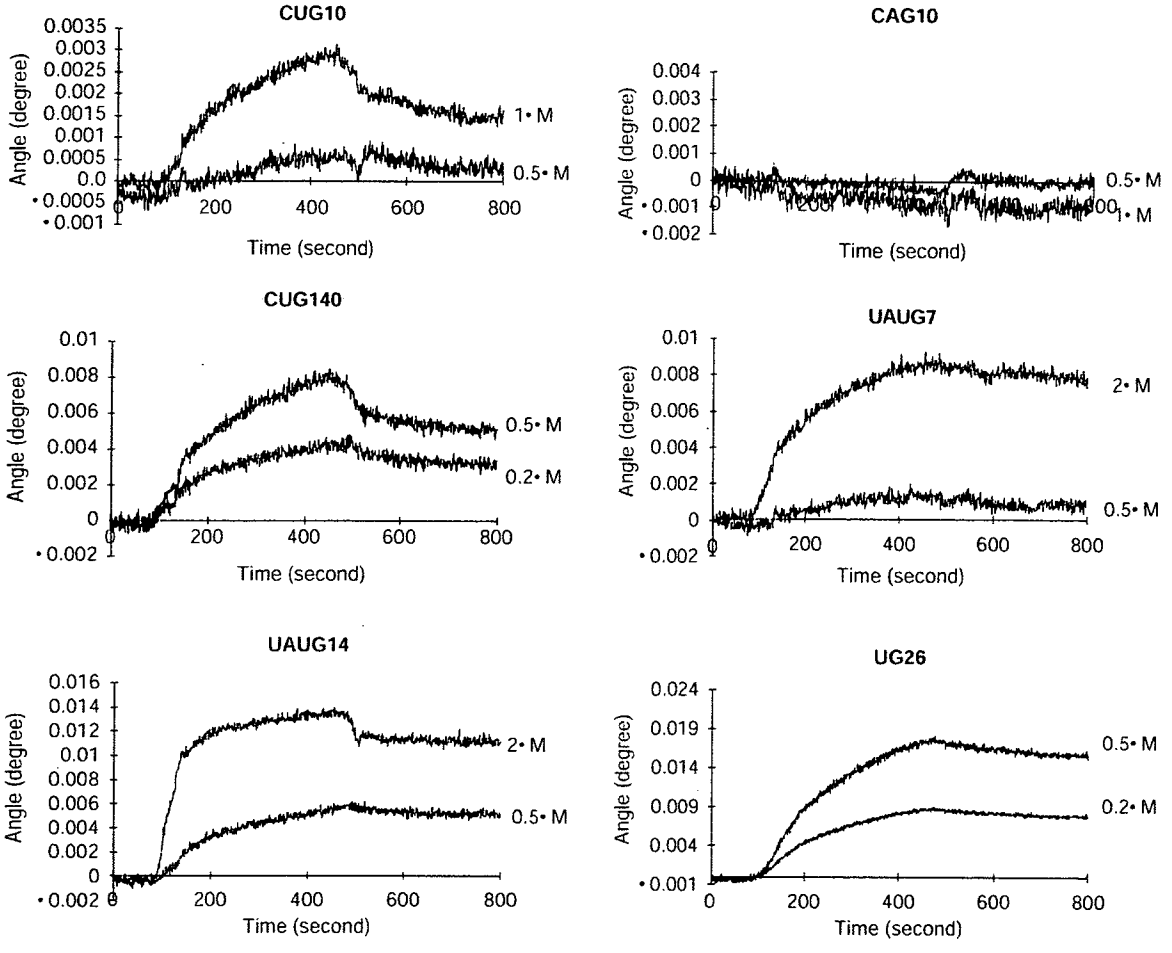

Fig. 3. Binding of various repetitive RNAs in the SPR analysis. SPR signals obtained with repetitive RNAs were monitored. Each RNA sequence is indicated above the panel. Concentrations (mM) represent that of injected RNA.

Table 1. Binding constants of CUG-BP with di-, tri- and tetra-nucleotide repeats.

Repeat	K_d (mM)	k_{ass} ($M^{-1}s^{-1}$)	k_{diss} (s^{-1})
(CA)15	–	–	–
(CAG)10	–	–	–
(CUG)10	27• 12	4.6• 2.5• 10^2	6.6• 0.3• 10^{-3}
(CUG)140	6.0• 2.0	2.1• 1.3• 10^3	9.2• 0.9• 10^{-3}
(UAUG)7	1.3• 0.1	2.1• 0.2• 10^3	2.6• 0.2• 10^{-3}
(UAUG)14	1.3• 0.3	2.6• 0.3• 10^3	3.4• 1.1• 10^{-3}
(UG)15	0.25• 0.10	9.0• 1.0• 10^3	2.3• 1.1• 10^{-3}
(UG)26	0.12• 0.05	9.6• 1.7• 10^3	1.1• 0.03• 10^{-3}
(UG)41	0.06• 0.02	3.1• 0.7• 10^4	1.9• 0.7• 10^{-3}

N = 3, mean• SE.

$(UGG)_{10}$ and $(UUG)_{10}$ showed similar binding to CUG-BP1, while $(CUG)_{10}$, which contains the same number of UG motifs, showed a 7-fold increased K_d value. This apparently suggests that CUG is not an optimal binding motif for CUG-BP1, despite its name.

Mutation Analysis of CUG-BP1 in a Yeast Three-Hybrid System—Although CUG-BP1 has three RRM RNA-binding domains, it is still unknown which RRM is responsible for binding to a UG repeat. Previously, we reported that these three RRMs redundantly contributed to binding to a UG repeat (15). To determine the RNA-binding domain of CUG-BP1 more specifically, we conducted a yeast three-hybrid assay using several RRM mutants, in which two conserved residues in the respective RRM were disrupted. Mutation of one of any RRMs reduced binding of CUG-BP1 to UG repeats (Fig. 4). Double mutants did not have substantial-binding ability. On the other hand, the results of deletion mutants indicated that the C-terminal region of CUG-BP1 has an important function to bind to UG repeats. N1 and N2 (or N3) mutants did not show ability to bind to UG repeats. In contrast, a deletion mutant of both RRM1 and RRM2 (C2) still showed binding to a UG repeat. In addition, the experiment showed that a small portion of CUG-BP1 linker region was also essential for efficient binding to UG repeats.

Table 2. K_d values of CUG-BP with di-, tri- and tetra-nucleotide repeats (30 nt.).

Repeat	Sequence (30 nt)	K_d (mM)
(UG)15	UGUGUGUGUGUGUGUGUGUGUGUGUGUG	0.25 • 0.1
(UG)15mut	UGUGUGUGUGUGUAUGUGUGUGUGUGUG	0.52 • 0.05
(UAUG)7	UAUGUAUGUAUGUAUGUAUGUAUGUAUGUA	1.3 • 0.1
(UUGG)7	GUUGGUUGGUUGGUUGGUUGGUUGGUUGGU	3.3 • 0.4
(UUUGGG)5	UUUGGGUUUGGGUUUGGGUUUGGGUUUGGG	–
(UA)15	UAUAUAUAUAUAUAUAUAUAUAUAUAUA	–
(UGG)10	UGGUGGUGGUGGUGGUGGUGGUGGUGGUGG	4.1 • 0.6
(UUG)10	UUGUUGUUGUUGUUGUUGUUGUUGUUGUUG	3.6 • 0.1
(CUG)10	CUGCUGCUGCUGCUGCUGCUGCUGCUGCUG	27.2 • 12
(CAG)10	CAGCAGCAGCAGCAGCAGCAGCAGCAGCAG	–

N = 3, mean • SE. (UG)15mut has UA in the seventh UG repeat (underlined).

