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Structurally Similar *Drosophila* α-Tubulins Are Functionally Distinct In Vivo

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We used transgenic analysis in *Drosophila* to compare the ability of two structurally similar α-tubulin isoforms to support microtubule assembly in vivo. Our data revealed that even closely related α-tubulin isoforms have different functional capacities. Thus, in multicellular organisms, even small changes in tubulin structure may have important consequences for regulation of the microtubule cytoskeleton. In spermatogenesis, all microtubule functions in the postmitotic male germ cells are carried out by a single tubulin heterodimer composed of the major *Drosophila* α-84B tubulin isoform and the testis-specific β2-tubulin isoform. We tested the ability of the developmentally regulated α85E-tubulin isoform to replace α84B in spermatogenesis. Even though it is 98% similar in sequence, α85E is not functionally equivalent to α84B. α85E can support some functional microtubules in the male germ cells, but α85E causes dominant male sterility if it makes up more than one-half of the total α-tubulin pool in the spermatids. α85E does not disrupt meiotic spindle or cytoplasmic microtubules but causes defects in morphogenesis of the two classes of singlet microtubules in the sperm tail axoneme, the central pair and the accessory microtubules. Axonemal defects caused by α85E are precisely reciprocal to dominant defects in doublet microtubules we observed in a previous study of ectopic germ-line expression of the developmentally regulated β3-tubulin isoform. These data demonstrate that the doublet and singlet axoneme microtubules have different requirements for α- and β-tubulin structure. In their normal sites of expression, α85E and β3 are coexpressed during differentiation of several somatic cell types, suggesting that α85E and β3 might form a specialized heterodimer. Our tests of different α-β pairs in spermatogenesis did not support this model. We conclude that if α85E and β3 have specialized properties required for their normal functions, they act independently to modulate the properties of microtubules into which they are incorporated.

INTRODUCTION

Eukaryotic cells use the α,β-tubulin heterodimer to construct diverse microtubule-based structures that are essential for cell shape, cell division, and cell motility. In addition to the tubulins, each microtubule structure has its own suite of constituent proteins. Many organisms express multiple tubulin isoforms, distinct but related proteins encoded in small multigene families. A key question is the role of differential tubulin gene expression in controlling the specificity of cellular microtubule function. There are two general answers possible to this question: regulated expression of equivalent isoforms allows temporal and spatial control over the amount of tubulin in different cell types, or, alternatively, each isoform has distinct properties, and thus differential expression of multiple tubulin genes provides functional diversity of heterodimers. The answer to the question depends on the kind of organism in which one asks the question (reviewed in Raff, 1994). In fungi, even quite divergent

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isoforms are functionally interchangeable (at least for growth under optimal laboratory conditions), suggesting that in these organisms the first answer is primarily the correct one (Schatz *et al.*, 1986; May, 1989; Kirk and Morris, 1993). For multicellular organisms, the situation is more complicated: patterns of tubulin gene expression coupled with functional tests reveal isoform specialization and differential gene expression as a means to control time, place, and amount of tubulin synthesis.

Functional specialization both of α- and β-tubulin isoforms has been documented in *Drosophila*. In a previous experiment, we asked whether the structurally divergent β3-tubulin isoform could replace the conserved sequence testis-specific β2-tubulin isoform in spermatogenesis (Hoyle and Raff, 1990). We found that β3 did not possess the assembly properties required for microtubule function in the male germ cells. β3 could provide wild-type function for one class of cytoskeletal microtubules, but it was unable to support axoneme assembly or other microtubule-mediated processes specific to spermatogenesis. Further experiments revealed features of the β2 sequence required for axoneme assembly and other microtubule functions in spermatogenesis (Fackenthal *et al.*, 1993, 1995; Hoyle *et al.*, 1995). Similarly, genetic analysis of *Drosophila* α-tubulin function has revealed that a divergent maternally expressed isoform, α67C, and the conserved sequence isoform, α84B, are both essential for early development (Matthews and Kaufman, 1987; Matthews *et al.*, 1993).

In this study we compared the functional capacity of the *Drosophila* α85E-tubulin isoform with that of the α84B-tubulin isoform by testing the ability of α85E to function in the male germ line. α84B is the predominant *Drosophila* α-tubulin, constitutively expressed in all cells; α85E is a minor isoform coexpressed with α84B in a restricted set of somatic cell types (Theurkauf *et al.*, 1986; Matthews *et al.*, 1989, 1990). Our experiments had two broad aims. The first was to test functional equivalency of closely related tubulins. Previous studies compared diverged sequence isoforms with conserved isoforms (e.g., β3 with β2 and α67C with α84B). Herein, we compared two α-tubulin isoforms that have distinct patterns of expression but are similar in sequence to each other (98% similar, 96% identical) and to major α-tubulins in other species. The second aim was to examine the functional significance of the fact that α85E and β3 are coexpressed in many somatic cell types. Both of these isoforms are primarily utilized in differentiating cells undergoing shape changes or cytoskeletal rearrangements, suggesting that they might have common distinctive features (Kimble *et al.*, 1989, 1990; Matthews *et al.*, 1990; Dettman *et al.*, 1996). Tests of microtubule function in the male germ line allowed us to determine whether the α85E-β3 heterodimer imparts properties on microtubule assembly distinct from those when α85E or β3 are paired with other isoform partners.

The data we present show that even though they are structurally very similar, *Drosophila* α84B and α85E have distinct functional capacities, suggesting that isoform specialization plays a key role in the control of microtubule assembly in complex metazoa. When it is the major α-tubulin isoform in the tubulin pool in the male germ cells, α85E can provide function for microtubule arrays such as spindles and cytoskeletal microtubules that are similar to those in other cells, but it causes dominant defects in assembly of the motile axoneme, a microtubule structure that is unique to the male germ line. Comparison of the dominant effects of α85E and β3 on axoneme assembly reveals that ectopic expression of either of the two heterologous isoforms disrupts distinct subsets of microtubules within the axoneme. Thus, different domains of α- and β-tubulin are required to generate the architecture of different microtubules within the axoneme. However, we found no compelling evidence for preferential formation or novel properties of a specific α85E-β3 heterodimer pair.

MATERIALS AND METHODS

Drosophila Stocks and Tubulin Mutations

Stocks were maintained at 25°C on a standard agar/molasses medium. *Drosophila* tubulin genes and mutant alleles are summarized in Lindsley and Zimm (1992). The gene that encodes the constitutive α84B-tubulin isoform is *αTub84B*; mutant alleles are designated $\alpha84B^{n}$. The α85E isoform is encoded by *αTub85E*; mutant alleles have not been isolated. The male germ line-specific β2-tubulin isoform is encoded by *βTub85D*; mutant alleles are designated $B2t^{n}$. The developmentally regulated β3-tubulin isoform is encoded by *βTub60D*.

