Suppression of Muscle Hypercontraction by Mutations in the Myosin Heavy Chain Gene of Drosophila melanogaster

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ABSTRACT

The indirect flight muscles (IFM) of Drosophila melanogaster provide a good genetic system with which to investigate muscle function. Flight muscle contraction is regulated by both stretch and Ca$^{2+}$-induced thin filament (actin + tropomyosin + troponin complex) activation. Some mutants in troponin-I (TnI) and troponin-T (TnT) genes cause a “hypercontraction” muscle phenotype, suggesting that this condition arises from defects in Ca$^{2+}$ regulation and actomyosin-generated tension. We have tested the hypothesis that missense mutations of the myosin heavy chain gene, Mhc, which suppress the hypercontraction of the TnI mutant held-up² (hdp²), do so by reducing actomyosin force production. Here we show that a “headless” Mhc transgenic fly construct that reduces the myosin head concentration in the muscle thick filaments acts as a dose-dependent suppressor of hypercontracting alleles of TnI, TnT, Mhc, and flightin genes. The data suggest that most, if not all, mutants causing hypercontraction require actomyosin-produced forces to do so. Whether all Mhc suppressors act simply by reducing the force production of the thick filament is discussed with respect to current models of myosin function and thin filament activation by the binding of calcium to the troponin complex.

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The indirect flight muscles (IFM) of Drosophila melanogaster provide a powerful genetic system with which to understand muscle function, structure, and development. As flight is not required for survival under laboratory conditions, many mutants have been obtained in the genes for the major sarcomeric proteins by selection for flightlessness or for a “wings-up” phenotype (see Bernstein et al. 1993; Vigoreaux 2001). A number of Drosophila muscle protein gene mutations that give a dominant or recessive flightless phenotype produce an additional recessive phenotype in which IFM fibers undergo an auto-destructive contraction after the muscles have developed normally (Kronert et al. 1995). This leads to separation and accumulation of fiber material to one or both attachment sites or to fiber bunching with detachment from both ends. Intracellularly, the phenotype is very variable but is characterized by disruption of the myofibrillar lattice as well as by bulging and shortening of individual sarcomeres. In some mutants (e.g., Mhc$^{33}$ and fln$^{n}$) the phenotype includes muscle protein proteolysis (Kronert et al. 1995; Reedy et al. 2000).

As the term “hypercontraction” has been used in muscle pathology to describe similar types of muscle damage arising from excessive contraction in mutant Caenorhabditis elegans (Korswagen et al. 1997; Garcia-Anoveros et al. 1998), in reperfused rat hearts (Duncan 1987; Bhatti et al. 1989; Monticello et al. 1996), in Duchenne muscular dystrophy (Valentine et al. 1989; Tay et al. 1992; Cozza et al. 2001), and in human muscle injuries (Roth et al. 2000; Finol et al. 2001), we have defined this Drosophila phenotype as “hypercontraction.” We make the important distinction, implicit in the above, that the term is used only where the muscles develop normally, or nearly so, before the muscle damage occurs.

Extant mutants exhibiting the phenotype include held-up² [hdp²; an allele of the wings-upA gene, which encodes Troponin-I (TnI)], ufp$^{001}$ [an allele of the upheld gene, which encodes Troponin-T (TnT)], some alleles of the IFM-specific actin gene, Act88F (An and Mogami 1996), three alleles of the myosin heavy chain gene, Mhc$^{c}$, Mhc$^{c'}$, and Mhc$^{26}$ (Kronert et al. 1995), and fln$^{n}$, an allele of the flightin gene, fln (Reedy et al. 2000). The fact that mutant alleles of the TnI and TnT genes produce hypercontraction suggests that defects in Ca$^{2+}$ regulation can produce this phenotype. The recovery of three missense mutants of the Mhc gene (Kronert et al. 1995) and, more recently, of an allele of flightin (Reedy et al. 2000), all of which cause hypercontraction, suggests that a single explanation for hypercontraction is unlikely. The extant hypercontracting Mhc alleles are restricted to a small, five-amino-acid region of the light meromyosin domain. It is through this domain that...
myosin dimerizes by the assembly of α-helical coiled-coil rods, which subsequently polymerize to form thick filaments. Flightin is likely a thick filament protein associated with the myosin rod domain (Vigoreaux et al. 1993; Reedy et al. 2000). These latter mutants suggest that structural defects within the sarcomere can also lead to hypercontraction. Since actin is the major component of the thin filament, the Act88F alleles that cause hypercontraction could produce either defects in the tropomysosin (Tn-Tm) complex regulation or, like the two thick filament proteins, structural defects of the sarcomere.

**TABLE 1**

Muscle fiber phenotypes caused by thin and thick filament hypercontracting mutations

<table>
<thead>
<tr>
<th>Genotype (protein)</th>
<th>Before 78 hr APF</th>
<th>After 78 hr APF</th>
<th>At eclosion</th>
<th>&gt;2 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>hdp² (TnI)</td>
<td>Normal</td>
<td>HC</td>
<td>HC</td>
<td>HC</td>
</tr>
<tr>
<td>up² (TnT)</td>
<td>Normal</td>
<td>HC</td>
<td>HC</td>
<td>HC</td>
</tr>
<tr>
<td>Act88F²¹⁶C (Actin)</td>
<td>Detached posterior fiber ends</td>
<td>HC initiated from posterior ends</td>
<td>PSC (one end detached)</td>
<td>HC</td>
</tr>
<tr>
<td>Act88F²¹⁶Q (Actin)</td>
<td>Loosely attached fiber ends</td>
<td>PSC detached ends</td>
<td>PSC (bunched)</td>
<td>HC</td>
</tr>
<tr>
<td>Act88F²³³O (Actin)</td>
<td>Wiggly fibers</td>
<td>Wiggly and detached fiber ends</td>
<td>PSC (bunched)</td>
<td>HC</td>
</tr>
<tr>
<td>Act88F²⁶⁶C (Actin)</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>PSC (pulled at posterior end)</td>
</tr>
<tr>
<td>Mhc⁹² (Myosin)</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>HC</td>
</tr>
<tr>
<td>flw⁷ (Fln)</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>HC</td>
</tr>
</tbody>
</table>

HC, hypercontracted fibers; PSC, partially hypercontracted fibers. All the genotypes scored were homo- or hemizygous (X-linked).

