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Construction of dystrophin fusion proteins to raise targeted antibodies to different epitopes

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For the study of the structure and function relationship of dystrophin, defective in DMD, and for diagnostic purposes it is important to dispose of antibodies against different parts of the protein. We have made five different constructs for the expression of fusion proteins containing parts of the four domains of dystrophin. Two different recombinant expression vectors, pATH2 and pEX1, were used. Rabbits were immunized with the fusion products and several polyclonal antibodies were raised. At a later stage, monoclonal antibodies were also raised to some of the fusion proteins. One polyclonal antibody, named P20 AB, is directed against the region covering amino acid sequence 1749–2248 or the nucleotide sequence 5456–6953 of the mRNA, which corresponds to the major deletion-prone region of the DMD gene. We show the particular value, sensitivity and specificity of the P20 AB in dystrophin analysis.

Dystrophin; pATH2 fusion protein; pEX1 fusion protein; Duchenne muscular dystrophy; P20 mutation hotspot; Polyclonal antiserum

1. INTRODUCTION

Duchenne muscular dystrophy (DMD) is a progressive lethal disorder, which affects 1:3500 newborn boys. The gene involved in DMD encodes a 427 kDa protein named dystrophin [1] and is the largest gene (2300 kb) known so far [2]. Dystrophin is present in low abundance (0.01% of total skeletal muscle protein) and is localized at the cytoplasmatic face of the sarcolemma [3–5]. In Duchenne patients dystrophin is absent from most (>98%) or all muscle cells. In muscle of Becker (BMD) patients dystrophin is present with altered size and/or at different levels [6]. In order to study the structure and function of dystrophin and carry out diagnostic studies on muscle of DMD/BMD patients and patients with related muscular disorders, antibodies against dystrophin are required. We therefore constructed fusion proteins with which we raised antibodies against various domains of dystrophin.

Dystrophin consists of four domains (Fig. 1): an N-terminal domain (A), a central rod-shaped triple-helical domain counting 24 repeat units of about 109 amino acids (B), a cysteine-rich domain (C) and a C-terminal domain (D) [7]. The domains A, B and C show homology with α-actinin, and A and B with spectrin as well, suggesting a cytoskeletal function for dystrophin [7], consistent with its location. The A domain is thought to bind actin. In the B domain of human dystrophin four hinge segments were identified, which may play a role in the flexibility of dystrophin in the cytoskeleton [8]. Domains C and D are highly conserved between human and chicken, suggesting an important role for this region of dystrophin in the interaction with other cellular components [9]. The D domain is supposed to be involved in the anchoring of dystrophin with glycoproteins in the plasmamembrane [10,11] and shows strong similarity with a dystrophin-like protein [12].

Five different cDNA fragments are selected for the construction of recombinant fusion proteins. Several antisera were raised using these fusion proteins as immunogens. One polyclonal antibody named P20 is directed against the dystrophin part which corresponds with the deletion-prone P20 region of the DMD gene [13]. About half of the deletions found in the DMD/BMD gene of Duchenne and Becker patients are breaking in this so called P20 hotspot [14]. The P20 AB has been characterized in detail and was found to be very specific and sensitive, and a valuable tool for dystrophin analysis. Similarly, the monoclonal antibody MANDRA1 raised against the distal DRA polypeptide [15], is now finding widespread use in the detection of distal dystrophin epitopes [16–18].

2. MATERIALS AND METHODS

2.1. *Plasmid constructions*

Human, fetal dystrophin cDNA clones were obtained from M. Koenig [7]. Nucleotide and amino acid sequence numbering are as in Koenig et al. [7] and are depicted in Fig. 1.

