Alternative splicing regulates the nuclear or cytoplasmic localization of dystrophin Dp71

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Abstract The subcellular distribution of Dp71 isoforms alternatively spliced for exon 71 and/or 78 was examined. The cDNA sequence of each variant was fused to the C-terminus of the green fluorescent protein and the constructs were transfected transiently in the cell lines HeLa, C2C12 and N1E-115. The subcellular distribution of the fused proteins was determined by confocal microscope analysis. The Dp71 isoform lacking the amino acids encoded by exons 71 and 78 was found exclusively in the cytoplasm whereas the variants containing the amino acids encoded by exon 71 and/or exon 78 show a predominant nuclear localization. The nuclear localization of Dp71 provides a new clue towards the establishment of its cellular function.

Key words: Dp71; Alternative splicing; Nuclear localization; Green fluorescent protein; Protein fusion

1. Introduction

Duchenne muscular dystrophy is an X-linked recessive disorder caused by mutations in the dystrophin gene located on the short arm of the chromosome at Xp21 [1]. Dystrophin is a 427 kDa (Dp427) rod-shaped protein consisting of four major domains: an N-terminal actin-binding domain, a large region of 24 spectrin-like triple helix repeats, a cysteine-rich region and a C-terminal domain [2–4]. The N-terminal domain binds to cytoplasmic actin while the cysteine-rich and C-terminal domains region are important for Dp427 interaction with a complex of transmembrane glycoproteins and cytoplasmic factors know collectively as the dystrophin-associated protein complex (DAPC) [5–7]. One protein of the complex, α-dystroglycan, has been shown to interact with the extracellular matrix protein, merosin [8]. Thus one of the proposed functions of Dp427 is to provide a structural link between the actin cytoskeleton, the sarcolemma, and the extracellular matrix.

The dystrophin gene is transcribed from at least seven different promoters located in different regions of the gene. Four of these promoters are located within introns and encode short dystrophin isoforms, named according to their molecular weight. There are three full-length dystrophins (Dp427) which contain unique N-termini fused to the actin-binding, rod, cysteine-rich and C-terminal domains [9,10]. Three dystrophin isoforms (Dp260, Dp140 and Dp116) lack the actin-binding domain, but contain a portion of the rod domain and retain the cysteine-rich and C-terminal domains [11–13]. The remaining isoform Dp71 has a unique seven residues N-terminus fused to the cysteine-rich and C-terminal domains [14,15]. Dp71 expression is regulated by a housekeeping type promoter located in intron 62 of the gene [16], which results in a wide variety of tissues containing this protein. It is present in brain, retina, lung, liver, testis, kidney and smooth muscle, and at low levels in fetal skeletal and cardiac muscle [17]. Recent reports have showed association of Dp71 with proteins of the DAPC such as β-dystroglycan [18], dystrobrevin [19] and syntrophin [20], however, Dp71 cannot functionally substitute for the full-length dystrophin. Although the expression of Dp71 in muscle of mdx transgenic mice restores the levels of dystrophin-associated proteins, it is not sufficient to alleviate symptoms of muscle degeneration [21,22]. This indicates that Dp71 is capable of interacting with the DAPC in a similar manner as dystrophin, but binding to the membrane per se is not sufficient to perform all the functional roles of dystrophin.

A further complication in the study of Dp71 function was raised from the identification of two alternative splicing sites within the 3′ end of its coding region, which involved exons 71 and 78 [23]. While the loss of exon 71 does not change the reading frame of the transcript, the loss of exon 78 does, resulting in the 13 C-terminal amino acids of dystrophin being replaced by 31 new amino acids with hydrophobic properties in the Dp71 protein product. The expression levels and the ratio of the alternatively spliced isoforms of Dp71 vary largely among the human adult tissues [23] and during the human neural development [24].

