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Detecting copy number changes in genomic DNA - MAPH and MLPA

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Chapter 2.2

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Duplications in the *DMD* gene are non-randomly distributed and can be complex rearrangements

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Abstract

Using Multiplex Amplifiable Probe Hybridization (MAPH) and Multiplex Ligation-dependent Probe Amplification (MLPA) we have screened different cohorts of Duchenne/Becker Muscular Dystrophy (DMD/BMD) patients for duplications. In an unselected series the duplication frequency was 8%; in a group of patients already screened for deletions and point mutations we found a duplication in 64% of cases. The majority were simple, contiguous duplications, however we detected 4 non-contiguous duplications, with two also including a triplication. In two instances the 3' end of the gene was affected, a region not usually screened by multiplex PCR. These mutations would therefore go undetected, whilst potentially disturbing the reading frame of the mRNA. This emphasizes the importance of screening the entire gene for rearrangements.

More than 50% of the duplications found were at the 5` end of the gene, whereas most deletions are found in the middle of the gene. A more detailed comparison of the regions affected showed that a duplication of exon 2 only was the single most common duplication found. Analysis of the breakpoints of 11 such cases revealed two recombination hotspots within intron 2, whereas the breakpoints within intron 1 were scattered. We propose that unequal crossing over between sister chromatids is not responsible for exon 2 duplications. Instead, a mechanism such as synthesis-dependent non-homologous end joining may be responsible. Assuming this also applies to other duplications within the gene, this may explain the different distributions seen for deletions and duplications.

Introduction

The Duchenne Muscular Dystrophy (*DMD*) gene is the largest known, spanning ~2.4 Mb of genomic sequence on Xp21 (Den Dunnen et al., 1992; Mandel, 1989). Mutations in the gene cause DMD, the most commonly inherited neuromuscular disorder, and Becker Muscular Dystrophy (BMD), the milder allelic form of the disease. The mutation spectrum within the gene is unusual in that deletions of one or more exons are found in ~65% of cases (Den Dunnen et al., 1989). These deletions are known to cluster in hot spot regions (Oudet et al., 1992; Forrest et al., 1987), and can easily be detected in males using simple PCR reactions. Two multiplex PCR kits of 9 exons each were developed (Beggs et al., 1990; Chamberlain et al., 1988), and together detect ~98% of all deletions. Duplication analysis and the determination of carrier status, however, require a quantitative method of analysis. Until recently the most commonly applied have been Southern blotting (Forrest et al., 1987) and quantitative multiplex PCR (Ioannou et al., 1992; Abbs and Bobrow, 1992). Southern blotting can cover all exons of the gene, but requires 6-8 blots to do this. This makes it time consuming, and it can be difficult to get blots of the necessary quality. Quantitative multiplex PCR has proven difficult to apply, and will miss mutations outside the hotspot regions. In addition, it implicitly assumes that the majority of duplications will be found within the deletion hotspots.

We recently reported the use of Multiplex Amplifiable Probe Hybridization (MAPH) for screening all 79 exons of the *DMD* gene for deletions and duplications in 2 reactions. Using this approach we were able to identify several small deletions and duplications not detected with other techniques, and showed that a duplication of exon 2, previously undescribed, was the single most common duplication found (White et al., 2002).

A similar technique, Multiplex Ligation-dependent Probe Amplification (MLPA) (Schouten et al., 2002), has become widely used for the detection of deletions and duplications in a variety of diseases (Taylor et al., 2003; Rooms et al., 2004; Slater et al., 2003). In comparison to MAPH, removal of any unbound probes is unnecessary, making the approach easier to perform.

In this report we have used both MAPH and MLPA to screen for duplications in different series of DMD/BMD patients.

Materials and Methods

Patients

All patients were diagnosed with either DMD or BMD by a medical specialist. DNA samples were obtained from blood samples using standard procedures.

Mutation Detection

All samples were screened for deletions and duplications with either MLPA or MAPH. The MLPA reaction was performed with the P034 and P035 kits from MRC-Holland (Amsterdam, the Netherlands). These sets contain probes for all 79 exons of the *DMD* gene as well as the cortical promoter (Dp427c). The reaction was performed following the protocol described in Schouten et al 2002. After the PCR amplification (33-35 cycles) 2 µl of product was mixed with 10 µl Hi Di formamide and 0.1 µl ROX 500 size standard, and separated on the ABI 3700 capillary sequencer (Applied Biosystems). MAPH was performed as described in White et al 2002. Peak data was derived with GeneScan and exported into Excel for analysis. Both MAPH and MLPA analysis was performed as described for MAPH in White et al 2002.

