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デュシェンヌ型筋ジストロフィーのアンチセンス治療法の開発に関する研究

平成１７年度　総括研究報告書

主任研究者　松尾　雅文

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厚生労働科学研究費補助金（こころの健康科学研究事業）
研究報告書

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I 総括研究報告

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（研究要旨）

デュシェンヌ型筋ジストロフィー(DMD)の治療法としてジストロフィン遺伝子のエクソンのスキッピングをスプライシング時に誘導する方法が注目されている。これは、DMD患者にみられるジストロフィンmRNAのアミノ酸読み取り枠のずれをエクソンのスキッピングを誘導することにより修正し、ジストロフィンを発現させるものである。私達はアンチセンスオリゴヌクレオチドを用いてジストロフィン遺伝子のエクソンのスキッピングを誘導することに世界で初めて成功するなど、本治療法開発において世界をリードしてきた。最近、新しく開発された修飾核酸(ENA)からなるアンチセンスオリゴヌクレオチドのRNA/ENAキメラが、従来のアンチセンスオリゴヌクレオチドに比較して飛躍的に高いエクソンスキッピング誘導能を有することを示す結果を世界で初めて得た。このためRNA/ENAキメラは、DMDの患者の治療に最適な核酸として大きく注目されている。

本研究は、RNA/ENAキメラの実用化を世界に先駆けて図るもので以下の研究を行うものである。①ジストロフィン遺伝子欠失のホットスポット領域にあるエクソンを対象として、そのエクソンのスキッピングを誘導する最適のRNA/ENAキメラを同定する。そのため、培養筋細胞に様々な合成RNA/ENAキメラを導入し、そのエクソンスキッピング誘導能を解析する。②同定した最適のRNA/ENAキメラをDMD患者培養筋細胞に導入し、導入細胞におけるジストロフィン発現を免疫組織化学的方法などを用いて検討する。そして、各DMD患者がそれぞれに有するエクソン欠失の型に対応した治療用のRNA/ENAキメラを決定する。

本研究は、RNA/ENAキメラを用いたエクソンのスキッピング誘導により、DMD患者培養筋細胞でジストロフィンの発現を世界で初めて実証するもので、DMD治療の確立を大きく促進するものである。本研究内容全般については、すでに本学部の医学倫理委員会にて承認を得ているものである。

Ⅱ分担研究報告

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本研究は神戸大学大学院医学系研究科の松尾雅文と竹島泰弘が共に分担研究者として共同して研究を推進してきた。本報告書は重複を避けるため2名の研究内容をまとめて記したものである。

A. 研究目的

本研究では、デュシェンヌ型筋ジストロフィー(DMD)患者が有する遺伝子欠失に対応したエクソンのスキッピングを誘導するのに最適なRNA/ENAキメラの探索を培養筋細胞を用いた系を用いてまず行う。ついで、RNA/ENAキメラを患者筋細胞に導入しジストロフィンmRNAが修正され、ジストロフィンが発現されることを検証する。

また、ジストロフィン異常症患者の分子病理を解明することにより、RNA/ENAキメラの探索の効率化をはかる。

B. 研究方法

1）培養筋細胞を用いた最適なRNA/ENAキメラの探索

ジストロフィン遺伝子の欠失のホットスポット領域にあるエクソンを標的として、そのエクソンのスキッピングを誘導する能力を有するRNA/ENAキメラを探索する。そのために、エクソンの配列と相補的な配列からなる候補RNA/ENAキメラを多数合成する。合成したRNA/ENAキメラを培養筋細胞に導入し、ジストロフィンmRNAを解析し、エクソンのスキッピングの誘導能を解析する2）患者由来培養筋細胞でのジストロフィン発現

先に明らかにした最適のRNA/ENAキメラを、患者培養筋細胞に導入する。導入した筋細胞で標的としたエクソンがスキッピングしたジストロフィンmRNAの産生をRT-PCR法で確認する。ついで、ジストロフィンmRNAでのエクソンのスキッピングを確認したのち蛍光免疫染色法を用いて培養細胞レベルでジストロフィンの発現を確認する。

3）DMD患者でのエクソンスキッピングの解析

ジストロフィン遺伝子のスプライシング制御機序の破綻はDMD/BMD患者で発見され、その破綻機序の解析は本研究のRNA/ENAキメラの決定に重要な情報をもたらす。そのため、本研究ではDMD/BMD患者の分子病理の解析を進めた

C. 研究成果

ジストロフィン遺伝子のエクソン51のスキッピングを誘導するRNA/ENAキメラの同定を重点的に実施した。エクソン51の配列に相補的な配列からなる18塩基のRNA/ENAキメラを各種合成した。そして、それぞれのRNA/ENAキメラを培養筋細胞に導入し、エクソン51のスキップした産物の検出を行った。多くの合成RNA/ENAキメラの中で、エクソンのスキッピングを誘導するものを同定した。

