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Expression of the dystrophin isoform Dp71 in differentiating human fetal myogenic cultures

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The dystrophin gene defective in Duchenne muscular dystrophy (DMD) is extreme in size and complexity with several promoters which direct expression of different isoforms in different tissues. In contrast with adult skeletal muscle which expresses 427 kDa dystrophin, fetal muscle tissue expresses the 71 kDa ubiquitous isoform Dp71 as well as 427 kDa muscle dystrophin. To examine Dp71 expression in fetal muscle further, we have monitored its expression pattern in differentiating myogenic cultures of human fetal muscle origin. The presence of transcripts initiated from the Dp71 promoter was demonstrated by quantitative RT–PCR. The level of transcript expressed from the Dp71 promoter did not change significantly during myogenic differentiation, consistent with the housekeeping nature of the promoter. Measurements to determine the stability of the Dp71 mRNA indicated that it has a half-life of ~20 h and, therefore, is somewhat more stable than the larger 14 kb muscle dystrophin mRNA ($t_{1/2}$ = 16 h). In contrast with the constant level of Dp71 transcript during myogenic differentiation, the level of Dp71 protein increased significantly, perhaps due to changes in translation efficiency or protein stability. These results demonstrate expression and posttranscriptional upregulation of Dp71 in human fetal myogenic cultures.

INTRODUCTION

Duchenne muscular dystrophy is an X-linked lethal disorder characterized primarily by progressive degeneration of skeletal muscle and caused by mutations in the dystrophin gene. This complex gene encodes a set of at least seven protein isoforms of various sizes and tissue distributions, each translated from a unique message initiated from one of the seven distinct promoters in the gene. Three high molecular weight isoforms (427 kDa), expressed in skeletal muscle (1), neurons of the cerebral cortex (2–4) and purkinje cells of the cerebellar cortex (2), are the result of transcripts initiated from three distinct promoters at the 5′ end of the gene, upstream of a common exon 2. Shorter isoforms of size 260 kDa (5), 140 kDa (6), 116 kDa (7) and 71 kDa derive from transcripts that contain unique first exons located in introns 29, 44, 55 and 62. The first three of these have tissue specificity for the retina, central nervous system and peripheral nerve respectively.

The shortest isoform, Dp71, is present in the liver cell line HepG2 and in many adult tissues including brain, liver, testis, lung and kidney but is not detectable in adult skeletal muscle (8,9). The transcripts are initiated from a promoter 8 kb upstream of exon 63 (9–11). Dp71 contains the cysteine-rich and C-terminal domains of dystrophin and is missing the N-terminal actin binding and spectrin-like repeat domains present in the largest isoforms (10). Seven amino acids are encoded by Dp71 exon 1 generating a novel N-terminus. The remainder of the amino acids are encoded by exons 63–79 with alternative splicing of exons 71 and 78 (12). Dp71 is undetectable in fetal brain but the levels increase during development and are detectable in adult brain. Conversely, Dp71 is expressed at detectable levels in fetal muscle but not in adult muscle tissue (13). The function of Dp71 is unknown but it localizes to the membrane of HepG2 cells (14) and when expression is forced in myogenic cells not expressing the 427 kDa dystrophin isoform it localizes to the membrane and restores the dystrophin-associated glycoprotein (DAG) complex to the membrane (15,16). Despite this, Dp71 is unable to correct the muscle defect in mouse models of DMD indicating that its function differs from dystrophin in muscle (15–16).

Since Dp71 is expressed in fetal, but not adult skeletal, muscle, its level of expression in cultured fetal myoblasts and differentiated myotubes is not readily predictable, and measurement of both mRNA and protein expression during myogenic differentiation in culture has not been reported. Such measurements are important, as expression in fetal myogenic cultures provides a system for study of the regulation and function of Dp71 in muscle; the primary tissue affected in individuals with DMD. Furthermore, previous studies have focused on muscle tissue which contains multiple cell types, the use of clonal human fetal myogenic cultures for these studies will clarify if Dp71 is expressed in this cell type. We monitored expression of Dp71 mRNA by quantitative RT–PCR and observed a significant level of transcript in fetal myoblasts but no increase in this level as cells fused to form multinucleated myotubes, a result consistent with the housekeeping nature of the Dp71 promoter. This was in contrast with dystrophin transcript levels which, as expected, increased during differentiation. The Dp71 mRNA half-life was measured to be 20 h, not grossly different from the dystrophin mRNA

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half-life of 16 h. In contrast with the relatively constant Dp71 mRNA levels, we observed an increase in the level of Dp71 protein, indicating posttranscriptional upregulation of Dp71 expression during fetal myotube formation.

