Myotonic dystrophy is associated with a reduced level of RNA from the DMWD allele adjacent to the expanded repeat

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Myotonic dystrophy is caused by the expansion of a CTG repeat sequence. The mechanism by which this expanded repeat produces the pathophysiology of myotonic dystrophy is not clear. It has been shown previously that expansion of the repeat produces allele-specific effects on transcripts from two genes, DMPK and SIX5. We have examined the effect of repeat expansion on the level of RNA from a third gene, DMWD. We have identified a polymorphism in this gene and developed a quantitative allele-specific assay for DMWD RNA levels, which we have applied to nuclear and cytoplasmic fractions of RNA from DM cell lines. We have found that the level of the DM-associated allele in the cytoplasm of DM cell lines is reduced by 20–50% compared with the wild-type allele, similar to the level of reduction found for SIX5 in allele-specific analysis. However, no such reduction is observed in RNA from the nuclear fraction of DM cell lines. This may reflect the complex nature of processing transcriptional units at the DM locus.

INTRODUCTION

Myotonic dystrophy (DM) is a complex neuromuscular disorder with an incidence of 1 in 8000 births. The clinical symptoms in adult patients are extremely variable. Mildly affected patients suffer from cataracts and myotonia while the more severely affected present with muscle weakness, cardiac abnormalities and diabetes. Congenitally affected DM patients display the most severe symptoms, which include mental retardation and sterility in males (1). Generally, there is a tendency for the disease to appear earlier in the female population, the repeat number varies between five and 37 copies. However, in DM individuals the repeat number is increased, with the mildly affected patients possessing 50–100 repeats and the more severely affected individuals having expansions extending to thousands of repeats (5).

The genetic mechanism by which the expanded trinucleotide repeat gives rise to the pathophysiology of DM remains undetermined. One possibility is that the repeat sequence (at either the DNA or RNA level) may interact with nuclear factors in trans. An alternative possibility is that repeat expansion affects the expression of DMPK and neighbouring genes. The DM interval is gene rich and we have shown that there are at least six genes within a 200 kb region surrounding the DM-associated repeat (6). The two genes flanking DMPK are SIX5 (previously called DMAHP), a homeodomain protein encoding gene (7) which is located immediately downstream of the triplet, and DMWD (previously called 59) (8), which is located immediately upstream of DMPK. These three genes are located close together, within an interval of 40 kb. Northern analysis has demonstrated stronger expression of DMWD (8) and its murine homologue (9) in brain and testis, thus raising the possibility that this gene may be responsible for the testicular atrophy and mental retardation which are symptoms of DM.

Allele-specific levels of SIX5 expression in DM cells and tissues have been measured in two studies (10,11), which exploited a coding polymorphism in exon C of this gene. Klepert et al. (10) reported that the level of SIX5 expression from the DM-associated allele was reduced to levels of ~20%, while Thornton et al. (11) demonstrated 20–60% reduction in the amount of RNA from the affected allele. In contrast, quantification of the gross levels of SIX5 RNA expression in DM and control cell lines revealed no significant differences (12). Gross level quantification of DMWD RNA expression also failed to demonstrate differences between the DM and control fibroblast cell lines (12).

The finding by others of allele-specific differences in SIX5 expression prompted us to re-examine the quantification of DMWD RNA using an allele-specific assay. We have identified a polymorphism in the 3′-UTR of DMWD and developed an assay that allows allele-specific quantification of DMWD RNA levels. In view of the difference in nuclear/cytoplasmic distribution of the wild-type and expanded repeat-containing transcripts of DMPK, we have also examined whether RNA from DMWD showed a similar distribution. Prior to quantification of DMWD transcripts, we fractionated the cells into nuclear and cytoplasmic compartments and compared the levels of RNA from each allele in each compartment. Thus, we have shown that in the cytoplasmic fraction of DM cell lines the level of RNA from the expanded repeat allele of DMWD is

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of the allele-specific primers, R-59G, was designed to be 13 bp longer compared with the level of DMWD RNA from the wild-type allele.

