Overexpression of 3'-Untranslated Region of the Myotonic Dystrophy Kinase cDNA Inhibits Myoblast Differentiation in Vitro*

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The genetic defect underlying myotonic dystrophy (DM) has been identified as an unstable CTG trinucleotide repeat amplification in the 3'-untranslated region (3'-UTR) of the DM kinase gene (DMK). Individuals with the most severe congenital form display a marked delay in muscle terminal differentiation. To gain insight into the role of DMK during myogenesis, we have examined the effect of DMK overexpression on the terminal differentiation of the murine myoblast cell line C2C12. We demonstrate that a 4–10-fold constitutive overexpression of DMK mRNA in myoblasts caused a marked inhibition of terminal differentiation. Surprisingly, this activity was mapped to a 239-nucleotide region of the 3'-UTR of the DMK transcript. When the DMK 3'-UTR was placed downstream of a reporter gene, the same inhibition of myogenesis was observed. Following the induction of differentiation of myoblast clones overexpressing the DMK 3'-UTR, the levels of myogenin mRNA were reduced by approximately 4-fold, whereas the steady state levels of mef-2c transcripts were not affected. These data suggest that overexpression of the DMK 3'-UTR may interfere with the expression of muscle-specific mRNAs leading to a delay in terminal differentiation.

Myotonic dystrophy (DM),¹ the most common form of inherited neuromuscular disease in adults, affects 1 in 8000 individuals globally. DM is an autosomal dominant and multisystemic disorder characterized mainly by myotonia and progressive muscle weakness and wasting (1). Importantly, skeletal muscle biopsies from adult DM patients display a marked atrophy and paucity of type I myofibers (1). Affected individuals present with a highly variable phenotype, ranging from an asymptomatic condition to a severe and frequently fatal congenital form. Congenital DM patients display marked hypotonia, neonatal respiratory distress, and severe mental retardation. Significantly, histochemical studies of muscle cross-sections from these patients have demonstrated the presence of immature myofibers (1–4). Furthermore, protein profile analyses have shown an altered content of contractile protein in these muscles, suggesting that the congenital form of DM is associated with a delay or an arrest of muscle maturation (2–4).

The genetic mutation for DM has been identified as an unstable CTG trinucleotide repeat in the 3'-untranslated region (3'-UTR) of a gene that encodes a serine/threonine kinase (5–9). This repeat has been found to be polymorphic on normal chromosomes, ranging from 5 to approximately 40 triplets. However, in over 98% of DM individuals, this region has been shown to be significantly amplified, ranging from approximately 50, in mild cases, to several thousand repeats in severely affected individuals. The degree of this amplification has been strongly correlated with disease severity (10–12).

The protein product encoded by the DM gene (DMK) displays high homology to the serine/threonine family of protein kinases (6, 8, 9, 13, 14). In agreement with this, it has been shown to have serine/threonine specific kinase activity (15). The DMK protein has been localized to sites of intercellular contacts such as the neuromuscular junctions in skeletal muscles and to the intercalated disks of heart (16, 17), and recently to the triad region of muscle fibers (15). However, the normal function of the protein is still unknown.

In research concerning the expression of the DMK gene, controversial observations have been reported on the effect of CTG amplification. Using different assays, overexpression (18) and reduced expression of DMK (19–22) have been observed in muscle tissues from congenital and adult DM tissue samples, respectively. Others have reported that the total activity of the DM locus was unaffected in DM patients but that the levels of DMK protein were elevated in skeletal muscle from severely affected individuals. The degree of this amplification has been strongly correlated with disease severity (10–12).

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In another analysis, Wang et al. (24) have shown that the levels of both mutant and wild type DMK mRNAs were markedly reduced in poly(A)⁺ RNA samples. These authors have proposed that the expanded CTG repeat may act as a dominant-negative mutation that affects RNA processing in trans. Recently, the expanded repeat has been shown to repress, in cis, the downstream gene DMAHP (25–26), suggesting its involvement in the disease process. However, it has been demonstrated that, when normalized to type I myosin heavy chain, the relative levels of DMK protein were elevated in skeletal muscle from DM individuals (15). Recently, targeted inactivation of the murine DMK gene has been shown to result in a normal phenotype in one case (27), whereas a late onset progressive muscle weakness has been demonstrated by Reddy et al. (28). Interestingly, no congenital DM phenotype has been observed in any of the null homozygous mice. Taken together these...
results suggest that the congenital phenotype is not mediated by a decrease in DMK expression.

Therefore, to gain insight into the role of DMK during myogenesis and the mechanism underlying DM, we have characterized DMK expression during the terminal differentiation of C2C12 myoblasts and investigated the effect of DMK overexpression on myogenesis. Our results show that DMK is expressed in various myoblast cell lines and slightly up-regulated during skeletal myogenesis in vitro. Importantly, a 4–10-fold DMK overexpression markedly inhibits myogenesis. Moreover, we demonstrate that this activity maps to the 3'-UTR of the DMK mRNA. In contrast to previous observations (29) where muscle-specific 3'-UTR enhanced differentiation, we show that overexpression of the 3'-UTR may function in a feedback loop and interfere with the expression of muscle-specific mRNAs required for muscle terminal differentiation.

