CONCLUSION

Duchenne muscular dystrophy is a fatal disease without any effective treatment. Recent studies opened a door to the establishment of molecular therapy for DMD.

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PEDIATRIC RESEARCH

Vol. 56, No. 5, 2004
Printed in U.S.A.

C-Terminal Truncated Dystrophin Identified in Skeletal Muscle of an Asymptomatic Boy with a Novel Nonsense Mutation of the Dystrophin Gene

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ABSTRACT

Mutations that cause premature stop codons in the dystrophin gene lead to a complete loss of dystrophin from skeletal muscle, resulting in severe Duchenne muscular dystrophy. Here, a C-terminally truncated dystrophin resulting from a novel nonsense mutation is shown for the first time to be localized to the muscle plasma membrane. An asymptomatic 8-y-old boy was examined for dystrophin in skeletal muscle because of high serum creatine kinase activity. Remarkably, no dystrophin labeling was seen with an MAb against the C-terminal domain, suggesting the presence of an early stop codon in the dystrophin gene. Labeling with an antibody specific to the N-terminal domain, however, revealed weak, patchy, and discontinuous staining, suggesting limited production of a truncated form of the protein. Molecular analysis revealed a novel nonsense mutation (Q3625X) as a result of a single nucleotide change in the patient's genomic DNA (C11081T), leaving 1.6% of dystrophin gene product unsynthesized at the C terminus. Dystrophin mRNA analysis did not show rescue of the nonsense mutation as a result of exon-skipping by an alternative splicing mechanism. This is the first report of an asymptomatic dystrophinopathy with a nonsense mutation in the dystrophin gene. (*Pediatr Res* 56: 1–5, 2004)

Abbreviations

BMD, Becker muscular dystrophy
CK, creatine kinase
DMD, Duchenne muscular dystrophy

Fnu

The severe Duchenne muscular dystrophy (DMD) and the more benign Becker muscular dystrophy (BMD) are allelic conditions characterized by progressive muscular degeneration and wasting accompanied by an elevation of serum creatine kinase (CK). DMD is a rapidly progressive disease, with those affected starting to show muscle weakness at ~4–5 y of age and losing the ability to walk independently before the age of 12 y. BMD has a slower rate of progression; affected individuals remain ambulatory beyond the age of 16 y, and a few may lead near-normal lives (1).

DMD and BMD are caused by mutation of the dystrophin gene, which encodes a 14-kb mRNA that consists of 79 exons. The gene is the largest in humans and covers >3000 kb on the X chromosome (2,3). DMD and BMD are the most common genetic muscle diseases, affecting >1 in 3,500 male births. Two thirds of DMD/BMD patients have deletion or duplication mutations of the dystrophin gene, and their clinical progression can be predicted by whether the deletion or duplication maintains (in-frame) or disrupts (out-of-frame) the translational reading frame (the reading-frame rule) (4). Dystrophin is absent from skeletal muscle of DMD, because the dystrophin that is produced is truncated as a result of the premature stop codon and therefore is unstable, whereas in BMD, dystrophin that contains internal in-frame deletions produces protein that can be detected (5).

Single-base nonsense mutations have been suspected in DMD patients who do not show deletion/duplication mutations. However, detection of such defects in individual DMD patients is very difficult as a result of the large size of the gene. More than 100 nonsense mutations have been reported at various points over a 14-kb length of the dystrophin mRNA (http://www.dmd.nl). Despite this wide variation in coding potential (0–98.6% of the full-length protein), these truncating mutations are associated with a surprisingly uniform severity of the DMD phenotype (6). However, a limited number of single-base nonsense mutations have been reported in patients with mild BMD that showed skipping of the exon encoding the mutation, thus producing an in-frame mRNA (7–11).

Dystrophin is a cytoskeletal protein that is implicated in membrane stability and in communication between the extra-

Received June 11, 2003; accepted November 14, 2003

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This work was supported by grants from the Ministry of Education, Science and Culture of Japan and a Research Grant for Nervous and Mental Disorders from the Ministry of Health and Welfare of Japan.

DOI: 10.1203/01.PDR.0000142734.46609.43

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cellular matrix and the inner cytoskeleton (12,13). The protein, which consists of 3685 amino acids, is divided into four distinct domains: an N-terminal domain, a large rod-like domain of 24 spectrin-like repeats that occupies >70% of its length, a cysteine-rich domain, and, finally, a C-terminal domain (2,14). Studies conducted on DMD/BMD patients suggest that the N-terminal, cysteine-rich, and C-terminal domains are essential for dystrophin's function (15,16). Notably, the C-terminal domain, which consists of 416 amino acids encoded by 13 exons, shows sequence similarity with only two other dystrophin-related proteins and is considered to exert dystrophin's specific function (14,17,18). In fact, in-frame deletions that extend into the C-terminal domain have been reported to result in DMD, whereas large in-frame deletions of the rod domain result in BMD (16).

Here we report a C-terminally truncated dystrophin caused by a mutation in an asymptomatic boy with high CK activity. We propose that nonsense mutations of the dystrophin gene can result in a wide variety of clinical phenotypes.

