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Detection of 98% of DMD/BMD gene deletions by polymerase chain reaction

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Summary. We describe oligonucleotide primer sequences that can be used to amplify eight exons plus the muscle promoter of the dystrophin gene in a single multiplex polymerase chain reaction (PCR). When used in conjunction with an existing primer set, these two multiplex reactions detect about 98% of deletions in patients with Duchenne or Becker muscular dystrophy (DMD, BMD). Furthermore, these primers amplify most of the exons in the deletion prone "hot spot" region around exons 44 to 53, allowing determination of deletion endpoints and prediction of mutational effects on the translational reading frame. Thus, use of these PCR-based assays will allow deletion detection and prenatal diagnosis for most DMD/BMD patients in a fraction of the time required for Southern blot analysis.

Introduction

Duchenne and Becker muscular dystrophies (DMD, BMD) are allelic neuromuscular disorders caused by mutations to the dystrophin gene at Xp21 (reviewed by Hoffman and Kunkel 1989). Because of the extremely large size of this gene (nearly 2,400 kb) the majority of mutations are intragenic deletions (~65%) or duplications (~5%) (Koenig et al. 1987; Forrest et al. 1988; Hu et al. 1988). The severe Duchenne form is caused by the absence of detectable dystrophin, which, at the DNA level, is usually a result of mutations that alter the translational reading frame leading to production of a truncated and presumably unstable dystrophin molecule. On the other hand, the milder BMD results from internal deletions or duplications that do not disrupt the reading frame, so partially functional protein can still be produced (Hoffman et al. 1988, 1989a; Monaco et al. 1988). Overall, about 92% of deletions conform to the "reading frame rule" (Koenig et al. 1989) with one notable exception being deletions of exons 3–7 (Malhotra et al. 1988). Interestingly, locations of deletions in the dystrophin gene are apparently nonrandom with a preponderance found in two "hot spot" regions at the 5′ terminus and in the distal half of the central rod domain around exons 44–53 (Koenig et al. 1987; Forrest et al. 1988).

The advent of polymerase chain reaction (PCR) technology is revolutionizing approaches to DNA analyses (Innis et al. 1990). Traditionally, deletions and duplications of the dystrophin gene have been detected by Southern blot analysis using cDNA probes (Koenig et al. 1987; Forrest et al. 1988; Hu et al. 1988). These studies require the use of up to seven different subcloned cDNA probes to examine at least two different restriction digests of genomic DNA. However, with the knowledge that most deletions are clustered in two regions, examination of only a subset of the ~70 exons is sufficient to detect a majority of deletions. Recently, Chamberlain et al. (1988) described a series of PCR primers that amplify six exons that are deleted in many DMD/BMD patients. This multiplex PCR analysis allows rapid (1–2 days) deletion detection on small quantities or suboptimal samples of genomic DNA. The addition of primers for three more exons makes the Chamberlain multiplex test informative in about 80% of DMD and BMD patients who have cDNA-detectable deletions (Chamberlain et al. 1990). Here, we describe primers for nine additional exons that, when used in conjunction with those of Chamberlain et al. (1988, 1990) detect all deletions that we know of in BMD patients and over 97% of deletions in patients with DMD (based on 329 deletions mapped by Koenig et al. 1989). Since most of the exons in the distal hot spot region (exons 44–53) are included in these two sets, accurate prognostic determinations can be made for many patients with deletions in this region. Thus, mutation detection, prenatal diagnosis, and even carrier detection for DMD/BMD can now be performed with considerably more ease than previously possible (reviewed by Beggs and Kunkel 1990a).

