The mdx mouse is a murine genetic equivalent of the human X-linked lethal disorder, Duchenne muscular dystrophy (DMD). A number of studies utilizing the mdx mouse have demonstrated the feasibility of gene therapy for this disorder. Many such studies require the ability to determine rapidly the mdx genotype of experimental animals. Previous methods described to identify the mdx allele require multiple manipulations which are technically demanding. We now describe a simple and rapid method to detect the mdx and wild-type alleles in crude mouse DNA samples, by the mdx-amplification-resistant mutation system (ARMS) assay. With this system we correctly identified the mdx status of various transgene-containing animals in a rapid and simple fashion. We discuss the utility of this system for many other studies utilizing the mdx mouse as a model system. © 1996 John Wiley & Sons, Inc.
Key words: Duchenne muscular dystrophy • mdx • amplification-resistant mutation system • transgene • gene therapy

THE mdx–AMPLIFICATION-RESISTANT MUTATION SYSTEM ASSAY, A SIMPLE AND RAPID POLYMERASE CHAIN REACTION-BASED DETECTION OF THE mdx ALLELE

ANDREA AMALFITANO, DO, PhD, and JEFFREY S. CHAMBERLAIN, PhD

The mdx mouse is a murine genetic equivalent of the human X-linked lethal disease Duchenne muscular dystrophy (DMD). Originally, the mdx mouse was detected based upon elevated serum levels of the muscle isoform of the enzyme pyruvate kinase.[1] Subsequently, with the breeding of the affected mice, the elevated pyruvate kinase levels were found to segregate as an X-linked recessive phenotype. Affected mice were also found to have degeneration and regeneration of the limb musculature of the mice, along with an extensive and progressive fibrosis of the diaphragm muscle.[1,6,9,13] Physiologic analysis of the mdx mouse demonstrated that the force- and power-generating capabilities of their skeletal muscles are significantly decreased when compared to wild-type animals.[6,9] Following the cloning of the human gene responsible for DMD, dystrophin, the mutation in the mdx mouse was found to be a C to T transition in exon 23 of the murine dystrophin gene, creating a nonsense codon resulting in premature termination of translation.[11] The expected truncated protein is not detectably expressed, in skeletal muscle, likely due to instability of the mRNA and the truncated protein.[4,7] Thus, the mdx mutation is in essence an X-linked recessive null mutation.

The nature of the mdx mutation has allowed our group to utilize transgenic mdx mice expressing full length or truncated forms of the dystrophin gene to study the feasibility of gene therapy for DMD.[6,9] Since the transgenes are usually injected into wild-type embryos, they must subsequently be bred onto the genetic background of mdx mice. To facilitate the transfer of newer versions of the dystrophin-expressing transgenes onto the mdx background, one must determine the genotype of an animal that has inherited the transgene construct. Previously we have described an allele-specific oligonucleotide (ASO)-based hybridization method to detect the mdx mutation.[2,5] Other methods available include those utilizing polymerase chain reaction (PCR) amplification of the region encompassing the mdx mutation, and either directly sequencing the PCR prod-

ucts or utilizing primers that incorporate a unique restriction site into the PCR product only if the wild-type allele is present.[5,10] For this latter strategy the PCR products must be purified and digested with the appropriate restriction enzyme, and the small diagnostic restriction products must be separated by polyacrylamide gel electrophoresis. Each of the above methods either requires multiple manipulations, the use of nonconvenient electrophoresis systems, carefully controlled hybridization conditions, and/or radiolabeled nucleotides. We now describe a simple PCR-based method to detect either the mdx or wild-type murine dystrophin alleles in crude mouse-tail DNA extracts. This method is based on the amplification-resistant mutation system (ARMS), a system previously utilized to detect point mutations in a number of other genes including alleles of the alpha-1-antitrypsin gene, and the various apolipoprotein E alleles.[8,12,14] Utilizing the mdx–ARMS assay, we have been routinely able to genotype the mdx status of crude mouse-tail DNA preparations rapidly after DNA isolation. We discuss the potential use of this assay system to facilitate future studies of DMD which utilize the mdx mouse as a model system.

MATERIALS AND METHODS

Mice. Wild-type C57/BL10 and mdx mice were originally obtained from the Jackson Laboratories, and have been maintained in our facilities in accordance with University of Michigan laboratory animal usage guidelines. All mdx animals utilized in our studies are maintained as a breeder colony of mdx males and homozygous mdx/mdx females. The mdx status of these mice are routinely confirmed by ASO screening, phenotypic analysis, and by the appropriate homozygous breedings. Transgenes were initially injected into heterozygous mdx/wild-type embryos at the University of Michigan Transgenic Animal Core Facility.