RESULTS

Identification of a G→T transition in DMWD

Single-strand conformation polymorphism (SSCP) analysis was used to screen DMWD for sequence variation using DNA from 27 healthy individuals. A single polymorphism in the 3'-UTR of DMWD was identified. This appeared to represent a bi-allelic system and two SSCP variants from each putative homozygous class were sequenced. The difference between these samples was a G→T change at nucleotide 2448 of the published sequence (GenBank accession no. L19267) (Fig. 1). The allele frequencies in British controls were 70.5% T and 29.5% G (14 individuals were TT, 10 GT and three GG). The DMWD DM-linked allele was determined following allele typing of one nuclear DM family and 102 DM patients. Among patients, the allele frequencies were 84% T and 16% G (69 patients were TT, 33 GT and none was GG). The DMWD DM-linked allele was shown to be a restriction enzyme site that could be used for the allele-specific quantification of DMWD expression. It was necessary, therefore, to develop an alternative assay. We employed two allele-specific oligonucleotides (59T and 59G) and one non-specific oligonucleotide (57L) for the PCR. The allele-specific primers differ at the terminal 3' base. Primer 59T has an A residue at the 3' end and only detects the 'T' allele. Primer R-59G has a C residue at the 3' end and detects the 'G' allele. To distinguish between the different PCR products, one of the allele-specific primers, R-59G, was designed to be 13 bp longer at the 5' end, thus allowing amplification of a larger product during the PCR. The longer primer was further destabilized by the addition of mismatches within the extended region. Thus, following the PCR, the ‘T’ allele product is 186 bp, whereas the ‘G’ allele product is 199 bp. The primer sequences are listed in Materials and Methods. A range of primer concentrations were tested on genomic DNA from both homozygotes and heterozygotes in an attempt to optimize the amplification of alleles without false priming. We found that when primer concentrations were adjusted to R-59G at 7 pmol (0.14 µM), 59T at 25 pmol (0.5 µM) and 57L at 10 pmol (0.2 µM), the amplification was allele specific. Figure 2a shows the PCR amplification of DNA from two different homozygotes and a heterozygote using these primer concentrations. The heterozygote result indicates that the amplification efficiency is the same for both alleles. Furthermore, to examine the sensitivity of the assay with different amounts of each allele in the template DNA, we performed a mixing experiment in which heterozygotes were reconstituted with different proportions of the two homozygote classes. PhosphorImager measurement of band intensities from gels such as in Figure 2b demonstrated that the proportion of each allele amplified reflected accurately the amount of DNA from each of the homozygote classes (data not shown).

Prior to the quantification analysis of RNA from both SIX5 and DMWD, preliminary experiments were performed to determine that the amplification was exponential at 26 and 28 cycles, respectively (data not shown). Complete digestion of the SIX5 PCR product following amplification as described above was also confirmed.

RNA expression of the DM-associated DMWD allele in DM fibroblast cell lines

Having established a sensitive and robust assay for the allele-specific quantification of DMWD, we analysed the relative abundance of RNA from each allele in DM and normal fibroblast cell lines. In view of the finding that DMPK transcripts from the normal and DM chromosome show different distributions in the nuclear and cytoplasmic compartments of DM tissues and cell lines (12,15), we have partitioned cells prior to RNA extraction and DMWD analysis. Three control and four DM individuals were used. Within the nuclear fraction no difference in the ratio of the RT-PCR products from the two alleles was found. However, within the cytoplasmic fraction, the level of the amplified product
derived from the DMWD DM-associated transcript was clearly reduced. Compared with the non-DM allele, RNA from the DMWD DM-associated allele was reduced by 20–50% (P < 0.024, two-sample t-test). There was no obvious correlation between the level of reduction and the length of repeat expansion. Figure 3a shows the results for allele-specific RT–PCR analysis of the RNA from the nuclear and cytoplasmic fractions of fibroblast cell lines. The quantification of bands from duplicate RTs, each set up as triplicate PCRs, are presented separately for each cellular compartment in Figure 3b.

