The myotonic dystrophy expanded CUG repeat tract is necessary but not sufficient to disrupt C2C12 myoblast differentiation

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Myotonic dystrophy type 1 (DM1) is a dominant neuromuscular disorder caused by a trinucleotide (CTG) repeat expansion. Mutant DMPK 3′-untranslated region (3′-UTR) transcripts aggregate in nuclear foci and are thought to impose dominant-negative effects by interacting with RNA binding proteins. We demonstrated previously that the mutant 3′-UTR RNA disrupted C2C12 myoblast differentiation, and that the CUG expansion was necessary for this effect. Several proteins are known to interact with the CUG tract or the region 3′ (distal) to it. Here, using a library of transfected C2C12 clones, we show that although transcripts containing a CUG expansion alone or a CUG expansion plus the distal region of the DMPK 3′-UTR accumulate into RNA foci, neither of these RNAs affect C2C12 myogenesis. Thus, RNA foci formation, and perturbation of any RNA binding factors involved in this process, are not sufficient to block myoblast differentiation. Interestingly, we found that transcripts containing expanded CUG tracts can form both nuclear and cytoplasmic RNA foci, demonstrating that factors involved in foci formation are present in the nucleus and cytoplasm. RNA analysis of myogenic markers revealed that the mutant DMPK 3′-UTR mRNA does not affect myoblast determination factors MyoD or Myf5, but significantly impedes upregulation of the differentiation factors myogenin and p21. C2C12 provide a good model to study adult muscle regeneration. Our observations in this system may be relevant to a regenerative response to continued muscle wasting in DM, and point to defects in early events in the myogenic response to muscle damage.

INTRODUCTION

Myotonic dystrophy (DM) is the most common form of muscular dystrophy among adults. Inherited as an autosomal dominant disorder, DM is characterized primarily by skeletal muscle myotonia, progressive muscle wasting and cardiac conduction defects (1). Non-muscle abnormalities can include cataracts, premature frontal balding, testicular atrophy and insulin resistance (1). Onset of DM typically occurs in early adulthood, but a more severe form of the disease, congenital myotonic dystrophy (CDM), affects patients from birth. Hallmark symptoms of CDM include neurological impairment, hypotonia and, importantly, defects in muscle development (1–3). The mutation at the DM1 locus (19q13.3), associated with 98% of DM cases, was identified as a trinucleotide (CTG) repeat expansion located in the 3′-untranslated region (3′-UTR) of the DM protein kinase (DMPK) gene (4–6).

It is unclear how the DM1 mutation causes disease, but accumulating evidence suggests that DM may be the result of several distinct molecular consequences of the CTG expansion, including DMPK haploinsufficiency, chromatin effects on neighboring genes and a trans-dominant effect of the mutant mRNA (reviewed in 7). Transcription of the mutant DMPK allele has been found to be unaffected (8), but the resulting transcripts, which contain an expanded CUG tract, aggregate in discrete foci in the nuclei of DM patient cells (9,10). The trans-dominant RNA model proposes that mutant DMPK mRNAs, concentrated in these nuclear foci, interact aberrantly with RNA-binding factors to cause deleterious effects. These could be mediated by sequestering RNA-binding proteins from their normal function, or by activating proteins not normally affected by the DMPK mRNA. Several RNA-binding proteins have been shown to interact with the DMPK 3′-UTR mRNA. These proteins interact with two regions of the 3′-UTR, either the CUG repeat tract, which forms a stable double-stranded RNA (dsRNA) hairpin at (CUG)$_{20}$ (11,12), or the sequences 3′ of the repeats (referred to as the distal region), CUG-BP (13), EXP (14) and PKR (15) are RNA-binding proteins that bind CUG repeats, whereas hnRNP C, PTB, PSF and U2AF (16) associate with the distal region (Fig. 1A).

