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The parental origin of *de novo* X-autosome translocations in females with Duchenne muscular dystrophy revealed by M27β methylation analysis

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Summary

The parental origin of 3 *de novo* X-autosome translocations in females with Duchenne Muscular Dystrophy (DMD) was studied by means of methylation analysis using the X-linked probe M27β. In all three the translocation was found to be paternal in origin. The parental origin of X-autosome translocations in females with and without DMD is compared with other structural abnormalities of the X and with autosomal translocations.

1. Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder affecting approximately 1 in 3300 males (Gardner-Medwin, 1980). It is caused by defects in the dystrophin gene located in the Xp21 region of the X chromosome (Jacobs *et al.* 1981; Davies *et al.* 1983; Clarke *et al.* 1986; Koenig *et al.* 1987). A small number of females are affected and 23 such patients have been found to have a *de novo* reciprocal X-autosome translocation with one breakpoint in the Xp21 region, the autosomal breakpoints being variable (Boyd *et al.* 1986). The X chromosome breakpoints have been shown to disrupt the dystrophin gene (Boyd *et al.* 1988), and this, together with non-random inactivation of the normal X-chromosome, results in expression of the disease phenotype. The parental origin of five such *de novo* X-autosome translocations has been determined using cytogenetic analysis, RFLP analysis of somatic cell hybrids or DNA analysis using probes from the dystrophin gene (Bjerglund-Nielsen *et al.* 1984; Kean *et al.* 1986; Ribiero *et al.* 1986; Bodrug *et al.* 1990). In all five cases the translocation was found to be paternal in origin.

We have studied the parental origin of X-autosome translocations in a further three female patients with DMD. In one case we have used a traditional approach based on the analysis of RFLPs associated with individual translocated chromosomes separated in somatic cell hybrids. For the other two patients, and also to confirm the findings for the first patient, we have used a relatively simple approach based on the detection of differential methylation of active and inactive X chromosomes. Patients with reciprocal X-autosome translocations invariably show inactivation of the normal X in all, or the great majority, of their cells. Therefore, identification of the parental origin of the active X chromosome also identifies the parental origin of the translocation. This can be achieved by routine Southern blot analysis using any probe (i) that shows differential methylation patterns on the active and inactive X chromosomes and (ii) for which the patient is heterozygous. X-linked probes that have been found to detect differential methylation on active and inactive X chromosomes include HPRT (Yen *et al.* 1984; Wolf *et al.* 1984*a*), G6PD, P3, Gdx (Wolf *et al.* 1984*b*; Toniolo *et al.* 1988), PGK (Keith *et al.* 1986) and P482·6 (Khalifa *et al.* 1990). These have been used to analyse X-activation patterns in Wiskott–Aldrich syndrome, (Greer *et al.* 1989), X-linked agammaglobulinaemia (Fearon *et al.* 1987) and fragile X mental retardation (Khalifa *et al.* 1990). However, these probes are all diallelic with a relatively low polymorphism information content and their use is often precluded because the patients of interest are not heterozygous.

Probe M27β, used in this study, has the advantage of being informative in over 90% of females (Fraser *et al.* 1989). M27β recognizes a highly polymorphic variable number tandem repeat (VNTR) sequence in the region Xcen–Xp12. It has been shown that the

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internal cytosine in the CCGG sequences lying on either side of the VNTR are methylated on the active X chromosome and hypomethylated on the inactive X chromosome (Boyd & Fraser, 1990). Methylation status is measured using the restriction enzyme *Msp*I, which cleaves both methylated and unmethylated CCGG sites, in combination with its isoschizomer *Hpa*II, which cuts only at unmethylated CCGG sequences. The pattern of methylation at the CCGG sites on either side of the VNTR is revealed by comparing the hybridization bands detected by M27β after digestion with *Msp*I and *Hpa*II. The active X chromosome is associated with the M27β allele which appears as a larger size fragment (or fragments) on digestion with *Hpa*II when compared with its *Msp*I counterpart, whereas the inactive X chromosome is recognised as the allele which generates a band of the same size in both *Msp*I and *Hpa*II digests. This is because *Msp*I cuts the methylated and unmethylated CCGG recognition sites on the active and inactive X chromosomes, whereas *Hpa*II does not cut the methylated flanking sites on the active X chromosome. Digestion of proband and parental DNA with *Msp*I, followed by Southern blot analysis with M27β, serves to identify the parental origin of the *Msp*I alleles. Parallel *Hpa*II digestion allows the methylation status of these alleles to be ascertained. Thus, analysis of the two separate enzyme digests demonstrates the parental origin of the active, translocated X chromosome.