MATERIALS AND METHODS

Plasmid Constructions—DMK expression vectors consisted of full-length or partial DMK cDNAs cloned into pcDNA3 (Invitrogen) using standard cloning protocols (30). Briefly, pCMV-DMK comprises a full-length DMK cDNA bearing both 5'- and 3'-UTRs in pcDNA3. pDΔ35 was obtained from B. Perryman (University of Colorado, Denver) and was generated by cloning a polymerase chain reaction-amplified fragment corresponding to the coding portion of the full-length DMK cDNA. pDΔ35 was generated by replacing the 5' KpnI-XhoI portion of pCMV-DMK by the corresponding segment of pDΔ35. Plasmid pK100R was obtained by in vitro mutagenesis of pCMV-DMK using the MutA-gene II kit (Bio-Rad) according to the manufacturer's instructions. A point mutation was introduced at amino acid residue 100, converting the conserved lysine residue of the ATP-binding site to an arginine. To construct HT3' and HT3'R, a 1.0-kilobase pair BamHI fragment encompassing the DMK 3'-UTR was cloned in both orientations, into the EcoRI site downstream of the hygromycin-tk hybrid gene encoded by pgtCMV/Hy-tk (31). The control plasmid HT-PGK was generated by cloning a 1.0-kilobase pair XhoI-HindIII fragment, comprising the mouse pgk 3'-UTR, into the SalI-HindIII sites of pgtCMV/Hy-tk. Plasmid pHtDar was generated by SacII-PstI digestion and religation of HT3', resulting in deletion of the CTG repeat region. Plasmids pHtΔ3' and pHtΔ5' were generated by blunt end cloning of polymerase chain reaction products encompassing nucleotides 2002–2278 and 2204–2726, respectively, of the human DMK cDNA (GenBank accession no. L19268).

Cell Culture, Transfections, and RNA Analysis—C3H10T1/2, C2C12, and NIH 3T3 cells were purchased from ATCC. C3H10T1/2 and NIH 3T3 fibroblasts were maintained in α-minimal Eagle’s medium supplemented with 10% fetal bovine serum and 2 mM glutamine. C2C12 myoblasts were grown in α-minimal Eagle’s medium containing 15% fetal bovine serum and glutamine. All cultures were maintained under 70–80% confluency and grown at 37 °C in a humidified atmosphere containing 5% CO2. For stable transfections, C2C12 cells were plated at 5 × 105 in 100-mm dishes 24 h prior to transfections. The cells were transfected by standard calcium phosphate precipitation (30) for 8 h and then re-fed with fresh growth medium. Following a 24-h incubation, G418 (Geneticin; Life Technologies, Inc.) or hygromycin B (Sigma) was added at final concentrations of 0.4 or 0.2 mg/ml, respectively. After a 14-day incubation, resistant clones (or pools) were isolated using cloning rings and grown for RNA, protein, and fusion analyses. To avoid the isolation of non-differentiating C2C12 clones, cells that had undergone 15 passages or less were used for each independent transfection experiment. For Northern blotting experiments, total RNA was extracted from confluent cultures of each individual clone and analyzed as described previously (18). For analysis of DMK expression during differentiation, C2C12 cultures were grown to 80% confluency and then transferred to differentiation medium (α-minimal Eagle’s medium containing 10% horse serum), and RNA was analyzed for DMK at various times as indicated. For myogenic conversion of 10T1/2 fibroblasts, 2 × 106 cells were plated in 35-mm dishes and grown for a further 24 h. The cultures were then treated with 3 μM 5-azacytidine (Sigma) for 24 h. The cells were then re-fed with fresh medium (day 0), and DMK expres-
Fig. 2. DM kinase cDNA plasmid constructs. A, DMK expression vectors carrying wild type or mutant full-length DMK (CMV-DMK and K100R, respectively). The position of the point mutation is shown by the asterisk. Expression vector in which the 5′-UTR or both the 5′- and the 3′-UTRs were deleted are denoted by DΔ5 and DΔ35, respectively. B, hygromycin-tk hybrid control vector (HT-C) or vectors carrying the DMK 3′-UTR (hatched bars) in the sense (HT3) or antisense (HT3R) orientation were used to analyze the effect of 3′-UTR overexpression on myoblast differentiation. An additional vector bearing the pgk 3′-UTR was constructed for use as a control (HT3PGK).

As a first step into the characterization of DMK expression during myogenesis, we determined DMK mRNA levels during the differentiation of C2C12 myoblasts. Cells were grown to 80% confluence and induced to differentiate in low serum medium. Total RNA was isolated at various times and analyzed for DMK expression. Northern blot analysis and normalization to pgk mRNA levels showed a modest increase (1.5- to 2-fold) in the steady state levels of DMK transcripts following transfer of the cells to differentiation medium (Fig. 1B). The major increase in DMK levels occurred 72–96 h following the induction of differentiation, suggesting that up-regulation follows the commitment to myogenic differentiation. Similarly, when the cells were allowed to differentiate following confluency in growth medium, DMK mRNA levels were found to increase approximately 2-fold during differentiation (Fig. 1C). Under low serum conditions, myoblast fusion was found to be approximately 37 and 60% after 2 and 4 days, respectively, in parallel cultures following myosin heavy chain immunostain (see “Materials and Methods”). For both time courses, myosin light chains 1 and 3 (mlc 1/3) and α-skeletal actin levels (data not shown) increased significantly, indicative of muscle cell terminal differentiation.