A number of observations support the idea that aberrant RNA–protein interactions have deleterious consequences in DM cells. First, overexpression of CUG-BP or mRNAs containing expanded CUG tracts has been shown to misregulate mRNA splicing (17). Secondly, PKR, a protein kinase that responds to dsRNA, is activated by RNA containing CUG expansions (15). Thirdly, EXP, the human homolog of Dro sophila muscleblind, binds CUG tracts proportionally to the number of repeats and accumulates in nuclear foci in patient cells (14). Evidence

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implicating the mutant RNA in DM pathogenesis comes from a cell culture model in which expression of the mutant DMPK 3′-UTR mRNA results in myoblast differentiation defects (18) similar to those seen in CDM, and from transgenic mice that express expanded CUG tracts (19). Mice that express a human skeletal actin transgene containing 250 CTG repeats develop skeletal muscle myotonia and myofiber abnormalities like those seen in DM patients.

We have used cultured C2C12 myoblasts to test the trans-dominant RNA hypothesis. C2C12 cells, derived from adult mouse skeletal muscle, readily differentiate in culture and thus provide a good model to study muscle regeneration (20). In order to repair or regenerate damaged muscle, myoblasts, derived from satellite cells located at the periphery of myofibers in both humans and mice, are recruited and undergo a process of myogenic differentiation and ultimately fuse to form new myotubes that are incorporated into the existing muscle (reviewed in 21). Previously, we reported that stable expression of a mutant (CTG)100 DMPK 3′-UTR fused to a lacZ reporter gene resulted in the aggregation of reporter transcripts into nuclear RNA foci and disrupted C2C12 myogenic differentiation (18). Importantly, in a screen for revertant clones that rescued cells from the differentiation defect, we recovered a spontaneous deletion event, which removed the distal region of the 3′-UTR and reduced the CUG tract from 200 to five repeats. Since these two regions (the CUG tract and the distal region) are known to interact with RNA-binding proteins, we next wanted to investigate whether these sequences mediated the disruption of myoblast differentiation.

To test this, we have characterized a new panel of stable C2C12 clones that express heterologous RNAs containing human DMPK 3′-UTR sequences. Consistent with our previous observations, a CUG expansion, in the context of the full-length 3′-UTR, was necessary to reproduce deleterious effects on C2C12 differentiation. Taken out of the context of the DMPK 3′-UTR, we found that an expanded CUG tract alone was sufficient to aggregate reporter transcripts into foci, but neither the CUG expansion nor the distal region were sufficient to inhibit C2C12 myogenesis. This is the first demonstration that the phenomenon of RNA foci formation caused by an expanded CUG tract is separable from a phenotype (myoblast differentiation defects) mediated by the mutant DMPK 3′-UTR RNA. Additionally, we show that the mutant RNA does not affect expression of the myogenic factors MyoD or Myf5, but dramatically affects upregulation of myogenin and p21 during the differentiation program. These results indicate that the mutant RNA exerts its deleterious effects in the early stages of myogenesis.

