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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

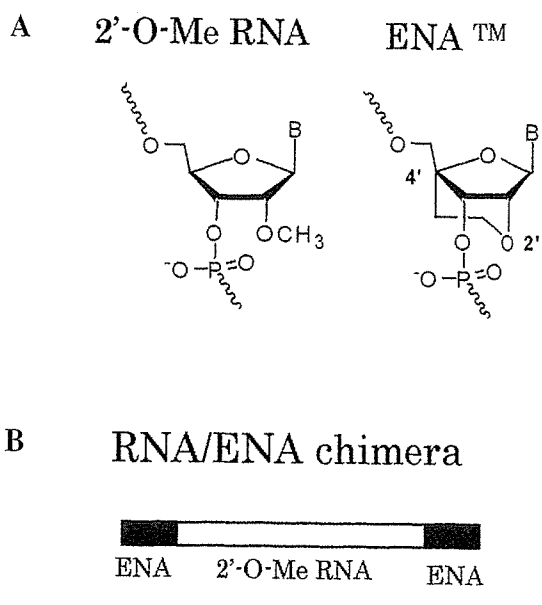

FIG. 1. RNA/ENA chimeras. (**A**) Structure of 2′-*O*-methyl RNA and ENA. (**B**) Scheme of RNA/ENA chimera. Black and white bars indicate stretches of ENA and 2′-*O*-methyl RNA, respectively.

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

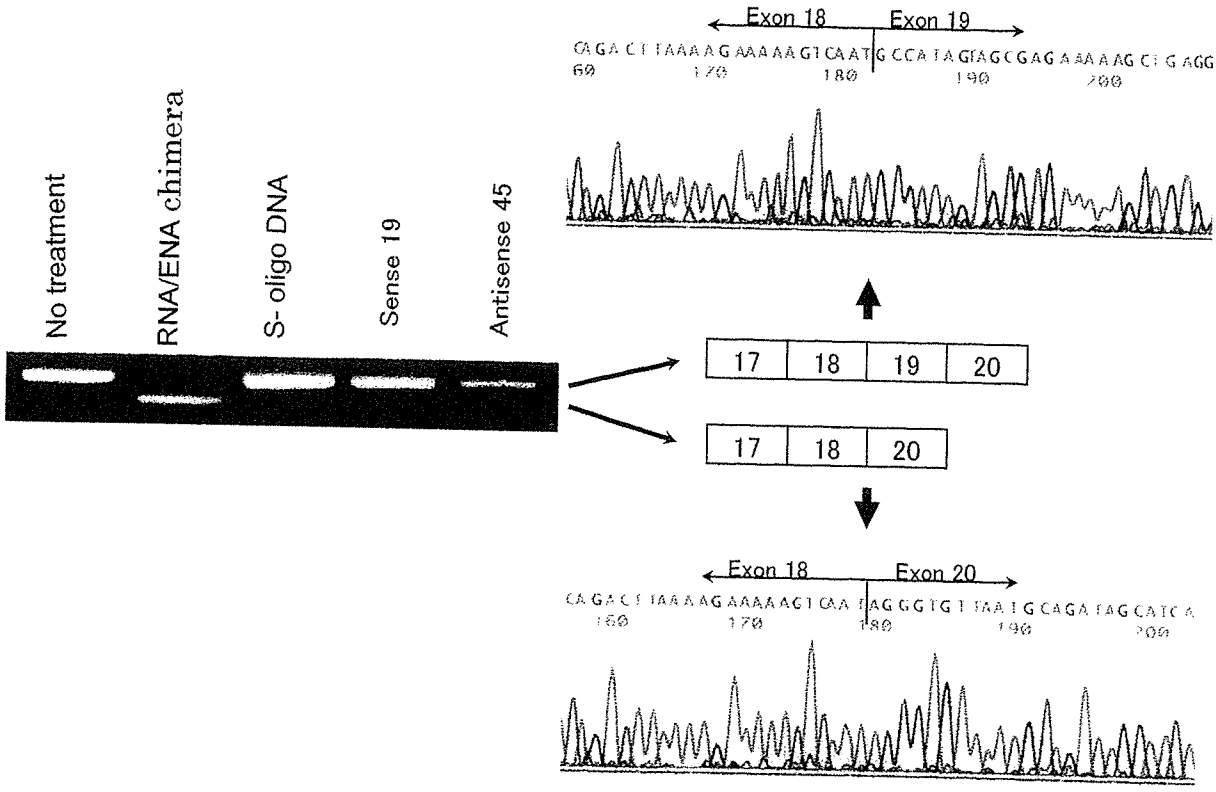

FIG. 2. Analysis of dystrophin mRNA. The ability of different oligonucleotides to induce exon 19 skipping was compared. DMD myocytes were incubated for 2 days at a concentration of 200 nM of each oligonucleotide separately, and the resulting dystrophin mRNA was analyzed. On amplification of the region 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 μg 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

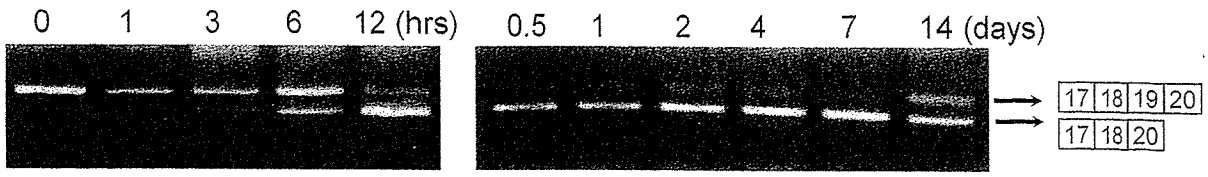

FIG. 3. Time course of exon 19 skipping. RT-PCR products of the region spanning exons 17–20 of the dystrophin genes are shown. Before and 1 hour after transfection, a single amplified product corresponding to the normal one was obtained. The smaller, exon 19-skipped fragment appeared 3 hours after transfection. 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

