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Dystrophin: The Protein Product of the Duchenne Muscular Dystrophy Locus

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Summary

The protein product of the human Duchenne muscular dystrophy locus (DMD) and its mouse homolog (mDMD) have been identified by using polyclonal antibodies directed against fusion proteins containing two distinct regions of the mDMD cDNA. The DMD protein is shown to be approximately 400 kd and to represent approximately 0.002% of total striated muscle protein. This protein is also detected in smooth muscle (stomach). Muscle tissue isolated from both DMD-affected boys and *mdx* mice contained no detectable DMD protein, suggesting that these genetic disorders are homologous. Since *mdx* mice present no obvious clinical abnormalities, the identification of the *mdx* mouse as an animal model for DMD has important implications with regard to the etiology of the lethal DMD phenotype. We have named the protein dystrophin because of its identification via the isolation of the Duchenne muscular dystrophy locus.

Introduction

The muscular dystrophies are a heterogeneous group of both human and animal hereditary diseases whose primary manifestation is progressive muscle weakness due to intrinsic biochemical defects of muscle tissue (Mastaglia and Walton, 1982). The most common and devastating of the human muscular dystrophies is the X-linked recessive Duchenne muscular dystrophy (DMD), first described in the mid-1800s (Meryon, 1852; Duchenne, 1868). Affecting approximately 1 in 3,500 boys, this genetic disorder exhibits no obvious clinical manifestation until the age of 3 to 5 years, when proximal muscle weakness is first observed. The ensuing progressive loss of muscle strength usually leaves affected individuals wheelchair-bound by the age of 11, and results in early death due to respiratory failure. Both the typical histological pattern of widespread degeneration and regeneration of individual muscle fibers in most skeletal muscle groups (Dubowitz, 1985) and high concentrations of soluble muscle-specific enzymes in serum are present in affected individuals long before the clinical onset of the disease, and can often be found in female carriers (Emery and Holloway, 1977). Despite many years of intensive research, the primary biochemical defect responsible for this disorder has remained elusive, as have any rational therapies to slow the progression of the disease.

With no effective treatments available, an animal model for this disease has long been sought to test possible therapies. Despite the availability of numerous muscular dystrophies in many different species (Harris and Slater, 1980), the lack of any information concerning the biochemical defect involved in both DMD and the putative animal models has made it difficult to equate any specific animal muscular dystrophy with DMD. An X-linked murine muscular dystrophy, *mdx*, was fortuitously discovered during screening of normal mouse serum enzymes in preparation for a mutagenesis screen (Bulfield et al., 1984). Given the general conservation of the X chromosome within mammalian species (Ohno, Becak, and Becak, 1964), the chromosomal location of *mdx* in mouse suggested that the *mdx* mutation might indeed represent the same biochemical defect as that manifested in X-linked human DMD. However, homozygous *mdx* mice exhibit little, if any, clinically detectable phenotype. Homozygous strains are easily maintained, showing only slightly reduced fecundity (Torres and Duchen, 1987), and develop no obvious muscle weakness (Tanabe, Esaki, and Nomura, 1986). Histologically, the persistent degeneration/regeneration characteristic of human DMD muscle fibers appears very similar in the *mdx* mouse, though the extensive connective tissue proliferation (fibrosis) evident in human DMD muscle groups appears to be largely absent in *mdx* muscle (Bridges, 1986). Though it could be argued that the *mdx* mutation represents a mild allele of the mouse DMD homolog (such as the rarer Becker allele of Duchenne), two ethylnitrosourea-induced (ENU) alleles of the *mdx* locus exhibit similar phenotypes, making it unlikely that all three represent Becker-like mutations (Chapman, personal communication).

The differences between the mouse *mdx* and human DMD phenotypes have been used as evidence against the significance of the common X-linked nature of the mutations. In apparent agreement with the phenotypic differences, the *mdx* mutation has been recently shown to be more closely linked genetically to markers neighboring the less severe and less common human X-linked Emery–Dreifuss muscular dystrophy (Avner et al., 1987). These two human X-linked dystrophies are on opposite ends of the human X chromosome, yet recent studies using portions of the cloned DMD locus as genetic markers in the mouse have placed the mDMD locus in the same chromosomal region as the *mdx* mutation (Chapman et al., 1985; Heilig et al., 1987; Brockdorff et al., 1987; Chamberlain et al., 1987).

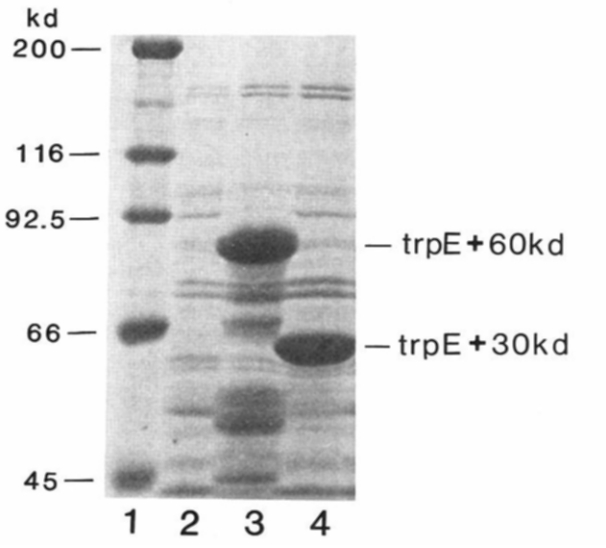

Figure 1. Insoluble Protein Fractions of Recombinant Bacterial Lysates

E. coli strain RR1 harboring either the parent plasmid vector (lane 2)(pATH2; Dieckmann and Tzagoloff, 1985) or one of its recombinants containing two different portions of the mDMD coding sequence (lane 3, trpE+60kd; lane 4, trpE+30kd) was induced with 3-B-indolacrylic acid and lysed, and insoluble proteins were isolated by centrifugation. Shown is 1% (~200 μg) of the insoluble protein obtained from a 100 ml culture of bacteria for each preparation. The trpE–mDMD fusion proteins are evident as the major protein species (~85% of total), at the expected molecular weights (trpE = ~33 kd). Protein size markers are indicated (lane 1).

A human cDNA clone representing a portion of the DMD transcript has been isolated on the basis of its conservation between mouse and man (Monaco et al., 1986). This partial cDNA was then used to isolate homologous cDNA sequences from mouse (Hoffman et al., 1987), and the entire 14 kb human coding sequence (Koenig et al., 1987). The mRNA product of this locus was detected only in muscle-containing tissues in both humans and mice (Monaco et al., 1986; Hoffman et al., 1987), and has been demonstrated to be expressed specifically in terminally differentiated myotubes in culture (Lev et al., 1987). In this paper we report the production of antibodies to the mDMD and DMD gene protein products. We describe the use of these antibodies to study the protein product of the Duchenne muscular dystrophy locus, called dystrophin, in tissues isolated from both normal and dystrophic mice and humans.

