Mutations affecting skeletal muscle myofibril structure in the zebrafish*

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Summary

We describe embryonic lethal mutations in the zebrafish, Brachydanio rerio, which affect organization of skeletal muscle myofibrils. The mutations, fab-I(b45) and fab-I(b126), were independently isolated from progeny of gamma-irradiated females. Each segregates as a single recessive gene; b45 is located about 23 map units from its centromere. The b126 mutation has a similar but slightly larger apparent gene–centromere distance and a less severe phenotype. The two mutations fail to complement, suggesting that they are allelic. Homozygous b45 mutant embryos are paralyzed, and their axial skeletal muscle cells are unstriated, containing severely disorganized myofibrillar components. Gel-electrophoretic comparisons of b45 mutant and wild-type muscle proteins failed to reveal absent or altered major myofibrillar proteins. Embryos genetically mosaic for b45 were also phenotypically mosaic, suggesting that the defect is cell-autonomous. We suggest that these mutations identify a gene required for proper organization of skeletal muscle myofibrils, and that the more severe mutation may represent a null allele.

Key words: zebrafish, muscle, myofibril, mutation.

Introduction

Although much is already known about the molecular structure of the contractile proteins of striated muscle fibers, little is known about how these proteins are assembled into filaments, and how the filaments are assembled into myofibrils. Some cytoskeletal elements may self-assemble (Davis, 1988), while others may require accessory proteins for assembly and maintenance of their structure (Pollard and Cooper, 1986). It seems likely that higher-order cytoskeletal structures, such as the myofibril, may require the interactions of a greater number of cytoskeletal elements and accessory proteins. In an attempt to identify such cytoskeletal elements, we have used a genetic approach to screen for mutations that disrupt the structure and function of striated muscle myofibrils.

Mutations that disrupt myofibrils in both Drosophila (Deak, 1977; Mogami and Hotta, 1981; Karlik et al. 1984; O’Donnell and Bernstein, 1988; Chun and Falkenthal, 1988; Beall et al. 1989) and C. elegans (Waterston et al. 1974; Epstein et al. 1986, 1974) have provided detailed information about assembly of these cytoskeletal specializations. In general, mutational analysis can be useful in probing assembly in two ways. Availability of a mutation that blocks myofibril assembly immediately suggests a protein element necessary for assembly, whether structural or enzymatic. Further, examination of mutant myofibrils may suggest the specific role of the mutant gene product in assembly. Once a locus has been identified in this way, various molecular techniques utilizing the mutant may be employed to isolate the relevant gene and protein for detailed study. Additionally, various alleles of these genes can be used to identify functional domains of both the gene and the protein.

This approach has proven useful for certain mutations in the nematode, C. elegans. Mutations at the unc-54 locus result in a defect in one of the myosin isoforms comprising thick filaments (Epstein et al. 1974). Absence of this protein leads to assembly of thick filaments that do not function correctly and are reduced in number, although they are the correct length. Mutant animals are paralyzed and have abnormal myofibrils. Studies of thick filaments in alleles of unc-54 that reduce that myosin isoform revealed interactions between myosin isoforms during thick filament assembly (Epstein et al. 1986). These mutants have increased our understanding of how thick filaments are assembled, and also of how thick filaments themselves contribute to the assembly of the mature myofibril.

In Drosophila, mutations affecting constituents of the
indirect flight muscles have been found (Deak, 1977; Mogami and Hotta, 1981). Such mutant individuals are often viable, but flightless, as the defect is tissue specific. One of these, $l_{fm}(3)7$, seems to result in a truncated muscle-specific actin protein (Karlik et al. 1984). The resultant myofibrils are not organized into sarcomeres, even though some thin filaments are present.

In contrast, genetic analysis has contributed little to our understanding of the assembly of myofibrils in vertebrates. Instead, we have relied on ultrastructural and histological evidence (Fischman, 1967; Peng et al. 1981; Dlugosz et al. 1984; Antin et al. 1986; Wang et al. 1988; Lin et al. 1989), and on evidence gained by examining the behaviour of labelled myofibrillar components in living cells (Sanger et al. 1984; Sanger et al. 1986). Additionally, new proteins that are components of vertebrate myofibrils have been identified with specific monoclonal antibodies and through biochemical techniques. The precise roles of many of these proteins, either in myofibril assembly or muscle contraction, remain unclear.

Ultrastructural and histological studies have provided a phenomenological description of myofibril assembly (Fischman, 1967; Peng et al. 1981). This process can be divided into several steps, represented by the sequential appearance of characteristic structures (Dlugosz et al. 1984). Mutations affecting proteins constituting these structures might, in effect, create an ablation of one of the structures hypothetically important for assembly. The resulting phenotype would clarify the role of the structure in myofibrillogenesis. Alternatively, mutational analysis may reveal functions of a protein not previously considered.

The zebrafish provides a convenient vertebrate system in which to study myofibrillar assembly through mutational analysis. First, the zebrafish is amenable to genetic analysis. Zebrafish have a generation time of 4 months, and each female can produce up to 100 eggs the day following fertilization by criteria described in the text, and the fraction of homozygous mutants scored. The EP mutation, mutant individuals were first identified among diploid parthenogenetic embryos produced by the 'early pressure' (EP) method (as described in Streisinger et al. 1986). Mutant embryos were identified at 24 h postfertilization by criteria described in the text, and the $fub-1$ mutants separated from any other lethal mutations that may have been induced in the same mutagenesis.