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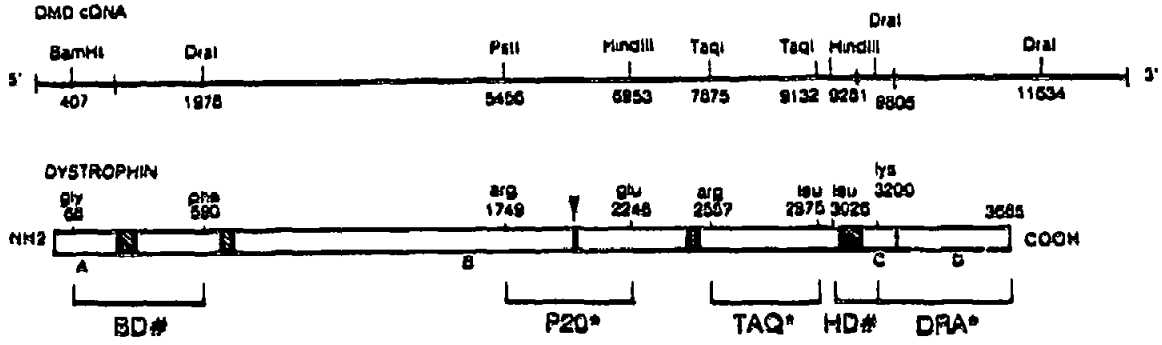

Fig. 1. Map of DMD cDNA restriction sites and number of dystrophin amino acids used for the construction of hybrid proteins. The pATH2 fusion proteins are denoted with * and the pEX1 fusion proteins with #. The four domains, four hinges and interdomain segment are according to Koenig and Kunkel [8] and are indicated by A, B, C, D, hatched boxes and an arrow, respectively.

2.2. *Construction of fusion protein genes*

Two different expression vectors pATH2 and pEX1 were used to obtain the in-frame (DMD) gene constructs. Expression of the constructs in *E. coli* yielded fusion products containing different dystrophin segments (Table I).

2.2.1. Construction of fusion proteins using the pATH2 vector

P20-pATH2 plasmid construct

A 1.5 kb fragment of DMD cDNA(47-4) [7] obtained after digestion with *PstI*(5456) and *HindIII*(6953) was subcloned into a partial (*PstI/HindIII*) digest of a bacterial expression vector pATH2 [19], which contains the first 969 nucleotides of the trpE gene. The resulting recombinant vector fused 33 kDa of the trpE protein to 59 kDa of dystrophin (amino acid sequence 1749–2248; Fig. 1), yielding fusion protein P20 (Table I).

TAQ-pATH2 plasmid construct

A 1.2 kb *TaqI* (7875–9132) fragment of cDNA(63-1), blunt-ended with DNA polymerase I (Klenow fragment), was inserted into the *SmaI* site of pATH2. The constructed fusion protein, named TAQ, contained 48 kDa of dystrophin (amino acid sequence 2557–2975; Fig. 1, Table I).

DRA-pATH2 plasmid construct

A 1.5 kb *DraI* (9805–11634) fragment of cDNA(63-1) was inserted into the *SmaI* site of pATH2. The obtained fusion protein, named DRA, includes the last 485 amino acids of dystrophin (amino acid sequence 3200–3685; Fig. 1, Table I).

2.2.2. Construction of fusion proteins using the pEX1 vector

BD-pEX1 construct

DMD cDNA(XJ10) was digested with *BamHI*(407)/*DraI*(1978) blunt-ended using DNA polymerase I (Klenow) and inserted into the *SmaI* site of pEX1 (Genofit Ltd), an expression vector containing the lacZ gene [20]. The resulting recombinant vector fused 108 kDa of β-galactosidase to 63 kDa of dystrophin (amino acid sequence 67–590), yielding fusion protein BD (Fig. 1, Table I). HD-pEX1 construct.

A 500 *HindIII*(9281)/*DraI*(9805) fragment of cDNA(63-1), blunt-ended by using DNA polymerase I (Klenow), was inserted into the *SmaI* site of pEX1. The resulting fusion protein, HD, contains 21 kDa of dystrophin (amino acid sequence 3026–3199; Fig. 1, Table I).

2.3. *Isolation of fusion proteins and use as immunogens*

The recombinant plasmid pATH2 was introduced into *E. coli* RR1 cells, which were grown according to Hoffman et al. [1]; induction of the plasmic encoding protein expression, harvesting of the cells and isolation of the fusion proteins were as described [14] except for the addition of 0.25 mM protease inhibitor PMSF during lysozyme treatment and a short sonication to shear the DNA after lysis of the cells.

