DUCHENNE MUSCULAR DYSTROPHY (DMD), the most common hereditary muscular disease, is a rapidly progressive muscle-wasting disease characterized by the absence of dystrophin in the muscle plasma membrane. Becker muscular dystrophy (BMD), on the other hand, is a clinically less severe form of the disease that often has only slight debilitating effects. DMD and BMD are allelic diseases caused primarily by various deletion mutations in the dystrophin gene. The clinical progression of DMD or BMD can be predicted from whether the deletion disrupts (out-of-frame) or maintains (in-frame) the translational reading frame of dystrophin mRNA (Monaco et al., 1988). Even though the molecular pathogenesis of DMD/BMD is fairly well understood, no effective treatment of DMD has been developed.

We have proposed a novel strategy for the treatment of DMD: changing a DMD-causing out-of-frame mutation into an in-frame mutation characteristic of BMD by inducing exon skipping (Takeshima et al., 1995; Pramono et al., 1996). In previous studies, we demonstrated that a 31-mer 2′-*O*-methyl RNA complementary to the splicing enhancer sequence of exon 19 of the dystrophin gene

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blocked *in vitro* splicing of intron 18 (Takeshima et al., 1995). We also have shown that transfection of phosphorothioate (S-) oligodeoxynucleotides (oligo DNA) consisting of the same sequence induced skipping of dystrophin exon 19 expressed in Epstein-Barr virus-transformed lymphoblastoid cells (Pramono et al., 1996). In addition, transfection of the S-oligo DNA into DMD myocytes that contained a deletion of exon 20 of the dystrophin gene induced exon 19 skipping successfully, leading to production of an in-frame dystrophin mRNA with simultaneous deletion of exons 19 and 20. This experiment, remarkably, led to expression of dystrophin (Takeshima et al., 2001).

Using the same strategy, an oligonucleotide consisting of 2′-*O*-methyl RNA with a phosphorothioate backbone was shown to induce skipping of exon 46 from mature dystrophin mRNA (van Deutekom et al., 2001), and in a subsequent study, skipping of exons 44, 49, 50, 51, and 53 was shown to be induced successfully using antisense oligonucleotides against the respective exon sequence (Aartsma-Rus et al., 2002). In these studies, induction of exon skipping led to expression of dystrophin in their respective dystrophin-deficient myocytes by correcting the translational reading frame (van Deutekom et al., 2001; Aartsma-Rus et al., 2002). These studies have confirmed modulation of dystrophin mRNA by exon skipping as a promising strategy for the treatment of DMD.

S-oligo DNA has been the standard choice for clinical application of antisense technology. The first antisense drug was approved in 1998 for the treatment of cytomegalovirus (CMV) retinitis in patients with AIDS (Persidis, 1999). Many antisense drugs have been created for the treatment of infectious or malignant diseases (Marwick, 1998; Coudert et al., 2001; Cripps et al., 2002; Tolcher et al., 2002; Oza et al., 2003). However, S-oligo DNA is associated with a variety of potentially toxic nonantisense effects (Stahel and Zangemeister-Wittke, 2003). In order to develop a less toxic antisense oligonucleotide, nucleic acids have been modified in various ways (Freier and Altmann, 1997; Micklefield, 2001). Recently, a novel nucleotide with an ethylene bridge between 2′-*O* and 4′-*C* of ribose (2′-*O*, 4′-*C*-ethylene-bridged nucleic acid [ENA™, Sankyo Lifetech Co., Ltd., Tokyo, Japan]) chemically synthesized and has been shown to be thermodynamically stable and highly nuclease resistant (Morita et al., 2001). Furthermore, ENA has been shown to have a high binding affinity for complementary RNA strands (Morita et al., 2002, 2003).

In treatment of DMD, lifelong administration of antisense oligonucleotides is mandatory. Therefore, more potent oligonucleotides that limit the side effects associated with repeated high-dose administration need to be developed. Here, we report the first evidence that the activity of an RNA/ENA chimera to induce exon 19 skipping of the dystrophin gene is 40 times stronger than that of S-oligo DNA, making it a promising candidate for use as a low-toxicity, high-potency oligonucleotide in the long-term treatment of DMD.

