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Sex-specific differentiation of a male-specific abdominal muscle, the Muscle of Lawrence, is abnormal in hydroxyurea-treated and in *fruitless* male flies

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SUMMARY

A prominent sex-specific abdominal muscle in male *Drosophila* is the Muscle of Lawrence (MOL), which is induced by male-specific innervation. We have examined MOL development in wild-type males, in males fed hydroxyurea to ablate the muscle precursors and in *fruitless* mutants, in which the MOL muscle develops aberrantly. One striking feature of MOLs in wild-type males was the presence of additional muscle nuclei compared with neighboring muscles or MOL-homologues in females. We tested whether muscle length and the sex-specific expression of a reporter gene depended critically on the number of nuclei present within a MOL fiber. MOL fibers developing from a reduced myoblast pool in hydroxyurea-affected hemisegments were recognizable by their attachment points and still contained more nuclei than did neighboring medial fibers, suggesting that these MOL fibers were able to actively recruit myoblasts nearly as well as wild-type MOLs. However, many of the hydroxyurea-affected MOL fibers were incapable of the normal male-specific expression of a muscle-specific reporter gene. We suggest that early events in MOL development, such as finding the correct muscle attachment points, are relatively insensitive to the number of MOL nuclei compared with later events, such as the sex-specific expression of a reporter gene.

In *fruitless* mutant males, MOL-position fibers are smaller and had substantially fewer nuclei compared to wild-type MOLs. Since the number and distribution of muscle precursors was the same in *fruitless* mutant and wild-type animals, we propose that one fru^+ function is to direct the male-specific recruitment of myoblasts into MOL-myotubes. However, $fruitless^+$ must have more than one role in MOL fiber development, since simple reduction in the number of muscle nuclei, as demonstrated by the hydroxyurea ablations, is insufficient to account for all of the MOL muscle phenotypes in *fruitless* mutant males.

Key words: *Drosophila*, myogenesis, sexual development, *fruitless*, Muscle of Lawrence

INTRODUCTION

One way to generate sexually dimorphic features is for homologous structures to undergo differential development in males and females. One example of this type of sex-specific differentiation is the formation of the Muscles of Lawrence (MOLs), a pair of large muscles found in the fifth abdominal segment of male *Drosophila* that have small homologues in the fifth abdominal segment of females and other abdominal segments of both sexes (Lawrence and Johnston, 1984). MOLs have two unusual features that govern their development. First, MOL fibers uniquely require male-specific innervation for their formation. This was demonstrated initially by elegant transplantation experiments (Lawrence and Johnston, 1986), and by nerve ablation experiments showing that denervation of the fifth abdominal nerve prior to metamorphosis prevents the formation of the MOL in adult males (Currie and Bate, 1995). Second, sexual differentiation of the MOL is independent of the activity of the *doublesex* gene, which constitutes the major output pathway for sexual development of somatic tissue (Taylor, 1992; for recent reviews: Baker, 1989; Belote, 1992; Burtis and Wolfner, 1992; McKeown and Madigan, 1992; Burtis, 1993; Taylor et al., 1994; Hall, 1994).

Adult muscle development has been well studied in *Drosophila* from the initial events during embryogenesis through to terminal differentiation during metamorphosis (for review see Bate, 1993). The myoblasts that found the dorsal, lateral and ventral muscles of the adult are set aside in the embryo and then divide during larval, pupal and early adult stages (Bate et al., 1991; Broadie and Bate, 1991). Midway through the pupal stage, the myoblasts migrate along the expanding adult epidermis and fuse to create multinucleate myotubes (Bate et al., 1991; Currie and Bate, 1991). These myotubes elongate, form their attachments to the epidermis and mature into muscle cells with concomitant high levels of expression of muscle-specific proteins, such as β3 tubulin (Currie and Bate, 1991).

It is unknown at what stage in adult muscle development sex-specific differences are expressed. Previously, it has been shown that the MOL first becomes recognizable by its size at around 48 hours of pupal development (Gailey et al., 1991). In this study, we show that MOL fibers in males have more muscle nuclei than do their neighboring medial fibers or their

homologues in females. The number of muscle precursors in the larva and the total number of muscle nuclei that contributed to the dorsal musculature were the same in male and female hemitergites suggesting that these differences were due to sex-specific differences in the distribution of myoblasts into myotubes.

We examined the relationship between the number of nuclei in MOL fibers and expression of MOL phenotypes by ablating myoblasts with hydroxyurea (HU) in wild-type male larvae. MOL fibers, recognizable by their attachment points, formed with fewer than half the average number of nuclei present in wild-type MOL fibers. We also evaluated the effect of reducing the number of muscle nuclei on the early male-specific expression of β-galactosidase in males with a *79B actin promoter-lacZ* reporter gene construct, another characteristic of MOL fibers (Courchesne-Smith and Tobin, 1989). Many recognizable MOL fibers in HU-affected hemisegments did not express β-galactosidase. Thus, the reduction in the number of nuclei within MOL fibers had less effect on earlier differentiative steps, such as forming appropriate attachment points, than on later steps, such as the male-specific expression of a reporter gene.

Mutations in *fruitless* (*fru*) lead to the abnormal development of MOLs (Gailey et al., 1991) and to the disruption of several male-specific reproductive behaviors in *Drosophila* (Gill, 1963; Hall, 1978; Gailey and Hall, 1989; Wheeler et al., 1989; Gailey et al., 1991). In most *fru* mutants, normal-sized MOL muscles failed to form, but the severity of the muscle defect depended on the individual genotype examined (Gailey et al., 1991). In the most severe *fru* mutant genotypes, the only muscle fibers in the MOL-position resembled neighboring nonsex-specific fibers in length and appearance. Because there were as many muscle fibers in mutant segments as in control segments, Gailey et al. (1991) proposed that MOL fibers were not deleted, but rather developed as small-sized muscles. Males mutant for other *fru* genotypes, such as fru^1 homozygotes or fru^1 over either one of two deficiencies, *Df(3R)P14* or $Df(3R)Cha^{M5}$, had no MOL-like muscle fibers but did have slightly longer and thicker muscles suggesting that these mutations were hypomorphic compared with the deficiency transheterozygotes. In the weakest genotype, fru^2 (originally called *P*[(w^+) *ARO-1*]), homozygous males often had muscles as large as MOLs in wild-type males.

Since *fru* mutations are the only non-homeotic mutations known to affect MOL differentiation, we examined these mutants to define the role of fru^+ in MOL myogenesis. In this study, we found that MOL-position fibers in *fru* mutant males have fewer muscle nuclei compared with wild-type MOL fibers. From our current understanding of the development of abdominal muscles in adult *Drosophila*, there are two ways that the fru^+ product might be expected to affect the number of nuclei present in adult muscles: (1) decreased proliferation of myoblasts in the larva and early pupa or (2) reduced fusion of myoblasts into myotubes. By comparing the number of myoblasts, identifiable as *twist*-positive cells, in control and several *fru* mutant genotypes, we showed that the fru^+ product does not affect the initial distribution or subsequent proliferation of myoblasts in larval stages. We propose that fru^+ controls the male-specific recruitment of myoblasts necessary to create full-sized MOL-fibers. However, based on the HU-ablations, the reduction in the number of nuclei in MOL-position fibers in *fru* mutants by itself is not sufficient to explain their small size suggesting that fru^+ has additional roles in MOL fiber development.