Mutagenesis and mutant screens

Clonal lines of zebrafish, free of lethal mutations, were mutagenized at the blastula stage by exposure to gamma-rays, as described previously (Walker and Streisinger, 1983; Grunwald et al. 1988). The recessive mutation $b_{26}$ was subsequently uncovered in haploid parthenogenetic progeny of irradiated adult females by fertilizing their eggs with sperm rendered genetically impotent by exposure to ultraviolet light. In the case of the $b_{45}$ mutation, mutant individuals were first identified among diploid parthenogenetic embryos produced by the 'early pressure' (EP) method (as described in Streisinger et al. 1986). Mutant embryos were identified at 24 h postfertilization by criteria described in the text, and the $fub-1$ mutants separated from any other lethal mutations that may have been induced in the same mutagenesis.

Mutant stocks

Mutant stocks were maintained by crossing heterozygous $fub-1$ females to clonal, lethal-free wild-type males. Heterozygous progeny were identified by one of the methods above, or by crossing to a fish already known to be heterozygous.

Half-tetrad analysis

To estimate gene-centromere linkage distance, diploid parthenogenetic progeny of females bearing either $fub-1$ mutation were produced by the EP method mentioned above, and the fraction of homozygous mutants scored. The EP treatment blocks the second meiotic division of the eggs, producing diploid embryos whose chromosomes are derived from sister chromatids. Genetic loci that have not been separated from their centromeres during meiotic recombination will be homozygous in embryos treated by EP. The fraction of such non-recombinant embryos is inversely proportional to the distance between the locus and its centromere (Streisinger et al. 1986).

Mosaic analysis

Mosaic embryos were generated as previously described...
(Streisinger et al. 1989). Two- or four-cell embryos from identified heterozygous b45 females were placed with 2 ml fish water into 1 cm diameter glass vials supported on a 6 cm high platform. This was inserted into the chamber of a Model M Gammator (Radiation International), and exposed to an estimated 200 Roentgens of gamma rays. Embryos thus treated were grown to 36–60 h, fixed in 10 % formaldehyde in phosphate-buffered saline, and individually screened for phenotypically mutant cells using Nomarski interference contrast optics.

**Light microscopy**

Living embryos were examined as previously described (Grunwald et al. 1988), with Nomarski interference contrast optics using a Zeiss Universal microscope. Birefringence of axial skeletal muscle was examined using polarized light microscopy done on the same microscope. Birefringence was maximized by positioning embryos appropriately relative to the plane of polarization. This was standardized in all observations by comparison to a known wild-type embryo placed in the same orientation in the same field of view.

**Electron microscopy**

Embryos were fixed, stained and embedded as described previously (Kimmel et al. 1981). Sections of about 100 nm thickness were cut with a glass knife, mounted on Formvar-coated hexagonal mesh grids, stabilized, stained with uranyl acetate and lead citrate, and examined with a Philips 300 electron microscope.

**Two-dimensional gel electrophoresis**

Two-dimensional gel electrophoresis was carried out according to the method of O’Farrell (1975) as modified by Garrels (1979a). Samples of wild-type and mutant sibling embryos were prepared simultaneously to reduce variability. Approximately twenty-five embryos at 24–36 h were washed three times in phosphate-buffered saline at 40°C. The heads and yolk were removed using a tungsten needle, and the remainder was quickly frozen in liquid nitrogen in a Corex thick-walled glass centrifuge tube. The frozen tails were ground to a powder using a stout fire-polished glass rod as a pestle. The mixture was frozen on dry ice and lyophilized. Samples were reconstituted at room temperature in 150 µl of a buffer containing 9.95 M Urea, 4.0 % NP-40, 2 % ampholytes, (LKB, pH 6–8 or 5–7), 100 mM dithiothreitol, and used immediately or stored at −70°C for up to one month. Samples were microcentrifuged before loading to remove any remaining unsolubilized material. The final protein concentration was approximately 1.6 mg ml⁻¹; 10–20 µl of sample was loaded onto each gel. Gels were silver-stained according to the method of Morrissey (1981).

**Electrophysiological measurements**

All electrophysiological measurements were made as previously described (Grunwald et al. 1988) except that experiments were carried out in a physiological saline composed of 115 mM NaCl, 3 mM KCl, 1.8 mM CaCl₂, 5 mM Hepes buffer, pH 7.2.

**Results**

Isolation of non-motile mutants

The mutation *fub-1 b(45)* was the first recognized (see below) among the parthenogenetic diploid offspring of an isogenic female that had been mutagenized with gamma rays at the late blastula stage of development (Walker and Streisinger, 1983). Homozygous parthenogenetic embryos were obtained by early pressure treatment (EP, see methods), which blocks meiosis II. A second mutation, called *fub-1 b(26)*, was later isolated during a screen of haploid embryos from a different stock of similarly mutagenized females. Both mutations were originally recognized because the mutant embryos were non-motile at 24 h.

In all, 225 mutagenized mothers were screened; sixty-six of these mothers produced a total of seventy-five separate recognizable mutations that were identified based on their distinctive embryonic phenotypes, although not all of these mutants were subsequently recovered and established as mutant lines. Out of these seventy-five observed mutants, seven were specifically non-motile at 24 h. These seven mutants had no other obvious phenotype. Of these seven, the two described in this paper had a myofibrillar phenotype detailed below. Of the phenotypes of the other five, four have not been examined in detail and one, *nic-l(b107)*, eliminates functional acetylcholine receptors (Westenberg et al. 1987).

