much interest to examine the expression pattern of dystrophin transcripts in XLDCM patients with mutations outside the 5' end of the DMD gene, such as in the patients reported by Oldfors et al. (1994) (a deletion affecting exons 6–13), by Towbin et al. (1994) (a mutation within exons 8–10), and by Franz et al. (1995) (a mutation around exons 27–30).

Our results, together with the report by Muntoni et al. (1995), also indicate that the sequence around the 5' end of the first muscle intron may be essential for the functions of the muscle promoter, because this region consistently is involved in these patients. In fact, Klamut et al. (1996) recently have identified a transcriptional enhancer within the first muscle intron of the human DMD gene. Further molecular and cellular biological studies on dystrophinopathy with the XLDCM phenotype will help us understand the functions of dystrophin promoters in the skeletal and cardiac muscles.

In summary, we showed up-regulation of the brain and Purkinje-cell forms of dystrophin transcripts not only in an atypical BMD patient (patient 1) with the XLDCM phenotype, but also in typical BMD patients (patients 2 and 3). We think that the other isoforms of dystrophin can modulate the clinical features and the course of dystrophinopathy, especially with regard to the XLDCM phenotype.

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Bilateral Retinoblastoma in a Male Patient with an X;13 Translocation: Evidence for Silencing of the RB1 Gene by the Spreading of X Inactivation

To the Editor:

We describe a male patient who has an X;13 translocation and bilateral retinoblastoma. DNA replication and methylation studies for this patient suggested that X inactivation had spread to chromosome 13 and had produced functional monosomy for genes on proximal 13q. Inactivation of both alleles of the RB1 gene in 13q14 comprises the two rate-limiting steps in the formation of retinoblastoma (Knudson 1975; Cavenee et al. 1983). In hereditary retinoblastoma, one allele is inactivated or

lost because of a germ-line mutation. In ~3% of patients, this is due to a cytogenetically visible deletion that includes 13q14 (Turleau et al. 1985). Owing to monosomy for a large chromosomal region, patients with 13q deletions also have mental retardation, growth retardation, and congenital anomalies (Brown et al. 1993). Retinoblastoma formation appears to be initiated by somatic loss or by inactivation of the second RB1 allele, in a retina precursor cell. The second event most often involves mitotic nondisjunction, mitotic crossing-over, or a structural gene mutation (Cavenee et al. 1983). In nonhereditary retinoblastoma, both RB1 alleles of a retina precursor cell must be inactivated during retinal development. In ~15% of such tumors, the RB1 CpG island is methylated (Greger et al. 1989, 1994; Sakai et al. 1991). In vitro and in vivo studies have suggested that methylation of the RB1 promoter reduces gene activity (Ohtani-Fujita et al. 1993; Greger et al. 1994). To date, RB1 promoter methylation has not been found in nontumor cells. It is known, however, that DNA methylation serves to regulate gene activity in normal cells (for review, see Razin and Shemer 1995). The role of DNA methylation has been studied best in genomic imprinting and in X inactivation. X inactivation spreads from the X inactivation center (XIC) at Xq13 throughout most of the X chromosome and seems to involve DNA methylation (for review, see Willard 1995). A similar spreading mechanism has been proposed for the imprinting of 15q11-13. Constitutional hypermethylation of the RB1 gene therefore might be expected to occur in translocations involving chromosome 13 and the X chromosome or in imprinted autosomal chromosome domains.

The patient was born to a G₄P₃ Hispanic mother by C-section, because of oligohydramnios, at the 37th wk of gestation. The APGAR scores were 6¹8⁵. The patient weighed 1.7 kg (<<5% of normal), with a length of 39 cm (<<5% of normal) and a head circumference of 30.5 cm (<<5% of normal). In addition to his small size, the baby had several major and minor congenital anomalies. These included a prominent occiput, micrognathia, hypertelorism, posteriorly rotated large ears and a long philtrum, bilateral hip dislocations, an imperforate anus with a vesicoureteral fistula, and a ventricular septal defect with tricuspid regurgitation. Chromosome analysis of cultured lymphocytes revealed an unbalanced X;13 translocation [46,XY,der(13)t(X;13)(q10q10)] in all cells, indicating the presence of extra X-chromosome material. Cytogenetically, this would imply a variant of Klinefelter syndrome, but the anomalies were not explained by this diagnosis. Males with an additional X chromosome usually are phenotypically normal at birth and often are not diagnosed until adolescence, when phenotypic features and infertility become evident. Although the entire long arm of chromosome 13 appeared