MATERIALS AND METHODS

Fly strains

Canton-S flies were used for the analysis of normal adult musculature and myoblasts during development. The descriptions of dorsal muscle groups in wild-type and HU-treated animals (see below) was made easier by examining animals that contained a *79B actin promoter-lacZ* gene construct (*P*[*79B actin-lacZ, ry*$^+$]; Courchesne-Smith and Tobin, 1989).

Most of the *fru* mutant stocks used in this study have been described by Hall (1978), Gailey and Hall (1989) and Gailey et al. (1991). Crosses between two deficiency stocks, *Df(3R)P14/ TM6B, Tb Hu e ca* and $Df(3R)Cha^{M5}$/*TM6B, Tb Hu e ca*, produced *fru* mutant flies with a severe phenotype (see Lindsley and Zimm, 1992, for descriptions of the markers). The original fru^1 is a small inversion; the distal breakpoint at 91B is uncovered when fru^1 is transheterozygous with $Df(3R)Cha^{M5}$ (Gailey and Hall, 1989) and is associated with the MOL mutant defect (Gailey et al., 1991). The fru^2 mutant stock, previously known as *P*[(w^+)*ARO-1*] (Taylor et al. 1994; Hall, 1994) was derived from a recently outcrossed line (Gailey et al., 1991). Two new P-element induced *fru* mutations, fru^3 and fru^4, were isolated in a screen for male steriles (Castrillon et al., 1993).

All fly stocks were raised on a diet of cornmeal, dextrose, agar and yeast which included propionic acid as a mold inhibitor. Fly cultures were kept in a humidified incubator at 25±1°C.

Abdominal muscle preparations

Dissections of adult abdomens were performed as described by Gailey et al. (1991). The abdomens were fixed for 1 hour in 4% paraformaldehyde in 0.1 M phosphate buffer and then incubated overnight in primary antibody (see below) in 0.1 M phosphate-buffered saline with 0.1% Triton X-100 and 2% normal goat serum (PBS-TX-NGS) with 0.1% sodium azide at room temperature. Following treatment with a biotinylated secondary antibody and ABC reagent (Vectakit, Vector Labs), diaminobenzidine (DAB; Sigma) was used for the final signal detection (Metcalf, 1985).

Two different antibodies were used on adult abdomens. For the nuclear counts, a monoclonal antibody (mAb) 8C5 (1:100; a gift from Dr Seymour Benzer) was used. To visualize MOL or MOL-position fibers in *P*[*79B actin-lacZ, ry*$^+$] flies, an anti-β-galactosidase antibody (1:10,000; Cappel Laboratories) was used.

Because of the number of muscle nuclei and close apposition of the fibers in the MOL, these fibers were drawn with a camera lucida attachment on a compound microscope with 100× oil immersion optics to ensure accuracy. Statistical analysis was performed using a one-way ANOVA with the Statgraphics program (Statistical Graphics Corporation).

Myoblast identification

Larval body walls from wandering stage third instar larvae were processed for immunohistochemisty (see above) using a polyclonal anti-twist antibody (1:1000, a gift from Dr Bruce Paterson).

Hydroxyurea ablations of myoblasts

Hydroxyurea (HU) was fed to larvae to kill myoblasts. HU has been shown to kill dividing cells when present at sufficiently high concentrations during S-phase of the cell cycle, but not to harm cells that are post-mitotic or in a different phase of the cell cycle (Furst and Mahowald, 1985; Truman and Booker, 1986; Broadie and Bate, 1991). Canton-S larvae were collected from timed egg collections as newly molted second or third instar animals within 1 hour of their

molt. At the appropriate times, staged larvae were placed on diet prepared with 5 mg/ml HU (see Broadie and Bate, 1991). After the HU treatment, larva were moved to normal food, in uncrowded conditions, and allowed to develop to the pharate adult stage (stage 15, Bainbridge and Bownes, 1981), then, abdomens were dissected, fixed and stained for nuclei with mAb8C5 according to the procedures listed above. Canton-S larvae were fed either during 12-20 hours of the second instar (late second instar group) or from 1-8 hours in the third instar (early third instar group) (see also, Broadie and Bate, 1991).

Because we were interested in cases where relatively few adult muscle nuclei remained, HU-affected fifth abdominal hemitergites were identified by counting the number of nuclei in all muscles (see above). We classified each hemitergite into one of four groups by comparing the number of nuclei in an experimental animal with the number of nuclei in untreated Canton-S males: group 1, no ablation where more than 183 nuclei were present, which is less that one standard deviation below the mean of wild-type males; group 2, partial ablations where from 156 to 182 nuclei were present, which is between one and two standard deviations from the mean of wild-type males; group 3, partial ablations where fewer than 155 nuclei were present, which is more than two standard deviations below the mean of wild-type males; group 4, complete ablation where no dorsal adult muscles were present (Tables 1 and 3).

To independently identify MOL fibers, late second instar (n=19), early third instar (n=39) and 0-24 hour third instar (n=19) *P*[*79B actin-lacZ, ry*$^+$] larvae were fed HU as described for Canton-S males and then labelled with anti-β-galactosidase antibodies (as above).

RESULTS

Pattern of adult musculature in the fifth abdominal segment

The simple pattern of dorsal longitudinal muscle fibers in each abdominal segment is interrupted in the fifth abdominal segment of male flies by a pair of large multi-fiber MOL muscles (Crossley, 1978; Miller, 1950; Lawrence and Johnston, 1984; Gailey et al., 1991; Taylor 1992; Currie and Bate, 1995). MOL fibers and their homologues in abdominal segments in males and females were identified by staining for β-galactosidase in the *P*[*79B actin-lacZ, ry*$^+$] line (Courchesne-Smith and Tobin, 1989). In pharate or newly eclosed males, only MOL fibers expressed β-galactosidase (Fig. 1A; Currie and Bate, 1995). On the day following eclosion, four to six fibers, the segmental homologues of MOL fibers in female and male abdomens, also expressed β-galactosidase (data not shown; D. A. Currie, personal communication; T.J.S. Merritt, personal communication). Two to four longitudinal muscle fibers arrayed next to the dorsal midline never stained for β-galactosidase in either males (Fig. 1A) or females (data not shown). Thus, the muscles found between the dorsal midline and the lateral insertion of the alary muscle are divided into two groups, MOL or MOL-homologue fibers and medial fibers.

MOL fibers contain more muscle nuclei than do neighboring fibers or MOL homologues in females

Consistent with their large size, more nuclei were present in MOL fibers than in medial fibers (see also Currie, 1991). MOL fibers had around 24 nuclei per fiber irrespective of whether fibers occupied positions 3, 4, 5, or 6 from the dorsal midline (Figs 1B, 2A). Only 13% of wild-type MOL fibers contained fewer than 20 nuclei (Fig. 1B, 2A). By comparison, medial fibers had on average 15 nuclei per fiber independent of their position. Only 4% of the medial fibers examined had more than 20 nuclei per fiber. As shown in Fig. 2A, there was nearly a two-fold range in the number of nuclei present in individual medial or MOL fibers.

