

ORIGINAL ARTICLE

Replacement of the myotonic dystrophy type 1 CTG repeat with 'non-CTG repeat' insertions in specific tissues

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ABSTRACT

Background Recently, curious mutations have been reported to occur within the (CTG)_n repeat tract of the myotonic dystrophy type 1 (DM1) locus. For example, the repeat, long presumed to be a pure repeat sequence, has now been revealed to often contain interruption motifs in a proportion of cases with expansions.

Similarly, a few de novo somatic CTG expansions have been reported to arise from non-expanded DM1 alleles with 5–37 units, thought to be genetically stable.

Aims and methods This study has characterised a novel mutation configuration at the DM1 CTG repeat that arose as somatic mosaicism in a juvenile onset DM1 patient with a non-expanded allele of (CTG)₁₂ and tissue specific expansions ranging from (CTG)₁₁₀₀ to 6000.

Results The mutation configuration replaced the CTG tract with a non-CTG repeat insertion of 43 or 60 nucleotides, precisely placed in the position of the CTG tract with proper flanking sequences. The inserts appeared to arise from a longer human sequence on chromosome 4q12, and may have arisen through DNA structure mediated somatic inter-gene recombination or replication/repair template switching errors. De novo insertions were detected in cerebral cortex and skeletal muscle, but not in heart or liver. Repeat tracts with –1 or –2 CTG units were also detected in cerebellum, which may have arisen by contractions of the short (CTG)₁₂ allele.

Conclusion This non-CTG configuration expands current understanding of the sequence variations that can arise at this hypermutable site.

INTRODUCTION

Myotonic dystrophy type 1 (DM1) is caused by the expansion of a CTG repeat located in the 3'-UTR of the *DMPK* gene on chromosome 19 (19q13.3). DM1 is inherited in an autosomal dominant fashion with disease-causing expansions of >50 up to 6550 repeats arising in one allele.¹ Expansion of the unstable repeat occurs through consecutive generations and in somatic tissues during the lifetime of an affected individual. However, the short non-expanded allele is genetically stable. The mechanism(s) involved in a non-expanded CTG repeat tract (5 to 35 units) reaching the status of a highly unstable sequence (>50 units) is poorly understood. Occasional expansion biased mutations of non-expanded CTG alleles in *DMPK* gene have been reported,^{2–4} usually involving the high

end of the normal size range (19–37 repeats), although in one recent case the normal allele that expanded was only 12 repeats expanding to >50 repeats.⁴ Aside from repeats expansions that arise through additions of more CTG units, the DM1 CTG tract has been shown to contain interspersed non-CTG units.^{5–7} Somatic mosaicism of the HD CAG tract (simultaneous presence of (CAG)₂₀, (CAG)₃₇ and (CAG)₄₇) has been reported.⁸ These rare events in DM1 and Huntington's disease (HD) have in some instances been detected in specific tissues.^{3 4 8} However, it should be noted such events are rare and alternative explanations, such as contamination, were not ruled out, and hence can be described as 'putative' CTG/CAG changes. Here, we report the presence of aberrant sequences that replace the CTG tract at the DM1 locus. These non-CTG configurations arise as somatic events in specific tissues, and appear to be derived from a region of chromosome 4.

METHODS

DNA extraction

UR-ADM9 DNA from different tissues was extracted as described in López Castel *et al.*¹

DNA amplification and electrophoresis

Amplification through the *DMPK* CTG repeat tract in UR-ADM9 samples was performed using PCR primers 409 (5'-GAAGGGTCCTTGTAGCCGGGAA-3') and 407 (5'-CAGAGCAGCGTCATGCACA-3') at an annealing temperature of 67°C for 2 min, over 30 cycles. Four per cent non-denaturing acrylamide gels and 1% agarose gels were run to resolve the PCR products.

Amplification using the primers designed based on the areas of homology found between the non-CTG insert in the skeletal muscle and cortex (3PC: 5'-CACCGGGTGGGTTACACC-3' and 5PSC: 5'-CTGGCCTTAGCCACGCCAC-3') was performed at an annealing temperature of 63° for 45 s, over 30 cycles. Products were run on a 1% agarose gel.

Sequencing

Chosen PCR products were sent routinely to the sequencing facility of The Hospital for Sick Children (The Centre for Applied Genomics) after a step of gel excision/cleaning (QIAquick PCR purification kit, Qiagen, Valencia, California, USA) and cloning (TOPO-TA 4.0 cloning kit, Invitrogen, Carlsbad, California, USA). A minimum of 10

clones were performed for each PCR product analysed. Each sequence was proofread against the electropherogram to avoid base misspelling.