RESULTS

Detection and splicing of Dp71 transcripts in fetal myogenic cultures

The Dp71 isoform previously characterized in other tissues is encoded by a transcript containing a novel exon 1 spliced to exons 63–79 of the dystrophin gene with alternative splicing of exons 71 and 78 (10–12). To determine whether Dp71 transcripts are expressed in human fetal myogenic cultures, myoblasts were plated at high density in growth medium, allowed to recover for 24 h, and then exposed to fusion medium to induce differentiation into multinucleated myotubes. Total RNA was isolated from cultures after 24 h in fusion medium and Dp71 mRNA was detected by RT–PCR. Template cDNA was made by reverse transcription using primer 79R in exon 79. Dp71 transcripts were subsequently amplified using the same reverse primer and the forward primer 741 in the novel Dp71 exon 1. The Dp71 amplification product expected from this reaction is 1879 bp if the RNA is not alternatively spliced. A single product of approximately this size was amplified from RNA of human fetal myogenic cultures (Fig. 1A).

Previous studies of Dp71 in other tissues have demonstrated alternative splicing of exons 71 and 78 which are 39 and 32 bp respectively. Deletion of these small exons would not be detected when amplifying nearly 1.9 kb as we did in the above PCR. To test for alternative splicing of exons 71 and 78 the PCR product in Figure 1A was gel purified and a portion reamplified using a primer pair that flanks exon 71 and another pair that flanks exon 78. With a forward primer in exon 69 (69F) and reverse primer in exon 72 (926) two products were visualized (Fig. 1B). One of these products aligns with the 335 bp control band which contains exons 70 and 71; the smaller and weaker band is ~296 bp, the size expected if exon 71 was not included in the transcript. With forward primer 837 in exon 77 and reverse primer 79R in exon 79 two bands of approximately equal intensity were visualized and these aligned with the two bands in the control lane that include and exclude exon 78 (Fig. 1C). This analysis of Dp71 isoforms expressed in cultured fetal muscle is consistent with the alternative splicing of Dp71 observed in other tissues (12).

Expression of Dp71 RNA during differentiation of fetal myogenic cultures

To monitor the expression profile of Dp71 and dystrophin transcripts during myogenic differentiation, clonal human fetal myoblasts were plated at high density, allowed to recover in growth medium for 24 h, then exposed to fusion medium to induce differentiation and dystrophin gene expression. Cell samples were collected 0, 10.5, 23.5, 29.8 and 37.4 h after the addition of fusion medium to the cultures and total RNA was isolated from each sample. Quantitative RT–PCR was used to monitor transcript accumulation. To quantitate RNA levels by RT–PCR accurately, we introduced an *in vitro* transcribed control RNA (cRNA) as a standard with which to compare the endogenous RNA. A control template (Dp71-1-79) for *in vitro* transcription (Fig. 2) was constructed by cloning part of the Dp71 cDNA, which included the novel exon 1 and exons 63–78 and a portion of exon 79 of the dystrophin gene, into a vector with a T7 promoter. A 118 bp insertion was introduced into exon 65 so that the products obtained for the control and endogenous RNAs following RT–PCR could be separated by gel electrophoresis.

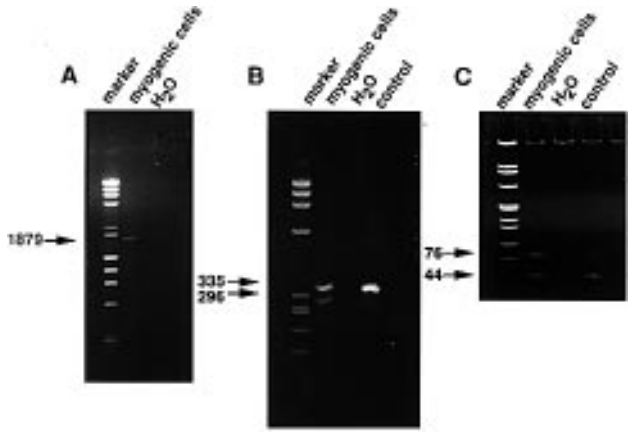

Figure 1. Detection of Dp71 transcripts and alternative splicing by RT–PCR. (**A**) Total RNA was prepared from a clonal human myogenic culture that had been exposed to fusion medium for 24 h. Template cDNA was made by reverse transcription from primer 79R (TGTGGAAAAGACTTCCTACATTG) using 3 µg of total RNA. One half of the cDNA reaction was amplified for 40 cycles using primer 741 (TCTAGAATTCATGAGGGAACAGCTCAAAGG which contained a terminal *Eco*RI restriction site not required for this particular experiment) in the novel Dp71 exon 1 and primer 79R located in exon 79. The product of ~1.9 kb is indicated by the arrow and is present in myogenic cells but absent in the lane in which template cDNA was replaced by H_2O. The marker is λ *Hin*dIII and ϕX174*Hae*III. (**B**) The product from (A) was gel-purified and 1/50 of the product was reamplified for 30 cycles using primer 69F (TACAGGAGTCTAAAGCACTT) in exon 69 and 926 (ATTGCGT-GAATGAGTATCATCGT) in exon 72. The two products are designated by arrows where the larger 335 bp product contains both exons 70 and 71 and is seen in the control lane in which a plasmid containing both exons was amplified. The smaller product is consistent with the size predicted when exon 71 is deleted. As a control the template cDNA was replaced with H_2O. Marker, ϕX174*Hae*III. (**C**) One fiftieth of the gel-purified product from (A) was re-amplified for 30 cycles using primer 837 (CCTTCCCTAGTTCAAGAGG) in exon 77 and primer 79R in exon 79. In the control lane a mixture of sequences in which exon 78 is known to be present or absent were amplified to show the expected product sizes of 76 and 44 bp when exon 78 is present or absent respectively (arrows). Both products are observed in myogenic cells but not when the template cDNA was replaced with H_2O. Marker, as in (B).