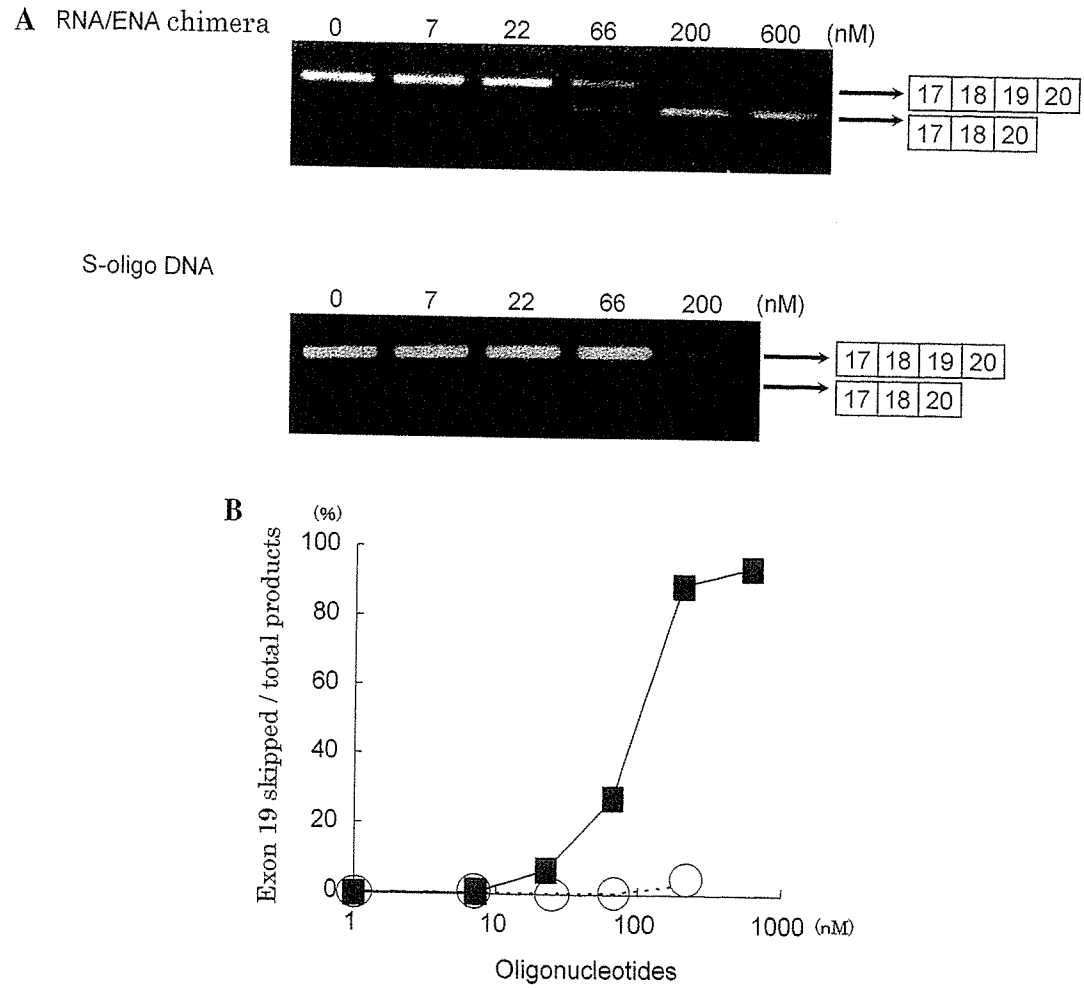

FIG. 4. Dose dependency of exon 19 skipping. **(A)** RFPCR product. Different amounts (0, 7, 22, 66, 200, and 600 nM) of RNA/ENA chimera were added to the culture medium. Only one product, corresponding to the normal, full-length cDNA, was amplified on treatment with 0 or 7 nM of the RNA/ENA chimera. 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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Chimeric RNA/Ethylene-Bridged Nucleic Acids Promote Dystrophin Expression in Myocytes of Duchenne Muscular Dystrophy by Inducing Skipping of the Nonsense Mutation-Encoding Exon

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ABSTRACT

Editing of dystrophin mRNA by induction of exon skipping, using antisense oligonucleotides, has been proposed as one way to generate dystrophin expression in Duchenne muscular dystrophy (DMD) patients. Here, antisense chimeric oligonucleotides consisting of RNA and a new modified nucleic acid are tested for activity to induce skipping of an exon containing a nonsense mutation. In a Japanese DMD case, a nonsense mutation (R1967X) due to a single nucleotide change in exon 41 of the dystrophin gene (C5899T) was identified. Oligonucleotides consisting of 2′-*O*-methyl RNA and a new 2′-*O*,4′-*C*-ethylene-bridged nucleic acid (ENA) were designed to bind the mutation site of exon 41, and their ability to induce exon 41 skipping in dystrophin mRNA was evaluated. Finally, among the specific oligonucleotides tested, an 18-mer RNA/ENA chimera was found to have the strongest activity, inducing exon 41 skipping in nearly 90% of dystrophin mRNA. Accordingly, nearly 90% of cultured myocytes were shown to be dystrophin positive by immunohistochemical analysis. Western blot analysis disclosed the presence of nearly normal-sized dystrophin up to 1 week after the transfection. Our results suggest that an RNA/ENA chimera can be used to express dystrophin in DMD.

OVERVIEW SUMMARY

Duchenne muscular dystrophy (DMD) is the most common and severe muscle-wasting disease characterized by dystrophin deficiency. Many attempts have been made to express dystrophin in DMD patients, but an effective treatment has not yet been established. In one Japanese DMD case, a nonsense mutation (R1967X) due to a single nucleotide change in exon 41 of the dystrophin gene (C5899T) was identified. Antisense chimeric oligonucleotides consisting of 2′-*O*-methyl RNA and a new modified nucleic acid are shown to induce skipping of the exon containing the nonsense mutation and to produce in-frame dystrophin mRNA in cultured DMD myocytes harboring the nonsense mutation. Furthermore, dystrophin was stained in more than 90% of transfected myocytes. It is proposed that new chemicals can be designed to induce skipping of other dystrophin exons, thereby making dystrophin expression possible in a broader spectrum of DMD cases.

INTRODUCTION

DUCHENNE MUSCULAR DYSTROPHY (DMD) is a rapid, progressive disease that usually results in death at about the age of 20 years, whereas Becker muscular dystrophy (BMD) is a clinically less severe form of the disease that often has only slight debilitating effects. DMD and BMD are allelic diseases caused by mutations in the dystrophin gene, which spreads over 3 Mb of the X chromosome and contains 79 exons encoded in a 14-kb-long mRNA (Ahn and Kunkel, 1993; Nishio *et al.*, 1994). Deletion mutations have been identified in two-thirds of DMD/BMD cases, and the clinical progression of DMD or BMD patients can be predicted from whether the deletion disrupts (out-of-frame) or maintains (in-frame) the translational reading frame of the mRNA (the reading frame rule) in more than 90% of DMD/BMD cases (Monaco *et al.*, 1988). Although nonsense mutations are thought to underlie many nondeletion DMD cases, the responsible nonsense mutation has been identified only in a limited number of cases because of the large size of the dystrophin gene.

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Although much progress has been made in the study of gene-replacement therapy for DMD, we are still a long way from achieving a clinically significant result (van Deutekom and van Ommen, 2003). Therefore, alternative strategies for DMD treatment, which retard the progression of clinical symptoms by converting DMD into the BMD phenotype, are now attracting much attention (Matsuo, 1996, 2002; Gebski *et al.*, 2003; van Deutekom and van Ommen, 2003). A naturally occurring example of this conversion has been demonstrated; in one case, skipping of an exon containing a nonsense mutation in the splicing enhancer sequence results in the production of a novel in-frame dystrophin mRNA, resulting in an internally deleted dystrophin molecule that functions sufficiently well to result in the milder BMD phenotype (Shiga *et al.*, 1997). So far, nonsense mutations in exons 25, 27, 29, and 72 have been shown to elicit skipping of the respective affected exon, resulting in BMD (Barbieri *et al.*, 1996; Shiga *et al.*, 1997; Melis *et al.*, 1998; Ginjaar *et al.*, 2000). These findings strongly support the possibility of treatment of DMD by phenotype conversion from DMD to BMD by artificial induction of exon skipping.

Artificial induction of exon 19 skipping in the human dystrophin gene was first reported using an antisense oligonucleotide against a polypurine exonic splicing enhancer (Matsuo *et al.*, 1991; Takeshima *et al.*, 1995; Pramono *et al.*, 1996). Furthermore, expression of an internally deleted dystrophin by induction of exon 19 skipping has been demonstrated in cultured myocytes from a DMD patient harboring an exon 20-deleted dystrophin gene (Takeshima *et al.*, 2001). Successful induction of exon skipping using antisense oligonucleotides against exonic polypurine sequences has been reported for several human dystrophin exons, resulting in an in-frame dystrophin mRNA successfully producing dystrophin protein (van Deutekom *et al.*, 2001; Aartsma-Rus *et al.*, 2002). All studies thus far have used antisense oligonucleotides with phosphorothioate backbones, the standard choice for such applications (Levin, 1999).

A novel nucleic acid consisting of 2′-*O*,4′-*C*-ethylene bridges (ENA) has been established as a highly nuclease-resistant and thermodynamically stable nucleic acid (Morita *et al.*, 2001). In a previous study, it was found that an antisense RNA/ENA chimera was 40 times as effective as a conventional phosphorothioate oligonucleotide in inducing exon 19 skipping (Yagi *et al.*, 2004).

Here, we report the identification of a nonsense mutation in exon 41 of the dystrophin gene accompanying novel tissue-specific alternative splicing of the exon. Remarkably, an RNA/ENA chimera was shown to promote dystrophin expression strongly via induction of exon 41 skipping in the patient's cultured myocytes.