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Table 1. Sequence of polymerase chain reaction (PCR) primers for ten exons of dystrophin

Pri[a]	Sequence (5'-3')	Pri	Sequence (5'-3')	PCR product size
PmF	GAAGATCTAGACAGTGGATACATAACAAATGCATG	PmR	TTCTCCGAAGGTAATTGCCTCCCAGATCTGAGTCC	535 bp
3F	TCATCCATCATCTTCGGCAGATTAA	3R	CAGGCGGTAGAGTATGCCAAATGAAAATCA	410
6F	CCACATGTAGGTCAAAAATGTAATGAA	6R	GTCTCAGTAATCTTCTTACCTATGACTATGG	202
13F	AATAGGAGTACCTGAGATGTAGCAGAAAT	13R	CTGACCTTAAGTTGTTCTTCCAAAGCAG	238
43F	GAACATGTCAAAGTCACTGGACTTCATGG	43R	ATATATGTGTTACCTACCCTTGTCGGTCC	357
47F	CGTTGTTGCATTTGTCTGTTTCAGTTAC	47R	GTCTAACCTTTATCCACTGGAGATTTG	181
50F	CACCAAATGGATTAAGATGTTCATGAAT	50R	TCTCTCTCACCCAGTCATCACTTCATAG	271
52F	AATGCAGGATTTGGAACAGAGGCGTCC	52R	TTCGATCCGTAATGATTGTTCTAGCCTC	113
60F	AGGAGAAATTGCGCCTCTGAAAGAGAACG	60R	CTGCAGAAGCTTCCATCTGGTGTTCAGG	139
49F[b]	GTGCCCTTATGTACCAGGCAGAAATTG	49R	GCAATGACTCGTTAATAGCCTTAAGATC	439

[a] Primers are named for the exon they amplify using numbering of Koenig et al. (1989). Pm is the muscle-specific promoter (underlined nucleotides create an artificial *Bgl*II site). F indicates forward and R, reverse relative to the coding sequence

[b] Primers 49F, R are not included in the multiplex reaction but are used separately with one or two other pairs as internal positive controls

Materials and methods

Oligonucleotide primer sequences and preparation

The sequences of ten pairs of PCR primers are listed in Table 1. Exon numbering is based on Koenig et al. (1989). Primers are derived from intron sequences and amplify the entire exon and from 5 to 176 base pairs (bp) of intron with the exception of those for exons 52 and 60, which are derived from cDNA (exon) sequences (Koenig et al. 1988). The promoter primers (Pm) amplify the entire muscle promoter and the first quarter of exon 1. Unpublished intron sequences were derived by Koenig et al. (1989) and are freely available upon request. Oligonucleotides were synthesized by the Biopolymers Laboratory, Howard Hughes Medical Institute, Harvard Medical School or were obtained unpurified (e.g. "machine grade") from Synthecell Corp. (Rockville, Md.). Full-length oligomers were purified on 20% denaturing polyacrylamide gels, eluted in 3 *M* NaCl, 10 m*M* Tris pH 8.0, 1 m*M* ethylene diamine tetraacetic acid (EDTA), ethanol precipitated, and washed extensively with 70% ethanol to remove excess NaCl. Primers were resuspended in water at 0.4 µg/µl and stored at −20°C.