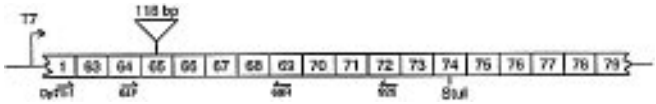

Figure 2. Schematic of control template Dp71-1-79 used for *in vitro* transcription. The control template contains the 3′ end of Dp71 exon 1 and exons 63–79 downstream of a T7 promoter. A 118 bp DNA fragment was inserted within exon 65 so the control RNA produced by *in vitro* transcription would differ slightly from the endogenous RNA. The exon numbers are shown in the rectangles representing the exons and follow the dystrophin cDNA nomenclature. Exon 1 represents Dp71 exon 1. Primers used to amplify transcripts initiated from the Dp71 promoter are designated Dp71-1 (TGCAGCCATGAGGGAACAGC) and 69R (GGAGTGCAATATTCCAC-CAT). Primers used to amplify both Dp71 and dystrophin transcripts are designated 64F (TTCTCAGCTTATAGGACTGCC) and 69R. The primer used for reverse transcription, designated 926, is shown.

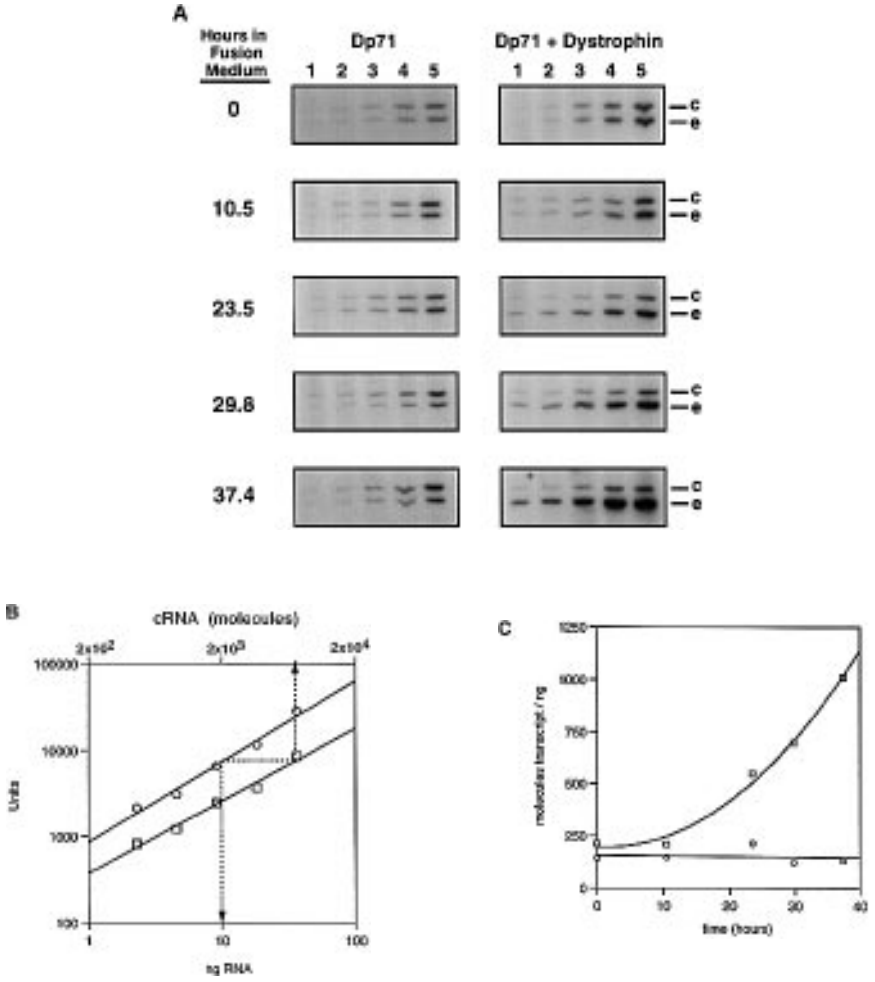

Figure 3. Expression of Dp71 and total (Dp71 plus dystrophin) RNA in differentiating fetal myogenic cultures. (**A**) Total RNA was isolated from myogenic cultures exposed to fusion medium for 0, 10.5, 23.5, 29.8 and 37.4 h. For each sample 365 ng of total cellular RNA was combined with 7.3×10^4 molecules of Dp71-1-79 cRNA and first strand cDNA was made by reverse transcription using primer 926 in exon 72. A dilution series of the cDNA (lanes 1–5) was amplified by PCR in the presence of an end-labeled primer and the control (c) and endogenous (e) products were separated by polyacrylamide gel electrophoresis. The 5% non-denaturing gels were then dried, exposed to X-ray film, and the resultant autoradiographs are shown. The gels in the Dp71 column show the control (c) and endogenous (e) products obtained at each time point following amplification using primers Dp71-1 and 69R. The gels in the Dp71+ dystrophin column show the results obtained when the same samples were analyzed using primers 64F and 69R. (**B**) To determine the absolute amount of transcript in each sample the amount of control (open squares) and endogenous (open circles) RT–PCR products (units as measured using a phosphorimager) were graphed versus the amount of cRNA (molecules) and total muscle RNA (ng). This graph indicates 6960 molecules of transcript per 10 ng of total RNA. This example plots the results from amplification of RNA isolated from a myogenic culture in fusion medium for 29.8 h with primers 64F and 69R. (**C**) The Dp71 and total transcript levels were determined by quantitative RT–PCR for each sample and are plotted against the time the myogenic culture was in fusion medium. The molecules of transcript/ng total cellular RNA obtained for transcripts initiated from the Dp71 promoter (open circles) and for total transcript (Dp71 + dystrophin) (open squares) are shown on the graph.

A measured amount of cRNA generated by *in vitro* transcription using the Dp71-1-79 template was combined with a measured amount of total muscle cell RNA. First strand cDNA was made by reverse transcription using a primer in exon 72, and this was amplified in two separate reactions using two different primer sets (Fig. 2). The first primer set was specific for Dp71 transcripts as the forward primer (Dp71-1) was located in the novel Dp71 exon 1 and the reverse primer (69R) was located in exon 69 of the dystrophin gene, producing a product of 888 bp from the endogenous transcript. The second primer pair amplified both Dp71 and full-length dystrophin transcripts as the forward primer (64F) was in exon 64 and the reverse primer (69R) was in exon 69 and these exons are present in both Dp71 and dystrophin transcripts.