*b45* and *b126* mutant individuals had a specific reproducible phenotype. Until late embryonic stages, (about 32 h) mutant embryos superficially resemble their wild-type siblings morphologically (Fig. 1), but not behaviorally. Homozygous mutants of either genotype (*b45* or *b126*) did not begin the stereotypic

![Fig. 1. 32 h wild-type and mutant zebrafish embryos appear superficially similar. Wild-type embryo (top) is very slightly longer than either *b45* (middle) or *b126* (bottom) homoyzygous embryos. In other respects, wild-type and mutant embryos appear similar in overall body form at this stage. Bar=0.5 mm.](image-url)
Spontaneous body movements that occur in wild-type embryos at about 18 h (Myers et al. 1986). Mutant embryos were easily distinguished from their wild-type siblings at this time by observing their motility using a dissecting microscope. Mutant embryos remained immotile, and did not respond vigorously to tactile stimuli, as did their wild-type siblings (but see below) (Grunwald et al. 1988). Mutant embryos died after about 7 d, during the early larval stage of development.

Mutant embryos were examined with Nomarski interference microscopy to further characterize the phenotype. Before about 36 h, mutant embryos were difficult to identify by criteria other than motility. At 36 h, mutant muscle cells had normal shape and size, and occupied characteristic locations within normal-appearing muscle segments (Fig. 2). At this time, wild-type axial muscle cells were striated. In contrast, mutant axial muscle was devoid of striations. Mutant muscle nuclei are clearly visible. Examination with a compound microscope revealed that fub-1(b45) muscle cells twitch very slightly by about 24 h. Such movement was not noticeable using a dissecting microscope at low magnification. Mutant embryos maintained in either fish water (see methods) or tissue culture medium (L-15 medium, 1 % fetal calf serum, 1 % fish embryo extract) for up to one week never showed wild-type motility or striation. After about four days mutant muscle cells become vacuolated and begin to die.

All skeletal muscle was affected. The extrinsic ocular muscles were also unstriated, as were the pharyngeal arch muscles. Other tissues that appeared affected by either mutation included the heart, which although beating was not pumping red blood cells and was smaller than normal. Thus, cardiac muscle may also be affected by these mutations, though this may be due to another linked mutation (see Discussion). Red blood cells did not enter the circulatory system of either mutant, probably because of diminished action of the heart. The pericardium was enlarged after 36 h in mutant embryos.

The remaining tissues of the mutants appeared unaffected; particularly, no alteration in the nervous system was observed. A well-characterized set of identified spinal motoneurons (Myers et al. 1986) were identified in the correct positions in b45 (data not shown), and these motoneurons functionally innervated their target muscle cells (Fig. 3). Synapses on mutant muscle cells thus appeared structurally and functionally normal.

Except for the specific differences mentioned above, development of mutant embryos proceeded according to the same schedule as that of their wild-type siblings. Both wild-type and mutant embryos displayed normal somite formation starting at 10.5 h, had a visible heartbeat at 24 h, began pigment formation at 30 h, and hatched at two to three days. It is interesting that nonmotile embryos can hatch; this is likely due to the action of secretions of the embryonic hatching gland located near the pericardium, in combination with mechanical stress exerted by the growth of the embryo. Wild-type embryos develop swim bladders at about 4 days; mutant embryos very occasionally developed swim bladders if maintained in the tissue culture medium mentioned above. After this point, wild-type embryos begin swimming and feeding and grow rapidly. Mutant embryos between four and seven days exhibited pronounced enlargement of the pericardium, and mutant cells appeared to be dying, becoming increasingly vacuolated. At about seven days, a heartbeat was no longer observed, and mutant embryos became increasingly misshapen as cells died. This cell death is likely not a specific consequence of the mutations as proliferating mutant cells can be maintained in culture as easily as wild-type cells (data not shown).

Heritability and genetic characterization

To establish that the phenotypes resulted from a heritable mutation, and to characterize the genetic behaviors of the defects, segregation was investigated in several types of crosses (Table 1). The phenotype of each mutation segregated as a single zygotic, fully penetrant, recessive trait. For the b45 mutation, about one-quarter of the embryos from crosses between putative heterozygous adults were of mutant phenotype. Also compatible with single gene segregation, about one-half of haploid progeny of heterozygous females displayed the mutant phenotype. In a third test of single gene segregation, heterozygotes were crossed with wild-type fish and approximately one-half of surviving progeny were heterozygous for b45; this also shows that b45 has no dominant effects on reproductive viability. Tests of segregation on the G-1 generation of b126 heterozygotes yielded similar results (Table 1b).

Mapping the mutations relative to the centromere

Half-tetrad analysis allowed us to obtain the gene–centromere map distance for each mutation. Pathenogenetic progeny were obtained by EP treatment (see methods) of eggs from fub-1(b45)/+ mothers and scored for the nonmotile phenotype (Table 2). Pathenogenetic homozygous b45 mutant embryos contain only half of the sister chromatids that did not undergo recombination between the gene and the centromere; the other half of nonrecombinant sister chromatids produce a like fraction (~26 %) of homozygous wild-type embryos. The remaining embryos (~48 %) contain recombinant chromatids and are heterozygous. This frequency of recombinant heterozygotes is proportional to the gene–centromere distance. Assuming complete chiasmatic interference (Streisinger et al. 1986), we calculated an apparent gene-centromere distance of approximately 29 map units (Haldane, 1919).