In order to demonstrate that the number of nuclei in MOL fibers was consistently greater than the number in medial fibers, we calculated a difference value for each animal. The average number of nuclei in medial fibers (positions 1+2) was subtracted from the average number of nuclei in MOL fibers (positions 5+6) for each segment (Table 1); a positive number denotes more nuclei in MOL fibers whereas a negative number denotes fewer nuclei in MOL muscle fibers compared with the number in medial fibers. For wild-type males, the difference

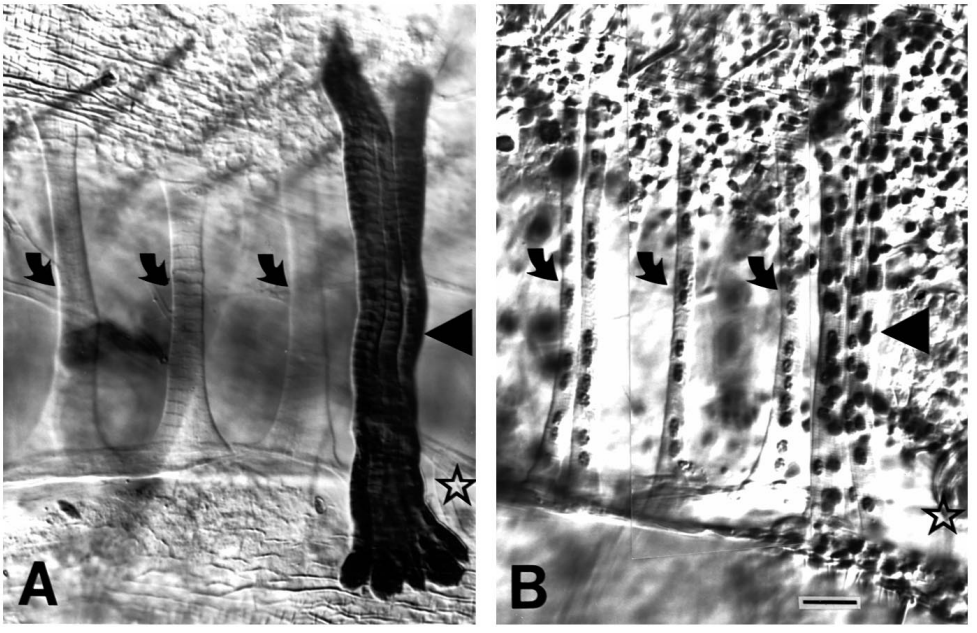

Fig. 1. Photomicrographs of the fifth abdominal hemitergite. (A) Newly eclosed *P*[*79B actin-lacZ, ry*$^+$] male labelled with an anti-β-galactosidase antibody showing that the large MOL fibers (arrowhead) are labelled but the three medial fibers (curved arrows) are unstained. (B) Photomontage of a Canton-S male hemitergite stained with mAb8C5 where the medial fibers are indicated by curved arrows and the MOL fibers with an arrowhead. Dorsal midline is to the left and the lateral attachment of the alary muscle is indicated by a star symbol in all figures. Bar is 20 μm for this and all subsequent photographs unless otherwise noted.

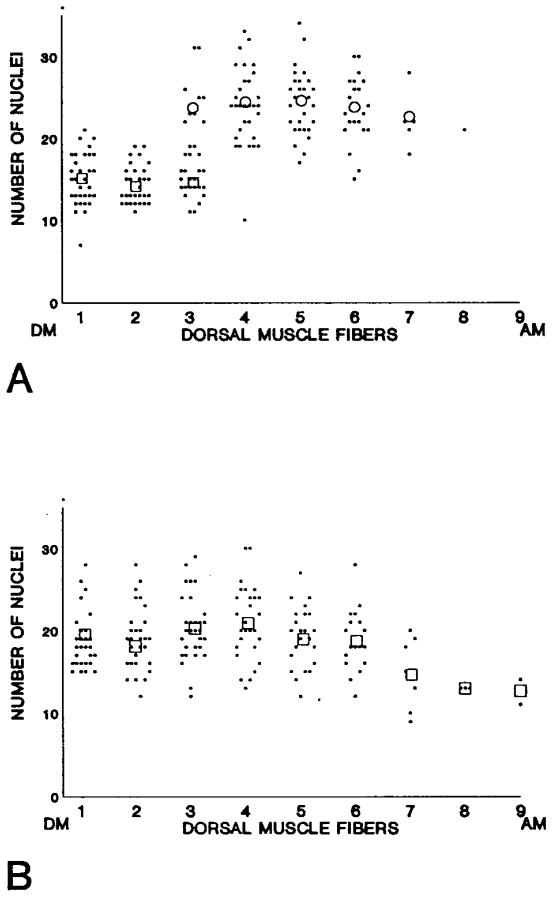

Fig. 2. Distribution of nuclei in fifth abdominal dorsal muscle fibers in wild-type animals. Counts were made from fifth abdominal hemisegments labelled with mAb8C5. (A) Scatterplot of nuclei in Canton-S male muscle fibers. The average number of nuclei is indicated by an open square for medial fibers and an open circle for MOL fibers. Fibers in position 3 may belong to the medial group or to the MOL group in a particular hemitergite. (B) Scatterplot of nuclei in Canton-S female muscles. The average of each fiber is indicated by an open square. DM, dorsal midline; AM, lateral alary attachment.

values were strongly positive, with a mean of nearly 10 additional nuclei in a MOL versus a medial fiber. Thus, the MOL group has nearly 20 to 50 more muscle nuclei than the medial group of fibers.

In wild-type females, all fibers between the dorsal midline and the alary insertion had the same average number of nuclei (Fig. 2B). Because medial and MOL-homologue fibers occupy comparable positions in females as medial and MOL fibers in males, we calculated an average difference value between medial fibers (positions 1+2) and MOL-homologue fibers (positions 5+6). There was no difference between the number of nuclei in medial fibers compared with the number in MOL-homologue fibers (0.9±1.0; mean ± s.e.m.).

Two sex-specific differences were found in the distribution of muscle nuclei among muscle fibers in the fifth abdominal tergite. Medial muscle fibers in females averaged 4-5 more nuclei than medial muscle fibers in males. Conversely, MOL-homologue fibers in females had 4-5 fewer nuclei than MOL fibers in males. The sex-specific differences in nuclei number

Table 1. Average difference values for wildtype and *fru* mutant males

Genotype	N	Average difference value MOL (fibers 5/6) - med (fibers 1/2)
Canton-S	18	9.9±0.9
Df(3R)P14/Df(3R)Cha^{M5}	10	–2.0±0.4†
fru^{1}	11	–0.3±0.3†‡
fru^{1}*/Df(3R)P14*	10	–1.4±0.8†
fru^{1}*/Df(3R)Cha*^{M5}	9	0.0±0.8†‡
fru^{2} (med-sized fibers)	6*	–1.9±0.8†
fru^{2} (MOL-sized fibers)	11*	1.4±0.6†‡
fru^{3}	9	–4.4±0.8†
fru^{4}	7	0.4±1.1†‡

Using abdominal preparations stained with mab8C5, the average number of muscle nuclei in medial fibers (positions 1 + 2) were subtracted from the number of nuclei in MOL fibers (positions 5 + 6). For each individual, unless noted, the muscle counts from both left and right sides of an individual were averaged together.