METHODS

Case. The proband (KUDN 02765682) was an 8-y-old boy. His family history disclosed no neuromuscular disease. He started to walk independently at 1 y of age, and his motor development was normal. He had a history of transient muscle weakness. At the age of 3 y, he complained of pain in the lower legs without any predisposing signs or symptoms and lost the ability to stand up and walk by himself. His serum CK was found to be 4901 IU/L (normal <169 IU/L). The muscle weakness persisted for 1 wk but disappeared spontaneously and completely.

During the following period, his serum CK remained elevated but showed a strong fluctuation in value, ranging from 1,607 to 21,100 IU/L. Despite his high CK, he did not show any muscle weakness. At the age of 5 y, he was referred to Kobe University Hospital for examination of his elevated serum CK activity. His mental development was normal. On physical examination, there was no Gower's sign, walking abnormality, or pseudohypertrophy of the legs. An electromyogram disclosed myogenic changes. A chest x-ray, electrocardiography, and echocardiography failed to reveal cardiac abnormalities. To clarify the cause of the elevation in serum CK and myogenic pattern in electromyogram, a quadriceps muscle biopsy was carried out after obtaining informed consent. The protocols of this study were approved by our ethical committee.

Immunohistochemical analysis. The muscle biopsy sample was examined pathologically and immunohistochemically. An indirect immunofluorescence analysis was performed using three dystrophin antibodies that recognize the N-terminal (NCL-Dys3), the rod (NCL-Dys1), and the C-terminal (NCL-Dys2) domains of dystrophin (Novocastra Laboratories, Newcastle upon Tyne, UK) (5,19). Furthermore, utrophin, β-dystroglycan, γ-sarcoglycan (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK), laminin α2 (Chemicon International Inc., Temecula, CA), and α-dystroglycan (Upstate Biotechnology, Lake Placid, NY) were also stained using their respective antibodies. Control skeletal muscle tissue was obtained with informed consent and was simultaneously stained with the same panel of antibodies. Western blot analysis of dystrophin using an MAb that recognizes the C-terminal domain was performed by Athena Diagnostics (Worcester, MA).

Analysis of the dystrophin gene. For mutational analysis of the dystrophin gene, blood samples were obtained from the index case and family members after obtaining informed consent. DNA was isolated by standard phenol-chloroform extraction methods. For screening for deletion mutations, 19 deletion-prone exons were amplified from the genomic DNA by PCR essentially according to methods described previously (20). Southern blot analysis using dystrophin cDNA as a probe was performed with *Hin*dIII restriction enzyme–digested DNA as a template, as described by Koenig *et al.* (21). For analyzing genomic mutations, the region that encompasses exon 76 was amplified by PCR with g76F:5'-GGAGGGCTTCTAAAGTAGG-3' as the forward primer and g76r:5'-ATGTCCCTGTAATACGACTCTACC-3' as the reverse primer under conditions described elsewhere (20).

Analysis of dystrophin mRNA. Reverse-transcription PCR (RT-PCR) was used to analyze the dystrophin mRNA expressed in lymphocytes or skeletal muscle as described by Roberts *et al.* (22,23). Full-length dystrophin cDNA was amplified as 10 separate, partially overlapping fragments and sequenced directly. For obtaining a fragment showing aberrant splicing, including exon 76 skipping, a region that encompasses exons 70–79 was amplified using a forward primer corresponding to a segment of exon 70 (70f:5'-CAGGAGAAGATGTTCGAGAC-3') and a reverse primer complementary to a segment of exon 79 (5f:5'-ATCATCTGCCATGTGGAAAAG-3').

Sequencing of the amplified product. The amplified product was purified and subjected to sequencing either directly or after subcloning into a pT7 blue T vector (Novagen, Madison, WI) (24). The DNA sequence was determined using an automated DNA sequencer (model 373A; Applied Biosystems, Foster City, CA).

RESULTS

For elucidating the cause of the elevation in serum CK, the biopsied muscle sample was examined pathologically. Microscopic examination disclosed slight dystrophic changes such as size variation in muscle fibers, fibers with central nuclei, and degenerated and regenerated fibers. Immunofluorescence staining for dystrophin revealed a complete absence of C-terminal domain labeling (Fig. 1). In contrast, both N-terminal and rod-domain staining was weak, patchy, and discontinuous along the plasma membrane (Fig. 1). These findings clearly indicated dystrophinopathy, but the patterns of dystrophin staining were not typical for either DMD or BMD. Western blot analysis of dystrophin using an antibody that recognizes the C-terminal domain of dystrophin revealed no significant bands (data not shown). These staining patterns indicate that a nonsense mutation in dystrophin is present in this patient, leading to production of a protein truncated somewhere upstream of the C-terminal epitope recognized by the aforementioned antibody. F1