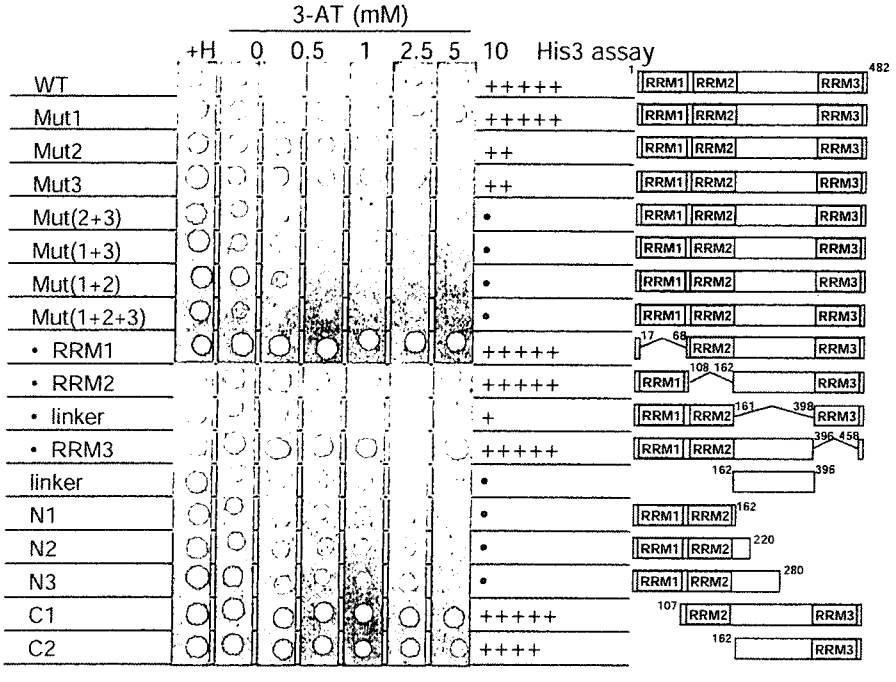

Fig. 4. Yeast three-hybrid analysis of CUG-BP1 mutants. Structures of CUG-BP1 mutants used in the assay are shown on the right. Mutated RRM motifs are indicated by hatched boxes. Results of the yeast three-hybrid HIS3 assay are shown in the middle. Wild-type or mutant CUG-BP1 constructs and the $(UG)_{24}$ expression vector were transformed into yeast cells. Transformants were seeded to test their viability on the selection plates, lacking histidine but containing 0.5, 1, 2.5, 5 or 10 mM 3-amino triazole (3-AT), a competitive inhibitor of histidine synthesis. Viability of yeast transformants was classified as follows: +++++, ++++, +++, ++, + means, viable with 10, 5, 2.5, 1 and 0.5 mM 3-AT, respectively and – means, not viable with 0.5 mM 3-AT.

DISCUSSION

CUG-BP1 Specifically Binds to (UG) Dinucleotide Repeat RNA in an SPR Assay—We previously reported that CUG-BP1 preferentially bound to UG dinucleotides in a yeast three-hybrid assay (15). In the current report, we used a SPR assay to determine actual binding constants of CUG-BP1 with RNAs in vitro. In the SPR assay, CUG-BP1 bound to $(CUG)_8$ and $(CUG)_{140}$ repeats, but not to a $(CAG)_{10}$ repeat. However, the dissociation constants obtained in our experiments between CUG-BP1 and $(CUG)_{10}$ or $(CUG)_{140}$ suggested that CUG-BP1 does not bind very strongly to the CUG triplet repeat in vitro or possibly under physiological conditions. The moderate binding between CUG-BP1 and CUG repeats in addition to differences in experimental systems may explain some of the previous controversial results.

In contrast to binding to the CUG repeat, CUG-BP1 bound strongly to UG dinucleotide repeats in the SPR assay. Subsequently, a comparative analysis using UG-like repetitive sequences (Table 2) revealed that CUG-BP1 prefers 'pure' UG dinucleotide repeats as binding targets, rather than other UG-rich sequences, such as $(UAUG)_7$ or $(UUG)_{10}$. Because longer UG repeats seemed to bind more strongly to CUG-BP1 (Table 1) and pure UG repeat tracts were sufficient for binding to CUG-BP1, we conclude that CUG-BP1 recognizes UG repeats as a

binding target. Importantly, a recent report showed that ETR-3, the closest CELF protein to CUG-BP1, recognized UG repeats as well as UGUU, which were identified from systematic evolution of ligands by exponential enrichment (SELEX) (21). These results suggest that these two CELF proteins have similar binding specificities. However, UGUU motifs were included in the $(UUG)_{10}$ repeat used in our study; binding was not as strong as to $(UG)_{15}$ in the assays. Thus, CUG-BP1 may bind more specifically to UG motifs than UGUU motifs. However, we cannot exclude that efficient recognition of UGUU motifs by CUG-BP1 requires a particular spacing of nucleotides between the UGUU motifs.

The Importance of RRMs in the Binding to UG Repeats—In our previous study, we examined the binding affinities of CUG-BP1 deletion mutants in a yeast three-hybrid assay and showed that no complete loss of RNA-binding ability of CUG-BP1 occurred when any of the three RRMs was singly deleted (15). This suggests redundant RNA recognition among the RRMs. We attempted to reveal more detail about the structural requirements of the RNA-binding abilities of CUG-BP1 using additional mutant proteins.

Deletion analyses indicated that the N-terminal fragment (containing RRM1 and RRM2) did not show ability to bind to UG repeats, although Mut3 had binding affinity to UG repeats. On the contrary, the C-terminal fragment (containing RRM3) of CUG-BP1 harbored UG repeat-binding abilities, although Mut(1 + 2) did not show any binding to UG repeats. These results indicate that a conformational change occurs in deletion mutants, and that a conformational and cooperative interaction of three RRMs is important for CUG-BP1 function.

Moreover, a deletion of linker (• linker) strongly reduced binding affinity to UG repeats. Taken that the linker region itself did not have RNA binding ability into account, this result indicates that the linker region is important for cooperative RNA-binding by both N- and C-terminus, possibly by modulating the conformation of the entire protein. Alternatively, it is conceivable that this linker region modulates the RNA-binding abilities or functions of CUG-BP1 by mediating multimer formation, as reported in a study of EDEN-BP, a CUG-BP1 ortholog in frogs (22).

Biological Implication of UG Binding of CUG-BP1—The characterization of the RNA-binding specificity of a protein is important in understanding its function or physiological role. In general, the presence of multiple binding motifs for an RNA-binding protein would enhance the probability of the protein binding to the RNA. From our results, it is predicted that the number of UG motifs in an RNA stretch will determine the affinity of CUG-BP1 for it. If this is so, the degree of influence on target RNAs of CUG-BP1 would be variable, depending on the content or length of a UG repeat. Indeed, we observed length-dependent binding of CUG-BP1 with UG repeats (Table 1).

Although there are many pure UG repeat-containing genes in the human genome (15), few of them have been analysed as targets of CUG-BP1. It would be of value to focus on such genes to obtain further insight into CUG-BP1 function. Several reports have described functional target sequences of CUG-BP1 (3–5). However, there is no previous example of a pure UG repeat as a functional target of CUG-BP1. Thus, it is of importance to examine the functional interaction between CUG-BP1 and UG-containing genes. Because CUG-BP1 may have pathogenic roles in DM, identification of its target genes may be a beneficial way to understand the molecular mechanism of this disease.

UG-binding ability is apparently a feature of CUG-BP1 (and ETR-3); another RNA-binding protein, TDP-43, also preferentially binds to a UG repeat (23). As TDP-43 is a splicing regulator, like CUG-BP1, there may be some functional connection, such as antagonism, between these proteins that bind to UG repeat-containing RNAs. In addition, functional competition or cooperation between CUG-BP1 and ETR-3 or other CELF proteins, which may share RNA substrates, would be an interesting area of future work toward understanding the importance of UG-binding proteins.

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Expression of MBNL and CELF mRNA transcripts in muscles with myotonic dystrophy

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Abstract

Myotonic dystrophy type 1 (DM1) is an autosomal dominant disorder that causes muscle wasting, myotonia, cardiac conduction abnormalities, and other multi-systemic symptoms. Current evidence supports a pathogenic mechanism involving aberrantly expanded CTG repeats in the 3′-untranslated region of the DM protein kinase (*DMPK*) gene. The repeats are thought to recruit various RNA-binding proteins such as muscleblind-like (MBNL) proteins into foci in the nuclei of DM cells, resulting in loss of function. However, aberrant regulation of transcription or subsequent RNA processing of MBNL-family mRNAs might also be part of the pathogenic mechanism of DM. We used real-time RT-PCR analysis to examine the possibility that MBNL mRNA expression is altered in DM1 patients. We also examined mRNA expression for members of the CUG-BP and ETR-3-like factor (CELF) family of RNA-binding proteins given that CELF proteins regulate alternative splicing and are also implicated in DM. We found that DM1 muscles displayed aberrant regulation of alternative splicing as reported previously; however, the levels of MBNL and CELF mRNA expression did not show any significant changes. Our results suggest that the expression and stability of the mRNA for these RNA-binding proteins are unaffected in DM1.

Keywords: Alternative splicing; CUG-BP- and ETR-3-like family; Muscleblind; Myotonic dystrophy

1. Introduction

Myotonic dystrophy (DM) is an autosomal dominant disorder and the most common form of muscular dystrophy affecting adults [1]. Multiple systems are affected in DM patients, and characteristic symptoms include muscle hyperexcitability (myotonia), cataracts, defects in cardiac conduction, mental retardation, and insulin resistance [1].

Two forms of DM have been identified thus far, DM1 and DM2. The gene affected in DM1 is *DM protein kinase* (*DMPK*) on chromosome 19q. This gene contains CTG trinucleotide repeats within its 3′-untranslated region (UTR) [2,3]. The expansion of this repeat has been known to trigger the pathogenesis of DM1 and interestingly, the number of repeats is thought to be correlated with symptom severity [3].

