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Monoclonal Antibodies against the Muscle-specific N-Terminus of Dystrophin: Characterization of Dystrophin in a Muscular Dystrophy Patient with a Frameshift Deletion of Exons 3–7

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Summary

The first three exons of the human muscle dystrophin gene were expressed as a β-galactosidase fusion protein. This protein was then used to prepare two monoclonal antibodies (mAbs) which react with native dystrophin on frozen muscle sections and with denatured dystrophin on western blots but which do not cross-react with the dystrophin-related protein, utrophin. Both mAbs recognized dystrophin in muscular dystrophy (MD) patients with deletions of exon 3, and further mapping with 11 overlapping synthetic peptides showed that they both recognize an epitope encoded by the muscle-specific exon 1. Neither mAb recognizes the brain dystrophin isoform, confirming the prediction from mRNA data that this has a different N-terminus. One Becker MD patient with a frameshift deletion of exons 3–7 is shown to produce dystrophin which reacts with the N-terminal mAbs, as well as with mAbs which bind on the C-terminal side of the deletion. The data suggest that transcription begins at the normal muscle dystrophin promoter and that the normal reading frame is restored after the deletion. A number of mechanisms have been proposed for restoration of the reading frame after deletion of exons 3–7, but those which predict dystrophin with an abnormal N-terminus do not appear to be major mechanisms in this patient.

Introduction

The Duchenne muscular dystrophy (DMD) gene on the human X chromosome contains 79 exons (Roberts et al. 1992) which together encode a 427-kD protein, dystrophin (Koenig et al. 1988). mRNAs for muscle and brain dystrophin isoforms have been identified, and they have different promoters and first exons (Nudel et al. 1989; Gorecki et al. 1992). Consequently, it has been predicted that the first 11 amino acids in muscle dystrophin will be replaced by either three or seven different amino acids in two brain isoforms, though these differences have not yet been demonstrated experimentally at the level of dystrophin protein. mRNA for the former brain isoform is found, together with muscle-form mRNA, in the cortex and hippocampus, while the latter isoform may be restricted to the Purkinje cells of the cerebellum (Gorecki et al. 1992). Much shorter products of alternative promoters at the 3′-end of the dystrophin gene ("apo-dystrophins") are also found in brain and other nonmuscle tissues (Blake et al. 1992; Hugnot et al. 1992; Lederfein et al. 1992).

The majority of mutations leading to DMD and Becker muscular dystrophy (BMD) are deletions of one or more complete exons (Koenig et al. 1989). When deletions occur without disrupting the reading frame, milder muscular dystrophy, BMD, is the usual result, and a correspondingly shortened form of dystrophin is produced. In contrast, when the reading frame is altered, the more severe muscular dystrophy, DMD, usually occurs with the production of little or no detect-

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able dystrophin (Koenig et al. 1989) or of truncated dystrophin without a C-terminus (Bulman et al. 1991; Helliwell et al. 1992; Recan et al. 1992), except in a small proportion of so-called revertant fibers where the reading frame appears to be restored (Nicholson et al. 1992).

Deletions of exons 3–7 are frequent exceptions to the reading-frame rule, and patients may display DMD, BMD, or intermediate phenotypes. In BMD patients with this deletion, there is clear evidence from using antibodies that much of the normal dystrophin sequence is present distal to the deletion, including a normal C-terminus, though epitopes encoded by exon 8 may be absent (Arahata et al. 1991). Evidence for restoration of the reading frame by alternative splicing of mRNA (exon skipping) has been found in two patients (Chelly et al. 1990*a*), though only at 1%–2% of the level of the out-of-frame mRNA. Gangopadhyay et al. (1992), however, found only the predicted exon 2/exon 8 junction in mRNA from seven BMD patients and suggested alternative mechanisms to account for the apparent production of functional dystrophin. These mechanisms were alternative promoters in intron 2, reinitiation at an internal AUG in exon 8, or ribosomal frameshifting near the deletion junction.

We now describe the production of muscle-specific mAbs which recognize the product of the first exon of the human or mouse dystrophin gene and distinguish between muscle and brain isoforms. Applications to studies of dystrophin in brain and to dystrophin analysis in an MD patient with a deletion of exons 3–7 are presented.

Patients, Material, and Methods

Patients

Patient 1 presented with muscle cramps at age 6 years. He was found to have an in-frame deletion of exons 3–9 by Southern blotting with the Cf27 probe (Read et al. 1988). Patient 2 presented with delayed walking at age 4 years and has an out-of-frame deletion of exons 3–7. Both patients had calf hypertrophy, increased creatine kinase levels, and no family history. They were provisionally diagnosed as BMD, rather than DMD, by detection of sarcolemmal dystrophin in all fibers by using C-terminal antibodies (see figs. 3 and 5). This is consistent with clinical and histological data, though the patients are too young for the latter criteria to be unequivocal. No DMD patients with deletion of exons 3–7 and with C-terminal dystrophin in all muscle fibers have been described, and all BMD patients studied (including severe BMD or intermediate cases) with this deletion do show dystrophin staining with C-terminal antibodies (10%–20% of normal levels; Arahata et al. 1991; Beggs et al. 1991; Gangopadhyay et al. 1992).

