

# The $\beta 3$ -Tubulin Gene of *Drosophila melanogaster* Is Essential for Viability and Fertility

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## ABSTRACT

We have previously shown that the  $\beta 3$ -tubulin gene of *Drosophila melanogaster* encodes a divergent isoform expressed in a complex developmental pattern. The  $\beta 3$  gene is transiently expressed in the embryo and again in the pupa at high levels in the developing musculature, and at lower levels in several different pupal tissues of ectodermal origin. Adult expression is confined to specific somatic cells in the gonads. In some of the cell types in which it is expressed,  $\beta 3$  is the sole or predominant  $\beta$ -tubulin, while in others the  $\beta 3$  protein is a minor component of the  $\beta$ -tubulin pool. The sites and timing of  $\beta 3$  expression demonstrated that  $\beta 3$ -tubulin is utilized primarily in cytoplasmic microtubule arrays involved in changes in cell shape and tissue organization, and suggested to us that this isoform may be functionally specialized. To determine whether the expression of the  $\beta 3$  gene is essential for normal development, and to examine the specific functions of this divergent isoform, we have generated mutations within the gene. We determined that the small deficiency *Df(2R)Px<sup>2</sup>*, which deletes the 60C5,6–60D9,10 region of chromosome 2, removes all of the  $\beta 3$  coding sequences, and that the distal breakpoint of the deficiency is approximately 2 kb upstream from the start of transcription of the  $\beta 3$  gene. We have generated a total of 31 ethyl methanesulfonate- or diepoxybutane-induced recessive lethal or visible mutations which map within the deficiency. These mutations define 12 new lethal complementation groups, which together with two previously identified visible mutations, altogether identify 14 genes in this interval of the second chromosome. A lethal complementation group comprising mutations in the  $\beta 3$ -tubulin gene ( *$\beta$ Tub60D*) was identified by rescue of their lethality by a wild-type copy of the gene introduced into the genome via *P* element-mediated germ line transformation. Analysis of the homozygous and transheterozygous phenotypes of the five  $\beta 3$  mutations recovered (alleles designated *B3t<sup>1</sup>–B3t<sup>5</sup>*) demonstrates that  $\beta 3$ -tubulin is essential for viability and fertility.

THE ability of eukaryotic cells to establish and maintain a wide variety of cell shapes is largely due to the plasticity of the cytoskeleton. Microtubules, a major component of the eukaryotic cytoskeleton, form many different structural elements, including cytoplasmic arrays, spindles, and the axonemes of cilia and flagella. The primary structural components of microtubules are the highly conserved  $\alpha$ - and  $\beta$ -tubulins. In nearly all eukaryotes these proteins are encoded by small multigene families, the different members of which show unique patterns of expression during development and differentiation. *In situ* localization of tubulin mRNAs and proteins has begun to give us a more defined understanding of the expression patterns of specific genes (LEWIS and COWAN 1988; KIMBLE, INCARDONA and RAFF 1989; MATTHEWS, MILLER and KAUFMAN 1990). Studies of this type have shown that expression of a particular gene may be restricted not only to particular tissues but to specific cell types within a tissue. For example, four

distinct  $\beta$ -tubulin genes have been shown to be expressed in the mammalian testis. Protein localization using antibodies specific for each of the different  $\beta$ -tubulin isoforms has shown that one of the genes is expressed only in the cells of the fibrous capsule, one is expressed only in Sertoli cells, and a third is germ line specific (LEWIS and COWAN 1988).

These studies contribute to our understanding of the complexities of tubulin gene expression, but do not indicate if expression of a particular gene is essential. While expression of genes that produce the predominate  $\alpha$ - or  $\beta$ -tubulin is probably required, minor components of the tubulin pool have been shown to be dispensable in *Saccharomyces cerevisiae* (SCHATZ, SOLOMON and BOTSTEIN 1986), *Aspergillus nidulans* (MAY *et al.* 1985; WEATHERBEE *et al.* 1985), and *Caenorhabditis elegans* (WOODS *et al.* 1989; DRISCOLL *et al.* 1989). Alternatively, the presence of a minor isoform may substantially influence the properties of the total cellular tubulin pool, as has been observed by D. REES, T. C. KAUFMAN and K. A. MATTHEWS (personal communication) for the divergent maternal *Drosophila*  $\alpha$ -tubulin isoform.

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We are examining  $\beta$ -tubulin expression and function in *Drosophila melanogaster*. The *Drosophila*  $\beta$ -tubulin gene family has four members, each of which, as in the vertebrate tubulin gene families, is expressed in a unique pattern with respect to developmental timing and tissue or cell type specificity (reviewed in FYRBERG and GOLDSTEIN 1990). The  $\beta 3$ -tubulin gene encodes a divergent isoform which is only 87–88% similar in sequence to the isoforms  $\beta 1$  and  $\beta 2$ , and to the major vertebrate  $\beta$  tubulins (RUDOLPH *et al.* 1987). Expression of  $\beta 3$ -tubulin first occurs transiently during mid-embryogenesis, from approximately 8–16 hr postfertilization (RAFF *et al.* 1982; NATZLE and MCCARTHY 1984; BIALOJAN, FALKENBURG and RENKAWITZ-POHL 1984). Embryonic  $\beta 3$  expression is restricted to the developing musculature, where it is the sole  $\beta$  tubulin isoform (LEISS *et al.* 1988; GASCH *et al.* 1988; GASCH, HINZ and RENKAWITZ-POHL 1989; KIMBLE, INCARDONA and RAFF 1989). Neither the  $\beta 3$  mRNA or protein has been detected during larval development. With the onset of pupal development, expression of  $\beta 3$ -tubulin resumes, but in contrast to embryonic expression, pupal expression occurs in a variety of tissues of both mesodermal and ectodermal origin (KIMBLE, INCARDONA and RAFF 1989).  $\beta 3$  expression persists in the adult only in specific somatic cells in the gonads. Although  $\beta 3$  is the only or predominant  $\beta$ -tubulin isoform in the developing musculature and in gonadal somatic cells, this isoform comprises only a minor part of the total  $\beta$ -tubulin pool in imaginal tissues, wing epidermal cells, and glial cells of the internal chiasma in the optic lobe.

The developmental pattern of  $\beta 3$  expression demonstrated that this divergent isoform is utilized primarily in assembly of arrays of cytoskeletal microtubules which mediate cell shape changes or tissue organization (KIMBLE, INCARDONA and RAFF 1989). We therefore suggested that the intrinsic functional capacity of the divergent  $\beta 3$ -tubulin isoform is specialized or restricted. This contrasts to the multiplicity of roles served by the two structurally conserved isoforms, the predominant isoform  $\beta 1$ -tubulin, and the testis-specific isoform  $\beta 2$ -tubulin, both of which have been shown to function in all morphological classes of microtubules, including spindles, cytoskeletal microtubules, and multiple-walled tubules of the centriole or axoneme, respectively (KEMPHUES *et al.* 1982; RAFF and FULLER, 1984; RUDOLPH *et al.* 1987; FULLER *et al.* 1988). Support for the hypothesis that  $\beta 3$  is functionally unique has been provided by the following experimental observations by HOYLE and RAFF (1990). Germ line expression of  $\beta 3$ -tubulin from a hybrid  $\beta 2\beta 3$  gene in  $\beta 2$  null males cannot supply all of the microtubule functions normally supported by the testis-specific isoform,  $\beta 2$ -tubulin. Second, expression of  $\beta 3$  from the hybrid gene in otherwise wild-

type males causes a dominant defect in axoneme assembly, with the result that such males are sterile if accumulation of  $\beta 3$  exceeds approximately 20% of the total testis  $\beta$ -tubulin pool.

It is therefore important to define the normal functions of  $\beta 3$ -tubulin in development, both to understand the molecular mechanisms which underlie the developmental processes in which this divergent isoform functions, and also to understand the overall functional role played by the presence in the genome of multiple copies for cytoskeletal proteins such as the tubulins. In order to do this, and to determine if expression of  $\beta 3$  is essential, we have begun genetic analysis of the  $\beta 3$ -tubulin gene. Here we present data demonstrating that expression of  $\beta 3$ -tubulin is essential for both viability and fertility.

## MATERIALS and METHODS

### Deficiency chromosomes and other *Drosophila* stocks:

Visible markers, deficiency chromosomes and balancer chromosomes used in this study are described in LINDSLEY and GRELL (1968). Deletions utilized in this study were the deficiency chromosome *Df(2R)Px<sup>2</sup>* (here referred to as *Px<sup>2</sup>*), which deletes the 60C5,6–60D9,10 region of chromosome 2, and *In(2LR)Px<sup>2</sup>* (here referred to as *Px<sup>2</sup>*), an inversion chromosome in which the 60B–60D1 region of chromosome 2 is deleted and region 21D1–22A3 is duplicated. The *Px<sup>2</sup>* deficiency is flanked proximally by *speck* (*sp*, map position 107.0) and distally by *Irregular facets* (*If*, map position 107.6). The only genes which have been previously mapped within the region uncovered by the *Px<sup>2</sup>* deficiency are the bristle mutation *Pin*, and the two allelic wing venation mutations *blistered* (*bs*) and *balloon* (*ba*). Second chromosome balancers used in this study were *In(2LR)CyO* (here referred to as *CyO*), which balances the entirety of chromosome 2, and *In(2R)bw<sup>Del</sup>*, which balances the right arm of chromosome 2.

