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Genetic Analysis of the *Drosophila* **β**3-Tubulin Gene Demonstrates That the Microtubule Cytoskeleton in the Cells of the Visceral Mesoderm Is Required for Morphogenesis of the Midgut Endoderm

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We have investigated the cellular basis for lethality of mutant alleles of the *Drosophila melanogaster* **β**3-tubulin gene, ***βTub60D.*** Lethal **β**3 mutations can be grouped into two classes: the most severe mutations (Class I alleles) cause death during the first larval instar, while weaker alleles (Class II) cause death in later larval stages or in early pupal development. Since **β**3 is not expressed during larval development, lethality of the Class I mutations must reflect essential functions of **β**3 in embryogenesis. **β**3-tubulin is zygotically expressed during midembryogenesis in the developing mesoderm, and the major site of **β**3 accumulation is in the developing muscles during myogenesis. We show that the embryonic pattern of **β**3 expression, including accumulation in the developing musculature, is conserved in other *Drosophila* species. However, we found that loss of **β**3 function does not cause discernible defects in either the ultrastructure or function of the larval muscle. Thus **β**3-tubulin is dispensable in its highest site of accumulation. Rather, the essential site of function of **β**3 in embryos is in cells of the visceral mesoderm. Lethality of Class I alleles is caused by defects in midgut morphogenesis and failure of gut function. Although the folding pattern is irregular and the gut is smaller than normal, a complete folded gut forms in mutant larvae, and the visceral muscle functions normally to move food through the gut. However, mutant larvae cannot absorb nutrients across the gut wall. Thus loss of **β**3 function in the mesoderm results in defects in the underlying endodermally derived layer of the gut. Our data provide an assay for cellular interactions between mesoderm and endodermal tissues and reveal a role for the microtubule cytoskeleton of the visceral mesodermal cells in differentiation of the endodermal cell layer of the larval gut. © 1996 Academic Press, Inc.

INTRODUCTION

Microtubules are an important component of the eukaryotic cytoskeleton. The major structural components of microtubules, the α- and β-tubulins, are encoded in multiple gene families, each member of which has a unique temporal and spatial pattern of expression. *Drosophila* has four β-tubulin genes that encode four distinct isoforms. In early *Drosophila* development, the microtubule cytoskeleton plays diverse roles both in prepositioning of developmental cues in the oocyte, and in the mitotic divisions and cytoplasmic reorganizations in the embryo (Theurkauf *et al.*, 1993, 1994). All of these early developmental functions are supported by microtubules assembled from the β1-tubulin isoform (β1), which is both maternally supplied and zygotically expressed (Raff *et al.*, 1982; Bialojan *et al.*, 1984; Gasch *et al.*, 1988; Buttgereit *et al.*, 1991). Expression of a second isoform, β3-tubulin (β3), does not begin until midembryogenesis, in cells that have ceased to divide and will undergo extensive cell shape changes (Raff *et al.*, 1982; Bialojan *et al.*, 1984; Leiss *et al.*, 1988; Gasch *et al.*, 1988; Kimble *et al.*, 1989). Dissection of the role of β3 would thus provide insight into the role of the microtubule cytoskeleton in cells undergoing morphogenetic changes in the final stages of differentiation.

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The β3 gene has a complex pattern of expression. In embryos, β3 is expressed at high levels in the developing musculature, where it is the single zygotically expressed β-tubulin (Gasch *et al.,* 1988; Leiss *et al.,* 1988; Kimble *et al.,* 1989), but the protein can no longer be detected in first or second instar larvae (Dettman, 1995; Dettman and Raff, in preparation). After 16 hr postfertilization, β3 message cannot be detected until the third instar larval molt (Bialojan *et al.,* 1984; Andres *et al.,* 1993), at which time the protein begins to accumulate in the male gonads (Gönczy *et al.,* 1992) and in imaginal discs (Kimble *et al.,* 1989). During pupal development, β3 is expressed in several tissues, including each of the developing muscles; expression persists in the adult only in the somatic cells of the gonads (Kimble *et al.,* 1989).

Although there may be some exceptions, in the embryo β3 is expressed primarily in cells of mesodermal origin (Leiss *et al.,* 1988; Kimble *et al.,* 1989). When expression of β3 begins after 8 hr of development, it is expressed in the cephalic, visceral, and somatic mesoderm. In addition to the pharyngeal, gut, and body wall musculature, β3 is expressed in nonmuscle cells derived from the mesoderm, including the macrophages (Leiss *et al.,* 1988), the transverse nerve exit glia (Gorczyca *et al.,* 1994), and the cap cells of chordotonal organs and the lymph and ring glands (Dettman and Raff, in preparation). The three distinct mesoderm layers may become determined early to perform distinct functions later. Cells of the cephalic mesoderm form the pharyngeal and foregut musculature and incorporate into several other organs in the head segments, such as support cells of sensory organs. The visceral mesoderm forms the gut muscles and also mediates events that allow the midgut to fold (Reuter and Scott, 1990). Cells of the somatic mesoderm give rise to body wall muscles and support cells of chordotonal organs.

The *βTub60D* gene, which encodes β3, is essential. Mutant alleles of *βTub60D* (*B3t*) cause lethality, male and female sterility, flightlessness, and defects in bristle structure—phenotypes consistent with known sites of β3 expression (Kimble *et al.,* 1990). To define the roles of microtubules in late events in embryogenesis, we investigated the cellular basis for lethality of *B3t* alleles. The most severe mutations, designated Class I alleles, cause death early in larval development, while less severe Class II alleles cause death much later in larval development, or during pupal development.

Our initial prediction was that the early-dying Class I mutants would reflect loss of β3 function in the somatic musculature, the embryonic tissue with the highest levels of β3 and in which β3 is the only zygotically expressed β-tubulin isoform (Bialojan *et al.,* 1984; Gasch *et al.,* 1988; Kimble *et al.,* 1989). That there are conserved general functions in both vertebrates and invertebrates for microtubules as essential cytoskeletal elements in myogenesis is supported by numerous ultrastructural and drug studies (Bischoff and Holtzer, 1968; Persitianis and Gregory, 1971; Crossley, 1972a,b; Warren, 1974; Toyama *et al.,* 1982; Tassin *et al.,* 1985; Reedy and Beall, 1993a,b). Moreover, we examined expression of the homologous isoform in other *Drosophila* species and determined that embryonic β3 expression in myogenesis has been conserved in evolution. We therefore speculated that the β3 isoform might have specialized functional properties required for myogenesis.

However, to our surprise, we found that β3 is dispensable for development and function of the larval muscle in *Drosophila melanogaster.* We have considered the possible meaning of evolutionarily conserved muscle expression of β3 in the light of this observation. We argue that β3 expression serves to increase the tubulin pool levels in myogenic cells, and that this confers a selective advantage for maintenance of muscle expression, but is not essential for the development of an individual larva. To define the functions of β3 that are essential in development, we examined other aspects of the mutant phenotypes. We found that lethality associated with β3 mutations results from abnormal development and function of the gut. Importantly, a major contribution to the lethality lies in the failure of the endodermal layer of the intestine to function correctly. This suggests that correct cytoarchitecture of the visceral mesoderm cells is important for the differentiation of the underlying endoderm.

MATERIAL AND METHODS

Flies

Drosophila stocks were maintained at 25°C on standard cornmeal/molasses/agar food. Visible markers, deficiency chromosomes, and balancer chromosomes used in this study are described in Lindsley and Zimm (1992) or FlyBase (1994). The deficiency chromosome *Df(2R)Px^2, bw sp^2* (designated *Px^2* throughout the text) deletes region 60C5,6–60D9,10 of chromosome 2, including *βTub60D,* the gene that encodes the β3-tubulin isoform (Kimble *et al.,* 1990). Second chromosome balancers used in this study were *CyO* (*In(2LR)O,Cy dp^{lvI} pr cn^2*) and *CyO-P*[y^+], the CyO balancer carrying a copy of the wild-type *yellow* gene in the *P*[y^+] transposon. All other stocks mentioned in the text were either generated by mutagenesis or obtained from the stock centers in Bloomington and Bowling Green or from other laboratories.

Isolation of B3t Alleles

Mutations in the *βTub60D* gene were recovered by F2 screens using standard methods of mutagenesis; mutant alleles are designated as *B3t^n*. The *B3t* lethal phenotype maps to the 60D region by recombination and is rescued with a P-element containing wild-type *βTub60D* genomic DNA (Kimble *et al.,* 1990). In addition to the five EMS-induced alleles described in our original study (*B3t$^{1-5}$*; Kimble *et al.,* 1990), we generated eight new alleles in this study, six of which were induced by EMS (*B3t$^{6-11}$*), one by gamma irradiation (*B3t^{sk}*) and one by P-element mutagenesis (*B3t^{p126}*). Screens were designed to isolate mutagenized chromosomes that failed to complement either the *Px^2* deficiency or *B3t^2*, the most severe lethal allele. Viable allele combinations were scored for fertility, bristle morphology, and ability to fly; flight tests were performed

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on flies that had not been anesthetized for at least 24 hr. Sterility, flightlessness, and defective bristle phenotypes remained tightly linked to *B3t* lethality for every allele isolated. $B3t^{P126}$ was generated by mobilization of an insertion of the P[*lacZ*] element in line *A1-2-26* isolated by Bier *et al.* (1989). The original P[*lacZ*] insertion is in the 5′ region of the β*Tub60D* gene and has no mutant phenotype but expresses *lacZ* in the same pattern as β3 (Dettman and Raff, in preparation). Southern analysis of the $B3t^{P126}$ chromosome revealed that the new position of the P-element insertion is within the first intron of β*Tub60D,* approximately 1 kb from the 5′ splice site; DNA rearrangements were not detected in any of the other alleles (Dettman, 1995). $B3t^{P126}/B3t^{2}$ animals die as pharate adults. All viable combinations with $B3t^{P126}$ are male sterile; in combinations of $B3t^{P126}$ with severe *B3t* alleles, including Px^{2}, surviving adults are male and female sterile and also exhibit bristle defects (Dettman, 1995). The *B3t* alleles are all recessive mutations that exhibit different degrees of loss of function; none of the alleles is a protein null. The mutations can be ordered from most to least severe as 2 > 3 > 10 > 9 = 6 > 8 = 7 > sk > 11 > 5 > 1 > 4 > P126 (Dettman, 1995).

Larval Lethal Phase and Feeding Experiments

Lethal phase and phenotype analysis was facilitated by use of the *CyO-P*[y^{+}] balancer to allow identification of the genotype of first instar larvae. When *B3t* alleles are balanced by *CyO-P*[y^{+}] in a *yellow* mutant background, larvae carrying the balancer chromosome have wild-type darkened mouth hooks (y^{+}), whereas larvae homozygous for the *B3t* allele have yellow mouth hooks (y^{-}). Eggs were collected on agar plates treated with penicillin/streptomycin to prevent bacterial growth in cultures of sickly mutant larvae (Sigma, 20 mg/ml). When culturing larvae, to prevent desiccation (to which *B3t* mutant larvae are particularly vulnerable), the underside of the tops of the plates were sprayed with a fine mist of water, and plates were sealed with parafilm and placed in humidity chambers inside incubators. The instar at which larvae died was determined by squashing dead larvae and observing the teeth on the mouth hooks (Bodenstein, 1965). Experiments to determine food intake and excretion were done by feeding larvae a yeast paste containing blue food dye and 1.4 nCi/mmol [^{35}S]methionine ([^{35}S]-met) as a tracer. Washed, individual larvae were placed on DE81 filters (Whatman, 2.4 cm diameter) and counted by liquid scintillation in 4 ml of Biosafe II (Research Products International). Drug feeding experiments for 20-OH ecdysone (Sigma) were done by dissolving the given dilution of ecdysone in a specified amount (dry weight) of autoclaved yeast. Larvae were transferred to fresh plates with a fresh dose of drug daily.