Preparation of Immunogen

A 1.98-kb *Eco*RI-*Bgl*II (partial digest) subfragment of the pCf27 fragment of dystrophin cDNA was isolated, and a 225-bp fragment (bases 209–433 of the sequence reported by Koenig et al. 1988) was amplified from this DNA by using oligonucleotide primers—5′-ATGCTTTGGGAAGAAGTAGAGGACTGT-3′ and 5′-GTTCAGGGCATGAACTCTTGTGGATCCTTT-3′—and PCR conditions described elsewhere (Love et al. 1991). The DNA from the PCR was incubated with Klenow enzyme, extracted with phenol/chloroform, purified in Seakem agarose (FMC Corporation), isolated by using Geneclean (Stratech), and treated with T4 polynucleotide kinase according to the manufacturer's instructions. After heating at 65°C for 10 min, the DNA was ligated with *Sma*I-digested, dephosphorylated pGEM 3Zf(−) by following standard procedures.

For expression, this plasmid was digested with *Bam*HI, and a 204-bp fragment was ligated with dephosphorylated, *Bam*HI-digested pEX2 vector. This fragment encodes amino acids 1–68 of dystrophin. The pEX2 construct was introduced into *Escherichia coli* POP2136 cells by electroporation, and individual ampicillin-resistant colonies were screened by PCR after alkaline denaturation (pH 10, at 95°C for 5 min) with pEX primers (5′-GGGGATTGGTGGCGACGACTCCTGG-3′ and 5′-CTAGAGCCGGATCGATCCGGTC-3′). Both strands of the cDNA insert from one clone were sequenced to verify the construct, and this clone (pEX2:dys1–3) was used for the production of fusion protein.

Log-phase cells were induced by raising the temperature to 42°C–44°C for 2–3 h, and insoluble inclusion bodies of fusion protein were solubilized in 2% SDS, 5% 2-mercaptoethanol, and 50 mM Tris-HCl pH 6.8. After centrifugation at 100,000 *g* for 20 min, the supernatant was purified by gel filtration on Ultragel AcA34 (LKB) according to a method described elsewhere (Nguyen thi Man et al. 1990*a*).

mAb Production

mAbs were produced by immunization of BALB/c mice and fusion of spleen cells with Sp2/0 myeloma cells according to a method described elsewhere

(Nguyen thi Man et al. 1990*a*). To distinguish between anti-dystrophin and anti-β-galactosidase antibodies, hybridoma culture supernatants were screened twice by enzyme-linked immunosorbent assay (Nguyen thi Man et al. 1990*a*) using microtiter plates coated with two different fusion proteins: the products of pEX2:dys1–3 and pEX2 alone. One hybridoma fusion produced over 500 colonies, 40 of which produced antibody against the dys1–3 part of the fusion protein and 152 of which produced antibody against β-galactosidase. Further screening was performed on these 40 colonies to select for binding to the 427-kD dystrophin band on western blots of mouse muscle extracts and to the sarcolemma in frozen human muscle sections. Only 3 of the 40 recognized authentic human dystrophin on western blots, and 2 of these were successfully established as cell lines by two rounds of limiting dilution cloning. The mAbs obtained, MANEX1A and MANEX1B, are IgG2a and IgG2b, respectively.

Western Blotting

Tissues were weighed, dropped into 4 vol of boiling SDS extraction buffer (10% SDS, 10% EDTA, 10% glycerol, 5% 2-mercaptoethanol, 50 mM Tris-HCl, pH 6.8), homogenized with a Silverson blender, boiled for 2 min, and centrifuged at 100,000 *g* for 20 min. Extracts (10 µl) were loaded onto 3%–12.5% gradient gels with a 3% stacking gel, and, after electrophoresis, proteins were transferred electrophoretically (BioRad Transblot) to nitrocellulose sheets (BA85; Schleicher and Schull) at 100 mA overnight in 25 mM Tris, 192 mM glycine, and 0.003% SDS.

Blots were blocked in 3% skimmed milk powder in incubation buffer (0.05% Triton X-100 in PBS [25 mM sodium phosphate pH 7.2, 0.9% NaCl]). After two 5-min washes in PBS, blots were incubated with mAb culture supernatant (1/100 dilution in incubation buffer, 1% horse serum, 1% FCS, 0.3% BSA) for 1 h at 20°C. After three 5-min washes with PBS, blots were incubated with biotinylated anti-mouse Ig and a peroxidase-avidin detection reagent (Vectastain ABC kit), according to the manufacturer's instructions (Vector Laboratories, Peterborough, UK). After four 5-min washes with PBS, substrate was added (0.4 mg diaminobenzidine (Sigma)/ml in 25 mM phosphate-citrate buffer pH 5.0 with 0.012% H_2O_2).