## MATERIALS AND METHODS

### *Oligonucleotides*

The conventional 31-mer S-oligo DNA (5′-GCCTGAGCTGATCTGCTGGCATCTTGCAGTT-3′) complementary to the splicing enhancer sequence in exon 19 of the dystrophin gene was chemically synthesized (Hokkaido System Science Co. Ltd, Sapporo, Japan) and used as a positive control, as this has been shown to induce exon 19 skipping in cultured myocytes (Takeshima et al., 2001). A new, modified nucleic acid with a 2′-*O*, 4′-*C*-ethylene bridge (ENA) was employed to increase the affinity to the complementary RNA sequence and the resistance to nuclease digestion (Morita et al., 2003) (Fig. 1). A chimeric oligonucleotide was designed, consisting of a 21-mer 2′-*O*-methyl RNA in the center of the sequence and a 5-mer ENA at both the 5′-end and 3′-end (5′-GCCTGagcugaucugcuggcaucuugCAGTT-3′: capital and lowercase letters represent ENA and RNA, respectively). This was generated using a DNA synthesizer (Applied Biosystems, Foster City, CA) as described previously (Morita et al., 2003). As negative controls, two RNA/ENA chimeras, each consisting of a central 21-mer 2′-*O*-methyl RNA sequence flanked by 5-mer ENA at

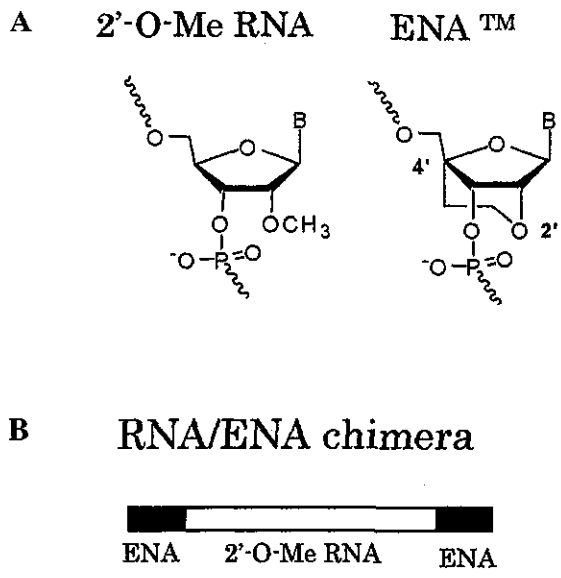


**FIG. 1.** RNA/ENA chimeras. **(A)** Structure of 2′-*O*-methyl RNA and ENA. **(B)** Scheme of RNA/ENA chimera. Black and white bars indicate stretches of ENA and 2′-*O*-methyl RNA, respectively.

the 5′-end and 3′-end, were synthesized, one with the sense strand sequence of the exon 19 splicing enhancer and the other complementary to a sequence in exon 45 (sense 19, 5′-AGATgccagcaGATC-3′; antisense 45, 5′-AATGCcauccTGGAG-3′).

*Myocyte culture*

A primary muscle cell culture was established from a muscle biopsy sample of a DMD patient with a deletion in exon 45 of the dystrophin gene after the patient gave informed consent. The muscle tissue was minced and dissociated in 5% trypsin for 30 minutes at 37°C, and isolated cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and 2% Ultroser-G (Ciphergen Biosystems, Fremont, CA). Myoblasts divided without differentiation in this medium. To induce muscle cell differentiation, medium was changed to differentiation medium, composed of DMEM supplemented with 2% horse serum (HS).