RESULTS

Establishment of stable C2C12 clones

To determine whether mRNA containing an expanded CUG tract plus the distal region (GFP+Δ 3′-UTR) or an expanded CUG tract alone (GFP+CUGs) can suppress myogenic differentiation, we established stable C2C12 myoblast clones that express green fluorescent protein (GFP) minigenes containing these sequences inserted into the 3′-UTR (Fig. 1B). For comparison, we also created clones that express GFP fused to a full-length DMPK 3′-UTR with either five CTG repeats (GFP+5′-UTR) or 200 CTG repeats (GFP+mut 3′-UTR), or a GFP gene without DMPK sequences (GFP) (Fig. 1B). Transfectants were isolated and screened for minigene expression by GFP fluorescence and RNA-FISH experiments that detect CUG foci. All clones that expressed RNA containing an expanded CUG tract (GFP+Δ 3′-UTR, GFP+CUGs and GFP+mut 3′-UTR) formed nuclear RNA foci, whereas control GFP and GFP+5′-UTR clones expressed GFP but did not form foci. Typical GFP and RNA-FISH results from each line are shown in Figure 2A–F. Clones that maintained stable expression of either GFP fluorescence or RNA foci over several passages were used for further analysis. To ensure that stable clones maintained myoblast identity, MyoD expression was checked in each positive clone by either northern blotting or immunocytochemistry (not shown). MyoD, which is expressed in cycling C2C12 cells, is a myogenic regulatory factor involved in the establishment and/or maintenance of myogenicity (22), and myogenic cells isolated from MyoD−/− mice show reduced differentiation potential in culture (23). We found no consistent effect on MyoD expression in proliferating cells specific to any of the minigenes. Therefore, to avoid
skewing results of differentiation assays, randomly isolated clones that did not express MyoD (total of five) were excluded from our analyses. Screening 19 independent clones transfected with the GFP control minigene yielded 15 clones that were positive for both GFP and MyoD expression. We also identified 14 out of 32 GFP+wt 3′-UTR, 17 out of 45 GFP+Δ 3′-UTR, 9 out of 52 GFP+CUGs and 11 out of 59 GFP+mut 3′-UTR clones that were positive for GFP and/or RNA foci and expressed MyoD.

Transcripts containing an expanded CUG tract can form cytoplasmic foci

Within each line of clones that formed nuclear RNA foci (GFP+Δ 3′-UTR, GFP+CUGs and GFP+mut 3′-UTR), we observed that some individual clones did not express detectable levels of GFP (suggesting efficient retention of the GFP transcript in the nucleus). However, the majority of clones in all three of these lines (24 out of 37 total) produced low levels of GFP protein, indicating that some portion of the GFP RNA was transported into the cytoplasm (Fig. 2). This could be due to saturation of the nuclear retention mechanism or incomplete retention of (CUG)200 RNAs. Interestingly, in all of the clones that formed nuclear RNA foci and expressed GFP protein, RNA-FISH experiments also detected foci in the cytoplasm (arrows in Fig. 2E). These foci were RNase A sensitive and DNase I resistant, indicating that they were comprised of RNA (data not shown). This is the first demonstration that the mutant DMPK 3′-UTR mRNA is capable of aggregating into cytoplasmic foci and suggests that the factors necessary for foci formation exist in both the nucleus and cytoplasm of C2C12 cells.

The expanded CUG tract and the distal region are not sufficient to disrupt myoblast differentiation

C2C12 cells have been used extensively as a model to study myogenesis because they readily differentiate into multinucleated myotubes when cultured in low serum differentiation media. We first evaluated the ability of our stable clones to form myotubes qualitatively by culturing the cells in differentiation media for 5 days and then analyzing expression of a myotube marker, myosin heavy chain (MHC), by indirect immunocytochemistry. Since the differentiation response of C2C12 cells is known to be sensitive to cell confluency, culture conditions and serum quality, each clone was tested in at least three independent experiments. We found that eight of 15 (53%) clones expressing the control GFP minigene formed MHC+ myotubes as effectively as untransfected parent C2C12 cells. It is unclear why only 50% of GFP control clones differentiated effectively, while the other 50% formed myotubes at reduced levels. It is possible that GFP expression, the process of selection and cloning, or heterogeneity in the parent C2C12 population could affect differentiation capability. Strikingly, however, we found that a similar percentage of clones differentiated at levels comparable to control C2C12 myoblasts among GFP+wt 3′-UTR clones (57%), GFP+Δ 3′-UTR clones (47%) and GFP+CUGs clones (45%). In all, 12 of 26 (46%) clones that contained RNA foci harboring an expanded CUG tract outside of the context of the full-length DMPK 3′-UTR were able to effectively differentiate into MHC+ myotubes. These results suggested that there were no qualitative differences in

