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Monoclonal antibodies targeted against the C-terminal domain of dystrophin or utrophin

Eric Fabbrizio, Jocelyne Léger, Monique Anoal, Jean J. Léger and Dominique Mornet

INSERM U 300, Physiopathologie Cellulaire et Moleculaire, Bâtiment K, Faculté de Pharmacie, Avenue Charles Flahault 34060, Montpellier, France

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The structure–function relationships of dystrophin, a protein which is absent or defective in patients with Duchenne or Becker muscular dystrophies, and utrophin can only be compared if specific antibodies are produced. We expressed C-terminal parts of dystrophin and utrophin in expression vectors. Mice were immunized with recombinant proteins and 26 monoclonal antibodies were produced and analyzed. Their respective epitopes were determined using other overlapping recombinant products. We observed antibody specificity towards 400 kDa dystrophin and/or utrophin protein bands, either by Western blot analysis or immunodetection in human skeletal (quadriceps) and smooth (uterus) muscles. These antibodies have been used to compare the relative abundance of both dystrophin and utrophin relative to the structures analyzed.

Dystrophin; Utrophin; Monoclonal antibody; Epitope mapping; Recombinant protein

1. INTRODUCTION

Duchenne muscular dystrophy (DMD) is a progressively degenerative lethal muscle disease promoted by the absence of dystrophin, a 400 kDa protein [1–3]. Becker muscular dystrophy (BMD) is a similar but milder disease due to the presence of an abnormal dystrophin. Dystrophin consists of four main domains characterized according to homology with other known cytoskeletal proteins [4–6]. The molecule is enriched at the sarcolemmal membrane [7] where it is anchored by its C-terminal part [8,9]. It is thought to have a structural and mechanical role [10,11] and to be involved in calcium ion channel regulation [12,13].

However, studies on BMD patients have revealed a size-altered dystrophin molecule [14] and the presence of another molecule (400 kDa), named DRP (dystrophin related protein). This new molecule, called utrophin, was recently sequenced and presents homology with dystrophin mainly in its N- and C-terminal domains [15]. The gene is located on an autosomal chromosome, locus 6q24 [16], opposite the dystrophin gene, locus Xp21 [17].

Correspondence address: D. Mornet, INSERM U 300, Physiopathologie Cellulaire et Moleculaire, Bâtiment K, Faculté de Pharmacie, Avenue Charles Flahault 34060, Montpellier, France.

Abbreviations: DMD, Duchenne muscular dystrophy; ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulfate; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycoether tetraacetic acid; PMSF, phenyl methyl sulfonyl fluoride.

In this study, two cDNA fragments derived from dystrophin or utrophin C-terminal sequences were expressed as fusion proteins and monoclonal antibodies produced. Two other subfragments allowed us to map their epitopes. Western blot analysis and immunodetection by optical microscopy methods were used to determine their specificity towards dystrophin or utrophin. Our data on some specific dystrophin monoclonal antibodies also revealed a 77 kDa product, called DP71, derived from the Xp21 gene [18].

2. MATERIALS AND METHODS

2.1. *Plasmid constructions*

Chicken skeletal dystrophin cDNA subclone 2 was obtained from Lemaire et al. [19]. The length of each produced fragment is indicated with a bar in Fig. 1. The different fragments were aligned according to their sequence homology, as shown by the relative positions of the bars. The characteristics of these fragments are given in Table I.

The pEX1 vector gave rise to a construction which corresponded to a fusion protein with C-terminal 303 residues of chicken dystrophin (aa 3,357–3,660) at the end of β-galactosidase. This fusion protein expressed in *E. coli* is hereafter designated as fusion protein H. The H_1 fusion protein (127 amino acids) spanned residues 3,357 to 3,484 and was produced by *Eco*RI/*Bam*HI digestion of the starting cDNA dystrophin material. The H_2 fusion protein (258 amino acids) spanned residues 3,357 to 3,615 and was obtained by *Nco*I/*Bam*HI digestion. Both vectors were religated after Klenow fragment treatment. The H_3 fusion protein contained the last 90 residues of dystrophin and was produced by cloning the *Pvu*II/*Hin*dIII fragment of H in the pMAL vector.