Following amplification of a dilution series of the cDNA, the control (c) and endogenous (e) products were separated by non-denaturing polyacrylamide gel electrophoresis (Fig. 3A). The amount of control and endogenous product was determined using the phosphorimager and graphed versus the amount of control RNA and total muscle RNA (Fig. 3B). The example in Figure 3B plots the results obtained using primers 64F and 69R to amplify both Dp71 and dystrophin transcripts in an RNA sample from a myogenic culture in fusion medium for 29.8 h. This analysis indicated ~696 molecules of transcript/ng total cellular RNA.

The ratio of endogenous (e) to control (c) RT–PCR products shows a modest decrease over time using primers Dp71-1 and 69R which amplify only Dp71 transcripts (Fig. 3A). In contrast, with primers 64F and 69R which amplify both Dp71 and dystrophin transcripts, the amount of endogenous RT–PCR product shows a significant increase relative to the control product over time

(Fig. 3A). A graph of the kinetics of Dp71 and total (Dp71 plus dystrophin) transcript accumulation clearly illustrates the modest decrease in the level of Dp71 transcript and the significant increase in total (dystrophin plus Dp71) transcript during myogenic differentiation (Fig. 3C). Presumably, the increase in total transcript levels is due to an increase in the 14 kb mRNA encoding 427 kDa dystrophin during myogenic differentiation.

Dp71 mRNA half-life in myogenic cultures

In other studies we measured the stability of the dystrophin mRNA in fetal myogenic cultures and obtained a half-life of 16 h (17). Since full length muscle dystrophin mRNA and Dp71 mRNA are both expressed in fetal myogenic cultures, and therefore in the same cellular environment, it was interesting to compare the stability of these transcripts which share many sequences at the 3′ end of the message. For these experiments fetal myogenic cultures were grown in fusion medium for 40 h and then exposed to 5 µg/ml actinomycin D, an inhibitor of transcription. Total RNA was prepared from cultures exposed to actinomycin D for 0, 1, 2, 4, 7, 11 and 23 h. The decay of the Dp71 mRNA was monitored by quantitative RT–PCR. Control RNA was prepared using the template Dp71-1-79 (Fig. 2). A measured amount of cRNA was combined with a measured amount of total RNA from the cultured cells and cDNA was made by reverse transcription using primer 926 in exon 72. The Dp71 mRNA and cRNA were amplified using the forward primer Dp71-1 in the first exon of Dp71 and the reverse primer 69R in exon 69. The decay of the endogenous Dp71 mRNA was monitored and the results are shown in Figure 4. The amount of endogenous RNA decreases modestly relative to the control RNA. Using a phosphorimager the amount of control and endogenous RNA was determined and the results used to calculate the absolute amount of Dp71 mRNA in each sample. This analysis was done for three different myogenic clones. The graph in Figure 5 plots the average percent maximum against the time the cells were exposed to actinomycin D. These results show that ~20 h are required for 50% of the Dp71 mRNA to decay, indicating this mRNA is somewhat more stable than the dystrophin mRNA.