DNA alignment and sequence location

Non-repetitive DNA sequences present in the repeat location were aligned against human, bacteria (*Escherichia coli*), and TOPO vector DNAs using the Blast2Align tool available at <http://www.ncbi.nlm.nih.gov/>.

DNA sequences found from the insert-specific primer PCRs were searched against the human genome using the BLAST tool at <http://www.ncbi.nlm.nih.gov/>.

Methylation status

The methylation status of the allele on which the non-CTG insert is located was carried out using *Sac*II digestion followed by multiplex PCR (as described in López Castel *et al*¹); 200 ng of UR-ADM9 patient samples were digested with 10 units of *Sac*II over night, then subjected to a multiplex PCR protocol. Multiplex amplification across the *DMPK* CTG repeat tract was performed using PCR primers 409 (5'-GAAGGGTCCTTG TAGCCGGGAA-3'), CTCFIa (5'-CTGCCAGTTCACAACCGCTCCGAG-3') and CTCFIIB (5'-AAAGCAAATTTCCCAGTAAGCAGGC-3'). Four per cent non-denaturing acrylamide gels were used to resolve PCR products.

RESULTS

During our analysis of CTG repeat length variations between DM1 patient tissues¹ we found that, while characterising the non-expanded allele by PCR, several aberrant PCR products arose in several tissues of a DM1 patient. The aberrant PCR products, in addition to the main PCR product, arose after the amplification of the short *DMPK* allele of a 44-year-old-woman (UR-ADM9) diagnosed with a juvenile form of DM1 disease (figure 1). This individual had expanded alleles ranging from 1100 to 6000 repeats.¹ The appearance of unexpected PCR products suggests their formation in only a fraction of the cells from the tissues affected. The detection was achieved in cerebral cortex, skeletal muscle, and cerebellum of the UR-ADM9 patient during the *DMPK* alleles repeat sizing in several tissues from up to eight different DM1 affected individuals (figure 1A).¹ The additional PCR fragments detected in the cortex and muscle were larger than the characterised (by sequencing) (CTG)₁₂ repeat length short allele, and shorter than that in the cerebellum. Such mosaicism in the shorter ranges was not detected in other tissues of the same individual nor in several tissues in other DM1 individuals (figure 1B).

Initially several faint extra PCR amplified fragments were detected in cerebral cortex, skeletal muscle, and cerebellum when resolved on non-denaturing acrylamide gels, which separates based upon both molecular weight and DNA secondary structure (figure 1A). The same PCR products resolved in agarose electrophoresis, relatively insensitive to structure (figure 1C), which suggested that a single additional product was present in these tissues with the largest in the cortex and the smallest in the cerebellum, while the other bands on acrylamide gels were likely heteroduplexes of the two differently sized PCR products.⁹⁻¹¹ Sequence analysis of these additional PCR products from all three tissues (see Methods) showed that six of 15 clones from cortex (40%) and six of 13 clones from skeletal muscle (46%) contained, as expected, larger sequences (a total of 63 and 46 base pairs, respectively) than the non-expanded repeat tract ((CTG)₁₂=36 base pairs). Strikingly, the sequences of these variants were completely devoid of CTG repeats except for the