We also tested the segregation of b126 among pathenogenetic progeny to determine the gene–centromere distance (Table 2). Of 342 embryos tested, 67 were mutant, a fraction of about 0.20, which implies a map distance of approximately 29 map units. This is significantly different (P<0.005, Table 2) than the 0.26 fraction of mutants for b45. It b45 and b126 are in fact allelic, any apparent small difference in gene–centromere distance between b126 and b45 may be observed
Mutant axial muscle cells lack striations. Sagittal views of living 36 h embryos. The wild-type embryo (top) has conspicuous cross-striated axial myotubes (m) at this time. Both b45 (middle) and b126 (bottom) homozygous embryos have axial myotubes which are devoid of striations. Myotubes of both mutants have the correct shape, a single myotube extending from one somite border to the next. Nuclei are easily seen in mutant myotubes but are obscured by cross-striations in wild-type. Observations of several hundred non-motile mutant embryos of each genotype were identical. Bar=20 μm.

either because of variability due to genetic background (Streisinger et al. 1986) of the animals, or because of an actual difference in map distance (see Discussion).

b45 and b126 fail to complement
b45 and b126 homozygotes are phenotypically similar and also have similar gene–centromere distances,
suggesting that the two mutations are allelic. To test this possibility, we examined offspring of crosses between b45 and b126 heterozygotes (Table 1). About 25% of the progeny from these crosses displayed the mutant phenotype, showing that the mutations failed to complement, and strongly suggesting that the two mutations are allelic.

Since the two mutations have similar effects and fail to complement, and to eliminate the possibility that the second mutation was not independent from the first because of an error in stock maintenance, it was necessary to confirm the independence of the two mutations. To test this possibility, we examined haploid mutant offspring of the originally mutagenized female...
Table 1. Segregation of fub-1(b45) and fub-1(b126) in standard crosses

<table>
<thead>
<tr>
<th>Parental genotype</th>
<th>Individuals tested (a)</th>
<th>Fraction heterozygous (b)</th>
<th>Fraction mutant (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Expected (c)</td>
<td>Observed (c)</td>
</tr>
<tr>
<td>(1a) fub-1(b45)/+ x fub-1(b45)/+ fub-1(b45)/+ + females fub-1(b45)/+ x +/+</td>
<td>Embryos</td>
<td>0.25</td>
<td>0.25 (791) (c)</td>
</tr>
<tr>
<td>(1b) G1 fub-1(b126)/+ x G1 fub-1(b126) (c) G1 fub-1(b126)/+ x fub-1(b45)/+</td>
<td>Embryos</td>
<td>0.25</td>
<td>0.26 (288) (c)</td>
</tr>
</tbody>
</table>

(a) Mutant embryos were identified at 24 h by their lack of motility. Adult genotypes were established by examining the phenotypes of their parthenogenetic progeny.

(b) The expected fractions were based on the segregation of a single recessive mutation. The observed fractions represent the fractions of embryos or adults observed for each category. The number of animals tested is shown within the parenthesis.

(c) The observed fractions did not differ significantly from the expected results (P>0.05) as calculated by the Chi-square test.

(d) Haploid embryos were generated by activating eggs from heterozygous females with sperm rendered genetically impotent with ultraviolet irradiation (Steisinger et al. 1981).

(e) G1 are the progeny of the cross of G0×++. G0 was the original female found to be carrying the mutation.

Table 2. Half-tetrad analysis of fub-1(b45) and fub-1(b126)

<table>
<thead>
<tr>
<th>Female population</th>
<th>Fraction mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>fub-1(b45):</td>
<td></td>
</tr>
<tr>
<td>Clonal genetic background (a)</td>
<td>0.27 (2270)</td>
</tr>
<tr>
<td>Nonclonal genetic background (b)</td>
<td>0.26 (3678)</td>
</tr>
<tr>
<td>Total</td>
<td>0.26 (5948)</td>
</tr>
<tr>
<td>fub-1(b126):</td>
<td></td>
</tr>
<tr>
<td>Clonal genetic background (d)</td>
<td>0.20 (342) (c)</td>
</tr>
</tbody>
</table>

(a) These fish were derived from mutagenesis of an original stock (C29), which had initially been made homozygous at all loci by heat-shocking haploid embryos during the first mitotic division (Steisinger et al. 1981).

(b) These fish are progeny of a cross of wild-type males of nonclonal background with clonal females carrying the b45 mutation.

(c) The expected fractions were based on the segregation of a single recessive mutation. The observed fractions represent the fractions of embryos or adults observed for each category. The number of animals tested is shown within the parenthesis.

(d) Haploid embryos were generated by activating eggs from heterozygous females with sperm rendered genetically impotent with ultraviolet irradiation (Steisinger et al. 1981).

fish from which b126 was obtained (termed G-0 (b126)). We found that mutants constituted significantly less (P<0.005) than 50% of the total progeny, making it unlikely that this fish carried the b45 mutation, and suggesting that she was mosaic in her germline for the bl26 mutation (Table 3, row 1). In support of this, less than half of the adult progeny of the G-0 b126 mother carried the mutation (P<0.05, Table 3, row 2). Additionally, crosses of identified b45 males with the G-0 (b126) female produced less than 25% phenotypically mutant offspring (P<0.005, row 3), also as expected for a mosaic germline. There are two alternative explanations for these observations: either b126 had an early embryonic lethal effect, or the G-0(b126) mother had a mosaic germ line. Since the next (G-1) generation of b126 produced about 25% phenotypically mutant offspring when crossed with other b126 or b45 heterozygotes, (Table 1), we concluded that b126 did not result in any embryonic lethal phenotype. From these data, we infer that the female G-0 (b126) had a mosaic germ line, and that the novel mutation fub-1(b126) arose in a single one of the 2–5 cells present at the blastula stage (Walker and Streisinger, 1983) which contributed to the germ line of G-0(b126). Thus, the two mutations, b45 and b126, arose independently; this independence is supported by the difference in phenotype between the mutations shown below.

b45 produces a more severe phenotype than b126

To determine whether there were any phenotypic differences between the two alleles and to further describe their genetic behavior, we made a detailed comparison among homozygotes of both mutant alleles, transheterozygous mutant offspring of crosses between alleles, and wild-type embryos. At 36 h, wild-type embryos display considerable motility and respond to tactile stimuli by rapidly contracting trunk and tail musculature (Eaton et al. 1977). Homozygous b45 embryos remained immotile at this time and did not respond to tactile stimuli. In contrast, b126 homozygotes at 36 h responded to tactile stimuli, although their movements were considerably weaker, with a smaller range of motion, than those of wild-type embryos. Mutant progeny of crosses between b45 and b126 fish resembled b126 homozygotes with regard to motility.