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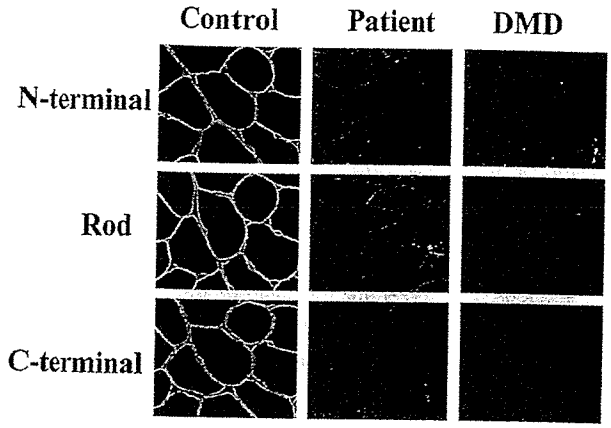

Figure 1. Immunofluorescence staining of biopsied muscle for dystrophin. The biopsied muscle was stained for dystrophin using antibodies against the N-terminal, rod, and C-terminal domains. Staining of N-terminal and rod domains was weak, patchy, and discontinuous. In contrast, no reactive material was visualized for the C-terminal domain (patient). In unaffected individuals, all domains were clearly stained along the plasma membrane (control) but not in DMD.

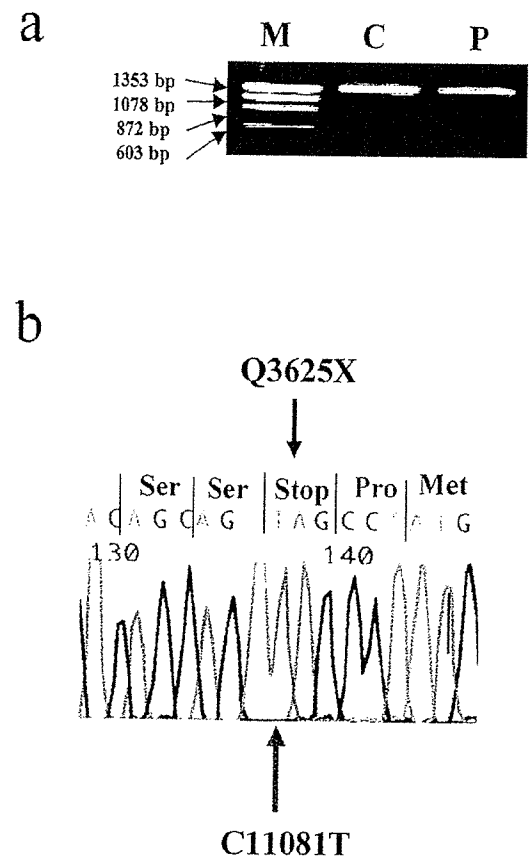

Figure 2. Analysis of dystrophin mRNA prepared from muscle. (*A*) The amplified product encompassing exons 70–79 is shown. One clearly visible product was obtained from the index case (P), and the size of the amplified product is the same as that of the control (C). M refers to a DNA size marker, *Hae*III-digested ϕX174 DNA. (*B*) Nucleotide sequence around the mutation site. Direct sequencing of the amplified product disclosed a single nucleotide change from C to T at nucleotide 11081 (C11081T). This nucleotide change converted a CAG codon to a TAG stop codon (Q3625X). Nucleotide and amino acid numbering are based on those presented by Koenig *et al.* (14)

For clarifying the molecular pathogenesis of the abnormal dystrophin, the dystrophin gene was scanned for mutations. Neither PCR amplification of 19 selected exons nor Southern blot analysis of the dystrophin gene revealed any gross gene rearrangements. The possibility of deletion mutation therefore seemed unlikely. To find a single-base mutation, we analyzed dystrophin mRNA extracted from peripheral lymphocytes using RT-PCR as described previously (22). Ten fragments covering the full-length dystrophin cDNA could be amplified as normal-sized products. Direct sequencing of a fragment that encompasses exons 70–79 disclosed a single nucleotide change: a transition from a cytosine to a thymine at nucleotide 11081 (C11081T) in exon 76 (14). The same nucleotide change (C11081T) was present not only in his muscle dystrophin mRNA (Fig. 2) but also in his genomic DNA (data not shown) (25). His mother was found to be a carrier of the same mutation (data not shown). Because sequencing of other fragments of dystrophin cDNA disclosed no other significant nucleotide changes, it was concluded that this mutation (C11081T) is the cause of the dystrophinopathy. The nucleotide change converted a CAG codon, which encodes glutamine at the 3625th amino acid position, to a stop TAG codon (Q3625X; Fig. 2). Therefore, a truncated dystrophin lacking 60 amino acids at its C terminus (1.6% of the total dystrophin sequence) was expected to be produced.