MATERIALS AND METHODS

Case

A 7-year-old Japanese boy without any family history of neuromuscular disorders was referred to Kobe University Hospital (Kobe, Japan) because of a high serum creatine kinase (CK) level (30,000 IU/liter; control, <200 IU/liter). He showed pseudohypertrophy of the calves, waddling gait, and difficulty in climbing stairs. Electromyogram (EMG) revealed a characteristic myogenic pattern, and chest X-ray and electrocardiogram (ECG) examinations failed to reveal other abnormalities. DMD was tentatively diagnosed. A quadriceps muscle biopsy was carried out to confirm this diagnosis after obtaining informed consent. Hematoxylin–eosin staining of biopsied muscle disclosed evidence of dystrophic changes. Immunohistochemical examination with antibodies recognizing various epitopes of the N-terminal, rod, and C-terminal domains of dystrophin (Adachi *et al.*, 2003) disclosed no staining for dystrophin, confirming the DMD diagnosis. The following study was approved by the medical ethics committee of Kobe University School of Medicine.

Mutational analysis

Reverse transcription-polymerase chain reaction (RT-PCR) or nested PCR (RT-nested PCR) was employed to analyze the dystrophin mRNA expressed in biopsied and cultured muscle cells or lymphocytes, respectively (Matsuo *et al.*, 1991; Adachi *et al.*, 2003). Full-length dystrophin cDNA was amplified as 10 separate, partially overlapping fragments (Roberts *et al.*, 1991). To characterize the mutation, a region encompassing exons 40–42 was amplified, using a forward primer corresponding to a segment of exon 40 (c40F, 5′-GGTATCAGTACAAGAGGCAGGCTG-3′) and a reverse primer complementary to a segment of exon 42 (c42R, 5′-CACTTCTAATAGGGCTTGTG-3′).

A DNA sample was extracted from whole blood obtained from the patient as described previously (Matsuo *et al.*, 1990). Southern blot analysis was performed with *Hin*dIII restriction enzyme-digested DNA as a template and a dystrophin cDNA fragment as a probe to screen for deletions (Koenig *et al.*, 1987). The region encompassing exon 41 was amplified with a forward primer corresponding to a segment of intron 40 (5′-TGGGTTATTGAGCGAGGAT-3′) and a reverse primer complementary to a segment of intron 41 (5′-TTTCTTGTGTCTTTAATTGGCA-3′). All PCR amplifications were performed under conditions essentially the same as those described previously (Surono *et al.*, 1999).

The PCR-amplified product was purified and subjected to sequencing either directly or after subcloning into a pT7 blue T vector (Novagen, Madison, WI) (Surono *et al.*, 1997). The DNA sequence was determined with a dye terminator cycle sequencing kit (Amersham Biosciences, Piscataway, NJ) with an automatic DNA sequencer (model ABI PRISM 310; Applied Biosystems, Foster City, CA).

Transfection of cultured myocytes with an RNA/ENA chimera

A primary muscle cell culture was established from the index case and from a normal control as described previously (Takeshima *et al.*, 2001), after informed consent was obtained. For antisense treatment, muscle cells were seeded in gelatin-precoated six-well plates. Myotubes were obtained from confluent myoblast cultures after 10–14 days of serum deprivation and were transfected with an RNA/ENA chimera (Yagi *et al.*, 2004). The RNA/ENA chimera was dissolved in 100 μl of Opti-MEM (Invitrogen, San Diego, CA) mixed with 6 μl of PLUS reagent (Invitrogen) and incubated for 15 min at room temperature. The incubated solution was mixed with 8 μl of Lipofectamine (Invitrogen) dissolved in 100 μl of Opti-MEM and incubated for 15 min. The mixture was then added to culture medium (800 μl of Opti-MEM) to a final RNA/ENA chimera

concentration of 200 pmol/ml (200 n*M*). After 3 hr of incubation, horse serum was added to 2% final concentration and the incubation was continued for 2 days, at which point the myocytes were harvested and RNA was extracted. In specified experiments incubation periods were varied.

Antisense oligonucleotides consisting of 2′-*O*-methyl RNA and ENA (Sankyo Lifetech, Tokyo, Japan) (RNA/ENA chimera) were synthesized with an automated DNA synthesizer (Applied Biosystems). To stabilize the ENA against 3′-exonucleases, 2-hydroxyethylphosphate groups were attached at the 3′ end of the ENA as reported previously (Koizumi *et al.*, 1997). At first, two 23-mer antisense oligonucleotides differing in the number of ENA residues they contained were synthesized to cover the mutation sites (Fig. 1). Each had five or seven ENAs at both their 5′ and 3′ ends and 2′-*O*-methyl RNA in their middle (ENA41WT5 and ENA41WT7) (Morita *et al.*, 2002). Second, three 18-mer RNA/ENA chimeras with five ENA residues at both the 5′ and 3′ ends (ENA41A, ENA41B, and ENA41C) were synthesized to cover the entire ENA41WT5 sequence and its flanking sequences (Fig. 1).

Dystrophin expression analysis

Total RNA was isolated at the indicated time from the cultured myocytes and cDNA was prepared from 2 μg of total RNA as described previously (Surono *et al.*, 1999). A fragment spanning from exons 40 to 42 of dystrophin mRNA was amplified by 30 cycle of PCR as described above. The amount of each amplified products was determined by measuring its density using FluorImage 585 (Amersham Biosciences).

Cultured myocytes were immunohistochemically analyzed and were processed for dystrophin analysis as described previously (Takeshima *et al.*, 2001). The following antibodies were applied: desmin polyclonal antibody (DakoCytomation, Carpinteria, CA) and three dystrophin antibodies recognizing the N-terminal (Dys-3), rod (Dys-1), and C-terminal (Dys-2) domains of dystrophin (Novocastra Laboratories, Burlingame, CA).

Western blot analysis of dystrophin was performed as described previously, using monoclonal antibody Dys-2 (Novocastra Laboratories) (Bertoni *et al.*, 2003).

RESULTS

Identification of a nonsense mutation

On diagnosis of the patient with DMD, we searched for a mutation in his dystrophin gene, but Southern blot analysis failed to reveal any exon deletion (data not shown). To analyze for fine mutations, dystrophin cDNA prepared from his lymphocytes was analyzed by nested PCR. All 10 fragments covering the entire dystrophin cDNA were amplified and directly sequenced (Roberts *et al.*, 1991). One fragment encompassing exons 36–45 showed an unclear sequencing result. To clarify this, the fragment encompassing exons 40–42 was amplified. Remarkably, two amplified products were obtained in different amounts (Fig. 2a). The size of the major product corresponded to that of the small-sized product from the control, whereas the minor product was the same size as the normal, major band in the control. Sequencing of the 224-bp major product from the index case revealed that exon 40 joined directly to exon 42, deleting the 183-bp-long exon 41 (exon 41$^-$ transcript) (Fig. 2c). Because exon 41 was present in genomic DNA, this major product was concluded to be an exon 41-skipped product. The production of an in-frame exon 41$^-$ transcript in a large fraction of dystrophin mRNA was expected to result in a BMD phenotype, because an exon 41$^-$ transcript would generate an internally deleted dystrophin protein (Shiga *et al.*, 1997). This molecular finding therefore was not compatible with the severe clinical phenotype of DMD in this patient.

The 407-bp minor product, in contrast, had the complete sequence from exons 40–42. Unexpectedly, a C-to-T transition at nucleotide 160 of exon 41 (nucleotide 5899 of dystrophin cDNA) was found (C5899T) (Fig. 2d). This mutation changed a CGA codon, which encodes an arginine, to a TGA stop codon at amino acid residue 1967 (R1967X). This nucleotide change was confirmed in his genome by direct sequencing of an amplified fragment encompassing exon 41 (data not shown), without revealing any other mutations that could be responsible for the splicing error. Therefore, the nonsense mutation within exon 41 was deemed to be the molecular basis of his DMD phenotype. However, the fact that mRNA containing a premature stop codon constituted a minor fraction of the dystrophin mRNA expressed in his lymphocytes (Fig. 2a) was not compatible with his severe DMD phenotype.

