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Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification

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ABSTRACT

The application of recombinant DNA technology to prenatal diagnosis of many recessively inherited X-linked diseases is complicated by a high frequency of heterogenous, new mutations (1). Partial gene deletions account for more than 50% of Duchenne muscular dystrophy (DMD) lesions, and approximately one-third of all cases result from a new mutation (2-5). We report the isolation and DNA sequence of several deletion prone exons from the human DMD gene. We also describe a rapid method capable of detecting the majority of deletions in the DMD gene. This procedure utilizes simultaneous genomic DNA amplification of multiple widely separated sequences and should permit deletion scanning at any hemizygous locus. We demonstrate the application of this multiplex reaction for prenatal and postnatal diagnosis of DMD.

INTRODUCTION

DMD is among the most common human genetic diseases, occurring approximately once in every 3,500 male births (6). Although there is no cure or effective therapy, highly accurate prenatal diagnosis and carrier detection is now possible *via* Southern analysis using DMD cDNA and genomic clones (2,5,7-9; P.Ward et al., manuscript in preparation). Unfortunately, there are several major limitations that prevent widespread and routine use of Southern analysis for DMD diagnosis. Exons of this gene are located on at least 65 genomic Hind III restriction fragments (2,10), which necessitates hybridization of Southern blots with 7-9 separate cDNA subclones to resolve each exon-containing restriction fragment for diagnosis of genomic alterations. Linkage analysis for DMD patients that do not display genomic alterations requires additional Southern blots using genomic clones as relatively few restriction fragment length polymorphisms (RFLPs) have been described that are detectable with the cDNAs. Furthermore, Southern analysis is an expensive, tedious, and time consuming technique that requires the use of radioisotopes making it unsuitable for routine use in clinical laboratories.

An alternative to Southern analysis for mutation diagnosis involves the polymerase chain reaction [PCR; (11)]. In this method specific regions of a

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gene can be amplified up to a million-fold from nanogram quantities of genomic DNA and then analyzed for the presence of mutant alleles either by direct DNA sequencing or by hybridization with allele-specific oligonucleotide (ASO) probes. These techniques have proven useful in the diagnosis of several diseases including ß-thalassemia (12), hemophilia A (13), sickle-cell anemia (14), and phenylketonuria (15).

The application of PCRs to DMD diagnosis has been limited since point mutations leading to DMD have not been identified. However, partial gene deletions occur in 50-60% of DMD patients and account for virtually all of the mutations that have been directly observed. These deletions typically span several hundred kilobases of the DMD locus and generally overlap 2 specific regions located approximately 0.5 and 1.2 megabases from the promoter (2,6,8). We have exploited these observations to develop a rapid and simple technique that is able to detect approximately 70% of all DMD gene deletions. In this method sequences flanking six deletion prone exons of the gene are simultaneously amplified via PCR. Any of these regions absent from patient DNA fail to amplify and are readily identified via agarose gel electrophoresis of the reaction products. Multiplex amplification reactions should be broadly applicable to the study of a number of problems. We demonstrate here the use of this technique for prenatal and postnatal diagnosis of DMD and discuss the advantages and logistics of integrating these methods into diagnostic laboratories.

MATERIALS AND METHODS

Isolation of genomic clones

Genomic clones were isolated from a human genomic library prepared in EMBL 3 (16; kindly provided by John Weis) and from a 49XXXXY library prepared in λ DASH (Stratagene; T. Webster, unpublished). The murine *Dmd* cDNA clone XD-1 has been described previously (17). Human cDNA probes were kindly provided by Dr. Louis M. Kunkel (2). Clones were isolated and characterized essentially as described previously (18). Exons were sequenced by the dideoxy chain termination method (19) following subcloning into M13mp18 and 19 (20) or pTZ18 and 19R (Pharmacia) using vector and insert specific synthetic oligonucleotide primers.