This truncation of dystrophin is compatible with the failure of the C-terminus–specific antibody to label the protein (Fig. 1), because this antibody recognizes amino acids 3669–3685, an epitope that is downstream of the premature stop codon (Q3625X). However, that dystrophin did stain positively with antibodies against its N-terminal and rod domains (Fig. 1) does not seem consistent with this truncation mutant, because other truncated dystrophin mutants have been found to be very unstable and undetectable immunohistochemically (4,6). In other cases, it has been hypothesized that the positive staining of dystrophin is the result of rescue of nonsense mutations by exon skipping or aberrant splicing (8,26). However, the RT-PCR–amplified product encompassing exons 70–79 disclosed only one visible band upon agarose gel electrophoresis (Fig. 2). In addition, both direct sequencing and sequencing after subcloning the product confirmed the presence of normal exon structure, indicating that only one mRNA was produced from the mutated gene. These observations do not support the possibility of exon-76 skipping or aberrant splicing.

Although the patient in our case harbored a Q3625X nonsense mutation, his clinical phenotype was unusually mild. Utrophin, a dystrophin-related protein, has been proposed to compensate for the function of dystrophin (27). Therefore, overexpression of utrophin might account for the clinical phenotype seen in the present case. The expression of utrophin was studied in muscle (Fig. 3) and was not found to be elevated in the index case in comparison with that typically seen in DMD. Therefore, enhanced expression of utrophin does not see, to be modifying the clinical phenotype. Furthermore, the dystrophin-dystroglycan axis was examined (Fig. 3). Laminin α2, an extracellular matrix protein, stained weakly. Neither α-dystro-

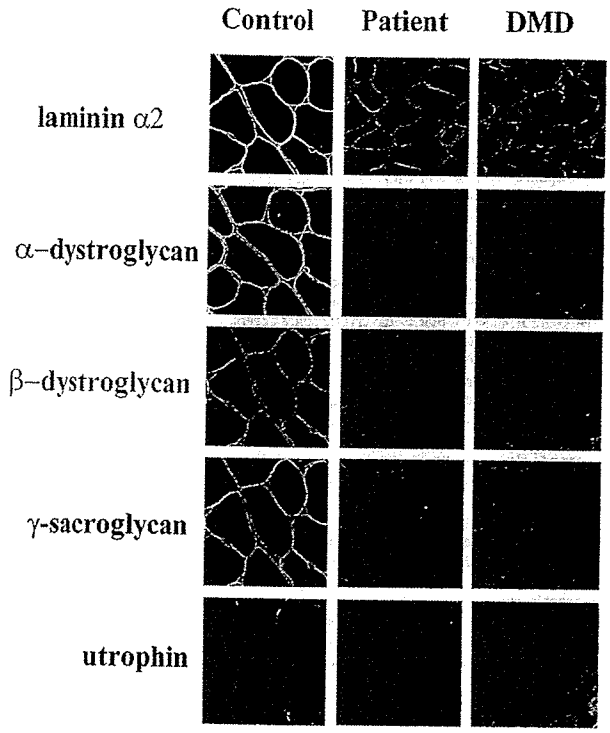

Figure 3. Immunofluorescence staining for dystrophin-associated and dystrophin-related proteins. The dystrophin-associated proteins α- and β-dystroglycan, γ-sarcoglycan, and laminin α2 were labeled with immunofluorescence. The staining patterns seen in the patient's muscle were similar to those observed in DMD. Also as in DMD, no labeling was seen for utrophin, a dystrophin-related protein.

glycan, an extracellular β-dystroglycan–binding protein, nor β-dystroglycan, a transmembrane dystrophin-binding protein, was stained. γ-Sarcoglycan, a member of the sarcoglycan complex, was stained very weakly. All of these staining patterns were similar to those found in DMD (Fig. 3), indicating no difference in the stabilization of the dystrophin-dystroglycan axis from that observed in DMD (Fig. 3). Therefore, no explanation for the mild phenotype was obtained through studies of protein staining

DISCUSSION

A novel nonsense mutation (Q3625X) in the dystrophin gene was identified in a Japanese boy who was as yet asymptomatic at the age of 8 y. Although a severe DMD phenotype would be expected to develop from his mutation type, his clinical course has been extraordinarily mild. The case has raised an important question to be answered: What is the mechanism that determines the severity of the dystrophic phenotype?

A somatic mosaic for a nonsense mutation has been shown to attenuate the clinical phenotype (28). However, this possibility seems to be excluded in the index case for the following reasons: *1*) the mutation was inherited through the mother, and *2*) a single genomic clone harboring C11081T was obtained not only from his lymphocytes but also from his muscle (data not shown). To rule out this possibility unequivocally, it is necessary to examine other muscle tissues, but this has not yet been done.

Another possible attenuating mechanism would be the modification of mRNA by either exon skipping or aberrant splicing, which would remove the nonsense mutation and produce a more complete dystrophin mRNA. Examples of exon skipping have been reported in nonsense mutations identified in exons 25, 27, 29, and 72 of the dystrophin gene (7–11), and aberrant splicing has been reported in intermediate dystrophinopathy (26). In our case, however, not only the RT-PCR product of dystrophin mRNA but also subcloning sequencing disclosed the existence of only one kind of mRNA consisting of a normal exon structure (Fig. 2). The possibility of either exon skipping or aberrant splicing thus was ruled out. Therefore, the discrepancy between genotype and phenotype could not be explained at the mRNA level.

