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Non-homologous recombination between Alu and LINE-1 repeats caused a 430-kb deletion in the dystrophin gene: a novel source of genomic instability

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Abstract Although large deletions in the dystrophin gene have been identified in more than two-thirds of Duchenne and Becker muscular dystrophy patients, the molecular mechanisms that lead to the generation of these deletions are largely unknown. Here, Alu and LINE-1 (L1) repetitive elements were shown to be present at one or other of the two ends, respectively, of a 430-kb deletion in the dystrophin gene. The breakpoint of the deletion, which stretches from exons 2 to 7, was defined more precisely by polymerase chain reaction (PCR) walking on introns 1 and 7. Finally, the region containing the breakpoint was amplified as a fragment of more than 10kb. Sequencing of the deletion endpoint revealed the presence of an Alu sequence in intron 1, 25kb downstream from the 3' end of exon 1 that was joined directly to an L1 sequence in intron 7, 4.5kb downstream from the 3' end of exon 7. The deletion was calculated to be 430kb. To our knowledge, this is a novel recombination event joining non-homologous Alu and L1 repeats, and is the largest known intrachromosomal deletion that is thought to involve repetitive genetic elements. Sequence characteristics around the breakpoint are discussed.

Key words Non-homologous recombination · Alu · L1 · Dystrophin gene · Deletion · Duchenne muscular dystrophy

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Introduction

Severe Duchenne and mild Becker muscular dystrophies (DMD/BMD) are caused by mutations in the dystrophin gene. The gene occupies 3000kb of the X chromosome and consists of 79 exons. DMD/BMD is the most common inherited muscle disease, affecting 1 in every 3500 boys, and it has been estimated that one-third of DMD/BMD cases result from new mutations in the dystrophin gene (Moser 1984). Two-thirds of DMD/BMD cases have large deletions (Koenig et al. 1987). More than 100 cases have been identified with deletion mutations in the dystrophin gene, and two deletion hot spots have been identified near the 5' end and in the central region of the gene (Koenig et al. 1987; Forrest et al. 1988). The presence of deletion hot spots in the gene suggests that a particular gene structure within them makes these regions prone to genetic instability. However, the molecular mechanisms underlying the generation of large deletions are largely unknown, because the huge size of the intron hampers molecular analysis of the genetic structure around the breakpoint.

Over one-third of the human genome consists of interspersed repetitive sequences that are primarily degenerate copies of transposable elements. Alu and LINE-1 (L1) sequences, which are present at more than 500,000 locations, are the two most common repeat elements identified on the human genome, representing more than 5% and 10% of the human genome, respectively (Smit 1996).

Homologous or non-homologous recombination events are sources of genome instability. Homologous recombination events between Alu repeats and between L1 repeats have been reported in 18 (Deininger and Batzer 1999) and 2 cases (Burwinkel and Kilimann 1998; Segal et al. 1999) of human disorders, respectively. Non-homologous recombination events involving one of these repeat elements at either end of a recombination site have also been reported in a limited number of mutations (Drechsler and Royer-Pokora 1996; Kumatori et al. 1998; Zhang and Zhang 1998; Deininger and Batzer 1999; Morgan et al. 1999). As far as we know, examples of recombination

events involving the fusion of Alu and L1 repeats have not been reported.

Deletions extending from exon 2 to exon 7 are among the most common deletion mutations in the dystrophin gene (Muntoni et al. 1994; Yuge et al. 1999). However, the molecular mechanism underlying the generation of these deletions remains unknown. In this report, we describe the first example of a non-homologous recombination event between Alu and L1 repeats as a cause of a 430-kb deletion in the dystrophin gene that is responsible for a case of BMD.