Determination of exon 2 duplication breakpoints.

MLPA probes were designed in intron 1 and intron 2, based on criteria outlined in White et al 2004. Probes within intron 1 ended with sequences allowing amplification with the MAPH primers; the intron 2 probes used the MLPA amplification sequences. All probes were combined in a single mix, with the reaction and analysis being performed as described previously (White et al., 2004).

Based on the estimated duplication borders primers for PCR amplification were designed using the Primer 3 program, with the forward primers in intron 2 and the reverse primers in intron 1. Long range PCR was performed using the Expand Long Template PCR system (Roche), and the resulting PCR products were separated on a 0.8% agarose gel by electrophoresis. Bands of interest were excised and purified using the QIAquick PCR purification kit (Qiagen), and sequenced by the Leiden Genome Technology Center (www.LGTC.nl).

MyoD infection, RNA isolation and RT-PCR analysis

Amniocytes from sample LM7 were forced into myogenesis by infection with a MyoD-containing adenovector as described previously (Havenga et al., 2002; Aartsma-Rus et al., 2003) . RNA isolation and RT-PCR analysis were performed as described (Aartsma-Rus et al., 2002). The full list of primers used is available on request.

Results

Different patient cohorts were screened for deletions and duplications in the *DMD* gene using either MLPA or MAPH. In the first series 120 patients had been screened for deletions within the hot spot regions using modified multiplex PCR kits. Rescreening these revealed 9 duplications, as well as several smaller deletions falling outside the hot spots (Lalic et al, manuscript in preparation). One of the duplications was a complex rearrangement, consisting of 2 distinct duplications and a triplication (Figure 1).

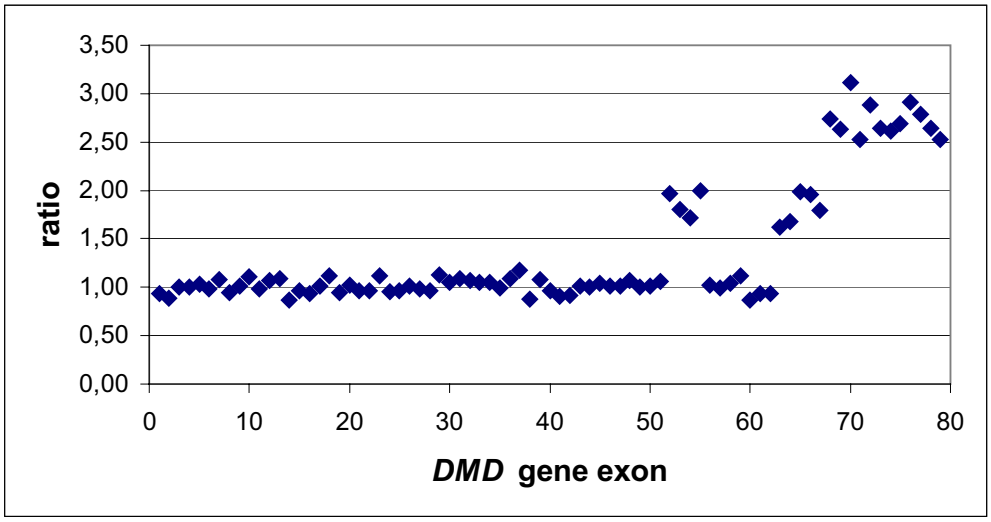

Figure 1. Graphical results based on MLPA analysis of a non-contiguous duplication/triplication.

The second series consisted of 50 patients that had been screened for point mutations and deletions. In this cohort we found 32 (64%) duplications. Non-contiguous rearrangements were seen in 3 samples, and a duplication of exon 2 was the single most common duplication seen, in this study making up 28% of all duplications found. The distribution of the remainder of the mutations was relatively even throughout the gene, but overall the mutations tended to cluster more in the 5' end of the gene. The duplications found in both study populations are listed in table 1.