PCR reation conditions and analysis

Genomic DNA was isolated from peripheral blood (generally 2–20 ml) anticoagulated with EDTA. Leukocyte nuclei isolation and subsequent DNA purification was essentially as described by Kunkel et al. (1977) except that the homogenization step was omitted. Following lysis by 0.5% sodium dodecyl sulfate (SDS) in 75 m*M* NaCl, 24 m*M* EDTA, nuclei were digested with 100 µg/ml proteinase K (Boehringer Mannheim, Indianapolis, Ind.) overnight at 37°C. After extraction, DNA was dialyzed against three changes of 0.5 m*M* EDTA, 10 m*M* Tris pH 7.6, 10 m*M* NaCl (High EDTA level in DNA samples may occasionally inhibit amplification by chelating magnesium, however, we have had no problems with adding 25 µl of DNA to a 50 µl reaction, e.g., 0.25 m*M* EDTA final concentration). PCR reactions using reagents from Perkin-Elmer Cetus GeneAmp kits (Norwalk, Conn.) contained 0.5 µl of each primer (e.g., ~0.5 µ*M*), 5 units *Taq* polymerase and 250 ng genomic DNA per 50 µl reaction. In practice, all primers for a given reaction were generally premixed and stored in aliquots. Each set of reactions was always prepared as a master mix and aliquots added to 0.5-ml microfuge tubes before addition of water and template DNA to a final volume of 50 µl. These were overlain with 25 µl mineral oil and incubated in either a Perkin-Elmer Cetus (Norwalk, Conn.) thermal cycler or an MJ Research (Watertown, Mass.) thermal controller as follows: 94°C, 7 min to denature, followed by 25 cycles of 94°C, 30 s (to denature); 65°C, 4 min (to anneal and elongate) with the last annealing/elongation step for 10 min. The nine primers described by Chamberlain et al. (1988, 1990) were used separately under reaction conditions described by those workers. PCR products were stored at 4°C for up to several days prior to analysis. Reaction products (15 µl) were separated on either 2% NuSieve (FMC BioProducts, Rockland, Me.) + 1% agarose or 1.4 agarose gels at 5 V/cm. Separate pipets were used for sample preparation and analysis, and at all stages PCR primers and reagents were rigorously segregated from reaction products and plasmids containing target sequences.

Results and discussion

In the course of several recent studies designed to test the reading frame hypothesis, intronic sequences surrounding many exons were examined and several hundred deletions were mapped (Koenig et al. 1987; Monaco et al. 1988; Baumbach et al. 1989). Using this information, Chamberlain et al. (1988) designed PCR primers to amplify six exons commonly deleted in DMD/BMD pa-

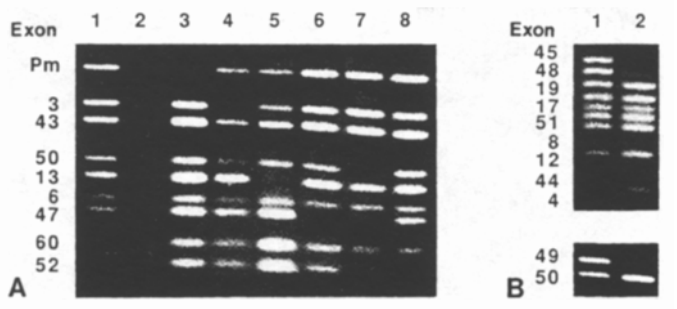

Fig. 1A, B. Analysis of dystrophin gene multiplex polymerase chain reactions (PCR). The exons represented by each PCR product are indicated on the *left*. **A** Analysis of DNA from a normal control *(lane 1)*, a DMD patient with complete deletion of the dystrophin gene *(lane 2)*, and DMD/BMD patients with various intragenic deletions *(lanes 3–8)*. Lack of amplification in *lane 2* serves as a negative control for contamination. The products in *lane 5* are from amplification of genomic DNA that was too degraded for Southern blot analysis. PCR primers are the multiplex mix described in Table 1. **B** Determination of deletion endpoints. Analysis of DNA from a control male *(lane 1)* and a BMD patient with an in-frame deletion of exons 45-49 *(lane 2)*. The *top panel* contains products of Chamberlain's primers (Chamberlain et al. 1990) and the *bottom panel* contains products of primers for exons 49 and 50 (Table 1). Amplification with the nine primers used in **A** gave a pattern similar to that in **A**, lane 6

tients (exons 8, 17, 19, 44, 45, and 48). With the recent addition of primers for three more exons (4, 12, and 51), this multiplex PCR test is predicted to detect about 80% of deletions that have been identified by Southern blotting with cDNA probes (Chamberlain et al. 1990). To raise the sensitivity of this PCR test, we have used the 273 deletions mapped by Koenig et al. (1989) as a guide in choosing nine more exons for a second multiplex reaction to be used in conjunction with the primers of Chamberlain et al. (1988, 1990). Our multiplex test contains primers that detect exons 3, 6, 13, 43, 47, 50, 52, 60, and the muscle promoter (Table 1). Together, examination of these eighteen exons allows detection of all 71 deletions found in patients with BMD or intermediate phenotypes and 197/202 deletions in patients with DMD for an overall sensitivity of 98.2% (Koenig et al. 1989). If we include data on a further 56 patients who were excluded from this study because of age or the presence of junction fragments, 322/329 deletions would have been detected (e.g., 97.9%).

