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PCR Analysis of Dystrophin Gene Mutation and Expression

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Abstract Duchenne muscular dystrophy (DMD) is caused by mutations that impair normal production of dystrophin in muscle and brain tissues. The dystrophin gene is expressed at extremely low levels in both humans and mice, which makes analysis of the 14kb mRNA a difficult task. In addition, 30% of all cases of DMD (and the genetic lesion in all three known *mdx* mouse models for DMD) are thought to arise from single base mutations, yet methods are not available to routinely identify and analyze these mutations and their effects on disease progression. We have been using the polymerase chain reaction (PCR) to analyze the expression of the murine dystrophin gene. A simple assay is described that distinguishes the murine dystrophin transcripts expressed from either the muscle or brain promoter. In addition, amplification of overlapping segments from the 5′ end of the murine transcript has enabled the identification of DNA sequence variations between wild-type and *mdx* mice. These results demonstrate that the mutation in the original strain of *mdx* mice is distinct from those in two newer *mdx* isolates and that three independently isolated *mdx* mutants are available for study of DMD.

Key words: *mdx* mice, Duchenne muscular dystrophy, chemical cleavage, polymerase chain reaction

Duchenne muscular dystrophy (DMD) is among the most common human genetic diseases and results from mutations that lead to abnormal expression of the protein dystrophin [1]. Approximately 60–70% of patients display major rearrangements of the dystrophin gene, while the remaining cases are presumed to result from point mutations. Dystrophin mRNA has been estimated to represent between 0.001–0.01% of the total mRNA in skeletal and cardiac muscle [2,3]. This low-level expression combined with the large (13.9Kb) size of the mRNA make analysis via northern blots of limited value. Quantitative analysis of the expression of this mRNA has been made in skeletal muscle and brain using the RNase A protection technique, and those studies indicate that dystrophin mRNA is approximately 10-fold more abundant in skeletal muscle than in brain and is reduced by approximately 80–90% from wild-type levels in tissues of the three known strains of the mouse DMD model, *mdx* [4–6]. However RNAse A protection can be a cumbersome and tedious technique for analyzing mRNA expression and is of limited value for DNA sequence variation analysis due to the inability to detect more than approximately 50% of all single nucleotide substitutions [7]. We have, therefore, applied PCR to analyze the expression pattern of this transcript and have used the amplified transcripts to search for sequence variations. This report describes the use of these methods to analyze the 5′ end of the murine dystrophin gene.

MATERIALS AND METHODS

Polymerase Chain Reaction

PCR amplifications were performed on a Perkin Elmer automatic thermocycler, in 50 μl reactions containing 200 μM dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl, 2 units *Taq* polymerase, 0.2 μM each primer, and first stand cDNA prepared from 100 ng of total RNA. Amplification was for 25–35 cycles using the step-cycle function. Conditions utilized were: an initial denaturation at 94°C for 5 min, followed by annealing for 30 s (usually at 62°C, but this temperature was optimized for each primer pair if necessary), exten-

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sion at 72°C for 1 min/1kb of sequence length, and denaturation at 94°C for 30 s. The extension time was quadrupled on the final cycle. Isolation of RNA, DNA sequence analysis, and cDNA synthesis were performed as described [8].