To see if the large number of exon 2 duplications was due to specific rearrangement hotspots we attempted to define the breakpoints in 11 samples, 10 from these studies and one identified previously (White et al., 2002). MLPA analysis with probes initially spaced ~20 kb apart throughout introns 1 and 2 showed that the breakpoints in intron 1 were scattered, whereas 10 of the 11 breakpoints in intron 2 were found in the first 40 kb. The largest duplication was maximally 220 kb, whereas the smallest duplication was at least 40 kb.

Sample	Mutation
D1	dup 02
D2	dup 02
D3	dup 02
D4	dup 02
D5	dup 02
D6	dup 02
D7	dup 02
D8	dup 02
D9	dup 02
D10	dup 02
D11	dup 02-11
D12	dup 03-04
D13	dup 03-11
D14	dup 03-44
D15	dup 03-06
D16	dup 5-18; trip 19-41; dup 42; trip 43-44
D17	dup 5-19; dup 38-41
D18	dup 06-07
D19	dup 08-13
D20	dup 08-13
D21	dup 08-29
D22	dup 08-44
D23	dup 12-30
D24	dup 17-18
D25	dup 22-25
D26	dup 29-43
D27	dup 37-43
D28	dup 43
D29	dup 45-55; 65-79
D30	dup 45-65
D31	dup 46-47
D32	dup 46-60
D33	dup 49-60
D34	dup 50-59
D35	dup 51
D36	dup 52-55; dup 63-67; trip 68-79
D37	dup 56-63
D38	dup 61
D39	dup 61-62
D40	dup 61-63
D41	dup 61-63
LM7	dup 52-62

Table 1. The duplications found in this study. The numbers refer to exons of the *DMD* gene; non-contiguous rearrangements are separated with a semi colon.

Given the apparent clustering of breakpoints within intron 2, more MLPA probes were designed within the first 40 kb of this intron. Retesting the 10 samples with apparently clustered intron 2 breakpoints showed that five of the samples had the intron 2 breakpoint within a 7 kb area, with the other five breakpoints clustering in a separate region of ~4 kb (Figure 2).

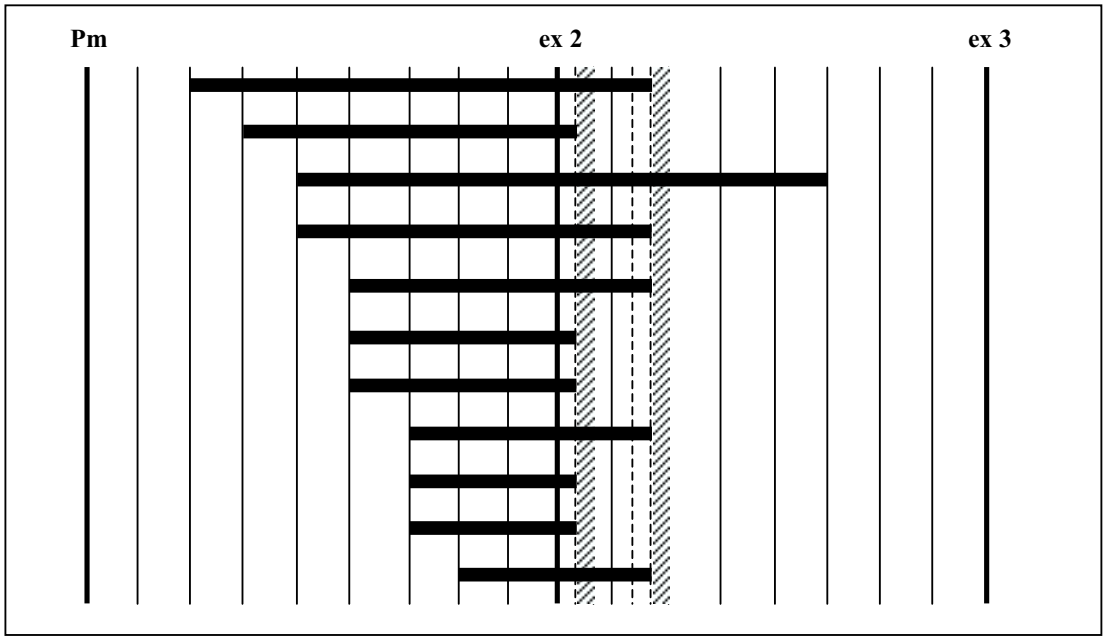

Figure 2. The minimum extent of the different exon 2 duplications, as determined by MLPA and long range PCR. The vertical bars indicate the approximate position of the MLPA probes; the shaded columns indicate the hotspots.