*Transfection of oligonucleotide*

On day 4 after induction of differentiation, DMD primary muscle cells were transfected with an RNA/ENA chimera. The RNA/ENA chimera was dissolved in 100 μl OptiMEM (Invitrogen, San Diego, CA) mixed with 6 μl Plus Reagent™ (Invitrogen) and incubated for 15 minutes at ambient temperature. The incubated solution was mixed with 8 μl Lipofectamine (Invitrogen) dissolved in 100 μl OptiMEM and incubated for 15 minutes. Then, the mixture was added to culture medium (800 μl OptiMEN) to a final RNA/ENA chimera concentration of 200 pmol/ml (200 nM). After 3 hours of incubation, HS was added to a 6% final concentration, and

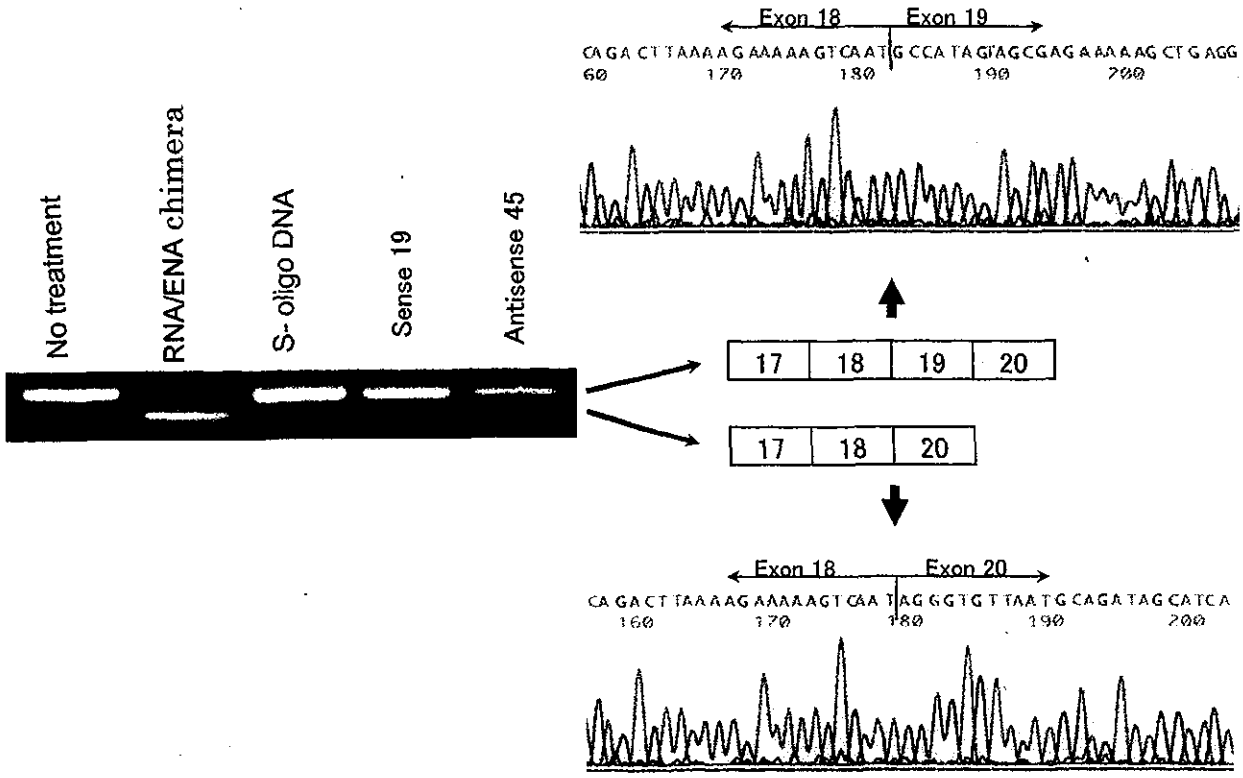


**FIG. 2.** Analysis of dystrophin mRNA. The ability of different oligonucleotides to induce exon 19 skipping was compared. DMD myocytes were incubated for 2 days at a concentration of 200 nM of each oligonucleotide separately, and the resulting dystrophin mRNA was analyzed. On amplification of the region spanning exons 17–20 of the dystrophin gene, two bands were visualized from the RNA/ENA chimera-treated myocytes. Sequence analysis of amplified products disclosed that the larger band corresponded to the normal, full-length fragment and the smaller band to the exon 19-skipped fragment. The exon structure of the amplified product is shown schematically at right, and the junction sequence of exon 18 is shown over or under the schematic. In contrast, only normal product was obtained when the cells were not treated. When they were treated with S-oligo DNA, a very faint band of exon 19-skipped fragment was obtained in addition to the strong normal-sized product. In order to examine the specificity of the RNA/ENA chimera, two other compounds (sense 19 and antisense 45) were synthesized and introduced into myocytes. In both treatments, the smaller product lacking exon 19 was not produced in myocytes, indicating the specificity of the experimental RNA/ENA chimera.

the incubation was continued for 2 days, at which point the myocytes were harvested and RNA was extracted. In specified experiments, the concentration of RNA/ENA chimera and the incubation period varied.