Our expression results with the various cell lines suggest that DMK may become up-regulated during myogenic determination (see above). To investigate this possibility, DMK expression was analyzed in the mesodermal precursor cell line C3H10T1/2 that has been demonstrated to differentiate predominantly into myoblasts as well as chondrocytes and adipocytes following a brief treatment with 5-azacytidine (33). As shown in Fig. 1D, the presence of the 82-kDa DMK isoform is apparent 4 days after treatment of 10T1/2 cells with 5-azacytidine. Similarly, DMK mRNA was found to be up-regulated following 5-azacytidine treatment, and this induction coincided with an increase in myoD mRNA in these cells (data not shown).

The results shown are from one representative experiment.
likely to be due to the presence of the other cell types. Therefore, our observations suggest that DMK is up-regulated during myogenic determination of precursor cells. Supporting this is the recent observation that DMK is expressed in murine somites of day 10.5 embryos (27), at which time the myogenic factors myoD and myf-5 are expressed (34).

Overexpression of DMK Inhibits the Terminal Differentiation of Cultured Myoblasts—Previous histological studies have shown a marked delay of muscle cell terminal differentiation (2–4). Our laboratory has shown that CTG expansion caused an increase of DMK mRNA steady state levels in muscle tissues from congenital DM individuals (18). These data suggest that DMK overexpression could directly inhibit or delay myogenesis in these patients. Our working hypothesis, therefore, was that DMK 3'-UTR Inhibits Myoblast Differentiation

**Fig. 3.** Expression of DMK mRNA and protein in myoblast clones and COS-1 cells. A, example of a Northern blot analysis of two independent myoblast clones (B6 and C4) isolated from CMV-DMK-transfected C2C12 cells. Normalization to myoD and pgk showed a 10-fold overexpression of DMK for both clones. Exposure time was for 24 h. B, Western (10 μg of total protein) blot analysis of the same B6 and C4 clones analyzed in A using anti-DMK-2 (17). Normalization to a parallel Coomassie Blue-stained gel shows no difference in DMK protein levels between C2C12 and clones overexpressing DMK mRNA. C, anti-DMK-2 immunoblot analysis showing expression of the various DMK constructs in COS-1 cells. COS-1 cells were transiently transfected with the indicated vectors (see Fig. 2) and analyzed for DMK expression 48 h following transfection. Panels I and II show the results of one experiment comparing the relative expression levels of CMV-DMK, K100R, DΔ35, and DΔ5. MTG-DMK represents a myc epitope-tagged DMK vector used in kinase activity assays. The relative expression levels for DΔ5, DΔ35, and K100RΔΔ5 (a mutant version of DΔ5), obtained in an independent experiment, are shown in panel III. DMK was not found to be expressed from CMV-DMK or K100R but was expressed equally well from all the other constructs. In all panels, DMK was detected with anti-DMK-2. D, fusion index determination of three DMK overexpressing clones. Following the induction of differentiation, C2C12 myoblasts or neomycin-resistant C2C12 clones displayed indices of about 35%, whereas DMK (CMV-DMK) overexpressing clones B6, D2, and C4 showed fusion indices of 6–10%. B6R represents a revertant population isolated from B6, which expresses 2-fold higher levels of CMV-DMK. All 24 clones tested exhibited reduced fusion indices, ranging from 5 to 14%, a 3–5-fold reduction relative to C2C12.
the dominant nature of the disease, and the ultimate effect on myogenesis, was due to the overexpression of the DM kinase gene and/or protein.

To this end, an expression vector, carrying a full-length human DMK cDNA was constructed. The cDNA, bearing 325 bp of 5'-UTR, the coding region, and the entire 3'-UTR, with 11 CTG repeats (14), was introduced into the pcDNA3 expression vector (Invitrogen) to generate pCMV-DMK (Fig. 2A). Transcription is under the control of the human cytomegalovirus promoter (CMV; Ref. 35). Plasmid DNA was transfected into C2C12, and 24 G418-resistant clones were randomly isolated and analyzed for DMK mRNA expression. When normalized to pGK, all the clones surveyed displayed a 4–10-fold higher level of DMK mRNA relative to untransfected C2C12 myoblasts (for example, those clones designated B6 and C4 shown in Fig. 3A) and were morphologically similar to the parent cell line (data not shown). In parallel, all clones were subjected to Western blot analysis and myoblast fusion assays under low serum conditions. Following myosin heavy chain or Giemsa staining of the fixed cultures, the fusion index (number of nuclei in the fusion product per 1000 nuclei) was determined 2 days after the induction of differentiation (37). However, Northern blot analysis of pCMV-DMK clones prior to differentiation did not show any differences in the levels of myoD mRNA (Fig. 3A), indicating that DMK exerts its effect downstream of myoD transcription. Furthermore, all the clones are still desmin-positive (not shown). Taken together, these observations suggest that overexpression of DMK does not affect the myogenic identity of these cells.

The apparent absence of increased DMK protein levels in our differentiation defective clones overexpressing DMK mRNA suggests that this phenotype is not mediated by the protein kinase itself but rather through, perhaps, one or both untranslated regions. Alternatively, a slight increase in DMK protein levels and/or activity which may not be readily detectable by Western blot analysis, or kinase activity assays, might be detrimental to myogenesis. To test these possibilities, we constructed additional DMK expression vectors and assayed differentiation in overexpressing clones. Initially, the conserved lysine of the ATP-binding site (38), at amino acid position 100, was converted to an arginine by in vitro mutagenesis (39) to generate pK100R (Fig. 2A). Kinase inactivation was verified using immunoprecipitation and histone H1 phosphorylation (data not shown) by comparing the kinase activity of DMK protein generated from c-myc epitope-tagged (40) pK100R and wild type DMK constructs expressed in COS-1 cells (Fig. 3C).