ジストロフィン遺伝子のエクソン51のスキッピングを誘導することにより治療し得る欠失のタイプは様々である。その中でジストロフィン遺伝子のエクソン52を欠失したDMDで本治療による効果を検討した。その結果、患者由来培養筋細胞でジストロフィンmRNAからエクソン51のスキッピングを誘導することに成功した。そこで、培養筋細胞のジストロフィン染色を行うとジストロフィン陽性細胞の検出が出来た。このことは、本RNA/ENAが患者の治療に応用し得ることを示した。

また、エクソン42に2塩基の欠失を有するDMDでジストロフィンmRNAの解析を行ったところ、エクソン42のスキッピングがあることを明らかにした。さらに、in vitroのスプライシング反応系でこの欠失した領域がスプライシングの制御に極めて重要な位置を占めていることを明らかにした。これはRNA/ENAキメラのデザインにおいて本領域を標的とすることの科学的証明となった。

D. 考察

本年度の研究においてはジストロフィン遺伝子のエクソン51のスキッピングを誘導する治療に用いられ得るRNA/ENAキメラの同定に成功した。また、エクソン42ではスプライシング促進配列を明らかにし、この配列に対するアンチセンスオリゴの投与が有効なことを示す結果を得た。したがって、今後本研究を推進することにより、より一層多くの症例で治療し得るRNA/ENAキメラの同定が可能になると考えられた。

本研究の成果は当初の予想通りに得られた。したがって、本研究課題は今後も当初の計画通りの成果が得られるものと考えられる。

E. 結論

本年度においてはジストロフィン遺伝子の欠失のホットスポット領域に存在するエクソンス51のスキッピング誘導能について検討し、DMD患者由来筋細胞でのジストロフィンの発現を誘導することに成功した。さらに、他のエクソンでもスプライシング促進配列を明らかにした。このように、本研究は順調に成果を挙げてきており今後継続して実施することにより、より一層大きな成果が挙げられるものと強く期待される。

F. 健康危険情報

特記事項なし

G. 研究発表

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H. 知的財産権の出願・登録状況

1. 特許取得
   なし
2. 実用新案登録
   なし
3. その他
   なし

研究成果の刊行に関する一覧表

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Becker Muscular Dystrophy

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INTRODUCTION

Becker muscular dystrophy (BMD) is an X-linked recessive inherited disease with a worldwide incidence of 1 in 35,000 male births. Becker muscular dystrophy is characterized by progressive muscle wasting but is distinguished by delayed onset, later dependence on wheelchair support, and longer life span from Duchenne muscular dystrophy (DMD) that follows severe progressive muscle wasting.

Dystrophin, the gene defective in not only BMD but also DMD, was isolated in 1986. Since then, genetic diagnosis of BMD has been done leading to better understanding of the disease process, and the difference between DMD and BMD can be explained at the molecular level by the reading frame rule.

CLINICAL DIAGNOSIS

Becker muscular dystrophy patients show normal growth and development in their early childhood. In BMD, affected men start to show disturbance of walking due to muscle weakness at 20s or over. He maintains to walk but his muscle strength gradually decreases. Dilated cardiomyopathy is sometimes an initial clinical sign for the diagnosis of BMD. The muscle weakness involved in BMD follows a mild downward course with patients living near normal lives.[1]

Serum creatine kinase (CK) is markedly increased. This marked elevation of serum CK is the most important hallmark for the diagnosis of BMD, but the level of elevation of serum CK is not so high compared to DMD. During the asymptomatic period, elevation of serum CK is the sole sign for BMD. Some BMD patients are identified accidentally because of elevations of AST or ALT which are commonly examined for liver function as serum CK elevation is accompanied with elevations of AST and ALT.

Becker muscular dystrophy should be included in the differential diagnosis of moderately elevated serum CK in males. Pathological examination of biopsied muscle consolidates the diagnosis of BMD.

GENE DIAGNOSIS

Becker muscular dystrophy is caused by mutations of the dystrophin gene that is also mutated in DMD.[2] Therefore, both DMD and BMD are sometimes called as dystrophinopathy. Furthermore, not only types but also locations of mutations identified in both BMD and DMD are quite similar.[3] For gene diagnosis of BMD both multiplex PCR and Southern blot analysis have been employed as in the genetic diagnosis of DMD.[4–6] Nearly two-thirds of mutations identified on the dystrophin gene are deletions or duplications occupying a single or multiple exons. Every mutation identified in BMD cases would be examined based on the following reading frame rule.[3] In the rest of the BMD cases it is rather difficult to identify the responsible mutations on the dystrophin gene as a single nucleotide change is supposed to be present. However, some point mutations that induced exon skipping have been reported.[7,8]