*In those cases where medial-sized fibers were found on one side and MOL-sized fibers on the other side, the right and left side were considered separately.

†Significantly different at $P \leq 0.05$ from Canton-S males using a one-way ANOVA with the Tukey HSD test for specific contrast.

‡Significantly different at $P \leq 0.05$ from *fru*^{3} males using a one-way ANOVA with the Tukey HSD test for specific contrast.

Table 2. Number of muscle nuclei in dorsal hemisegments of wildtype and *fru* mutant animals

Genotype	Sex	Nuclei count medial + MOL group	Nuclei count hemitergite
Control	XY	115.7±3.0 (N=17)	205.4±10.6 (N=6)
	XX	114.4±4.4 (N=14)	195.8±4.0 (N=5)
Df(3R)P14/ Df(3R)Cha^{M5}	XY	101.1±3.9 (N=10)*	183.3±11.0 (N=4)
fru^{1}	XY	83.4±3.1 (N=11)*,†	148.5±3.3 (N=5)*
fru^{1}*/Df(3R)P14*	XY	93.9±3.5 (N=10)*,†	166.0±7.6 (N=4)*
fru^{1}*/Df(3R)Cha*^{M5}	XY	92.7±3.5 (N=9)*,†	149.3±6.3 (N=4)*
fru^{2}	XY	106.8±3.5 (N=15)	173.3±10.1 (N=5)

In abdominal preparations stained with the mab8C5 antibody, the number of muscle nuclei were determined for the set of muscles located between the lateral alary attachment and the dorsal midline (medial + MOL group) and for the total number of nuclei within a dorsal hemisegment for each of the genotypes listed. Both the right and left sides from an individual were averaged together.

*Significantly different from control male and female values at $P \leq 0.05$ using a one-way ANOVA with the Tukey test for specific contrast.

†Significantly different from *fru*^{2} male values at $P \leq 0.05$ using a one-way ANOVA with the Tukey test for specific contrast.

found between the two muscle groups reflect the unequal partitioning of an equivalent pool of myoblasts into medial and MOL fibers in males. Sex-specific differences were not found in the total pool of muscle nuclei contributing to the dorsal musculature (Table 2). Nor were there differences in the fraction of this pool (56% in males and 58% in females) used to create the medial and MOL or MOL-homologue muscles in males and females (Table 2).

Recognizable MOLs form with a fraction of the normal number of nuclei but these reduced-nuclei MOLs do not express all normal MOL functions

To test directly whether the length of MOL fibers depended solely on the number of muscle nuclei, we reduced the number of myoblasts available to create the adult muscula-

Table 3. Percentage of total and partial ablations in the fifth abdominal hemitergite

Hydroxyurea treatment period	N	Group 1 no ablation	Group 2 partial ablation	Group 3 partial ablation	Group 4 complete ablation
Canton-S late 2nd instar	35	54.3	11.4	20.0	14.3
Canton-S early 3rd instar	33	54.5	21.2	24.2	0.0

Canton-S males were fed hydroxyurea as larvae at the times indicated and allowed to develop until the pharate adult stage when the abdomens were processed for immunohistochemistry with the mab8C5 antibody. Counts were made of the number of nuclei in the fifth abdominal hemisegment; in these animals each hemisegment was treated independently. The distribution of cases into the four groups is described in Materials and Methods.

ture by feeding hydroxyurea to late second and early third instar larvae (see Materials and Methods for details). To ensure that all fifth abdominal HU-affected hemitergites were detected, nuclei counts were made in abdomens labelled with a nuclei-specific antibody. To interpret the data, all of the cases were divided into four groups ranging from no ablation to complete ablation based on the total number of muscle nuclei present (Table 3).

Ablation groups in which nuclei number was reduced by less than 25% (groups 1 and 2) showed normal-sized MOL fibers (data not shown). In about 10% of group 2 hemitergites, MOLs were separated into two distinct sets of two or three fibers and in a few cases, only two MOL fibers were present rather that the normal three to five. Neither of these two conditions were seen in any untreated males. The entire adult musculature was ablated in group 4, which occurred in 14% of the animals fed in the late second instar (Table 3; see also Broadie and Bate, 1991).

Hemitergites with group 3 partial ablations did show effects on MOL and medial fiber development. In these cases, there were severe reductions in the total number of nuclei, ranging from 75% to 25% of the mean number of nuclei in wild-type males (Table 3). At least one recognizable MOL fiber was produced in 14 of the 15 group 1 hemitergites. These MOL fibers had normal or nearly normal posterior attachment points, but frequently failed to reach their usual anterior attachment points. These fibers were shorter than wild-type MOLs, but were distinctly longer than medial fibers. The nuclear distribution across the dorsal hemisegment in four examples of group 3 partial ablations are shown in Fig. 3A-D. In cases where only one-quarter to one-half of the wild-type number of nuclei remained, medial and MOL fibers contained the fewest nuclei; medial fibers were present with as few as one or two nuclei and MOL-type muscles formed with as few as six to eight nuclei, about a quarter of the average number of nuclei in wild-type MOLs (Figs 3A-C, 4A). When the number of nuclei in a group 3 hemisegment was greater than one-half the normal number, more nuclei were found in both muscle groups (Fig. 3D). The striking feature of these cases was that MOL fibers always had more nuclei compared with their medial fiber neighbors; the average difference value for hemitergites with group 3 ablations was 6.2 ± 0.8 (mean ± s.e.m.), nearly two-thirds the value obtained for wild-type MOLs (Table 1 and Fig. 4A).

In two group 3 hemitergites, no MOL fibers were detected. In the first case, only six fibers were present with a total of 12 nuclei; two extremely small fibers, which could not be identified, were found medial to the alary attachment and four fibers were located more laterally. In the other case, a complete nuclear count was not possible, but four of a total of eight fibers in the hemisegment were located between the dorsal midline and the alary insertion, about half the expected number of fibers in this region. Each fiber had 10-11 nuclei which should have been sufficient to have produced a visible MOL-type fiber. These two examples may represent cases with regional ablations of one or more myoblast nests as opposed to reduc-

