The dystrophin associated protein complex in zebrafish


1Division of Genetics, Children’s Hospital, Boston, MA, USA, 2Department of Genetics, Harvard Medical School, Boston, MA, USA, 3Howard Hughes Medical Institute, Boston, MA, USA and 4Division of Hematology/Oncology, Children’s Hospital Boston, MA, USA

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Many cases of muscular dystrophy in humans are caused by mutations in members of the dystrophin associated protein complex (DAPC). Zebrafish are small vertebrates whose bodies are composed predominantly of skeletal muscle, making them attractive models for studying mammalian muscle disorders. Potential orthologs to most of the human DAPC proteins have been found in zebrafish by database screening. Expression of the sarcoglycans, dystroglycan and dystrophin has been confirmed by western blotting. Immunohistochemical and biochemical techniques localize these proteins to the muscle cell membrane in adult zebrafish. Morpholino (MO) experiments designed to inhibit the translation of dystrophin mRNA produce juvenile zebrafish that are less active than zebrafish injected with control morpholinos. Western blot analysis of the dystrophin morpholino-injected zebrafish shows concurrent reduction of dystrophin and the sarcoglycans, suggesting that these proteins, like those in mammals, are part of a complex whose integrity is dependent on dystrophin expression. These results indicate that the zebrafish is an excellent animal model in which to approach the study of dystrophin and its associated proteins.

INTRODUCTION

Dystrophin is a large protein (427 kDa) (1) that tethers the membrane-bound dystrophin associated protein complex (DAPC) to the intracellular cytoskeleton (2,3). The core DAPC is comprised of eight proteins including one intracellular protein (dystrophin), six transmembrane proteins (α-, β-, γ-, δ-sarcoglycan, sarcospan, and β-dystroglycan), and one extracellular protein (α-dystroglycan) (reviewed in 4). In addition to these core proteins, merosin, filamin C, dystrobrevin, biglycan and a number of other proteins have also been shown to interact with the DAPC. Mutations in many of the DAPC constituents have been shown to cause various forms of muscular dystrophy (reviewed in 4). For example, Duchenne’s and Becker’s Muscular Dystrophy are caused by mutations in the dystrophin gene (5) whereas Limb Girdle Muscular Dystrophy (LGMD) Types 2C–F are caused by mutations in the sarcoglycan genes (6–10).

Much of the study of dystrophin and its associated proteins has been accomplished using animal models of muscular dystrophy. Naturally occurring dystrophin deficiencies have been identified in mouse (mdx) (11,12), cat (13,14) and dog (15,16) and these animals have been used to model Duchenne’s Muscular Dystrophy. Additional mouse models for most of the LGMDs have also been created by gene targeting (reviewed in 17). These animal models have been an invaluable resource for dissecting the pathogenesis of disease and have also served as good models to test potential therapeutic interventions. In addition to these vertebrates, orthologs of the DAPC proteins have also been identified in flies (18) and worms (19–21). Although these organisms have musculature which is somewhat different than that of mammals, these invertebrates can be readily manipulated and grown in large quantities allowing one to genetically analyze mutations either causing or correcting muscle disease.

The zebrafish is rapidly emerging as an excellent model for many human disease traits (22). The fish’s body is composed predominantly of skeletal muscle, making this vertebrate attractive for studying muscle protein function. Its relatively small size makes the zebrafish very useful for genetic studies requiring large numbers of animals. To aid in these studies, the zebrafish genome can also be manipulated from diploid to haploid (reviewed in 23), thereby making these organisms

*To whom correspondence should be addressed at: Division of Genetics, Children’s Hospital Boston, Enders Rm 570, 300 Longwood Ave, Boston, MA 02115, USA. Tel:+1 6173558200; Email: kunkel@enders.tch.harvard.edu

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attractive for screening recessive traits like many of the LGMDs. Currently, there are no methods in zebrafish to directly target specific genes, but morpholinos offer a quick way to assess the phenotype of a zebrafish lacking a specific protein (24). Morpholinos are oligonucleotide analogs that bind specific mRNAs to disrupt translation of those transcripts (25,26).

Others have also recognized the attractiveness of zebrafish in the study of muscle disease. Recent reports have localized zebrafish dystrophin to the muscle cell membrane in adult fish (27) and a partial dystrophin clone has been mapped to Linkage Group (LG) 1 (28). In situ hybridization experiments have shown that dystrophin is expressed early in development predominantly at the borders of the muscle somites (29). In addition, a β-dystroglycan ortholog has also been isolated and subsequently localized by immunohistochemistry to the myosepta in the developing embryo (30). At 48 h post fertilization, zebrafish embryos injected with morpholinos directed against β-dystroglycan were phenotypically bent (30). Electron microscopy revealed that these fish had less defined muscle fibers with a reduced number of sarcomeres (30), all potential symptoms of muscular dystrophy.

Because of their transparency early in development, the zebrafish embryo has also been used to study both heart and somite development. Genetic screens have been used to identify pickwick-1 (m171), a recessive lethal mutation which causes poor heart contraction in developing zebrafish embryos (31). The causative gene was then mapped to the titin locus (31). Titin is an extremely large protein which has been shown to span from the M- to the Z-line in the muscle sarcomere. Mutations in titin are linked to a form of muscular dystrophy, cardiomyopathy in human dystrophin (31,32). In addition to titin, troponin T has also been isolated in zebrafish and shown to be critical for sarcomere function in cardiac muscle cells (33). In zebrafish, mutations in troponin T are recessive lethal due to lack of development of a functional heart.