READING-FRAME RULE OF DMD/BMD

Although both DMD and BMD patients have been shown to have deletion or duplication mutations of the dystrophin gene, the extent of the deletion does not always correlate with the severity of the disease: some BMD patients with mild symptoms have deletions encompassing numerous exons, whereas some DMD patients with severe symptoms lack only a few exons. In some cases, the long deletions resulting in BMD and the short deletions resulting in DMD may even overlap. The reading-frame rule explains the difference between DMD and BMD as follows: in DMD the translational reading frame of the dystrophin mRNA is shifted after a deletion or duplication mutation whereas it is maintained in BMD.[3] According to the reading-frame rule, BMD patients with long deletions are able to produce dystrophin mRNA that would still direct the production of an internally truncated semifunctional protein. Shorter deletions harbored by severe DMD patients, on the other hand, would bring together exons that, when spliced, would change the translational reading frame in the mRNA, such that a

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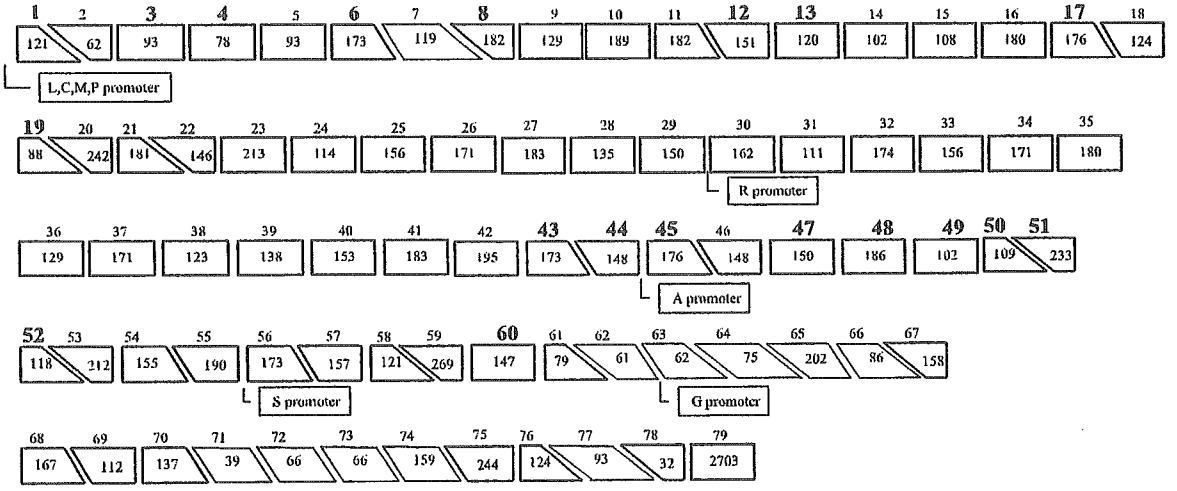

Fig. 1 Schematic description of the exon structure of the dystrophin gene. The dystrophin gene consisting of 79 exons (boxes) and at least eight alternative promoters (boxes under the lined boxes) is schematically described. Numbers over the box indicate exon number, the bold numbers being the exons that are examined by multiplex PCR. Quadrilaterals and parallelograms indicate in-frame exons (type 0 exons). Trapezoids indicate out-of-frame exons (type 1 or type 2 exons).

premature stop codon is created. This rule predicts that milder BMD patients would produce a smaller semifunctional protein whereas DMD patients would either produce a severely truncated dystrophin lacking the entire C-terminal region or would not produce dystrophin at all.

Subsequent gene analyses have shown that over 90% of the deletion–duplication mutations that cause BMD maintain the dystrophin mRNA reading frame whereas those causing DMD are frameshifts.[9] Accordingly, point mutations identified in DMD are nonsense mutations[10] except in rare DMD cases with missense mutations.[11,12]

Considering that molecular therapy for DMD to change the reading frame from out-of-frame to in-frame has been proposed,[13,14] it is important to see the resulting translational reading frame of dystrophin mRNA after the identification of a deletion or duplication mutation. Exons of the dystrophin gene are classified into three types according to the number of nucleotides encoded in the exon (Fig. 1): 1) in-frame exon that encodes nucleotides of multiples of 3 (type 0 exon); 2) two out-of-frame exons that have nucleotides of multiples of 3+1 or 2 (type 1 exon or type 2 exon, respectively). Among the 79 exons, 40, 18, and 21 exons are classified into types 0, 1, and 2 exons, respectively. In cases with deletion/duplication of the dystrophin gene the reading frame can be determined as described in Fig. 1. Cases having a deletion of a type 2 exon, e.g., exon 45, should be DMD based on the reading frame rule. Although gene diagnosis of DMD/BMD has been conducted, not all DMD/BMD cases have been examined for its reading frame.