The DM-associated SIX5 allele is reduced in DM fibroblast cell lines

Since the results of our previous gross level quantitative analysis of SIX5 in fibroblasts (12) differed from those obtained by Thornton et al. (11) and Klesert et al. (10), we re-analysed the same DM samples we had used previously and exploited the SIX5 coding sequence polymorphism (11) in our allele-specific analysis. This polymorphism, a C→T transition at nucleotide 4143, is located within exon 3 of SIX5 (GenBank accession no. X84813) and eliminates a Cac8I restriction site in the less common allele. Complete linkage disequilibrium was demonstrated for the more common allele of SIX5 (Cac8I-digested allele) with the DM mutation (11). Six DM cell lines and one control were informative for this polymorphism. The relative abundance of RNA from each allele was assessed in both the nuclear and the cytoplasmic fractions of these cell lines using quantitative RT–PCR. Following the amplification of a 251 bp PCR fragment with primers Cac81R and Cac81L2, the product was digested with Cac8I restriction enzyme to allow differentiation between the two alleles (251 and 101 bp). Figure 4a shows an example of the allele-specific analysis of transcripts from SIX5 in the nuclear and cytoplasmic fractions. Figure 4b shows the combined results from two RTs with three separate amplifications of each. These findings are broadly similar to those reported by Thornton et al. (11) and Klesert et al. (10), with a 20–50% reduction in the RNA level from the SIX5-associated allele. It appears that, in the cytoplasmic fraction of fibroblasts, the level of reduction in RNA from the DM-linked allele of SIX5 is similar to the level of reduction observed for RNA from the DM-linked allele of DMWD. The reduction in level of the DM-linked SIX5 allele is greater for samples DM2, DM4, DM5 and DM6, which have repeat sizes between 3 and 5.5 kb, compared with samples DM1 and DM3, which have expansions of 240 bp and 1.2 kb, respectively. Clearly, the results for the nuclear fractions differ between DMWD and SIX5.

Both DMWD and SIX5 are affected in the same DM patient

In order to directly compare the effect of repeat expansion on both SIX5 and DMWD cytoplasmic RNA, we screened for individuals who are doubly informative for the polymorphisms at both loci. Only one DM fibroblast cell line (DM10) was informative for both of the DMWD and SIX5 polymorphisms. Allele-specific analysis of transcripts from both genes was performed separately within the cytoplasmic RNA fraction of DM10 and compared with RNA from a control cell line. The results of this analysis are shown in Figure 5. The degrees of reduction in the cytoplasmic levels of RNA from the DM-associated alleles (allele 2) of both DMWD and SIX5 for patient DM10 are very similar. There is a 50% reduction in the RNA level of the DM-linked alleles of both genes. This is the first demonstration that following repeat expansion within a DM patient, the expression of both genes may be similarly affected.

DISCUSSION

The primary objective of the work described here was to examine the effect of repeat expansion on the level of DMWD RNA using an allele-specific assay. We provide evidence that repeat expansion affects the levels of DMWD RNA from the DM chromo-
Human Molecular Genetics, 1999, Vol. 8, No. 8

Some. This finding is consistent with data from other groups showing that expansion of the CTG repeat could affect the expression of adjacent genes through changes to local chromatin structure. Wang et al. (16) found that the CTG repeat creates a very strong nucleosome positioning signal which increases with repeat length expansion and this may profoundly alter the chromatin structure (16,17). Otten and Tapscott (18) showed that the presence of large repeat expansions eliminate a DNase I hypersensitive site which is located 3' of the triplet repeat sequence. Hence, it is possible that the accessibility of nuclear proteins, such as transcription factors, to the interval adjacent to the repeat could also change, affecting the control of gene expression. Other studies have shown that multiple hairpins are likely to occur on CTG repeat-containing stretches while the DNA is unwound for transcription (19). Consequently, if a complex structure on the single-stranded DNA is encountered, the polymerase may stall and possibly dissociate.