MATERIALS AND METHODS

**Fly strains:** All chromosome and gene symbols unless specifically mentioned are as described in FlyBase (http://flybase.bio.indiana.edu/). Canton-S was used as the control in all the experiments unless specified. The Y⁵⁷ and Y⁹⁷ transgenic lines express a myosin heavy chain polypeptide lacking the head (or motor) domain and were gifts from S. I. Bernstein and R. M. Cripps (Cripps et al. 1999). The Mhc suppressors of hdp², Mhc²¹, Mhc²⁰¹, Mhc²⁰⁷, and Mhc²⁰² were obtained from A. Ferrus. flw⁷ is described in Reedy et al. (2000). All flies used for experiments are 2–4 days old unless otherwise indicated. All stocks and crosses were maintained at 25°C on a yeast-sugar-agar medium.

**Isolation, mapping, and sequencing of dominant Mhc suppressor mutations:** Ethyl methanesulfonate (EMS) mutagenesis and isolation of dominant suppressors of the hdp² wings-up phenotype were as described in Prado et al. (1995). Mutants mapping to the second chromosome were assigned as Mhc suppressors by noncomplementation with lethal Mhc alleles, including Df(2L)H20 (except Mhc²⁰²<X>), and designated as Mhc⁸<X>, where X is an allele identifier. They are referred to as St(2)X mutations in the text. Oligonucleotide primers used...
Figure 1.—Polarized light micrographs of hypercontracting IFM. (A) IFM of wild-type fly. A star indicates one of the DLMs. (B) hdp2 showing IFM hypercontraction; fibers are bunched to the cuticle. Arrow indicates the bunched fibers at one end of the thorax. (C) DLMs of the Act88FR28C fly just before the eclosion showing partially hypercontracted phenotype. Note the initiation of hypercontraction at the attachment site of the fibers (arrowhead). (D) Late Act88FR28C showing the DLMs pulled to the center (arrows). (E) up101 showing the partially pulled DLMs (arrowhead). (F) hdp2/Y; Y97 partial suppression of the hdp2 phenotype with a copy of the myosin headless construct. (G) hdp2/Y; Mhc10+/H11001 showing that a reduction in MHC partially suppresses the hdp2 phenotype. (H) hdp2/Y; Mhc10+/+, Y97, a copy of Mhc10, and the headless myosin construct completely suppress the hdp2 hypercontraction (star). In all frames the anterior fly thorax is at the left corner, and the dorsal side is toward the top. All flies are 2–4 days old unless otherwise indicated and at the same magnification. Bar, 0.125 mm.

RESULTS

The hypercontracted IFM phenotype: Hypercontraction can take a number of forms. Table 1 summarizes the thick and thin filament protein mutants showing hypercontraction and the developmental stage at which this phenotype occurs. In many mutants hypercontraction ends with the muscles parted, or seemingly so, in the middle with the bulk of the fiber bunched at one or both attachment sites (Figure 1B). In other mutants the fibers separate from the attachment sites and bunch in the middle of the fiber (Figure 1, C and D). On the basis of these characteristics, only a handful of IFM mutants are classified as hypercontracted. In some mutants, Act88FR28C and Act88FR330Q (Figure 1D), the phenotype is less extreme in terms of either the degree of muscle shortening or the number of fibers showing the...
TABLE 2

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Wing phenotypes (%)</th>
<th>Muscle phenotypes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Upheld    Down    Normal</td>
</tr>
<tr>
<td>hdp2/Y</td>
<td>50</td>
<td>100        0         0</td>
</tr>
<tr>
<td>up101/Y</td>
<td>50</td>
<td>86         6         8</td>
</tr>
<tr>
<td>Mhc13</td>
<td>50</td>
<td>56         40        4</td>
</tr>
<tr>
<td>fln0</td>
<td>50</td>
<td>16         24        60</td>
</tr>
<tr>
<td>Mhc10/Mhc10</td>
<td>50</td>
<td>52         42        6</td>
</tr>
<tr>
<td>Mhc10/H11001</td>
<td>50</td>
<td>4          0         96</td>
</tr>
<tr>
<td>Y97/Y97</td>
<td>45</td>
<td>56         30        14</td>
</tr>
<tr>
<td>Y97/+</td>
<td>32</td>
<td>0          44        56</td>
</tr>
<tr>
<td>Mhc10/+; Y97/+</td>
<td>51</td>
<td>49         51        0</td>
</tr>
<tr>
<td>hdp2/Y; Mhc10/+; Y97/+</td>
<td>55</td>
<td>13         0         87</td>
</tr>
<tr>
<td>up101/Y; Y97/+</td>
<td>45</td>
<td>100        0         0</td>
</tr>
<tr>
<td>hyb10/Y; Y97/+</td>
<td>58</td>
<td>100        0         0</td>
</tr>
<tr>
<td>up101/Y; Mhc10/+; Y97/+</td>
<td>45</td>
<td>22         18        60</td>
</tr>
<tr>
<td>Mhc3+/Mhc20</td>
<td>72</td>
<td>76         24        0</td>
</tr>
<tr>
<td>Mhc3+/H11001</td>
<td>82</td>
<td>51         49        0</td>
</tr>
<tr>
<td>Mhc3/+; Mhc20</td>
<td>58</td>
<td>12         26        62</td>
</tr>
<tr>
<td>Y57; Mhc20/Mhc10; fln0/fbn0</td>
<td>50</td>
<td>20         24        56</td>
</tr>
</tbody>
</table>