Using long range PCR (see materials and methods) it was possible to precisely define the sequence at the breakpoint in 4 of the samples, indicating that these rearrangements were in tandem. All of the breakpoints had an insertion or deletion of one or more nucleotides at the breakpoint junction.

An isolated sample tested was derived from amniocytes, and showed a deletion of exon 63 at the mRNA level. Exon 63 was present in the genomic DNA, and sequence analysis did not reveal any mutation that might affect splicing. Analysis with MLPA showed that the mutation at DNA level was in fact a duplication of exons 52-62. In order to gain a greater insight as to how such a rearrangement could lead to the skipping of an exon we performed further RT-PCR analysis. By amplifying different fragments it was possible to show that the rearrangement juxtaposed the duplicated exon 62 onto the original exon 64 (Figure 3).

Discussion

We have screened DMD/BMD patients for duplications using the MAPH and MLPA techniques. The patients could be divided into different cohorts, the first unselected and the second where deletions and

point mutations had already been excluded. Duplications were found in 8% of the first group, an overall duplication frequency comparable with what other studies have found (White et al., 2002; Den Dunnen et al., 1989). In patients that had already been screened for deletions and point mutations we detected duplications in 64% of cases. As these samples have not been tested at the RNA level, it is likely that the majority of the remaining patients have mutations affecting splicing. Studies have shown that mutations deep in intronic sequences can activate cryptic splice sites (Beroud et al., 2004), changes that can only be detected by mRNA analysis (Roest et al., 1993; Tuffery-Giraud et al., 2004).