Elevated expression of utrophin has been speculated to convert a severe phenotype to a mild one without affecting dystrophin expression (27). However, level of utrophin expression was the same in our case as in DMD (Fig. 3). Furthermore, the staining of proteins encompassing the dystrophin-dystroglycan axis was identical to that seen in DMD (Fig. 3). These similarities show that protein-level changes do not underlie the observed phenotypic differences.

The truncated dystrophin produced in the index case seems to be unusually stable, as demonstrated by the weak but significant staining of the N-terminal and rod domains (Fig. 1). This may be because the truncated dystrophin retains functionally important binding sites, such as actin binding sites in the N-terminal and rod domains (29,30), a β-dystroglycan binding site in the cysteine-rich domain (31), and syntrophin and dystrobrevin binding sites and a phosphorylation site in the C-terminal domain (Fig. 4) (32–35). In fact, it has been demonstrated that dystrophin lacking the amino acids encoded by exons 71–78 is stable in muscle membranes of the *mdx* mouse, an animal model of DMD (36). However, this hypothesis is not

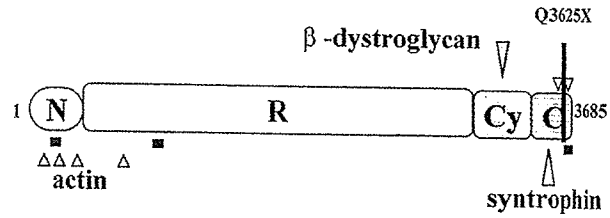

Figure 4. Dystrophin domain structure. Dystrophin consists of 3685 amino acids and is divided into four domains: the N terminus (box N), the rod (box R) and cysteine-rich (box Cy) domains, and the C terminus (box C). Three actin-binding sites have been identified in the N terminus (ABS1 18–37 amino acids, ABS3 86–120 amino acids, and ABS2 128–149 amino acids), and one site (amino acids 1416–1880) has been found in the rod domain (all four sites indicated by arrowheads). In the cysteine-rich domain (amino acids 3115–3263), dystrophin interacts with transmembrane β-dystroglycan (inverted triangle), which in turn binds to α-dystroglycan. Syntrophin binds to a region in the C-terminal domain (3446–3481; open triangle), and a serine at the 3552nd residue and threonine at the 3675th residue serve as phosphorylation sites (open arrowheads). Antibodies that recognize the N-terminal, rod, and C-terminal domains react to amino acid residues 321–494, 1181–1388, and 3669–3685, respectively (bars). The novel nonsense mutation (Q3625) is located at the end of the C-terminal domain (vertical bar). Numbers at both ends of the boxes indicate amino acid residues. The figure is not drawn to scale.

supported by a previous report that Q3625X, a nonsense mutation just 10 amino acids downstream of the one reported here (Q3635X), gave rise to clinically typical DMD (6) (Fig. 4). Furthermore, examination of laminin alpha 2, α- and β-dystroglycans, and γ-sarcoglycan disclosed no difference in their staining patterns between our case and DMD (Fig. 3), indicating that augmented stabilization of these proteins does not contribute to the mildness of the phenotype. Clearly, further study is required to clarify these complex results.

Activation of transcription of the dystrophin gene may lead to overproduction of dystrophin mRNA. It has been proposed that an abnormality in a transcription factor(s) or in its binding site in the promoter of the dystrophin gene is a factor in phenotypic severity. In fact, mutation of the MYF6 gene results in a severe phenotype of BMD (37). However, modifier(s) that make the phenotype mild have not been reported to date (38,39), although *mdx* mice characterized by dystrophin deficiency do not show a severe DMD phenotype (40). We are now following up the index case, and a future study analyzing not only the dystrophin gene but also other genes may clarify the molecular mechanism explaining his mild phenotype.

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Nucleic Acids Symposium Series No. 48 297-298

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.

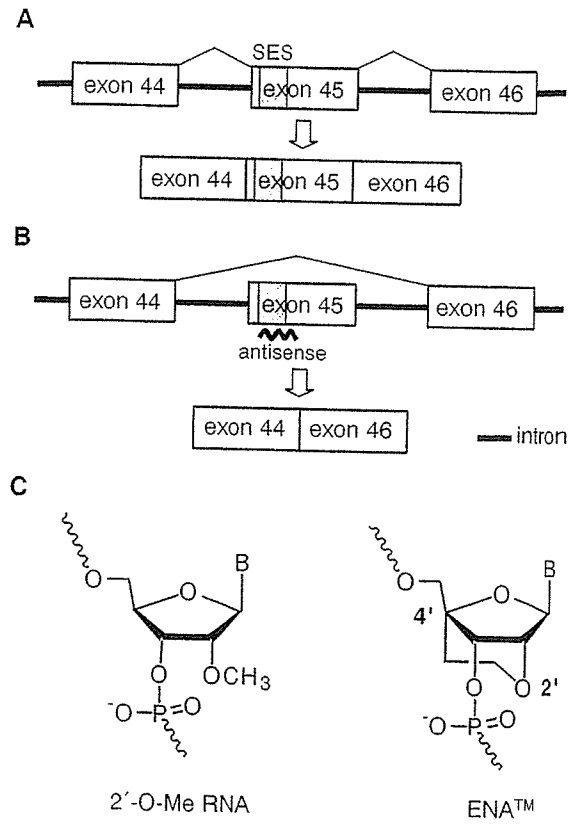

Figure (A), (B) Mechanism of exon-skipping of pre-mRNA using antisense molecules and (C) structures of 2'-O-Me RNA and ENA™

