Two *Drosophila* Beta Tubulin Isoforms Are Not Functionally Equivalent

Henry D. Hoyle and Elizabeth C. Raff

Institute for Molecular and Cellular Biology and Department of Biology, Indiana University, Bloomington, Indiana 47405

Abstract. We have tested the functional capacity of different beta tubulin isoforms in vivo by expressing β3-tubulin either in place of or in addition to β2-tubulin in the male germ line of *Drosophila melanogaster*. The testes-specific isoform, β2, is conserved relative to major metazoan beta tubulins, while the developmentally regulated isoform, β3, is considerably divergent in sequence. β3-tubulin is normally expressed in discrete subsets of cells at specific times during development, but is not expressed in the male germ line. β2-Tubulin is normally expressed only in the postmitotic germ cells of the testis, and is required for all microtubule-based functions in these cells. The normal functions of β2-tubulin include assembly of meiotic spindles, axonemes, and at least two classes of cytoplasmic microtubules, including those associated with the differentiating mitochondrial derivatives. A hybrid gene was constructed in which 5′ sequences from the β2 gene were joined to protein coding and 3′ sequences of the β3 gene. *Drosophila* transformed with the hybrid gene express β3-tubulin in the postmitotic male germ cells. When expressed in the absence of the normal testis isoform, β3-tubulin supports assembly of one class of functional cytoplasmic microtubules. In such males the microtubules associated with the membranes of the mitochondrial derivatives are assembled and normal mitochondrial derivative elongation occurs, but axoneme assembly and other microtubule-mediated processes, including meiosis and nuclear shaping, do not occur. These data show that β3 tubulin can support only a subset of the multiple functions normally performed by β2, and also suggest that the microtubules associated with the mitochondrial derivatives mediate their elongation. When β3 is coexpressed in the male germ line with β2, at any level, spindles and all classes of cytoplasmic microtubules are assembled and function normally. However, when β3-tubulin exceeds 20% of the total testis beta tubulin pool, it acts in a dominant way to disrupt normal axoneme assembly. In the axonemes assembled in such males, the doublet tubules acquire some of the morphological characteristics of the singlet microtubules of the central pair and accessory tubules. These data therefore unambiguously demonstrate that the *Drosophila* beta tubulin isoforms β2 and β3 are not equivalent in intrinsic functional capacity, and furthermore show that assembly of the doublet tubules of the axoneme imposes different constraints on beta tubulin function than does assembly of singlet microtubules.

MICROTUBULES are the basic cytoskeletal element from which many different subcellular structures, diverse in both morphological and functional complexity, are constructed. A major unanswered question is to what extent the structural and functional characteristics of different microtubule arrays are determined by intrinsic differences in the properties of different tubulin isoforms. The evolutionary conservation across species of distinct isoforms, as well as the differential patterns of expression of different members of tubulin gene families, suggests that at least in some cases different isoforms may be functionally specialized. However, it has heretofore not been possible to demonstrate unambiguous differences in the functional capacity associated with given isoforms in vivo (see discussions in Raff et al., 1987; and Monteiro and Cleveland, 1988). In cases where multiple isoforms are coexpressed in a cell, all of the isoforms appear to be intermingled, at least to some extent, in all of the microtubule populations in the cell (Bond et al., 1986; Lewis et al., 1987; Paul et al., 1987; Lopata and Cleveland, 1987). Similarly, the microtubule literature is replete with examples in which heterologous tubulins can be incorporated into functional microtubules in vivo: thus, bovine brain tubulin introduced by injection into cultured mammalian cells (Keith et al., 1981; Saxton et al., 1984), sea urchin embryos (Salmon et al., 1984), or *Drosophila* embryos (Kellog et al., 1988) is coassembled with endogenous tubulin into functional spindles and cytoplasmic microtubules. In *Aspergillus*, it has been experimentally demonstrated that two divergent beta tubulin isoforms are in fact functionally interchangeable (May, 1989). Thus the question of why multiple tubulin isotypes exist has remained unanswered.

In this paper we have considered this question with regard to the *Drosophila* beta tubulins, a gene family consisting of four members. The patterns of gene expression and localization of the protein product have been defined for three of the

members. β1-Tubulin, encoded on the right arm of the second chromosome at polytene band position 56, is the predominant *Drosophila* beta tubulin in all but a few tissues; the known exceptions include the developing musculature of embryos and pupae (Gasch et al., 1988; Kimble et al., 1989), the ovarian follicle cells (Gasch e al., 1988), and the postmitotic cells in the male germ line (Kemphues et al., 1982). β2-Tubulin, on the right arm of the third chromosome at position 85D, is expressed only in postmitotic cells in the male germ line (Kemphues et al., 1979, 1980, 1982, 1983). β3-Tubulin, on the right arm of the second chromosome at position 60D, is expressed in a complex temporal and spatial pattern in a number of somatic cell types in embryos, pupae, and certain adult tissues (Raff et al., 1982; Bialojan et al., 1984; Gasch et al., 1988; Kimble et al., 1989). β3-Tubulin is the predominant isoform in the transient microtubule populations in the cells of the developing musculature in embryos and pupae and in the ovarian follicle cells (Gasch et al., 1988; Kimble et al., 1989). However, in at least some cell types β1 and β3 are coexpressed, as in the developing wing blade, although the two isoforms appear to be differentially localized in this tissue (Kimble et al., 1989). β3 is also expressed in the imaginal discs shortly after pupariation, in a small set of cells within the developing optic lobe, and in a specific set of somatic cells within the testis (Kimble et al., 1989). The sites of β3 expression suggest that this isoform is primarily used in specialized cytoplasmic microtubules involved in determination of cell shape or tissue arrangement (Kimble et al., 1989).

Protein sequences, for the most part derived from nucleic acid sequences, are now known for many beta tubulins. From examination of the large and highly conserved beta tubulin families of different vertebrate species, Sullivan and Cleveland (1986) defined two variable regions of the beta tubulin molecule of potential functional importance: an internal variable region near the amino terminus comprising amino acids 35–57, and the carboxyl terminus, which is unique for each isotype. Biochemical data have demonstrated the importance of the carboxyl-terminal region of the beta tubulin molecule for determining specific properties of microtubule assembly. The carboxyl region is exposed both in the alpha, beta tubulin dimer and in intact microtubules (Blose et al., 1984; Breitling and Little, 1986; Sackett and Wolff, 1986), and it is to this region of the molecule that a number of microtubule-associated proteins bind (Serrano et al., 1985; Littauer et al., 1986; Maccioni et al., 1986, 1988; Ginzburg and Littauer, 1988).

Among the *Drosophila* beta tubulins, the major isoform β1 and the testis-specific isoform β2 are 95% similar in amino acid sequence both to each other and to the major vertebrate beta tubulin isoforms, while β3 is a divergent protein only 88% similar to the other two *Drosophila* isoforms and to the major vertebrate isoforms (Rudolph et al., 1987; Michiels et al., 1987). In addition, certain structural features distinguish β3 not only from β1 and β2 but from other beta tubulins as well (Rudolph et al., 1987). First, following amino acid 56 at the end of the internal amino variable region, β3 possesses an additional 6 amino acids not found in any other beta tubulin to date. Second, β3 has cysteine at position 130 (corresponding to position 124 in other beta tubulins). Three minor vertebrate isoforms also have cysteine at this position (Sullivan and Cleveland, 1986), but in all other beta tubulins this residue is serine or alanine, including *Drosophila* β1 and β2 (Michiels et al., 1987; Rudolph et al., 1987), the other vertebrate isoforms (Sullivan and Cleveland, 1986; Wang et al., 1986; Monteiro and Cleveland, 1988), and the beta tubulins of *Chlamydomonas* (Youngblom et al., 1984) and fungi (Neff et al., 1983; Hiraoka et al., 1984; May et al., 1987).

We have addressed the functions of *Drosophila* beta tubulin isoforms within the context of microtubule function during spermatogenesis. Of the three isoforms expressed in the testis, β1 and β2 are similar not only in primary structure but in the fact that both are used to assemble multiple sets of microtubules in the male germ line, including both the singlet tubules of the spindle and of the cytoskeleton and tubules with shared walls in the centriole (β1) and in the axoneme (β2). β3 is markedly different both in structure and in the cellular context of its function. The time and place of expression of β2 and β3 in the testis are illustrated schematically in Fig. 1. Both the patterns of expression and the differences in primary structure suggest that these two isoforms serve very different roles in microtubule assembly. The testis-specific isoform β2-tubulin is expressed solely in the postmitotic germ cells but has multiple microtubule functions. β2 is first synthesized in mature primary spermatocytes just before meiosis, and is then required for all subsequent sets of microtubules including the meiotic spindles, cytoplasmic microtubules, and the axonemes of the sperm tail flagella (Kemphues et al., 1979, 1980, 1982, 1983). The divergent-sequence isoform β3 is not expressed in the germ line. β3 is present only in the two somatic cells which enclose each gonial cell during the entire course of its differentiation to ultimately form 64 spermatozoa (Kimble et al., 1989). These cells never subsequently divide (Lindsley and Tokuyasu, 1980), but during spermatogenesis undergo radical shape changes, suggesting that extensive rearrangements of the cytoskeleton must take place. In the experiments described below we have assessed the effects of expressing β3 in the male germ line. Our results show that these two isoforms are not equivalent in intrinsic functional capacity. When expressed in the absence of β2-tubulin, β3 protein is capable of supporting assembly of at least one class of functional cytoplasmic microtubules; furthermore, when coexpressed with the endogenous testis isoform, β3 does not interfere with spindle or cytoplasmic tubule assembly. However, when β3 is present above a threshold level it causes a specific defect in the assembly of the doublet tubules of the axoneme, resulting in dominant male sterility.

Materials and Methods

Construction of a Transformation Vector Containing a Wild-Type β2 Gene

The 4.55-kb Sal I–Xba I fragment of wild-type β2 genomic sequence (Rudolph et al., 1987, and Fig. 2) was inserted into pUChsneo', created from the *Drosophila* transformation vector pUChsneo (Steller and Pirrotta, 1985) by replacing the polylinker sequence containing the Sal I, Bam HI, Sma I, and Eco RI sites with the pUC 19 polylinker sequence containing the Sal I, Xba I, Bam HI, Sma I, Kpn I, Sac I, and Eco RI sites. Two transformed *Drosophila* lines with this copy of the wild type β2 gene inserted in the genome were obtained. For the experiments reported here, an insert at 61C on the left arm of the third chromosome was used; we refer to this insert as β2-Neo.

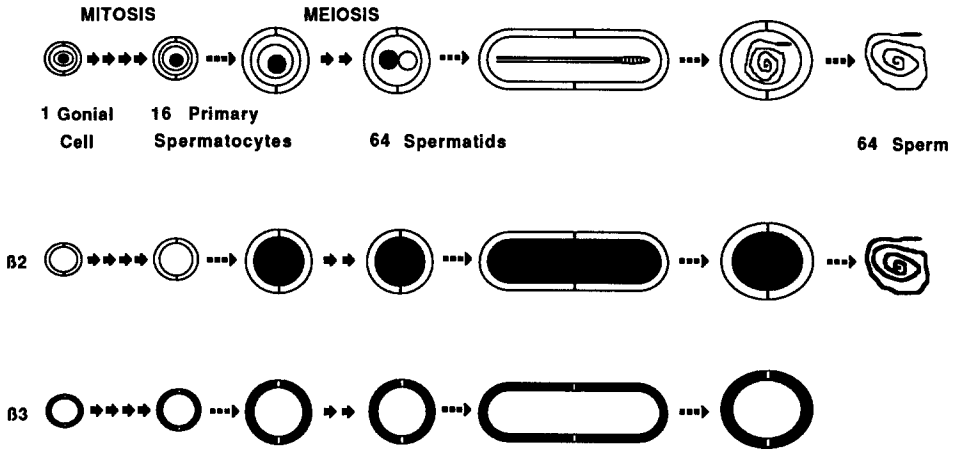

Figure 1. Expression of β2- and β3-tubulin in the testis. (*Top*) The developmental process of spermatogenesis is illustrated diagrammatically (see Lindsley and Tokuyasu, 1980): two somatic cells enclose a single primary gonial cell at the time of its formation. These two cyst cells enclose the developing germ cells derived from the gonial cell until the entry of the mature sperm into the seminal vesicles. For simplicity in this diagram only a single germ cell is illustrated at each developmental stage. The primary gonial cell undergoes 4 mitotic divisions, giving rise to 16 primary spermatocytes. These undergo meiosis to form 64 haploid spermatids. Differentiation of the spermatids involves the assembly of the axoneme, shaping and maturation of the sperm nucleus, and elongation of the mitochondrial derivative formed by coalescence of all of the mitochondria in each spermatid. The mature 1.8-mm long spermatids are individualized, coiled, and then released from the cyst cells into the seminal vesicles as mature motile sperm. (*Middle*) The testis-specific β2-tubulin is expressed solely in the postmitotic germ cells and is required for all postmitotic microtubule functions (Kemphues et al., 1982). The time and place of β2 expression is indicated in this diagram by the black filled-in regions. Our previous biochemical and genetic analysis of beta tubulin function in the testis showed that β1-tubulin is the only beta tubulin present in the early mitotic and growth stages of germ cell development, but is then replaced before meiosis by β2 as the major testis isoform (Kemphues et al., 1982). It should be noted that our data in that study did not distinguish whether β1 may persist in germ cells past the primary spermatocyte stage. If β1 does persist, it does not support microtubule assembly in these cells, since in the testes of males which are homozygous for loss of function mutations in the β2 gene, there are no microtubules present in postmitotic germ cells (Kemphues et al., 1982, 1983). The one exception are the basal bodies that were earlier formed as centrioles during the mitotic divisions, and that we therefore concluded are assembled from β1. Consistent with this hypothesis, we have since found that using a monoclonal antibody specific to β1 (Piperno and Fuller, 1985), we can detect very small amounts of β1 on Western blots of proteins present in mature motile sperm isolated from the seminal vesicles (unpublished data). (*Bottom*) Endogenous expression of β3 in the testis is confined to the somatic cyst cells (shown here in black) that enclose the developing germ cells (Kimble et al., 1989). We have not excluded the possibility that β1 is coexpressed at some level with β3 in the cyst cells, but our antibody localization data suggest that β1 may not be present in these cells. During spermatogenesis, the cyst cells in which β3 is expressed never divide but undergo dramatic changes in size and shape. The two cyst cells initially enclose a single gonial cell of ~10 μm in diameter; this cell gives rise to 16 mature primary spermatocytes each ~30 μm in diameter, entailing a concomitant 55-fold increase in the surface area enclosed by the two cyst cells. Eventually, before individualization of the spermatids, the same 2 cyst cells are enclosing 64 spermatids each ~0.6 μm in diameter but nearly 2 mm long, then during individualization and coiling of the maturing spermatids, the surface area encompassed by the cyst cells is reduced again.