The nine reaction products range in size from 113 to 535 bp and can all be resolved on a single gel (Fig. 1A). The lack of amplification in a patient with complete deletion of the dystrophin gene (Hoffman et al. 1989b) confirms that these are specific products of the dystrophin locus as well as serving as a negative control to ensure that there is no contamination. Examination of DNA from patients with known partial deletions has further confirmed that the absence of a given PCR product is always correlated with deletions detected by Southern blotting (not shown). Finally, we have never detected any apparent deletions in individuals who do not have neuromuscular disease.

Several recent studies have shown a high degree of correlation (> 95%) between clinical severity and the effect of deletions on the translational reading frame for patients with deletions in the hot spot region of exons 44–53 (Koenig et al. 1989; Gillard et al. 1989). Since approximately 75% of deletions are in this region of the gene, prognostic determinations would be possible for many patients if the deletion endpoints could be determined. Figure 1B illustrates such an analysis using both our and Chamberlain's primer mixes plus primers for exon 49.

There are several potential artifacts and pitfalls to the use of these multiplex PCR reactions. It is very important to remove all the NaCl after ethanol precipitating the purified primers. If each pair works independently but the mixture of nine pairs does not, a simple ethanol reprecipitation in 0.3 *M* sodium acetate is usually sufficient to allow multiplexing of these primers. Because the target sequences are small, PCR will often work on DNA that is too degraded for Southern blotting, although in these cases, the larger sized products may appear fainter than smaller ones (Fig. 1A, lane 5). Additionally, suboptimal DNA or amplification conditions may also result in larger products not being amplified while medium-sized ones may be fainter then usual and this may be accompanied by unusually large amounts of "primer dimer." These conditions are usually apparent because the "missing exons" do not form a contiguous deletion. Furthermore, correlation of results from both primer sets may also serve as an internal control. Finally, it is essential to run positive (e.g., normal male) and negative (e.g., no DNA) controls with each set of reactions to ensure that all primers are active and that no contamination has occurred. In rare cases where the entire dystrophin gene is apparently deleted, an internal control of primers for another locus is necessary to ensure that the DNA can be amplified.

The ability to perform deletion analysis by PCR has tremendous advantages over traditional Southern blot based techniques. Rapid DNA isolation protocols (Kawasaki 1990) designed for PCR analysis can save considerable time: a complete analysis can be done with as little as 1 μg of DNA (or 100 μl of blood) in only 1 or 2 days. These attributes make PCR ideal for prenatal diagnosis where time and sample quantity may be at a premium. The new primer sequences presented here bring the sensitivity of these tests up to 98%, thus virtually eliminating the need for Southern blotting for the 65% of patients with deletions. Because of the robustness of PCR, studies can even be done on fixed and embedded specimens such as might be available for deceased patients who have living relatives at-risk for DMD/BMD. In our experience, we have correctly identified duplications in three males, and deletions in seven carrier females simply by visual inspection of relative band intensities. However, for this analysis, the quality of the PCR reactions must be optimal with none of the artifacts described above.

Deletion detection provides accurate information for genetic counseling for the 65% of DMD/BMD patients with deletions. For the remaining families, linkage analysis using polymorphic markers is still necessary. Recently, PCR-based assays have become available for sev-

eral dystrophin polymorphisms (Roberts et al. 1989; Beggs and Kunkel 1990b) and continuing development of assays for new polymorphisms should soon allow DNA diagnosis to be performed entirely by PCR.

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