We integratively assessed the function of alternative versions of a region near the N terminus of Drosophila muscle myosin heavy chain (encoded by exon 3a or 3b). We exchanged the alternative exon 3 regions between an embryonic isoform and the indirect flight muscle isoform. Each chimeric myosin was expressed in Drosophila indirect flight muscle, in the absence of other myosin isoforms, allowing for purified protein analysis and whole organism locomotory studies. The flight muscle isoform generates higher ATPase rate than the embryonic isoform. Exchanging the embryonic exon 3 region into the flight muscle isoform decreased ATPase rates to embryonic levels but did not affect actin sliding velocity or flight muscle ultrastructure. Interestingly, this swap only slightly impaired flight ability. Exchanging the flight muscle-specific exon 3 region into the embryonic isoform increased actin sliding velocity 3-fold and improved indirect flight muscle ultrastructure integrity but failed to rescue the flightless phenotype of flies expressing embryonic myosin. These results suggest that the two structural versions of the exon 3 domain independently influence the kinetics of at least two steps of the actomyosin cross-bridge cycle.

Myosin powers muscle shortening by converting the chemical energy of ATP into actin movement through conformational changes in the myosin head. Developmental stage and muscle-specific myosin isoforms have distinct rates of ATP hydrolysis and propel actin at velocities that strongly correlate with muscle contractile properties (1–3). Differences in myosin isoform function have been well characterized in many species (4). However, the mechanism by which variation in specific structural regions of the myosin heavy chain (MHC) contributes to isoform-specific properties is not well understood, especially in striated muscle isoforms (5, 6).

Studies aimed at defining structural regions of MHC that are responsible for setting isoform-specific properties have focused on two flexible surface loops that connect the proteolytic domains of the myosin head (25/50-kDa, loop 1; and 50/20-kDa, loop 2) (for review see Murphy and Spudich (6)). Altering these loops in some myosin types influences ATPase rates and actin sliding velocities (7, 8). In mammalian smooth muscle and scallop and carp muscle isoforms, the pattern of Mhc RNA alternative splicing suggests that these loops contribute to differences in myosin functional properties; in some cases this has been supported by in vitro chimeric studies (9–12). However, in other organisms such as Drosophila melanogaster, these two surface loops are invariant among all muscle myosin isoforms (13).

In Drosophila, all structural variation between muscle MHC isoforms is confined to four discrete regions of the S-1 head (Fig. 1), to the S-2 hinge region, and to the C-terminal tailpiece (13). The discrete domain variation in Drosophila myosin isoforms, which differs from the widespread variation observed in most vertebrate striated isoforms, highlights areas that must set isoform-specific functional properties. Structural variation in these regions is generated by alternative splicing of mRNA transcripts from the single Drosophila Mhc gene (14–16). This mechanism produces at least 15 myosin isoforms that are expressed in a wide variety of muscle types including slow embryonic and larval body wall muscle and the very fast indirect flight muscle (17, 18).

We recently compared the functional properties of two Drosophila myosin isoforms that differ in all four S-1 variable regions, the indirect flight muscle isoform (IFi) and a major embryonic body wall muscle isoform (EMB) (19). Expression of the EMB isoform in a Drosophila myosin null background results in a flightless phenotype, impaired jump ability, poor ambulation, and difficulty in mating (20). EMB myosin isolated from the transgenic line propels actin at one-tenth the velocity in the in vitro motility assay and has one-fourth the basal Mg-ATPase rate but does not differ in unitary step size compared with IFi (19). Thus the functional differences between the two isoforms arise primarily from kinetic differences in the cross-bridge cycle and must be caused by variation in one or more of the alternative exon-encoded regions.

