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Molecular analysis of X-autosome translocations in females with Duchenne muscular dystrophy

Sharon E.Bodrug[1,3], Jeanette J.A.Holden[2], Peter N.Ray[1] and Ronald G.Worton[1]

[1]Genetics Department and Research Institute, Hospital for Sick Children, and Department of Molecular and Medical Genetics, University of Toronto, 555 University Avenue, Toronto, Ontario, M5G 1X8 and [2]Cytogenetics and DNA Research Laboratory, Ongwanada, and Department of Psychiatry, Queen's University, Kingston, Ontario, K7M 8A6, Canada
[3]Present address: The Walter and Eliza Hall Institute of Medical Research, Post Office, Royal Melbourne Hospital, Melbourne, Australia

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To further an understanding of the mechanism of constitutional chromosomal rearrangement, the translocation breakpoints of two X-autosome translocations carried by females with Duchenne or Becker muscular dystrophy have been mapped, cloned and sequenced. Breakpoints were mapped to specific introns within the dystrophin gene and intron sequences spanning the two breakpoints were cloned and used as probes to identify DNA fragments containing the translocation junctions. The junction-containing fragments were cloned after amplification by inverse PCR or single-specific-primer PCR. Sequence through the junctions and the autosomal regions spanning the breakpoints identified the mechanism of rearrangement as non-homologous exchange with minor additions or deletions (0–8 nucleotides) at the breakpoints. Paternal origin of these X-autosome translocations, coupled with evidence for non-transmission of X-autosome translocations through male meiosis suggested that the translocations were the result of a post-meiotic rearrangement in spermiogenesis.

Key words: Chromosome rearrangement/Duchenne muscular dystrophy/inverse PCR /non-homologous recombination/X-autosome translocation

Introduction

Duchenne muscular dystrophy (DMD) and its milder counterpart Becker muscular dystrophy (BMD) are allelic X-linked disorders characterized by progressive muscle wasting. The defective gene at Xp21 has been cloned (reviewed by Worton and Thompson, 1988) and the protein product, dystrophin, has been identified (Hoffman *et al.*, 1987). Females with DMD and BMD are rare, but 22 cases have been described with an X-autosome translocation that disrupts the short arm of the X chromosome at band Xp21 (Boyd *et al.*, 1986). In each case the disease was expressed as a result of non-random inactivation of the normal X chromosome, with variation in severity of the disease likely depending on the percentage of active normal X chromosomes in the muscle (Boyd *et al.*, 1986). Parental origin of a number of the translocations has been determined, and to date eight out of eight are paternal in origin (Bodrug *et al.*, 1990; Robinson *et al.*, 1990).

High resolution cytogenetic analysis of nine X-autosome translocations in affected females suggested that the translocation breakpoints might be scattered over a 3000–4000 kb region of band Xp21 (Boyd and Buckle, 1986). In fact, all of 12 breakpoints examined at the molecular level have now been shown to lie within the 2300 kb dystrophin gene (Boyd *et al.*, 1988; Bodrug *et al.*, 1989, 1990), and five have been mapped to a specific intron. These include a t(X;5) and a t(X;11) translocation with breakpoints in intron 1, a t(X;21) and a t(X;1) with breakpoints in intron 7 and a t(X;4) with breakpoint in intron 51 (Bodrug *et al.*, 1989,1990).

Most information concerning mechanism of chromosome translocation has been derived from analysis of somatic cell rearrangements that occur in leukemias and lymphomas. In contrast, constitutional translocations may arise in the germ line, possibly by quite different mechanisms. The X-autosome translocations in females with DMD have provided the first opportunity to study germ line translocations. To date, only the t(X;21) translocation breakpoint has been cloned and sequenced; at the site of exchange both the X and chromosome 21 were found to be missing a small amount of DNA, and there was no sequence homology between the two chromosomes at the exchange points (Bodrug *et al.*, 1987). There was, however, a 4 bp motif at the site of the breakpoint on both the X and chromosome 21 that we speculated might be implicated in triggering the non-homologous exchange.

In this paper we provide further information on the mechanisms involved in constitutional translocation. We establish the parental origin and map position of a t(X;2)(p21;q37) (Holden *et al.*, 1986) translocation breakpoint. For this translocation and the t(X;4)(p21;q35) mentioned above, we have cloned PCR-amplified translocation junctions and the autosomal regions flanking the breakpoints. Sequence analysis of these regions indicated that both translocations were the result of non-homologous recombination, and in contrast with the t(X;21) translocation breakpoint, did not indicate a common sequence motif that may have been involved in the generation of the rearrangements. Considering the consistent paternal origin of X-autosome translocations affecting the dystrophin gene and the general lack of transmission of X-autosome translocations through meiosis in the male we have suggested that translocations causing Duchenne and Becker muscular dystrophy in females result from post-meiotic non-homologous recombination in spermiogenesis.

Results

Breakpoint mapping

The breakpoint of the t(X;4) translocation has been previously mapped within intron 51 [exon designations as

defined by Koenig *et al.* (1989)] (Bodrug *et al.*, 1990). To map the t(X;2) translocation exchange points in the two translocation-derived chromosomes, der(X) and der(2), somatic cell hybrids generated from cells of patient TM were first characterized by Southern blot analysis using the polymorphic probes 754 and 99-6. These probes flank the dystrophin gene on the centromeric side and telomeric side respectively, and TM is heterozygous at both loci. This allowed identification of der(X)-containing hybrid clones with only one fragment detected by the probe 754, and der(2)-containing hybrid clones with only one fragment detected by the probe 99-6 (data not shown), mapping the t(X;2) breakpoint between these two markers. An intragenic probe, J-66, from the 3′ half of the DMD gene, hybridized with DNA from the der(X)-containing hybrid (Figure 1A, lane 2), thereby mapping the breakpoint to the 3′ end of the gene.

The breakpoint was further localized using the 0.7 kb *Bam*HI fragment of cDMD 9-14, a cDNA fragment from the 3′ end of the transcript. In Figure 1B, *Hin*dIII digests were probed with the cDNA fragment, and seven *Hin*dIII exon-containing bands were identified in the control lanes (lanes 6 and 10). The cross-hybridizing mouse bands found with this probe are shown in lane 9. The der(X) hybrid in lane 7 shows the presence of five of the seven human specific bands (representing exons 58 to 63). Exons 64 and 65, not seen in the der(X) or der(2) (lane 8) hybrids, could have been deleted, but the intensity of the bands in the translocation carrier, TM (lane 6), and the control (lane 10) indicated that this was unlikely. It was later confirmed that the non-selectable der(2) hybrid carried the der(2) chromosome in a minority of cells, producing a signal too weak to be detected with the cDNA probe. Thus, the t(X;2) breakpoint occurred within intron 63.