The gene affected in DM2 is *zinc-finger protein 9* (*ZNF9*). This gene contains CCTG tetranucleotide repeats in intron 1, and as in DM1, expansion of this repeat is believed to be the cause of this disease [4]. The most strongly supported pathogenic hallmark is that the expanded repeat-containing mRNA transcribed from the altered *DMPK* and *ZNF9* genes forms foci that are retained within the nuclei of DM cells [4–6]. Given that DM1 and DM2 have phenotypic overlap in spite

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of the different loci of *DMPK* and *ZNF9*, this finding suggests that the expanded repeats themselves cause DM. Indeed, transgenic mice expressing human skeletal actin containing expanded CUG repeats manifest some DM symptoms, including myotonia [6].

The nuclear foci found in DM cells appear to recruit certain RNA-binding proteins, thus disrupting the proper functions of these proteins (loss of function) [7,8]. One family of RNA-binding proteins thus affected is the muscleblind (MBNL) family, consisting of MBNL1, MBNL2, and MBNL3 in humans [8]. Each of these proteins has been shown to co-localize with RNA foci in both DM1 and DM2 cells [8]. The MBNL proteins are zinc-finger proteins that bind to both CUG and CCUG repeats [9,10], and act as regulators of alternative splicing of certain genes that are strongly implicated in some of the symptoms of DM1. In particular, the myotonia and insulin resistance of DM1 are caused by defects in chloride channel and insulin receptor (IR) proteins, respectively, that arise because the MBNL protein is trapped in the nuclear foci and thus suffers loss of function [11–13]. Importantly, MBNL1-knockout mice exhibit some DM symptoms such as myotonia and cataracts [11], which strongly supports the involvement of MBNL proteins in the pathogenesis of DM1.

Another group of proteins strongly implicated in DM pathogenesis is the CUG-BP and ETR-3-like factor (CELF) family, which regulates alternative splicing [14–19], translation [20,21], and deadenylation [22]. Upregulation of CUG-BP protein expression has been implicated in the DM1 mechanism [14,23], suggesting that abnormal activation of CUG-BP might be involved in DM1 pathogenesis. In addition, some studies have suggested a strong involvement of CELF in DM pathogenesis, indicating that CUG-BP transgenic mice exhibit DM-like symptoms [23,24]. CUG-BP and other CELF members can regulate alternative splicing of various pre-mRNAs that are important in DM1 pathogenesis, including pre-mRNAs for IR and cardiac troponin T (cTNT) [16,24–28].

Loss of function of MBNL proteins [11,13] and altered activity [23,24,29] or localization of CELF proteins [30] have been suggested as possible pathogenic mechanisms in DM. However, aberrant regulation of transcription or subsequent RNA processing of MBNL- and CELF-family mRNAs might also be part of the DM mechanism. To our knowledge, however, this has not been previously addressed.

The functions of non-coding RNA have been the subject of increased attention. These functions include roles in DNA replication, chromosome maintenance, and regulation of transcription, as well as RNA processing, translation, and mRNA stability. One study reported that expanded CUG repeats in *DMPK* tend to form a double-helical RNA hairpin, which could be a source of microRNA and/or small interfering RNA. It also noted that MBNL contains a sequence that is almost complementary to the CUG repeats of *DMPK* [31]. These findings suggest that the CUG repeats in *DMPK* mRNA may silence expression of MBNL1, although this mechanism has not been confirmed experimentally.

In the present study, we examined whether altered expression of MBNL and CELF proteins has any relevance for understanding DM pathogenesis. Using RT-PCR, we compared the expression of MBNL and CELF mRNA in DM1 patients and non-DM individuals to determine whether expression is indeed altered in DM1.

2. Materials and methods

2.1. Tissue samples

Biopsy materials were obtained from the biceps (brachii muscle) or quadriceps (femoris muscle) of 20 DM1 patients (11 males and 9 females, 11–68 years old) and 12 confirmed non-DM individuals with no histological abnormality. All samples were stored at −80 °C. Clinically, all DM1 samples had muscle weakness with myotonia. Myotonic discharge that was detectable by EMG. Onset was during childhood or adolescence except for a 21-year-old patient who had congenital onset. Pathologically, all DM1 samples showed myopathic change with variation in fiber size. Some displayed fibers with internalized nuclei (>5%; 16/20 patients, 90%), type 1 fiber predominance (10/20, 50%), endomysial fibrosis (16/20, 80%), adipose tissue replacement (6/20, 30%), sarcoplasmic mass (7/20, 35%), and pyknotic nuclear clumps (17/20, 85%). Relatively extensive fibrosis was seen in three patients and mild adipose tissue replacement in two.

All biopsy materials used in this study were acquired with informed consent.

2.2. Real-time RT-PCR

Total RNA was isolated from the biopsy samples using Concert™ Cytoplasmic RNA Reagent (Invitrogen, Carlsbad, CA), treated with DNase, and purified by standard phenol–chloroform extraction and isopropanol precipitation. cDNA synthesis was performed using a template consisting of 100 ng total RNA and the ThermoScript™ RT-PCR System (Invitrogen) with a mixture of oligo$(dT)_{20}$ and random hexamers on a 10-μl scale. The cDNA was then diluted 50-fold with sterile water, and 10 μl of diluted cDNA was used for the real-time RT-PCR measurements.

All primers used for generation of a standard curve template and for the quantification of IR-A, IR-B, GAPDH, β-actin, HPRT, MBNL and CELF mRNAs were designed using Primer 3 software (Whitehead Institute for Biomedical Research, Totowa, NJ). The primer sequences (shown $5' \rightarrow 3'$) were as follows. IR-A:

TGCTG CTCCT GTCCA AAGAC and GAGAT GGACT GGGGA CGAAA; IR-B: TTCGT CCCCA GAAAA ACCTC and CACCG TCACA TTCCC AACAT; GAPDH: GAGTC AACGG ATTTG GTCGT and AATGA AGGGG TCATT GATGG; β-actin: GACAG GATGC AGAAG GAGAT TACT and TGATC CACAT CTGCT GGAAG GT; HPRT: TGAGG ATTTG GAAAG GGTGT and CATCT CGAGC AAGAC GTTCA; MBNL1: GGGTT TGTTG GTTTC ACTG and TGTCC CGAAT TGGTG TGA; MBNL2: CCACC ACGCC TGTTA TTGTT and CCCTG CATAC CTCCA GTTTG; CUG-BP: CTGGA AGCCA GAAGG AAGGT and GCAGG TCCTG ATCAC CAAAC; ETR-3: CAGGG TGATG TTCTC TCCAT TT and GCCTC GACTC AGCCC ATC.

Total RNA from skeletal muscle (BD Biosciences, Franklin Lakes, NJ) was reverse-transcribed to synthesize the cDNA, which was then used as the template for the standards. Three housekeeping genes were chosen as calibration standards: β-actin, HPRT, and GAPDH.

cDNA calibrators were prepared by PCR amplification of HEK293 cell cDNA with the primers listed above. The resulting PCR products yielded unique bands by agarose gel electrophoresis and were purified by gel extraction using GeneEluteTM Agarose Spin Columns (Sigma, St. Louis, MO) followed by standard phenol–chloroform extraction. The concentrations of these calibrators were determined using a spectrophotometer (NanoDrop Technologies, Wilmington, DE). Concentrations were calibrated from 1.0×10^{-5} to 1.0×10^{-9} μg/μl by serial 10-fold dilutions. The PCR products above were cloned into pGEM®-T-Easy vector (Promega, Madison, WI) and their sequences were confirmed with the CEQTM 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA).

Real-time PCR was performed on a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) using SYBR Premix Ex Taq™ (TaKaRa Bio, Tokyo, Japan). The thermal profile consisted of an initial incubation at 95 °C for 10 s followed by 40 cycles at 95 °C for 5 s and 60 °C for 34 s. To assure specific amplification, dissociation temperatures were measured after each run. The 7300 Real-Time PCR System software was used to determine the crossing points for the individual samples, including those for the calibration standards. The expression levels of the target genes were normalized relative to expression of the β-actin gene.

For each run, data acquisition and analysis was performed using the 7300 Real-Time PCR System software. As all samples were available only in a limited amount, the mean values and p-values were determined by Student's t-test, but not the variance.

2.3. Splicing assays

cDNAs produced as described above were used as templates for the RNA splicing assay. PCR was performed using the primers and thermal conditions described below. For the cTNT splicing assay, the forward and reverse primers (shown 5′ → 3′) were ATAGA AGAGG TGGTG GAAGA GTAC and GTCTC AGCCT CTGCT TCAGC ATCC, respectively; 35 cycles of amplification were performed, each consisting of 30 s at 96 °C, 30 s at 63 °C, and 30 s at 72 °C, followed by a final 5 min extension at 72 °C. For the IR splicing assay, the forward and reverse primers were CCAAA GACAG ACTCT CAGAT and AACAT CGCCA AGGGA CCTGC, respectively; 35 cycles of amplification were performed, each consisting of 30 s at 96 °C, 30 s at 60 °C, and 30 s at 72 °C, followed by a final 5 min extension at 72 °C. The PCR products were resolved on a 10% polyacrylamide gel that was stained with ethidium bromide and analyzed using an LAS-3000 luminescence image analyzer (Fujifilm, Tokyo, Japan).