Several α-tubulin mutations were used in this study: 1) $Df(3R)Scx^{4}$, a deficiency chromosome that deletes the region 84B3–84D1–2, including the *α84B*-tubulin gene (Hazelrigg and Kaufman, 1983). For simplicity, we refer to this chromosome as *Df(α84B)*. 2) EMS-induced recessive lethal mutations in the *α84B* gene, $\alpha84B^{2}$, $\alpha84B^{5}$, and $\alpha84B^{6}$, isolated and described by Matthews and Kaufman (1987) and a recessive viable male sterile allele, $\alpha84B^{nc33}$, isolated as a second-site noncomplementing mutation for recessive loss-of-function mutations in the germ line-specific β2-tubulin gene (Raff and Fuller, 1984). All of these are hypomorphic alleles that encode stable protein products electrophoretically indistinguishable from wild-type α84B. The $\alpha84B^{nc33}$ allele was molecularly characterized as a substitution of methionine for a highly conserved valine at residue 177 in the α84B protein (Hays *et al.*, 1989); the phenotype in spermatogenesis is described in this study. The molecular lesions are not known for the other *α84B* mutations. Animals that are homozygous or hemizygous for the weakest allele, $\alpha84B^{nc33}$, are viable and male sterile (females are sterile or very weakly fertile). The most severe allele $\alpha84B^{5}$ is homozygous and hemizygous lethal and is also lethal in combination with all other recessive lethal alleles, but in combination with $\alpha84B^{nc33}$, it yields viable but sterile adults. Other recessive lethal alleles are also viable but sterile in combination with $\alpha84B^{nc33}$. In addition, the allele combination $\alpha84B^{2}/\alpha84B^{6}$ also yields viable but sterile adults.

β-Tubulin mutations used in this study are EMS-induced recessive male-sterile mutations in the β2-tubulin gene that have been previously characterized at the phenotypic and molecular levels

(Fuller *et al.*, 1987, 1988; Rudolph *et al.*, 1987; Fackenthal *et al.*, 1995): $B2t^{null}$ is a protein null allele, and $B2t^{6}$, $B2t^{7}$, and $B2t^{8}$ are three alleles that encode stable electrophoretic β2-tubulin variants with different defects in microtubule assembly.

Transgenic Drosophila *Stocks with Altered Tubulin Gene Expression*

We utilized a number of *Drosophila* stocks carrying transgenic inserts that result in altered tubulin gene expression in the male germ cells. Stocks carrying the following P-element inserts have been characterized previously: 1) p[α84B], a transgenic insert carrying a wild-type copy of the *α84B* gene generated by Matthews *et al.* (1993). In the experiments reported herein, we used a stock carrying p[α84B] on the second chromosome. 2) p[β3*], a transgenic insert that supports expression of the β3-tubulin isoform in the male germ line, directed by the β2 gene 5′ regulatory sequences (Hoyle and Raff, 1990; Hoyle *et al.*, 1995). In the experiments reported herein, we used a stock carrying the third chromosome p[β3*] insert IIIC that yields β3 expression at a level such that males of genotype *p[β3*], $B2t^{+}$/$B2t^{+}$* are fertile, whereas males of genotype *p[β3*], $B2t^{+}$/p[β3*], $B2t^{+}$* are sterile.

New transgenes generated for this study are p[α85E] that supports expression of α85E-tubulin in the postmitotic male germ cells and p[AS-α84B], an antisense sequence that depresses the endogenous male germ line α84B pool. These constructs use regulatory elements from the germ line-specific β2-tubulin gene and are diagrammed in Figure 1; details of their construction are given below. The constructs were introduced into the *Drosophila* genome by P-element transformation, carried out as described previously (Hoyle and Raff, 1990; Fackenthal *et al.*, 1993). We generated multiple genomic insertions for each of the constructs; only homozygous viable lines were retained. In our experiments, we used several different inserts of each construct; thus, we are confident that the resulting phenotypes result from expression of the inserted sequences and do not reflect site of insertion effects.

Construction of p[α85E]. To test tubulin function, it is necessary to achieve the correct developmental pattern of expression and also the correct tubulin pool size. We previously demonstrated that a 4.5-kb genomic β2-tubulin fragment contains sequences sufficient to support full wild-type expression of β2-tubulin (Figure 1a; Hoyle and Raff, 1990), and it has been shown that sequences contained within the 5′ noncoding region of the β2-rescuing fragment are both necessary and sufficient to drive expression of other β-tubulins and reporter constructs (Michiels *et al.*, 1989, 1991; Hoyle and Raff, 1990; Fackenthal *et al.*, 1993; Hoyle *et al.*, 1995) with the correct developmental timing and localization in the postmitotic male germ cells. The 3′ noncoding region of the rescuing fragment is required for the correct level of β-tubulin expression (Hoyle *et al.*, 1995). A male germ line expression vector that drives the expression of test sequences with the same developmental pattern and level as the β2 gene was generated from the genomic *Sal*I-*Xba*I fragment by removal of the *Apa*I-*Eco*RI fragment containing the β2 coding sequence. The *Apa*I site at position +157 was trimmed and converted to an *Eco*RI site by the addition of *Eco*RI linkers. Subsequent digestion with *Eco*RI followed by religation removed all β2-coding sequences 5′ to the *Eco*RI site at position +1542. The β2 3′ sequence flanking the test sequence thus contains the last 13 codons of the β2 gene, the two adjoining β2 stop codons, the entire β2 3′ untranslated sequence, and 3′ flanking genomic sequence (the α85E sequence used to make p[α85E] contains its own stop codon). After deletion of the β2 coding sequence, the *Sal*I-*Xba*I fragment was cloned into pCaSper4 (Pirrotta, 1988) to produce the male germ line expression vector (Figure 1a). p[α85E] was generated by cloning the 1860-bp *Dra*I-*Hin*cII fragment of a genomic α85E clone (Figure 1b; Matthews *et al.*, 1989) into the male germ line expression vector. As supplied by D. Miller, the *Dra*I site at position +68 (28 bp 5′ to the AUG start codon) had been converted to an *Eco*RI site. The 3′ *Eco*RI site was acquired by subcloning the α85E sequence into Bluescript (Stratagene, La Jolla, CA) using the α85E *Hin*cII site at position +1928, allowing subsequent use of the adjacent *Eco*RI site in the Bluescript vector.