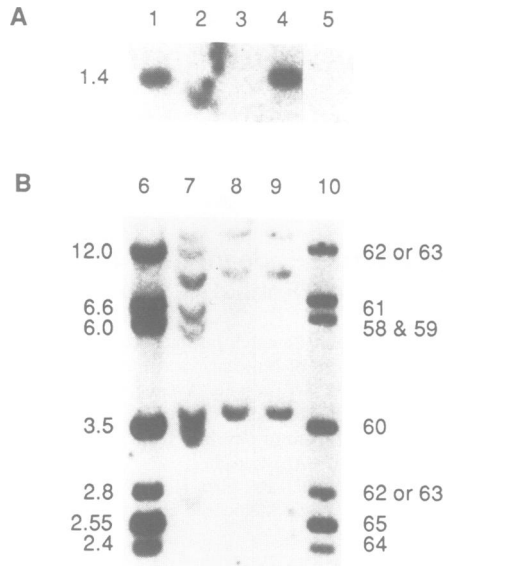

Fig. 1. Mapping t(X;2) translocation in DMD gene. (**A**) Southern blot of DNA digested with *Pst*I and fractionated on a 0.8% agarose gel, followed by hybridization with the probe J-66. (**B**) Southern blot of DNA digested with *Hin*dIII and fractionated on a 0.9% agarose gel. The blot was probed with the 0.7 kb *Bam*HI fragment of cDMD 9-14. Exon numbers are labelled on the right. The order of exons is adapted from Koenig *et al.* (1989) based on unpublished data from our laboratory on one deletion patient and Southern analysis with fragments of the 0.7 kb cDNA. Size of bands in kb are labelled on the left of each blot. Lanes: (1) male control, (2 and 7) der(X)t(X;2) hybrid, (3 and 8) der(2)t(X;2) hybrid, (4 and 10) female control, (5 and 9) mouse(RAG), (6) t(X;2) translocation carrier TM.

Identification of translocation junction fragments

In an analysis of DNA from the t(X;2) and t(X;4) translocation carriers, we were unable to identify a translocation junction fragment using the cDNA probes, indicating that the breakpoint did not occur within, or immediately adjacent to, an exon of the DMD gene. To obtain intron sequences closer to each translocation junction, a XXXXY phage library was screened with oligonucleotide probes from the exons that flanked the two breakpoints. To determine if any of the hybridizing phage clones contained a region of the X chromosome that crossed the breakpoint, riboprobes from the T3 and T7 promoters at the ends of the phage clones were hybridized to DNA from the hybrids carrying the translocation-derived chromosomes. One clone containing exon 64 crossed the t(X;2) breakpoint, and a clone containing exon 52 crossed the t(X;4) breakpoint. Restriction maps of these clones were constructed (Figure 2A and B), and multiple fragments from each phage were used as probes on Southern blots to confirm the genomic collinearity of the map.

To identify the t(X;2) translocation breakpoint position within the phage clone, a restriction fragment (EX0.6 in Figure 2A) was used to probe Southern blots of DNA from the translocation carrier, TM. Figure 3A shows the junction fragments detected in TM. The unrearranged 3.6 kb *Pst*I fragment is seen in a normal control (lane 1) and in TM (lane 2). The der(2) junction fragment of 3.4 kb is seen in TM and the der(2) hybrid (lane 4), while the der(X) junction fragment of 6.0 kb is seen in TM and the der(X) hybrid (lane 3). Restriction maps based on similar analyses with a variety of enzymes were constructed for the der(X) and der(2) chromosomes (Figure 2A).

To identify the t(X;4) translocation breakpoint position within the phage clone, restriction fragments (HX1.1 and X0.6 in Figure 2B) were used to probe Southern blots of DNA from the translocation carrier, EH. Figures 3B and 3C show the junction fragments detected in EH. In Figure 3B the unrearranged *Eco*RI fragment of 6.2 kb is seen in a normal control (lane 5) and in EH (lane 6). The der(4) junction fragment of 7.5 kb is seen in EH and the der(4) hybrid (lane 8). In Figure 3C the unrearranged *Sst*I fragment of 24 kb is seen in the normal controls (lanes 9 and 11) and EH (lane 10), while the der(X) junction fragment of 16 kb is seen only in EH (lane 10). Restriction maps based on similar analyses with a variety of enzymes were constructed for the der(X) and der(4) chromosomes (Figure 2B).

PCR amplification

Full molecular characterization of a translocation required sequencing DNA fragments spanning the junctions on both derivative chromosomes, as well as DNA fragments from the normal X chromosome and autosome at the site of exchange. As an alternative to cloning these DNA segments by genomic library screening, inverse PCR (IPCR) (Ochman *et al.*, 1988; Triglia *et al.*, 1988) and single-specific-primer PCR (SSP-PCR) (Shyamala and Ames, 1989) were used to amplify the translocation junctions based on sequence from the X chromosome only. The specific amplification schemes are shown in Figures 4A–D for the t(X;2) translocation and