3. Results

3.1. Aberrant splicing in DM1

We used RT-PCR to examine several genes already known to undergo abnormal alternative splicing to determine whether aberrant regulation of alternative splicing was evident in the study samples. Cardiac troponin T (cTNT) is known to shift from an immature isoform to a mature isoform during heart development and it has been reported that cardiac tissues and skeletal muscle from DM1 patients display an inappropriate retention of fetal exon 5 of cTNT [32]. Our RT-PCR analysis using RNA isolated from 20 DM patients and 12 non-DM individuals showed significant promotion of exon 5 inclusion in DM skeletal muscle (Fig. 1a), thus supporting the previous findings. Furthermore, as was also reported previously, inclusion of exon 5 was notably promoted in DM tissues, with a 1.8-fold increase in the average ratio of exon 5 inclusion (Fig. 1b).

Alternative splicing of the 36-nucleotide exon 11 of the insulin receptor (IR) gene yields two isoforms, IR-A (exon 11 removed) and IR-B (exon 11 retained). The IR-B isoform is predominant in the insulin-responsive tissues that are responsible for glucose homeostasis, such as adipose tissues, liver, and skeletal muscle whereas in skeletal muscle from DM1 patients, the IR-A isoform is most common [14]. This switching of IR isoforms has not been observed in other myopathies [14]. We found that retention of IR exon 11 was strongly suppressed in DM1 patients, whereas IR-B was predominant in non-DM individuals. The average ratio of exon 11 retention decreased from 0.56 (non-DM) to 0.26

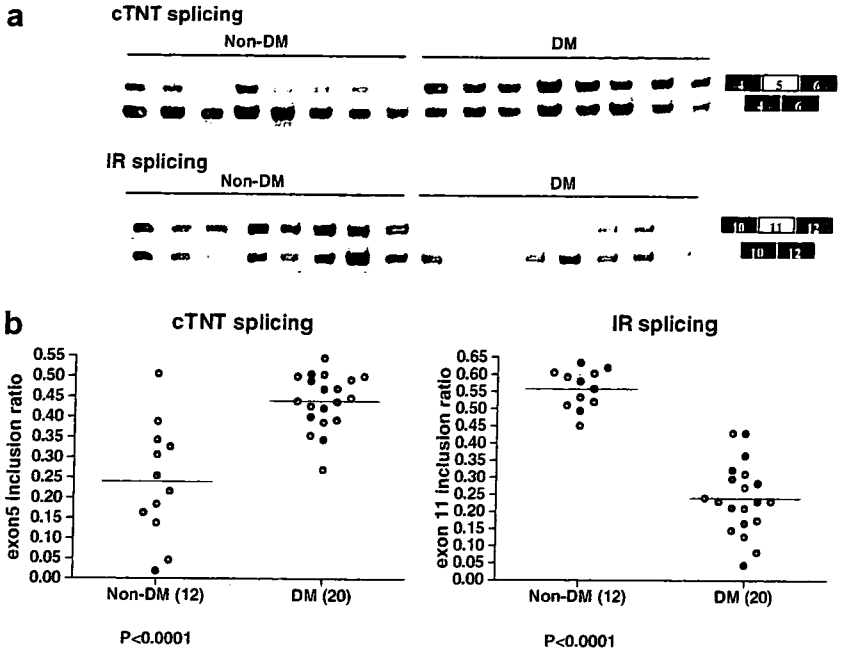

Fig. 1. Alternative splicing of cardiac troponin T (cTNT) and insulin receptor (IR) RNA. (a) Splicing products obtained by RT-PCR amplification of RNA isolated from non-DM control ($n = 8$) and DM1 ($n = 8$) biopsy samples. The exon utilization for each splicing product is shown. (b) Quantification of the RT-PCR products was performed among 20 DM1 samples and 12 non-DM individuals by using LAS-3000 luminescence image analyzer (Fujifilm). The graphs show the ratio of the alternative exon inclusion of cTNT and IR.

(DM; Fig. 1b). Therefore, the biopsy tissues used in this study manifested aberrant regulation of alternative splicing – a characteristic feature of DM pathogenesis.

3.2. Expression of mRNAs for MBNL1, MBNL2, CUG-BP, and ETR-3

An important consideration in interpreting the observed changes in splicing patterns is that both MBNL loss of function and CELF activation could explain our results [12]. For example, the retention of cTNT exon 5 is promoted by CELF but repressed by MBNL proteins. Conversely, the retention of IR exon 11 is promoted by MBNL but repressed by CELF proteins. Therefore, we sought to determine whether the observed altered splicing patterns resulted from loss of MBNL function and/or activation of CELF function.

To determine the degree of change detectable by this method, we measured the expression of insulin receptor isoform B (IR-B), which has been shown by gel electrophoresis to be downregulated in DM patients (Fig. 1). The results indicated that, as predicted, the DM patients expressed significantly less IR-B compared to the non-DM group (Fig. 2a). However, nine DM samples (out of 20) showed particularly high levels of downregulation such that their expression levels did not reach the range in which the accuracy of this method is assured by the standard curve. However, when these data were omitted from the analysis, this quantification method still successfully showed a 2- to 3-fold difference in expression levels, thus testifying to the suitability of this method for our experiment. Expression of IR-A was also measured and no significant differences were observed between non-DM and DM patients (Fig. 2a), as was expected from the results shown in Fig. 1.

We also performed real-time PCR analysis to measure the expression of MBNL and CELF mRNA. Total RNA was extracted from 20 DM and 12 non-DM biopsy samples, and the RNA was reverse-transcribed using a mixture of oligo(dT) and random hexamers. To allow for rigorous calibration of the data, we also examined mRNA expression for three commonly used housekeeping proteins, β-actin, GAPDH, and hypoxanthine–guanine phosphoribosyl transferase (HPRT). Comparing the relative mRNA level of each protein to that of the other two mRNAs allowed us to identify the gene with the most stable expression. We found that while the HPRT/GAPDH and β-actin/GAPDH ratios exhibited significant variation, the HPRT/β-actin ratio remained relatively constant among all smples. This indicated that HPRT and β-actin were reliable housekeeping genes; therefore, we selected the β-actin gene for normalization of the real-time PCR data.

We found no significant differences between DM and non-DM individuals with respect to mRNA levels for MBNL1, MBNL2, CUG-BP, and ETR-3 using real-time PCR (Fig. 2b), suggesting that the expression or stability of these mRNAs is not affected in DM. However, as shown in Fig. 2b, substantial individual variation