to be present, there was concern that functional monosomy for proximal 13q might have caused the anomalies and might have placed the baby at risk for the development of retinoblastoma. Therefore, an ophthalmologic consultation was obtained. In close proximity to the optic nerve, a peripapillary exophytic tumor measuring 2 disc diameters was present in the left eye. The patient was transferred to a second facility for laser ablation of the tumor. In the 3 d between the initial examination and the transfer, the tumor increased in size by 50%. In addition, a second tumor focus was detected. The patient received both laser therapy and chemotherapy with carboplatin. At 4 mo of age, retinoblastoma was identified near the ora in the right eye, and both tumors were treated by cryotherapy. In addition to multiple surgeries for the tumor, he also required gastrostomy tube feeding and a tracheotomy to control respiratory difficulties secondary to tracheomalacia. At 5½ mo of age, the left eye was found to be filled with a hazy media with a greenish yellow cast. The retina was detached. A magnetic resonance imaging examination of the brain showed diffuse atrophy of the cerebral hemisphere, with more atrophy in the left hemisphere than in the right. There was an enlargement of the lateral and the third ventricles. The left eye was enucleated, since there was no chance of recoverable vision and since regrowth of the tumor in that eye could not be excluded. A pathological examination revealed retinoblastoma that filled the vitreous and was continuous with the detached retina. Results of cytogenetic studies of the tumor were identical to those of cytogenetic studies of the peripheral blood. At 13 mo of age, no new tumors were present, but the tumor near the ora was larger. Cryopexy was applied in a triple freeze-thaw technique involving all of the tumor and the surrounding area. Two weeks later, the patient was admitted for sepsis. He went into cardiac arrest and failed to respond to resuscitative efforts. The parents declined the performance of an autopsy. At the time of his death, the patient had moderate/severe developmental delay.

The translocated chromosome in this patient is a centric fusion of the long arm of a chromosome 13 and the long arm of an X chromosome (fig. 1A). FISH studies indicated that the centromere of this chromosome contained material from both chromosome 13 and the X chromosome (not shown). The XIST locus was present on both the normal X chromosome and the derivative chromosome, and the RB1 locus was present on both the normal chromosome 13 and the derivative chromosome (not shown). Replication-time studies indicated that the derivative chromosome was late replicating in all cells visualized (fig. 1B). Late replication appeared to spread through the translocated chromosome, from the long arm of the X chromosome through the centromere of chromosome 13 and continuing to proximal 13q14.

Figure 1 A, G-banded chromosome 13 and derivative X;13 chromosome. B, BrdUrd-stained derivative X;13 chromosome adjacent to chromosome 13. The arrow indicates the transition region between the late-replication area and the early replication area of chromosome 13 (13q14).

To determine the methylation status of the RB1 gene, in peripheral blood cells of the patient, we used the methylation-sensitive restriction enzyme *Bss*HII, which cuts once within a 6.1-kb *Sac*I fragment spanning the promoter and exon 1 (Greger et al. 1989, 1994; Sakai et al. 1991). Aliquots of genomic DNA (2 μ g) were digested with *Sac*I + *Bss*HII. To control for complete digestion by use of the rare cutting enzyme *Bss*HII, 200 μ g of a 186-bp cloned DNA fragment from the EXT1 gene containing a single *Bss*HII site (H. J. Lüdecke and B. Horsthemke, unpublished data) were added to genomic DNA. After digestion, an aliquot of the restriction mixture was analyzed on a 2% agarose gel. In each case, complete digestion of the cloned DNA into fragments of the expected size (111 bp and 75 bp) was observed. For Southern blot analysis of the RB1 gene, the DNA fragments were separated on a 0.8% agarose gel, transferred to a nylon membrane, and hybridized with a 921-bp PCR product spanning the promoter and exon 1 of the RB1 gene (Lohmann et al. 1991). In DNA from a normal control (fig. 2, lane 3), the 6.1-kb fragment was unmethylated and cleaved into two fragments of 4.3 kb and 1.8 kb. In DNA from a hypermethylated retinoblastoma (fig. 2, lane 2; patient OM in Greger et al. 1994), the 6.1-kb *Sac*I fragment was completely resistant to digestion by *Bss*HII. In DNA from the patient (fig. 2, lane 1), ~50% of the *Sac*I fragments were not cut by *Bss*HII. The addition of more enzyme did not change the Southern pattern (not shown). In contrast to genomic DNA, a 921-bp PCR product spanning the *Bss*HII site was cut to completion (not shown). These results indicate that partial cleavage of the genomic DNA from the patient was not due to a *Bss*HII polymorphism but was due to hypermethylation of one gene copy per cell.