Design of the mdx–ARMS Oligonucleotides. We have previously sequenced the 3'-region of murine dystrophin intron 22 immediately 5' of exon 23.[5] The mdx mutation is a C to T transition within exon 23[11] at position 3203 relative to the full-length murine dystrophin cDNA sequence that we previously submitted to Genbank (See Fig. 1).[3] The sense primer p9427 (5'-AACTCATCAAATATGCGTGT-TAGTG-3') was designed to be complimentary to sequences located within intron 22; therefore it will hybridize only with mouse genomic DNA sequences, and not with any dystrophin cDNA-derived transgenes. There are two reverse primers utilized in the mdx–ARMS assay. The first primer, p259E (5'-GTCACTCAGATAGTTGAAGCCATTTAA-3'), was designed such that its 3' nucleotide is complimentary only to the corresponding mdx nucleotide, and is therefore a mismatch for the corresponding wild-type nucleotide at that position, while the 3' nucleotide of the reverse primer p260E (5'-GTCACTCAGATAGTTGAAGCCATTTAG-3') is complimentary only to the wild-type sequence and is mismatched for the mdx allele. To further prevent incorrect extension by Taq polymerase when the mdx primer has incorrectly annealed with the wild-type sequence, a second destabilizing mismatch immediately 5' to the 3' mismatches was introduced into both reverse primers. This additional mismatch is especially important since the A-C (purine/pyrimidine) mismatch is expected to be more likely to allow inappropriate primer extension by the Taq polymerase than other types of mismatches.[8] The introduced second mismatch, however, does not interfere with extension when a correct annealing has occurred at the 3' terminus of the primers (see Results).

PCR Reactions. Individual 0.5-in. mouse-tail samples were digested at 55°C in 600 µL of 10

```
                    p9427-Forward
             ┌─────────────────────────►
5'-ttgatatgaa tgaaactcat caaatatgcg tgttagtgta aatgaacttc tatttaattt


  intron│exon                        T=mdx allele
    22  │ 23                         ▲
     tga│GGCTCTG CAAAGTTCTT TGAAAGAGCA ACAAAATGGC TTCAACTATC TGAGTGACAC-3'
                             3'-AATTTACCG AAGTTGATAG ACTCACTG-5'  =p259E
                             3'-GATTTACCG AAGTTGATAG ACTCACTG-5'  =p260E
```
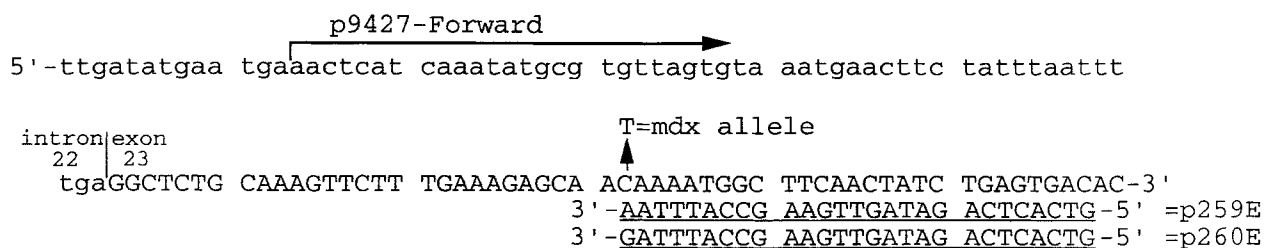
FIGURE 1. The sequence of the 3' end of murine dystrophin intron 22 (Genbank accession #: U56724) and the 5' end of exon 23 of the murine dystrophin gene are displayed.[3,5] The location of the forward primer, p9427, and the reverse primers p259E and p260E are depicted. Note the location of the intron 22/exon 23 border in relation to the forward and reverse primers. The location of the C to T transition responsible for the mdx phenotype is noted and is located at position 3203 of the full-length murine dystrophin cDNA sequence.[3,11] The sequences of p9427, p259E, and p260E are also shown.