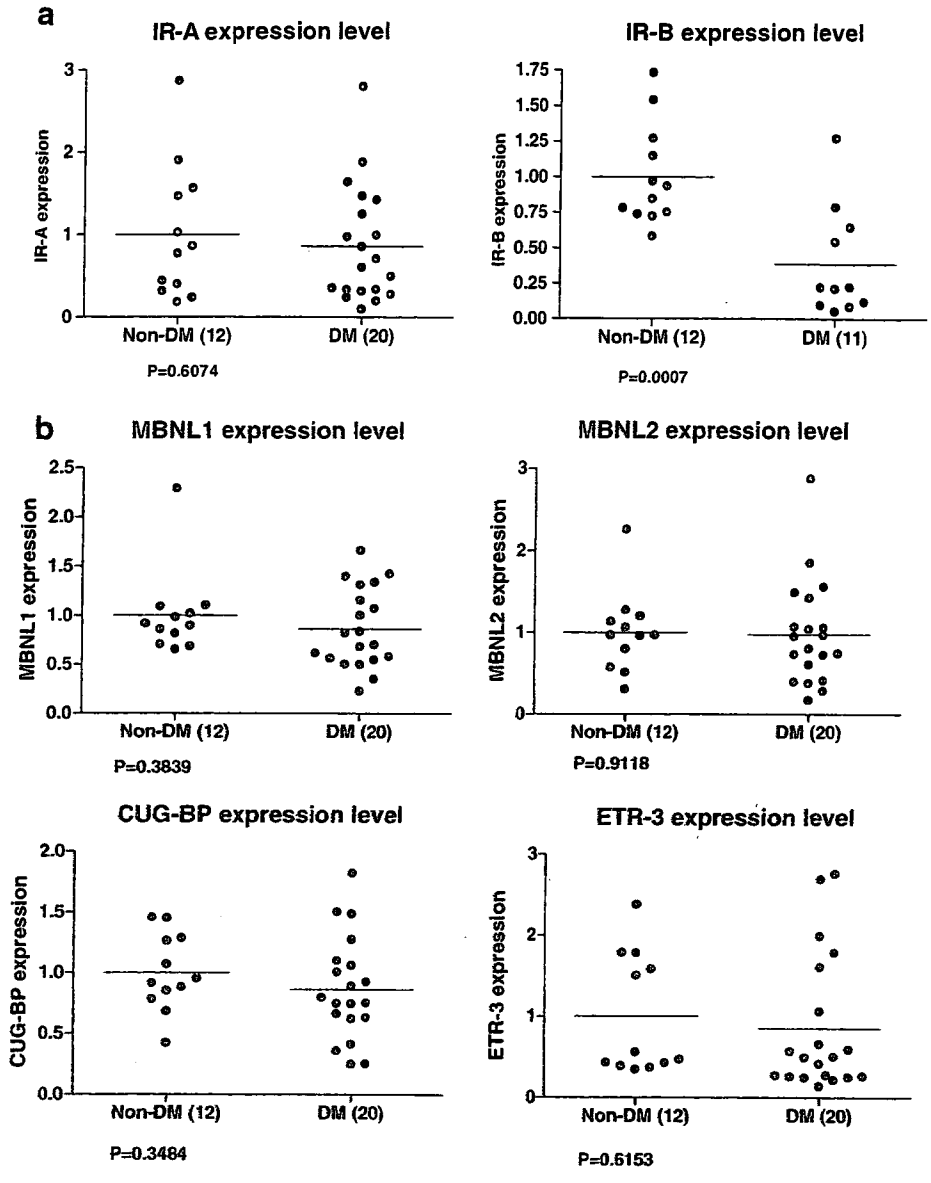

Fig. 2. (a) Expression of isoform A and B of insulin receptor in DM and non-DM samples determined by Real-time PCR. (b) Real-time RT-PCR analysis of relative expression levels of MBNL1, MBNL2, CUG-BP, and ETR-3 mRNAs in 21 DM-patients and 12 non-DM individuals. All data were normalized relative to β-actin mRNA expression. The values shown in the graphs have been divided by the average value for non-DM individuals.

in levels of these mRNAs was observed. We had predicted that the CELF and MBNL mRNA levels would be correlated with the extent of altered splicing seen in DM patients; however, our results did not support this hypothesis. Instead, DM individuals exhibiting significantly increased retention of cTNT exon 5 did not necessarily display significant abnormalities in IR splicing. Furthermore, mRNA expression of the four RNA-binding proteins examined in this experiment did not result in a significant change in IR splicing (data not shown).

The mRNA expression levels for the remaining MBNL and CELF proteins were so low that quantifying them was technically difficult in both DM and non-DM individuals. Previous reports have also described low expression levels for these proteins in muscles of normal individuals [33,34].

4. Discussion

The objective of this study was to determine whether a difference exists in mRNA expression levels of MBNL and CELF proteins between DM and non-DM muscles. Measurement by real-time RT-PCR showed that mRNA expression levels for these proteins were not significantly altered in DM patients. However, splice variants of MBNL proteins were not distinguished in this experiment because the primers for the real-time PCR assay were designed to amplify a fragment common to

all the known variants. MBNL1 has at least nine variants [10], some of which lack zinc-finger motifs and therefore probably do not interact with mRNA. The relevance of the other variants remains largely unknown. The observation that the MBNL1 isoform with exon 5 retained localizes in nuclei, where alternative splicing takes place, and therefore it has the largest potential (i.e., the most opportunity) to influence alternative splicing is noteworthy (Kino et al., unpublished data). Thus, the abundance ratio of these variants may be altered in DM1, which may influence MBNL1 function in cells.

We also found that levels of CUG-BP and ETR-3 mRNA were not significantly different for DM and non-DM individuals. During heart development, CUG-BP and ETR-3 undergo strong downregulation, whereas MBNL1 and MBNL2 expression is maintained [35]. Our findings suggest that expression of CUG-BP and ETR-3 mRNA is regulated normally throughout muscle development in DM patients. Whether the expression of CUG-BP and ETR-3 is altered at the protein level in DM patients is thus an important question. While increased levels of CUG-BP protein have been observed in DM1 tissues [14,23], this increase could be a result of altered posttranscriptional processing affecting translation, phosphorylation, or proteolytic degradation rather than altered regulation of transcription or mRNA stability.

In summary, our data confirmed the occurrence of aberrant splicing regulation in DM1 patients and demonstrated that these abnormalities are not associated with any altered expression of MBNL or CELF mRNA.

Acknowledgments

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Congenital neuromuscular disease with uniform type 1 fiber and *RYR1* mutation

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ABSTRACT

Background: Congenital neuromuscular disease with uniform type 1 fiber (CNMDU1) is a rare form of congenital myopathy, which is pathologically diagnosed by the presence of more than 99% of type 1 fiber, with no specific structural changes. Its pathogenic mechanism is still unknown. We recently reported that almost all patients with central core disease (CCD) with ryanodine receptor 1 gene (*RYR1*) mutations in the C-terminal domain had type 1 fibers, nearly exclusively, in addition to typical central cores.

Objective: To investigate whether CNMDU1 is associated with *RYR1* mutation.

Methods: We studied 10 unrelated Japanese patients who were diagnosed to have CNMDU1 based on clinical features and muscle pathology showing more than 99% type 1 muscle fibers. We extracted genomic DNA from frozen muscles and directly sequenced all 106 exons and their flanking intron–exon boundaries of *RYR1*.

Results: Four of 10 patients had a heterozygous mutation, three missense and one deletion, all in the C-terminal domain of *RYR1*. Two missense mutations were previously reported in CCD patients. Clinically, patients with mutations in *RYR1* showed milder phenotype compared with those without mutations.

Conclusion: Congenital neuromuscular disease with uniform type 1 fiber (CNMDU1) in 40% of patients is associated with mutations in the C-terminal domain of *RYR1*, suggesting that CNMDU1 is allelic to central core disease at least in some patients.

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Congenital neuromuscular disease with uniform type 1 fiber (CNMDU1) was first described in 1983.[1] It is a rare disorder pathologically characterized by the exclusive presence of type 1 muscle fiber (>99%) without any specific structural abnormality such as cores, nemaline bodies, or centrally placed nuclei. Clinically, it shares common features with congenital myopathy; including early onset, mild proximal muscle weakness, hypo- or areflexia, normal creatine kinase levels, and myopathic electromyography findings. So far, at least 12 cases have been reported.[1-10] However, its genetic cause and molecular pathomechanism are still unknown.

We are aware of a rare existence of CNMDU1 case with a family history of central core disease (CCD) in our own series[6] and in the previous report.[7] In addition, our recent study on CCD revealed that patients with a heterozygous C-terminal mutation in the gene encoding ryanodine receptor 1 (*RYR1*) have nearly exclusively type 1 fibers, in addition to well-demarcated, mostly singular and centrally located, "typical" cores,[11]

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suggesting a tight relationship between uniform type 1 fiber and *RYR1* mutations, especially those in the C-terminal domain. We therefore hypothesized that CNMDU1 may be caused by *RYR1* mutation.

METHODS Subjects. All clinical materials used in this study were obtained for diagnostic purpose with informed consent. Ten unrelated Japanese patients (seven boys and three girls) were diagnosed to have CNMDU1 among 9,300 frozen muscle biopsies diagnosed at National Center of Neurology and Psychiatry (NCNP) from 1976 to 2005. The diagnosis was established based on clinical and pathologic findings of muscle specimens consistent with CNMDU1 described previously.[1] Clinical features of the patients were assessed by the information provided by the physicians. Pathologic features of all patients were independently evaluated by three authors. A battery of histochemical stains was performed on biopsied muscle specimens from all patients, including hematoxylin and eosin, modified Gomori–trichorome, nicotinamide adenine dinucleotide-tetrazolium reductase, and myosin ATPase. We counted the total number of muscle fibers and that of each fiber type in one section to accurately calculate the percentage of type 1 fibers. Muscle sample for electron microscopic analysis was available only in Patient 3.

Patients underwent muscle biopsy because of hypotonia since birth (4/10) or delayed motor milestones (6/10). Age at biopsy varied from 5 months to 13 years with a mean age of 3.3 ± 3.8 years old (mean ± SD, n = 10).