One variable S-1 head region in Drosophila is encoded by alternative versions of exon 3. It is located near the N terminus of the protein, between the ATP hydrolysis site and the pivot point of the lever arm, and corresponds to residues 69–116 of chicken skeletal MHC (Fig. 1). The two alternative versions of exon 3 differ in 18 of 48 amino acids, 12 of which are nonconserved substitutions (13). Exon 3b is expressed primarily in adult muscles, including the indirect flight muscle, and a few external embryonic body wall muscles (17, 18). Exon 3a ap-
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EXPERIMENTAL PROCEDURES

Myosin DNA Construct Preparation—To determine the functional role of the MHC region encoded by exon 3, we constructed two P element-based transgenes. The first transgene, IFI-3a, allows expression of only the "embryonic" version of exon 3 (3a) in all MHC isoforms, including the IFI isoform. It was constructed by replacing the entire Mhc genomic clone (20) with cDNA encoding exon 3b. Specifically, an ApaI and AseI 4.7-kb fragment of Mhcemb containing exons 1–12 was subcloned into pBluescript KS. Exon 3a was removed from the subclone by digestion with PstI (restriction site in exon 2) and StuI (restriction site in exon 4). Digestion of an adult cDNA with the same restriction enzymes created a DNA fragment containing exon 3b. This fragment was ligated into the subclone, and the subclone insert was ligated back into digested Mhcemb.

Both clones were ligated into a CaSpeR vector behind the Mhc promoter, which drives high levels of myosin expression in all muscle types (21). All ligation sites were sequenced, and appropriate restriction digests were carried out to ensure transgene fidelity.

Transformation of Drosophila with Chimeric Mhc—Drosophila were transformed by injecting the chimeric exon 3 plasmids at 0.6 mg ml⁻¹ along with 0.05 mg ml⁻¹ of York modified glycerol (without Triton X-100 and glycerol). After sedimentation by centrifugation for 5 min in a benchtop microcentrifuge tube as described in Swank et al. (19). After sedimentation by centrifugation for 5 min in a benchtop microcentrifuge tube, the IFMs were resuspended and washed in 300 μl of York modified glycerol (without Triton X-100 and glycerol).
Following a 5-min centrifugation, the IFM pellet was homogenized in 200 μl of TRIZol (Life Technologies), and total RNA was isolated according to the manufacturer’s instructions. The final RNA pellet was dissolved in 20 μl of diethyl pyrocarbonate/H2O.

An aliquot of total RNA (4.75 μl) was used for cDNA synthesis in a volume of 10 μl of reverse transcriptase buffer containing 10 mM dithiothreitol, 10 μl of RNasin, 10 μl of Moloney murine leukemia virus reverse transcriptase, and 5 μmol of an exon 7a reverse primer (5′-TACAAGACAGAAATGCTT-3′) or an exon 7d reverse primer (5′-AGAGGAACGACATCCTCT-3′). After incubation for 1 h at 37 °C, the reaction was terminated by heating at 95 ºC for 10 min.

PCR was performed with 1 μl of the cDNA and 0.4 μmol of the primers using Ready-to-Go PCR beads (Amersham Biosciences). The primer sequences were 3a (5′-ATCCGAGAGGGTGAAA-3′) with 7d and 3b (5′-GAGATCTGCTTGGCAGA-3′) with 7a. Touchdown PCR (25) was performed as follows. After a 3-min incubation of the primers and single-stranded cDNA at 95 °C, 2 cycles consisting of 45 s at 94 ºC, 1 min at 60 ºC, and 2 min at 72 ºC were performed. This was followed by a series of cycles, each repeated once, where the annealing temperature, initially 60 ºC, was progressively decreased by 1 ºC until an annealing temperature of 54 ºC was reached. The last cycle, i.e. 45 s at 95 ºC, 1 min at 54 ºC, and 2 min at 72 ºC, was repeated 25 times. PCR products were analyzed on a 1% agarose gel.

Myosin Expression Levels—To determine the amount of myosin expressed by the transgenes in the Mhc10 background, one-dimensional SDS-PAGE was performed (26). At least 3 upper thoraces were individually homogenized in separate 60-μl aliquots of sample buffer. Ten μl of homogenate from each sample was loaded into a single lane on a 7.5% gel. Coomassie Blue-stained gels were digitally photographed (Eagle Eye II, Stratagene), and relative protein amounts were determined using gel-scanning macros available with the public domain NIH Image software package. The myosin to actin ratio for each lane was determined and normalized to that of wild type (yw) flies.