Immunohistochemistry

Frozen muscle sections (6–8 µm) were allowed to attach to untreated glass slides and were stored at −70°C. After both incubation for 30 min at 20°C with mAb culture supernatants, diluted 1+3 with PBS, and three 5-min washes with PBS, sections were incubated with the second antibody for 30 min at 20°C. The second antibody (1/20 dilution in PBS containing 1% horse serum, 1% FCS, 0.3% BSA) was FITC-labeled anti-mouse Ig (DAKOpatts). After three 5-min washes in PBS, slides were mounted in 70% glycerol in PBS. Slides were examined with a Leitz (Leica) epifluorescence photomicroscope with appropriate filters and were photographed for a fixed time (60 s) by using Kodak Tri-X Pan film.

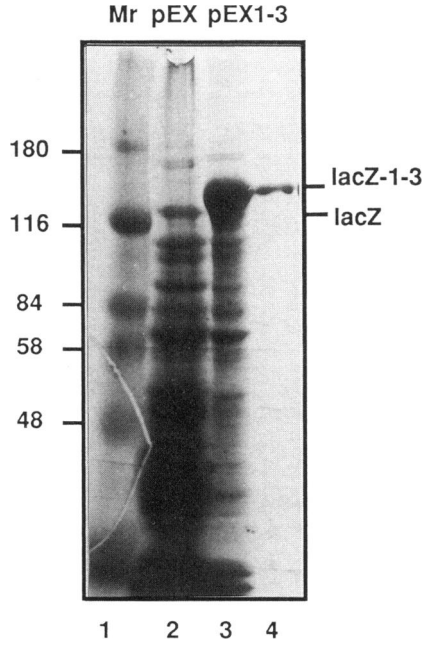

Figure 1 Induction and purification of the bacterial fusion protein immunogen. *Escherichia coli* POP2136 cells transformed with the pEX2:dys1–3 construct (lane 3) are compared with cells transformed with pEX2 alone (lane 2). Lane 4 shows the purified fusion protein (lacZ-1–3) after gel filtration and ethanol precipitation, while lane 1 contains prestained Mr markers (Sigma). After SDS-PAGE, the gel was stained with Coomasie Brilliant Blue R250.

Results

Figure 1 shows the expression and purification of the β-galactosidase/dys1–3 fusion protein. After induction of pEX2:dys1–3, a 127-kD fusion protein (lacZ-1–3) is overexpressed in the total *Escherichia coli* extract (lane 3), while pEX2 plasmid alone produces the smaller β-galactosidase product (lacZ, 119 kD) in lane 2 (itself migrating slightly more slowly than the 116-kD β-galactosidase molecular-weight marker in lane 1). This fu-