In addition, DMK expression vectors in which the 5'-UTR (pDΔ5) or both UTRs have been deleted through polymerase chain reaction amplification of the coding region (pDΔ5; Fig. 2A) were also tested (see Table I). All three vectors were transfected into C2C12 myoblasts, and 12 individual clones were analyzed in each case, as described above. As for the full-length construct, all the clones isolated from pK100R-transfected cells expressed high levels (up to 10-fold) of DMK mRNA encoding the inactive kinase (for example clones K5 and K6 shown in Fig. 4A, expressing 7- and 8-fold, respectively). Similarly, these clones, as well as transfected COS-1 cells (Fig. 2C), did not show any appreciable increase in DMK protein levels (Fig. 4B). However, the fusion index of these clones was also markedly reduced compared to control cultures (Fig. 4C and Table I). The use of a DMK catalytic mutant in these experiments indicates that active kinase is not required to confer the observed polyphenotype.

In marked contrast to pK100R-transfected clones, C2C12 cells transfected with pDΔ5 expressing well in COS-1 cells (Fig. 2C), showed high levels of DΔ5-specific mRNA and were found to express 3–5-fold higher levels of DMK protein when compared with C2C12 (Table I and data not shown). Interestingly, clones overexpressing DΔ5 mRNA displayed fusion indices that were similar to control cultures

| Table I: Phenotypes obtained with plasmid constructs used in this study |
|------------------|----------------|------------------|------------------|
| **Vector** | **DMK 3'-UTR** | **mRNA** | **DMK protein** | **Number of clones surveyed** | **Inhibition of differentiation** |
|------------------|----------------|------------------|------------------|
| pCMV-DMK | + | 4–10 | 1–2 | 24 | + |
| pΔΔ5 | + | 3–10 | 2–5 | 12 | + |
| pK100R | + | 4–10 | 1–2 | 12 | + |
| pDΔ35 | - | 2–8 | 3–5 | 12 | - |
| pH-T-C | - | 5–10 | 1* | 5 | - |
| pH-T3 | - | 5–10 | 1 | 24 | + |
| pH-T3R | + | 5–10 | 1 | 12 | - |
| pH-T7PGK | - | 5–10 | 1 | 5 | - |
| pH-TAR | + | ND | 1 | 12 | + |
| pH-TΔ3’ | + | ND | 1 | 12 | + |
| pH-TΔ5’ | + | ND | 1 | 12 | - |
| C2neo’ | - | 1 | 1 | 5 | - |

a HT (hygromycin-tk)-derived vectors did not encode DMK protein and expressed wild type levels of DMK protein.

b HTΔAR contains DMK 3'-UTR lacking the repeat region.

c HTΔAR contains DMK 3'-UTR lacking sequences downstream of the repeat.

d HTΔAR contains DMK 3'-UTR lacking sequences upstream of the repeat.

e ND, not determined.
DMK 3'-UTR Inhibits Myoblast Differentiation

The Inhibition of Myogenesis by DMK Is Mediated by the 3'-UTR—The findings that the only DM mutation identified to date maps to the 3'-UTR of the DMK transcript (reviewed in Ref. 41) and the recent implication of specific 3'-UTRs in the control of myoblast differentiation (29, 42) prompted us to examine whether the DMK 3'-UTR alone could function in our assay as an inhibitor of myoblast fusion. To test this, the DMK 3'-UTR, including 255 bp of coding region upstream of the termination codon, was inserted downstream of tgCMV/Hy-tk (pHT3'; Fig. 2B) encoding a hybrid gene conferring resistance to hygromycin B and sensitivity to gancyclovir (31). Sequence analysis confirmed that the expression of truncated DMK product from this vector is unlikely since no initiation codons are observed in the proper DMK reading frame and that termination codons are present in the other two frames (data not shown). This hybrid vector was then introduced into C2C12 myoblasts, and a total of 24 hygromycin-resistant clones were isolated and analyzed for hy-tk expression by Northern blotting. These clones were found to express the hy-tk gene at levels that were comparable to pCMV-DMK or pK100R (for example those shown in Fig. 5A). When subjected to differentiation assays, similar to DΔ5 clones, cultures of HT3'-transfected cells exhibited a marked reduction in their fusion index (Fig. 5B) supporting the hypothesis that overexpression of the DMK 3'-UTR alone is sufficient to cause a block of myogenesis. As a control, the same 3'-UTR segment was cloned in the reverse orientation (pHT3'R; Fig. 2B), and 12 hygromycin-resistant clones were assayed for fusion. All of these clones exhibited fusion indices that were comparable to control cultures consisting of wild type C2C12 myoblasts or hygromycin-resistant cells that had been transfected with vector DNA alone (pHT-C; Fig. 5B), suggesting that the effect is sequence-specific.

As an additional control, the 3'-UTR of the mouse phosphoglycerate kinase (pgk) cDNA (43) was also cloned into pgtCMV/Hy-tk to generate pHTPGK (Fig. 2B). As for pHT3'R clones, hygromycin-resistant myoblast clones isolated following transfection with pHTPGK fused equally well when compared with control cultures (Fig. 5B), suggesting that the observed reduction in fusion index for HT-3' clones is not likely due to toxicity by the overexpressed mRNA.