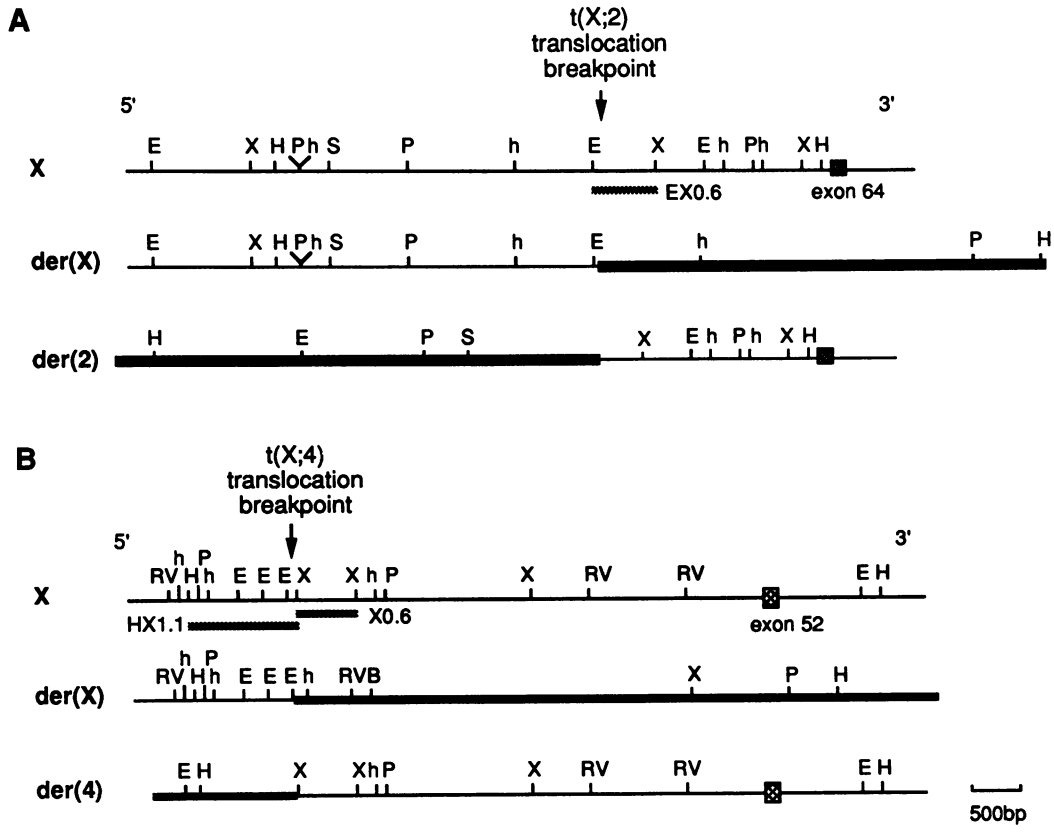

Fig. 2. Restriction maps of the chromosomes involved in the t(X;2) (**A**) and t(X;4) (**B**) translocations. Thin lines represent DNA from the X chromosome, while thick lines represent DNA from the autosomal regions. The 5′ to 3′ direction of the DMD gene on the X chromosome is indicated, as are the approximate locations of exons 64 (A) and 52 (B) within the phage clones. Subclones EX0.6 (A), HX1.1 and X0.6 (B), which were used as probes in Southern blot analysis to identify junction fragments and generate maps of the derivative chromosomes, are indicated by shaded blocks below the maps of the X chromosome. E-*Eco*RI, h-*Hinc*II, H-*Hin*dIII, P-*Pst*I, RV-*Eco*RV, S-*Sst*I, X-*Xba*I

4E−H for the t(X;4) translocation. Oligonucleotide primers for the amplification of the four junctions, the der(X) and der(2) of the t(X;2) translocation and the der(X) and der(4) of the t(X;4) translocation, were chosen from the sequence of the X chromosome in the region of the t(X;2) and t(X;4) breakpoints (Figure 4). This X chromosome sequence, derived from plasmid subclones of the phage clones described above, is presented in Figures 5A [t(X;2) translocation] and 6A [t(X;4) translocation].

The sequence of the four amplified junctions allowed identification of the precise breakpoints in the X chromosome and also provided new sequence from the autosomal side of each junction. This autosomal sequence allowed chromosome 2 and 4 primers to be chosen for amplification from normal chromosomes of the autosomal regions surrounding the translocation breakpoints (Figure 4). Successful amplification of the expected size of PCR product, using primers generated independently from two different junction sequences, was considered to be confirmation that the amplified products were from the chromosomes 2 and 4 regions spanning the breakpoints. The autosomal sequence surrounding the t(X;2) and t(X;4) breakpoints, which was obtained from cloned PCR products, is presented in Figures 5B and 6B, respectively.

To confirm that each autosomal fragment was disrupted by the translocation, non-repetitive fragments from chromosome 2 (0.23 kb *Hpa*I−*Bal*I fragment) and chromosome 4 (0.6 kb PCR product) were used to probe blots containing DNA from the translocation carriers. The

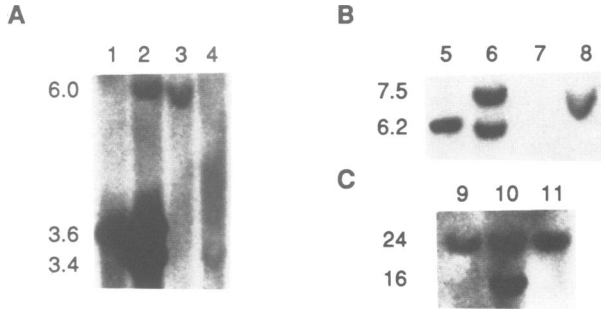

Fig. 3. Identification of junction fragments of the t(X;2) (A) and t(X;4) (B and C) translocations. (**A**) Southern blot of *Pst*I digested DNA separated on a 0.7% agarose gel, followed by hybridization with the probe EX0.6. (**B**) Southern blot of *Eco*RI digested DNA separated on a 0.7% agarose gel, followed by hybridization with the probe X0.6. (**C**) Southern blot of *Sst*I digested DNA separated on a 0.6% agarose gel, followed by hybridization with the probe HX1.1. Size of bands in kb are labelled on the left of each blot. Lanes: (1, 5, 11) female control, (2) t(X;2) translocation carrier TM, (3) der(X)t(X;2) hybrid, (4) der(2)t(X;2) hybrid, (6 and 10) t(X;4) translocation carrier EH, (7) der(X)t(X;4) hybrid, (8) der(4)t(X;4) hybrid, (9) male control.

der(X) and/or der(autosome) junction bands identified were identical to those shown by an X chromosome probe that spans each junction, providing this confirmation.

Junctional sequences

To understand fully the nature of the sequence changes that resulted from the translocations, it was necessary to eliminate