As a first step in understanding the functional meaning of Dp71 alternative splicing, we examined the subcellular distribution of the different Dp71 splicing isoforms expressed as fusion proteins with green fluorescent protein (GFP). We report that alternative splicing determines the nuclear or cytoplasmic distribution of Dp71. The Dp71 isoform lacking the

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Abbreviations: DAPC, dystrophin-associated protein complex; GFP, green fluorescent protein; NPC, nuclear pore complex; NLS, nuclear localization signal

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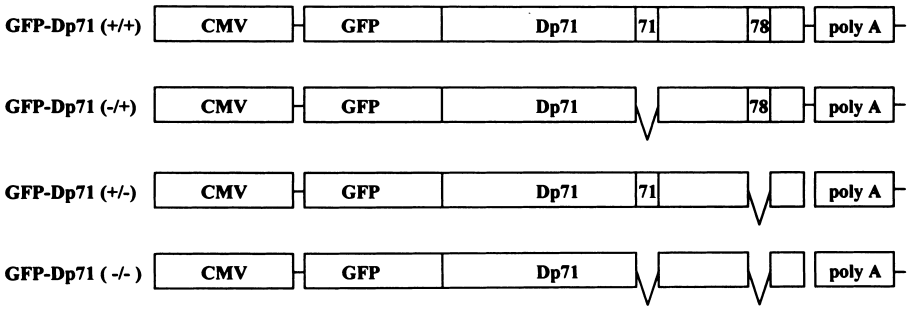

Fig. 1. Schematic illustration of the GFP-Dp71 gene fusion constructs. The cDNA sequences corresponding to Dp71 isoforms were fused to the C-terminus of GFP. The alternatively spliced exons of Dp71 are denoted by breaks in the solid horizontal lines. The constructs contain the cytomegalovirus promoter (CMV) and the polyadenylation signal (poly (A)) of BGH.

amino acids encoded by exons 71 and 78 was found exclusively in the cytoplasm whereas the variants containing the amino acids encoded by exon 71 and/or exon 78 show a predominant nuclear localization. These results indicate that alternative splicing of Dp71 modulates its subcellular localization and that the amino acids encoded by either exon 71 or exon 78 participate in the nuclear transport of Dp71.

2. Materials and methods

2.1. Plasmid constructions

To generate GFP-Dp71 fusion proteins, cDNA clones corresponding to Dp71 isoforms alternatively spliced out for exon 71 and/or exon 78 [23] were digested with *Eco*RI and subcloned in the correct reading frame into the *Eco*RI site of the expression vector pQBI25 (Quantum Biotechnologies Inc.). The resulting plasmids contained Dp71 cDNAs fused to the C-terminus of GFP (Fig. 1) and are under the control of the cytomegalovirus promoter. All constructs were verified by restriction enzymes digest analysis and sequencing.

2.2. Cell cultures

HeLa cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% neonatal serum, 25 U/ml penicillin and 25 μg/ml streptomycin. C2C12 cells were grown in DMEM supplemented with 10% fetal bovine serum, 25 U/ml penicillin, 25 μg/ml streptomycin and 1 mM sodium pyruvate. N1E-115 were grown in DMEM supplemented with 10% fetal bovine serum, 25 U/ml penicillin and 25 μg/ml streptomycin.

2.3. Western blot

At 48 h post-transfection, cells were lysed in extraction buffer containing a protease inhibitor cocktail (Boehringer). Equal amounts (30 μg) of total cellular protein were loaded per well, size fractionated on 3–10% polyacrylamide/sodium dodecyl sulfate gels and transferred onto nitrocellulose membrane (Gibco) as previously described [25]. Blots were incubated overnight in the presence of monoclonal anti-dystrophin antibody, H5A3 (1:500) [26], and developed using the ECL Western blotting analysis system (Amersham).

2.4. Transfection and confocal microscopy analysis

Cells plated on coverslips were transfected with 5 μg of each GFP-Dp71 construct pre-mixed with 5 μl of Plus reagent and 8 μl of lipofectamine (Gibco) in serum free medium. After a 5 h incubation period, the transfection mixture was removed by washing with DMEM and cells were maintained in DMEM supplemented with serum for 48 h. For phalloidin staining, cell were fixed in 3% paraformaldehyde for 15 min at room temperature, permeabilized in 0.3% Triton X-100 for 5 min and then incubated for 5 min with Texas Red-phalloidin (Molecular Probes). After washing, coverslips were mounted on microscope slides with VectaShield (Vector laboratories Inc.). GFP and Texas Red staining were visualized on a Nikon epi-fluorescence microscope coupled with a laser scanning confocal system Bio-Rad MRC 600. From each image, 9–12 Z-sections (0.2–0.5 μm tick) were taken starting from the top of the cell.