For some experiments, in order to have a visible marker to identify prelethal homozygous *Px<sup>2</sup>* embryos, a stock obtained from T. C. KAUFMAN was used which carries on the second chromosome both the *Px<sup>2</sup>* deficiency and the recessive lethal pair-rule mutation *paired* (*prd*), located on the left arm of chromosome 2 in polytene region 33C (NUSLEIN-VOLHARD and WIESCHAUS 1980; NUSLEIN-VOLHARD, KLUDING and JURGENS 1985). A recombinant stock was also generated which carries *prd*, *Px<sup>2</sup>*, and the second chromosome  $\beta 3$  transgene insert *P[w<sup>+</sup> $\beta 3$ ]<sub>2</sub>*. Homozygous *prd* embryos exhibit defective segmentation and are thus distinguishable from normally segmented sibs by 8–9 hr of development, but remain viable until the end of embryonic development. The *prd* phenotype could thus be used as a marker for homozygous *Px<sup>2</sup>* embryos.

The multiply marked *b If, b pr c px sp*, and *sp<sup>2</sup> bs<sup>2</sup>* stocks were obtained from the Mid-America *Drosophila* Stock Center at Bowling Green. The *w* stock was obtained from M. MUSKAVITCH, Indiana University, Bloomington, Indiana. The stock *dp b Px<sup>4</sup>/In(2LR)Gla* was obtained from the National Institute of Environmental and Health Sciences at Research Triangle Park, North Carolina. All other stocks were obtained from the *Drosophila* Stock Center at Indiana University, Bloomington. All stocks were maintained at 25° on standard cornmeal/molasses/agar food.

**Nomenclature for mutations in the  $\beta 3$ -tubulin gene.** According to the nomenclature established for the *Drosophila*

ila tubulin genes (reviewed in FYRBERG and GOLDSTEIN 1990), each  $\alpha$ - or  $\beta$ -tubulin gene can be designated according to its cytological position in the polytene chromosome bands and to the isoform which it encodes. Thus the designation for the  $\beta 3$ -tubulin gene, based on our localization of the gene by genomic Southern blot analysis presented below, is  $\beta\text{Tub60D}$ ; the symbol for mutations in the  $\beta 3$  gene is  $B3t$ , with different alleles designated by superscripts, i.e.,  $B3t^1$ ,  $B3t^2$ , etc.

**Genomic Southern analysis:** Genomic DNA for Southern blot analysis was isolated from batches of about 500 embryos, using a procedure slightly modified from that described by G. RUBIN (personal communication). Dechorionated embryos were homogenized in 100  $\mu\text{l}$  of 0.1 M Tris-HCl, pH 9.0; 0.1 M EDTA; 1% SDS (wt/vol); 0.5% diethyl pyrocarbonate in an eppendorf microcentrifuge tube. After incubating for 30 min at 70°, 8.0 M potassium acetate was added to a final concentration of 1.0 M and the homogenate was incubated at 0° for 30 min. Cellular debris was removed by spinning the tubes at maximum speed in the microcentrifuge for 15 min at 4°. The genomic DNA in the supernatant was precipitated by adding an equal volume of isopropanol and spinning the tubes in the microcentrifuge at 25° for five minutes. DNA pellets were resuspended in TE (10 mM Tris, pH 8; 1 mM EDTA). The samples were treated with RNase A at a final concentration of 5 ng/ $\mu\text{l}$  for 30 min at 37°, followed by precipitation in 2 mM spermine. DNA pellets were washed in spermine extraction buffer (75% ethanol[vol/vol]; 0.3 M sodium acetate; 10 mM magnesium acetate), dried and then resuspended in 10  $\mu\text{l}$  of H<sub>2</sub>O and immediately digested with the indicated restriction enzyme. To obtain DNA from embryos homozygous for the  $Px^2$  and  $Px^4$  deficiency chromosomes, 2–4-hr egg collections were taken from heterozygous parents, and the embryos allowed to develop at 25° for 20–22 hr. Unhatched embryos were collected, dechorionated in 50% bleach/0.02% triton (vol/vol) and washed copiously with 0.02% triton. Embryos homozygous for the deficiency chromosomes  $Px^2$  or  $Px^4$  can be distinguished from their heterozygous and wild-type siblings by their lack of trachea and malformed malpighian tubules, and were manually selected under a dissecting microscope. Embryos were frozen in liquid N<sub>2</sub> and stored at –80° until the DNA was prepared. Genomic DNA from wild-type (*Oregon R*) and heterozygous  $Px^2$ /*CyO* adults was isolated according to the method in AUSUBEL *et al.* (1987) for preparation of genomic DNA from mammalian tissue, except that an RNase treatment and spermine precipitation were done prior to the restriction digest. Concentration of DNA preparations was measured on a DNA minifluorometer (Hoefer Scientific Instruments); 7.5  $\mu\text{g}$  DNA was used for each restriction digest. Genomic DNA samples were electrophoresed on agarose gels and blotted to Zeta Probe membrane (Bio-Rad) using the alkaline blotting procedure supplied by the manufacturer. Probes were prepared by gel-purifying double stranded genomic clones and labelling them with [<sup>32</sup>P]dCTP using a random primed DNA labeling kit (U.S. Biochemical Corp.) according to the manufacturer's instructions.

**Isolation of mutations:** Mutations which map to the  $Px^2$  deficiency were recovered in a standard F<sub>2</sub> screen. Males homozygous for marked second chromosomes were mutagenized by exposure to 0.0125 M ethyl methanesulfonate (EMS) or 0.05 M diepoxybutane (DEB) in 2.5% sucrose for 24 hr. For the EMS screens (A and B), homozygous *b If* males were mutagenized. For the DEB screens, males homozygous for either *cn bw* (screen D) or *b pr c px sp* (screens C and E) were mutagenized. Mutagenized males were mated to virgin females doubly balanced for the right arm of the

second chromosome (genotype *In(2R)bw<sup>VDel</sup>,b/CyO*). Embryos were collected for 5 days, transferring the adults to fresh food bottles daily. Virgin daughters were collected and mated en masse to  $Px^2$ /*CyO* males for 2–3 days, after which individual females plus several males were transferred to vials and allowed to lay eggs for 5 days at 25°. For crosses that yielded no progeny heterozygous for the mutagenized chromosome and the  $Px^2$  deficiency ( $2^*/Px^2$ ), the balanced sibs ( $2^*/CyO$ ) were retested by crossing to  $Px^2$ /*CyO*. Mutations that retested as being lethal in combination with the deficiency chromosome were stocked by mating the  $2^*/CyO$  progeny.

A total of 5234 chromosomes were screened, yielding 43 mutations that were lethal or gave a visible phenotype in combination with the  $Px^2$  deficiency. To determine if the new mutations mapped to the 60C/D region, the mutations were scored by recombination for linkage to the visible markers *sp* or *If*, which flank the deficiency proximally and distally, respectively. Twelve of the mutations were readily separable from the markers by recombination, and thus are unlikely to lie within the 60C/D interval. Thirty-one of the mutations that were lethal or gave a visible phenotype in combination with the  $Px^2$  deficiency also mapped to the 60C/D region by recombination. These mutations were studied further as described below.

**Construction of a transformation vector containing the  $\beta 3$  gene and recovery of transformed strains:** To identify the complementation group that represents mutations in the  $\beta 3$ -tubulin gene, an 11-kb fragment containing the wild-type  $\beta 3$  gene from the genomic clone M5B3 described in RUDOLPH *et al.* (1987) was subcloned into a slightly modified version of the pW8 vector (KLEMENZ, WEBER and GEHRING 1987). Modification of the pW8 polylinker and insertion of the  $\beta 3$  genomic sequences are described in the text (see Figure 4). Strains carrying the  $\beta 3$ -transgene ( $P[w^+\beta 3]$ ) were recovered by *P* element-mediated germ line transformation following the procedure of SPRADLING and RUBIN (1982) as described in HOYLE and RAFF (1990). DNA (1 mg/ml) was injected into *w;ry<sup>506</sup>P[ry<sup>+</sup> $\Delta 2-3$ ](99B)/+* embryos; the source of transposase activity is the defective *P* element [*ry<sup>+</sup> $\Delta 2-3$* ] (ROBERTSON *et al.* 1988).

Three independent transformed lines were recovered. The insert location of the  $\beta 3$  transgene in each transformed strain was determined by recombination mapping. The insert in the  $P[w^+\beta 3]2$  strain mapped to the second chromosome within 2 map units of *curved* (*c*, map position 75.5). The inserts in the  $P[w^+\beta 3]3a$  and  $P[w^+\beta 3]3b$  strains both map to the third chromosome to the left of *Kinked* (*Ki*, map position 47.6). The  $P[w^+\beta 3]2$  and  $P[w^+\beta 3]3a$  strains produce homozygous viable progeny and were used in subsequent studies.

**Immunocytology:** Embryos stained with the affinity purified polyclonal antiserum specific for  $\beta 3$ -tubulin were fixed and stained as is described in KIMBLE, INCARDONA and RAFF (1989).

**Lethal phase analysis:** For all of the mutations that map within the  $Px^2$  deficiency, lethal phases of hemizygous mutant animals were determined by examining at the light microscope level progeny of crosses in which one parent was  $2^*/CyO$  and the second parent was  $Px^2/In(2LR)bw^{VDel}$ . Animals that carry both balancer chromosomes are viable. Females used in these crosses were collected as virgins and held for 2–3 days before mating. Males and females were allowed to mate for at least 24 hr before transfer into egg collection bottles; a 4–6-hr prelay was followed by a single 2-hr collection. Embryos were maintained at 25° for 24–30 hr, at which time the percent of total embryos that had not hatched was determined. The unhatched embryos were

collected, dechorionated and scored under the dissecting scope for the extent of development. Animals which hatched were monitored to determine the subsequent extent of development. Lethal phase analysis of animals carrying  $\beta 3$ -tubulin mutations in the presence or absence of the  $\beta 3$  transgenes was done in the same manner. For those combinations of alleles which resulted in pupal lethality, examination of pupae that failed to eclose was done within 2 days of the time when the last of their siblings emerged.