Primer and cDNA Sequences

PCR primers were synthesized on an Applied Biosystems model 380B DNA Synthesizer and were used following deprotection without purification. Primers were dissolved in H_2O at a concentration of approximately 250 μM and stored at −70°C. Working stocks were diluted to 10 μM with H_2O and stored at 4°C for up to six months. Primers were synthesized to correspond to the murine dystrophin cDNA sequence and were approximately 25 bases in length, with G/C base compositions between 40% and 60%. The complete murine dystrophin cDNA sequence will be reported elsewhere [3]. PCR primers were designed to enable overlapping amplification of various segments of the murine dystrophin gene. The segments are defined by the overlapping regions amplified with the following PCR primer pairs (sequences are listed 5′–3′). Region 1A (exons 1 through 7): 5′ primer brain exon 1, AAACA GCTGG CATGG AAGAT GAAAGA; 5′ primer muscle exon 1, TGGGA AGAAG TAGAG GACTG TTATG; 3′ primer (in exon 7), GAAGG CATGT TCCAG TCTTT GGGTG. These primer pairs amplify a 606bp fragment [muscle transcript] or a 587 bp fragment [brain transcript]. Region 1b (mRNA bases 727 to 1806): 5′ primer, GAATG CTCTT ATCCA TAGTC ACAGG; 3′ primer, GCTGT TGCAT GATCA CCGCT GGATTC. Region 2 (bases 1792 to 2621): 5′ primer, TGATC ATGCA ACAGC TGCTT TGGAAG; 3′ primer, CAGCA TTAAC ACCCT CATTT GCCATC. Region 3 (bases 2618 to 3433): 5′ primer, GCTGA AAGTA TCAGA CAAGC TTCAG; 3′ primer, AACAT CAACT TCAGC CATCC ATTTC. C) Region 4 (bases 3281 to 4340): 5′ primer: CTGTC AGAAT TTGAA GAGAT TGAGG; 3′ primer, CAGAC TGGAT ACTCT GTTCA AGCAAC. Region 5 (bases 4199 to 5210): 5′ primer, TATTG GCACA GACTC TTACA GATGG; 3′ primer, CTACT CTGGA GGTGA CAGCT ATCCA. Primers to amplify mouse exon 23 (site of original *mdx* mutation): 5′ primer, GCTCT GCAAA GTTCT TTGAA AGAGC; 3′ primer, CTGGC ATATT TCTGA AGGTG CTTTC. ASO probes for original *mdx* mutation: wild-type probe, AGAGC AACAA AATGG; mutant sequence probe, AGAGC AATAA AATGG. ASO hybridizations were performed in 5X SSPE at 42°C, with 0.5% Sodium Dodecyl Sulphate (SDS), and 0.1 mg/ml herring sperm DNA. Washes were performed at 42°C in 2X SSPE plus 0.1% SDS.

RESULTS AND DISCUSSION

Sequence analysis of the murine dystrophin mRNA has enabled the synthesis of PCR primers for amplification of the transcript from various tissues. Amplification uses reverse transcribed total RNA as a template and requires as little as 100 ng of cDNA. We have observed that efficient amplification of this rare mRNA necessitates that the amplified region be less than about 1.5 kb in length. Therefore, a series of PCR primers were synthesized that each amplify short, overlapping segments of the transcript. These amplified transcripts can either be sized directly to examine mRNA splicing or utilized as templates for mutation detection. The sequence of the PCR primers is listed in Methods, as is a description of the mRNA segments amplified by each primer set.

Analysis of Dystrophin Expression During Development

Figure 1 demonstrates that dystrophin expression can be detected in both muscle and brain tissues as early as mouse embryonic day 14, at which time the transcript is easily amplifiable to visible (and clonable) quantities. These experiments utilized PCR primers specific for region 2 of the mRNA (see Methods), and these primers amplify an identical region of the transcript in both tissue types. Similar levels of product were obtained with muscle and brain RNAs isolated from 14 day mouse embryos (Fig. 1), although a greater yield of the PCR product was consistently obtained from the muscle samples at later stages of development. The accumulation of dystrophin mRNA increased throughout development until approximately 2 weeks following birth (Fig. 1 and unpublished observations). In contrast, Northern analysis failed to detect dystrophin accumulation in the embryonic brain and was unable to reveal relative variations in the mRNA levels during development due to the extreme low level of expression [2]. Dystrophin mRNA levels in the adult brain and in *mdx* skeletal muscle are at the lower limit of detection via Northern analysis, and the mRNA ap-

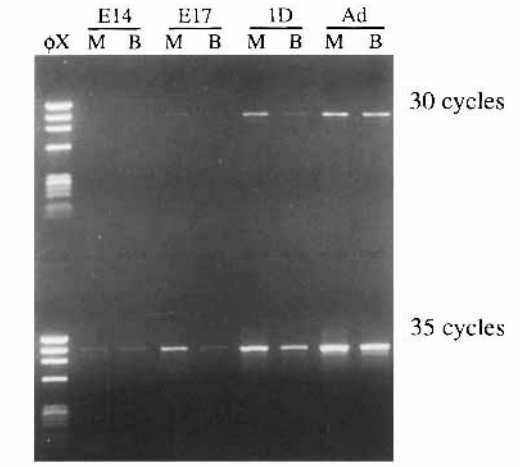

Fig. 1. PCR amplification of first strand cDNA prepared from C57B1/10 mouse skeletal muscle **(M)**, or brain **(B)** total RNA. **E14, E17, 1D,** and **Ad** refer to PCR templates prepared from 14-day or 17-day embryos, or neonatal or adult animals, respectively. Shown is a photograph of a 1.2% agarose gel through which 5 μl of the 50 μl PCR reaction was electrophoresed. The PCR templates were cDNA prepared from 100 ng of total RNA and were amplified for 30 cycles (top) or for 35 cycles (bottom), using the PCR primers for region 2 of the mRNA (see Methods). The size standard is Hae III-digested φX174.