Immunocytochemistry

Preparation and purification of antibodies and immunolocalization experiments were done as described in Kimble *et al.* (1989). Antibodies used in this study were anti-β3, an affinity purified polyclonal antiserum specific to *Drosophila* β3-tubulin (Kimble *et al.,* 1989); anti-β1, a monoclonal antiserum specific to *Drosophila* β1-tubulin prepared by M. T. Fuller (Fuller and Raff, unpublished data) that recognizes only the β1 isoform on both Western blots and in *Drosophila* tissues (unpublished data and Fig. 2); and anti-Mhc, a polyclonal antiserum specific to myosin heavy chain (Young *et al.,* 1991).

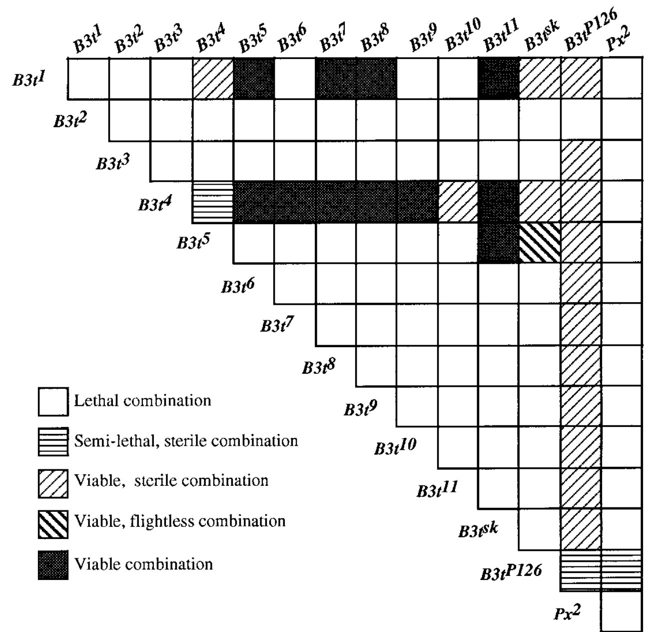

FIG. 1. Interallelic complementation matrix for *B3t* alleles at 25°C showing allele combinations that are lethal, semilethal, and viable. Viable adult animals carrying combinations of *B3t* mutant alleles exhibit other phenotypes. Phenotypes of alleles $B3t^{1}$–$B3t^{5}$ are described in detail in Kimble *et al.* (1990); phenotypes of new alleles isolated in this study are described under Materials and Methods and Results. Allele combinations that are viable or partially viable at 25°C are semi-lethal or lethal at 29°C, and some phenotypes are also more severe at 29°C than at 25°C, indicating that developmental processes involving β3 are intrinsically temperature-dependent.

Electron Microscopy

Samples were prepared and analyzed by transmission electron microscopy as described by Fackenthal *et al.* (1993).

RESULTS

Lethal Mutations in the β3-Tubulin Gene Cause Death during Larval Development

Figure 1 shows an *inter se* complementation matrix for the β3 mutations. All of the *B3t* alleles result in recessive lethality, with the exception of $B3t^{4}$ and $B3t^{P126}$, semilethal alleles for which homozygous viable adults are sterile. A number of interallelic *B3t* mutant combinations are also viable or partly viable; animals carrying these allele combinations exhibit defects in fertility, bristle morphology, and flight, revealing essential β3 functions during pupal development.

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TABLE 1
Analysis of Lethal *B3t* Alleles

B3t mutant allele	Lethal larval instar	No. days as larva	No. animals observed
Class I alleles			
$B3t^2$	L1	2–3	100
$B3t^6$	L1	5	30
$B3t^7$	L1	5	45
$B3t^8$	L1	3	55
$B3t^9$	L1	5	64
$B3t^{10}$	L1	3	54
$B3t^{11}$	L1	3	38
Class II alleles			
$B3t^1$	L3/P	>5	30
$B3t^5$	L3/P	>5	30
$B3t^{sk}$	L3/P	>5	30

Note. The larval lethal phases at 25°C are shown for Class I and Class II *B3t* lethal alleles. "Lethal larval instar" is the final instar that mutant larvae reach before death: L1, first instar, L3/P, some die as third instar larvae and some die as pupae. "No. days as larva" is the average number of days that a larva of the given genotype survives in the lethal instar. (Compare to the normal developmental time course at 25°C: hatching, 1 day post egg lay; time in larval instars: L1, 1 day; L2, 1 day; L3, 3 days; pupariation, 5 days posthatching; eclosion to adult, 10 days). The alleles $B3t^3$, $B3t^4$, and $B3t^{P126}$ shown in Fig. 1 were not included in this study, because $B3t^3$ carries a tightly linked secondary embryonic lethal mutation, and $B3t^4$ and $B3t^{P126}$ are semiviable male sterile alleles (see Kimble *et al.,* 1990, and Materials and Methods).

Lethal *B3t* alleles fall into two classes (Table 1). Class I mutants hatch and live for several days as first instar larvae but never grow and fail to molt into the second instar. The time elapsed before death varies, depending on the strength of the particular allele and the conditions under which the larvae are reared. Given optimal culture conditions, mutant larvae homozygous for more severe Class I alleles ($B3t^2$, $B3t^8$, $B3t^{10}$, and $B3t^{11}$) die in the second or third day after hatching; animals homozygous for weaker Class I alleles ($B3t^6$, $B3t^7$, and $B3t^9$) do not die until the third to fifth day after hatching, but they also rarely molt into the second instar. The most severe Class I allele is $B3t^2$. Most homozygous or hemizygous $B3t^2$ larvae die in the second day after hatching, with $B3t^2$ hemizygotes appearing more sickly than $B3t^2$ homozygotes before death.

Class II alleles comprise a less severe class of mutations that exhibit multiphasic lethality, dying late in the third instar or during pupal development. The stage of development reached by Class II alleles also depends on the strength of the allele and the culture conditions of larvae. Three of the five weakest lethal alleles defined by *inter se* complementation ($B3t^1$, $B3t^5$, and $B3t^{sk}$) make up the Class II mutants. The weakest Class II allele is $B3t^1$; rare viable or manually eclosed $B3t^1$ homozygotes are weak, short-lived, sterile, flightless, and have severe bristle defects (Kimble *et al.,* 1990), similar to phenotypes exhibited by animals with other viable allele combinations (Fig. 1).

β3 Is the Major Isoform in Embryonic Muscle of Drosophila melanogaster, and Muscle Expression of the β3 Homolog Is Conserved in D. simulans and D. virilis

Previous studies showed that β3 is the predominant β-tubulin isoform in developing muscle cells (Gasch *et al.,* 1988; Kimble *et al.,* 1989; Buttgereit *et al.,* 1991). In late stage embryos, zygotic expression of the β1 gene supports accumulation of high levels of β1 in the developing nervous system and in the segmental muscle attachment cells, the apodemes, but the β1 gene is not zygotically expressed in other tissues (Gasch *et al.,* 1988; Buttgereit *et al.,* 1991). However, abundant levels of both β1 mRNA and protein are preloaded into the egg during oogenesis, and significant levels of maternally supplied tubulins persist until the end of embryogenesis (Raff *et al.,* 1982; Raff, 1984; Gasch *et al.,* 1988; Buttgereit *et al.,* 1991; Theurkauf, 1992; Dettman, Hoyle, and Raff unpublished data). Thus myogenic cells descend from cells wherein β1 is maternally loaded. We used a monoclonal antibody specific to the β1 isoform to see if β1 is present along with β3 in differentiated muscle cells. As shown in Fig. 2, the lateral body wall muscles of each segment exhibit high levels of staining with the β3-specific antibody (Fig. 2A) but not with the β1-specific antibody (Fig. 2B). We observed specific high-level β1 staining in the apodemes, consistent with the zygotic expression in these cells demonstrated by Buttgereit *et al.* (1991). We did not detect β1 staining in embryonic muscle above the generalized background caused by the presence of β1 throughout the embryo (Figs. 2B and 2C). These data confirm the previous observation that β3 is the predominant β-tubulin isoform in developing muscle and demonstrate that the developing body wall muscles do not contain high levels of β1. However, the generalized β1 staining in late embryos may obscure low-level β1 staining in muscle; thus our results do not preclude the possibility that some maternally derived β1 persists in the muscle cells. The data we present below show that maternally derived β1 is present in the initial stages of myogenesis.

The accumulation of high levels of β3 in the developing musculature was suggestive that β3 has an important role in this tissue. As shown in Fig. 3, a second important observation consistent with this hypothesis is our finding that the localization pattern of the anti-β3 antibody is conserved in other species of *Drosophila.* We expected that *Drosophila* β3 might have homologs in other species, in analogy with the conserved β-tubulin isotype classes in vertebrates, and as has been demonstrated for the testis-specific β2 isoforms in *D. melanogaster* and *D. hydeii* (Michiels *et al.,* 1987). We stained embryos from a closely related species (*D. simulans*) and a more distantly related species (*D. virilis*) with

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anti-β3. The staining pattern shows that each species has a β3 homolog expressed in a pattern similar to that of the *D. melanogaster* β3 isoform (Fig. 3A). In both species, anti-β3 stained body wall muscles (Figs. 3B–3F), pharyngeal muscles (Figs. 3B and 3D), gut muscles (Fig. 3D, not shown for *D. simulans*), and the dorsal vessel (Fig. 3B, not shown for *D. virilis*). Myoblasts were also stained in both *D. virilis* and *D. simulans* embryos (data not shown), as we have observed for *D. melanogaster* (Dettman and Raff, unpublished observations). We also observed staining in macrophages (Figs. 3C and 3F) and cells in the anterior-most portion of the embryo (Figs. 3B and 3D) in *D. simulans* and *D. virilis* embryos, similar to that in *D. melanogaster* (Dettman and Raff, in preparation), demonstrating that β3 expression is also conserved in nonmuscle tissues.