Results

Production of mDMD Fusion Proteins

The DNA and predicted amino acid sequences for 4.3 kb (30%) of the Duchenne muscular dystrophy (DMD) gene has been previously presented for cDNA clones isolated from human fetal skeletal muscle and for the mouse cardiac muscle homolog of the human DMD locus (mDMD; Hoffman et al., 1987; Koenig et al., 1987). These cDNAs were found to be highly conserved between mice and humans, exhibiting over 90% similarity at both the DNA and amino acid levels. To study the mouse and human protein product of the DMD locus, polyclonal antibodies were produced against large regions of the mouse mDMD polypeptide.

Two different regions of the mouse heart DMD cDNA were fused to the 3′ terminus of the E. coli *trpE* gene by using the expression vector pATH2 (Dieckmann and Tzagoloff, 1985). The two regions represent the majority of the mouse heart cDNA sequence previously described (Hoffman et al., 1987), but do not overlap. One construction resulted in the fusion of approximately 30 kd of mDMD protein to the 33 kd trpE protein, while the second employed roughly 60 kd of the mDMD protein. Since the trpE protein is insoluble, quantitative yields of induced fusion proteins were obtained simply by lysing of the cells and precipitation of insoluble proteins. As shown in Figure 1, novel insoluble fusion proteins of the expected size were produced; they were not present in lysates from bacteria containing the pATH2 vector alone.

Production of Antisera

Both fusion proteins were purified by preparative SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) and used to immunize rabbits and sheep (see Experimental Procedures). Rabbits were immunized with electroeluted, "native" (free from SDS) insoluble antigen, while sheep were immunized with SDS–polyacrylamide gel slices containing denatured antigen. In order to monitor the titer and specificity of immune sera, dot blots of each antigen were made on nitrocellulose by using the insoluble fractions of bacterial lysates harboring either the parent plasmid vector (pATH2) or a recombinant fusion-protein plasmid. Each antigen solution was loaded such that the amount indicated refers to the amount of trpE protein in each dot (Figure 2).

The DMD portion of the smaller fusion protein (trpE+30kd) proved to be highly antigenic. Greater than 95% of rabbit antibodies (rabbit serum; Figure 2A) were found to be directed specifically against the DMD portion of the polypeptide, though this portion represented only half of the fusion protein. Similarly, approximately 90% of the sheep antibodies were directed specifically against the DMD portion of this fusion protein (data not shown). The larger trpE+60kd fusion protein also proved to be quite antigenic, with a 1:1000 dilution of primary sera having antibody titers nearly equal to the limits of the detection system (~1 ng antigen), though a much larger proportion of antibodies were directed against the trpE protein (sheep serum shown in Figure 2B; rabbit serum results similar but not shown).

To ensure that any protein species identified by the antisera was due to recognition by antibodies specific for he DMD portion of the fusion proteins, rabbit and sheep antibodies directed against the 30 kd antigen and sheep antibodies directed against the 60 kd antigen were affinity-purified (Figure 2). Affinity purification of the rabbit antibodies directed against the mDMD portion of the fusion protein was facilitated by the insolubility of the partially purified fusion protein. By simple resuspension of crude insoluble protein fractions (shown in Figure 1) in immune se-

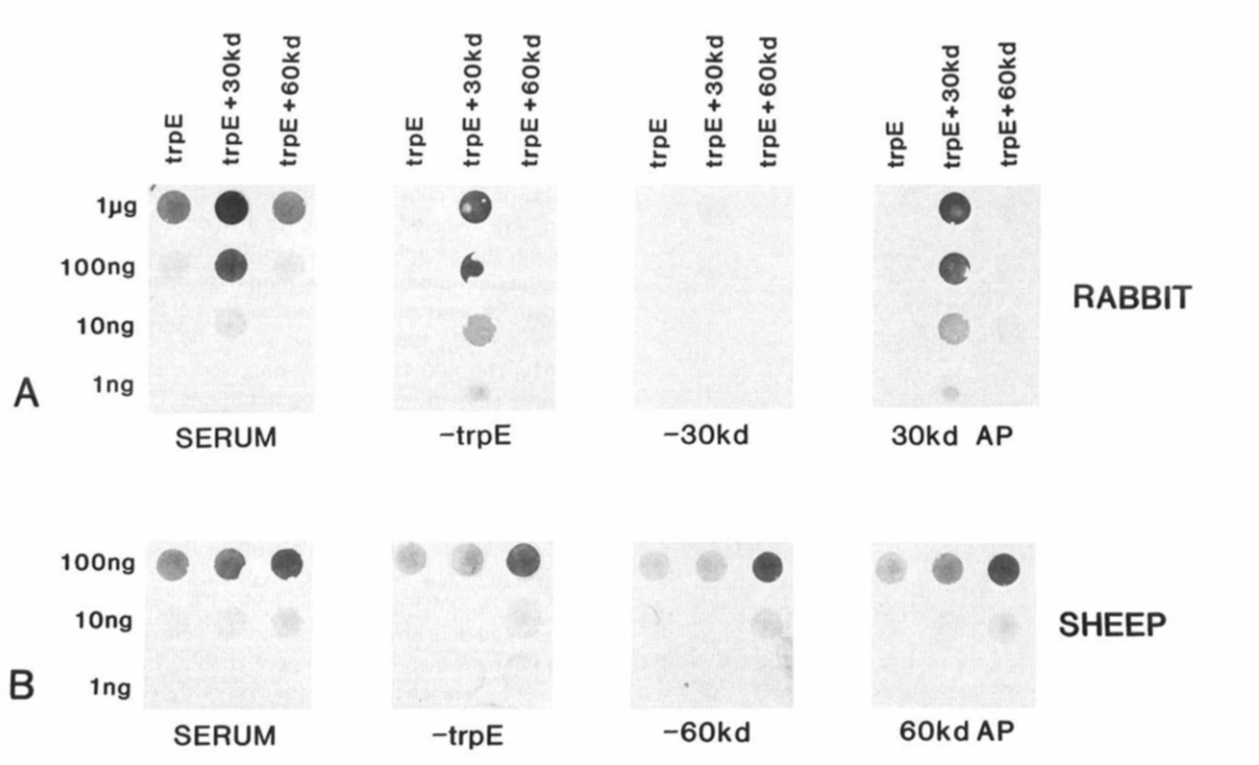

Figure 2. Specificity and Titer of Antisera Raised against the mDMD Fusion Proteins

Antigen dot blots were prepared on nitrocellulose by using the insoluble protein fractions shown in Figure 1. The amounts indicated refer to the relative amount of trpE protein in each dot.