**Analysis of rescued mutants:** The fertility of animals carrying  $\beta 3$  mutations which survived to adulthood in the presence of the  $\beta 3$  transgene was assessed by mating tests. The rescued mutant adults were maintained with appropriate mates for at least 1 week, at which time the cross was scored for the presence of progeny and the rescued adults recovered and tested for the ability to fly. Animals that could not fly were assessed for the ability to jump.

## RESULTS

**Chromosomal localization of the  $\beta 3$  gene:** The  $\beta 3$ -tubulin gene was previously localized by *in situ* hybridization to the right arm of the second chromosome in the 60C/D region of the polytene chromosomes (NATZLE and MCCARTHY 1984). We examined tubulin synthesis patterns during mid-embryogenesis in prelethal embryos homozygous for various deficiency chromosomes (our unpublished data); these studies suggested that the  $\beta 3$  gene was removed by the  $Px^2$  deficiency, in which the relatively small region 60C, 5–6 to 60D, 9–10 is deleted (LINDSLEY and GRELL 1968). The proximal portion of the region deleted in the  $Px^2$  deficiency is also deleted in the  $Px^4$  chromosome, which removes region 60B–60D1 (LINDSLEY and GRELL 1968).

To confirm that the  $Px^2$  deficiency deletes the  $\beta 3$ -tubulin gene and to localize the gene within the deficiency, SOUTHERN (1975) analysis was performed on genomic DNA isolated from several lines of *Drosophila*, including embryos homozygous for either  $Px^2$  or  $Px^4$ , as shown in Figure 1. The  $\beta 3$  gene structure and the probes used in genomic DNA blots are diagrammed in Figure 1A; results are shown in panels B and C.

When blots of restricted DNA were hybridized with probe 2 (a 5.4-kb genomic fragment that contains exons 2–4 and sequences 3' to the  $\beta 3$  gene), the only hybridization seen in the  $Px^2/Px^2$  lane can be accounted for by cross hybridization to the other  $\beta$ -tubulin genes of *Drosophila* (Figure 1C) and to a small amount of contamination by DNA from non  $Px^2$  chromosomes, while  $\beta 3$  sequences are clearly identified in DNA from wild-type (*OreR*) and  $Px^4/Px^4$  animals and from the *CyO* chromosome (Figure 1, B and C). For DNA preparations, 500–600 18–22-hr-old  $Px^2/Px^2$  embryos were collected from egg lays from heterozygous parents. Homozygous embryos were identified on the basis of their prelethal phenotype (absence of trachea and malformed malpighian tubules). The faint hybridization at 5.4 kb in the  $Px^2/Px^2$  lane indicates

that a small number of nonhomozygous embryos were included in the genomic DNA preparation. To confirm that the difference in the hybridization of probe 2 to  $Px^2/Px^2$  and *OreR* DNA was not due to differences in the amount of DNA loaded on the gel, probe 2 was stripped from the blot and the blot was then rehybridized with a labeled 7.0-kb genomic clone which contains the structural gene for  $\beta 1$  tubulin. This probe showed strong hybridization to the expected 7.0-kb  $\beta 1$  band in both the *OreR* lane and the  $Px^2/Px^2$  lane (data not shown). Densitometry of the 7.0-kb  $\beta 1$  bands showed that the  $Px^2/Px^2$  genomic DNA loaded onto the gel equaled 50% of the *OreR* DNA loaded onto the gel. The difference in the DNA loaded may account for the difference in cross hybridization of probe 2 from the  $\beta 3$  gene with  $\beta 1$  sequences in the two lanes in panel C. We found that the extent of cross-hybridization of genomic  $\beta 3$  probes to other  $\beta$ -tubulin genes was also very sensitive to the time and conditions of hybridization and washing, and was somewhat variable from experiment to experiment.

When probe 1 (a 4.6-kb genomic fragment that contains the first exon and sequences 5' to the gene) was hybridized to blots containing DNA from  $Px^2/CyO$  animals, two bands of hybridization that are not due to cross hybridization to other  $\beta$ -tubulin genes were seen (Figure 1B). The 5.3-kb band which shows strong hybridization is derived from the *CyO* balancer chromosome. The second band (7.4 kb) is slightly smaller than the 8.7-kb band seen in DNA from a wild-type strain of *Drosophila* (*OreR*), and gives a much weaker level of hybridization. The weak hybridization of the probe to the 7.4-kb band could occur if the fragment was the result of partial digestion. Alternatively, the weak hybridization could indicate that this fragment does not contain all of the sequences present in the probe, suggesting that one of the break-points of the deficiency falls within this fragment. To determine which of these is correct, samples of  $Px^2/CyO$  DNA were digested with other restriction enzymes and then blotted and hybridized with probe 1. In DNA digested with *Bam*HI or *Sal*I, a novel sized band which hybridized weakly to probe 1 was seen, but no new bands were observed in *Sac*I-digested DNA (data not shown). Analysis of the sizes and relative hybridization intensity of the novel bands indicates that one of the break points of the  $Px^2$  deficiency lies approximately 2.0 kb upstream of the transcription start site, near the two *Sac*I restriction sites (Figure 1A).

Regardless of which probe is used, strong hybridization is seen to  $Px^4/Px^4$  DNA (Figure 1, B and C), showing that the  $Px^4$  deficiency does not remove the  $\beta 3$  structural gene. Interestingly, the  $Px^4$  chromosome carries the same polymorphic *Eco*RI site that is present 5' of the  $\beta 3$  gene in the *CyO* chromosome but not in

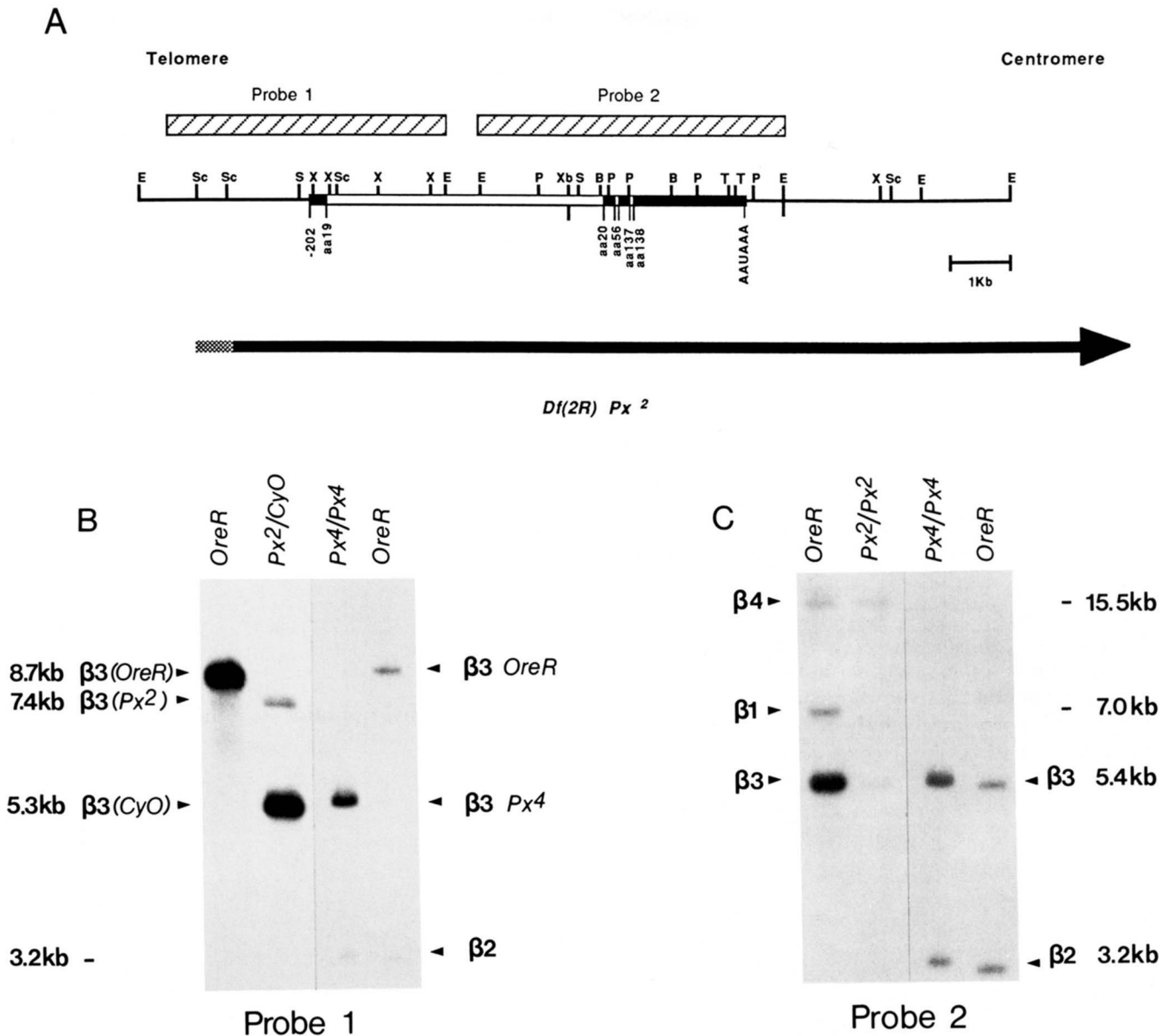
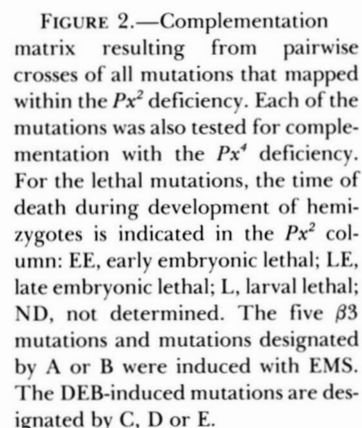