In other types of mutations, nonsense mutations are expected to be identified in DMD. However, nonsense mutation that should result in DMD phenotype has been identified in BMD cases,[8,15] where exon skipping is shown as a mechanism that modified clinical phenotype. Furthermore, BMD has been shown to have a nonsense mutation in in-frame exons.[16–18] Detailed analysis of genotype–phenotype correlation would lead a better understanding of molecular mechanism of dystrophinopathy.

PATHOLOGICAL DIAGNOSIS

The pathological examination of biopsied skeletal muscle confirms the diagnosis of BMD. Immunohistochemical analyses of normal muscle demonstrate that dystrophin is present along with muscle cell membranes. Muscle from BMD patients contains reduced amounts of dystrophin that is stained discontinuously and patchy along the muscle cell membranes.[19] Western blot analysis using dystrophin antibody reveals a band corresponding to 427 kDa, close to the predicted size of dystrophin, in extracts of normal muscle tissue. Shorter or lower amount of dystrophin is detected in muscle extracts from patients with BMD.

Dystrophin contains 3685 amino acids organized in four domains: N-terminal actin binding, triple helical rod, cystein-rich, and C-terminal domains. The internally truncated dystrophin identified in BMD maintains both N-terminal and C-terminal domains, but lacks some of the 24 repeat sequences of triple helical rod domain. Therefore, dystrophin is stained when antibody recognizing either N-terminal or C-terminal domains is used, but in

some cases no dystrophin is stained as in DMD when antibody recognizing rod domain is employed.[20]

TREATMENT

For BMD patients, supportive therapies such as rehabilitation or ventilator support are clinically employed, but no effective way to improve the clinical course is available. Gene therapy has been considered a cure for BMD but no clinically applicable way has been established.

CONCLUSION

Becker muscular dystrophy is a mild muscle wasting disease and characterized by dystrophin abnormality in skeletal muscle. Currently, no effective treatment is available although a molecular understanding of BMD developed well.

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Duchenne Muscular Dystrophy

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INTRODUCTION

Duchenne muscular dystrophy (DMD) is a common inherited disease with a worldwide incidence of 1 in 3500 male births. DMD is a lethal disorder of childhood characterized by progressive muscle wasting. Affected individuals are wheelchair-bound by the age of 12 and succumb to cardiac or respiratory failure in their mid to late 20s.

Dystrophin, the gene defective in DMD was isolated in 1986. Since then, genetic diagnosis of DMD has been done leading to better understanding of the disease process. Based on the molecular pathogenesis of DMD, molecular therapies for DMD have been proposed.

CLINICAL DIAGNOSIS

Patients with DMD show normal growth and development in their early childhood. In DMD, affected boys start to show disturbance of walking and frequently fall because of muscle weakness at 4 to 5 years of age. Patients are shown to have a positive Gower's sign wherein the child climbs up his thighs to extend the hips and push his trunk up. He manages to walk but his muscle strength gradually decreases. He loses the ability to climb up stairs. Lumbar lordosis becomes more exaggerated and the waddling gait increases. Patients usually are wheelchair-bound by the age of 12. Muscle wasting progresses as the patients get older until finally respiratory or cardiac failure develops due to muscle wasting.

Serum creatine kinase (CK) is markedly increased 50 times more than the normal range in infantile DMD. This marked elevation of serum CK is the most important hallmark for the diagnosis of DMD. During the asymptomatic period, elevation of serum CK is the sole sign for DMD. Some DMD patients are accidentally identified due to elevations of AST or ALT, which are commonly examined for liver function, because serum CK elevation is accompanied with elevations of AST and ALT.

GENE DIAGNOSIS

Duchenne muscular dystrophy is caused by mutations of the dystrophin gene.[1] The dystrophin gene is 3000 kb in size and consists of 79 exons encoding a 14-kb mRNA.[2,3] At least eight alternative promoters that are regulated in a tissue- or development-specific manner have been identified on the dystrophin gene. The unusually high incidence of DMD in all human populations could be simply a reflection of the enormous mutation target size of the gene, but the recombination rate is reported to be four times the rate expected for a gene of this size.[4] Nearly two-thirds of mutations identified on the dystrophin gene are deletions or duplications occupying a single or multiple exons, with the rest of the DMD cases having other types of mutations including point mutations. Remarkably, deletion mutations have been localized to two deletion hot spots of the dystrophin gene, the 5′ and the central regions.

Multiplex PCR Analysis

Currently, multiplex PCR analyses that amplify deletion-prone exons are used as the first step for gene diagnosis. Two sets of PCR amplification are used to screen 19 deletion-prone exons (exons 1, 3, 4, 6, 8, 12, 13, 17, 19, 43–45, 47–52, and 60).[5,6] Using this method of examination, one finds that nearly half of the DMD cases are shown to have deletion mutations. Therefore, the rest of the cases need further examination to identify the responsible mutation in the dystrophin gene.