Multiplex genomic DNA amplification

DNA was prepared from lymphoblasts, cultured amniotic fluid cells, or chorionic villus specimens using an Applied Biosystems model 340A DNA extractor. Synthetic oligonucleotide primers were prepared by phosphoramidite

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chemistry (21) on an Applied Biosystems model 380B DNA synthesizer. Primers were purified by electrophoresis on 20% polyacylamide gels, followed by electroelution and desalting on a NENsorb column (Dupont). We have not attempted multiplex amplification with unpurified primers although we have recently observed that purification is not always necessary when amplifying a single sequence. Reaction conditions were modified from Kogan et al (13) to permit multiplex amplification as follows. Primers (1μM each final concentration) were annealed at 55° for 45 sec, 210 sec extension times and 10 units Taq polymerase (Cetus) were used, 500ng DNA was added as template, and amplification was performed for 25 cycles (except as indicated) on a Perkin-Elmer/Cetus automatic thermocycler using the 'step-cycle' function. On the final cycle extensions were performed for ten min. Reaction products can be stored at 4° for up to two months prior to analysis on agarose gels. Optimal resolution was obtained by electrophoresing 15μl of the 100μl reactions through 1.4% agarose gels at 3.7V/cm for 100 min. Reaction mixes containing everything except template and enzyme appear quite stable at -70°, although we have not attempted storage for longer than one month. Manual amplifications using glycerin filled heat blocks required slightly different conditions. Those reactions were annealed at 47° for 30 sec., and extended for 180 sec.

RESULTS

Isolation of DMD genomic clones

Development of DNA amplification based diagnostic methods required the isolation of genomic clones from deletion prone regions of the DMD gene. Since this gene is extremely large [> 2Mb (2, 22-24)] and contains at least 60 exons, we sought to direct our library screening by using cDNA subclones known to hybridize with exons frequently deleted in DMD patients. Three cDNA probes were used to screen two human genomic libraries, and four of the isolated clones were used in the present study. Exon 17 of the DMD gene was isolated using the murine DMD cDNA XD-1 (17), which hybridizes with the DXS 164 (pERT 87) locus (2). Two additional exons were isolated with the human DMD cDNA probe 7 (2). As the complete exon/intron structure of the DMD gene is not yet known, it is not clear how many additional exons lie 5′ of these two. These exons are within the 4.1 kb and 0.5 kb HindIII fragments detectable with cDNA probe 7 (2). Comparison of these exon sequences with the human DMD cDNA sequence (25) reveals that the 4.1 kb fragment lies 5′ of the 0.5 kb HindIII fragment, and that these are adjacent exons on the reported DMD cDNA. The fourth region cloned for these studies was isolated with human DMD cDNA probe

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A

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B

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C

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D

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Figure 1: Nucleotide sequence of four DMD gene exons and flanking introns. Exon sequences are in upper case, and intron sequences are in lower case. Arrows indicate the location and 5′ - 3′ orientation of primers used for amplification reactions. Forward arrows are above the sequence to which they correspond, reverse arrows are below the sequence to which they are complementary (Table 1). (A) exon 17; EMBL/Genbank accession # X13045. (B) 4.1 kb HindIII fragment exon detectable with cDNA probe 7 (2); EMBL/Genbank accession # 13046. (C) 0.5 kb HindIII fragment exon detectable with probe 7; EMBL/Genbank accession # X13048. (D) 1.2/3.8 kb HindIII fragments exon detectable with probe 8 (2); EMBL/Genbank accession # X13047. The nucleotide differing from the reported cDNA sequence (25) is underlined. All splice sites have been confirmed by sequencing the exons both 5′ and 3′ of the ones shown (JSC unpublished).

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Table 1. Summary of DMD gene multiplex amplification primer sets.

Exon and Size[1]	Primer Sequence[2]	Amplified[3]	Deleted[4]
A. Exon 8 182bp; (probe 1b)	F- GTCCTTTACACACTTTACCTGTTGAG R- GGCCTCATTCTCATGTTCTAATTAG	360 bp	11.3%
B. Exon17 178bp; (probe 3)	F- GACTTTCGATGTTGAGATTACTTTCCC R- AAGCTTGAGATGCTCTCACCTTTTCC	416 bp	9.4%
C. Exon 19 88bp; (probe 3)	F- TTCTACCACATCCCATTTTCTTCCA R- GATGGCAAAAGTGTTGAGAAAAAGTC	459 bp	10.3%
D. 4.1Kb HindIII 148bp; (probe 7)	F- CTTGATCCATATGCTTTTACCTGCA R- TCCATCACCCTTCAGAACCTGATCT	268 bp	4.0%
E. 0.5Kb HindIII 176bp; (probe 7)	F- AAACATGGAACATCCTTGTGGGGAC R- CATTCCTATTAGATCTGTCGCCCTAC	547 bp	8.4%
F. 1.2/3.8Kb HindIII 186bp; (probe 8)	F- TTGAATACATTGGTTAAATCCCAACATG R- CCTGAATAAAGTCTTCCTTACCACAC	506 bp	18.2%
		Total[5]:	37%

