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RESEARCH ARTICLE

Duplications in the *DMD* Gene

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The detection of duplications in Duchenne (DMD)/Becker Muscular Dystrophy (BMD) has long been a neglected issue. However, recent technological advancements have significantly simplified screening for such rearrangements. We report here the detection and analysis of 118 duplications in the *DMD* gene of DMD/BMD patients. In an unselected patient series the duplication frequency was 7%. In patients already screened for deletions and point mutations, duplications were detected in 87% of cases. There were four complex, noncontiguous rearrangements, with two also involving a partial triplication. In one of the few cases where RNA was analyzed, a seemingly contiguous duplication turned out to be a duplication/deletion case generating a transcript with an unexpected single-exon deletion and an initially undetected duplication. These findings indicate that for clinical diagnosis, duplications should be treated with special care, and without further analysis the reading frame rule should not be applied. As with deletions, duplications occur nonrandomly but with a dramatically different distribution. Duplication frequency is highest near the 5′ end of the gene, with a duplication of exon 2 being the single most common duplication identified. Analysis of the extent of 11 exon 2 duplications revealed two intron 2 recombination hotspots. Sequencing four of the breakpoints showed that they did not arise from unequal sister chromatid exchange, but more likely from synthesis-dependent nonhomologous end joining. There appear to be fundamental differences therefore in the origin of deletions and duplications in the *DMD* gene. Hum Mutat 27(9), 938–945, 2006. Published 2006 Wiley-Liss, Inc.[†]

KEY WORDS: DMD; duplications; genomic rearrangements; MLPA; MAPH

INTRODUCTION

The routine detection of deletions and duplications in the diagnosis of genetic disease has long been neglected. When these rearrangements are rare, this omission is not a significant problem, however, surprising results can be obtained when their existence is first tested (for example in breast cancer [Hogervorst et al., 1995]).

X-linked diseases have historically been a positive exception; detection of deletions in male patients is simplified by the absence of a signal from the second chromosome. This promoted detection of deletions in disorders like Duchenne/Becker muscular dystrophy (DMD, MIM# 310200; BMD, MIM# 300376) [Koenig et al., 1987], Pelizaeus-Merzbacher disease (PMD; MIM# 312080) [Raskind et al., 1991], and steroid sulfatase deficiency (STS; MIM# 308100) [Yen et al., 1987]. Still, even in X-linked diseases the detection of duplications in patients as well as the determination of carrier status in females is much more demanding. A clear example of this is DMD/BMD, in which early reports suggested that duplications occurred at a frequency of up to 10% [Hu et al., 1988; den Dunnen et al., 1989], whereas other studies did not report any such rearrangements [Koenig et al., 1987; Darras et al., 1988].

Recently, several new techniques have been developed, in particular multiplex amplifiable probe hybridization (MAPH) [Armour et al., 2000] and multiplex ligation-dependent probe amplification (MLPA) [Schouten et al., 2002], which have simplified the detection of quantitative changes considerably. The availability of these techniques has revived the interest in the detection of deletions and duplications in a range of diseases, including DMD/BMD [White et al., 2002; Schwartz and Duno, 2004; Janssen et al., 2005; Dent et al., 2005; Lalic et al., 2005]. Application of these technologies, testing all 79 exons of the *DMD* gene individually, confirmed the presence of all previously detected deletions and revealed a significant number of new deletions (up to 5%) and duplications (5–8%).

In this article we describe 118 duplications that we have recently detected in unrelated BMD/DMD patients. Among these we observed several exceptional cases, including noncontiguous duplications, complex rearrangements involving (partial) triplica-

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tions, and a case where a single-exon deletion in the mRNA surprisingly turned out to be caused by a multiexon duplication at the genomic level.

MATERIALS AND METHODS

Samples

All patients were diagnosed with either DMD or BMD by a medical specialist, and DNA was isolated from blood of the patient or mother using standard procedures. The samples were derived from three sources. The first group were patients that had already been screened for deletions and point mutations using direct sequence analysis [Flanigan et al., 2003] and that had subsequently been tested with either MAPH or MLPA. The second cohort of patients is described in detail elsewhere [Lalic et al., 2005], and was screened with MLPA. The third group consisted of patients or mothers of patients in whom a duplication had been detected with Southern blotting; MLPA was performed to confirm the presence of a duplication and to exactly determine its breakpoints.