(A) Immune serum from a rabbit immunized with the trpE+30kd antigen in insoluble form (see Experimental Procedures). The first filter shows the alkaline phosphatase staining after incubation with unprocessed immune serum diluted 1:1000, followed by incubation with alkaline phosphatase conjugated goat anti-rabbit second antibody (rabbit serum). Greater than 95% of the antibodies in the unprocessed serum are seen to be directed specifically against the DMD portion of the fusion protein. Antibodies specific for the trpE portion of the fusion peptide were then removed, with the resulting serum showing no apparent remaining reactivity for the trpE protein (−trpE). The immune serum with the antibodies against the 30 kd protein removed is shown to contain little remaining reactivity with the fusion protein (−30 kd). 30kd antigen–antibody complexes were disassociated, with the resulting supernatant showing a high titer of antibodies directed specifically against the DMD portion of the fusion peptide (30kd AP [affinity-purified]).

(B) The same affinity purification protocol as used in (A) was applied to immune serum from a sheep immunized with denatured trpE+60kd antigen.

rum, antibodies against the trpE protein were eliminated (−trpE; Figure 2A) and antibodies specific for the mDMD protein isolated (30kd AP). The immune serum that had been absorbed with both the trpE and trpE+30kd antigens showed very little remaining reactivity with either of these antigens (−30kd; Figure 2A). The resulting affinity-purified antibody (30kd AP) had a titer above the limits of the detection system (1 ng) when a 1:1000 dilution was used (Figure 2A). The sheep antisera against this same fusion protein were affinity-purified in the same manner, with greater than 95% of the resulting affinity-purified immunoglobulins being directed specifically against the 30 kd DMD antigen (not shown). In the case of the sheep antiserum directed against the 60 kd antigen, the same affinity purification protocol was used but appeared to be much less efficient (Figure 2B; −trpE, −60kd, 60kd AP).

Identification of the Protein Product of the Duchenne Muscular Dystrophy Locus

Total protein samples were isolated from mouse (fresh) and human (frozen) tissues by direct solubilization of tissues in 10 volumes of gel loading buffer (100 mM Tris, pH 8.0; 10% SDS; 10 mM EDTA; 50 mM DTT). Alternatively, Triton X-100 insoluble fractions were isolated from human and mouse tissues by homogenization in 0.25% Triton X-100 using a Waring blender at full speed, and by pelleting of insoluble proteins. The protein concentrations in the Triton-insoluble fractions were quantitated by using the Bio-Rad protein assay, while the protein concentration of the directly SDS-solubilized tissues was estimated based on the starting mass of the tissue used. All protein samples (50 µg) were separated by electrophoresis on 3.5% to 12.5% gradient SDS–polyacrylamide gels (Laemmli, 1970) using a 3.0% stacking gel, and transferred to nitrocellulose (Towbin, Staehelin, and Gordon, 1979). Identical nitrocellulose blots of the separated proteins were incubated with affinity-purified rabbit antibodies directed against the 30 kd antigen (Figure 3A), affinity-purified sheep antibodies directed against the 60 kd antigen (Figure 3B), and affinity-purified sheep antibodies directed against the 30 kd antigen (Figure 3C), each at a 1:1000 dilution. Immune complexes were detected by using either ^{125}I-protein A (Figure 3A) or alkaline phosphatase conjugated donkey anti-sheep IgG second antibody (Figures

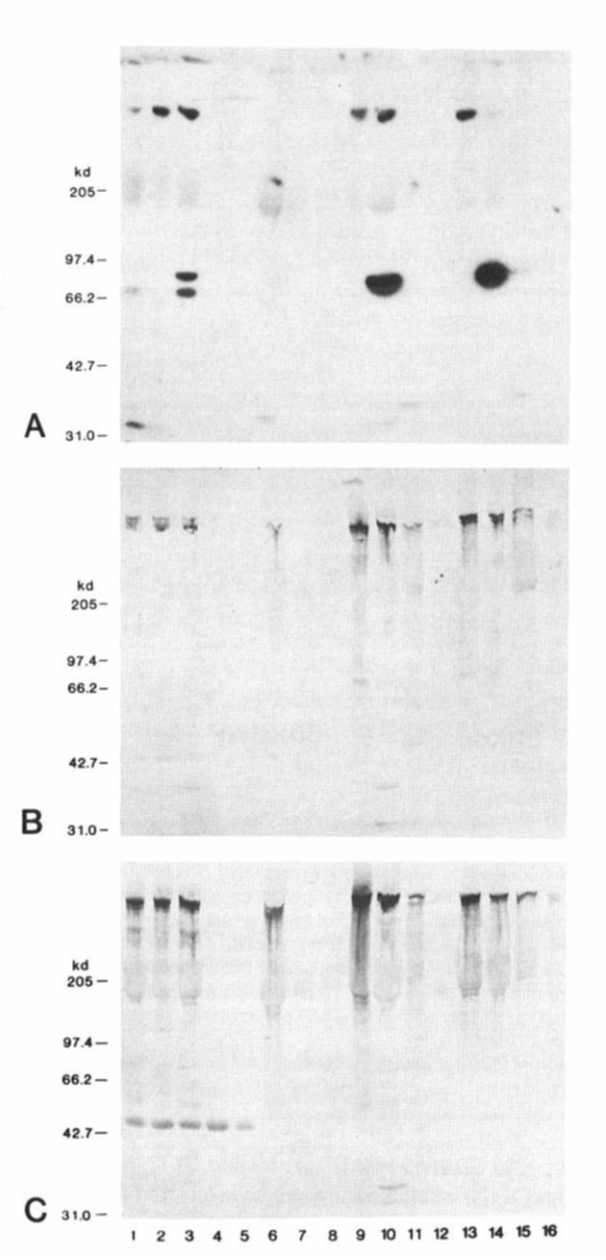

Figure 3. Identification of the Protein Product of the DMD Locus

Mouse (fresh) or human (frozen) tissues were either directly solubilized and denatured in gel loading buffer or were first homogenized with 0.25% Triton X-100, and Triton-insoluble proteins were isolated. Protein samples (50 μg) were fractionated on 3.5%–12.5% gradient SDS–polyacrylamide gels (Laemmli, 1970), transferred to nitrocellulose (Towbin, Staehelin, and Gordon, 1979), and incubated with affinity-purified rabbit antibodies directed against the 30 kd cardiac mDMD (A), sheep antibodies directed against the 60 kd antigen (B), or sheep antibodies directed against the 30 kd antigen (C). Immune complexes were visualized by using either ^{125}I-protein A (A), or alkaline phosphatase conjugated donkey α-sheep IgG (B and C). Lanes are as follows: 1, human adult skeletal muscle; 2, human newborn cardiac muscle; 3, human newborn skeletal muscle (psoas); 4, human DMD-affected skeletal muscle (patient 1); 5, human DMD-affected skeletal muscle (patient 2); 6, Triton-insoluble extract of adult human skeletal muscle; 7, Triton-insoluble extract of DMD-affected skeletal muscle (patient 1); 8, Triton-insoluble extract of DMD-affected skeletal muscle (patient 2); 9, normal mouse heart; 10, normal mouse skeletal muscle; 11, normal mouse stomach; 12, normal mouse brain; 13. Triton-insoluble extract of normal mouse heart; 14. Triton-insoluble extract of normal mouse skeletal muscle; 15, Triton-insoluble extract of normal mouse stomach; 16, Triton-insoluble extract of normal mouse brain.