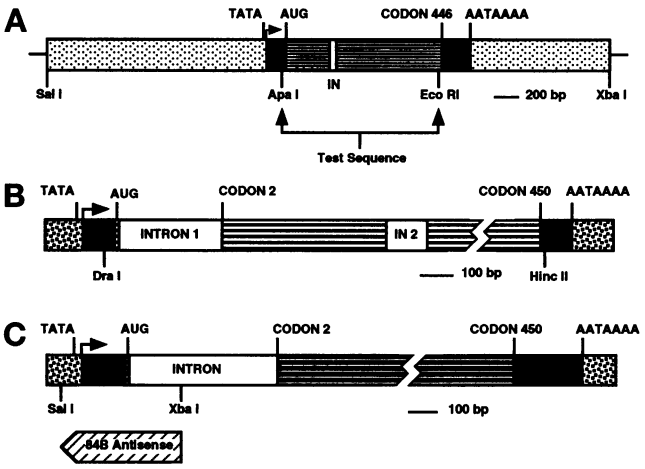

Figure 1. Transgenic constructs that support altered α-tubulin expression in the postmitotic male germ line. Diagrams show *Drosophila* tubulin gene sequences used to generate the transgenic constructs made in this study: coding regions, horizontally hatched; transcribed but nontranslated regions, solid; intron sequences, open; nontranscribed genomic sequences, fine stipples (β2 gene in a) or large stipples (α-tubulin genes in b and c). (a) The 4.5-kb genomic fragment of the β2-tubulin gene that we have previously shown is sufficient to support full wild-type expression in the postmitotic male germ line (Hoyle and Raff, 1990; Hoyle *et al.*, 1995). As described in MATERIALS AND METHODS, the β2-coding sequence was replaced by the test sequences shown in b and c; the resulting transgenic constructs were introduced into the *Drosophila* genome by P-element-mediated transformation. (b) The *Drosophila α85E*-tubulin gene. The *Dra*I-*Hin*cII fragment from the *α85E* gene was inserted into the male germ line expression vector to generate the p[α85E] transgene that supports expression of α85E-tubulin in the postmitotic male germ cells. (c) Top, *Drosophila α84B*-tubulin gene. Bottom, α84B antisense sequence (hatched). The 395-bp *Sal*I-*Xba*I fragment from the *α84B* gene was cloned into the male germ line expression vector to generate the p[AS-α84B] transgene that supports expression of an antisense RNA that disrupts expression of α84B in the postmitotic male germ cells.

We have previously shown that autosomal insertions of transgenic constructs using the β2 gene regulatory sequences support expression at levels equivalent to that of the wild-type β2 gene, whereas X chromosome insertions yield variable and relatively low levels of expression, consistent with the hypothesis that some or all X chromosome-linked genes are inactivated early in spermatogenesis (Hoyle *et al.*, 1995). Similarly, we found that only low levels of α85E synthesis were supported by X chromosome insertions, whereas autosomal insertions yielded levels equivalent to that of the endogenous tubulin genes (Table 1). In this study we used only autosomal p[α85E] insertions. Of the homozygous viable autosomal lines obtained, most were fertile, albeit with reduced fecundity compared with wild-type males. To examine the phenotype when α85E is in excess over α84B, we constructed stocks carrying multiple copies of p[α85E] by recombination. To avoid possible effects of homozygosity for multiple sites of insertion, we concentrated our phenotypic analysis on animals that carry two or three different insertions of p[α85E].

Construction of the Antisense Vector p[AS-α84B]. A 395-bp *Sal*I-*Xba*I Fragment of α84B (Matthews *et al.*, 1989) was placed in an antisense orientation in the male germ line expression vector. This

α84B sequence extends from position −62 to position +233 of the *α84B* gene and includes the first exon (141 bp of 5′ untranslated region plus the AUG start codon) and 192 bp of the first intron (Figure 1c). The *Sal*I and *Xba*I sites were converted into *Eco*RI sites by restriction endonuclease digestion, trimming, and the addition of *Eco*RI linkers. This modified α84B sequence was cloned into the *Eco*RI site of the male germ line expression construct to produce p[AS-α84B]. The p[AS-α84B] construct effectively reduces the level of α84B tubulin in the male germ line.

Consistent with our previous results (Hoyle *et al.*, 1995; and see above), the X chromosome insertions of p[AS-α84B] obtained in the original transformation experiments yielded only low levels of antisense expression, as inferred from the level of reduction of α84B. However, for the genetic manipulations necessary to generate males carrying the desired gene copy number of p[α85E] in a decreased α84B background, we needed to use X chromosome insertions of the antisense construct. We reasoned that there might be regions on the X chromosome that escape early inactivation in spermatogenesis (as is known to occur in mammals) and that if so, we would expect both the *white* gene and the antisense gene contained in the P-element to exhibit higher levels of expression when inserted into such a location. We therefore used a stable source of the P-element transposase (Robertson *et al.*, 1988) to mobilize a low-level-expressing X insert and screened for high-level X chromosome expressers by selecting new X inserts with marked increase in eye color, denoting increased *white* gene expression. We do not know whether our hypothesis about X chromosome function in spermatogenesis is correct or not, but this strategy was successful. We obtained three new X chromosome insertions of p[AS-α84B] that showed both increased eye pigmentation and concomitant increased levels of antisense expression, as indicated by the extent of reduction of α84B. In the experiments reported herein, we used both autosomal p[AS-α84B] insertions and the high-expressing X chromosome insertions, which reduced α84B expression in the male germ cells only slightly less than the autosomal insertions (Tables 1 and 2). The difference in expression level is reflected in the fact that males carrying the high-expressing X chromosome insertions were weakly fertile, whereas males homozygous for autosomal insertions were sterile.

Determination of Male Fertility

In *Drosophila*, sperm motility is required for exit of mature sperm from the testes into the seminal vesicles (see discussion in Hoyle *et al.*, 1995); thus, fertility can be scored both by production of motile sperm and mating tests. Sperm production was scored in males held apart from females from shortly after eclosion for 5 to 7 d. All fertility tests were carried out at 25°C, since male fertility is intrinsically temperature dependent. Different fertility classes were defined as follows: Fertile, most males are fertile and sperm production and fecundity in mating tests are equivalent to that of wild-type males; fertile, reduced fecundity (Fertile, R. F.), most males are fertile and sperm production and fecundity are variable but reduced compared with wild type; weakly fertile (W. Fertile), many males are fertile but some males are sterile and sperm production and fecundity of the fertile males are significantly reduced compared with wild type; very weakly fertile (V. W. Fertile), many males are sterile but some are fertile and males that are fertile produce very small amounts of sperm and give very few progeny in mating tests; Sterile, most or all males do not produce any sperm and no progeny are produced in mating tests.

Table 1. Tubulin synthesis in the postmitotic male germ cells is proportional to tubulin gene dose

	No. of copies of α-tubulin genes in test males				Total α gene dose	β2 gene dose	Ratio α/β2 gene dose	Ratio α/β2 synthesis (n)	Fertility phenotype
	p[α85E]	p[AS-α84B]	p[α84B]	α84B$^+$					
a:	—	—	—	2	2	2	1.0	1.1 (39)	Fertile
b:	—	—	—	2	2	1	2.0	2.3 (2)	Fertile, R. F.
c:	—	—	—	1	1	2	0.5	0.62 (8)	Fertile, R. F.
d:	—	—	—	1	1	1	1.0	1.1 (1)	W. fertile
e:	—	—	1	1	2	2	1.0	0.95 (4)	Fertile
f:	—	—	1	2	3	2	1.5	1.3 (5)	Fertile
g:	—	—	2	2	4	2	2.0	2.1 (2)	Fertile
h:	—	1(X)	—	2	<2	2	<1.0	0.65 (13)	Fertile, R. F.
i:	—	1(A)	—	2	<2	2	<1.0	0.60 (8)	W. fertile
j:	—	1(A)	—	1	<1	2	<0.5	0.44 (2)	Sterile
k:	—	2(A)	—	2	≪2	2	≪1.0	0.47 (4)	Sterile
l:	1	—	—	1	2	2	1.0	1.0 (5)	W. fertile
m:	2	—	—	2	4	2	2.0	2.4 (6)	Fertile, R. F.
n:	4	—	—	2	6	2	3.0	3.6 (6)	Sterile