The last expressed fusion protein inserted into the *Bam*HI site of vector pEX3 was obtained according to the procedure described by Khurana [20] and contained 227 residues from the C-terminal end of utrophin, i.e. amino acids 3,161* to 3,388* (*: relative to the human utrophin sequence). This fragment is hereafter designated as K.

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2.2. *Purification of recombinant proteins*

The pEX recombinant proteins were purified as described previously [21].

The pMAL recombinant protein was produced as specified by the manufacturer (OSI). Briefly, induction of the recombinant protein with 0.3 mM IPTG was performed for 2 h when OD (600 nm) reached 0.6. Sonication of bacterial pellets resuspended in lysis buffer (phosphate buffer 10 mM, NaCl 30 mM, Tween 20 0.25%, β-mercaptoethanol 10 mM, EDTA 10 mM, EGTA 10 mM, pH 7) and centrifugation at 8,000 × *g* for 25 min after the addition of NaCl 0.5 M (final concentration) allowed us to obtain a soluble fraction which was loaded after 4-fold dilution on an amylose resin column. The protein was eluted with maltose 10 mM contained in the column phosphate buffer (10 mM, NaCl 0.5 M, β-mercaptoethanol 10 mM, EGTA 1 mM, pH 7).

2.3. *Antibody production*

Two sets of antibodies were produced from selective fusion proteins expressing either C-terminal dystrophin (H protein with residues 3,357 to 3,660) or C-terminal utrophin (K protein with residues 3,161* to 3,388*) sequences in *E. coli*.

2.4. *SDS-PAGE and immunoblots*

Human skeletal tissue and purified fusion proteins were homogenized in 100 mM Tris HCl, pH 8.0, 5% SDS containing 10 mg/ml PMSF, 1 mg/ml leupeptin, 10 mg/ml soybean trypsin inhibitor, 1 mg/ml iodoacetamide and boiled for 5 min in the presence of an equal amount of loading buffer. Samples were applied on 0.75 mm SDS-polyacrylamide gradient gel containing 25% glycerol with no stacking gel. Western blot conditions were carried out as previously described [22,23].

2.5. *Immunodetection by optical microscopy*

Transverse cryostat sections of human skeletal (quadriceps) and smooth (uterus) muscles were treated with specific dystrophin or utrophin antibodies and then revealed with peroxidase-linked antibodies.

3. RESULTS AND DISCUSSION

3.1. *Expression of fusion proteins*

The different produced fusion proteins corresponding to different parts of dystrophin or utrophin are shown in Fig. 1. They were characterized by polyacrylamide gel electrophoresis. The expected sizes of each recombinant protein (Table I) were always compatible with the calculated molecular weight (M_r). For pEX constructions, the 108 kDa part corresponding to β-galactosidase was fused to the cloned sequences. For pMAL construction, the 42 kDa part corresponding to the maltose binding protein was fused to the dystrophin sequence.

3.2. *Characterization of the raised antibodies*

H and K fusion proteins provided us with 10 and 16 different monoclonal antibodies, respectively. Each were analyzed by ELISA titration curves and Western blots against both H or K antigens. Only 3 antibodies (designated H 4B9, H 12G9 and H 2A12) were specific to the Xp21 dystrophin locus since they detected only the H protein and did not react with the K protein. Their location was further restricted to H_1 as they detected both H_1 and H_2 proteins but not H_3 protein. Due to the sequence homology between dystrophin and utrophin, two potential sites for these antibodies were thought to correspond to residues 3,414–3,421 and