Examination of wild-type embryos with polarized light microscopy at 36 h revealed that axial muscle is highly birefringent due to the ordered array of myofilaments in striated muscle. When observed under polar-
Table 3. Mosaicism of fub-1(b126) G0 germ line

<table>
<thead>
<tr>
<th>Parental genotype</th>
<th>Individuals tested</th>
<th>Expected nonmosaic mutant frequency (b)</th>
<th>Observed mutant frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0 fub-1(b126)/+ females (a)</td>
<td>Haploid embryos</td>
<td>0.50</td>
<td>0.30 (56) (c)</td>
</tr>
<tr>
<td>G0 fub-1(b126)/++/+</td>
<td>Adult heterozygotes (d)</td>
<td>0.50</td>
<td>0.35 (43) (c)</td>
</tr>
<tr>
<td>G0 fub-1(b126)/++×fub-1(b45)/+</td>
<td>Embryos</td>
<td>0.25</td>
<td>0.18 (323) (c)</td>
</tr>
</tbody>
</table>

(a) G0 is the female fish that was originally gamma irradiated as a blastula, and which was subsequently found to be mutant.
(b) This represents the fraction expected if the germ line cells of G0 are not mosaic for fub-1(b126).
(c) These numbers are significantly different from 0.50 and 0.25, respectively, as determined by the Chi-square test. [Chi-square=8.80 (P<0.005); =3.93 (P<0.05%); =8.13 (P<0.005)].
(d) Heterozygous adults were identified by crossing with identified b126 adults of the opposite sex and scoring for nonmotile fish at 24 h.

Fig. 4. Low magnification comparison of two mutant alleles with wild-type fish at 36 h by bright-field microscopy (left) and polarized light microscopy (right) shows differences in degree of perturbation of axial muscle. (A) Wild-type (top) and b45 homozygous (bottom) embryos. Note smaller eye and larger pericardium in the mutant. These features are common in fish mutant at other loci and may not be specific to fub-1 but rather a general effect of lethal mutations. In polarized light microscopic view of same embryos, axial striated muscle of wild-type fish is highly birefringent; this birefringence is reduced in the b45 embryo below. (B) A b126 homozygous embryo. Notice that the axial muscle of the mutant embryo displays nearly as much birefringence as that of wild-type. (C) This mutant embryo is a result of a cross between b45 and b126 heterozygous parents; like B above, this embryo also is nearly as birefringent as wild type. Bar=0.5 mm.
that, while the mutant phenotypes are generally similar, \textit{b}45 is more severe in every respect than \textit{b}126; this difference further supports the conclusion that \textit{b}45 and \textit{b}126 are independent alleles.

\textbf{Characterization of mutant myofibrils}

To determine the cause of nonmotility and lack of striation in mutant embryos, and to ascertain that the two mutations cause defects of the same nature but differing severity, we examined wild-type and mutant axial muscle with transmission electron microscopy. All observations were made at 36 h, a time when mutant fish display the phenotype strongly yet remain free of obvious cell death.

Transverse sections through a midbody somite of a wild-type embryo (Fig. 5A) exhibited the standard features of striated muscle including a well-defined sarcoplasmic reticulum surrounding myofibrils, and the characteristic hexagonal packing of thin and thick filaments. Longitudinal sections of the same regions clearly showed myofibrils with sarcomeres in register and the details of sarcomeres such as the A-, I-, M-, and Z-bands.

In contrast, typical midbody transverse sections from a \textit{b}45 homozygote (Fig. 5B) revealed no continuous sarcoplasmic reticulum and showed very little hexagonal packing. The ratio of thick to thin filaments in such sections of mutant muscle appeared the same as that in sections of wild-type muscle, although the disorder of mutant myofilaments rendered them difficult to count. Longitudinal sections of \textit{b}45 homozygous mutant muscle at this axial level displayed no discernible myofibrils, and arrangements of myofilaments in such sections were not distinguishable from those in transverse sections. Close inspection of such micrographs revealed that mutant muscle often contained thick and thin filaments in close association with one another; small areas of nearly normal hexagonal filament packing were sometimes observed. Thus myofilaments were present but incorrectly oriented with respect to the long axis of the cell. The orientation of myofilaments was not completely random: areas of coherence were found, in which groups of associated filaments ran in the same direction. However, the orientation from group to group with varied considerably with respect to the long axis of the cell. Other poorly oriented features of striated muscle could be readily found, such as Z-band material. The sarcoplasmic reticulum was not continuous; instead it appeared as a number of isolated vesicles interspersed among the myofibrils. Very rarely a sarcoplasmic reticular 'triad' was found, sometimes associated with Z-band material. Similar micrographs were obtained from \textit{b}45 haploid embryos (data not shown). These ultrastructural observations show that the \textit{b}45 mutations affected the way in which the filamentous components of myofibrils were ordered, either through a defect in assembly, a defect in maintenance of order, or a defect in a structural protein.