Testis tubulins were displayed on 2D gels and [^{35}S]methionine incorporation was quantified as described in MATERIALS AND METHODS. The endogenous *α84B*-tubulin gene is indicated by α84B$^+$; males with two copies are wild type at the *α84B* locus, and males with one copy are genotype *α84B$^+$/Df(α84B)*. All males express the germ line-specific β2-tubulin as the sole β-tubulin in the postmitotic male germ cells. Males with two copies of the β2 gene are wild type at the β2 locus, and males with one copy are genotype $B2t^+/B2t^{null}$. The α/β2 tubulin synthesis ratio for each genotype is the average obtained in multiple experiments; the number of gels (n) analyzed is shown in parentheses. Fertility phenotypes (defined in MATERIALS AND METHODS) were also determined for each genotype; see Table 2 for details. Experiments a–d, data for males with different gene doses of the wild-type endogenous germ line tubulins expressed in the postmitotic male germ cells (data for wild-type males in experiment a includes males from wild-type stocks plus siblings of other experimental test males). Experiments e and f, data for males with additional copies of the wild-type *α84B* gene carried on the p[α84B] transgene generated by Matthews *et al.* (1993). Experiments h–k, data for males in which *α84B* expression in the postmitotic male germ cells is depressed by the antisense sequence expressed from the p[AS-α84B] transgene. Experiments l–n, data for males that express α85E in the postmitotic male germ cells from the p[α85E] transgene.

Antibodies

We used the following antibodies to detect α-tubulins: 1) A commercially available anti-α-tubulin monoclonal antibody (Amersham N356, Amersham, Arlington Heights, IL) that strongly recognizes *Drosophila* α84B but gives only very weak staining of α85E (Matthews *et al.*, 1990). We refer to this antiserum as anti-α84B. 2) Two anti-α85E-specific antisera: We used the polyclonal antiserum generated by Matthews *et al.* (1990), directed to a fusion peptide containing the 12 C-terminal amino acids of α85E. This antiserum recognizes α85E but does not bind to α84B. Because the original antiserum was in short supply, for this study another similar polyclonal antisera specific for α85E was generated (Research Genetics, Birmingham, AL), directed to a 13-amino acid synthetic peptide representing the α85E C terminus. These antisera have very similar properties and we refer to them collectively as anti-α85E. 3) A polyclonal antiserum generated by Matthews *et al.* (1990) directed to a fusion peptide containing the 21 C-terminal amino acids of α85E. This antiserum exhibits preferential binding to α85E but recognizes both α85E and α84B. We refer to this antiserum as anti-α85E/α84B. 4) An antiserum generated by Piperno and Fuller (1986) specific to acetylated α-tubulins (Piperno clone 6–11B-1, Sigma T6793, Sigma).

To detect β-tubulin, we used a commercial anti-β-tubulin monoclonal antibody (Amersham N357) that recognizes all *Drosophila* β-tubulins (Kimble *et al.*, 1989; Hoyle and Raff, 1990). In addition, in some experiments we used a commercial anti-actin monoclonal antibody (Amersham N350).

Two-Dimensional (2D) Gel Electrophoresis and Antibody Staining

For gel analysis of testis proteins, testes were dissected from adult males within 5 d of eclosure and labeled for 1 h with [^{35}S]methionine. Labeling, sample preparation, 2D gel electrophoresis, and transfer to nitrocellulose were done as described previously (Hoyle *et al.*, 1995). Protein samples for 2D gels of total testis proteins were prepared from testes from five males. Sperm protein samples were prepared from mature motile sperm isolated from the seminal vesicles of 10 males held away from females for 7 to 10 d. To provide a radioactive marker for comparison of the position of testis and sperm proteins on the gels, sperm gel samples also included [^{35}S]methionine-labeled testis proteins equivalent to one-tenth of a testis; this amount of testis proteins is not detectable on blots by antibody staining. To achieve maximal resolution of α- and β-tubulins, an "expanded scale" isoelectric focusing dimension was used, consisting of a pH range generated by an ampholyte mixture of one part pH 3.5–10 ampholytes and two parts pH 5–6 ampholytes (Serva Ampholines, Serva, Heidelberg, Germany). In this gel system, *Drosophila* α-tubulins separate into a closely spaced doublet in the SDS dimension, as has been described previously (Raff *et al.*, 1982; Matthews *et al.*, 1989); the split in the SDS dimension is variable and its basis is not known. Antibody staining of gel blots was done by following the Vectastain protocol (Vector Laboratories, Burlingame, CA). Proteins were visualized using horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA).

Table 2. Effect of the size and α-tubulin isoform composition of the germ line tubulin pool on male fertility

No. of copies of α-tubulin genes in test males					Fertility phenotype (fraction of males that produce sperm)
A. Males that express only α84B-tubulin in the germ line					
	p[AS-α84B]	p[α84B]	α84B$^+$	Total *α84B* gene dose	
a:	—	—	2	2	Fertile (138/138)
b:	—	2	2	4	Fertile (52/52)
c:	—	—	1	1	Fertile, R. F. (61/71)
d:	1(X)	—	2	<2	Fertile, R. F. (45/51)
e:	1(A)	—	2	<2	W. fertile (40/75)
f:	1(X)	—	1	<1	Sterile (0/66)
g:	1(X)	1	1	<2	Fertile, R. F. (40/41)

B. Males that express both α85E-tubulin and α84B-tubulin in the germ line						
	p[α85E]	p[AS-α84B]	α84B$^+$	Total α84B gene dose	Ratio α85E:α84B	
a:	1	—	2	2	0.5	Fertile (229/232)
b:	2	—	2	2	1	Fertile, R. F. (203/260)
c:	1	—	1	1	1	W. fertile (35/70)
d:	2	—	1	1	2	Sterile (0/62)
e:	4	—	2	2	2	Sterile (0/67)
f:	2	1(X)	2	<2	>1	Sterile (5/80)
g:	2	1(A)	2	<2	>1	Sterile (0/70)

α-Tubulin genes expressed in the male germ line are designated as in Table 1; all males were wild type at the β2 locus. Fertility phenotypes were determined by scoring sperm production and fecundity in mating tests as described in MATERIALS AND METHODS. A, Fertility of males in which the size of the germ line tubulin pool was varied by changing the gene dosage for α84B-tubulin. The total tubulin pool size is wild type in males in which the total *α84B* gene dose is two or greater and is depressed relative to wild type in males in which the total *α84B* gene dose is less than two. B, fertility of males that express both α85E and α84B in the postmitotic male germ cells. The total tubulin pool size is equivalent to wild type in all males shown, since the total α-tubulin gene dose (sum of *p[α85E]* gene dose and total *α84B* gene dose) is at least two. In addition to the genotypes shown above, males of all other genotypes tested in which the ratio of α85E:α84B is >1 were sterile.