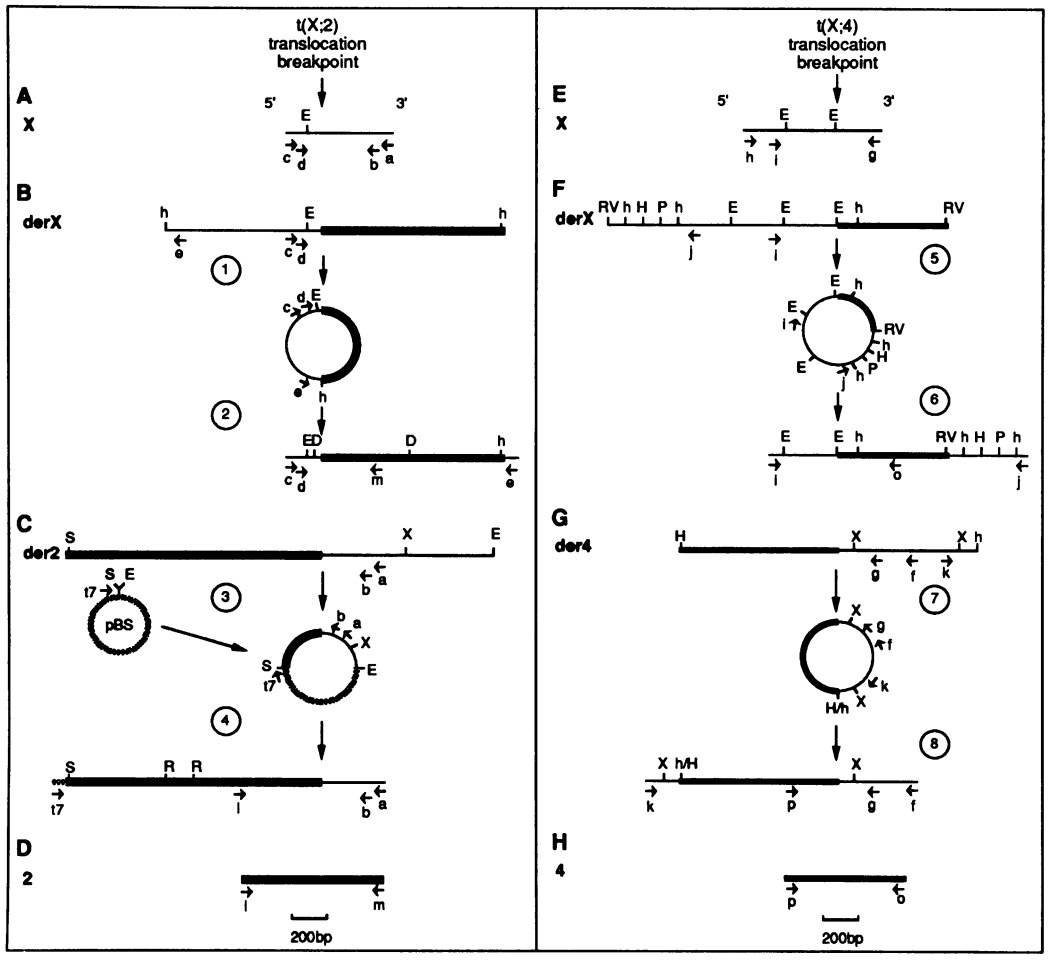

Fig. 4. PCR amplification of the t(X;2) (A−D) and t(X;4) (E−H) translocations. (**A**) Direct PCR amplification of X chromosomal DNA from the father of TM. DNA (0.5 μg) was amplified using primers a and c (buffer 2; 50 μl). After 30 cycles (94°C 90 s, 58°C 2 min, 72°C 3 min) the product was re-amplified using primers b and d. After 35 cycles (94°C 90 s, 55°C 2 min, 72°C 3 min) the 0.5 kb PCR product was sequenced directly. The 5′ to 3′ direction of the DMD gene on the X chromosome is indicated. (**B**) PCR amplification of der(X) DNA from TM. DNA (10 μg) was digested with *Hin*cII (h) and *Xba*I (X), and the DNA between 1.7 kb and 2.0 kb was size-selected after separation on an agarose gel (*Xba*I was included only to reduce the size of the corresponding fragment from the unrearranged X to below the size-selected range). Step 1: The DNA was ligated under conditions favouring circularization [<3.8 μg/ml using 1 μl of ligase (IBI-2U/μl) per 20 μl ligation] (Collins and Weissman, 1984). Step 2: One tenth of the ligation mix was amplified with primers c and e (buffer 1; 100 μl). After 35 cycles (94°C for 2 min, 60°C for 90 s, 72°C for 5 min) the product was re-amplified using primers d and e under the same conditions. The 1.2 kb PCR product was digested with *Eco*RI (E) (linker on primer e and internal site) and cloned into the plasmid Bluescript (pBS-Stratagene). A 0.6 kb *Dra*I (D) fragment was subcloned for sequencing. (**C**) SSP-PCR amplification of der(2) DNA from TM. DNA (14 μg) was digested with *Eco*RI and *Sst*I (S) then treated with calf intestinal phosphatase. DNA was separated on an agarose gel, and the fragments between 2.1 kb and 2.7 kb were purified from the gel. The plasmid pBS (12.5 μg) was similarly digested. Step 3: The size-selected and plasmid DNAs were ligated. Step 4: One tenth of the ligation mix was amplified with primers t7 and a (buffer 1; 50 μl). After 60 cycles (94°C 1 min, 55°C 2 min, 72°C 5 min) a size selected fraction (~1.9 kb) of the PCR reaction was re-amplified with primers t7 and b. The re-amplification was initiated with a low stringency first cycle followed by a further 30 cycles (same conditions). The 1.9 kb PCR product was digested with *Eco*RI (linkers on primers b and t7) and cloned into pBS. A 0.9 kb *Eco*RI−*Rsa*I (R) fragment was subcloned into pBS for sequencing. (**D**) Direct PCR amplification of chromosome 2 DNA from TM and her father. DNA from TM (1 μg) or her father (0.5 μg) was amplified using primers l and m (buffer 1; 50 μl). After 25 cycles (94°C 1 min, 58°C 1 min, 72°C 2 min) the 0.8 kb PCR product from TM was digested with *Eco*RI and cloned into the *Eco*RI site of pBS for sequencing. The PCR product from her father was sequenced directly. (**E**) Direct PCR amplification of X chromosome DNA from the father of EH. DNA (1 μg) was amplified using primers g and h (buffer 2; 50 μl). After 30 cycles (94°C 90 s, 58°C 2 min, 72°C 3 min) the product was re-amplified using primers g and i (buffer 2; 50 μl). After 35 cycles (94°C 90 s, 55°C 2 min, 72°C 3 min) the 0.55 kb PCR product was sequenced directly. The 5′ to 3′ direction of the DMD gene on the X chromosome is indicated. (**F**) IPCR amplification of der(X) DNA from EH. DNA (10 μg) was digested with *Eco*RV (RV). The DNA was separated on an agarose gel, and the fragments between 1.6 kb and 1.9 kb were purified from the gel. Step 5: The DNA was ligated under conditions favouring circularization (as in Step 1). Step 6: One third of the ligation mix was amplified with primers i and j (buffer 1; 100 μl). A low stringency first cycle was followed by 35 cycles (94°C 2 min, 58°C 90 s, 72°C 5 min). The 1.4 kb PCR product was digested with *Pst*I (P) (linker on primer i and internal site), cloned into pBS, and sequenced from primer i. In addition, a 0.9 kb *Eco*RI−*Pst*I fragment and a 0.6 kb *Hin*cII fragment were subcloned into pBS for sequencing. (**G**) IPCR amplification of der(4) DNA from the der(4)-containing hybrid. Hybrid cell DNA (15 μg) was digested with *Hin*dIII (H) and *Hin*cII, followed by a fill-in reaction with Klenow. The DNA was separated on an agarose gel, and fragments between 1.5 kb and 2.0 kb were purified from the gel. Step 7: The DNA was ligated under conditions favouring circularization (as in Step 1). Step 8: One fifth of the ligation mix was amplified with primers f and k (buffer 1; 100 μl). A low stringency first cycle was followed by 35 cycles (94°C 2 min, 60°C 90 s, 72°C 5 min). Re-amplification using primers g and k went through a low stringency first cycle followed by 35 cycles (94°C 2 min, 58°C 90 s, 72°C 5 min). The 1.3 kb PCR product was digested with *Eco*RI (linker on primers g and k) and cloned into pBS. A 1.0 kb *Xba*I fragment was subcloned for sequencing. (**H**) Direct PCR amplification of chromosome 4 DNA . DNA from EH (1 μg) or her father (1 μg) was amplified using primers o and p (buffer 1; 50 μl). After 25 cycles (94°C 90 s, 50°C 2 min, 72°C 3 min) the 0.6 kb product from EH was digested and cloned into the *Eco*RI site of pBS for sequencing. Re-amplification of the PCR product from her father used primers **o** and **p** (buffer 1; 50 μl). After 35 cycles (94°C 90 s, 48°C 2 min, 72°C 3 min) the product was sequenced directly.

