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Distal transcript of the dystrophin gene initiated from an alternative first exon and encoding a 75-kDa protein widely distributed in nonmuscle tissues

J. P. Hugnot*, H. Gilgenkrantz*, N. Vincent*, P. Chafey*, G. E. Morris†, A. P. Monaco‡, Y. Berwald-Netter§, A. Koulakoff§, J. C. Kaplan*, A. Kahn*, and J. Chelly*‡

*Institut Cochin de Génétique Moléculaire, Institut National de la Santé et de la Recherche Médicale U129, 24 rue du Fg Saint Jacques, 75014 Paris, France; †The North East Wales Institute, Deeside, Clwyd, CH5 4BR, Wales, United Kingdom; ‡Imperial Cancer Research Fund, Institute of Molecular Medicine, J. Radcliffe Hospital, Oxford OX3 9DU, United Kingdom; and §Biochimie Cellulaire, Collège de France, 75231 Paris Cedex 05, France

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ABSTRACT **A transcript generated by the distal part of the Duchenne Muscular Dystrophy (DMD) gene was initially detected in cells where the full size 14-kilobase (kb) messenger RNA is not found at a significant level. This transcript, ≈4.5 kb long, corresponds to the cysteine-rich and carboxyl-terminal domains of dystrophin. It begins with a novel 80- to 100-nucleotide exon containing an ATG start site for a new coding sequence of 17 nucleotides in-frame with the consecutive dystrophin cDNA sequence from exon 63. This result suggests the existence of a third promoter that would be localized about 8 kilobases upstream from exon 63 of the *DMD* gene. The distal transcript is widely distributed but is absent in adult skeletal and myometrial muscle. It is much more abundant in fetal tissues. With an antibody directed against the dystrophin carboxyl terminus, the protein corresponding to this transcript was detected as a 70- to 75-kDa entity on Western blots. It was found in all tissues analyzed except in skeletal muscle. It was not found in lymphoblastoid cells from a Duchenne patient with a complete deletion of the dystrophin gene. The role and subcellular localization of this protein is not known. It may explain extramuscular symptoms exhibited by some Duchenne patients.**

The huge X-linked *DMD* gene, which is abnormal in Duchenne and Becker muscular dystrophies (1, 2), is controlled by different promoters in different tissues (3, 4). A promoter active in skeletal muscle, heart, and smooth muscle and a neuron-specific promoter (5, 6), which is located 90 kilobase pairs (kbp) upstream of the muscle-specific promoter (7), control the production of two 14-kilobase (kb) mRNA species (8) that is translated into 427-kDa proteins (9) differing in their N-terminal ends. In addition, several forms generated by alternative splicing, some of them being tissue-specific or developmentally regulated, have been detected (3, 10, 11). Most of them involve the 3′ coding part of the transcripts.

In this paper, we report the characterization and expression analysis of a newly discovered transcript of the *DMD* gene, probably similar to the species recently reported by Bar *et al.* (12). This mRNA, referred to as the "distal transcript," is about 4.5 kb long and shares with the dystrophin mRNA those exons that are located downstream of exon 62. Thus, it encodes the last two domains of the protein—i.e., the cysteine rich and C-terminal domains. Its 5′ end consists of a new first exon spliced to the dystrophin exon 63 and located between exons 62 and 63 of the *DMD* gene. This alternative first exon contains a consensus ATG translation initiation codon in phase with the dystrophin gene sequence. The open reading frame codes for a 70- to 75-kDa protein that is detected in several tissues. Transcripts containing the alternative first exon are widely distributed in adult and fetal tissues and cell lines, but are absent from adult skeletal muscle and myometrium.

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MATERIAL AND METHODS

RNA Extraction. Total RNA was extracted from different cell lines and frozen tissues by the method of Chirgwin *et al.* (13). The amount of RNA used for reverse transcription and amplification reactions (cDNA PCR) was determined by spectrophotometry and corrected by computerized image analysis (Vilber Lourmat, Marne la Valleé, France) after loading on an agarose electrophoresis gel stained with ethidium bromide.