Microtubule Arrays Precede Myofibril Formation and Are Associated with Growing Myofibrils in Embryonic Body Wall and Gut Muscle

Assembly of arrays of microtubules has been shown to accompany sarcomere formation in a number of vertebrate and invertebrate species (Persitianis and Gregory, 1971; Crossley, 1972a,b; Warren, 1974; Toyama *et al.,* 1982; Tassin *et al.,* 1985; Reedy and Beall, 1993a,b). As shown in Fig. 4, ultrastructural examination of developing larval muscle in wild-type *Drosophila* embryos revealed that in myogenic cells, microtubules form in arrays parallel to the eventual muscle fibers. Microtubule assembly precedes sarcomere formation (Fig. 4A), as has been observed in other species. In later stages, microtubules are intermingled among growing sarcomere elements (Figs. 4B and 4C). Microtubules remain associated with growing myofibrils, but the number of microtubules with each myofibril decreases as the islands of thick and thin filaments increase in diameter (Fig. 4C). We examined sections of muscle from first, second, and third instar larvae; few microtubules persist after sarcomeres form. We only rarely observed microtubules in muscle cells of first instar larvae and never observed microtubules in association with myofibrils in second and third instar larvae (not shown). Thus, muscle-associated microtubules are transient and are disassembled after completion of myogenesis, consistent with the transient accumulation of β3. The temporal course and placement of microtubules in *Drosophila melanogaster* are consistent with a role for microtubules in the orientation or organization of sarcomere formation, as has been postulated for myogenesis in other species, and as also appears to be the case during development of the flight muscles during *Drosophila* pupal development (Reedy and Beall, 1993a,b). During development of the flight muscles, however, the microtubule cytoskeleton is very highly organized, unlike the seemingly unpatterned microtubule arrays that we observed during embryonic myogenesis. Correspondingly, the sarcomeres in the *Drosophila* larval muscles are also much less tightly organized than those in the adult flight muscles; the larval musculature exhibits considerable variability in size and spacing of thick and thin filaments and Z lines (Fig. 4D).

FIG. 2. β1-tubulin does not accumulate in the body wall musculature. (A) Stage 16 wild-type embryo stained with anti-β3. β3 accumulates in the dorso-lateral body wall muscles (small arrowheads) but is not detectable in the apodemes (large arrowhead). (B and C) Stage 16 wild-type embryo stained with anti-β1. Higher magnification (B) shows that β1 is accumulated in the apodemes and in clusters of neuronal cells adjacent to the body wall muscles (large arrowheads), but is not accumulated above background levels in the dorso-lateral body wall muscles. Generalized staining of β1 is also visible in the gut and other tissues. Lower magnification (C) shows the overall localization pattern of β1 in the late embryo. β1 is present at high levels in the tissues in which it is zygotically expressed (nervous system and apodemes), but maternally derived β1 is also present throughout the embryo. Bar in A represents 0.03 mm, for A and B. Bar in C is 0.1 mm.

FIG. 3. β3 homologs accumulate in the embryonic mesoderm of *D. virilis* and *D. simulans* in a pattern similar to that in *D. melanogaster.* (A) Stage 16 wild-type *D. melanogaster* embryo stained with anti-β3. The predominant site of accumulation is in the somatic musculature; β3-staining can also be seen in the pharyngeal and gut muscles (arrowheads). (B and C) *D. simulans* embryos stained with anti-β3. Substantial accumulation of the β3 homolog in the somatic muscles can be seen in both embryos. Arrowheads in B indicate anti-β3 staining in the pharyngeal muscles (left) and the dorsal vessel (right). Arrowheads in C indicate anti-β3 staining in macrophages. (D–F) *D. virilis* embryos stained with anti-β3. Accumulation of the β3 homolog in the body wall muscles can be seen in all embryos; the focal plane in E shows the pattern of the somatic musculature. The arrowhead in D indicates staining in the pharyngeal muscles; arrowheads in F indicate staining in the macrophages. Embryos are oriented with the anterior end to the left. Bar in A represents 0.08 mm. Bar in F is 0.06 mm, for B–F.

FIG. 6. β3 expression in the visceral mesoderm and the apposition of the visceral mesoderm and endoderm during formation of the midgut. Embryos are stained with anti-β3, showing the accumulation of β3 in the visceral mesoderm (large arrowheads) that surrounds the columnar endodermal cells of the gut (small arrowheads). The yolk mass (Y) is visible in the lumen of the gut. (A) Early stage 14 embryo, prior to constriction of the midgut. The visceral mesoderm cells are also columnar at this stage. (B) Later stage 14 embryo, when constriction of the midgut is proceeding, and the visceral mesodermal cells have flattened. Large arrowheads indicate accumulation of β3 at the site of midgut constriction. Bars are 0.08 mm.

FIG. 7. $B3t^2$ larvae have morphologically abnormal midguts. Larvae tested were fed yeast paste mixed with blue food dye to visualize gut morphology. All larvae are first instar larvae, oriented with their anterior ends to the top of the page. (A) Wild-type larva, showing normal midgut morphology. (B and C) Examples of smaller midguts typical of Class I mutant larvae: (B) $B3t^2$ homozygote. (C) $B3t^2/Px^2$ larva. Bar in A represents 0.08 mm, for all panels.

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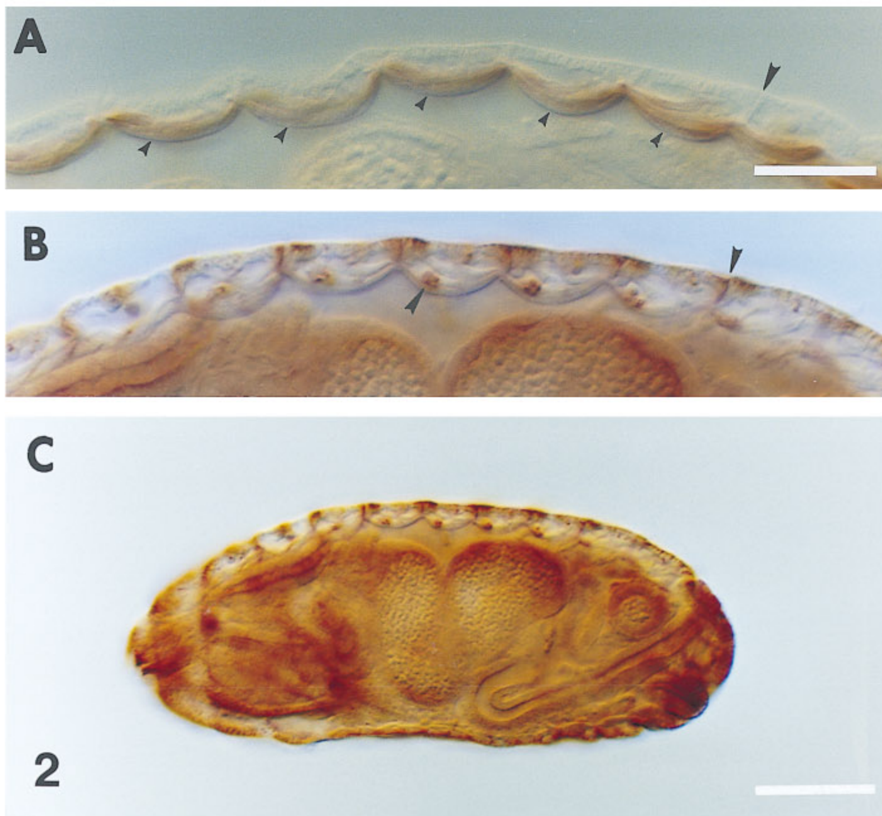
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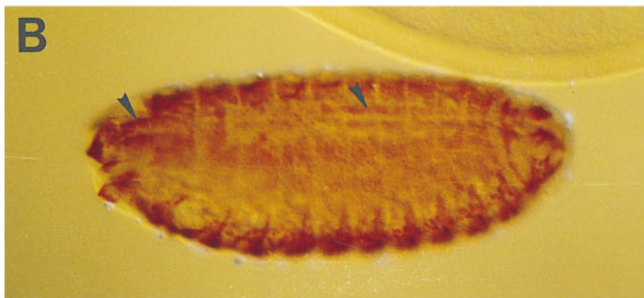
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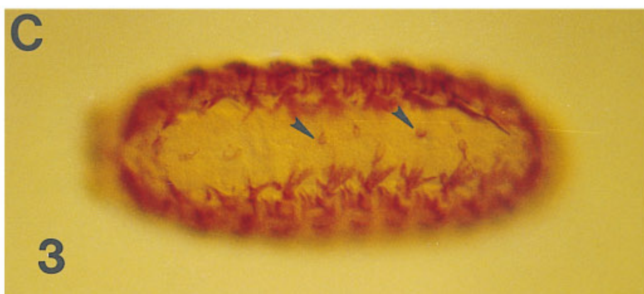
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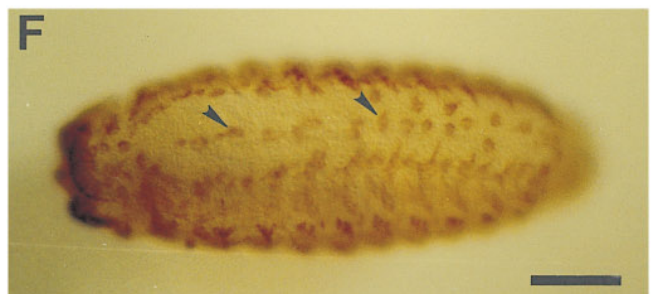
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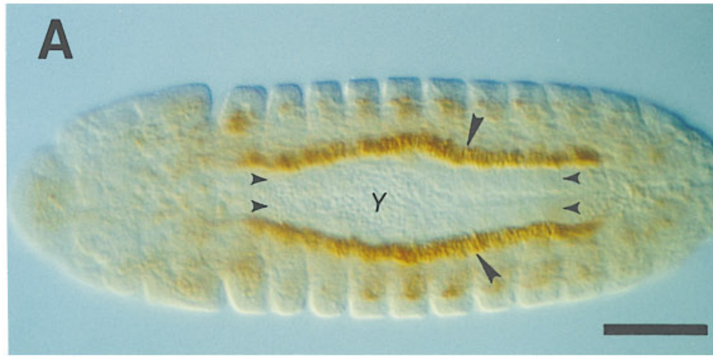
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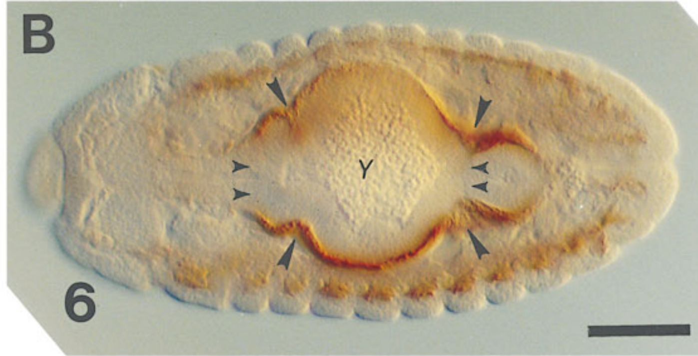
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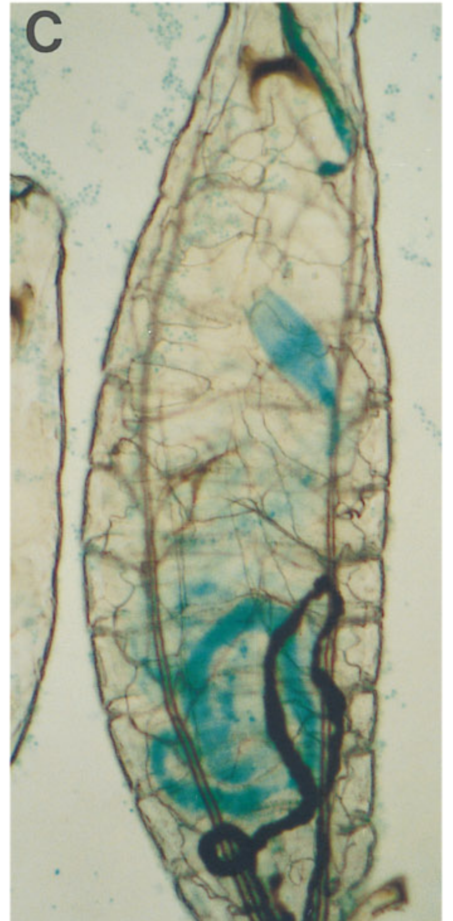
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Myogenesis and Sarcomere Integrity Are Normal in Homozygous Px^2 Embryos and in B3t Mutant Larvae and Adults