2. Materials and Methods

(i) *Subjects*

Patient S. W. has an X;1 (p21;p34) translocation with the X chromosome breakpoint in the 5′ region of the dystrophin gene (Lindenbaum *et al.* 1979; Boyd *et al.* 1988; Meitinger *et al.* 1988). Replication banding showed the normal X chromosome to be late replicating, and thus presumably inactive, in all 75 cells studied from blood leucocyte cultures. This finding was confirmed on the lymphoblastoid cell line used for DNA analysis.

Patient M. B. L. has an X;5 (p21;p35·3) translocation (Jacobs *et al.* 1981) with the X chromosome breakpoint also in the 5′ region of the dystrophin gene, but with a different X-breakpoint from that of patient S. W. (Bodrug *et al.* 1989). Replication banding showed the normal X to be late replicating in all 100 cells studied from blood leucocytes and in 576 of the 586 skin fibroblasts examined.

Patient L. C., has a *de novo* X;9 (p21·2;q21·3) translocation. Replication banding showed the normal X to be late replicating in all 100 blood leucocytes studied and in the 60 skin fibroblasts examined.

(ii) *Construction and characterisation of somatic cell hybrids retaining the translocation chromosomes from the X;1 patient*

Somatic cell hybrids were constructed between the lymphoblastoid cell line established from the X;1 patient and the mouse cell line, RAG, and hybrids selected in HAT medium using standard protocols (Choy *et al.* 1982; Munro *et al.* 1985). After a series of sub-cloning experiments a hybrid WAG 8-1 was isolated in which the only human chromosome present was the derivative (X) (Fig. 1). After back-selection of a different hybrid population in 6-thioguanine, a second hybrid, WAG21R-11, was isolated which retained the derivative(1), plus several other human

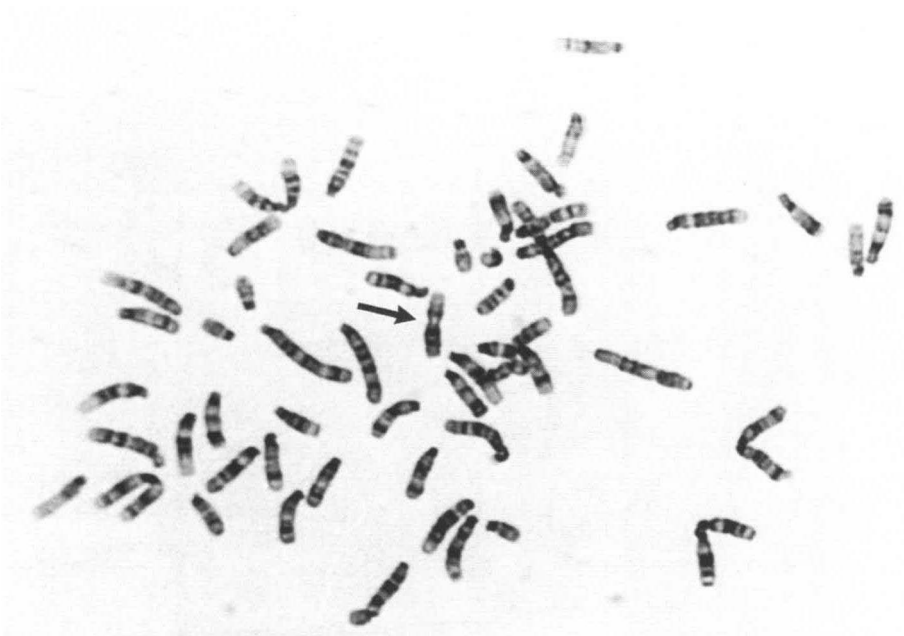

Fig. 1. Metaphase chromosome preparation of WAG 8-1, trypsin-Giemsa banded. The human derivative (X) is arrowed.

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autosomes (including an intact chromosome 1) in the absence of the derivative (X) and inactive X chromosome. Hybrid karyotypes were analysed by trypsin-Giemsa banding and *in situ* hybridization with human and mouse DNA.