Dystrophin and Dp71 protein accumulation during differentiation of fetal myogenic cultures

To examine the expression profile of the Dp71 protein during differentiation, total protein extracts were prepared from myogenic cultures. Protein accumulation was examined using Western blots stained with the monoclonal antibody Mandra 1 which detects both dystrophin and Dp71. The protein samples were prepared from the same clone and simultaneous with the RNA samples used for the quantitative RT–PCR experiments shown in Figure 3. The signal was revealed by ECL (enhanced chemiluminescence) and exposure to X-ray film (Fig. 6A).

The 427 kDa dystrophin protein is present at low levels (barely detectable by ECL) in myoblasts at time 0 but increases with time in fusion medium (Fig. 6A). The dystrophin protein accumulation profile was estimated by densitometric analysis of the autoradiograph and the results are shown in Figure 7. This analysis indicates a significant increase in dystrophin protein following the addition of fusion medium. The protein detected in Figure 6A is similar in size to the 427 kDa dystrophin protein detected in adult skeletal muscle which has been included as a control in this

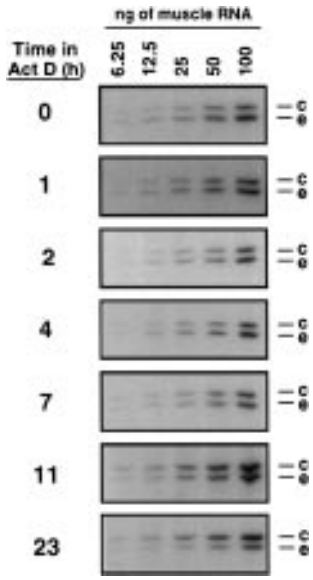

Figure 4. Measurement of Dp71 mRNA stability in myogenic cultures. Myogenic clones expressing Dp71 were exposed to 5 µg/ml actinomycin D and the decay of the Dp71 transcript was monitored by quantitative RT–PCR. Total RNA was isolated from muscle cultures 0, 1, 2, 4, 7, 11 and 23 h following addition of actinomycin D. For each sample, 1.0 µg of total RNA was combined with 2×10^5 molecules of cRNA synthesized from the control plasmid Dp71-1-79. The RNA was reverse transcribed using the reverse primer 926 in exon 72 and a serial dilution of the cDNA was amplified for 25 cycles using primers Dp71 exon 1 and 69R. A total of 1×10^6 c.p.m. of end-labeled primer was included in each amplification reaction. The RT–PCR products were separated on a 5% non-denaturing polyacrylamide gel and an autoradiograph of the gel is shown. The larger amplification product is generated from the control RNA (c) and the smaller product is generated from the endogenous (e) Dp71 mRNA.

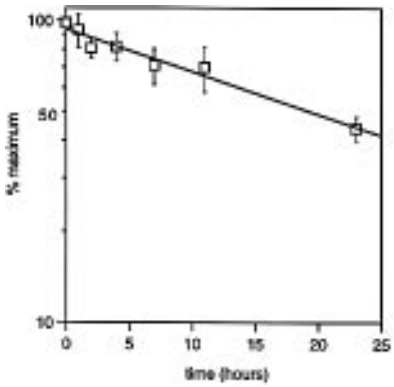

Figure 5. Half-life of the Dp71 mRNA in fetal myogenic cultures. Quantitation of the Dp71 mRNA in cultures treated with actinomycin D was done as described in Figure 3. Three separate clones were analyzed. The average percent maximum is plotted against the time the cells were exposed to actinomycin D. Regression analysis indicated that ~20 h are required for 50% of the transcript to decay.

analysis. The delay of ~23 h (Fig. 7) is a result of the fact that the first mature dystrophin mRNA appears at ~21 h after induction of differentiation (18).

The 71 kDa isoform is also present at low levels at time 0, and it also increases with time as shown in Figure 6A. Densitometric analysis of the autoradiograph indicates an at least 10-fold