FIGURE 1.—Southern analysis of genomic DNA showing localization of the  $\beta 3$  gene within the  $Px^2$  deficiency. (A) Restriction map of the genomic region which includes the structural gene for  $\beta 3$ -tubulin. The solid lines indicate 5' and 3' flanking regions. The filled boxes indicate sequences included in the  $\beta 3$  mRNA, and the open boxes indicate introns. The solid bar below the map indicates the portion of the gene that is deleted in the  $Px^2$  chromosome with the arrowhead pointing toward the proximal side of the deficiency. The shaded portion of the bar indicates the approximate position of the distal breakpoint of the deficiency. The restriction fragments used as probes are indicated by the cross-hatched boxes above the map. Restriction sites are indicated as follows: B, *Bam*HI; E, *Eco*RI; P, *Pst*I; S, *Sal*I; Sc, *Sac*I; T, *Taq*I; X, *Xho*I; Xb, *Xba*I. (B) and (C) Autoradiograms of Southern blots containing DNA isolated from the *Ore* R,  $Px^2/CyO$ ,  $Px^4/Px^4$ , and  $Px^2/Px^2$  strains and digested with *Eco*RI. The strain of flies from which the DNA was isolated is indicated above each lane. The origin and size of bands corresponding to hybridization to the  $\beta 1$ -,  $\beta 2$ -,  $\beta 3$ - and  $\beta 4$ -tubulin genes from each of the chromosomes are identified next to each lane. The probe used for each set of blots is indicated at the bottom of the figure.

the *Ore*R chromosome. Since the  $Px^4$  deficiency overlaps  $Px^2$  proximally, these data place the  $\beta 3$  gene near the distal breakpoint of the  $Px^2$  deficiency in the 60D9,10 region, and show that the orientation of the  $\beta 3$  gene is distal to proximal on the chromosome.

**Recovery and analysis of mutations within the  $Px^2$  deficiency:** As described in MATERIALS AND METHODS, the  $Px^2$  deficiency was used to select 31 recessive lethal or visible mutations which map to the 60C-D region. Of these, 16 also failed to complement the  $Px^4$  defi-

ciency. *Inter se* complementation analysis of the mutations resulted in the complementation matrix shown in Figure 2. The mutations define 13 complementation groups, eight of which are represented by multiple alleles. Twelve of these are new lethal complementation groups identified in this screen. We also isolated additional alleles of the gene represented by the visible wing blade mutations *blistered* and *balloon*, which were previously mapped within the  $Px^2$  deficiency (LINDSLEY and GRELL 1968), and which appear to be allelic



The complementation groups containing the mutations isolated in this screen are shown schematically in Figure 3. The new *blistered* alleles we recovered (designated as complementation group 1) are homozygous viable and exhibit wing blade defects. All the other mutations we recovered are recessive lethal

### Identification of mutations in the $\beta 3$ -tubulin gene:

A vector containing a wild type copy of the  $\beta 3$  gene was constructed and introduced into the genome by *P* element-mediated transformation (see MATERIALS AND METHODS). The construction of the  $\beta 3$  transgene is shown schematically in Figure 4. In the diagram, the solid lines show flanking sequences, open boxes show introns, and sequences present in the  $\beta 3$  mRNA are shown by the cross-hatched boxes. The amino acid codon positions at the intron boundaries and the end of translation (RUDOLPH *et al.* 1987), and the mRNA polyadenylation site and probable start site of transcription (GASCH *et al.* 1988) are indicated. GASCH *et al.* (1988) observed some heterogeneity in the start site for  $\beta 3$  transcription, but their analysis placed the

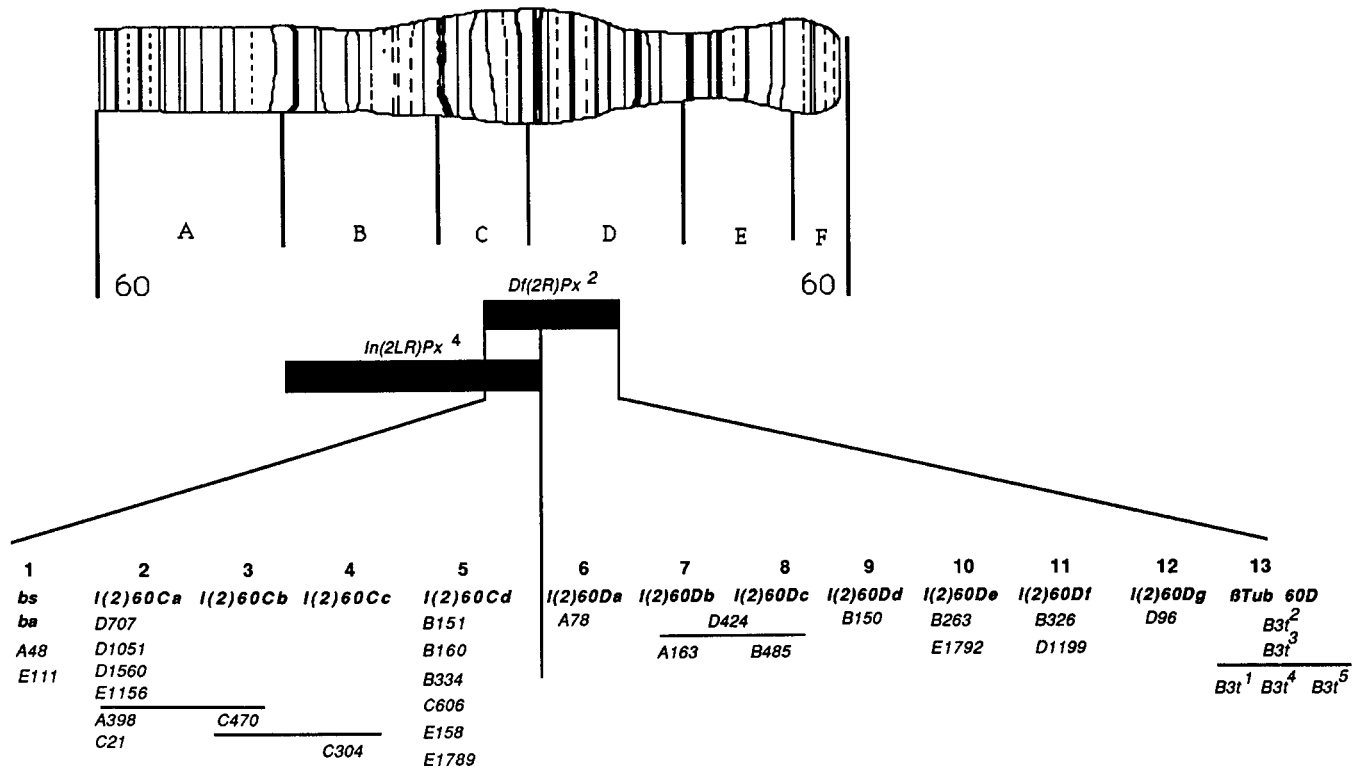


FIGURE 3.—Distribution of EMS- and DEB-induced mutations within the  $Px^2$  and  $Px^4$  deficiencies. A schematic representation of the tip of the right arm of chromosome 2 is shown. The solid boxes below the chromosome indicate the extent of material deleted in the  $Px^2$  and  $Px^4$  chromosomes. The mutant alleles of each gene identified in this screen are diagrammed below. The complementation groups within each deficiency interval were not ordered and are here numbered arbitrarily. The unusual complementation pattern among the five  $B3t$  mutations (complementation group 13) is discussed in the text.

most upstream site of transcription initiation at an adenosine residue 202 bases preceding the start of translation, as indicated in the diagram. The final transformation construct includes  $\beta 3$  genomic sequences comprising approximately 2.5 kb of 5' flanking sequence, the entire transcribed portion of the  $\beta 3$  gene, including the 4.5-kb first intron, and approximately 600 bp of 3' flanking sequence. Two independent transformed lines with homozygous viable inserts of the  $\beta 3$  transgene on the second and the third chromosomes ( $P[w^+\beta 3]2$  and  $P[w^+\beta 3]3a$ , respectively) were used in analysis of the  $\beta 3$  mutations.

To determine whether additional genes are encoded within the 10 kb of genomic DNA used in the transformation construct, all sequenced regions were checked for open reading frames displaying the codon usage bias seen in other *Drosophila* genes (MARUYAMA *et al.* 1986). No significant open reading frames, other than the  $\beta 3$  coding region, are present. Nearly half of the sequences in the transgene are contained in the large 4.5-kb first intron. This intron has been shown by GASCH, HINZ and RENKAWITZ-POHL (1989) to contain regulatory elements required for proper embryonic expression of the  $\beta 3$ -tubulin gene, but the regulatory sequences have not yet been defined, nor has the intron yet been sequenced in its entirety. In order to be sure that no additional transcripts are encoded

by sequences within the intron, we isolated the 4.4-kb *SacI-SalI* restriction fragment which contains all of the unsequenced portion of the 4.5-kb first intron and used the intron fragment to probe an RNA blot containing total RNA isolated from *Drosophila* at different stages throughout development. The intron probe did not hybridize with any RNA species on this blot, indicating that no other readily detectable RNAs are transcribed from the genomic sequences used in the transformation construct (data not shown). A probe which hybridizes to the moderately abundant 0.6-kb mRNA for the ribosomal protein RP49 (O'CONNELL and ROSBASH 1984) was included in the hybridization mix as a control; hybridization was observed to the RP49 message. Although we can not rule out the possibility that a rare RNA is transcribed from sequences in the  $\beta 3$  gene intron, we should have been able to detect any mRNA that represented at least 0.0005–0.002% of the total mRNA pool, based on the specific activities of the two probes and the level of signal due to hybridization to the RP49 mRNA. Data presented below show that the  $\beta 3$  transgene supports expression of  $\beta 3$ -tubulin, that mutations in only one complementation group are rescued by the transgene, and that the phenotypes of the rescued mutations are consistent with the pattern of  $\beta 3$  expression. We therefore conclude that the rescue