In contrast, electron micrographs of \textit{b}126 homozygote embryonic muscle at 36 h (Fig. 5C) revealed that, in transverse sections, many areas of associated, hexagonally arrayed thick and thin filaments were present. Many myofilaments could also be found which were incorrectly oriented. Electron micrographs of horizontal or sagittal sections of \textit{b}126 homozygote axial muscle showed myofilaments that were mostly ordered parallel to the long axis of the myotube; transverse sections were easily distinguishable from longitudinal sections. Many myofibrillar features were recognizable, such as consecutive Z-bands with aligned thick and thin filaments, and triads of the sarcoplasmic reticulum, although A-, I-, and M-bands were not distinguishable (see also Fig. 6). These elements often formed incomplete sarcomeres of correct length arrayed periodically along disorganized myofibrils. Many myofilaments were present that were not obviously part of any coherent structure. Micrographs of haploid \textit{b}126 embryos appeared the same as those of \textit{b}126 homozygous diploids (data not shown). Additionally, embryos of the genotype \textit{b}126/\textit{b}45 appeared ultrastructurally similar to \textit{b}126/\textit{b}126 homozygotes (Data not shown). In sum, these data show, in agreement with the light microscopic data described above, that \textit{b}126/\textit{b}45 myofibrils more closely resemble wild-type myofibrils than \textit{b}45/\textit{b}45 myofibrils; the \textit{b}126 mutation is less severe.

\textbf{The \textit{b}126 mutation differentially affects a subpopulation of axial muscle fibers}

Detailed ultrastructural observation of \textit{b}126 homozygotes revealed that a subpopulation of muscle fibers was less severely affected at 36 h than other muscle cells (Fig. 6). These fibers are the most lateral in the myotome and lie just under the skin. Myofibrils with many consecutive sarcomeres were evident in these myotubes, and were nearly wildtype in appearance, though still somewhat disorganized. These features were not often found in deeper axial muscle cells in the same embryo. These cells in \textit{b}45 homozygotes did not appear less severely affected than more medial cells. The distinct ultrastructural morphology of superficial myotubes in wild-type zebrafish embryos has been previously described (Waterman, 1969). These observations substantiate the conclusion that the superficial muscle is constituted from a separate subpopulation of muscle cells at 36 h; we propose that these cells be called 'superficial myotomal muscle cells'.

\textbf{The mutation may be cell autonomous}

Since environmental cues are known to be important for muscle differentiation, we examined the possibility that the defect in \textit{fub-1} is caused by an environmental, rather than cell-autonomous, defect. To discover if the action of (\textit{b}45) was cell autonomous or resulted from a defect in intercellular interaction originating in another cell, we constructed genetically mosaic animals by gamma irradiating two-cell embryos from a cross between a heterozygous female and a homozygous wild-type male. This occasionally produced an animal that contained phenotypically mutant as well as wild-type muscle cells (Fig. 7): the precise individual cellular genotype is unknown. We assume that gamma irradiation renders one of the two cells present at the
Fig. 5. Mutant myofilaments are incorrectly oriented. Transmission electron micrographs of wild-type and mutant muscle at 36h. Transverse (top) and longitudinal (bottom) sections. (A) Sections of wild-type axial trunk muscle. Note typical features of skeletal muscle myofibrils, such as hexagonal array of thick and thin filaments within a myofibril (mf) seen in transverse section. The longitudinal section shows prominent sarcomeres with A-, I-, M-, and Z-bands (z), and triads of the sarcoplasmic reticulum (r).

In typical mosaic embryos (Fig. 7), many individual unstriated myotubes were observed scattered in a background of wild-type myotubes, although occasional patches of three or more adjacent phenotypically mutant cells were also found. This mixed distribution of the mutant cells was consistent with the observed distribution of clones of cells labelled with lineage tracer dyes during the blastula stage (Kimmel
Fig. 5. (B) Similar sections of a b45 homozygote. The thick (m) and thin (a) filaments are incorrectly arranged relative to the long axis of the cell; no sarcomeres are observed. Occasional disorganized Z-band material is seen (z). Thick and thin filaments are generally associated with each other in groups of myofilaments of similar orientation. At least three such oriented groups (1–3) can be seen in either of these sections; they may represent attempts of mutant myofilaments to associate into myofibrils. The inset shows a higher-magnification detail of a section through associated thick and thin filaments. Note that longitudinal and transverse sections are not distinguishable. Mutant myofilaments often appear to have a larger diameter than wild-type myofilaments; this may be due to greater accessibility of mutant myofilaments to lead citrate stain.
and Warga, 1987). The number of mutant myotubes observed in mosaics irradiated at the two-cell stage was estimated to be less than one-half the total muscle cells. This may be because the event causing mosaicism occurred after replication at the two-cell stage; thus only one-fourth of all cells would be homozygous (Streisinger et al. 1989). It is also possible that the defect was not observable in multinucleate myotubes, which contained a wild-type nucleus as well as one or more mutant nuclei: about 25% of myotubes have more than
Fig. 6. Myofibrils in superficial muscle of bl26 embryos appear less severely affected than those in deeper muscle. An electron micrograph of a horizontal section of a 36 h bl26 homozygous mutant embryo. A superficial muscle cell (s) contains a properly oriented myofibril with many consecutive sarcomeres. Z-bands are designated by arrows. The sarcomeres comprising this myofibril are incomplete: no A bands are apparent. Myofilaments in deeper cells (d) are more poorly organized. Bar=1 μm.

Additionally, clones giving rise to mutant myoblasts may have a disadvantage during development compared to wild-type clones.

We observed that phenotypically mutant cells could be found surrounded by wild-type neighbors within a single region of a single muscle segment. This distribution would not be observed if the mutant phenotype was due to a defect in a diffusible factor. Since neighboring cells on the same side of the horizontal myoseptum within a muscle hemisegment receive input from the same primary motoneuron (Westerfield et al. 1986), it is unlikely that the muscle phenotype results from a neuronal abnormality. Thus, mosaic analysis suggests that the defect is likely not due to absence or alteration of a diffusible element and is not correlated with innervation. The results suggest that the defect is cell autonomous. However, without independent linked genetic markers, we cannot rule out the possibility that the phenotype is caused by a deficit in more...
subtle intercellular interactions among closely neighboring cells.