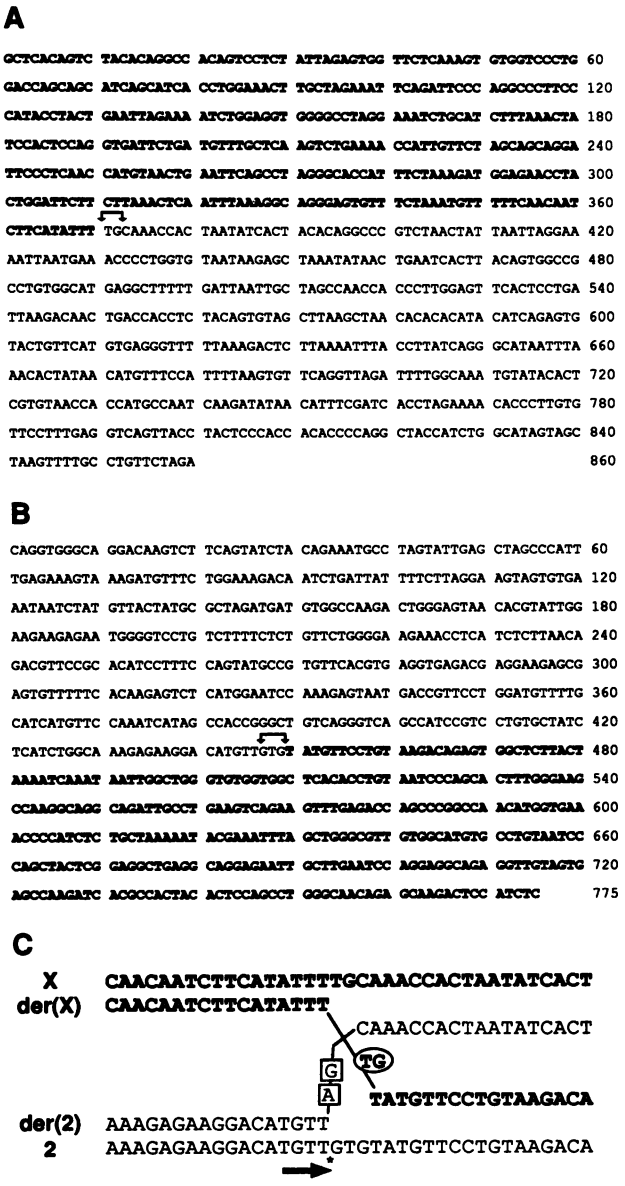

Fig. 5. Sequence of t(X;2) translocation chromosomes. (**A**) Sequence from the unrearranged X chromosome surrounding the t(X;2) breakpoint, obtained from plasmids which were cloned from phage. (**B**) Sequence from the unrearranged chromosome 2 surrounding the t(X;2) breakpoint, obtained by sequencing of four plasmid clones generated from three PCR reactions. (A and B) The sequence in bold type is identical to that of the der(X) chromosome, obtained from four clones generated from three PCR reactions, each from an independent ligation. The sequence in plain type is identical to that of the der(2) chromosome, obtained from four clones from one PCR reaction. Due to the uncertainty as to the exact location of the translocation (as discussed in the text), the sequence defined by the arrows indicates the range over which the translocation may have occurred. (**C**) Diagram of the origin of the nucleotides on the translocated chromosomes. The sequence in bold type is from the X and der(X) chromosomes, while that in plain type is from der(2) and 2 chromosomes. The circled nucleotides could have originated from either the X chromosome or chromosome 2. The boxed nucleotides are potential additions of unknown origin as discussed in the text. The bold arrow indicates the location of a topo I consensus sequence [rat liver and wheat germ topo I consensus: 5′-A/C/T, C/G, A/T, T*-3′] (Been *et al.*, 1984). * indicates the location of cutting by the topoisomerase within the consensus sequence.

those changes that may have resulted from individual heterogeneity. Since it was established that both translocations originated in the father's DNA (data below),

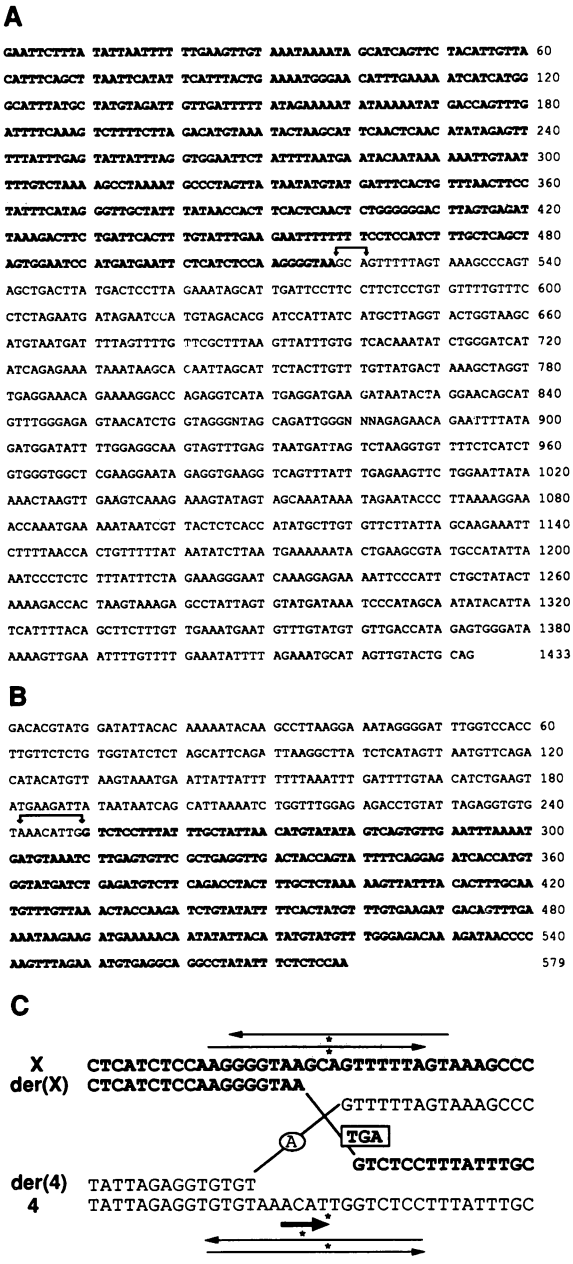

Fig. 6. Sequence of t(X;4) translocation chromosomes. (**A**) Sequence from the unrearranged X chromosome surrounding the t(X;4) breakpoint, obtained from plasmids which were cloned from phage. (**B**) Sequence from the unrearranged chromosome 4 surrounding the t(X;4) breakpoint, obtained from four clones generated from three PCR reactions. (A and B) The sequence in bold type is identical to that of the der(X) chromosome, obtained from five clones generated from two PCR reactions from one ligation. The sequence in plain type is identical to that of the der(4) chromosome, obtained from five clones generated from one PCR reaction. Due to the uncertainty as to the exact location of the translocation (as discussed in the text), the sequence defined by the arrows indicates the range over which the translocation may have occurred. (**C**) Diagram of the origin of the nucleotides on the translocated chromosomes. The sequence in bold type is from the X and der(X) chromosomes, while that in plain type is from der(4) and 4 chromosomes. The circled nucleotide could have originated from either the X chromosome or chromosome 4. The boxed nucleotides are additions of unknown origin. The bold arrow indicates the location of a topo I consensus sequence (as for Figure 5C). The long thin arrows indicate the location of topo II consensus sequences [70% match to vertebrate (chicken) topo II consensus sequence: 5′-A/G, N, T/C, N, N, C, N, N, G, T/C * N, G, G/T, T, N, T/C, N, T/C-3′ (Spitzner and Muller, 1988). * indicates the location of cutting by the topoisomerase within the consensus sequence.

