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Scaled-down genetic analysis of myotonic dystrophy type 1 and type 2

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ABSTRACT

Types 1 and 2 myotonic dystrophy are neuromuscular disorders caused by genomic expansions of simple sequence repeats. These mutations are unstable in somatic cells, which leads to an age-dependent increase of expansion length. Studies to determine whether changes in repeat size may influence disease severity are limited by the small amount of DNA that can be recovered from tissue biopsies samples. Here we used locked nucleic acid oligonucleotide probes and rolling circle amplification to determine length of the expanded repeat in sub-microgram quantities of genomic DNA. These methods can facilitate genetic analysis in cells and tissues obtained from individuals with myotonic dystrophy.

1. Introduction

The myotonic dystrophies are dominantly-inherited disorders caused by expanded triplet or tetramer repeats. Myotonic dystrophy type 1 (DM1) results from expansion of a CTG repeat in the 3′ untranslated region of *DMPK* [1]. In myotonic dystrophy type 2 (DM2), the expanded repeat is a CCTG tetramer in intron 1 of *ZNF9* [2]. These mutations are unstable when transmitted through successive generations of a family [3]. They are also unstable in somatic cells, so that heterogeneity and length of the expansion increase over time in an individual [4,5].

Few studies have addressed the natural history of somatic instability in DM-affected tissues. Expansion lengths in DM1 muscle biopsy tissues were 2- to 13-fold larger than in leucocytes from the same individuals [6–9]. It is unclear, however, whether the onset or progression of myopathy is determined by changes of repeat length in skeletal muscle. Studies of tissue biopsy samples are limited by several factors, including the inability to amplify highly expanded repeats by PCR [10], the requirement of 5–10 μg of DNA for a conventional Southern blot [11] (equivalent to 15–30 mg of muscle tissue), and, in the case of DM2, somatic heterogeneity that is so extreme that the hybridization signals from expanded alleles can drop below detection threshold, resulting in false negative results of Southern blots [12]. To enhance genetic analysis under conditions where DNA samples are limiting and size of the expansion is large, we developed an alternate detection system that employs digoxigenin (DIG)-labeled $(CAG)_7$ or $(CCTG)_5$ probes composed of locked nucleic acids (LNA), coupled in some cases with rolling circle amplification (RCA) of DNA.

2. Materials and methods

2.1. DNA preparation

DNA was isolated by phenol/chloroform extraction following by ethanol precipitation as described previously [8]. Twenty one postmortem samples from patients with classical DM1 and three samples from congenital DM1 were examined. DM1 fibroblasts were obtained from the Coriell Institute. DNA from needle muscle biopsies was obtained from four patients with DM2. Leucocyte DNA was obtained from two classical DM1, 17 DM2, and fifteen normal individuals. These studies were approved by the local institutional review board. All study participants provided informed consent.

2.2. Southern blot

Genomic DNA was digested with BglI, HaeIII, AluI, DpnII, or MwoI for DM1 analysis, or with HaeIII and AluI for DM2 analysis. Fragments were resolved on 0.8% agarose gels buffered with 40 mM Tris–acetate, 1 mM EDTA for 4 h at 6 V/cm for RCA products, or on 0.5% gels for 24 h at 1 V/cm for genomic DNAs, and then transferred overnight onto nylon membranes (Roche) by alkaline transfer. Blots were fixed at 120 °C for 20 min and then hybridized for 4 h at 70 °C with 10 pmol/ml DIG-labeled $(CAG)_7$ (5′-gcAgCagcAgCagCagcAgca-3′) for DM1 or $(CCTG)_5$ (5′-cCTgccTgcCTgccTgcCTg-3′) for DM2 (upper case letter indicates position of LNA nucleotide) in hybridization buffer [5× SSC, 1% block solution (Roche), 0.1% *N*-lauryl sarcosine, 0.02% sodium dodecyl sulfate]. After wash-

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ing to high stringency (0.5× SSC at 70 °C), blots were developed with alkaline phosphatase-conjugated anti-DIG antibody using CDP-Star substrate according to manufacturers' instructions (Roche), followed by 2–30 min exposure to BioMax XAR film (Kodak). For conventional Southern analysis, blots were hybridized with ^{32}P-labeled random-primed probe p5B1.4 [13], a 348 nt *DMPK* fragment that is adjacent to the CTG repeats. These blots were analyzed by using a Typhoon 9600 phosphorimager (GE Health Care).