Subjects and methods

Genomic DNA was extracted from whole blood drawn from a 6-year-old Japanese boy suffering from BMD, and from his family members. The boy was diagnosed with BMD on the basis of high serum creatine kinase (CK) levels, electromyogram analysis, and muscle biopsy. Both his mother and his older sister showed elevated serum CK levels. His maternal uncle was diagnosed with idiopathic dilated cardiomyopathy at the age of 21, and underwent heart transplantation.

The presence of a deletion mutation in the dystrophin gene was examined in 19 deletion-prone exons that are clustered within two deletion hot spots and which were amplified by PCR, using primer sets described previously (Chamberlain et al. 1988; Beggs et al. 1990). In order to determine the borders of the deletion, the genomic region encompassing exons 2 or 7 was PCR amplified (Dwi Pramono et al. 2000) and dystrophin cDNA was analyzed as described before (Hagiwara et al. 1994). The breakpoints within introns 1 or 7 were examined by successive PCR amplification reactions, and four and five regions were sub-

sequently amplified from introns 1 and 7, respectively (Fig. 1). In order to clone the breakpoint, PCR was carried out using primers that hybridized close to the breakpoints of the deletion: a forward primer in the sequence of intron 1 and a reverse primer complementary to the sequence of intron 7. A fragment of more than 10kb was amplified. This product was digested with the restriction enzyme *SauIII*AI. Finally, sequences around the breakpoint were determined by sequencing the sub-cloned fragments. PCR was carried out essentially as described before (Narita et al. 1993). Primers used in this study are listed in Table 1. PCR-amplified products or digested fragments subcloned into pT7 blue T vector (Novagen, Madison, WI, USA) were sequenced using a Taq dye termination cycle sequencing kit (Perkin Elmer Applied Biosystems, Norwalk, CT, USA) with an automatic DNA sequencer (model ABI Prism 310 Genetic Analyzer; Perkin Elmer Applied Biosystems), as described previously (Surono et al. 1999).

Results

Nineteen deletion-prone exons of the dystrophin gene were examined in the index case, by PCR amplification. Exons located in the central deletion hot spot were amplified as normal. In the 5' region, however, exons 3, 4, and 6 could not be amplified, whereas exons 1 and 8 could be amplified, suggesting the presence of a deletion spanning from exons 3 to 6. In order to determine the border of the deletion, the flanking exons, 2 and 7, were examined. An amplification product corresponding to these two exons could not be obtained. Considering that exons 1 and 8 were present, the index case must have a deletion spanning from exons 2 to 7 (Fig. 1). Analysis of dystrophin cDNA prepared from the index case revealed that the 3' end of exon 1 was joined

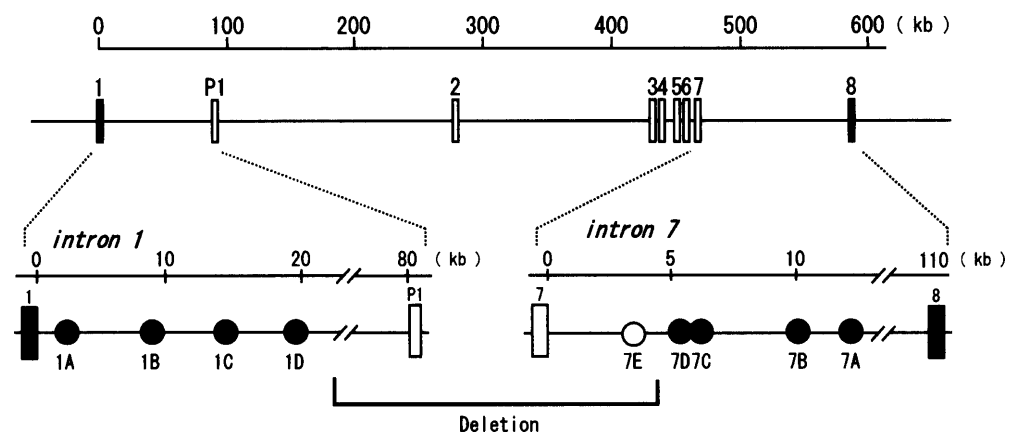


Fig. 1. Physical map of the 5' region of the dystrophin gene. The deletion of the genomic region in the 5' region of the dystrophin gene was examined by polymerase chain reaction (PCR) amplification, as indicated by *boxes* and *circles*. Numbers over each *box* indicate the exon number. *P1* indicates the P-promoter and the first exon region. *Horizontal lines* indicate introns. *Filled* and *open boxes* represent exons and deleted exons, respectively. *Open* and *filled circles* on intron se-