Because neither of the two dystrophin mRNAs expressed in his lymphocytes could explain his phenotype, dystrophin mRNA extracted from his skeletal muscle was analyzed to clarify the molecular basis of the complete absence of dystrophin in his skeletal muscle. In contrast to lymphocytes, a single normal-sized product was obtained by amplification of the fragment encompassing exons 40 to 42 (Fig. 2b). Sequencing of this product disclosed normal exon structure, but the presence of the same C5899T mutation as was found in his genomic DNA. This indicated that all the dystrophin mRNA expressed

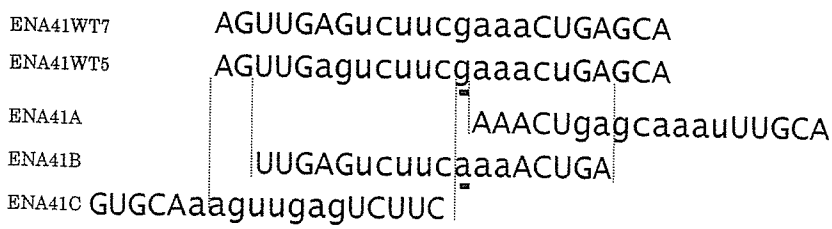

FIG. 1. Sequences of RNA/ENA chimeras. ENA41WT5 and ENA41WT7 are complementary to the wild-type sequence, whereas ENA41B is complementary to the mutation sequence. Upper case and lower case letters indicate ENA and 2′-*O*-methyl RNA monomers, respectively. Underlined letters represent the mutation site.

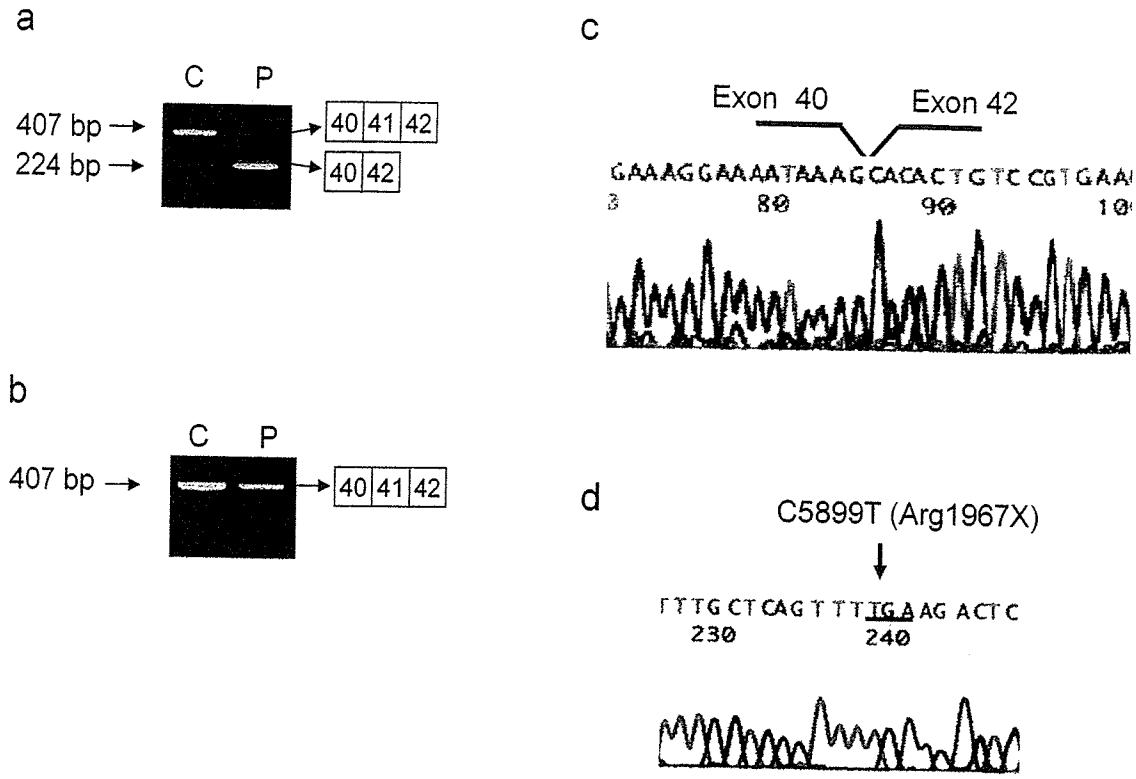

FIG. 2. Amplification of dystrophin cDNA encompassing exons 40 to 42. (**a**) Amplified products from lymphocytic cDNA. From the control (C), one major band (407 bp) and an additional minor band (224 bp) were amplified. From the patient (P), two amplified products were obtained: the major band (224 bp) corresponded to the small-sized product seen in the control, whereas the minor band was the same size as the major product in the control. On the right, the exon structure of each band is schematically described. (**b**) Amplified product from skeletal muscle. Only one 407-bp product was obtained from both the control (C) and the patient (P). No alternative splicing product (224 bp) was obtained. (**c**) Sequencing of the small-sized product. Sequencing of the small-sized product revealed that the 3′ end of exon 40 (5′-TAAAG-3′) joined directly to the 5′ end of exon 42 (5′-CACAC-3′), skipping entirely over exon 41 (183 bp). (**d**) Sequencing of the normal-sized product. Sequences of the normal-sized product revealed normal exon structure and the presence of a C-to-T transition at nucleotide 160 of exon 41 (C5899T), changing the wild-type CGA codon, which encodes arginine, to a TGA nonsense codon (underlined).

in his skeletal muscle encodes a premature stop codon (R1967X), thus giving rise to his DMD phenotype.

Induction of exon 41 skipping

Alternative splicing to remove exon 41 was first identified in normal lymphocytes (Fig. 2a). It was found that this alternative splicing was enhanced in the patient's lymphocytes (Fig. 2a), probably because of a single nucleotide change (C5899T) that disrupted the splicing enhancer sequence in exon 41. Therefore, we hypothesized that blocking the sequence around the mutation site with an antisense oligonucleotide would induce exon 41 skipping in his muscle cells, resulting in the expression of an internally deleted dystrophin protein. This hypothesis was verified by a two-step experiment: (1) an antisense oligonucleotide that induces exon 41 skipping was developed by testing in cultured myocytes, and (2) dystrophin expression in cultured myocytes from the patient was examined in the presence of this antisense oligonucleotide.

To find the most effective antisense oligonucleotide, we first designed two 23-mer antisense oligonucleotides covering the mutation site, consisting of 2′-*O*-methyl RNA residues flanked by either five or seven ENAs at both the 5′ and 3′ ends (RNA/ENA chimera) (Fig. 1, ENA41WT5 and ENA41WT7) (Yagi *et al.*, 2004). They were transfected individually into myocytes from the normal control and the patient and, 24 hr after the transfection, the fragment of the resulting dystrophin mRNA spanning exons 40 to 42 was analyzed by RT-PCR. Remarkably, the production of exon 41$^-$ transcript was enhanced by the transfection (Fig. 3a). This indicated that both of the chimeras were able to induce exon 41 skipping. In the patient's myocytes, the density ratio of exon 41$^-$ band to normal band was higher in ENA41WT5-treated myocytes compared with ENA41WT7-treated myocytes. This indicated that ENA41WT5, with five ENAs at each ends, more effectively induced exon skipping than did ENA41WT7, which had seven ENAs at each end (Yagi *et al.*, 2004). Therefore, the RNA/ENA chimera with five ENAs at each end was determined to be suitable to carry out further study of exon 41 skipping.