Shown is the 400 kd, low abundance protein species recognized by all three antibodies in all normal muscle-containing tissues. Smaller, cross-reactive protein species are detected by antibodies raised against the 30 kd DMD antigen in either rabbit (A) or sheep (C). The size and location of myosin and biotinylated molecular weight markers are indicated.

3B and 3C) (sheep IgG binds very poorly to protein A). All antibodies detected a large molecular weight, apparently low abundance protein species calculated to be approximately 400 kd in total solubilized human and mouse skeletal and cardiac muscle (lanes 1–3, 9–10). The higher resolution of the alkaline phosphatase staining (Figures 3B and 3C) resolved this protein into doublets or triplets, though the slightly smaller bands most likely represent degradation products since there has been no evidence to date for alternatively spliced isoforms of the DMD mRNA (Koenig et al., 1987; Hoffman et al., 1987; Burghes et al., 1987). The 400 kd species was also clearly evident in mouse smooth muscle (stomach) (lane 11), though at a level substantially lower than that found in cardiac and skeletal muscle (lanes 9–10). The same apparent protein species was detectable in mouse brain at an extremely low level (Figures 3B and 3C, lane 12). Though transcriptional studies of the DMD gene in mice and humans (Monaco et al., 1986; Hoffman et al., 1987) were unable to identify DMD gene transcription in brain, the results presented here are completely compatible given the much greater sensitivity of the Western analysis used for protein detection relative to the Northern analysis used for mRNA detection of the large transcript. Further studies are required to determine whether the apparent low level of the DMD protein in brain is due to expression in smooth muscle or in other cell types.

The 400 kd protein species recognized by all antibodies was generally Triton-insoluble, though it appeared to be associated more strongly with the myofibrillar matrix fraction in cardiac muscle than it did in either skeletal muscle or smooth muscle (Figure 3, lanes 9–11, 13–15). This protein was not detectable in the SDS-solubilized (lanes 4–5) or Triton-insoluble (lanes 7–8) fractions of skeletal muscle samples from two boys affected with DMD. The deficiency of this protein in the DMD-affected boys is particularly evident in Figure 3C, where the presence of a cross-reactive 50 kd Triton-soluble protein serves to verify the equal protein content of the normal vs. DMD lanes (lanes 1–5).

Though sheep antibodies directed against the larger 60 kd DMD antigen (Figure 3B) recognized solely the 400 kd protein in both mouse and human tissues, additional protein species were clearly detected by rabbit and sheep antibodies raised against the 30 kd antigen (Figures 3A and 3C). However, none of the smaller proteins recognized by the sheep α-30kd antibodies were similarly recognized by the rabbit α-30kd antibodies, indicating that these smaller proteins represent cross-reactive protein species and are not themselves products of the DMD locus in either mice or humans. Though it appears that the amount of the DMD protein varies in different tissues depending on the anti-

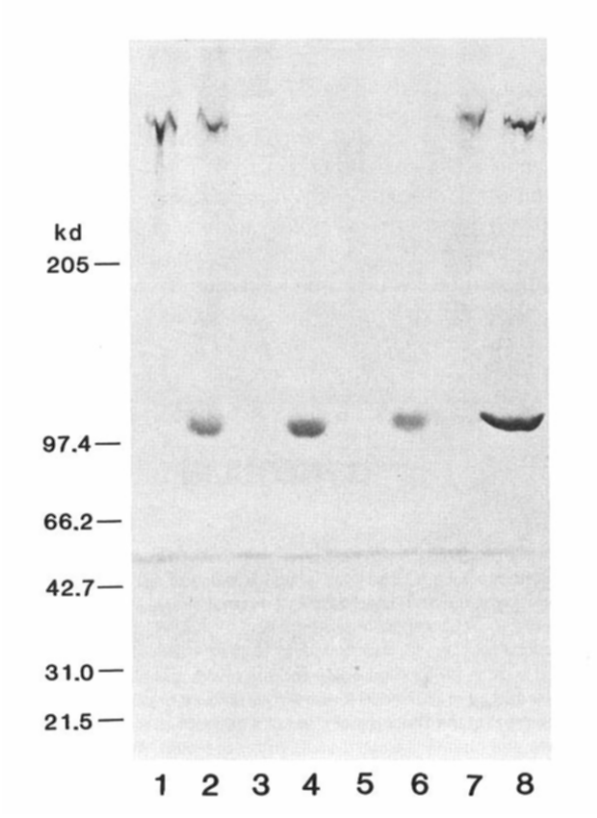

Figure 4. Analysis of *mdx* Mice

Freshly dissected heart and skeletal muscle tissues from normal, *mdx*, mdx^{467}, and homozygous *Tr* mice were solubilized and denatured in gel loading buffer, and aliquots (50 μg) were fractionated on 3.5%–12.5% gradient SDS–polyacrylamide gels. Proteins were transferred to nitrocellulose and incubated with a mixture of rabbit (anti-30kd antigen) and sheep (anti-60kd antigen) anti-DMD antibodies, and the immune complexes were detected by using alkaline phosphatase conjugated second antibodies. Lanes are as follows: 1, homozygous *Tr* heart; 2, *Tr* skeletal muscle; 3, mdx^{467} heart; 4, mdx^{467} skeletal muscle; 5, *mdx* heart; 6, *mdx* skeletal muscle; 7, normal mouse heart; 8, normal mouse skeletal muscle.

The 400 kd protein species recognized by both sets of antibodies is evident in both normal and *Tr* mice, but is absent from both alleles of *mdx* mice. The more abundant 90 kd cross-reactive protein species recognized by rabbit antibodies directed against the 30 kd antigen (as shown in Figure 3A) is seen at equal levels in the skeletal muscle of all mice. This cross-reactive protein has an apparent molecular weight of 100 kd, which is probably a better representation of the size than the 90 kd weight calculated in Figure 3A. The size and location of myosin and biotinylated molecular weight markers are indicated.

body used (see Figure 3A vs. Figure 3B), this is assumed to be an artifact due to the increased contrast of the autoradiographic exposure of Figure 3A compared to the alkaline phosphatase staining of Figures 3B and 3C. Indeed, a comparison of Figures 3B and 3C, both of which employ immunochemical staining, indicates that antibodies raised against the two different antigens recognize the same 400 kd protein in equal relative abundances.

Analysis of *mdx* Mice

Skeletal and cardiac muscle was dissected from normal, *mdx* (Bulfield et al., 1984), mdx^{467} (V. Chapman, personal communication), and homozygous *Tr* (a severe neuropathological disorder; Falconer, 1951; Henry, Cowen, and Sidman, 1983) mice and solubilized directly in gel loading buffer as described above. Protein samples were separated by electrophoresis on 3.5% to 12.5% gradient polyacrylamide gels and analyzed as above, by using a cocktail of sheep α-60kd and rabbit α-30kd antibodies. As shown in Figure 4, the 400 kd protein species was present in the skeletal and cardiac muscle of both normal (lanes 7–8) and *Tr* (lanes 1–2) mice. The detected protein appeared the same with this cocktail of antisera as it did with each antiserum separately (shown in Figure 3), indicating that the two antibodies raised against different antigens recognized the same protein. Both antibodies failed to detect the 400 kd protein in muscle tissues isolated from mice harboring either allele of the *mdx* mutation (lanes 3–6).