quences represent deleted and retained regions, respectively. 1A, 1B, 1C, and 1D are located at nearly 1, 8, 16, and 20kb downstream from the exon 1, respectively. 7A, 7B, 7C, 7D, and 7E are 13, 10, 7, 5, and 4kb downstream from exon 7, respectively. Enlarged maps of introns 1 and 7 are shown below the full genomic map. *Bracket* represents a deletion region

Table 1. List of primers prepared in this study

| Fragment | Forward primer(f) | Reverse primer(r) |
|----------|-------------------------|--------------------------|
| 1A | GATGAATCCCAATGACTCAC | ACTAAATCAGGATTCCCCAG |
| 1B | CTGTGAATATTACCAAAATG | CAGGTTAGCCCAGTTCTTAGCT |
| 1C | CCAGTCTAACCAACCTATTCATA | GATTGTCATGTACATACAAGC |
| 1D | CCTCACCTAGTACTAGCTTAGA | ACACCTATACTGCCCTAGTAAG |
| 7A | TTCTGGGCAGTTGGAACCCAG | AACAAATCAGCCCCTAGGGG |
| 7B | GGCTGCAAATAAGATGAGGATTC | TTTGGAGAGTAGCCAACATCATG |
| 7C | GAACTTGACCACTTGGCTCC | CCAGAGCTGAGCTCAAGTAC |
| 7D | GGCTGCTGCCTTTCTTCAG | GGAGCCAAGTGGTCAAGTTC |
| 7E | GTAGGTTGACGAGCCTGTAATC | GCGATAAGATGCTCTTTCAATTGG |
| 7F | CCAGAAGACAGCCTTTTGCC | GGTAGGAGGGGATACTATCGTG |

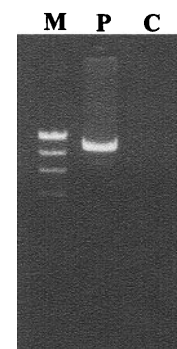
directly to the 5' end of exon 8 (data not shown), confirming the deletion of exons 2 to 7.

Because deletions of exons 2 to 7 of the dystrophin gene have already been reported in several BMD cases in different ethnic group (Muntoni et al. 1994; Yuge et al. 1999), it was proposed that the deletion endpoint includes a genomic structure or element that predisposes this region to deletion. To examine this proposal, genomic sequences around the deletion endpoint were analyzed. First, the deletion endpoint intron 1 was defined more precisely. The Purkinje promoter/exon 1 (P-promoter) region, which is one of alternative promoters and is present about 80kb downstream from exon 1 (Gorecki et al. 1992), was analyzed by PCR amplification. However, no amplified product was obtained, suggesting that the deletion endpoint is between exon 1 and P1 (Fig. 1). Subsequently, successive fragments of intron 1, starting from the 3' end of exon 1, were examined by PCR amplification. Using the available sequence of intron 1, we designed four sets of primers (1A, 1B, 1C, and 1D) (Fig. 1, Table 1). All four regions, located 1, 8, 16, and 20kb from the 5' end of exon 1, respectively, could be amplified from the patient's DNA, as well as from the control DNA (data not shown). Therefore, the deletion did not affect at least 20kb of the 5' region of intron 1.