Construction of Transformation Vectors Containing Hybrid Beta Tubulin Genes

*β2β3**. A chimeric gene was constructed consisting of β2 5′ untranslated sequences and the first 19 codons of β2 joined to β3 sequences starting at β3 codon 20 and including 3′ untranslated sequences. The fusion between β2 and β3 genomic sequences was carried out on a construct consisting of 2 kb of genomic β2 sequence from a 5′ Eco RI site to a Bgl II site at β2 codon 102 joined to a β3 fragment extending from a Bam HI site 57-bp upstream of β3 codon 20 (the 5′ codon of β3 exon 2) to a Pst I site at β3 codon 45 (see Fig. 2). The cloning vector pTZ 18U was used to generate single-stranded template following the Genescribe experimental protocol (United States Biochemical Corporation, Cleveland, OH). The resulting 247 bp of sequence between β2 codon 19 and β3 codon 20 were removed by site-directed mutagenesis as described by Zöller and Smith (1982) using a synthetic 66-base DNA oligomer representing β2 codons 13–19 and β3 codons 20–34. After mutagenesis the fusion sequence was verified by dideoxy sequencing (Sanger et al., 1977). Using pUChsneo′ as a vector, a second construct was made containing the β2 promoter sequence from the Sal I site used in constructing β2-Neo to a BamH I site 974 bp downstream of β2 codon 19, joined to a β3 fragment starting at a Bgl II site 341 bp upstream of β3 codon 20 and extending 3′ to an Eco RI site 1,530 bp 3′ of the β3 stop codon. The sequences of this construct from the Apa I site of the β2 gene to the 5′ Pst I site of the β3 gene were then replaced with the fusion sequence where β2 codon 19 is adjacent to β3 codon 20. The hybrid gene is designated *β2β3** and its protein product as *β3**. The hybrid gene inserted in the pUChsneo′ vector is designated *β2β3**-Neo. The Sal I and Kpn I sites of *β2β3**-Neo were used to reinsert the *β2β3** fusion gene into the Xho I and Kpn I sites in the polylinker of the *Drosophila* transformation vector pW8 (Klemenz et al., 1987), producing *β2β3**-pW8. Multiple transformed lines were obtained with both *β2β3**-Neo and *β2β3**-pW8. The *β3** produced by the hybrid gene is identical to that of wild-type β3 except in two positions in the first 19 amino acids, where the sequences of β2 and β3 have two conservative amino acid substitutions, ile/leu at residue 7, and gly/ala at position 18 (Rudolph et al., 1987). *β3** is identical in electrophoretic properties to wild-type β3-tubulin (Raff et al., 1982; Kimble et al., 1989) and binds to an antibody generated to the unique β3 carboxyl terminus (Kimble et al., 1989).

β2β3. As described below, the chimeric protein *β3** had an unexpected dominant phenotype in microtubule function. To be sure that this phenotype did not result from the two amino acid differences between *β3** and wild-type β3, a second construct was made that allowed wild-type β3 protein to be expressed in the male germ line. This gene is designated *β2β3*, and its protein product is wild-type β3. As described below, the phenotype of β3 protein expressed ectopically in the male germ is identical to that of *β3**. Thus there is no apparent functional result of the conserved amino acid changes present in *β3**, relative to wild-type β3.

The *β2β3* construct was derived from *β2β3** by using a β3 cDNA clone described in Rudolph et al. (1987). The 5′ Eco RI site of the β3 clone was converted to an Apa I site using the linker tailing protocol (Bethesda Research Laboratories, Gaithersburg, MD). The 160-bp Apa I–Acc I fragment of *β2β3**-pW8 (see Fig. 2) was then replaced with a 210-bp β3 cDNA Apa I–Acc I fragment where the Acc I site in both fragments represents the same position in the β3 gene. The final fusion gene contains the testis-specific β2 promoter and transcription start site as well as all but 20 bp of the β2 mRNA leader sequence, which have been replaced by 65 bp of β3 mRNA leader immediately 5′ to the β3 translation start site. The coding sequence is entirely β3 and the gene is designated *β2β3* (see Fig. 2). The 4.5-kb first

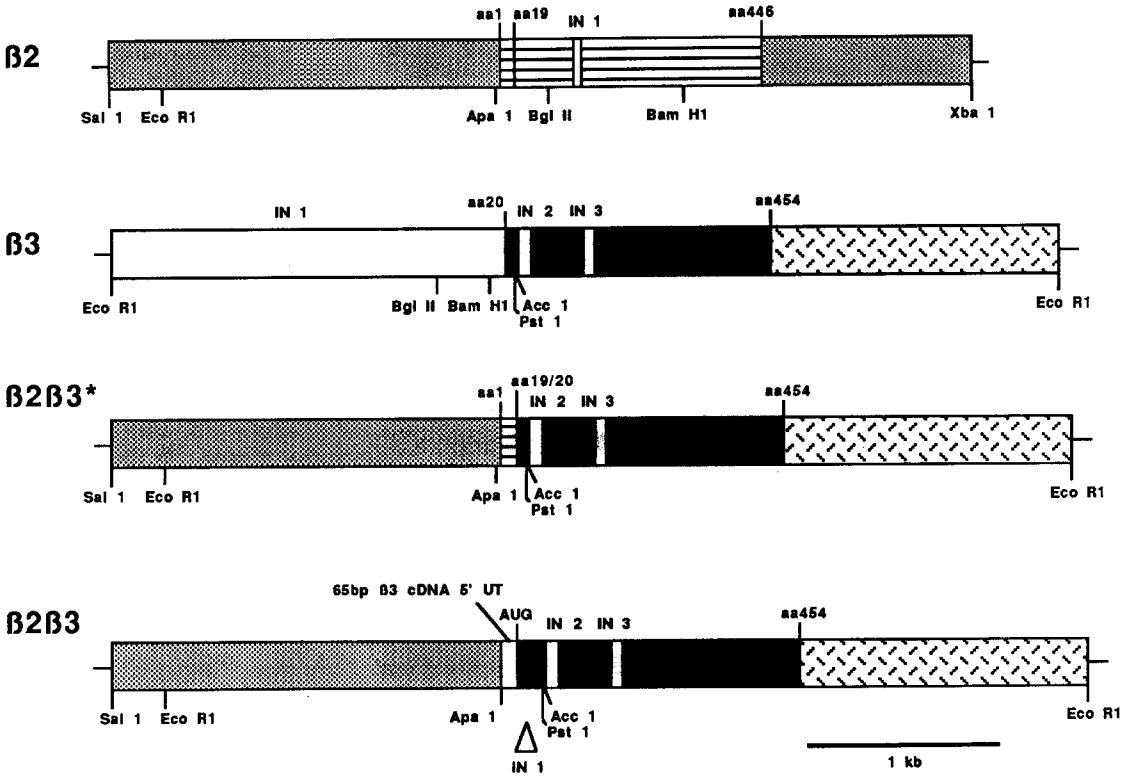

Figure 2. Construction of β2-Neo and the hybrid β2β3 genes. Sequences used to transform *Drosophila* stocks with the wild-type β2-tubulin gene, the β2β3* gene or the β2β3 gene. The diagrams are divided into intron, exon and nontranslated flanking sequences. Intron (*IN*) and codon (*aa*) positions are marked above each figure. The restriction sites used in constructing β2-Neo, β2β3* and β2β3 are marked below each diagram. (*β2*) The 4.55-kb genomic β2-tubulin sequence from Sal I to Xba I cloned into pUChsneo′ (see Materials and Methods) and designated β2-Neo. The shaded boxes represent nontranscribed and transcribed sequences flanking the β2 coding sequence, shown in striped boxes. The single β2 intron is represented by an unfilled box. The β2-tubulin sequence from Sal I to codon 19 was used in the β2β3 fusion gene. (*β3*) A 5.03-kb Eco RI fragment of genomic β3-tubulin originally subcloned as DTB3 (Natzle and McCarthy, 1984). The large unfilled box represents 1,720 bp of the first intron. The remaining 2,780 bp of the first intron and the first exon of the β3 gene are not contained in this clone. The solid boxes represent β3 coding sequence, with the small second and third introns shown by unfilled boxes. The patterned box represents 3′ flanking nontranslated and nontranscribed sequences. The β3 sequence from codon 20 to the 3′ Eco RI site was used in the β2β3 fusion gene. (*β2β3**) The 5.14-kb β2β3* fusion gene. The fusion gene was cloned into pUChsneo′ (Steller and Pirrotta, 1985; see Materials and Methods) and pW8 (Klemenz et al., 1987). The protein product of these genes is designated β3*. (*β2β3*) The 5.19-kb β2β3 fusion gene. The gene was derived from β2β3* (see Materials and Methods). The protein product is wild-type β3.

intron of the β3 gene is absent in β2β3, as it is in β2β3*, while the sequences 3′ to coding are exactly the same in β2β3 and β2β3*.

P Element Transformation

β2-Neo and β2β3*-Neo vectors (Fig. 2) were injected into Oregon R embryos. β2β3*-pW8 and β2β3-pW8 were injected into homozygous *white*$^-$ embryos. Injection of *Drosophila* embryos was done essentially as described by Spradling and Rubin (1982). A solution of 500 μg/ml vector DNA was used for each injection. The defective P element pHSπ coinjected at 250 μg/ml (Steller and Pirrotta, 1986) or the stable third chromosome P element Δ2-3 described by Robertson et al. (1988) was the source of transposase. Microinjection was performed at 18–22°C; injected animals were transferred to standard food at 25°C. Adult pW8 injectees were mated to *white*$^-$ flies on standard food at 25°C. Adult Neo injectees were held 2 d on standard food and then mated with Oregon R flies on 0.9 g of instant *Drosophila* food (formula 4-24; Carolina Biological Supply Co., Burlington, NC), which had been rehydrated with 3 ml of an aqueous solution of 1.2 mg/ml G418 (Gibco Laboratories, Grand Island, NY) and tamped into a solid mass before use. Yeast was added to each vial just before use. Untransformed flies showed no survival at 0.8 mg/ml G418.

Recovery of pW8 transformants was as previously described by Klemenz et al., 1987. Transformants raised at 25°C could be identified by eye coloration resulting from constitutive expression from the hsp 70 promoter controlling the *white*$^+$ gene. The ability to recover transformants at 25°C was critical since temperatures above 29°C adversely affect male fertility. The degree of eye color varied among different transformed lines; in general pW8-transformed lines with more eye pigmentation were found to be expressing more β3 or β3* protein. Homozygous transformants showed an increase in eye pigmentation compared to heterozygotes. In addition, pW8 transformants show decreased or no pigmentation in the ocelli and testes, a fact that was useful as a second marker.

Additional sites of insertion of the hybrid genes in the genome were produced by introducing p-element transposase with the stable third chromosome p-element Δ2-3 as described by Robertson et al. (1988). A number of different inserts for each hybrid gene were used in the studies reported here, and are identified by the chromosome in which they are inserted (e.g., β2β3*(IIa), β2β3(IIIb), etc.).

Stocks were constructed which carried the hybrid genes in a $\beta2^{null}$ background by appropriate crosses for X and second chromosome inserts or by recombination for third chromosome inserts. A stock of the third chromosome β2-Neo insert in a $\beta2^{null}$ background was made by first generating by recombination a chromosome containing both the β2-Neo insert and at the endogenous β2-tubulin locus, the mutation $\beta2t^8$, which expresses an electrophoretically distinguishable β2 variant (Rudolph et al., 1987; Fuller et al., 1987). In the resultant stock (β2-Neo, $\beta2t^8$) we could therefore distinguish electrophoretically the protein expressed by β2-Neo from the protein expressed at the endogenous β2 locus. This stock was then used to generate by recombination chromosomes containing both the third chromosome β2-Neo insert and $\beta2^{null}$. The $\beta2^{null}$ mutation used is an ethyl methanesulfonate–induced mutation in the β2 locus isolated by K. A. Matthews (Department of Biology, Indiana University, Bloomington, IN) which results in failure to produce β2-tubulin protein (described in Fuller et al., 1988).

Table I. A Wild-type β2-Tubulin Gene Inserted into the Genome by Transformation Supports Normal Male Fertility

Genotype	Number of $\beta2^+$ genes	Fertility
$\beta2^{null}/\beta2^{null}$	0	−
$\beta2^{null}/\beta2^+$	1	+
β2-Neo, $\beta2^{null}/\beta2^{null}$	1	+
$\beta2^+/\beta2^+$ (wild type)	2	++
β2-Neo, $\beta2^{null}$/β2-Neo, $\beta2^{null}$	2	++
β2-Neo, $\beta2^{null}/\beta2^+$	2	++
β2-Neo, $\beta2^+/\beta2^+$	3	++
β2-Neo, $\beta2^+$/β2-Neo, $\beta2^+$	4	++
$B2t^8/B2t^8$	0	−
$B2t^8/\beta2^{null}$	0	−
$\beta2^+/B2t^8$	1	+
β2-Neo, $B2t^8/\beta2^{null}$	1	+
β2-Neo, $\beta2^{null}/B2t^8$	1	+
β2-Neo, $B2t^8$/β2-Neo, $B2t^8$	2	+
β2-Neo, $\beta2^+/B2t^8$	2	++
β2-Neo, $B2t^8/\beta2^+$	2	++

The genotypes of the males tested are given with respect to β2-tubulin genes, designated as follows: $\beta2^+$, a wild type allele at the endogenous β2 locus; β2-Neo, a wild-type β2 gene inserted in the genome by transformation; $B2t^8$, a recessive male sterile mutation at the β2 locus; $\beta2^{null}$, a null mutation at the β2 locus. Fertility was assessed by scoring for production of motile sperm and by mating tests: sterile males (−); fertile males (+, ++ indicate relative fecundity).

Drosophila Stocks

Stocks were maintained at 25°C to minimize temperature effects on male fertility. *Drosophila* visible mutations and balancers are described in Lindsley and Grell (1968).

Determination of Male Fertility

Male fertility was assessed both by mating tests and by scoring for the presence of motile sperm in the seminal vesicles. In *Drosophila* males, only mature motile sperm enter the seminal vesicles; nonmotile or otherwise defective sperm are retained within the testis and degraded (Lindsley and Tokuyasu, 1980). Production of motile sperm is thus the best indication of the successful completion of spermatogenesis. We scored for sperm production by collecting young adult males and holding them without females at 25°C for 4 d or more and then dissecting them to determine the presence or absence of sperm in the seminal vesicles; males of genotypes that produced mature motile sperm were fertile in mating tests. It should be noted that not all fertile males produce the same amount of sperm. For example, as we have previously described, males with only one functional wild-type β2-tubulin allele (represented in Table I by genotype $\beta2^+/\beta2^{null}$) produce fewer mature sperm than wild-type males and thus produce fewer progeny per male (Kemphues et al., 1982, 1983; Fuller et al., 1988). Males with the β2-Neo insert produced similar amounts of sperm and numbers of progeny as males with the same number of functional wild-type β2 genes.

Two-dimensional Gel Electrophoresis

Testes from seven or fewer young males were dissected in DM buffer (insect Ringer's solution [Ephrussi and Beadle, 1936] plus 1% polyethylene glycol), transferred to ZD medium (Wyss and Bachmann, 1976) containing 9 μM [^{35}S]methionine at 7.5 mCi/ml and incubated at room temperature for 1 h, then transferred to 30 μl Laemmli sample buffer (Laemmli, 1970), and boiled for 3 min. Two-dimensional gel electrophoresis and transfer to nitrocellulose was as described in Kimble et al. (1989).

Quantitation of Beta Tubulin Levels

Levels of [^{35}S]methionine-labeled beta tubulins in testes were quantified by densitometric scanning of autoradiographs of two-dimensional gels. Exposures were taken with preflashed Kodak RP film and scanned using a Quick Scan RD densitometer (Helena Laboratories, Beaumont, TX). Exposures were kept within a signal range which gave a linear response in timed control experiments. The signals from the hybrid gene products were compared with that of β2, if present, and to actin.

Pulse-Chase Experiments

Testes from 14 males less than 5 d posteclosion were dissected in DM buffer, transferred to ZD media containing 9 μM [^{35}S]methionine at 7.5 mCi/ml and incubated for 1 h. Testes were then washed twice with 25 vol of ZD media containing 1 mM cold methionine and transferred to 500 μl of the same media for the chase. For the zero time point, 14 testes were immediately transferred to 25 μl Laemmli sample buffer (Laemmli, 1970). At each subsequent time point during the chase incubation four testes were transferred to 25 μl Laemmli sample buffer and heated to 95°C for 3 minutes before storage at −70°C. Dissection, labeling, and chase were performed at room temperature (24–25°C). Sample preparation and two-dimensional gel electrophoresis was done as described in Kimble et al. (1989).