An isolated sample (54242) was obtained by amniocentesis. This had been requested as the pregnant woman had a brother who died from DMD, and no mutation had been detected. DNA was isolated from amniocytes using standard procedures.

Mutation Detection

MAPH was performed as described [White et al., 2002]. MLPA was performed with the P034 and P035 kits from MRC-Holland (Amsterdam, The Netherlands) as described [Lalic et al., 2005]. Mutations are reported following current recommendations [den Dunnen and Antonarakis, 2000] in relation to a coding DNA reference sequence (NM_004006.1), counting the A of the translation initiation coding as nucleotide 1.

Determination of Exon 2 Duplication Breakpoints

Synthetic MLPA probes were designed in intron 1 and intron 2 of the *DMD* gene, based on the criteria outlined in White et al. [2004]. The DMD gene sequence was obtained from the Human working draft (http://genome.ucsc.edu; May 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 [White et al., 2004]. Additional probes were subsequently designed in the first 40 kb of intron 2 for fine mapping. Sequences of all probes used are available on request. Based on the estimated extent of each duplication, primers for PCR amplification across the duplication breakpoint were designed using the Primer 3 program [Rozen and Skaletsky, 2000], with forward primers in intron 2 and reverse primers in intron 1. Long-range PCR was performed using the Expand Long Template PCR system (Roche, Woerden, the Netherlands), 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, Venlo, the Netherlands), and sequenced by the Leiden Genome Technology Center. Breakpoint sequences have been submitted to GenBank (www.ncbi.nlm.nih.gov/Genbank/; accession numbers DQ013877–DQ013880).

Muscle Differentiation (MyoD) Vector Infection, RNA Isolation, and RT-PCR Analysis

Amniocytes from sample 54242 were forced into myogenesis by infection with a MyoD-containing adenovector as described [Roest et al., 1996; Havenga et al., 2002]. RNA isolation and RT-PCR analysis were performed as described [Aartsma-Rus et al., 2002].

RESULTS

Different patient cohorts were screened for deletions and duplications in the *DMD* gene using either MLPA or MAPH. The first series consisted of 39 patients that had already been screened for point mutations and deletions. In this cohort we found 34 duplications (87%). Noncontiguous rearrangements were seen in three samples. A duplication of exon 2 was the single most common duplication, in this cohort making up 26% of all duplications found. The distribution of the remainder of the rearrangements was relatively even, but overall the duplications clustered toward the 5′ end of the gene.

The second series of 123 unrelated patients had been screened for deletions within the hotspot regions using modified multiplex PCR kits. Rescreening these with MLPA revealed nine duplications, as well as several smaller deletions that were not detected by multiplex PCR [Lalic et al., 2005]. One of the duplications was a complex rearrangement consisting of two noncontiguous duplicated regions and a triplicated region.

The third series consisted of 74 samples in which a duplication had been detected with Southern blotting, either in the index patient or (when no patient material was available) in a carrier mother. MLPA was used to confirm the duplication and more precisely define its extent. Again the majority of duplications were in the 5′ end of the gene, with the most common being a duplication of exons 3–7. The duplications found in all three cohorts are listed in Table 1.

The most frequent change detected was a duplication of exon 2. Additionally, a large number of duplications begin in intron 2 and extend further downstream in the gene (exon 3 and further). To determine whether this was due to specific rearrangement hotspots, we attempted to define the breakpoints in 11 exon 2 duplication cases, 10 from these studies and one (DL120.7) identified previously [den Dunnen et al., 1989; White et al., 2002]. MLPA analysis with probes initially spaced ~20 kb apart throughout introns 1 (191 kb) and 2 (170 kb) showed that the breakpoints in intron 1 were relatively scattered, whereas 10 of the 11 breakpoints in intron 2 were found in the first 40 kb.