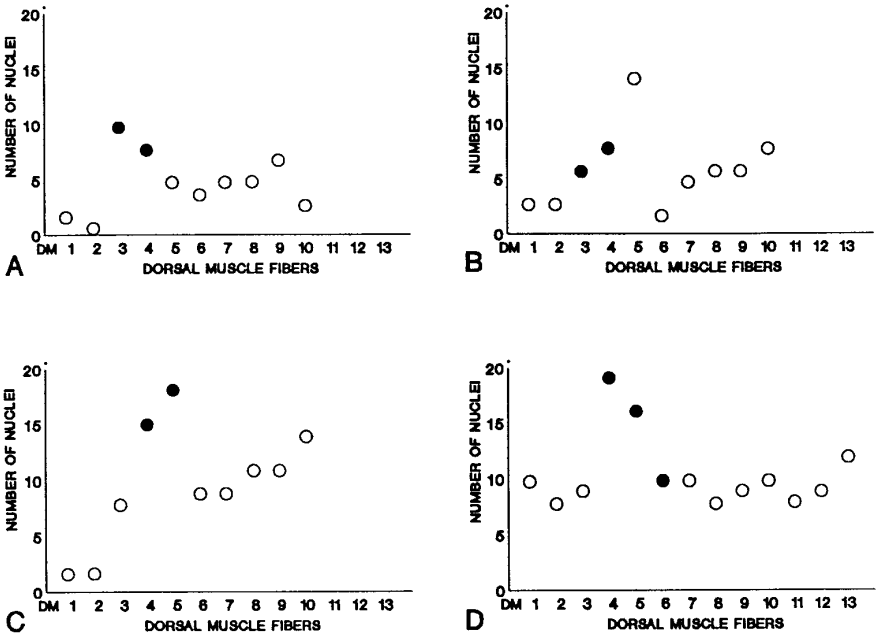

Fig. 3. Distribution of nuclei in muscles of group 3 partial HU ablations. All of these males were fed HU as late second instars and then the abdomens were dissected and labelled with mAb8C5 when they had developed into pharate adults. The MOL fibers are indicated by filled circles. (A) 50 nuclei per hemitergite, the medial and MOL fibers are shown in Fig. 4A; (B) 61 nuclei; (C) 99 nuclei; (D) 138 nuclei.

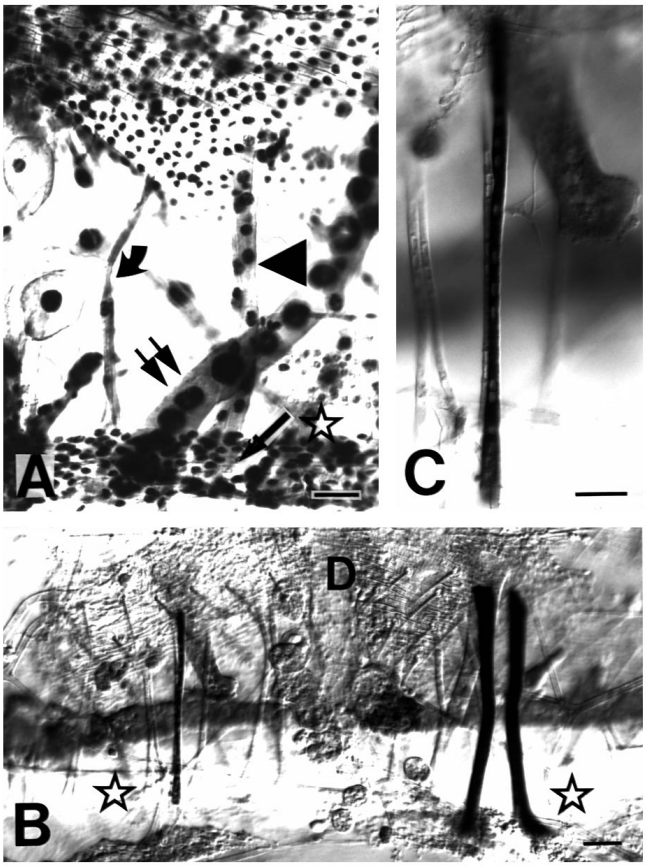

Fig. 4. Photomicrograph of fifth abdominal hemitergites in HU-treated males. (A) Group 3 Canton-S male hemitergite stained with mAb8C5. The distribution of nuclei is shown in Fig 3A. Two closely apposed medial fibers (curved arrow) have 2 and 1 muscle nuclei, respectively, and the two MOL fibers underneath the larval oblique muscle (double arrowheads) have 10 and 8 nuclei, respectively. The posterior attachment of the MOL fibers (straight arrow) is close to that of the retained larval oblique muscle, which normally degenerates a few hours after eclosion. (B) Photomicrograph of the fifth tergite of a *P*[*79B actin-lacZ, ry*$^+$] male that had been fed hydroxyurea as a late second instar and dissected and stained with anti-β-galactosidase as a pharate adult. On the left side, two MOL fibers are present. On the right hemitergite are two pairs of normal-sized MOL fibers but split into two groups. D, dorsal midline. Bar, 40 μm. (C) Higher magnification photomicrograph of the MOL fibers in the HU-affected hemitergite in B. One of the fibers stains heavily for the DAB reaction but the other fiber is very lightly stained. The stained MOL fiber has 14 nuclei and the weakly stained MOL fiber has 9 nuclei.

tions of some but not all myoblasts within a nest (Broadie and Bate, 1991).

Because MOL fibers formed male-like attachments even with fewer nuclei than normal, we evaluated whether another male-specific feature of MOL development, the early onset of β-galactosidase expression in *P*[*79B actin-lacZ*] males, was affected in ablated hemisegments. Unexpectedly, several recognizable MOL fibers failed to express β-galactosidase. In the early third instar treatment group, 82% (*n*=39 hemitergites) had normal-sized MOL fibers that expressed β-galactosidase. The remaining six hemitergites did not have any β-galactosidase-positive fibers, although in five cases, one or two fibers were recognizable as MOLs from their deeper posterior attachment points. The one remaining hemitergite had 3 fibers between the dorsal midline and the alary attachment but lacked any MOL fiber. In all cases, the contralateral MOL muscle was β-galactosidase positive. Where MOL fibers were unstained, it was not possible to accurately count the number of nuclei, but the fibers were thin indicating that they did not contain many nuclei. In *P*[*79B actin-lacZ, ry*$^+$] males fed as late second instars (*n*=19 hemisegments), 11% of the hemisegments had complete ablations of the adult musculature. All remaining cases from the second instar and all of the cases from the 24-hour third instar treatment group had MOL fibers that expressed β-galactosidase. Among these hemitergites were two exceptional cases. In one case, a single thin MOL fiber was present, which expressed β-galactosidase and had 17 nuclei (data not shown). In the second case, two, closely apposed MOL fibers were visible; the MOL fiber with 14 nuclei stained strongly for β-galactosidase, but the neighboring MOL fiber with only 9 nuclei stained only extremely weakly (Fig. 4B,C). Thus, only a subset of MOL fibers from HU-affected hemitergites, recognizable by their attachment points, expressed the full range of detectable MOL-specific phenotypes.

In *fru* males, MOL-position fibers have fewer muscle nuclei than MOL fibers in wild-type males

Because there is gradation in MOL muscle phenotype in various *fru* mutants (Gailey et al., 1991), we expected that the relationship between the number of nuclei and muscle size in *fru* mutants would conform to one of two possibilities. MOL-position fibers in *fru* mutants might have the same number of nuclei as wild-type MOLs, but be unable to produce a normal-sized MOL. Alternatively, MOL-position fibers in *fru* mutants might have fewer muscle nuclei and thus be limited to making smaller MOLs. To distinguish between these alternatives, we examined several *fru* mutant genotypes encompassing the full range of MOL phenotypes. The existence of MOL-position fibers in *fru* mutants was confirmed in *P*[*79B actin-lacZ, ry*$^+$] *fru*^{1} recombinant males which had four to six β-galactosidase-positive fibers more laterally than two to four medial fibers (data not shown).