To access the applicability of using zebrafish to model muscular dystrophy, we began by investigating whether zebrafish express orthologs of the dystrophin-associated proteins. Database searches of the zebrafish genomic sequence identified specific regions which could encode peptides homologous to many of these proteins. Expression of the sarcoglycans, dystrophin and dystroglycan was confirmed in zebrafish by immunoblot analysis. As in mammals (34), the zebrafish DAPC localizes to the muscle cell membrane. Using a morpholino knock-down approach, we describe a bent and less active phenotype for zebrafish lacking dystrophin early in development. Although not completely understood, a similar phenotype has been previously described for β-dystroglycan knock-down embryos (30). In addition to bending, zebrafish injected with anti-dystrophin morpholinos also show reduced expression of other DAPC proteins suggesting that these proteins are part of a large complex. These morpholino experiments suggest that bending and reduced activity are good indicators of dystrophin deficiency in the fish. This phenotype can be used to screen mutant fish lines in future genetic screens with the hope that fish genetically expressing this phenotype will possess mutations in dystrophin and/or its associated proteins.

RESULTS

Zebrafish muscle appears morphologically similar to mammalian muscle

Zebrafish muscle structure was initially evaluated by staining 10 μm cross-sections of adult zebrafish with hematoxylin (stains nuclei blue) and eosin (stains cellular proteins pink; Fig. 1). As reported previously (27), zebrafish muscle fibers were found to be relatively large with peripherally located nuclei similar to that of mammalian muscle. Based on their location, fast muscle fibers were identified by their location between the spine and skin whereas the slow fibers were located subcutaneously (35). The many blue nuclei present amongst the slow fibers are likely to be circulating blood cells.

Zebrafish have genes capable of expressing DAPC orthologs

Previous studies have shown by both immunoblot analysis and immunohistochemical staining that zebrafish express the dystrophin protein in skeletal muscle (27,28). While the C-terminus of the zebrafish dystrophin orthologue has been cloned (28), the N-terminal actin binding section and most of the central rod domain have yet to be identified. To expand these earlier studies, sequences of dystrophin and the other DAPC proteins were used to electronically screen the unfinished Sanger Center Zebrafish genome database to search for potential orthologs. Strong homology (greater than about 50% identity) was found to approximately one-third of the exons encoding dystrophin, the sarcoglycans and most of the other DAPC-associated proteins (data not shown). Overwhelmingly, the homologous zebrafish sequences had the same intron–exon boundaries as their human counterparts, suggesting that the homologous exons were specific (data not shown).

Cloning the 5′ end of the zebrafish dystrophin gene

As previously mentioned, the 5′ end of the zebrafish dystrophin gene had not yet been identified, but was required for the morpholino experiments described below. Homology searches for zebrafish orthologs found a genomic trace file capable of expressing a peptide with high homology (85% identity at the protein level) to human dystrophin exon 2. This DNA sequence was used to design primers to PCR amplify the 5′ end of the dystrophin gene from a zebrafish pGAD10 cDNA library (Clontech). Briefly, nested PCR was performed using primers specific to the vector and others directed against the putative exon 2 of zebrafish dystrophin. Following PCR, the fragment was sequenced and found to contain vector sequence, a novel insert, and the remaining portion of dystrophin exon 2 (Fig. 2A). The novel insert was located at the predicted exon1–exon 2 splice junction (Fig. 2A) suggesting that exon 1 of zebrafish dystrophin had been isolated. While this exon contained minimal direct homology to dystrophin exon 1 from other organisms (Fig. 2B, see AA in bold text), some offset homology was identified to exon 1 of the human muscle isoform (Fig. 2B, see underlined amino acids).

Interestingly, when zebrafish dystrophin exon 1 was compared with the first exon of human utrophin (Fig. 2B),
there was significant homology (36% amino acid identity versus 18% for dystrophin). In mammals, utrophin is a dystrophin homolog (44% identity) expressed at the sarcolemma early in development, but later localizes to both the myotendinous junctions and the neuromuscular synapses (36,37). When overexpressed, utrophin can partially rescue dystrophin mutations in mice (38). Interestingly, when the zebrafish dystrophin exon 2 amino acid sequence was compared, it was more like human dystrophin (85% identity) than utrophin (75% identity), suggesting some uncertainty as to the identity of the cloned fragment. To ensure that exon 1 of the zebrafish dystrophin was cloned, radiation hybrid mapping was used and both dystrophin exons 1 and 2 co-mapped to the ‘z5058’ marker on linkage group (LG) 1 (Fig. 2C). In agreement with the previously published dystrophin location (28), the DNA encoding exon 64 of zebrafish dystrophin was also mapped, and it positioned near the ‘unp1862’ marker also on LG 1 (Fig. 2C). It is estimated that the ‘z5058’ and ‘unp1862’ markers are separated by 1 Mb, a distance consistent with that of dystrophin exons 1 and 64 in mammals (39).