Protein Isolation and Purification—Myosin was isolated as described by Swank et al. (19). Briefly, 120 sets of dorsolongitudinal IFMs (DLMs) were dissected from Mhc10 Drosophila expressing the myosin transgene of interest. After incubation in a glycerol and Triton X-100 solution, myosin was extracted and purified by a series of high salt suspensions and low salt precipitations. Myosin purity was confirmed on a 10% polyacrylamide gel. Myosin protein amounts were quantified by absorbance at 280 nm using an extinction coefficient of 0.53 cm−1 for 1 mg ml−1.

Actin was isolated from dissected DLMs as described by Razzaq et al. (1999). Purified F-actin was resuspended in assay buffer (AB), which contained 25 mM imidazole, pH 7.4, 25 mM KCl, 4 mM MgCl2, 1 mM EGTA, and 1 mM diethotheitol (27). Actin was quantified by absorbance at 290 nm minus absorbance at 310 nm using an extinction coefficient of 0.62 cm−1 for 1 mg ml−1. For the motility assay, actin was labeled with Alexa Fluor 488 phallolidin (19).

For actin-activated ATPase assays, chicken actin was used instead of Drosophila actin because Drosophila actin yields were too low to be practical. Chicken pectoralis actin was prepared as in Pardee and Spudich (28). Aliquots of G-actin were frozen at −80 °C, and polymerized before use.

In Vitro Motility and ATPase Assays—Actin sliding and basal ATPase assays were performed on isolated myosin immediately following purification. Methods and analyses were identical to those previously (19). In some experiments smooth muscle tropomyosin (smTM) (chicken gizzard, Sigma) was bound to Drosophila IFM actin by mixing at a 1:1 molar ratio. In this case, smTM (100 nM) was included in the solutions added to the flow cell after actin addition to prevent smTM dissociation from actin. Motility assays were conducted at 22–23 °C, and filament movement was recorded onto videotape.

Actin-activated ATPase assays were performed in 0.15 ml of assay solution consisting of 10 mM imidazole, pH 6.0, 20 mM KCl, 0.1 mM CaCl2, 1 mM MgCl2, 1 mM [γ-32P]ATP, and myosin to a final concentration of 70 nM. Calcium in this buffer does not affect basal or actin-activated Mg-ATPase rates. Actin concentration was varied from 0 to 2 μM. Myosin was preincubated with actin in the assay solution (minus ATP) for 10 min at 22–23 °C. The reaction was started with the addition of 15 μl of 10 mM [γ-32P]ATP and was terminated 30 min later with the addition of 50 μl of 1.8 N HClO4. This reaction period was determined to lie in the linear portion of the time versus P, production curve. P, production was monitored as described in Swank et al. (19). For each myosin form, Vmax and actin-activated K0.5 values for actin were computed by fitting all data points from one myosin preparation with the Michaelis-Menten equation. Vmax and K0.5 values from multiple preparations were averaged (n of at least 3) to give mean ± S.E.

Ultrastructure Analysis—To determine the effect of the exon 3 transgenes on assembly and stability of myofibrils, we analyzed cross-sections and longitudinal thin sections of IFMs using transmission electron microscopy as described previously (29).

Flight Assays—Flight ability was assayed by observing whether a fly was capable of flying up (U), horizontally (H), down (D), or not at all (N) when released in a Plexiglas flight chamber. An attractant light was suspended above the chamber as wild type flies normally fly up toward a light source (30). Flight index equals 6U/T + 4H/T + 2D/T + 0NT, where T is the total number of flies tested. At least 100 flies were assayed for flight ability from each line of each transgene expressed in the Mhc10 null background. All flight assays were performed at 22 ºC with 2-day-old flies. Student’s t test and one-way analysis of variance were used for most statistical analysis with p values <0.05 considered statistically significant.