In addition to the selection of hygromycin-resistant clones overexpressing the DMK 3'-UTR, the use of the pgtCMV/Hy-tk vector allows for the selective killing of these clones by gancyclovir treatment. Consequently, clones that have lost the expression of the 3'-UTR through reversion and loss of thymidine kinase expression can be isolated and assayed for differentiation. Two clones overexpressing the 3'-UTR were grown in the presence of gancyclovir for 21 days, and resistant colonies were isolated. When assayed for differentiation, two independent resistant clones isolated from gancyclovir-treated cultures of HT3'-2 and HT3'-5 (see Fig. 5A) displayed myogenic indices that were comparable to those of control cultures (Fig. 5B). When replated in medium containing hygromycin B, resistant clones were isolated at a frequency of about 10−4, suggesting that loss of the hygromycin B resistance phenotype occurred at a relatively high frequency. These data strongly suggest that determination of DΔ5 and DΔ35-transfected myoblasts. Fusion index comparison between DΔ5, DΔ35 representative clones, and C2C12 cells. DΔ35 clones overexpressing DMK mRNA and protein fused as well as C2C12, whereas DΔ5 clones showed reduced fusion potential.

Fig. 4. Expression of DMK mRNA and protein in K100R-expressing myoblast clones. A, example of a Northern blot analysis of DMK mRNA expressed from C2C12 cells or K100R-transfected clones, K5 and K6. DMK was found to be expressed 7- and 8-fold higher than the endogenous DM kinase mRNA, respectively. Exposure was for 24 h. B, Western blot analysis of DMK protein expression in K5 and K6 clones using anti-DMK-2. C, fusion index determination for four K100R-transfected clones. Following 48 h in differentiation medium, fusion indices were determined as described (see “Materials and Methods”) and compared with wild type C2C12 cells or C2neo. In 11/12 clones overexpressing mutant DMK mRNA, reduced fusion indices were observed. A single clone, expressing DMK at levels that were comparable to C2C12, was found to fuse as well as control cells (not shown). D, fusion index
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Fig. 5. Inhibition of myoblast differentiation by DMK 3'-UTR. A, overexpression of hy-tk hybrid mRNA carrying the DMK 3'-UTR (HT3') in myoblast clones. The membrane was probed with a hy-tk fragment and exposed to x-ray film for 24 h. Probing of the same filter with a DMK 3'-UTR probe showed an identical pattern of expression in addition to the endogenous DMK mRNA. Normalization to pgk levels and endogenous DMK showed that hy-tk was expressed 10-fold higher than the endogenous DMK mRNA in HT3 clones. Clones transfected with HT3 R or HT3 PGK expressed similar levels of hy-tk hybrid mRNA (not shown). B, fusion index measurement on myoblast clones transfected with various hy-tk constructs. Clones overexpressing the DMK 3'-UTR in the sense orientation (HT3') showed a reduced fusion potential when compared with C2C12 cells, hygromycin-resistant C2C12 control clones (HT-C), or control HTPGK clones. Gancyclovir-resistant clones (Gc-2 and Gc-5) obtained through negative selection were found to fuse more than the parent clones HT3'-2 and HT3'-5. For plasmid pH3, 24 clones were assayed and 12 clones for pH3 R and 5 clones for pHTPGK. Two examples of such clones are shown.

Fig. 6. Effect of DMK 3'-UTR overexpression on myogenin and mef-2c expression. C2C12 control cultures or 3'-UTR overexpressing clones (HT3'-1 and HT3'-2) were grown to 80% confluency and then switched to differentiation medium for 48 h and analyzed for myogenin and mef-2c mRNA expression. (-) represents cultures in growth medium; (+) indicates cultures in differentiation medium for 2 days. Myogenin expression was found to be reduced by 75%, whereas mef-2c expression was unaffected.

The DMK 3'-UTR is sufficient to mediate the observed inhibition of myoblast terminal differentiation in transfected C2C12 cultures.

Overexpression of DMK 3'-UTR Reduces Myogenin, but Not mef-2c mRNA Steady State Levels—The differentiation of myoblasts in vitro has been shown to be associated with a rapid induction of myogenin, a member of the basic helix-loop-helix family of transcriptional activators (reviewed in Ref. 34). Interestingly, myogenin is also required for the formation of myotubes and myofibers in vivo (44, 45), suggesting that it plays a central role in the terminal differentiation of myoblasts. Therefore, we investigated the effect of DMK 3'-UTR overexpression on myogenin gene expression following the induction of differentiation. Clones HT3'-1 and HT3'-2 were grown to 80% confluency and transferred to differentiation medium. Total RNA was prepared 48 h later and analyzed for myogenin expression. After normalization to pgk levels, Northern blot analysis demonstrated that myogenin mRNA levels were reduced by about 75% in clones overexpressing the 3'-UTR (Fig. 6) compared with C2C12 cells. Similarly, these clones showed a marked reduction in myogenin protein levels (not shown). The RNA was subsequently probed for mef-2c, another early regulator of myogenesis, and a member of the MADS box family of proteins (34). Interestingly, mef-2c mRNA was found to be induced at levels that were similar to that of C2C12 control cultures (Fig. 6), indicating that the 3'-UTR may affect the steady state levels of subsets of mRNAs, as hypothesized by others (24).