Figure 2. Minigene expression in stable C2C12 clones, and its effect on myotube formation. GFP fluorescence and RNA-FISH experiments were used to identify transfected clones that were positive for minigene expression. Representative results are shown in (A–F). Transcripts containing expanded CUG tracts (expressed in GFP+Δ 3′-UTR, GFP+CUGs and GFP+mut 3′-UTR) aggregated into nuclear foci (red) (D–F). Many of these clones expressed low levels of GFP protein (green) and formed cytoplasmic RNA foci as well (arrowheads in E). Nuclei are stained blue with DAPI. C2C12 differentiation into myotubes was assessed in each positive clone by MHC immunocytochemistry (G–L). A similar percentage of clones expressing GFP, GFP+wt 3′-UTR, GFP+Δ 3′-UTR and GFP+CUGs formed myotubes (red) as effectively as untransfected (Unt.) C2C12 (G–K). However, myobute formation was significantly reduced in most GFP+mut 3′-UTR clones (L).
myotube formation between the populations of GFP, GFP+wt 3'-UTR, GFP+Δ 3'-UTR and GFP+ΔUGs clones. In contrast, only two of 11 (18%) GFP+mut 3'-UTR clones were found to effectively form MHC+ myotubes, supporting previous results that the mutant 3'-UTR can suppress C2C12 differentiation (18). Representative MHC immunocytochemistry results from each line are shown in Figure 2G-L.

The results from our MHC assays suggested that expression of an expanded CUG tract alone or in combination with the distal region does not have the same deleterious effects on myogenic differentiation as the intact mutant DMPK 3'-UTR. To quantitatively test the effect of each GFP minigene on C2C12 differentiation, we used the upregulation of creatine kinase (CK) enzyme activity as a sensitive molecular marker of the differentiation program. Due to the inefficiencies of measuring CK activity during differentiation in all 66 positive clones, we took two alternative approaches for this analysis. First, since we isolated nine independent GFP+CUGs clones, we analyzed each of these and nine representative clones from each of the other lines. The representative clones were selected proportionally based on the MHC immunocytochemistry results (i.e., since 57% of the GFP+wt 3'-UTR clones formed myotubes effectively, we randomly selected five clones that differentiated effectively and four that showed reduced myotube formation). The second approach was to analyze a combined pool of all positive clones within a line (see Materials and Methods for a description of the clone pools). Since the GFP+Δ 3'-UTR clones express RNAs containing both of the regions of the mutant DMPK 3'-UTR RNA we were testing (expanded CUG tract and distal region), we generated a pool of GFP+Δ 3'-UTR clones rather than the GFP+CUGs clones. This pool was compared to pools of GFP+wt 3'-UTR and GFP+mut 3'-UTR clones. The set of representative clones and the clone pools were both analyzed to be confident the results accurately reflected the population of clones established and to ensure bias was not introduced by either approach.

CK activity was measured after 0, 1, 3 and 5 days of differentiation. The average fold increase in CK activity measured in each line (n = nine representative clones) and untransfected C2C12 cells (n = five experiments) is presented in Figure 3A. To test whether any of the minigenes had a statistically significant effect on C2C12 differentiation, CK activity in each set of representative clones was compared to untransfected myoblasts at each time point. This analysis revealed no significant differences between the control C2C12 cells and the clones expressing GFP, GFP+wt 3'-UTR, GFP+Δ 3'-UTR or GFP+CUGs at any of the time points. Only the GFP+mut 3'-UTR clones were significantly different (P = 0.019), and only on day 5. We found similar results analyzing the clone pools. CK activity was significantly reduced in the GFP+mut 3'-UTR pool on day 5 compared to activity in the GFP+wt 3'-UTR and GFP+Δ 3'-UTR pools (Fig. 3B).