Quantitation of Testis Tubulins

Western blots reflect the relative contribution of different isoforms to the total tubulin pool in transgenic males (e.g., see Figure 2). However, because of differences in the affinities of different antibodies and variability of staining reactions, antibody staining is unreliable for accurate quantitation of different species in the tubulin pool. We used incorporation of [^{35}S]methionine into testis proteins to examine tubulin synthesis; comparison of antibody staining to synthesis levels showed that the heterologous α85E isoform exhibits stability comparable to that of the endogenous α84B isoform, just as we previously demonstrated for heterologous β-tubulins expressed in the male germ line (Hoyle and Raff, 1990; Hoyle *et al.*, 1995). Therefore, by measuring [^{35}S]methionine incorporation, we could quantify the relative contributions of α85E and α84B to the tubulin pool in males with different gene doses for the two isoforms. Testis tubulins from males of desired genotypes were displayed on 2D gels, and the [^{35}S]methionine signals were quantified with a Molecular Dynamics PhosphorImager system (Sunnyvale, CA). To control for gel conditions, samples from wild-type males were electrophoresed in parallel with samples from experimental males in all experiments. The ratio of α/β2 tubulin synthesis in testes of males of each genotype was determined as the ratio of the signal for [^{35}S]methionine incorporation into each species, after dividing the raw value for the signal in α-tubulins by 0.61 to normalize for the difference in methionine content in the two subunits (α84B and α85E each contain 11 methionine residues per molecule, compared with 18 methionine residues in β2).

Cytology and Ultrastructure

Light microscopic analysis of testis preparations and transmission electron microscopy were performed as described previously (Hoyle *et al.*, 1995).

RESULTS

Expression of the α85E-Tubulin Isoform in the Drosophila *Male Germ Line: Posttranslational Modification Patterns of α85E Differ from the Endogenous Germ Line α84B Isoform*

To compare the functional properties of α85E with α84B, we expressed the α85E isoform in the male germ line, a tissue not required for viability of the fly, that offers the opportunity to examine the ability of α85E to support multiple microtubule arrays with distinct architectures and functions. The transgenic constructs utilized are diagrammed in Figure 1. We generated transgenic stocks that express the α85E coding sequence in the male germ line, driven by regulatory sequences from the male germ line-specific β2-tubulin gene (Figure 1, a and b). Data presented below show that autosomal insertions of the p[α85E] transgene support expression of α85E-tubulin in the postmitotic male germ cells at levels equivalent to endogenous testis tubulins.

We used several approaches to generate males with different amounts of α85E relative to α84B in the germ-cell tubulin pool (see MATERIALS AND METHODS for details): 1) We manipulated the amount of α85E expressed by using stocks carrying multiple copies of the p[α85E] transgene. 2) We reduced the number of functional copies of the α84B-tubulin gene by using a deficiency chromosome or mutant α84B alleles (Matthews and Kaufman, 1987; Hays *et al.*, 1989). 3) Since the α84B-tubulin gene is ubiquitously expressed and essential for viability of the fly, we could not eliminate α84B in the testis genetically. To further reduce α84B in the postmitotic male germ cells, we used an antisense construct expressed exclusively in the male germ line (Figure 1c). Because of the close similarity of the coding sequences of α84B and α85E, it was not feasible to use a cDNA-based antisense construct; we therefore designed an antisense to interrupt processing of the primary α84B transcript. To our knowledge, this kind of approach has not been previously reported in *Drosophila*; as we show below, p[AS-α84B] causes significant reduction in testis expression of α84B.

Figure 2 shows accumulation of tubulins in testes and sperm of transgenic males in which α85E is coexpressed with α84B in the germ line. As we previously showed for the endogenous α84B (Raff and Fuller, 1984), both the primary α85E synthetic product and a major modified form are accumulated in the total germ line tubulin pool (Figure 2b). The modified form is the major species incorporated into mature sperm (Figure 2c). Previous studies in several systems, including *Drosophila*, have shown that the α-tubulin incorporated into the motile axoneme is acetylated (L'Hernault and Rosenbaum, 1985; Piperno and Fuller, 1985; le Dizet and Piperno, 1987). The acetylation site is lysine 40, a conserved residue present in most α-tubulins, including both *Drosophila* α84B and α85E. Figure 3 shows that the modified form of α85E, like that of α84B, is acetylated. Acetylation neutralizes the basic lysine side chain; consistent with this, in the isoelectric-focusing dimension of 2D gels, the modified form of α85E migrates to a position one charge more negative that the primary synthetic α85E product (Figures 2c, right, and 3a). The major modified form of α84B, however, migrates two charges more negative than the primary synthetic α84B product (Figures 2b, top left, and 3b), suggesting that α84B undergoes another modification in addition to acetylation. In addition, staining by the anti-acetylated α-tubulin antiserum revealed that there are multiple modified forms of α84B in the accumulated tubulin pool (Figure 3, right), although only the major modified form is present in sufficient amount to be stained by the anti-α84B antiserum (Figure 2, compare b and c).

Another major posttranslational modification of α-tubulin is glutamylation (Eddé *et al.*, 1990, 1991; Audebert *et al.*, 1993; Bré *et al.*, 1994; Fouquet *et al.*, 1994). Multiple glutamyl residues may be added to the carboxyl group of glutamic acid residues in the α-tubulin C terminus; each glutamyl residue adds a negative charge to the protein, generating a series of modified forms with successively more acid isoelectric focusing points. Our preliminary data suggest that the