In all of the *fru* mutant genotypes examined, we found that there were fewer muscle nuclei in MOL-position fibers than in MOL fibers from wild-type males. In the most severely affected mutants, such as *Df(3R)P14*/*Df(3R)Cha*^{M5} males, MOL-position muscles in the dorsal region were the same size as medial muscles (Fig. 5; Gailey et al., 1991). In the scatterplot of nuclei counts from *Df(3R)P14*/*Df(3R)Cha*^{M5} males (Fig. 6A), medial fibers in positions 1, 2, and 3 had on average 15 nuclei per fiber, similar to the average number of nuclei found in wild-type medial group fibers (Fig. 2A). Muscles in positions 4, 5 and 6 had slightly fewer nuclei per fiber (Fig. 6A). Only 3% of the MOL-position fibers had more than 20 nuclei per fiber. We computed the mean difference value between fibers at positions 5 and 6, corresponding to MOL-position fibers, and those at positions 1 and 2, corresponding to medial fibers. The average difference value was slightly negative confirming that in these mutants MOL-position fibers had fewer nuclei than medial fibers (Table 1).

In *fru* mutants, which produced no full-sized MOL fibers and generally have only small sized MOL-position fibers (Gailey et al., 1991), the average number of nuclei for fibers

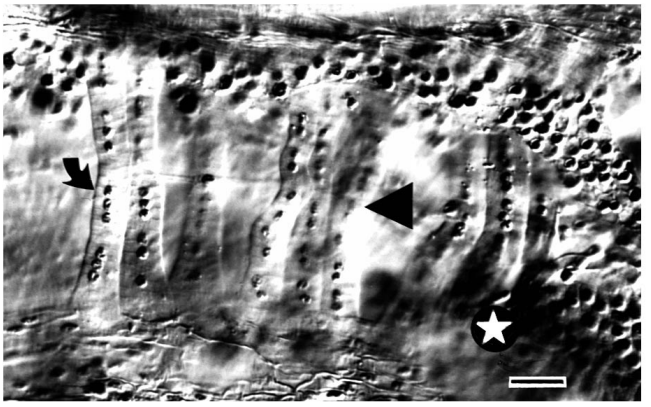

Fig. 5. *Df(3R)P14*/*Df(3R)Cha*^{M5} male hemitergite stained with mAb8C5. All muscles are the same size. Medial fibers 1 (curved arrow) and 2 have 12 and 13 nuclei, respectively, and MOL-position fibers 5 and 6 (arrowhead) have 11 and 13 nuclei, respectively.

in the various medial to lateral positions was similar to that found in the double deficiency *fru* mutants. In *fru*^{1} homozygotes, *fru*^{1}/*Df(3R)P14* and *fru*^{1}/*Df(3R)Cha*^{M5} transheterozygotes, MOL-position fibers (4, 5, and 6) had nearly the same number of nuclei as medial fibers (1, 2, and 3; Fig. 6B). The average difference values calculated for these mutant genotypes showed that medial and MOL-position fibers had roughly the same number of nuclei (Table 1). In two other *fru* mutant genotypes, *fru*^{3} and *fru*^{4}, where no MOL or intermediate-sized fibers formed (Castrillon et al 1993; data not shown), the average number of muscle nuclei per fiber was greater than for other *fru* mutants (Fig. 6B), but even in these animals, there were fewer nuclei in MOL-position fibers than medial fibers (Fig. 6B, Table 1). The large negative difference value for *fru*^{3} animals was due primarily to the greater number of nuclei in medial fibers not to an unusually severe reduction in the number of nuclei in MOL-position fibers (Fig. 6B).

In the weakest genotype, *fru*^{2} homozygous males, a normal-sized MOL often forms (Gailey et al., 1991). On average, the number of nuclei in large-sized MOL fibers was slightly larger than small-sized MOL fibers in the same positions (Fig. 6B). When the difference value was calculated for *fru*^{2} MOL or intermediate-sized fibers, there were slightly more nuclei than in medial muscles; but these fibers still had significantly fewer nuclei than wild-type MOL muscles ($P \leq 0.05$; Table 1). Indeed, the mean difference value for *fru*^{2} MOL fibers compared with small MOL-position fibers in *fru*^{2} or in other *fru* genotypes was not significant (Table 1).

Since there were fewer nuclei in MOL-position fibers in *fru* mutants, we determined whether there were fewer muscle nuclei altogether in the hemitergite. Several mutant genotypes, *fru*^{1} homozygotes, *fru*^{1}/*Df(3R)Cha*^{M5} and *fru*^{1}/*Df(3R)P14* transheterozygotes, had fewer muscle nuclei than did Canton-S males, but *fru*^{2} homozygotes and *Df(3R)P14*/ *Df(3R)Cha*^{M5} transheterozygotes were not different from wild-type males (Table 2). Between 56-61% of the total muscle nuclei were distributed in the medial two groups of muscles in *fru* mutants similar to the proportion contributed to these muscle in wild-type males, indicating that MOL-position muscles in *fru* mutants did not exist in a specially disadvantaged region within the hemisegment.

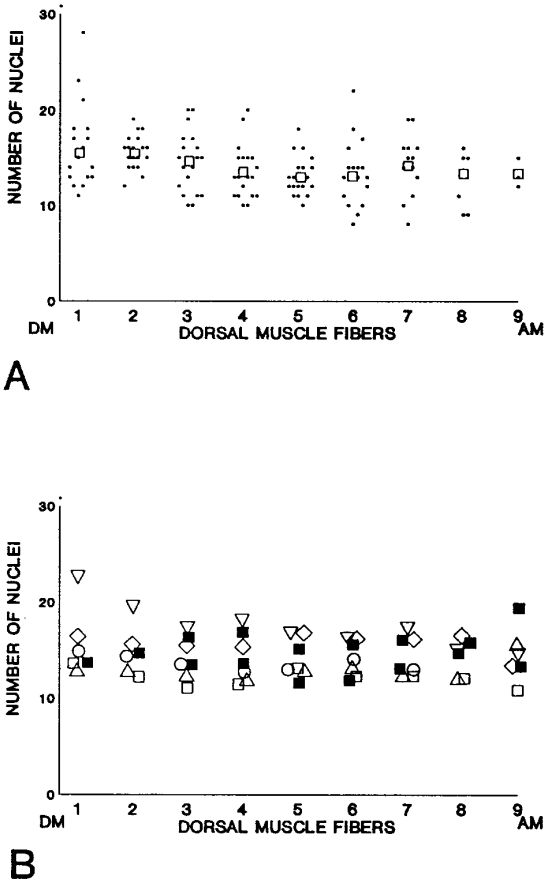

Fig. 6. Distribution of nuclei in fifth abdominal dorsal muscle fibers in *fru* mutant males. (A) Scatterplot of *Df(3R)P14*/ *Df(3R)Cha*^{M5} males as described for Fig. 2. The average number of muscle nuclei per fiber is denoted with an open square since it is not possible to distinguish medial fibers from potential MOL-equivalent fibers. (B) Average number of muscle nuclei found in different *fru* mutant alleles. Symbols for the *fru* mutant genotypes are: (■) *fru*^{2}/*fru*^{2}; (□) *fru*^{1}/*fru*^{1}; (○) *fru*^{1}/*Df(3R)P14*; (△) *fru*^{1}/*Df(3R)Cha*^{M5}; (▽) *fru*^{3}/*fru*^{3}; (◇) *fru*^{4}/*fru*^{4}. In *fru*^{2} homozygotes, MOL fibers can be divided into large MOL fibers, similar to the size of normal MOL fibers, and small MOL fibers and the average of large MOL fibers was always higher than that for small MOL-sized fibers. Standard error bars for each point were at most the size of the symbol.