(iii) *DNA extraction and analysis*

Total genomic DNA was extracted from peripheral blood or cultured lymphoblasts using standard techniques. 5 μg samples of DNA were digested to completion with the restriction enzymes *Msp*I and *Bam*I, or *Hpa*II and *Bam*HI. Simultaneous digestion with *Bam*HI helps to resolve the higher molecular weight *Hpa*II fragments into discrete bands (Boyd & Fraser, 1990). The digested fragments were separated by electrophoresis through 0·8% agarose and transferred to Hybond N (Amersham) membranes by Southern blotting (Maniatis *et al.* 1982).

Membranes were prehybridized overnight at 42°C in 3 × SSC, 50% formamide, 10 × Denhardt's solution, 20 μg/ml denatured salmon sperm DNA and 2% SDS. They were then hybridized overnight at 42°C in 3 × SSC, 50% formamide, 2 × Denhardt's solution, 5% dextran sulphate, 2% SDS and 20 μg/ml denatured salmon sperm DNA. The probe M27β was labelled with [^{32}P]dCTP by random hexanucleotide priming (Feinberg & Vogelstein, 1983). After hybridization, filters were washed in 3 × SSC and 0·1% SDS for 3 × 5 min at room temperature, 3 × 5 min at 65°C, dried and exposed to X-ray film.

3. Results

The results of probing *Msp*I/*Bam*HI and *Hpa*II/*Bam*HI digests of DNA with M27β are shown in Figures 2–4. The *Msp*I digests show all the females in this study to be heterozygous (Fig. 2, tracks 1,3; Fig. 3, tracks 3,5; Fig. 4, tracks 1,3). In each case, the parental origin of the two alleles in each proband is evident. *Hpa*II digestion of DNA extracted from the mothers of the probands M. B. L. and L. C. shows that bands corresponding to both the alleles detected with *Msp*I are present, but with a reduced intensity, and larger bands are present which do not occur in the *Msp*I digests (Fig. 3, track 4, Fig. 4, track 4).

Methylation of CCGG sequences around the M27β site has therefore occurred on both X chromosomes in these females with a normal karyotype. This observation is consistent with methylation of the CCGG recognition sequences flanking the locus detected by M27β on the active X chromosome but not on the

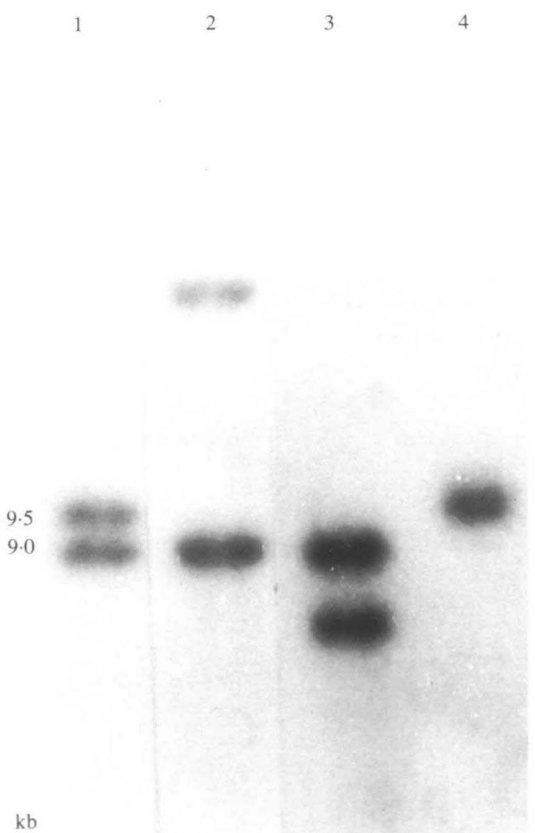

Fig. 2. Southern blot analysis of the (X;1) translocation with probe M27β. Tracks 1 and 2, *Msp*I/*Bam*HI and *Hpa*II/*Bam*HI digests respectively of patient S. W. Note the absence of the 9·5 kb band representing the allele on the active X chromosome in track 2. Tracks 3, and 4 are *Msp*I digests of the proband's mother and the hybrid WAG 8-1 which retains the der (X) of S. W. The 9·5 kb band is absent in the mother's DNA and is therefore inherited from her father. The presence of the 9·5 kb band in WAG 8-1 demonstrates that it lies on the active, translocated X chromosome.