### *mRNA analysis*

A fragment spanning exons 17–20 of dystrophin mRNA was analyzed. RNA was isolated from the cultured myocytes, and cDNA was prepared from 2 $\mu$g total RNA as described previously (Matsuo et al., 1991). PCR amplification of cDNA spanning exons 17–20 was performed using the following primers: forward, 5′-GCA TGC TCA AGA GGA ACT TCC-3′; reverse, 5′-TAG CAA CTG GCA GAA TTC GAT-3′. To analyze the full-length dystrophin mRNA, 10 partially overlapping fragments spanning the entire coding region of the dystrophin mRNA were amplified as described previously (Roberts et al., 1991).

### *DNA sequencing*

The PCR-amplified products were subcloned into the pT7 blue T vector (Novagen, Madison, WI) and sequenced using a Taq dye termination cycle sequence kit (Perkin-Elmer Applied Biosystems, Norwalk, CT) with an automatic DNA sequencer (model ABI Prism 310 Genetic Analyzer) (Perkin-Elmer Applied Biosystems), as described previously (Surono et al., 1999).

## RESULTS

### *Exon skipping by RNA/ENA chimera*

The ability of the RNA/ENA chimera to induce skipping of exon 19 of the dystrophin gene was examined. Synthesized RNA/ENA chimera was added at 200 nM to the culture medium of myocytes, and 2 days later, dystrophin mRNA expressed in DMD myocytes was analyzed. Reverse transcriptase PCR (RT-PCR) amplification of the region spanning exons 17–20 revealed two product bands consisting of a weak, large one and a strong, small one (Fig. 2). Sequence analysis of the smaller product disclosed that the 3′-end of exon 18 was directly joined to the 5′-end of exon 20, indicating that exon 19 had been skipped, whereas the large band represented exons 17, 18, 19, and 20 in their entirety. This result shows that the RNA/ENA chimera has the ability to induce exon 19 skipping in more than half of the dystrophin mRNA. In contrast, treatment with the conventional S-oligo DNA (200 nM) caused faint visualization of the smaller band (Fig. 2), indicating only weak exon 19-skipping activity.

### *Specificity of RNA/ENA chimera*

To study the effect of the applied RNA/ENA chimera on the splicing of other exons in dystrophin mRNA, we amplified 10 different fragments from the 14-kb full-length dystrophin cDNA. All 10 fragments except for the 1 encompassing exon 19 were amplified and gave normal-sized bands, indicating that our chimeric oligonucleotide did not induce the skipping of any other exons (data not shown). To analyze further the specificity of the construct, we generated two other RNA/ENA chimeras with different sequences. One had exactly the same sequence as the sense strand of the splicing enhancer of dystrophin exon 19 (sense 19), and the other was complementary to a sequence within exon 45 (antisense 45). Neither of these oligos induced skipping of exon 19 (Fig. 2). Therefore, it was concluded that the original RNA/ENA chimera specifically induced exon 19 skipping.

### *Time dependency of exon skipping*

Incubation of the cultured myocytes for 2 days with the RNA/ENA chimera was sufficient to induce exon 19 skipping in the majority of the dystrophin gene transcripts (Fig. 2). In order to study more closely the time course of this process, however, we varied the incubation time from 0 hour to 14 days. Before and 1 hour after the transfection, dystrophin mRNA analysis disclosed only