All the genotypes are flightless. HC, hypercontracted muscle phenotype; PHC, partially hypercontracted; N, normal/suppressed muscle phenotype; n, number of flies scored.

phenotype (Figure 1E), and we refer to this as partial hypercontraction (Naimi et al. 2001).

For Mhc3, Mhc3+, Mhc20, and fln0, the hypercontraction phenotype develops progressively during the first 24 hr after adult eclosion (Kronert et al. 1995; Reedy et al. 2000); for hdp2, we have shown (Naimi et al. 2001) that the IFM form normally up to 78 hr after puparium formation (APF), when twitching of the pupal legs is first observed and the IFM begin to shorten and break, a process that is complete at, or just before, eclosion.

Why is this hypercontraction phenotype produced at different developmental stages? Although the development and maturation of myofibrils continue for a few hours after eclosion, it is likely that functional myofibrils are formed by 75 hr APF (Reedy and Beall 1995; Reedy et al. 2000). Thus the Tnl mutation, hdp2, causes a pupal development of the phenotype while the four mutants of two thick filament proteins, MHC and FLN, lead to the progressive adult phenotype. We have investigated whether this correlation extends to other hypercontracting muscle protein mutations. The up101 mutation shows normal development until 78 hr APF when, like hdp2 (Table 1), the IFM begin to hypercontract, a process that is complete by eclosion with the exception of a few flies that still remain partially hypercontracted (Figure 1E; Table 2). Actin Act88F268D and Act88F28C mutants show almost normal myofibril development until 78 hr APF with the initiation of fiber detachment from one end (Figure 1C), followed by fibers bunching to one end of the thorax (not shown) or in the middle (Figure 1D). Act88F268D develops “wavy” fibers but partially hypercontraction (Naimi et al. 2001). For Mhc6, Mhc13, Mhc19, and fln0, the hypercontraction percontracts only after 78 hr APF, while in Act88FR95C partial hypercontraction develops only after eclosion (Table 1).

Hypercontraction is suppressed by reducing the amount of functional myosin heads: The appearance of hypercontraction in muscles that have developed normally suggests a priori that forces developed by actomyosin crossbridges cause the damage. We began by extending the experiment of Beall and Fyrberg (1991) on the hypercontraction of hdp2, but rather than removing all the myosin (which also removes the thick filament and myofibrillar lattice), we used two “headless” Mhc-expressing transgenic lines, Y57 (on the first chromosome) and Y97 (on the third chromosome; Cripps et al. 2000). Thus the Tnl mutation, hdp2, causes a pupal development of the phenotype while the four mutants of two thick filament proteins, MHC and FLN, lead to the progressive adult phenotype. We have investigated whether this correlation extends to other hypercontracting muscle protein mutations. The up101 mutation shows normal development until 78 hr APF when, like hdp2 (Table 1), the IFM begin to hypercontract, a process that is complete by eclosion with the exception of a few flies that still remain partially hypercontracted (Figure 1E; Table 2). Actin Act88F268D and Act88F28C mutants show almost normal myofibril development until 78 hr APF with the initiation of fiber detachment from one end (Figure 1C), followed by fibers bunching to one end of the thorax (not shown) or in the middle (Figure 1D). Act88F268D develops “wavy” fibers but partially hypercontracts only after 78 hr APF, while in Act88F28C partial hypercontraction develops only after eclosion (Table 1).

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In combination with the IFM-specific Mhc20 null allele, the Y97 construct acts as a dose-dependent suppressor of hdp2 (Figure 1, F–H; Figure 2, E–J). In hdp2 flies, although the muscle and myofibrils develop normally before 78 hr APF, by eclosion the fibers are completely pulled apart (Figure 1B). Hypercontraction completely disrupts the myofibrillar lattice, leaving fields of disor-
ordered thick and thin filaments (Figure 2D) in which misaligned Z-bands and M-lines are seen. Few sarcomeres are seen and these are short (see Table 3) and often have a bulging appearance (Figure 2C). A slight suppression of the hypercontraction phenotype of hdp2 occurs due to hdp2; Mhc10/+ (Figure 1G and Figure 2, G and H). In Mhc10/+ flies the removal of one functional Mhc gene copy will lead to ~60% of wild-type myosin accumulation Overall genotypes that suppress the wings-up phenotype also suppress IFM hypercontraction, but in individual flies that is not invariably the case. Flies with hdp2/Y; Mhc10/+ or hdp2/Y; +/+; Y97 genotypes with partially suppressed hypercontraction show the full range of wing position from "wings up" to "wings held beside the abdomen" to "normal wing position" (Table 2), as do Mhc10/Mhc10 homozygotes, which never show hypercontraction. In general we find in these and other studies that the correlation between the wings-up and IFM hypercontraction phenotypes is poor.