RESULTS

Generation of Transgenic Lines—We generated multiple transgenic Drosophila lines expressing chimeric myosins with alternative exon 3 regions exchanged between the EMB and IIF isoforms. We injected 522 yw embryos with the EMB-3b P element vector to produce over 10 independently transformed lines. We injected IIF-3a into 730 yw embryos to yield 3 independently transformed lines. The probability of a P element-mediated insertion event decreases exponentially as the size of the plasmid increases (23). Thus the difference in transformation frequency between the IIF-3a plasmid (30.5 kb) and EMB-3b (17.5 kb) was expected. We also used the embryo injection protocol to produce three additional EMB lines (Mhcemb) that are identical to those described in Wells et al. (20) (Table I).

One of the three IIF-3a transgene insertions mapped to the third chromosome. This makes it useful for MHC studies, as the transgene can readily be crossed with stocks carrying Mhc mutations on the second chromosome. The other two lines were not useful in that one was homozygous lethal and the other mapped to the second chromosome. We generated an additional line by genetically moving (jumping) the P element construct from the second chromosome to the third. Lines with trans-
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Verification of Transgene Transcription and Protein Expression—We confirmed that the Mhc transgenes were transcribing the appropriate mRNA by using RT-PCR. cDNA created from RNA isolated from wild type IFM contains alternative exons 3b and 7a, whereas cDNA from the IFM of EMB flies contains exons 3a and 7d. cDNA created from RNA isolated from the IFM of IFI-3a flies was checked to verify the presence of embryonic exon 3a with adult exon 7d, a combination not found in IFM of IFI-3a flies was checked to verify the presence of embryonic exon 3a with adult exon 7d, a combination not found in IFM of IFI-3a flies. cDNA created from RNA isolated from wild type IFM contains alternative exons 3b and 7a, (Fig. 2, lanes 3–5). The 3a primer verified that exon 3 was exchanged, and the 7d primer verified the IFI MHC background. A band was not produced in PCRs using primers to exons 3b and 7a (Fig. 2, lanes 3–5). The expected inclusion of the IFI-specific versions of exons 9, 11, and 15 was verified by RT-PCR and restriction enzyme digestion (data not shown).

cDNA generated from RNA isolated from EMB-3b lines produced the expected band at 800 bp in PCRs using primers to exons 3a and 7d (Fig. 2A, lanes 1 and 2). The 3a primer verified that exon 3 was exchanged, and the 7d primer confirmed the EMB background. A band was not produced in PCRs using primers to exons 3b and 7a (Fig. 2B, lanes 3–5). The expected inclusion of the IFI-specific versions of exons 9, 11, and 15 was verified by RT-PCR and restriction enzyme digestion (data not shown).

Verification of Embryonic Exon 3—We sequenced Mhc cDNA cloned from IFM of EMB-3b flies to verify that the flies expressed the EMB version of exon 3 (3a) in conjunction with the IFI version of exon 7 (7d). A 790-bp band in the EMB-3b lanes verified that the flies expressed the IFI version of exon 3 (3b) in conjunction with the EMB version of exon 7 (7d).

Verification of Transgene Transcription and mRNA Synthesis by RT-PCR—A, primers to exons 3a and 7d were used in PCRs with cDNA templates prepared from mRNA from IFM of different transgenic fly lines. Lanes correspond to the following Drosophila lines or markers: lane 1, yw/; lane 2, Mhc10; lane 3, IFI-3a line 49; lane 4, IFI-3a line 7; lane 5, IFI-3a line 223; lane 6, 1-kb DNA ladder (1,018-bp fragment, 506-bp fragment); lane 7, EMB-3b line 72; lane 8, EMB-3b line 91; lane 9, EMB-3b line 157. The 800-bp band in the IFI-3a lanes confirmed that these flies expressed the EMB version of exon 3 (3a) in conjunction with the IFI version of exon 7 (7d). B, primers to exons 3b and 7a were used in PCRs with cDNA templates prepared from the same Drosophila lines as above. A 790-bp band in the EMB-3b lanes verified that the flies expressed the IFI version of exon 3 (3b) in conjunction with the EMB version of exon 7 (7d).