**Zebrafish express DAPC proteins**

To evaluate whether zebrafish express DAPC proteins, immunoblot analysis was performed on zebrafish extracts using antibodies directed against mammalian DAPC proteins (Fig. 3A and B). Protein extracts from the trunks of 4-day-old (Fig. 3A) and adult (Fig. 3B) zebrafish were prepared. In agreement with previously published results (27,28), immunoblot analysis of zebrafish muscle using polyclonal antibodies raised against human dystrophin reveals a large protein of approximately 400 kDa (Fig. 3A, lane 2 and 4). Similarly, antibodies raised against human β-sarcoglycan, δ-sarcoglycan, β-dystroglycan, and actin all recognized zebrafish proteins of a molecular weight similar to their mammalian counterpart (Fig. 3B, lanes 1–2, 3–4, 5–6, 7–8). Interestingly, the β-sarcoglycan antibody detected two prominent proteins in adult zebrafish and mouse (Fig. 3B, lanes 1–2). Since RH experiments map β-sarcoglycan to two different locations (data not shown), it is possible that zebrafish contain two orthologs for this gene with differing molecular weights. Because only the smaller band (~36 kDa) was detected in later immunoblots from zebrafish embryo protein extracts, this isoform was labeled as β-sarcoglycan in Figure 3B, even though the larger band may also be a β-sarcoglycan isoform.

Antibodies directed against other DAPC and muscle proteins were also tested, but failed to detect zebrafish orthologs, even though those genes appear to exist in the zebrafish genome (data not shown). These antibodies include: z-sarcoglycan (Novocastra), sarcospan (40), FLNC (41) and integrin β7 (Santa Cruz). The z-sarcoglycan and integrin β7 antibodies are monoclonal and their exact epitopes might not be well conserved in zebrafish.

**Zebrafish DAPC proteins co-localize to the muscle cell membrane**

The DAPC proteins localize to the muscle cell membrane in mammals. If this complex exists and functions in a similar way in zebrafish, fish DAPC proteins would also be expected to be found at the skeletal muscle membrane. Zebrafish DAPC proteins were localized by immunohistochemistry using cross-sections of adult zebrafish stained with dystrophin, β-sarcoglycan, δ-sarcoglycan or actin primary antibodies (Fig. 4). FITC (fluorescein isothiocyanate)-conjugated secondary antibodies were used to label the primary antibodies. The FITC tag fluoresces at 520 nm and is visible as a green glow after excitation. Using this tag, membrane-bound proteins would show a green border along the sarcolemma. Immunohistochemical analysis shows that the dystrophin protein colocalizes with β- and δ-sarcoglycan at the muscle cell membrane (Fig. 4), whereas actin predominantly stains the myofilaments which are viewed as small dots on muscle cross-sections. The fluorescent line going through the β-sarcoglycan panel is likely to be the transverse myosepta, a location shown previously to express dystrophin and β-dystroglycan (29). Control zebrafish sections labeled with the secondary antibody alone showed no significant staining (Fig. 4), suggesting that the fluorescence seen in the other panels was specific.

Co-localization of the DAPC proteins at the sarcolemmal membrane suggests that the complex may be similar to that found in mammals. In mammals, these proteins co-purify in membrane preparations of skeletal muscle (42). Analogous membrane preparations were made from adult zebrafish by cell fractionation to prepare either cytoplasmic (supernatant) or membrane (pellet) enriched fractions. Immunoblot analysis using approximately 9 μg of protein (total cell lysate, supernatant or pellet), showed that the DAPC components dystrophin, the sarcoglycans (β, δ, and γ), and β-dystroglycan all co-purify predominantly in the membrane fraction (Fig. 5, lanes 4, 8, 12 and data not shown), whereas the cytosolic proteins calpain-3 (a muscle-specific protease) and actin co-purify predominantly in the cytoplasmic fractions (Fig. 5, lane 14 and data not shown). Because of the large size of dystrophin, this protein was subject to partial proteolytic cleavage during fractionation resulting in a slight decrease in size (Fig. 5, compare lanes 2 and 4). In addition, the dystrophin antibody also detects a prominent band in the concentrated supernatant (Fig. 5A, lane 3). This band is also found in other dystrophin immunoblots (see Fig. 8A). Based on its size, this protein is likely to be the myosin heavy chain, a heavily expressed protein with some homology to dystrophin.
Figure 2. Isolation of the 5' end of zebrafish dystrophin cDNA. (A) Nested PCR was performed using primers specific for both zebrafish dystrophin exon 2 and also the pGAD10 cloning vector containing the zebrafish cDNA library. The cloned PCR fragment was sequenced and the encoded peptide predicted using MacVector software. (B) Sequence comparison of dystrophin exons 1 and 2. The Genbank accession number is shown for each of the three tissue specific dystrophin exon 1 isoforms. Direct homology is indicated in bold text whereas offset homology is underlined. (C) Using radiation hybrid panels, zebrafish dystrophin exons 1 and 2 co-mapped with exon 64 to linkage group (LG) 1.
Down-regulation of dystrophin in zebrafish by morpholino injection