Since the Px^2 chromosome deletes the β*Tub60D* gene, we anticipated that in homozygous Px^2 embryos we could examine muscle formation in the absence of microtubules and that the defects in morphogenesis or function of the muscles would reveal the specific function played in myogenesis by the β3-based microtubule cytoskeleton. We knew that loss of β3 function does not prevent formation of muscle, since in prelethal terminal stage Px^2 homozygotes, somatic muscles are present and moreover can undergo coordinated rhythmic contractions along the body segments, albeit with much less vigor than in prehatching wild-type animals (Kimble *et al.,* 1990; this study). We therefore examined the organization of the muscle fibers in homozygous Px^2 embryos and in *B3t* mutants by immunolocalization and scanning electron microscopy (not shown). We fixed embryos laid by heterozygous Px^2 or $B3t^n$ parents and stained them with anti-Mhc, an antibody directed at muscle myosin (see Materials and Methods). We examined muscle myosin staining in populations of stained embryos, one quarter of which will be the homozygotes for the mutant allele being tested. We observed no defects in the muscle fiber pattern in any of the embryos in any of the genotypes examined. We used SEM to examine the organization of the body wall muscle in homozygous Px^2 embryos at the terminal stage of development (prehatching) and in *B3t* mutant larvae. For each of the genotypes examined, muscles of the correct size and morphology were organized correctly in the typical fiber pattern for each hemisegment. Thus overall muscle development is not disrupted by loss of β3 function.

We then examined myofibrillar development at the ultrastructural level in Px^2 homozygotes to determine if there were any defects in the cytoarchitecture of individual sarcomeres formed in the absence of β3. To identify Px^2 homozygotes at the time when myogenesis occurs during midembryogenesis, the *prd,* Px^2 stock was utilized as described in Kimble *et al.* (1990). The *prd* segmentation defect allows identification of homozygous *prd,* Px^2 embryos, but the *prd* mutation does not affect the structure of muscle fibers within a given segment. Our results were unexpected. We analyzed a large number of sections of somatic muscle representing multiple homozygous *prd,* Px^2 embryos. Muscles in *prd,* Px^2 embryos were indistinguishable from muscles in wild-type embryos (Figs. 4E and 4F). We could identify no intrinsic abnormalities in the sarcomeres of the *prd,* Px^2 embryos either in longitudinal or cross sections. Neither the length of the sarcomeres nor the organization in cross section were appreciably different in the *prd,* Px^2 embryos compared to wild-type controls, based on counts of thick and thin filaments in cross sections and measurements of the length of the sarcomeres in longitudinal sections (data not shown). Not only was the structure of sarcomeres in homozygous *prd,* Px^2 embryos normal, but microtubules were present in all of the developing muscles we examined (Figs. 4E and 4F). We also examined the ultrastructure of body wall, gut, and pharyngeal muscle in first instar larvae hemizygous for $B3t^2$, the most severe recessive lethal allele. As predicted from the homozygous Px^2 phenotype, the muscles in $B3t^2/Px^2$ larvae were indistinguishable from those of wild-type controls (Figs. 4G and 4H). We did not observe any qualitative difference between wild-type controls and β3 mutants.

The results described above were such a surprise to us that we constructed another *prd,* Px^2 stock and repeated our analysis in order to eliminate the possibility that the original stock had broken down and that in the first experiments the *prd* embryos we had examined were in fact not homozygous for Px^2. The results were the same in the second experiment: Muscle in homozygous *prd,* Px^2 embryos was normal. The only difference between wild-type animals and animals deficient for β3 that we could discern in either experiment was that there were fewer microtubules in the muscle cells of Px^2 homozygotes than in wild-type controls. This observation was consistent with our expectation that the size of the tubulin pool in myogenic cells would be considerably diminished when β3 is absent.

Since microtubules are still present in myogenic cells in the absence of β3 expression, there must be another source of β-tubulin. β1-tubulin is the only likely candidate, since β1 and β3 are the only isoforms expressed at the appropriate time in development (Raff *et al.,* 1982; Gasch *et al.,* 1988). Of the remaining two β-tubulin isoforms, β2 is exclusively expressed in the male germ line (Kemphues *et al.,* 1979, 1982; Hoyle *et al.,* 1995) and β4 is first expressed only during late embryogenesis and is not detectable in muscle by immunolocalization (H. B. Diaz and E. C. Raff, unpublished observations). Once the muscles have formed, we did not detect high levels of β1 in muscle in wild-type embryos (Fig. 2B). We considered the possibility that residual β1-tubulin from earlier stages in development might be preferentially stabilized when β3 is not expressed. To determine if β1-tubulin is present above wild-type levels in muscle in Px^2 homozygotes, we collected stage 16 and 17 embryos from Px^2/CyO parents and stained them with anti-β1. There was not a population of embryos with preferential accumulation of β1 (data not shown). We therefore concluded that β1 accumulation and/or stability is not enhanced in Px^2 homozygotes. Thus, in the absence of β3 expression, assembly of microtubules must be supported by the maternally derived β1 that normally persists from earlier stages in development.

Formation of muscle fibers begins during early stage 13 and is complete by stage 16 (Campos-Ortega and Hartenstein, 1985; and see Fig. 4). We observed that microtubule assembly in the myogenic cells begins in stage 12 (Figs. 4A and 4E). For homozygous Px^2 embryos, therefore, the question is the amount of maternally derived β1 protein, and hence the size of the available tubulin pool, that is present when microtubule assembly commences in the initial stages of myogenesis. Available data show that the ma-

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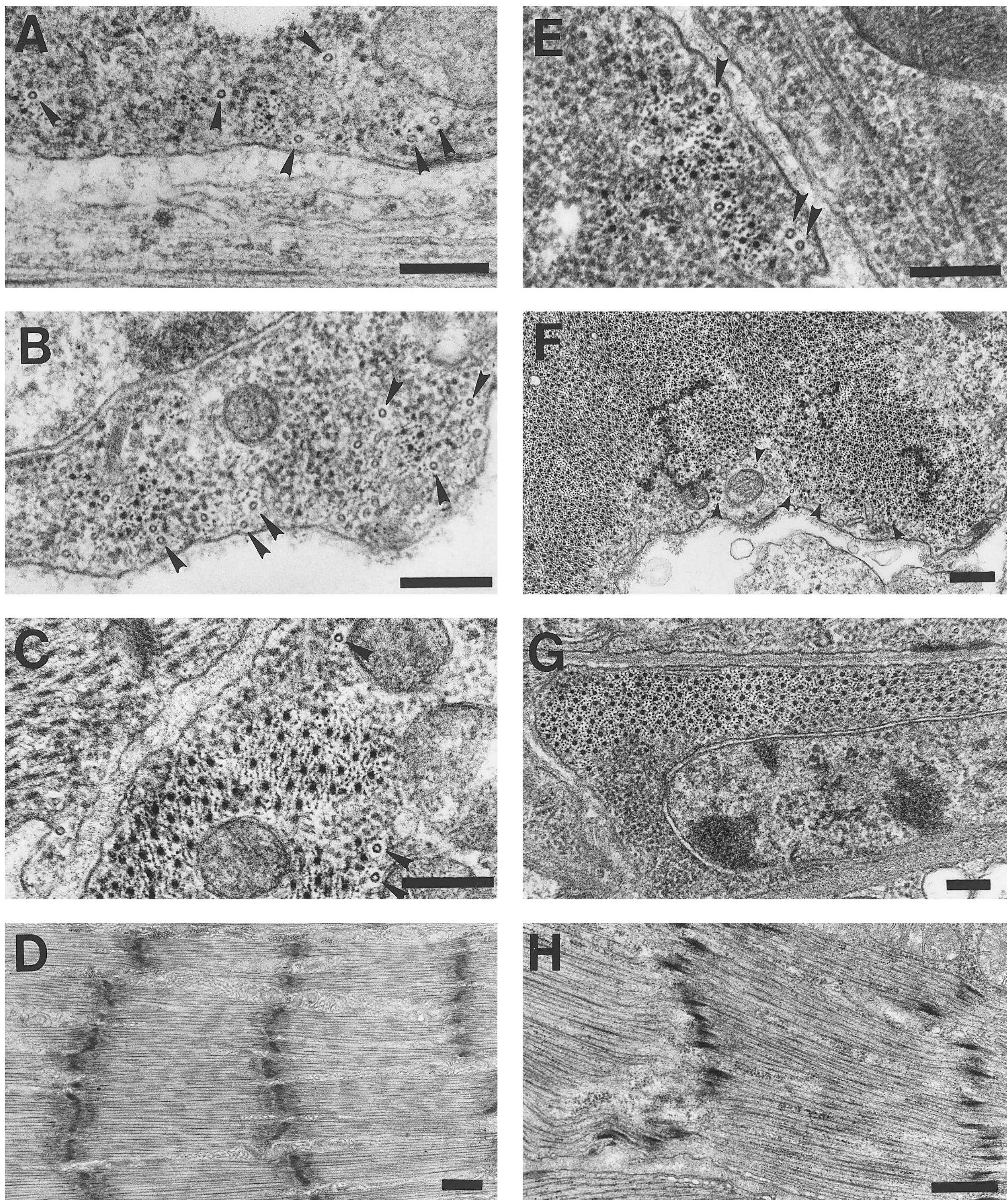

FIG. 4. Ultrastructure of developing somatic muscle in wild-type embryos and embryos deficient in β3 function. (A–C) Representative cross sections showing the distribution of microtubules in developing wild-type body wall muscle. Cross sections of developing gut muscle are similar in appearance to that of body wall muscle. (A) Developing muscle cell in a stage 12 wild-type embryo. Microtubules (arrowheads) can be seen both in association with forming bundles of myofibrils and also in areas of the cytoplasm devoid of myofibrillar structure. All microtubules observed were oriented parallel to the developing myofibrils. (B) Stage 16 embryo; microtubules (arrowheads) are associated with larger bundles of thick and thin filaments. (C) Late stage 17 embryo in which myofibrils fill most of the cytoplasm of muscle cells. Fewer microtubules are present, but some microtubules (arrowheads) are associated with the periphery of the developing myofibril. (D) Longitudinal section of wild-type larval muscle. Larval somatic muscle is less well organized than the highly organized indirect flight muscle (Reedy and Beall, 1993a,b). As illustrated here, larval muscles exhibit variable size and spacing of thin and thick filaments in cross