Given the apparent clustering of breakpoints within intron 2, additional MLPA probes were designed to refine their mapping. Retesting the 10 samples showed that six of them had the intron 2 breakpoint within an ~7-kb region, with the other four breakpoints clustering in a separate ~6-kb region (Fig. 1). Using long-range PCR we were able to obtain a fragment containing the duplication junction from four patients (two from each hotspot) and determine the breakpoint sequence (GenBank DQ013877–DQ013880). In each hotspot the two breakpoints were ~1 kb apart. Three of the four breakpoints had an insertion of one or more nucleotides at the junction (Fig. 2). One breakpoint was within a complex multinucleotide repeat, and in all four cases the breakpoints were characterized by a low GC content. We could not detect any significant sequence homology around the breakpoints.

An unexpected genomic rearrangement was detected in sample 54242, derived from a prenatal diagnosis. As no mutation was found after initial analysis (multiplex PCR), a MyoD analysis was performed [Roest et al., 1996; Havenga et al., 2002] and we detected a deletion of exon 63 at the cDNA level. Exon-specific PCR showed that this exon was present in the genomic DNA, and sequence analysis did not reveal any mutation that might affect splicing. Surprisingly, MLPA analysis revealed a duplication

TABLE 1. Duplications Identified in This Study

Rearrangement	Mutation	Independent cases	Patient IDs
dup 2	c.32-?_93+?dup	13	1397, 55880, 58303, 43093, 43110, 43051A, DRVH1037, 42989, 43135, 43141, 43123, 42966, B104+
dup 2-7	c.32-?_649+?dup	3	1215, 53830, 2018
dup 2-11	c.32-?_1331+?dup	3	1308, 61191, 42960*
dup 3	c.94-?_186+?dup	1	54013
dup 3-4	c.94-?_264+?dup	3	1239, 53829, 43160
dup 3-6	c.94-?_530+?dup	1	DRVH1122
dup 3-7	c.94-?_649+?dup	5	1202, 1567, 53475, 62262, 43073
dup 3-9	c.94-?_960+?dup	1	61117
dup 3-11	c.94-?_264+?dup	1	B119
dup 3-12	c.94-?_1482+?dup	1	50174
dup 3-13	c.94-?_1602+?dup	1	42587*
dup 3-16	c.94-?_1992+?dup	1	2027
dup 3-18	c.94-?_2292+?dup	1	50233
dup 3-38	c.94-?_5448+?dup	1	56365
dup 3-44	c.94-?_6438+?dup	1	B40+
dup 5	c.265-?_357+?dup	1	59824
dup 5-7	c.265-?_649+?dup	2	1057, 50786
dup 5-18; trip 19-41; dup 42; trip 43-44	c.[265-?_2292+?dup; 2293-?_5922+?tri; 5923-?_6117+?dup; 6118-?_6438+?tri]	1	43067
dup 5-19; dup 38-41	c.[265-?_2380+?dup; 5326-?_5922+?dup]	1	42391*
dup 6-7	c.358-?_649+?dup	2	58691, 43119
dup 7	c.531-?_649+?dup	2	52522, 57368
dup 8-9	c.650-?_960+?dup	2	1008, 58586
dup 8-13	c.650-?_1602+?dup	2	DRVH1060, 43154
dup 8-16	c.650-?_1992+?dup	1	43092
dup 8-29	c.650-?_4071+?dup	1	DC0042
dup 8-30	c.650-?_4233+?dup	1	51500
dup 8-44	c.650-?_6438+?dup	2	58064, B108
dup 10-11	c.961-?_1331+?dup	2	1221, 59448
dup 10-17	c.961-?_2168+?dup	1	1520
dup 12	c.1332-?_1482+?dup	1	1118
dup 12-13	c.1332-?_1602+?dup	1	1147
dup 12-30	c.1332-?_4233+?dup	1	DRVH106
dup 13-19	c.1483-?_2380+?dup	1	1494
dup 13-29	c.1483-?_4071+?dup	1	2060
dup 14-17	c.1603-?_2168+?dup	1	58304
dup 14-34	c.1603-?_4845+?dup	1	58845
dup 14-42	c.1603-?_6117+?dup	2	2093, 2145
dup 16-34	c.1813-?_4845+?dup	3	2008, 2076, 2138
dup 16-41	c.1813-?_5922+?dup	1	2148
dup 17	c.1993-?_2168+?dup	1	55390
dup 18-23	c.2169-?_3162+?dup	1	58686
dup 18-27	c.2169-?_3786+?dup	1	56283
dup 18-37	c.2169-?_5325+?dup	1	59307
dup 22-25	c.2804-?_3432+?dup	1	DRVH1032
dup 22-41	c.2804-?_5922+?dup	1	2099
dup 29-43	c.3922-?_6290+?dup	1	43104
dup 33-44	c.4519-?_6438+?dup	1	56904
dup 37-43	c.5155-?_6290+?dup	1	43027
dup 43	c.6118-?_6290+?dup	1	43164
dup 44	c.6291-?_6438+?dup	2	1150, 56539
dup 44-55	c.6291-?_8217+?dup	1	1565
dup 44-57	c.6291-?_8547+?dup	1	1206
dup 45	c.6439-?_6614+?dup	3	1164, 1178, 1254
dup 45-47	c.6439-?_6912+?dup	2	1465, 58429
dup 45-50	c.6439-?_7309+?dup	1	1117
dup 45-52	c.6439-?_7660+?dup	1	56942
dup 45-55	c.6439-?_8217+?dup	1	1592
dup 45-55; dup 65-79	c.[6439-?_8217+?dup; 9362-?_13749+?dup]	1	43000
dup 45-56	c.6439-?_8390+?dup	1	1176
dup 45-65	c.6439-?_9563+?dup	1	DRVH1053
dup 46-47	c.6615-?_6912+?dup	2	1320, 43189
dup 46-60	c.6615-?_9084+?dup	1	43050
dup 48-50	c.6913-?_7309+?dup	1	1370
dup 49-60	c.7099-?_9084+?dup	1	B110+
dup 50-55	c.7201-?_8217+?dup	1	1012