Mapping of the 3'-UTR Inhibitory Region Excludes the CTG Repeat—To define the sequence elements that mediate the inhibition of myoblast terminal differentiation, a series of three expression vectors carrying various portions of the 3'-UTR (pHTAR, pHTΔ3', pHTΔ5'; Fig. 7A) were transfected into C2C12 cells, and a total of 12 hygromycin B-resistant clones were isolated from independent transfections and tested for
expression potential. As shown in Fig. 7B, clones expressing a mutant 3′-UTR lacking the repeat region (pHTAR) or all sequences downstream of the repeat (pHTAS′) displayed reduced fusion potential similar to HT3′ clones (Fig. 8). However, clones expressing an mRNA in which the 239-bp region upstream of this repeat (pHTAS′) displayed reduced fusion indices in contrast to clones expressing the 3′-UTR without the 239-bp most 5′-region (pHTAS′). HTAS′ clones fused as well as control cultures. These data indicate that the inhibitory activity maps to a 239-bp region 5′ to the CTG repeat. The graph shows one representative fusion index experiment of two examples of clones expressing each of the vectors described in A. The bars denoted by HTAS-R, HTAS-p, and HTAS-p′ represent the average fusion index ± S.E. obtained in one experiment for pools of hygromycin B-resistant cells obtained from an independent transfection experiment.

**Discussion**

As a first step into the characterization of DMK expression during myogenesis, we analyzed its steady state levels in myogenic cell lines and during differentiation of C2C12 murine myoblasts. DMK was found to be expressed in all myogenic lines analyzed and at much lower levels in P19 stem cells, suggesting that it is up-regulated during myogenic determination. Supporting this hypothesis, we have found that DMK was up-regulated significantly in C3H10T1/2 mesodermal precursors treated with 5-azacytidine or transfected with a myoD expression vector. Similarly, when P19 stem cells or normal human fibroblasts (as well as DM fibroblasts) are transfected with myoD, up-regulation of DMK is observed (46, 47).

Following the induction of differentiation, DMK mRNA and protein levels were observed to be increased by about 1.5 to 2-fold relative to uninduced C2C12 cells which is considerably less than what has been observed for other muscle-specific structural or regulatory genes. The observed increase in DMK levels during differentiation was found to occur 72–96 h following the onset of differentiation, at which time the expressions of various muscle differentiation markers such as myogenin, mef-2c, and creatine kinase have reached their peak and the differentiation programs are well underway, suggesting that DMK does not play an early role in differentiation per se. This pattern of expression is similar to that of mrf-4, a member of the basic helix loop helix family, postulated to have a significant role in the maturation of myotubes (34, 48, 49).

**Inhibition of Myoblast Terminal Differentiation by the DMK 3′-UTR**—We have previously shown that DMK mRNA steady state levels were increased 4–6-fold in muscle tissues from severely affected congenital DM patients (18). In addition, others (1–4) have reported that skeletal muscles from these individuals display immature myofibers and altered contractile protein profiles, indicative of a delay in muscle terminal differentiation.

Our data show that overexpression of a full-length DMK cDNA in myoblasts markedly reduces the fusion potential of C2C12 cells, a result consistent with the in vivo observation of delayed muscle differentiation in congenital DM patients (see Table I). We have determined that this activity maps to the 3′-UTR of DMK, as elevated DMK protein levels or active kinase was not required. Furthermore, this activity could be conferred to a heterologous gene using chimeric drug resistance DMK 3′-UTR constructs. However, it is still unknown whether the inhibition of differentiation by the 3′-UTR is accompanied by increased cell death in these cultures. In light of the observation that myofiber atrophy is a characteristic of adult DM patients, experiments designed to look at apoptosis in these cultures are underway.

This inhibitory activity was mapped to a region of the 3′-UTR that excludes the CTG repeat but which is highly conserved between human and mouse (14). In view of the fact that the CTG region is not conserved between the latter two species, it is likely that the conserved segment mediates the inhibitory effect. However, one potentially conflicting observation is the fact that DMK mRNA levels are slightly increased during myoblast differentiation, whereas elevated expression of DMK 3′-UTR in myoblasts interferes with differentiation. One explanation is that high levels of DMK mRNA (3′-UTR) in myoblasts, prior to the induction of differentiation, are detrimental to the differentiation process. Alternatively, a 2-fold increase in DMK mRNA levels during differentiation is not sufficient to delay myogenesis. Supporting this is the observation that revertant populations of CMV-DMK expressing low levels of DMK mRNA differentiated as well as wild type myoblasts (Fig. 3D).

Recently, it was reported that overexpression of DMK protein, using a human cDNA lacking the 3′-UTR, enhanced the skeletal muscle phenotype of the smooth muscle-like cell line BC3H1 as evidenced by increased myogenin expression (50). Interestingly, in contrast to wild type cells, BC3H1 clones overexpressing the DMK 3′-UTR did not induce expression of myogenin or other differentiation-specific markers, supporting our observations. However, in contrast to Busch et al. (50) overexpression of DMK protein in C2C12 cells did not have any effect on their differentiation potential. This could be due to the
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Fig. 8. Average fusion index and standard error from the mean for all clones surveyed. The average fusion index for all the clones analyzed (for each DMK expression vector; see Table I) was graphed with the standard error from the mean. The average indices were calculated from two independent determinations for each clone. A total of at least 1500 nuclei was counted for each fusion index (see “Materials and Methods”). Reduced fusion potential was observed in clones overexpressing the DMK 3′-UTR.