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ternally derived $\beta1$ pool is substantial. Using *in situ* hybridization to localize $\beta1$ transcripts, Gasch *et al.* (1988) showed that up to stage 13 there are high levels of $\beta1$ message in all tissues of the embryo; furthermore, they observed that in stage 13 embryos, when accumulation of zygotic $\beta1$ begins in neural tissue, a number of other tissues, including mesodermal tissue, continued to exhibit high levels of $\beta1$ transcripts. Using a $\beta1$-specific antibody and $\beta1$-lacZ reporter gene constructs, Buttgereit *et al.* (1991) examined both maternal and zygotic $\beta1$ expression; they showed that new zygotic transcription of the $\beta1$ gene is limited to neuronal tissues and the apodemes, but that stage 13 embryos have significant levels of $\beta1$ protein in all tissues of the embryo. Additional evidence for the perdurance of maternally derived tubulins during embryogenesis is provided by studies of α-tubulins. There is no deficiency chromosome available that deletes the $\beta1$ gene; however, we were able to examine the maternal tubulin pool using embryos homozygous for deletions that remove the gene for the major embryonic α-tubulin, α84B. Like $\beta1$, α84B is both maternally and zygotically expressed (Theurkauf *et al.*, 1986; Matthews and Kaufman, 1987; Matthews *et al.*, 1989). In a previous study, we found that in homozygous deficiency embryos, residual α-tubulin synthesis from maternal message could still be detected in terminal prehatching stage 17 embryos (Raff, 1984), consistent with the *in situ* results of Gasch *et al.* (1988) for $\beta1$. During our present study, we repeated our previous experiment, but also used Western blots to examine the total amount of maternally derived α-tubulin (i.e., preloaded maternal protein plus new protein synthesized in the zygote from maternal message) present in homozygous deficiency embryos in which no new zygotic gene transcription could occur. We found that terminal prehatching embryos contain substantial levels of maternally derived α-tubulin, resulting in a total tubulin pool that we estimate to be more than half that in wild-type embryos (Hoyle and Raff, unpublished data). Theurkauf (1992) obtained similar results for the minor α67C-tubulin isoform; he demonstrated that maternally synthesized α67C protein is detectable to the very end of embryogenesis, even though there is neither zygotic transcription of the α67C gene nor any zygotic translation of maternally loaded α67C message in the embryo (Matthews *et al.*, 1989, 1993). From all of the above data, we conclude that the myogenic cells are born with a significant pool of $\beta1$ derived from maternally loaded protein and protein synthesized in the zygote from maternal transcripts.

In addition to the developing muscles in the embryo, $\beta3$ is also expressed in the pupa during formation of all of the adult muscles (Kimble *et al.*, 1989). Corresponding to the higher degree of organization both of the preceding microtubule cytoskeleton and of the sarcomeres themselves (Reedy and Beall, 1993a, b), tubulin requirements during formation of the adult musculature appear to be more stringent than in development of the larval muscles. Although $\beta3$ is dispensable for larval muscle, it is essential for development of at least a subset of adult muscles, since two of the viable combinations of *B3t* alleles are flightless. However, as in the larval musculature, $\beta3$ does not appear to be required for the overall organization or sarcomeric architecture of the adult muscles. We examined the ultrastructure of the six major indirect flight muscles (IFM) of flightless $B3t^1$/$B3t^1$ and $B3t^5$/$B3t^{sk}$ adults; organization and structure of the sarcomeres in the flightless mutants could not be distinguished from wild type (not shown). It is possible that there are structural defects in other muscles that are also involved in flight. However, since loss of $\beta3$ function does not disrupt morphogenesis of the IFM, which are the most complex set of adult muscles, and since the flightless animals can perform other muscle movements, including walking or jumping and moving the wings, this seems unlikely. Rather, we speculate that muscle function or coordination necessary for flight may simply be most sensitive to subtle perturbations of myogenesis. Since muscle attachment is microtubule-mediated (Reedy and Beall, 1993b), one possibility is that the inability of $\beta3$ mutants to fly might result from subtle defects in muscle attachment, so that the muscles fail to develop the tension required for the muscle vibration necessary for flight. For example, flightless mutations in the *Drosophila* muscle-specific *α-actinin* gene manifest a defect only in the terminal sarcomeres that attach the muscle to the cuticle (Fyrberg *et al.*, 1990).

Somatic Muscle Functions Normally in B3t Mutant Larvae

Homozygous Px^2 embryos develop in the absence of $\beta3$ and therefore represent the most severe possible $\beta3$ mutant phenotype. Since there are no apparent ultrastructural or organizational defects in the somatic muscle of Px^2 homozygotes, it is clear that $\beta3$ is not essential to achieve correct architecture of the larval musculature. It was possible that although apparently normal morphologically, the muscle of *B3t* mutant larvae might be functionally abnormal, e.g., as

section and myofibers with sarcomeres containing irregularly spaced thick filaments and interrupted Z lines in longitudinal sections. (E and F) Cross sections of developing muscle from homozygous *prd, Px^2* embryos. Myofibrils appear to develop normally and microtubules are present within the cytoplasm of developing muscle. (E) Developing muscle in a stage 12 *prd, Px^2* embryo in which small islands of myofibrils are in close association with parallel microtubules (arrowheads), as in early stages in wild-type muscle development. (F) Low-magnification view showing fully developed body wall muscle in a stage 17 *prd, Px^2* embryo. As in wild type, the myofibrils fill almost the entire space of the cytoplasm. Microtubules (arrowheads) and Z-band material are also present in this field. (G and H) Representative sections of muscle from newly hatched $B3t^2$/Px^2 larvae. The ultrastructure is similar to that of wild-type muscle in both cross sections (G) and longitudinal sections (H). Bars represent 0.2 μm in D and H, 0.5 μm in all other panels.

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appears to be the case for the flight muscle in flightless *B3t* mutant adults. We therefore tested somatic muscle function in mutant larvae hemizygous or homozygous for the most severe allele, *B3t*^{2}. Hemizygous *B3t*^{2} larvae were videotaped in motion to observe locomotion and contractions of the segmental body wall muscles. Newly hatched *B3t*^{2} hemizygotes were able to contract their body wall muscles normally and move at the same rate as wild-type control larvae. Additional tests to examine other aspects of muscle movements in larvae developed from embryos defective in β3 function were modeled on assays for the larval phenotypes of mutations in the *foraging* and *α-actinin* genes of *Drosophila* (deBelle *et al.,* 1989; Roulier *et al.,* 1992). Mutant larvae were examined in behavioral assays to measure the time taken to complete various muscular movements that require coordination of different muscle contractions. Our expectation was that if the function of some or all of the body muscles were defective, then the mutant animals would not be able to perform them, or would take longer to perform these movements than wild-type larvae. As shown in Fig. 5, all tests showed that the somatic muscles function normally in *B3t*^{2} mutant animals.

We first measured the time required for an individual larva to roll onto its ventral side after it was manually rolled onto its dorsal side (Fig. 5A). The time taken to roll over varied from animal to animal even in wild-type larvae, but there was no difference in the average time for mutant and control larvae of similar sizes to roll over, nor did we observe any difference in the manner in which they rolled over. As wild-type larvae grew, they took longer to roll over (not shown), corresponding to the large size difference between first and third instar larvae.

In the second test, "the maggot circus," we measured the half-time elapsed for groups of mutant larvae to move 3 cm from the center of a hard agar plate to the edge, providing a test of whether mutants could control the coordination of their muscle contractions in forward motion (Fig. 5B). In the first two days of larval life, mutants were able to reach the outer edge of the plate at a rate equal to that of control larvae. As mutant larvae aged they became increasingly sluggish and moved much more slowly than larger wild-type larvae of the same age; however, we interpreted this to be the result of a general prelethal state rather than paralysis or a specific loss in muscle coordination.

The third test was to measure the proportion of time individual mutant larvae "rest" (i.e., cease making food scraping movements) while feeding on wet yeast on a hard agar plate (Fig. 5C). Class I mutant larvae fail to grow any larger than the size of newly hatched larvae, even though they live for up to 3 days after hatching. We speculated that failure to grow might reflect inability to perform the muscle movements necessary for scraping food during feeding. However, similar to the other tests, although observations of feeding behavior revealed a general decline over time in the vitality of *B3t* mutant larvae, we did not observe any specific defect in the ability to perform feeding movements or in the amount of time spent feeding. Wild-type larvae and recently hatched *B3t*^{2} larvae rest very little during feeding. However, aging mutant larvae rest for increasing amounts of time.

The data in Fig. 5 show that somatic muscle function and coordination are not intrinsically defective in larvae hemizygous for *B3t*^{2}. Since *B3t*^{2} is not a protein null allele, these experiments may not reflect the condition of larval muscles that have developed in the complete absence of β3 function. However, the phenotype resulting from loss of β3 function cannot be more severe than the phenotype of animals homozygous for the *Px*^{2} deficiency, which deletes 12 other essential genes in addition to the β3 gene (Kimble *et al.,* 1990). *Px*^{2} results in lethality during stage 17, the final stage of embryonic development. Thus, the only possible lethal phase for the hypothetical *B3t* null allele is between late stage 17 and after hatching to the L1 stage. Both the somatic and visceral muscles are morphologically normal in *Px*^{2} homozygotes. Moreover, somatic muscle in *Px*^{2} homozygotes is functional at least to some extent, since prelethal embryos undergo muscle movements, including rhythmic contractions along the body wall. Thus our data unambiguously show that β3 is not required either for embryonic myogenesis or for the basic features of larval muscle contraction and coordination. Since we do not know whether *B3t*^{2} is a complete loss of function allele, we cannot eliminate the possibility that the quality of muscle function might be diminished by a true β3 null mutation. Indeed, the *B3t* adult flightless phenotypes demonstrate that perturbing β3 function can affect muscle function without causing gross defects in sarcomere architecture. However, since somatic muscle function is normal in *B3t*^{2} mutant animals, the lethal phenotype reveals that β3 has an essential role in some other developmental process. In order to understand why *B3t* mutants die, we had to define the other role(s) of β3 in embryogenesis.

The key feature of the Class I lethal phenotype is the failure to grow coupled with terminal arrest at the first larval instar. Starvation has been shown to cause larval arrest in other species of insects (Wigglesworth, 1972). We therefore examined the effects of starvation on wild-type larvae, as an approach to understanding the failure of Class I mutants to grow or molt. We found that starving wild-type larvae remain arrested in the first instar, similar to *B3t* Class I mutant larvae. Starving wild-type larvae continued to actively forage throughout the period of the experiment and did not exhibit the rapid decline in vitality shown by *B3t* mutant larvae. As long as starving larvae remained hydrated, most lived for many days; starving larvae up to 10 days old were able to recover and complete development after they were placed back onto food. Thus starving wild-type larvae are more robust than *B3t* mutant larvae. However, in our behavioral tests for muscle function, starving wild-type larvae behaved similarly to *B3t* mutant larvae (Figs. 5B and 5C).

