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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.

Keywords

Myotonic dystrophy; repeat length; RCA; LNA

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 to 10 µg of DNA for a conventional Southern blot [11] (equivalent to 15 to 30 mg of muscle tissue), and, in the case of DM2, somatic heterogeneity that is so extreme that that hybridization signals from expanded alleles can drop

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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).

Materials and methods

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, seventeen DM2, and fifteen normal individuals. These studies were approved by the local institutional review board. All study participants provided informed consent.

Southern Blot

Genomic DNA was digested with BglII, 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 hours at 6V/cm for RCA products, or on 0.5% gels for 24 hours at 1V/cm for genomic DNAs, and then transferred overnight onto Nylon Membranes (Roche) by alkaline transfer. Blots were fixed at 120°C for 20 minutes and then hybridized for 4 hours 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 washing 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 to 30 minutes exposure to BioMax XAR film (Kodak). For conventional Southern analysis, blots were hybridized with ³²P-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).

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 minutes, 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 hours. The RCA reactions were performed in a total volume of 30 µl containing 1mM dNTP, 0.3 µM primers, 1x phi29 buffer, 2x 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'-CACAGACCATTCTTTCTTTTCGGCCAGGCTG*A*G-3'; start primer 2, 5'-CATTCCTCGGTATTTATTGTCTG*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 phosphothioate bonds.

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 ³²P-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 ³²P-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.

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 are shown in Fig. 2B). Similar to DM1, expanded CCTG repeats were detected from as little as 50 ng of genomic DNA (Fig. 2C).

Previously we found that CTG repeat expansions were 2- to 13-fold greater in skeletal muscle biopsies than in peripheral blood cells from the same individual with DM1 [8]. It is unknown whether individuals with DM2 show similar somatic instability that leads to larger expansions in muscle than in leucocytes. To address this question, we examined DNA from needle muscle biopsy samples. The expanded repeats were greater than 36 kb (> 9000 repeats) in each of four DM2 muscles that we analyzed (two examples are shown in Fig. 2D). Although the gel system does not precisely quantify length of fragments in this size range, the results indicate that DM2 expansions are considerably larger in muscle than in leucocytes from the same individual.

These results support the feasibility of detecting large DM1 and DM2 expansions using CAG- or CCTG-repeat LNA probes. However, for moderately expanded repeats, such as those in DM1 peripheral blood, the signal-to-noise ratios would be lower due to fewer probe binding sites in the target allele and a higher background of short fragments from other genomic locations. In line with these expectations, we found that CTG expansions less than 750 repeats

were difficult to detect using CAG-repeat probes. We therefore carried out isothermal amplification to enrich for sequences at the DM1 locus prior to the Southern blot. Previously we reported that rolling circle amplification (RCA) with Phi29 DNA polymerase can amplify circular DNAs containing CTG repeats of up to 6 kb [16]. We digested genomic DNA and then added ligase under dilute conditions that favor the formation of DNA circles over concatamers. For the DM1 locus this produces a target circle that contains CTG repeats + 807 bp of *DMPK* flanking sequence. To enhance sequence selectivity of the amplification, the initial RCA was primed on the non-template strand, and then a biotinylated “capture primer” was added. The capture primer is complementary to the extension products from the initial primers, leading to formation of a highly branched extension product. These products were then pulled down using streptavidin-coated magnetic beads, and subjected to further isothermal amplification by addition of *DMPK*-specific sense and antisense primers plus fresh RCA reagents. The highly-branched product of the second amplification was cut to its unitary length by *AvrII*, and then analyzed by Southern blot using the DIG-labeled (CAG)₇ LNA probe. Distinct signals of expanded CTG repeats (up to 2500 repeats), in addition to the normal allele, were detected in DM1 samples, whereas no expansion signal was observed in normal samples (Fig. 3A, left). We confirmed specificity of the signals for the *DMPK* gene, by stripping and re-probing with ³²P-labeled *DMPK* probe (Fig. 3A, right). We also examined the minimum amount of initial DNA required for this RCA reaction. An expansion of 1300 repeats was effectively amplified and clearly detected from as little as 20 ng of cerebellum DNA (Fig. 3B).

Discussion

An important unanswered question in myotonic dystrophy, and in other repeat expansion disorders, is whether the onset or progression of symptoms depends on somatic instability of the expanded repeat. It would be useful to know, for example, whether stabilization of the repeat could prevent or delay onset of the disease. Myotonic dystrophy provides a unique opportunity to address this question because it is possible to obtain serial samples of muscle tissue during the life of an individual, including pre-symptomatic or later stages of the disease process. The enormous size of the expansion, however, creates a challenge for performing the genetic analysis.

