Emerin deletions occurring on both Xq28 inversion backgrounds

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Emery–Dreifuss muscular dystrophy (EMD) is an X-linked disorder characterized by contractures, progressive weakness and cardiomyopathy. EMD is caused by mutations in the 2 kb emerin gene that is located within human Xq28. Emerin is immediately distal to the 26 kb filamin gene, and flanking the filamin–emerin region are two large inverted repeats. This entire region previously has been found to be inverted in ~20% of X chromosomes, presumably mediated by the inverted repeats. Only one complete emerin deletion has been reported previously. It was found to be due to a complex rearrangement involving the inverted repeats which partially duplicated filamin. We report here two additional EMD patients who have large deletions of 20 and 34 kb, respectively. Unlike the previously reported deletion, these deletions appear to be simple deletions, with each breakpoint junction showing only 2 bp of overlap, suggesting an end-joining mechanism. However, the two deletions were found on each of the two inverted backgrounds. The 20 kb deletion includes the entire emerin gene and extends well into most of the distal inverted repeat. In contrast, the 34 kb deletion occurs on the inverted X chromosome and extends centromeric, well beyond the proximal inverted repeat. In addition, at least three nearby putative genes detected by previous sequence analysis are deleted among these patients but without obvious deviation from a typical EMD phenotype. Similarly to the previously reported deletion, filamin remains intact in these two deletions. All three deletions involve distinct breakpoints within the 4.7 kb filamin–emerin intergenic region, suggesting that loss of filamin is a lethal event. Thus, the close proximity of filamin to emerin may place constraints upon potential emerin deletions and probably accounts for the rarity of complete emerin deletions in EMD patients.

INTRODUCTION

Emery–Dreifuss muscular dystrophy (EMD) is an X-linked recessive disorder characterized by progressive muscle weakness, contractures of the elbows, Achilles tendons and post-cervical muscles, and cardiomyopathy (1–3). The 2 kb emerin gene responsible maps to a well characterized, gene-rich region on Xq28 where >280 kb of sequence has been described (4–6; accession Nos L44140, Z49258 and Z46936). Immediately proximal to emerin is the 26 kb filamin (FLN1) gene which encodes a 280 kDa actin-binding protein (7,8). Flanking the FLN1–emerin region are two large (11.3 kb) inverted repeats with >99% sequence identity (Fig. 1 A, ref. 6).

Emerin mutations identified to date include a few missense mutations, and the majority are nonsense, splice site or small deletions/insertions that ultimately result in premature translation termination and loss of emerin protein (4,5,9–11). We recently described the first complete emerin gene deletion that also resulted in a partial duplication of the nearby FLN1 gene (12). This DNA rearrangement appeared to be the result of mispairing of the two conserved and nearly identical repeats flanking the FLN1–emerin region, followed by double recombination among one set of mispaired repeats and internal sequences. Moreover, characterization of this rare DNA rearrangement led to the discovery of a more common result of mispairing of the inverted repeats: recombination leading to a benign inversion of the 48 kb FLN1–emerin region. Indeed, it was shown that nearly 20% of human X chromosomes present this inversion (Fig. 1A and B).

Here we describe two additional emerin gene deletions of ~20 and 34 kb, respectively. Unlike the previous deletions, these appear due to an end-joining of double strand breaks. However, each occurs on either inverted background. Although each deletion extends either telomeric or centromeric to emerin, the emerin deletion on either of the inverted polymorphic chromosomes leaves the FLN1 gene unaffected.
Figure 1. Diagrams of the genomic region surrounding the emerin gene for each inversion polymorphism. (A) The genomic region surrounding the emerin gene as described by Chen et al. (6). The 219 kb of contiguous sequence that describes most of this region is available at accession No. L44140, and the sequence of an overlapping cosmid (14B7) which extends 40 kb towards the centromere is available at accession No. Z49258. (B) The genomic region surrounding the emerin gene in the inverted polymorphism. The shaded rectangles represent emerin, the unshaded rectangles are FLN1, and the direction of transcription for each gene is indicated by small arrows. The thick black arrows represent the 11.3 kb inverted repeats flanking emerin, and the circle represents the centromere. Pertinent restriction sites for EcoRI (E), BamHI (B) and BglII (Bg) are shown, and fragment sizes in kilobases are indicated below. Probes used in Southern blot experiments are indicated by thick black lines, and the dashed lines indicate the regions within the proximal inverted repeat that cross-hybridize to probe C. (C) Southern blot of 10 µg each of genomic DNA from patients BS and AB as well as a control female (C) that is heterozygous for the inversion polymorphism hybridized to probe F.