DNA from the father of each of the translocation carriers was used as template to amplify the X and autosomal sequences surrounding the translocation junctions. The PCR products were sequenced directly, and no polymorphic differences between the sequence of the fathers' chromosomes, the translocation carriers' chromosomes, or the phage clones from the XXXXY library, were found in 100 bp on either side of the breakpoints.

The junction sequences of the t(X;2) translocation chromosomes are shown in Figure 5C. In the model presented in this figure, there are two nucleotides (TG) on the der(X) chromosome with junctional homology. These nucleotides could have originated from either the X chromosome or chromosome 2. On the der(2) chromosome there has been an addition of either an A or an AG of unknown origin. If the TG on the der(X) did not originate from the X chromosome, the G on the der(2) chromosome could have originated from the X chromosome. Depending on the origin of the der(X) and der(2) junctional nucleotides, the X chromosome has lost from zero to two nucleotides, while chromosome 2 has lost from one to three nucleotides in the generation of the translocated chromosomes.

The junction sequences of the t(X;4) translocation chromosomes are shown in Figure 6C. In the model presented in this figure, there is one nucleotide (A) on the der(4) chromosome with junctional homology. This nucleotide could have originated from either the X chromosome or chromosome 4. On the der(X) chromosome there has been an addition of three nucleotides (TGA) of unknown origin. Depending on the origin of the A with junctional homology, the X chromosome has lost two or three nucleotides, while chromosome 4 has lost seven or eight nucleotides in the generation of the translocated chromosomes.

The sequence of the regions flanking the t(X;2) and t(X;4) translocations from the X chromosome, chromosome 2 and chromosome 4 is presented in Figures 5A and B, and 6A and B. Comparison of these sequences with those in GenBank did not show any notable homology. Comparison with those involved in another translocation in a muscular dystrophy female (Bodrug *et al.*, 1987) did not indicate a common sequence motif that might be involved in the translocation process. Further analysis of the sequences indicated an Alu sequence on chromosome 2, oriented away from the translocation breakpoint with its 5′ end ~50 nucleotides from the breakpoint. No other Alu or LINE homologies were noted. A search for sequences thought to be involved in recombination or rearrangments included a search for chi sequences, minisatellite sequences, Z-DNA sequences and topoisomerase I and II (topo I and topo II) sites. The only homologies evident were topo I and topo II sites. Predicted topoisomerase cut sites on the unrearranged chromosomes which would potentially cut in the region of the junction are indicated in Figures 5C and 6C. The topoisomerase sites over the rest of the sequences (Figures 5A and B, and 6A and B) showed a random distribution with the expected number of cut sites based on the degeneracy of the consensus sequences.

Parental origin

The t(X;2) translocation carrier was analyzed with X-linked polymorphic markers flanking and within the DMD gene to determine in which parent the *de novo* translocation originated. Table I provides a summary of the results with all informative probes tested. In every case the paternally derived marker is found in the hybrid carrying one of the two translocation-derived chromosomes, indicating paternal origin of the t(X;2) translocation. The t(X;4) translocation was previously shown to be of paternal origin (Bodrug *et al.*, 1990).

Discussion

There are many possible mechanisms by which translocations could occur, and these are summarized in Figure 7. In somatic cells, translocations may be the result of a homologous exchange (H) between repetitive elements, or a non-homologous exchange (NH) as a result of random breakage and reunion (r) or a non-random event (nr) such as an enzyme cleavage. In the germ line, the situation is more complex since translocations occurring in male or female germ cells may occur by different mechanisms, and a translocation may occur during one of the mitotic divisions prior to meiosis, during meiosis, or during post-meiotic development. The best information available concerning mechanism of translocation has been derived from analysis of somatic cell rearrangements that occur in lymphoid malignancies where translocation is often the result of aberrant immunoglobulin gene or T cell receptor gene rearrangements (reviewed by Haluska *et al.*, 1987). The Philadelphia chromosome translocation found in leukemia is less well understood, although Alu sequences have been identified on one of the two chromosomal partners in the few cases studied, suggesting that homologous recombination is not the general mechanism of formation (van der Feltz *et al.*, 1989).

The X-autosome translocations in females with DMD provide the first opportunity to study exchange mechanisms in germ line translocations. Analysis of the sequences surrounding the t(X;2) and t(X;4) translocation breakpoints showed no potential sequence alignment between the translocations which would indicate a common sequence important in the generation of translocations. The CGGC motif found at the t(X;21) translocation junction (Bodrug

Table I. Parental origin of t(X;2) translocation

Probe	Size of polymorphic bands (kb)				
	father	patient	mother	der(X) hybrid	der(2) hybrid
D2	6.6	6.0/6.6	6.0/6.6	–	6.6
p87-15*Taq* I	3.3	3.1/3.3	3.1/3.1	3.3	–
58-1	4.0	2.5/4.0	2.5/4.0	4.0	–
CX5.7	3.5	3.5/7.0	3.5/7.0	3.5	–

et al., 1987) was not apparent in the DNA surrounding the translocation breakpoints reported here, and is not likely to be involved in a general mechanism of translocation. The only repetitive sequence identified was a single Alu sequence about 50 bp from the junction on chromosome 2, this distance making it unlikely that the Alu has played a role in the generation of this translocation. Purine–pyrimidine tracts (presumably in the Z-DNA form) and a chi-like octamer (strongly conserved in human minisatellites) have recently been implicated in the generation of oncogene translocations (Boehm *et al.*, 1989 and Krowczynska *et al.*, 1990, respectively), but no such sequences were found in the vicinity of the t(X;2), t(X;4), or t(X;21) translocations. Our sequence analysis leads to the conclusion that all three X-autosome translocations were generated by a non-homologous recombination event. Non-homologous recombination also appears to underlie the molecular mechanism of dystrophin gene deletions (Love *et al.*, 1991) and duplications (Hu *et al.*, 1991) that are frequently found in patients with Duchenne and Becker muscular dystrophy.

A common feature of all three dystrophin gene translocations was deletion and addition of a few nucleotides at the site of rearrangement. The deleted regions ranged in size from 0 to 2 nucleotides in the t(X;2) translocation to 70 nucleotides in the t(X;21) translocation while the addition of random bases involved at most three nucleotides. This contrasts markedly with dystrophin gene duplications where the rearrangements are 'clean' in that no sequences are lost or gained at the site of the recombination event (Hu *et al.*, 1991).