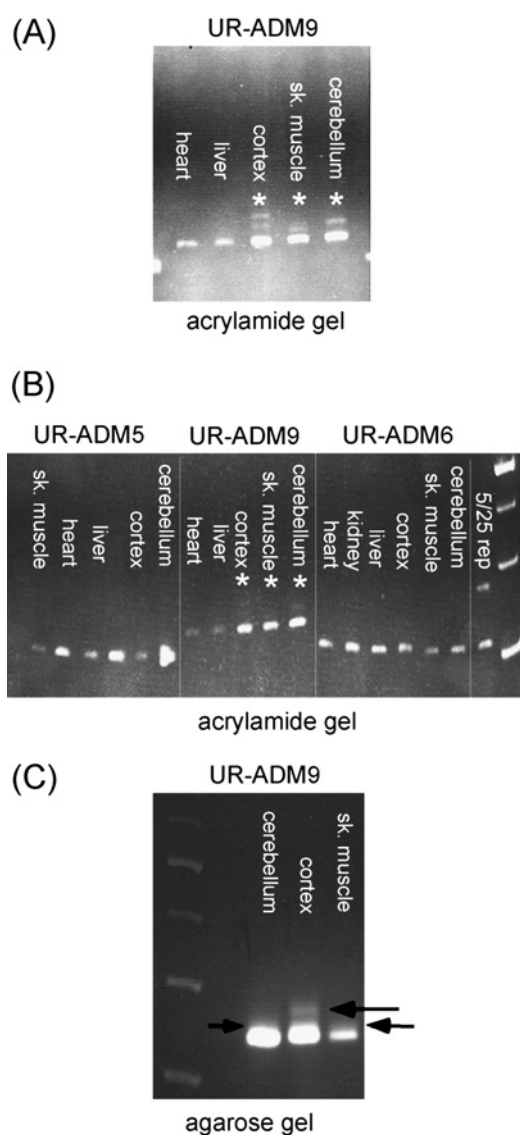


Figure 1 Detection of extra bands after short myotonic dystrophy type 1 (DM1) allele PCR amplification in UR-ADM9 DM1 patient. Products of PCR amplification (30 rounds) were analysed by: (A) Non-denaturing acrylamide gel showing additional bands in cortex, skeletal muscle and cerebellum in UR-ADM9 patient. (B) No detection of extra bands in other DM1 patients from the same study⁶ (*detection of extra bands); (C) Agarose gel in UR-ADM9 cerebellum, cortex, and skeletal muscle tissues to compare the appearance of additional bands versus acrylamide gel methodology.

first 5'-CTG unit (figure 2A). In the cerebral cortex the complete 5'-(CTG)-3' appeared to have been replaced with a sequence containing the first CTG followed by a 60 base 5'-CTG-repeat-free sequence (figure 2A). In the muscle the complete 5'-(CTG)-3' appeared to have been replaced with a sequence containing only the first CTG followed by a 43 base 5'-CTG-repeat-free sequence (figure 2A). Interestingly, a comparison between the muscle or cortex insertion sequences revealed high levels of sequence identity at the beginning and end: complete alignment (100%) of the first 15 bases and strong alignment (81%) in the last 16 bases (13 of 16) (figure 2B). Inserts began with a CTG and ended with a TG, presumably belonging to the first and last CTG units. The flanking *DMPK* sequence also PCR amplified in these samples was perfectly aligned with the known gene sequence (GeneID 1760; figure 2A) in all 28 clones. The rest of

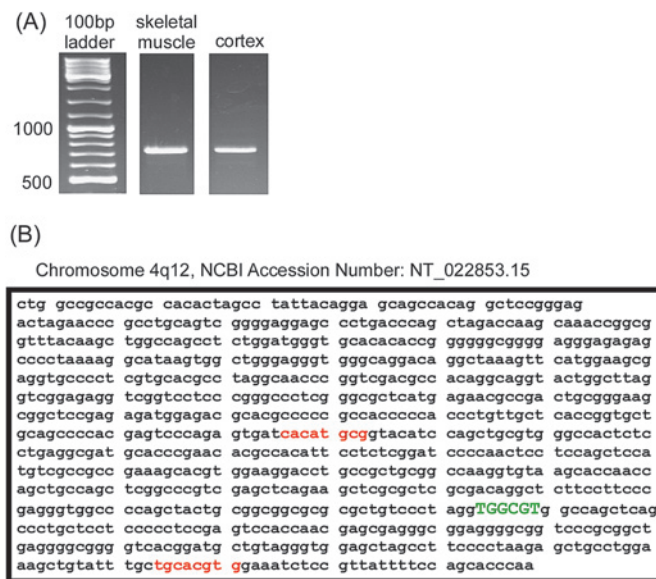


Figure 3 Characterisation of the PCR products of the insert specific designed primers. (A) PCR amplification of UR-ADM9 skeletal muscle and cortex DNA with the primers designed from the homology between the non-CTG insert in both tissues revealed a product approximately 800 bp in length. Primers were imperfectly hybridised at the ends of the sequence. (B) Sequencing and a BLAST search determined that the PCR product came from chromosome 4q12. Red text indicates locations of purine/pyrimidine tracts able to form Z-DNA.¹⁶ Bold green text shows a polymerase α mutation hotspot site.^{17–18} Mutant sequences were deposited to GenBank (accession numbers JF697199, JF697200 and JF697201).