Quantitative cDNA PCR. PCR was performed as described in detail by Chelly *et al.* (10, 14). The internal standard used here was the L-pyruvate kinase mRNA amplified from rat liver.

Nuclease S1 Protection Assay. This assay was performed with a single-stranded, quasi-end-labeled probe as described by Cognet *et al.* (15).

RNA Blot-Hybridization Analysis. After electrophoresis in a 20 mM methylmercury(II)/1% (wt/vol) agarose gel, Northern blot analysis was carried out as described by Bayley *et al.* (16).

Anchored PCR. The 5′ sequence of the distal transcript was obtained by anchored PCR as described (17). cDNA synthesis was performed by primer extension of an oligonucleotide complementary to nucleotides 10,055–10,075. The cDNA was then 3′-tailed by terminal deoxynucleotidyltransferase (Boehringer Mannheim), under conditions designed to add about 15–30 dGMP residues. PCR amplification was performed with a 3′ complementary oligonucleotide spanning nucleotides 9885–9904 of the dystrophin sequence. The 5′ PCR primers and conditions have been described by Loh *et al.* (17). The PCR products were size-fractionated by 1% (wt/vol) agarose gel electrophoresis, electroeluted, digested with *Not* I restriction enzyme, and then cloned in the *Not* I/*Hin*cII sites of Bluescript KSII(−) plasmid (Stratagene).

Mapping and Cloning of a Genomic Fragment Including the Alternative Exon. Cosmids, isolated by using 63.1 dystrophin cDNA clone (position 7880 to the end) (18) as a hybridization probe on Imperial Cancer Research Fund flow-sorted chromosome X cosmid filter (19), were used to map and clone the genomic region of interest. Analysis of these cosmids by hybridization with the oligonucleotide probe allowed isolation of two overlapping cosmids (DMD3′-8 and DMD3′-12) that contained the sequence specific to the distal transcript. To define the position of the alternative exon, the cosmids were restriction-mapped with *Sac* I, *Sac* II, *Hin*dIII, and *Eco*RI. Southern blots were hybridized with probes derived from the exon corresponding to the 12-kb *Hin*dIII genomic

fragment detected by the 63.1 dystrophin cDNA probe and the alternative exon, respectively (see *Results*). To sequence the genomic fragment, *Hin*dIII fragments of cosmid DMD3′-8 were cloned into *Hin*dIII-cut Bluescript KSII(−) plasmid. Recombinant colonies hybridizing with a distal transcript-specific oligonucleotide probe were further analyzed and sequenced.

Cell Culture. Cultures of mouse brain neurons and astroglial cells were prepared and maintained as described (5, 20, 21).

Protein Analysis. Immunoblotting was performed essentially as described by Nicholson *et al.* (22) with the following modifications. The samples were fractionated on 1-mm SDS/polyacrylamide gel by using a 3% stacking gel and a 5–15% gradient-resolving gel. Mandra-1 is a primary monoclonal antibody against the end of the C-terminal domain of dystrophin (amino acid residues 3558–3684) (G.E.M., unpublished data).

RESULTS

Detection of a Different Transcript of the Dystrophin Gene. To investigate simultaneously several parts of the dystrophin gene transcript in different tissues and cell lines, we coamplified by cDNA PCR two fragments of the dystrophin mRNA, one corresponding to exons 1 and 3 (fragment A, positions 209–331) and the other encompassing several exons of the distal part (fragment B, positions 9811–10,516) (Fig. 1 *Upper*). Quantitative analysis performed after 15 cycles of amplification showed that the 5′ fragment A was detected only, as expected, in muscle and in spleen (in this tissue, the expression of dystrophin is related to the smooth muscle content of blood vessels), whereas the distal fragment B was reproducibly detected in addition in cultured hepatoma and lymphoblastoid cells, in which dystrophin is not expressed (Fig. 1 *Lower*). The absence of amplification of these two fragments from two lymphoblastoid cell lines of patients bearing different deletions—a deletion of the entire dystrophin gene (patient MJ, deletion 1) and a deletion from exon 8 to the end of the gene (deletion 2)—ruled out the possibility of a contamination or of the amplification of a related transcript encoded by another gene. Since the limits between exons in this region are not precisely known, we also verified that no genomic DNA could be amplified with these primers. This indicates that they are indeed located in two different exons (data not shown). These results suggest that the distal part of the dystrophin gene is transcribed in significant amounts in the two cell lines, whereas the proximal part is not.