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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American Journal of Pathology, Vol. 164, No. 5, May 2004

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

Supported by grants-in-aid for Research on Nervous and Mental Disorders (13B-1) and for Research in Brain Science (H12-brain-028) from the Ministry of Health, Labor, and Welfare of Japan; a grant-in aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (14770106); and a grant-in-aid from the Nakatomi Foundation.

Accepted for publication January 29, 2004.

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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 β, 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-μm 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.			
5 PC3	M60921	↑	↑	→	(m) CTCGAGATGAGCCACGGGAAGAGAAC	(m) GCGGCCGCCTAGCTGGAGACGGCCATCA	(m) 476
6 mc7	AJ278191	↑	N.D.	→	(h) ATGAGCCACGGGAAGGAAGGGAAC	(h) GGGTCAGCTCGCTGGGCAGC	(h) 304
7 CGI 61	AF151819	↑	→	→	GACGGCACGTGTGACGAGTG	CCTCGCTGTCTTCTTCACTC	422
					CCTCCTTAGCAAAGCTGAATG	TGCAGCATCCAAATCCAGTC	393
8 Thymosin β4	M34043	↑	↑	→	(m) CCGGAATTCATGTCTGACAAACCCG	(m) GCTCTAGATTACGATTCGCCAGCTTG	(m) 135
9 EST-MNCb4008 Telomeric repeat binding	BF168890	↑	→	↑	(h) CAACCATGTCTCTGAGAAACCCG	(h) GATTCGCCTGCTTGCTTCTCC	(h) 135
					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	
13 Selenoprotein P	X99807	↑	↑	→	(m) CCGGAATTCGAGAGCCAAGGCCAAAGC	(m) GCTCTAGATTAGTTTGAATGTCATTTC	(m) 1085
					(h) CAATGTGGAGAAGCCTGGGG	(h) AGATGTGTGTATTTAATCG	(h) 312
14 GARG16 Phosphatidic acid	Q64828	↑	↑	→	(m) GCGAAGGCTCTGAAAGTGG	(m) GGGGTAACTCAGAATGCAGGGTTC	(m) 908
					(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 μg 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 μl of 10 μmol/L cardiotoxin (Wako), and they were frozen in isopentane precooled in liquid nitrogen. Ten-μm cryostat longitudinal sections were prepared and fixed in 4% paraformaldehyde in PBS (pH 7.4) and treated with 1 μg/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 β4 (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-