Next, we determined the optimal sequence for induction of exon skipping by designing three overlapping 18-*mer* RNA/ENA chimeras covering the sequence of ENA41WT5 and its flanking sequences (Fig. 1, ENA41A, ENA41B, and ENA41C). Each of the three was examined for its ability to induce exon

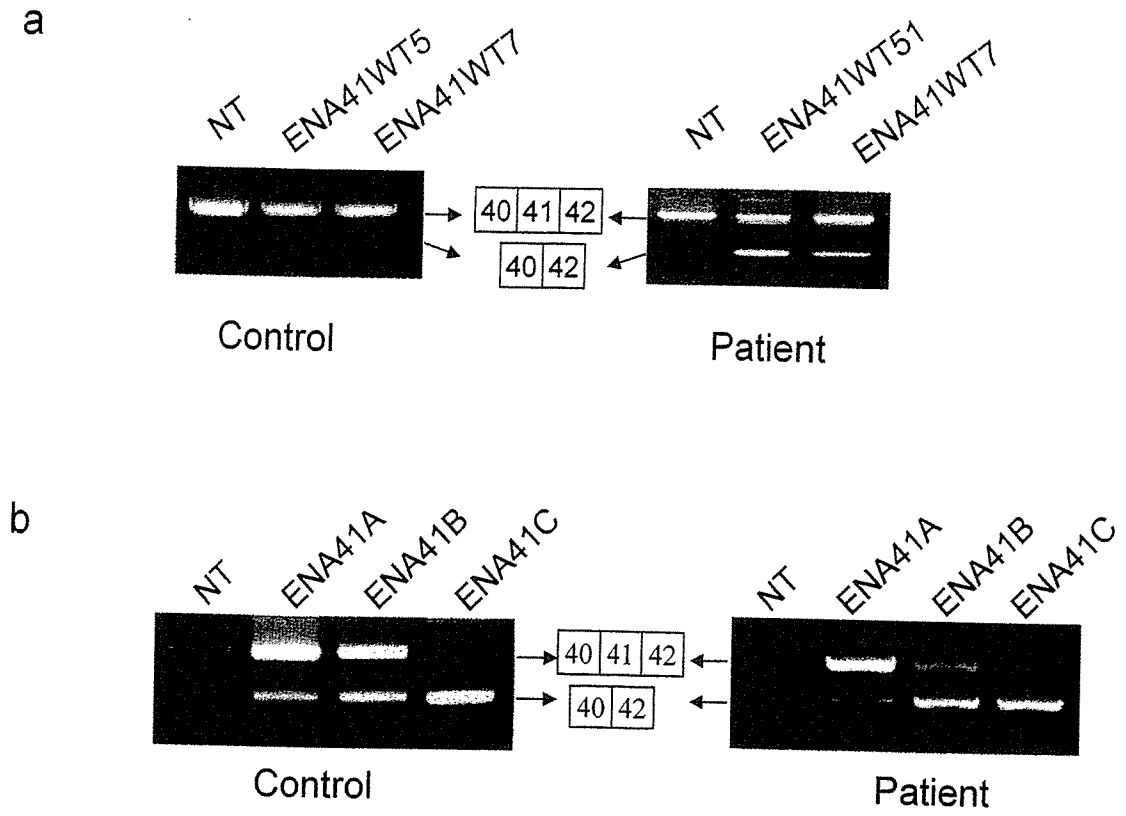

FIG. 3. Effects of transfection of RNA/ENA chimeras. (**a**) Transfection of ENA41WT5 or ENA41WT7. The region from exon 40 to exon 42 of the dystrophin mRNA transcript was amplified from cDNA obtained from normal control- and patient-derived myocytes 24 hr posttransfection. Remarkably, an extra product lacking exon 41 was obtained in myocytes from both the control (*left*) and the patient (*right*) on treatment with either ENA41WT5 or ENA41WT7. However, the density of the extra band obtained after treatment with ENA41WT5 was higher than that seen after ENA41WT7 transfection in the patient. In the control, on the other hand, the band densities under these two conditions were identical. The exon structure of the amplified product is represented schematically between the panels. Lanes NT, ENA41WT5, and ENA41WT7 represent nontreated myocytes and myocytes treated with antisense ENA41WT5 and ENA41WT7, respectively. (**b**) Transfection of ENA41A, ENA41B, or ENA41C. RT-PCR products encompassing exons 40 to 42 are shown. On treatment with either ENA41A, ENA41B, or ENA41C, an extra product lacking exon 41 was obtained in myocytes from both the control (*left*) and the patient (*right*), with ENA41C yielding the most dense product band. The exon composition of the amplified product is represented schematically between the panels. Lanes NT, ENA41A, ENA41B, and ENA41C represent nontreated myocytes and myocytes treated with ENA41A, ENA41B, and ENA41C, respectively.

41 skipping in myocytes of both the control and the DMD patient. All three oligonucleotides were able to induce exon 41 skipping, but the most dense band of exon 41$^-$ product was recovered from myocytes treated with ENA41C (Fig. 3b). ENA41B had less activity than did ENA41C, but was more active than ENA41A (Fig. 3b). From the density of product bands it was calculated that about 70 or 90% of mRNA were missing exon 41 on treatment of the patient's myocytes with ENA41B or ENA41C, respectively (Fig. 3b). No other abnormal splicing was observed, although the full length of dystrophin cDNA was examined. These results, taken together, indicate that induction of exon 41 skipping is dependent on the sequence of the applied oligonucleotide. In accordance with this, ENA41B, which was designed to be perfectly complementary to the mutated sequence, showed higher exon 41 skipping activity in DMD patient myocytes than in normal control myocytes, which harbor the mismatched, wild-type sequence (Fig. 1).

Expression of dystrophin

After induction of exon 41 skipping was accomplished successfully in the patient's myocytes using ENA41B and ENA41C, dystrophin expression in these cells was examined. Immunohistochemical staining with an antibody recognizing the C-terminal domain of dystrophin revealed dystrophin-positive cells transfected with each of these two chimeras (Fig. 4a). The efficiency of conversion from dystrophin-negative to -positive cells was determined by quantification of the number of dystrophin-positive cells against desmin-positive cells among the ENA41B- or ENA41C-treated cultures. On average, approximately 75 or 89% of desmin-positive myocytes became dystrophin positive after ENA41B or ENA41C transfection, respectively (Fig. 4b). This conversion ratio is well correlated with the production of exon 41$^-$ transcript (Fig. 3b).