Patients 2 and 4 had family history of neuromuscular disease. The father of Patient 2 had muscle weakness of unknown origin. Regarding Patient 4, the father was previously diagnosed to have CCD and the brother had similar clinical manifestations to the patient, although muscle sample was not available. None of the patients had past or family history of malignant hyperthermia (MH) or MH susceptibility. Nine had perinatal history: poor fetal movement (5/9), asphyxia (4/10), hypotonia (7/9), and weak suck (8/9). Six had respiratory distress; five of them experienced acute respiratory distress requiring mechanical ventilation; three (Patients 5, 7, and 8) had asphyxia at birth, two (Patients 6 and 10) developed infection during childhood, and one (Patient 1) had wheezing during neonatal period. All had muscle weakness and delayed motor milestones. Skeletal deformity (9/10), myopathic facies (7/9), and high arched palate (7/9) were also frequently observed. Patient 9 had exotropia. Five had mental retardation. Brain imaging was performed in these five patients, and we have seen dilatation of the ventricles (Patients 7 and 8) and brain atrophy (Patient 9). Patients 5 and 6 had no abnormality. Patient 8 had an episode of interventricular hemorrhage in the perinatal period. Moreover, no patient had epileptic episode, and Patient 9 showed normal EEG findings. All patients showed hypo- or areflexia. Serum creatine kinase level was within normal range in all. Only one patient (Patient 5) underwent muscle biopsy twice initially at 5 months and later at 2 years 9 months, both providing the same diagnosis (CNMDU1). The detailed clinical information of Patients 4,[5] 6,[10] and 9[4] was previously described elsewhere.

Mutation analysis. Genomic DNA was extracted from muscle biopsy samples according to standard protocols.[12] All 106 exons of *RYR1* and their flanking regions (GenBank GeneID 6261) were amplified and directly sequenced as described previously.[11,13] We also analyzed DNA from two patients with congenital myopathy with marked type 1 fiber predominance (96% and 97%) but without any other specific pathologic abnormalities such as type 1 fiber atrophy, nemaline body, centrally placed nuclei, and cores. DNA samples from 150 subjects apparently without any neuromuscular disorders and those from 2 patients with congenital myopathy with marked type 1 fiber predominance were used as controls. We also performed mutation screening in exons 1 to 4 of *FKBP1A* (GenBank Gene ID 2280), encoding FK506-binding protein 1A (12 kd), and exons 14 to 17 and 25 to 27 of *CACNA1S* (GenBank Gene ID 779), encoding the α_1S subunit of L-type voltage-dependent calcium channel or dihydropyridine receptor, both of which span the *RYR1*-interacting region. DNA from family members was available only in Patient 1. In addition, we extracted genomic DNA from paraffin-embedded muscles of the original three patients[1] and attempted to directly sequence the C-terminal domain, exons 90 to 106 of *RYR1*.[14]

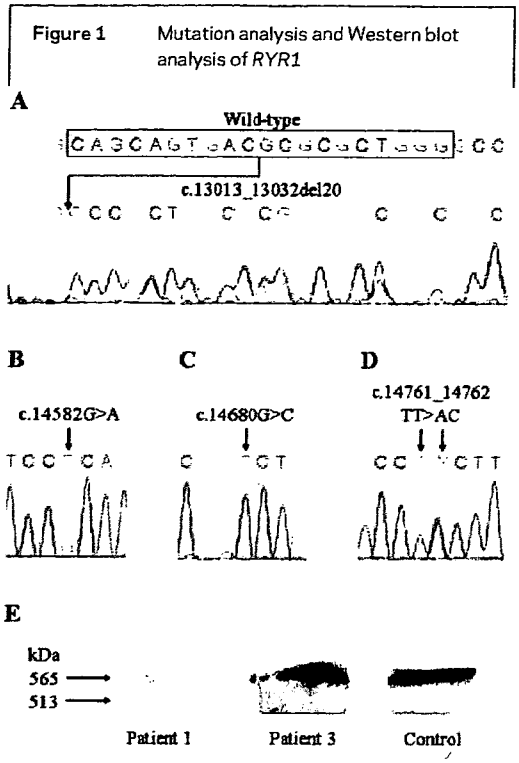
Total RNA was extracted from biopsied muscle using standard technique and reversely transcribed into cDNA using SuperScript III First-Strand Synthesis System for reverse transcription PCR (Invitrogen, Carlsbad, CA). Four overlapping fragments spanning exons 89 to 106 (nucleotides 12,220 to 12,819, 12,719 to 13,419, 13,351 to 14,350, 14,251 to 15,170) were amplified from the first-strand cDNAs.

PCR-amplified fragments were directly sequenced using BigDye Terminator v3.1 Cycle Sequencing kits on ABI3100 automated Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were analyzed with the SeqScape program in comparison with the reference sequences of *RYR1*, *FKBP1A*, and *CACNA1S*.

To rule out the presence of polymorphisms, we sequenced all the exons carrying novel sequence variations in 150 control DNAs without any known neuromuscular disorder. We also used the Japanese Single Nucleotide Polymorphisms database for checking common gene variations in the Japanese population.[15]

To confirm the mutations of Patients 1 and 4, the PCR products of mutant *RYR1* were cloned into plasmid pGEM-T (Promega, Madison, WI) and direct sequencing of the cloned fragments was performed.

Western blot analysis of RYR1 protein. Solubilized proteins (approximately 50 μg) from five slices (6 μm thick) of frozen muscles (Patients 1 and 3) were loaded onto 5% sodium dodecyl sulfate polyacrylamide gels, electrophoresed, transferred onto polyvinylidene fluoride membranes, and hybridized with primary mouse monoclonal anti-RYR antibody (34C; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA; 1:100).[16] A horseradish peroxidase- goat anti-mouse IgG (Zymed Laboratories, San Francisco, CA) was used as secondary antibody. The immunoreactive bands on the membrane were visualized using Las-1000 Pro (Fujifilm, Tokyo) by enhanced chemiluminescence (Amersham Bioscience Buckinghamshire, UK) as recommended by manufacturer. The intensity of the RYR bands was quantified by densitometric analysis using Quantity One (PDI, Huntington, NY). One muscle sample without any neuromuscular disorder was used as control.



(A) A novel deletion mutation of c.13013_13032del20 (p.A4338fs) is identified in exon 91 in Patient 1. Deleted nucleotides (enclosed in box) are shown in wild-type sequence. (B) In Patient 2, a known missense mutation c.14582G→A (p.R4861H) is detected in exon 101. (C) A novel missense mutation of c.14680G→C (p.A4894P) is identified in Patient 3 in exon 102. (D) In Patient 4, substitution of two consecutive nucleotides c.14761_14762TT→AC (p.F4921T) is identified in exon 102. Arrows mark the site of mutation. (E) Western blot analysis of muscles from Patient 1, Patient 3, and control. All show a 565-kd band corresponding to the predicted size of full-length *RYR1* (upper arrow). Only Patient 1 has a smaller sized band (about 513 kd) corresponding to the truncated *RYR1* mutant (lower arrow).

RESULTS **Mutation screening.** Four of 10 patients (40%) had heterozygous sequence variations predicted to change amino acids in the C-terminal domain of RYR1 (figure 1, A through D). In Patient 1, we identified a 20-bp deletion (c.13013_13032del-CAGCAGTGACGCGCGCTGGG, p.A4338fs) in exon 91, which resulted in a premature stop codon at the 4,575th amino acid. The presence of the deletion mutation was confirmed by sequencing of the cloned fragments. No family members including the parents and siblings carried the mutation, suggesting that the mutation in Patient 1 is a de novo mutation. Patient 2 had a missense mutation (c.14582G→A, p.R4861H) in exon 101, which had been previously reported in patients with CCD.[14] In Patient 3, a novel missense mutation of c.14680G→C (p.A4894P) was identified in exon 102. Patient 4 had a substitution of two consecutive nucleotides (c.14761_14762TT→AC, p.F4921T) in exon 102, which was previously reported in his father with CCD.[6,11] We confirmed the two-nucleotide change in one allele and the absence in the other by sequencing of the cloned fragments in Patient 4. All three amino acids replaced by missense mutations, R4861, A4894, and F4921, were highly conserved across the *RYR1* species including human, pig, rabbit, mouse, frog, and *C. elegans* (data not shown). These substitutions were not found in either 300 Japanese control chromosomes or in the Japanese Single Nucleotide Polymorphisms database.[15]

The substitution c.11266C→G (p.Q3756E) in exon 79 previously reported as nonpathogenic[17] was found in 4 of 10 patients: 1 with *RYR1* mutation and 3 without *RYR1* mutation. This substitution was also reported in the Japanese population diversity to be 11.4% in the Japanese Single Nucleotide Polymorphisms database . Fifteen silent single-nucleotide polymorphisms were also identified (data not shown). For the six patients without *RYR1* mutation, we were able to amplify and sequence the C-terminal domain of RYR1 in muscle cDNA and confirmed the absence of any mutation including aberrant splicing.

No mutations were found in either *FKBP1A* or *CACNA1S*. Two patients with congenital myopathy with marked type 1 fiber predominance did not have any mutation in *RYR1*.

We also tried to sequence the C-terminal domain of RYR1 using DNA from the original three patients first reported to have CNMDU1.[1] However, as only paraffin-embedded muscles were available, the quality of DNA did not allow us to successfully amplify the regions except for exons 96 and 100 wherein no mutation was found.