2.3. Gene-selective rolling circle amplification (RCA) of CTG expansion in DMPK

Genomic DNA was digested with StyI and then circularized with T4 DNA ligase (NEB). For the DM1 locus this created a circular DNA containing 807 bp plus the CTG repeat. Using 40 ng of circularized DNA, RCA was initiated with three primers that hybridized to the antisense strand of *DMPK* (start primers). After two hours, a biotinylated "capture" primer was added. This primer is complementary to the sense strand of *DMPK*, and anneals to the extension products initiated by start primers. After 30 min, the extension products from the capture primer were pulled down on Dynabeads MyOne Streptavidin C1 (Invitrogen). After washing, fresh RCA reagents and final amplification primers (one hybridizing to the sense strand, the other to the antisense strand) were added and incubated for 14 h. The RCA reactions were performed in a total volume of 30 μl containing 1 mM dNTP, 0.3 μM primers, 1× phi29 buffer, 2× BSA, and 10 units of phi29 DNA polymerase (NEB) at 37 °C. RCA products were digested with AvrII for Southern blot. Primer sequences were start primer 1, 5′-CACAGACCATTTCTTTCTTTCGG-CCAGGCTG∗A∗G-3′; start primer 2, 5′-CATTCCTCGGTATTTATTG-TCTG∗T∗C-3′; start primer 3, 5′-CAAAGCTTTCTTGTGCATGA∗C∗G-3′; capture primer, 5′-CTCGGAGCGGTTGTGAACTG-3′; final RCA primer 1, 5′-AAACGTGGATTGGGGTTGTT∗G∗G-3′; and final RCA primer 2, 5′-GACTCGCTGACAGGCTACA∗G∗G-3′. Asterisks indicate position of phosphorothioate bonds.

3. Results

We postulated that short probes comprised of CAG repeats may provide sensitive detection of DM1 expansions because many copies of probe can hybridize to each expanded allele. We also postulated that favorable hybridization characteristics of LNAs [14,15] may enhance the sensitivity and specificity of CAG-repeat probes. Consistent with this concept, the $(CAG)_7$ LNA probe detected expanded DM1 alleles by Southern blot using 200–300 ng of genomic DNA (Fig. 1B, left). Serial dilution of input DNA showed that expanded repeats could be detected from as little as 50 ng of cardiac muscle DNA (Fig. 1C).

Several findings support the specificity of hybridization signals under these conditions. First, when genomic DNA was digested with restriction enzymes that cleave at a distance from the repeat tract, the fragments detected by $(CAG)_7$ LNA probes corresponded exactly with those detected by a conventional ^{32}P-labeled probe (Fig. 1B, right). The latter probe hybridizes to single-copy sequence that flanks the expanded repeat. Note that 3–6 μg of genomic DNA was required to obtain signal with the ^{32}P-labeled probe, and this amount still did not clearly reveal the size heterogeneity of these highly expanded alleles (Fig. 1B, lane 3 of right and left panel). Second, when genomic DNA was digested with different restriction enzymes, the size of the expanded allele in DNA from DM1 heart was invariant for each of three different 4-bp restriction enzymes (DpnII, AluI, and HaeIII) (Fig. 1D). This result fits with expectations that cleavage with these 4-bp restriction enzymes eliminates nearly all of the flanking sequence, resulting in fragments that have similar size because they are comprised almost entirely of expanded repeats. By contrast, signals were not observed after digestion by MwoI, a 4-bp restriction enzyme that cleaves CTG repeats. Finally, expanded alleles were detected from each of 24 DM1 autopsy tissue samples that we analyzed, but not in leucocyte DNA samples from eight normal controls.