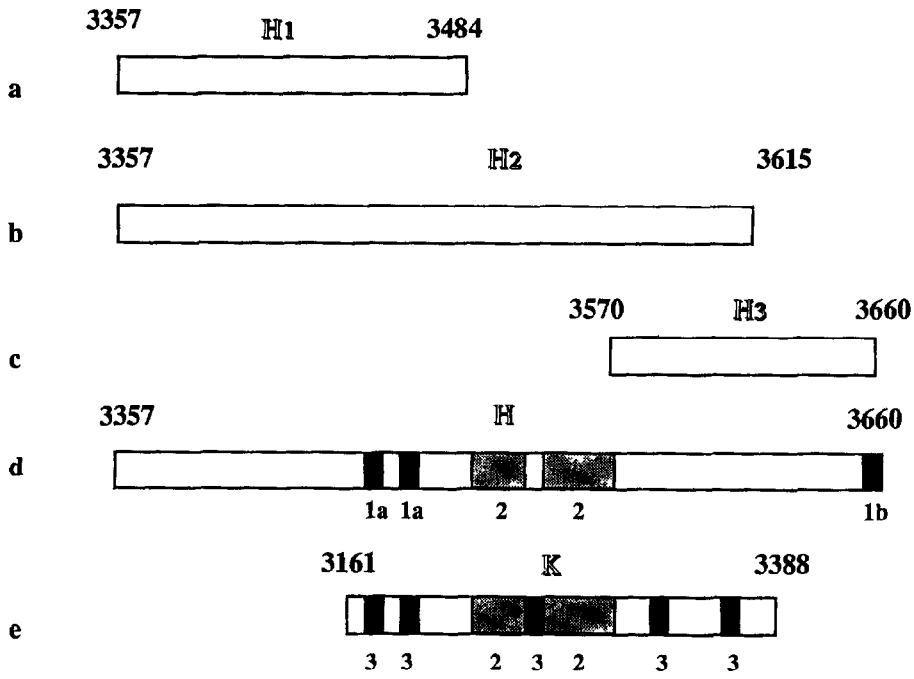

Fig. 1. Linear diagram of dystrophin and utrophin C-terminal domain constructions. Hl (lane a), H2 (lane b), H3 (lane c), H (lane d) and K (lane e) constructions were aligned according to their sequences. Localization of the specific monoclonal antibodies sites are indicated by solid boxes. Black boxes labelled with number 1 means that the antibodies were specific to the Xp21 locus. Dark-dashed boxes labelled with number 3 means that the antibodies were specific to the 6q24 locus. Clear-dashed boxes labelled with number 2 means that the antibodies were specific to either locus Xp21 or 6q24. For the H, H_1, H_2 and H_3 fragments, the sequence numbering was given according to the chicken dystrophin sequence. For the K fragment, the sequence numbering was given according to the human utrophin sequence and noted with an asterisk.

Table I

Characteristics of the different dystrophin and utrophin recombinant protein C-terminal parts obtained

Expression vector	Fusion protein	Insert cDNA (bp)	Amino acid sequence	Mw (kDa)	Protein size
pEX1	H	1,050	3,357 –3,660	33.3	141.3
pEX1	H_1	381	3,357 –3,484	14	122
pEX1	H_2	774	3,357 –3,615	28.4	136.4
pMAL	H_3	408	3,570 –3,660	9.9	51.9
pEX3	K	681	3,161*–3,388*	24.9	132.9

The cDNA fragments used for H and H_3 cloning include a small part which is not translated. This explains the difference observed in length with the deduced amino acid sequence. * corresponds to the numbering of the recently reported human utrophin sequence [15].

3,459–3,466. They belonged to the group that we called 1a (Table II and Fig. 1, lane d). A commercially available antibody (Dys2; no. 4 on Table II) was also found to be specific to the Xp21 dystrophin molecule since it detected only H and H_3 fragments. It was classified as belonging to group 1b (Fig. 1, lane d), since it was directed against the dystrophin C-terminal 17 residues [24], a region which did not provide us with any antibodies.

Among the 16 antibodies which were produced from K protein, 9 were specific to the 6q24 utrophin locus since they only detected K protein. They belonged to group 3 (Table II). According to the sequence homology between dystrophin and utrophin, five potential sites for these antibodies could correspond to residues 3,161*–3,175*, 3,210*–3,217*, 3,295*–3,305*, 3,351*–3,356* and 3,372*–3,387* (Fig. 1, lane e).

Other antibodies were designated as K′ or H′ according to their parental origin and belonged to group 2 (see Table II). They detected both H and K proteins but did not detect H_1 or H_3 proteins. This allowed us to map their epitopes on residues 3,485–3,518 and 3,530–3,570 on the H fragment and on residues 3,261*–3,294* and 3,307*–3,345* on K fragment (Fig. 1, lanes d and e). We noticed that all group 2 antibodies always showed better titrations with K than with H fusion proteins.

One antibody of each group was used for immunological detection of dystrophin and utrophin by Western blot analysis and histochemical detection in human tissues to check the efficiency of the above monoclonal antibodies relative to their specificity.

