**Short communications**

**An informative polymorphism detectable by polymerase chain reaction at the 3’ end of the dystrophin gene**

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**Summary.** A fragment that contains a (CA)n sequence from the 3’ untranslated region of the dystrophin gene can be amplified by the polymerase chain reaction and shows length polymorphism in a Caucasian population. The two common alleles differ by 4 bp. This new genetic marker has a heterozygosity of about 35% and is typed more rapidly than a conventional restriction fragment length polymorphism. Its localisation at the 3’ end of the dystrophin gene makes it a useful tool for diagnostic applications in families with Duchenne/Becker muscular dystrophy, and for the analysis of intragenic recombination.

**Introduction**

The cloning of genomic and cDNA sequences corresponding to the dystrophin gene has provided essential tools for the prenatal and carrier diagnosis of Duchenne (DMD) and Becker muscular dystrophy. About 50%–65% of the cases are caused by deletions that can be detected by Southern blotting using a set of cDNA probes that span the whole gene (Koenig et al. 1987; Forrest et al. 1988; Worton and Thompson 1988; Lindløf et al. 1989). About 80%–90% of these deletions can now be detected by polymerase chain reaction (PCR) using 9 pairs of oligonucleotide primers (Chamberlain et al. 1988; J. S. Chamberlain, personal communication). This is the method of choice for screening probands for deletions and for prenatal diagnosis, since it is rapid and can be performed on a very small amount of fetal DNA. However, it is informative in only about 50% of the families.

Carrier diagnosis in families with deletions can be performed by dosage analysis of the region deleted in the proband. Dosage analysis in Southern blots requires optimal conditions; very good quality blots are necessary, with even transfer and hybridisation, low background, the presence of internal standards and typing at least in duplicate. Carrier diagnosis can also be based on the tracking of abnormal junction fragments; however, these are rarely detected by normal Southern blotting with cDNA probes. They can be detected with high efficiency by pulsed field (or field inversion) gel electrophoresis (Den Dunnen et al. 1987), but this is a demanding technique for routine work in a diagnostic laboratory.

Indirect tracking of the mutation with intragenic restriction fragment length polymorphisms (RFLPs) is still essential for families where no deletion is detected, and can increase the reliability of carrier diagnosis in families with deletions, given the problems of dosage analysis. Many RFLPs have been identified, i.e. those detected by genomic probes in the Pert87 and XJ regions (Kunkel et al. 1986; Thompson et al. 1986) that define informative haplotypes, the RFLPs detected by probes J. Bir, P20 and J66 (Monaco et al. 1987; Van Ommen et al. 1987; Wapenaar et al. 1988) and, more recently, RFLPs detected by cDNA probes (Darras and Francke 1988). The interpretation of RFLP results suffers, however, from the possibility of intragenic recombination between the RFLP analysed and the mutation; this may lead to diagnostic errors. A recent large scale study by Chen et al. (1989) gave an estimate of 3% recombination between the XJ RFLPs (DXS206) and the J. Bir RFLP (DXS270) that are separated by about 600 kb, i.e. only one fourth of the dystrophin gene length.

To overcome this problem and to detect recombination events, it would be necessary to analyse RFLPs at the two ends of the dystrophin gene. Most known RFLPs are present within a region 500–800 kb from the 5’ end (XJ and Pert region) and few are near the 3’ end of the gene, i.e. the PstI RFLP detected by J66, which is difficult to type because of the similarity of allele sizes, and a TaqI RFLP detected by the cDNA probe 11–14 (Van Ommen et al. 1987; Darras and Francke 1988). Extragenic RFLPs on the 3’ side are sometimes used but these are genetically far away from the dystrophin gene, with about 10% recombination for C7 (DXS28) and even more for 99.6 (DXS41) (Dorkins et al. 1985; Chen et al. 1989); moreover, they cannot distinguish between intragenic and extragenic recombination events. There is thus still a need for informative polymorphisms at both ends of the dystrophin gene. We report here on a length polymorphism located at the extreme 3’ end of the gene, in the 3’ untranslated region; it has a heterozygosity of 37% and is rapidly typed by the polymerase chain reaction.

**Materials and methods**

Two oligodeoxynucleotide primers MZ-18 [position 11654; Koenig et al. 1988: AAGAAAAAGATTGAAACTAAAGTTGCTT] and MZ-19 [position 11789: GGATGCAAAACAAATGGCGTGGCTCTCTCA] that flank a (CA)n sequence were synthesised using a Gene Assembler (Pharmacia). Prim-
ers were purified by HPLC on a reverse phase column (Millipore-Waters). In some experiments, primer NB94 [position 11693: GTTGTITATAAAAACCCCTAATAAAAAACAAAAC] was used instead of MZ-18, which generates smaller fragments (96 bp).