TABLE 1. Continued

Rearrangement	Mutation	Independent cases	Patient IDs
dup 50-59	c.7201-?_8937+?dup	1	B92+
dup 51	c.7310-?_7542+?dup	2	56120, 43020
dup 51-55	c.7310-?_8217+?dup	1	54694
dup 52-55	c.7543-?_8217+?dup	1	1010
dup 52-55; dup 63-67; trip 68-79	c.[7543-?_8217+?dup; 9225-?_9807+?dup; 9808-?_13749+?tri]	1	B129+
dup 52-60	c.7543-?_9084+?dup	1	52443
dup 52-62	c.7543-?_9224+?dup	2	1439, 54242
dup 53	c.7661-?_7872+?dup	1	B18+
dup 56-63	c.8218-?_9286+?dup	1	B109+
dup 57-60	c.8391-?_9084+?dup	1	1382
dup 58-63	c.8548-?_9286+?dup	1	1033
dup 61	c.9085-?_9163+?dup	1	42987*
dup 61-62	c.9085-?_9224+?dup	1	43169
dup 61-63	c.9085-?_9286+?dup	1	43099

*The numbers in the first column refer to exons of the *DMD* gene; a semicolon separates noncontiguous rearrangements. The mutations are described based on reference sequence NM_004006.1, with the first base of the Met-codon counted as position 1. Samples marked with an asterisk have been previously reported in Dent et al. [2005]. Samples marked with a plus sign have been previously reported in Lalic et al. [2005]. Four of the exon 2 duplication breakpoints were sequenced; the precise mutation description is given in Figure 2.

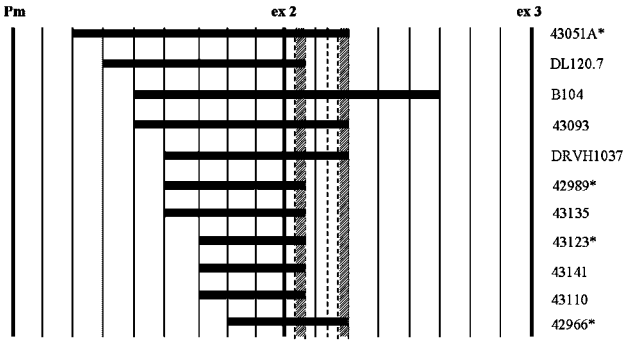

FIGURE 1. **The maximum extent of the 11 different exon 2 duplications examined, as determined by MLPA and long-range PCR. Each vertical bar indicates the approximate position of an MLPA probe. The shaded columns indicate the two hotspot regions identified. For samples indicated with an asterisk, the breakpoint was PCR amplified and sequenced.**

of exons 52–62. To determine how a duplication at DNA level could lead to the deletion of an exon on RNA level we performed a detailed RT-PCR analysis, focusing on the duplication borders.