Table II

ELISA titration of the different antibodies obtained and their classification into 3 Groups

	Antibodies	ELISA H	ELISA K	Group
1	H12G9	1/1,300	–	1a
2	H2A12	1/1,000	–	1a
3	H4B9	1/800	–	1a
4	Dys2	1/1,500	–	1b
5	H′2H11	1/10,000	1/2,900	2
6	H′10A3	1/200	1/700	2
7	H′3E7	1/240	1/1,100	2
8	H′4A1	1/420	1/4,200	2
9	H′4C10	1/150	1/100	2
10	H′5A3	1/1,100	1/3,000	2
11	K′14H9	1/100	1/3,750	2
12	K′4A8	1/600	1/1,200	2
13	K′4D9	1/220	1/800	2
14	K′5B1	1/100	1/2,250	2
15	K′5B4	1/480	1/1,000	2
16	K′7G12	1/7	1/14	2
17	K′8E11	1/2	1/400	2
18	K′8G9	1/7	1/14	2
19	K14A7	–	1/2	3
20	K14G6	–	1/260	3
21	K2A4	–	1/2	3
22	K2B12	–	1/200	3
23	K4C6	–	1/1,800	3
24	K7H9	–	1/60	3
25	K8C6	–	1/2	3
26	K9D6	–	1/325	3
27	K9E12	–	1/850	3

Group 1a and 1b antibodies specifically recognized dystrophin, Group 2 antibodies recognized both proteins and Group 3 specifically recognized utrophin.

3.3. *Western blot analysis*

Crude skeletal muscle extracts from a BMD patient were used for Western blotting. This previously analyzed patient [25] had a 300 kDa reduced-size dystrophin and also a 400 kDa protein band corresponding to utrophin. This was clearly demonstrated with each monoclonal antibody of each group, as shown in Fig. 2. Group 2 (H′5A3) showed a faint 400 kDa utrophin band and a strong 300 kDa dystrophin band. Group 1a (H12G9) showed only the 300 kDa reduced-size dystrophin band and Group 3 (K4C6) showed only the 400 kDa utrophin faint band.

Crude brain and skeletal muscle extracts from rat were also obtained and used for Western blotting. Anal-

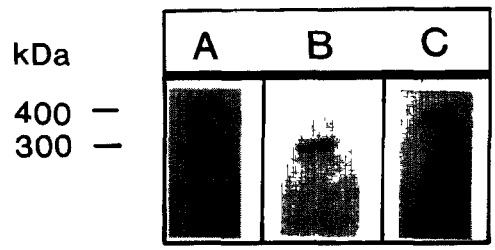

Fig. 2. Western blots for dystrophin and utrophin detection. Comparison of Group 1 (B), Group 2 (A) and Group 3 (C) antibodies on previously used BMD crude skeletal muscle extract [18,22].

ysis with H′ or H antibodies revealed, in the brain but not in the skeletal muscle, the 400 kDa dystrophin band and also a 77 kDa protein band (Fig. 3). This band corresponded to the small transcript product of the dystrophin gene which has already been described [26] and called DP71.

3.4. *Immunohistological analysis*

Dystrophin specific antibodies stained the human skeletal muscle cell (Fig. 4B) and the smooth muscle cell peripheries (Fig. 5B). Detection was performed using two specific antibodies (left panel H12G9, right panel H4B9) and gave similar patterns. Utrophin specific antibodies also stained the human skeletal muscle cell (Fig. 4C) and the smooth muscle cell peripheries (Fig. 5C). Each specific utrophin antibody (left panel K4C6, right panel K9E12) gave faint detection in comparison to dystrophin antibodies in the skeletal muscle cells and all cells, regardless of their size, were underlined by utrophin antibodies. Both dystrophin and utrophin antibodies stained with a rather similar pattern the smooth muscle fibres, opposite the skeletal muscle fibres. A very strong staining was observed with the vessel wall which was even more intense in the human smooth muscle (Fig. 5C) than in the human skeletal muscle (Fig. 4C). These differences might not result from the different accessibility of the epitope sites since two antibodies of each group gave the same result but could reflect the natural abundance of the antigens present in these tissues. The NMJ were also stained with these antibodies (not shown) and allowed us to confirm that both dystrophin and utrophin were present in this particular structure in agreement with recent reports [25,27].