In order to narrow down the deletion endpoint in intron 7, five regions, from 7A to 7E, in intron 7 were examined by PCR amplification. Regions 7A, 7B, 7C, and 7D, but not 7E, could be amplified (data not shown). These results indicated that the deletion endpoint is between regions 7D and 7E, which are 5kb and 4kb downstream from the 3' end of exon 7, respectively (Fig. 1).

Finally, we attempted to amplify the deletion endpoint using a primer set comprising a forward primer in intron 1 (1D f) and a reverse primer in intron 7 (7D r) (Table 1). A product of more than 10kb was amplified (Fig. 2). A fragment of the same size was also amplified from the genomic DNA of the mother of the index case, who was considered to be a carrier (data not shown). This indicated that the amplified product was from the recombination region; therefore, the amplified product was subjected to sequence analysis, using restriction enzyme-digested fragments. In the fragment containing the breakpoint, the sequence of the 5' portion of the fragment was completely homologous to the sequence of intron 1 (Genbank AL049643), while the

Fig. 2. PCR amplified product spanning from intron 1 to 7. An amplified fragment of more than 10kb was obtained from the patient's genomic DNA (P), but not from control (C), when primers on intron 1 (1D f) and 7 (7D r) were used. M represents a size marker, which is *HincII*-digested ϕ X174 phage DNA (Toyobo, Osaka, Japan)



remaining 3' portion of the fragment was completely identical to the sequence of intron 7 (Genbank U60822) (Fig. 3). These results indicated that the deletion end points were 25kb downstream from the 3' end of muscle exon 1 and 4.5kb downstream from the 3' end of exon 7 (Fig. 1). The size of the deletion was calculated to be 430kb, according to the approximate mapping data (Coffey et al. 1992).

One kb of sequences on each side of the deletion breakpoints in intron 1 and 7 were then examined (Fig. 4). It was found that 296bp encompassing the deletion endpoint of intron 1 was homologous to the Sg member of the Alu repeat family. The 3' breakpoint in intron 7 was located within the sequence of open reading frame 1 of an L1 element. However, the DNA sequences flanking the 5' and 3' breakpoints in the wild-type dystrophin gene are not homologous (Fig. 3). Thus, we concluded that a non-homologous Alu-L1 recombination event is likely to be responsible for this deletion. Significantly, the 26-bp core sequence of Alu repeats that has been reported to trigger recombination and to cause the frequent involvement of Alu elements in gene rearrangements (Rudiger et al. 1995) was identified 34nt upstream from the breakpoint (Fig. 3).

Possible matrix attachment region (MAR) sites in introns 1 and 7 were also analyzed, using the MAR-Finder program of Singh et al. (1997). The region around the breakpoints included several MAR motifs (Fig. 4). Secondary structure analysis (Zuker 1989) revealed that the breakpoint in intron 1 was in the middle of the hairpin-loop structure formed by two complementary sequences: CAGGATG upstream of the breakpoint and the down-

stream complementary CATCCTG (Fig. 3). Remarkably, the sequence CAGG that was originally found in a deletion hot spot in the murine MHC gene cluster (Steinmetz et al. 1987), and the oppositely orientated CCTG sequence that was frequently observed in deletion hot spots of the globin