Basal and Actin-activated ATPase Rates—Similar to our initial measurements (31), basal Ca- and Mg-ATPase rates for IFI were 1.7- and 2.1-fold faster than EMB myosin, and IFI actin-activated ATPase rates were almost 2-fold greater than EMB (Table II). The degree of actin activation above basal was 11-fold for IFI and 15-fold for EMB.

All ATPase rates for IFI-3a were markedly decreased compared with IFI rates. Basal Mg-ATPase and Ca-ATPase rates decreased to 50 and 34% of IFI, respectively, and Vmax values of actin-activated ATPase decreased to 42% of IFI (Table II and Fig. 3A). These ATPase rates are similar to those of EMB but lower than EMB for Ca-ATPase. The switch appeared to decrease K0.5 for actin, but this was not statistically significant (p = 0.167, Student’s t test). These results suggest that the embryonic exon 3 region, 3a, is sufficient to set EMB ATPase rates.

Surprisingly, the IFI version of exon 3 had little or no influence on ATPase rates when incorporated into EMB. The basal Mg-ATPase and Vmax values of actin-activated ATPase of EMB-3b were not significantly different from that of EMB, although EMB-3b Ca-ATPase rates were significantly increased compared with EMB (Table II and Fig. 3B). The K0.5 values of EMB for actin were not altered by the exon 3 exchange.

In Vitro Actin Sliding Velocity—IFI myosin moves actin 9-fold faster than EMB (19). To determine whether the exon 3 region influences this property, we measured the rates at which the two chimeras moved actin in the in vitro motility assay. The velocity at which EMB-3b propelled actin filaments was 5.4-fold faster than EMB (Fig. 4A and Table III). However, actin propelled by IFI-3a moved at the same velocity as IFI-propelled actin (Fig. 4B).

Previously, we observed that binding smTM to actin (smTM-actin) increased the velocity at which EMB myosin moved actin filaments by 5.7-fold. However, smTM-actin moved at the same velocity over IFI myosin or slightly slower compared with actin (19). To determine whether this effect is linked to the exon 3 region, we tested the effect of smTM-actin on velocity in motility assays with IFI-3a and EMB-3b myosin. smTM-actin moved 1.4-fold faster over EMB-3b myosin than actin alone (Table
lar hyperbola, transgenic line were fit with the Michaelis-Menten equation (rectangular hyperbola, y = a/bx + x). All basal Mg-ATPase levels and actin-activated Mg-ATPase rates of IFI-3a myosin compared with IFI did not render the IFI-3a lines flightless. However, flight indexes of the EMB-3b lines were all significantly lower (p < 0.02, Student’s t test). Interestingly, the decrease in actin velocity of EMB-3b myosin was increased to more closely resemble IFI velocity. Actin-stimulated myosin Mg-ATPase activity. The rate at which myosin hydrolyzed Mg-ATPase was measured over an actin range of 0–2 μM. A, IFI and IFI-3a actin-activated Mg-ATPase rates, B, EMB and EMB-3b actin-activated Mg-ATPase rates. All data points from at least three preparations are plotted. Basal Mg-ATPase levels were subtracted prior to plotting the data. All data points for each transgenic line were fit with the Michaelis-Menten equation (rectangular hyperbola, y = a/bx + x).

In contrast, both actin and smTM-actin moved over IFI-3a myosin at the same velocity. This result demonstrates that exon 3 is not responsible for the smTM effect.

IFI Myofilament Assembly and Ultrastructure Maintenance—As is the case for expression of the EMB isoform in IFM (20), expression of the chimeric myosins did not disrupt myofibrillar assembly. Furthermore, the myofibrillar ultrastructure of each of these lines was identical to wild type at the pupal stage (compare Fig. 5, A and B) instead of resembling the structurally distinct embryonic muscle.