Western blot analysis. To know whether the truncated protein is expressed in Patient 1, we performed Western blot analysis. As expected, the muscle from Patient 1 showed two bands: a 565-kd band of predicted size of wild-type RYR1 protein and a 513-kd band, which is the predicted size of mutant *RYR1* (figure 1E, left lane). The lower bands were not observed in samples from the other patient and control (figure 1E, center and right lanes).

Clinical features. None of the four patients with C-terminal mutations in *RYR1* showed mental retardation (table). Moreover, no severe clinical incident during the perinatal stage was observed in this group. As described above, two patients had family history of neuromuscular disease and the father of Patient 4 was reported to have CCD.[6]

Table Clinicopathologic features of patients with and without *RYR1* mutations

Patient	1	2	3	4	5	6	7	8	9	10
Nucleotide changes	c.13013_13032del20	c.14582G>A	c.14680G>C	c.14761_14762TT>AC						
Protein mutations	p.A4338fs	p.R4861H	p.A4894P	p.F4921T						
Exons	91	101	102	102						
Age at biopsy/sex	3 y 5 mo/M	6 mo/M	6 y/F	2 y 9 mo/M	5 mo/M	7 mo/M	8 mo/M	11 mo/F	3 y 10 mo/M	13 y/F
Age at last clinical examination	8 y	6 mo	6 y	11 y	2 y 9 mo	5 y 3 mo	3 y 5 mo	5 y 5 mo	3 y 10 mo	21 y
Family history		+	NA	+	NA					
Poor fetal movement			+		NA	+	+	+	+	
Asphyxia					+	+	+	+		
Infantile hypotonia	NA		+		+	+	+	+	+	+
Poor sucking	+	+	+		+	NA	+	+	+	+
Respiratory distress	+				+	+	+	+		+
Muscle weakness	G	+	P	P	G	G	G	P	P	P
Delayed motor milestones	+		+	+	+	+	+	+	+	+
Mental retardation					+	+	+	+	+	
Facial muscle involvement	+		+		NA	+	+	+	+	+
High arched palate	+		NA		+	+	+	+	+	+
Skeletal deformity	FC	JC	Lo, HD		FC	FC, Sc, HD	Sc, JC	Sc, JC	JC	Sc
Biopsied muscle	BB	QF	AL	BB	NA	BB	BB	NA	QF	BB
Total no. of muscle fibers	1,770	1,887	1,709	1,609	1,665	2,056	1,546	1,414	1,558	1,252
Type 1 fibers, n (%)	1,757 (99.3)	1,886 (99.9)	1,709 (100)	1,609 (100)	1,663 (99.9)	2,949 (99.7)	1,537 (99.4)	1,408 (99.6)	1,557 (99.9)	1,249 (99.8)
Type 2 fibers, n, 2A/2B/2C	0/11/2	0/0/1	0/0/0	0/0/0	0/0/2	0/0/7	0/1/8	0/0/6	0/0/1	0/2/1
Internal nuclei, %	0.4	0.2	0.2	0.2	1.7	0.2	0.4	4.0	0.2	0.3
Endomysial fibrosis	Mild	Minimal	Mild	Mild	Mild	Mild	Marked	Moderate	Minimal	Minimal

G = generalized; P = proximal; FC = funnel chest; JC = joint contracture; HD = hip dislocation; Lo = lordosis; Sc = scoliosis; (+) = present; (−) = absent; BB = biceps brachii; QF = quadriceps femoralis; AL = adductor longus; NA = no information was available.

In contrast, five of six patients without mutations in *RYR1* had mental retardation except Patient 10. Severe respiratory distress, with asphyxia or infection necessitating mechanical support, was observed in five patients. None had family history of any neuromuscular disease. Myopathic facies and high arched palate were predominant in this group.

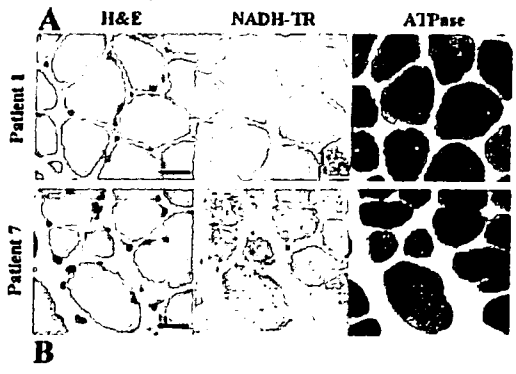
Among patients with and without mutations, there was no difference in the presence of muscle weakness, delayed motor milestones, or skeletal deformity.

Pathologic findings. The mean age of biopsy between patients with and without mutations (3.2 ± 2.3, n = 4; 3.2 ± 4.9 years, n = 6) did not differ; all patients showed fiber size variation, regardless of the *RYR1* mutations (table; figure 2). Endomysial fibrosis was mild except in two patients without *RYR1* mutations (Patients 7 and 8). There were no necrotic or regenerating fibers, although a small number of fibers with internally placed nuclei were seen. No group atrophy was noted. No nemaline bodies, ragged-red fibers, or rimmed vacuoles were seen. Intermyofibrillar network was well organized in all fibers without any core or core-like structure. Type 1 fibers comprise more than 99% of fibers. A small number of type 2 fibers were seen except in two patients (Patients 3 and 4), even though the percentage was less than 1%. These type 2 fibers were either type 2B or 2C, and no type 2A fibers were observed.

On electron microscopy, none of 50 fibers observed showed either loss of mitochondria or disorganized myofibrillar structure such as Z-line streaming.

DISCUSSION This is the first genetic study for CNMDU1. In 4 of 10 patients (40%), we identified a heterozygous mutation in the C-terminal domain of the gene encoding RYR1, which is vir-

Figure 2 Muscle biopsy



(A) Histochemistry. Patient 1 is a boy age 3 years 5 months (upper panel) with a mutation in *RYR1*. There is mild endomysial fibrosis. Patient 7 is an 8-month-old boy (lower panel) without *RYR1* mutation. Marked fibrosis is observed. In both patients, pathologic findings show marked variation in fiber size, well-organized intermyofibrillar network, and with all fibers composed of type 1 on myosin ATPase staining at pH 4.2. Bar = 20 μm. (B) Electron micrograph. Neither Z streaming nor loss of mitochondria is seen.
Bar = 5 μm

tually exclusively expressed in the skeletal muscle, forming the homotetrameric structures in the sarcoplasmic reticulum membrane, and functions as a Ca^{2+} release channel.[18] As *RYR1* mutations have been associated with three different diseases (CCD, multiminicore disease, congenital myopathy with cores and rods) and MH,[19-22] therefore, CNMDU1 may be the fifth disease linked to *RYR1* mutations.

Among four mutations that we identified, c.14582G→A (p.R4861H) was previously associated with CCD in Europeans,[14] and the c.14761_14762TT→AC (p.F4921T) was previously reported in the father of Patient 4, who had CCD.[11] Two other mutations, c.13013_13032del20 (p.A4338fs) and c.14680G→C (p.A4894P), were novel ones. The 20-bp deletion mutation is predicted to cause a frame-shift, leading to a premature stop codon and removal of the C-terminal 464 amino acid residues from the protein.

The predicted transmembrane helices have been described as M1 to M10.[23,24] However, the recent in vitro study suggested that M1 to M4 regions are actually located in the cytosol and that only M5 to M10 are the transmembrane domains.[25] According to this model, the deletion mutation identified in Patient 1, which was predictably located between M3 and M4, should truncate the protein after the M3 region, losing all transmembrane domains. Furthermore, the previous study showed that the mutant *RYR1* truncated after M3 region can still exist in the cytosol even without being anchored to the membrane.[25] Indeed, the truncated RYR1 protein was present in the patient's muscle as confirmed in Western blot analyses. Our results raise a possibility that the truncated *RYR1* mutant may somehow be associated with the wild-type *RYR1* and disrupt its function. However, the limited amount of the sample did not allow further investigation to clarify the interaction between wild-type and mutant *RYR1*.

Interestingly, c.14680G→C (p.A4894P) affects the same nucleotide and amino acid site with c.14680G→A (p.A4894T), which was found in the MH patient in our previous study.[13] Pathologically, the MH patient with p.A4894T had a normal mosaic pattern of fiber type distribution and not uniform type 1 fiber. Core-like structure was observed in only a few fibers. Proline differs from other amino acids in its structure of imino acid; that is, the side chain of proline forms a cyclic structure.[26] Therefore, a single amino acid change from alanine to proline may lead to a different structural and thereby functional change in RYR1 from that in p.A4894T, resulting in uniform type 1. It is an interesting issue as to whether p.A4894P mutation is also associated with MH. However, no sample was available for in vitro contraction[27] or calcium-induced calcium release test[28] in Patient 3.