[1] Each exon is designated A,B,C,D,E, or F. When known the exon number is listed, when not known the size of the genomic Hind III fragment that the exon is located on is listed. Also shown is the human DMD cDNA probe that hybridizes with each exon (2), as well as the size of the exon in base pairs (bp).
[2] Shown is the sequence in 5'-3' orientation for the PCR primers used to amplify each region. F: forward primer, hybridizes 5' of the exon; R: reverse primer, hybridizes 3' of the exon.
[3] The size of the amplified fragment obtained with each primer set.
[4] The percentage of DMD patients analyzed that are deleted for each indicated exon. This data is derived from references 2 and 6.
[5] The percentage of DMD patients analyzed that are deleted for any of the exons A-F (2,6). This number is less than the sum of the individual exon deletion frequencies as many deletions encompass multiple exons.

8 (2). Sequence analysis revealed the exon on these clones to overlap both the 1.2 kb and 3.8 kb Hind III restriction fragments detectable with probe 8. This exon sequence contained a single base mismatch from the reported human cDNA sequence (Fig 1D, underlined) which results in an amino acid change from glutamine to lysine. It is not yet known whether this represents a cloning artifact or a polymorphism. Figure 1 shows the DNA sequences of these four exons and their flanking introns.

Two previously published exon/intron sequences have also been incorporated into this study. The first is exon 8 (26) and the second is exon 19 (27). These exons are located on 7.5 kb (exon 8) and 3.0 kb (exon 19) HindIII restriction fragments detectable with cDNA probes 1b, and 3, respectively (2). Analysis of data obtained from DNA deletions that were observed in a group of over 200 DMD patients indicates that one or more of the six exons referred to above is missing from 70% of the deletions studied (2,6).

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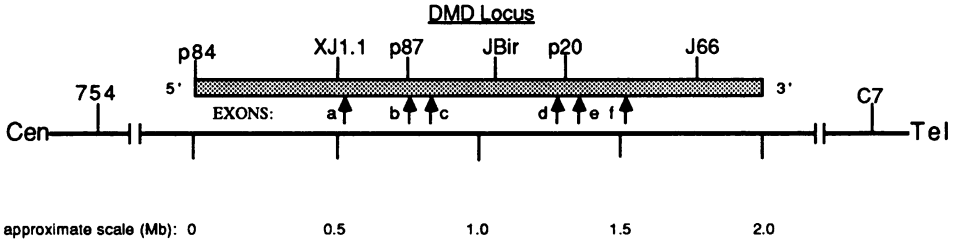

Figure 2: Schematic representation of the DMD gene. The approximate size of the locus, the position of the amplified fragments, and the location of the genomic probe markers are inferred from references 2,22-24,26,27,40,41. (a) exon 8; (b) exon 17; (c) exon 19; (d) exon from Fig. 1B; (e) exon from Fig. 1C; (f) exon from Fig. 1D.

Development of multiplex genomic DNA amplification reactions

25-28 base oligonucleotide primers were synthesized to correspond to sequences flanking these six DMD exons. Primers were chosen to overlap or lie outside of exon splice site regions to ensure that a deletion that removed a splice site but left the exon intact would be detectable. This also provided greater flexibility in choosing the size of the region to be amplified, facilitating resolution of each fragment by agarose gel electrophoresis following multiplex reactions. The location of the sequences chosen are shown in Figure 1 for the four exons cloned in this study. Table 1 shows the sequence of all 6 primer sets, the size of each exon and the amplified fragment obtained, a description of each exon, and the frequency that each has been observed to be deleted in patients studied. For the remainder of this manuscript these exons will be referred to as A,B,C,D,E, and F (Table 1). Figure 2 shows a schematic representation of the DMD locus illustrating the relative location of each of these six exons which span approximately 1 megabase of the gene.

Exon 17, the initial isolate, was used to demonstrate that deletions could be detected via PCR and that the reactions could successfully detect deletions in amniotic fluid cells (28). However, PCR amplification of single exons would require a cumbersomely large number of reactions for analysis. We therefore combined multiple primer sets in a single reaction. Addition of each extra primer set frequently required modification of primer annealing temperatures, time of annealing, polymerase extension times, and the amount of enzyme required. The conditions that permitted multiplex amplification with all six primer sets are listed in materials and methods.