a total sequence of 858 bp (figure 3B). Sequencing was carried out on both the clones of direct PCR products of skeletal muscle, cortex, and cerebellum, as well as the gel purified and cloned PCR products, with three clones of each separate tissue having been sequenced. Each clone contained at least a partial sequence, with a BLAST search revealing that each sequence mapped to the same location within the human genome (chromosome 4q12) (accession # NT_022853.15).^{19–21} The sequence contained motifs capable of forming unusual DNA structures, including Z-DNA (figure 3B¹⁶), as well as containing a pol- α mutation hotspot site,^{17–18} which may suggest a tendency to be recombinogenic, thereby permitting it to be inserted into the DM1 CTG repeat tract region.¹⁵

Although close in size with the short repeat allele, we initially cannot discard the expanded allele as the source of these aberrant products. To determine whether the non-CTG insertions arose on the expanded or non-expanded DM1 alleles, we took advantage of known epigenetic marks specific to the expanded allele. Initially we considered using the adjacent markers that could discern DM1 alleles. Unfortunately, in patient ADM9 the upstream *Bpm1* polymorphism in exon 10²² is homozygous and the downstream *Cac8I* polymorphism in exon 3 of the *Six5* gene that is in complete linkage disequilibrium with the DM1 CTG expansion²³ is very far (~5 kb) from the CTG repeat and rich in G+C content, making PCR amplification impossible. However, we recently demonstrated the presence of CpG methylation on the expanded but not the non-expanded allele in many DM1 patient tissues.¹ In patient UR-ADM9 the cortex showed high levels of CpG methylation upstream of the CTG repeat spanning the adjacent methyl-sensitive *SacII* restriction site. However, the cerebellum was relatively free of methylation. We used the methyl-specific multiplex PCR assay that we had

devised to assess the methylation status of the *SacII* site 42 bp upstream of the CTG tract (figure 4A). Forward and reverse primers were placed on opposite sides of the CTG repeat, and therefore were only capable of amplifying across the non-expanded CTG tracts, as well as the short non-CTG insertions. (No primer sets were able to amplify across the expanded allele due to the very large expansions they harboured; skeletal muscle (with (CTG)=3700–4300), cortex (with (CTG)=4000–5500), and cerebellum (with (CTG)=1100–1500 and 3800–4600) of this individual¹). Using two upstream primers, one that did and one that did not cover the *SacII* site, permitted determination of the methylation status of the *SacII* site in the non-expanded allele (figure 4A). PCR amplification revealed that in the absence of *SacII* digestion two major PCR products, both from the non-expanded allele, were evident. Also evident were the slower migrating products, representing the non-CTG insertions in the muscle and cortex DNAs and the CTG length stutter products in the cerebellum (figure 4B, see grey arrowhead). Successful *SacII* digestion before PCR amplification, an indication of a non-methylated template, considerably reduced the production of the PCR products that encompassed the *SacII* site. The loss of the non-expanded CTG allele, the non-CTG insertion, and the CTG stutter PCR products supports the conclusion that the DNA templates from which these were amplified were not methylated at the *SacII* site.

Together these results could suggest that the non-CTG insertions arose on the non-expanded DM1 allele or that they arose on the subset of expanded alleles that was free of methylation or that some time following insertion the expansion-specific methylation was lost.