However, the two chimeric transgenes differed dramatically in their impact on myofibril ultrastructure maintenance. IFI-3a myofilament protein arrangement remained highly ordered and identical to IFI for at least 2 weeks following eclosion (Fig. 5, C and D). In contrast, EMB-3b myofibrils showed disrupted filament packing in 2-day-old Drosophila, and the normally rigid hexagonal arrangement of thick and thin filaments was disturbed (Fig. 5E). The characteristic oval shape of the periphery of the myofibril was less defined. Viewed longitudinally, the EMB-3b myofibrils appeared cracked and frayed because of gaps between thick and thin filaments and/or loss of thick and thin filaments. This phenotype became progressively worse with age (Fig. 5F).

However, compared with EMB, EMB-3b myofibril ultrastructure was markedly improved (compare Fig. 5, E and F with G and H). EMB expression in IFM causes severe cracking and fraying of myofibrils and disruption of thick and thin filament packing. The basic sarcomere pattern is disrupted at 2 days, as Z-line material alignment is abnormal. Whereas EMB-3b myofibrils showed similar structural degeneration, this occurred at a much slower rate than EMB. For example, at 2 weeks of age, myofibrils in EMB fibers were no longer discernible; only a random mixture of thick and thin filaments remained (Fig. 5H). In contrast, at 2 weeks of age distinct sarcomeres were still discernible in EMB-3b fibers, with the majority of thick and thin filaments still in their normal location (Fig. 5F). Thus, the exchange of the IFI exon 3 region into EMB partially rescues the degenerative ultrastructural phenotype.

Flight Ability—Drosophila expressing the EMB isoform in an Mhc10 background are flightless (20). Incorporating the exon 3 region from IFI did not rescue flight (Fig. 6), even though actin velocity of EMB-3b myosin was increased to more closely resemble IFI velocity. Interestingly, the decrease in ATPase rates of IFI-3a myosin compared with IFI did not render the IFI-3a lines flightless. However, flight indexes of the IFI-3a lines were all significantly lower (p = 0.002, one-way analysis of variance) than flight indexes of IFI lines as follows: IFI-3a line 223, 3.0 ± 0.2; line 7, 2.9 ± 0.3 versus IFI line 1, 3.9 ± 0.2; IFI line 2, 4.0 ± 0.2. Specifically, the ability of IFI-3a Drosophila to fly up appears impaired compared with IFI (Fig. 6).

**DISCUSSION**

Drosophila MHC isoforms EMB and IFI differ structurally in only four regions of the myosin S-1 head (13). The IFI isoform moves actin in vitro 9-fold faster than EMB, has 1.7-fold higher actin-activated ATPase Vmax values, and 2-fold higher basal Mg- and Ca-ATPase rates (Table II) (19, 31). Making use of the powerful genetic techniques available with the Drosophila MHC system, we now show that the exon 3-encoded variable region near the N terminus can influence all the properties known to differ between the two native isoforms.

The two alternative versions of exon 3 did not affect the same functional property when exchanged between the native iso-
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Fig. 4. Actin sliding velocity when propelled in vitro by myosin from transgenic Drosophila. A, actin sliding velocity increases when the IFI exon 3 region is exchanged into the EMB isoform (EMB-3b). B, IFI and IFI-3a do not differ in actin sliding velocity.

Table III

In vitro actin sliding velocity

| Isoform   | Actin velocity (μm s⁻¹) | smTM-actin velocity |
|-----------|--------------------------|---------------------|
| IFI       | 6.4 ± 0.7 (4)            | 5.7 ± 0.8 (4)       |
| IFI-3a    | 6.5 ± 0.7 (5)            | 6.2 ± 0.6 (3)       |
| EMB       | 0.7 ± 0.1 (4)            | 4.0 ± 0.7 (5)       |
| EMB-3b    | 3.8 ± 1.0 (4)            | 5.2 ± 0.8 (3)       |

* Statistically different from EMB with actin (p < 0.001, Student’s t test).