The recombinant vectors pEX1 were introduced into *E. coli* POP2136 cells, grown in NZY at 30°C to log phase and induced for 90 min at 37°C, harvested (10 min at 500×*g*) and lysed in STE (15% sucrose; 50 mM Tris-HCl, pH 8; 50 mM EDTA) with lysozyme (10 mg/ml) containing Triton X-100 (0.2%). The suspension containing the insoluble fusion proteins was sonicated for 15 s to shear the DNA and centrifuged (12,000×*g* for 15 min at 4°C); the pellet was resuspended in PBS and stored at −70°C. Between 5 and 10 mg of the different fusion proteins were isolated from 100 ml induced bacterial culture. The protein concentrations were determined (Bio-Rad protein assay). The fusion proteins were used to immunise rabbits (New Zealand White) according to Harlow and Lane [21]. Purification of the fusion proteins before their use as an antigen was not found to be a prerequisite.

2.4. *Immunohistochemical staining*

Muscle tissue was either fixed directly at room temperature overnight in methanol/acetone/acetic acid/water (35:35:5:25, v/v/v/v) followed by dehydration and embedding in paraplast (Monoject) [22] or frozen directly in isopentane chilled in liquid nitrogen and stored at −70°C until use. For immunohistochemical staining, 5 μm-thick sections of muscle tissue were immunostained as described by Moorman et al. [23]. The sera were diluted in PBS.

2.5. *Gel electrophoresis and Western blotting*

Extraction and sampling of proteins from muscle biopsies were according to Hoffman et al. [1] except that we used 10× sampling buffer. Gel electrophoresis, protein transfer and antibody incubation were as described by Wessels et al. [24]. Fusion proteins were electrophoretically separated on SDS-polyacrylamide gels using a 4% stacking gel and a 7.5% resolving gel. To determine the molecular weights, prestained molecular weight markers (Sigma) were used. To identify the α-actinin polypeptide a monoclonal antibody against α-actinin (Sigma) was used.

Table I

Insert sizes DMD cDNA, deduced amino acid sequences and corresponding fusion protein size

Name fusion protein	Expression vector	Size (kb) insert (cDNA)	Amino acid sequence	Size (kDa) fusion protein
BD	pEX1	1.6	68–590	169
P20	pATH2	1.5	1749–2248	91
TAQ	pATH2	1.3	2557–2975	81
HD	pEX1	0.5	3026–3199	128
DRA	pATH2	1.4	3200–3685	89

3. RESULTS AND DISCUSSION

3.1. *Expression of fusion proteins*

In this study two different bacterial expression vector systems, pATH2 and pEX1, were used (Table I). The positions of the resulting fusion proteins on the dystrophin molecule are depicted in Fig. 1 and their analysis is shown in Fig. 2. The expression of pATH2 fusion proteins was induced biochemically: besides the predominant bands in agreement with the expected size of the fusion proteins, some background proteins of the bacterial host, *E. coli* RR1 and/or proteolytically degraded fusion proteins were coprecipitated (Fig. 2: lanes 3, 4 and 5). Coprecipitation also occurred in another host strain, *E. coli* 1046 (not shown). Therefore, new constructs were made in the pEX expression system [20]. The pEX expression system makes use of a temperature-inducible promoter and shows predominantly the fusion protein bands (Fig. 2: lanes 7 and 8). In this expression system hardly any degradation product was present and/or the amount of bacterial proteins in the insoluble protein fraction was rather low. These results show that the pEX expression system gives a higher yield of fusion protein compared with the pATH2 system.

The insoluble fusion protein fractions obtained with both systems could be used directly as immunogens in rabbits.