A much smaller skeletal muscle–specific cross-reactive polypeptide recognized by the rabbit antibodies raised against the 30 kd antigen (Figure 3A) appeared at equal levels in all mice, and serves to verify the quantity and quality of protein loaded in the skeletal muscle lanes. On the gradient gel in this experiment this smaller protein species has an apparent molecular weight of 100 kd, which probably represents a more accurate determination than that shown in Figure 3A.

Relationship between the DMD Protein and Nebulin

Nebulin, a large molecular weight, high abundance myofibrillar protein (Wang, 1985), has recently been implicated as being a candidate for the primary product of the DMD gene (Wood et al., 1987). To compare nebulin levels to those of the DMD protein, tissue samples from normal and DMD-affected human individuals and from normal, *mdx*, and *Tr* mice were directly solubilized in gel loading buffer, the proteins fractionated on 3.5% SDS–polyacrylamide gels, and the gels processed as above. Identical nitrocellulose blots were incubated with affinity-purified rabbit antibody directed against the 30 kd antigen (Figure 5A), or with guinea pig anti-rabbit nebulin (Figure 5B), followed by incubation with ^{125}I-protein A. As expected from the previous experiments, the anti-DMD antibodies recognized a 400 kd protein species in normal human skeletal muscle (Figure 5A, lanes 6 and 9) and in normal and *Tr* mouse skeletal and cardiac muscle (lanes 1–2, 10–11). This protein species was not detectable in human DMD muscle biopsies (Figure 5A, lanes 7–8), in either allele of *mdx* mouse (lanes 4–5, 12–13), or (in this experiment) in normal mouse brain (lane 3). The cross-reactive 100 kd skeletal muscle–specific protein species normally detected by the rabbit α-30kd antibodies used (see Figures 3A and 4) was run off the 3.5% gels, and is therefore not seen.

The anti-nebulin antibodies detected the expected abundant, skeletal muscle–specific protein species of approximately 500 kd (Wang, 1985; Hu, Kimura, and Maruyama, 1986; Locker and Wild, 1986), though this represents the first reported immunological evidence for the apparent absence of nebulin in cardiac muscle. Comparison of the autoradiographic exposure times and the signal intensities of the 400 kd DMD protein to those of

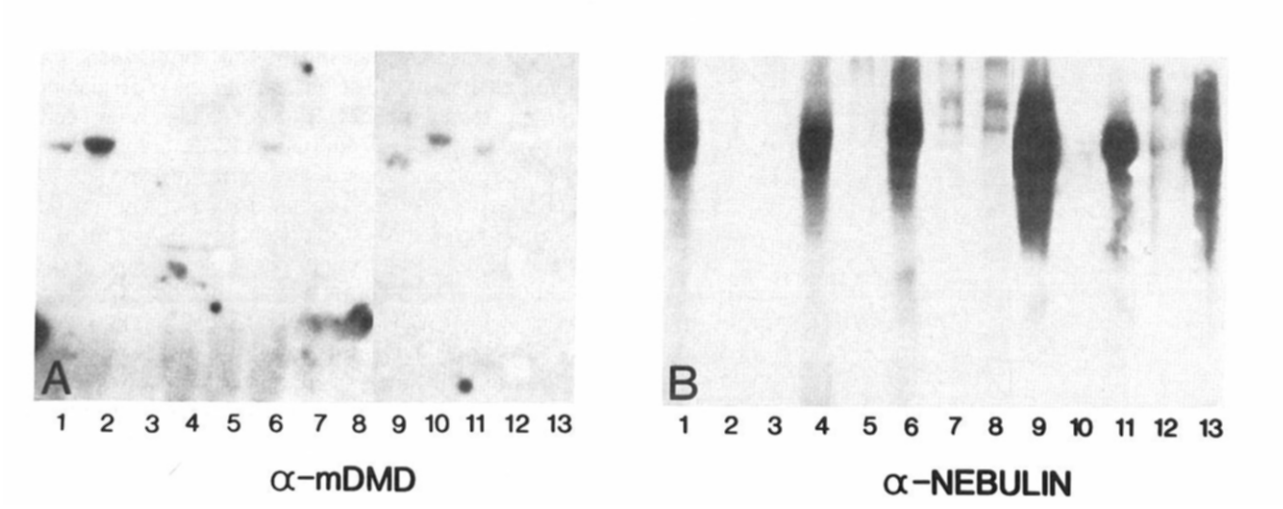

Figure 5. Comparison of DMD Protein Levels and Distribution with Those of Nebulin

Mouse (fresh) and human (frozen) tissues were solubilized in sample loading buffer, with aliquots (50 μg) fractionated on 3.5% SDS–polyacrylamide gels. Proteins were transferred to nitrocellulose and incubated with antibodies directed against the 30 kd DMD antigen (A) or anti-nebulin antibodies (B), followed by detection of bound IgG with ^{125}I-protein A. Lanes are as follows: 1, normal mouse skeletal muscle; 2, normal mouse heart; 3, normal mouse brain; 4, *mdx* skeletal muscle; 5, *mdx* heart; 6, normal human skeletal muscle; 7, DMD skeletal muscle (patient 1); 8, DMD skeletal muscle (patient 2); 9, normal human skeletal muscle; 10, homozygous *Tr* heart; 11, *Tr* skeletal muscle; 12, *mdx*^{467} heart; 13, *mdx*^{467} skeletal muscle.
The primary anti-DMD protein antibody and the detection system used for (A) is the same as for Figure 3A, and the levels and size of the DMD protein appear similar. The apparent size difference of dystrophin between lanes 9 and 10 in (A) is due to the larger amount of protein loaded in lane 9, such that the migration of the DMD protein is distorted by nebulin (B). In contrast to the DMD protein, nebulin appears as a larger (500 kd; Wang, 1985), more abundant, skeletal muscle–specific protein species in all mouse and human skeletal muscle samples tested. Autoradiography in (A) was for 3 days; (B), for 2 hr. Comparison of (A) and (B) shows that the DMD protein is approximately one-thousandth the level of nebulin.

nebulin indicated that the DMD protein was approximately one-thousandth the level of nebulin (Figure 5). Since nebulin has been calculated to represent about 3% of myofibrillar protein (and thus 1% of total muscle protein) (Wang, 1985), the DMD protein can be estimated to represent approximately 0.001% of total muscle protein.

Nebulin is evident, though greatly reduced, in the human DMD muscle samples tested (Figure 5B, lanes 7–8). On the other hand, nebulin appeared at normal levels in both alleles of *mdx* mouse (lanes 4–5, 12–13). This immunological data provides conclusive evidence that nebulin and the DMD protein are indeed distinct proteins, and therefore indicates that nebulin cannot be the protein product of the DMD locus.