Table 1. Southern blot analysis of normal and EMD patient DNAs

<table>
<thead>
<tr>
<th>Probe</th>
<th>Size (kb) of hybridizing fragment(s) with EcoRI and BamHI digests</th>
<th>EcoRI</th>
<th>BamHI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control AB BS Control AB BS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>3.0 + 2.1 – –</td>
<td>7.3</td>
<td>–</td>
</tr>
<tr>
<td>B</td>
<td>3.7 6.0 13 1.3 + 1.0 (2x)</td>
<td>1.3</td>
<td>10.5</td>
</tr>
<tr>
<td>C</td>
<td>17 + 7.6a 17 + 7.6a 12 + 4.8a 12</td>
<td>10.5 + 4.8a</td>
<td></td>
</tr>
</tbody>
</table>

*Cross-hybridizing fragments from proximal inverted repeat, see Figure 1.

RESULTS

Unrelated patients AB and BS were initially identified as emerin-negative by Western analysis of buffy coat extract (data not shown). Southern blot analysis revealed complete emerin gene deletions in both patients using the emerin cDNA as a probe (Table 1, Fig. 1). To characterize these deletions further, Southern blot analysis was performed using genomic DNA fragments isolated from cosmids encompassing the emerin region as probes (Fig. 1). Hybridization of probe B to EcoRI- and BamHI-digested DNA from patient BS showed the absence of normal sized DNA fragments and the presence of novel 13 kb EcoRI and 10.5 kb BamHI fragments (Table 1, Fig. 1A). When this blot was stripped and hybridized with probe C, the normal 17 kb EcoRI and 12 kb BamHI hybridizing fragments were absent, and the 13 and 10.5 kb fragments previously detected using probe B were again observed. Probe C also identified the cross-hybridizing fragments (7.6 kb EcoRI and 4.8 kb BamHI) located within the first inverted repeat. These results indicated that patient BS probably had an ∼20 kb deletion on the more common inversion polymorphic of Xq28, centromere–FLN1–emerin–telomere, with the proximal and distal deletion breakpoints contained within the regions encompassed by probes B and C, respectively.

In patient AB, hybridization of probe B to EcoRI- and BamHI-digested DNA also revealed altered restriction patterns, with the absence of the normal 3.7 kb EcoRI fragment and the presence of a novel 6.0 kb EcoRI fragment and the normal 1.3 kb BamHI fragment only (Table 1, Fig. 1B). Using probe C, the normal 17 kb EcoRI and 12 kb BamHI fragments were observed; however, the cross-hybridizing fragments from the proximal inverted repeat were not. These data therefore predicted that patient AB had a >20 kb deletion on the less common inversion background of Xq28, centromere–emerin–FLN1–telomere, with the distal breakpoint contained within the region encompassed by probe B and the proximal breakpoint occurring within sequences centromeric to the first inverted repeat.

A diagnostic Southern blot subsequently was performed to verify the orientation of the FLN1–emerin region in both patients (Fig. 1). BglII-restricted DNA hybridized with a 3′ FLN1 cDNA probe.
Figure 2. Diagram of the emerin deletions in patients BS (A) and AB (B). The shaded and unshaded rectangles represent the emerin and FLN1 genes, respectively. The small arrows inside represent the direction of transcription for each gene. The thick black arrows are the inverted repeats, and the circle represents the centromere. The region that was deleted in each patient is indicated by the triangle, and the small arrows below represent the primers used for PCR amplification of the deletion junction in each patient. The sequence surrounding each breakpoint is also shown (middle strand), along with the normal sequences located proximal (top strand) and distal (bottom strand) to emerin. Homology between the normal and junction sequences is indicated by shading, and the underlined portions indicate topoisomerase recognition sites.

(probe F) showed a 17.5 kb band in patient BS and a 30.3 kb band in patient AB, which confirmed the orientation centromere–FLN1–emerin–telomere in BS and centromere–emerin–FLN1–telomere in AB (Fig. 1C).

Analysis of Southern blot data (described above) as well as PCR experiments designed to delineate the deletion boundaries (data not shown) ultimately allowed generation of primers which amplified DNA fragments spanning the deletion junctions in both patients (Fig. 2). Primers 45.0F and 66.0R, located ~4.8 kb proximal and 14 kb distal to emerin, respectively (on the FLN1–emerin polymorphism), amplified a 795 bp fragment in patient BS DNA (data not shown). Similarly, primers JF and 45.4F, located ~28 kb proximal and 4.4 kb distal to emerin, respectively (on the emerin–FLN1 polymorphism), amplified a 392 bp fragment in patient AB DNA (data not shown). No amplification was detected using either set of primers in control DNA.