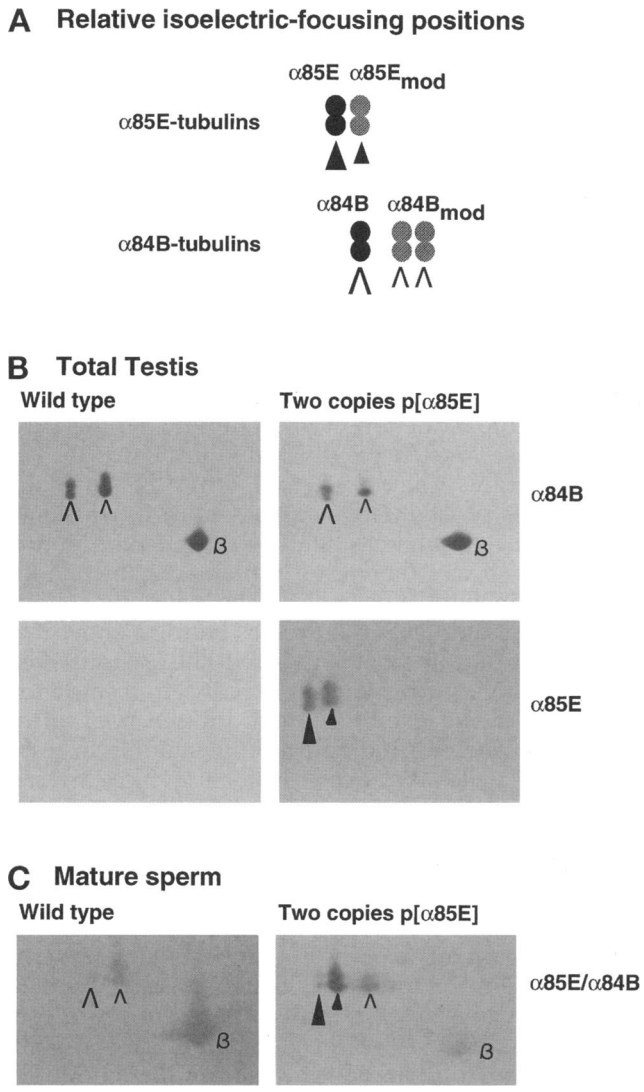

Figure 2. Tubulins in testes and mature sperm of wild-type males and transgenic males that express both α85E and α84B in the germ line. (a) Diagram of the position of α-tubulins in the isoelectric focusing dimension of 2D gels, showing the relative positions of the primary synthetic product and posttranslationally modified forms of α85E and α84B. In the diagram and all figures, the acidic region of the gel is to the right. All of the forms of α85E and α84B display the same relative mobility in the SDS dimension; thus, in 2D gels, the primary synthetic product of α84B comigrates with the posttranslationally modified form of α85E. As discussed in MATERIALS AND METHODS, the *Drosophila* α-tubulins separate on 2D gels into a closely spaced doublet in the SDS dimension, resulting in the dumbbell-shaped spot shown. The following convention, as illustrated, is used in all figures to designate the α-tubulin species present: closed arrowheads, α85E; open arrowheads, α84B. For each α-tubulin isoform, large arrowheads indicate the position of the primary synthetic product, and small arrowheads indicate the position of posttranslationally modified forms. Upward facing arrowheads indicate antibody-stained α-tubulins on Western blots of 2D gels. In figures in which autoradiograms of [^{35}S]methionine incorporation into testis tubulins are shown, downward facing arrowheads indicate the positions of newly synthesized α-tubulins. In some of the gels, the positions of β2-tubulin (β) and actin (A) are also indicated for reference. Antibodies specific to α85E, α84B, α85E/α84B, β-tubulin, and actin are described in MATERIALS AND METHODS. (b and c) Portions of 2D gels showing the tubulin region of Western blots of 2D gels stained to show α-tubulins and β2-tubulin as indicated. (b) Western blots of total testis proteins in wild-type males (left) and transgenic males that have two copies of p[α85E] in addition to the two endogenous copies of the wild-type *α84B* gene (right) stained with anti-α84B and anti-β (top) or anti-α85E (bottom). Total testis proteins contain both the primary synthetic α-tubulin products and modified forms. In wild-type males, only a slight trace of α85E is present due to the α85E expressed in the somatic cyst cells from the endogenous *α85E* gene (not significantly detectable by antibody staining; see bottom left). The α85E accumulated in testes of transgenic males (bottom right) reflects the germ line expression from the p[α85E] transgene. In transgenic males, the accumulated pool in the germ line contains both α84B and α85E; the total tubulin pool is constant (compare β2 staining in top), but the amount of α84B is correspondingly decreased when both α85E and α84B are expressed (compare α84B signal in top left and top right). (c) Western blots of proteins in mature motile sperm isolated from the seminal vesicles of fertile males stained with anti-α85E/α84B and anti-β. Left, sperm proteins from wild-type males; right, sperm proteins from males with two copies of p[α85E] and two wild-type *α84B* genes. It has been shown previously that the β2 gene regulatory sequences drive expression only in the postmitotic germ cells (Michiels *et al.*, 1989, 1991; Hoyle and Raff, 1990; Hoyle *et al.*, 1995). Germ line expression of α85E is confirmed by its presence in motile sperm of fertile transgenic males. Modified forms of both α85E and α84B are the majority of α-tubulins incorporated into the motile sperm tail axoneme.

differences in electrophoretic mobilities of the modified forms of α84B and α85E may be explained by differences in glutamylation state. The fact that α84B and α85E are handled differently in the male germ cells demonstrates that the two isoforms are biochemically distinct. The phenotypic analysis presented below documents that they also have distinct functional properties.

Tubulin Synthesis in the Male Germ Line Is Proportional to Gene Copy Number: An Antisense Sequence Directed to Unprocessed α84B Message Reduces α84B Synthesis

To test the capacity of the α85E isoform to function in the male germ line, we had to first determine that we could reliably manipulate the size and isoform composition of tubulin pools in the postmitotic male germ cells. We have previously shown that the level of β2-tubulin synthesis in the male germ cells reflects transcription levels and is directly proportional to gene copy number (Hoyle *et al.*, 1995). As illustrated in Figure 4, top, autoradiograms of 2D gels of testis proteins from transgenic and wild-type males showed that α-tubulin synthesis is likewise proportional to gene dose (also compare the autoradiograms in Figure 3). We quantified testis tubulin pools in males of different genotypes, as described in MATERIALS AND METHODS. These data are summarized in Table 1 and confirm that both α- and β-tubulin synthesis in

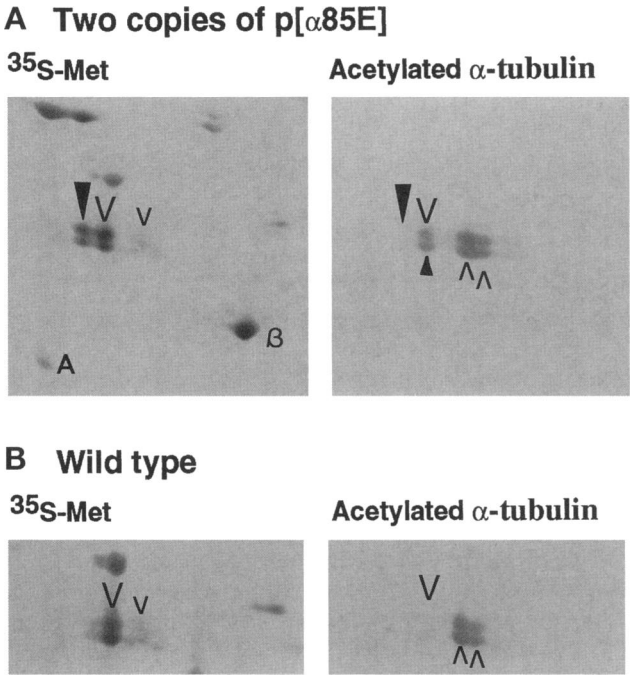

Figure 3. Acetylated forms of α85E and α84B in the male germ line. Left, autoradiograms of the tubulin region of blots of 2D gels of testis proteins, showing [^{35}S]methionine incorporation during a 1-h labeling period. Right, same gels stained with the anti-acetylated α-tubulin antibody (Piperno and Fuller, 1985). Designations for the primary synthetic products and modified forms of α-tubulins are as described in Figure 2a. For reference, the positions of the primary α85E and α84B synthetic products are also indicated on the antibody-stained blots. (a) Testis proteins in transgenic males with two copies of p[α85E] and two wild-type *α84B* genes. The autoradiogram (left) shows synthesis of α85E and α84B. A trace of modified α84B is also visible on the autoradiogram, representing modification that occurred during the labeling period. (Any modified α85E present comigrates with the primary α84B synthetic product.) Positions of the germ line-specific β2 isoform (β) and actin (A) are also indicated. Staining of total testis tubulins with anti-acetylated α-tubulin antibody (right) shows the modified form of α85E plus two major modified forms of α84B. The acetylated form of α85E migrates to the same position as the primary synthetic α84B product (the stained gel in b demonstrates that this antibody does not recognize the unacetylated primary α-tubulin synthetic products). (b) Testis proteins in control wild-type males. The autoradiogram (left) shows synthesis of α84B; as above, a trace of modified α84B is also labeled. Staining for acetylated α-tubulin (right) shows accumulation of the two major acetylated forms of α84B.

the male germ cells directly parallels gene dose. Furthermore, in wild-type males, α- and β-tubulins are synthesized at equimolar levels in the male germ cells [as is also the case in embryos (Raff *et al.*, 1982)]. The rate of synthesis of α85E from a single insert of p[α85E] is the same as the rate of α84B synthesis from either a single copy of the endogenous wild-type gene or a single insert of the p[α84B] transgene. Thus, the regulatory elements of the constitutive α84B gene and the male germ line-specific β2 gene drive expression in the male germ cells at equivalent levels.