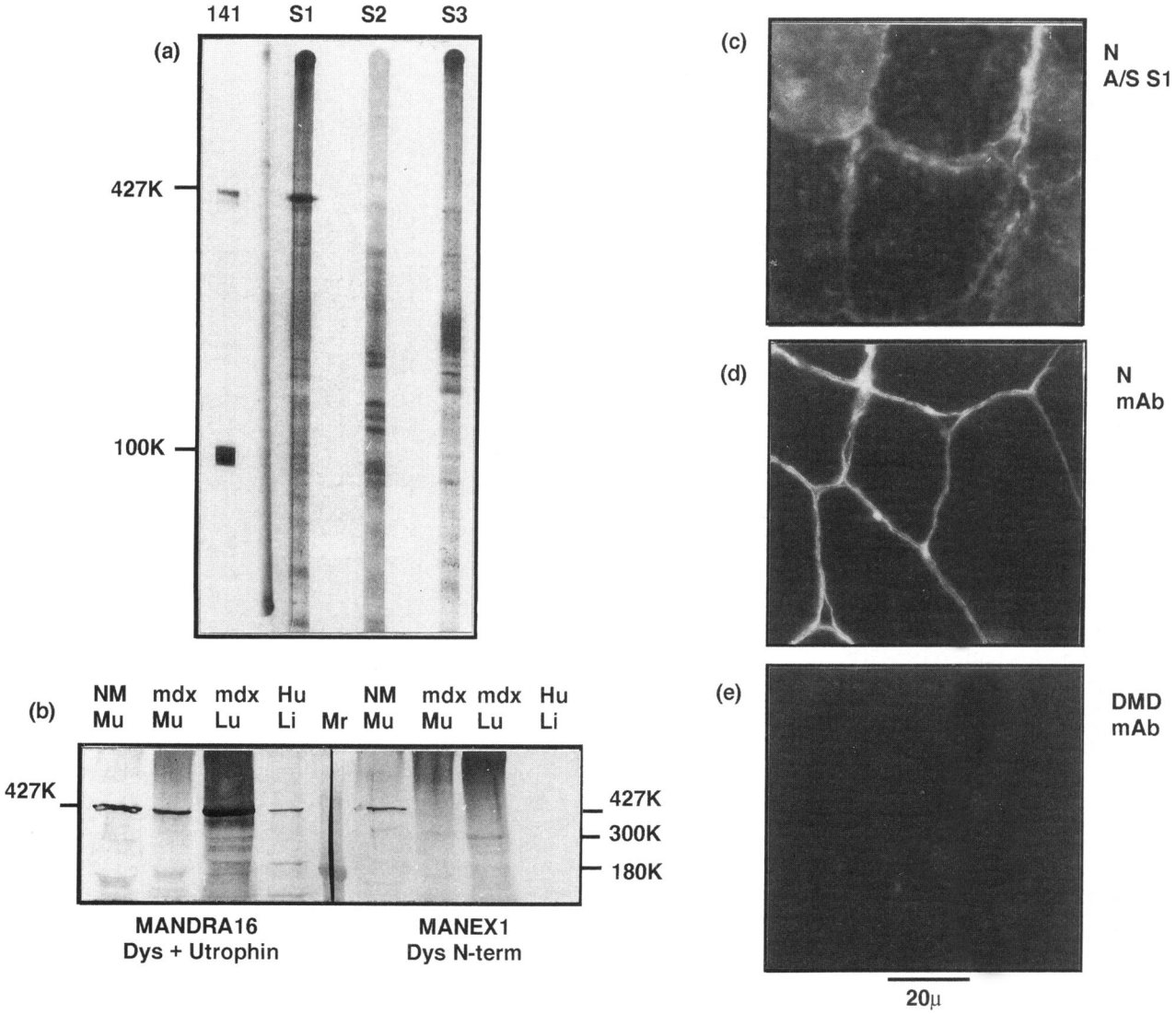

Figure 2 mAb production and specificity. (*a*), Extract of human muscle subjected to SDS-PAGE as a horizontal strip and with antibodies applied to the western blot in the vertical lanes of a miniblotter (Immunetics, Boston). The mAb, MANDYS141, which reacts with both dystrophin and α-actinin (Nguyen thi Man et al. 1990*b*) was used for the first lane to provide Mr markers. The mouse antisera (S1–S3) raised against pEX2:dys1–3 fusion protein were used at 1:100 dilution. (*b*), Binding of MANEX1 mAbs to dystrophin, but not utrophin, on western blots. A western blot of extracts of normal mouse muscle (NMMu), *mdx* mouse muscle (mdxMu), or lung (mdxLu) or adult human liver (HuLi) was cut into two through the prestained Mr markers (Sigma), and each half was developed using either MANEX1A (produced from the mouse S1) or MANDRA16 (a mAb which binds to the C-terminal domains of both dystrophin and utrophin [Nguyen thi Man et al. 1992]) as primary antibody. (*c*)–(*e*), Immunofluorescence analysis of muscle sections. When mouse antiserum S1 is used as primary antibody, a faint sarcolemmal staining is seen on all fibers, with some punctate internal staining in (*c*). Some fibers (e.g., at the top corner) show a more general internal staining. MANEX1A mAb gives sarcolemmal staining on normal human sections, (*d*), but no staining on muscle from a DMD patient, (*e*).

sion protein was purified (lane 4) by a single gel filtration step in the presence of SDS (Nguyen thi Man et al. 1990*a*).

Three BALB/c mice were immunized with this fusion protein (2 × 100 μg/mouse), and figure 2(*a*) shows that only one mouse responded by producing antibody against dystrophin at 427 kD on western blots of total human muscle extracts (lane S1). This antiserum also stained the sarcolemma of mouse muscle frozen sections, though (*a*) the membrane staining was rather

weak and (*b*) internal staining of fibers also occurred (fig. 2[*c*]). With antisera from the other two mice, the 427-kD band was very weak (fig. 2[*a*], lanes S2 and S3), and sarcolemmal staining was not detectable (not shown).

Two mAbs, MANEX1A and MANEX1B, were produced from the first mouse (S1), and these show greatly improved specificity on muscle sections (fig. 2[*d*]). Absence of sarcolemmal staining in DMD muscle confirms specificity for dystrophin (fig. 2[*e*]). Both mAbs bind to the 427-kD dystrophin band in normal mouse muscle, as well as in human muscle, but not in muscle from the *mdx* mouse, which lacks dystrophin (fig. 2[*b*]). Neither mAb cross-reacts with utrophin, the autosomal dystrophin-related protein, in either *mdx* mouse lung or human liver (fig. 2[*b*]), but a faint band of about 300 kD is detected in *mdx* muscle and lung. This protein is present in many human, normal mouse, and *mdx* mouse tissues, though amounts are lower in liver and muscle, and it is recognized by both mAbs (data not shown).