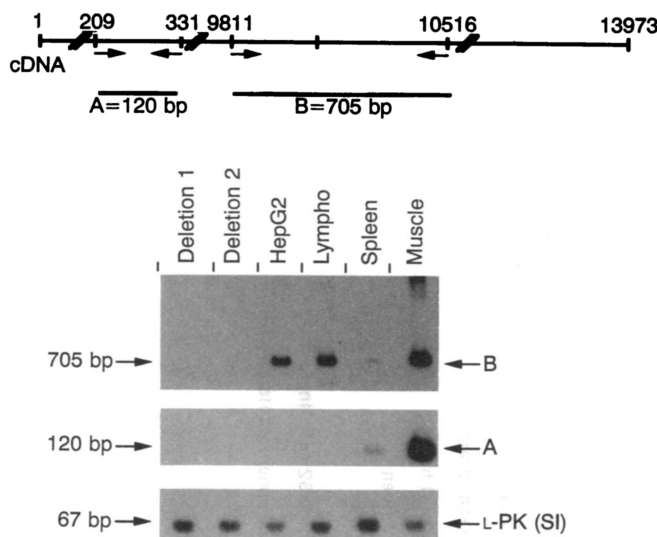

FIG. 1. Characterization of a nonmuscular transcript produced by the distal part of the dystrophin gene. (*Upper*) Position of the different primers on dystrophin mRNA and size of the amplified fragments. Nucleotide position numbers are on top. (*Lower*) Autoradiographs of a Southern blot of the cDNA PCR products obtained after 15 cycles. Hybridization was performed with an internal 5′-radiolabeled oligonucleotide probe. Total RNA was extracted from lymphoblasts of a patient with a chromosome X deletion that removed the whole dystrophin gene (lane Deletion 1; patient MJ), lymphoblasts from a patient with a chromosome X deletion that removed the distal part of the dystrophin gene (lane Deletion 2), Hep G2 hepatoma cell line (lane HepG2), control lymphoblasts (lane Lympho), spleen (lane Spleen), and skeletal muscle (lane Muscle). SI, internal standard; L-PK, L-pyruvate kinase transcript.

Table 1. Position of dystrophin mRNA fragments analyzed by cDNA PCR in nonmuscular cells

Fragment	Nucleotide position	Result after amplification
1	Brain exon 1–exon 2	−
2	209–331	−
3	1017–1197	−
4	6465–6670	−
5	7366–7450	−
6	9365–9490	−
7	9811–9907	+
8	9811–10,516	+
9	10,520–10,855	+
10	10,785–11,307	+

To further characterize the divergence region between the dystrophin mRNA and this new transcript, cDNA PCR amplification of different fragments of the dystrophin mRNA was carried out with several couples of primers (see Table 1). With couples of primers localized in the spectrin-like and the N-terminal regions, fragments of the expected size were observed only in muscle and spleen. Similar results were obtained with primers that allowed amplification of the sequence corresponding to the junction between the spectrin-like region and the cysteine-rich domain (positions 9365–9490). In contrast, fragments localized in the cDNA sequence corresponding to the cysteine-rich and C-terminal domains of dystrophin (fragments 7, 8, 9, and 10; Table 1) gave strong signals not only in muscle but also in lymphoblastoid and hepatoma cells. Altogether, these results indicate that the divergence between these two transcripts is located between positions 9365 and 9811. In addition, cDNA PCR amplification showed in a lymphoblast sample the presence of a signal of the same size as in muscle as well as shorter species that resulted from alternative splicing, suggesting the existence of a distal transcript-specific species. One of these alternatively

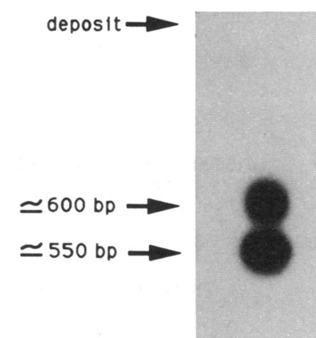

FIG. 2. Detection of the anchored PCR products. Autoradiograph of a Southern blot of the anchored PCR products electrophoresed on a 1% (wt/vol) agarose gel. Hybridization was performed with a 5′-radiolabeled dystrophin primer (positions 9461–9482).