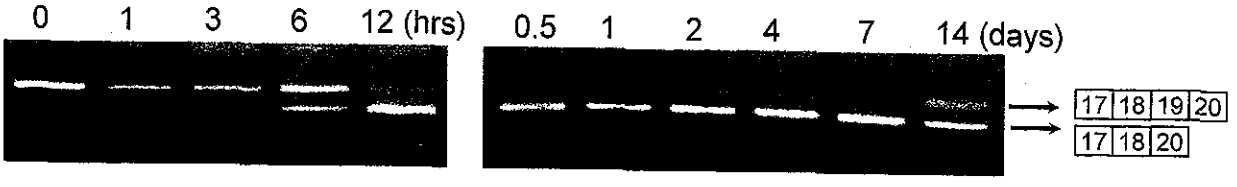


**FIG. 3.** Time course of exon 19 skipping. RT-PCR products of the region spanning exons 17–20 of the dystrophin genes are shown. Before and 1 hour after transfection, a single amplified product corresponding to the normal one was obtained. The smaller, exon 19-skipped fragment appeared 3 hours after transfection. Lengthening the incubation time increased the density of the smaller product. The relative ratio of the smaller product to the normal one reached a peak at 12 hours and stayed at a plateau until day 7. The band corresponding to the exon 19-skipped product was still present as a major band on day 14 after transfection. The exon structure of the amplified product is represented schematically at right, and the numbers at the top represent the hour and day after transfection with the RNA/ENA chimera.

one normal-sized product. Remarkably, the smaller band, representative of exon 19 skipping, appeared at 3 hours, in addition to the normal band (Fig. 3). By lengthening the incubation time, the smaller band became more intense, indicating increased production of exon 19-skipped dystrophin mRNA. The relative ratio of the smaller band to the normal one reached its peak at 12 hours and stayed at a plateau until day 7. Even on day 14, the band representing the exon 19-skipped transcript was more intense than that corresponding to the full-length product (Fig. 3). These results indicate that the effect of the RNA/ENA chimera was dependent on the period of incubation and persisted for at least 14 days.

*Dose dependency of exon skipping*

To estimate the effective dose of the RNA/ENA chimera, different amounts of it were added to the culture medium, ranging from 7 to 600 nM. After 2 days of incubation at each concentration, dystrophin mRNA production was analyzed. Without treatment with the RNA/ENA chimera, a single band corresponding to the full-length fragment was visualized (Fig. 4A). Two amplified bands, one corresponding to the full-length fragment and the other representing skipping of exon 19, were obtained at a dose of 22 nM of the RNA/ENA chimera. This latter product was also observed at higher concentrations of the