Morpholinos were designed to target the dystrophin initiation translation start-site based on the 5′ end of the dystrophin cDNA sequence (Fig. 2A). Two anti-sense (the knock-down morpholinos), a sense (control), an inverted (control), and the standard Gene-Tools control morpholino (Fig. 6A) were injected separately into zebrafish embryos at the 1–4 cell stage and the developmental progress of these fish compared with uninjected fish. Injection of high concentrations (~50 ng per embryo) of the sense and anti-sense morpholinos were found to cause non-specific brain necrosis as evidenced by a delay in development, brain defects and the slowing (or stoppage) of blood flow through the circulatory system. To rule out non-specific effects of necrosis caused by morpholino over-injection, morpholino concentrations (5–10 ng) were chosen at one-fifth the level which would cause visible necrosis. Injected embryos were analyzed until 5 days post-fertilization (dpf), the predicted limit of morpholino stability and also a time-period similar to that used in previous studies (43). At day 1, both the control and anti-dystrophin morpholino injected fish developed normally such that the muscle somites were visible, the heart was beating and blood was flowing normally through the embryo. At this stage, there were no visible differences between the dystrophin and control morpholino-injected fish. Once the fish hatched out of the chorion, many of the anti-sense dystrophin morpholino fish were curved or bent (Fig. 6B). Interestingly, curving appeared to be a permanent condition whereas bending was more transient and seemed to result from the fish adopting the most energy-efficient conformation laying on the bottom of the Petri dish. In addition to bending and curving, the dystrophin morphants also appeared less motile than control fish, although activity was a difficult phenotype to quantitate. Generally, at 4 days post-fertilization, each anti-dystrophin-injected fish moved once per minute, whereas each of the control morpholino-injected fish moved every 3 s, or 20 times as often. Although the dystrophin anti-sense 2 (‘Dys2’) morpholino was used for most of these experiments, the dystrophin anti-sense 1 morpholino also inhibited activity and caused bending, but to a lesser degree. While the standard and inverted control injected embryos showed no significant differences relative to uninjected embryos, injection of the sense control morpholino slightly inhibited dystrophin expression and was only used in early experiments. The phenotypic results of approximately seven different injection experiments have been grouped and tabulated in Table 1. While it is currently unknown exactly why some fish exhibit a combination of different phenotypes, it is possible that differences in phenotype can be attributed to small changes in injection amounts, the location and time of the injection, or even due to small genetic differences found in the AB strain of these experiments. As bending was observed more often in later experiments (last two experiments had 25/31 curved or bent), it is possible that the injection efficiencies improved with our expertise in performing the injections.

Dystrophin morpholino injected zebrafish were then analyzed by both immunohistochemistry and western blot analysis to determine if there was truly a reduction of dystrophin protein in the injected animals. Immunohistochemical analysis using the
anti-dystrophin MANDRA-1 antibody (Fig. 7), an antibody which has been used previously to specifically bind dystrophin in zebrafish (30), showed strong expression of dystrophin predominantly at the myosepta (Fig. 7, see green FITC staining at the white arrow) in both the uninjected and standard control morpholino 5 day-old embryos. In the anti-sense injected embryos, there was a marked reduction in the levels of dystrophin at this same location (Fig. 7, compare green staining at white arrows in the anti-sense versus standard control injected fish). Identical results were also obtained with the anti-dystrophin ‘6–10’ antibody (data not shown), a reagent used previously in immunoblot experiments (Figs 3 and 5). In some immunohistochemical experiments, δ-sarcoglycan (Fig. 7) and β-sarcoglycan (data not shown) expression levels also appeared reduced in the dystrophin anti-sense morpholino injected fish although the non-quantifiable nature of the assay prohibits stating this conclusively. Interestingly, the actin staining pattern appears more punctate in the bent dystrophin morpholino-injected fish (Fig. 7, see actin labeled panels), suggesting that the organization of the sarcomeres may be altered, a characteristic of dystrophic muscle.

To confirm the immunohistochemistry results and to investigate the levels of protein expression, western blot analysis was performed. Protein extracts were prepared from uninjected and morpholino injected zebrafish, separated by SDS-PAGE and subsequently transferred to nitrocellulose. Immunoblot analysis was performed using two different anti-dystrophin antibodies. The anti-dystrophin polyclonal ‘6-10’ antibody has been described previously whereas the ‘DysC’ antibody is a peptide antibody designed specifically against the zebrafish C-terminus (Genbank Accession Number AAK38376). Both antibodies show marked reduction of dystrophin in the anti-dystrophin morpholino injected zebrafish (Fig. 8A, see arrow and compare lanes 4–5 with 1–3 and compare lanes 9–10 with 6–8). Although western blots are not quantitative, an estimation of the degree of dystrophin reduction was made by comparing the amount of dystrophin protein with a background normalization band (designated as either ‘n1’ or ‘n2’ in Fig. 8B). From this analysis, it was determined that dystrophin expression was down-regulated approximately 70% in the anti-sense morpholino injected embryos. This reduction was found regardless of whether the

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**Table 1. Phenotypic summary of dystrophin morpholino injection experiments.** This table contains phenotypic data gathered from seven separate experiments performed at different times. The normal and bent phenotypes are shown in Figure 6, whereas ‘On side’ refers to inactive fish laying on their sides. All data was observed from 4 to 6 days after injection. ‘DysAS2’ is the dystrophin anti-sense 2 morpholino, ‘DysAS2-Inv’ is the inverted dystrophin anti-sense 2 morpholino, and ‘Std Control’ is the standard control morpholino (see Methods).