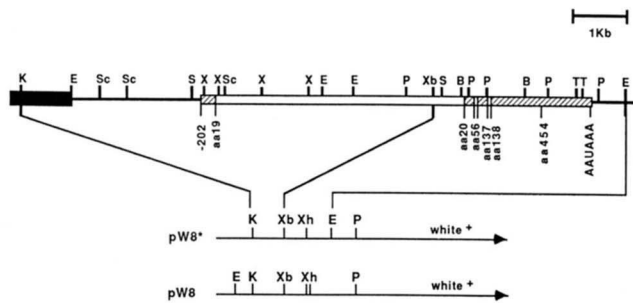


FIGURE 4.—Construction of the  $\beta 3$  transgene  $P[w^+\beta 3]$ . DNA fragments from the M5B3 genomic clone of  $\beta 3$ -tubulin (shown at the top) were subcloned into a modified pW8 vector (designated pW8\*) for transformation into *Drosophila* strains. The pW8\* vector was generated by first destroying the *EcoRI* cloning site in the original vector and then replacing the *XhoI* to *PstI* fragment of the pW8 polylinker with a synthetic DNA fragment that consisted of restriction sites for *XhoI*, *EcoRI* and *PstI*. Only the polylinker regions for the original and modified vectors are shown. Genomic fragments from the M5B3 clone were inserted into pW8\* in a two-step cloning procedure. The 3.4-kb *XbaI* to *EcoRI* fragment which contains most of the coding region and approximately 600 bp of 3' flanking sequence was inserted first. The second fragment inserted was the 7.6-kb *KpnI* to *XbaI* fragment that begins within the left arm of the lambda vector and contains about 2.5 kb of 5' flanking sequence, the first exon and most of the first intron. The arrow head at the right indicates the direction of transcription of the hsp70-driven *white* minigene that serves as a selectable marker for transformation; the  $\beta 3$  gene in the construct is transcribed in the same direction. In the map of M5B3, the filled boxes represent lambda vector, the solid lines indicate 5' and 3' flanking sequences, the cross-hatched boxes represent sequences present in the  $\beta 3$  mRNA and open boxes represent intron sequences. Restriction sites are indicated as follows: B, *BamHI*; E, *EcoRI*; K, *KpnI*; P, *PstI*; S, *Sall*; Sc, *SacI*; T, *TaqI*; X, *XhoI*; Xb, *XbaI*.

of the mutant phenotypes supported by the  $\beta 3$  transgene described below indeed results from expression of  $\beta 3$ -tubulin.

That the  $\beta 3$  transgene supports embryonic expression of  $\beta 3$ -tubulin in the proper temporal and spatial pattern was demonstrated by immunolocalization of  $\beta 3$ -tubulin in the developing musculature of 12–16-hr-old embryos, using the  $\beta 3$ -specific antiserum described by KIMBLE, INCARDONA and RAFF (1989). Figure 5 shows the results of these experiments. The mutation *paired* (*prd*), which results in a segmentation defect (NUSSLEIN-VOLHARD and WIESCHAUS 1980; NUSSLEIN-VOLHARD, KLUDING and JURGENS 1985), was used as an embryonic marker, since animals homozygous for the *Px*<sup>2</sup> deficiency alone cannot be distinguished morphologically from their heterozygous or wild type sibs until several hours after the time of  $\beta 3$ -tubulin synthesis. (It should be noted that 12–16-hr-old embryos of genotype *prd*, *Px*<sup>2</sup>/*CyO* and *CyO*/*CyO* are also morphologically indistinguishable from wild type.) Figure 5A shows two sibling embryos produced from *prd*, *Px*<sup>2</sup>/*CyO* parents. The embryo on the lower right side of figure 5A is a homozygous *prd*, *Px*<sup>2</sup>/*prd*, *Px*<sup>2</sup> embryo, as identified by the abnormal segmentation pattern. This embryo showed no specific staining

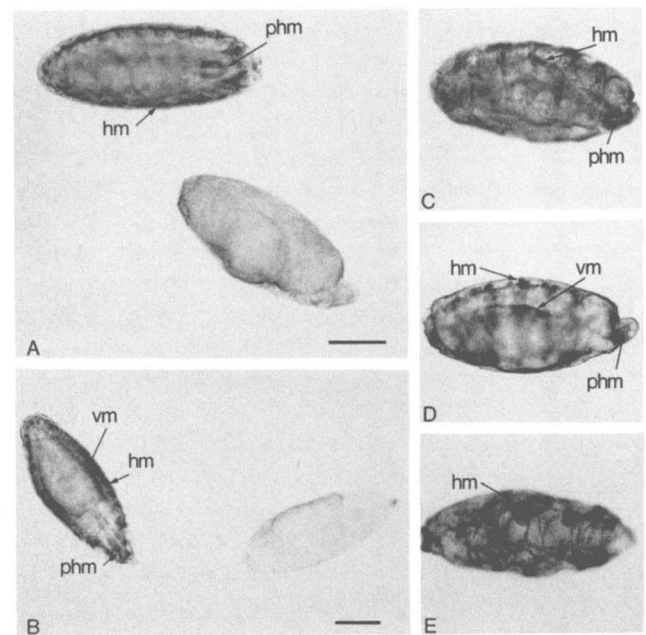


FIGURE 5.—Immunoperoxidase staining of whole embryos probed with the anti- $\beta 3$ -tubulin antibody. All embryos were fixed 12–16 hr after fertilization. Embryos in which  $\beta 3$  is expressed exhibit high levels of staining with the anti- $\beta 3$ -tubulin antibody in all of the mesodermally derived muscle groups. Embryos were photographed in focal planes which allowed staining in specific muscle types to be seen, as indicated by arrows: hm, hypodermal muscle; vm, visceral muscle; phm, pharyngeal muscle. (A) and (B) The embryos on the bottom right of each panel are *prd*, *Px*<sup>2</sup>/*prd*, *Px*<sup>2</sup> embryos in which  $\beta 3$  is not expressed. The embryos on the upper left of each panel are morphologically normal siblings which exhibit normal wild type  $\beta 3$  staining. (C) An embryo of genotype *prd*,  $P[w^+\beta 3]2$ , *Px*<sup>2</sup>/*prd*,  $P[w^+\beta 3]2$ , *Px*<sup>2</sup> showing  $\beta 3$  staining in the musculature. (D) An embryo of genotype *prd*, *Px*<sup>2</sup>/*prd*, *Px*<sup>2</sup>;  $P[w^+\beta 3]3a/P[w^+\beta 3]3a$  showing  $\beta 3$  staining. (E) An embryo of genotype *prd*/*prd* showing  $\beta 3$  staining. The embryos in panels A and B are oriented anterior to the right and posterior to the left. The *prd*, *Px*<sup>2</sup>/*prd*, *Px*<sup>2</sup> embryos in panels A and B are oriented dorsal to the bottom of the page and ventral to the top of the page. The embryos in panels C–E are oriented anterior to the right, posterior to the left, dorsal to the bottom of the page and ventral to the top of the page. The bar in panel A represents 100  $\mu$ m and shows the magnification for panels A, C, D and E. The bar in panel B represents 100  $\mu$ m.

with the affinity purified anti- $\beta 3$ -tubulin antibody. This result is consistent with the genomic southern results which show that the *Px*<sup>2</sup> deficiency removes the structural gene for  $\beta 3$ -tubulin. The left side of Figure 5A shows an adjacent morphologically normal sib (genotype *prd*, *Px*<sup>2</sup>/*CyO* or *CyO*/*CyO*). In this focal plane specific localization of  $\beta 3$ -tubulin can be seen in this embryo in both the pharyngeal and body wall or hypodermal musculature, in the typical pattern that we have previously observed for normal  $\beta 3$ -tubulin expression (KIMBLE, INCARDONA and RAFF 1989). The wild-type pattern of staining in this embryo demonstrates that the conditions of fixation and staining of these embryos were adequate for proper staining to be observed. Figure 5B shows a pair of slightly older embryos from the same experiment, showing that the

homozygous *prd*,  $Px^2/prd$ ,  $Px^2$  embryo does not express  $\beta 3$ , while in its morphologically normal sibling, wild-type  $\beta 3$  localization can be seen in all three muscle groups, the pharyngeal, hypodermal and visceral muscles.

Both transgene inserts  $P[w^+\beta 3]2$  and  $P[w^+\beta 3]3a$  support  $\beta 3$ -tubulin expression with correct timing and localization, as demonstrated by anti- $\beta 3$  staining of embryos carrying the transgenes in a *prd*,  $Px^2$  background. Figure 5C shows an embryo produced from parents of genotype *prd*,  $P[w^+\beta 3]2$ ,  $Px^2/CyO$ . This embryo is homozygous for the *prd*,  $P[w^+\beta 3]2$ ,  $Px^2$  chromosome. In this focal plane, specific staining can be observed both in the hypodermal and the pharyngeal musculature. Figure 5D shows an embryo homozygous for both *prd*,  $Px^2$  and the third chromosome insert  $P[w^+\beta 3]3a$ . Again, specific staining in the hypodermal and pharyngeal musculature is clearly observed in this focal plane. The morphologically normal siblings of the embryos in Figure 5, C and D, also exhibited the normal wild type pattern of  $\beta 3$  staining. For comparison, the staining due to expression of the endogenous wild type  $\beta 3$  gene in the musculature of embryos homozygous for *prd* alone is shown in Figure 5E; the staining pattern is the same as in the homozygous *prd*,  $Px^2$  embryos which express  $\beta 3$ -tubulin solely from the transgene. Because of the morphological defects in homozygous *prd* embryos, it is difficult to see both body wall and pharyngeal musculature staining in the same focal plane. However, staining in both muscle types was observed in embryos of all the genotypes shown in Figure 5, C, D and E.