**Mhc suppressor mutations of hdp2 hypercontraction:** Six dominant EMS-induced suppressor mutations of the hdp2 wings-up phenotype were recovered on the second chromosome from a screen of 25,000 progeny. Five mapped to the region between black (48.5) and cinnabar (57.5), which includes the Mhc gene. Four suppressors, Su(2)A, Su(2)B, Su(2)C, and Su(2)F, are Mhc alleles by their failure to complement the recessive lethality of Mhc1 and Df(2)H20. Mhc1 is a null allele due to a 1-kb internal deletion (O’Donnell and Bernstein 1988); Df(2)H20 spans from 36A8-9 to 36F1 (Steward and Nusslein-Volhard 1986), which includes the Mhc gene. Lethality of these four Mhc suppressors as homozygotes or in heterozygous combination with either Mhc1

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**Figure 2.**—Electron micrographs of hdp2 myofibrils suppressed by combinations of Mhc10 and the Y97 headless myosin transgenic insert. (A) Wild-type, longitudinal section (LS). (B) Wild-type, transverse section (TS). Myofibrils showing highly ordered myofibrillar lattices and borders. (C) hdp2 LS from newly eclosed flies. Sarcomere length is greatly reduced with streaming of Z- and M-bands (arrows). (D) hdp2 TS disruption of the myofibrillar borders and lattices (arrow); star indicates the region where thick and thin filament integrity is still preserved. (E and F) hdp2/Y; Y97 partial suppression of the hdp2 phenotype with a copy of the Y97 headless construct. Sarcomere structure is slightly improved. (G and H) hdp2/Y; Mhc10/+ . Partial suppression of the hdp2 phenotype by reduction of MHC. Breaks within the sarcomeric lattice are evident (arrow) and sarcomere length is shorter than normal. (I and J) hdp2/Y; Mhc10/+; Y97. One copy of Mhc10 and one copy of the Y97 headless myosin construct completely suppress the hdp2 hypercontraction. Sarcomere length is nearly normal and there are minimal breaks and disruptions of the myofibrils; however, increased gaps filled with sarcoplasmic material separate the myofibrils (arrowheads). M, M-band. Z, Z-Band. Myo, myofibril. Bar, 1 μm for all the LS (all are of same magnification) and 0.5 μm for all the TS (all are at same magnification).
TABLE 3
Sarcomere lengths (in micrometers) in suppressed muscle fibers

<table>
<thead>
<tr>
<th>Canton-S</th>
<th>hdp(^{2})</th>
<th>2B/+</th>
<th>hdp(^{2}); 2B/+</th>
<th>2F/+</th>
<th>hdp(^{2}); 2F/+</th>
<th>2D/2D</th>
<th>hdp(^{2}); 2D/+</th>
<th>hdp(^{2}); 2D/2D</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.9 ± 0.3</td>
<td>1.0 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td>2.4 ± 0.1</td>
<td>3.2 ± 0.2*</td>
<td>2.4 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td>1.8 ± 0.1</td>
<td>2.6 ± 0.2</td>
</tr>
</tbody>
</table>

LENGTHS OF THE SARCOMERE WERE MADE BY MEASURING THE DISTANCE BETWEEN NEIGHBORING Z-DISCs FROM ELECTRON MICROGRAPHS. MEAN LENGTHS ARE CALCULATED FROM MEASUREMENTS OF 30 SARCOMERES FROM THREE DIFFERENT SAMPLES.

*a Indicates nonsignificant difference (Student’s t-test) from the wild type. All other genotypes show significant differences in the length of the sarcomeres (at P < 1%) with respect to Canton-S and hdp\(^{2}\).

TABLE 4
Suppression of hypercontraction mutant phenotypes with newly isolated missense mutations in myosin head

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Upheld</th>
<th>Down</th>
<th>Normal</th>
<th>HC</th>
<th>PHC</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mhc(^{2})/+</td>
<td>41</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Mhc(^{2})/+*</td>
<td>56</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Mhc(^{2})/Mhc(^{2})</td>
<td>85</td>
<td>8</td>
<td>0</td>
<td>92</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>hdp(^{2})/Y; Mhc(^{2})/+</td>
<td>92</td>
<td>49</td>
<td>0</td>
<td>51</td>
<td>10</td>
<td>85</td>
<td>5</td>
</tr>
<tr>
<td>hdp(^{2})/Y; Mhc(^{2})/+</td>
<td>53</td>
<td>28</td>
<td>0</td>
<td>72</td>
<td>0</td>
<td>11</td>
<td>89</td>
</tr>
<tr>
<td>hdp(^{2})/Y; Mhc(^{2})/+</td>
<td>68</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>hdp(^{2})/Y; Mhc(^{2})/+</td>
<td>68</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>hdp(^{2})/Y; Mhc(^{2})/+</td>
<td>29</td>
<td>52</td>
<td>14</td>
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<td>100</td>
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</tr>
<tr>
<td>hdp(^{2})/Y; Mhc(^{2})/+</td>
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<td>0</td>
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<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>hdp(^{2})/Y; Mhc(^{2})/+</td>
<td>56</td>
<td>7</td>
<td>0</td>
<td>93</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Mhc(^{2})/Mhc(^{2})</td>
<td>55</td>
<td>4</td>
<td>38</td>
<td>58</td>
<td>0</td>
<td>35</td>
<td>65</td>
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<tr>
<td>Mhc(^{2})/+; (f)w(^{0})</td>
<td>50</td>
<td>22</td>
<td>0</td>
<td>78</td>
<td>0</td>
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<td>100</td>
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</tbody>
</table>