Numerous studies have defined major roles for mRNA 3′-UTRs. Specific motifs found in the 3′-UTR of several classes of transcripts have been shown to mediate post-transcriptional mRNA stability or have been demonstrated to be involved in the control of translation (reviewed in Ref. 53). Recently, Rastinejad and Blau (29) have identified novel functions in the control of translation (reviewed in Ref. 53). Recently, DMK nullizygous mice have been demonstrated to display only mild myopathy at an advanced age (27, 28). In addition, Jansen et al. (27) have shown that transgenic animals overexpressing DMK showed enhanced neonatal mortality, a hallmark of congenital DM. However, again, these mice did not present with classical DM symptoms. These data suggest that the mechanism underlying the pathophysiology of DM is much more complex than a simple dosage phenomenon and is likely to be dependent on the presence of the CTG repeat. Our results suggest that accumulation of the mutant DMK mRNA, as previously reported in DM individuals (52), may contribute significantly to the DM phenotype, independently of the protein product. Taken together, these results suggest that the dominant inheritance pattern of DM may not be due to changes in DMK protein levels or activity but rather be the result of a cis or trans dominant effect of the amplified repeat in the 3′-UTR.

In situ hybridization studies have demonstrated that the mutant DMK mRNA accumulates in the nuclei of fibroblasts and muscle cells isolated from adult DM patients (52). These studies, however, were not able to specifically distinguish between altered pre-mRNA processing or transport of the mutant DMK mRNA. Recently, Timchenko et al. (54) have isolated an RNA-binding protein that interacts with the CUG repeat of the DMK mRNA. Taken together, these observations support the hypothesis that the expanded repeat may alter the normal metabolism of the mutant transcript through a protein-dependent mechanism.

Combined with the observations of DMK overexpression in DM muscle tissue (15, 18), these results suggest that the CTG repeat may lead to the accumulation of mutant DMK mRNAs and/or full-length mutant heteronuclear RNAs, and this could exert a significant effect on the disease process such as the delay of muscle maturation in congenital DM patients.

The binding of protein factors to the CTG repeat may interfere with normal DMK mRNA processing or transport, and its accumulation in the nuclei of these cells may lead to a competition for 3′-UTR binding sites involved in the export or processing of differentiation-specific mRNAs. As shown by Timchenko et al. (54), the CTG repeat is a target site for such factors. In addition, 5′ adjacent sequences might be the binding site(s) for trans-acting factors and, in the disease state, a neighboring expanded repeat may prevent secondary DMK mRNA processing subsequent to the binding of these factors to sequences lying upstream, resulting in a titration of the primary binding factor. In any case, expression of high levels of a wild type 3′-UTR such as the one used in the present study could produce a similar effect in cultured myoblasts by sequestering processing factors (54) which might be required for the metabolism of other classes of mRNA.

Four major basic helix loop helix proteins (MyoD, myogenin, Myf-5, and Mrf-4/Myrf-6/herculin) have been demonstrated to play central roles in the control of myoblast differentiation and differentiation (34). Our results show that clones overexpressing DMK 3′-UTR do not fuse but express normal levels of myoD mRNA. However these clones display reduced levels of myogenin mRNA following the onset of differentiation, suggesting that the DMK 3′-UTR may interfere with the expression of differentiation-specific mRNAs. Interestingly, the same clones did not show altered expression of mef-2c following the induc-
tion of differentiation. It is then possible that the DMK 3'-UTR affects the expression of specific subsets of genes whose products are involved in differentiation or other cellular processes, suggesting that it may in fact affect the activity of specific mRNA-binding factors.

Clearly, our data demonstrate that the inhibition of terminal differentiation in C2C12 myoblasts is due to overexpression of DMK. We are also grateful to Drs. Mike McBurney and Mike Rudnicki for the pgk and mlc 1/3 cDNAs, respectively. We thank Dr. Eric Olson for providing the myogenin and myf-2c probes.