mmol/L Tris-Cl (pH 7.5), 400 mmol/L NaCl, 100 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.6% sodium dodecyl sulfate (SDS), and proteinase K at 0.6 mg/mL. A saturated NaCl solution (167 µL) was added and mixed thoroughly, followed by centrifugation in a minifuge at full speed for 10 min at room temperature. One volume of cold 95% ethanol was mixed with the supernatant and minifuged at room temperature for 20 min. The pelleted DNA was rinsed with 70% ethanol, air dried, and resuspended in 200 µL of 10 mmol/L Tris-Cl (pH 8.0), 1 mmol/L EDTA. Approximately 50–200 ng of crude tail DNA extract was used in each set of 15-µL PCR reactions. The PCR reaction mixture included 2 ng/mL of each of the appropriate forward and reverse primers, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl$_2$, 0.001% gelatin, 1 unit of Taq DNA polymerase, and 0.34 mmol/L of each of the four deoxynucleotide triphosphates (dNTPs). A Perkin–Elmer 9600 thermocycler was programmed with the following cycling parameters: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 53–57°C for 30 s, and Taq polymerase extension at 72°C for 20 s. A final extension for 10 min at 72°C was followed by storage at 4°C. The 105-bp DNA products of the PCR reactions were electrophoresed through 4% agarose gels (3 parts NuSieve agarose: 1 part conventional agarose) and photographed after ethidium bromide staining.

RESULTS

The ARMS assay is based on the principle that a single nucleotide change can be discriminated dur-

ing a simple PCR reaction.[8] By designing oligonucleotide primers which differ only by a single 3' nucleotide, single nucleotide changes in the complimentary position of a DNA sample can be rapidly identified.[8,12,14] This discrimination is facilitated by the fact that the Taq polymerase does not have a 3'-exonucleolytic proofreading activity. Our laboratory has previously described and modified ASO hybridizations to genotype the mdx status of animals carrying various dystrophin-expressing transgenes.[2,5] We now demonstrate the ability to determine the mdx genotype of transgene-bearing animals utilizing a simple set of PCR reactions. For each DNA sample to be analyzed, two sets of PCRs are utilized. One PCR set utilizes oligonucleotide primers p9426 and p259E as forward and reverse primers, which only allow amplification of the mdx allele; the other PCR utilizes the forward primer p9426 and reverse primer p260E to amplify only the wild-type allele. With each set of PCRs performed, appropriate positive and negative control samples must always be analyzed simultaneously, so as to be sure that both sets of PCRs proceeded correctly. Without positive and negative controls, false-negative results may be assigned incorrectly to those reactions with no evidence of amplification products. To analyze the specificity of the oligonucleotide primers for allowing amplification of only the mdx or wild-type alleles, mdx–ARMS assays were performed using a range of annealing temperatures (see Fig. 2). Utilizing crude mouse-tail DNA samples isolated from animals of known genotype, we demonstrated that the mdx–ARMS assay correctly identified the mdx allele status in each of the control animals. Note the lack of amplification of
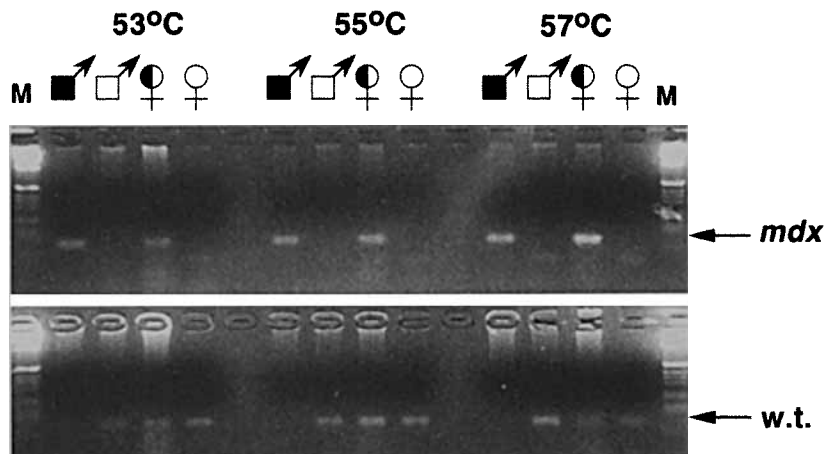

FIGURE 2. Samples of previously genotyped mouse-tail DNA were analyzed by the mdx–ARMS assay as described in the text. The top row shows the PCR amplification products produced with the mdx-specific reverse primer p259E and p9427 after electrophoresis through a 4% agarose gel. The bottom row displays the PCR amplification products generated by the wild-type (w.t.) specific reverse primer p260E and p9427. Each set of mouse DNA samples was analyzed using the indicated annealing temperature. The diagnostic 105-bp PCR products are indicated by arrows. Symbols: box = male, circle = female, shading indicates presence of an mdx allele.

the 105-bp product from any of the negative control samples, even when utilizing annealing temperatures that varied between 53 and 57°C (see Fig. 2). The mdx allele-specific primers allowed amplification of the mdx allele at even lower temperatures (i.e., as low as 49°C); however the wild-type specific primers failed to allow consistent amplification of the wild-type allele at lower temperatures (data not shown). While we have never had a false-positive result in over 100 mdx–ARMS attempts, we have noted that the annealing temperature is the most critical aspect of this assay. It is critical to determine the optimum annealing temperature on each individual thermocycler used for this type of assay; similar machines have slightly different heating/cooling profiles, which may impact upon the reliability of the system.

