Detection of Carrier Status in Duchenne and Becker Muscular Dystrophies by Quantitative Polymerase Chain Reaction and Allele-Specific Oligonucleotides

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Detection of carriers of Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD), in the deletion cases, involves calculating gene dosage from Southern blots. We show that the analysis of dosage can also be made from the polymerase chain reaction (PCR) with use of allele-specific oligonucleotides (ASOs). The deletion-prone exons are amplified, transferred to a membrane, and hybridized with ASOs complementary to the exons; the autoradiographic bands are then quantified with a densitometer. After determining the quantitative conditions of the amplification reaction, we were able to identify deletions in a DMD/BMD carrier female. The determination of carrier status via PCR removes several of the technical limitations of Southern analysis and is also cost- and labor-effective.

Additional Keyphrases: gene probes · heritable disorders

Duchenne muscular dystrophy (DMD) is an X-linked recessive progressive muscle-wasting genetic disorder that affects ~1 of 3500 to 5000 newborn males (1).2 Both DMD and the milder allelic Becker muscular dystrophy (BMD) result from mutations in the dystrophin gene. The 14-kb coding sequence of the dystrophin gene has been cloned (2). Use of cDNA probes has shown that molecular deletions account for ~65% of the mutations in DMD and BMD (3–6). Carrier detection in the deletion cases involves assessing gene dosage by quantitative Southern-blot analysis, whereby one determines whether the female at risk exhibits no reduction (noncarrier status) or 50% reduction (carrier status) in hybridization intensity in those bands that are deleted in the affected male (7). The dosage determinations permit direct analysis for carrier status and eliminate the inherent problems of the restriction-fragment-length-polymorphism technique (e.g., recombinations, noninformative meioses, unavailability of family members, and sporadic mutations). To further increase the accuracy of the dosage analysis, we quantify the autoradiographic bands by scanning with a densitometer (8).

Although dosage analysis has significantly improved carrier studies, particularly in the isolated cases of the disease, there are technical limitations. Dosage analysis of Southern blots requires optimal conditions; very good quality blots are necessary, with uniform transfer and hybrid-

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2 Nonstandard abbreviations: DMD, Duchenne muscular dystrophy; BMD, Becker muscular dystrophy; PCR, polymerase chain reaction; ASO, allele-specific oligonucleotide; and SDS, sodium dodecyl sulfate.

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ization and low background. To obtain this high quality, we have found that ~20% of the Southern blots have to be repeated, increasing time and labor. Rather than directly comparing single bands, band ratios are calculated as a means of decreasing the error caused by differences in the amount of DNA in each lane. The normal control ratio is established by comparing in an unaffected female a band lacking in the patient against a band present in the patient (which serves as an internal control). A ratio in a female (at risk) that is approximately half the control ratio indicates that she has a single copy of the band deleted in the patient and therefore is a carrier.

Depending on the extent of the deletion, the restriction fragments involved in the deletion, and the specific cDNA probe that identifies the deletion, one may be extremely limited as to what bands can be used in the control ratio. We have found that bands >10 kb and <0.5 kb typically result in weaker intensities and are not always adequate for scanning purposes. Mao and Cremer (9) recently stated that, for deletions in the center of the gene (cDNA 8 hybridizations), they prefer to make a statement regarding carrier status only if at least one of the strong hybridizing fragments (7, 3.8, 3.7, or 3.1 kb) is deleted in the patient. Furthermore, the difference between one or two copies is relatively easy to detect but differences between two and three copies, or sometimes three or four copies, in the case of a duplication or comigrating bands can be very difficult. Finally, because of the extent of a deletion in an affected individual, no hybridizing bands may be detected with a cDNA probe, so that comparison of hybridization bands within a lane is not possible in these cases.

Given these limitations, we investigated the possibility of using quantitative polymerase chain reaction (PCR) to determine dosage (10). After identifying the deletion in the affected individual, specific DMD exons of the female relative are amplified (11), hybridized with allele-specific oligonucleotides (ASOs) and used in the dosage ratio calculation. The purpose of this paper is to evaluate the technique and illustrate its application through an actual case study.

Materials and Methods

Blood specimens were collected from patients with DMD or BMD at the Ohio State University Hospital. Diagnosis was based on clinical symptomology, above-normal activities of CK, and muscle histology. Genomic DNA was extracted by a salting-out procedure (12) from leukocytes harvested from whole blood anticoagulated with EDTA. DNA concentrations were determined with a spectrophotometer and by monitoring the intensity of ethidium bromide staining on a test gel.