HC, hypercontracted; PHC, partially hypercontracted; N, normal/suppressed; n, number of flies scored.
*Flighted genotypes; all other genotypes are flightless.
Abnormal myofibrillar structure in hdp2 IFM suppressed by myosin suppressor mutations: Su(2)B completely suppresses fiber hypercontraction as seen in polarized light, except for some thinning of the dorsal-longitudinal muscle (DLM) fibers (Figure 4A) in a few flies. Electron micrographs of hdp2/Y; Su(2)B/+ flies (Figure 4, B and C) show a complete recovery of wild-type myofibrillar structure, although the sarcomere length remains slightly, but significantly, shorter than that of wild type (Table 3). hdp2 hypercontraction is completely suppressed by Su(2)F; sarcomere structure is comparable to wild type (Figure 4E) except that mean sarcomere length remains significantly shorter than that of wild type (Table 3) and a few days after eclosion muscle fibers become thin in many areas and contract (Figure 4D). In cross sections the periphery of the myofibrils show loosely packed thick and thin filaments (Figure 4F), quite similar to hdp2 myofibrils when they start to hypercontract (Figure 2D).

Su(2)D heterozygotes partially suppress hdp2. Half of the flies still show a wings-up phenotype and >80% of them have a partially suppressed muscle phenotype (Table 4). The fibers are thin and hypercontract from the posterior ends of the thorax (Figure 5A). Sarcomere structure is improved compared to hdp2 (Figure 5B), but sarcomere length is barely half that of wild type (Table 3) and at the periphery the myofibrillar lattice is perturbed, suggesting that hdp2 suppression is only partial at this level (Figure 5C). Homozygous Su(2)D completely suppresses hdp2 hypercontraction (Figure 5D; Table 4) but still fails to restore wild-type sarcomere length (Table 3); homozygous Su(2)D sarcomeres are significantly shorter than those of wild type. For 6–7 days after eclosion the muscle structure remains completely normal, but after this the central myofibrillar lattice becomes disordered (Figure 5, E and F), with characteristic Z-band streaming and gaps in the lattice. We have previously reported on a similar age-related progressive myopathy with hdp2 and the Tm2 suppressor mutant, D53 (Naimi et al. 2001). Electron micrographs of myofibrils from the Mhc suppressors as hetero- or homozygotes (in the case of 2D) without hdp2 have a completely wild-type appearance (data not shown).

Suppression by the myosin suppressors of other behaviors affected by hdp2: The hdp2 mutation is in the constitutively expressed exon 5 of the TnI gene. Its effects on other muscle groups have been observed as changes in behavior (adult jumping and walking and larval crawling and feeding), including an age-dependent myopathy of the legs, associated with ultrastructural defects (Naimi et al. 2001). All the new myosin suppressors fully suppress the effects of hdp2 on walking (Figure 6) and all other behaviors (data not shown), consistent with their position within constitutive Mhc gene exons. In Su(2)D heterozygotes the hdp2 walking behavior is partially suppressed while in homozygotes the suppression is complete. Suppressor Su(2)B itself completely in homozygotes (Table 4). Results from complementation analysis of Su(2)D and Mhc are ambiguous because Mhc+/+ flies are viable and show partial IFM hypercontraction (Nongthomba and Ramachandra 1999). Su(2)D maps to position 54.2 on chromosome 2, i.e., within the Mhc region. Sequencing of the constitutive and IFM-specific Mhc exons of homozygous flies following PCR showed a single base pair change (G→A) leading to an amino acid change from glycine to serine in codon 413 (416), close to the Su(2)B suppressor mutant (Figure 3). Su(2)E is a very weak suppressor for both wing and muscle phenotypes of hdp2, making it difficult to map. We have not included more detailed data on this mutation.

FIGURE 3.—The locations of the new suppressor mutations (blue) and those previously reported (red) within the atomic structure (2MYS.PDB) of chicken myosin S1 (Rayment et al. 1993b) depicted using Protein Explorer (http://www.umass.edu/microbio/chime/explorer). Suppressors Su(2)A, Su(2)B, and Su(2)C show the same amino acid change P401S (404 in chicken skeletal muscle myosin). Suppressor Su(2)F is the mutation A462T (465) in the same actin-binding loop. Su(2)D leads G413S (416). Mutations D1 (D625G) and D43 (A261T) are as described in Kronert et al. (1999) and occur in the actin-binding loop and near the ATP-binding pocket, respectively. Exon 7 (orange) is alternatively spliced in D41 as a result of a 2-bp insertion. D62 causes an eight-amino-acid deletion near the actin-binding loop and is not shown, as the loop is not seen in this atomic structure (see Kronert et al. 1999 for more details).
shows a progressive myopathy of the leg muscles, but hdp\textsuperscript{2}/Y; Su(2)B/+ walks as well as wild type (Figure 6).

**Headless transgenic construct and newly isolated myosin suppressors suppress other hypercontracting alleles:** If the IFM hypercontraction phenotype requires actomyosin force production, then \textit{a priori} all myosin suppressor mutations, including the transgenic headless myosin constructs, should suppress all those mutations that can generate the phenotype. We have tested this by making genotypes containing hypercontracting alleles and the Mhc suppressors and/or the headless myosin constructs. Both headless myosin constructs (Y97 and Y57) suppress the up\textsuperscript{100}, Mhc\textsuperscript{23}, and fln\textsuperscript{0} genes in a dose-dependent manner (Table 2). A single copy of Y57 is enough to suppress fln\textsuperscript{0} hypercontraction in the absence of any full-length endogenous MHC. Fiber morphology looks normal although with less birefringence, possibly a result of the highly disrupted myofibrillar organization at eclosion (data not shown). Such myofibrillar disruption is usually seen a few days after eclosion in fln\textsuperscript{0} mutants and then only after hypercontraction has occurred (Reedy et al. 2000). The fln\textsuperscript{0} fiber phenotype is also suppressed by a copy of the Mhc mutation Su(2)B (Table 4), indicating that force is required for fln\textsuperscript{0} hypercontraction.