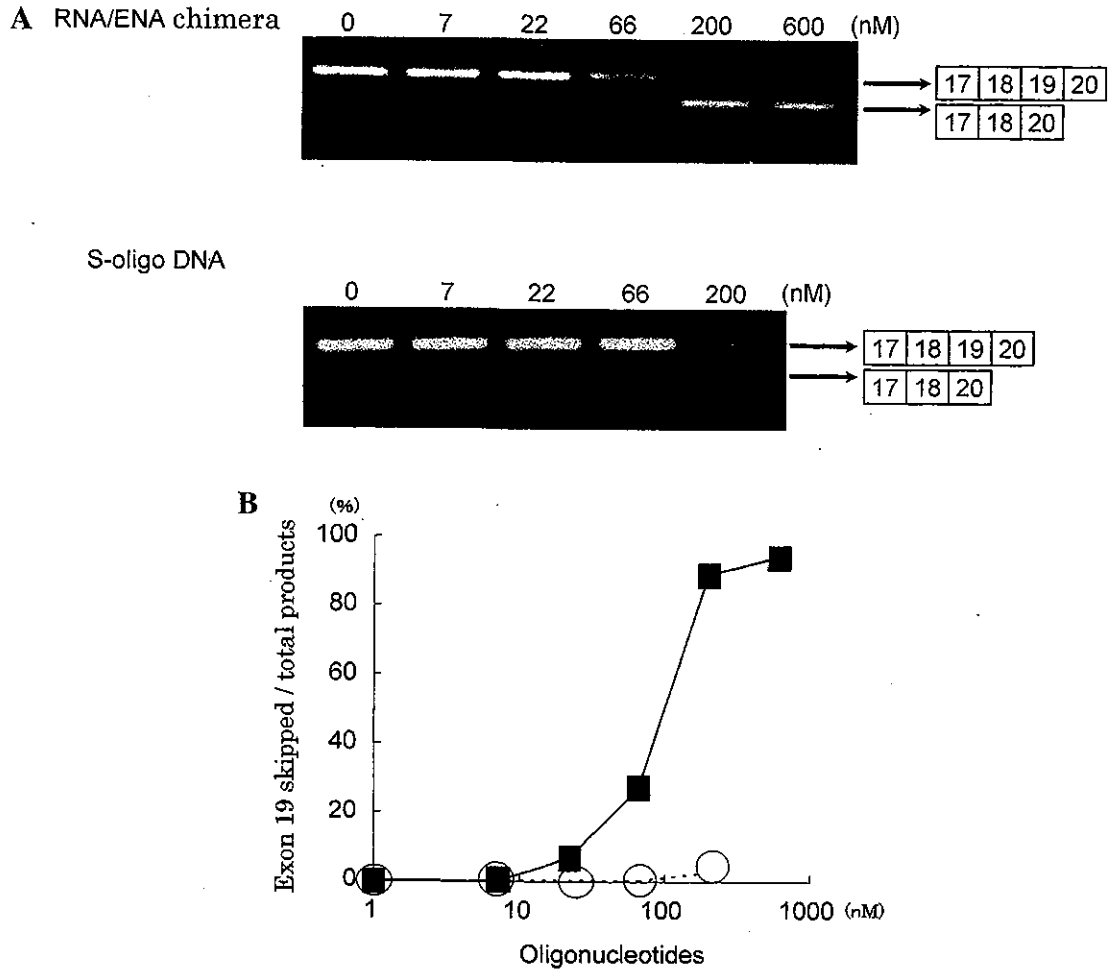


**FIG. 4.** Dose dependency of exon 19 skipping. **(A)** RFPCR product. Different amounts (0, 7, 22, 66, 200, and 600 nM) of RNA/ENA chimera were added to the culture medium. Only one product, corresponding to the normal, full-length cDNA, was amplified on treatment with 0 or 7 nM of the RNA/ENA chimera. The smaller product, lacking exon 19, was observed after treatment with 22, 66, 200, and 600 nM of the RNA/ENA chimera. In the case of S-oligo DNA treatment, on the other hand, only the 200 nM dose gave rise to this smaller product. The exon structure of the amplified product is shown schematically at right, and the numbers at the top represent the oligonucleotide concentrations (in nM). **(B)** Ratio of exon 19-skipped product to the total product. The ratio of skipped product to total product was calculated from the intensities of the product bands and plotted against the concentration of either the RNA/ENA chimera (squares) or S-oligo DNA (circles). The RNA/ENA chimera has more than 40 times stronger activity than the S-oligo DNA.

RNA/ENA chimera (66, 200, and 600nM) (Fig. 4A). The intensity of the small band increased with increasing concentrations of the RNA/ENA chimera, reaching a virtual plateau at 200 nM, indicating the dose dependency of exon 19 skipping. In contrast, the exon-skipped product was not visible even at a dose of 66 nM of the S-oligo DNA, becoming barely visible only at 200 nM (Fig. 4A). Remarkably, no RT-PCR product was obtained from material prepared from myocytes treated with 600 nM S-oligo DNA, demonstrating the toxicity of the S-oligo DNAs. The ratio of the exon 19-skipped product to the total product was calculated by measuring the intensities of the two product bands (Fig. 4B). It was found that >90% of the dystrophin mRNA had skipping of exon 19 at 200 nM or higher concentrations of the RNA/ENA chimera. This activity was calculated to be 40 times stronger than that of the S-oligo DNA.

These results indicate that this novel RNA/ENA chimera has the ability to induce exon 19 skipping and that this activity is more than 40 times stronger than that of conventional S-oligo DNA.