For the 5′ junction, a specifically designed RT-PCR (Fig. 3) showed the presence of transcripts joining exon 63 to exon 52, both deriving from an upstream promoter as well as from the Dp71 promoter present in intron 62. For the 3′ duplication junction our analysis identified transcripts lacking exon 63, again originating from an upstream and the Dp71 promoter (Fig. 3). These data indicate that this duplication is in fact noncontiguous, most probably derived from a duplication/deletion event. It should be noted that in this case the finding does not influence the deduced effect on the reading frame (and expected phenotype), since both the juxtaposition of exons 62 and 63 to exon 52 will disrupt the reading frame. However, in other cases it is easily possible that the reading frame could be altered in an unexpected manner, with the consequence that an incorrect prognosis is made.

DISCUSSION

Although duplication mutations in the *DMD* gene were reported to be relatively frequent early on [den Dunnen et al., 1989], the considerable effort (and associated cost) to detect them made them largely neglected. This situation has changed only recently with the development of MAPH [Armour et al., 2000] and MLPA [Schouten et al., 2002], two easy and versatile methods for the detection of both deletions and duplications. We have now screened several hundred DMD/BMD patients using these techniques. In an unscreened cohort, duplications were found in 7% of cases, an overall duplication frequency comparable with what previous studies had suggested [den Dunnen et al., 1989; White et al., 2002; Schwartz and Duno, 2004; Janssen et al., 2005]. In patients that had been screened for deletions and point mutations we detected duplications in 87% of the cases. As these samples have not been tested at the RNA level, it is likely that the majority of the remaining cases have mutations affecting splicing [Beroud et al., 2004].

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]. Data collected in the Leiden Muscular Dystrophy Database (LMDp) (www.dmd.nl) confirm that duplications in the majority of cases have a grandpaternal origin. As a consequence, duplications present more frequently as familial cases and the recurrence risk is high. On the other hand, the chance of a combination of a duplication and a germline mosaicism are rather low. It has 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 specific rearrangements, depending on the type or stage of cell division. For the *NF1* gene, it was reported that the extent of the deletion is dependent on whether the rearrangement occurs during mitosis or meiosis [Kehrer-Sawatzki et al., 2004].

Several different mechanisms are known to cause genomic rearrangements [Hu et al., 1991; Shaffer and Lupski, 2000; Helleday, 2003]. For example, intrachromatid looping can occur through the interaction of palindromic sequences, with the intervening sequence being deleted. However, this mechanism is not expected to generate 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