On the basis of the findings that the translocated chromosome was late replicating in all cells and that one copy of the RB1 gene was methylated, we propose that the RB1 gene on the translocated chromosome had been inactivated by the spreading of X inactivation into 13q and that this epimutation represents the first genetic event involved in the formation of retinoblastoma in our patient. Late replication and DNA methylation are

hallmarks of the inactive X chromosome. Inactivation is initiated at the XIC in Xq13, involves a regulatory RNA (XIST), and spreads through most of the X chromosome. CpG islands found to be associated with the 5' end of constitutively expressed genes are methylated on the inactive, but not the active, X chromosome.

X inactivation in somatic cells of female individuals usually is random. This is not true in females with balanced X autosomal translocations. In the majority of cells of such individuals, the translocated X chromosome remains active, whereas the normal X chromosome is inactive. It generally is assumed that the non-random X inactivation observed in such cases is the result of a selection process operating against cells in which X inactivation has spread into the autosome and has inactivated autosomal genes (Willard 1995). In our patient, replication-time studies indicated non-random X inactivation, with the translocated chromosome being inactive in all cells. Theoretically, a need for functional copies of genes on Xp might have selected against the survival of embryonic cells with an inactive X chromosome.

To our knowledge, the patient described here represents the first case of a male with an X autosomal translocation who developed retinoblastoma. We are aware of four previous reports of X;13 translocations associated with retinoblastoma (Nichols et al. 1980; Ejima et al. 1982; Kajii et al. 1985; Ponzio et al. 1987). All of these patients were females. In the first three cases, the translocation breakpoints were on Xp and 13q12-q13, and the derivative X chromosome was late replicating in the majority of cells (Nichols et al. 1980; Ejima et al. 1982) or in a minority of cells (Kajii et al. 1985). In the patient described by Ponzio et al. (1987), the breakpoints were at Xq12 and 13q31, and the normal X chromosome was late replicating in all cells studied.

Figure 2 DNA methylation analysis. DNA was digested with *Sac*I + *Bss*HII and was analyzed by Southern blot hybridization with a probe spanning the promoter and exon 1 of the RB1 gene. Lane 1, Peripheral blood cell DNA from the patient. Lane 2, DNA from a hypermethylated retinoblastoma. Lane 3, Peripheral blood cell DNA from a normal control. Lane 1 contains $1.5 \times -2 \times$ as much DNA as is contained in lanes 2 and 3, as estimated by ethidium bromide staining of the gel (not shown). Approximately 50% of the 6.1-kb *Sac*I fragments of the patient's DNA were resistant to cleavage by *Bss*HII. Owing to the high G + C content, the probe crosshybridizes to rDNA, which is present in 200–400 copies per haploid genome, and to other fragments unrelated to the RB1 gene (Greger et al. 1989, 1994; Belka et al. 1991).

It was postulated that a cell line with a late-replicating derivative chromosome 13, which contained the XIC and RB1 loci, was present in the very early stages of development and then was lost.

Nichols et al. (1980) were the first to suggest that, in X;13 translocations, band 13q14 may be functionally monosomic owing to the spreading of X inactivation, thus becoming a predisposing factor for the onset of retinoblastoma. Mohandas et al. (1982) isolated, in mouse-human cell hybrids, the inactive *der(X)* chromosome of the patient described by Nichols et al. (1980) and determined that the gene for esterase D, which maps close to the RB1 gene, was not expressed. This result provided biochemical evidence for the inactivation of an autosomal gene by the spreading of X inactivation. In our case, we have investigated the RB1 gene itself and have provided direct evidence for methylation of the CpG island. This suggests that the spreading of X inactivation into autosomal chromatin involves the methylation of CpG dinucleotides within CpG islands, as in the X chromosome.

Of the four previous cases of retinoblastoma involving an X;13 translocation, three of the four female patients did not exhibit multiple congenital anomalies. In one case, however, the patient also had incontinenti pigmenti (Kajii et al. 1985). This was assumed to have resulted from the interruption of the IP gene at Xp11.2 by the translocation. In contrast to these cases, the translocation in our patient was a centric fusion of Xq and 13q. X inactivation had spread through the centromere and had affected 13q10-q14. As judged from the replication data, inactivation had not spread beyond 13q14. Inactivation of the entire 13q region likely would have been lethal to the early embryo. It is not clear why the spreading of inactivation might have ended in midchromosome.