Antibody Staining

Immunostaining of whole testes and Western blots of two-dimensional gels was done as described in Kimble et al. (1989). Antibodies used were a β3-specific antibody previously described in Kimble et al. (1989) and a com-

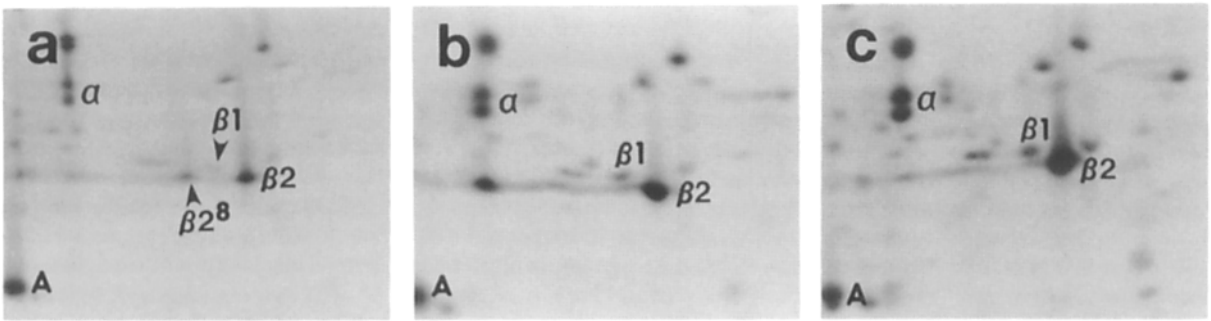

Figure 3. Expression of the β2-Neo gene yields wild-type β2-tubulin. Dissected testes were labeled with [^{35}S]methionine and the proteins separated by IEF in the first dimension and by SDS-PAGE in the second dimension. The basic end of the first dimension is to the left. *A*, actin; α, α-tubulin. (*a*) Testes from fertile males homozygous for β2-Neo, a 4.55-kb β2 genomic sequence inserted at position 61C, and homozygous for $\beta2t^8$, an electrophoretic variant of β2-tubulin. (*b*) Testes from fertile males homozygous for β2-Neo and homozygous for the β2 null mutation ($\beta2^{null}$). All of the β2-tubulin produced in this genotype is due to expression of the β2-Neo gene. (*c*) Testes from fertile males homozygous for β2-Neo and homozygous for the endogenous β2 gene, showing comigration of β2-tubulin expressed by the β2-Neo gene and by the wild-type allele.

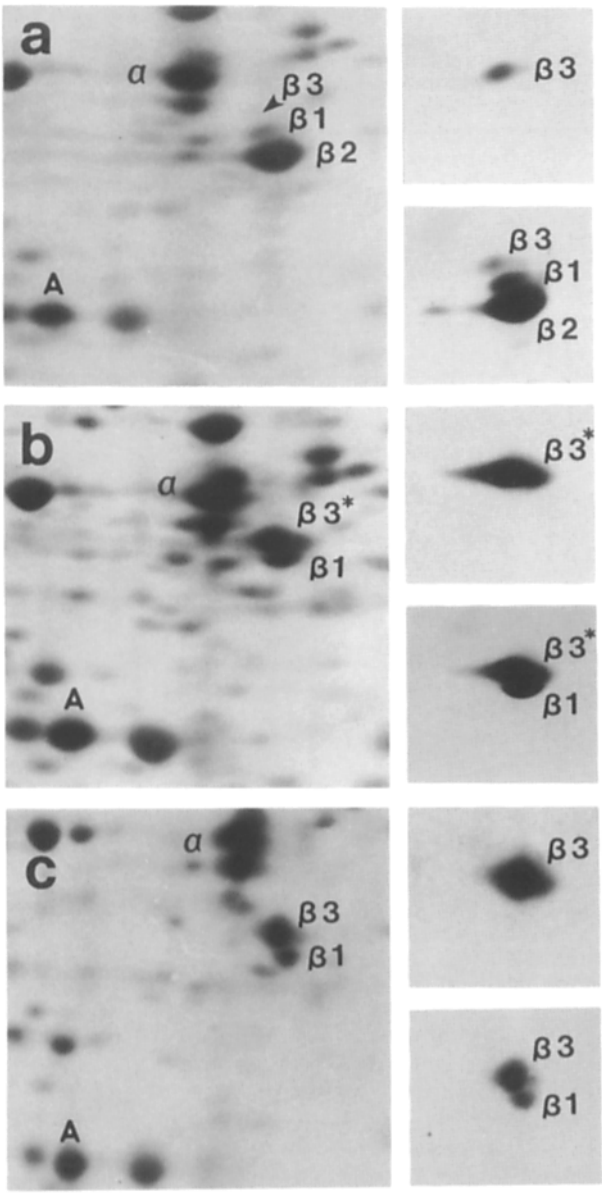

Figure 4. Expression of the hybrid β2β3 genes and the endogenous β3 gene in testes. Labeled proteins from testes were separated by IEF and PAGE, transferred to nitrocellulose and probed sequentially with a β3-specific antibody (anti-β3) and with a commercial β-tubulin antibody, which reacts with all β-tubulins present in testes. For each panel the left side shows the autoradiogram, top inset shows β3 tubulin, and bottom inset shows total β-tubulins. Antibody staining in *a* and *b* was visualized with horseradish peroxidase. Antibody staining in *c* was visualized with alkaline phosphatase. *A*, actin, α, α-tubulin. (*a*) Wild-type testes. (*Left*) Autoradiogram of labeled proteins. Synthesis of endogenous β3-tubulin is barely detectable. (*Top right*) Same filter stained with anti-β3 to detect accumulated rather than newly synthesized proteins. The staining represents β3-tubulin present in the somatic cyst cells (see Figs. 1 and 6). (*Lower right*) Same filter restained with commercial anti-β, which detects β1-, β2-, and β3-tubulins. (*b*) Testes from sterile males homozygous for β2β3*(IIIb) and homozygous for $\beta2^{null}$ This insert expresses β3*-tubulin at ~15% of the level of β2-tubulin expression in wild type. (*Left*) Autoradiogram of labeled proteins. (*Top right*) Same filter stained with anti-β3. (*Lower right*) Same filter restained with commercial anti-β. β2-Tubulin is not detectable in the $\beta2^{null}$. The amount of β1-tubulin staining is approximately equal to that in wild-type males while the amount of β3 staining is considerably greater, representing the β3*-tubulin synthesized from the hybrid gene. Note that β3* and β3-tubulin comigrate. (*c*) Testes from sterile males homozygous for $\beta2^{null}$ and homozygous for β2β3(IIIc). This insert expresses a level of β3-tubulin similar to the level of β3* shown in *b*. (*Left*) Autoradiogram of labeled proteins. (*Top right*) Same filter stained with anti-β3. (*Lower right*) Duplicate filter stained with commercial anti-β.

mercial anti-beta tubulin (Amersham Corp., Arlington Heights, IL), which exhibits approximately equal affinity to β1, β2, and β3. Staining with horseradish peroxidase-conjugated secondary antibody (HyClone Laboratories, Logan, UT) was done following the Vectastain ABC protocol (Vector Laboratories, Inc., Burlingame, CA). Staining with alkaline phosphatase-conjugated secondary antibody (Zymed Laboratories, Inc., San Francisco, CA) was done using the protocol of Harlow and Lane (1988).

Microscopy Methods

Determination of spermatogenic phenotype at the light microscope level was done by examination of live or orcein-stained testis squashes under phase-contrast optics as previously described (Kemphues et al., 1982; Fuller et al., 1987, 1988). Testes were prepared for electron microscopy as described in Fuller et al. (1987).

Results

A Wild-Type β2-Tubulin Gene Inserted into the Genome by Transformation Supports Correct Expression of β2-Tubulin and Normal Male Fertility

Fig. 2 diagrams the wild-type β2 gene sequences contained in the β2-Neo construct. Data summarized in Fig. 3 and Table I show that when inserted into the *Drosophila* genome by transformation, β2-Neo supports the expression of β2-tubulin at wild-type levels in the testis and that the β2-tubulin expressed from the inserted gene is stable and supports all of the normal functions of β2. Fig. 3 shows autoradiograms of two-dimensional gels of [^{35}S]methionine-labeled testis proteins from males that express β2 from the transformed copy of the gene. Fig. 3 *a* shows tubulins in testes of fertile males homozygous for both β2-Neo and for the recessive male sterile β2-tubulin mutation $\beta2t^{8}$ at the endogenous β2 locus. The β2 variant encoded by the $\beta2t^{8}$ allele is electrophoretically distinct from β2 but is synthesized at a level equivalent to that of wild-type β2 (Fuller et al., 1987, 1988; Rudolph et al., 1987). Fig. 3 *b* shows tubulins in testes of fertile males homozygous for both β2-Neo and $\beta2^{null}$, an EMS-induced null mutation in the endogenous β2 locus (see Materials and Methods). Fig. 3 *c* shows tubulin in testes from fertile males homozygous for both β2-Neo and the wild-type β2 tubulin. The tubulin expressed by the β2-Neo gene and the wild-type allele comigrate. Two-dimensional gel analysis of total proteins from embryos, ovaries, and brains from flies homozygous for β2-Neo did not reveal synthesis of β2-tubulin, indicating that the inserted gene in transformants is expressed in the correct testis-specific manner. The sequences contained in the β2-Neo construct thus include all elements necessary for correct wild-type expression of β2-tubulin.

β2-Neo was tested in a number of genetic combinations with wild type β2 alleles, the mutation $\beta2t^{8}$, and the β2 null mutation. As shown in Table I, we found that the β2-Neo gene supports normal male fertility and can entirely substitute for a wild-type β2 allele. The overall phenotype of sper-

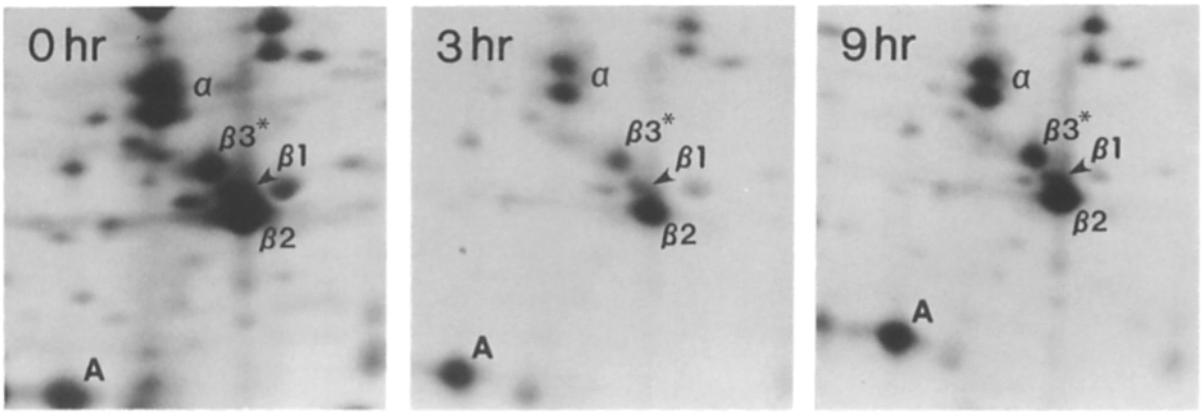

Figure 5. The protein product of the hybrid β2β3* gene is stable. Dissected testes were pulse-labeled with [^{35}S]methionine for one hour and then incubated in culture medium containing excess unlabeled methionine for 0, 3, or 9 h. Labeled proteins were then separated by two-dimensional gel electrophoresis and transferred to nitrocellulose filters. Testes were from fertile males homozygous for β2β3*(IIa) and homozygous for $\beta 2^{+}$. The ratio of β3*-tubulin to β2-tubulin remains constant throughout the 9-h chase. Additional experiments using testes from sterile males homozygous for β2β3*(IIa) and homozygous for the $\beta 2^{null}$ mutation demonstrate that β3*-tubulin is also stable over 9 h in the absence of β2-tubulin (not shown). This β2β3* insertion expresses a similar level of β3*-tubulin expression as that shown in Fig. 4 *b*. *A*, actin; α, α-tubulin.

matogenesis as assessed by light and electron microscopy was the same in males with β2-tubulin produced from the inserted β2-Neo gene as in males with wild type β2 gene(s). Over-production of β2-tubulin in males homozygous for both β2-Neo and the endogenous wild-type β2 gene did not adversely affect sperm morphology or male fertility.

Hybrid Tubulin Genes Inserted into the Genome by Transformation Support Production of Stable Protein in the Male Germ Line

The hybrid genes β2β3 and β2β3* diagrammed in Fig. 2 were constructed as described in Materials and Methods. In the hybrid genes, 2.3 kb of 5′ sequences from a β2 genomic clone containing the putative β2 promoter region were juxtaposed to genomic β3 sequences. Both hybrid genes contain the two small introns from the β3 gene (intron 2 of 62 bp and intron 3 of 56 bp, the latter corresponding in position to the sole 59 bp intron of the β2 gene). The 4.5-kb first intron of the β3 gene is eliminated (Rudolph et al., 1987), and none of the fusion gene constructs contain the β3 promoter elements found in the first intron by Gasch et al. (1989). The mRNA product for both of the hybrid genes is a chimeric RNA consisting of 5′ untranslated sequences from the β2 gene and 3′ untranslated sequences from the β3 gene. In the β2β3 mRNA, 20 bp of the β2 untranslated sequence just 5′ to the start of translation have been replaced with 65 bp of β3 mRNA leader sequence. The protein product (β3) of the β2β3 hybrid gene is identical to wild-type β3, while the protein product (β3*) of the β2β3* hybrid gene is a chimeric protein consisting of the first 19 amino acids for β2 followed by the remainder of the β3 protein. The chimeric β3* protein thus differs from wild-type β3 in two conservative amino acids (Rudolph et al., 1987): At position 7, β2 and β3* have isoleucine and wild-type β3 has leucine, and at position 18, β2 and β3* have glycine and wild-type β3 has alanine. For each hybrid gene, we have examined multiple lines carrying the hybrid gene inserted at different sites in the genome. We have found that the expression properties and the phenotype in microtubule function of the protein products of β2β3 and β2β3* are identical. These findings are documented below in Figs. 4–12. The identical results obtained with hybrid genes that express either wild-type β3 or the chimeric β3* protein are not surprising. The changes in the two amino acids represent conservative substitutions that would be expected to serve equivalent roles within the proteins. These substitutions are seen in other beta tubulins: the major *Drosophila* isoform, β1, has isoleucine at position 7, as in β2, and alanine at position 18, as in β3 (Michels et al., 1987). In vertebrates, the predominant testes isoform (mouse β3, chicken β3) and a brain specific isoform (mouse β4, human β5) both have leucine at position 7 and alanine at position 18, as in *Drosophila* β3 (Sullivan and Cleveland, 1986; Wang et al., 1986). It is clear that the alternate amino acids at these positions are compatible with multiple tubulin functions.