<table>
<thead>
<tr>
<th>Morpholino</th>
<th>Normal</th>
<th>Curved</th>
<th>Bent</th>
<th>On side</th>
<th>Notes</th>
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<tbody>
<tr>
<td>Uninjected</td>
<td>244 (99%)</td>
<td>1 (&lt;1%)</td>
<td>1 (&lt;1%)</td>
<td>Few</td>
<td>1 (&lt;1%) with large heart</td>
</tr>
<tr>
<td>Dys-AS2</td>
<td>28 (16%)</td>
<td>75 (42%)</td>
<td>13 (7%)</td>
<td>32 (18%)</td>
<td>~30 (17%) others inactive</td>
</tr>
<tr>
<td>DysAS2-Inv</td>
<td>123 (98%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>2 (2%)</td>
<td></td>
</tr>
<tr>
<td>Std control</td>
<td>57 (88%)</td>
<td>0 (0%)</td>
<td>3 (5%)</td>
<td>3 (5%)</td>
<td>2 (3%) with large hearts</td>
</tr>
</tbody>
</table>
fish exhibited the bent phenotype or were assayed as straight (Fig. 8A, compare lane 5 with lane 4), thereby suggesting that fish lacking dystrophin could potentially show a spectrum of phenotypes.

As a control for protein loading, the same protein extracts used to assay dystrophin expression levels were also separated on SDS-PAGE gels and stained with commassie R-250 (Fig. 8B). As relatively equal staining intensities were found in each of the samples (Fig. 8B), this suggested that equal concentrations of protein were being analyzed in the immunoblots shown in both Figures 8A and 9.

In mammals, the stability of other DAPC proteins is dependant on dystrophin expression and its measure at the cell membrane. For example, patients with Duchenne's Muscular Dystrophy also show decreased levels of the sarcoglycans and other DAPC proteins when dystrophin is mutated (44). A similar observation was made in the anti-sense dystrophin morpholino-injected fish such that there was a reduction of β-, γ- and δ-sarcoglycan (Fig. 9A, compare lanes 4–5 with lanes 1–3 (~55% reduction), compare lanes 9–10 with lanes 6–8 (~60% reduction), and compare lanes 14–15 with lanes 11–13 (~25% reduction)) but near normal or slightly elevated expression of actin and calpain-3 (Fig. 9B, compare lanes 9–10 with lanes 6–8 (~15% increase), and compare lanes 14–15 with lanes 11–13 (~25% increase)), two proteins whose levels are not reduced in the absence of dystrophin in mammals. Interestingly, the levels of β-dystroglycan were quite variable though. In this experiment, its levels seemed unchanged [Fig. 9B, compare lanes 4–5 with lanes 1–3 (~7% decrease)], whereas in another experiment (data not shown), there was clear reduction of β-dystroglycan in the dystrophin morpholino-injected fish. As in mammals, these results show that the stability of the zebrafish DAPC is also dependant on dystrophin expression.

**DISCUSSION**

In vertebrate skeletal muscle, the DAPC is thought to link the extracellular matrix to the intracellular cytoskeleton (2). Orthologs of proteins in the human dystrophin-associated protein complex (DAPC) have been identified in many organisms including worms (45), flies (18), dogs (15), chickens (46), and mice (1). Orthologs of the DAPC are clearly present in the zebrafish (27,30). Zebrafish are rapidly emerging as an excellent animal model of vertebrate development and genetics. Other groups have also recognized the applicability of using zebrafish to study muscle as there are recent reports analyzing dystrophin and β-dystroglycan in these animals (27–30). By homology searching the genomic zebrafish sequence, it is apparent that zebrafish also contain genes that encode many of the sarcoglycans (data not shown). Western blot analysis confirms expression of the DAPC orthologs in zebrafish (Fig. 3) and immunohistochemical analysis of zebrafish muscle cross-sections localizes the DAPC to the sarcolemmal membrane (Fig. 4). Biochemical fractionation shows that DAPC proteins predominantly purify in the membrane enriched fraction (Fig. 5). These data suggest that the DAPC exists in zebrafish as a distinct membrane associated complex analogous to that found in mammalian skeletal muscle.

In mammals, the following dystrophin isoforms exist: muscle Dp427 (47), brain Dp427 (48), purkinje Dp427 (49), retinal Dp260 (50), brain and kidney Dp140 (51), Schwann cell Dp116 (52) and the more ubiquitous Dp71 (53). Most of the smaller dystrophin isoforms are N-terminal truncations of Dp427 transcribed from internal promoters (reviewed in 54). While the significance of each of the different isoforms is unknown, mutations in muscle Dp427 have been linked to Duchenne's Muscular Dystrophy. In mammals, Dp427 is transcribed from three different tissue specific promoters.
These transcripts differ by only their first exon, which is small ($\leq 12$ AA) and not well conserved (Fig. 2B). Using PCR and primers highly homologous for zebrafish dystrophin exon 2, only one isoform of dystrophin was cloned from a zebrafish cDNA library (Fig. 2A). Since muscle is the most common tissue expressing dystrophin, it is likely that this clone represents the 5’ end of the muscle specific isoform and, while slightly offset, some homology was observed between exon 1 of fish dystrophin and exon 1 of the human muscle dystrophin isoform.