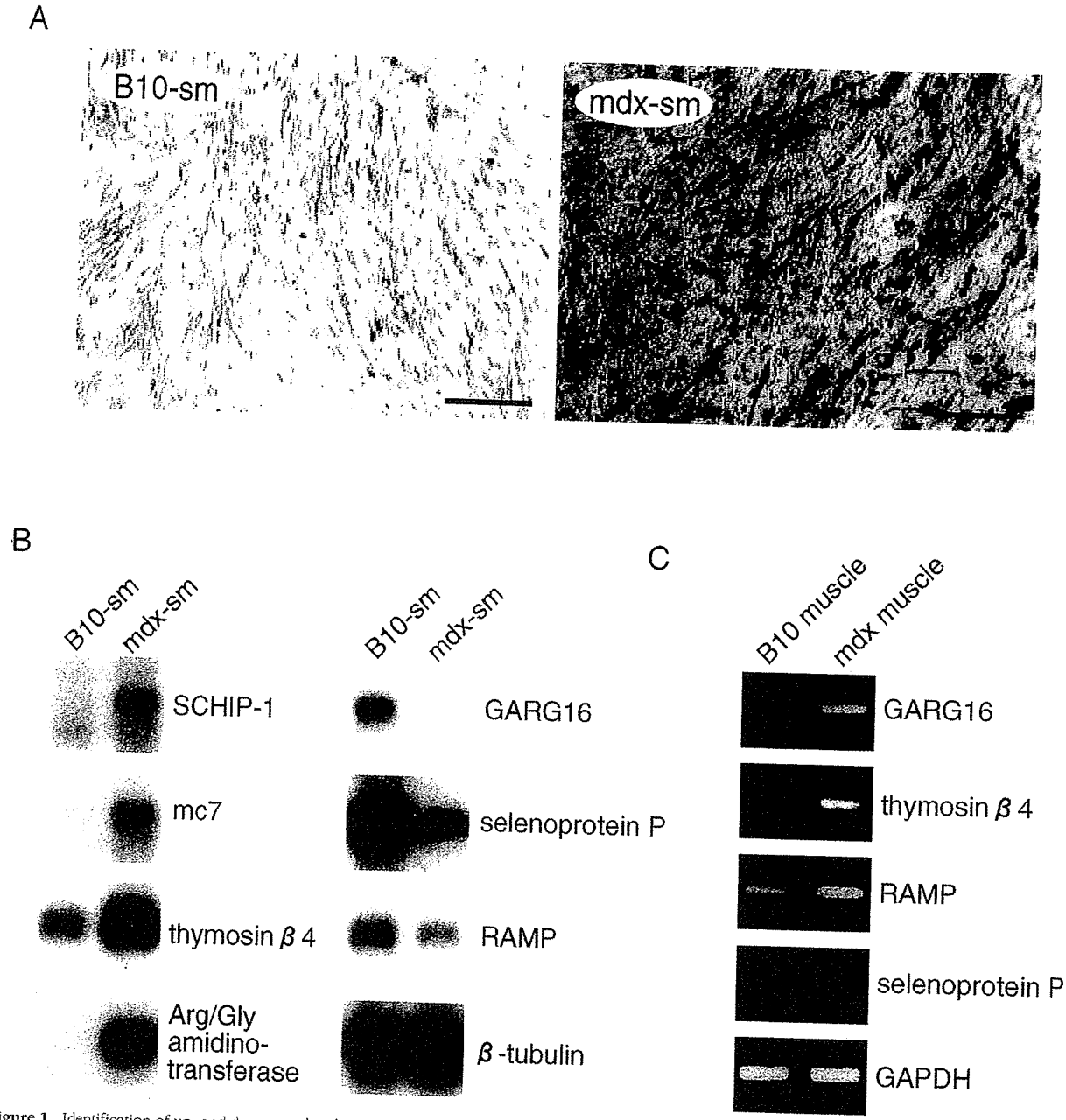

Figure 1. Identification of up- and down-regulated genes in the *mdx*-derived skeletal muscle cell line. **A:** Extent of spontaneous myogenic differentiation in the B10-sm and mdx-sm myoblastic cell lines derived from B10 and *mdx* mice, respectively. Cells cultured in differentiation-inducing conditions 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.

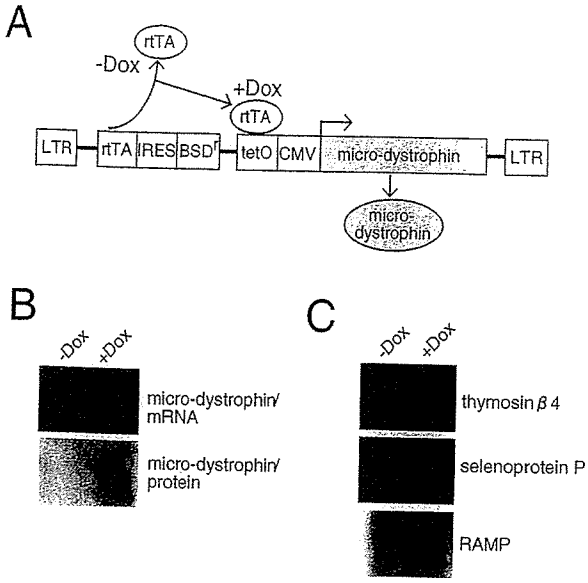

Figure 2. Effect of enforced expression of microdystrophin protein in mdx-sm cell line. **A:** Structure of the pLRT vector carrying a mouse micro-dystrophin gene under the Dox-inducible promoter. **B:** Dox-inducible expression of microdystrophin mRNA and protein in mdx-sm cells transfected with pLRT-microdystrophin. **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