As an example of the utility of the mdx–ARMS assay, we have used the system to ascertain the mdx status of mice that have integrated into their genomes various transgenes we have previously constructed.[6,9] Figure 3 demonstrates the ability of the mdx–ARMS assay to identify the genotype of transgenic offspring. In this example a female mouse known to be heterozygous for the mdx and wild-type alleles was mated to a previously genotyped mdx male mouse. The first mating produced 7 transgenic offspring of which 2 females were found by mdx–ARMS to be homozygous for the mdx mutation; sub-

sequent breeding of the offspring and histopathologic examination confirmed the assigned genotypes (data not shown). In the second mating, 2 female transgenic offspring were isolated and identified as mdx/mdx homozygotes by the mdx–ARMS assay. The latter result demonstrated another useful feature of the mdx–ARMS assay: the transgene that integrated into the genomes of those mice contained a full-length dystrophin cDNA molecule, but since the forward PCR primer is complimentary to intronic DNA sequences, no amplification products from the integrated dystrophin cDNA were generated.

DISCUSSION

Use of the mdx mouse as an animal model for the human disease DMD has been invaluable, not only enabling investigation of the pathogenesis of the disease, but also for testing the potential for gene therapy of this lethal disorder. We and others have demonstrated that many of the characteristics of the mdx mouse are consistently observed and can be reliably measured. These same parameters can be analyzed to determine if a mode of intervention has had any effect on the mdx phenotype.[6,9] One very useful form of investigation includes the isolation of mdx mice that express transgenes encoding the full-length and truncated forms of the dystrophin gene itself (or other proteins) to ascertain their impact on the mdx phenotype. We have developed the mdx–
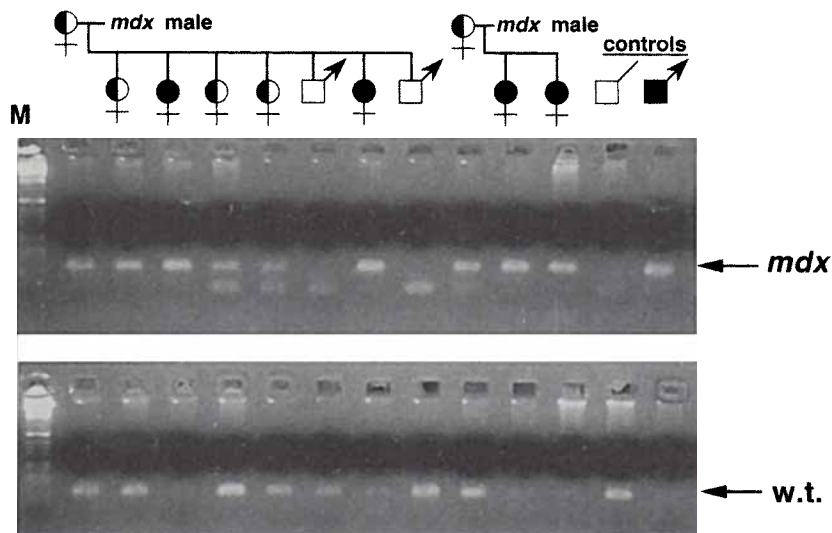

FIGURE 3. Crude samples of mouse DNA isolated from transgenic mice analyzed by the mdx–ARMS assay. The genotypes of offspring derived from matings between transgenic heterozygous mdx/wild-type (w.t.) females with mdx males is depicted. In the first mating pair, 2 of the 7 transgenic offspring were found to be mdx/mdx homozygotes; in the second mating pair, both transgenic offspring are mdx/mdx homozygous females. Control reactions are also included in both sets of mdx–ARMS reactions. The diagnostic 105-bp PCR products are indicated by the arrows (note the presence of dimerized primers in some of the lanes: utilizing 4% NuSeive agarose gels allows resolution of these products away from the diagnostic 105-bp PCR products). Symbols: box = male, circle = female, shading indicates presence of an mdx allele.