Sequence analysis of these deletion junction PCR products revealed the exact locations of the deletion breakpoints in both patients. In patient BS, sequence breakpoints were identified 4.5 kb proximal to emerin, within the FLN1–emerin intergenic region, and 13.5 kb distal, within a repetitive Alu located in the distal region of the second inverted repeat (Fig. 2A). In patient AB, a sequence breakpoint was also identified within the FLN1–emerin intergenic region, and the proximal deletion breakpoint was located 13 kb centromeric to the first inverted repeat (Fig. 2B). At each deletion junction, only two bases of sequence homology were identified. Although, in the databases queried, no extensive sequence homology was found associated with the proximal and distal normal sequences surrounding any of the breakpoints, we did note some topoisomerase recognition sites [(G/C)(A/T)T] in close proximity to the breakpoints (13). Therefore, a non-homologous or illegitimate mechanism of recombination may have occurred for both deletions.

Thus, patient BS revealed a 20,297 bp deletion on the inverted polymorphic background of centromere–FLN1–emerin–telomere which encompassed the entire emerin gene and extended toward the telomere to include most of the distal inverted repeat (Fig. 2A). Patient AB, on the other hand, showed a 34,090 bp deletion on the inverted polymorphic background of centromere–emerin–FLN1–telomere which also encompassed the entire emerin gene and extended towards the centromere to include sequences beyond the proximal inverted repeat (Fig. 2B).

DISCUSSION

This report describes the complete emerin gene deletions of two unrelated EMD patients, BS and AB. These deletions were shown to occur on an inverted polymorphic background of Xq28 that encompasses the emerin and filamin genes (12). Patient BS was found to have a >20 kb deletion on the more common inversion background of centromere–FLN1–emerin–telomere that extended telomeric to include most of the distal inverted repeat. In contrast, patient AB was shown to have a >34 kb deletion on the less common inversion background of centromere–emerin–FLN1–telomere that extended towards the centromere to include sequences beyond the proximal inverted repeat.
The deletions described in this report occur within a gene-rich region of Xq28 where 13 known genes and six candidate have been located (6). Pertinent to this study, three of the six candidate genes, CVG-1, CVG-2 and CVG-4, identified by GRAIL and found to be associated with CpG islands, are located in regions deleted in patients BS and AB. CVG-2 and CVG-4 are exact copies and are located within the proximal and distal 11.3 kb inverted repeats, respectively. CVG-1 is located immediately proximal to the first inverted repeat. Therefore, in patient AB, CVG-1 and CVG-2 are also deleted and, in patient BS, CVG-4 is deleted. Despite the loss of these potential genes, both patients displayed only typical EMD, suggesting that these candidate genes may be pseudogenes, although a CVG-1 transcript has been identified in a PCR-based test (6), or that loss of one or more of these genes does not result in an obvious phenotype.

Emerin gene deletions are relatively rare. In addition to a few missense mutations, 48 of the 52 EMD mutations characterized to date have been nonsense, splice site or small deletions/insertions that predict the absence of functional emerin (4,5,9–11; see also the Emery–Dreifuss Muscular Dystrophy database at http://www.path.cam.ac.uk/emd). There are four complete emerin gene deletion patients identified [the initial patient reported by Small et al. (12), the two reported here and an unpublished and uncharacterized deletion in the EMD database submitted by C. Muller]. Thus, from the EMD database, 56 unique mutations leading to EMD have been characterized. The vast majority (93%) are changes that lead to a null mutation (52 of 56). However, only 6% are large deletion of emerin. While this may reflect the relatively small size of emerin, it also appears to be the result of constraints placed upon such deletions by the nearby FLN1 gene. Each of the three characterized emerin deletions shows distinct deletion breakpoints that occur within the 4.7 kb FLN1-emerin intergenic region. Within this region, deletion breakpoints for patients BS and AB were found within 250 bp of each other, located 4.5 kb proximal to emerin and 194 bp from FLN1 exon 1 in patient BS and 4.3 kb distal to emerin and 398 bp from FLN1 exon 1 in patient AB. The deletion breakpoint for patient CM (12) is located closer to emerin, ~1.4 kb proximal. Furthermore, for each deletion, the filamin gene remains unaltered. (For patient CM, FLN1 is partially duplicated with one copy remaining intact.) These results suggest that only DNA rearrangements maintaining FLN1 function are viable and that emerin deletions are relatively rare due to the necessity of maintaining FLN1 function. Consistent with this hypothesis is the fact that filamin, a 280 kDa actin-binding protein, has been shown to be essential for cell motility (7,14).