Relative Cellular Abundance of the DMD Protein

The protein product of the DMD locus has been previously calculated to be in very low abundance on the basis of mRNA levels in muscle tissue (Hoffman et al., 1987). In order to measure more directly the cellular abundance of the DMD protein, the amount of this protein in heart was quantitated. Known quantities of DMD fusion proteins were denatured and fractionated on 3.5%–12.5% gradient SDS–polyacrylamide alongside 100 μg of solubilized mouse heart. Proteins were transferred to nitrocellulose, incubated with sheep antibodies directed against the 60 kd DMD antigen, and then detected by using alkaline phosphatase conjugated second antibodies. As shown in Figure 6, the antiserum reacts only with the antigen to which it was raised and not with the 30 kd fusion protein, indicating that all immunostaining is due to antibodies specific for the DMD portion of the trpE+60kd fusion protein. The signal exhibited for the 400 kd DMD protein in 100 μg of total cardiac protein corresponds to approximately 2 ng of the partially purified antigen to which the antibody was raised (Figure 6, lanes 3–4). Thus, by this measurement, the DMD protein comprises approximately 0.002% of total muscle protein.

Discussion

Recent reports have substantiated the correlation of recently described human cDNA sequences to the Duchenne muscular dystrophy locus in humans (Monaco et al., 1986; Monaco and Kunkel, 1987; Burghes et al., 1987; Koenig et al., 1987). Indeed, the fact that a large proportion of affected individuals exhibit small deletions within the genomic locus covered by cloned cDNAs indicates that these sequences represent the human DMD gene. The mouse homolog of the human DMD locus has been shown to reside on the mouse X chromosome by both genomic DNA analysis (Monaco et al., 1986) and cDNA analysis (Hoffman et al., 1987; Chamberlain et al., 1987; Heilig et al., 1987; Brockdorff et al., 1987). The human and mouse DMD cDNAs have been shown to be greater than 90% homologous over the entire amino-terminal one-third of the protein (~130 kd), diverging only upstream of their common translation initiation codon (Hoffman et al., 1987; Koenig et al., 1987). The predicted amino acid sequences of this portion of the human and mouse DMD proteins indicated that the protein might serve a highly conserved structural role in the myofiber (Hoffman et al., 1987). By the raising of antibodies to in vitro engineered fusion proteins containing portions of the mDMD protein (30 kd and

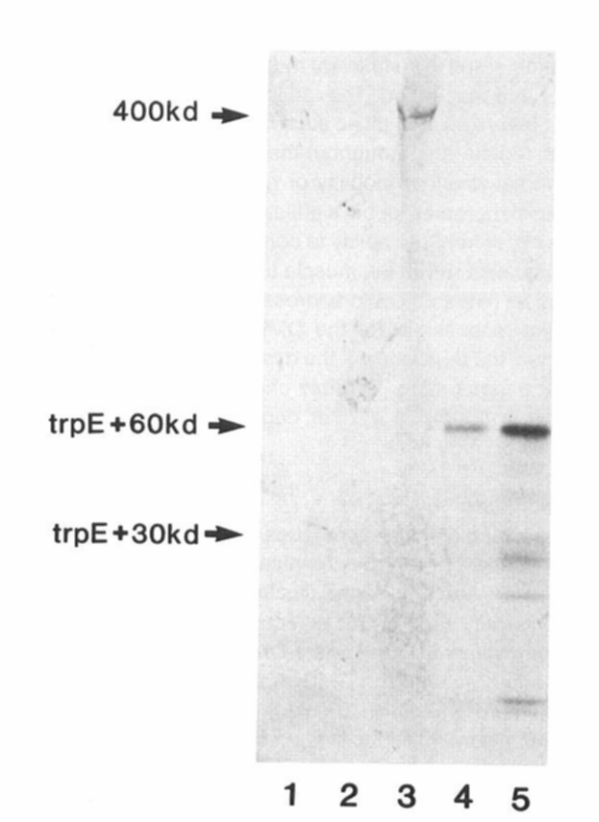

Figure 6. Quantitation of DMD Protein Levels in Normal Heart

Aliquots of bacterial lysates containing DMD–trpE fusion proteins (see Figure 1) were quantitated, solubilized in gel loading buffer, and fractionated on 3.5%–12.5% SDS–polyacrylamide gels alongside 100 μg of solubilized mouse heart. Fractionated proteins were transferred to nitrocellulose and incubated with antibodies (sheep) directed against the 60 kd DMD antigen, followed by immune complex detection with alkaline phosphatase conjugated donkey anti-sheep second antibody. Lanes are as follows: 1, trpE+30kd (2 ng); 2, trpE+30kd (10 ng); 3, normal mouse heart (100 μg); 4, trpE+60kd (2 ng); 5, trpE+60kd (10 ng). The affinity-purified antibody directed against the trpE+60kd antigen is seen to react specifically with this antigen and with the 400 kd DMD protein product. The signal intensity of the 400 kd DMD protein is seen to correspond to approximately 2 ng of partially purified antigen, indicating that the DMD protein represents approximately 0.002% of total heart protein. This calculation has an inherent uncertainty due to the variable transfer efficiencies of proteins of different sizes, though the antigenicity of these proteins should be identical because of their identical amino acid sequence.

60 kd), the protein product of the DMD locus was identified in both mouse and human muscle. The protein identified by these antibodies fulfills the following requirements expected of the primary gene product disrupted in DMD. First, the mRNA product of the mouse and human DMD loci has been estimated to be 16 kb in length (Monaco et al., 1986; Hoffman et al., 1987). This estimated size was revised to 14 kb with the complete cloning of the human cDNA (Koenig et al., 1987). The protein species described in this paper has been estimated to be approximately 400 kd, a size that is in general agreement with the translation of a mRNA of 14 kb. Second, the mRNAs corresponding to both the human and mouse DMD loci have been found to represent 0.01–0.001% of total muscle mRNA, as evidenced by both clone frequency in cDNA libraries and abundance relative to α-tubulin mRNA (Hoffman et al., 1987). The abundance of the identified protein agrees with the mRNA abundance, as evidenced by abundance relative to nebulin (Figure 5) and direct quantitation in mouse heart (Figure 6). Third, the expression pattern of the mDMD gene has been studied at the mRNA level, with the large DMD transcript detectable only in striated and possibly smooth muscle (Monaco et al., 1986; Hoffman et al., 1987; Burghes et al., 1987). The 400 kd protein was clearly detected in skeletal and cardiac muscle, with smaller amounts in stomach (smooth muscle) (Figure 3). Fourth, the primary amino acid sequence of the amino-terminal 30% of the DMD protein has been shown to exhibit features common to many structural proteins, being highly conserved and rich in α-helix (Hoffman et al., 1987; Koenig et al., 1987). In agreement with this hypothesis, the DMD protein was found to be largely Triton-insoluble, suggesting an association with the myofibrillar matrix (Figure 3, lanes 13–15). Fifth, muscle biopsies from two DMD-affected individuals contained no detectable 400 kd protein (Figures 3 and 5), consistent with the molecular analysis of the DMD gene which has shown that most DMD-affected individuals possess null mutations of the DMD locus (Koenig et al., 1987). Sixth, antibodies raised in both rabbit and sheep against fusion peptides encoded by two separate, distinct regions of the DMD cDNA recognize the same protein species, as evidenced by the identical size, abundance, and tissue distribution of the detected protein. Taken together, this evidence validates the specificity of the described antibodies for the DMD gene product, and thereby substantiates the identification of this protein as the primary biochemical defect in Duchenne muscular dystrophy. Since we know of no previously reported protein that shares the abundance, sequence, or size characteristics of the DMD protein, and since this protein was identified by molecular genetic studies of patients affected with Duchenne muscular dystrophy, we have named this protein dystrophin.