To determine whether that part of dystrophin encoded by exon 3 is necessary for mAb binding, muscle sections from a 6-year-old BMD patient with an in-frame deletion of exons 3–9 were examined. Figure 3 shows that both MANEX1 mAbs bind to dystrophin in the plasma membrane of the frozen sections and that the mAbs must therefore recognize epitopes encoded by exon 1 and/or exon 2. Epitope mapping with 11 overlapping synthetic peptides (fig. 4[*a*]) confirmed that both mAbs bound to the first two peptides (fig. 4[*b*]) and thus require dystrophin encoded by exon 1. The two tryptophan residues, Trp3 and Trp4, appear to be essential for binding. A very weak reaction was also observed with peptides 8–11, especially when the spots were reprobed with the same mAb (not shown). This may be due to low affinity recognition of the Trp residue in all four peptides (fig. 4), but the possibility of some contribution to the epitope by exon 2–encoded residues cannot formally be ruled out.

Immunofluorescence microscopy of a 4-year-old BMD patient with a frameshift deletion of exons 3–7 and low dystrophin levels revealed a clear reaction of sarcolemmal dystrophin with MANEX1 mAbs, as well as with central rod and C-terminal mAbs (fig. 5). As in both normal muscle and that of the patient shown in figure 3, the intensity of sarcolemmal staining in figure 5 is similar for all three epitopes, suggesting that most, if not all, of the dystrophin mRNA is transcribed from the normal muscle promoter to produce a normal exon 1–encoded N-terminus. The coproduction of small amounts of dystrophins without the normal N-terminus cannot be ruled out, but the results suggest that mechanisms other than alternative promoters or internal AUG codons have a major role in correcting the frameshift.

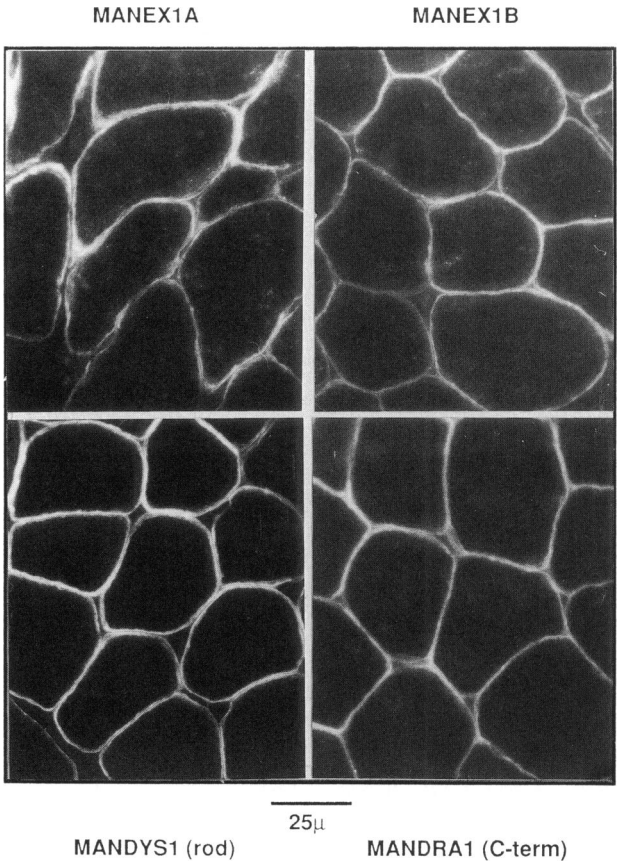

Figure 3 mAbs which bind to dystrophin from BMD patients with a genetic deletion of exon 3. This 6-year-old patient has an in-frame deletion of exons 3–9 and reasonably high dystrophin levels. MANDYS1 (Nguyen thi Man et al. 1990*a*) and MANDRA1 (Nguyen thi Man et al. 1992) are dystrophin-specific central rod and C-terminal mAbs, respectively.