The staining experiments thus clearly show that the  $\beta 3$ -tubulin gene is not expressed in embryos which are homozygous for the  $Px^2$  deficiency and that in embryos, the  $\beta 3$  transgene is expressed in the proper temporal and spatial fashion. The rescue experiments described below show that the transgenes support  $\beta 3$  expression in at least some pupal tissues. However, since the  $\beta 3$  mutations are fully recessive, but cannot be fully phenotypically rescued by a single copy of either of the  $\beta 3$  transgene inserts, our data suggest that expression from the  $\beta 3$  transgene construct is not at full wild type levels in either embryos or pupae.

The 15 mutations which were recovered in the distal region of the  $Px^2$  deficiency and were therefore candidates for mutations in the  $\beta 3$ -tubulin gene were tested for rescue of lethality by  $P[w^+\beta 3]3a$  or  $P[w^+\beta 3]2$ . Only members of a single complementation group were rescued by the transgene, identifying these mutations as alleles of the  $\beta 3$  gene (designated as complementation group 13 since genomic Southern analysis showed the  $\beta 3$  gene to be close to the distal breakpoint of  $Px^2$ ). A single copy of either transgenic insert rescues the hemizygous lethality of  $B3t^4$ . Rescue was not observed for hemizygotes for any of the other mem-

bers of the complementation group. The homozygous lethality of two members of this complementation group,  $B3t^1$  and  $B3t^5$ , can be rescued by the transgene. No rescue was observed for the homozygotes for the remaining two alleles,  $B3t^2$  and  $B3t^3$  (however, as discussed below,  $B3t^3$  appears to carry an additional closely linked lethal mutation). The identification of the mutations in complementation group 13 as  $B3t$  alleles was confirmed by rescue by the transgenes of the lethality of most of the interallelic combinations of mutations in this complementation group.

**Phenotypic Analysis of mutations in the  $\beta 3$ -tubulin gene:** The *inter se* complementation pattern of the five  $\beta 3$ -tubulin mutations, and the effects of the transgenes on the terminal phenotypes of the interallelic combinations are summarized in Figure 6. As shown in Figure 6A, all of the  $B3t$  mutations are lethal in combination with  $Px^2$ . Although lethal as a hemizygote,  $B3t^4$  is viable but male sterile as a homozygote, suggesting that this allele is a weak hypomorph. The most severe alleles are  $B3t^2$  and  $B3t^3$ , which are lethal in combination with each other as well as with the other three members of the complementation group. The  $B3t^1$ ,  $B3t^4$  and  $B3t^5$  mutations, however, all complement each other as interallelic heterozygotes. This complex complementation pattern obtained for the  $\beta 3$  alleles resembles the interallelic complementation previously observed among different mutations in the gene for the *Drosophila* testis-specific isoform  $\beta 2$ -tubulin (KEMPHUES, RAFF and KAUFMAN 1983; RAFF and FULLER 1984), and for *Drosophila*  $\alpha$ -tubulin mutations (MATTHEWS and KAUFMAN 1987). None of the  $\beta 3$  alleles we have isolated encode electrophoretic variants of  $\beta 3$ -tubulin; experiments to address the levels and localization of protein produced from the mutant alleles in both embryos and pupal tissues are in progress. However, the complementation data clearly indicate that  $B3t^1$  and  $B3t^5$ , as well as the homozygous viable  $B3t^4$  allele, encode mutant proteins which retain a considerable degree of function.

In embryos,  $\beta 3$  is the sole  $\beta$ -tubulin isoform expressed in the developing musculature (LEISS *et al.* 1988; GASCH *et al.* 1988; GASCH, HINZ and RENKAWITZ-POHL 1989; KIMBLE, INCARDONA and RAFF 1989), suggesting that in this tissue  $\beta 3$  functions in the transient microtubule scaffold involved in organization of the developing sarcomeres (CROSSLEY 1978). If  $\beta 3$  expression in the embryo is essential, the most severe phenotype resulting from loss of  $\beta 3$  function might be embryonic lethality resulting from defective differentiation of the larval musculature. The lethal phenotype could obviously be no more severe than that of the  $Px^2$  deficiency. The external morphology of embryos homozygous for  $Px^2$  (*i.e.*, missing all of the fourteen or more genes in this region of the genome) appears normal through mid to late embry-

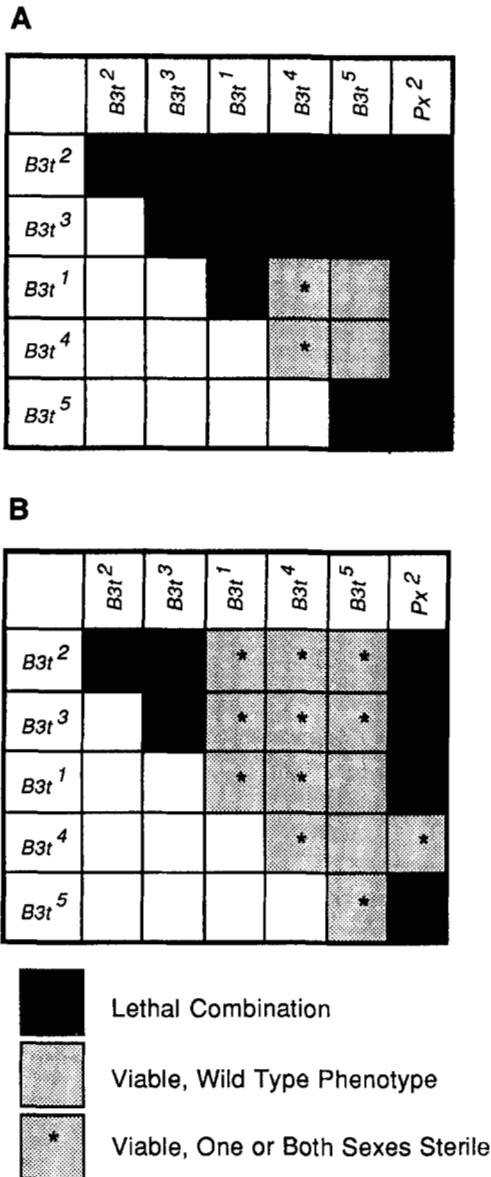


FIGURE 6.—Matrices showing complementation (A) among mutations in the  $\beta 3$ -tubulin gene and (B) among the  $\beta 3$ -tubulin mutations in the presence of two copies of the  $\beta 3$  transgene. In the crosses used to obtain the progeny of the indicated genotype in panel B, one parent carried the second chromosome transgene  $P[w^+\beta 3]2$  and the indicated  $\beta 3$ -tubulin allele, balanced over  $CyO$ . The second parent carried the indicated  $\beta 3$ -tubulin allele balanced over  $CyO$  and was homozygous for the  $P[w^+\beta 3]T3a$  transgene on the third chromosome. The resulting viable adult progeny carried the indicated combination of  $B3t$  alleles and one copy of each of the two  $\beta 3$  transgenes  $P[w^+\beta 3]2$  and  $P[w^+\beta 3]3a$ . Solid squares indicate nonviable combinations and shaded squares indicate allele combinations for which some viable adults were obtained. Shaded squares with a star (\*) indicate sterility of one or both sexes of the rescued adults.

ogenesis, but at 24 hr, when their normal sibs are hatching, these embryos exhibit some visible morphological defects, including lack of trachea and malformed malpighian tubules and mouthparts, do not exhibit normal movements in response to physical stimulation, and do not hatch. Light microscopic ex-

amination of sections of terminal  $Px^2$  homozygous embryos revealed defects and/or disorganization in many tissues. We have not as yet examined the tissue organization or ultrastructure of muscles in animals homozygous for  $Px^2$  or for the  $\beta 3$  mutations. It is clear, however, that  $\beta 3$  is not essential for muscle formation *per se*, since at least some muscle tissue is present in homozygous  $Px^2$  embryos, and some prelethal animals exhibit some muscle movements. However, compared to the strong rhythmic waves of contraction of the body wall and gut musculature which occur in normal pre-hatching animals, the muscle movements exhibited by  $Px^2$  homozygotes are infrequent, feeble, and uncoordinated.

None of the  $\beta 3$  mutations we have isolated thus far causes embryonic lethality. The effective lethal phase for all  $B3t^m/Px^2$  hemizygotes is during the larval period. Similarly, animals homozygous for  $B3t^2$  or  $B3t^5$  also die during larval development, as do most animals carrying nonviable  $B3t$  mutant allele combinations. Although homozygotes for the  $B3t^3$  chromosome die as late embryos, this most likely indicates that this chromosome carries a second recessive lethal mutation closely linked but not within the  $Px^2$  deficiency (we have as yet been unable to separate the embryonic lethality by recombination). The most likely explanation of the delayed lethal phase of  $\beta 3$  mutant animals relative to  $Px^2$  homozygotes is that even the most severe alleles,  $B3t^2$  and  $B3t^3$ , retain some  $\beta 3$  function. Consistent with this interpretation is the fact that death of animals carrying lethal combinations of  $\beta 3$  mutant alleles occurs throughout the larval period, and appears to depend somewhat on culture conditions, animals fed on soft food tending to survive longer. Alternatively, it may be that even in the absence of embryonic  $\beta 3$ -tubulin function, the muscles can form and function well enough for the embryo to hatch and begin to feed, in which case, other gene(s) removed by the deficiency would be responsible for the embryonic lethality of deficiency homozygotes.