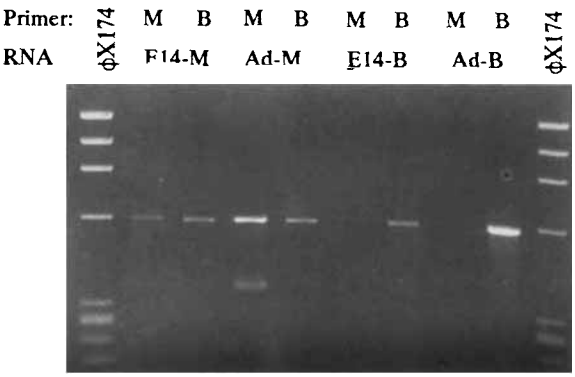

Fig. 2. PCR amplification of first strand cDNA prepared from C57B1/10 mouse skeletal muscle **(M)**, or brain **(B)** total RNA. In each case the PCR reaction utilized a common primer in exon 7, and an exon 1 primer corresponding to the muscle exon 1 (M) or the brain exon 1 (B). Symbols and PCR conditions were as in Figure 1, except that PCR was for 35 cycles.

pears to be the same size in all tissues from normal and mutant *mdx* mice [4,9]. Subtle size differences that arise from alternatively spliced exons or from point mutations that lead to exon skipping would not be revealed by Northern analysis of this 13.9 kb transcript. However, PCR amplification of dystrophin transcripts from all three strains of *mdx* mice demonstrated that the the transcripts are of normal size through the 5′ 5.2 kb of the mRNA (data not shown).

It has previously been reported that the rat and human dystrophin genes utilize a separate promoter in muscle and brain tissue [10,11]. Figure 2 shows the results of an analysis of the 5′ end of the murine transcript. For this experiment, a PCR primer corresponding to sequences within the rat brain exon 1 was used to amplify mouse transcripts from muscle and brain tissues at various stages of development. The results demonstrate that two first exons are also utilized in the mouse and that these exons are expressed in both skeletal muscle and in brain tissue. Dissection of the mouse embryos was performed using a dissecting microscope, and care was taken to avoid non-targeted tissues. It remains a possibility that small amounts of non-muscle tissues were isolated with the muscle from the embryonic samples, although pure tissue types were easily isolated from adult animals. Furthermore, the muscle transcript could not be efficiently amplified from kidney or spleen, indicating that vascular smooth muscle was not the source of the muscle transcript in brain tissue. These results indicate that the transcripts are not expressed as true tissue specific isoforms, although they are each predominantly expressed in either muscle or brain tissues. Similar results were obtained with human tissues [12].

Identification of DNA Sequence Variations in Dystrophin Transcripts

Mutations that affect expression of dystrophin will be detectable by analysis of the mRNA, with the exception of promoter mutations. We have been exploring the use of a scanning technique to identify point mutations in the 2.5 million base pair dystrophin gene. The original *mdx* isolate has already been shown to contain a point mutation (a premature stop codon; [13]), and Northern, Southern, and RNase A protection analyses indicate the newer mutants (designated 467 and 551, [5]) also are likely to contain coding region point mutations [4,9]. To identify these lesions we have amplified dystrophin mRNA from wild-type mice (C57B1/10) and from each of the three *mdx* mutants and have used these amplified transcripts for heteroduplex analysis to detect sequence variations.

Our initial trials of these methods have focused upon the 5′ end of the mRNA. The procedure involves isolation of total RNA from appro-

priate tissues of each mouse strain, copying it into single-stranded cDNA, and amplifying via 35 cycles of PCR (see Methods for primer sequences and PCR conditions). The amplified cDNA is excised from an agarose gel and used for heteroduplex analysis. In this case we have incorporated ^{32}P into the wild-type transcripts, which are then annealed with a 10- to 15-fold excess of mutant transcript. Following chemical mismatch cleavage analysis [14], the heteroduplexes are analyzed on a sequencing type acrylamide gel to detect cleavage products. Cleavage of a heteroduplex into smaller fragments indicates the presence of a base mismatch, whose location can be inferred from the size of the products observed on the gel. A similar approach has been used to identify point mutations in the human ornithine transcarbamylase gene [15]. Chemical cleavage is capable of detecting virtually 100% of all possible DNA sequence mismatches in a heteroduplex, and this approach, therefore, should enable detection of point mutations in a dystrophin gene mRNA [1].