† Statistically different from EMB with actin (p = 0.001, Student’s t test).

‡ Statistically different from EMB-3b with actin (p = 0.045, paired t test).

forms. The EMB version of exon 3 decreased all IFI ATPase rates, but did not influence IFI actin sliding velocity. The IFI version of the exon 3 region increased EMB actin sliding velocity but did not alter EMB ATPase rates, except for the non-physiological Ca-ATPase rate. These results suggest that the exon 3 region can independently influence at least two kinetic rate constants of the actomyosin cross-bridge cycle.

The EMB version of the exon 3-encoded region converted the actin-activated ATPase rates of IFI to EMB levels. This indicates that 3a may be the only EMB-specific region needed to set EMB ATPase kinetics. In a previous study of the variable region encoded by exon 11, termed the “converter” domain, we were surprised to find that the EMB converter increased the Vₘₐₓ value of the actin-activated ATPase when exchanged into the IFI isoform (31). The exon 3a region, and/or an EMB-specific version of the other 2 variable regions encoded by exons 7 and 9, must set EMB ATPase rates by overriding the opposing effect of the EMB converter on Vₘₐₓ values. From its location in the molecule (Fig. 1), it is plausible that the exon 7-encoded region might also influence ATPase rates (13). Future studies of regions encoded by exons 7 and 9 plus studies of coordinately exchanged alternative regions will determine whether exon 3a is the only exon that can specify EMB ATPase rates.

The values obtained in the actin-activated Mg-ATPase assays should be interpreted cautiously in light of the fact that these assays were performed with chicken actin, due to the technical limitations of isolating large amounts of actin from Drosophila IFM. However, the 2-fold difference in the actin-activated Mg-ATPase rate for IFI-3a compared with IFI is the same fold difference seen in the absence of actin. This strongly suggests that the reduced ATPase activity is a change in the inherent properties of the chimeric myosin molecule that is independent of actin isoform present.

The EMB version of the exon 3-encoded region increased EMB in vitro actin sliding velocity 5-fold (from 0.7 to 3.8 μm s⁻¹). This value is still lower than the velocity of IFI, i.e. 6.4 μm s⁻¹. Thus at least one more IFI exon needs to be coordinately exchanged along with exon 3 into EMB to achieve IFI actin velocity. Previously, we found that alternative exon 11 had a major impact on actin velocity (32). Substitution of the IFI converter into EMB increased actin velocity to 5.4 μm s⁻¹ (32). Simply adding the actin sliding velocity for the IFI exon 3 region in the EMB backbone (3.8 μm s⁻¹, Table II) to the 4.7 μm s⁻¹ augmentation observed with the IFI converter would be more than sufficient to increase actin velocity to that of IFI.

Interestingly, the EMB version of the exon 3-encoded region (3a) did not decrease motility when inserted into IFI, as one might have expected based on our results with the EMB-3b chimera. Thus either exon 3a has no influence on velocity or another alternative exon must be exchanged in concert with exon 3a for it to decrease actin velocity. Perhaps the strong positive influence of the IFI converter on velocity (32) inhibits exon 3a from decreasing the actin sliding velocity of IFI. In support of this concept, a recent study (33) suggests that portions of the exon 3- and 11-encoded areas interact during cross-bridge steps involved in ADP release, steps thought to set unloaded actin velocity.

All muscle types studied so far have similar ATPase to muscle shortening velocity ratios, i.e. a tightly “coupled” positive correlation is observed when ATPase activity is plotted against maximum muscle shortening velocity (34). Similarly, vertebrate myosin isoforms have a tightly coupled ATPase to actin sliding velocity ratio (1). Our exon 3 chimeras appear to alter this correlation. IFI-3a had a decreased actin-activated ATPase rate without a corresponding decrease in actin sliding velocity compared with IFI, whereas EMB-3b had a much higher actin sliding velocity than EMB without a corresponding increase in Vₘₐₓ of actin-activated ATPase activity. Furthermore, we found previously (31) that the chimeras made by exchanging alternative versions of exon 11 between IFI and EMB are also uncoupled. Of these 4 “uncoupled” chimeras, only EMB-3b is native to Drosophila as it is expressed in a few of the embryonic body wall muscles (17). If myosin chimeras with uncoupled ATPase to velocity ratios are not typically expressed, this
would support a selective pressure for tight coupling of myosin ATPase and velocity. Alternatively, the ATPase to velocity ratios of the IFI or EMB isoform(s) may be atypical, resulting in apparent uncoupling of some chimeras. ATPase and velocity measurements of other native Drosophila myosin isoforms are needed to address this issue.