Midgut Morphology and Gut Function Are Defective in β3 Mutant Larvae, but Visceral Muscle Functions Normally

The partial phenocopy of the Class I phenotype by starvation of wild-type larvae suggested that mutant larvae are

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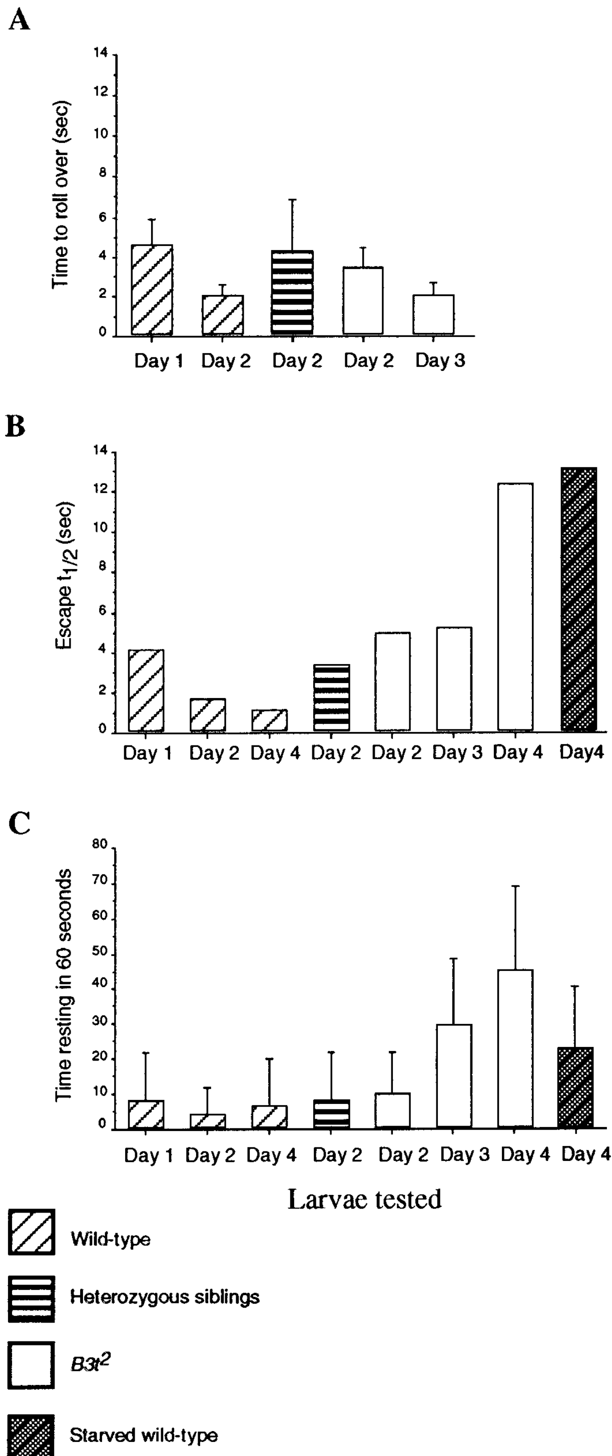

FIG. 5. The function of the body wall muscle in Class I mutant larvae is normal. (A) Comparison of the ability of larvae to roll over from the dorsal to ventral side. Ten larvae were tested three times successively and the average time is represented by the bar. (B) Comparison of the ability of larvae to wander from the center to the edge of a hard agar plate. In this test, no food was present; thus larvae exhibit foraging behavior. Groups of 20 larvae were released in the center of the plate and the time was measured for half of the larvae to traverse the 3-cm radius of the plate. (C) Comparison of the time spent resting during a 60-sec time period by larvae feeding on agar plates completely covered by wet yeast paste. Ten larvae were tested and the average time is represented by the bar. For all experiments, the genotypes of the larvae tested were wild type, diagonally striped bars; morphologically and phenotypically normal siblings of $B3t^2$ mutant larvae (genotype $B3t^2/+$ or $Px^2/+$), horizontally striped bars; $B3t^2/Px^2$ mutant larvae, open bars; starved wild-type larvae, darkened diagonally striped bars. The age of the larvae tested is given in days. Day 1 animals were newly hatched, first instar larvae. On subsequent days $B3t^2/Px^2$ mutant larvae and starved wild-type larvae remain arrested in the first instar stage and at the same size as newly hatched wild-type larvae. The developmental stage for control wild-type larvae and normal $B3t^2$ heterozygous siblings on Day 2 was second instar; Days 3 and 4, third instar. Error bars show 95% confidence interval.

living under starvation conditions. We therefore examined gut development and function to determine whether loss of $\beta3$ expression in the visceral mesoderm might be the basis for the lethal phenotype. In the developing gut, microtubules are assembled into at least two distinct arrays: those that accompany morphogenesis of the gut musculature (Kimble *et al.*, 1989) and those that mediate constriction of the midgut (Reuter and Scott, 1990). As shown in Fig. 6, the localization of $\beta3$ in the visceral mesoderm during development of the gut is consistent with a role in both of these processes. $\beta3$ accumulates throughout the visceral mesoderm, but is not expressed in the underlying endoderm tissue (Fig. 6A). Subsequently, $\beta3$ accumulates at the sites of midgut constriction (Fig. 6B). Absence of $\beta3$ function, however, does not result in failure to form a complete folded gut. We observed that Px^2 homozygous embryos have a fully extended and constricted gut tube. Bilder and Scott (1995) have shown that in Px^2 homozygous embryos the central gut constriction forms in a more posterior position than in wild-type embryos but is otherwise normal; the midgut phenotype is localized to the distal, β*Tub60D*-containing, portion of the deficiency.

As shown in Fig. 7, to visualize the morphology of the fully developed gut, we fed dyed food to first instar wild-type (Fig. 7A) and *B3t* mutant larvae (Figs. 7B and 7C). All of the *B3t* mutant larvae were capable of both filling the gut and excreting. However, in most cases the overall pattern of folding of the midgut in mutant larvae was irregular, and the size of the gut smaller than normal. The extent of the defect in gut morphology varied among $B3t^2$ larvae, ranging from a very small gut to an almost normal gut. The larvae pictured in Figs. 7B and 7C represent typical examples of the range in gut morphology in hemizygous and homozygous $B3t^2$ larvae. Since *B3t* mutant larvae could move food through the gut, if the defects in gut morphology and size were the sole basis for the lethality, we would expect that the phenotype of individual larvae would exhibit similar variability with respect to growth and time of survival; that

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is, we would expect animals with nearly normal guts to be able to grow significantly more than animals with the smallest guts. This is not the case; all *B3t*^{2} mutant larvae remain the same size as newly hatched larvae, whereas wild-type larvae exhibit significant growth by the end of the first day of larval life. Thus, defects in gut morphogenesis *per se* cannot explain the lethality of the β3 mutations. We therefore examined the ability of the gut to function in *B3t* mutant larvae.

As shown in Fig. 8, we quantitated food uptake and movement through the gut and the capability for nutrient absorption by feeding larvae a mixture of yeast and food dye containing [^{35}S]met as a tracer. The results showed that *B3t*^{2} mutant larvae are unable to absorb nutrients. In the first experiment (Figs. 8A and 8B), *B3t*^{2} mutant and wild-type larvae were fed labeled food for 12 hr and then fed unlabeled food for another 6 hr to chase the gut contents; uptake of label thus represents absorption of the [^{35}S]met from the food. *B3t*^{2} mutant larvae absorbed significantly less than wild-type larvae. In further experiments, we followed nutrient absorption over time (Figs. 8C–8E). Differences between wild-type larvae and mutants in the amount of labeled food taken up could be detected by 60 min (Fig. 8C). In these experiments, larvae were counted while their guts contained labeled food; thus the initial amount of labeled food taken up reflects the amount of [^{35}S]met in a full gut, allowing us to estimate the difference in the size of the gut of *B3t*^{2} mutant larvae compared to wild-type controls. Consistent with the variation in the size of the gut in mutant larvae (Fig. 7), the initial intake of [^{35}S]met was variable, but on average, the amount of [^{35}S]met taken up by *B3t*^{2} mutant larvae by 60 min was about half that by wild-type larvae (Fig. 8C). After the gut was initially filled, the total amount of label per larva reflects the amount in the gut plus the amount of [^{35}S]met that was absorbed across the gut wall. Over time, the increasing difference in the total [^{35}S]met taken up by mutant and wild-type larvae is much greater than the relative differences in gut size, and thus demonstrates the failure of *B3t* mutant larvae to absorb [^{35}S]met from the food (Figs. 8D and 8E).

One possibility was that *B3t* mutant larvae absorb less [^{35}S]met than wild-type larvae simply because the visceral muscles are not able to pump food through their guts. We used two experimental approaches to test this possibility. Our results showed that *B3t* mutant larvae can move food through the gut normally; thus, gut dysfunction caused by the Class I β3 mutations does not result from defects in the visceral musculature. First, to measure excretion, and thus the rate at which the visceral muscles can move food through the gut, we fed mutant and wild-type larvae yeast paste mixed with blue food dye and [^{35}S]met and then did a "chase" experiment in which we monitored the loss of counts that accompanied excretion of the dyed food after the larvae were transferred to unlabeled food. Newly hatched mutant larvae excreted labeled food at a rate similar to that of wild-type larvae (Fig. 8F).

In a second set of experiments, we measured the time required for newly hatched to 1-day-old homozygous *B3t*^{2}/*B3t*^{2} or hemizygous *B3t*^{2}/*Px*^{2} larvae and their phenotypically wild-type sibs (genotype *B3t*^{2}/+ or *Px*^{2}/+) to fill and empty their guts of dyed food. First, we transferred groups of mutant larvae and normal sibs of the same age to blue-dyed yeast and measured the time required to fill the gut with blue-dyed food. Mutant larvae could move food all the way to the posterior end of the gut as rapidly as their normal sibs: within 30 min, 26 of 28 mutant larvae and 33 of 39 wild-type larvae had blue food through the entire length of the gut tube. This result indicates that visceral muscle functions normally in the mutants, and in addition provides another test of the function of the musculature required for performing the movements necessary for feeding. We monitored the ability of the visceral muscles to keep food moving through the gut tube by feeding mutant larvae and sibs on blue food for 1 to 3 hr, then transferring larvae with completely filled guts to undyed yeast paste and measuring the time required for them to excrete the blue yeast. In these experiments we tested groups of larvae of the same age in four paired sets of 10–20 larvae each. Mutant larvae emptied their guts of dyed food only slightly slower than their normal sibs, and there was no difference in the ability of *B3t*^{2} homozygotes and hemizygotes to move food through the gut. We could not detect a significant decrease in blue dye in the gut in either mutant or wild-type larvae in the first hour after transfer to white yeast. Thereafter, we could follow movement of the blue food in the gut as it was replaced by undyed yeast; as the larvae fed, blue food was rapidly displaced from the anterior region of the gut, with concomitant "dilution" of the blue color in the posterior region of the gut, followed by clearing of blue food from the gut. Loss of blue dye from the gut was the result of bona fide excretion (and not, for example, due to metabolism of the dye), since after transfer to undyed food, the feeding larvae left little trails of blue excrement in the white yeast paste. Mutant larvae were somewhat slower than wild-type larvae in achieving a marked decrease in the amount of blue dye in the gut, but by 3 hr, of a total of 54 mutant larvae and 54 sibs tested, 41 of the mutant larvae had cleared substantially all of the blue food from their guts, compared to 47 of their normal sibs. We also considered the possibility that gut muscle function declined sharply in mutant larvae after hatching (i.e., much more rapidly than the slow decline in somatic muscle function we observed). We showed that this was not the case by refeeding larvae on blue yeast after they had cleared their guts in the first round; they refilled the gut as rapidly as newly hatched larvae, and there was no difference between wild-type and mutant larvae. Similarly to their decline in tests of somatic muscle function, as mutant larvae aged, they excreted food more slowly, indicative of their general decline. However, mutant larvae are still feeding and moving food through the gut normally during the time in which their sibs exhibit significant growth and undergo molting. Thus the failure to absorb nutrients is not due to failure of the visceral musculature.