There is no correlation between the minigene expression level and effects on myogenic differentiation

Minigene RNA expression was characterized in a randomly selected set of clones by northern blot analysis. Probes against the DMPK 3'-UTR and GFP were used to show that each minigene produced transcripts containing the appropriate sequences. Transcript heterogeneity was rare and most often observed in cells expressing GFP+mut 3'-UTR (Fig. 4A). This was likely due to instability or heterogeneity of the CTG repeats since the same transcripts were recognized by probes against both the proximal and distal regions of the DMPK 3'-UTR (not shown). To test whether high levels of minigene expression affected C2C12 differentiation, relative steady-state levels of GFP minigene RNA were determined by normalizing to β-actin. In addition, we determined minigene expression ranged from 0.1 to 10 times the levels of endogenous mouse dmpk mRNA in proliferating cells (data not shown). However, we found no correlation between expression level and myogenic differentiation (Fig. 4B), indicating that the effect of GFP+mut 3'-UTR expression on myogenesis is due to the RNA sequence rather than simply the overexpression of foreign RNA or GFP protein.

Timing the effects of the mutant DMPK 3'-UTR mRNA

Many key events in the C2C12 differentiation pathway from myoblasts to myotubes are well established (24) (Fig. 5A). We next wanted to determine which step(s) of the myogenic
program are affected by the mutant 3′-UTR RNA. Our previous analyses suggest that late stages of differentiation, CK activation on day 5, MHC expression and cell fusion, are reduced in cells expressing the mutant RNA. Therefore, we undertook an analysis of molecular markers of earlier stages of C2C12 differentiation (MyoD, Myf5, myogenin and p21). MyoD is expressed in proliferating myoblasts and is required for proper function and differentiation of adult skeletal muscle stem cells (25). Myf5, also expressed in cycling myoblasts, has been shown to compensate for the lack of MyoD in myogenic determination in knockout mice (26). Myogenin upregulation, which begins within hours of removing growth factors, is one of the initial events in the differentiation program (24). Subsequently, the induction of cyclin-dependent kinase inhibitors, such as p21 (27), withdraw cells from the cell cycle. This is followed by expression of muscle specific genes such as MHC and eventually myoblast fusion.

Steady-state mRNA levels of each marker were assessed in a subset of the GFP+wt 3′-UTR, GFP+Δ 3′-UTR and GFP+mut 3′-UTR clones (n = 5 each), chosen proportionally according to MHC immunocytochemistry results. RNA was analyzed at 24 h time points, starting with cycling myoblasts (day 0) through the first 4 days of differentiation, on RNA slot-blot and northern blots (Fig. 5B). In each group of representative clones (Fig. 5C), the average expression levels of MyoD and Myf5 during differentiation did not vary significantly from untransfected C2C12 cells. However, the induction of both myogenin and p21 mRNA was dramatically reduced in the GFP+mut 3′-UTR clones on days 3 and 4, and as compared to the GFP+wt 3′-UTR clones and control C2C12 myoblasts. This effect was not observed in the GFP+Δ 3′-UTR clones, adding further evidence that RNA molecules containing the distal region of the DMPK 3′-UTR and an expanded CUG tract do not affect C2C12 differentiation. Analysis of RNA from the clone pools revealed expression patterns of each marker that were quite similar to the averages of the five representative clones (Fig. 5D). Together, these data suggest the mutant DMPK 3′-UTR mRNA does not affect genes involved in maintaining myoblast identity (MyoD or Myf5) in cycling myoblasts, but prevents upregulation of the differentiation factors myogenin and p21.

**DISCUSSION**

It has been proposed that mutant DMPK transcripts may mediate deleterious effects by interacting aberrantly with RNA binding proteins. We reasoned that mutant DMPK 3′-UTR RNA may disrupt C2C12 myogenesis through such interactions. The expanded CUG tract and distal region were attractive candidates to test in our C2C12 model since all binding proteins known to associate with the DMPK 3′-UTR RNA have been shown to interact with these sequences in vitro. EXP proteins, which bind expanded CUG repeats, are upregulated during C2C12 differentiation (14) and required for terminal muscle differentiation in Drosophila (28). PKR, a protein kinase that can be activated by CUG hairpins (15), has been implicated in C2C12 differentiation (29). Furthermore, other DMPK 3′-UTR binding proteins (CUGBP, hnRNPC, PTB, U2AF and PSF) are expressed in myoblasts (13,16), making it possible that altering the function of any of these could play a role in the differentiation phenotype. However, in this study we found that transcripts containing a mutant CUG tract and the distal region had no significant effects on markers of C2C12 myogenesis (Figs 3 and 5), suggesting that any in vivo interactions between these sequences and RNA binding factors are not sufficient to inhibit C2C12 differentiation.

