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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

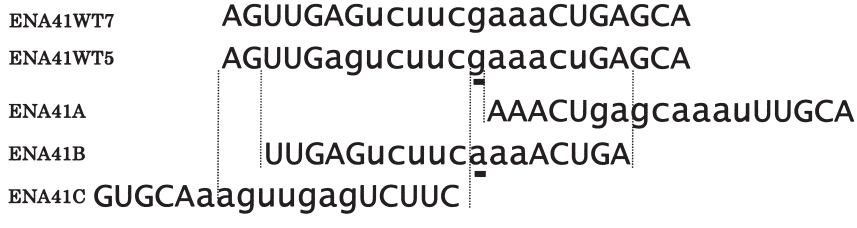

FIG. 1. Sequences of RNA/ENA chimeras. ENA41WT5 and ENA41WT7 are complementary to the wild-type sequence, whereas ENA41B is complementary to the mutation sequence. Upper case and lower case letters indicate ENA and 2′-*O*-methyl RNA monomers, respectively. Underlined letters represent the mutation site.

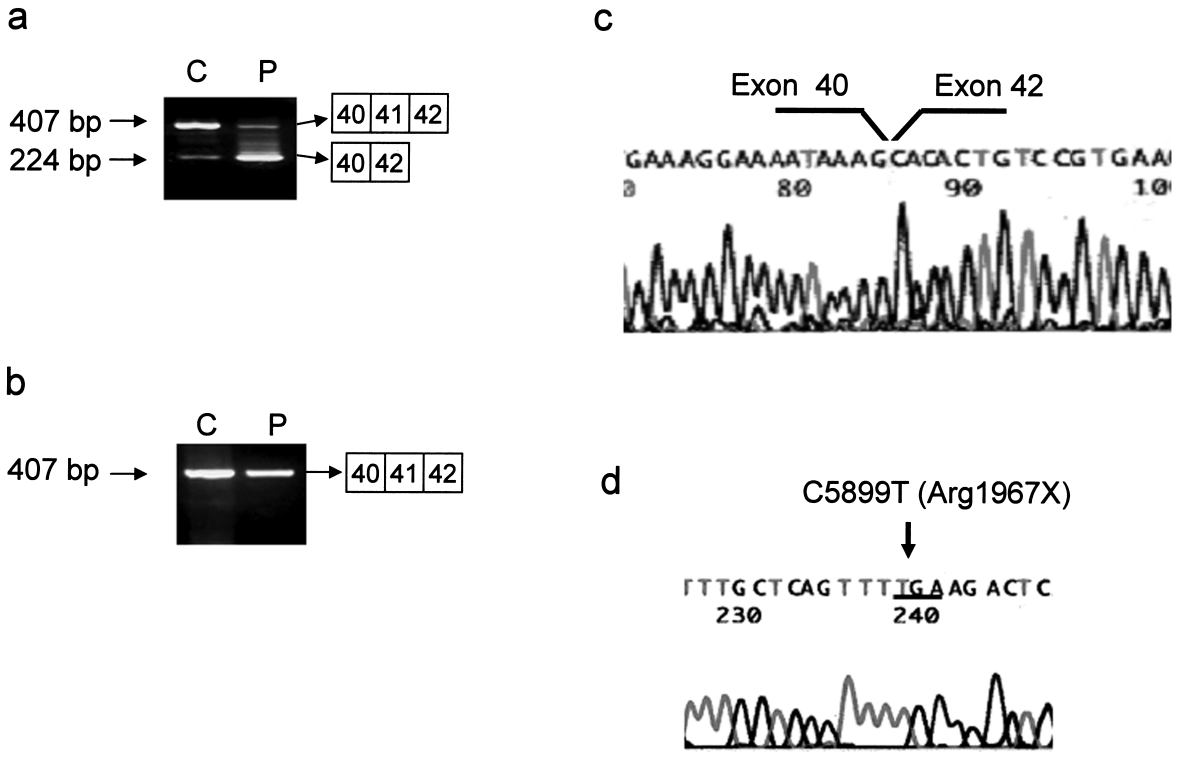

FIG. 2. Amplification of dystrophin cDNA encompassing exons 40 to 42. (**a**) Amplified products from lymphocytic cDNA. From the control (C), one major band (407 bp) and an additional minor band (224 bp) were amplified. From the patient (P), two amplified products were obtained: the major band (224 bp) 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