Southern Blot Analysis

To examine the deletion/duplication in every exon of the dystrophin gene, Southern blot analysis is used, using segments of the dystrophin cDNA as probes.[7] Two-thirds of DMD patients are shown to have recombination events of deletions or duplications spread in one or more exons at the genomic DNA level.[8] However, Southern blot analysis not only needs high-quality DNA and radioisotope, but it is also time-consuming.

Detection of Fine Mutation

In DMD cases that have no large recombination event, identification of the causative mutation remains a laborious goal because of the difficulty in detecting a single point mutation in the 3000-kb-sized gene. To

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facilitate the identification of mutations in the dystrophin gene, more than 99% of which is made up of introns, dystrophin mRNA that is 100 times smaller than the dystrophin gene has been analyzed.[9] Analysis of dystrophin mRNA expressed in lymphocytes leads to not only identification of rare genomic mutations, but also to disclosures of nonauthentic alternative splicing.[10–12] In addition, several ways to identify small mutations have been proposed.[13–17] In the advent of recent advances in mutation analysis techniques, more than 90% of DMD cases are shown to have mutations in the dystrophin gene.[18,19]

PATHOLOGICAL DIAGNOSIS

The pathological examination of biopsied skeletal muscle confirms the diagnosis of DMD. Immunohistochemical analyses of normal muscle demonstrate that dystrophin is present along with muscle cell membranes. In DMD, dystrophin is missing from skeletal muscle.[20] Western blot analyses using dystrophin antibody reveals a band corresponding to 427 kDa, close to the predicted size of dystrophin, in extracts of normal muscle tissue, whereas no protein can be detected in DMD.

TREATMENT

For DMD patients, supportive therapies such as rehabilitation or ventilator support are clinically employed but no effective way to improve the clinical course is available. Since the discovery of the dystrophin gene, gene therapy is now considered an attractive way to cure the disease. The main aim of DMD gene therapy is to establish a way to inject constructed dystrophin genes consisting of partial- or full-length cDNA joined to an appropriate promoter. Although much progress has been made in this field of study, we still seem to be a long way from achieving a clinically significant result. As an alternative for gene transfection, molecular therapies have been studied including antisense oligonucleotide treatment[21–23] or translational readthrough treatment using gentamicin.

Antisense Oligonucleotide Treatment

An alternative strategy for DMD treatment is to retard the progression of the clinical symptoms, i.e., to convert DMD into the BMD phenotype. Theoretically, this therapy can be done by changing a frame-shift mutation causing DMD into an in-frame mutation characteristic of BMD by modifying the dystrophin mRNA. Artificial induction of exon skipping with antisense oligonucleotides is a way to make the out-of-frame dystrophin mRNA in-frame. Artificial induction of exon 19 skipping using an antisense oligonucleotides against the splicing enhancer sequence has been reported,[24] and this treatment was shown to produce dystrophin expression in exon-20-deleted DMD myocytes.[23] Disruption of the splicing enhancer sequence to induce exon skipping was further evidenced by the fact that in the nonsense mutation of exon 27 the dystrophin gene resulted in exon 27 skipping, producing an in-frame dystrophin mRNA.[25] In addition, another natural example causing conversion of DMD to BMD was identified in a nonsense mutation in exons 25 and 29.[26,27] Furthermore, BMD has been shown to have a nonsense mutation in in-frame exons.[28,29]

Antisense oligonucleotides against a purine-rich sequence have been used to induce skipping of exons 44, 45, 46, 49, 50, 51, or 53.[22,30,31] In these studies, induction of exon skipping led to the expression of dystrophin in their respective dystrophin-deficient myocytes by correcting the translational reading frame. Recently, double exon skipping of exon 43 and 44 or exon 45 and 51 has been induced.[32] This extends the application of the antisense oligonucleotide treatment to more varieties of deletion mutations of the dystrophin gene.

Phosphorothioate DNA has been the standard choice for the clinical application of antisense technology.[33–37] However, phosphorothioate DNA is associated with a variety of potentially toxic non-antisense effects.[38] In order to develop less toxic antisense oligonucleotides, nucleic acids have been modified in various ways.[39,40] Recently, morpholino modified oligonucleotides were shown to be delivered to muscle cells efficiently.[41] Furthermore, the chimera of 2′-*O*-methyl RNA and 2′-*O*, 4′-*C*-ethylene-bridged nucleic acid (ENA) was shown to induce exon 19 skipping of the dystrophin gene 40 times stronger than the conventional phosphorothioate oligonucleotides.[42]

Translational Readthrough of Stop Codon

Aminoglycoside antibiotics have been suggested as possible therapeutic interventions for treating patients who carry a nonsense mutation because of the ability of these antibiotics to lead translational readthrough of stop codons. To evaluate whether aminoglycosides can be used to suppress the nonsense mutation in a human DMD case, four DMD/BMD cases with various stop codon sequences were tested once daily with intravenous gentamicin at 7.5 mg/kg/day for 2 weeks. However, the full-length dystrophin protein was not detected in posttreatment muscle biopsies.[43] The possible reason for the failure of gentamicin treatment in human cases is the difference in efficiency of aminoglycoside-induced readthrough among the different types of nonsense mutations.