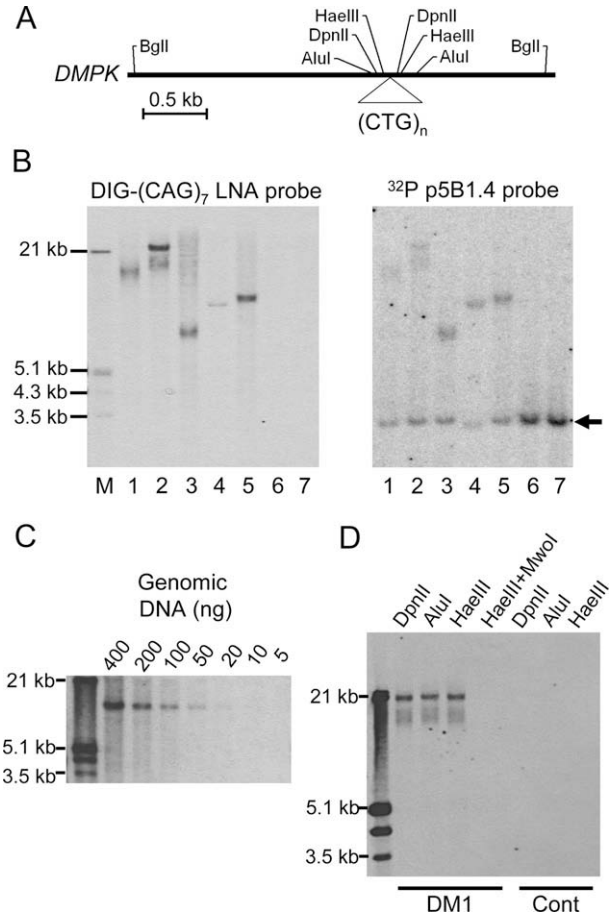

Fig. 1. (A) Restriction map of the DM1 locus. For each restriction enzyme, the cleavage site closest to the CTG repeat is indicated. (B) Southern blot analysis of genomic DNA digested by BglI. Left panel shows DNA probed with digoxigenin (DIG)-labeled $(CAG)_7$ locked nucleic acid (LNA) probe. Lane 1, DM1 skeletal muscle (300 ng of DNA); lane 2, DM1 heart (300 ng); lane 3, DM1 cerebellum (200 ng); lane 4, DM1 fibroblast cells containing 2000 repeats (300 ng); lane 5, congenital DM1 (CDM) heart (300 ng); lanes 6 and 7, normal leukocytes (300 ng); M indicates DIG-labeled molecular weight marker. Right panel shows the same DNA samples analyzed with ^{32}P-labeled p5B1.4 probe. Lane 1, DM1 skeletal muscle (4 μg of DNA); lane 2, DM1 heart (4 μg); lane 3, DM1 cerebellum (3 μg); lane 4, DM1 fibroblast cells containing 2000 repeats (4 μg); lane 5, CDM heart (4 μg); lanes 6 and 7, normal leukocytes (6 μg). The arrow indicates the normal *DMPK* allele (3.3 kb). (C) Southern blot showing serial dilution of genomic DNA obtained from congenital DM1 heart, hybridized with DIG-labeled $(CAG)_7$ LNA probe. Genomic DNA was digested with HaeIII. The repeat expansion is clearly detected in 50 ng of genomic DNA. (D) Southern blot of cardiac DNA digested with different restriction enzymes. Expanded alleles are almost same size after digestion with each of three different 4-base cutters (DpnII, AluI, and HaeIII). Signals were not detected after digest with MwoI, a restriction enzyme that cuts within the CTG tract. No signal was seen in control samples.

Next we used a $(CCTG)_5$ LNA probe to examine CCTG-repeat length in DM2. For these blots, genomic DNA was co-digested with two different 4-bp restriction enzymes in order to improve resolution (flanking sequence is reduced to 250 bp) and decrease background (most of the genome is reduced to small fragments). Southern blots showed expanded repeats in 500 ng of DNA extracted from peripheral blood cells from individuals with DM2 ($n = 17$), but not in healthy controls ($n = 7$) (representative blots