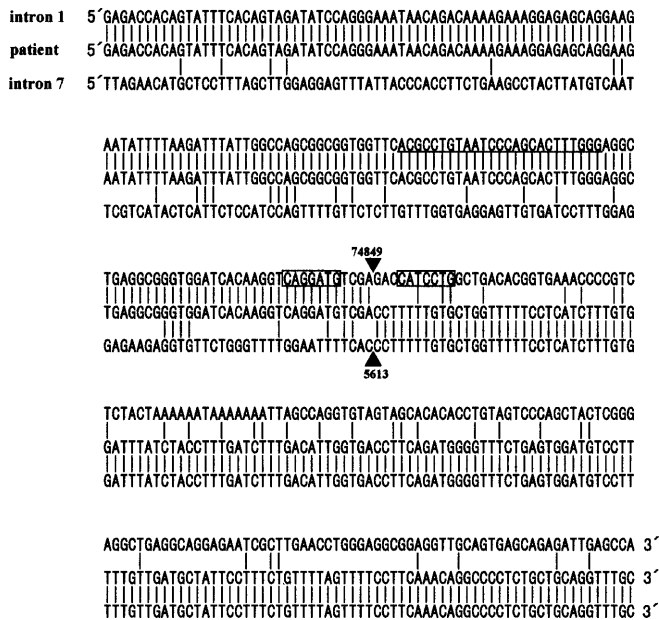


Fig. 3. DNA sequence spanning the deletion junction with the corresponding intron sequences. The intron 1 sequence (*top line*) (Genbank AL049643), the sequence containing the breakpoint (*middle line*), and the intron 7 sequence (*bottom line*) (Genbank, U60822) are aligned. The bars between the sequences indicate positions at which they are identical. Filled arrowheads indicate the deletion breakpoint. Numbers over and under the arrowheads indicate the nucleotide numbers of the end of the homologous region to intron 1 (Genbank AL0496439) and intron 7 (Genbank, U60822), respectively. The 26-bp core sequence of Alu repeats is underlined. The boxed segments indicate regions containing the hairpin structure in which two 7-bp stretches of DNA on the same strand are complementary

genes (Krawczak and Cooper 1991) were identified in these complementary sequences.

In addition, the sequence TTTT, the putative polymerase mutation hot spot that is frequently found around deletion breakpoints (Krawczak and Cooper 1991), was observed several times near the 3' breakpoint in intron 7. Finally, sequences homologous to the topoisomerase II binding and cleavage consensus sequences were found in both introns 1 and 7, especially near the breakpoint of intron 1. However, a topoisomerase I cleavage site was not identified near the recombination point (Fig. 4).

Discussion

Although the approximately 3000-kb dystrophin gene is a large target for mutations, the mechanisms that promote the observed high frequency of spontaneous mutations in this gene are not well understood. In this report, we propose that the molecular basis of a deletion spanning exons 2 to 7 of the dystrophin gene is a non-homologous recombination event between an Alu repeat in intron 1 and an L1 repeat in intron 7 (Fig. 4). The data demonstrate that deletion mutations are not necessarily produced by homologous DNA misalignments, as noted previously (McNaughton et al. 1998).

Southern blot analysis indicated the presence of two deletion hot spots in the dystrophin gene (Koenig et al. 1989). Den Dunnen et al. (1989) proposed that two of the longer introns, 7 and 44, might contain factors that predispose them to deletion. Previously, we pointed out that one small deletion in the dystrophin gene was caused by a palindromic structure near the deletion junction (Matsuo et al. 1990). However, molecular analyses of the endpoints of large deletions have been performed in only a limited number of cases. Alu or L1 repeats have been identified at either end of several deletions (Ehrenpreis et al. 1991; Love et al. 1991; Baldrich et al. 1992; Pizzuti et al. 1992; McNaughton et al.