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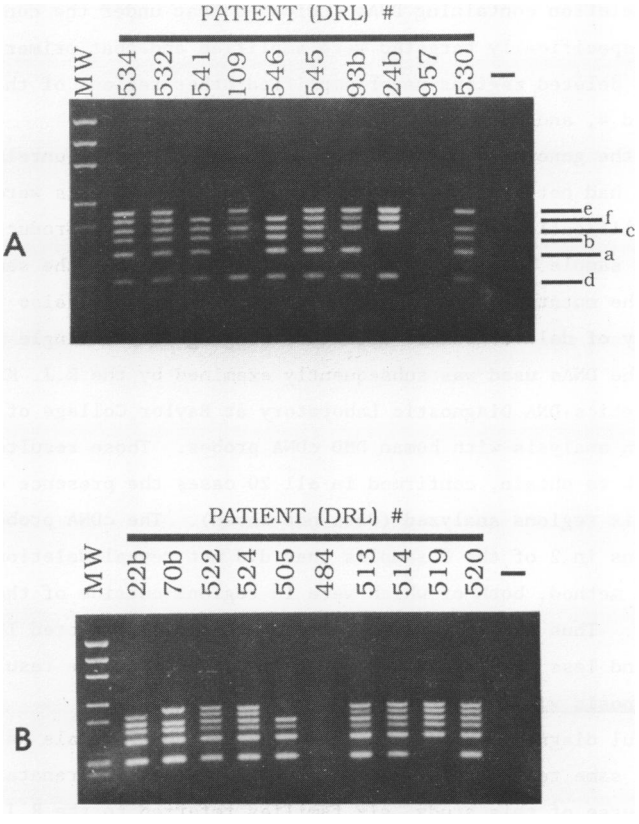

Figure 3. Multiplex DNA amplification of lymphoblast DNA from 20 unrelated male DMD patients. A and B show two sets of ten samples each. DRL # refers to the R.J. Kleberg Center for Human Genetics Diagnostic Research Laboratory family number. MW: HaeIII-digested ϕ X174 DNA. - : no template DNA added to the reaction. To the right of Panel A is indicated which amplified fragment corresponds to each region indicated in Fig. 2. Each sample was amplified as described in materials and methods then stored at 4° until analysis. Shown is an ethidium bromide stained 1.4% agarose gel through which 15μl of each 100μl reaction was electrophoresed.

Deletion detection using DNA from male DMD patients

DNA from a number of male patients containing deletions previously delineated using human cDNA probes were initially used as templates for multiplex amplifications. The results demonstrated that deletions involving any or all of the six regions assayed were detectable via multiplex amplifications and that the relative location or size of the deletions did not affect the results (data not shown). Analysis of amplification products utilizing

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control and deletion containing DNAs indicated that under the conditions used only regions specifically targeted were amplified and that primer sets corresponding to deleted regions never amplified other regions of the genome (Figures 3 and 4, and data not shown).

To test the general utility of this method DNA from 20 unrelated male DMD patients that had not been characterized by Southern analysis were analyzed by multiplex amplification. Figure 3 shows the amplification products obtained with each DNA sample. Deletions were detected in 12/20 of the samples, thus identifying the mutations in 60% of the patients. Figure 3 also illustrates that a variety of deletions were detected, ranging from a single region to all 6. Each of the DNAs used was subsequently examined by the R.J. Kleberg Center for Human Genetics-DNA Diagnostic Laboratory at Baylor College of Medicine using Southern analysis with human DMD cDNA probes. Those results, which took a further week to obtain, confirmed in all 20 cases the presence or absence of each of the six regions analyzed (data not shown). The cDNA probes detected small deletions in 2 of the 8 samples that did not reveal deletions via the amplification method, both of which were in regions outside of the six originally analyzed. Thus the multiplex method successfully detected 12/14 deletions (86%) and less than one day was required to obtain the results.

Prenatal diagnosis *via* multiplex amplification

Successful diagnosis of deletions in the DMD gene of male patients led us to ask if the same techniques could be utilized for rapid prenatal diagnosis. During the course of this study, six families referred to the R.J. Kleberg Jr. Center for Human Genetics were chosen for analysis. In each case a female who had previously given birth to an affected male requested prenatal diagnosis of a fetus. DNA was prepared from cultured amniotic fluid cells and used as template for multiplex amplification in parallel with lymphoblast DNA isolated from the affected male in each family (Figure 4). Identical deletions were detected with the fetal and affected male DNAs in 2 of the six families, diagnosing the fetuses as affected. In two cases a deletion was detected in the affected male DNA but not with the fetal DNA, diagnosing the fetuses as unaffected. No deletions were detected in either the fetal or affected male DNAs in the remaining 2 cases.