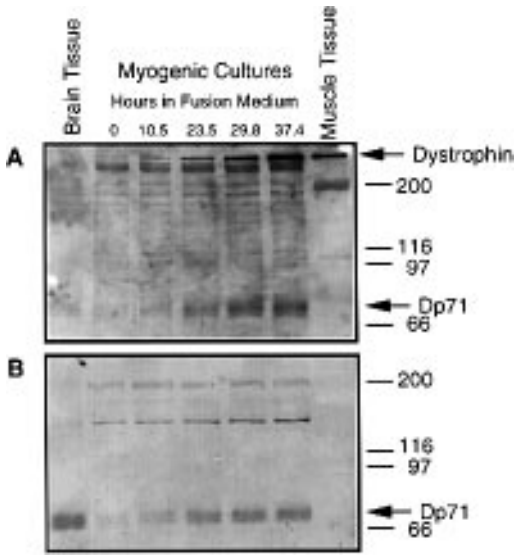

Figure 6. Western blot analysis of dystrophin and Dp71 protein accumulation during differentiation of fetal myogenic cultures. Human fetal myoblasts were plated at high density in growth medium, allowed to recover for 24 h and then transferred to fusion medium to induce expression of the dystrophin gene with differentiation. Protein extracts were prepared from cells harvested at various times (0, 10.5, 23.5, 29.8 and 37.4 h) after addition of fusion medium to the culture. Proteins were separated by SDS–polyacrylamide gel electrophoresis using a 7% gel, transferred to nitrocellulose, and stained with (**A**) the monoclonal antibody Mandra 1 which detects both Dp71 and dystrophin and (**B**) the polyclonal antibody NME-1 which is specific for Dp71. Protein extract from adult brain and muscle (10 µg) was included as controls in the first and last lanes respectively. This experiment used 60 µg protein extract from cultured myogenic cells.

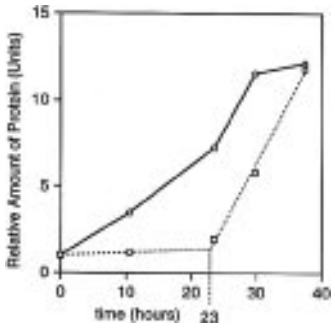

Figure 7. Kinetics of dystrophin and Dp71 protein accumulation during fetal myogenic differentiation in culture. Densitometry was used to estimate the relative change in dystrophin (open squares) and Dp71 (open circles) protein levels in the experiment shown in Figure 6. In each case the value obtained at time 0 was set to 1. An estimation of when dystrophin protein starts to accumulate is indicated by the dashed line.

increase in Dp71 protein during the first 38 h in fusion medium (Fig. 7). To confirm this result the same samples were analyzed using an antibody specific to the N-terminus of Dp71 (Fig. 6B). This antibody revealed a profile of Dp71 expression identical to that obtained using Mandra 1 (Fig. 6B). These results indicate that while Dp71 mRNA levels show a modest decline during fetal myogenic differentiation, the protein levels increase at least 10-fold, providing evidence for post-transcriptional upregulation of Dp71 expression.

Consistent with the RT–PCR results we detect multiple isoforms of the Dp71 protein in cultured fetal muscle. Other faint bands representing very low level proteins, non-specific interactions, or degradation products are detected in these samples. This may be due to the long exposure time used in an effort to detect the 427 kDa dystrophin protein early in differentiation.

DISCUSSION

The initial characterization of Dp71 by others established that the protein contains only the cysteine-rich and C-terminal domains of dystrophin and that it is expressed in most tissues except adult skeletal muscle (8–9). More recent studies have detected Dp71 in fetal muscle by Western blot (13) and at very low levels in adult skeletal muscle tissue by Western blot analysis of immuno-precipitated proteins (19). Since muscle tissue contains non-myogenic cell types it is difficult to assess the cellular origin of the Dp71 protein in these studies. We have shown expression of both Dp71 protein and transcript in clonal cultures containing only myogenic cells, indicating that Dp71 is expressed in human fetal myogenic cells. This is consistent with a previous report that the Dp71 transcript is expressed at low levels in the rat myogenic cell line L185 (20).

Our analysis of Dp71 expression during myogenic differentiation indicated that the transcript level was relatively constant over the first 38 h of this process. This was not surprising as studies of the Dp71 promoter have shown it to be a housekeeping type promoter, high in GC content with several potential Sp1 binding sites and no TATA box (11). This type of promoter is expected for genes expressed in many different tissues and is not likely to be responsive to differentiation signals. In contrast with the Dp71 transcript, the level of full-length (14 kb) dystrophin transcript increased sharply following differentiation. This result is similar to that obtained in differentiating rat glial cultures where the Dp71 transcript remains constant while the level of dystrophin transcript increases (20). This increase is consistent with transfection studies showing that expression from the muscle promoter, which contains several myogenic regulatory sequences, is upregulated during myogenic differentiation (21). The RT–PCR strategy we designed to assay Dp71 RNA used primers in exon 72 or 79 for reverse transcription and therefore would not have detected the apo-dystrophin-3 transcript which contains dystrophin exons 63–70 (22).

Using actinomycin D we have shown that the Dp71 mRNA has a half-life of ~20 h, a value somewhat greater than that obtained for muscle dystrophin mRNA ($t_{1/2}$ = 16 h) (17) in the same cellular environment. The fact that these values are not grossly different supports the concept that Dp71 and dystrophin transcript accumulation profiles differ primarily because of differential regulation at the promoters for these transcripts.