We therefore conclude that *B3t*^{2} mutant larvae have an

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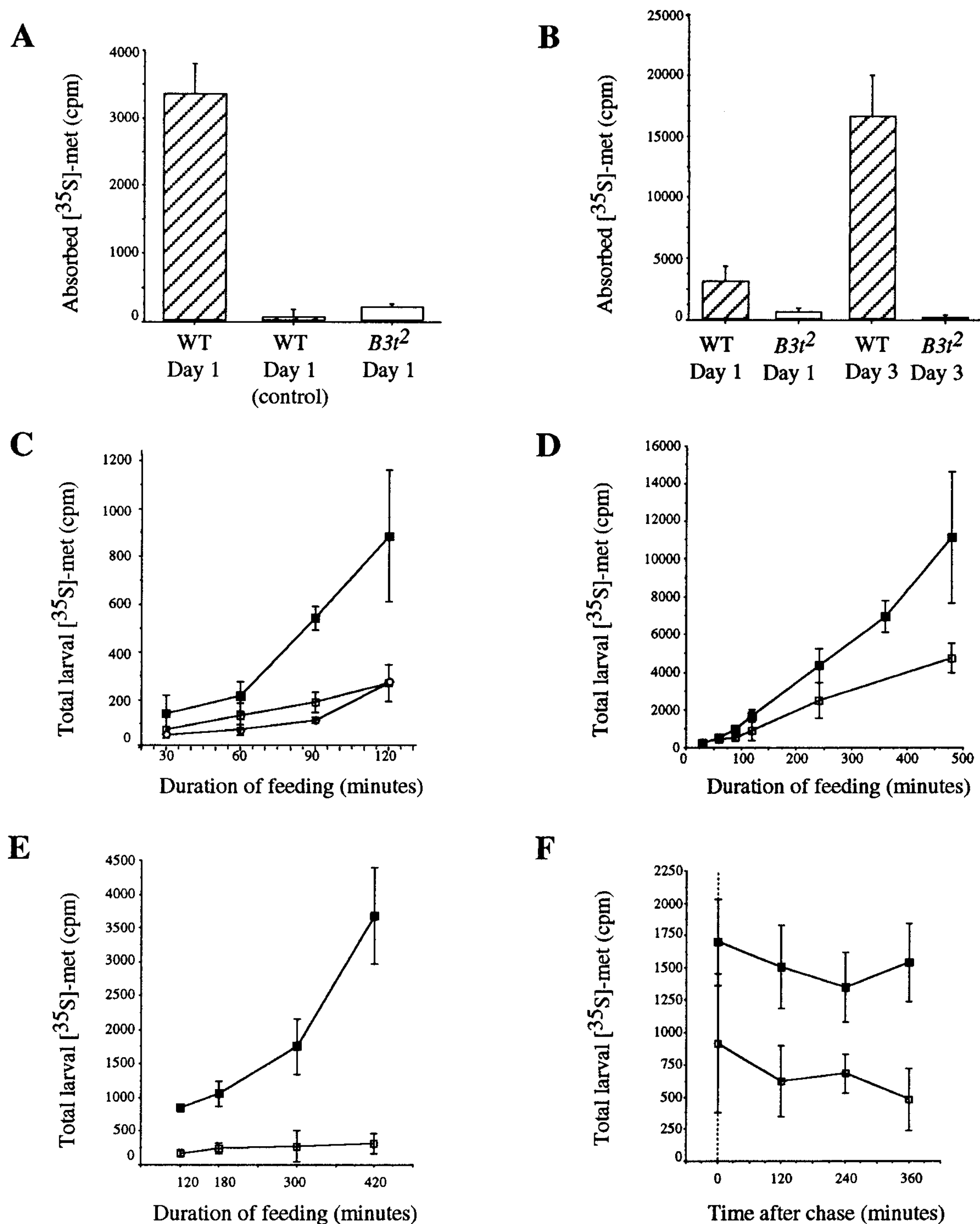

FIG. 8. *B3t²* larvae can move food through the gut, but absorption of nutrients is defective. (A and B) Results from two separate experiments in which the capability for absorption of nutrients from the gut was monitored by uptake of [^{35}S]met from the food. Larvae were fed yeast paste containing [^{35}S]met for 12 hr, washed, and allowed to feed for 6 hr on nonradioactive yeast paste (chase), and [^{35}S]met uptake was determined. Symbols are wild-type larvae, diagonally striped bars; homozygous *B3t²* larvae, open bars. Day 1 animals were newly hatched, first instar larvae. On subsequent days homozygous *B3t²* mutant larvae remain arrested in the first instar stage and remain the same size as newly hatched wild-type larvae. Day 3 wild-type larvae are in the third instar. Twenty larvae were counted for each time point, except for Day 3 *B3t²* larvae, in which five were counted. (A) includes a control experiment showing background counts obtained for wild-type larvae fed on yeast with no [^{35}S]met added. Error bars show 95% confidence intervals. (C–E) Time course experiments quantifying total uptake of [^{35}S]met in newly hatched wild-type larvae (solid squares) compared to newly hatched homozygous *B3t²* mutant larvae (open squares) or 2-day-old *B3t²* mutant larvae (open circles). Fifty to 100 larvae were placed on food containing [^{35}S]met and 5 (C) or 10 (D and E) larvae were removed at each time point, washed, and counted. (F) Excretion by wild-type larvae (solid squares) and *B3t²* homozygous larvae (open squares). Larvae were fed [^{35}S]met-containing food for 4 hr, and then placed on unlabeled food. Five larvae were removed at each time point, washed, and counted. The upturn in the last time point for wild-type larvae reflects the reconsumption of labeled excretion products. Error bars (C–F) indicate the range on the graphs for one standard deviation unit.

intrinsic defect in absorption of nutrients across the gut wall. Since this is a function of the endodermal tissue, the inability of *B3t* mutants to absorb nutrients must be a consequence of defects in the endodermal cells of the gut. We do not as yet know the cellular basis for gut dysfunction. However, our data clearly demonstrate a required role for the microtubule cytoskeleton in cells of the mesoderm in determining the correct function of the endoderm cells. A body of previous data has shown a role for gene expression in the mesoderm in transcriptional activation of endodermal genes (Immerglück *et al.,* 1990; Reuter *et al.,* 1993; Reuter, 1994). Our data provide a functional link in development between morphogenesis of gut mesoderm and gut endoderm.

DISCUSSION

The Drosophila $\boldsymbol{\beta}$*3-Tubulin Subunit Is Dispensable in Its Highest Site of Embryonic Expression but Is Essential for Larval Development*

A paradigm in developmental genetics is that the pattern of expression of a given gene can provide a clue to the function of the gene product. We approached our study of the *Drosophila* $\beta 3$-tubulin isoform within this conceptual framework. We knew that $\beta 3$ is an essential gene (Kimble *et al.,* 1990). Since the predominant site of accumulation of $\beta 3$ in embryos is in the developing somatic musculature (Gasch *et al.,* 1988; Kimble *et al.,* 1989), and since it is a general postulate that transient arrays of microtubules guide sarcomere assembly, our starting premise was that $\beta 3$ has an essential role in embryonic myogenesis. However, we found that both microtubule assembly and subsequent sarcomere morphogenesis proceed in the absence of $\beta 3$. We therefore had to address several new questions about myogenesis and the biological meaning of the $\beta 3$ expression pattern before we could get back to our original question for the $\beta 3$ mutants—why do they die?

First, when $\beta 3$ is absent, what is the source of the tubulin that is used in the microtubules during somatic myogenesis? Our data confirmed that $\beta 3$ is the major β-tubulin in developing muscle; moreover, we showed that $\beta 1$ does not accumulate to high levels in developing muscle, either in wild-type embryos or in embryos in which $\beta 3$ is not expressed. In wild-type embryos, proliferating myoblasts begin to synthesize $\beta 3$ just before they begin to fuse (Dettman and Raff, unpublished observations), and microtubule assembly commences during early myogenesis in stage 12 embryos (Fig. 4A). However, maximal accumulation of $\beta 3$ protein does not occur until much later, at stage 15–16, when the muscle pattern is established (Kimble *et al.,* 1989, and see Fig. 3A). Since significant levels of maternal $\beta 1$ are present at stage 13 (Gasch *et al.,* 1988; Buttgereit *et al.,* 1991), it is likely that the β-tubulin pool utilized during early stages of myogenesis in wild-type embryos is composed of both $\beta 1$ and $\beta 3$, and then $\beta 3$ takes over as the predominant β-tubulin in the muscle cells. Our results show that in the absence of $\beta 3$ synthesis, the levels of maternally derived $\beta 1$ are sufficient to support early myogenic events, consistent with our observation that in *B3t* mutants, fewer microtubules are assembled in each muscle cell. Thus the requirements for microtubules in generating the larval musculature appear to be minimal. Our results are consistent with the model that microtubules are only required during the initial formation and orientation of sarcomeres, and thereafter the sarcomeric cytoskeleton can grow using the initial small islands of thick and thin filaments as guides.

That sufficient microtubule assembly occurs in myogenic cells with a significantly lowered cellular tubulin pool seems surprising, but we have observed another precedent. In the *Drosophila* male germ line, several different microtubule-mediated events are required for sperm production. We have demonstrated that although some sets of microtubules are sensitive to perturbations in the level of the tubulin pool, other microtubule arrays are incredibly robust to a substantial decrease in the tubulin pool (Raff, 1994; Fackenthal *et al.,* 1993, 1995; Hoyle *et al.,* 1995; Hoyle, Hutchens, and Raff, unpublished data).

If $\beta 3$ is not essential for development of the somatic musculature, the next question is the meaning of the conservation of embryonic muscle expression of $\beta 3$ in both *D. simulans* and the more distantly related species, *D. virilis.* Maintenance of this expression pattern through approximately 50 million years of evolutionary separation in itself suggests a selective value for $\beta 3$ expression in somatic muscle. There are several plausible reasons why a nonessential functional component of a gene's expression may be maintained (Erickson, 1993). For $\beta 3$, one possibility is that embryonic muscle expression is mechanistically linked to expression in other tissues where $\beta 3$ is essential. Our data show that there is an essential role for $\beta 3$ in the adult flight muscle. If muscle expression of $\beta 3$ is governed by regulatory elements that drive expression in all muscles, then a requirement for $\beta 3$ in the IFM would result in maintenance of $\beta 3$ in forming larval muscles. However, the incomplete data available are not wholly consistent with this model. In the embryo, two enhancers act separately to regulate $\beta 3$ expression in somatic and visceral muscle (Gasch *et al.,* 1989; Hinz *et al.,* 1992). Thus there may not be general ''muscle regulators.''