Each of the three emerin deletions characterized so far appears to be the consequence of non-homologous recombination, as evidenced by only 2 bp of sequence homology at each deletion junction. Previous studies have shown that spontaneous deletions are often associated with junctions with very short regions of homology, and it has been suggested that these short sequence homologies facilitate the joining of broken ends (15,16). Although the mechanisms creating the deletions in patients AB and BS remain unclear, Sakagami et al. (17) have shown evidence for homology-associated non-homologous recombination that appears to promote illegitimate recombinations in mammalian cells. In support of this hypothesis, the unstable gene-rich region surrounding the iduronate-2-sulfatase gene (IDS) on human Xq27.3–q28 contains large blocks of inverted homologous sequences and has been shown to be involved in recombinations resulting in deletions or rearrangements of the IDS gene (18–20). It has been suggested that these frequent IDS rearrangements could be promoted by homology-associated non-homologous recombination whereby mispairing of large duplicated regions in Xq27.3–q28 predisposes the IDS locus to illegitimate recombination (21). We have shown previously that mispairing of the inverted repeats surrounding the emerin and filamin genes does occur (12); therefore, the deletions described in this report may also be the consequence of a similar mechanism. In conclusion, the large 20 and 34 kb deletions of Xq28 causing EMD described in this report illustrate the complexity of DNA rearrangements that can occur on a polymorphic inverted background.

**MATERIALS AND METHODS**

**Patients**

Both BS and AB presented as sporadic cases with typical EMD phenotypes. However, subsequent analysis confirmed X-linked inheritance in both families. Only in patient BS were we able to ascertain prior generations, confirming the maternal origin of the deletion. Patient BS exhibited contractures of the neck, elbow and Achilles tendons, as well as muscle weakness and wasting beginning at age 5. Cardiomyopathy, present as bradycardia, was treated by the insertion of a pacemaker at age 9. Patient AB exhibited contractures of the elbows at age 10 and later, with muscle weakness and wasting. Cardiomyopathy was also apparent as a low heart rate (30 beats per min) at age 26.

**Southern analysis**

Genomic DNAs for Southern blot analysis were prepared from lymphoblastoid cell lines and/or blood samples (Qiagen). Ten µg of each DNA was digested with 20–40 U of the appropriate restriction enzyme (Gibco, BRL) and electrophoresed through 0.8% agarose gels for 24 h at 35 V. To separate large sized fragments, as shown in Figure 1C, DNAs were electrophoresed through 0.6% agarose gels for 30 h at 45 V. DNA was transferred to Hybond N+ (Amersham), hybridized with [32P]dCTP randomly labeled probe (Megaprime DNA Labeling Kit, Amersham) and washed according to the manufacturer’s recommendations. Probe A was the 1.1 kb emerin cDNA (4), probe B was a 3.7 kb EcoRI fragment isolated from cosmID 220D6 (22), probe C was an 8.1 kb EcoRI–BstEII fragment isolated from cosmID 58ES (22) and probe F was a 1.4 kb EcoRI fragment that was the 3’ end of the FLN1 cDNA (7). To suppress hybridization to repetitive sequences, probes were pre-annealed at 65°C to total human DNA at C0 values of 50.

**PCR and sequence analysis**

For patient BS, primers used for amplification of the deletion junction were 45.0F (5’-CCT GAG AGC GAC CGG TGA CCG ATG AC-3’) and 66.0R (5’-GGG GGA ACT GTG AGT CCA CCT-3’). For patient AB, the deletion junction was amplified using primers JF (5’-GCA CCC TCA GCA TGG TGT CTG AG-3’) and 45.4F (5’-CAC AAG GAT CCG TGG CCT CTG AG-3’). PCR conditions were as follows: 200 ng of genomic DNA was amplified in a 20 µl reaction volume with 60 mM Tris–HCl, 15 mM ammonium acetate, 2.0 mM MgCl2, 200 µM dNTPs, 5 pmol of each primer and 2 U of Tag polymerase at pH 8.5 for primers 45.0F and 66.0R and pH 9.5 for primers JF and
REFERENCES