When the antisense construct p[AS-α84B] is present, α84B synthesis is significantly reduced. One copy of p[AS-α84B] in an otherwise wild-type male reduces the synthesis rate to approximately the same as that in a male hemizygous for the endogenous *α84B* gene. The residual synthesis of α84B in males that have two copies of the antisense construct or males that are hemizygous for the α84B tubulin gene and also carry the antisense construct must in the main reflect synthesis from α84B message that persists from the earlier mitotic stages, prior to turn on of the β2 promoter. The fact that the effect of one copy of p[AS-α84B] can be rescued by one copy of p[α84B] shows that the antisense construct acts specifically on α84B synthesis.

The specific reduction of α84B-tubulin synthesis by the antisense construct is illustrated in Figure 4b. The autoradiograms (Figure 4b, top) show that in males that express p[AS-α84B], synthesis of α84B is considerably reduced, whereas synthesis of β2-tubulin and actin, as well as other testis proteins, is unchanged from that in wild-type males. The antibody-stained blots of the same gels (Figure 4b, bottom) show that when the α-tubulin pool is reduced, the accumulation of β2-tubulin is correspondingly reduced, resulting in a decrease in the size of the total tubulin pool in the germ cells. The reduced anti-tubulin staining in testis proteins of males that express p[AS-α84B] clearly reflects a change in the tubulin pools and is not due to different protein loads on the gels or to an overall reduction in testis proteins, as shown by the equivalent levels of actin in the samples from wild-type and transgenic males (compare actin staining in Figure 4b, bottom). Comparison of the antibody staining patterns in wild-type males and males that express p[AS-α84B] effectively visualizes the utility of the antisense construct for reducing α84B levels, but we want to point out that the very marked reduction in the amount of anti-tubulin staining of testis proteins of males that express the antisense construct gives a somewhat misleading impression, in that it invites underestimation of the actual pool. Quantitation from [^{35}S]methionine incorporation (see Figure 4b, top), a more reliable measure of the relative testis tubulin pool sizes, shows that, on average, the tubulin pool is reduced to about half the wild-type level in males that express one autosomal copy of the antisense construct in an otherwise wild-type background (e.g., Table 1, compare experiments i and a).

The total tubulin pool accumulated in the *Drosophila* male germ cells reflects the amount of the limiting subunit (see Raff, 1994; Hoyle *et al.*, 1995). Examination of the tubulin pools in the antisense construct-expressing males shows that when the α subunit is the limiting subunit, excess β-tubulin turns over, even though it is synthesized at the normal rate. We have previously shown that the reciprocal is true; i.e., when the amount of stable β2-tubulin is limiting, excess α-tubu-

lin turns over (Kemphues *et al.*, 1982; Raff and Fuller, 1984). The total amount of tubulin in the pool will thus depend on the amount of the limiting subunit present, while the isoform composition of the pool will reflect the relative synthesis rate of each tubulin species. Thus, for males in which both α85E and α84B are present (e.g., Table 1, experiments l–n), the total tubulin pool will be at the same level as in a wild-type male (i.e., limited by the amount of the β2 subunit), and the α-tubulin pool will be composed of the α85E and α84B isoforms in relative amounts proportional to the gene dose of each isoform. This can be seen in the gels in Figure 2b. Both the wild-type males and transgenic males carrying the p[α85E] construct have two copies of the β2 gene; thus in the transgenic males that express four α-tubulin genes, the limiting subunit in the pool is β2. When both α85E and α84B are synthesized at the level equivalent to that of α84B in a wild-type male, the total amount of α84B is correspondingly diminished, relative to the amount in wild-type males in which α84B is the only α species present (i.e., compare α84B staining in Figure 2b, top).

Functional Consequences of Expression of α85E in the Male Germ Line: α85E Can Support Multiple Classes of Microtubules in the Male Germ Line but Causes Dominant Defects in the Substructure of the Singlet Microtubules of the Axoneme

The data discussed above showed that we could satisfactorily manipulate the composition of the α-tubulin pool in the postmitotic male germ cells. We then examined microtubule-mediated processes in spermatogenesis when α85E was present at various ratios to α84B. Formation and differentiation of the haploid spermatids is directly dependent on four sets of microtubules: the meiotic spindles, two specialized arrays of cytoskeletal microtubules that mediate nuclear shaping and elongation of the mitochondrial derivative, and assembly of the motile sperm tail axoneme (for review, see Tates, 1971; Lindsley and Tokuyasu, 1980; Raff, 1994). In addition, another process requires microtubule function for the successful production of mature sperm: alignment of the developing spermatids within each syncytial bundle, and later, the individualization of each differentiated spermatid. At the completion of meiosis, the newly formed haploid spermatids are oriented so that they face toward the seminal vesicle, with all of the nuclei grouped together. The spermatids remain aligned throughout differentiation; the mature spermatids then become individualized in a process of membrane investment that proceeds from the nucleus to the tip of the sperm tail. Even if they are otherwise normally formed, only spermatids with their nuclei properly aligned at the proximal end of the cyst are individualized; hence, spermatid alignment is crucial for fertility.