**Other myosin suppressors of hdp\textsuperscript{2} also suppress up\textsuperscript{100}:** Kronert et al. (1999) described four Mhc suppressors of hdp\textsuperscript{2} in which the mutants localized within the myosin head domain. These mutations were dominant flightless in combination with hdp\textsuperscript{2} (Prado et al. 1995) but fully or partially flighted in heterozygous condition without hdp\textsuperscript{2}, indicating that the mutant myosins can assemble into normal myofibrils. We find that these mutations completely suppress the hypercontraction phenotype in flies up\textsuperscript{100} and up\textsuperscript{100},+/-, hdp\textsuperscript{2} (Table 5). The degree of suppression appears to relate to how severely a mutation affects the molecule; the intragenic deletion mutations (D41, D62) suppress more strongly than either point mutation (D1 or D45). Similar results were obtained with fln\textsuperscript{0}. All four myosin alleles suppress hyperconstr-
Figure 5.—Suppression of hdp2 hypercontraction by mutations in Mhc S1. (A) Polarized light micrograph of 2-day-old hdp2/Y; Mhc20+/+ flight muscles. Fibers are broken at many places, particularly in E and F of DLM fibers (star). (B and C) Electron micrographs of hdp2/Y; Mhc20+/+ myofibrils. Sarcomeres appear disrupted and shorter; the periphery of the myofibrils (Myo) is loosely packed with disrupted thick and thin filament lattices (arrowhead). (D) Polarized light micrograph of 10-day-old hdp2/Y; Mhc20+/+ showing completely normal fibers. (E and F) LS and TS of 10-day-old hdp2/Y; Mhc20+/+ showing age effects [2- to 3-day-old flies show completely normal myofibrils (not shown)]. Normal length sarcomeres show disruptions and gaps (arrows) that are clearly visible in the centers (arrows) of myofibrils (Myo), which is different from the hdp2/Y; Mhc2+/+ suppressor. Anterior-posterior axis running from left to right for the thoraces. Bar, 0.134 mm for A and D; 1 μm for B and E; and 0.5 μm for C and F.

Mhc suppressors cause hypercontraction in the absence of a functional troponin complex: The vou4A allele hdp1 causes a missplicing of the IFM-specific transcript and no functional TnI is produced (Barbas et al. 1993). The result is that the IFM fail to form and only small muscle remnants are seen in the thoraces of later pupal and adult stages (Figure 7A; Beall and Fyrberg 1991; Barthmaier and Fyrberg 1995). A priori an absence of TnI is expected to prevent thin filament inhibition of muscle contraction. Unregulated contraction during myogenesis will lead to the observed phenotype. We have used this circumstance to explore whether the MHC produced in myosin suppressor homozygotes produces sufficient force in vivo to cause the muscle destruction previously seen in hdp1 flies. These experiments were performed to resolve the issue (see below) as to whether the Mhc suppressor mutations affect only force production or the role of the myosin in the muscle activation processes itself, since some current models (reviewed in Gordon et al. 2000) suggest that binding of the myosin head to the actin of the thin filament plays a role in muscle activation. The results (Table 6; Figure 7) show that in hdp1 flies lacking myosin, hdp1/Y; Mhc2+/+; Mhc2+/+; Mhc2+/+; and by are as yet not completed, for any of the suppressors (Mhc3) and the Mhc2 null mutant. However, in hdp1 genotypes containing Mhc suppressors heterozygous with Mhc2+, i.e., hdp1/Y; Mhc2+/+, hypercontraction occurs (Figure 7C), although in some cases it is only partial (alleles Su(2)B, Su(2)F, and D62; see Figure 7B). These latter results suggest that these alleles show the largest reduction in force production so that, even in the absence of normal muscle regulation, in a single gene dose they can barely produce sufficient force to cause hypercontraction.

DISCUSSION

Our aim was to test the following proposals: that actomyosin force generation is required to produce the hy-
percontraction phenotype and that force reduction explains the action of Mhc suppressors. Additionally, we intended to distinguish between these proposals, also made by Kronert et al. (1999), and their alternative explanation that Mhc suppressors may indicate direct interactions between the myosin head and the TnI component of the troponin complex.

If the role of the myosin head in hypercontraction is to produce the forces that destroy the fibers in response to aberrant regulation of contraction or when the sarcomeric structure is compromised, then all hypercontracting mutants should be suppressed by reductions in myosin head concentration. In addition, suppression by Mhc alleles should be neither allele nor gene specific. The headless Mhc gene construct suppresses the hypercontraction muscle phenotypes of the hdp², up⁰, Mhc²⁰, and fln0 mutants in an Mhc gene dose-dependent manner consistent with this expectation. In addition, all of the Mhc suppressors described previously (Kronert et al. 1999) and newly described here suppress the hypercontraction of all these hypercontracting mutants. There is no evidence for gene or allele specificity in the known Mhc suppressors. Although the fiber morphology shows complete suppression by the headless myosin constructs, normal sarcomeric structure is not restored. This is not surprising since the headless myosin does not produce wild-type myofibrils when expressed alone or in combination with wild-type myosin (Cripps et al. 1999). Incomplete suppression of structural aspects suggests that reduced force production is not sufficient to allow normal myofibrillogenesis or to prevent microdamage within the sarcomeres.