CONCLUSION

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

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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-

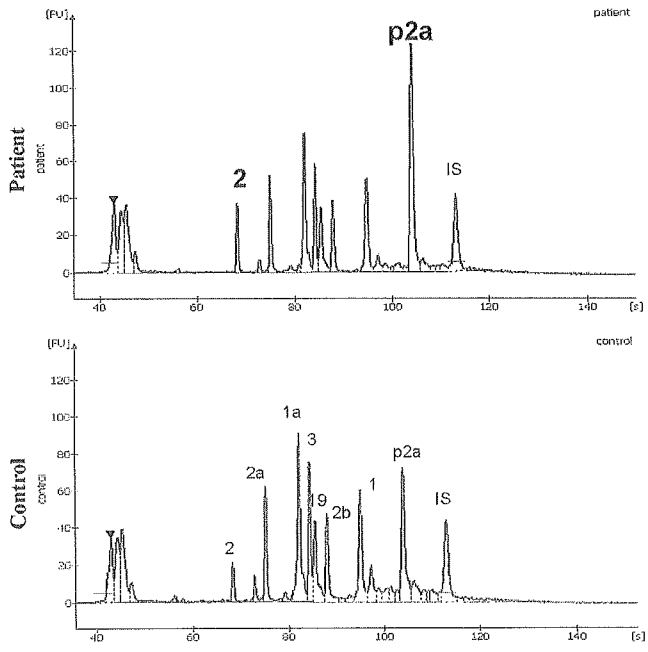

Fig. 1 Quantification of PCR products. Capillary electrophoretic patterns of PCR products are shown. Eight genomic regions were coamplified in one PCR reaction, and the products were separated using capillary electrophoresis. The position of each amplified product of exons 1, 1a, 2, p2a, 2a, 2b, 3, and 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