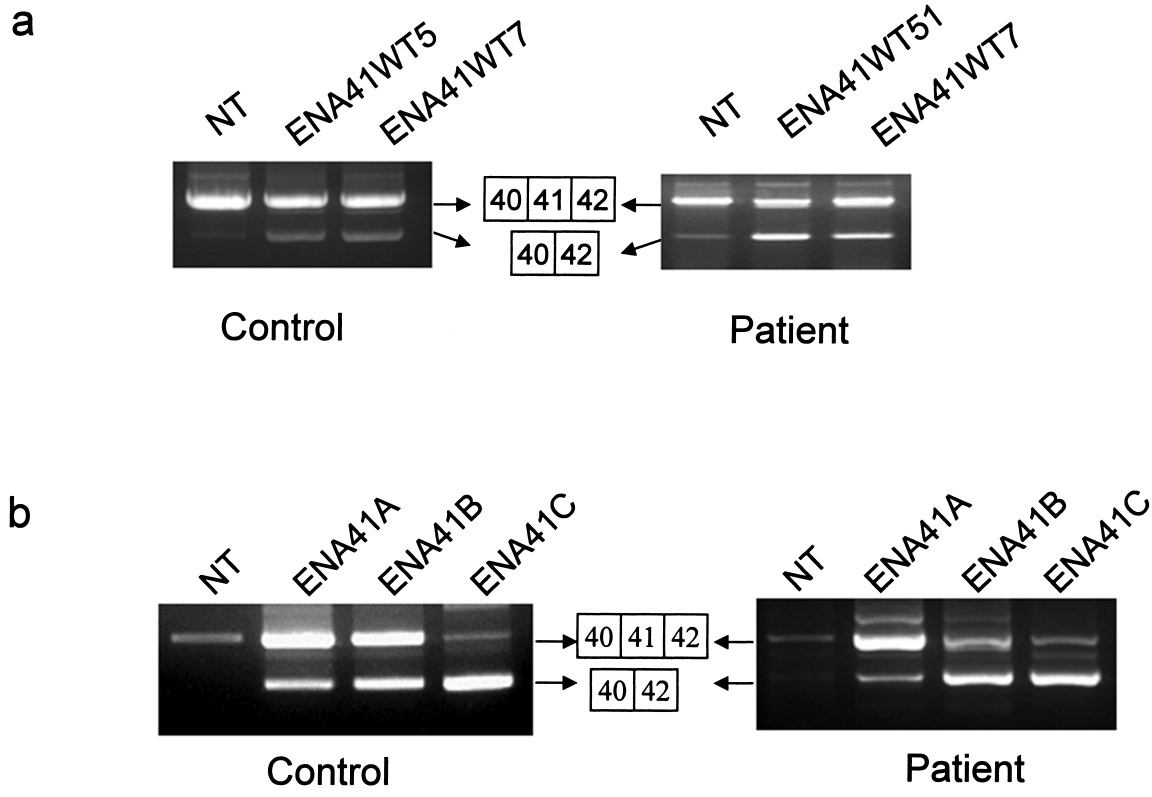

FIG. 3. Effects of transfection of RNA/ENA chimeras. (**a**) Transfection of ENA41WT5 or ENA41WT7. The region from exon 40 to exon 42 of the dystrophin mRNA transcript was amplified from cDNA obtained from normal control- and patient-derived myocytes 24 hr posttransfection. Remarkably, an extra product 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).