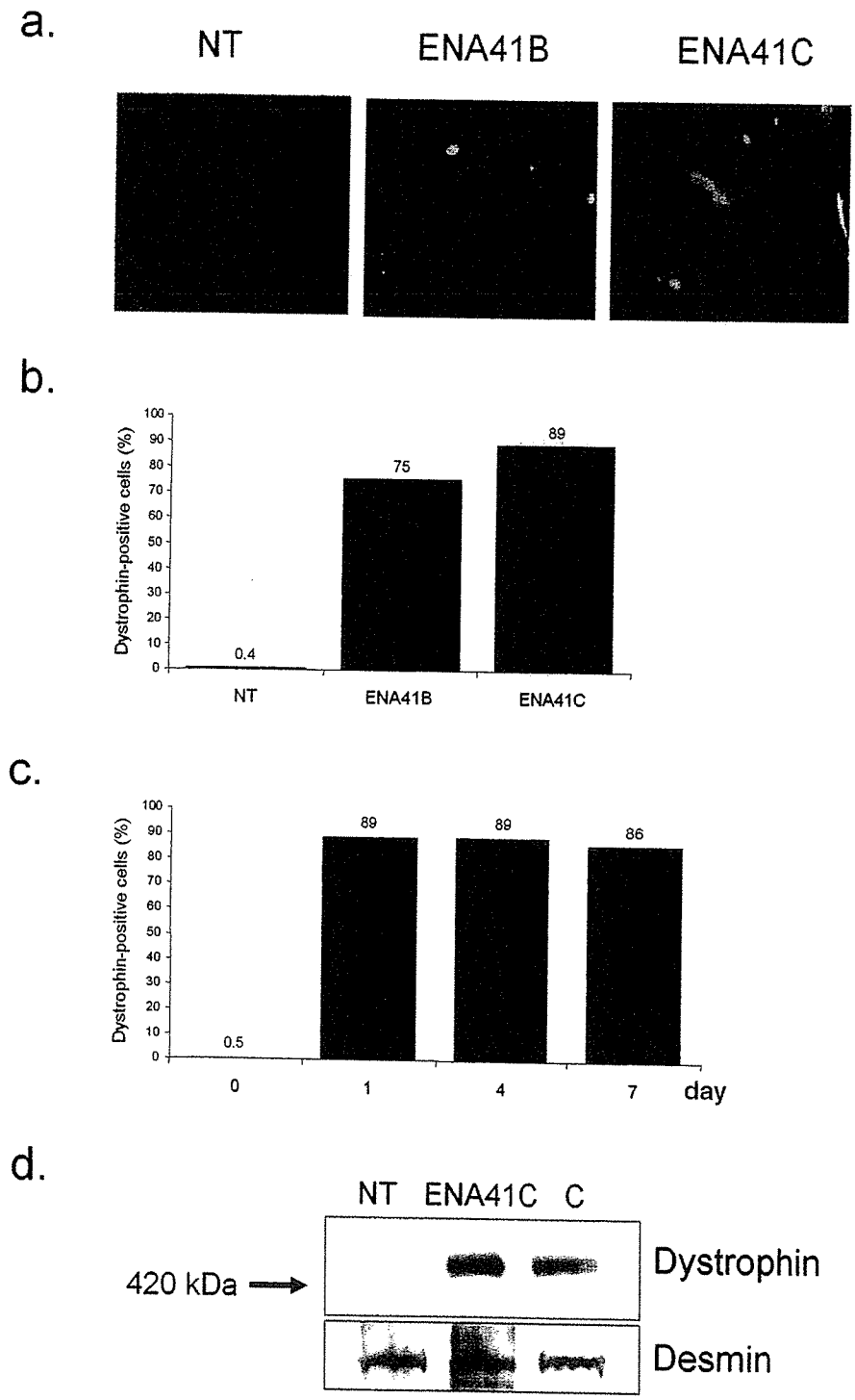

FIG. 4. Dystrophin analysis of RNA/ENA-treated myocytes from a patient. (**a**) Dystrophin staining. No dystrophin signals could be detected in untreated cells (NT) stained with Dys-2, whereas clear, mainly cytoplasmic, dystrophin signals could be detected after treatment with either ENA41B or ENA41C. (**b**) Quantification of dystrophin-positive myocytes. The number of dystrophin-positive myocytes was determined in nontreated myocytes and myocytes treated with ENA41B or ENA41C, respectively, and the percentage of dystrophin-positive cells relative to desmin-positive cells was calculated and is shown above each column. (**c**) Quantification of the percentage of dystrophin-positive myocytes treated with ENA41C over 7 days posttransfection. The number of dystrophin-positive cells was determined on the indicated days. The percentage of dystrophin-positive cells relative to desmin-positive cells was calculated and is shown above each column. (**d**) Western blot analysis of ENA41C-treated myocytes. A clear dystrophin signal was detected 7 days posttransfection of ENA41C (lane ENA41C). The detected dystrophin appeared to have the same molecular weight as control dystrophin (lane C). To demonstrate consistent protein loading, blots were additionally stained with an antibody against desmin. Lane NT represents nontreated myocytes.

Expression of dystrophin was followed up until day 7 in ENA41C-transfected myocytes. The percentages of dystrophin-positive myocytes were 89, 89, and 86% of cells on days 1, 4, and 7, respectively (Fig. 4c). Seven days after oligonucleotide transfection, dystrophin expression was confirmed by Western blot analysis. Dystrophin could be detected with a monoclonal antibody recognizing its C-terminal domain (Fig. 4d). However, the expressed dystrophin, which should lack 61 amino acids, was difficult to differentiate from the control dystrophin, based on molecular weight alone.

Our results show that RNA/ENA chimeras against an exon 41 sequence can induce exon 41 skipping in myocytes. More than 90% of the dystrophin mRNA treated with these chimeric oligonucleotides lacked exon 41, and the resulting exon 41 transcript led to the production of dystrophin in treated myocytes. These results indicate that RNA/ENA chimeras may be applied to clinical use for treatment of nonsense mutations in exon 41 of the dystrophin gene.

DISCUSSION

In this study, we identified a nonsense mutation (C5899T) in the dystrophin gene of a Japanese DMD patient through isolation of a novel dystrophin mRNA lacking exon 41. We have identified dystrophin gene mutations in more than 200 Japanese DMD/BMD cases by analyzing dystrophin mRNA expressed in lymphocytes (Hagiwara *et al.*, 1994; Shiga *et al.*, 1997; Surono *et al.*, 1999; Adachi *et al.*, 2003; Ito *et al.*, 2003; Yagi *et al.*, 2004). From these cases, we have succeeded in discovering more than 20 nonsense mutations. However, no secondary splicing errors had been observed in any case, except for one in which the patient had a BMD phenotype (Shiga *et al.*, 1997). Thus far, reported nonsense mutations in the dystrophin gene have been classified into two types: one induces a secondary splicing error, and the other does not. The former is rare and has been limited to cases showing the BMD phenotype (Barbieri *et al.*, 1996; Shiga *et al.*, 1997; Melis *et al.*, 1998; Ginjaar *et al.*, 2000). In the index case, a severe DMD phenotype developed even though an in-frame mRNA was produced. This is because weak exon 41 skipping was induced in his skeletal muscle, although a large fraction of the dystrophin mRNA in his lymphocytes did lack exon 41. Secondary errors of splicing due to a single nucleotide change have been claimed to disrupt exonic splicing enhancer sequences (Shiga *et al.*, 1997). Because exon 41 skipping was enhanced in the patient's lymphocytes, which harbor the C5899T point mutation, we propose that this mutation lies within an exonic splicing enhancer sequence (Fig. 2).

The C5899T mutation had been reported four times in countries other than Japan (see Leiden Muscular Dystrophy Pages, www.dmd.nl); this is the fifth such report. Our result further confirms a general recurrence of the C5899T mutation, suggesting that it is a mutational hot spot in the dystrophin gene despite the fact that most of the nonsense mutations seen thus far in the dystrophin gene have been unique. Clinically, our case and three others showed a DMD phenotype, whereas the fifth expressed a phenotype intermediate between DMD and BMD (www.dmd.nl). The phenotypic differences among these cases may be due to the degree of activation of alternative splicing in the individual patient's skeletal muscle. Therefore, it may be appropriate to analyze the mRNA expressed in muscle to determine a genotype–phenotype correlation in R1967X patients, even though the mutation itself can be identified simply by analyzing lymphocyte mRNA.

In this report, we have identified a novel alternative splicing product that lacks exon 41 in lymphocytes obtained from a normal control subject (Fig. 2a). However, this alternative splicing was observed very weakly in control skeletal muscle (Fig. 2b). This suggests the existence of a difference in splicing regulatory mechanisms between lymphocytes and skeletal muscle, a phenomenon that has been reported previously in the context of dystrophin pre-mRNA (Ito *et al.*, 2003). Remarkably, low levels of this alternative splicing were observed in untreated cultured myocytes from the control and the patient (Fig. 3). It has been demonstrated that splicing factors function to facilitate exon definition and are implicated in cell-specific and developmentally regulated alternative splicing (Norgren *et al.*, 1994; Hastings and Krainer, 2001; Strasser and Hurt, 2001; Lam and Hertel, 2002). Alternative splicing of exon 41may thus be dependent on tissue- or development-specific splicing factors. Further study is required to clarify this difference in splicing regulation.

Currently, induction of exon skipping using antisense oligonucleotides has attracted much attention as a plausible therapy for DMD (Matsuo, 1996; Takeshima *et al.*, 2001; Matsuo, 2002; Gebski *et al.*, 2003; van Deutekom and van Ommen, 2003). However, most experiments conducted thus far have employed phosphorothioate DNA analogs as monomers. Although increased resistance of phosphorothioate oligonucleotides to nucleases has been shown, they exhibit several disadvantages, including a low binding capacity relative to complementary nucleic acids and nonspecific binding to proteins (Guvakova *et al.*, 1995), which can cause toxic side effects that limit their clinical application (Levin, 1999). Several groups have focused on developing various types of modified oligonucleotides (Freier and Altmann, 1997; Manoharan, 1999; Nielsen, 1999; Summerton, 1999; Zhang *et al.*, 2000; Morita *et al.*, 2001). The latest such development, ENA, is expected to have better antisense activity than 2′,4′-BNA (bridged nucleic acid)/LNA (locked nucleic acid) (Morita *et al.*, 2003). In a previous study, it was shown that an RNA/ENA chimera was 40 times as active as a phosphorothioate oligonucleotide in inducing exon 19 skipping in dystrophin (Yagi *et al.*, 2004).