It is interesting to note the very small amount of dystrophin present in total mouse brain tissue. A 30% incidence of mental retardation has been observed in boys afflicted with DMD (Zellweger and Hanson, 1967). It is tempting to speculate that the observed mental retardation could be a direct consequence of dystrophin absence, though the variable penetrance of this phenotype would argue against this. It is also interesting that, to date, both the mRNA and the protein have been found only in terminally differentiated cells (Hoffman et al., 1987; Lev et al., 1987; this paper). Given the greater than 2 million base pair size of the genomic DMD locus in humans (Monaco and Kunkel, 1987; Koenig et al., 1987), it would take more than 24 hr for RNA polymerase II to transcribe a single mRNA molecule from the DMD gene (Ucker and Yamamoto, 1984). If it is assumed that DNA replication and mRNA transcription cannot take place simultaneously, then only cells that are mitotically inactive for longer than 24 hr would be capable of transcribing the DMD gene, thus limiting the production of the DMD protein to predominantly mitotically inactive cells.

Our results concerning the absence of dystrophin in tis-

sues isolated from two alleles of *mdx* mice are particularly provocative. The lack of any detectable muscle weakness in *mdx* mice has led to the past hypotheses that the original *mdx* mutation represents either a mild allele of the mouse DMD homolog (Bulfield et al., 1984) or the homolog of the less severe human Emery–Dreyfuss dystrophy (Avner et al., 1987). We have found that two different mutant alleles of the *mdx* gene appear to lack the mDMD gene product, indicating that *mdx* and DMD most likely represent the same genetic disorder. Although the molecular defect in *mdx* has not been detected by DNA analysis (Chamberlain et al., 1987; L. M. Kunkel, unpublished data), the deficiency of dystrophin in *mdx* mice is presumed not to be a secondary consequence of a nonhomologous genetic disorder for the following reasons. First, the homology of the *mdx* and DMD loci is consistent with the linkage data (Bulfield et al., 1984; Chapman et al., 1985; Heilig et al., 1987; Brockdorff et al., 1987; Chamberlain et al., 1987). Second, the deficiency of dystrophin in *mdx* mice appears to be disease-specific, as muscle samples from homozygous Trembler mice, afflicted with a severe neuropathological disorder (Henry, Cowen, and Sidman, 1983), exhibit wild-type levels of this protein. Third, though it could be argued that the absence of the mDMD product represents a generalized degradation of muscle proteins in *mdx* muscle, nebulin, which is regarded as one of the more labile muscle proteins (Wang, 1985; Sugita et al., 1987), is detectable at wild-type levels in *mdx* skeletal muscle (Figure 5B). Indeed, the normal levels of nebulin protein observed in mouse *mdx* contrasts to the severely reduced levels observed in human DMD patient muscle (Wood et al., 1987; Sugita et al., 1987). Such differences might be indicative of a more active role of endogenous proteases in human DMD muscle fibers, and could possibly explain some of the differences in the clinical phenotype.

By equating the mouse *mdx* and human DMD loci, an animal model is now available for DMD. The differences in the clinical manifestation of the same primary biochemical defect in mice and in humans might be explained by differences in secondary biochemical effects or histological changes. Histologically, both DMD and *mdx* muscle exhibit nearly identical patterns of myofiber degeneration and regeneration (Dubowitz, 1985; Bridges, 1986), a process that is probably a direct consequence of dystrophin deficiency in myofibers. The *mdx* muscle, however, never develops the extensive connective tissue proliferation (endomysial fibrosis) that is characteristic of human DMD muscle tissue, especially in the later stages of the disease (Dubowitz, 1985; Tanabe, Esaki, and Nomura, 1986; Torres and Duchen, 1987). This indicates that the prominent fibrosis in human DMD muscle is probably an indirect or secondary consequence of dystrophin deficiency. Perhaps the extensive endomysial fibrosis in human DMD muscles results in the impairment of the ability of individual muscle fibers to regenerate. This would mean that muscle fiber number would progressively decrease as the connective tissue content of each muscle group increases—a process that is, in fact, observed (Cullen and Fulthorpe, 1975; Watkins and Cullen, 1985). Such a process could ultimately result in insufficient muscle fiber numbers for mobility and respiration. The muscle fibers of *mdx* mice, on the other hand, exhibit no such fibrosis and retain the ability to regenerate throughout the life of the mouse, posing no threat to either mobility or normal life span. Possible rational therapies for boys afflicted with DMD might therefore result from the ability to control the connective tissue proliferation within the muscle tissue. Alternatively, future medical research could address the primary biochemical defect responsible for the DMD and *mdx* phenotypes, namely the deficiency of the dystrophin protein leading to fiber degeneration. Possible chemical agents that might result in a slowing of fiber degeneration could then be tested on *mdx* mice.

Conclusion

Molecular biological techniques have led to the identification of the primary biochemical defect in an important hereditary human disease, Duchenne muscular dystrophy. The identification of this defect was based solely on the chromosomal location of the DMD locus. The antibodies produced against the DMD protein product, dystrophin, should prove useful in the diagnosis and characterization of this disorder. As more is understood about the role of dystrophin in normal muscle function, rational therapies for the many boys affected with this fatal disease will, we hope, emerge. Many of these therapies could be tested on the *mdx* mouse model for this disease.