2.5. Flow cytometric analysis

At 48 h post-transfection, cells were removed from six well plates by trypsin treatment, washed twice with phosphate-buffered saline (PBS) and resuspended in 500 μl of PBS. Fluorescent emission of cells expressing GFP-Dp71 protein fusions was analyzed with a FACSort (Becton Dickinson Immunocytometry System, San José, CA, USA) using a 488–515 nm band pass filter. Data were acquired and analyzed with CellQuest software (Becton Dickinson).

3. Results

To determine the subcellular localization of Dp71 protein isoforms, four different Dp71 cDNAs, alternatively spliced for exons 71 and/or 78 [23], were fused with the 3′ end of the GFP gene (Fig. 1). GFP-Dp71 gene fusions are under the control of the cytomegalovirus promoter. To examine the expression of the proteins synthesized from the hybrid genes, human HeLa cells were transfected with each of the fusion genes and equal aliquots of cell lysates were analyzed by Western immunoblot using an anti-dystrophin monoclonal antibody H5A3 which recognizes epitopes encoded by exons 73 and 74 [26]. Taking into account that GFP and Dp71 have

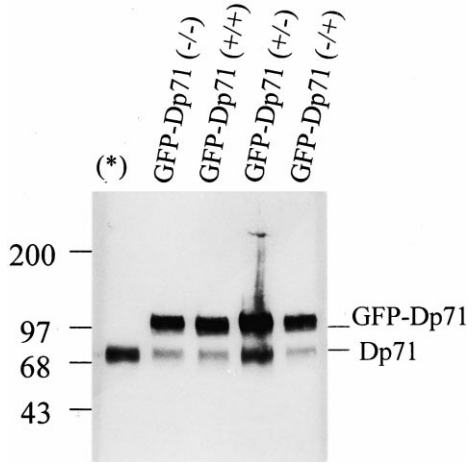

Fig. 2. Transient expression of the GFP-Dp71 protein fusions in HeLa cells. Cell lysates from transfected HeLa cells were analyzed 48 h post-transfection by Western blotting using an anti-dystrophin monoclonal antibody, H5A3. *, untransfected cells. The + and − symbols in brackets represent the presence or absence of exons 71 and/or 78, respectively. Numbers at the left denote protein molecular weight markers in kDa. The position of endogenous Dp71 and GFP-DP71 fusion proteins is indicated at the right.