ARMS assay as a rapid and simple method to determine the mdx genotype of the transgenic mice.

New strains of mice with targeted knockouts of genes encoding proteins known to interact with dystrophin are being isolated by several labs. It will be of interest to breed such knockout lines onto the mdx background, to ascertain how the lack of the various genes impact upon the mdx phenotype. In addition, breeding the mdx allele onto mouse strains deficient for various components of the immune system may also be helpful in elucidating the role of the different arms of the immune response in the pathogenesis of the mdx phenotype. The mdx–ARMS assay will be very useful in the rapid identification of these new mouse strains. Finally, discrimination between the wild-type and mdx alleles could be useful in studies of myoblast transfer into mdx mice; i.e., detection of the wild-type allele present in the donor myoblasts. Other methods have been designed to identify the mdx allele, including the ASO hybridization analysis as well as a PCR-based method requiring PCR product purification, restriction enzyme digestion, and polyacrylamide gel electrophoresis.[2,5,10] Both methods require multiple steps that can be technically challenging and time-consuming. We have successfully utilized the mdx–ARMS assay for rapid genotyping of the mdx and wild-type alleles of the murine dystrophin gene in a simple PCR-based method utilizing conventional gel electrophoresis, without the need for extensive sample DNA purification, organic extraction, differential hybridization, or enzyme digestion.

REFERENCES

1. Bulfield G, Siller WG, Wight PA, Moore KJ: X chromosome-linked muscular dystrophy (mdx) in the mouse. Proc Natl Acad Sci USA 1984;81:1189–1192.

2. Chamberlain JS, Farwell NJ, Ranier JE, Cox GA, Caskey CT: PCR analysis of dystrophin gene mutation and expression. J Cell Biochem 1991;46:334–336.

3. Chamberlain JS, Pearlman JA, Muzny DM, Civetello A, Farwell NJ, Malek R, Powaser P, Reeves AA, Lee C, Caskey CT: Mouse dystrophin cDNA Sequence. Genbank accession number M68859.

4. Chamberlain JS, Pearlman JA, Muzny DM, Gibbs RA, Ranier JE, Reeves AA, Caskey CT: Expression of the murine Duchenne muscular dystrophy gene in muscle and brain. Science 1988;239:1416–1418.

5. Chamberlain JS, Phelps SF, Cox GA, Maichele AJ, Greenwood AD: PCR Analysis of Muscular Dystrophy in mdx Mice, in Partridge T (ed): Molecular and Cell Biology of Muscular Dystrophy. London, Chapman & Hall, 1993, pp 167–189.

6. Cox GA, Cole NM, Matsumura K, Phelps SF, Hauschka SD, Campbell KP, Faulkner JA, Chamberlain JS: Overexpression of dystrophin in transgenic mdx mice eliminates dystrophic symptoms without toxicity. Nature 1993;364:725–729.

7. Hoffman EP, Brown RH Jr, Kunkel LM: Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell 1987;51:919–928.

8. Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C, Kalsheker N, Smith JC, Markham AF: Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucl Acids Res 1989;17:2503–2516.

9. Phelps SF, Hauser MA, Cole NM, Rafael JA, Hinkle RT, Faulkner JA, Chamberlain JS: Expression of full-length and truncated dystrophin mini-genes in transgenic mdx mice. Hum Mol Genet 1995;4:1251–1258.

10. Shrager JB, Naji A, Kelly AM, Stedman HH: A PCR-based assay for the wild-type dystrophin gene transferred into the mdx mouse. Muscle Nerve 1992;15:1133–1137.

11. Sicinski P, Geng Y, Ryder-Cook AS, Barnard EA, Darlison MG, Barnard PJ: The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. Science 1989;244:1578–1580.

12. Stavljenic-Rukavina A, Sertic J, Salzer B, Dumic M, Radica A, Fumic K, Krajina A: Apolipoprotein E phenotypes and genotypes as determined by polymerase chain reaction using allele-specific oligonucleotide probes and the amplification refractory mutation system in children with insulin-dependent diabetes mellitus. Clin Chim Acta 1993;216:191–198.

13. Torres LF, Duchen LW: The mutant mdx: inherited myopathy in the mouse. Morphological studies of nerves, muscles and end-plates. Brain 1987;110:269–299.

14. Wenham PR, Newton CR, Price WH: Analysis of apolipoprotein E genotypes by the amplification refractory mutation system. Clin Chem 1991;37:241–244.