All of the hybrid gene inserts we have examined support expression of stable protein in the male germ line. Fig. 4 shows two-dimensional gels of total testis proteins from wild-type males (*a*), males that carry the β2β3*(IIa) hybrid gene insert (*b*), and males that carry the β2β3(IIIb) hybrid gene insert (*c*). As discussed above, in pupal and adult testes β3-tubulin is normally expressed at low levels in the somatic cells that enclose each cyst of syncytially developing germ cells (Kimble et al., 1989, and see Fig. 1). The endogenous β3 is detectable in the testes of wild-type males by antibody staining of blots of gels of testis proteins, but either not at all or only at very low levels on autoradiograms showing [^{35}S]methionine incorporation into testis proteins (Fig. 4 *a*). The amount of the divergent isotype expressed from the hybrid genes is thus the "excess" β3 seen in the gels in the lower two panels.

For all of the hybrid gene inserts we have thus far recovered, the level of protein synthesis in the testis is less than the normal level of β2-tubulin. The majority of the inserts yielded β3* or β3 at a level comparable to that of the inserts illustrated in Fig. 4, *b* and *c*. A few of the hybrid gene inserts yield higher levels, while some of the inserts produce lower levels. The reduced amounts of protein produced from the hybrid genes relative to normal β2 levels are not due to

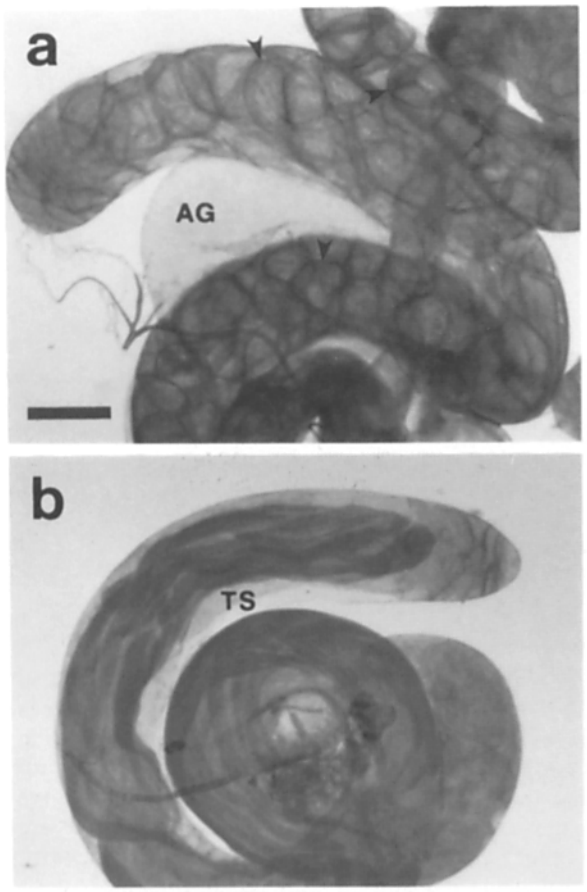

Figure 6. Antibody localization of endogenous β3-tubulin and β3*-tubulin in testes. (*a*) Testes from a sterile male homozygous for $\beta 2^{null}$ stained with an antibody specific for β3-tubulin (anti-β3). The somatic cyst cells (*arrows*) surrounding each spermatid cyst stain with anti-β3, but the germ cells within each cyst do not stain. The accessory gland (*AG*) comprised entirely of somatic tissue also does not stain with anti-β3. (*b*) Testes from a sterile male homozygous for β2β3*(IIa) and homozygous for $\beta 2^{null}$ stained with anti-β3. The spermatids within each cyst show considerable elongation and now stain with anti-β3; however, elongation is not as extensive as in wild-type testes (compare Fig. 7, *a*, *b*, *c*, and *d*). There is no anti-β3 staining of the testes sheath (*TS*). Anti-β3 also does not stain the seminal vesicle, ejaculatory duct or accessory gland (not shown). Bar, 0.1 mm.

instability of the protein. Pulse-chase experiments such as those shown in Fig. 5 demonstrate that β3*-tubulin exhibits comparable stability to β2-tubulin, which, as we previously showed, is extremely stable (Kemphues et al., 1982). Antibody staining of equivalent two-dimensional blots shows that β3 accumulates to the same level as β3* when the rates of synthesis, as seen by [^{35}S]methionine incorporation, are comparable. Since β2, β3, and β3* are stable, autoradiograms such as those shown in Figs. 4 and 5 reliably reflect the relative amounts of β tubulins in the testis protein pool, so long as the exposures are in the linear range of the x-ray film.

The divergent isotype expressed by the hybrid genes is localized in the male germ line, the normal site of β2-tubulin expression. This is shown in Fig. 6 by antibody localization in whole testes using the β3-specific antibody. Fig. 6 *a* shows localization of endogenous β3 in the testis of a male homozygous for the $\beta 2^{null}$ mutation. In the absence of β2, spermatid differentiation and elongation fail to occur, and the cysts in the testis therefore remain round (Kemphues et al., 1982). β3 is present only in the somatic cells that enclose each cyst of germ cells, as in wild-type males (see also Kimble et al., 1989). Fig. 6 *b* shows localization of β3* in the testis of a male that is homozygous both for the $\beta 2^{null}$ allele at the β2 locus and for the β2β3*(IIa) insert. This is the same insert illustrated in Fig. 5, and supports β3* synthesis at a level comparable to the insert illustrated in Fig. 4 *b*. A similar degree of elongation is seen in testes of males homozygous for β2β3(IIIb) and for $\beta 2^{null}$ (see Fig. 7 *c*). Two things are clear: the divergent isotype is present in the developing germ cells, and even in the absence of β2 supports a considerable degree of spermatid elongation. The latter point is discussed in further detail below.

The 5′ sequences from the β2 gene thus appear to be sufficient to confer correct tissue specificity of expression of the hybrid gene, as confirmed by Northern blot analysis and immunolocalization using the β3-specific antibody (data not shown). This observation is consistent with the recent identification by Michiels et al. (1989) of a 14-bp sequence in the β2 promoter responsible for testis specificity. It is not clear at present whether the relatively low level of expression from the hybrid gene inserts we recovered reflects an intrinsic property of the hybrid genes or their transcripts, or is due to position effect of the sites of chromosomal insertion. Experiments are in progress to address this question.

In the Absence of β2-Tubulin Expression, the Divergent Isotype Supports Assembly of One Class of Functional Cytoplasmic Microtubules in Differentiating Spermatids

As illustrated in Fig. 6 *b*, expression of the divergent isotype as the sole beta tubulin in the postmitotic male germ line supports considerable spermatid elongation. The extent of spermatid elongation depends on the level of expression, as illustrated by the late stage spermatids in phase micrographs of squash preparations from live testes shown in Fig. 7. Fig. 7 *a* shows late stage spermatids in a sterile male homozygous for $\beta 2^{null}$ (i.e., neither β2 nor the divergent isotype is expressed). In such males, spermatids undergo an abortive differentiation, but fail to elongate so that the cysts containing the spermatids remain round (see also Fig. 6 *a*). Fig. 7 *b* shows elongating spermatids in a fertile male that has one β2β3 gene and one functional wild-type β2 gene. The spermatids in this male appear identical to those in a wild-type male. Fig. 7 *c* shows spermatids in a sterile male that is homozygous for $\beta 2^{null}$ and β2β3(IIIb). Fig. 7 *d* shows spermatids in a sterile male which is also homozygous for $\beta 2^{null}$ but has one copy of β2β3*(IIa). Fig. 7 *e* shows spermatids in a sterile male homozygous for both $\beta 2^{null}$ and β2β3*(IIa). Comparison of Fig. 7, *d* and *e* shows that spermatid elongation due to the divergent isotype is considerably improved when the gene dose is doubled. Comparison of Fig. 7, *c* and *e* shows that at similar levels of expression, both β3 and β3* support the same degree of elongation in the absence of endogenous β2.

In fertile males, three morphogenetic events occur during differentiation of the haploid spermatids: the axoneme is as-

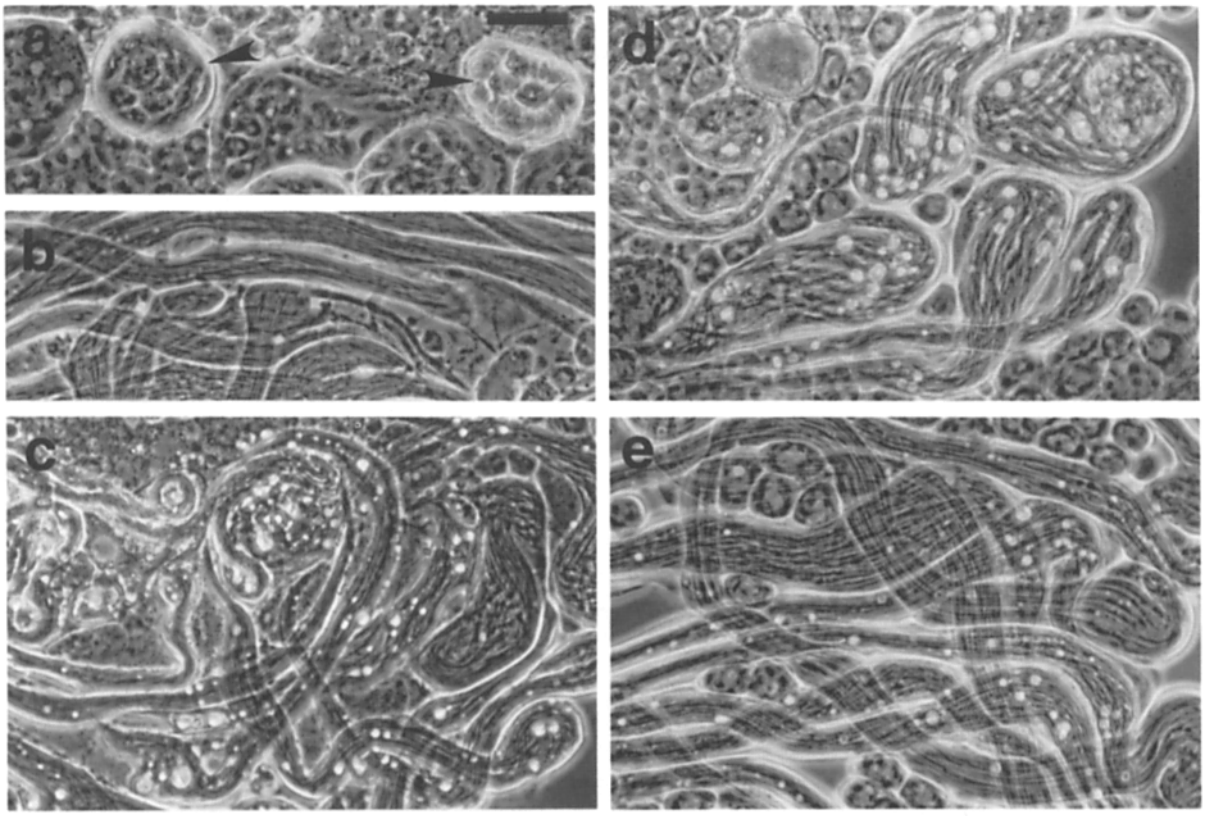

Figure 7. Elongating spermatids in males that express the divergent isotype in the germ line. Late-stage spermatids are shown in light micrographs of live testis squash preparations viewed by phase contrast optics. (*a*) Cysts of late stage spermatids (*arrows*) in a sterile male homozygous for the $\beta 2^{null}$ mutation (with no $\beta 2\beta 3$ insert). In the absence of $\beta 2$, the spermatids do not elongate and the cysts remain round (see also Fig. 6 *a*). (*b*) Elongated late-stage spermatids in a fertile male which has one $\beta 2\beta 3$*(IIa) insert and one $\beta 2^{+}$ gene. Spermatids appear identical to those in a wild-type male. (*c*) Elongated late-stage spermatids in a sterile male that is homozygous for both the $\beta 2\beta 3$(IIIb) insert and the $\beta 2^{null}$ mutation. Elongation is less than seen in $\beta 2$ wild-type (*b*) but considerably improved over $\beta 2^{null}$ (*a*). The level of $\beta 3$ expressed by $\beta 2\beta 3$(IIIb) is comparable to that expressed by $\beta 2\beta 3$*(IIa). The extent of elongation is also comparable (compare *c* and *e*). (*d*) Elongated late-stage spermatids in a sterile male that has one $\beta 2\beta 3$*(IIa) insert and is homozygous for the $\beta 2^{null}$ mutation. A significant degree of spermatid elongation has occurred (compare with *a*). (*e*) Elongated late-stage spermatids in a sterile male that is homozygous both for the $\beta 2\beta 3$*(IIa) insert and for the $\beta 2^{null}$ mutation (same insert as in *d*). Although elongation is not equivalent to wild type, there is considerable improvement over that in males who have only a single $\beta 2\beta 3$ insert (compare with *d*). Bar, 50 μm.

sembled to its final mature length of 1.8–2 mm. Concomitantly, the spherical mitochondrial derivative differentiates and is elongated so that it stretches along the entire length of the axoneme. In addition, the nucleus is shaped to form the needle-like mature sperm head. Males that express the divergent isotype in the absence of $\beta 2$ produce no motile sperm and thus are sterile. However, as shown in Fig. 8, the divergent isotype supports assembly and function of the cytoplasmic microtubules that are normally associated with the membranes of the mitochondrial derivatives during their elongation. The spermatid elongation supported by the divergent isotype when it is expressed in the absence of $\beta 2$ is entirely due to the single process of the differentiation and elongation of the mitochondrial derivatives, which in this experimental situation proceeds in the absence of meiosis and the other subsequent morphogenetic processes, which in wild-type males are also mediated by the testis-specific $\beta 2$ isoform.