Western blots were uninformative, for what we suspect to be technical reasons. In *both* patients, with frameshift *and* in-frame deletions, a shortened dystrophin of the expected size (approximately 400 kD) was detected with rod and C-terminal mAbs but not with MANEX1 mAbs (results not shown). Absence of the N-terminus, however, would be inconsistent with the very clear immunofluorescence results (figs. 3 and 5). Abnormal junctions (exons 2–10, in this case) are believed to produce dystrophins with structural perturbations leading to increased susceptibility to proteolytic degradation during SDS extraction and western blot-

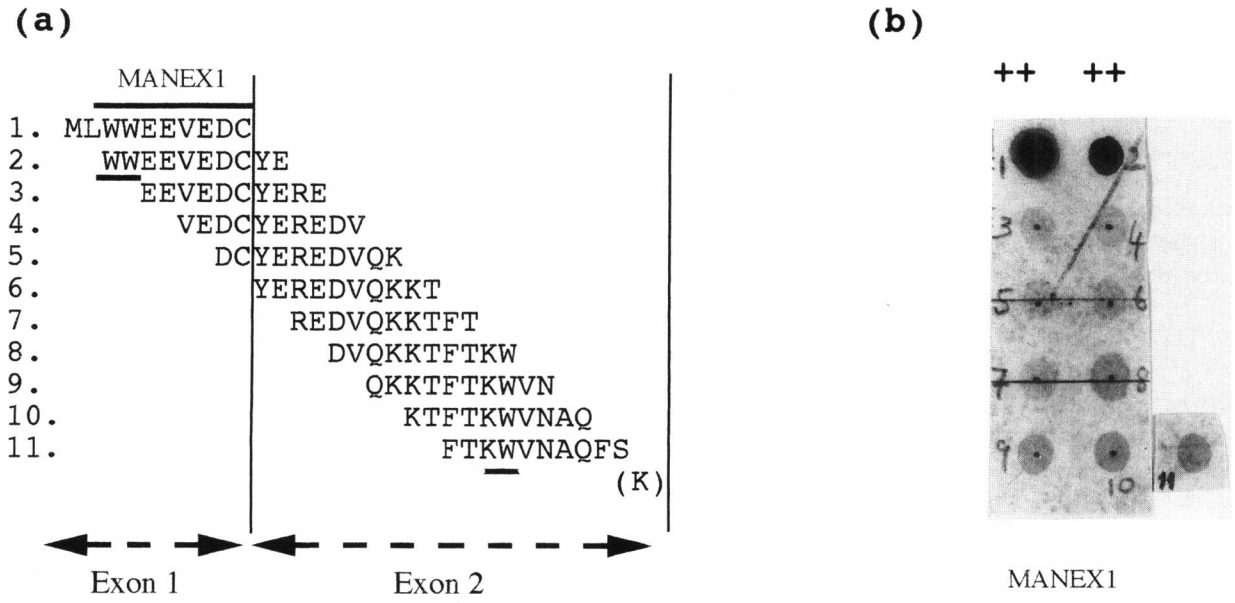

Figure 4 Epitope mapping of MANEX1 mAbs by using synthetic peptides. The 11 peptides shown in (*a*) were synthesized by conventional F-moc chemistry as "spots" on a cellulose membrane support, by using a SPOTS kit according to the manufacturer's instructions (Cambridge Research Biochemicals, Northwich, UK). After synthesis, membranes were developed with mAbs as for western blotting. Binding of MANEX1A to peptides 1 and 2 only is shown in (*b*); MANEX1B gave the same result (not shown). Amino acids which *may* be involved in each epitope (see text) are marked with one horizontal bar, while two Trp residues which are *essential* for the epitope are also underlined in peptide 2. Exon boundaries are based on the data of Koenig et al. (1988). The exon 1/exon 2 boundary is drawn between Cys10 and Tyr11 in (*a*), though the first nucleotide encoding Tyr11 is actually the last nucleotide of exon 1.

ting (Beggs et al. 1992), so loss of the MANEX1 epitopes in this way (or, less likely, as a direct consequence of structural changes near the N-terminus) may account for the anomalous western blot result. A similar technical problem has been reported for the extreme C-terminus of dystrophin (Nicholson et al. 1992). The problem is less evident with normal full-length dystrophin, which shows strong binding with MANEX1 mAbs.

Studies of dystrophin mRNA transcripts in rodent brain have shown that an alternative promoter with a different first exon is used in neuronal cells (Nudel et al. 1989; Barnea et al. 1990; Chelly et al. 1990*a*). The predicted consequence of this is that the first 11 amino acids of muscle dystrophin should be replaced by 3 different amino acids in brain dystrophin (Nudel et al. 1989). Consistent with this, neither mAb detects any dystrophin band at 427 kD in human brain extracts, though a 427-kD protein is detected by mAbs against the dystrophin rod and C-terminal domains (fig. 6). Both N-terminal mAbs detect in brain a 300-kD band which is not detected by the other dystrophin-specific mAbs, suggesting that it is a cross-reactive protein rather than an alternative product of the dystrophin gene. Only the C-terminal mAbs detect apo-dystrophin-1, a product of alternative transcription of the 3′ end of the dystrophin gene. This shows that the last 13 amino acids of dystrophin are not involved in mAb binding, since they are replaced by 31 different amino acids in apo-dystrophin-1 (Lederfein et al. 1992).