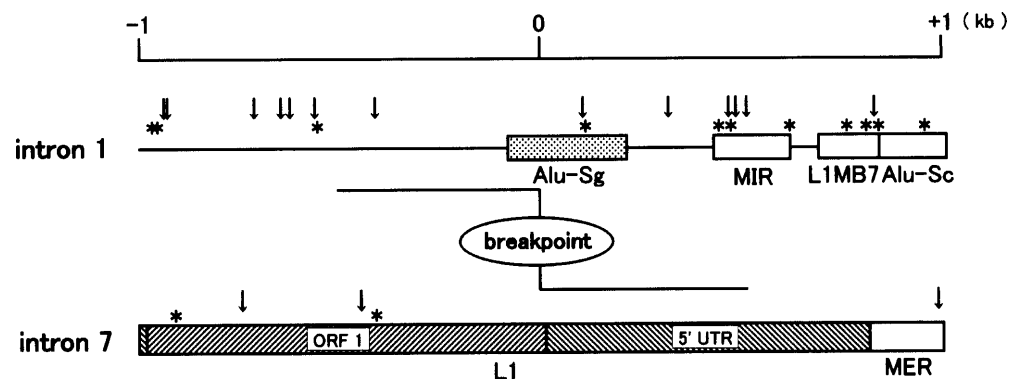


Fig. 4. Physical DNA map of the region around the deletion breakpoint. The organization of 2 kb of intron 1 and intron 7 is shown. Lines indicate introns. Boxes represent the repetitive genomic sequences as marked below. MIR and MER represent mammalian-wide interspersed repeats and medium reiteration repeats, respectively. Dotted and shaded boxes are the Alu-Sg in intron 1 and the L1 repeat

in intron 7 that are joined together. ORF1 and 5' UTR represent open reading frame 1 and the 5' untranslated region of L1 repeat, respectively. Vertical arrows indicate topoisomerase II recognition sites (GTNWAYATTNATNNR). Asterisks show matrix attachment region (MAR) motifs (ATATTT)

1998), suggesting that Alu or L1 repeats could be factors that predispose the dystrophin gene to instability. The large number of Alu elements within the human genome provides abundant opportunities for unequal homologous recombination events between Alu repeats, resulting in human diseases (Kornreich et al. 1990; Burwinkel and Kilimann 1998; Deininger and Batzer 1999). However, only one example of Alu-Alu recombination has been reported in the dystrophin gene, the deletion of exons 6 to 7 (McNaughton et al. 1998).

The other major repeat unit in the human genome is the L1 element. Unequal homologous recombination events between two neighboring L1 sequences are an established cause of genome instability (Burdwinkel and Kilimann 1998), and L1 elements frequently contribute to recombination by diverse mechanisms (Segal et al. 1999). Here, we report the first example of non-homologous recombination between Alu and L1 elements.

Chromatin structure may bring distant termini close together as chromatin loops that span about 50 to 100 kb attach to the fibers of the chromosomal scaffold at MARs. It has been proposed that four large deletions arose in the human beta-globin gene cluster from the loss of integral numbers of chromatin loops anchored to the nuclear matrix during DNA replication (Vanin et al. 1983). In the present study, the presence of many MAR motifs near the breakpoint (Fig. 4) suggested that the 430-kb deletion may have involved at least four chromatin loops. The physical conformation of this region of the dystrophin gene may be an important factor in the etiology of the deletions (McNaughton et al. 1997). Because we identified several putative topoisomerase II consensus sequences in both introns 1 and 7 (Fig. 4), we suppose that this enzyme was instrumental in creating the chromatid exchange events. Supporting this supposition, a topoisomerase II consensus sequence has been identified in several breakpoints of non-homologous recombination (Hu et al. 1991; Krawczak and Cooper 1991; Blok et al. 1995).

It has been proposed that deletions are generated by the formation of a hairpin loop structure in a single strand of DNA, followed by enzymatic degradation at unpaired regions within the loop (Robinson et al. 1997). Furthermore, it has been proposed that the sequences on either side of the deletion must have a high degree of intra-strand base complementarity. In apparent agreement with this, the 7-bp complementary sequences that flank the breakpoint in intron 1 in the deletion studied here may be one factor that predisposes this region to recombination (Fig. 3).

Intron 7 of the human dystrophin gene is unusually large (110 kb), and a substantial number of deletion endpoints have been identified within it (McNaughton et al. 1997). Defined deletion endpoints are dispersed throughout intron 7, including several at different sites within L1 repeats. The L1 sequence in this report, which is near the 5' end of intron 7 (Fig. 1) is different from those described previously.

Because we could not identify any single cause for the recombination event described here, we propose that it is generated by a combination of the above-mentioned sequence characteristics of the two introns that are involved.

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