3.2. *Characterization of the antibodies raised*

3.2.1. Polyclonal antisera. A number of very sensitive rabbit antisera are raised with the trpE fusion proteins: the 30 kDa and 60 kDa antisera raised by Hoffman et al. [1] and the P20 and TAQ antisera in our study. The P20 AB reacts both with native and denatured protein and can be used at very high serum dilutions on sections (1:500–1000) as well as on Western blots (1:1,000) (Figs. 3 and 4). Using P20 AB, fixed sections of normal mouse and human muscle tissue showed a clear staining of the sarcolemma (Fig. 3A) and in sections of DMD muscle tissue no dystrophin was observed (Fig. 3B). On Western blots, P20 AB identified the 427 kDa dystrophin band besides a minor band of 95 kDa. This appears due to some α-actinin crossreaction (Fig. 4: lane 1), since the 95 kDa band on blots was also recognized by a monoclonal antibody against α-actinin (not shown), while in the muscle extract of DMD patients the dystrophin band was absent and the 95 kDa band present (Fig. 4: lane 2). This observation indicates the sharing of antigenic determinants between α-actinin and this region of the dystrophin B domain. For diagnostic purposes the amount of α-actinin identified with P20 AB has turned out to be a valuable indicator for the amount of muscle proteins present in the muscle extracts.

The polyclonal antisera BD and TAQ also identified a major dystrophin band next to a 95 kDa α-actinin band (no shown). In total 20 rabbits were immunized with the various fusion proteins. So far most successful immunizations were obtained using fusion proteins from the B domain: the 30 kDa and 60 kDa fusion proteins from Hoffman et al. [1] (not shown) and our P20 and TAQ fusion proteins (Fig. 1).

3.2.2. Monoclonal antibodies. Fusion proteins containing the deletion prone area of the B domain and the D domain of dystrophin P20 and DRA, respectively (Fig. 1), were also used to generate monoclonal antibodies [15,25]. Some P20 monoclonal antibodies identified

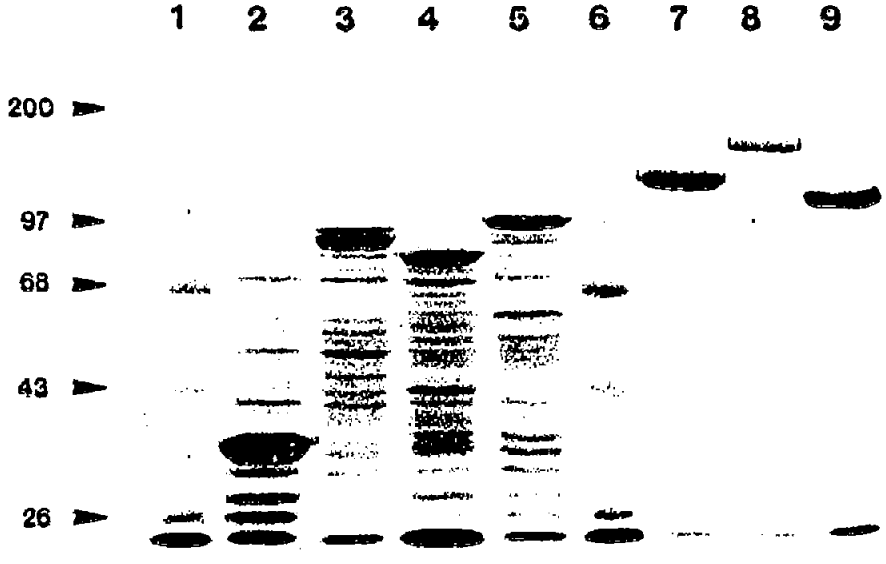

Fig. 2. A 7.5% SDS-PAGE of five different fusion proteins. Lanes 3, 4 and 5 are loaded with trpE hybrid proteins: DRA, TAQ and P20, respectively. Lanes 7 and 8 are loaded with pEX1 fusion proteins, HD and BD. In lanes 1 and 6 molecular weight markers are shown and in the lanes 1 and 9, trpE and β-galactosidase, respectively.