Due to inherent clone to clone differences and variability in the response of C2C12 myoblasts to induction of myogenesis by culture conditions, we characterized several independent clones from each line and analyzed clone pools in order to be confident of the effects of the RNA sequences tested. The differentiation response in each of our assays was only significantly affected in the population of GFP+mut 3′-UTR clones. Initially, we were concerned that we isolated only nine GFP+UGTs clones. We thought it may prove difficult to be confident about differences between the GFP+UGTs and GFP+Δ 3′-UTR clones if the distal region was necessary to reproduce the defective differentiation phenotype. We were also concerned about the size of the CUG expansion [(CUG)100 instead of (CUG)200 due to cloning similarity restrictions] in the...
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GFP+CUGs clones. However, these two concerns were inconsequential as the pool of GFP+∆3′-UTR clones that expressed RNAs containing 200 CUG repeats (similar to the GFP+mut 3′-UTR clones) did not show significant differences in myogenic behavior compared to control C2C12 cells. This indicates that although necessary, the (CUG)200 tract is not sufficient to disrupt C2C12 myogenic differentiation.

The clumping of CUG RNA in nuclear foci could affect gene expression by disrupting the splicing, processing or export pathways of a subset or all RNAs. Such effects on programmed gene expression could explain why cells expressing the mutant 3′-UTR RNA fail to complete the differentiation process. The results presented here argue against the process and/or effects of nuclear foci formation alone being responsible for defects in myogenesis. The GFP+∆ 3′-UTR and GFP+CUGs clones did not show consistent differentiation defects, but formed nuclear foci indistinguishable from cells expressing the intact mutant 3′-UTR. Additionally, in these clones, we observed foci in the nuclei of differentiated myotubes (not shown). We also found that RNAs harboring a CUG expansion can aggregate into cytoplasmic foci, indicating that any factors required for foci formation are present in the cytoplasm (Fig. 2). Among the GFP+∆ 3′-UTR and GFP+CUGs clones, these cytoplasmic foci, like nuclear foci, appear to have no adverse effects on C2C12 proliferation or differentiation.

To begin to understand the mechanism(s) by which the mutant RNA obstructs myogenesis, it was essential to identify events in the pathway that were affected. Since C2C12 differentiation depends on a complex network of signaling cascades and abrupt changes in gene expression, the mutant RNA could

Figure 5. The mutant DMPK 3′-UTR mRNA disrupts the upregulation of myogenin and p21. (A) Diagram of temporal events in myoblast differentiation. The removal of growth factors (GF) stimulates the induction of myogenin (Myg), which is followed by cell cycle withdrawal, expression of muscle specific genes and cell fusion. (B) Representative northern blot results from individual clones and untransfected control C2C12 cells showing expression of MyoD, Myf5, Myg and p21 during four days of differentiation. (C) RNA slot blot results showing the average relative RNA expression level of each marker (normalized to GAPDH expression) in representative clones (n = 5 for each line) and untransfected C2C12 (n = 4 experiments). These analyses indicated that average MyoD and Myf5 expression was not significantly affected in any line, whereas myogenin and p21 upregulation was blunted on days 3–4 specifically in GFP+mut 3′-UTR clones (P < 0.014). (D) Slot blot analyses of clone pools showed a similar pattern of expression for each marker.
interfere with any number of critical molecular events. For example, differentiation could proceed normally until the point of cell fusion, which could be selectively blocked. Alternatively, it could be cell cycle withdrawal that is restricted. Another possibility is that MyoD levels could be compromised during differentiation. Recent reports show that specific degradation of MyoD mRNA during C2C12 myogenesis is associated with differentiation defects (30,31). To address this issue, we measured RNA levels of markers expressed at various stages of differentiation. The average expression levels of MyoD and Myf5, myogenic determination factors present in proliferating myoblasts, were not dramatically different between control C2C12 cells and clones that express the mutant RNA. In contrast, the upregulation of myogenin, an early differentiation factor, and p21, a marker of cell cycle withdrawal, was significantly reduced in GFP+mut 3'-UTR clones (Fig. 5). Since we analyzed steady-state RNA levels, it is not clear whether this reduction of myogenin and p21 is at the transcriptional or post-transcriptional level. Taken together, our analyses of differentiation markers (Myg, p21, CK and MHC) suggest that myoblasts expressing the mutant 3'-UTR RNA enter the myogenic pathway and behave normally through the first 2–3 days of differentiation, but then fail to progress any further. These results provide, for the first time, molecular targets and time points for the trans effect of the mutant 3'-UTR RNA on muscle differentiation.