Amplification was accomplished in the presence of 150 ng of each oligonucleotide primer and 2.5 U of Taq polymerase (Cetus-Perkin Elmer, Norwalk, CT) in a final volume of 100 μL in the following solution: 0.5 mmol of deoxynucleotide triphosphates, 3 mmol of MgCl2, 67 mmol of Tris (pH 8.8), 16.6 mmol of ammonium sulfate, 6.7 μmol of EDTA, and 10 mmol of 2-mercaptoethanol per liter. The DMD primer sequences have been previously described (11). Amplification was performed at 55 °C as the annealing temperature, 72 °C as the extension temperature, and 94 °C as the denaturation temperature in a thermal cycler (Ericomp, San Diego, CA). For quantitative analysis we subjected the samples to 10–16 cycles. For visual deletion analysis, via ethidium-bromide-stained gel, amplification was carried out for 30 cycles. Optimal resolution was obtained by electrophoresing 20 μL of the 100-μL reaction mixtures through 25 g/L agarose gels.

The PCR products were transferred to a nylon membrane (Zetabind; CUNO, Inc., Meriden, CT) by Southern blotting with a solution of 0.5 mol of NaOH and 0.6 mol of NaCl per liter (13, 14). The filter was prehybridized at 37 °C in the following mixture: 5 × SSC (1 × SSC = NaCl, 0.15 mol/L; sodium citrate, 150 mmol/L), sodium phosphate buffer (50 mmol/L, pH 6.5), 5 × Denhardt's (Ficoll 1.0 g/L, polyvinylpyrrolidone 1.0 g/L, bovine serum albumin 1.0 g/L), and containing 1 g of sodium dodecyl sulfate (SDS) and 250 mg of yeast RNA per liter. Hybridization was performed in the same solution at 37 °C, in which two 32P-end-labeled ASOs were added, complementary to the amplified DNA sequences. The filter was washed first in 5 × SSC, followed by a second wash in 2 × SSC, 5 g/L SDS solution at 45 °C for 15–30 min. Autoradiography was performed for ~18 h at ~70 °C. Multiple exposures of each filter were made to ensure that the results on the film to be quantitated were in the linear range. The ASOs used for the detection of the dystrophin exons are described in Table 1. The degree of hybridization was assessed by using a CS-9000 scanning densitometer (Shimadzu Corp., Kyoto, Japan). The area under the peak represents the intensity of each hybridization band.

Results

Analytical Performance

Range: Because the extension product of each primer serves as a template for the other primer, each cycle essentially doubles the amount of the DNA product produced in the previous PCR cycle. This results in the exponential accumulation of the specific fragment, up to several millionfold in a few hours. To obtain quantitative results, one must measure the PCR products during the exponential phase of the amplification process. This occurs when the primers, nucleotides, and Taq polymerase are in a large excess so that the template to be quantitated is amplified in each cycle. In our experience, after the number of cycles (25–30) adequate to make visible the PCR products on an ethidium-bromide-stained gel is complete, the PCR reaction is no longer in the quantitative range. Therefore, one must determine the appropriate number of PCR cycles for each primer set.

To do this, we amplified exons 8 and 19 from a series of identical samples, containing 1 μg of DNA, from eight to 16 cycles. We removed 20-μL aliquots of amplified DNA samples from different cycle numbers, ran these on an agarose gel, Southern blotted, hybridized with the corresponding ASOs, and scanned with the densitometer. The autoradiograph and a graph displaying densitometric band intensity vs the cycle number are shown Figure 1. Linearity was maintained through 15 cycles but the curve started to plateau slightly at cycle 16. The 8/19 exon ratio remained constant over the entire range of cycles (CV = 6.2%).

Table 1. Description of DMD Allele-Specific Oligonucleotides Used

<table>
<thead>
<tr>
<th>Exon</th>
<th>Sequence (5'–3')*</th>
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<tbody>
<tr>
<td>ATCCAGGAAGTGAAATGTTG</td>
<td>8</td>
</tr>
<tr>
<td>TTCAAAAACTGCAAAGTGCC</td>
<td>19</td>
</tr>
<tr>
<td>GAAATAATCCAGCAATCTCA</td>
<td>45</td>
</tr>
<tr>
<td>AAGGTGGAAGACCTGTGAAG</td>
<td>48</td>
</tr>
</tbody>
</table>
*The sequences are derived from reference 16.
linearity was observed for all dystrophin exons amplified over a range of 8–15 cycles.