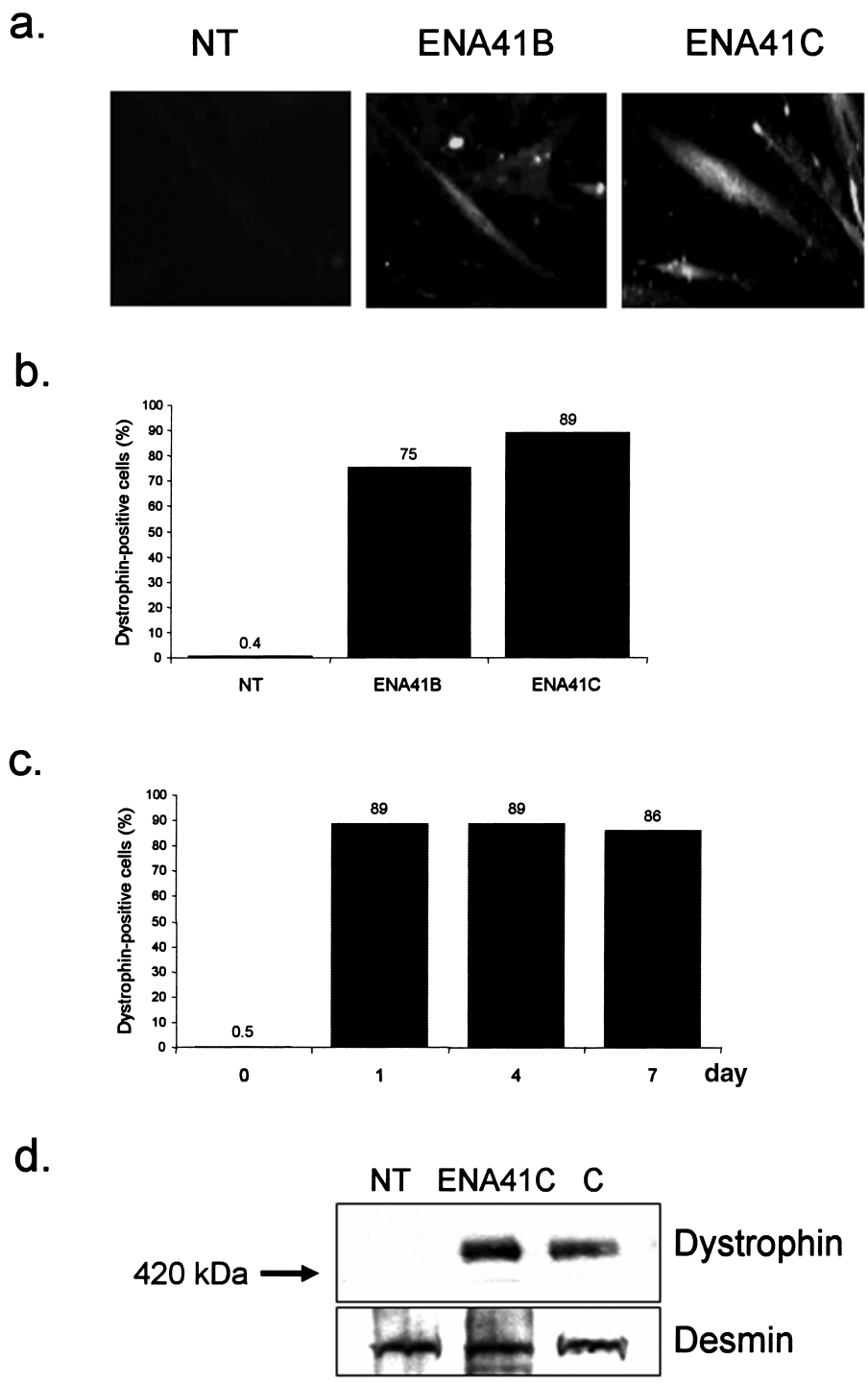

FIG. 4. Dystrophin analysis of RNA/ENA-treated myocytes from a patient. (**a**) Dystrophin staining. No dystrophin signals could be detected in untreated cells (NT) stained with Dys-2, whereas clear, mainly cytoplasmic, dystrophin signals could be detected after treatment with either ENA41B or ENA41C. (**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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