In pupae,  $\beta 3$  is again transiently expressed at high levels at the beginning of differentiation of each set of adult muscles, and thus appears to play a similar role in the development of the adult muscles as it does during muscle differentiation in the embryo (KIMBLE, INCARDONA and RAFF 1989). However, in the pupa, in addition to muscle expression,  $\beta 3$  is also transiently expressed at lower levels in a variety of tissues of ectodermal origin (KIMBLE, INCARDONA and RAFF 1989).  $\beta 3$  is expressed in all of the imaginal discs at the onset of pupal development, and during mid to late pupal development in a specific subset of cells in the optic lobe of the brain and in the developing wing blades. Finally, during pupal development  $\beta 3$  expression also begins in somatic cells in the gonads, the follicle cells of the ovary and the somatic cyst cells of

TABLE 1  
Adult phenotypes of  $\beta 3$ -tubulin mutations

Genotype of Viable adults	First day of Eclosion <sup>a</sup>	Percent of adults that survive <sup>b</sup>	Flight <sup>c</sup>	Fertility	
				Female	Male
Adult viable combinations of <i>B3t</i> alleles					
<i>w; B3t<sup>1</sup>/B3t<sup>2</sup>; +</i>	1–2	100	++	F	F
<i>w; B3t<sup>1</sup>/B3t<sup>2</sup>; +</i>	1–2	100	++	F	F
<i>w; B3t<sup>1</sup>/B3t<sup>4</sup>; +</i>	1–2	100	++	F	S
<i>w; B3t<sup>4</sup>/B3t<sup>4</sup>; +</i>	1–2	45	++	F	S
Adult viable with the β3 transgene present					
<i>w; B3t<sup>4</sup>/P[w<sup>+</sup>β3]2, Px<sup>2</sup>; +</i>	ND	<91	ND	ND	S
<i>w; P[w<sup>+</sup>β3]2, B3t<sup>4</sup>/B3t<sup>4</sup>; P[w<sup>+</sup>β3]3a/+</i>	1–2	100	++	F	S
<i>w; B3t<sup>2</sup>/B3t<sup>2</sup>; P[w<sup>+</sup>β3]3a/+</i>	3–4	<30	+	F	F
<i>w; P[w<sup>+</sup>β3]2, B3t<sup>1</sup>/B3t<sup>1</sup>; P[w<sup>+</sup>β3]3a/+</i>	ND	61	+ to ++	WF	S
<i>w; P[w<sup>+</sup>β3]2, B3t<sup>1</sup>/B3t<sup>2</sup>; P[w<sup>+</sup>β3]3a/+</i>	2	6	–	WF	S
<i>w; P[w<sup>+</sup>β3]2, B3t<sup>1</sup>/B3t<sup>2</sup>; P[w<sup>+</sup>β3]3a/+</i>	6	15	–/+	S	S
<i>w; P[w<sup>+</sup>β3]2, B3t<sup>1</sup>/B3t<sup>2</sup>; P[w<sup>+</sup>β3]3a/+</i>	4	9	–	ND	S
<i>w; P[w<sup>+</sup>β3]2, B3t<sup>4</sup>/B3t<sup>2</sup>; P[w<sup>+</sup>β3]3a/+</i>	4	42	–/+	S	S
<i>w; P[w<sup>+</sup>β3]2, B3t<sup>2</sup>/B3t<sup>2</sup>; P[w<sup>+</sup>β3]3a/+</i>	6	3	–	S	S
<i>w; P[w<sup>+</sup>β3]2, B3t<sup>2</sup>/B3t<sup>2</sup>; P[w<sup>+</sup>β3]3a/+</i>	5	9	–/+	S	S

<sup>a</sup> First day of eclosion of  $\beta 3$  mutant adults relative to beginning day of eclosion of heterozygous sibs.

<sup>b</sup> The % of individuals of each genotype that survived to adulthood was determined as the fraction of the number of adults eclosed to the number expected. For each cross, the number expected was one-third of the total progeny from the cross.

<sup>c</sup> Ability to fly: ++, normal flight; +, flight for short distances only; –/+, could not fly but could jump; –, could not fly or jump.

<sup>d</sup> F, fertile; WF, weakly fertile (few progeny produced); S, sterile.  
ND, not determined.

the testis; these cell types are the sole sites where  $\beta 3$  expression persists in the adult.

Because of the complex pattern of expression of  $\beta 3$ -tubulin during pupal development, a variety of phenotypes might result from disruption of  $\beta 3$  function in the pupa. This is in fact what we observe. For example, most homozygous  $B3t^1$  animals and animals of the genotype  $B3t^1/B3t^2$  die during larval development, but some survive to the pupal stage. The fraction of animals of these genotypes that survive to the pupal stage and the extent of subsequent pupal development depends on the culture conditions. Under standard culture conditions (i.e., when larvae are raised on agar containing cornmeal and molasses), 21% of  $B3t^1/B3t^1$  animals survived to the pupal stage. Of these, approximately two-thirds form an apparently normal pupal case but fail to undergo subsequent development, and by 24 hr post-pupariation most of the larval tissues have histolyzed. When  $B3t^1$  homozygotes are raised on yeast paste, most of the animals pupate and develop to pharate adults but do not emerge from the pupal case. The dependence of the lethal phase on culture conditions is consistent with the hypothesis that defective  $\beta 3$  function results in defective muscle development, leading to larvae with reduced ability to burrow into the medium and feed when grown on normal agar-based food. When dissected from their pupal cases, prelethal homozygous  $B3t^1$  pharate adults resulting from raising larvae on yeast paste exhibited decreased thoracic muscle mass relative to wild-type animals, as well as one or

more pairs of deformed legs. Rarely, homozygous  $B3t^1$  adults do eclose. Such flies are weak and short-lived, and exhibit a variety of abnormalities which reflect the pupal pattern of  $\beta 3$  expression: they are sterile, walk poorly, cannot fly, and exhibit minor defects in wing venation.

The phenotypes for  $\beta 3$  mutant allele combinations which allow survival to adulthood are shown in Table 1. Animals of genotypes  $B3t^1/B3t^2$ ,  $B3t^4/B3t^2$ , and  $B3t^1/B3t^4$  are fully viable, while only about half of  $B3t^4/B3t^4$  animals survive to adulthood. All of the surviving adults of these genotypes are able to fly, indicating normal muscle development. Females of all of these genotypes are fertile.  $B3t^1/B3t^2$  and  $B3t^4/B3t^2$  males are fertile, but  $B3t^1/B3t^4$  and  $B3t^4/B3t^4$  males are sterile. In these males, overall testis morphology is normal, but no motile sperm are produced. We do not yet know the precise molecular basis for the male sterility, but preliminary light and electron microscopic examination of the  $B3t^4/B3t^4$  phenotype suggests the possibility that cytoskeletal interactions are disrupted between the syncytium of developing germ cells and the two somatic cyst cells which enclose the syncytium and in which  $\beta 3$ -tubulin is expressed (R. W. DETTMAN and E. C. RAFF, unpublished data).

**Rescue of the  $\beta 3$ -tubulin mutations by the  $\beta 3$  transgene:** The *inter se* complementation pattern of the  $\beta 3$  mutations in the presence of the  $\beta 3$  transgenes is shown in Figure 6B; the adult phenotypes of combinations of the  $\beta 3$  alleles which are viable in the presence of the transgenes are summarized in Table

1. To examine the effects of two copies of the transgene, complementation crosses were carried out such that for each cross one parent was  $w;B3t^m,If/CyO$ ;  $P[w^+\beta3]3a/P[w^+\beta3]3a$ , and the second parent was  $w;P[w^+\beta3]2,B3t^m,If/CyO$ ;  $+/+$ . Thus progeny examined for rescue carried one copy of the  $\beta3$ -transgene on the second chromosome and one copy on the third chromosome. This was done because homozygosity for the transgenes themselves had a somewhat deleterious effect on viability, presumably due to the sites of insertion.

$B3t^4$  is the only allele for which the transgene allows survival of hemizyotes to adulthood. In the presence of two copies of the transgene,  $B3t^4$  homozygotes are fully viable, but remain male sterile. One copy of the transgene allowed survival of some  $B3t^1$  and  $B3t^5$  homozygotes to adulthood, and allowed some  $B3t^2$  animals to undergo pupariation. Rescue of the lethality of other combinations of  $\beta3$  alleles required two copies of the transgene. However, even two copies of the transgene allowed only partial survival of adults of the genotypes  $B3t^1/B3t^2$ ,  $B3t^1/B3t^3$ ,  $B3t^4/B3t^2$ ,  $B3t^4/B3t^3$ ,  $B3t^5/B3t^2$ , and  $B3t^5/B3t^3$ . The rescue data for these allele combinations indicate that  $B3t^2$  has the most severe effect on viability.

The rescued adults were tested for ability to fly and for fertility. All adults having genotypes that result in viable adults in the absence of the transgene are able to fly. The rescued  $B3t^1$  and  $B3t^5$  homozygotes exhibit decreased ability to fly relative to wild type animals. None of the rescued transheterozygous adults can fly, although  $B3t^1/B3t^3$ ,  $B3t^3/B3t^4$ , and  $B3t^3/B3t^5$  animals are capable of rather prodigious leaps. We speculate that if the transient  $\beta3$ -based microtubule arrays present during myogenesis are defective, the resultant muscle may be structurally or functionally defective, resulting in loss of the ability to fly. In the  $B3t^2/B3t^1$ ,  $B3t^2/B3t^4$ , and  $B3t^2/B3t^5$  adults, which not only did not fly but also did not walk well, the thoracic muscle mass was clearly less than in normal animals.