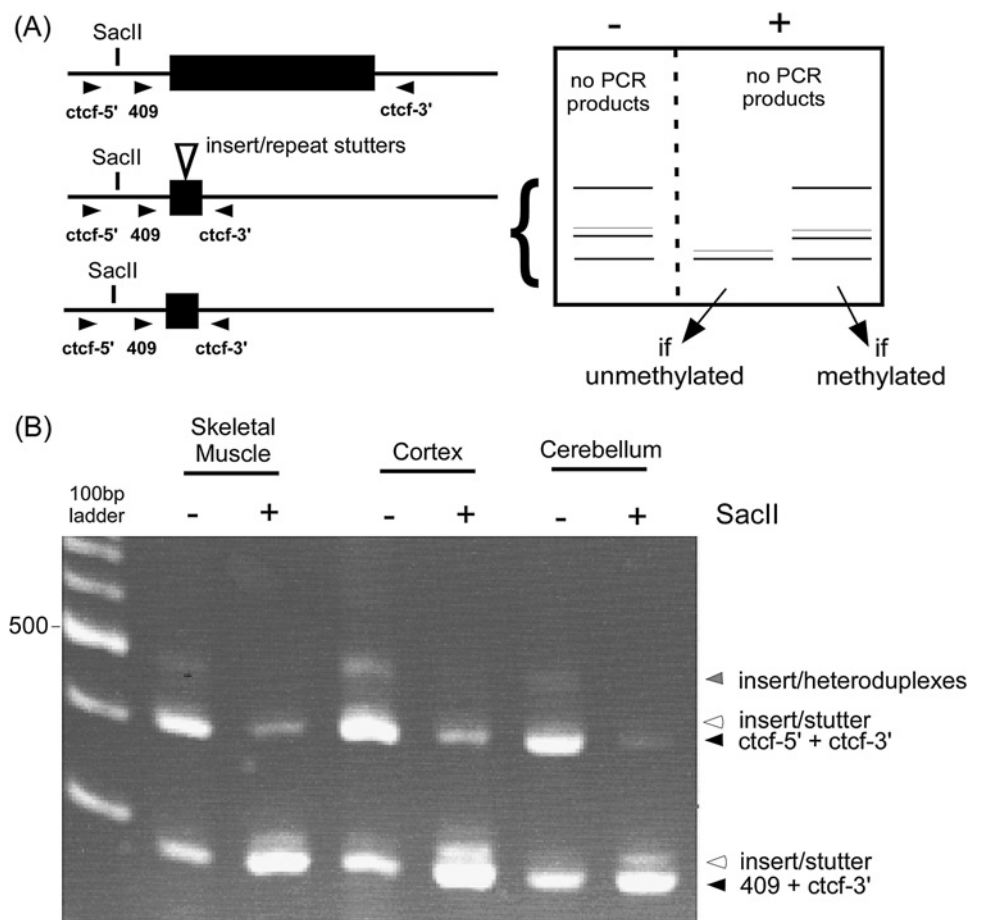
DISCUSSION

In this study we have observed a DM1 allele that has completely lost the CTG tract, retaining only the first CTG and the TG of the very last CTG unit. Previously, the complete loss of all CGG repeats at the *FMR1* gene was reported, but this also incurred loss of flanking sequences, and appeared to have occurred during transmission.²⁴ In our DM1 sample the loss of the CTG tract did not incur any changes in flanking sequences and arose as a somatic insertion mutation. Insertions into repetitive sequences have not been reported; however, SCA31 was recently reported to have arisen through the insertion of an unstable repetitive element.²⁵ It is noteworthy that there are several reports claiming apparent DM1 allele length mosaicism, that have been interpreted to be the result of small CTG length alterations of the non-expanded allele.²⁶ However, sequence analysis of these presumed CTG length changes was not performed. Our findings suggest other reports of presumed CTG or CAG length changes^{2–4 26–28} may actually be insertions at the repeat of non-CTG sequences, much as we described herein. Thus, the insertion mutations we report here may be more common than suspected.

It is curious that these non-CTG somatic insertions arose in some, but not all, tissues. Previously, somatic CTG expansions were present at higher levels in the cardiac muscle than in peripheral blood mononuclear cells.⁴ Tissue preference for somatic length heterogeneity at the DM1 repeat and the HD locus has been reported.^{8 27} We previously reported, for this same juvenile onset DM1 individual, a range of heterogenous CTG expansions in the heart (with (CTG)=4100–5300 and 6000), liver (with (CTG)=4200–4600), skeletal muscle (with (CTG)=3700–4300), cortex (with (CTG)=4000–5500), and cerebellum (with (CTG)=1100–1500 and 3800–4600) for this individual.¹ The presence of non-repeat insertions in cortex and muscle, and

Mutational mechanisms

Figure 4 Assessment of methylation allele specificity. (A) *SacII* methylation sensitive digestion combined with multiplex PCR amplification was performed, using two upstream primers and one downstream primer to produce two PCR products from only the non-expanded CTG allele, that either did or did not encompass the methylation sensitive *SacII* site present upstream of the CTG tract. The insert, or stutter PCR products of the skeletal muscle, cortex, or cerebellum, are visible as lighter grey bands in the schematic. (B) Following *SacII* digestion the elimination of only the PCR product encompassing the *SacII* site revealed that this site on the allele in which the non-CTG insert is found was not methylated in either the cortex or the skeletal muscle. The same is true of the stutter found in the cerebellum sample.



limited CTG length variations of the short allele in cerebellum, with absence of variant short products in the heart and liver, does not offer any obvious pattern. While such variant DM1 configurations may be present at extremely low levels in other tissues, they are not detectable using the bulk PCR methods used herein. The significance, if any, of the tissue selections is unknown.