Discussion

The MANEX1 mAbs should be useful for immunohistochemical analysis of muscular dystrophy patients, since they should detect nearly all truncated and internally deleted mutant dystrophins (deletions of exon 1 are rare; Koenig et al. 1989). We have used them to characterize the dystrophin made by one BMD patient with a frameshift deletion of exons 3–7. Some patients are evidently able to restore the reading frame and produce an internally deleted and partly functional dystrophin, but others appear to be insufficiently able to do this, and they display the severe, DMD phenotype. Chelly et al. (1990*a*) found, in two BMD patients, evidence that exon skipping distal to the deletion produced alternatively spliced mRNAs with restored reading frames, though levels were low (1%–2%), and some

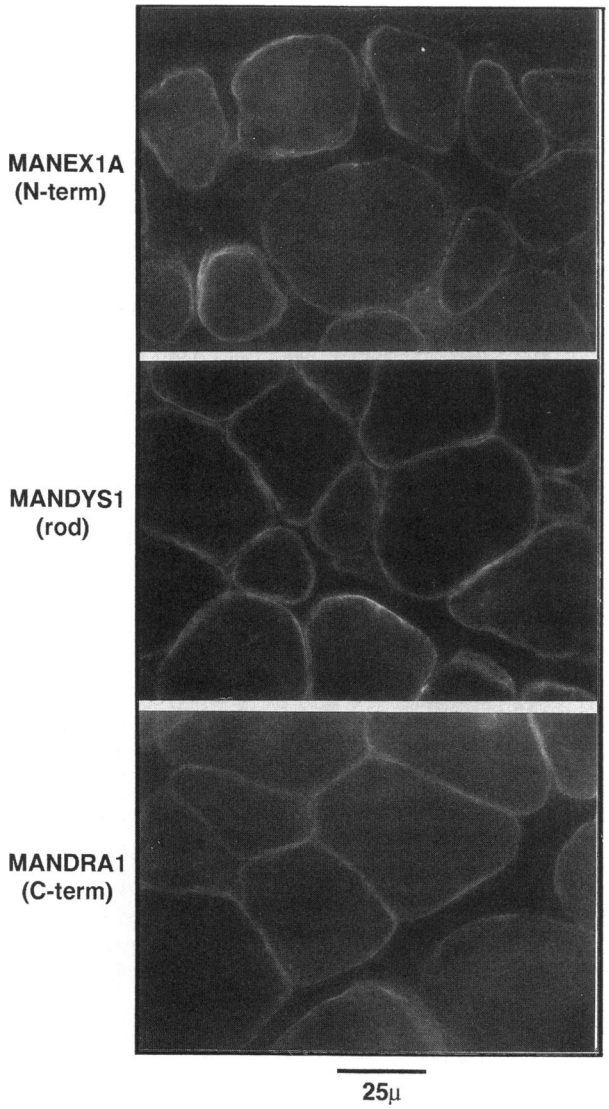

Figure 5 Presence of a normal N-terminus of dystrophin in a BMD patient with a frameshift deletion of exons 3–7. The patient was 4 years old. MANDYS1 and MANDRA1 are the dystrophin-specific central rod and C-terminal mAbs, respectively.

up-regulation mechanism would be required for these mRNAs to account for all dystrophin production. In seven other BMD patients with this deletion, Gangopadhyay et al. (1992) were unable to find even such low levels of alternative mRNAs. Some of the alternative mechanisms suggested by Gangopadhyay et al. (1992), such as use of an alternative promoter in either intron 2 or intron 7 or an internal AUG codon in exon 8, do not appear to be dominant in the BMD patient whom we have studied. The binding of MANEX1 mAbs suggests that most, if not all, of the dystrophin of our BMD patient with the deletion of exons 3–7 is produced from the normal muscle promoter and begins with the muscle-specific exon 1 (fig. 5). Ribosome frameshifting to restore the reading frame in the exon 2/exon 8–spliced mRNA remains a possibility (Gangopadhyay et al. 1992), though it is not clear why this should be specific for deletion of exons 3–7. More detailed characterization of the mutant dystrophins, using mAbs against the products of exons 2 and 8–10, in addition to MANEX1, may be necessary to solve this problem. Our results do not rule out the possibility that more than one type of mutant dystrophin is present at the sarcolemma. The staining intensity with MANEX1 mAbs, however, is similar to that with mAbs distal to the deletion for both the in-frame deletion of exons 3–9 (fig. 3) and the deletion of exons 3–7 (fig. 5), suggesting that, if any dystrophins without a normal N-terminus are present, they are likely to be minor components. Although western blot data on the deletion of exons 3–7 alone might suggest a different conclusion, similar results even with an in-frame deletion (exons 3–9) and the established possibility of proteolysis at abnormal junctions (Beggs et al. 1992), leading in this case to loss of the N-terminal amino acids, lead us to conclude that the immunofluorescence data on native dystrophin in situ are more reliable. Nicholson et al. (1992) reached similar conclusions by using an mAb against the extreme C-terminus of dystrophin; this mAb detected dystrophin by immunofluorescence on patient biopsies but usually failed to do so on western blots.