## DISCUSSION

Antisense oligonucleotides have been attracting much attention as a new tool for the treatment of DMD (Takeshima et al., 1995; Pramono et al., 1996; van Deutekom et al., 2001; Aartsma-Rus et al., 2002). We have shown that S-oligo DNA induces exon 19 skipping and leads to increased expression of dystrophin (Takeshima et al., 2001). In this report, we have shown for the first time that a chimeric RNA/ENA antisense oligonucleotide has 40-fold higher activity in inducing exon 19 skipping compared with the conventional S-oligo DNA (Fig. 4).

As S-oligo DNA possesses increased resistance to nucleases, it is commonly used clinically, such as in the treatment of malignant or infectious diseases (Marwick, 1998; Persidis, 1999; Cripps et al., 2002; Stahel and Zangemeister-Wittke, 2003). However, S-oligo DNA exhibits several disadvantages, including relatively poor binding to complementary nucleic acids and significant nonspecific binding to proteins (Guvakova et al., 1995; Levin, 1999), causing toxic side effects that limit its clinical application (Levin, 1999). Because DMD requires long-term therapy, a less toxic agent needs to be developed.

In addition to nucleotides with a phosphorothioate backbone, several modified nucleic acids have been created (Freier and Altmann, 1997; Micklefield, 2001), the most recent of which is ENA. The introduction of ENA into an oligonucleotide raises the $T_m$ by 5.2°C of the hybrid formed with a complementary DNA or RNA. Indeed, ENA has been shown to possess a high binding affinity for complementary RNA strands and to be more nuclease resistant than natural DNA and other modified nucleic acids (Morita et al., 2002).

Our data suggest that the use of ENA/RNA hybrid oligonucleotides can reduce the effective dosage in antisense therapy by at least 40 times relative to S-oligo DNA (Fig. 4B). We also expect that these RNA/ENA chimeras will exhibit substantially less toxicity at therapeutic doses. One of the most sought after properties in oligonucleotides used clinically is their stability in biologic media. Current protocols using S-oligo DNA in humans require drug administration more than once a week because of its relatively short lifetime. The RNA/ENA chimera examined in this study, on the other hand, showed activity for up to 14 days of incubation, the longest time point tested (Fig. 3), indicating that its therapeutic administration would be necessary at most only once every 2 weeks. For such conditions as DMD that require long-term treatment, the long therapeutic life-span of this novel oligonucleotide and the lower dosage frequency it allows make it a very attractive option.

The efficacy and safety of antisense oligonucleotides as a therapeutic tool critically depend on the specificity of their binding to their targets. In this study, RNA/ENA chimeras with sequences corresponding to the sense strand of the exon 19-splicing enhancer or an antisense strand complementary to exon 45 of the dystrophin gene did not induce skipping of exon 19 (Fig. 2). In addition, the antisense exon 19 RNA/ENA chimera that we used to skip exon 19 did not affect the splicing of other exons in the dystrophin gene. These results indicate that the RNA/ENA chimera acts in a sequence-specific manner and could be used as an effective and safe antisense agent.

The RNA/ENA chimera used in this study consisted of a 21-mer 2′-*O*-methyl RNA flanked by 5-mer ENAs at the 5′-end and 3′-end. This chimera was the most effective inducer of exon 19 skipping among several chimeric compounds consisting of 2′-*O*-methyl RNAs and ENAs that were tested (data not shown), confirming a previous report that ENA modification at both ends of a standard oligonucleotide makes the final construct very stable (Morita et al., 2003).

In previous studies, antisense oligonucleotides have been used to repress gene function by destabilizing mRNA with RNase H digestion (Stahel and Zangemeister-Wittke, 2003). In this study, antisense oligonucleotides were employed to block the binding of nuclear protein to the splicing enhancer sequence (Ito et al., 2001; Takeshima et al., 2001). The binding of the RNA/ENA chimera to the splicing enhancer sequence is competitive with that of nuclear protein (Blencowe, 2000). Considering that ENA has high binding affinity to the RNA sequence (Morita et al., 2001), it is well understood that the RNA/ENA chimera showed strong activity to induce exon skipping.

In conclusion, our RNA/ENA chimera is a much stronger inducer of exon 19 skipping than is the conventional S-oligo DNA. Our results suggest a novel method to develop effective antisense drugs. Further experiments to extend the application of chimeric RNA/ENA in skipping of dystrophin exons are in progress.

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