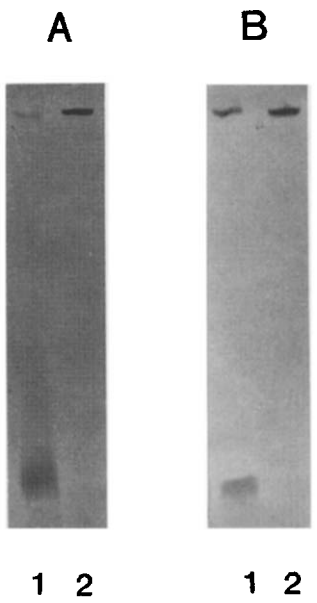

Fig. 3. Western blots for dystrophin and DP71 detection. Comparative detection of the 400 kDa dystrophin band and the 77 kDa band, called DP71, on rat brain (1) or skeletal muscle (2) crude extracts. (A) Group 2 antibody (H′5A3), (B) Group 1 antibody (H12G9).

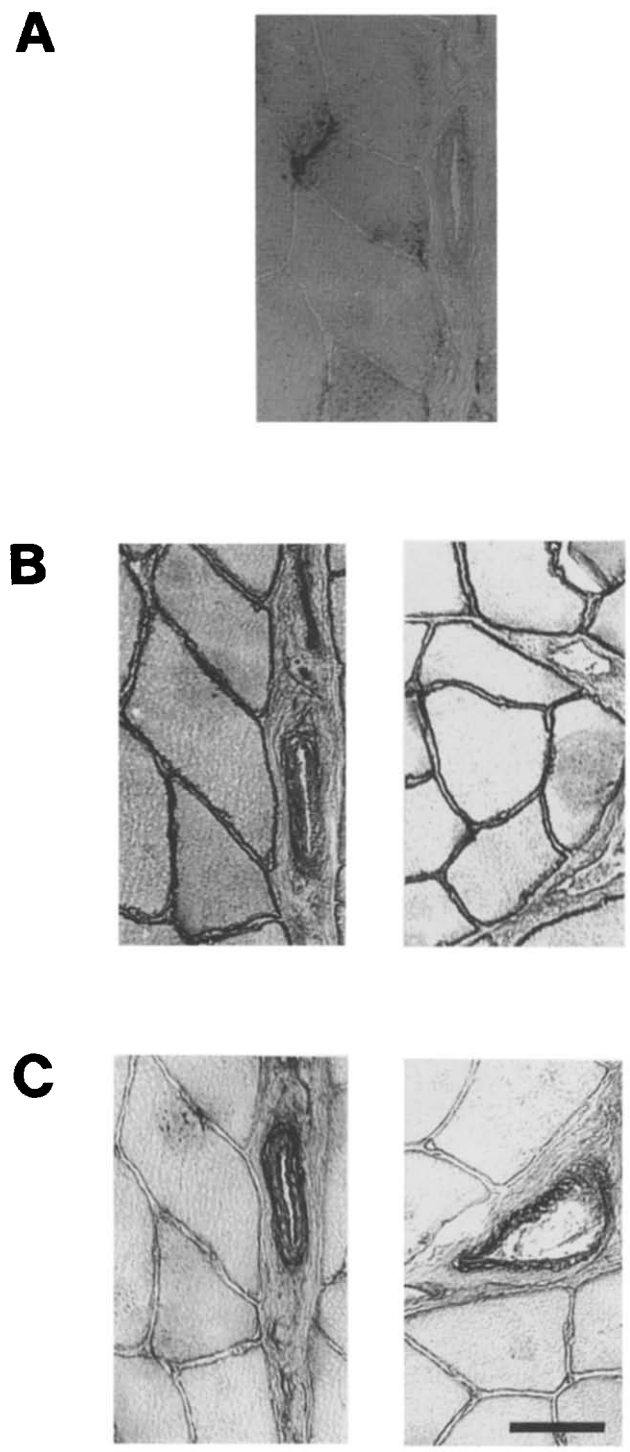

Fig. 4. Immunofluorescence detection of dystrophin and utrophin in human skeletal muscle (quadriceps). (A) PBS control, (B) Xp21 specific monoclonal antibody: left panel H12G9, right panel H4B9, (C) 6q24 specific monoclonal antibody: left panel K4C6, right panel K9E12. Bar corresponds to 20 μm.