Fig. 8 *a* shows early stages of spermatid differentiation in a wild-type male: axoneme morphology is not yet completed; the mitochondrial derivative is composed of two nearly equally sized components, in one of which electron dense paracrystalline material has begun to accumulate; and cytoplasmic microtubules are present in association with the mitochondrial membranes. In later stages (Fig. 8 *b*), axoneme assembly is complete; the major component of the mitochondrial derivative is nearly filled with electron dense paracrystalline material; and the microtubules associated with the mitochondrial derivatives are no longer present. Fig. 8 *c* shows early stage spermatid differentiation in a sterile male with one copy of $\beta 2\beta 3$(IIIb) and homozygous for $\beta 2^{null}$. In testes of such males, axonemes are not present. However, the cytoplasmic microtubule array near the mitochondrial membranes is present and the mitochondrial derivatives elongate, although their morphology is often somewhat abnormal. In males of this genotype, the amount of $\beta 3$ in the testis protein pool is about half that illustrated in Fig. 4 *c*, i.e., ~8% the amount of $\beta 2$-tubulin in a wild-type male. In testes of males that have higher levels of $\beta 3$ or $\beta 3$*, mitochondrial differentiation and elongation is improved. This is illustrated in Fig. 8 *d*, which shows a cross section through early to middle stage spermatids in a sterile male with three $\beta 2\beta 3$* inserts (genotype $\beta 2\beta 3$*(IIa)/+; $\beta 2\beta 3$*(IIIb), $\beta 2^{null}$/ $\beta 2\beta 3$*(IIIc), $\beta 2^{null}$). This genotype yields $\beta 3$* at ~30% the

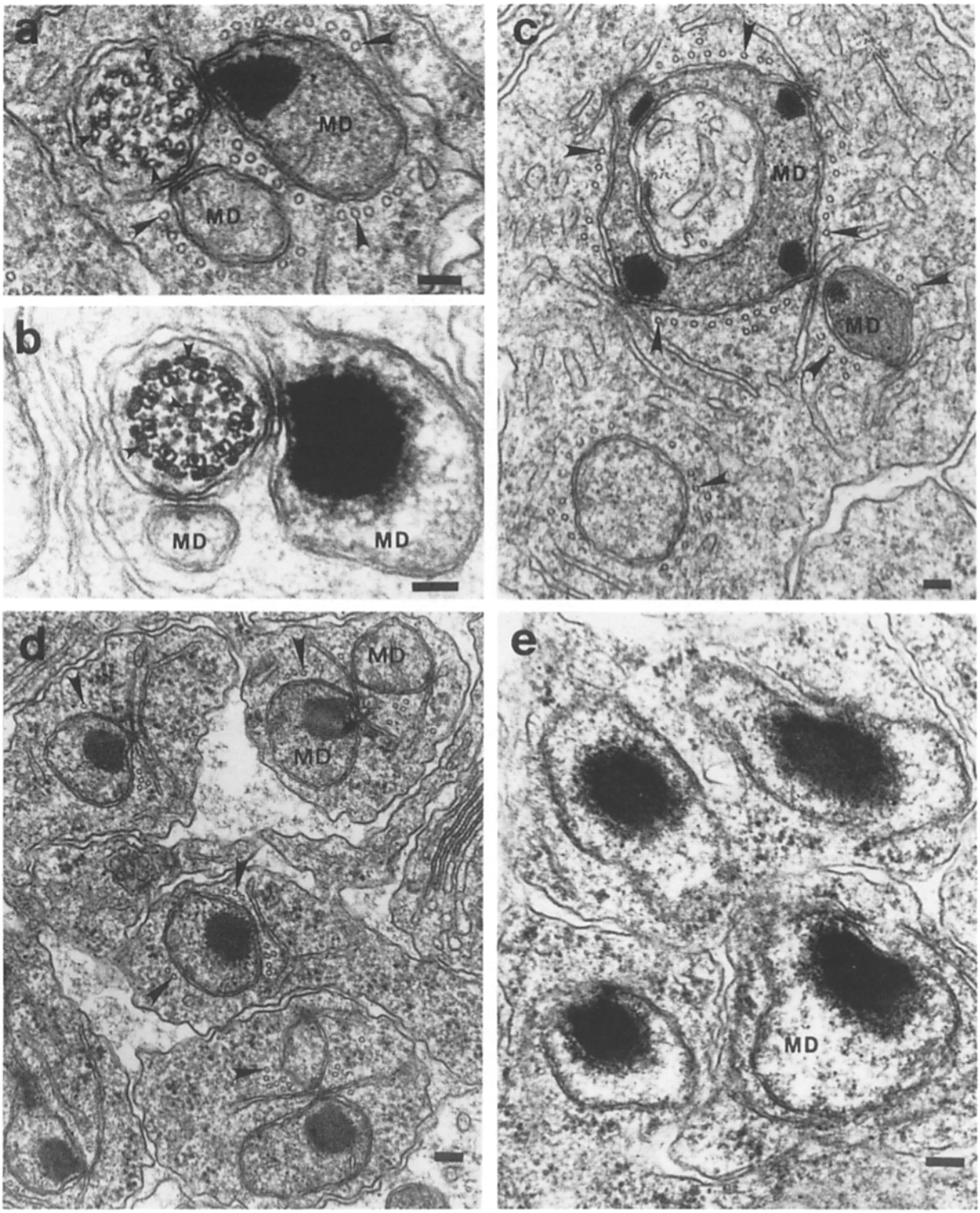

Figure 8. Microtubules in elongating spermatids in males that express the divergent isotype in the absence of β2-tubulin. Electron micrographs of cross sections through developing spermatids are shown. (*a*) Cross section through the testis of a wild-type male showing an elongating spermatid in an intermediate stage of development. The 9 + 2 tubules of the axoneme are present, and the accessory tubules (*small arrows*) are in the process of assembly from the B tubule of each doublet. The elongating mitochondrial derivative (*MD*) is composed of two elements; electron-dense paracrystalline material has begun to accumulate in the major element. Numerous cytoplasmic tubules (*large arrows*) are associated with each element of the mitochondrial derivatives. (*b*) Cross section through the testis of a wild-type male showing a nearly mature spermatid. The axoneme shows the completed morphology of the mature sperm. The lumen of the central pair microtubules and of each accessory tubule is filled with electron dense material, giving the appearance of a "dot" in the center of each of these tubules (*arrows*). Accumulation of the electron dense paracrystalline material in the major element of the mitochondrial derivative (*MD*) is now completed. At this stage, cytoplasmic microtubules are no longer present. (*c*) Cross section through the testis of a sterile

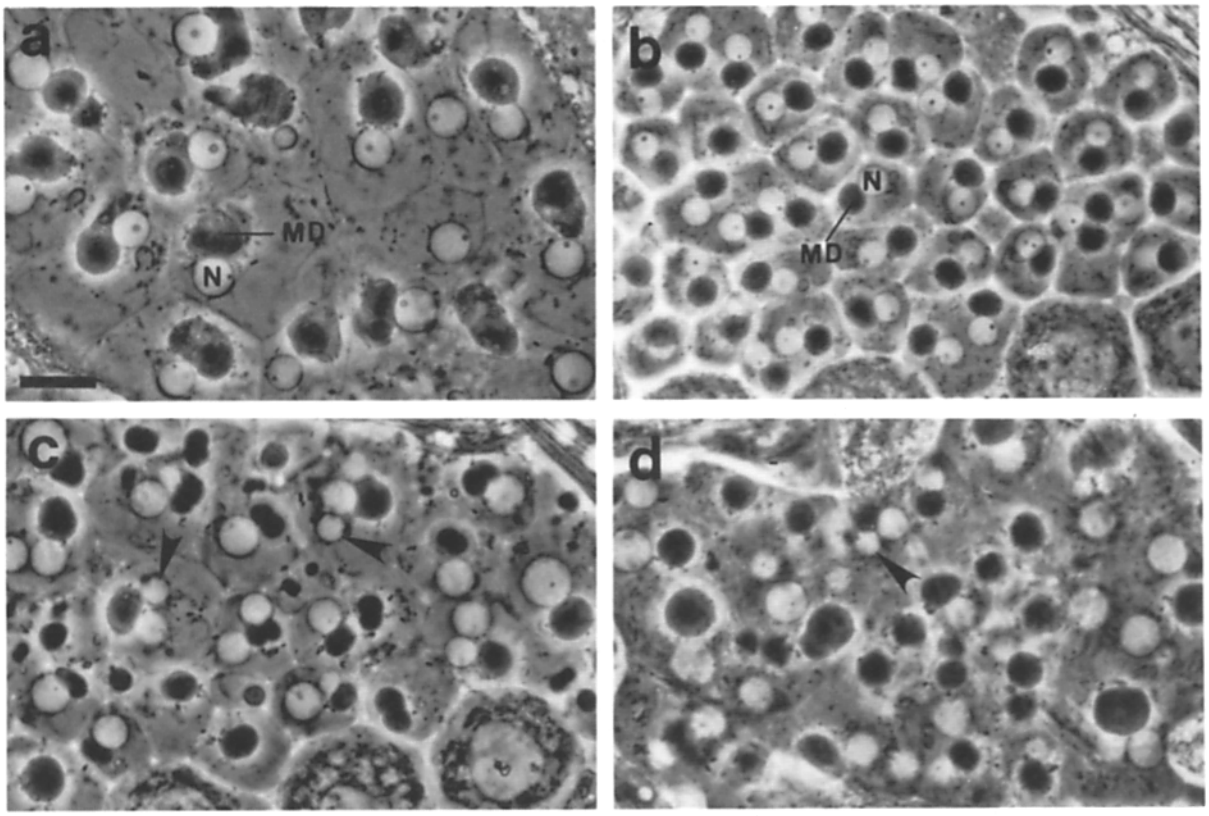

Figure 9. Early spermatids in males that express the divergent isotype in the germ line. Early stage spermatids are shown in light micrographs of live testis squash preparations viewed by phase-contrast optics. (*a*) A complete cyst of early spermatids in a sterile male that has one $\beta2\beta3$*(IIa) insert and is homozygous for the $\beta2^{null}$ mutation. This cyst contained 16 large nuclei (*N*), of which 15 are shown here. Both the number and size of the nuclei indicates that they are tetraploid, reflecting the failure of meiotic spindle function. (Spermatid nuclear size in *Drosophila* is proportional to chromosome content [Hardy, 1975]; the letter *N* in *a* and *b* can be used for a direct comparison of size.) The mitochondria have coalesced but the resultant mitochondrial derivatives (*MD*) are aberrant in both morphology and their association with the nuclei. These spermatids are identical in appearance to spermatids in males homozygous for $\beta2^{null}$ but with no $\beta2\beta3$ insert. (*b*) Portion of a cyst of 64 early spermatids in a fertile male that has one $\beta2\beta3$*(IIa) insert and one $\beta2^{+}$ gene. These spermatids appear identical to those in a wild-type male. Each spermatid has a white haploid spherical nucleus (*N*) and a spherical dark mitochondrial derivative (*MD*) of approximately equal size. (*c*) Portion of a cyst of early spermatids in a sterile male which is homozygous both for the $\beta2\beta3$*(IIa) insert and for the $\beta2^{null}$ mutation. Many of the nuclei are tetraploid, as in the male in *a*, but a few spermatids show evidence of abortive meiosis (*arrows*), as indicated by the nuclei of varying sizes. (*d*) Portion of a cyst of early spermatids in a sterile male which is homozygous both for the $\beta2\beta3$(IIIb) insert and for the $\beta2^{null}$ mutation. The phenotype of early spermatids in *c* and *d* is identical. Bar, 20 μm.

level of $\beta2$ in a wild-type male, or 60% of the $\beta2$ level in a fertile male of genotype $\beta2^{+}/\beta2^{null}$. We have never observed axonemes in males of the genotype shown in Fig. 8 *d*, but in such males the arrangement of the two components of the mitochondrial derivative is nevertheless nearly normal, and the derivatives show a wild-type association with cytoplasmic microtubules. These microtubules are assembled and disassembled at normal developmental times. Fig. 8 *e* shows a late stage spermatid in which the electron dense paracrystalline material in the mitochondrial derivatives has accumulated to

male which is homozygous the $\beta2^{null}$ mutation and carrying one copy of the $\beta2\beta3$(IIIb) insert, showing a spermatid at approximately the same stage as that in *a*, as estimated by the development of paracrystalline material in the elongating mitochondrial derivative (*MD*). No axonemes are present. Mitochondrial derivative morphology is abnormal, but the associated cytoplasmic microtubules (*arrows*) are present, similar to wild type. (*d*) Cross section through the testis of a sterile male that carries three $\beta2\beta3$* inserts (one copy each of $\beta2\beta3$*(IIa), $\beta2\beta3$*(IIIb), and $\beta2\beta3$*(IIIc)) and is homozygous for the $\beta2^{null}$ mutation. The developing spermatids are at approximately the same stage as that in *a*, as estimated by the development of paracrystalline material in the major mitochondrial derivative (*MD*). No axonemes are present, but at this dosage of the divergent isotype (approximately twice that in the male shown in *c*; see text), the morphology and arrangement of the mitochondrial derivatives is nearly normal. Associated cytoplasmic microtubules (*arrows*) are present. (*e*) Cross section through the testis of a sterile male that is homozygous both for the $\beta2\beta3$*(IIIc) insert and for the $\beta2^{null}$ mutation. The developing spermatids are mature, as in *b*, as estimated by the development of paracrystalline material in the major mitochondrial derivative (*MD*). Axonemes are absent, but the mitochondrial derivative morphology is similar to wild type. Cytoplasmic microtubules are no longer present. The level of $\beta3$* in this genotype is approximately the same as that in the genotype shown in *d* (see text). Bars, 100 nm.

the same extent as in the wild type male in Fig. 8 *b*, and the cytoplasmic microtubules have disappeared, as occurs in wild-type males. The genotype of the male in Fig. 8 *e* is +/+; β2β3*(IIIc), $\beta2^{null}$/β2β3*(IIIc), $\beta2^{null}$, in which the level of β3* is comparable to that in the genotype shown in Fig. 8 *d*. The phenotype of males that express β3* in the absence of β2 suggests to us that the mitochondrial associated cytoplasmic microtubules are directly involved in mediating the differentiation and elongation of the mitochondrial derivatives.

The cytoplasmic microtubules transiently associated with elongating mitochondrial derivatives are the only microtubules that we have observed in the spermatids of males that express the divergent isotype but not β2. Electron microscope surveys of testes of males that express the divergent isotype but not β2 reveal no axoneme assembly, at least at the levels at which it is produced in the testes of males that have up to three hybrid gene inserts (Fig. 8, *c*, *d*, and *e*). We have observed one instance each of high levels of β3 and β3* protein directing assembly of doublet microtubules in the absence of endogenous β2 tubulin. These apparent abortive axonemes were in the correct position relative to the mitochondrial derivative but consisted of only two or three doublets each. It should be noted, however, that our present data does not eliminate the possibility that there may be some very short axonemes extending a few microns from the basal body, as serial sectioning revealed occasionally occurs in males homozygous for the assembly defective β2-tubulin variant encoded by the mutation $\beta2t^{8}$ (Fuller et al., 1988).

In males in which the divergent isotype is expressed in the absence of β2, at any of the dose levels we have been able to test, shaping of the spermatid nuclei fails to occur (see Fig. 11 *a*). We have not determined as yet whether the cytoplasmic microtubules that normally mediate nuclear shaping fail to assemble in such males, or whether they are assembled but fail to function, as for example that which occurs in the males homozygous for $B2t^{8}$ (Fuller et al., 1987, 1988).

A similar question remains for the meiotic spindle. As illustrated in Fig. 9, normal meiosis does not take place in males in which the divergent isotype but not β2 is expressed. In a normal male, the meiotic divisions of the syncytial cysts of 16 primary spermatocytes results in the formation of 64 haploid spermatids. Each spermatid nucleus is associated with a mitochondrial derivative of approximately the same size as the nucleus; this typical morphology of newly formed spermatids is diagnostic for the fidelity with which meiosis has occurred (see discussion in Fuller et al., 1988). Fig. 9 *a* shows early spermatids in a male that has one β2β3*(IIa) gene and is homozygous for $\beta2^{null}$. Spermatids in this male appear identical with those in males homozygous for $\beta2^{null}$ but with no hybrid gene. This phenotype represents the absence of meiotic spindle function: chromosomes replicate and condense, but meiosis and cytokinesis fail to occur, resulting in the formation of 16 tetraploid spermatids with correspondingly large, and sometimes morphologically aberrant, mitochondrial derivatives (Kemphues et al., 1982; Fuller et al., 1988). Fig. 9 *b* shows newly formed spermatids in a fertile male that has one β2β3*(IIa) gene and one functional β2 gene. Spermatids in this male appear identical to those in a wild-type male. (The phenotype of males that coexpress the divergent isotype and β2 is discussed in detail below). Fig. 9 *c* shows early spermatids in a male homozygous for $\beta2^{null}$ and for β2β3*(IIa) gene. Fig. 9 *d* shows early spermatids in a male homozygous for $\beta2^{null}$ and for β2β3-(IIIb). The meiotic phenotype in Fig. 9, *c* and *d* is somewhat ameliorated as compared to that in *a*, but most spermatids contain 4N nuclei, indicating that meiotic spindle function is still extremely defective. Although increasing the dose of the divergent isotype from one to two genes did result in some improvement in the meiotic phenotype, males with three genes did not show a significant improvement over males with two genes. Examination of orcein-stained testes confirmed that meiotic chromosome separation is rare in males in which the divergent isotype but not β2 is expressed. Thus the meiotic spindle is either not assembled or if so, it is not functional.