Extra nucleotides at the point of circularization have been observed following transfection of linear SV40 DNA suggesting a non-homologous recombination mechanism that includes a stage with free DNA ends (Roth *et al.*, 1989). Roth and Wilson (1988) suggested that DNA ends may have arisen from errors of DNA metabolism (i.e. replication, repair, recombination or transcription) or from DNA damage, while joining of DNA ends may involve either direct ligation or repair synthesis primed by homology of a few nucleotides at the free termini. Thode *et al.* (1990) proposed an 'alignment protein' which would align free DNA ends allowing primed repair of gaps and ligation. Junctions of germ line deletions in the β-globin gene showed characteristics similar to Roth's experimentally-derived junctions (Henthorn *et al.*, 1990), suggesting that Roth's results could be extrapolated to naturally-occurring rearrangements. Our results would further support this.

The finding of paternal origin for the t(X;2) and t(X;4) translocations is of particular interest, since six other translocations in females with DMD or BMD are also of paternal origin (Bodrug *et al.*, 1990; Robinson *et al.*, 1990). A further four X-autosome translocations, not associated with DMD, have been studied and all are of paternal origin (Robinson *et al.*, 1990). The probability of finding 12 out of 12 cases of paternal origin by chance is $(1/2)^{12}$ or 2×10^{-4}.

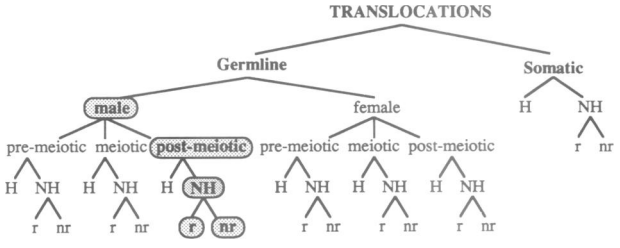

Fig. 7. Hierarchy of possible mechanisms of chromosomal translocations. H: homologous, NH: non-homologous, r: random, nr: non-random. Shaded circles represent the most likely mechanism for X-autosome translocation. Refer to the text for a complete description.

Among *de novo* chromosome rearrangements in general, 84% are of paternal origin, suggesting that preferential male origin may be a reflection of the greater opportunity for error during the many mitotic divisions in spermatogenesis (Olson and Magenis, 1988). It is tempting to suggest that error during a mitotic division in spermatogenesis is the reason for the preferential paternal origin of the 12 X-autosome translocations. However, X-autosome translocations in man or mouse invariably lead to male sterility, with an inability to pass on the translocation (Mattei *et al.*, 1982; Madan, 1983; Russell, 1983; Chandley, 1988). Meiotic studies on sterile human males with reciprocal X-autosome translocations have demonstrated spermatogenic arrest at meiosis I (Faed *et al.*, 1982; Quack *et al.*, 1988). Furthermore, in mice X-autosome translocations may be generated by mutagenesis of postspermatogonial germ cells, but not by mutagenesis of spermatogonia (Russell, 1983), suggesting that spermatogonia with an X-autosome translocation are not viable. Based on these results, Chandley (1990) has proposed that *de novo* X-autosome translocations of paternal origin originate as post-meiotic events, a model that implies greater opportunity for post-meiotic rearrangement in male germ cells than in female germ cells. Consistent with this is the fact that female meiosis is completed after fertilization allowing little or no time for post-meiotic chromosomal rearrangement, whereas in the male post-meiotic development of spermatocytes to mature sperm (spermiogenesis) lasts ~27 days (Erickson, 1990).

During spermiogenesis there is little or no legitimate replication, repair or recombination, with transcription being the only active process (Willison and Ashworth, 1987; Erickson, 1990). It is possible that DNA breakage leading to translocation occurred in genes which are transcriptionally active in spermiogenesis, but only a limited number of genes are known to be transcribed at that time (Erickson, 1990). The dystrophin gene is transcribed at a low level in testis (Nudel *et al.*, 1989), but there is no evidence for or against transcription during spermiogenesis.

Topoisomerases I and II have both been implicated in illegitimate recombination because of their ability to make transient cuts in DNA (Bae *et al.*, 1988; Champoux and Bullock, 1988). These enzymes are crucial for DNA condensation, and condensation of DNA into the sperm head is a major event during spermiogenesis (Hecht, 1989). Topo II has been reported in non-replicating and transcriptionally inactive late spermatids of chicken (Roca and Mezquita, 1989) suggesting that it might modulate the topology of DNA during the nucleo-histone–nucleo-protamine transition at the end of spermiogenesis. A search for topo I and topo II consensus sequences at the breakpoint regions of the three translocations indicated that consensus sequences for topoisomerase cleavage occurred within some, but not all, of the breakpoint regions. Evidence for topo I and II cleavage playing a role in chromosomal rearrangement also exists for dystrophin gene duplications where cleavage sites were found within one nucleotide of the breakpoint in all four breakpoints of two duplications studied (Hu *et al.*, 1991).

Figure 7 presents a model of the various pathways leading to chromosomal rearrangement. From the data presented here we conclude that X-autosome translocations affecting the dystrophin gene occur primarily, if not exclusively, in the male germ line by a post-meiotic non-homologous recombination event (the shaded path in Figure 7). We suggest that the initial breakage of the translocated chromosomes may be a random event, or in some cases may involve a non-random event such as a topoisomerase cleavage. The breakage is followed in most cases by degradation of the free DNA ends, followed by the addition of extra nucleotides to the free ends. The last step of the process must involve ligation of the free and modified ends to inappropriate partners, perhaps brought into close proximity during the extreme condensation of the DNA during spermiogenesis. It is possible that this illegitimate recombination mechanism is the only mechanism by which X-autosome translocations arise, although analysis of more events will be the test. For reciprocal translocations not involving the X chromosome this mechanism is likely to be only one of a number of mechanisms since these events can presumably happen in stages of germ cell development other than spermiogenesis.

Materials and methods

Translocation carriers

Translocation carrier TM is a female with BMD and a *de novo* translocation t(X;2) (p21.2;q37) (Holden *et al.*, 1986). She is 21 years old and not yet confined to a wheelchair. Translocation carrier EH is a 6 year old female with a *de novo* translocation t(X;4)(p21;q35) whose disorder has not yet been classified as DMD or BMD (Bodrug *et al.*, 1990). Lymphoblast cell lines from TM and EH have been submitted to the Human Genetic Mutant Cell Repository (HGMCR) (Camden, NJ, USA) and assigned identification numbers GM11024 and GM11025, respectively.