Sensitivity: We amplified a dilution series for several of the DMD exons to establish the relation between input target DNA and hybridization intensity. Densitometric scans showed a linear relation between input DNA (which ranged from 0.5 to 3.0 µg) and hybridization signal for several dystrophin exons. However, the signal produced is a function not only of the amount of DNA, but also of the exposure time and the specific activity of the ASO. Because these factors result in proportional changes in the band intensities, quantification of the ratios has been shown to be maintained over a wide range of exposure times (depending on the age of the isotope). The results for exon 45 are shown in Figure 2. We determined that the products resulting from the PCR were proportional to the input target DNA over a range of input concentrations of target sequence. This is important, given the variations in the starting DNA concentrations that may occur between patients and controls.

Precision: Within-run precision of the dosage ratio was calculated from nine PCR amplifications and ASO hybridizations of DMD exons 8 and 48 (Figure 3). The autoradiogram was scanned and we determined the CV to be 15%. Calculated CVs from other dosage ratios have ranged from 5% to 20%. We have observed similar values for precision when scanning from Southern blots. Although the results show some imprecision in the PCR dosage ratios, the precision is adequate for distinguishing hemizygous (half band intensity) from the homozygous samples (normal band intensity). We have not observed overlap between the ratios for normal controls and DMD carriers. However, as a means of reducing any possible overlap and ensuring the accuracy of the dosage analysis, we commonly amplify several appropriate exons and use multiple ratios as an internal confirmation of the results. This is a distinct advantage of performing dosage from PCR hybridizations because, given the extent of the deletion and the specific cDNA hybridization, one often cannot use multiple ratios when performing dosage from Southern blots.

Case Study

Southern-blot analysis of DNA from a DMD patient showed a large molecular deletion for exons 10–41 of the DMD gene. Figure 4 shows a photograph of an ethidium-bromide-stained gel after amplification of exon 8 and 19 in

Fig. 1. Effect of cycle number on the amplification of DMD exons 8 and 19
Autoradiograms of exon 8 and 19 ASO hybridizations (A); exon 8 (B) (r = 0.99), and exon 19 (C) (r = 0.99)

Fig. 2. Relationship between PCR amplification and DNA concentration of exon 45 (cycle number = 12)
Autoradiograms of exon 45 hybridization (A); exon 45 (B) (r = 0.99)

Fig. 3. Precision of the PCR amplification: autoradiogram of nine replicate PCR amplifications/ASO hybridizations of exons 8 and 48

Fig. 4. Multiplex DNA amplification of exons 8 and 19
Shown is a 25 g/L ethidium-bromide-stained agarose gel, through which 20 µL of each 100-µL amplification reaction is run. Amplification products are in base pairs (bp). Lane 1: molecular mass markers. Lane 2: blank. Lane 3: nonaffected control. Lane 4: proband. Lane 5: mother
the patient, his mother, and an unaffected control. Although there was no family history of DMD, the mother had above-normal concentrations of creatine kinase (EC 2.7.3.2) on three separate occasions, strongly suggesting positive carrier status. As shown, exon 19 is clearly deleted for the child, but it is difficult to see whether the mother is also carrying the deletion. To accumulate viable PCR products, it is usually necessary to amplify for at least 20 cycles. However, because the PCR reaction plateaus at high cycle numbers, we have not been able to definitively determine carrier status from an ethidium-bromide-stained gel. Therefore, we amplified exons 8 and 19 in the mother, proband, and a normal control for 12 cycles, hybridized them with the corresponding ASOs, and scanned the autoradiograms (Figure 5). The 19/8 exon ratio in the mother is 0.3, approximately half the normal control ratio (0.6). Therefore, we conclude that the mother is carrying the deletion and is positive for DMD carrier status, thus supporting her above-normal creatine kinase results. We have found the quantitative PCR technique to be extremely reliable, because all family carrier determinations (10 families during this study) have subsequently been confirmed by Southern dosage analysis. We elected to show a case in which the mother was positive for DMD carrier status; however, the results of Southern blots and quantitative PCRs have been in agreement for noncarriers also.

Discussion

There being at present no cures for DMD or BMD, the accurate identification of carriers is of the utmost importance. The determination of carrier status via PCR has several advantages. The amplification of only two specific exons reduces the background problems often present on Southern blots. We have not had to repeat any of the ASO hybridizations for high background or unresolvable autoradiographic bands, unlike our experience with the Southern analysis. Furthermore, this assay requires less DNA, can be performed more rapidly than Southern analysis, and is both cost- and labor-effective. However, for reliable quantification of the amount of DNA, the range of concentrations of template and the number of amplification cycles must be determined such that they stay within the exponential phase of the PCR. Therefore, we had to define the range of concentrations that gave an exponential amplification over a defined range of cycle numbers. The conditions were relatively liberal, and the PCR reaction could be accurately quantified over a range of cycle numbers and starting DNA template concentrations. Moreover, as demonstrated, the assay could clearly differentiate the carrier state in a DMD carrier mother.