The Drosophila system not only permits insight into the effect of structural changes on the molecular properties of myosin but also has the unique advantage of showing how changes in myosin influence muscle ultrastructure and function. Based on our structural examination of the chimeric exon 3 IFMs, and previous work with exon 11 chimeras (32), we conclude that when overall transgenic myosin properties are not drastically different from IFI, the result is normal myofibril ultrastructure and a functional muscle capable of supporting flight. Expressing IFI-3a instead of IFI myosin caused no obvious ultrastructural abnormalities and resulted in only a slight decrease in Drosophila flight ability at 22 °C. However, the impaired ability to fly up suggests that the decreased ATPase rate results in lower power generation by the IFMs. Whereas myofibrils in the EMB-based transgenics (EMB, EMB-3b, and EMB-IC) show normal assembly properties, their degeneration and failure to support flight indicate that the myosin isoforms are incompatible with the contractile protein complement and architecture of the IFMs. Alterations in myo-
sin kinetics and abnormal actomyosin interactions likely account for the observed degeneration rather than disuse atrophy, because mutants that lack contractile function maintain wild type ultrastructure (35, 36).

Structural variation in exon 3 may influence the mechanical properties of muscle fibers and the biochemical and biophysical properties of isolated myosin by changing the mechanical or kinetic properties of myosin. It is unlikely that the exon 3 exchange influences myosin step size, although this could have accounted for the increased actin sliding velocity of EMB-3b. Previously, we found no difference in myosin unitary step size between the IFI and EMB isoforms (19) or between IFI and IPI-EC (31). Rather, different amino acid interactions from alternative versions of exon 3 regions likely influence the rates of local myosin conformational changes associated with rate-limiting cross-bridge steps. The rate-limiting steps of the cycle are generally thought to be release of products (2). Although the exon 3 region is close to the nucleotide-binding site, it is not sufficiently close to directly influence product release. Instead, it probably indirectly influences local structural changes that are linked to product release. There is ample evidence for discrete movements of several myosin subdomains, some of which have been correlated with specific steps of the cross-bridge cycle (2, 37). Because the exon 3 exchanges caused independent changes in ATPase and velocity, the two versions of exon 3 must be able to influence independently at least two separate myosin conformational changes. The EMB version of the exon 3 region likely affects a conformational change associated with detachment and ADP release, based on optical spectroscopic signals (38). Furthermore, Burghardt et al. (40) propose that amino acids 84 and 85 (encoded by exon 3) interact with the converter region and/or lever arm during or following the power stroke, based on optical spectroscopic signals from extrinsic probes bound to rabbit S1. Prior to the MgADP state, exon 3 only interacts with other portions of the catalytic domain, but in the MgADP and rigor states, it is in close proximity to the converter domain and the portion of the lever arm adjacent to the catalytic domain.

It will be interesting to determine whether other organisms show functional variation in the same structural regions that are encoded by alternative exons in Drosophila myosin. Animals that require very fast myosin isoforms, approaching the speed of the flight muscle isoform, may use similar mechanism and would be good candidates for such investigations. Our work and that of others (41–43) indicate that modifications of other structural domains, in addition to loops 1 and 2, can produce similar alterations in myosin kinetics. Thus one must be cognizant that fine-tuning of the myosin motor may not be accomplished by the same mechanism in different organisms or, indeed, in different isoforms of the same organism.

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