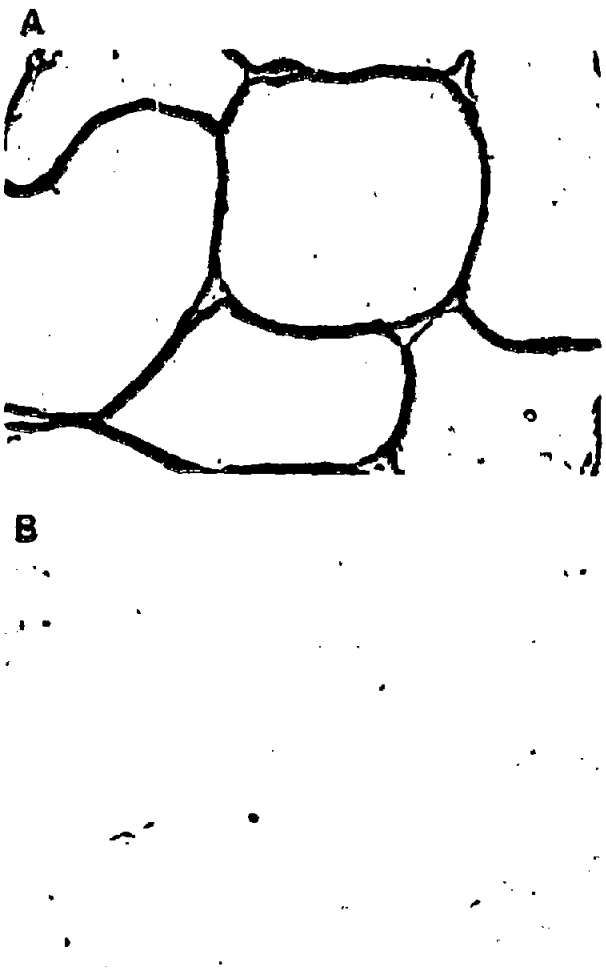

Fig. 3. Sections of human fixed muscle cells incubated with P20 AB (1:1,000). A. normal human muscle; B. DMD muscle. Magnifications ×325.

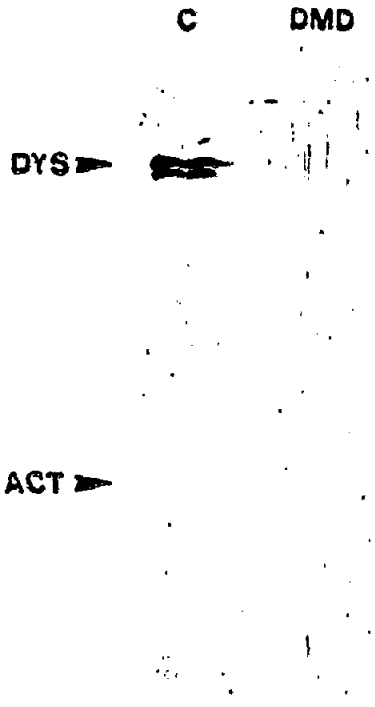

Fig. 4. A Western blot of muscle extracts separated on a 4–7% gradient gel SDS-PAGE and incubated with P20 AB (1:1,000). C: normal, adult muscle tissue; DMD, DMD muscle tissue.

both dystrophin and α-actinin, underscoring the homology of their B domains [25].

Two classes of monoclonal antibodies could be discriminated using the fusion protein DRA as immunogen: one type binding exclusively to dystrophin and another type binding to a cross-reacting dystrophin-like protein [15]. Besides the improvement of using monoclonal antibodies for dystrophin analysis, this observation also shows the importance of using fusion proteins as immunogens. Thus antibodies may be raised, which also identify dystrophin-like proteins. The dystrophin-exclusive antibody MANDRAI has since found widespread use as a specific reagent for the COOH terminus of dystrophin [16–18].

A panel of sensitive antisera against different parts of dystrophin is of great value for diagnostic purposes. Such a set of antibodies may pinpoint the localization of the mutation in the DMD gene [26]. This is of special interest in those DMD/BMD families (35%) for which DNA analysis does not currently show detectable abnormalities of the DMD gene. In protein analysis, truncated dystrophins can be identified [26,27], which may guide to further examination of the region of interest for small mutations leading to frameshift and/or early termination of the DMD gene [27].

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