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REFERENCES
1. Harper, P. S. (1989) Myotonic Dystrophy, 2nd Ed., W. B. Saunders Co., Philadelphia.
2. Farkas-Barger, E., Barbet, J. P., Dancea, S., Wehrle, R., Checoury, A., and Dulac, O. (1996) J. Neurol. Sci. 143, 145–159.
3. Sarnat, H. B., and Silbert, S. W. (1976) J. Neurol. Sci. 32, 195–202.
4. Sarnat, H. B., and Silbert, S. W. (1976) J. Neurol. Sci. 32, 195–202.
5. Hinterberger, T. J., Sassoon, D. A., Rhodes, S. J., and Konieczny, S. F. (1991) Genes Dev. 5, 2050–2061.
6. Van der Ven, P. F. M., Jansen, G., van Kuppevelt, T. H. M. S., Perryman, M. H., Lupa, M., Dunne, P. W., ter Laak, H. J., Jap, P. H. K., Veerkamp, J. H., Epstein, H. F., and Wieringa, B. (1993) Hum. Mol. Genet. 2, 765–770.
7. Whiting, E. J., Waring, J. D., Tamaki, K., Somerville, M. J., Himc, M., Staines, W. A., Ikeda, J.-E., and Korneluk, R. G. (1995) Hum. Mol. Genet. 4, 281–286.
8. Saiboun, L. A., Mahadevan, M. S., Narang, M., Lee, D. S. C., Surti, L. C., and Korneluk, R. G. (1993) Genet. 4, 340–348.
9. Fu, Y.-H., Friedman, D. L., Richards, S., Pearlman, J. A., Gibbs, R. A., Pizzuti, A., Ashizawa, T., Perryman, M. B., Scarlata, G., Fenwick, R. G., Jr., and Caskey, C. T. (1993) Science 260, 235–238.
10. Hofmann-Radvanyi, H., Lavedan, C., Rabes, J.-P., Savoy, D., Daros, C., Johnson, K., and Junien, C. (1993) Hum. Mol. Genet. 2, 1263–1266.
11. Koga, R., Nakao, Y., Kurano, Y., Tsukahara, T., Nakamura, A., Ishiura, S., Nonaka, I., and Arakata, K. (1994) Biochem. Biophys. Res. Commun. 202, 377–385.
12. Krahe, R., Ashizawa, T., Abbruzzesse, C., Roeder, E., Carango, P., Giaconelli, M., Funcan, V. L., and Tapp, S. J. (1997) Hum. Mol. Genet. 6, 402–406.
13. Jansen, G., Groenen, P. T. J. A., Bachner, D., Jap, P. H. K., Coerwinkel, M., Hameister, H., and Wieringa, B. (1996) Hum. Mol. Genet. 5, 599–608.
14. Thorton, C. A., Wymer, J. P., Simmons, Z., McClain, C., and Mosley, R. T., III (1997) Nat. Cell. Biol. 10, 479–490.
15. Kesler, T. R., Otten, A. D., Bird, T. D., and Tapp, S. J. (1995) Hum. Mol. Genet. 4, 189–196.
16. Van der Ven, P. F. M., Jansen, G., van Kuppevelt, T. H. M. S., Perryman, M. H., Lupa, M., Dunne, P. W., ter Laak, H. J., Jap, P. H. K., Veerkamp, J. H., Epstein, H. F., and Wieringa, B. (1993) Hum. Mol. Genet. 2, 1889–1894.
17. Reddy, S., Smith, D. B. B., Rich, M. M., LeBlond, S., Earle, P., Davis, B. M., Tran, K., Rayburn, H., Bronson, R., Bros, D., Balice-Gordon, R. J., and Housman, D. (1996) Nat. Cell. Biol. 13, 325–335.
18. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
19. Lupton, S. D., Brunton, L. L., Kalberg, V. A., and Orelli, B. (1991) Mol. Cell. Biol. 11, 3374–3378.
20. Bader, D., Masaki, T., and Fischman, D. A. (1982) J. Cell Biol. 85, 763–770.
21. Konieczny, S. F., and Emerson, C. P., Jr. (1984) Cell 38, 791–800.
22. Olson, E. N., and Klein, D. (1993) Gene Dev. 1–7.
23. Seed, B., and Aruffo, A. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 3365–3369.
24. Black, R. A., and Hall, Z. W. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 124–128.
25. Song, K., Wang, Y., and Sassoon, D. (1992) Nature 360, 477–481.
26. Tester, H., and Sefton, B. (1991) Methods Enzymol. 200, 423–462.
27. Ellison, M. J., and Hochstrasser, M. (1991) J. Biol. Chem. 266, 21150–21157.
28. Wieringa, B. (1994) Hum. Mol. Genet. 3, 1–7.
29. Rastinejad, F., Conboy, M. J., Rando, T. A., and Blau, H. M. (1993) Cell 75, 1107–1117.
30. Adra, C., Boer, P. H., and McBurney, M. W. (1987) Gene (Amst) 60, 65–74.
31. Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esumi, E., Li, S., Nonaka, I., and Nabeshima, Y. (1993) Nature 364, 532–535.
32. Verucchi, J. M., Morris, J. H., Vianan, J. L., Olson, E. N., and Klein, W. J. (1995) J. Cell. Biol. 126, 536–573.
33. Otten, A. D., and Tapp, S. J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5465–5469.
34. Skerjanec, I. S., Slack, R. S., and McBurney, M. W. (1994) Mol. Cell. Biol. 14, 845–8459.
35. Hinterberger, T. J., Sassoon, D. A., Rhodes, S. J., and Konieczny, S. F. (1991) Cell. Biol. 115, 1107–1117.
36. Bush, E. W., Taif, C. S., Meixell, G. E., and Perryman, M. B. (1996) J. Biol. Chem. 271, 458–552.
37. Tanbaum, M. B., Smith, C. W., Jumaa, S., Grant, J. W., Endo, T., Andreadis, A., and Nadal-Ginard, B. (1989) J. Cell. Biol. 108, 1798–1806.
38. Taneja, K. L., McBurney, M., Schallinger, M., Housman, D., and Singer, R. H. (1991) J. Cell. Biol. 126, 8530–8542.
39. Belasco, J., and Brawerman, G. (1993) Control of Messenger RNA Stability, Academic Press, San Diego.
40. Timchenko, L. T., Miller, J. W., Timchenko, N. A., Devore, D. R., Datar, K. V., Lin, L., Roberts, R., Caskey, C. T., and Swanson, M. S. (1996) Nucleic Acids Res. 24, 4407–4414.