If suppression of hypercontraction by Mhc alleles is by reduced force production, then the stronger suppressors should be those Mhc alleles with more extreme phenotypes. Suppression by the new Mhc alleles is complete by the criterion of fiber structure in each case and most are stronger suppressors than the earlier ones (Kronert et al. 1999) where suppression was incomplete. Some of these earlier suppressors now fly and homozygotes survive, a feature originally true only for D1 (Kronert et al. 1999). Stronger hdp² suppressors have a more extreme phenotype with respect to myosin function. So Su(2)B and Su(2)F have embryonic lethal and dominant flightless phenotypes, whereas Su(2)D is viable and flighted on its own, but only partially suppresses hdp². The D1 suppressor is at odds with this relationship between suppression and myosin dysfunction; it is homozygous viable and flighted, even allowing the flight of some hdp²/Y; D1/+ flies (Table 5).

The Mhc suppressor mutations all occur in the head domain (Figure 5). D1 (D625G, chicken myosin S1 numbering system) and D62 (a 24-bp in-frame deletion) are in the actin-binding loop (Kronert et al. 1999); Su(2)B (P404S) and Su(2)D (G416S) are located fairly close together in a region known to be involved in actin binding (Raymond et al. 1993a; Uyeda et al. 1994; Rovner et al. 1995). D45 (A261T) and D41 (a 2-bp insertion that affects splicing) cause changes near the ATP-entry and the ATP-binding sites (Kronert et al. 1999), while Su(2)F (A465T) is also close to the ATP-binding site. The mutant residues do not form the single cluster expected if they affect a specific binding of the myosin head to a component of the Tm-Tn complex, perhaps TnI, as suggested by Kronert et al. (1999), nor has any such interaction been detected in the large volume of research on thin filament regulation (see review by Gordon et al. 2000). However, all the mutant residues, including the new Mhc suppressors, are in head regions important for actin binding and nucleotide exchange/hydrolysis, supporting the argument that all the mutants affect the crossbridge cycle and force production, changes sufficient to explain suppression.

Su(2)B (P404S) is next to residue R405, a hot spot for mutations causing human hypertrophic cardiomyopathy (HCM). Arginine 405 is part of a myosin loop that could directly interact with actin (Raymont et al. 1995). Myosins from myopathy patients move actin filaments with decreased velocity in an in vitro motility assay (Cuda et al. 1993) and muscle fibers display diminished power output (Lankford et al. 1995), although more recent in vitro studies of the same HCM myosins.
showed enhanced myosin activity (PALMITER et al. 2000;
YAMASHITA et al. 2000). The partial suppression of hdp2
hypercontraction by Su(2)B suggests that mutations in
this region of the myosin molecule can reduce force in vivo, but do not directly address changed functions in
the HCM mutations.

An absence of clustering of suppressor mutations and
the fact that their effects can be explained by effects on
myosin ATP hydrolysis and actin interactions reducing
force production is not consistent with the proposed
direct interaction between myosin and TnI (KRONERT et al. 1999). However, current models (reviewed in GORDON et al. 2000) suggest that myosin is involved in both
force production and thin filament regulation. GEEVES
and LEHRER (1998) have developed a model of thin
filament regulation based on the kinetic studies of
MCKILLOP and GEEVES (1993) in which the Tm-Tn complex
can exist in three states on the F-actin thin filament
core. In the absence of calcium, TnI binding to actin
holds the Tm-Tn complex in the “closed” state in which
the myosin-binding site is occluded, preventing myosin
from binding actin. Calcium released into the muscles
following neural stimulation binds to TnC, which under-
goes a conformational change that alters its relationship
with TnI, resulting in release of TnI binding from actin.
This represents the “blocked” state, but in this state
small movements of the Tm/Tn across the F-actin sur-
face allow small numbers of myosin heads to bind F-actin, leading to the displacement of this complex to the
“open” state. In this state myosin heads can bind to
any available actin “target” site and muscle activation is
achieved. In this model, therefore, a small fraction of
myosin heads play an important role in the blocked to
open transition. In doing so they bind to F-actin. There
is no reason to assume that this binding is in any way
different from the binding of a myosin head that also
produces force. It is thus formally very difficult to deter-
mine whether the Mhc mutations that suppress hdp2 do
so by affecting regulation rather than force production.
The reduced ability of the myosin suppressor mutants
to hypercontract the IFM in the absence of a functional
regulatory system (hdp3) and any wild-type myosin cer-
tainly suggests that the suppressor mutations produce
less force, but does not allow us to deduce that this is
the primary effect of these mutants when acting as
suppressors. At present we cannot perform with Dro-
sophila proteins the type of sophisticated biochemical
and biophysical experiments needed to resolve this
issue. However, considerable progress has been made
recently with the purification and assay of Drosophila
IFM actin (RAZZAQ et al. 1999; SCHMITZ et al. 2000) and
myosin (SWANK et al. 2001, 2002), including ATPase
assays, in vitro motility, single molecule studies, and
rapid kinetics.