The number of myoblasts is unaffected in *fruitless* mutants

Adult dorsal longitudinal muscles derive from dorsal groups of myoblasts, detected as twist-positive cells, located near the larval dorsal oblique muscles 1, 2 and 3 (Bate et al., 1991; Currie and Bate 1991; Broadie and Bate, 1991). Fewer adult muscle nuclei, such as found in many *fru* mutant males, might result from a reduced number or abnormal distribution of these founder populations of larval myoblasts or to later events, such as myoblast fusion.

twist-positive cell counts in wild-type and *fru* mutant larvae show that both genotypes have the same number of myoblasts. In wild-type wandering third instar larvae, 18 twist-positive cells were found distributed among the three dorsal groups in the fifth abdominal hemisegment of both males and females (Table 4; see also Broadie and Bate, 1991). Occasionally,

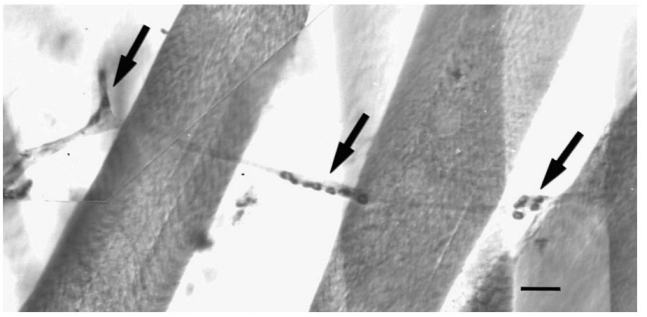

Fig. 7. Dorsal hemisegment of a *Df(3R)P14*/ *Df(3R)Cha*^{M5} male third instar larvae. The abdomen was labelled with an anti-twist antibody. There are three nests of myoblasts distributed along the intersegmental nerve between the dorsal oblique muscles. Dorsal midline is to the right.

myoblasts were not clearly collected into three nests but were distributed along the intersegmental nerve or merged into two groups. Male *Df(3R)P14*/*Df(3R)Cha*^{M5} larvae had the same number and distribution of twist-positive cells as wild-type male and female larvae (Table 4, Fig. 7). The lateral and ventral myoblast nests, which contribute to the lateral and ventral adult muscles, were also present in these and the other *fru* mutant larvae examined (data not shown) indicating that the original embryonic patterning of the adult musculature was unaffected by mutations in *fruitless*.

Homozygous *fru*^{1} and *fru*^{2} mutant larvae have one-third to one-half as many twist-positive cells as wild-type animals (Table 4). These differences were significant when compared to wild-type or double-deficiency males. In *fru*^{1} and *fru*^{2} males, the cluster of twist-positive cells associated with larval muscle DA3, the most lateral group, was commonly the smallest cluster, often having only two cells. When *fru*^{1}/*Df(3R)P14* or *fru*^{1}/*Df(3R)Cha*^{M5} males were examined, the number of myoblasts was not significantly different from wild-type males (Table 4), making it likely that the reduction in the number of myoblasts is a background effect due to homozygosis of the *fru*^{1} chromosome, not to a direct *fru* mutant effect.

Table 4. Number of twist-expressing cells in wandering third instar larvae

Genotype	Sex	N	Mean ± s.e.m.
Canton-S	XY	9	17.2±0.7
	XX	8	17.9±0.8
Df(3R)P14/*Df(3R)Cha*^{M5}	XY	13	18.8±0.8
fru^1	XY	14	11.2±0.7*,†
fru^1/*Df(3R)P14*	XY	6	16.1±1.5
fru^1/*Df(3R)Cha*^{M5}	XY	7	15.7±0.8
fru^2	XY	6	9.9±1.0*,†

Counts were made of the number of twist-expressing cells in the fifth abdominal segment of male (XY) and female (XX) larvae stained with anti-twist antibody and processed for immunohistochemistry. Both right and left sides from an individual were averaged together.

*Significantly different at $P \leq 0.05$ from Canton-S male values using a one-way ANOVA where the Tukey test provided for specific contrast.

†Significantly different at $P \leq 0.05$ from *Df(3R)P14*/*Df(3R)Cha*^{M5} male values using a one-way ANOVA where the Tukey test provided specific contrast.

DISCUSSION

Sex-specific differences in muscle differentiation in the fifth abdominal segment

In this study we have shown that male-specific MOL fibers consistently contain ten more muscle nuclei than neighboring medial fibers, commensurate with their larger size. This differential is maintained over a two-fold range in the number of nuclei present within individual medial and MOL fibers. In contrast, female MOL-homologue and medial muscles are the same size and contain equivalent numbers of muscle nuclei. No sex-specific differences were found in the number of larval myoblasts or in the combined number of nuclei present within the adult medial and MOL or MOL-homologue muscles. Sex-specific recruitment of nuclei into MOL fibers appears to result from the unequal allocation of a common pool of myoblasts during the formation of medial and MOL muscles rather than a special set of male-specific myoblasts.

A sex-specific increase in nuclei in MOL fibers might depend on one of three different developmental processes. First, simply lengthening the time period for myoblast fusion to create MOL myotubes would lead to an increase in the number of nuclei found in MOL compared to medial fibers. In embryos, muscle size is proportional to the number of myoblasts that have fused, which in turn depends on the length of the fusion period (Bate, 1990). Second, male MOL fibers might actively recruit myoblasts that were destined to contribute to medial group muscles or other nearby muscles. Third, some combination of developmental processes such as male-specific muscle nuclei divisions in MOL myotubes and apoptosis of previously fused medial myotube nuclei could account for the numerical disparity between MOL and medial muscles. The period of myoblast division in the adult overlaps with the period of fusion but no evidence of male-specific divisions in MOL myotubes has been detected in pupae that have been exposed to 5-bromodeoxyuridine to label nuclei in S-phase (D. A. Currie, personal communication; B. J. Taylor unpublished observations).