Table II. The Effect on Spermatogenesis of Coexpression of the Divergent Isotype and β2-Tubulin in the Germ Line

Gene dose		
β2β3*:β2	β2β3:β2	Phenotype
0:2	0:2	Fertile
0:1	0:1	Fertile
1:2	1:2	Fertile
1:1	1:1	Fertile
2:2	2:2	Fertile
3:2	3:2	Sterile
4:2	4:2	Sterile
2:1	2:1	Sterile

The genotypes tested are given with respect to β2β3-tubulin or β2β3*-tubulin hybrid genes inserted on the second and third chromosomes and to the β2-tubulin allele at the β2 locus on the third chromosome. The amount of the divergent isotype produced from each of the inserts used in this experiment is equal to ~15–20% the amount of β2-tubulin produced by a wild-type β2 allele, as determined from autoradiograms of [^{35}S]methionine incorporation into testis proteins (see Fig. 4).

The failure of the divergent isotype alone to support the assembly of spermatogenic microtubules other than those involved in mitochondrial derivative elongation may be due in part to the relatively low levels of protein produced from the hybrid gene inserts. However, we think this is probably not so, because we have recently been able to obtain males in which the divergent isotype is present in the germ line at levels equivalent to the β2 level in a fertile $\beta2^{+}$/$\beta2^{null}$ male. The phenotype in these males is the same as described here for males in which the divergent isotype is present at somewhat lower levels, i.e., even at higher levels, the divergent isoform alone supports only the mitochondrial-associated tubules, and when co-expressed with the normal isoform causes dominant disruption of axoneme morphology.

Coexpression in the Germ Line of the Divergent Isotype with the Endogenous Testis-specific Isoform β2 Results in Dominant Male Sterility at a Threshold Level of Approximately 20% of the Total Beta Tubulin Pool

The phenotype of males in which the divergent isotype is coexpressed with β2-tubulin unambiguously demonstrates that the divergent isotype, although capable of supporting assembly of functional cytoplasmic microtubules, is not functionally equivalent to the normal testis isoform. In testes of males in which the divergent isotype and β2 are coexpressed, all categories of spermatogenic microtubules are present.

The presence of the divergent isotype at any level that we have been able to observe does not significantly interfere with the normal function of β2 in assembly of the singlet tubules of the meiotic spindle or of the cytoskeleton. However, in males in which the divergent isotype comprises 20% or more of the testis beta tubulin pool, the protein acts in a dominant way to interfere with the normal function of β2-tubulin in assembly of the doublet tubules of the axoneme, resulting in male sterility.

We tested the effect of different relative amounts of the divergent isotype and β2 proteins in the testis tubulin pool by examining the fertility of males with different numbers of hybrid genes and functional wild-type β2 genes. As described in Materials and Methods, the relative amounts of testis proteins were estimated by scanning autoradiograms of gels similar to those shown in Fig. 4. The inserts carried in the genomes of the males used to compile the data in Table II yield an amount protein from a single hybrid gene equal to ~15% the amount of β2-tubulin produced from a single functional wild-type β2 allele in the same male. (This is the level of expression we observe from the majority of the inserts we recovered, including the inserts illustrated in Figs. 4, 5, 6, and 7.) The ability of males with different amounts of β3 or β3* and β2 to produce mature motile sperm was determined as described in Materials and Methods. The results are summarized in Table II: males in which the level of the divergent isotype in the testis comprises <20% of the total testis beta tubulin pool are fertile. Moreover, we found that in such males, the divergent isotype is incorporated into axonemes in approximately the same ratio to β2 as present in the testis, as shown by two-dimensional gels of proteins present in functional mature sperm isolated from the seminal vesicles. However, males in which the level of the divergent isotype is 20% or more of the total testis beta tubulin pool produce no mature motile sperm and are sterile, even if they have two copies of the wild-type β2 gene. Sterility is not simply due to the expression of excessive beta tubulin in the testis, since males with two fusion gene inserts and one functional β2 gene are also sterile. Similarly, as seen in Table I, four copies of the wild-type β2 gene do not adversely affect male fertility.

It is clearly the ratio of the divergent isotype to β2 in the testis tubulin pool which is important, not the absolute amount of beta tubulin. Thus, for the fusion gene inserts that yield protein levels below 20% of the β-tubulin pool, males with equal numbers of fusion gene and $\beta2^+$ genes are fertile. Similarly, males with a 2:1 ratio of fusion gene inserts and $\beta2^+$ genes are sterile. As can be seen in Table II, the crucial ratio of the divergent isotype relative to β2 for male fertility is represented by males that have three fusion gene inserts and two functional wild-type genes; in such males the amount of the divergent isotype is ~25% that of β2, i.e., 20% of the total beta tubulin pool. For the observations reported in Table II, 150 males of the 3:2 gene dose ratio were scored; none produced any mature sperm, nor did any males with higher fusion gene/β2 gene dose ratios. In contrast, all males of the genotypes scored as fertile produced mature motile sperm. Also consistent with the conclusion that it is the ratio of the divergent isotype to β2 that determines fertility or sterility is the observation that males that carry fusion gene inserts that yield higher levels of the divergent isotype exhibit male sterility at lower copy numbers of the hybrid gene. For example, males carrying one copy of $\beta2^+$ and one copy of β2β3*(IIIc), the insert illustrated in Fig. 12 *d*, are sterile.

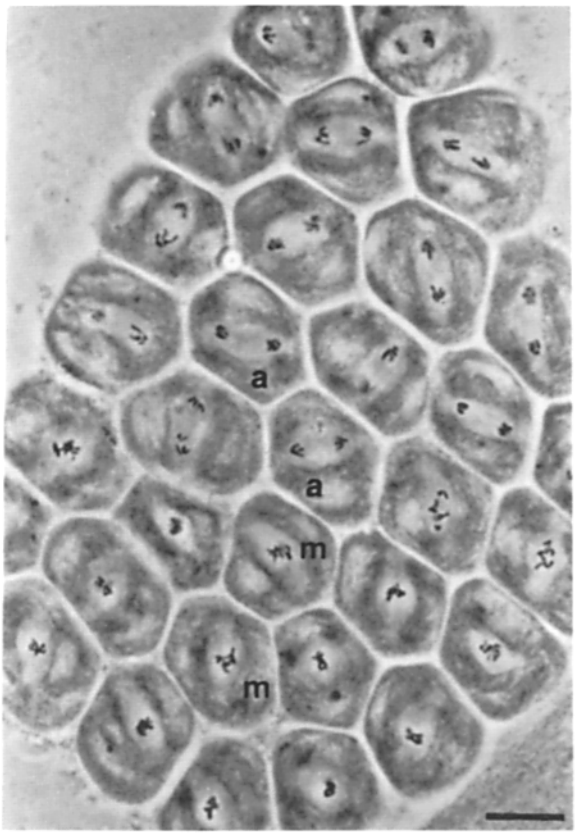

Figure 10. Meiosis in males that express both β3*-tubulin and β2-tubulin in the germ line. A complete cyst in meiosis II is shown in a light micrograph of an orcein-stained testis from a sterile male that has two β2β3* inserts (one copy each of β2β3*(IIIa) and β2β3*(IIIb)) and one $\beta2^+$ gene. This meiotic cyst looks the same as in a wild-type male. Note the typical "wave" of meiosis from metaphase (*m*) to anaphase (*a*). Bar, 20 μm.

When Coexpressed with β2-Tubulin, the Divergent Isotype Does Not Cause Significant Defects in the Assembly and Function of Meiotic or Cytoplasmic Microtubules

In addition to the assembly of the axoneme, β2-tubulin is required for assembly of the meiotic spindles and of at least two classes of cytoplasmic microtubules, those involved in elongation of the mitochondrial derivatives and those required for shaping of the mature sperm nucleus. As shown above in Fig. 8, the divergent isotype alone is sufficient to support mitochondrial derivative differentiation and elongation; likewise, this process appeared to be normal in males which express both the divergent isotype and β2.

Meiotic function was assessed by light microscope examination of live and orcein-stained testis squashes using phase contrast optics. As shown in Fig. 10, nearly all cells in meiotic cysts were normal in sterile males in which both the divergent isotype and β2 are expressed, at any ratio. Likewise, the morphology of most early spermatids resulting from the meiotic divisions was also normal. Thus we con-

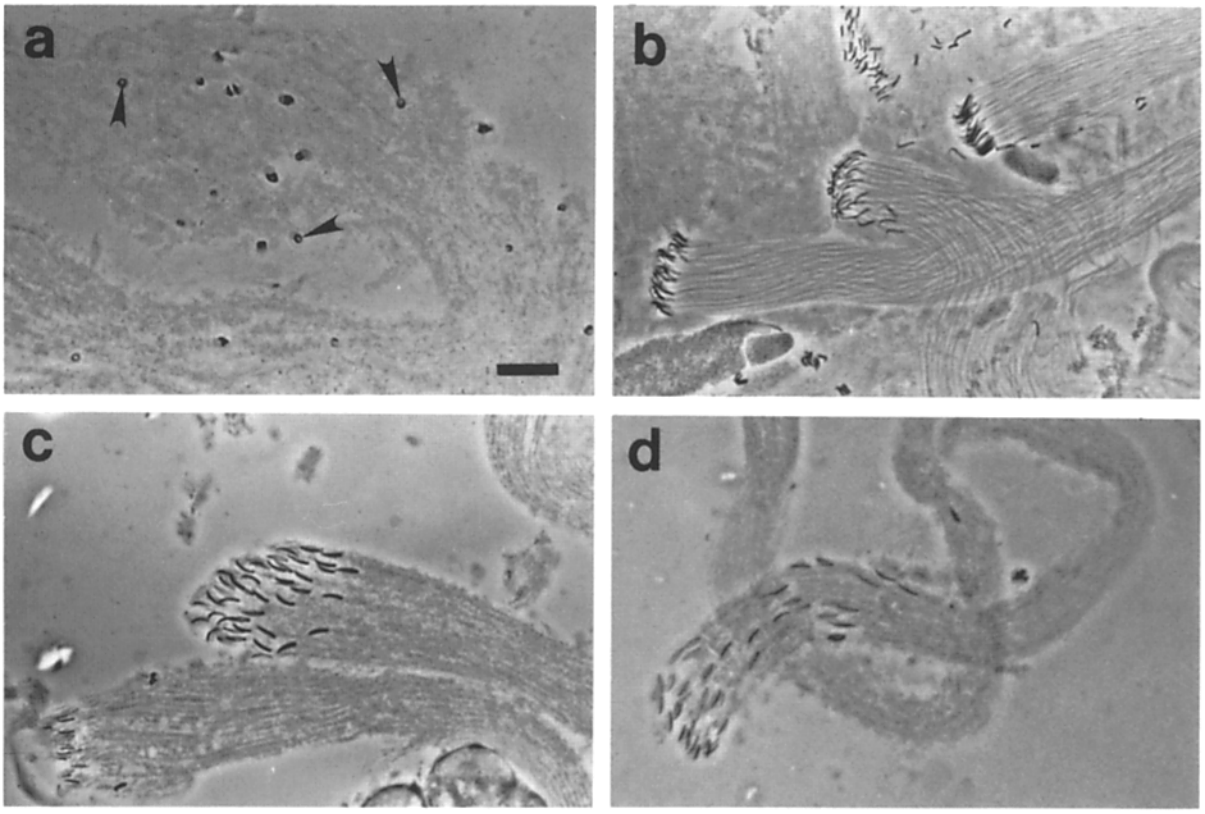

Figure 11. Nuclear shaping and alignment in males that express β3-tubulin in the germ line. Nuclei of elongating spermatids are shown in light micrographs of orcein-stained testes. (*a*) Elongated spermatids in a sterile male that is homozygous for β2β3(IIIb) and is homozygous for the $\beta 2^{null}$ mutation. The nuclei (*arrows*) remain round and unshaped, and also fail to be aligned within the bundle of spermatids. Multiple sizes indicate nuclei of differing ploidy were formed during meiosis (see Fig. 9, *b–d*). (*b*) Elongated spermatids in a fertile male that has two β2β3 inserts (one copy each of β2β3(IId) and β2β3(IIIb)) and two $\beta 2^{+}$ genes (i.e., is wild type at the β2 locus). Nuclei are both correctly shaped and aligned at the end of the bundles, and appear the same as in a wild-type male. The sperm heads in the bundles shown here are in a nearly mature state of shaping. Individualization of spermatids is complete, giving a highly striated appearance to the contents of each bundle (compare with *a*, *c*, and *d*). (*c*) Elongated spermatids in a sterile male that has three β2β3 inserts (one copy of β2β3(IId) and homozygous for β2β3(IIIb)) and two $\beta 2^{+}$ genes (i.e., wild type at the β2 locus). At this ratio of β3 to β2, nuclei become correctly shaped and in some bundles, some heads are misaligned. However, the degree of misalignment is within the range seen in wild-type β2 testes. The sperm heads in the bundles shown here are in an intermediate stage of shaping. The bottom bundle is older than the top bundle and the sperm heads have condensed to the mature size seen in *b*. (*d*) Elongated spermatids in sterile males which have four β2β3 inserts (homozygous for both β2β3(IId) and β2β3(IIIb)) and two $\beta 2^{+}$ genes, i.e., wild type at the β2 locus. At this ratio of β3 to β2, the spermatid bundles show significant defective alignment. Occasional spermatid bundles show no defect in alignment (not shown). Bar, 20 μm.

clude that the presence of the divergent isotype does not significantly interfere with the normal meiotic function of β2.

The fourth requirement for β2-tubulin is during the differentiation of the mature sperm head. Although the mechanism by which shaping of the nucleus occurs has not yet been elucidated, it is clear that this process is mediated by a set of microtubules assembled along one side of the nucleus shortly after axoneme assembly and mitochondrial derivative elongation have begun (Kemphues et al., 1982, 1983; Fuller et al., 1987). When the divergent isotype is coexpressed with β2, at any ratio, normal nuclear shaping occurs.