The patient described here may not be representative of all BMD patients with the deletion of exons 3–7, though previous studies have not revealed major differences between such patients, at the mRNA level (Chelly et al. 1990*a;* Gangopadhyay et al. 1992). The potential for differences in expression clearly exists in patients with this deletion, however, since DMD and intermediate phenotypes can also be produced. The possibility that even different BMD patients express different mutant dystrophins cannot yet be ruled out, and more exon-specific mAbs will be necessary to investigate it fully.

Both mAbs detect a protein of approximately 300 kD in human brain (fig. 6), mouse muscle and lung (fig. 2[*b*]), and most other tissues (data not shown). This is not a dystrophin isoform, since it is not detected by dystrophin rod and C-terminal mAbs (fig. 6). It may be a chance cross-reaction at a single shared epitope. Absence of sarcolemmal staining by MANEX1 mAbs in *mdx* and DMD muscle shows that dystrophin—and

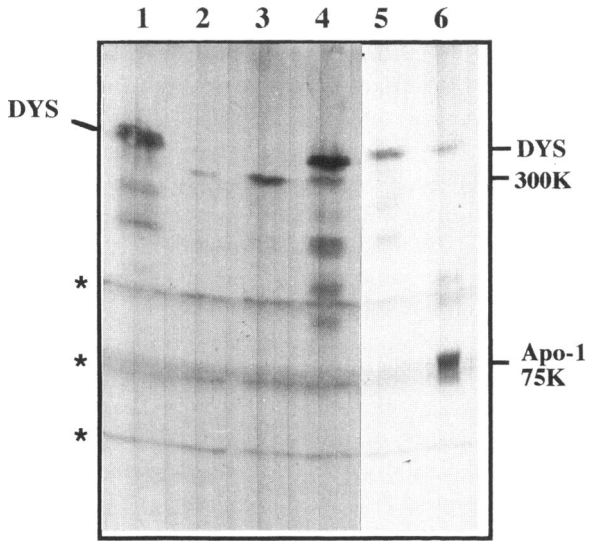

Figure 6 Failure of MANEX1 mAbs to detect dystrophin in brain. An extract of a 0.5-g slice from the cerebral cortex of normal human brain (stored at −70°C for several years postmortem) was subjected to SDS-PAGE as a strip, and mAbs were applied to the western blot in alternate vertical lanes of a miniblotter (lane 1, MANDYS1, dystrophin rod; lane 2, MANEX1B; lane 3, MANEX1A; and lane 4, MANCHO3, utrophin-specific [C-terminus] [Nguyen thi Man et al. 1991]; lane 5, MANDYS106, dystrophin rod [Nguyen thi Man et al. 1992], and lane 6, MANDRA5, dystrophin C-terminus [Nguyen thi Man et al. 1992]). The horizontal bands across and between all lanes (*) are cross-reactions of the Vectastain detection system.

not the 300-kD protein—is responsible for the membrane staining in figures 3 and 4.

The major mRNA product of the 5′ end of the DMD gene in rat (Nudel et al. 1989), mouse (Chelly et al. 1990*b*), and human (Feener et al. 1989) brain is transcribed from a brain-specific promoter and is predicted to produce a brain isoform of dystrophin in which the first 11 amino acids (exon 1) of muscle-type dystrophin are replaced by 3 different amino acids. The existence of such a protein product, however, has not yet been clearly demonstrated. Our observation that human brain dystrophin, identified by specific mAbs against the rod and C-terminal regions, is not detected by MANEX1 mAbs (fig. 6) is clearly consistent with the proposed isoform. Although muscle-type mRNA can be detected in brain, possibly in glial or smooth-muscle components, the brain-type mRNA is expressed mainly in neuronal cells (Chelly et al. 1990*b*; Bies et al. 1992) and is known to be present in cerebral cortex from which the brain extract shown in figure 6 was prepared.

The successful production of a mAb against an exon 1–encoded dystrophin epitope by using a fusion protein immunogen of over 1,000 amino acids is somewhat remarkable, since only 10 amino acids of dystrophin are encoded entirely by exon 1. Only one of three mice, however, responded to the immunogen in this way (fig. 2[*a*]). The third and fourth amino acids of dystrophin (Trp3 and Trp4) are essential for the MANEX1 epitope. These are very close to the junction with the lacZ gene product in the immunogen, but the MANEX1 mAbs nevertheless recognize authentic dystrophin in both native and denatured forms. It is of interest that two hydrophobic tryptophan residues appear to be easily accessible to antibody in native dystrophin.

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