For RNA analysis, the development of isothermal linear amplification has allowed scale-down of analysis procedures to a point where DM-related abnormalities can be assessed using minimally invasive muscle biopsy techniques. Here we have asked whether DNA analysis can be scaled down to a similar extent, which potentially could broaden the scope of DM research and improve the feasibility of serial sampling. For the highly expanded repeats that exist in skeletal muscle, we found that a 100-fold scale down can be achieved using LNA probes that target the repeat expansion. Moreover, it seems likely that additional sensitivity can be achieved with further refinements in probe design, hybridization conditions, and detection methods.

By using 4-base restriction enzymes to extensively cleave genomic DNA, and LNA probes that have improved ability to discriminate target versus off-target binding [15], we have detected DM1 alleles with high specificity using short probes comprised of triplet or tetramer repeats. As compared to conventional Southern blots, the current method has advantages of lower requirement for input DNA, improved resolution of fragment size due to reduced flanking sequence, simple hybridization and wash procedures, non-radioactive detection, and higher intensity of hybridization signals. Taken together, these characteristics may enhance the ability to make inferences about somatic heterogeneity from the distribution of hybridization signal in a single lane. For example, in samples of DM1 cerebella we observed highly heterogeneous expansions that we were unable to detect previously by conventional methods. Further investigation of this bimodal repeat expansion may provide additional clues about cell-specific differences in repeat instability. For samples that show a wide range of

expansion sizes, however, a method to correct the hybridization signal for greater probe binding to larger fragments may become necessary.

LNA-repeat probes also have several limitations. Expanded repeats detected by this method do not necessarily arise from the DM1 or DM2 loci. While this does not pose a limitation for analysis of somatic heterogeneity in individuals with known DM1 or DM2, the method is not appropriate as a stand-alone diagnostic test for DM1 or DM2, unless combined with some other method that is locus specific, such as, repeat-primed PCR [17]. Of course, this limitation could potentially be exploited to identify novel repeat expansions that cause muscular dystrophy or other genetic disorders. Another limitation is that with current conditions we were unable to detect DM1 expansions below a threshold of around 750 repeats, unless the target region for first enriched by rolling circle amplification. In DM2 we were unable to determine the lower limit of expansion size for detection by LNA-repeat probes because highly expanded alleles were observed in all leukocyte and muscle samples that we analyzed (n = 21, where the shortest expansion was 3000 repeats). Thus, while this method may eliminate the problem of false negative DM2 Southern blots due to extreme somatic heterogeneity [12], it is nevertheless possible that it may overlook DM2 expansions that are unusually short. An additional limitation is that LNA-repeat probes will not be sensitive to CCG and CTC interruptions in DM1 expansions [18] unless the restriction enzymes are specifically selected to cleave within these regions.

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Figure 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 ³²P-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.

Figure 2.

A Restriction map of the genomic locus encompassing the CCTG repeat in *ZNF9*.

B Southern blot of leukocyte DNA from DM2 patients and controls using DIG-labeled $(CCTG)_5$ LNA probe. Five hundred ng (*left*) or three μ g (*right*) of genomic DNA digested with AluI and HaeIII were loaded in each well. Lanes 1–4 are from patients with DM2, lanes 5–7 are from healthy controls. Asterisk indicates nonspecific signal, M indicates DIG-labeled molecular weight marker.

C Southern blot showing serial dilution of DM2 leukocyte DNA hybridized with DIG-labeled $(CCTG)_5$ LNA probe. The repeat expansion is detected in 50 ng of genomic DNA.

D Repeat length comparison between muscle and leucocyte DNA from two patients with DM2. The repeat size was considerably larger in muscle than blood for both patients.

Figure 3.

A Southern blot analysis of rolling circle amplification (RCA) products, probed with DIG-labeled (CAG)₇ LNA probe (*left*), or ³²P-labeled DMPK probe (*right*). Lane 1, DM1 fibroblast cells containing 900 repeats; lane 2, DM1 cerebellum; lanes 3 and 4, DM1 leukocytes from two patients; lanes 5, 6, and 7, leukocytes from three normal controls. Arrows indicate normal alleles (807 bp plus repeats). M, DIG-labeled molecular weight marker.

B Southern blot of RCA products from serially diluted template DNA of DM1 cerebellum. The products were probed with DIG-labeled (CAG)₇ LNA probe. Expanded repeats are observed in the RCA products from 20 ng of template DNA.