During spermatid differentiation in a wild-type male, the heads of all 64 elongating spermatids in each bundle are aligned together against the cyst cell toward the distal end of the testis (Lindsley and Tokuyasu, 1980). The mechanism by which alignment is achieved is not known. Microtubule function may be directly or indirectly involved in this process. For example, no alignment occurs in males homozygous for the recessive male sterile β2 mutations $B2t^{8}$ and $B2t^{7}$, which encode stable but defective β2 variants (Fuller et al., 1987, 1988). In addition, in males that have only a single functional wild type β2 allele, most spermatid nuclei are shaped normally, but many fail to become properly aligned at the end of the bundle (Kemphues et al., 1982, 1983). Misaligned spermatids fail to be individualized and therefore never reach the seminal vesicles as mature sperm. The defective alignment appears to be the reason for the decreased fecundity of $\beta 2^{+}/\beta 2^{null}$ males relative to wild-type males. Because fertile males with only a single functional β2 allele exhibit significant problems in nuclear alignment, the effect of the divergent isotype on nuclear shaping and alignment can best be seen by examining males with two wild-type β2 alleles and varying numbers of hybrid gene inserts, as shown in Fig. 11, *b*, *c*, and *d*. Fertile males with two β2β3 inserts and two wild-type β2 genes appear identical to wild-type in both nuclear shaping and alignment (Fig. 11 *b*). In sterile males with three β2β3 inserts and two wild-type β2 genes, nuclear shaping is normal and the degree of misalignment is

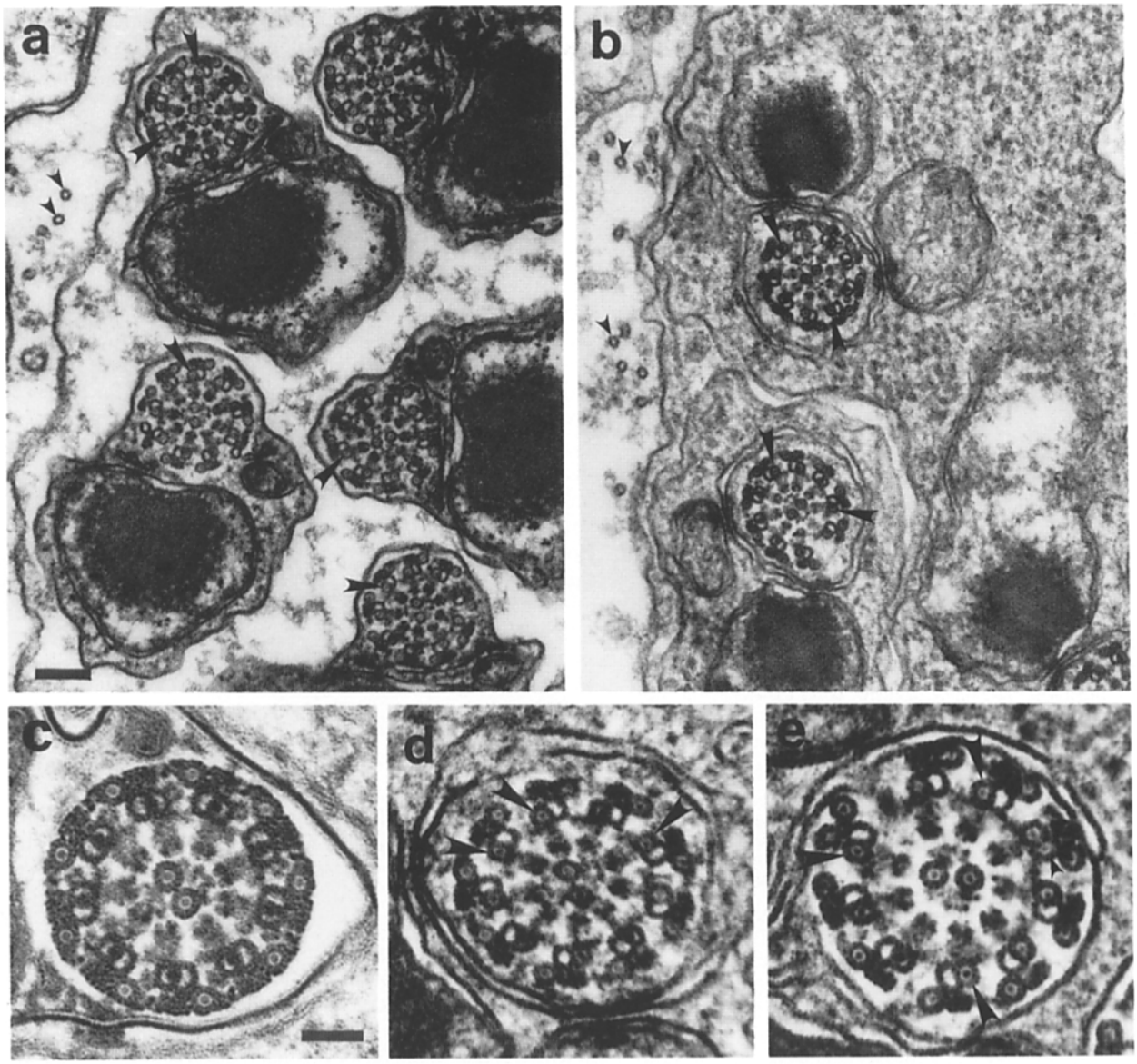

Figure 12. Sperm flagellar axonemes in males that express both the divergent isotype and β2-tubulin in the germ line. Electron micrographs of cross sections through developing spermatids are shown. (*a*) Mature individualized spermatids, each with a mature axoneme and associated mitochondrial derivative, in the testis of a sterile male that carries four β2β3* inserts (homozygous for both β2β3*(IIa) and β2β3*(IIIa)) and two $\beta2^+$ genes, i.e., is wild type at the β2 locus. The morphology of the spermatids is normal except that in the axonemes the electron dense dot is present not only in the the lumen of the central pair and of the accessory tubules but also in the lumen of the A tubule in most of the doublet tubules (*large arrows*). This section includes a region of one of the somatic cyst cells that encloses the cyst of developing spermatids and that are the site of endogenous β3 expression; cytoplasmic microtubules can be seen in cross section within the cyst cell (*small arrows*). (*b*) Mature spermatids just before individualization in a sterile male that carries three β2β3 inserts (one copy of the β2β3(IIIb) insert and homozygous for β2β3(IId)) and two copies of $\beta2^+$, i.e., is wild-type at the β2 locus. Spermatid morphology is normal except for the presence of the electron dense dot in the lumen of the A tubule (*large arrows*). The section also includes a region of a somatic cyst cell which expresses endogenous β3 tubulin; cytoplasmic microtubules can be seen in cross section within the cyst cell (*small arrows*). (*c*) Axoneme of a mature individualized spermatid in the testis of a fertile male that carries one copy of the β2β3(IIc) insert and one copy of the $\beta2^+$ gene. The appearance of this spermatid is the same as wild type. Spermatids of males of this genotype are occasionally seen with dots in one or two A tubules (not shown). (*d*) Axoneme of a mature spermatid in the testis of a sterile male that has one β2β3*(IIIc) insert and has one $\beta2^+$ gene and thus the same β3*/β2 ratio as in *a*. Similar to the genotype shown in *a*, the electron-dense dot is present in the lumen of the A tubule in some of the doublet tubules in the axonemes of males of this genotype. In the axoneme shown, seven of the nine doublets have filled in A tubules (*large arrows*). The cyst in which this spermatid is contained is beginning to degenerate, indicating that this is the terminal phenotype for the axoneme of this spermatid. (*e*) Similar to *b*. Axoneme of a mature spermatid in the testes of a sterile male that carries four copies of the β2β3 gene (homozygous for both β2β3(IId) and β2β3(IIIb)) and two copies of $\beta2^+$, i.e., is wild type at the β2 locus. The electron dense dot is present in the lumen of the A tubule of each doublet (*large arrows*). In addition, the lumen of a B tubule shows a rare instance of filling with the electron dense dot (*small arrow*). Bars: (*a* and *b*) 100 nm; (*c–e*) 50 nm.

within the range seen in wild-type $\beta2^+$ males (Fig. 11 *c*). In sterile males with four $\beta2\beta3$ inserts and two wild-type $\beta2$ genes, most nuclei are shaped normally, but in many cysts, some nuclei fail to be aligned at the end of the bundle (Fig. 11 *d*). Thus the higher ratios of the divergent isotype to $\beta2$ result in problems in nuclear alignment, similar to those seen in fertile $\beta2^+/\beta2^{null}$ males. The alignment problem is not in itself sufficient to account for the sterility of males that coexpress the divergent isotype and $\beta2$.

When Coexpressed with β2-Tubulin, the Divergent Isotype Causes a Specific Defect in Axoneme Assembly

The dominant male sterility caused by expression of the hybrid gene is caused by interference of the divergent isotype in the assembly of axonemes. When the divergent isotype is present, the doublet tubules of the sperm tail axoneme exhibit a distinctive morphological feature normally present only in the singlet tubules of the axoneme. The morphology of the axoneme of the mature wild-type *Drosophila* sperm tail flagellum is illustrated in Fig. 8 *b*. In addition to the typical "9 + 2" arrangement of nine doublet tubules surrounding a central pair of singlet microtubules, the axoneme contains an outer set of singlet "accessory" microtubules. During axoneme assembly an accessory microtubule is initiated as a projection from the B tubule of each doublet tubule, as can be seen in Fig. 8 *a*. (In each doublet tubule, the A tubule, which bears the dynein arms responsible for axoneme motility, is a complete tubule composed of 13 protofilaments with morphology similar to that of singlet microtubules, while the B tubule is composed of 10 protofilaments and shares the wall of the A tubule.) In the mature axoneme the accessory microtubules are complete singlet microtubules, separate from the B tubule of the doublet and embedded in an "eyebrow" of electron-dense material (Fig. 8 *b*). A striking feature of the mature wild-type *Drosophila* axoneme is that the lumen of each of the central pair and of the accessory microtubules is filled with electron-dense material, which in cross section appears as a "dot" within each of these tubules (Fig. 8 *b*).

As shown in Fig. 12, the major defect in morphology which we observe in the testes of males in which the divergent isotype is coexpressed with $\beta2$ is that the lumen of the A tubule of the doublet tubules also becomes filled with electron dense material. Fig. 12 *a* shows mature individualized spermatids in the testes of a sterile male with four $\beta2\beta3$* gene inserts and two wild-type $\beta2$ genes. In these spermatids, virtually all of the doublet tubules in all of the axonemes have filled A tubules. Fig. 12 *b* shows a similar axoneme in a sterile male that has two wild-type $\beta2$ genes and three copies of the $\beta2\beta3$ gene. In both cases the divergent isotype represents approximately a quarter of the total testis beta-tubulin pool. Males of both of these genotypes fail to produce any motile sperm.

Fig. 12, *a* and *b* each show a portion of a somatic cyst cell that expresses endogenous $\beta3$ tubulin. The cytoplasmic microtubules seen in the cyst cells do not have filled lumens although they presumably contain endogenous $\beta3$ tubulin. In the spermatid syncytium which these cyst cells surround, the central pair and the accessory microtubules have filled normally and the A tubules have filled abnormally due to the presence of $\beta3$ tubulin expressed in the germline by the hybrid gene inserts. Thus in the presence of the divergent isoform, the identity of the doublet tubules in the axonemes is altered so that the doublet tubules acquire morphological characteristics normally present only in the singlet tubules of the axoneme.

We have found that the number of axonemes that exhibit doublet tubules with filled A tubules, and the number of filled A tubules in each axoneme, roughly corresponds with the level of the divergent isotype, relative to that of $\beta2$. Fertile males that express the divergent isotype exhibit some filled A tubules, but we do not observe axonemes with the extensive filling that occurs in sterile males. Fig. 12 *c* shows a mature individualized sperm axoneme present in the seminal vesicles of a fertile male with one wild-type $\beta2$ gene and one $\beta2\beta3$(IIc) insert (which expresses the divergent isotype in levels comparable to that shown in Fig. 4 *c*). Such males produce mature motile sperm in near wild-type amounts. None of the A tubules in Fig. 12 *c* have filled lumens although occasionally sperm present in the seminal vesicles have one or two filled A tubules. We do not know if such sperm are motile or if a few nonmotile sperm present in a cyst may be passively carried into the seminal vesicle when the majority of the sperm in a cyst are motile. Fig. 12 *d* shows an axoneme from a sterile male carrying a single copy of the $\beta2\beta3$*(IIIc) gene and a single copy of the $\beta2^+$ gene. The ratio of $\beta3$* to $\beta2$ in the total β-tubulin pool is ~30%, similar to the ratio in Fig. 12 *a*. Fig. 12 *e* shows an axoneme from a sterile male homozygous for both $\beta2\beta3$(IId) and $\beta2\beta3$(IIIb) and homozygous for $\beta2^+$. The ratio of $\beta3$ to $\beta2$ in the total β-tubulin pool is twice that shown in Fig. 12 *c* and approximately the same as the ratio of $\beta3$* to $\beta2$ in Fig. 12 *d*. Fig. 12, *d* and *e* clearly show normal filling of the central pair and accessory microtubules and abnormal filling of the A tubule. Fig. 12 *e* also shows an extremely rare instance of filling in the lumen of a B tubule. The filling of the A tubules of the doublets is the only consistent defect we observe in spermatogenesis in males that coexpress the divergent isotype and $\beta2$. We therefore conclude that the dominant sterility caused by the divergent isotype results from this defect in axoneme assembly. The most likely explanation for the sterility is that axonemes in which a critical number of doublet tubules become filled are not motile, and hence do not enter the seminal vesicles, since it appears that the intrinsic motility of the mature spermatids is needed for entry into the seminal vesicles (Lindsley and Tokuyasu, 1980). A similar morphological phenotype occurs in males homozygous for *B2t*^{6}, an EMS-induced recessive male sterile $\beta2$ mutation in which a stable, slightly acidic $\beta2$ variant is produced. Likewise in this mutation, filling of the doublet tubules correlates with male sterility (Fuller et al., 1988).

Discussion

In this paper we have addressed the functional capacity of two *Drosophila* beta tubulin isoforms, $\beta2$ and $\beta3$, which differ significantly in sequence. The testes-specific isoform, $\beta2$, is conserved relative to the major class of metazoan β-tubulins while the developmentally regulated isoform, $\beta3$, is divergent. As summarized in Fig. 1, both are expressed in the testis, but in very different cellular contexts. We have examined the consequences to microtubule function in spermatogenesis of the expression of hybrid genes in which $\beta2$ promoter elements direct testes-specific expression of the

divergent isotype. The data we have presented demonstrate two important findings: (*a*) for the first time we have presented unambiguous and definitive evidence for functional specialization among different tubulin isotypes; and (*b*) we have shown that there are different structural constraints over the assembly of different classes of microtubules.

When the divergent isoform is expressed in the male germline in the absence of the endogenous testes isoform, it supports assembly of a single class of microtubules, a transient cytoskeletal array associated with elongating mitochondrial derivatives. It is possible that assembly of this class of microtubules reflects the intrinsic assembly properties of the divergent isoform, since in the tissues in which it is normally expressed, β3-tubulin appears to function primarily in cytoskeletal arrays, often transient, involved in cell shape changes or tissue organization (Kimble et al., 1989). These results also demonstrate that the remarkable elongation of the mitochondrial derivative is directly mediated by these transient cytoskeletal microtubules. In males that express only the divergent isoform, spermatids undergo extensive elongation due entirely to mitochondrial derivative elongation. In a wild-type male, mitochondrial derivatives undergo differentiation from an initial 6.5 μm spherical membrane stack to a 0.2 μm × 1.8-2 mm structure in the mature sperm. No mitochondrial derivative elongation occurs at all in the absence of microtubules (Kemphues et al., 1982, 1983; Raff and Fuller, 1984; Fuller et al., 1987, 1988). We have also observed that in a meiotic mutant in which spermatogenic structures are spatially disorganized, mitochondrial derivatives frequently are not associated with axonemes but nevertheless undergo normal differentiation and elongation (Raff, E.C., unpublished data). This is consistent with the hypothesis that it is the associated cytoplasmic tubules, and not the axonemes, that support mitochondrial derivative elongation. We can not at present suggest the mechanism by which these cytoplasmic microtubules might mediate mitochondrial derivative elongation, but it is interesting to note that a number of investigators have recently documented extensive tubulovesicular movements along microtubules mediated by ATPase motor proteins such as kinesin (Dabora and Scheetz, 1988; Lee and Chen, 1988; Vale and Hotani, 1988).

When it is coexpressed with β2 in the postmitotic germ line, the divergent isotype does not interfere with the normal functions of β2-tubulin in the assembly of either meiotic spindles or the cytoplasmic microtubules involved in mitochondrial elongation and nuclear shaping. In contrast, however, the divergent isoform acts as a dominant "poison" of axoneme assembly, resulting in the failure to produce functional motile sperm if it is present above a critical threshold level. In particular, the divergent isotype causes a specific axoneme defect that suggests confusion of morphological identity of different categories of axoneme tubules, even when the endogenous subunit β2 is present in normal amounts. In such males the doublet tubules of the axoneme acquire morphological features normally characteristic of the singlet central pair and accessory microtubules of the axoneme, perhaps reflecting the wild-type function of β3-tubulin in assembly of singlet cytoplasmic microtubules.