Somatic cell hybrids

t(X;2) translocation. $2-4\times10^6$ lymphoblastoid cells from TM were fused with 2×10^6 mouse RAG cells. Fusion was induced with Sendai virus (800 HAU) or with 42% PEG 8000 and 15% DMSO. Growth and selection of clones containing the der(X) chromosome or the der(2) chromosome, but not the unrearranged X chromosome, was as previously described (Bodrug *et al.*, 1990). Detailed chromosome analysis to determine which other chromosomes were present in the hybrids was not performed. The der(X)-containing hybrid has been submitted to the HGMCR; identification number GM11022.

t(X;4) translocation. Somatic cell hybrids that contained either the der(X) or the der(4), but not the unrearranged X chromosome, have been described (Bodrug *et al.*, 1990). The der(X)-containing hybrid has been submitted to the HGMCR; identification number GM11023.

Probes

Polymorphic probes flanking the DMD gene included 754 (DXS84) (Hofker *et al.*, 1986), centromeric to the DMD gene, and 99-6 (DXS41) (Aldridge *et al.*, 1984) on the telomeric side. Intragenic probes included J-66 (DXS268) (Van Ommen *et al.*, 1987) and a 0.7 kb *Bam*HI fragment of cDNA probe cDMD 9-14 (American Type Culture Collection) (Koenig *et al.*, 1987).

DNA analysis

DNA was prepared from cell cultures or blood by standard procedures. Restriction digests were done on 5 μg of human or mouse DNA, or 10 to 15 μg of hybrid DNA. Southern blots were hybridized with ^{32}P-radiolabelled probes (Feinberg *et al.*, 1984). Probes containing repeats were pre-annealed with sheared placental DNA (Sealey *et al.*, 1985). Hybridization of Southern blots was carried out overnight at 42°C in 50% formamide, 3×SSC, 0.05 mol/l $NaP0_4$, 1% SDS, 0.5% (w/v) non-fat milk powder, 0.3 mg/ml sheared herring sperm DNA, 10% dextran sulphate, and 1 to 3×10^6 c.p.m./ml of labelled probe. Washing conditions varied depending on the probe used. Autoradiography was at −70°C for 1−7 days.

Genomic library

A *Sau*3A partial digest of DNA from an individual with four X chromosomes, karyotype 49, XXXXY (HGMCR, GM1202B) was size selected and ligated into the phage vector λDash (Stratagene). 8.5×10^5 recombinant phage were plated and transferred to Hybond-N (Amersham). Oligonucleotide probes for library screening were ^{32}P-end-labelled using T4 polynucleotide kinase. Hybridization of library filters was carried out overnight at 62°C or 64°C in 6×SSC, 0.5% (w/v) non-fat milk powder, 1% SDS and 1×10^6 c.p.m./ml of labelled oligonucleotide. Washing of filters was in 6×SSC at 41°C or 46°C. Autoradiography was at −70°C for 1−3 days. DNA was prepared from plaque-purified hybridizing clones by the method of Grossberger (1987). Labelled RNA probes (riboprobes) were generated from the T3 or T7 promoters at the ends of the phage clones by incorporating [^{32}P]rUTP with T3 or T7 polymerase, respectively (Stratagene λDash instruction manual).

The sequence of oligonucleotides used to screen the library corresponded to the sequence of the exons flanking the translocation breakpoints. This sequence was chosen from the published cDNA sequence of the DMD gene (Koenig *et al.*, 1987). The sequence of the oligonucleotide for exon 52 was 5′-AAGAACTCATTACCGCTGCCC-3′ (nucleotides 7790−7811) and of the oligonucleotide for exon 64 was 5′-TTCTCAGCTTATAGGACTG-CC-3′ (nucleotides 9514−9535).

Polymerase chain reaction

General reaction conditions. PCR reactions were carried out in a DNA thermal cycler from Perkin-Elmer Cetus. Two buffers were used: buffer 1 is as described by Kogan *et al.* (1987) but without the addition of DMSO; buffer 2 contained 50 mM KCl, 10 mM Tris−HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin and 200 μmol of dNTPs. *Taq* DNA polymerase (Perkin-Elmer Cetus) was used at 2.5 units per 50 μl reaction volume. Primers were added at 11 pmol of each primer per 50 μl reaction volume. All PCR reactions included an extra 7 min extension at 72°C after the final cycle. Some PCR reactions as indicated with 'low stringency first cycle' went through one first cycle at a reduced stringency in which the mixture was denatured at 94°C for 4 min, annealed at 22°C for 2 min, and extended at 72°C for 7 min.

Primers. Primers with a superscript contain an extra 7 or 9 nucleotides at the 5′ end which contain an *Eco*RI (e) or *Pst*I (p) restriction site (underlined). All sequences are given in the 5′ to 3′ direction. The location of each of the primers is indicated in Figure 4.

a: CCTCACATGAACAGTACACTCTGA
b^{e9}: GACGAATTCGTTAGCTTAAGCTACACTGTAGAG
c: CTCCAGGTGATTCTGATGTTTGCT
d^{e9}: GACGAATTCTTGTTCTAGCAGCAGGATTCCCTC
e^{e9}: GACGAATTCTCTGTATACCAACACCATCCTGTG
f: AGCCACCCACAGATGACAAAACAC
g^{e9}: GACGAATTCTATGATCCCAGATATTTGTGACAC
h: ATCATCATGGGCATTTATGCTATG
i^{p9}: GACCTGCAGGACCAGTTTGATTTTCAAAGTCTT
j^{p9}: GACCTGCAGAACCATCCTCATTGTCTTCCACTT
k^{e9}: GACGAATTCAGCGTATGCCATATTAAATCCCTC
l^{e7}: CGAATTCCAGGTGGGCAGGACAAGTCTT
m^{e7}: CGAATTCGAGATGGAGTCTTGCTCTGTT
o^{e7}: CGAATTCTTGGAGAGAAATATAGGCCTG
p^{e7}: CGAATTCGACACGTATGGATATTACACA
t7^{e9}: GACGAATTCAATACGACTCAGTATAGGGCGAAT

Amplification of junctions. Translocation junctions were amplified by inverse PCR (IPCR) (Ochman *et al.*, 1988) or by single-specific-primer PCR (SSP-PCR) (Shyamala *et al.*, 1989) according to the scheme described in Figure 4.

Sequencing

Direct sequencing was carried out by the method of Winship (1989) after purification of the amplified DNA (Heery *et al.*, 1990). A modified procedure of Sanger's dideoxy sequencing of plasmids was carried out on double-stranded DNA (Hattori and Sakaki, 1986) using Sequenase (United States Biochemical Corporation).

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Note added in proof

The sequence data reported here are available from the EMBL/GenBank/DDBJ databases under the following accession numbers: M62508, chromosome 2, 775 bp; M62509, chromosome 4, 579 bp; M62510, X chromosome, part of intron 63, 860 bp; M62511, X chromosome, part of intron 51, 1433 bp; M62512, derivative (X) chromosome from t(X;2)(p21;q37), 36 bp; M62513, derivative (2) chromosome from t(X;2)(p21;q37), 37 bp; M62514, derivative (X) chromosome from t(X;4)(p21;q35), 36 bp; M62515, derivative (4) chromosome from t(X;4)(p21;q35), 31 bp.