We favor the second alternative. In HU-affected hemitergites with severe reductions in the number of myoblasts, the differential in the number of nuclei per MOL fiber compared to the number of nuclei per medial fiber is nearly as great as that in normal fibers. Under these conditions where, effectively, no myoblast surplus exists, the difference between MOL and medial fibers would be expected to diminish or disappear if due only to a passive lengthening of the fusion period. The severe reduction of nuclei in medial fibers, compared to longitudinal muscles lateral to the MOL, (see Fig. 3D) supports an active process in MOL fibers operating at the expense of medial fibers, within a general lateral to medial progression in the formation of abdominal muscle fibers.

MOL fiber phenotypes are not equally affected by reductions in the number of nuclei the fiber contains

We had anticipated that there would be a strict lower limit to the number of nuclei needed to produce either a medial or a MOL fiber. From the range of nuclei number in wild-type animals, medial fibers appeared to need at least seven nuclei, and small MOL fibers at least 15. From examination of HU-

affected hemitergites, we were astonished to discover that medial muscles with one and two nuclei were able to stretch to their normal attachment sites even though these fibers were exceedingly thin. Easily recognizable MOL fibers, with deeper posterior attachments than medial muscles, formed with as few as six nuclei, about one-quarter the average number of nuclei in wild-type muscles. There were too few cases to determine whether MOL fibers developing with fewer than six nuclei would be indistinguishable from medial muscles. At the other end of the scale, the formation of full-sized MOL muscles required closer to twenty nuclei in both wild-type or HU-treated males.

From the HU ablations with control and *P*[*79B actin-lacZ, ry*$^+$] males, it is apparent that the number of MOL fibers and the expression of β-galactosidase from the *79B actin* promoter were more sensitive to the number of nuclei than was the outgrowth of muscle fibers toward their attachment points. In none of the severely affected hemisegments were more than two MOL-type fibers found compared with three to five fibers present in wild-type animals. Additionally, recognizable MOL fibers did not always express β-galactosidase. Even fibers of equivalent size had extremely divergent reporter expression that correlated with the number of nuclei present (see Fig. 4B,C). We interpret the lack of β-galactosidase staining in five other MOL fibers in HU-treated *P*[*79B actin-lacZ, ry*$^+$] males as due to a lack of sufficient muscle nuclei to promote muscle maturation.

An alternative explanation for the loss of β-galactosidase expression in HU-treated males is the death of the MOL-specific motorneurons, which would prevent the induction of the MOL (Lawrence and Johnston, 1986; Currie and Bate, 1995). A direct effect of HU on motorneurons seems improbable since abdominal motorneurons for skeletal muscles in holometabolous insects are generated in the embryo and so should be resistant to the HU treatment (e.g. Taylor and Truman, 1974; Giebultowicz and Truman, 1984; Thorn and Truman, 1989; Truman and Booker, 1986; Broadie and Bate, 1991; B. J. Taylor, unpublished observations). Indeed, in our experiments, even smaller MOL fibers were recognizable as MOLs by their attachment points suggesting that they had been innervated by MOL-specific motorneurons.

Thus, the steps in muscle development that appear to be the most dependent on the number of nuclei are involved in terminal differentiation. Attachment to the epidermis by developing abdominal muscles is an event that occurs about midway during the pupal stage (Currie and Bate, 1991; Gailey et al., 1991) followed by terminal differentiation. The inability of MOL fibers with fewer nuclei to respond appropriately perhaps reflects a failure to reach this fully differentiated state. Further examination of the MOL fibers in HU-affected hemitergites would be needed to demonstrate whether general muscle cell differentiation, such as the accumulation of glutamate receptors at the muscle endplate or the expression of other muscle-specific proteins, was also reduced, or whether only the sex-specific differences associated with MOL development were affected in fibers having a fraction of the normal complement of nuclei. Our results indicate that the formation of the proper attachment points and the expression of a late stage muscle-specific reporter gene are independent phenomena that have different requirements for the number of muscle nuclei.

MOL-position fibers in *fru* mutants do not exhibit the male-specific recruitment of myoblasts

Considering the small size of MOL-position fibers in severe *fru* mutants, one of our initial expectations was that fewer muscle nuclei would be present in MOL-position fibers concomitant with their reduced size. Indeed, MOL-position fibers in nearly all *fru* mutants had either the same or slightly fewer muscle nuclei than did neighboring medial fibers (see Table 1), a significant difference compared to the relationship between MOL and medial fibers in wild-type males. These results suggest that one role of the *fru*$^+$ product is to mediate the male-specific recruitment of nuclei into MOL fibers. The *fru* mutant effect appears to be specific to MOL muscle development and not a generalized inability to form dorsal abdominal muscles.

We propose that the first developmental stage where *fru*$^+$ acts to generate a normal MOL muscle occurs during the fusion of myoblasts into MOL myotubes. Since the number of *twist*-positive cells in *fru* mutant larvae was the same as in wild-type larvae, there appears to be no *fru* mutant effect at early stages of myogenesis. Subsequent divisions by these myoblasts in the pupal stage generated a sufficient pool of myoblasts to have generated a normal-sized MOL in at least two of the strong *fru*$^-$ genotypes when compared to the HU-ablation treatments (see Tables 2 and 3). In addition, the medial and MOL-position fibers in *fru* mutant males used the same fraction of the total muscle nuclei pool as did medial and MOL fibers in wild-type males. The remaining step in muscle development which would affect the final number of nuclei in muscles is fusion, leading us to the conclusion that in *fru* mutant males, MOL-position fibers were apparently unable to actively recruit additional myoblasts.

The reduction in the number of nuclei in MOL-position fibers in *fru* mutant males is not sufficient to account for the small size of these fibers. In one of the five *fru* mutant genotypes tested, *fru*^{1}/*Df(3R)P14*, the total number of nuclei fell within the range found for the HU-treatment group 2 partial deletions. Two other *fru* mutant genotypes, *fru*^{1}/*fru*^{1} and *fru*^{1}/*Df(3R)Cha*^{M5}, had total nuclei counts similar to the HU-treatment group 3 partial deletions. Normal sized or reduced MOL fibers were present in virtually all of the HU-treated hemitergites whereas MOL-position fibers were no larger than medial fibers in virtually all of the severe *fru* mutant genotypes. In the genotype, *fru*^{2}, where normal sized MOL fibers form, the number of either total muscle nuclei or the number of nuclei in MOL-position fibers is not different from other *fru* genotypes where MOLs do not form. On the basis of the distribution of nuclei into MOL and medial fibers in hemisegments where the number of total nuclei had been reduced to that of various *fru* mutants, we would have expected that MOL fibers would have been able to sequester essentially normal numbers of nuclei and that medial fibers in these mutants would have had even fewer nuclei than found in the medial fibers of wild-type males.

Our findings support a dual role for *fru*$^+$ activity in the development of the MOL. The first role is in the male-specific recruitment of additional myoblasts to the MOL myotubes and in an additional, independent step, *fru*$^+$ product leads to the elongation of MOL fibers to their appropriate attachment points. Due to the unusual dependence of MOL development on innervation by male-specific motorneurons, *fru*$^+$ may act either in the MOL-specific motorneuron, in the generation of

the male-specific inductive activity, or in the myoblasts to mediate the response to the male-specific inductive signals.

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