The consequences of expression of the divergent isotype in the *Drosophila* male germ line are very different from the usual case in which microtubule function is unaffected by the presence of heterologous subunits. These results are also in contrast to the results obtained when chimeric beta tubulins comprised of chicken-yeast sequences were expressed in mouse cells in culture (Bond et al., 1986; Friedovich-Keil et al., 1987). These authors estimated that the chicken-yeast chimeric protein comprised a minimum of 10% of the total beta tubulin pool, and found that the chimeric protein was incorporated into endogenous microtubules and did not appear to adversely affect microtubule function. The differences in our results from those obtained with the chimeric chicken-yeast protein could be due to the fact that in the *Drosophila* testis, unlike in the mouse cell system, the heterologous protein was required to participate in axoneme assembly. That the divergent *Drosophila* isotype can support assembly of functional cytoplasmic microtubules but is a dominant inhibitor of axoneme assembly shows that assembly of the doublet tubules of the axoneme imposes more stringent constraints on beta tubulin function than does assembly of the singlet microtubules of the spindle and of the cytoskeleton, and in addition demonstrates that the *Drosophila* isoforms β2 and β3 are not equivalent in intrinsic functional capacity.

Perhaps the most general insight to be gained by the unexpectedly complex phenotype resulting from expression of the divergent isotype in the testes is related to our original question of why beta tubulin isotypes exist. The similarity of the testes-specific isoform β2 to the major isoform β1 suggests that the origin of β2 may have been via regulatory pressures rather than functional specifications (see Raff et al., 1987, for a discussion). The high degree of protein sequence conservation of β2 and β1 both with each other and with the major vertebrate isoforms may represent selective constraints reflecting the fact that all of these isoforms are used in a variety of microtubule assemblies including both singlet and multiple-walled tubules. The divergence of the β3 sequence may represent decreased selective pressure on this isoform because it is under less stringent functional constraint, or, conversely, may represent positive selection for the particular functions of this isoform. Taken together, however, our results suggest that β2 function in spermatogenesis could be replaced by β1, the other conserved isoform in *Drosophila*. Experiments to test this hypothesis are currently in progress.

We thank Mary Kimble and William Saxton for the critical reading of this manuscript and for many lively and helpful discussions of the data. We thank Mary Kimble for help with antibody staining of whole testes and Jeffrey Hutchens for expert technical assistance with two-dimensional gels. We thank F. Rudolf Turner, Joan Caulton, and Dorothy Barone for the electron microscopy in this study. We thank K. A. Matthews for the null mutation in the β2 tubulin gene used in this study.

This work was supported by a United States Public Health Service research grant HD16739 to E. C. Raff and by a postdoctoral fellowship from the Indiana University Institute for Molecular and Cellular Biology to H. Hoyle.

Received for publication 12 July 1989 and in revised form 8 May 1990.

References

Bialojan, S., D. Falkenberg, and R. Renkawitz-Pohl. 1984. Characterization and developmental expression of β tubulin genes in *Drosophila melanogaster*. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:2543–2548.

Blose, S. H., D. I. Melter, and J. R. Feramisco. 1984. 10-nm filaments are induced to collapse in living cells microinjected with monoclonal and polyclonal antibodies against tubulin. *J. Cell Biol.* 98:847–858.

Bond, J. F., J. L. Fridovich-Keil, L. Pillus, R. C. Mulligan, and F. Solomon. 1986. A chicken-yeast chimeric β-tubulin protein is incorporated into mouse

microtubules in vivo. *Cell.* 44:461–468.
Breitling, F., and M. Little. 1986. Carboxy-terminal regions on the surface of tubulin and microtubules. *J. Mol. Biol.* 189:367–370.
Dabora, S. L., and M. P. Scheetz. 1988. The microtubule-dependent formation of a tubulovesicular network with characteristics of the ER from cultured cell extracts. *Cell.* 54:27–35.
Ephrussi, B., and G. Beadle. 1936. A technique of transplantation for *Drosophila*. *Am. Nat.* 70:218–225.
Fridovich-Keil, J. L., J. F. Bond, and F. Solomon. 1987. Domains of β-tubulin essential for conserved functions in vivo. *Mol. Cell. Biol.* 7:3792–3798.
Fuller, M. T., J. H. Caulton, J. A. Hutchens, T. C. Kaufman, and E. C. Raff. 1987. Genetic analysis of microtubule structure: a β-tubulin mutation causes the formation of aberrant microtubules in vivo and in vitro. *J. Cell Biol.* 104:385–394.
Fuller, M. T., J. H. Caulton, J. A. Hutchens, T. C. Kaufman, and E. B. Raff. 1988. Mutations that encode partially functional β2 tubulin subunits have different effects on structurally different microtubule arrays. *J. Cell Biol.* 107:141–152.
Gasch, A., U. Hinz, D. Leiss, and R. Renkawitz-Pohl. 1988. The expression of β1 and β3 genes of *Drosophila melanogaster* is spatially regulated during embryogenesis. *Mol. Gen. Genet.* 211:8–16.
Gasch, A., U. Hinz, and R. Renkawitz-Pohl. 1989. Intron and upstream sequences regulate the expression of the *Drosophila* β3 tubulin gene in the visceral and somatic musculature respectively. *Proc. Natl. Acad. Sci. USA.* 86:3215–3218.
Ginzburg, I., and U. Z. Littauer. 1988. Dissimilarities in microtubule structures are revealed at the C-terminal region of β-tubulin. *Protoplasma.* 145:141–144.
Hardy, R. W. 1975. The influence of chromosome content on the size and shape of sperm heads in Drosophila melanogaster and the demonstration of chromosome loss during spermiogenesis. *Genetics.* 79:231–264.
Harlow, E., and D. Lane. 1988. *Antibodies: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 406–407.
Hiraoka, Y., T. Toda, and M. Yanagida. 1984. The NDA3 gene of fission yeast encodes β-tubulin: a cold sensitive nda3 mutation reversibly blocks spindle formation and chromosome movement in mitosis. *Cell.* 39:349–358.
Keith, C. H., J. R. Feramisco, and M. Shelanski. 1981. Direct visualization of fluorescein-labelled microtubules in vitro and in microinjected fibroblasts. *J. Cell Biol.* 88:234–240.
Kellog, D. R., T. J. Mitchison, and B. M. Alberts. 1988. Behavior of microtubules and actin filaments in living *Drosophila* embryos. *Development.* 103: 675–686.
Kemphues, K. J., R. A. Raff, T. C. Kaufman, and E. C. Raff. 1979. Mutation in a structural gene for a β-tubulin specific to testis in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA.* 76:3991–3995.
Kemphues, K. J., E. C. Raff, R. A. Raff, and T. C. Kaufman. 1980. Mutation in a testes-specific β-tubulin in *Drosophila*: analysis of its effects on meiosis and map location of the gene. *Cell.* 21:445–451.
Kemphues, K. J., T. C. Kaufman, R. A. Raff, and E. C. Raff. 1982. The testes-specific β-tubulin subunit in *Drosophila melanogaster* has multiple functions in spermatogenesis. *Cell.* 31:655–670.
Kemphues, K. J., E. C. Raff, and T. C. Kaufman. 1983. Genetic analysis of β2t, the structural gene for a testis-specific β-tubulin subunit in *Drosophila melanogaster*. *Genetics.* 105:345–356.
Kimble, M., J. P. Incardona, and E. C. Raff. 1989. A variant β-tubulin isoform of *Drosophila melanogaster* (β3) is expressed primarily in tissues of mesodermal origin in embryos and pupae, and is utilized in populations of transient microtubules. *Dev. Biol.* 131:415–429.
Klemenz, R., U. Weber, and W. J. Gehring. 1987. The white gene as a marker in a new P-element vector for gene transfer in *Drosophila*. *Nucleic Acids Res.* 15:3947–3959.
Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
Lee, C., and L. B. Chen. 1988. Dynamic behavior of endoplasmic reticulum in living cells. *Cell.* 54:37–46.
Lewis, S. A., W. Gu, and N. J. Cowan. 1987. Free intermingling of mammalian β tubulin isotypes among functionally distinct microtubules. *Cell.* 49:539–548.
Lindsley, D. L., and E. L. Grell. 1968. Genetic variations of *Drosophila melanogaster*. Carnegie Institute of Washington Publication 627. Carnegie Institute of Washington, Washington, DC. 471 pp.
Lindsley, D. L., and K. T. Tokuyasu. 1980. Spermatogenesis. *In* The Genetics and Biology of Drosophila. Vol. 2. M. Ashburner and T. R. F. Wright, editors. Academic Press Inc., New York. 225–294.
Littauer, U. Z., D. Giveon, M. Thierauf, I. Ginzburg, and H. Ponstingl. 1986. Common and distinct tubulin binding sites for microtubule-associated proteins. *Proc. Natl. Acad. Sci. USA.* 83:7162–7166.
Lopata, M. A., and D. W. Cleveland. 1987. In vivo microtubules are copolymers of available β tubulin isotypes: Localization of each of six vertebrate β tubulin isotypes using polyclonal antibodies elicited by synthetic peptide antigens. *J. Cell Biol.* 105:1707–1720.
Maccioni, R. B., L. Serrano, J. Avila, and J. R. Cann. 1986. Characterization and structural aspects of the enhanced assembly of tubulin after removal of its carboxyl terminal domain. *Eur. J. Biochem.* 156:375–381.
Maccioni, R. B., C. I. Rivas, and J. C. Vera. 1988. Differential interaction of synthetic peptides from the carboxyl-terminal regulatory domain of tubulin with microtubule-associated proteins. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:1957–1963.
May, G. S. 1989. The highly divergent β tubulins of *Aspergillus* are functionally interchangeable. *J. Cell Biol.* 109:2267–2274.
May, G. S., M. L. Tsang, H. Smith, S. Fidel, and N. R. Morris. 1987. Aspergillus nidulans beta-tubulin genes are unusually divergent. *Gene (Amst.).* 55:231–243.
Michiels, F., D. Falkenburg, A. M. Müller, U. Hinz, U. Otto, R. Bellmann, K. H. Glätzer, R. Brand, S. Bialojan, and R. Renkawitz-Pohl. 1987. Testes-specific β2 tubulins are identical in *Drosophila melanogaster* and *D. hydei* but differ from the ubiquitous β1 tubulin. *Chromosoma (Berl.).* 95:387–395.
Michiels, F., A. Gasch, Kaltschmidt, and R. Renkawitz-Pohl. 1989. A 14 bp promoter element directs the testis specificity of the Drosophila β2 tubulin gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1559–1565.
Monteiro, M. J., and D. W. Cleveland. 1988. Sequence of chicken cβ7 tubulin: Analysis of a complete set of vertebrate β-tubulin isotypes. *J. Mol. Biol.* 199:439–446.
Natzle, J.E., and McCarthy, B.J. 1984. Regulation of *Drosophila* α and β-tubulin genes during development. *Dev. Biol.* 104:187–198.
Neff, N. F., J. H. Thomas, P. Grisafi, and D. Botstein. 1983. Isolation of the β-tubulin gene from yeast and demonstration of its essential function *in vivo*. *Cell.* 33:211–219.
Paul, E. C. A., A. Roobol, K. E. Foster, and K. Gull. 1987. Patterns of tubulin isotype synthesis and usage during mitotic spindle formation in *Physarum*. *Cell Motil. Cytoskel.* 7:272–281.
Piperno, G., and M. T. Fuller. 1985. Monoclonal antibodies specific for an acetylated form of alpha-tubulin recognize the antigen in cilia and flagella from a variety of organisms. *J. Cell Biol.* 101:2085–2094.
Raff, E. C., M. T. Fuller, T. C. Kaufman, K. J. Kemphues, J. E. Rudolph, and R. A. Raff. 1982. Regulation of tubulin gene expression during embryogenesis in *Drosophila melanogaster*. *Cell.* 28:33–40.
Raff, E. C., and M. T. Fuller. 1984. Genetic analysis of microtubule function in Drosophila. *In* Molecular Biology of the Cytoskeleton. G. G. Borisy, D. W. Cleveland, and D. B. Murphy, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 293–304.
Raff, E. C., B. Diaz, H. D. Hoyle, J. Hutchens, M. Kimble, R. A. Raff, J. E. Rudolph, and M. Subler. 1987. The origins of multiple gene families: are there both functional and regulatory restraints? *In* Development As an Evolutionary Process. R. A. Raff and E. C. Raff, eds. Alan R. Liss, Inc., New York. 203–238.
Robertson, H. M., C. R. Preston, R. W. Phillis, D. M. Johnson-Schlitz, W. K. Benz, and W. R. Engels. 1988. A stable genomic source of *P* element transposase in *Drosophila melanogaster*. *Genetics.* 118:461–470.
Rudolph, J. E., M. Kimble, H. D. Hoyle, M. A. Subler, and E. C. Raff. 1987. Three Drosophila beta-tubulin sequences: a developmentally regulated isoform (β3), the testes-specific isoform (β2), and an assembly-defective mutation of the testes-specific isoform ($\beta2t^8$) reveal both an ancient divergence in metazoan isotypes and structural constraints for beta-tubulin function. *Mol. Cell. Biol.* 7:2231–2242.
Sackett, D.L., and J. Wolff. 1986. Proteolysis of tubulin and the substructure of the tubulin dimer. *J. Biol. Chem.* 261:9070–9076.
Salmon E. D., R. J. Leslie, W. M. Saxton, M. L. Karow, and J. R. McIntosh. 1984. Spindle microtubule dynamics in sea urchin embryos. Analysis using a fluorescein-labeled tubulin and measurements of fluorescence redistribution after laser photobleaching. *J. Cell Biol.* 99:2165–2174.
Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463–5467.
Saxton, W. M., D. L. Stemple, R. J. Leslie, E. D. Salmon, M. Zavortink, and J. R. McIntosh. 1984. Tubulin dynamics in cultured mammalian cells. *J. Cell Biol.* 99:2175–2186.
Serrano, L., E. Montejo De Garcini, M. A. Hernandez, and J. Avila. 1985. Localization of the tubulin binding site of the tau protein. *Eur. J. Biochem.* 153:595–600.
Spradling, A. C., and G. M. Rubin. 1982. Transposition of Cloned P elements into *Drosophila* germ line chromosomes. *Science (Wash. DC).* 218:341–347.
Steller, H., and V. Pirrotta. 1985. A transposable P vector that confers selectable G418 resistance to *Drosophila* larvae. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:167–171.
Steller, H., and V. Pirrotta. 1986. P transposons controlled by the heat shock promoter. *Mol. Cell. Biol.* 6:1640–1649.
Sullivan, K. F., and D. W. Cleveland. 1986. Identification of conserved isotype-defining variable region sequences for four vertebrate β-tubulin polypeptide classes. *Proc. Natl. Acad. Sci. USA.* 83:4327–4331.
Vale, R. D., and H. Hotani. 1988. Formation of membrane networks in vitro by kinesin-driven microtubule movement. *J. Cell Biol.* 107:2233–2241.
Wang, D., A. Villasante, S. A. Lewis, and N. J. Cowan. 1986. The mammalian β-tubulin repertoire: hematopoietic expression of a novel, heterologous β tubulin isotype. *J. Cell Biol.* 103:1903–1910.
Wyss, C., and G. Bachmann. 1976. Influence of amino acids, mammalian serum and osmotic pressure on the proliferation of *Drosophila* cell lines. *J. Insect Physiol.* 22:1581–1586.
Youngblom, J., J. A. Schloss, and C. D. Silflow. 1984. The two β-tubulin genes of *Chlamydomonas reinhardtii* code for identical proteins. *Mol. Cell. Biol.* 4:2686–2696.
Zöller, M. J., and M. Smith. 1982. Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for the production of point mutations in any fragment of DNA. *Nucleic Acids Res.* 10:6487–6500.