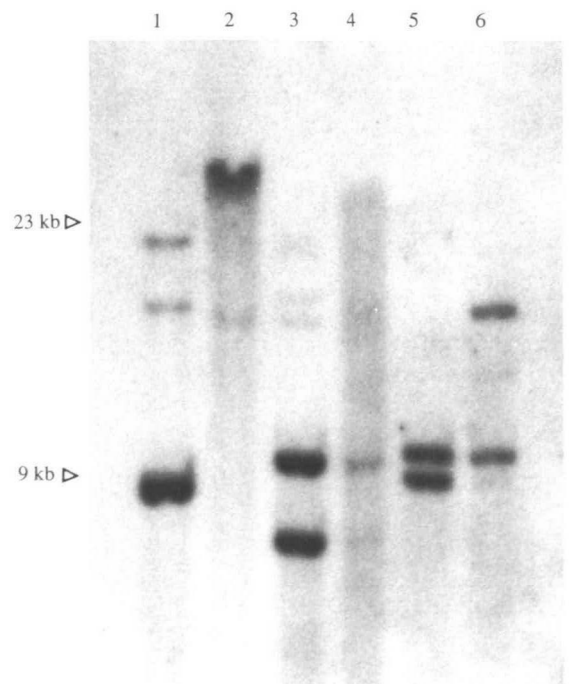

Fig. 3. Southern blot analysis of the patient M. B. L. with probe M27β. Tracks 1, 3 and 5 are *Msp*I/*Bam*HI digests and tracks 2, 4 and 6 are *Hpa*II/*Bam*HI digests of paternal blood DNA, maternal blood DNA and proband lymphoblast DNA respectively.

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inactive X chromosome as reported previously (Boyd & Fraser, 1990; Brown *et al.* 1990). However, in all three probands only one band remains in the same position in both *Msp*I and *Hpa*II digests, the other band being represented by larger fragments after *Hpa*II digestion (Fig. 2, track 1,2; Fig. 3, track 5,6; Fig. 4, track 1,2). Methylation is therefore non-random and in all three probands the methylated M27β allele is paternal in origin. As M27β analysis recognises methylation on active X chromosomes and the translocated X has been shown to be active, the translocation must therefore be of paternal origin in all three cases.

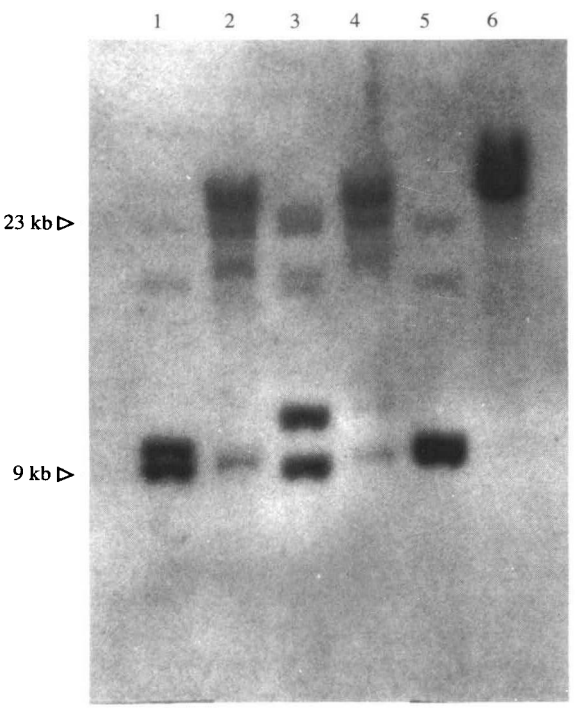

Fig 4. Southern blot analysis of patient L. C. with probe M27β. Tracks 1, 3 and 5 are *Msp*I/*Bam*HI digests and tracks 2, 4 and 6 are *Hpa*II/*Bam*HI digests of the proband, mother and father respectively.

The M27β methylation analysis of the X;1 patient confirmed results from somatic cell genetic studies (Figs. 1 and 5). This patient was found to be heterozygous at the DXS85 locus in Xp22 which is detected by the probe 782 (Mandel *et al.* 1989). The patient's mother was homozygous for the 14·0 kb allele whereas the patient's father and the hybrid WAG21R-11 were positive for the 7·0 kb allele. This finding, together with that obtained from the M27β analysis of the hybrid WAG 8-1 (Fig. 2), demonstrates the paternal origin of this translocation.

4. Discussion

The differential methylation detected by probe M27β in active and inactive X chromosomes has enabled the parental origin of three X-autosome translocations to be established. This probe has the advantage of exhibiting heterozygosity in over 90% of females. However, we encountered one problem when analysing DNA extracted from blood, namely that the bands present in the *Hpa*II digests were very much less intense than expected. This is a consistent finding in our hands and suggests either that blood DNA is extremely refractory to *Hpa*II digestion because of the co-purification of inhibitory factors or because only a minor population of cells in peripheral blood have unmethylated sites at M27β on the inactive X chromosome. In contrast, analysis of the methylation pattern in DNA from cultured lymphoblasts of patients S. W. and M. B. L. showed the bands to be of approximately equal intensity in digests using *Msp*I and *Hpa*II (Figs. 2 and 3).