Morpholino experiments directed against dystrophin mRNA show both specific reduction of the dystrophin protein (Figs 7-9).
and 8) and repression of other DAPC proteins (Fig. 9). In mammals, the integrity of the DAPC is dependant on dystrophin expression and its localization near the sarcolemmal membrane (44). Reduction of many of the same DAPC proteins in fish lacking dystrophin suggests that, as in mammals, the DAPC exists as a distinct complex. Zebrafish embryos injected with dystrophin morpholinos also produced fish which were approximately 20 times less active than uninjected fish or fish injected with the standard or inverted control morpholinos (see Results). In addition, many (see Table 1) of these dystrophin morpholino-injected fish were curved or bent (Fig. 6B). While the exact nature of the cause of this phenotype is unknown, the dystrophin morphant was bent in a manner similar to that seen in zebrafish with muscular dystrophy induced by injecting β-dystroglycan morpholinos (30). Since dystrophin and β-dystroglycan are both expressed at the myosepta early in zebrafish development (29), it is possible that the loss of these proteins could destabilize muscle fiber interactions at this location during critical stages of development. It is also possible that reduction of dystrophin expression could effect notochord development, a structure that when altered in zebrafish can cause bending (58). While full-length dystrophin was not detected in the notochord, the presence of Dp71 (29) and β-dystroglycan (30) suggests that members of the DAPC may contribute to notochord development. Finally, it is possible that some of the morphant phenotypes are the result of non-specific toxicity effects, which is a unique characteristic for each morpholino. For example, curving (a permanent condition) could potentially mask bending (a more transient condition).

The dystrophin morpholino experiments yielded zebrafish with a more severe phenotype than that seen in either the DMD mouse or hamster models. Although zebrafish are presumed to have duplicated approximately 20% of their genes (59), mammals evolved after fish and may require additional factors for function. It is also possible that zebrafish express only one dystrophin/utrophin ortholog. Interestingly, translational database searches of zebrafish ESTs using the C-terminal sequences of both human dystrophin (AA 2581–3685) and utrophin (AA 2581–3433) show expression of predominantly only one ortholog, suggesting this may be the case. As such, the phenotype of a dys- fish would therefore be similar to that of mouse lacking both utrophin and dystrophin. The utr-/dys- mice have been generated and show a more severe muscular dystrophy having progressive muscle weakness with kyphosis (a progressive curvature of the spine) (60), some of the characteristics seen in the dystrophin morpholino-injected fish.

The dystrophin morpholino experiments also showed that dystrophin expression was reduced by approximately 75% in zebrafish embryos injected with dystrophin anti-sense morpholinos (Fig. 8A). As morpholinos inhibit protein translation by binding a specific transcript, it is possible that morpholino dilution at 4–6 dpf could allow for 25% expression of the
dystrophin gene. As full-length mammalian dystrophin is transcribed from three different promoters, it is more likely that the remaining dystrophin represents either brain and/or purkinje Dp427 since the morpholinos used in these anti-dystrophin experiments were designed specifically against exon 1 of the muscle Dp427 isoform (Fig. 6A). In support of this hypothesis, immunohistochemical analysis of muscle isolated from anti-dystrophin morpholino-injected embryos shows almost complete loss of the dystrophin protein at the myosepta (Fig. 7).

As a small vertebrate, zebrafish are an ideal organism for modeling skeletal muscle disorders. As in mammals, these fish express DAPC orthologs which assemble as part of a large complex at the muscle cell membrane. When dystrophin expression is disrupted during zebrafish development by morpholino injections at the 1–4 cell stage, there is clear perturbation of embryonic development (Fig. 6) and concomitant reduction of dystrophin and other DAPC proteins (Figs 7–9). As zebrafish lack sex chromosomes, the autosomal location of dystrophin suggests that the DMD phenotype would be transmitted as a recessive disorder. Fortunately, zebrafish can be genetically manipulated from diploid to haploid making future screens for recessive traits feasible. By scoring zebrafish for bending, reduced activity or down-regulation of the DAPC proteins, it is likely that mutations can be found in genes which cause muscular dystrophy in fish. Currently, an estimated one-third of the muscular dystrophy causative genes have not been identified, suggesting that genetic screens in the fish could isolate novel candidate genes.

**MATERIALS AND METHODS**

**Western blot analysis**

Western blot analysis was performed as previously described (61). For separation of large proteins (>100 kDa), 3–8% Tris–acetate gradient gels were used and electrophoresis accomplished at 130 V for approximately 2 h. Following separation, the proteins were transferred to 0.45 µm nitrocellulose in a submerged transfer apparatus according to the instructions provided by the manufacturer (Invitrogen). For separation of smaller proteins (<100 kDa), 4–12% Tris–glycine gradient gels (Invitrogen) were used and electrophoresis accomplished at 130 V for approximately 2 h. Following separation, the proteins were transferred to 0.45 µm nitrocellulose using a semi-dry transfer apparatus according to the instructions provided by the manufacturer (Bio-Rad).

All antibodies used have either been described previously or are commercially available with the exception of ‘DysC’. ‘DysC’ is a rabbit antibody generated for us by Bethyl Laboratories against the following peptide: NH2–DDELSPTQDASTGLEDVIE–COOH. This sequence is derived from the published zebrafish C-terminus (Genbank Accession Number AAK38376, amino acids 1060–1079) (28) and was selected as being antigenic using the MacVector analysis software.