A second possibility is that $\beta 3$ plays a role in the morphogenesis of the somatic muscle that is important in determining fitness but that is not demonstrable under laboratory growth conditions, or in the time course of laboratory observations. In wild-type embryos, zygotic expression of $\beta 3$ considerably boosts the level of the tubulin pool in the myogenic tissue, and when the tubulin pool is bigger, there are more microtubules in the myogenic tissue. This might be the selective role for $\beta 3$. Even though a small number of microtubules can support adequate muscle development for an individual animal grown in the laboratory, in the wild, animals whose muscles develop in the presence of

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more microtubules may be at an advantage over animals whose muscles develop with fewer microtubules. Expression of the β3 gene may not bring an essential specialized functional property to myogenesis, but may serve to regulate the size of the tubulin pool. We think that this explanation may be the most likely for the evolutionary conservation of the embryonic β3 expression pattern.

*Disruption of **β**3-Tubulin Function Reveals That the Cytoskeleton of Visceral Mesodermal Cells Is Essential for Morphogenesis of the Endoderm*

Our results show that the essential requirement for β3 expression is in the visceral mesoderm and that β3 has multiple roles in morphogenesis of the larval gut. Loss of β3 function results in defects in midgut morphogenesis, and perhaps in some variability in the robustness of visceral muscle function. However, while these factors may contribute to the severity of the mutant phenotype, the proximal cause of lethality in the Class I *B3t* mutants is defective function of the endodermal tissue of the gut. Previous studies have demonstrated an inductive relationship between gene expression in the visceral mesoderm and the endoderm (Immerglück *et al.,* 1990; Reuter *et al.,* 1993; Reuter, 1994). Our data reveal that disruption of cell structure in one layer can affect developmental events in the other layer.

There are two direct roles for β3 in visceral mesoderm cells, but for which β3 is at least partly dispensable. First, we have shown that transient cytoskeletal microtubules accompany sarcomere formation in the visceral muscles; however, myogenesis is little if at all affected by disruption of β3 function. Second, microtubules are involved in constriction and folding of the midgut. Reuter and Scott (1990) observed dense microtubule arrays in the three sites of constriction of visceral mesoderm closest to the yolk mass, beginning at stage 14. Since β3 is expressed in these cells and since the gut is often abnormally shaped in β3 mutants, it is likely that β3 is required in gut constriction furrow microtubules. Gut constriction microtubules are assembled later than the early myogenic microtubules; their function may be optimal in the presence of β3 because maternal β1-tubulin is more depleted by this time. However, since a complete and folded (albeit abnormally) gut forms in Px^2 homozygotes, assembly of the gut constriction microtubules is not dependent on the presence of β3.

The gut defects in Class I β3 mutations reveal an unexpected and essential role for the β3-containing microtubule cytoskeleton in visceral mesoderm cells in directing morphogenesis of the midgut endoderm. To form the midgut, endodermal cells migrate from two sites to form a single luminal epithelial cell layer that is surrounded by the mesodermally derived muscle cell layer (Reuter *et al.,* 1993). After cell migration is completed, cells in both layers expand into contiguous layers of columnar cells that interact to fold the midgut, as shown in Fig. 6A. After folding of the midgut, endodermal and mesodermal cells flatten surrounding the yolk. The change in configuration of the mesodermal cells during the initial stages of gut folding is shown in Fig. 6B. Throughout these processes, endodermal cells must differentiate, since the final endodermal layer of the larval midgut contains multiple cell types (Dimitriadis and Kastritsis, 1984). Several events in midgut morphogenesis could require cytoskeletal mediated cell–cell interactions. Reuter and colleagues (1993) have shown that cell migration and the establishment of the midgut epithelium requires the visceral mesoderm. They propose that the mesodermal layer provides developmental cues required for cell migration. These signals could be provided either by secreted molecules or transmembrane cell adhesion proteins in the overlying mesodermal cells. In addition, they postulated that since proteins such as Notch and Delta are expressed in both the mesoderm and the endoderm, they could be essential components of a mechanism that mediates the close association and transient cell shape changes of endodermal and mesodermal cells. The placement of such signals and the ability of cells to change shape may depend on the proper function of the cytoskeleton in mesodermal cells.

Utilization of the β3 isoform to satisfy tubulin requirements in cells where the microtubule cytoskeleton is involved in mediating cell–cell interactions is not unique to the interactions between mesoderm and endoderm. Analysis of the male sterility phenotype of viable *B3t* mutant adults has revealed a similar requirement for β3 in determining somatic cell–germ cell interactions. In the testis, β3 is expressed in the somatic cells that enclose each cyst of developing germ cells (Kimble *et al.,* 1989). We have shown that loss of β3 function in the somatic cells disrupts crucial developmental events in the germ cells (Raff and Dettman, unpublished observations). We hypothesize that just as there are cell signaling and attachment molecules, such as Notch and Delta, that mediate many different cell–cell interactions, there may also be generalized cytoskeletal components, of which β3 is one, that function in such contexts in numerous cell types.

*The Phenotypes of Class I and Class II **β**3 Mutations Suggest Additional Developmental Roles for **β**3 in Sensory Perception and Ecdysis*

Other factors in addition to starvation resulting from gut dysfunction must contribute to the *B3t* mutant phenotype, since *B3t* mutant larvae are less vigorous and die much sooner than starving wild-type larvae. Class I *B3t* mutant larvae exhibit behavioral abnormalities that suggest a role for β3 in sensory perception. Recently hatched mutant larvae feed actively, but they invariably begin to exhibit wandering behavior even when they are still in the presence of a source of food. This behavior does not appear to be similar to the wandering behavior of wild-type third instar larvae prior to pupariation; we did not observe any other behavioral or physical characteristics of the onset of pupariation, such as tanning or hardening of the cuticle, in the wandering Class I larvae. Rather, even when food is present, the behavior of the mutants appears similar to the foraging behavior

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exhibited by starving wild-type larvae, suggesting that Class I *B3t* mutant larvae cannot sense the presence of food. This might be the result of defects in the ability to detect food via chemosensory organs or to sense that the gut is filled via stretch receptors. We have identified two sites of zygotic β3 expression in which defective microtubule function might account for the abnormal behavior of Class I mutants. We have observed accumulation of β3 in the support cells of the chordotonal organs, the mechanosensory organs in the body wall, and also in sets of previously undefined cells in the anterior-most portion of the embryo, which we postulate may be sensory organs (Dettman and Raff, in preparation). A precedent for such a role for microtubules can be found in the touch receptor cells in *C. elegans,* in which the expression of the *mec7* β-tubulin gene is required for assembly of the array of microtubules that serve as rigid cytoskeletal elements whose deformation mediates mechanochemical signal transduction (Savage *et al.,* 1989). However, in *Drosophila,* loss of β3 does not completely abolish touch sensitivity, since even prelethal homozygous Px^2 embryos respond to touch by undergoing muscle contraction, nor did we observe any structural abnormalities in the chordotonal organs in *B3t* mutants (Dettman and Raff, in preparation).

The Class II *B3t* lethal phenotype reveals a requirement for β3 in ecdysis. At death, Class II larvae often possess multiple mouth parts, suggesting difficulty in shedding the cuticle after a molt, and small spots of tanned cuticle, showing incomplete entry into the pupal stage. The steroid hormone ecdysone, produced by the ring gland, is an essential signal that induces larval molts and pupariation in insects (Wigglesworth, 1972). There are two possible clues to a relationship between developmental processes involving ecdysone and β3. We have found that β3 is expressed in the ring gland (Raff and Dettman, in preparation), and there is also evidence that β3 is an ecdysone-responsive gene (Bruhat *et al.,* 1990; Andres *et al.,* 1993). Thus β3 might function in microtubule-mediated events required for generating or responding to ecdysone. We examined the effects of including 1–5 mg/ml ecdysone in the diet of wild-type or mutant larvae, amounts shown by Garen *et al.* (1977) to bypass larval arrest in *ecdysone* mutants. Ecdysone had a deleterious effect on the development of wild-type larvae: 80% of animals died as larvae, exhibiting pleiotropic defects, including improper shedding of the cuticle during molting and cuticular tanning, similar to the lethal phenotypes of Class II mutant larvae. Addition of ecdysone to the food of homozygous Class II larvae had no effect on most larvae, but some larvae died earlier than the normal lethal period. The Class II phenotype thus more closely resembles the effect of excess rather than insufficient levels of hormone.

We tested whether exogenous ecdysone could overcome the developmental arrest caused by starvation by rearing starving, arrested wild-type larvae on a glycerol solution containing ecdysone. None of these larvae molted. Thus exogenous ecdysone is not sufficient to induce molting in the absence of growth. As expected, given these results, addition of ecdysone to the food also had no effect on Class I mutant larvae. Although the Class I lethality results from developmental defects that are ''upstream'' of the defects in ecdysis revealed by the Class II phenotypes, loss of β3 function in the responsible cells may be a contributing factor in the Class I lethality.

What Is the General Role for β3 in Development?

The complex developmental pattern of expression of β3, coupled with our finding that β3 is not essential in a number of cell types in which it is expressed, raises the question of the specific functions that β3 performs in the microtubule cytoskeleton. Differential expression of multiple tubulin genes may provide an organism with a repertoire of tubulin isoforms, each of which has perhaps subtly distinct assembly properties, selected in evolution for optimization of microtubule function in different cellular contexts. Alternatively, different isoforms might be functionally equivalent but differential expression might serve to allow control over the time, place, and amount of tubulin synthesis in different cell types. In fungi, even structurally diverged isoforms have been shown to be functionally interchangeable *in vivo* (Schatz *et al.,* 1986; May, 1989), whereas developmental studies in *Drosophila* have provided evidence for functional specialization of tubulin isoforms (Hoyle and Raff, 1990; Hoyle *et al.,* 1995; Matthews *et al.,* 1993). We cannot eliminate the possibility that β3 has properties that may be specialized for some subset of its sites of expression; in fact we suspect that this is likely. However, we would argue that our observations on the utilization of β3 in development are consistent with the possibility that the general role for β3 expression may be to provide a way for cells to control tubulin pool size. First, in previous experiments, we showed that β3 is considerably restricted in its functional capacity relative to the β2-tubulin isoform utilized in the male germ line (Hoyle and Raff, 1990; Hoyle *et al.,* 1995). Second, in its normal sites of expression, β3 is expressed in terminally differentiated cells that require microtubules to mediate cell shape changes and tissue interactions. β3 expression may serve to boost the tubulin pool in such cells and would be essential only in cells in which accumulation of other β-tubulins is below some critical threshold. This hypothesis is a testable one: If the role of β3 in other cells is primarily to augment the tubulin pool, then just as myogenic cells are capable of utilizing the pre-existing β1 pool, expression of another β-tubulin isoform should be able to fulfill β3's roles in cells where β3 is normally essential. This is precisely what has been observed in fungi, where an ''essential'' tubulin isoform may be replaced by another, structurally diverged isoform so long as the tubulin pool is maintained above some minimal level (Schatz *et al.,* 1986; May, 1989). We are currently testing the ability of the β1 isoform to rescue mutant phenotypes that result from loss of β3 function.

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