The frequency and timing of these aberrant insertions is unclear. We speculate that the unique nature of the mutant alleles makes it extremely unlikely that these mutations occurred more than once in the lifetime of the patient. Likewise, *de novo* mutations were detected in cerebral cortex and skeletal muscle, but not in heart or liver. Only two mutant alleles were detected, and although they must have occurred *de novo* at some point, it remains unclear if they actually occurred in those tissues or in a precursor lineage at an earlier developmental stage. The level of mosaicism of such novel species has not been determined rigorously. However, considering that they are readily detectable using the bulk PCR methods applied herein, we suggest that their occurrence is likely to be around 10% of the cells in the cortex and muscle.

As to how the DM1 insertion mutations arose we can only speculate. Such events may have arisen on the expanded or the non-expanded alleles, through events involving only one or both alleles. Short size alterations may have arisen through insertions into the non-affected CTG allele, or through catastrophic contractions and insertions into the expanded allele. Previously, a germline contraction of the expanded DM1 allele to non-affected lengths was reported,²⁹ a transmission event that appeared to arise by recombination between the two DM1 alleles. Similarly, somatic mosaicism of the HD CAG tract was shown to arise by the contraction of the expanded (CAG)47 to

(CAG)37 rather than by the expansion of the normal (CAG)20 allele.⁸ While we are unable to definitively assign which allele incurred the non-CTG insertions, our analyses—considering the facility of the expanded repeat to form slipped-DNA structures³⁰ and the absence of CTG region mutations in non-DM1 individuals—suggest that these insertions arose on the CTG expanded allele. Recent studies in yeast have suggested that other expanded repetitive elements can facilitate gene conversion events and such events may be facilitated by unusual DNA structures, double- or single-strand breaks, and the capturing of single-strand regions.³¹ Another possible mechanism is a template switching event, where a replication fork at the expanded CTG tract may arrest, and through multiple microhomology mediated template switches with the chromosome 4 region (or other), may incur a series of insertions and aberrant nucleotide incorporations—resulting in a novel sequence at the DM1 CTG tract region.^{32–33} Such obscure mutation configurations, although rare, have been observed in model systems and may involve unusual DNA structures.^{17–18 31–35} The likely source of the DM1 insert sequence on chromosome 4 is enriched in G+C content and can form unusual DNA structures, with this region having also been associated with sequence deletions in tumours.^{19–21} The insertion at the DM1 repeat in two different tissues with sequence arising from the same region of chromosome 4 argues for the close nuclear localisation of this region with the DM1 region of chromosome 19. As to whether such events can lead to the non-CTG insertions observed here remains to be determined.

In conclusion, we detected a CTG-free configuration at the DM1 CTG repeat location that completely replaced the CTG tract. These non-CTG configurations arise as somatic events in

specific tissues, and appear to be derived from a region of chromosome 4. Strikingly, these de novo mutations did not contain the CTG repeat. The DM1 repeat tract has recently been described as presenting configurations distinct from the pure CTG trinucleotide motif. Interruptions by non-CTG repeat units like CCG and GGC through the DM1 CTG tract have been linked with altered CTG repeat stability.^{5–7} However, like those interruptions, it is unknown if the non-repeat configuration observed herein is a product of instability of the larger pure tract. Interruptions in the DM1 tract have been suggested in one family to be associated with altered clinical presentation.⁷ The impurity of the repeat will essentially deplete CUG repeats within the toxic *DMPK* transcript and may well affect its ability to bind and sequester RNA splicing factors such as MBNL1 which specifically binds CUG, as has been suggested.⁷ Similarly, *DMPK* transcripts with a complete absence of CUG repeats, as in the alleles observed herein, would also be expected to have altered toxic effects (if any). While the clinical significance of these distinct configurations of the DM1 repeat tract awaits future analysis, their existence advises against presuming a pure repeat when interpreting changes in nucleotide sizes of the DM1 CTG region.

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Competing interests None.

Ethics approval This study was conducted with the approval of the University of Rochester.

Contributors All experiments were performed by MMA and ALC.

Provenance and peer review Not commissioned; externally peer reviewed.

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