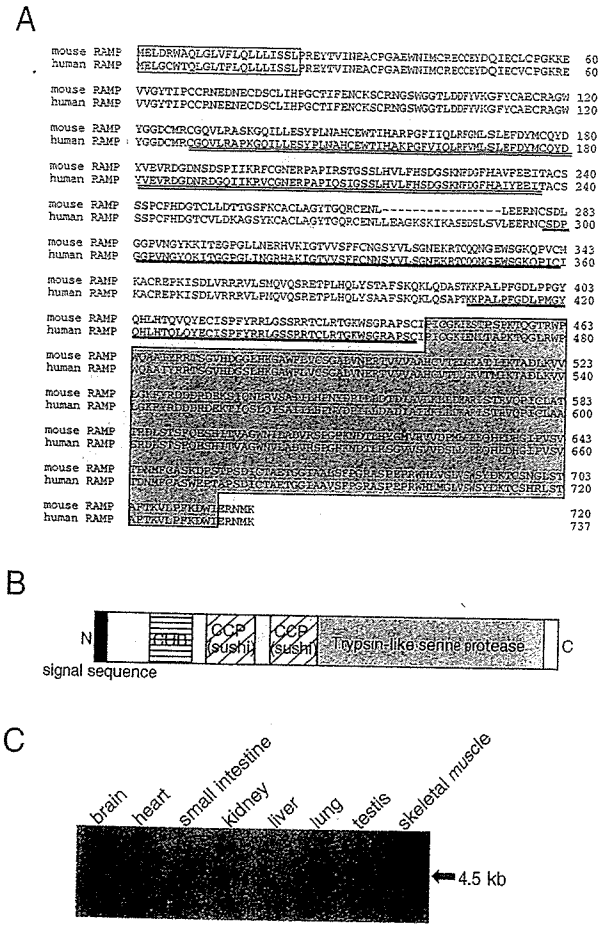

Figure 3. Sequence and tissue distribution of RAMP. **A:** Sequences of human and mouse RAMP proteins. Predicted signal sequences are **boxed**. CUB and CCP/Sushi domains are indicated by a **double line** and a **solid line**, respectively. The trypsin-like serine protease domain is shown by a **light 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.

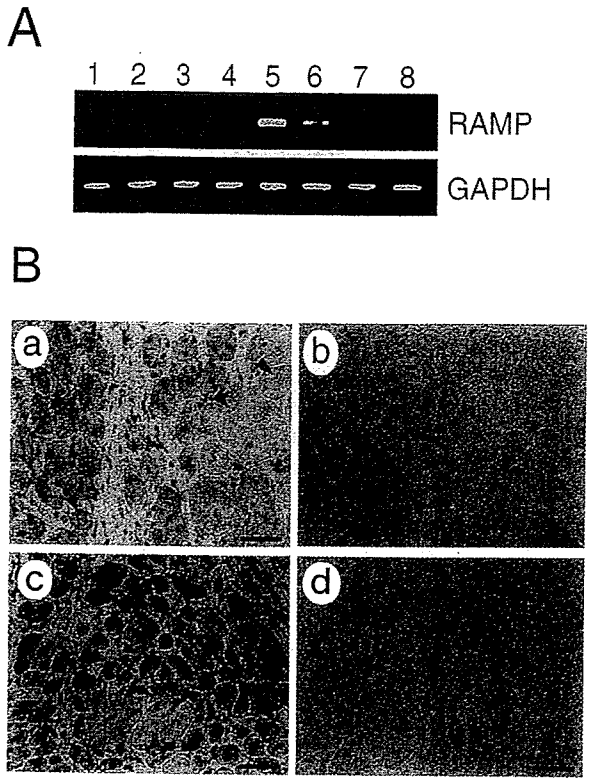

Figure 4. Up-regulation of RAMP mRNA expression in skeletal muscle after injury. **A:** Gastrocnemius muscle of mice was dissected 5 hours (**lane 1**) or 1 (**lane 2**), 2 (**lane 3**), 3 (**lane 4**), 4 (**lane 5**), 7 (**lane 6**), 10 (**lane 7**), or 14 (**lane 8**) days after injuring the muscle 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.

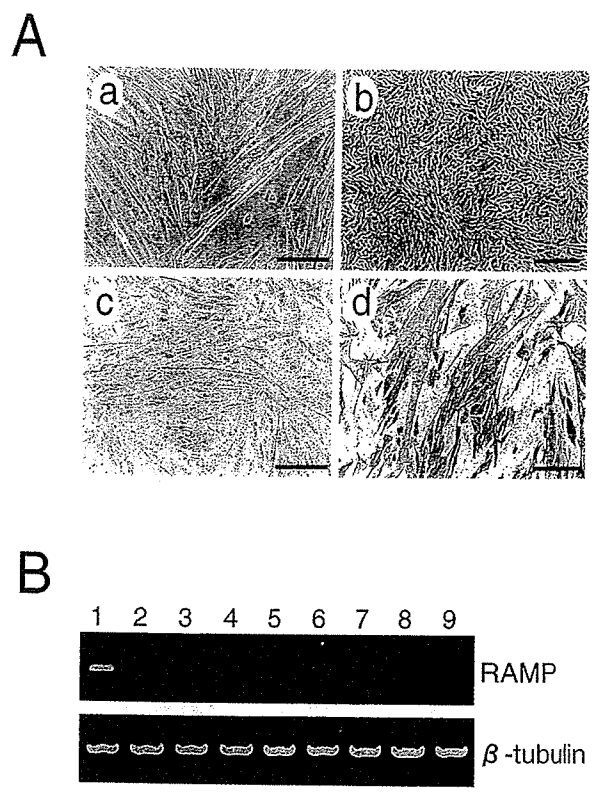

Figure 5. Expression of RAMP mRNA in cell lines derived from human BMD and DMD patients. **A:** Myotube differentiation capacity of BMD-sm1, a representative cell line derived from a BMD patient's skeletal muscle. The morphology is shown by phase contrast microscopy of the BMD-sm1 cell line cultured 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.

Acknowledgments

We thank Drs. Yuko Miyagoe-Suzuki and Shin-ichi Takeda (National Institute of Neuroscience) for valuable advice; and Drs. Norman Drinkwater (University of Wisconsin Medical School) and Masatoshi Hagiwara (Tokyo Medical and Dental University) for providing us with the pMESVts and pLRT-X retrovirus vectors, respectively.

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