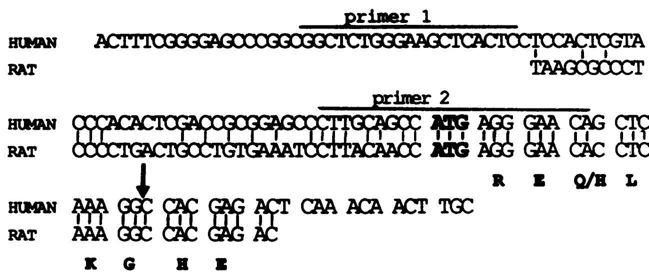

FIG. 3. Sequences of the human and rat distal alternative exon. The putative initiation codon is represented in boldface letters. The arrow indicates the exonic limit between the cysteine-rich domain coding region and the additional exon. Sequence identity between the two species is indicated by vertical bars. The sequences of the distal exon-specific primers 1 and 2 are indicated by horizontal lines. The deduced amino acid sequence beginning at the ATG codon is represented in the one-letter code.

spliced species corresponds to the 32-nucleotide exon deletion found by Bar *et al.* (12) in one liver cDNA clone.

To confirm the presence of a transcript in lymphoblasts, we performed a nuclease S1 protection assay with cloned fragment B (Fig. 1 *Upper*) as a probe. This fragment was protected not only in muscle but also in lymphoblasts (data not shown).

In Northern blot analysis with fragment B (Fig. 1 *Upper*) as a probe, we detected in lymphoblastoid and hepatoma cells a transcript migrating just below 28S RNA, which corresponds to a length of about 4.5 kb.

Cloning of the 5′ End of the Distal Transcript. To obtain more details on the 5′ end of this distal transcript, we used the anchored PCR technique (17) on a lymphoblast RNA sample. The amplification products consisted mainly of two bands hybridizing with an internal oligonucleotide specific to the dystrophin gene (Fig. 2). After cloning and sequencing, we found that the largest fragment contained the dystrophin cDNA until position 9431, preceded by a 80- to 100-nucleotide-long new sequence (Fig. 3). The sequence of the second band revealed that it was artefactual, since it did not contain the poly(dGMP) stretch and, thus, was most likely generated by internal priming of the anchored primer during the PCR procedure.

The new sequence seems to be an additional exon rather than an unspliced intron, since the sequence at the junction does not show an intron acceptor site. An ATG codon in good agreement with the Kozak consensus initiation sequence (23) and in frame with the dystrophin reading frame is seen 20 nucleotides upstream from the divergence point.

Expression of the Distal Transcript. cDNA PCR with a primer situated in the additional exonic sequence was negative in patient MJ, indicating that it corresponds to a part of the dystrophin gene (Fig. 4 *Upper*).

We used the same couple of primers to quantify the distal transcript in various adult and fetal tissues, and cell cultures. The results are shown in Fig. 4 *Lower*, and the quantification is reported in Table 2. The distal transcript is particularly abundant in fetal tissues (brain, heart, liver, and stomach). It is also present to a lesser extent in various adult tissues and cultured cells but is absent from adult myometrium and leg skeletal muscle.

Mapping of the Alternative Exon Within the Dystrophin Gene and Nucleotide Sequence Surrounding This Exon. Southern blot of a cosmid DMD3′-8 *Hin*dIII digest hybridized with oligonucleotide probes specific to the exon 63 (GenBank M86887) of the dystrophin cDNA, and the alternative exon showed that this cosmid contains the two exons (data not shown). Restriction mapping of cosmids DMD3′-8 and DMD3′-12, combined with oligonucleotide probe-specific hybridization with both probes, allowed localization of the new exon about 8 kb upstream from the exon 63 (Fig. 5 *Left*).