But if the mutant 3'-UTR inhibits differentiation, then why were two GFP+mut 3'-UTR clones isolated which effectively formed myotubes? It is possible that the genetic background in these cells compensated for the deleterious effects of the RNA. For example, MyoD expression was the highest (2–3-fold higher than control C2C12) in the GFP+mut 3'-UTR clone that differentiated most effectively. Thus, perhaps high levels of MyoD, or the upregulation of one of the many MyoD target genes, can ameliorate the effects of the mutant DMPK 3'-UTR RNA.

Myoblast differentiation and fusion is essential for muscle regeneration and maintenance. Muscle weakness and progressive wasting are the most debilitating aspects of DM and may result from an inadequate regenerative response to muscle damage. It is therefore notable that histologic analysis of damaged muscle from DM patients shows a paucity of regeneration (1). Our C2C12 model may provide a useful system to further study the effects of the mutant DMPK RNA on the muscle regeneration response. From the experiments presented here, we conclude that while a full-length mutant 3'-UTR is capable of disrupting myoblast differentiation by blunting expression of the early differentiation factors myogenin and p21, transcripts containing the distal region and/or expanded CUG tract do not have this effect. It is of interest to note that although transgenic mice expressing a chimeric transcript containing a CUG expansion alone form nuclear RNA foci and develop myotonia and histologic changes of DM, they show no evidence of muscle weakness or wasting (19). We propose that the expanded CUG tract is necessary to anchor mutant transcripts in nuclear RNA foci, but then an additional combination of DMPK 3'-UTR sequences or perhaps the intact 3'-UTR is required for in vivo interactions with trans-acting factors that mediate the myogenic defect.

**MATERIALS AND METHODS**

**GFP minigene constructs**

pcDNA-PGK GFP minigenes were created by removing the CMV promoter fragment from pcDNA3.1/Myc-His B (Invitrogen) and inserting a cassette containing the PGK promoter and GFP sequences. Similarly, a Rosa 26 promoter fragment (32) and an EFGP cDNA (Clontech) were used to generate pcDNA-R26EGFP. Human DMPK 3'-UTR fragments were then inserted 3' of the GFP stop codon in both pcDNA-PGK GFP and pcDNA-R26EGFP. The DMPK 3'-UTR sequences were: (i) a full-length wild-type 3'-UTR with five CTG repeats (GFP+wt 3'-UTR); (ii) a full-length mutant 3'-UTR with approximately 200 CTG repeats (GFP+mut 3'-UTR); (iii) a 3'-UTR fragment in which a deletion removed the first 155 bp of the proximal, but left the 200 CTG repeat tract and distal region intact (GFP+Δ 3'-UTR); (iv) a tract of 106 CTG repeats with 37 bp of 5' and 40 bp of 3' flanking DMPK sequence (GFP+Δ 3'-UTR). The integrity of each construct was confirmed by automated DNA sequencing.