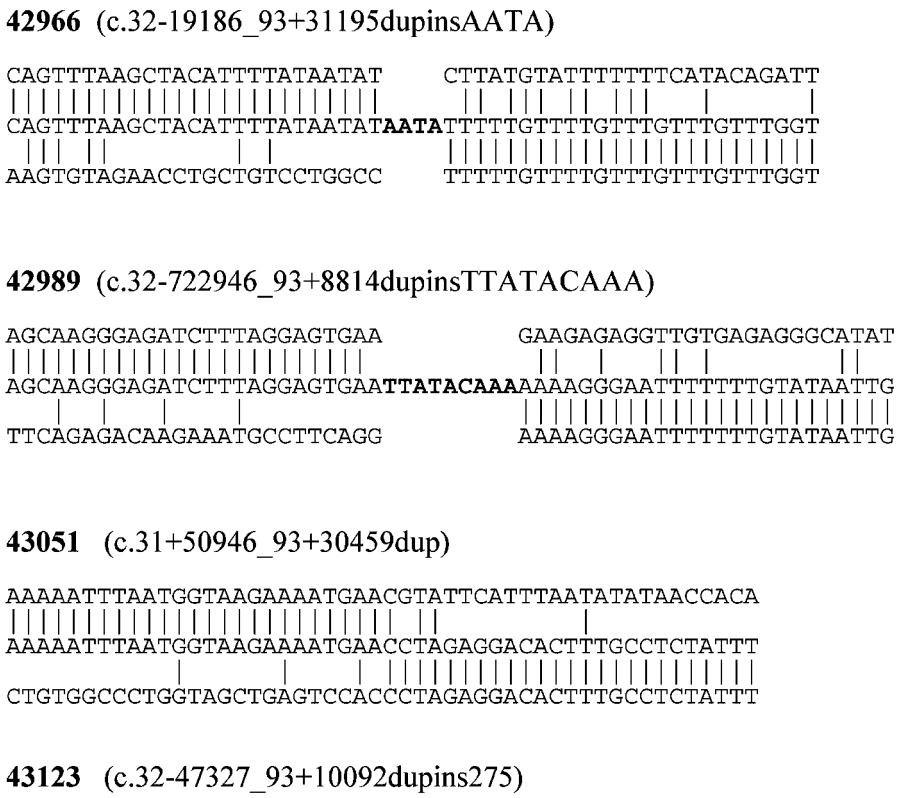

FIGURE 2. **Three of the four exon 2 duplication breakpoints that were sequenced. For each sample the middle line is the breakpoint sequence, with homology to the genomic sequence above and below indicated by vertical bars. Inserted nucleotides at the junction are indicated in bold. The mutations are described based on reference sequence NM_004006.1, with the first base of the Met-codon counted as position 1. Sample 43123 had a breakpoint within a complex multinucleotide repeat, making a precise characterization impossible (as this region is likely to be polymorphic).**

responsible for many genomic disorders, via nonallelic homologous recombination between low-copy repeats [Ji et al., 2000; Emanuel and Shaikh, 2001; Stankiewicz and Lupski, 2002]. Unequal crossing over between Alu repeats has been described within several genes, including *DMD* [Hu et al., 1991; Prior et al., 1997; Deininger and Batzer, 1999]. It has been suggested that duplications within the *DMD* gene are primarily caused by unequal crossing over [Hu et al., 1989, 1991].

The most frequently occurring duplication observed in the *DMD* gene is a duplication of exon 2 (Table 2). If unequal crossing over were the mechanism involved then deletions of exon 2 would be expected to occur relatively frequently. However, a deletion of exon 2 only 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, or 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 less likely that an individual with a smaller deletion would be nonviable. A more likely explanation is that the mechanism responsible for the duplication does not involve unequal crossing over, although it should be noted that the different possibilities are not mutually exclusive.

To investigate this question, we precisely mapped the 11 exon 2 duplication breakpoints and sequenced four of them. This study revealed the addition of multiple nucleotides in three of the four breakpoint junctions, and no significant sequence homology was seen directly around the junction. This is consistent with nonhomologous end joining (NHEJ) as the causative process generating these duplications. Scattered breakpoints is another characteristic feature of NHEJ, yet two distinct hotspots of ~6 kb and ~7 kb were seen, containing four and six of the intron 2 breakpoints, respectively. One possible mechanism that supports these apparently contradictory findings 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. This mechanism has recently been proposed for duplications of *PLP1*, another gene on the X-chromosome [Woodward et al., 2005].

If the repair can proceed in either direction, then one would expect a similar number of duplications starting in intron 2, i.e., seen as beginning at 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 frequency to exon 2 duplications.