Membranes were incubated with the following antibodies in a cocktail of 1 × PBS, 0.1% Tween-20, and 5% non-fat dry milk (NFDM): ‘DysC’ (1:333 dilution), Dystrophin ‘6–10’
(1:300 dilution) (62), Dystrophin ‘2–6’ (1:300 dilution) (63), β-sarcoglycan (Novocastra; 1:20 dilution), δ-sarcoglycan (gift of V. Nigro; 1:300 dilution) (64), γ-sarcoglycan (Novocastra; 1:20 dilution), β-dystroglycan (Novocastra; 1:25 dilution), calpain-3 ‘12A2’ (Novocastra; 1:25 dilution), calpain-3 ‘11B3’ (Novocastra; 1:13 dilution), and actin ‘A2066’ (Sigma; 1:300 dilution). Membranes were incubated with primary antibodies for 1 h at 25°C then washed twice quickly and three times for 10 min in a solution of 1 × PBS with 0.1% Tween-20.

An anti-rabbit conjugated goat HRP secondary antibody (1:5000 dilution) (Jackson ImmunoResearch Lab) was used to detect the following primary antibodies: ‘DysC’, ‘6–10’, ‘2–6’, ‘δ-sarc’, and actin ‘A2066’. An anti-mouse conjugated goat HRP secondary antibody (1:5000 dilution) (Jackson ImmunoResearch Laboratory) was used to detect the following primary antibodies: ‘β-SG’, ‘γ-SG’, ‘β-DG’, calpain-3 ‘12A2’, and calpain-3 ‘11B3’. The membranes were incubated with 1:5000 dilution of secondary antibody in a solution of 1 × PBS with 0.1% Tween-20 and 5% NFDM for 1 h at 25°C before
washing twice quickly and three times for 10 min in a solution of 1 x PBS with 0.1% Tween-20. The blots were incubated with Western Lighting Chemiluminescence Reagent Plus (Perkin Elmer) as described by the distributor and exposed to film for up to 40 min.

For quantitation purposes, film was scanned into Photoshop (Adobe) and tiff files were imported into QuantityOne (Bio-Rad). The image was inverted and rectangles were drawn around the indicated bands. A background rectangle of the same size was drawn and used to subtract the background signal. Signal comparisons were made as described in the text.

PCR cloning the 5′ end of dystrophin

A zebrafish cDNA library prepared from 1-month-old adult fish was purchased from Clontech and the plasmid library was amplified on sixty 24 × 24 cm plates. The pooled pGAD10 plasmid library was purified from the bacteria using Qiagen kits as described by the manufacturer (Qiagen). Approximately 10 ng of the pGAD10 plasmid cDNA library was used as the PCR template in the first of two nested PCR reactions. Nested primers were designed to the pGAD10 vector surrounding the PCR template in the end of dystrophin. The primers were: pGAD10F (5′-CTA TTC GAT GAT GAA GAT ACC CCA-3′), pGAD10F2 (5′-AAA AGA GAT CTC TCG AGG ATC C-3′), FishDysEx2A (5′-ACT GTG ATG TTA TCC ATT TGG TG-3′) and FishDysEx2B (5′-TTG GTG AAG GTT TTC TTT TGA ACG-3′). PCR reactions using the pGAD10F and FishDysEx2A primers were at 35 cycles of 1 min at 94°C, 2 min at 52°C, and 1 min at 72°C using a GC 2 PCR kit (Clontech) and 10 ng of pGAD10 library template. Nested PCR reactions were performed by purifying the first PCR reaction with a Qiagen PCR Cleanup Kit to remove the first set of primers and then using an aliquot (diluted 1:100) of this reaction as the template for the second PCR reaction. The second nested PCR reaction with the pGAD10F2 and FishDysEx2B primers was performed under the same PCR conditions as described for the first reaction. The finished PCR reaction was purified with a Qiagen PCR Cleanup Kit and prepared for sequencing.

Sequencing

All sequencing was performed by the Mental Retardation Research Center Sequencing Core Facility in Children’s Hospital Boston. Sequencing reactions were prepared with approximately 50 ng of PCR product with 10 pm of primer in a 12 μl reaction volume. DNA was fluorescently labeled and analyzed on ABI 373 and 377 fluorescent automated sequencers.

Gene mapping

Potential exons of zebrafish orthologues to human DAPC components were identified by electronic database searching the unfinished Sanger Center zebrafish genomic DNA database using the BLAST algorithm and the published human DAPC protein sequences. To evaluate whether homologous sequences were part of a specific gene, sequences of DNA which could be translated into peptides with high homology to one or more human DAPC exons were radiation hybrid mapped on the zebrafish T51 radiation hybrid (RH) panel (65) using techniques described previously (66). For example, genomic trace files for zebrafish dystrophin exons 1, 2, 59 and 64 were identified by homology against the Sanger Center’s unfinished zebrafish genomic DNA sequence. Primers were designed against these sequences and used in PCR reactions with the T51 RH panel as the template. PCR reactions were analyzed for product by agarose gel electrophoresis with PCR product indicating the retention of zebrafish DNA in the hamster cells. SAMapper (Stanford Human Genome Center) (67) was used to obtain LOD scores and map distances to known zebrafish markers.