IFM hypercontraction appears to develop from either
misregulation of muscle contraction (hdp2, up300) or
structural defects arising from reduced sarcomeric in-
tegrity (Mhc0, Mhc1, fln0). It may be significant that in
the former mutant group hypercontraction occurs dur-
ing late pupal stages and is complete shortly after eclo-
sion (NAIMI et al. 2001), while in the latter the phen-
type arises during the first day or so of adult life
(KRONERT et al. 1995). On this basis different hypercon-
tracting Act88F alleles may affect regulation or sarco-
meric integrity. Despite the different etiology the same
Mhc suppressors suppress both groups. This does not

\begin{table}
\centering
\caption{Suppression phenotypes of hdp2 and up300 with D series mutant suppressors of KRONERT et al. (1999)}
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
Genotype & Wing phenotypes (%) & Flight (%) & \\
& n & Normal & Upheld & Down & U & H & D & N \\
\hline
hdp2; Mhc201/+ & 94 & 93 & 7 & 0 & 0 & 17 & 56 & 27 \\
hdp2; Mhc201/+ & 82 & 94 & 2 & 4 & 0 & 0 & 17 & 83 \\
hdp2; Mhc201/+ & 41 & 93 & 7 & 0 & 15 & 7 & 34 & 44 \\
hdp2; Mhc201/+ & 64 & 84 & 16 & 0 & 0 & 0 & 3 & 97 \\
up301; Mhc201/+ & 45 & 78 & 18 & 4 & 0 & 0 & 18 & 82 \\
up301; Mhc201/+ & 40 & 80 & 15 & 5 & 0 & 0 & 10 & 90 \\
up301; Mhc201/+ & 47 & 96 & 4 & 0 & 9 & 57 & 17 & 17 \\
up301; Mhc201/+ & 31 & 100 & 0 & 0 & 0 & 0 & 10 & 90 \\
up301;+/+, hdp2 & 46 & 96 & 4 & 0 & 0 & 8 & 92 & \\
u p301;+/+, hdp2; Mhc201/+ & 41 & 100 & 0 & 0 & 29 & 32 & 17 & 22 \\
up301;+/+, hdp2; Mhc201/+ & 36 & 89 & 11 & 0 & 0 & 0 & 6 & 94 \\
u p301;+/+, hdp2; Mhc201/+ & 52 & 100 & 0 & 0 & 23 & 52 & 25 & 0 \\
u p301;+/+, hdp2; Mhc201/+ & 43 & 100 & 0 & 0 & 0 & 0 & 26 & 74 \\
\hline
\end{tabular}

U, flies flying up toward a light source; H, horizontal; D, down; N, flightless. n, number of flies tested. Also see KRONERT et al. (1999) for details on these mutations.

* Shows hypercontraction muscle phenotype; all other genotypes show normal muscle morphology under polarized light.
\end{table}
allow us to distinguish between force production and regulation of muscle contraction as the primary effect of the mutant myosins. With few exceptions (see Naimi et al. 2001) hypercontraction phenotypes are restricted to the IFM. It is intriguing that this phenotype, which presumably arises from excessive shortening, shows up in the IFM, muscles where contraction is typically isometric.

Not all Mhc alleles that cause myosin dysfunction are suppressors. Two exceptions are MhcΔ and MhcΔ2, which have hypercontraction phenotypes themselves. In both, the mutant amino acids are within the myosin α-helical coiled-coil domain that associates to form the thick filaments. MhcΔ acts as an enhancer of the hdpΔ phenotype as hdpΔ/Y; MhcΔ/+ males are lethal as young larvae. However, this allele on its own produces only a hypercontraction phenotype in adults and the synthetic lethality is almost certainly due to epistatic interactions. The MhcΔ mutation substitutes G200 with aspartate (G200D); residue G200 is at the beginning of a helix that interacts with bound nucleotide (Kronert et al. 1999). MhcΔ myosin must be able to produce sufficient force for hypercontractive destruction of the muscle, but also affect regulation so that the phenotype occurs during late pupal/early adult life. The occurrence of hypercontraction at this time is consistent with an effect on regulation. How can MhcΔ affect regulation? One possibility is that it does so by altering myosin kinetics so that a fraction of heads remains bound, keeping the thin filament in the “open” state in the absence of calcium. The MhcΔ mutation (Y832H, a mutation in the myosin lever arm) is recessive lethal and, like MhcΔ, its survival as a wild-type heterozygote is severely reduced in combination with hdpΔ (Kronert et al. 1999), so it is an enhancer of hdpΔ. How a lever arm mutation achieves this is not clear. The lever arm binds the essential and regulatory light chains, so MhcΔ may affect regulation rather than compromising its force-producing capacity.

Since most Mhc missense mutants that reduce the efficacy of myosin function suppress hypercontraction, they will not be very informative about troponin/tropomyosin complex function. However, the small number of mutations with unexpected interactions with troponin mutations (e.g., MhcΔ and MhcΔ) are likely to be informative about troponin/tropomyosin complex function.

<table>
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<th>Genotype</th>
<th>n</th>
<th>Upheld</th>
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All the genotypes are flightless. HC, hypercontracted; PHC, partially hypercontracted; N, normal/suppressed. x, representing any suppressor (all produce the same data). *No muscle fibers are visible; see text for details.
ciently isolates Drosophila Mhc mutations with important effects on myosin function.

Many human familial myofibrillar myopathies of skeletal and cardiac muscle have been linked with sarcromeric proteins (reviewed in Coonar and McKenna 1997; Redwood et al. 1999; Seidman and Seidman 2001) including myosin, actin, tropomyosin, and the troponins. The hypertrophic cardiomyopathies show very variable penetrance and expressivity, which are likely to have a genetic component (Coonar and McKenna 1997). The study of interacting mutations in Drosophila, where muscle structure and sarcomeric proteins are very similar to their vertebrate counterparts, should shed light on the direct effects of muscle dysfunction and on the genetic interactions that are important in the occurrence of human muscle disease.

We thank Sandy Bernstein and Richard Cripps for providing the transgenic lines containing the headless myosin constructs, Alberto Ferrus for the original myosin hdp suppressor lines, and Meg Stark for her excellent EM work. This research was supported by the BBBSRC (UK; J.C.S.) and the National Science Foundation (J.O.V.).

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