The parental origin of the X-autosome translocation in eight females with DMD has now been investigated

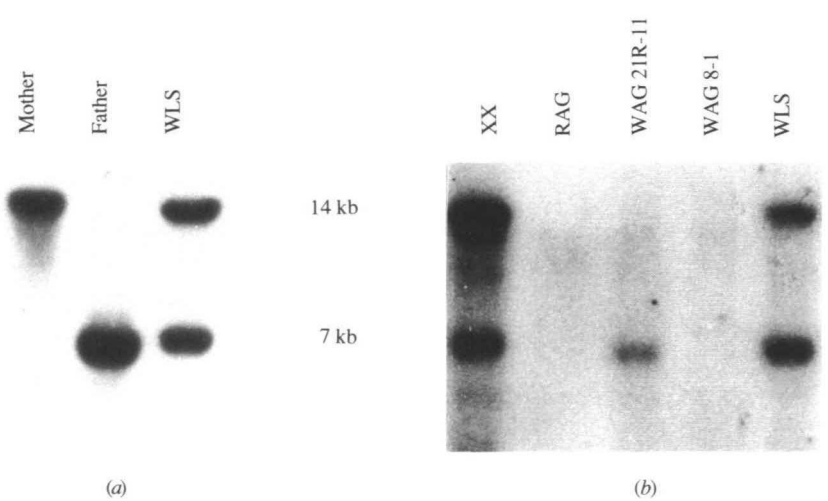

Fig. 5. Demonstration of the paternal origin of the translocation chromosome of the X;1 patient by somatic cell genetic analysis. Southern blots of *Eco*RI digested DNA probed with 782. From left to right: (*a*) mother, father and patient S. W. (WLS) (*b*) human female control (XX), mouse control (RAG), hybrid retaining der(1) (WAG 21R-11), hybrid retaining der(X) (WAG 8-1), patient S. W. (WLS).

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and, in all cases, the translocation was found to be paternal in origin (Bodrug *et al.* 1990; present report). The parental origin of a further four X-autosome translocations, none of which is associated with DMD, is known (Chamberlin & Magenis, 1980; Pai *et al.* 1980; D. O. Robinson, unpublished observations) and again all are of paternal origin. The probability of all twelve translocations having the same parental origin by chance is less than 1 in 4000. Thus preferential paternal origin appears to be a feature of all balanced X-autosome translocations and is not restricted to those associated with DMD.

We recently studied the parental origin of nineteen structurally abnormal X chromosomes in patients with Turner's syndrome (Jacobs *et al.* 1990). Eleven structurally abnormal chromosomes were found to be paternal in origin and eight maternal, a figure not significantly different from the 1:1 ratio expected from either a mitotic or a meiotic origin. Our data suggest therefore that X-autosome translocations may have a different mechanism of origin from structural abnormalities involving only the X.

Olson & Magenis (1988) summarized the data available on the parental origin of different types of structural abnormalities. Among the data they presented there were fourteen apparently balanced *de novo* reciprocal translocations involving two autosomes, eleven being of paternal origin and three of maternal origin. Thus there seems to be a less marked paternal bias in the origin of inter-autosome translocations than that observed for X-autosome translocations. The preferential paternal origin of both X-autosome and inter-autosome translocations is based on very small numbers of cases. However, if substantiated by further data, it must reflect a difference between male and female gametogenesis, where the majority of such mutations presumably occur. There are very many more pre-meiotic mitotic divisions in the male than in the female and, if errors at these divisions are responsible for the majority of abnormalities, the higher likelihood of males giving rise to such translocations is easily understood. However, Bodrug *et al.* (1990) argued cogently that X-autosome translocations present in pre-meiotic cells are selected against during meiosis and do not complete gametogenesis, thus the origin of X-autosome translocations must be meiotic or gametic rather than in mitotic spermatogonia. A similar type of selection has been reported for some inter-autosome translocations (Chandley, 1988). Therefore it seems plausible that the increased propensity for such mutations to originate in the male must be related either to sex differences in the process and outcome of meiosis itself, or to male gametes being more prone to chromosome breakage and exchange than those of the female.

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