The allele-specific quantification of DMWD RNA levels within the nuclear fractions of DM fibroblast cell lines indicates that there is no difference in the relative amount of RNA from each allele. However, quantification of cytoplasmic RNA reveals a relative reduction in the level of RNA from the DM-linked DMWD allele. The level of reduction ranges between 20 and 50% when compared with the non-DM allele. In contrast, for SIX5 there is a reduction in the relative amount of RNA from the DM-associated allele in both the nuclear and cytoplasmic compartments of DM fibroblast cell lines. The relative reduction in the level of the DM-linked SIX5 transcript also ranges between 20 and 50%, similar to that found for DMWD in the cytoplasm. A cell line from one of our DM patients is doubly informative for polymorphisms in DMWD and SIX5. Within the cytoplasmic fraction of this cell line DMWD and SIX5 RNA levels are reduced to a similar extent.

For the quantification of SIX5, the four samples with larger expansions (3–5.5 kb) showed a greater allele-specific reduction than did the two samples with smaller expansions (240 bp–1.2 kb). There was no obvious correlation between repeat length and level of DMWD reduction, based on the analysis of

Figure 4. Allele-specific analysis of SIX5 transcripts. (a) Phosphorimages of the RT–PCR products of the two SIX5 alleles from the nuclear and cytoplasmic fraction of control and DM cell lines. Samples are presented as triplicate RT–PCRs from one control and six DM cell lines (DM1, DM3, DM4, DM2, DM5 and DM6). Each RT was amplified in triplicate and is shown with the corresponding negative control. The marker, pBR322 digested with MspI, is shown in lane M. (b) Quantitative analysis of SIX5 allele 2 calculated as a ratio of the two alleles. Each point represents the average of triplicate PCRs from two independent RT reactions. Error bars for each sample are shown.

Figure 5. Allele-specific analysis of DMWD (left) and SIX5 (right) transcripts from doubly informative DM (DM10) and control (C1) cell lines. The quantitative results for the nuclear and cytoplasmic levels of RNA from the DMWD and SIX5 DM-linked alleles from DM10 are shown, illustrating the difference between DMWD and SIX5. For DMWD the reduction in level of the DM-linked transcript in the cytoplasm was not mirrored in the nucleus. N, nuclear; C, cytoplasmic. The analysis was based on triplicate PCRs from duplicate RTs.
four DM samples with large expansions. Such a correlation may only become apparent using a larger sample size with a greater range of repeat expansions.

The results presented here differ from our gross level quantification of DMWD and SIX5 RNA, which failed to demonstrate any significant difference in the level of these transcripts in cell lines from patients and controls (12). This indicates that the more sensitive allele-specific analysis detects changes in RNA level which are not detectable in gross level analysis. Thornton et al. (11), using an allele-specific assay on RNA from cultured myoblasts and various DM tissues (skeletal muscle, heart and brain), showed that the DM-linked SIX5 transcript is reduced to levels of 20–60% when compared with RNA levels from the non-DM allele. These results are similar to those of Klesert et al. (10), who also reported a significant decrease in the SIX5 transcript associated with the DM chromosome.

The extent of the allele-specific reduction in RNA from SIX5 is similar in both nuclear and cytoplasmic compartments, consistent with the notion that there is reduced transcription from the DM-linked allele. However, the result for DMWD quantification differs from that seen for SIX5. Although there is a significant reduction in the level of RNA from the DM-linked allele of DMWD in the cytoplasmic fraction, there is no corresponding reduction in the nuclear fraction. This indicates that export of transcripts for gene DMWD (like those for DMPK) may also be affected in cis by expansion of the repeat. This observation may reflect unique aspects of the transcriptional processing of DMWD that are not shared by SIX5. The precise point of transcriptional termination for DMWD has not been mapped. Two weak putative polyadenylation sites are present at positions 2101 and 2147 of the published DMWD sequence (8) and mouse transcripts have been documented that terminate at comparable places in the mouse homologue dmwd (formerly called DMR-N9). However, some DMWD transcripts have been identified that extend beyond these weak polyadenylation signals.