The multiple congenital anomalies of our patient were likely the result of functional monosomy for genes on chromosome 13, perhaps from 13q10-q14. The clinical features of patients with partial monosomies for 13q vary. The majority of such individuals with retinoblastoma associated with congenital anomalies have terminal deletions of chromosome 13 distal to 13q12. The clinical features include a high forehead, bulbous nose, long philtrum, and a large mouth with a thin upper lip. Although our patient had some of the features described in other patients with deletions in 13q (high forehead and long philtrum), he had additional features, including micrognathia, hypertelorism, vesicourethral reflux, and a cardiac anomaly. Two patients with retinoblastoma and interstitial deletions of chromosome 13 [del(13)(q12.3q21.1)] have been described elsewhere (Montegi et al. 1983). The clinical features were similar to those of our patient and included parietal bossing, a long philtrum, microg-

nathia, and tetralogy of Fallot. The second interstitial deletion [46,XX,del(13)(q12q22)] was associated with microcephaly, micrognathia, and cleft palate (Petit et al. 1979).

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Meiotic Drive at the Myotonic Dystrophy and the Cone-Rod Dystrophy Loci on Chromosome 19q13.3

To the Editor:

The apparently conflicting observations of a high new-mutation rate at the myotonic dystrophy (DM) locus on chromosome 19q13.3 and of a founder effect for DM chromosomes led researchers to invoke the influence of meiotic drive at this locus. Two studies (Carey et al. 1994; Gennarelli et al. 1994) suggested such an effect in male meioses, whereas one study (Shaw et al. 1995) found evidence for segregation distortion in female meioses. In the October 1996 issue of the *Journal*, Leeflang et al. demonstrated convincing evidence that, if such an effect exists in male meioses, it must operate postejaculation, presumably influencing sperm motility

or sperm survival. In so doing, the authors also reviewed the literature both supporting and opposing the influence of the action of meiotic drive at the DM locus. However, they appear to have missed a report from our laboratory (Evans et al. 1994) of a similar observation for dominant cone-rod dystrophy (CORD2), a form of retinal degeneration that also maps to chromosome 19q. The data from the study of the CORD2 locus suggest segregation distortion in female meioses. The most recent locus refinement for CORD2 (Bellingham et al., in press) places it in an interval 0.8–2.4 Mb distal to the DM locus, on the metric FISH map of Gordon et al. (1995). Is it not possible that the close proximity of these two loci, both of which apparently have such an unusual pattern of inheritance, is more than a coincidence?

There appear to be five hypotheses to explain this observation.

1. It is possible that this is indeed no more than a coincidence. Several reports of anomalous segregation for other human conditions exist in the literature, although most of these were tentative and remain unconfirmed. These conditions include split-hand/split-foot malformation (Stevenson and Jennings 1960), retinoblastoma (Munier et al. 1992), aniridia (Shaw et al. 1960), Alport syndrome (Shaw and Glover 1961), and postaxial polysyndactyly (Orioli 1995). Evidence for segregation distortion of alleles for several blood-group markers also has been observed (Palaniappan et al. 1996). Nevertheless, meiotic drive in humans remains a relatively rare phenomenon, so this hypothesis seems unlikely.

2. It is not unreasonable to speculate that these diseases may be allelic, since DM patients do have some visual symptoms (Burian and Burns 1967). However, these symptoms are clinically very different from the symptoms of CORD2. In addition, in the CORD2-linked family, there is a crossover between the phenotype and the DM expansion, which also is seen with two other markers that are distal to DM and proximal to CORD2 (data not shown). This hypothesis therefore is excluded.

3. A third hypothesis would suppose that gamete selection at both loci operates on the alleles of the DM locus. If the mutation in the CORD2 family arose on a chromosome containing a DM allele of 19 repeats, which is one of the founder chromosomes first postulated by Imbert et al. (1993), then patients with CORD2 would carry a predisposition to DM but not the disease itself. CORD2 then would be selected in gametes not because of any allele at this locus but because it was in linkage disequilibrium with the DM predisposing allele. However, the linked DM allele in the CORD2 family is in fact the smallest, most common allele—that is, the