The use of independent promoters within the same gene to activate transcription in different tissues is a common regulatory mechanism. Independent promoters are used less frequently to drive expression of vastly different isoforms in the same tissue, but one other example is the gene encoding myosin light chain kinase and its smaller isoform telokin (23). This mechanism may be used less commonly, than for instance, multigene families, because nested transcription units active in the same gene may tend to cause promoter occlusion. This has been observed for the ADH gene in *Drosophila* (24), which contains two closely linked promoters. Our results indicate that in the case of the dystrophin

gene an increase in expression from the upstream dystrophin muscle promoter does not significantly alter expression from the downstream Dp71 promoter. Perhaps the great distance between the promoters due to the large size of the dystrophin gene prevents such effects.

In these studies we also examined expression of the Dp71 and dystrophin proteins during fetal myogenic differentiation in culture. Protein levels were monitored by Western blot analysis using the monoclonal antibody Mandra 1 which detects both proteins. Dystrophin protein levels are very low initially and begin to increase at ~23 h after exposure of the cultures to fusion medium (Fig. 7). This is consistent with our previous studies which showed that 16 h are required for transcription of this gene (>2300 kb) and that under our culture conditions mature transcript begins to accumulate ~21 h after exposure to fusion medium (18).

In contrast with the relatively constant levels of Dp71 transcript during myogenic differentiation, the level of Dp71 protein increased significantly over this same time period. Since transcript levels stay fairly constant the mechanism by which protein levels increase may involve changes in translational efficiency. Alternatively, the effective stability of the protein may change during differentiation. The effective stability is determined by two components, the intrinsic stability and a dilution factor dependent on the rate of cell division (25). During the process of differentiation cells stop dividing and if the protein is stable it will appear to accumulate as it is no longer diluted by cell division.

Studies of Dp71 expression in mouse indicate that although the protein is expressed in fetal muscle the levels decrease and become undetectable in adult muscle tissue. Therefore, although the level of Dp71 protein increases during the early formation of multinucleated myotubes, it is downregulated as the animal matures indicating another level of regulation *in vivo*.

As observed for other tissues (10–12), Dp71 is alternatively spliced for exons 71 and 78 in cultured fetal muscle. Our analysis of the Dp71 transcript in these cultures indicated that a portion of Dp71 mRNAs are missing exon 71. This deletion does not change the reading frame and therefore results in removal of 13 amino acids from the protein (10). Similarly, a portion of transcripts are missing exon 78, the penultimate exon. In this case the reading frame is changed and the last 13 amino acids are replaced by 31 amino acids (10). Our Western blot analysis confirmed the presence of multiple Dp71 isoforms at the protein level in these cultures. Western analysis of Dp71 expressed from a transgene in an *mdx* myoblast cell line identified a doublet where the larger protein was shown to be phosphorylated (15) and treatment with alkaline phosphatase gave a single small band. Therefore some of the Dp71 isoforms we observed by Western blot may be due to differences in phosphorylation.

The 427 kDa dystrophin protein expressed in muscle is localized to the muscle sarcolemma (26). Muscle dystrophin binds cytoskeletal actin at its N-terminus (27) and associates with a membrane-associated glycoprotein complex through the cysteine-rich C-terminal domains (28–30). One member of the dystrophin-associated glycoprotein (DAG) complex interacts with a component of the extracellular matrix (31) and therefore dystrophin links the intra- and extracellular matrices across the muscle membrane. It has been proposed that through these interactions dystrophin may stabilize the muscle membrane and prevent rupture induced by repeated rounds of contraction. Clearly, Dp71 would not play such a role as it does not contain the actin-binding domain of dystrophin. Alternatively, Dp71 may play a role in cell to cell contact or may help organize the glycoprotein complex (13). Dp71 will go to the membrane and restore the glycoprotein complex in muscle of *mdx* mice indicating that it can interact with components of the complex (15). Studies have shown that some components of the DAG complex bind to domains of dystrophin that are present in Dp71. For example, the syntrophins bind to amino acids in exons 73 and 74 (32,33) and β-dystroglycan binds directly to residues of the cysteine-rich and C-terminal domains (34). Furthermore, alternative splicing may modulate the nature of the proteins that associate with Dp71. Since Dp71 is expressed in myoblasts and newly formed myotubes its expression overlaps with dystrophin and may compete for proteins like these that interact with the cysteine-rich C-terminal domains. The precise role of Dp71 in fetal muscle is not known but its presence in the primary tissue affected in DMD patients makes the observation interesting and potentially significant.

MATERIALS AND METHODS

Human myogenic cell culture

Clonal populations of myogenic cells were prepared from muscle of a 10 week human fetus. The procedures to prepare clonal human myoblast cultures have been described (21). A myogenic clone was expanded at low density in cloning medium [F12 medium (Gibco-BRL) containing 20% fetal bovine serum and one Bullet Kit (Clonetics) which includes 0.5 ml human epidermal growth factor (10 μg/ml), 5.0 ml insulin (100 mg/ml), 0.5 ml GA-1000 (gentamycin at 50 mg/ml and amphotericin at 50 μg/ml), 5.0 ml Fetuin (50 mg/ml), 0.5 ml dexamethasone (0.39 mg/ml) and 5.0 ml bovine serum albumin (50 mg/ml)]. The cells were then plated at high density in cloning medium, allowed to recover for 24 h, and then transferred to fusion medium (alpha minimal essential medium; Gibco-BRL) containing 16 mM glucose, 2% fetal bovine serum and 100 U/ml penicillin and 0.1 mg/ml streptomycin, to induce differentiation into multinucleated myotubes.