Southern analysis confirmed the presence or absence of the six regions in every case (data not shown) demonstrating that multiplex amplification works well with amniotic fluid cells and that the deletions were not masked due to amplification from any contaminating maternal cells (see below). In one of the two families where no deletion was detected via amplification (DRL # 469),

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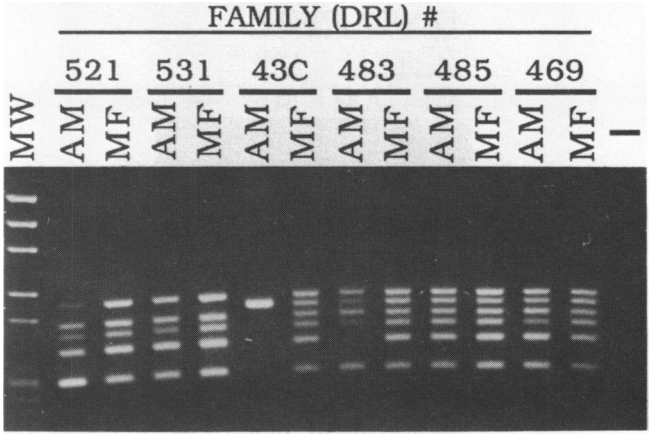

Figure 4. Multiplex DNA amplification for prenatal diagnosis of DMD. Shown are the results of amplification using DNA from an affected male (AM; lymphoblast DNA) and a male fetus (MF; cultured amniotic fluid cell DNA) from six different families. Analysis was as described in Figure 3. Both the affected male and the fetal DNAs of DRL #s 521 and 531 display a deletion of region f (Fig. 2) diagnosing these fetuses as affected. In DRL # 43C the affected male is deleted for all regions except f, while the fetus is unaffected. The affected male in DRL # 483 is deleted for region a, while the male fetus is unaffected. Neither of the samples from DRL #s 485 or 469 displayed a deletion with this technique. Analysis was as described in Fig. 3.

Southern analysis did detect a small deletion located outside of the six regions amplified in both the affected male and fetal DNAs. Therefore multiplex amplification detected 2 of 3 deletions in the fetal samples providing a diagnosis of affected, two were diagnosed as normal, and two were indeterminate (only one of which was further clarified by Southern analysis).

Frequently chorionic villus specimens (CVS) rather than amniotic fluid cells are used for DNA diagnosis. Since CVS are potentially contaminated with greater amounts of maternal tissue than are amniotic fluid cells we tested whether multiplex amplification from CVS derived DNA might fail to detect deletions due to amplification from the normal allele of any contaminating decidual DNA. Chorionic villus specimens received by the R.J. Kleberg Center for Human Genetics for diagnostic studies are microscopically dissected to remove decidual tissue prior to study, and cytogenetic analysis rarely detects any maternal XX karyotypes in dissected male specimens.

Multiplex amplification was performed with fetal DNA prepared from two chorionic villi and with lymphoblast DNA from an affected brother from one of the families. No living affected male was available for the second case.

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Figure 5. Multiplex DNA amplification from CVS DNA. Both the affected male (AM; lymphoblast DNA) and the male fetus (MF; CVS DNA) from DRL # 92 display a deletion of regions e and f (Fig. 2), diagnosing the fetus as affected. CVS DNA from DRL # 120 did not display a deletion with this technique, and no living affected relative was available for comparison. Samples were analyzed as described in Fig. 3.

Figure 5A demonstrates that a deletion was detected in DRL # 92 fetal DNA and in DNA from his affected brother, but not in the DRL # 120 fetal DNA. Southern analysis with cDNA probes confirmed the veracity of these results, but detected a deletion of two exons located outside of the amplified regions of the DRL # 120 fetal DNA. Thus although the DRL # 120 deletion was not detectable with this method the results with the DRL # 92 sample indicate that maternal DNA did not lead to a false positive amplification under the conditions used.