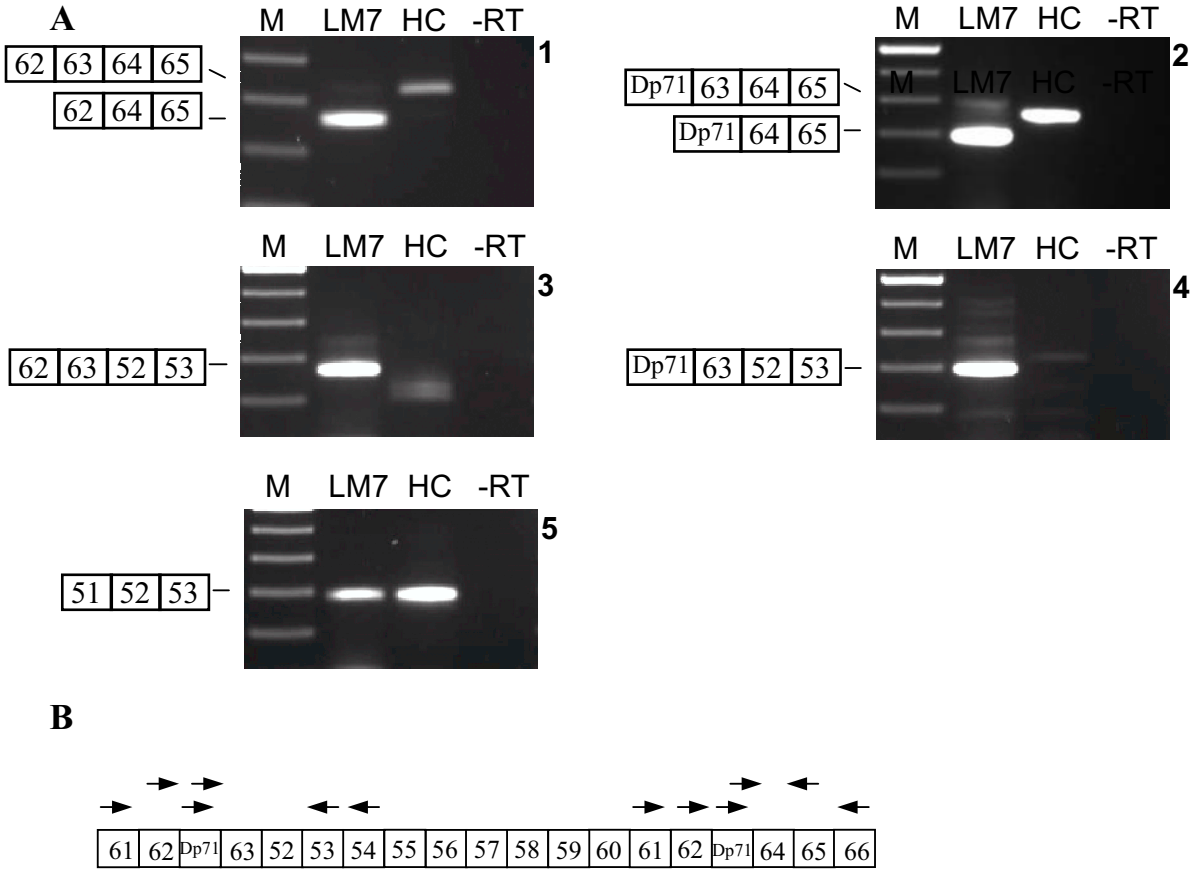

Figure 3 – An example of how a duplication at DNA level can lead to an apparent deletion at RNA level. A. RT-PCR analysis of LM7 and a healthy control (HC) encompassing the duplication breakpoints of LM7 in full-length dystrophin (Dp427m) and Dp71 transcripts. Using primers flanking exon 63 a shorter fragment lacking exon 63 was detected for LM7 when compared to the control, both in the Dp427m and Dp71 transcripts (upper two panels). Using a reversed primer in exon 53 in combination with forward primers in exon 62 (third panel) or in the first exon of Dp71 (fourth panel) a clear band containing exon 62 and 63, or the Dp71 first exon spliced to exon 52 could be produced for LM7, whereas only aspecific bands were observed for the control. Using the same cDNA and reversed primer, a fragment could be amplified for both LM7 and the control with forward primers in exon 51 (lower panel). M is 100 bp size marker, -RT is negative control.
B. The order of the exons in the duplicated region, with the arrows indicating the different primers that were used in determining the borders of the rearrangement.

Combining the data in this report with all other duplications previously described, and comparing these with the most common deletions, shows a marked difference in distribution of the two types of rearrangements (table 2). More than 50% of duplications are located in the 5' end of the gene, whereas the most common deletions cluster almost exclusively between exons 45-52. The reason for this difference is not clear, but the disparity, coupled with the far greater incidence of deletions, suggests that there are fundamental differences in the origins of the two types of rearrangements in the *DMD* gene.

It has been previously described for several genes on the X chromosome, including the *DMD* gene, that deletions are predominantly maternally inherited, whereas duplications mostly originate in the male germline (Hu et al., 1990; Grimm et al., 1994). It has also been shown that the distribution of mutations in the DMD gene when comparing mosaic and nonmosaic cases is significantly different (Passos-Bueno et al., 1992). It is likely that different chromosomal regions show varying levels of susceptibility to rearrangement, depending on the type or stage of cell division. This has been described for rearrangements in the NF1 gene, where the extent of the deletion is dependent on whether the rearrangement occurs during mitosis or meiosis (Kehrer-Sawatzki et al., 2004). The specific mechanism responsible for the rearrangement may also be dependent on factors such as cell type and position in the cell cycle.

ranking	deleted exon(s)	%	ranking	duplicated exon(s)	%
1	45-47	7.0	1	2*	8.5
2	45	5.3	2=	3-7	3.5
3	48-50	5.1	2=	8-9	3.5
4	45-48	4.3	4=	2-7	3.0
5	45-50	4.0	4=	51	3.0
6	51	3.9	6=	3-11	2.0
7	44	3.5	6=	8-13	2.0
8	49-50	2.8	8=	3-4	1.5
9=	3-7	2.3	8=	9-14*	1.5
9=	45-52	2.3	8=	17	1.5
11	46-47	2.3	8=	22-25*	1.5
12	50	2.2	8=	43	1.5
13	45-49	2.0	8=	44	1.5

Table 2. The most frequently occurring deletions and duplications. It is clear that the region most commonly deleted is between exons 45-52; in contrast the duplications occur more frequently in the 5' end of the gene. A * indicates a duplication that has not been described as a deletion. Data from The Leiden Muscular Dystrophy pages (LMDp), based on the DMD_deldup database at 2004/10/26 (Data kindly provided by Ivo Fokkema).