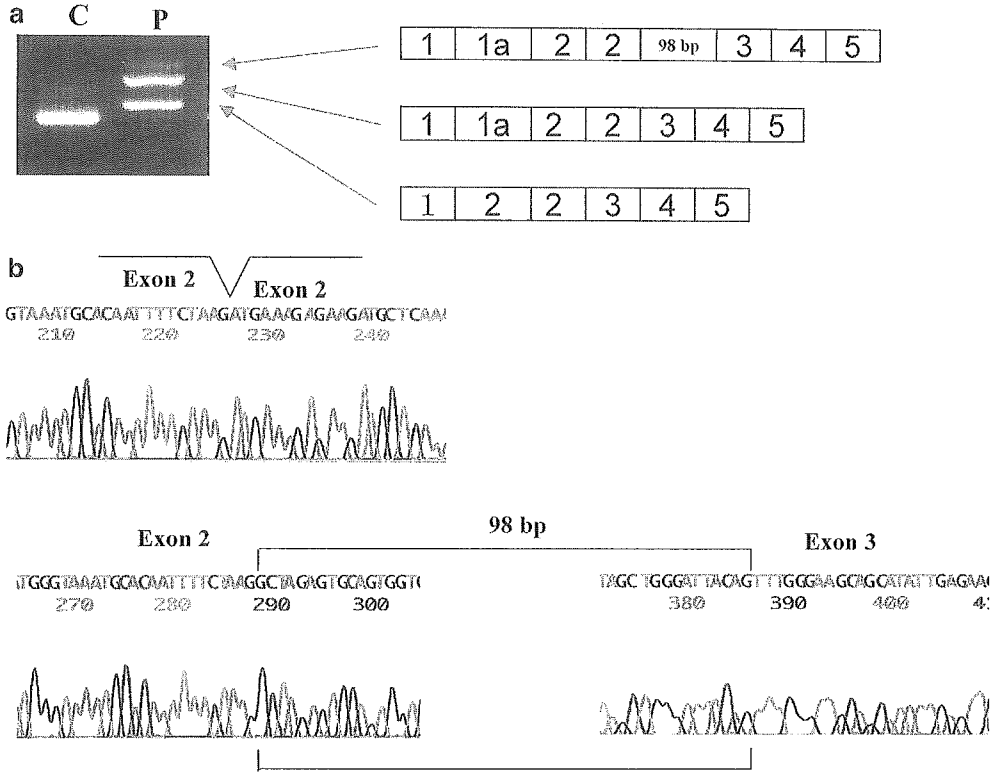

Fig. 2 Reverse transcription (RT)-PCR amplification of a fragment comprising exons 1–5 from lymphocyte mRNA. **a** RT-PCR products: Three PCR products were visualized on the gel from the index case—one barely visible, and two others clearly visible and with equally dense bands (*P*). One discrete amplified product was obtained from the control (*C*). A schematic representation of exon organization in the amplified fragments is shown at the *right* of the products. *C* and *P* refer to control and index case, respectively. **b** A partial sequence of each product is shown. Sequences joining the double exon 2 are shown (*upper*). The 3′ terminal sequence of exon 2 (CTAAG) is joined precisely to the 5′ end of the sequence of exon 2 (ATGAA). Partial sequences at the junctions of the inserted sequence and its flanking authentic exons are shown (*lower*). The terminal sequence of exon 2 (CTAAG) is joined precisely to the 5′ end of the 98-bp inserted sequence (GCTAG), and at its 3′end (TACAG), the unknown sequence is joined precisely to the 5′ end of the sequence of exon 3 (TTTGG)

oning. Sequencing of the smallest fragment revealed a sequence of tandem exon 2 sequences between exons 1 and 3 (Fig. 2). Sequencing of the middle-sized band revealed an insertion of exon 1a between exon 1 and 2 in addition to duplication of exon 2. The sequence of the largest fragment revealed the same exons as in the middle-sized one, but, remarkably, an unidentified 98-bp sequence was found to be inserted precisely between tandem exon 2 and exon 3 (Fig. 2). Since all three dystrophin mRNAs contained tandem exon 2 sequences, we concluded that the index case had a duplication of exon 2. This mutation created a premature stop codon at 15th codon of the duplicated exon 2 sequence and was determined as a cause of DMD.

The fact that the 98-bp sequence was inserted precisely between exons 2 and 3 led us to speculate that the sequence could be a retained intron or an unknown exon. A BLAST search of the 98-bp sequence revealed an identical sequence in the 3′ region of intron 2 (bases 10151–10054 of GenBank AL121880). The 98-bp sequence was located 82 kb downstream from exon 2a and 29 kb upstream of exon 3 (Fig. 3). Remarkably, the AG and GT dinucleotides that are absolutely conserved at the splice acceptor and donor sites of all introns, respectively, were identified immediately adjacent to the 5′ and 3′ ends of the 98-bp sequence (Fig. 3). The Shapiro probability scores for splice acceptor and donor sites were 0.77 and 0.74, respectively (Shapiro and Senapathy 1987). Furthermore, the sequence TATTAAT, a perfect match to the branch-point consensus sequence, was identified 84 bp upstream of the novel sequence (Fig. 3). A polypyrimidine tract was also identified between the putative branch point and the splice acceptor site (Fig.3). Splicing enhancer sequences have been identified in exon sequences and are critical for proper incorporation of exons into mRNA (Schaal and Maniatis 1999). The splicing enhancer sequence within the 98-bp sequence was examined by ESE Finder (Cartegni et al. 2003). The heptanucleotide CTCCCGG in the middle of exon 2b has a score (3.74) higher than the threshold score (1.95) for SF2/ASF binding, and we consider it a strong candidate for a splicing enhancer. Since the 98-bp-inserted sequence exhibited all of the characteristics typical of a genomic exon and was inserted between authentic dystrophin exons, we refer to it as the novel exon 2b.