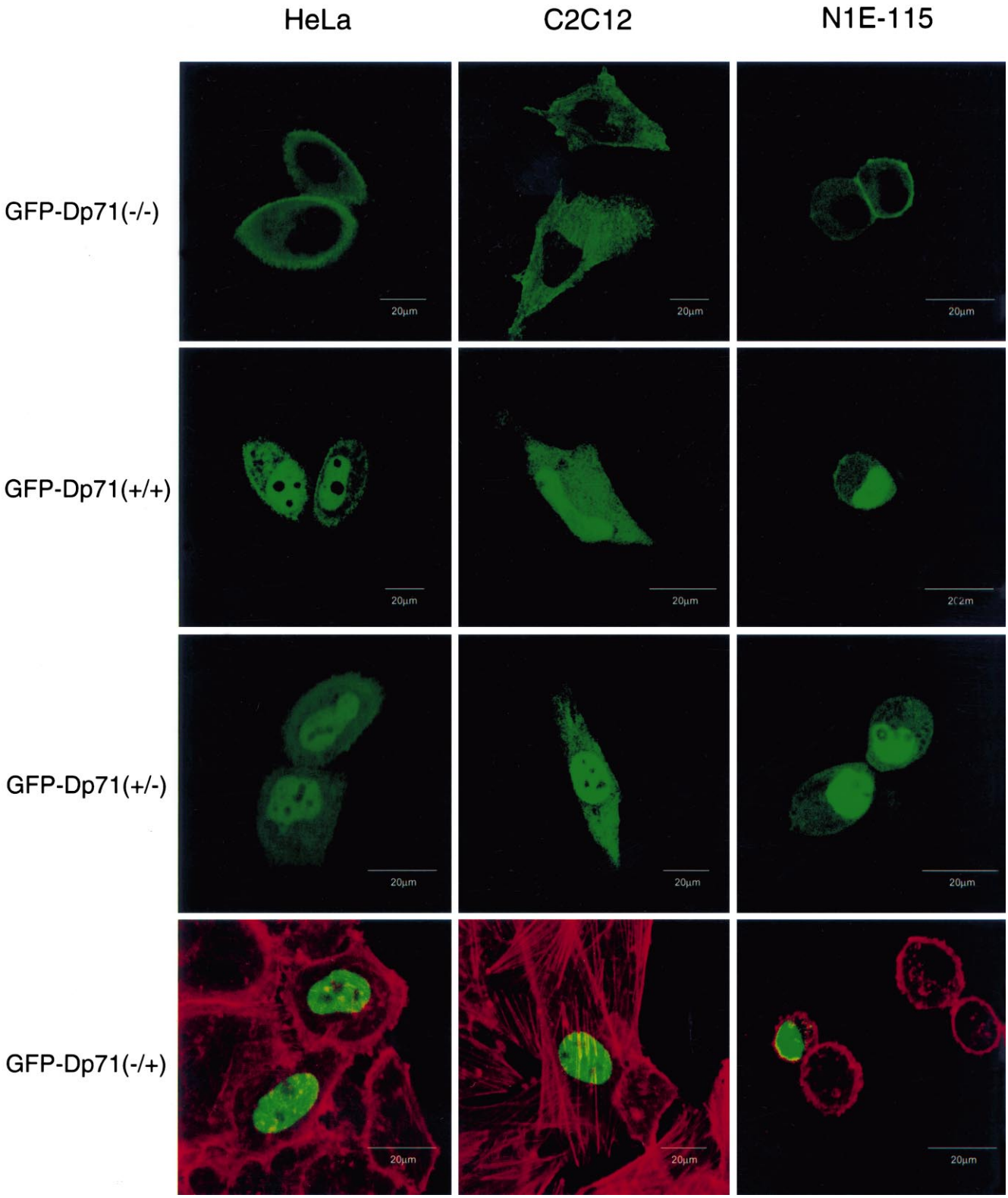

Fig. 3. Subcellular localization of Dp71 spliced isoforms. Plasmid expression constructs encoding GFP-tagged Dp71 isoforms were transfected into the cell lines indicated at the top of the figure. The + and − symbols in brackets represent the presence or absence of exons 71 and/or 78, respectively. Cells transfected with the construct GFP-Dp71 (−/+) were fixed and stained with Texas Red-phalloidin to visualize cytoplasmic actin. Images of representative cell fields were captured 48 h post-transfection on an epifluorescence confocal microscope.

Table 1
Quantification of GFP-Dp71 gene fusions expression using FACS analysis

	Int	Subcellular localization
GFP-Dp71 (−/+)	26.94 ± 2.55	N
GFP-Dp71 (+/−)	26.54 ± 2.10	N/C
GFP-Dp71 (+/+)	27.56 ± 2.36	N/C
GFP-Dp71 (−/−)	28.43 ± 2.97	C

Int denotes the mean ± S.D. of fluorescence intensity. N, nuclear localization; C, cytoplasmic localization.

molecular weights of 28 and 70 kDa, respectively, the protein fusions would have molecular masses of approximately 100 kDa. As shown in Fig. 2, a 70 kDa band that corresponds to the endogenous Dp71 was observed in both transfected and untransfected cells (lanes 1–5). Additionally, a fusion protein band of the expected molecular mass was produced in substantial amount from each GFP-Dp71 plasmid (lanes 2–5). No fusion protein band was detected in lysates from untransfected cells, thereby demonstrating the specificity of the immunodetection. Also, these results demonstrated that the GFP portion did not affect the accumulation of the fusion proteins.