The MZ-19 primer was end-labelled for 90 min at 37°C in a volume of 25 μl containing 50 pmol (0.5 μg) primer, 0.125 mCi γ 32-P-ATP at 5000 Ci/mmol, 10 mM MgCl₂, 5 mM DTT, 50 mM TRIS pH 7.6 and 2 μl T4 polynucleotide kinase (20 units). It was further used without removing unincorporated nucleotides.

Polymerase chain reaction (PCR) was carried out in a volume of 50 μl containing 200 ng genomic DNA, 25 pmol (0.25 μg) each unlabelled primer, 1.2 pmol end labelled MZ-19 primer, 200 μM each dNTP, 50 mM KCl, 10 mM TRIS, pH 8.3, 1.5 mM MgCl₂, and 1U Taq polymerase (Perkin Elmer, Cetus). Samples were subjected to 15–20 cycles consisting of 1 min at 94°C (denaturation), 40 sec at 65°C (annealing) and 40 sec at 72°C (elongation), using a Perkin Elmer Cetus thermocycler. The last elongation was lengthened to 10 min.

PCR products were precipitated with ethanol. Precipitates were dried and resuspended in 10% 91 formamide, 0.5% xylene cyanol, 0.5% bromophenol blue, 20 mM EDTA. Aliquots (4 μl) were electrophoresed on 8% polyacrylamide, 50% urea sequencing gels. The precipitation step can be avoided when a larger number of amplification cycles is used. Gels were electrophoresed at 20 mA/1.6 kV for 4 h, dried and exposed to film for 1–3 days.

Results and discussion

The sequence of the dystrophin cDNA established by Koenig et al. (1988) reveals the presence, in the 3’ untranslated region from position 11726 to 11758, of a region with tandem repeats of the CA dinucleotide, (CA)₈–TA–(CA)₇, surrounded by an AT-rich region. It was recently reported that such sequences (termed microsatellites) very frequently show polymorphic variation in the number of CA (or TG) repeat units; this can be detected after DNA amplification by electrophoresis on a denaturing sequencing polyacrylamide gel (Weber and May 1989; Litt and Luty 1989). We chose the two primers MZ-19 (a 30-mer) and MZ19 (a 25-mer) that flank the (CA)n microsatellite (Weber and May 1989; Litt and Luty 1989). Samples were subjected to 25 cycles consisting of 1 min at 94°C (denaturation), 40 sec at 58°C (annealing) and 1 min at 72°C (elongation), using a Perkin Elmer Cetus thermocycler. The last elongation was lengthened to 10 min.

PCR products were precipitated with ethanol. Precipitates were dried and resuspended in 10% 91 formamide, 0.5% xylene cyanol, 0.5% bromophenol blue, 20 mM EDTA. Aliquots (4 μl) were electrophoresed on 8% polyacrylamide, 50% urea sequencing gels. The precipitation step can be avoided when a larger number of amplification cycles is used. Gels were electrophoresed at 20 mA/1.6 kV for 4 h, dried and exposed to film for 1–3 days.

Fig. 1. Length polymorphism of an amplified DNA fragment including a (CA)n repeat block from the 3’ untranslated region of the dystrophin gene. DNA was amplified and analysed using genomic DNAs from unrelated females. Alleles are designated A1, A2 and A3. The length differences (4 bp between A1 and A2, 12 bp between A2 and A3) were determined by comparison with sequencing reactions run in parallel (not shown)

The new polymorphic marker that we have characterised should became a very useful addition to the panel of RFLPs within the dystrophin gene because of its localisation at the extreme 3’ end of the gene. When informative, it will allow the detection of intragenic recombination events and will profitably replace the extragenic markers DXS28 or DXS41 that are far away. An added advantage is the very rapid typing. Amplification and electrophoresis can be performed in a day, and exposure usually requires one or two days. This probably could be accelerated by raising the number of amplification cycles. One should note that the heterozygosity of this polymorphism (37%) is lower than that observed on average for microsatellites (Weber and May 1989; Litt and Luty 1989) because of the presence of only two common alleles in Caucasians. Contrary to other cases described where successive alleles differ by one dinucleotide repeat (2 bp), here they differ by 4, 12 and 14 bp; intermediates have not been observed.

In addition to the diagnostic applications, this polymorphism might be used to evaluate the recombination frequency in the distal half of the dystrophin gene. The data of Chen et al. (1989) suggest a comparatively high recombination frequency between the XJ region and J. Bir (3% for about 600 kb, i.e. about 5 times the expected rate), but no data are available for the region distal to J. Bir. In particular, it should be interesting to see whether the distal region that contains the hot spot of deletion (around P20; Wapenaar et al. 1988) also shows increased recombination. Finally, this polymorphism could be used to investigate new mutation events (deletions or duplications) in order to ascertain what proportion of these events are linked to meiotic recombination.

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