We did not find any *RYR1* mutation in six patients in our cohort, suggesting the presence of another causative gene for CNMDU1 and the genetic heterogeneity of the disease, even though there still remains a possibility that mutations may exist in unexamined regions such as the majority of introns. We did not find any *RYR1* mutation in two patients having congenital myopathy with marked type 1 fiber predominance in which type 1 fibers account for less than 99%, suggesting that the *RYR1* mutation in the C-terminal domain may be tightly associated with uniform type 1 fiber, namely, >99% type 1 fibers, albeit a greater number of

patients are needed to make a definite conclusion.

We could amplify only two exons in the C-terminal domain in DNA of the patients first reported to have CNMDU1.[1] Although the original patients were clinically similar to our patients with *RYR1* mutation, in terms of early onset, mild muscle weakness, delayed motor milestones, and pathologic features, their age at the time of biopsy (ages 9 and 12) was higher in comparison with our patients, raising the possibility that the original patients may have had a genetically distinct disorder.

Excitation–contraction (EC) uncoupling caused by *RYR1* mutation is thought to be closely associated with CCD.[29] In vitro studies have shown that two RYR1-binding proteins, FKBP1A and CACNA1S, directly participate or modulate EC coupling in skeletal muscle.[30,31] In addition, 1% of MH patients have mutations in the RYR1-binding region in CACNA1S.[32] Therefore, we sequenced *FKBP1A* and *CACNA1S*, but we did not find a mutation in any patient, suggesting that these genes may not or only rarely be associated with CNMDU1.

In our study, CNMDU1 patients with *RYR1* mutations have mild clinical features compared with those without mutations, in terms of poor fetal movement, asphyxia, infantile hypotonia, respiratory distress, mental retardation, myopathic facies, and high arched palate. This supports the idea that CNMDU1 may be genetically heterogeneous. Most remarkably, none of the patients with *RYR1* mutations had mental retardation, whereas five of six patients without *RYR1* mutations had it. Three of five patients had ventricular dilatation or brain atrophy on brain imaging, suggesting that the mental retardation might occur with a perinatal history of asphyxia or another primary abnormality of unknown origin.

Regarding pathologic findings, CNMDU1 patients either with or without mutations in *RYR1* had similar myopathic changes: mild to marked variation in fiber size. The majority of type 2 fibers, albeit few in number, found in our patients were type 2C, indicating that mature type 2 fibers are even fewer. Patients without *RYR1* mutations had more pathologic variation than those with mutations, suggesting that those without mutations might have genetically different causes.

Solely from the clinical features, it is difficult to differentiate between CNMDU1 patients with *RYR1* mutations and CCD patients with C-terminal mutation in *RYR1*. Both groups of patients show muscle weakness and delayed motor milestones. The frequency of asphyxia, mental retardation, myopathic facies, high arched palate, and skeletal deformities is similar. Furthermore, uniform type 1 fiber is a characteristic pathologic finding in both groups.[11] Two mutations of c.14582G→A (p.R4861H) and c.14761_1476-2TT→AC (p.F4921T) were identified in both CNMDU1 and CCD patients, and all the patients showed type 1 fiber uniformity despite the absence or presence of cores. This result suggests that type 1 fiber uniformity is closely associated with C-terminal *RYR1* mutation. Although additional study is required, there still remains a possibility that CNMDU1 and CCD are closely related diseases, regardless of the presence or absence of cores.

In support of this notion, the father of Patient 4 had CCD, while no cores were observed in the patient's sample.[6] A similar family case was also reported: a 4-month-old girl had CNMDU1 in a family with CCD due to p.Y4864C mutation in exon 101 of *RYR1*.[7] In both families, CNMDU1 was identified in younger children, whereas CCD was found in older family members. These findings suggest that the core may be formed later in the course of disease at least in some patients. Alternatively, cores may not be formed in CNMDU1 patients for factors that are yet to be known.

The fact that we were unable to find distinct pathologic changes other than type 1 fiber uniformity can be due to many possibilities. One is that CCD and CNMDU1 may be a part of a spectrum, as mentioned above. Interestingly, in all familial cases including the one previously reported by others, adults had CCD, whereas children showed CNMDU1, suggesting that cores might not be present in their early lives. In fact, age at biopsy in CNMDU1 patients with C-terminal mutations (3.2 ± 2.3 years, n = 4) was more than 1 year lower than that in CCD patients with C-terminal mutations (4.4 ± 3.0 years, n = 14) in our series,[11] although there is a significant overlap between the two age groups. Nevertheless, we have never found a case with muscle pathology falling between CNMDU1 and CCD, that is, uniform type 1 fiber with cores only in a few fibers. In addition, electron microscopic study of our patient (albeit only one was available) did not show any sign of core formation. These observations may cast some doubt on the notion that CNMDU1 and CCD are part of a spectrum.

Another possibility is that CNMDU1 is actually CCD, and the absence of cores in CNMDU1 may be attributed to the site of sampling. This,

however, is less likely because we have sampled a wide range of sites. MRI studies in CCD have confirmed a distinct pattern of muscle involvement, mostly involving muscle of the lower extremities.[33] In our series, even biceps brachii, which is relatively spared based on clinical examination, actually shows core formation (data not published). The third possibility is that CNMDU1 is a distinct entity, and thus the absence of other pathologic findings may not be influenced by the choice of area sampled.

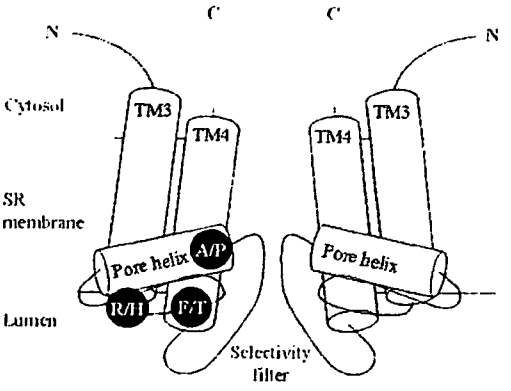
Previously, we have identified 14 CCD patients with heterozygous C-terminal mutation in *RYR1*.[11] Combining this number with that from this study, 4 of 18 patients (22%) with a heterozygous C-terminal mutation are associated with CNMDU1, suggesting that CNMDU1 may not be a rare condition at least among those with the *RYR1* mutation in the C-terminal domain.

The pathomechanism for the development of uniform type 1 fiber is still unknown. Skeletal muscle fiber type formation is thought to be regulated by nerve control and succeeding intracellular signal transduction including Ca^{2+} release that lead to transcriptional activation of fiber type-specific genes. In slow muscle fibers, the lower amplitude and longer duration of Ca^{2+} transition facilitate activation of calcineurin, the Ca^{2+}/calmodulin-dependent phosphatase, which dephosphorylates and activates two kinds of transcription factors; nuclear factor of activated T cell (NFAT) and myocyte enhancer factor 2 (MEF2) resulted in significant activation of slow myosin heavy chain 2 gene (slow *MyHC2*) promoter, expressed in avian skeletal muscle.[34-36] In contrast, in fast fibers, high-amplitude calcium sparks induced by infrequent phasic firing of the motor nerves are insufficient to keep activation of calcineurin. When calcineurin is inactivated, phosphorylated NFAT cannot enter the nucleus and the slow fiber-specific program is down-regulated, resulting in the predominant transcription of genes encoding fast fiber-specific proteins.[37]

The inhibition of RYR1 by ryanodine treatment has been reported to induce the activation of slow *MyHC2* promoter in fast muscle fibers via NFAT- and MEF2-dependent transcriptional pathway.[38] It strongly suggests that the loss of function of the RYR1 channel contributes to slow *MyHC2* gene expression. This naturally raises a possibility that the C-terminal mutations found in this study may cause loss of function of RYR1, which leads to the activation of slow fiber-specific program in fast fibers.

In fact, all missense mutations found in CNMDU1 in this study are located in the pore-forming segment of RYR1 (figure 3). This pore-forming segment is located close to the luminal end of the RYR1 channel and is supposed to form the selectivity filter, which plays a critical role in the selection of permeating ions.[39,40] According to this hypothetical model, binding of ryanodine would be expected to change the conformation of this selectivity filter and the inner conduction pore.[41] Therefore, mutations in the pore-forming segment may well alter the interaction of the pore helix and the selectivity filter. Loss of function of RYR1 may explain the mechanism of type 1 fiber uniformity as in the model of RYR1 inhibition by ryanodine, although further investigations are necessary to elucidate the precise pathomechanism for CNMDU1.

Figure 3 Location of mutation sites in the pore-forming region of RYR1 protein



Putative positions of the three missense mutations (filled circle) of *RYR1* in congenital neuromuscular disease with uniform type 1 fiber found in this study are shown. The putative pore-forming segments from two RYR1 monomers are illustrated as TM3 (outer helix), TM4 (inner helix),[39] same as M8 and M10,[23] and the pore helix connected together.[39] The selectivity filter is indicated. R4861H and A4894P are located at each end of TM3 and the pore helix, whereas F4921T is situated at the beginning of TM4.

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