To further explore potential problems associated with contaminating maternal DNA reconstruction experiments with deleted and normal DNA were performed. We have observed that low level contamination of DNA samples with non-deleted DNA is not apparent at early rounds of amplification, but that as the level of amplification of the test DNA approaches saturation the contaminating DNA continues to be amplified and eventually can produce similar quantities of reaction products as does the test DNA. In addition to the number of cycles performed, the relative amount of contamination from exogenous or maternal DNA will also affect the results. To estimate the amount of contamination which can be tolerated before ambiguous results are obtained we mixed a partially deleted DNA sample with various amounts of non-deleted DNA and then performed increasing cycles of multiplex amplification. Figure 6 demonstrates that levels up to 3-5% contamination can be tolerated when amplification is limited to 25 cycles. Fewer cycles do not produce sufficient

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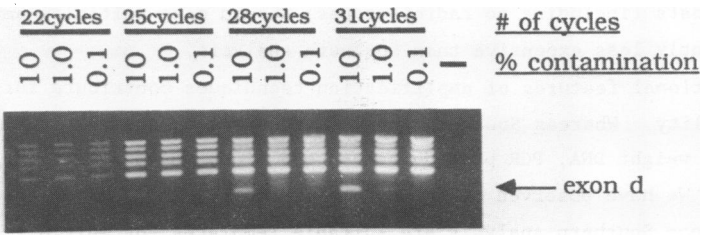

Figure 6. Effect of contamination on multiplex amplification. 500ng of DNA from DRL # 519 (Fig. 3B) was mixed with 50ng (10%), 5ng (1%), or 0.5ng (0.1%) of normal control female (46XX) DNA prior to amplification. Analysis was as described in Fig. 3 except that 15μl aliquots were removed from each reaction after the indicated number of amplification cycles and stored on ice until analysis. DRL # 519 is deleted only for region d. By 25 cycles the sample with 10% contamination has begun to display the exon d fragment. Note that the contaminant contains two X chromosomes; exogenous male DNA would be expected to produce half the observed signals.

quantities of DNA for visual analysis, whereas additional cycles allow detection of decreasingly smaller quantities of contaminating DNA.

DISCUSSION

Multiplex amplification methods have recently been integrated into the DMD diagnostic protocols of the R.J. Kleberg Center for Human Genetics at Baylor College of Medicine. Currently all DNA samples received for analysis are initially analyzed by multiplex amplification. This procedure requires five hours from start-up to photography of results, and as currently practiced eliminates the need for further Southern analysis of approximately 37% of the samples (Table 1). Cases in which deletions are not detected are subsequently examined via Southern analysis using full-length DMD cDNAs subdivided into 7-9 separate probes. Those samples that do not display genomic alterations with cDNAs must then be examined by Southern analysis with multiple genomic probes using family member DNAs digested with numerous restriction enzymes for linkage analysis (29). Complete Southern analysis of samples not diagnosed via multiplex amplification requires one to two weeks for hybridization of multiple blots with up to 16 separately labelled probes for each family referred for diagnosis. The ability to eliminate 37% of the cases from the necessity for Southern analysis clearly illustrates the tremendous savings of time and effort which can be achieved *via* a one day multiplex amplification protocol. While it is difficult to estimate the dollar savings which would result, the reduction in labor from one or two weeks to less than one day, and the lower

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reagent costs (including no radioisotopes) should make multiplex amplification significantly less expensive than Southern analysis.

Additional features of amplification techniques contribute further to their utility. Whereas Southern analysis requires extensively purified high molecular weight DNA, PCR procedures can use crude and partially degraded samples. We have observed that DNA samples too degraded to produce detectable signals upon Southern analysis are suitable templates for multiplex amplification (data not shown). PCR procedures require only that the average molecular weight of template DNAs be slightly greater than the largest fragment amplified. This feature of the technique should prove advantageous for the analysis of low quality DNA resulting from suboptimal purification, storage, or shipping. Procedures have also been described for PCR amplification from paraffin embedded biopsy samples stored for years at room temperature (30). Such samples should allow retrospective examination of DMD cases diagnosed before the isolation of DMD clones, which can facilitate future prenatal diagnosis involving other family members. In addition future pregnancies within non-carrier DMD families where a lesion was thought to have arisen *via* new mutation can be rapidly screened by PCR amplification of a region deleted from the gene of the affected male to guard against rare cases of mosaic germ line mutations (31,32).