In this report, an 18-mer RNA/ENA chimera (ENA41C) showed the strongest induction of exon 41 skipping, resulting in an in-frame dystrophin mRNA that led to the successful production of an internally deleted dystrophin protein (Fig. 4). The induction of exon 41 skipping by an antisense oligonucleotide was first reported with a 19-mer, AoN41, that was complementary to a purine-rich sequence within exon 41, which led to exon skipping in less than 40% of dystrophin mRNA (Aartsma-Rus *et al.*, 2002). Our RNA/ENA chimera achieved much higher activity, generating exon 41^- transcripts in 90% of the dystrophin mRNA. This may be due to two factors: (1) ENA41C may have a stronger affinity for the complementary RNA sequence; and (2) the sequence targeted in this study is more critical for proper splicing than the purine-rich sequence. These data, which demonstrate the efficacy of this RNA/ENA chimera in producing internally deleted, but nonetheless pres-

ent, dystrophin molecules, suggest that it may be a promising avenue for antisense drug therapy. Further experiments to reveal other potential targets for RNA/ENA chimeras in dystrophin mRNA transcripts are in progress.

Our new RNA/ENA chimera could be used for the treatment of DMD in patients harboring a mutation in exon 41, of which there are now eight. Two of the eight mutations are 1- or 11-nucleotide deletions, and the remaining six are nonsense mutations at three different sites. It is proposed that our RNA/ENA chimera can be used in these eight patients to convert their clinical phenotypes from DMD to BMD. In future new chemicals can be designed to induce skipping of other exons located in deletion hot spots, thereby making dystrophin expression possible in a broader spectrum of DMD cases.

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ORIGINAL ARTICLE

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A novel cryptic exon identified in the 3′ region of intron 2 of the human dystrophin gene

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Abstract The dystrophin gene, which is mutated in Duchenne muscular dystrophy (DMD), is the largest known human gene and is characterized by the huge size of its introns. Intron 2, the second largest intron, is 170-kb long and has been shown to include a 140-bp cryptic exon (exon 2a) in its 5′ region. The rest of this intron has no known function. In this study, we find that another cryptic exon, located in the 3′ region of intron 2, is activated in a promoter- or tissue-specific manner. An unknown 98-bp insertion precisely between exons 2 and 3 was identified in one of the dystrophin mRNAs from lymphocytes of a DMD patient with a duplication of exon 2. This 98-bp sequence, located in the 3′ region of intron 2, was found to possess a branch point, acceptor and donor splice-site consensus sequences, and an exonic splicing enhancer sequence, and thus is a novel exon, which we named "exon 2b." In lymphocytes, exon 2b incorporation was detected in the muscle-specific, promoter-driven transcript. Five of 20 normal human tissue mRNAs, including cardiac and skeletal muscle mRNAs, were confirmed to contain a fragment extending from exon 1 to exon 2b by reverse transcription PCR amplification, indicating that exon 2b is activated in a tissue-specific manner. This provides a clue to a novel cause of dystrophinopathy.

Introduction

The human dystrophin gene, which is defective in patients with Duchenne or Becker muscular dystrophy (DMD/BMD), spans approximately 3,000 kb of the X-chromosome and encodes a 14-kb transcript consisting of 79 exons (Ahn and Kunkel 1993; Nishio et al. 1994). Genomic structural analysis disclosed at least eight alternative promoters over the entire dystrophin gene, producing tissue-specific dystrophin isoforms (Ahn and Kunkel 1993; Nishio et al. 1994). Consequently, more than 99% of the gene sequence is comprised of introns and has been considered functionless. An alternative promoter identified within the largest intron, the 250-kb intron 44, regulates the expression of a tissue-specific dystrophin isoform, and this isoform has been suggested to be required for normal intellectual development (Bardoni et al. 2000; Felisari et al. 2000). In contrast, the second largest intron, the 170-kb-long intron 2, has been shown to contain a cryptic exon, exon 2a, in its 5′ region, but the physiological role of exon 2a is still unknown (Dwi Pramono et al. 2000). Recently, a part of the 5′ region of intron 2 was shown to be incorporated into dystrophin mRNA due to an activating mutation in the splice donor site of an embedded weak exon (exon p2a) (Yagi et al. 2003). So far, two tiny segments of the huge intron 2 have been shown to be incorporated into dystrophin mRNA, leaving nearly 170 kb uncharacterized.

Splicing is the process that removes introns from pre-mRNA thereby producing mature mRNA consisting of only exons. The presence of well-defined cis elements, namely, the 5′ and 3′ splice sites and the branch point, is necessary but not sufficient to define intron–exon boundaries in pre-mRNA (Senapathy et al. 1990). Pseudoexons that match splice-site consensuses have been identified in introns, but their inclusion in mRNA is prevented by silencer elements (Sironi et al. 2004). However, unconventional splicing defects often occur at exons with weak homology to canonical splicing sequences, leading to dystrophinopathies (Tuffery-Giraud et al. 2003; Yagi et al. 2003).

Complex patterns of alternative splicing of the 5′ region of the dystrophin gene have been reported (Chelly et al. 1991; Reiss and Rininsland 1994; Torelli

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and Muntoni 1996; Surono et al. 1997). The translational reading frame rule explains genotype–phenotype correlation in dystrophinopathy; i.e., out-of-frame deletion of the dystrophin gene results in severe DMD while in-frame deletions result in mild BMD (Chelly et al. 1991; Winnard et al. 1992, 1995). However, many dystrophinopathy cases with deletions in the 5′ region of the dystrophin gene have been shown to be exceptions to this rule, and alternative splicing has been considered to be a factor leading to such an exception by changing the translational frame (Muntoni et al. 1994).

We have analyzed dystrophin mRNA expressed in peripheral lymphocytes from more than 100 cases of dystrophinopathy. Here, we identify an unknown sequence inserted into a dystrophin transcript in a case with exon 2 duplication of the dystrophin gene, and we find that the sequence is a novel cryptic exon (exon 2b) located in the 3′ region of intron 2 of the dystrophin gene. Exon 2b is incorporated into mRNA in a promoter- or tissue-specific manner. This provides a clue to a novel cause of dystrophinopathy.

Patient and methods

Case

A 5-year-old Japanese boy was referred to the Kobe University Hospital for the genetic diagnosis of DMD. He was the first-born boy, and his family history disclosed no muscular disease. At age 4, he was shown to have an extremely high level of serum CK (13,750 IU/l, normal: 56–248 IU/l) and was clinically diagnosed as DMD. Physical examination disclosed mild calf hypertrophy, and he showed Gowers' sign. Chest X-ray and ECG were normal. All analysis was done after obtaining informed consent from his parents.

Analysis of genomic DNA

Genomic DNAs were isolated from lymphocytes of DMD patients and a normal male individual using a Wizard genomic DNA extraction kit (Promega Corporation, Madison, WI, USA). Conventional PCR amplification was employed to find deletion mutations in 19 deletion-prone exons of the dystrophin gene (Chamberlain et al. 1988; Beggs et al. 1990). PCR was performed essentially, as described previously (Matsuo et al. 1991). To examine the entire dystrophin gene, Southern blot analysis of the patient's genomic DNA was performed using *Hae*III-digested cDNA fragments as probe. A genomic region encompassing the 98-bp inserted sequence (exon 2b) was amplified using primers derived from the flanking sequences (Table 1). The copy number of exons was assessed by semiquantitative, multiplex PCR. Seven segments in the 5′ region of the dystrophin gene, including exon 1; exon 1a; exon 2, a pseudoexon (exon p2a) in intron 2 (Yagi et al. 2003); exon 2a; exon 2b; and exon 3 were amplified in one PCR reaction together with the exon-19-encompassing region. Amplification was carried out in a total volume of 20 μl containing 400 ng of genomic DNA, 2 μl 10X Ex Taq Buffer (Takara Bio Inc., Kyoto, Japan), 2 μl of 2.5 mM dNTPs, 5 pmol of each primer, and 1U of Ex Taq Polymerase (Takara Bio Inc., Kyoto, Japan). PCR cycling conditions were as follows: an initial denaturation at 94°C for 5 min followed by 20 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, extension at 72°C for 2 min, and a final extension at 72°C for 5 min. To quantify the amplified products, 1 μl of each reaction mixture mixed with 5 μl of the loading buffer solution containing size markers (15 and 1,500 bp) was analyzed by capillary electrophoresis (Agilent 2001 Bioanalyzer with DNA 1000 Lab Chips, Agilent Technologies, Palo Alto, CA, USA). The amount of each PCR product was quantified by measuring the peak area and calculating the ratio of this area to that of exon 19. The sequences of the primers used in this study are listed in Table 1. All