The nucleotide sequence of the region downstream from the alternative exon shows a donor splice site in agreement with the consensus sequence. The sequence upstream from

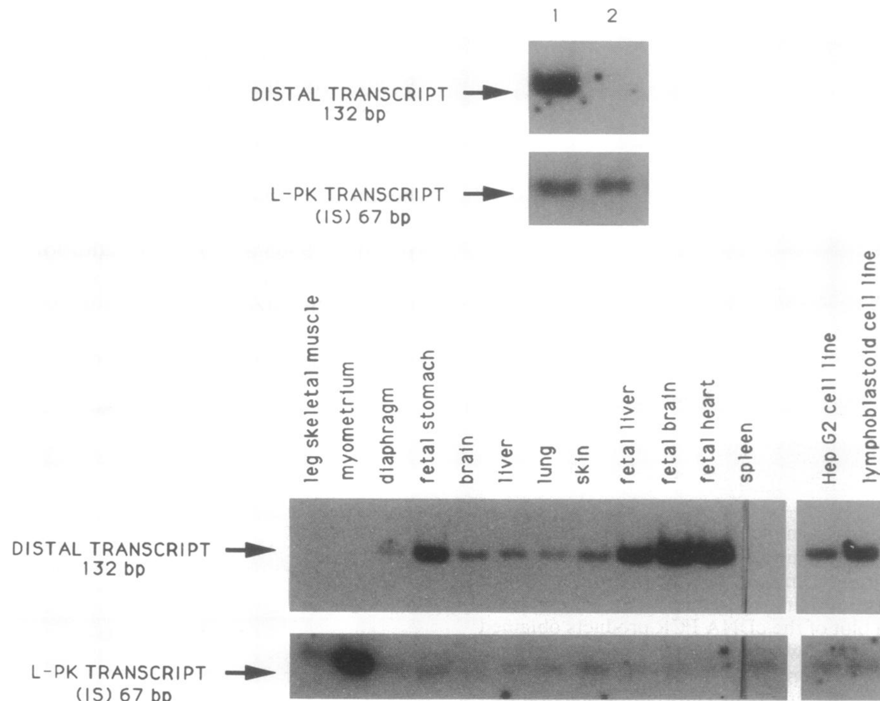

FIG. 4. Expression of the distal transcript in human tissues and cell lines. Autoradiographs of Southern blots of the cDNA PCR products obtained after 15 cycles are shown. The distal-transcript-specific primer 1 (see Fig. 3) and a primer spanning nucleotides 9461–9482 were used for the amplification. The oligoprobe used is the 5′ radiolabeled distal primer 2 (see Fig. 3). IS, internal standard; L-PK, L-pyruvate kinase. (*Upper*) Total RNA was extracted from lymphoblastoid cell lines of a normal subject (1) and patient MJ (2). (*Lower*) Total RNA was extracted from indicated tissues and cell cultures. The fetal tissues are from a 3.5-month-old fetus.

Table 2. cDNA PCR quantitation of the distal transcript

Samples	% of distal transcript
Tissue	
Fetal brain	100
Fetal heart	90
Fetal liver	60
Fetal stomach	60
Skin	20
Brain	20
Lung	15
Liver	15
Diaphragm	10
Spleen	5
Myometrium	<1
Leg skeletal muscle	<1
Cell lines	
Lymphoblastoid	55
Hep G2	30

this exon revealed a C+G-rich region without TATA box or A+T-rich element (Fig. 5 *Right*).

Determination of the Sequence of the 5′ End of the Rat Distal Transcript. Several couples of primers were used to perform cDNA PCR under low-stringency hybridization conditions with total rat liver RNA as a template. We then directly sequenced the band that hybridized with an internal oligonucleotide probe after a step of gel purification. This rat sequence, shown in Fig. 3, is highly homologous to the human sequence downstream from the ATG codon except for one amino acid—a glutamine residue in the human sequence that is replaced by a histidine in the rat. In contrast, the sequence upstream from the ATG is not conserved.