To date we have identified two DNA sequence variations in the murine dystrophin gene with this approach. The first is located at the 5′ end of the mouse mRNA and resulted in a cleavage product being detected with heteroduplexes formed between amplified transcripts from wild-type and the two new *mdx* mutants, 467 and 551 (data not shown). Sequence analysis of the PCR products obtained from wild-type and mutant mice revealed that the cleavage product resulted from a C to T sequence change in mouse dystrophin exon 7 (position 835 of the cDNA sequence [data not shown]; the sequence was confirmed on multiple independent subclones of the PCR product). This base change is present in both the new strains of *mdx* mice, but does not change the encoded amino acid (cysteine). This sequence variation is therefore a polymorphism and not the mutation in the newer *mdx* strains. However, this nucleotide substitution can be used to distinguish the wild-type from the *mdx* 467 and 551 transcripts and provides a convenient assay to distinguish the newer *mdx* isolates from the original mutant.

The second region of sequence divergence was within segment 4 of the mRNA (see Methods). Heteroduplexes formed between amplified dystrophin mRNA from the original *mdx* mouse and wild-type transcript produced a cleavage product after treatment with hydroxylamine, resulting in two fragments of 586 and 230 base pairs (data not shown). The size of the products obtained with the original *mdx* isolate suggested that the previously reported *mdx* mutation was the source of the base mismatch that led to heteroduplex cleavage. To confirm that this was the mutation, we analyzed the amplified transcripts obtained from the wild-type and the *mdx* mice via hybridization with allele specific oligonucleotide (ASO) probes corresponding to the wild-type [3] and the original *mdx* mouse sequences [13]. For this experiment the PCR products obtained from amplification of region 4 of murine dystrophin mRNA from a wild-type mouse, two separate *mdx* animals, and *mdx* 467 and 551 animals were electrophoresed on an agarose gel and examined via Southern analysis. Figure 3 displays two autoradiograms obtained from sequential hybridization of the PCR products with 15 base ASO probes. These results confirm the sequence of the reported *mdx* mutation and demonstrate that the newer mutants contain independently isolated mutations and are not simply new or re-isolates of the original mutation. This strategy represents a simple assay whereby the original *mdx* mutant can be distinguished from the newer alleles and enables the genotype of the mutant mice to be definitively distinguished from the control animals. This latter point is of importance in that the *mdx* mice have a very mild phenotype and it can be quite difficult to distinguish the mutant from the control animals. PCR amplification and ASO hybridization also provide a way to monitor the tissue specific expression of an exog-

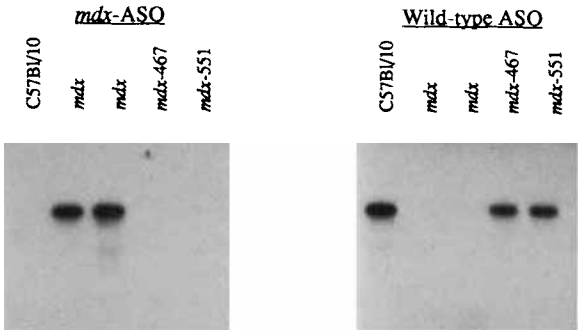

Fig. 3. Genotyping of the dystrophin gene in different strains of mice. Region 3 of the dystrophin mRNA was amplified via 30 cycles of PCR, and approximately 10 ng of amplified transcript was separated on an agarose gel and transferred to a nylon membrane. The membrane was then sequentially hybridized with oligonucleotides corresponding to the wild-type or *mdx* mutant sequence. The results confirm the reported sequence of the *mdx* mutation and demonstrate that the two new *mdx* alleles are derived independently from the original mutant.

enous dystrophin gene introduced into *mdx* animals either by gene transfection or by myoblast transplantation. Although the assay shown in Figure 3 utilized RNA (copied into cDNA) as the template for amplification, we have also successfully amplified and genotyped the mutant region using genomic DNA. The sequence of the PCR primers used for genomic DNA amplification are listed in the Materials sections.

These results demonstrate that a scanning technique can be applied to detect DNA sequence variations in a large gene expressed at very low levels. Although the major murine dystrophin mRNA is 13.9 kb, such a length of DNA can be scanned with approximately 10–14 separate PCR reactions, using the method of chemical mismatch cleavage. Such an approach should enable the detection of virtually any point mutation in the > 2 million base pair dystrophin gene, without necessitating examination of the intron sequences of the gene. These methods should be of value in analyzing the expression of wild-type dystrophin genes introduced into a mutant background and will assist in analyzing the effects of myoblast transplantation and gene therapy treatments for DMD.

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