**Transfections and cell culture**

To establish stable clones, C2C12 cells were transfected with 1 µg of linearized plasmid, carrying a GFP minigene and a neomycin resistance marker, using Lipofectamine Plus reagent (Gibco BRL). Forty-eight hours after transfection, cells were transferred to media containing 0.8 mg/ml G418 (Cellgro) to select for stable integration of the plasmid. Both pcDNA-PGK GFP and pcDNA-R26EGFP constructs were used in an attempt to generate clones with a wide range of expression levels. Between 24 and 48 G418 resistant colonies were isolated from each transfection. Colonies were expanded and screened for minigene expression by RNA-FISH and GFP fluorescence. C2C12 myoblasts were maintained at sub-confluence in growth media containing 10% cosmic calf serum (HyClone) in DMEM (Cellgro). Stable clones were maintained in G418 selective media.

Clone pools were generated by combining an equal number of cells from each positive clone within a given line (GFP+wt 3'-UTR, GFP+Δ 3'-UTR and GFP+mut 3'-UTR). GFP fluorescence and RNA-FISH confirmed appropriate minigene expression in each pool and MHC immunocytochemistry revealed that the GFP+wt 3'-UTR and GFP+Δ 3'-UTR pools effectively formed myotubes, whereas the GFP+mut 3'-UTR pool did not.

For differentiation studies, cells were grown to 80–90% confluence and then cultured in differentiation media containing DMEM plus 2% equine serum (HyClone) for 5 days. Differentiation media was changed every 48 h.

**RNA-FISH and GFP fluorescence**

Myoblasts grown on coverslips were fixed in 4% paraformaldehyde for 15 min at room temperature and stored in 70% ethanol at 4°C. A CY3-conjugated (CAG)$_{10}$ oligonucleotide probe (Operon) was used for RNA-FISH experiments performed as described previously (9,18). CY3 and GFP fluorescent signals were visualized using an Olympus IX 50 microscope with epifluorescence. Images were captured with a SPOT II digital camera (Diagnostic Instruments, Inc.)
and then assembled using Photoshop (Adobe) and Canvas (Deneba Systems) software.

**Immunocytochemistry**

Cells grown in 24-well plates were cultured in differentiation media for 5 days and then fixed as described above. After rehydration in 1× PBS, the cells were incubated with monoclonal MY32 antibody against myosin heavy chain (Sigma) for 1 h in 1× PBS + 1% BSA at 37°C. After three washes with 1× PBS, the cells were incubated with a Texas Red-conjugated anti-mouse secondary antibody (Jackson Laboratories) for 1 h at 37°C. Cells were then washed again and nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (Sigma). Samples were visualized and documented as described above.

**Creatine kinase assays**

Myoblasts were seeded in 24-well plates at a density of 2.5 x 10^5 cells per well. The next day (day 0), cells were induced to differentiate by replacing growth media with differentiation media. Cell extracts were harvested after 0, 1, 3 and 5 days in differentiation media by lysing cells in 1× PBS + 0.1% Triton X-100 at room temperature for 10 min. CK activity in each lysate was analyzed on the Dimension Clinical Chemistry System (Dade Behring) and normalized to total protein concentration (Bio-Rad). At least four measurements (duplicates from at least two experiments) were made for each representative clone and each clone pool. The Wilcoxon rank sum test was used for statistical analyses.

**RNA analysis**

Total RNA was extracted from cells lysed in guanidinium thiocyanate buffer (4 M guanidinium thiocyanate, 20 mM sodium acetate pH 5.4, 0.5% sarcosyl) by ultracentrifugation through a 5.7 M CsCl density gradient. For northern blots, 10 µg of RNA on to a membrane using a Bio Dot SF micro-apparatus (Bio-Rad) and normalized to total protein concentration (Bio-Rad) and normalized to total protein concentration (Bio-Rad). At least four measurements (duplicates from at least two experiments) were made for each representative clone and each clone pool. The Wilcoxon rank sum test was used for statistical analyses.

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