Detection of a 70- to 75-kDa Protein Product with a Monoclonal Antibody Directed Against the C-Terminal Domain of Dystrophin. The new transcript described above should code for a protein containing the cysteine-rich and the C-terminal domain of dystrophin. Using a monoclonal antibody directed against the end of this region (Mandra-1), we showed in Western blot experiments that a protein of 70–75 kDa is present in lymphoblastoid cells of a normal subject but is absent from patient MJ's cells (Fig. 6). This protein is present in brain and in cultures of brain astroglial and neuronal cells but is absent in leg skeletal muscle (Fig. 6). It is also present in liver and Hep G2 cells (data not shown). This protein appears sometimes as a doublet, suggesting the presence of isoforms. Antibodies directed against the spectrin-like or N-terminal domains did not detect this dystrophin gene product (data not shown).

DISCUSSION

Using cDNA PCR with primers in the distal part of the dystrophin gene, we unexpectedly found significant amounts of transcript in nonmuscle cells. Since this result was obtained after 15 cycles of amplification, it cannot correspond to illegitimate transcripts that are not detectable at this number of cycles (24). Bar *et al.* (12) have reported that nonmuscle tissues express a dystrophin distal transcript that shares with the dystrophin mRNAs the sequences encoding the last two protein domains. We confirm these results and demonstrate that, in addition, this transcript differs qualitatively from the dystrophin mRNAs by a unique 5′ sequence transcribed from an additional exon located between exons 62 and 63 of the dystrophin gene. This additional exon is 80–100 nucleotides long and is precisely spliced with exon 63. It contains a putative translation initiation codon that is in good agreement with the Kozak consensus sequence [GCCATGA instead of GCCATGG (23)] and is in phase with the dystrophin reading frame. This ATG codon and flanking nucleotides are well conserved in rat (ACCATGA) as is the downstream coding sequence. In contrast, the sequence upstream from the ATG initiator is not homologous in human and rat, as is often observed for 5′ untranslated regions of a given mRNA in different species. The length of the transcript detected by Northern blot analysis of lymphoblast RNAs is about 4.5 kb, which could correspond to 1.8 kb of the coding dystrophin mRNA sequence—from exon 63 to the stop codon—plus the unique 5′ sequence (about 0.1 kb) and the 3-kb-long 3′ untranslated region. Bar *et al.* (12) have reported a similar mRNA species with a size of 6.5 kb. Further investigations are needed to explain this size discrepancy.

The exon that is specific to the distal transcript is localized at 8 kb preceding exon 63 of the dystrophin gene. Since it has been detected by anchored PCR, it is likely to correspond to exon 1 of the distal messenger. Therefore, we may speculate that the 5′ flanking genomic sequence constitutes a potential promoter whose main structural characteristics should be discussed in the light of its pattern of expression in various tissues. The first 104 5′ flanking nucleotides (positions −78 to −182 with respect to the ATG initiator) are 83% G+C-rich, while the G+C ratio is only 64% in the transcribed sequence (nucleotides −78 to +20). In contrast, a homopyrimidine–homopurine stretch of 28 nucleotides is located upstream from the G+C-rich region between positions −248 and −276. The G+C-rich flanking region contains three putative binding sites for the Sp-1 factor or related proteins but no TATA box (see Fig. 5 *Right*). At positions −104 to −114 (i.e., 32 nucleotides upstream from the most proximal putative start site), we find an AGCTGTGAGCT palindrome.