The generation of new specific monoclonal antibodies directed against dystrophin or utrophin will be essential to prevent misinterpretations concerning localization of these proteins and to carry out more precise

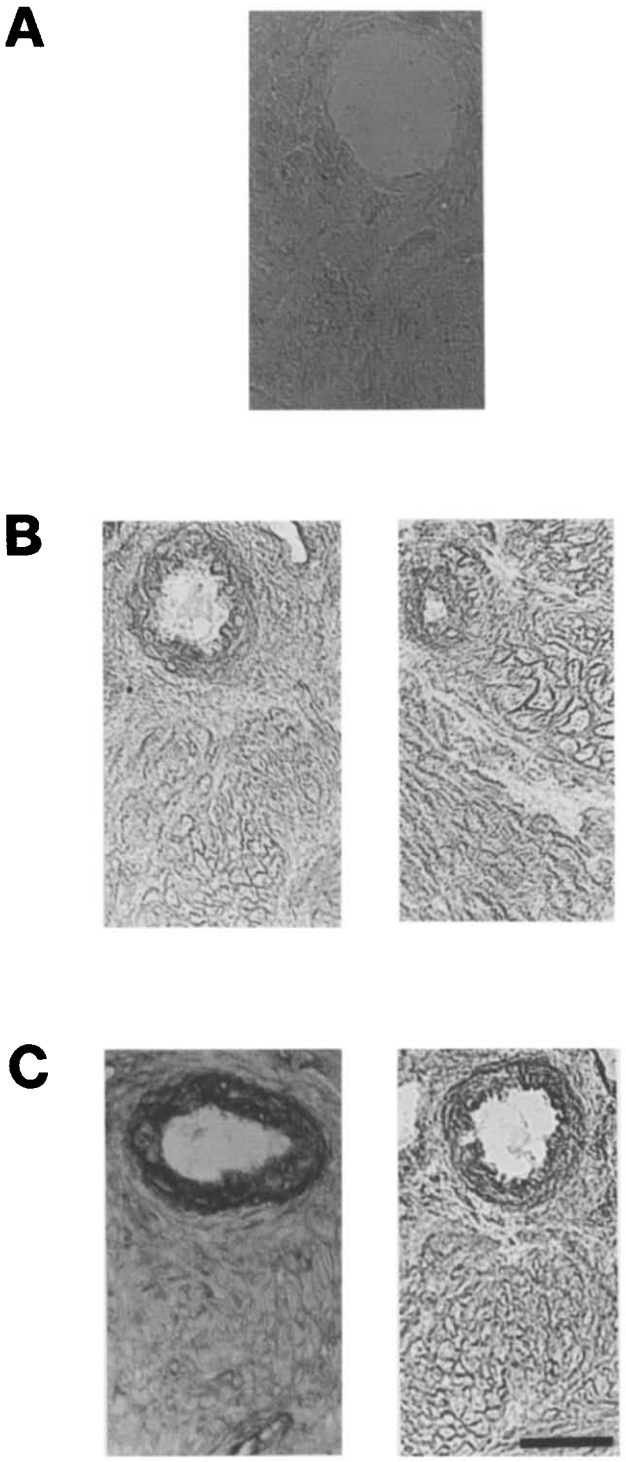

Fig. 5. Immunofluorescence detection of dystrophin and utrophin in human smooth muscle (uterus). (A) PBS control, (B) Xp21 specific monoclonal antibody: left panel H12G9, right panel H4B9, (C) 6q24 specific monoclonal antibody: left panel K4C6, right panel K9E12. Bar corresponds to 20 μm.

diagnostic studies on muscles of BMD/DMD patients and patients with related muscular disorders. But the fact that specific dystrophin monoclonal antibodies also reacted with DP 71, especially in non-muscle tissues such as brain, might make immmunofluorescence analysis difficult to interpret because of the lack of information concerning DP71 distribution within cells.