RNA preparation

Total RNA was isolated from myogenic cultures as described (35). RNA concentration was determined from OD_{260} nm.

Construction of the control template Dp71-1-79

The plasmid Dp71-1-79 shown in Figure 2 contains the last 27 bps of Dp71 exon 1 followed by dystrophin cDNA sequences 9433–11806 which includes dystrophin exons 63–78 and a portion of exon 79. There is a 118 bp fragment from the *Hae*III phi X ladder (Gibco-BRL) inserted in exon 65. It was constructed from the plasmid Dp71-118 which is a Bluescript vector (Stratagene) containing dystrophin exons 63–70 following the Dp71 first exon and a 118 bp insertion in exon 65. To make Dp71-1-79, the plasmid Dp71-118 was digested with *Acc*III and *Nde*I to remove dystrophin cDNA sequences downstream of 9996. A 1.8 kb *Acc*III–*Nde*I insert containing dystrophin cDNA sequences 9996–11806 was isolated from the plasmid p9-14 (ATCC 57677) and ligated into the above plasmid.

Preparation of cRNA by *in vitro* transcription

Dp71-1-79 plasmids were isolated from bacteria by alkaline lysis and purified using equilibrium centrifugation in CsCl (36). Purified plasmid was linearized using *Stu*I and control RNA (cRNA) was generated by *in vitro* transcription from a T7 promoter using the T7 MEGAscript kit (Ambion) as described (18). The concentration of cRNA was determined from OD_{260} nm.

Quantitative RT–PCR

Quantitative RT–PCR required a first step to produce cDNA by reverse transcription of samples containing a measured amount of cRNA and total muscle cell RNA. This was followed by PCR of a dilution series of these samples using a radiolabeled primer. The detailed conditions used for reverse transcription and PCR have been described (37). The RT–PCR products were separated by non-denaturing polyacrylamide gel electrophoresis. The gels were dried and autoradiograms obtained. A phosphorimager and imagequant software (Molecular Dynamics) were used to quantitate the amount of control and endogenous RT–PCR products.

Preparation of protein extracts

Cultured myogenic cells were washed twice with PBS-CIT and scraped into 1 ml of fresh PBS-CIT. The cells were collected by centrifugation and resuspended in 200 µl of solubilization buffer (0.1 M Tris, pH 8.0, 1% SDS, 10 mM EDTA, 10 mM DTT, 0.01 mM PMSF, 5 µg/ml aprotinin, 0.1 mM benzamidine, 0.5 µg/ml leupeptin, 0.5 µg/ml trypsin–chymotrypsin inhibitor and 0.025 µg/ml pepstatin A). Cells were then passed through a 25 gauge needle three times, boiled for 2 min and centrifuged for 4 min at 10 000 r.p.m. The supernatant was aliquoted and stored at –80°C.

Powder of frozen muscle tissue and brain tissue were made using a mortar and pestle pre-cooled on dry ice. Crushed tissue (~50–100 mg) was added to 500 µl of solubilization buffer and vortexed. The solution was passed through a 25 gauge needle three times, boiled for 2 min and centrifuged for 4 min at 10 000 r.p.m. The supernatant was aliquoted and stored at –80°C.

Western blot analysis

Protein concentration was determined by the Lowry assay (38). Protein extract from cultured myogenic cells (60 µg) and protein extract from muscle tissue and brain tissue (10 µg) were size-fractionated by gel electrophoresis using a 7% SDS–polyacrylamide gel. Proteins were transferred to nitrocellulose using the Mini-Transblot Electrophoretic Transfer Cell (BioRad). Non-specific binding sites were blocked by shaking the blot in TBS-T [10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% (v/v) Tween-20] containing 5% dry skim milk overnight at 4°C. The blot was then washed once in TBS-T for 15 min at room temperature and twice for 5 min with fresh buffer at room temperature. The primary antibody was diluted (1:1000 for NME-1 and 1:300 for Mandra1) in TBS-T containing 1% dry skim milk and the blot incubated for 2 h at room temperature in this mixture. The monoclonal antibody Mandra1 was generously provided by G. E. Morris and detects an epitope within amino acids 3558–3684 in the C-terminal domain of dystrophin (39). It does not detect utrophin and has been shown to detect the originally described isoform of Dp71 which is missing exons 71 and 78 of the dystrophin gene (10). The polyclonal antibody NME-1 is specific to Dp71 exon 1 (G. Y. Dally and P. N. Ray, manuscript in preparation). The membrane was then washed as described above and incubated in TBS-T containing 1% dry skim milk and a secondary antibody linked to horseradish peroxidase (Amersham) diluted 1:2000. The signal was revealed by enhanced chemiluminescence (Amersham).

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