Experimental Procedures

Plasmid Constructions

The predicted amino acid sequence has been determined from the cDNA sequence for the amino-terminal one-third of the Duchenne muscular dystrophy gene product in both mice and humans (Hoffman et al., 1987; Koenig et al., 1987). Two different regions of the mouse sequence were fused to the E. coli *trpE* gene as follows, with the predicted number of amino acids being deduced from the DNA sequence.

trpE+60kd

The mouse DMD cDNA (Hoffman et al., 1987) was restricted at the unique SpeI site, blunt-ended with Klenow, and then digested with HindIII in the 3′ polylinker. The excised cDNA fragment of 1.4 kb was gel-purified and ligated to pATH2 (Dieckmann and Tzagoloff, 1985), which had been digested with SmaI and HindIII. Recombinants were identified by colony hybridization to random primer extended (^{32}P) insert (Feinberg and Vogelstein, 1983), and verified by subsequent plasmid DNA restriction analysis. The resulting plasmid construction fused the trpE protein (33 kd) to 410 amino acids (~60 kd) of the mDMD protein, and corresponds to position 1.3 kb to 2.7 kb on the equivalent human cDNA map (Koenig et al., 1987).

trpE+30kd

The most 3′ end of the mouse cDNA currently available (Hoffman et al., 1987) was restricted at its unique nonmethylated XbaI site and at the BamHI site in the 3′ polylinker. The excised 700 bp fragment was ligated to pATH2 digested with XbaI and BamHI as described above. This plasmid construction fused the trpE protein to 208 amino acids (~30 kd) of the mDMD protein, and corresponds to position 3.7 kb to 4.4 kb on the equivalent human cDNA map (Koenig et al., 1987).

Induction and Purification of Fusion Proteins

Plasmid constructions were maintained in E. coli RR1, which was grown as suggested by A. Tzagoloff (unpublished data) except that 200 μg/ml of tryptophan was used as a supplement to all media. Induction with 3-B-indolacrylic acid (IAA), harvesting, and initial purification of trpE fusion proteins was as described by Dieckmann and Tzagoloff

(1985). Between 15 and 25 mg of insoluble protein was obtained from 100 ml of induced bacterial culture, of which approximately 85% was estimated to be the desired fusion protein (Figure 1). Between 2 and 5 mg of insoluble protein was solubilized by boiling in SDS, and then size-fractionated on preparative SDS–polyacrylamide gels (Laemmli, 1970). The fusion proteins were visualized by rinsing of the gels in distilled water for 5 min followed by immersion in cold 0.25 M KCl, with the appropriate protein band then being excised.

For rabbit immunizations, fusion proteins were then purified by electroelution into dialysis sacs, followed by precipitation with 5 volumes of acetone to remove SDS. Protein pellets, which also contained coprecipitated glycine, were resuspended in sterile 10 mM Tris (pH 8.0), and the protein concentration was determined (Bio-Rad protein assay on extensively sonicated aliquots).

For sheep immunizations, gel slices containing SDS-denatured antigen were sent to Polyclonal Seralabs (Cambridge, MA).

Antibody Production

New Zealand white female rabbits were immunized according to the following schedules: 1, intravenous injection (10 μg) with weekly boosts; 2, intradermal using 10 μg of fusion protein emulsified with Freund's complete adjuvant, with 10 μg boosts every 3 weeks using Freund's incomplete adjuvant; 3, intradermal as above (2) using 50 μg of fusion protein, with 100 μg boosts.

One rabbit was immunized with each fusion peptide according to each of the schedules. The titers and specificity of the antibodies produced in each rabbit were constantly monitored by enzyme-linked immunoassays performed on nitrocellulose dot blots of insoluble protein fractions such as those shown in Figure 2. The best immune responses were obtained by using the trpE+30kd polypeptide with immunization protocols 1 and 3 above, with >95% of the antibodies produced being specifically against the mDMD portion of the fusion peptide, and with titers greater then the sensitivity of the ELISA assay system when using a 1:1000 dilution of crude serum 4 weeks after immunization. The trpE+60kd antigen took much longer (12 weeks) to evoke an immune response in rabbits, with the resulting sera showing a low specificity for the DMD portion of the fusion protein (not shown).

A single sheep was immunized with each antigen in the form of SDS-denatured protein in polyacrylamide gel slices. Approximately 1 mg of fusion protein was used per immunization. The initial immunization was with Freund's complete adjuvant, with boosts using incomplete adjuvant at days 14 and 28. Injections were at multiple sites both intramuscularly and subcutaneously in lymph node areas. Serum was collected at day 50.

Antibody Purification

Approximately 3 mg of partially purified trpE protein (insoluble fraction; see above) was reprecipitated, resuspended in 10 mM Tris (pH 8.0), and then precipitated again. The pellet was resuspended in 1.5 ml of immune serum, incubated on ice for 1 hr, centrifuged to pellet the trpE–antibody immune complexes, and discarded. The supernatant was then mixed with approximately 3 mg of partially purified fusion protein (insoluble fraction) that had been washed as above. After incubation on ice, the mDMD–antibody immune complexes were precipitated by centrifugation. The pellet was then resuspended in 500 μl of 0.2 M glycine (pH 2.3), incubated on ice for 5 min to disassociate the immune complexes, and centrifuged at 4°C to precipitate the insoluble antigen. The supernatant containing the purified immunoglobulins was neutralized with 50 μl Tris (pH 9.5), and either stabilized with BSA (fraction V)(5 mg/ml) or dialyzed extensively against phosphate-buffered saline (PBS).

Western Blotting

Mouse (fresh) or human (frozen) tissues were homogenized in 10 volumes of gel loading buffer (Sugita et al., 1987) by using a motorized Teflon tissue homogenizer. The protein concentration of the solubilized tissues was approximated based on the weight of the tissues used. Mouse skeletal muscle samples used were total hind limb muscle.

Triton X-100 insoluble proteins were prepared by homogenization of fresh or frozen tissues in a Waring blender at high speed for 30 sec in buffer consisting of 10 mM HEPES (pH 7.2), 5 mM EGTA, 1 mg/ml PMSF, 1 mM iodoacetamide, 1 mM benzamidine, 0.5 mg/ml aprotinin, 0.5 mg/ml leupeptin, 0.25 mg/ml pepstatin A, and 0.25% Triton X-100. Triton-insoluble proteins were precipitated and then resuspended in buffer without Triton X-100, and the protein concentration was determined (Bio-Rad protein assay). Aliquots were diluted with gel loading buffer, and 50 μg was used per lane. Protein samples were heated to 95°C for 2 min, centrifuged, and electrophoretically fractionated on 0.75 mm SDS–polyacrylamide gels (Laemmli, 1970), by using a 3% stacking gel and either a 3.5% or 3.5%–12.5% gradient resolving gel.

Fractionated proteins were transferred to nitrocellulose (Towbin, Staehelin, and Gordon, 1979), and the filters were dried. Dried filters were blocked in 5% nonfat dry milk in TBST (10 mM Tris, pH 8.0; 500 mM NaCl; 0.05% Tween-20). All immunological reagent dilutions and filter washes were done in TBST. The affinity-purified anti-mDMD antibodies, affinity-purified second antibodies (Sigma), and guinea pig anti-rabbit nebulin antisera were diluted 1:1000 in TBST prior to use. Affinity-purified ^{125}I-protein A was from Amersham, and was used at 5 uCi per 20 ml of TBST. Biotinylated molecular weight markers were purchased from Bio-Rad, and were visualized by using protocols and reagents supplied by the manufacturer. Myosin was easily visualized on all filters by immunostaining ghosts due to the abundance of this protein.

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