As discussed previously, for those genotypes that are viable in the absence of the transgene, all adults are fertile, except for the  $B3t^4/B3t^4$  and  $B3t^4/B3t^1$  males. The presence of the  $\beta3$  transgene did not rescue the sterility of these males. Among the rescued genotypes,  $B3t^5/B3t^5$  females and males are both fertile, while  $B3t^1/B3t^1$  and  $B3t^2/B3t^1$  females are weakly fertile. All other rescued flies, both male and female, are sterile. (It was not possible to definitively test fertility of  $B3t^2/B3t^4$  females, as these adults died within 24 hr of eclosion.) We do not know the molecular basis for loss of female fertility in the rescued  $\beta3$  mutant adults. Defective  $\beta3$  function in the follicle cells might lead to defects in the ability of the follicle cells to transport yolk to the developing oocytes, a process which GUTZEIT (1986) has shown requires

microtubules. Another possibility is that the follicle cells are defective in deposition of the vitelline membrane or chorion (MAHOWALD and KAMBYSELLIS 1980). The number of mature oocytes produced by the weakly fertile and sterile females varied from none ( $B3t^2/B3t^5$ ) to nearly normal numbers ( $B3t^3/B3t^5$ ). Although the  $B3t^3/B3t^5$  females had mature oocytes in their ovaries, no eggs were laid by these females, and the chorion morphology of oocytes from dissected ovaries was abnormal, showing "frayed" chorionic appendages.

## DISCUSSION

The divergent protein sequence and complex pattern of expression of the  $\beta3$  isoform originally led us to speculate that this isoform might have functional properties in microtubule assembly distinct from the predominant *Drosophila* isoform  $\beta1$  and the testis-specific isoform  $\beta2$ , both of which are of a conserved sequence type relative to other major metazoan  $\beta$  tubulin isotypes, and both of which function in all morphological classes of microtubules, including spindles, cytoskeletal microtubules, and multiple-walled tubules of the centriole or axoneme, respectively (KEMPHUES *et al.* 1982; RAFF and FULLER 1984; RUDOLPH *et al.* 1987; FULLER *et al.* 1988). We have recently compared the functional potential of the divergent  $\beta3$  isoform with that of the testis-specific isoform  $\beta2$ -tubulin by constructing and transforming into the genome  $\beta2\beta3$  hybrid genes which bring expression of  $\beta3$  under control of the  $\beta2$ -tubulin promoter and thus allow the expression of  $\beta3$  in the male germ line. These studies have confirmed that there are intrinsic differences between the microtubule assembly properties of these two isoforms *in vivo* (HOYLE and RAFF 1990). We found that when  $\beta3$ -tubulin is coexpressed in the male germ line with  $\beta2$ -tubulin, it does not interfere with the assembly and function of any category of singlet microtubules including the meiotic spindle or cytoskeletal microtubules, but causes a dominant defect in morphology of the doublet tubules of the sperm tail axoneme, resulting in dominant male sterility. When  $\beta3$ -tubulin is expressed in the male germ line in the absence of  $\beta2$ -tubulin, it supports assembly of a single class of transient cytoplasmic microtubules, a category of microtubules resembling that in which  $\beta3$  normally functions.

The  $\beta3$ -tubulin mutations we have isolated will allow us to begin a detailed analysis of the specific role(s) *in vivo* normally fulfilled by this divergent  $\beta$ -tubulin isoform, and will allow us to determine if  $\beta3$  functions are specialized, or perhaps merely restricted, relative to the multiple functions of the conserved sequence isotypes. The  $\beta3$  mutations result in a diverse set of phenotypes which directly reflect the complex developmental pattern of  $\beta3$  expression we have previously

described (KIMBLE, INCARDONA and RAFF 1989). Our results demonstrate that embryonic expression of the  $\beta 3$  gene in the developing musculature is essential for subsequent survival of the hatched larva, while later expression of  $\beta 3$  in multiple tissues in the pupa and in the somatic cells of the adult gonad is required for a number of differentiative events. Male fertility is particularly sensitive to lesions in  $\beta 3$ , but development of the adult musculature, female fertility, and imaginal disc development are also sensitive to disruption in  $\beta 3$  function. Wing blade development appears to be only minimally affected by lesions in  $\beta 3$ , and we have not to date observed any phenotype attributable to disruption of  $\beta 3$  function in the optic lobe (where  $\beta 3$  expression occurs during mid pupal development in the cells of the internal chiasmata, a structure for which the function is not known).

The  $\beta 3$  transgene supports only partial rescue of the  $\beta 3$  mutant phenotypes. Although the  $\beta 3$  construct inserted into the genome contains 2.5 kb of 5' flanking sequences and 0.6 kb of 3' flanking sequences, as well as all of the  $\beta 3$  transcribed sequences, including the three introns, it appears that the transgene does not contain all of the elements required for full wild type expression of the  $\beta 3$  gene. GASCH, HINZ and RENKAWITZ-POHL (1989) have shown that the  $\beta 3$ -tubulin gene has complex *cis*-acting regulatory requirements for embryonic expression. These authors found that proper expression of the  $\beta 3$  gene in the developing embryonic musculature requires sequences greater than 600 bp upstream of the gene, and that sequences within the 4.5-kb first intron are required for visceral mesoderm expression. Our data suggest that the  $\beta 3$  transgene supports proper spatial and temporal regulation of embryonic  $\beta 3$  expression, but does not yield wild type levels of protein in the embryo.

Failure of complete rescue of the adult mutant phenotypes may be due to failure of the transgene to support wild type levels of pupal  $\beta 3$  expression and/or failure of  $\beta 3$  expression in some of its normal sites of expression in pupal tissues. However, the rescue data suggest that in the pupa, the  $\beta 3$  transgenes support at least some degree of  $\beta 3$  expression in early imaginal discs, muscle, testis, ovary, and wing blade. First, that expression of the  $\beta 3$  gene in the imaginal discs during the white prepupa stage is essential is suggested by the phenotype of the  $B3t^1$  homozygous animals that form pupal cases but fail to develop further. In  $B3t^1$  stocks which carry one or more copies of the  $\beta 3$ -transgene, pupae of this type do not occur, suggesting rescue by early pupal  $\beta 3$  expression. Second, although we have not been able to rescue the intrinsic male sterility of the adult viable  $B3t^4/B3t^4$  and  $B3t^1/B3t^4$  animals, the associated spermatogenic defects are alleviated in the presence of the transgene

(R. W. DETTMAN and E. C. Raff, unpublished data). Third, that there is some expression of  $\beta 3$  from the transgene inserts in the developing wing blade is suggested by the fact that the rare surviving  $B3t^1$  homozygous adults exhibit wing venation defects, a phenotype not observed in rescued adults. In the wing blades the  $\beta 3$  protein is a minor component of the total  $\beta$ -tubulin pool, of which  $\beta 1$ -tubulin is the predominant isoform. It is possible that expression of  $\beta 3$ -tubulin in the wing may be dispensable.

Evidence for expression of the transgene in developing adult musculature and the follicle cells in the ovary is somewhat indirect, and derives from the observation that rescued adults of some genotypes are viable but exhibit impaired ability to fly and female as well as male sterility. The residual defects in muscle and follicle cell function may reflect low levels of expression from the transgene in tissues which require high levels of  $\beta 3$  for normal development. Both mRNA and protein localization studies suggest that  $\beta 3$  is the predominant  $\beta$ -tubulin expressed in the developing adult musculature and in the ovarian follicle cells (LEISS *et al.* 1988; GASCH *et al.* 1988, GASCH, HINZ and RENKAWITZ-POHL 1989; KIMBLE, INCARDONA and RAFF 1989). Our antibody localization studies suggest that this may also be true in the somatic cyst cells in the testis, but this has not been definitively demonstrated.

The phenotype that we expected to see for a mutation which results in complete loss of  $\beta 3$  function, but have not obtained, is embryonic lethality or death at or shortly after the time of hatching. It may be that even in the absence of  $\beta 3$  expression, muscle development in the embryo is sufficient for survival into the larval period. More likely, however, is that all of the alleles we have so far isolated encode proteins that retain enough  $\beta 3$  function to allow sufficient development of the musculature for larval survival, but the resultant larvae are weak and have difficulty feeding. The phenotypes of the mutations support this, since for some allele combinations the time of lethality is dependent on culture conditions, and since lethality occurs over an extended period in larval development, even for  $B3t^2$ , the most severe allele. Second, in rescue experiments, for some combinations of alleles, rescued adults did not begin emerging for up to 6 days after their heterozygous siblings had begun to eclose (Table 1). This could be due to slow development of the rescued flies. Alternatively, it is possible that the larvae are weak and are only able to survive once the food has been softened by the burrowing and feeding activity of their heterozygous siblings, allowing these weak larvae to feed.

Taken together, the lethal phases, *inter se* complementation pattern, and rescue phenotypes of the  $\beta 3$  mutations suggest that  $B3t^2$  and  $B3t^3$  are strong hy-

pomorphic alleles, while  $B3t^1$  and  $B3t^5$  are intermediate hypomorphs, and  $B3t^4$  is a weak hypomorph. The five  $\beta 3$  mutations differ considerably in the severity of their effect on development. Moreover, the relative severity of the effect a particular allele has on one aspect of development may be greater or less than its effect on a second aspect of development. When the five alleles are ranked as to their effect on viability, the order of the alleles from most severe to least severe is  $B3t^2 > B3t^3 > B3t^5 > B3t^1 > B3t^4$ . When the alleles are ranked according to their effect on fertility, the order is  $B3t^3 > B3t^2 > B3t^4 > B3t^1 > B3t^5$ . Both the differential effects on different aspects of development and the complex complementation pattern seen among the five  $\beta 3$  mutations are similar to the phenotypes observed for other *Drosophila* tubulin mutations, including mutations in the testis-specific  $\beta 2$ -tubulin isoform (KEMPHUES, RAFF and KAUFMAN 1983; RAFF and FULLER 1984), and in the major alpha tubulin isoform (MATTHEWS and KAUFMAN 1987). The complex phenotypes may reflect the different functional interactions—between  $\alpha$ - and  $\beta$ -tubulin to form a functional heterodimer, and between the  $\alpha, \beta$ -tubulin heterodimer and a variety of different microtubule-associated proteins—required for participation of a single tubulin isoform in a variety of different microtubule arrays.

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