As more sequence data for the DMD gene becomes available additional primer sets can be added to the assay to increase the frequency of deletion detection. With 6 primer sets we detected deletions in 54% (15/28) of the samples analyzed in this study, or 79% (15/19) of the deletions observed with cDNA probes. Examination of the frequency that individual exons were observed to be deleted from the DNA of over 200 randomly surveyed male DMD patients (2,6) indicates that the multiplex amplification procedure would have detected deletions in 37% of those patients, or 70% of the frequency observed with cDNA clones. The higher detection rate displayed in Figures 3-5 presumably reflects a normal variation which should be expected from analyzing the smaller number of cases referred to our laboratory for this study. The deletion data previously obtained from DMD patients indicates that by cloning and subsequently including 2 more exons in this assay [exon 4 and the 3.7 kb HindIII fragment detectable with human cDNA probe 8 (2)] a deletion detection rate of almost 90% would be achieved (2,6). Inclusion of other additional exons would result in very small increases in detection frequency, as most deletions encompass numerous exons and the few that do not are usually located in deletion prone regions of the gene (2,6).

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The multiplex amplification technique can also be adapted to polymorphism detection for linkage analysis. We have observed that intron sequences contain a high frequency of polymorphisms, most of which do not occur at restriction enzyme recognition sites (unpublished observation). These polymorphisms can be detected via amplification of intron containing sequences (as is done with this method) followed by hybridization with ASO probes, or in the case of RFLPs, via restriction enzyme digestion (1,13,14). Techniques are currently under development which should permit polymorphism detection directly during PCR amplification (RAG, manuscript in preparation). Integration of these future modifications could permit analysis of almost all DMD diagnostic cases via amplification based techniques.

The rapidity and simplicity of these methods should permit routine DMD diagnosis at clinical laboratories without the need for highly trained research personnel. Currently we are using this assay in a kit form. Primers and buffer are pre-mixed and aliquoted into tubes for storage at -70°. For analysis a tube is thawed, DNA and enzyme are added, the reaction is performed on an automatic thermocycler (Perkin Elmer/Cetus), and the results are determined by agarose gel electrophoresis. The use of fluorescent primers will permit automatic analysis of reaction products on DNA sequencing apparatus such as is available from Applied Biosystems (33). We envision that clinical laboratories could routinely screen DNA samples for deletions, then forward only those cases in which deletions were not detected to a research oriented DNA diagnostic laboratory for Southern analysis. In extremely rare cases where every amplified region is deleted (e.g. DRL # 957 and 484, Fig. 3) the deletion should be confirmed *via* Southern analysis.

Successful use of these multiplex amplification methods requires that DNA from maternal or exogenous origin not interfere with deletion detection. We have demonstrated that cultured amniotic fluid cells and chorionic villus specimens dissected of decidual tissue produce clearly manifested deletions as long as amplification reactions do not approach saturation (Figs. 4 and 5). Furthermore, after 25 cycles of amplification levels of non-deleted DNA at up to 3-5% of the total do not interfere with interpretation of the results (Fig. 6). As with any sensitive technique careful pipetting of reagents is required, and we routinely use separate pipettors to mix reactions than the ones that are used to load gels. This latter precaution is taken to prevent the possibility that trace aerosol contamination of pipettors by amplification products will serve as an efficient template for future reactions.

The techniques we have applied to the study of DMD should be generally

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applicable to any hemizygous locus susceptible to deletions. Examples include hypoxanthine phosphoribosyltransferase (Lesch-Nyhan syndrome; 1); steroid sulfatase (x-linked ichthyosis; 34); orthinine transcarbamylase (OTC deficiency;35); and the pseudoautosomal sex-chromosome regions (36). Homozygous deletions including those leading to growth hormone deficiency (37) and somatic neoplasia such as Wilm's tumor and retinoblastoma (38,39) would also be detectable. As currently applied these techniques do not detect heterozygous deletions due to amplification from the non-deleted allele. However, reaction conditions producing uniform amplification of each fragment might be applicable to carrier detection of heterozygous (including DMD) gene deletions. Multiplex amplification should also simplify the screening of blood samples by permitting simultaneous assaying for numerous parasitic or viral nucleic acid sequences. Finally, implementation of fully automatic multiplex amplification protocols could eventually lead to routine screening of new born infants to diagnose a high percentage of the cases of deletion prone genetic diseases arising from new mutations.

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QIAGEN Exhibit 1022.0014

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QIAGEN Exhibit 1022.0015

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QIAGEN Exhibit 1022.0016