Several different mechanisms are known to cause rearrangments (Shaffer and Lupski, 2000; Helleday, 2003; Hu et al., 1991). For example, intrachromatid looping can occur through the interaction of palindromic sequences, with the intervening sequence being deleted. This mechanism is not expected to produce a duplication. In contrast, unequal crossing over is expected to produce deletions and duplications at an equal frequency. This mechanism has been demonstrated to be responsible for many genomic disorders, via nonallelic homologous recombination between low copy repeats (Emanuel and Shaikh, 2001; Ji et al., 2000; Stankiewicz and Lupski, 2002). Unequal crossing over between Alu repeats has also been described within several genes, including *DMD* (Prior et al., 1997; Deininger and Batzer, 1999; Hu et al., 1991). The 3 duplication breakpoints in the *DMD* gene that had previously been described showed that all were tandem duplications. In one case homologous recombination between Alu elements was seen, the other 2 cases appeared to be due to non-homologous end joining (NHEJ), possibly mediated by topoisomerases. It was suggested that the rearrangements were the result of unequal crossing over between sister chromatids (Hu et al., 1991; Hu et al., 1990; Hu et al., 1989) .

The most frequently occurring duplication described to date in the *DMD* gene is of exon 2. Our initial analysis of the size and location of 11 unrelated exon 2 duplication cases showed that they differed in size. Sequencing of four of the breakpoints of the exon 2 duplications showed the removal or addition of one or more nucleotides at the junction, consistent with NHEJ. Scattered breakpoints is another characteristic feature of NHEJ, yet two distinct hotspots of ~4 kb and ~7 kb were seen, each containing five of the intron 2 breakpoints. This suggests that the event initiating the duplication occurred in intron 2.

It is interesting to note that a deletion of only exon 2 has never been reported. There are several possible reasons for this. It could be that such deletions do occur, but do not result in a severe DMD phenotype. It is also possible that such a mutation is somehow embryonic lethal. These explanations seem unlikely, as there is no obvious reason why an out of frame deletion should not lead to a DMD-like phenotype, when other deletions in the same region do. Likewise, larger deletions encompassing exon 2 have been described in DMD patients, making it unlikely that an individual with a smaller deletion in the same region would be non-viable before birth. A more feasible explanation is that the mechanism responsible for the duplication does not involve unequal crossing over. An example of such a mechanism is synthesis dependent NHEJ (Helleday, 2003). This will result in a tandem duplication at the site of a double stranded break, without unequal crossing over taking place. If the repair can procede in either direction, then one would also expect a similar number of duplications starting in intron 2, i.e. extending from exon 3. As can be seen in table 2, this is in fact the case, where duplications starting at exon 3 occur at a similar frequently to exon 2 duplications. Alternative mechanisms for duplication and deletion formation may therefore underly the differences seen in distribution and parent-of-origin of the two mutation types.

We found 4 cases involving non-contiguous duplications and/or triplications, suggesting multiple mutational events. The chance of 2 independent duplications is low, and it is more likely that the initial duplication was larger, covering the full extent of the different rearrangments. It is known that duplications can be unstable, with reversion to the normal situation (Helleday et al., 1998; Monnat et al., 1992; Hu et al., 1990). It is possible that this reversion will not always be absolute, leading to unusual, non-contiguous rearrangements. Notably in two of these cases a duplication would have been detected in both samples using probes located in the hotspot regions, yet the complete extent of the rearrangements would not have been identified. Indeed, in the case of sample D29, the duplication of exons 45-55 is expected to be an in-frame mutation, which is presumed to lead to a BMD-type phenotype. This patient in fact has a DMD phenotype, presumably as the more 3' duplication disrupts the reading frame. Despite non-contiguous rearrangements being rare events, the fact that they have been shown to occur reinforces the importance of screening the entire gene, in particular when the reading frame rule is going to be used prognistically.

The application of the reading frame rule to duplications assumes that the duplication is in tandem. Although this has been the case in all the *DMD* gene duplication breakpoints described to date (Hu et al., 1991), the consequence of a duplication on the RNA molecule may not always be as simple. An example of this was a duplication of exon 52-62 seen in sample LM7. The actual effect of this mutation at RNA level was an apparent skip of exon 63. This is of importance not only in the predictive diagnosis of disease progression, but also for targetted gene therapy (van Deutekom et al., 2001; Lu et al., 2003). In addition, it is theoretically possible that the apparent duplication is in fact a transposition, which, if outside the *DMD* gene, would have no effect on the reading frame.

In conclusion, we show here that duplications within the *DMD* gene are distributed differently compared to deletions, and can be complex rearrangements. In addition, we provide evidence that the mechanism involved in generating the exon 2 duplications, and by extension other duplications as well, does not involve unequal crossing over between sister chromatids.

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