Next, to study the localization pattern of each GFP-Dp71 fusion protein in living cells, HeLa cells were transfected with the different expression vectors and observed by confocal laser scan microscopy. As a control, expression of GFP from the plasmid pQBI25 was verified in parallel. As previously reported [27], GFP protein alone was equally distributed between the cytoplasm and the nucleus (data not shown). Since GFP has a molecular weight of 29 kDa, this protein is able to enter the nucleus passively through the nuclear pore complex (NPC). The Dp71 isoform lacking the amino acids encoded by exons 71 and 78 (−/−) showed a punctuate labeling throughout the cytoplasm with the cellular margins more intensely stained (Fig. 3). The isoforms generated from constructs containing exon 71 and exon 78 (+/+) or preserving exon 71 but not exon 78 (+/−) showed a fluorescence accumulation in both the cytoplasm and the nucleus (Fig. 3). Judging from the contrast of fluorescence between the cytoplasm and the nucleus, the distribution of these Dp71 variants seems to be predominantly nuclear. The remaining isoform, lacking amino acids encoded by exon 71 and preserving those encoded by exon 78, localized exclusively to nuclei (Fig. 3).

To ascertain whether the subcellular localization of Dp71 variants is independent of species and/or cell type, we analyzed their pattern of distribution in two other cell lines of different origin, C2C12, a mouse myogenic clone, and N1E-115, a mouse neuroblastoma cell line. The protein localization pattern of the Dp71 splicing variants was identical to that found with HeLa cells: isoforms −/− and −/+ showed exclusive cytoplasmic and nuclear accumulation, respectively, whereas the isoforms +/− and +/+ presented the majority of the signal in the nucleus with a comparative weak signal in the cytoplasm (Fig. 3). These results indicate that the absence of amino acids encoded by exons 71 and 78 confers a cytoplasm distribution of Dp71 and that the presence of amino acids encoded by exons 71 and/or 78 confers nuclear localization on Dp71. Yet, the construct containing exon 78 solely has a higher nuclear import efficiency than those containing exon 71 or both, exon 71 and exon 78. To rule out the possibility that the presence of the isoforms +/+ and +/− in the cytoplasm is due to the saturation of the nuclear import mechanism by overproduction of these proteins, we determined the expression of each GFP-Dp71 gene fusion by flow cytometric analysis. It was found that the mean intensities of fluorescence of transformed cells were similar among the four fusion proteins (Table 1).

4. Discussion

It has been established that Dp71 is alternatively spliced for exons 71 and/or 78. The deletion of exon 71 does not change the reading frame and therefore results in removal of 13 amino acids from the protein. On the contrary, the absence of exon 78 does change the reading frame replacing the last 13 amino acids with a hydrophobic C-terminus consisting of 31 unique residues [23]. Alternatively spliced transcripts of Dp71 are expressed at varying levels in a number of human adult tissues [23]. At the protein level, the use of isoform-specific antibodies, that distinguish between the hydrophilic and the hydrophobic C-termini, showed wide variation among tissues in both the level of Dp71 expression and in the ratio of the two C-terminus variants (P. Ray, unpublished results).

In this study, we analyzed the pattern of subcellular distribution of GFP-tagged Dp71 isoforms alternatively spliced for exon 71 and/or 78. We found that alternative splicing determines the subcellular localization of Dp71 in the HeLa, C2C12 and N1E-115 cell lines. The isoform of Dp71 lacking the amino acids encoded by exon 71 and exon 78 appeared to be exclusively cytoplasmic, while an isoform generated from a construct containing exon 78 but not exon 71 presented nuclear localization. The remaining two isoforms (+/− and +/+) showed predominant nuclear localization with a weak signal in the cytoplasm. It seems that the presence of the amino acids encoded by either exon 71 or 78 in the Dp71 protein changes its cellular localization drastically from the cytoplasm to the nucleus. Previous studies have shown that Dp71 is associated with the plasma membrane of Hep2 cells and muscle tissue of mdx mice [22,28]. Recently, Howard et al. [29] reported that Dp71 localizes to stress fiber-like structures in myogenic cultured cells. None of these studies reported the presence of Dp71 in the nucleus. One possible explanation for this discrepancy is that the GFP moiety mislocalized Dp71 to the nucleus, however, this is unlikely since the size of GFP-Dp71 fusion proteins (100 kDa) is well above the exclusion limit of the NPC (< 40 kDa). Therefore, without a specific transport mechanism through the NPC, the fusion proteins would be excluded from the nucleus [30,31], instead, we observed a nuclear accumulation of GFP-Dp71 isoforms containing the protein domains encoded by exon 71 and/or 78. In support of these results, it has been found that the endogenous Dp71, expressed in PC12 cells, shows a predominant nuclear localization when it is derived from transcripts containing exon 78 and a cytoplasmic localization when it is generated from transcripts alternatively spliced for exon 78 (F. Marquez et al., article in preparation). Nuclear localization of Dp71 does not seem to be a restricted phenomenon present only in certain cell types, since it was observed in the three cell lines studied.