We have characterized several cDNAs that terminate at a genomic poly(A) tract which is located ~500 bp upstream of DMWD (8). The original RNA could be longer as the cDNA may be produced by internal priming from this poly(A) tract. Furthermore, Jansen et al. (20) have shown that following the functional inactivation of dmpk in the mouse, which involved removal of the gene 5’ end, a read-through transcript for dmwd was produced which extended to, and included, the neo cassette of the targeting construct. Thus, there is good evidence to support the existence of transcripts of DMWD, which may extend into DMPK. If these transcripts were to include the expanded repeat, they would also show nuclear retention, similar to that observed for DMPK. Such an effect would serve to mask, in the nucleus, any deficit of RNA transcribed from the DM-linked DMWD allele. Nevertheless, any deficit would be apparent in the cytoplasm. Clearly, further work is needed to examine this possibility.

An alternative explanation for the reduced levels of RNA from DMWD within the cytoplasmic fraction of DM cell lines might be that the repeat mutation operates in trans, affecting the post-transcriptional regulation of this gene. Phillips et al. (21) have shown that repeat expansion affects the cellular distribution of the CUG-binding protein (CUG-BP), which in turn affects alternative splicing. However, it is not readily apparent how this mechanism might produce an allele-specific effect for DMWD in the cytoplasmic fraction only.

The results presented here indicate that in the cytoplasmic fraction of DM fibroblasts repeat expansion produces a reduction in the level of RNA from the DM-linked allele of DMWD. These data add to the growing body of evidence indicating that DM is a multigene disorder. DMWD represents the third gene, after DMPK and SIX5, to show reduced RNA levels as a consequence of repeat expansion in DM tissues and cell lines. Further studies will be required to establish the precise biological role of DMWD and the extent to which it is involved in the pathophysiology of DM.

**MATERIALS AND METHODS**

**DMWD genomic PCR and mutation detection**

SSCP was used to detect sequence polymorphisms within DMWD. Blood was collected in EDTA tubes from 24 healthy individuals and DNA extraction performed using the nucleon II genomic DNA extraction kit (Scotlab, Strathclyde, UK) according to the manufacturer’s instructions. For each individual, 100–150 ng of DNA was used as template in PCR reactions (25 μl total). Using primers 56R and 57L a 506 bp genomic fragment within exon 5 of DMWD was amplified. The reaction conditions were 94°C for 3 min, followed by 34 cycles of 94°C for 1 min, 60°C for 25 s and 72°C for 45 s, with a final extension step of 72°C for 10 min. An aliquot of 10 μl of the PCR product from each individual was digested with ApaI restriction endonuclease to allow resolution within an SSCP gel. For each sample, 3 μl of formamide buffer (98% formamide, 10 mM EDTA pH 8.0, 0.05% bromophenol blue, 0.05% xylene cyanol) was added to the digested PCR product. The mix was then denatured at 94°C for 5 min and cooled rapidly on ice for 3 min. The single-strand products (6 μl) were resolved by electrophoresis through a 0.4 mm MDE gel (FMC) in a 40 cm vertical gel apparatus. The gel was electrophoresed at 3 W (constant power) for 1 h in 0.6x TBE buffer at 4°C. A standard method of silver staining was used to detect band shifts (22). Individuals were typed according to the banding pattern displayed on the SSCP gel.

PCR products from homozygous individuals were purified using the QIAquick PCR purification kit (Qiagen, Chatsworth, CA). Direct sequencing of the PCR products with primer 56R was carried out in an ABI 373 sequencer using the dye terminator sequencing kit. Sequencing data revealed a polymorphic T→G transition at nucleotide 2448 (GenBank accession no. L19267).

**Patient cell lines**

For the genotype analysis of SIX5 (GenBank accession no. X84813) DNA was extracted from the panel of normal control and DM fibroblast cell lines in our laboratory to determine which individuals were heterozygous for the Cac81 polymorphism in exon 3 (11). One control and six DM cell lines were found to be informative for the polymorphism. Cell lines that were heterozygotes for the DMWD polymorphism were determined by SSCP analysis of the extracted DNA. Three controls and four DM cell lines were shown to be heterozygous for the polymorphism. Allele-specific analysis was performed separately for SIX5 and DMWD. The approximate triplet repeat expansion sizes for the DM individuals are 8 kb for DM7, 9.5
kb for DM8, 7.2 kb for DM9 and 2.9 and 10 kb for DM10. The expansion sizes of patients DM1 (240 bp), DM2 (3.5 kb), DM3 (1.2 kb), DM4 (3 kb), DM5 (5.5 kb) and DM6 (5.5 kb) have been published previously on (12). Repeat expansion sizes were measured on batches of cells that were within two to three passages of the batches on which RNA quantification was performed.