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REFERENCES

[1] Koenig, M., Monaco, A.P. and Kunkel, L.M. (1988) Cell 53, 219–228.
[2] Hoffman, E.P., Brown, R.H. and Kunkel, L.M. (1987) Cell 51, 919–928.
[3] Koenig, M. and Kunkel, L.M. (1990) J. Biol. Chem. 265, 4560–4566.
[4] Hammonds, R.G. (1987) Cell 51, 1.
[5] Koenig, M., Monaco, A.P. and Kunkel, L.M. (1988) Cell 53, 219–228.
[6] Noegel, A.A., Witke, W. and Schleicher, M. (1987) FEBS Lett. 221, 391–396.
[7] Ohlendieck, K., Ervasti, J.M., Snook, J.B. and Campbell, K.V. (1991) J. Cell. Biol. 112, 135–148.
[8] Ervasti, J.M. and Campbell, K.P. (1991) Cell 66, 1–20.
[9] Yoshida, M. and Ozawa, E. (1990) J. Biochem. 108, 748–752.
[10] Hutter, O.F., Burton, F.L. and Bovell, D.L. (1991) J. Muscle Res. Cell Motil. 12, 585–589.
[11] Mencke, A. and Jockush, H. (1991) Nature 349, 69–71.
[12] Turner, P.R., Fong, P., Denetclaw, W.F. and Steinhardt, R.A. (1991) J. Cell. Biol. 115, 1701–1712.
[13] Franco Jr., A. and Lansmann, J.B. (1990) Nature 344, 670–673.
[14] Hoffman, E.P., Fischbeck, K.H., Brown, R.H., Johnson, M., Medori, R., Loike, J.D., Harris, J.B., Waterson, R., Brooke, M., Spechts, I., Kupsky, W., Chamberlain, J., Caskey, T., Shapiro, F. and Kunkel, L.M. (1988) N. Engl. J. Med. 318, 1363–1368.
[15] Tinsley, J.M., Blake, D.J., Roche, A., Fairbrother, U., Riss, J., Byth, B.C., Knight, A.E., Kendrick-Jones, J., Suthers, G.K., Love, D.R., Edwards, Y.H. and Davies, K.E. (1992) Nature 360, 591–593.
[16] Love, D.R., Hill, D.F., Dickson, G., Spurr, N.K., Byth, B.C., Marden, R.F., Walsh, F.S., Edwards, Y.H. and Davies, K.E. (1989) Nature 339, 55–58.
[17] Koenig, M., Hoffman, E.P., Bertelson, C.J., Monaco, A.P., Feener, C. and Kunkel, L.M. (1987) Cell 50, 509–517.
[18] Bar, S., Barnea, E., Levy, Z., Neuman, S., Yaffe, D. and Nudel, U. (1990) Biochem. J. 272, 557–560.
[19] Lemaire, C., Heilig, R. and Mandel, J.L. (1988) EMBO J. 7, 4157–4162.
[20] Khurana, T.S., Hoffman, E.P. and Kunkel, L.M. (1990) J. Biol. Chem. 265, 16717–16720.
[21] Eldin, P., Le Cunff, M., Diederich, K.W., Jaenicke, T., Cornillon, B., Mornet, D., Vosberg, H.P. and Leger, J.J. (1990) J. Muscle Res. Cell Motil. 11, 378–391.
[22] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4357.
[23] Pons, F., Augier, N., Heilig, R., Leger, Joc., Mornet, D. and Leger, J.-J. (1990) Proc. Natl. Acad. Sci. USA 87, 7851–7855.
[24] Nicholson, L.V.B., Davidson, K., Falkous, D.G., Harwood, C., Odonnel, E., Slater, C.R. and Harris, J.B. (1989) J. Neurol. Sci. 94, 125–136.
[25] Pons, F., Augier, N., Leger, Joc., Robert, A., Tome, F.M.S., Fardeau, M., Voit, T., Nicholson, L.V.B., Mornet, D. and Leger, J.J. (1991) FEBS Lett. 282, 161–165.
[26] Lederfein, D., Levy, Z., Augier, N., Mornet, D., Morris, G., Fuchs, O., Yaffe, D. and Nudel, U. (1992) Proc. Natl. Acad. Sci. USA 89, 5346–5350.
[27] Bewick, G.S., Nicholson, L.V.B., Young, C., Odonnell, E. and Slater, C.R. (1992) Neuroreport 3, 857–860.