Comparison of mutant and wild-type proteins
To investigate whether major myofibrillar proteins were altered in the mutant, we compared wild-type and b45 mutant tail proteins from 24–36 h embryos on two dimensional polyacrylamide gels (O'Farrell, 1974; Garrels, 1979a). We used only b45 and not b126 mutant tails because b45 resulted in a more severe phenotype, and thus is likely to be the result of a more easily observable protein difference. Tails have a relatively higher proportion of muscle than other areas of the embryo: we estimate that tails of 24–36 h fish are about 70% muscle by volume. Examination of typical two-dimensional gels (Fig. 8) (pH 5–7, 10% acrylamide) of mutant and wild-type proteins showed that the patterns of spots were virtually indistinguishable. Comparisons of many different gels from this stage revealed no consistent differences, although small differences could be observed in individual experiments. Similar results were obtained with one-dimensional gels (data not shown). Identity of some of these proteins (summarized in Table 4) was surmised on the basis of similarity in electrophoretic mobility of myofibrillar proteins characterized in other organisms (see for example Garrels, 1979b) or expected mobility based on physical properties. Proteins that could be tentatively identified according to electrophoretic mobility included titin, nebulin, myosin heavy chain, alpha-, beta-, and gamma-actins, and alpha- and beta- tropomyosins. Presence of desmin and myosin heavy chain were confirmed by staining mutant and wild-type embryos with monoclonal antibodies directed against those proteins (data not shown). Additionally, several antibodies were used to screen protein blots (data not shown) from both wild-type and mutant embryos; again, no differences were observed. These antibodies were against myosin heavy chain, alpha-actinin, beta-tubulin, vinculin and tropomyosins.

Discussion
We have described two recessive mutations, b45 and b126, that affect myofibrillar organization. These mutations fail to complement, have similar gene–centromere distances and have similar phenotypes. Thus, both mutations have been tentatively assigned to the same locus, fub-1. Embryonic fish homozygous for fub-1 have myotubes that contain severely disorganized myofibrils. Ultrastructural and gel electrophoretic evi-
Table 4. Evidence for proteins present in fub-1(b45)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Electrophoretic mobility (a)</th>
<th>Sections (b)</th>
<th>Western blot</th>
<th>Structure/function</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wt</td>
<td>mut</td>
<td>wt</td>
<td>mut</td>
</tr>
<tr>
<td>Actin</td>
<td>+</td>
<td>+</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>Myosin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(d)</td>
</tr>
<tr>
<td>Myosin Heavy chain</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>nt</td>
</tr>
<tr>
<td>Myosin Light chain</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>nt</td>
</tr>
<tr>
<td>Beta-tubulin</td>
<td>+</td>
<td>+</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>Vinculin</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>Tropomyosin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>nt</td>
</tr>
<tr>
<td>Desmin</td>
<td>nt</td>
<td>nt</td>
<td>+</td>
<td>(i)</td>
</tr>
<tr>
<td>Titin</td>
<td>+</td>
<td>(j)</td>
<td>+</td>
<td>nt</td>
</tr>
<tr>
<td>Nebulin</td>
<td>+</td>
<td>(k)</td>
<td>+</td>
<td>nt</td>
</tr>
</tbody>
</table>

nt = not tested, or, in the case of gels, not unambiguously identified.

(a) Electrophoretic mobility was assessed either by one- or two-dimensional polyacrylamide gel electrophoresis. Identity of proteins was assigned based on either expected mobility inferred from physical properties, or comparison to similar experiments done in other vertebrate systems (see for example Garrels, 1979).
(b) These were 25 μm vibratome sections of 24 h zebrafish embryos prepared according to Trevarrow, 1988.
(c) Calcium ATPase activity was assayed histologically according to Brumback and Leech, 1984.
(d) Antibodies against chick myosin heavy chain were a generous gift from Dr Howard Holtzer. All Western blots were carried out as described in Towbin et al. 1979.
(e) Antibodies against chick alpha-actinin (Lin, 1982) were purchased from Amersham Biochemicals.
(f) Antibodies against chick beta-tubulin (Blose et al. 1982) were purchased from Amersham Biochemicals.
(g) Antibodies against chick vinculin were a generous gift of Dr S. Craig (Pardo et al. 1983).
(h) Antibodies against chick tropomyosin were the generous gift of Dr J. J.-C. Lin (Lin et al. 1985).
(i) Antibodies against chick desmin were the generous gift of Dr D. Fischman.
(j) Titin and nebulin were identified by their mobility on low percentage one-dimensional polyacrylamide gels (K. Wang, 1979, 1982).

Discussion

Eleven proteins were identified that are present in fub-1(b45) mutants. Double mutant analysis of fub-1(b45) and fub-1(bl26) suggests that the two mutations are allelic. The possible candidates for the two mutations are actin, myosin, alpha-actinin, tropomyosin, desmin, beta-tubulin, vinculin, and nebulin. It is not possible to determine the exact molecular identity of any of these proteins, as antibodies against them were not available. However, the presence of multiple proteins suggests that the mutations are affecting multiple components of the muscle cell. The possibility that the mutations are affecting more than one gene cannot be ruled out, as both can be present in a single muscle cell. Further, the b126 mutation is mosaic, as expected if the mutation first occurred in that individual. Further, the b126 mutant phenotype is less severe.

Observations of b126 homozygotes revealed that superficially located axial muscle cells were less affected than the rest. Detailed examination showed that these superficial muscle cells contained well-oriented myofibrils with many poorly organized sarcomeres. This finding suggests that these muscle cells constitute a separate subpopulation, and that fub-1+ acts differentially in separate populations of muscle cells.