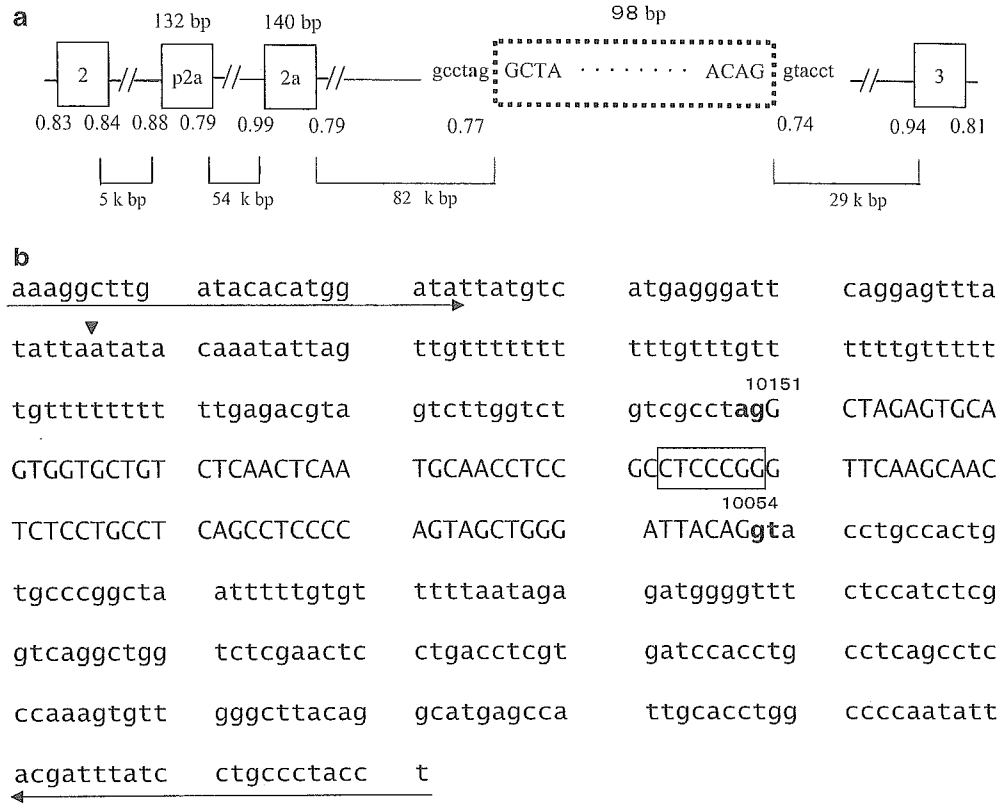

Fig. 3 Genomic structure and sequence. **a** Schematic description of intron 2 of the dystrophin gene. Intron 2 is the second largest intron (170 kb). One cryptic exon (exon 2a) is located 59 kb downstream from the 3′ end of exon 2. One pseudoexon (exon p2a) is 5 kb downstream of exon 2. The 98 bp of the unidentified sequence is identical to the intron 2 sequence (nt 10151–10054, GenBank AL121880) located 82 kb downstream of exon 2a and 29 kb upstream of exon 3. Remarkably, two nucleotides, both upstream and downstream of the sequence, are AG and GT dinucleotides that are conserved at splicing acceptor and donor sites, respectively. Their Shapiro probability scores for splice donor and acceptor sites are 0.77 and 0.74, respectively. *Boxes* and *lines* indicate exons and introns, respectively. The *dotted box* indicates the novel cryptic exon, *brackets* indicate the size, and the *numbers under the boxes* show the Shapiro's splicing probability scores at splicing sites. **b** Genomic nucleotide sequences of the inserted 98-bp sequence and its flanking introns are shown. The 98 bp of exon 2b are shown in *uppercase* letters, and the 139 and 184 bp upstream and downstream of the exon 2b sequence, respectively, are shown in *lower case* letters. Absolutely conserved AG and GT (*bold*) dinucleotides are present at the boundaries between exon 2b and its flanking regions. The branch point was identified 84 bp upstream of exon 2b within the consensus sequence tattaat (*inverted triangle*). Between exon 2b and the branch point, a polypyrimidine tract was identified. The exonic splicing enhancer sequence is *boxed*. *Horizontal arrows* indicate the locations and directions of primers. *Superscripted reference numbers* indicate numbered nucleotides of AL121880 (GenBank)

As we supposed that a genomic mutation located near exon 2b activated the incorporation of exon 2b in the index case, we examined the genomic sequence encompassing exon 2b. A total of 421 bp of genomic DNA was PCR amplified, but the sequence of the product was completely normal (data not shown), thus, no genomic mutation contributing to the activation of exon 2b was found.

Although tandem exon 2 sequences were identified in the case's dystrophin mRNA, only one exon 2b was identified. This fact suggested two possibilities: (1) a single copy of exon 2b was present in his genome, or (2) exon 2b was duplicated but only one copy was incorporated. To explore this, the copy numbers of exon 2b and nearby exons were examined by coamplification of the regions encompassing exons 1, 1a, 2, p2a, 2a, 2b, 3, and 19 (Fig. 1). The amplified products were quantified. The ratio of the peak area of exon 2b to that of exon 19 from the case was the same as that of the male control, indicating the presence of a single copy of exon 2b in the case's genomic DNA. We conclude that a single exon 2b downstream of the duplicated exon 2 was activated. In addition, the exon p2a to exon 19 peak area ratio was twice that of the control while those of exons 1a and 2a were the same as the control. This indicated that the duplicated region extended from exon 2 to exon p2a but not to exon 1a or 2a. The size of the duplication was calculated to be at least 5.5 kb (Fig. 3).

Though we have analyzed dystrophin mRNAs in peripheral lymphocytes obtained from more than 100 dystrophinopathy cases, exon 2b incorporation has never before been identified (Matsuo et al. 1991; Hagiwara et al. 1994; Surono et al. 1999; Adachi et al. 2003; Yagi et al. 2003). In order to see whether exon 2b activation is common to all exon 2 duplication mutations, we analyzed dystrophin mRNA from lymphocytes of