Hematoxylin and eosin staining

Adult zebrafish were anesthetized in 0.3% tricaine solution for 2–10 min, coated in OCT (Optimal Cutting Temperature Compound, Sakura) and frozen in liquid nitrogen-cooled 4-methyl butane. Ten micron cross-sections were cut at −20°C using a Microm HM 505 E cryostat, transferred to silane-coated microscope slides, and fixed by immersing in methanol for 3 min before equilibrating in 1 × PBS at 4°C overnight. Slides were washed for 10 min in ddH2O and stained with Harris’ Alum Hematoxylin (Harleco) with 4% acetic acid for 5 min at room temperature with shaking. Sections were washed with acetic alcohol (0.5% HCl in 70% ethanol) for 30 s, rinsed under tap water again for 2 min. The slides were washed for 10 min in ddH2O and stained with a 1:100 dilution of primary antibody (polyclonal goat antisera to human dystrophin exons 1, 2, 59 and 64, 1:100 in 1% bovine serum albumin (w/v) in 10% PBS) at 4°C overnight. Slides were washed for 10 min in ddH2O and dehydrated in 70% ethanol for 1 min and then dehydrated in 100% ethanol twice for 1 min and then left at room temperature to dry for 15 min before mounting in cytoseal 60. Slides were viewed using a Zeiss Axioplan II microscope under white light and images obtained with a Zeiss MC 80 camera.

Immunohistochemical analysis

Both adult and embryonic (3–5 day old) zebrafish were anesthetized in 0.3% tricaine solution for 2–10 min, coated in OCT (Optimal Cutting Temperature Compound, Sakura) and frozen in liquid nitrogen-cooled 4-methyl butane. Ten micron sections were cut at −20°C using a Microm HM 505 E cryostat, transferred to silane coated microscope slides, and fixed by immersing the slides in methanol for 3 min before equilibrating in 1 × PBS at 4°C overnight.

To block non-specific binding of the antibodies, sections were incubated for 30 min at room temperature in PBS+10% goat serum. Following blocking, the slides were incubated overnight at 4°C using the same antibodies described above for western blot analysis. Slides were washed three times in 1 × PBS and sections were incubated with FITC-conjugated goat secondary antibodies (Jackson ImmunoResearch
Laboratory) at a 1:50 dilution for 1 h at room temperature. Slides were washed three times in 1 × PBS before mounting in 25 μl vectashield. Slides were analyzed using a Zeiss Axioplan II microscope with a triple filter and the images stored using either IP Lab or OpenLab Software.

Membrane preparations

Membrane preparations were made as described previously (42). In summary, zebrafish cell lysates were prepared by grinding adult anesthetized zebrafish bodies using a liquid nitrogen-cooled mortar and pestle. Before grinding, the head, fins and internal organs of the zebrafish were removed. Each gram of ground zebrafish was resuspended in 5 ml of resuspension buffer (10% sucrose, 0.5 mM EDTA, pH 7.2, 1 mM PMSF) and homogenized with pestle B of a 40 ml dounce homogenizer at 4°C. The lysate was centrifuged at 4°C at 12 000 g to pellet lysosomes and mitochondria. The supernatant was then centrifuged at 105 000 g for 1 h at 4°C to pellet microsomes. The supernatant (cytoplasmic fraction) was frozen on dry ice and stored at −80°C. The pellet was washed with half pellet volume 150 mM Tris, pH 7.5, 0.5 M KCl to remove non-specifically associated proteins. The washed pellets (membrane fraction) were frozen on dry ice and stored at −80°C. Protein concentrations were determined by Bradford Assay for equal protein loading. When analyzing the proteins by SDS–PAGE or western blot analysis, the protein samples were resuspended in SDS–PAGE loading buffer [final concentration of approximately 20 mM Tris–HCl, pH 6.8, 3% (v/v) glycerol, 0.6% (w/v) SDS, 0.22 M 2-mercaptoethanol, 0.004% (w/v) bromophenol blue], boiled for 5 min, vortexed and then analyzed by electrophoresis on 3–8% Tris–acetate gel for large proteins (<100 kDa) or on 4–12% Tris–glycine gels for small proteins (>100 kDa). Samples were transferred to nitrocellulose and analyzed by western blot as described above.

Morpholino experiments

The following two different morpholinos against the 5’ end of the zebrafish dystrophin mRNA sequence were designed (Gene Tools): dystrophin anti-sense 1 (5’-GCC ATG ACA TAA GAT CCA AGC CAA C) and dystrophin anti-sense 2 (5’-TTG AGT CCT TTA ATC CTA CAA TTT T). The following control morpholinos were used: standard control (5’-CCCT TTC ACC TCA GTC ACC TAA ATT TAT A; Gene Tools), dystrophin sense (5’-AAA ATT GTA TGA TAA AAG GAC TCA A; complement of dystrophin anti-sense 2), and dystrophin inverted (5’-TTT TAA CAT CCT AAT TCC CTG AGT T; inversion of dystrophin anti-sense 2). Zebrafish were injected at the 1–4 cell stage with approximately 10 ng of morpholino. The eggs were incubated in 28.5°C fish water for 3–5 days and analyzed for a phenotype. Photographs of the embryos were taken with a Nikon Coolpix 995 camera mounted on a Nikon Eclipse TS100 microscope and the pictures imported into Photoshop™. Embryos were harvested by euthanizing with 0.3% tricaine at days 3 and 5 and frozen in OCT for immunohistochemical analysis (described above) or resuspending in SDS-loading dye and boiling for western blot analysis (described above).

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