Deletions have been shown to account for ~65% of the DMD and BMD cases. Because most of the deletions (~98%) can be identified with the multiplex PCR (15), one can now also perform carrier studies by using PCR amplification. Upon determining the extent of the deletion in affected subjects, one selects the specific exons (an exon that is deleted and an exon that is present in those affected) and performs amplification and ASO hybridizations in the females at risk and in controls. Although we have utilized only four ASOs in this report, the sequence of the cDNA has been published (16) and one can use any number of ASOs derived from exon sequences. We therefore recommend using multiple ratios as an internal control for carrier studies. We have found the method to be reliable and are currently using it for all carrier studies for which the affected male has a deletion.

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References
Determination of Reference Values for Serotonin Concentration in Platelets of Healthy Newborns, Children, Adults, and Elderly Subjects by HPLC with Electrochemical Detection

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We adapted a high-performance liquid chromatographic method with electrochemical detection (Clin Chim Acta 1984;139:1-12) to the determination of platelet-serotonin. We used this method to determine platelet serotonin reference values in a healthy population, measuring platelet serotonin concentration in the following subjects: 31 newborns (16 girls, 15 boys); 41 children (11 girls, 30 boys), ages 20 months to 15 years; 56 adults (26 women, 30 men), ages 20 to 58 years; and 20 elderly subjects (16 women, four men), ages 65 to 94 years. There was no significant difference in platelet serotonin concentration between sexes in each age group. However, significant changes (P < 0.001) were observed between the newborns (mean ± SD: 1.67 ± 0.74 nmol/10^6 platelets) and the children (4.09 ± 1.04) or the adults (3.81 ± 0.87). Moreover, the platelet serotonin concentration in the elderly subjects (2.57 ± 1.12) was significantly (P < 0.001) lower than in the adults and children and significantly higher (P < 0.01) than in the newborns. Such age-related differences must be taken into consideration when data from neurological or psychiatric patients and control subjects are compared.

Additional Keyphrases: age-related effects · neuroactive amines · reference interval

Studies over the past 15 years have demonstrated that blood platelets can serve as a peripheral model for the central serotonin (5-hydroxytryptamine, 5-HT) presynaptic nerve terminals because the platelets accumulate, store, and release serotonin in a manner analogous to the central serotonergic synaptosomes (1, 2).

Because blood platelets can be easily obtained, the concentration of serotonin in platelets has been extensively measured as an indirect index of central serotonergic function in various psychiatric or neurological diseases, in basal conditions (3, 4), and during treatment with various drugs known for interfering with the central uptake or release of serotonin (5).

However, interpretation of the above studies can be difficult because of the lack of reference values for platelet serotonin concentration at various ages. The aim of this work was, therefore, to determine these values in a healthy population of newborns, children, adults, and elderly subjects of both sexes.

Materials and Methods

Instrumentation

Platelets in whole blood and in platelet-rich plasma (PRP) were counted with an HPC 52 platelets counter (Hycel, Rennes, France).

The chromatographic system is described in Gagnieu et al. (6); however, we used a sample processor WISP 712, instead of a U6K injector (both from Waters Associates, Milford, MA).

Reagents

The mobile phase consisted of sodium acetate buffer (0.15 mol/L, pH 4.0), containing 150 mL of methanol and 0.5 μmol of EDTA per liter. The buffer was filtered through a 0.45-μm (pore size) membrane filter (Millipore, Bedford, MA) and degassed.

The acetic acid and sodium acetate used to prepare the mobile phase were both Normapur grade, from Prolabo (Paris, France); chromatographic-grade methanol was from Merck (Darmstadt, F.R.G.).

A reference stock solution (400 μmol/L) of 5-HT (Sigma Chemical Co., St. Louis, MO) was prepared in distilled de-ionized water and stored at 4 °C for as long as six months.

On the day of the experiment, a standard solution of 5-HT, 1 μmol/L, was prepared by diluting the stock solution in distilled water.

Samples

Sample collection: Except for the newborns who had their umbilical cord blood withdrawn at the time of the birth, blood was collected by venipuncture, between 0900 and