**RNA extraction and cDNA synthesis**

Cytoplasmic and nuclear RNA were isolated from cultured fibroblast cells using Notidet P-40 by applying the method described previously (6). All RNA samples were treated with RNase-free DNase I (10 U/ml; Stratagene, La Jolla, CA) for 30 min at 37°C in a 40 µl total reaction volume. The RNA samples were then purified using the RNeasy kit (Qiagen) according to the manufacturer’s instructions. For the cDNA synthesis reaction 1–1.5 µg of the DNase I-treated RNA was used as template in a 20 µl reaction containing 0.003 U of random hexamers (Pharmacia) and 200 U of MMLV reverse transcriptase (RT) (Gibco BRL) incubated at 37°C for 1 h. First strand synthesis was performed in duplicate on all samples and parallel samples were analysed without the addition of RT to establish that products were derived from RNA and not DNA. Two microlitres of each RT reaction (and the –RT control) were used as template for three independent PCR analyses (six replicates in total).

**Allele-specific analysis: SIX5**

Oligonucleotide CacR (25 pmol) was end-labelled with [γ-32P]ATP using T4 polynucleotide kinase (NBL Gene Sciences, Northumberland, UK). Amplification of cDNA was carried out using 1/30th of the end-labelled Cac81R in a 25 µl PCR reaction containing 10 pmol of each oligo (Cac81R and Cac81L2), 200 µM dNTPs and 0.5 U of Taq DNA polymerase (Boehringer Mannheim, Basel, Switzerland) with the buffer supplied. The PCR conditions were 94°C for 3 min followed by 28 cycles of 94°C for 1 min, 66°C for 20 s and 72°C for 20 s. To monitor the level of RNA from each SIX5 allele, 5 µl of the PCR product were digested with 1.5 U of Cac8l (New England Biolabs, Beverly, MA), the supplier’s buffer and 0.1 mg/ml BSA in a total 15 µl reaction. The digest mix was incubated overnight at 37°C. Alleles were separated onto a 6% polyacrylamide–urea gel. The gel was dried and exposed to PhosphorImager plates (Molecular Dynamics, Sunnyvale, CA). Quantification of each allele was performed using ImageQuant v.1.1 (Molecular Dynamics).

**Allele-specific analysis: DMWD**

Oligonucleotide 57L (25 pmol) was end-labelled as above. Amplification of the cDNA was performed in a reaction containing the allele-specific primers R-59G and 59T at concentrations of 7 and 25 pmol, respectively. In addition to the standard components of the PCR reaction, 10 pmol of oligo 57L and 1/30th of the labelled 57L primer were added. The amplification conditions were 94°C for 3 min followed by 26 cycles of 94°C for 1 min, 64°C for 20 s and 72°C for 20 s. PCR products were loaded onto 6% polyacrylamide–urea gels and electrophoresed at 60 W (constant power) for 2 h in 1× TBE. The gel was then treated and analysed as above.

**Oligonucleotide primer sequences**

Cac81R, 5'-CCCTGCACTGAAAGCCAGAAG-3';
Cac81L2, 5'-TGGTGACAGCAAGCCAGAAG-3';
56R, 5'-GGAAACCCCCCTCAAGTTCCTCCTTG-3';
57L, 5'-CCAGGTTGAGCGGTTCTCCAGAGTG-3';
59T, 5'-CTCTATCGAATCCCTGGTGT-3';
R-59G, 5'-TGGAGGTTGCTACACCTCATTAAAGTG-3'.

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**REFERENCES**