Genetic considerations

Both mutations segregate as a single locus in standard tests of assortment. These data do not rule out the possibility that either mutation affects more than one tightly linked gene; this is not unlikely, as both mutations were produced by gamma-rays, which may often produce large deletions in zebrafish (Chakrabarti et al. 1983). This consideration becomes more important if the closely linked genes are related in function. Both mutations have similar (but not identical) gene–centromere distances, have similar phenotypes and they fail to complement. In complementation tests, one-quarter of the progeny were non-motile, and had the less severe (b126) phenotype. These observations strongly suggest that the two mutations are allelic; there remains the small possibility that they are not mutant alleles of the same gene, but rather mutations affecting two interacting proteins encoded by closely linked genes. This question could be explored by using existing fub-1 mutants to isolate new noncomplementing point mutations and can be resolved conclusively by detailed molecular characterization of the relevant proteins. Assuming that the two mutations are in fact allelic, some observations suggest that b45 may represent a null allele. First, b126 results in a less severe phenotype. Second, b126 haploids resemble b45/b126 diploids. If b45 is indeed a null allele, then the mutation b126 may result in a partial loss of function. It will be interesting to obtain additional alleles to further substantiate this hypothesis.
The observation that \(b45\) and \(bl26\) have slightly (but significantly) different gene-centromere distances is intriguing. Since the frequency of recombination over physical DNA length has not yet been determined for zebrafish, the magnitude of this difference is unknown. The observed difference may be due merely to a difference in genetic background (Striesinger et al. 1986). Alternatively, the difference may be due to differences in the mutations themselves. We would observe these results if \(b45\) is a large deletion including the \(fub-1\) locus as well as more proximal sequences, and \(bl26\) is a smaller, more distal mutation covering the \(fub-1\) locus. This idea is consistent with the observation that \(b45\) is phenotypically more severe than \(bl26\).

The \(fub-1\) locus appears to be easily mutable, since the two alleles were found after screening only 225 mutagenized mothers. This result may be a reflection of the presumably large lesions made by gamma-rays. It will be interesting to measure the specific locus mutation frequency using chemical mutagens. It is encouraging that non-motile phenotypes arise so frequently in zebrafish. This may be partly due to the ease in recognizing this phenotype. This consideration enhances the attractiveness of using this system to study vertebrate muscle development.

**Nature of the wild-type protein**

We observed that phenotypically wild-type and mutant cells were intermingled in genetically mosaic animals. The very existence of phenotypically mosaic animals makes it unlikely that the defect is due to a defective element originating in a remote cell. This kind of myofibrillar defect has been described in the case of the \(c\) mutation in axolotl which results in defective cardiac myofibrils: the defect can be rescued by contact with wild-type endoderm (Davis and Lemanski, 1987). Since \(b45\) mutant cells are found dispersed among wild-type cells in a mosaic zebrafish embryo, and neighboring cells within a given region of a somite are innervated by the same motoneuron, it is unlikely that the putative gene product is made by motor neurons. Thus, mosaic analysis of \(fub-1\) suggests that the defect is cell autonomous. However, defects in subtle interactions between neighboring cell types cannot be ruled out.

It is interesting to note that mutant myotubes have the correct shape and orientation within the myotome. This suggests that the defect is not the result of a nonspecific cytoskeletal disruption.

Mutations affecting myofibril structure in *C. elegans* and *Drosophila* have similar ultrastructural phenotypes and in some cases the relevant protein has been identified. In these examples, there is a defect in a major myofibrillar component: flight muscle specific actin (Karlik et al. 1984) or myosin (O'Donnell and Bernstein, 1988; Chun and Falkenthal, 1988; Beall et al. 1989) in the case of *Drosophila* and myosin heavy chain and paramyosin (Waterston et al. 1977) in *C. elegans*. However, it appears that the defect in \(fub-1\) is not in a similar myofibrillar protein for three reasons. First, extensive two-dimensional gel electrophoretic comparisons of mutant and wild-type tail proteins exhibit no consistent differences. Of course, the resolution of this technique may not be adequate, and a small change in a protein may remain undetected. Second, ultrastructural examination of mutant muscle shows that all of the conspicuous filamentous elements of the myofibril are present but not correctly oriented in relation to the long axis of the cell. In the less severe allele \(bl26\), elements of myofibrils are present and better aligned in relationship to each other and the cell axis. Third, ultrastructural examination of mutant muscle from various *C. elegans* and *Drosophila* mutations that affect muscle-specific actin or myosin heavy chain show that the myofilamentous components that remain are still largely oriented parallel to the long axis of the cell (Epstein et al. 1974; Karlik et al. 1984; O'Donnell and Bernstein, 1988; Chun and Falkenthal, 1988; Beall et al. 1989). This last observation also suggests that \(fub-1\) acts earlier in myofibrillar assembly than the *C. elegans* and *Drosophila* mutations.

The mutation appears to be specific to muscle cells; other tissues are not obviously affected and, in particular, motoneurons make functional connections in mutant embryos. The defect in mutant myofibril structure may be due to incorrect initial assembly of myofibrils, incorrect maintenance of an initially correct structure, or a defect in a structural protein. Although the defect is likely not the result of a large change in an easily identifiable myofibrillar protein, more subtle defects cannot be ruled out. Careful examination of myofilament assembly in mutant and wild-type embryos to determine when the defect first becomes apparent may resolve which of these alternatives is the case. Observations described elsewhere (Felsenfeld and Curry, 1988; Felsenfeld, 1988) show that \(fub-1\) mutant myofibrils are aberrant from the start of myofibril assembly.

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