We also observed several noncontiguous duplications, with two also involving partial triplications. The instability of duplications has been reported, with reversions to the normal situation occurring [Hu et al., 1990; Monnat et al., 1992; Helleday et al., 1998]. When such a reversion is not absolute, an unusual, non-contiguous rearrangement will remain. Notably, in two of the noncontiguous cases described here, a duplication could have been detected using probes located in the hotspot regions, yet the complete extent of the rearrangements would not have been identified. Obviously this has consequences when applying the reading frame rule [Monaco et al., 1988]. In the case of sample 43000, the duplication of exons 45–55 would be concluded to be

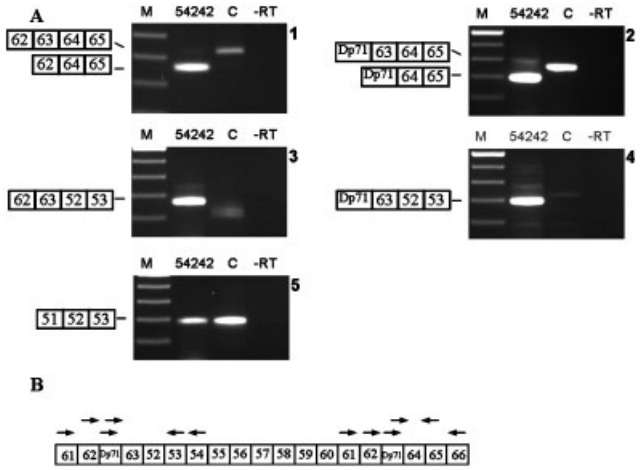

FIGURE 3. **An example of how a duplication at DNA level can lead to an apparent deletion at RNA level. A: RT-PCR analysis of sample 54242 and a control (C) encompassing the duplication breakpoints of 54242 in full-length dystrophin (Dp427m) and Dp71 transcripts. Using primers flanking exon 63, a shorter fragment lacking exon 63 was detected for 54242 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) only 54242 produced a clear band containing exon 62 and 63, or the Dp71 first exon spliced to exon 52, respectively. Using the same cDNA and reversed primer, a fragment could be amplified for both 54242 and the control with forward primers in exon 51 (lower panel). M = 100-bp size marker; −RT = 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.**

TABLE 2. **Most Frequently Occurring Deletions and Duplications in the *DMD* Gene***

Ranking	Deleted exon(s)	%	Ranking	Duplicated exon(s)	%
1	45–47	7.0	1	2	8.6
2	45	5.3	2 =	2–7	3.3
3	48–50	5.1	2 =	8–9	3.0
4	45–48	4.3	4 =	3–7	2.7
5	45–50	4.0	4 =	51	2.3
6	51	3.9	6 =	3–4	2.3
7	44	3.5	6 =	44	2.0
8	49–50	2.8	8 =	8–13	1.7
9 =	3–7	2.3	8 =	2–11	1.3
9 =	45–52	2.3	8 =	3–11	1.3
11	46–47	2.3	8 =	5–7	1.3
12	50	2.2	8 =	6–7	1.3
13	45–49	2.0	8 =	17	1.3

*The region most commonly deleted lies between exons 45–52. In contrast, the duplications occur more frequently near the extreme 5′ end of the gene. Noteworthy is that the reciprocal deletion of the most common duplication (exon 2) has never been detected. Data taken from the DMD deletion/duplication database at the Leiden Muscular Dystrophy website (LMDp).

in-frame, with an expected BMD-type phenotype. This patient in fact has a DMD phenotype, presumably as the more 3′ duplication of exons 65–79 disrupts the reading frame. Despite noncontiguous rearrangements being rare events, the fact that they have been shown to occur reinforces the importance of screening the entire gene.

We observed another case that underlined the above observation. Application of the reading frame rule to duplications assumes that the duplication is in tandem. However, most duplications have not been analyzed at the RNA level, and we found a clear example in which the assumption that the duplication is in tandem turned out to be incorrect. Sample 54242 had a duplication of exons 52–62, with the unexpected finding of an exon 63 deletion at the RNA level. Only detailed RNA analysis was able to show that the RNA in fact did contain a duplication (exon 52–62), but that the duplication was more complicated than expected and in fact not contiguous. This case shows that an accurate ascertainment of the consequences of a duplication is of importance not only in the prognostic diagnosis regarding disease progression (DMD or BMD phenotype), but also for targeted gene therapy [van Deutekom et al., 2001; Lu et al., 2003]. It should also be noted that, theoretically, it is possible that a duplication is in fact a transposition, which, if outside the *DMD* gene, would have no effect at all on the reading frame.

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

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