Table 1 Primer sequences

Target region	Forward primer	Reverse primer
Exon 1	SQQPmF: TAGACAGTGGATACATAACAAATGCATG	SQQPmR: TTCTCCGAAGGTAATTGCCTCCCAGATCTGAGTCC
Exon 1a	SQQ1aF: GAGCTATTTGCCACTTTTACCG	SQQ1aR: GGCCTGTTAGAAAGTGACATTC
Exon 2	SQQ2F: AAAAGAAAACATTCACAAAATGGG	SQQ2R: GTGTATCTTTGCCATATCTTCTGC
Exon P2a	int2SKF: TTCCATTTTCTCCGCAGCCC	int2SKR: GCATCATCAGCAAAACCTTCCG
Exon 2a	g2aF: TAGAGTTATCCTAGAGAGGTGG	g2aR: TCACGTGCATCATCCAGCAAC
Exon 2b	g2bF: AAAGGCTTGATACACATGGATA	g2bR: AGGTAGGGCAGGATAAATCGT
Exon 3	SQQ3F: TCATCCGTCATCTTCGGCAGATTAA	SQQ3R: CAGGCGGTAGAGTATGCCAAATGAAAATCA
Exon 19	SQQ19F: TTCTACCACATCCCATTTTCTTCCA	SQQ19R: GATGGCAAAAGTGTTGAGAAAAAGTC

PCR oligonucleotide primers were synthesized off site (Hokkaido System Science Co. Ltd., Sapporo, Japan).

Analysis of dystrophin transcripts

Total RNA was isolated from peripheral lymphocytes, as previously described (Matsuo et al. 1991). A fragment encompassing exons 1–5 of dystrophin mRNA was analyzed by reverse-transcription (RT), seminested PCR. The first PCR was done to amplify the region comprising exons 1–8 using primers located in each exon (M1: ATGCTTTGGTGGGAAGAAGTAG and c8R: TGTTGAGAATAGTGCATTTGATG, respectively) followed by the second amplification of a fragment comprising exons 1–5 (primer c5R: TGCCAGTGGAGGATTATATTCCAA), as described previously (Suminaga et al. 2002).

To examine the promoter specificity of exon 2b incorporation, fragments stretching from promoter-specific exon 1 to exon 2b were amplified from lymphocyte cDNA. PCR primers were designed to detect promoter-specific transcripts. PCR detection of transcripts from the L, M (exon 1), C, or P promoters was performed using different exon-1-specific forward primers (L1: ACTGACACATAGAGTAAC, C1: TTGATTTGTTACAGCAGCCAACTTAT, M1, and P1: CCAGGTTTACCATACCCCATAGA, respectively). A reverse primer for exon 2b (ex 2b: GGAGGTTGCATTGAGTTGAG) was used in combination with one of each of the unique exon-1-specific primers. cDNA corresponding to 0.2 μg of the RNA samples was subjected to PCR amplification.

To examine the efficiency of exon 2b activation in different tissues, fragments spanning from exon 1 to exon 2b and from exon 1 to exon 5 were amplified from cDNA prepared from total RNA from 20 human tissues (adrenal gland, brain cerebellum, whole brain, fetal brain, fetal liver, heart, kidney, liver, whole lung, placenta, prostate, salivary gland, skeletal muscle, spleen, testis, thymus, thyroid gland, trachea, uterus, and spinal cord; BD Biosciences, San Jose, CA, USA). cDNA corresponding to 0.2 μg of each RNA sample was subjected to PCR amplification. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was amplified as a control. PCR reactions (20 μl) contained 1 μl of cDNA and 200 nM of each primer and 250 μM dNTPs. PCR was performed using 2 μl of 10× Ex Taq buffer (Takara Bio Inc., Kyoto, Japan) and 1 U of Ex Taq polymerase (Takara Bio Inc., Kyoto, Japan). A 5-min, 94°C denaturation step was followed by 30 cycles of PCR (94°C denaturation for 0.5 min, 60°C annealing for 0.5 min, 72°C extension for 0.5 min) followed by extension at 72°C for 7 min. A 10-μl sample of each PCR reaction was separated on an agarose gel containing 0.2 mg/ml of ethidium bromide, prior to photography.

DNA sequencing

For DNA sequencing, amplified products were separated by electrophoresis in low-melting-point agarose gels. Bands of amplified products were cut out, and the DNA was purified. The purified DNA was subcloned into vector pT7 (Novagen, Inc., Madison, WI, USA) and the inserted DNA was sequenced using an automated DNA sequencer (model 373A, Perkin–Elmer Applied Biosystems Inc., Norwalk, CT, USA).

Results

PCR amplification of the selected 19 exons of the dystrophin gene disclosed neither deletion mutations in the index case nor any deletions or duplications detected by the conventional Southern blot analysis. However, PCR amplification of the region encompassing exon 2 appeared to result in a larger amount of amplified product from the case than from the control. This was quantified using capillary electrophoresis. In the coamplified products of the exon 2 and 19 encompassing regions, the ratio of exon 2 peak area to exon 19 peak area of the index case was twice that of the control (0.76 versus 0.34; Fig. 1). This indicated that exon 2 was duplicated in the genome of the index case.

In order to examine the duplication of exon 2 at the mRNA level, dystrophin mRNA expressed in the patient's peripheral lymphocytes was analyzed. The region encompassing exons 1–5 was amplified by RT nested PCR. Remarkably, one barely visible, weak band and as well as two major, equally dense bands were obtained (Fig. 2). Each of the bands was sequenced after subcl-

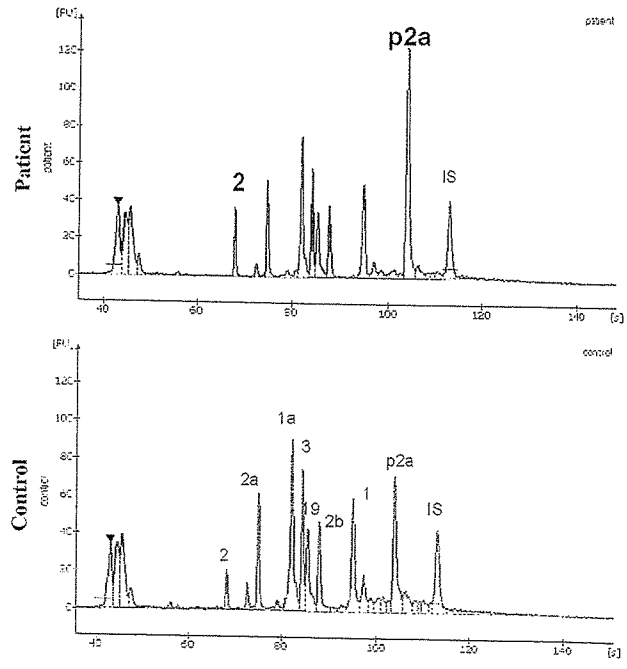

Fig. 1 Quantification of PCR products. Capillary electrophoretic patterns of PCR products are shown. Eight genomic regions were coamplified in one PCR reaction, and the products were separated using capillary electrophoresis. The position of each amplified product of exons 1, 1a, 2, p2a, 2a, 2b, 3, and 19 is marked above its peak (*lower*). The peak area of exons 2 and p2a is nearly double in the patient (*upper*). IS refers to 1,500-bp marker