All of these characteristics are compatible with the genome region that precedes the novel exon being a ubiquitous promoter whose properties are currently being investigated. Consistent with the structure of this putative promoter, the distal transcript is accumulated almost ubiquitously, although with quantitative differences in expressing cells and tissues, since the abundance is maximal in fetal tissues and in actively proliferating cultured cells such as lymphoblasts and hepatoma Hep G2 cells. However, it remains to determine why the distal transcript is not appreciably accumulated in

5′ 3′
Sac I Sac II Sac I Sac I Sac I
8 kb
1 kb
new alternative first exon
exon 63

−276 CTTTCTCTTTTCTTTTTCCTTTCCGTTTTGAAAGCCCAACTGCCAGTCTC
−226 TGGGTGTCCGGGTTCCCAGGTCCCTAGGTGGGCGAGCCGACACACGCCCG
−176 CCGCGTCTGGGGGCAGCGCCCCCTCCCCGGCCCGCCCGCGCCGGCTCCTC
−126 CGCAGTGCTTTCAGCTGTGAGCTTGGGCGGCGGCGGCGGCGGCGCTCCAC
−76 TTTCGGGGAGCCCGGCGGCTCTGGGAAGCTCACTCCTCCACTCGTACCCA
−26 CACTCGACCGCGGAGCCCTTGCAGCC**ATG**AGGGAACAGCTCAAAGGGTAA
25 GTGGATCGCGGCCC

FIG. 5. Characterization of the genomic region containing the alternative exon specific to the distal transcript. (*Left*) Restriction map for two enzymes of a genomic fragment containing the alternative exon. (*Right*) Nucleotide sequence of the genomic region surrounding the alternative exon. Consensus binding sites for the Sp-1 factor are boxed. The arrow indicates the 5′ end of the longest new exon found during cloning. The putative initiation codon is represented in boldface letters. The numbers on left indicate the position relative to this codon. The donor splice site is underlined.

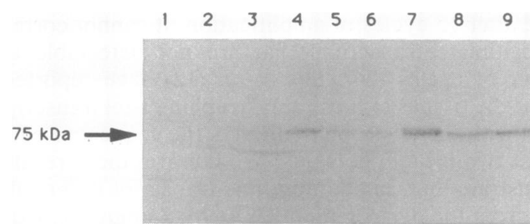

FIG. 6. Detection of a 70- to 75-kDa protein by immunoblot analysis with monoclonal antibody Mandra 1. The proteins were extracted from: rat leg skeletal muscle (lane 1); lymphoblastoid cells of patient MJ (lane 2); lymphoblastoid cells of a normal subject (lane 3); cultures of mouse brain neuronal cells at 22 days of culture (lane 4), 15 days of culture (lane 5), and 7 days of culture (lane 6); cultures of mouse brain astroglial cells (lane 7); rat brain (lane 8); and mdx (dystrophin-deficient mouse) mouse brain (lane 9). The arrow indicates the 70- to 75-kDa protein encoded by the dystrophin gene.

adult skeletal muscle and in smooth muscle. Transcriptional interference (25) between the muscular and the distal promoter could be one of the mechanisms responsible for this extinction.

The translation product of the distal mRNA is expected to possess 607 amino acid residues shared with dystrophin and a 6-amino-acid-long unique N-terminal peptide with polar characteristics (Arg-Glu-Gln-Leu-Lys-Gly). Its predicted molecular weight (70 kDa) fits well with the mobility of a protein that we have observed on Western blot with a monoclonal antibody specific to the carboxyl domain. This 70- to 75-kDa protein is not recognized by antibodies directed against other dystrophin domains and is found in the same cells and tissues as those accumulating the distal transcript; it is absent in skeletal muscle and present in liver, brain, lymphoblastoid cells, and Hep G2 cells. In addition, both the distal transcript and the 70- to 75-kDa protein are absent in patients with dystrophin gene deletions involving the C-terminal domains. Therefore, the 70- to 75-kDa protein is most likely the product of the distal transcript.

The putative physiological role of the 70- to 75-kDa protein is unknown. Since it is relatively abundant in brain, where, according to Bar *et al.* (12), the distal transcript is expressed at least at the same level as the dystrophin transcript, it may play a role in brain cell functions. In fact, this function could be common in glial cells and neurons. Further studies will establish whether there is a correlation between synthesis of the 70- to 75-kDa protein and the mental retardation recognized in as much as 1:3 of the Duchenne muscular dystrophy patients (26).

Note Added in Proof. The products described in this paper are probably identical to the species recently reported by Blake *et al.* (27) and Lederfein *et al.* (28).

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