Our data suggest the existence of a pathway that allows Dp71 to migrate into the nucleus by an active transport through the NPC. Selective nuclear import requires energy, physiological temperature, a nuclear localizing signal (NLS) and soluble transport machinery [30,31]. Two of the best char-

acterized NLS motifs are the simian virus 40 large T-antigen NLS (also known as the classical NLS) and the nucleoplasmin bipartite NLS. The classical NLS contains a stretch of basic amino acids whereas the bipartite NLS is composed of two basic stretches separated by a sequence of 10–12 amino acids [30]. A protein carrying the classical or the bipartite NLS is bound by a cytoplasmic receptor that consists of importins α and β. Importin α serves as adaptor able to bridge the binding between proteins carrying different NLS sequences and importin β. Importin β targets the complex to the NPC and the complex is then translocated into the nucleus. Upon entering the nucleus, the complex is dissociated by binding of Ran-GTP to importin β [30,31].

According to our results, it could be proposed that two different NLSs are present in Dp71, the strongest one in the protein domain encoded by exon 78 and the other one within the stretch of amino acids encoded by exon 71. A computational analysis of the entire amino acid sequence of Dp71 failed to detect a consensus NLS motif, nevertheless, it is possible that the Dp71 protein sequence contains atypical NLS motifs. Previously, several examples of proteins with NLS sequences that do not conform to the classical NLS have been identified including the HIV-I Tat protein [32], the heat shock protein Hsp70 [33] and the cell adhesion and signaling protein β-catenin [34]. Nuclear import of the first two proteins is believed to occur by a novel mechanism distinct from that mediated by importins, whereas the nuclear translocation of β-catenin is facilitated by its direct interaction with the nuclear pore proteins [32–34]. Alternatively, it could be possible that Dp71 enters into the nucleus through its association with other proteins that facilitate its access to the soluble transport machinery, as reported for a number of nuclear proteins [30]. Further studies are necessary to unequivocally establish the presence of at least one active NLS in the protein sequence of Dp71.

Protein import into the nucleus has several levels of regulation, one of which is protein phosphorylation. Phosphorylation can induce nuclear translocation by releasing the cargo from a cytoplasmic anchor or by promoting the association of the cargo with its import receptor [30]. Protein phosphorylation can also inhibit nuclear import by inducing an intramolecular change that masks the NLS sequence or by decreasing the affinity of the cargo for its import receptor [30]. In this respect, it has been shown that dystrophin is phosphorylated at serine and threonine residues by endogenous protein kinases [35,36] although the exact sites have not yet been mapped. Likewise, Dp71 was found to be phosphorylated in skeletal muscle of mdx mice transgenic for Dp71 [22]. Sequence analysis of Dp71 revealed two potential phosphorylation sites in the C-terminal region, the first located in exon 78 and the second in exon 79 [35,36]. Therefore, it is interesting to speculate that nuclear import of Dp71 isoforms, generated from transcripts bearing exon 78, is modulated by means of phosphorylation.

While it is difficult to envisage a role for Dp71 in the nucleus, it should be emphasized that a member of the DAPC complex, β-dystrobrevin and the recently characterized syntrophin-binding protein, SAT (syntrophin-associated serine/threonine kinase) have been found in the nuclei of hippocampal pyramidal neurons [19,37]. Hence, our data and the data of other authors raise the possibility that a DPC-like complex may be present in the nucleus. Clearly, establishing a role for these proteins in the nucleus warrants further investigation.

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