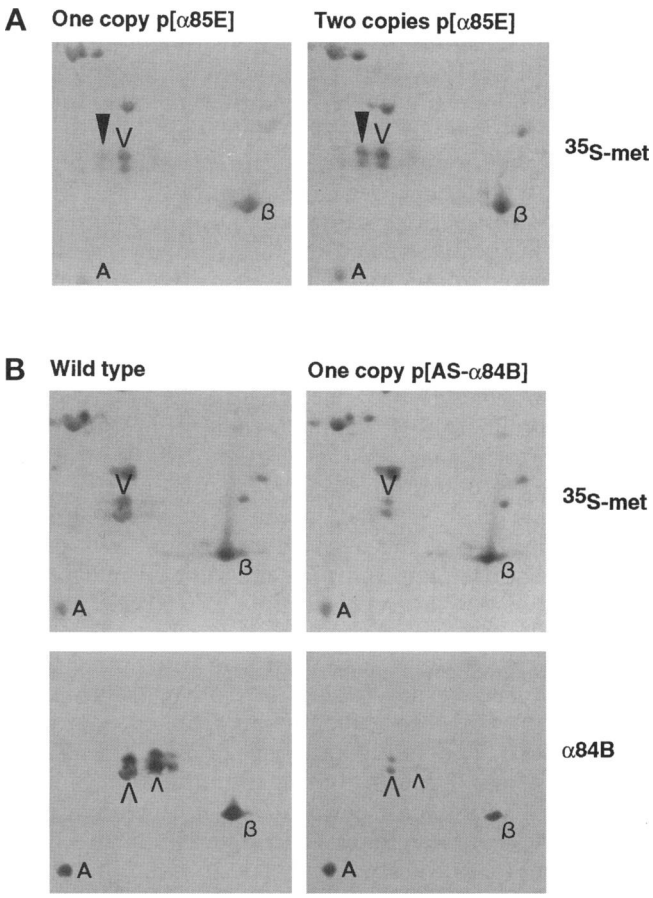

Figure 4. Synthesis and accumulation of tubulins in the testes of wild-type males and transgenic males that express α84B plus either p[α85E] or p[AS-α84B]. The tubulin region of 2D gels of testis proteins of males of the indicated genotype is shown. Designations for α-tubulins are as described in Figure 2a. The positions of the germ line-specific β2 isoform (β) and actin (A) are also indicated. (a) Autoradiograms of 2D gels showing [^{35}S]methionine incorporated into total testis proteins in males that have two copies of the endogenous *α84B* gene and either one copy (left) or two copies (right) of the p[α85E] transgene. α85E synthesis is proportional to gene dose. (b) Testis proteins in wild-type males (left) and transgenic males that have two copies of the endogenous *α84B* gene and one autosomal insert of the p[AS-α84B] antisense transgene (right). Top, autoradiograms showing [^{35}S]methionine incorporation; bottom, same gels stained to show α84B-tubulin, β-tubulin, and actin. Comparison of the [^{35}S]methionine signal in α84B with the signal in other protein spots in wild-type males (top left) and in transgenic males (top right) shows that the antisense specifically reduces synthesis of α84B but has no effect on synthesis of β2-tubulin, actin, or other testis proteins. Visualization of the accumulated tubulin pool by antibody staining (bottom) reveals that when the α-tubulin pool is reduced, the level of β2-tubulin is correspondingly diminished, resulting in a decrease in the total tubulin pool. Total testis proteins are not reduced overall in the transgenic males, as indicated by the actin staining used as an internal loading control.

We have previously established that *Drosophila* spermatogenesis is sensitive to both the amount and isoform composition of the tubulin pool in the male germ

cells and that different microtubule functions are differentially sensitive to perturbations of tubulin structure and of the level of the tubulin pool (Kemphues *et al.*, 1982; Fuller *et al.*, 1987; Fackenthal *et al.*, 1993, 1995; Raff, 1994; Hoyle *et al.*, 1995). To interpret the spermatogenesis phenotype caused by expression of α85E, we had to take both of these factors into consideration. All microtubule functions can potentially be affected by changes in tubulin structure, but axoneme assembly and nuclear shaping are the most sensitive. Alignment and individualization of spermatids are extremely sensitive to changes in the normal tubulin pool size, whereas meiosis and spermatid differentiation are much less sensitive.

As shown in Table 2, fertility is compromised in two categories of males. First, decreasing the size of the germ line testis tubulin pool by decreasing the functional gene dose of α84B in itself adversely affects fertility because of the disruption of spermatid alignment and subsequent individualization. In males that are otherwise wild type, fertility is reduced if the functional *α84B* gene dose in the germ line is less than two, and males with less than one functional *α84B* gene dose are sterile (Table 2A). To avoid affects on male fertility resulting solely from reduced pool size, we carried out phenotype analysis of α85E expression in males of genotypes in which the tubulin pool level in the postmitotic male germ cells is equivalent to that in wild-type males. Expression of α85E in the male germ line disrupts spermatogenesis even in males with wild-type tubulin pool levels. Males in which the amount of α85E in the germ line tubulin pool exceeds that of α84B are sterile (Table 2B).

The data presented in Figures 5 and 6, and Table 3 document the distinctive phenotypes in spermatogenesis of males with reduced but otherwise wild-type tubulin pools and males that express α85E in the germ cells. Reducing the tubulin pool by decreasing the *α84B* gene dose reduces fertility by disrupting spermatid alignment, thereby resulting in production of fewer individualized sperm. However, as long as there is a tubulin pool sufficient to support any microtubule assembly, meiosis and the microtubule-mediated processes of spermatid differentiation occur normally. In males with severely decreased tubulin pools, not all spermatids assemble full-length axonemes, but the morphology of all of the axonemes that are assembled is wild type. In contrast, in males in which α85E is the predominant α-tubulin in the germ cells, sterility results from defective axoneme substructure.

Spermatid alignment is illustrated in Figure 5. Misalignment of spermatids in males with only one copy of the *α84B* gene is shown in Figure 5a. Spermatid misalignment is attributable to reduction of the tubulin pool resulting from the decreased *α84B* gene dose and not to loss of function of other genes also removed in the deletion chromosome, since spermatid misalignment also occurs in males that express the antisense construct from the p[AS-α84B] transgene in a wild-type α84B background (Figure 5, b and c) and in males that carry viable but male-sterile combinations of *α84B* mutations (our unpublished results). Moreover, both spermatid alignment and fertility can be rescued in males of all of these genotypes by supplying wild-type copies of the *α84B* gene with the p[α84B] transgene (Figure 5, d and e, and Table 2). Rescue of spermatid misalignment also provides a functional assay that confirms the specificity of action of the antisense construct on α84B.

We found that the fertility of males in which α85E is expressed primarily depends on the ratio of α85E: α84B in the germ line tubulin pool, although there was some dependence on the absolute amount of α84B. Males with two copies of each gene are fertile, albeit of reduced fecundity compared with wild-type, whereas males with one copy of each gene are only weakly fertile (Table 2B, compare experiments b and c). The difference in sperm production by males of these two genotypes reflects spermatid misalignment in males hemizygous for α84B. In males that are wild type for α84B, spermatid alignment is normal in males with one or two copies of p[α85E]; alignment is perturbed only if the amount of α85E exceeds that of α84B. As shown in Figure 5f, sterile males with four copies of p[α85E] in a wild-type α84B background consistently exhibited defects in spermatid alignment, although the misalignment defects were not severe enough in themselves to cause sterility (compare with Figure 5e). The simplest interpretation of the phenotype illustrated in Figure 5f is that α85E cannot provide full function for alignment, as is consistent with other data on α85E function (Table 4 and see Figure 8). However, males of this genotype have a total of six α-tubulin genes, and we cannot eliminate the possibility that spermatid alignment might be intrinsically sensitive to substantial overexpression of α-tubulin. We know that spermatid alignment is not perturbed in males with four copies of the wild-type *α84B* gene, but the transgenic stocks that we presently have available do not allow us to test the effect of six copies of wild-type *α84B* gene. Another possibility is that the site of insertion effects resulting from homozygosity for both of two p[α85E] inserts adversely affects spermatid alignment, but we think this is unlikely since homozygosity for either insert alone does not cause failure of alignment.

At any dose we have examined, α85E does not perturb meiotic spindles or the cytoplasmic microtubules involved in nuclear shaping or mitochondrial differentiation. Figure 5g illustrates a normal cyst of haploid spermatids produced by meiosis in a male of the same genotype as the male in Figure 5f (four copies of p[α85E] in a wild-type α84B background).

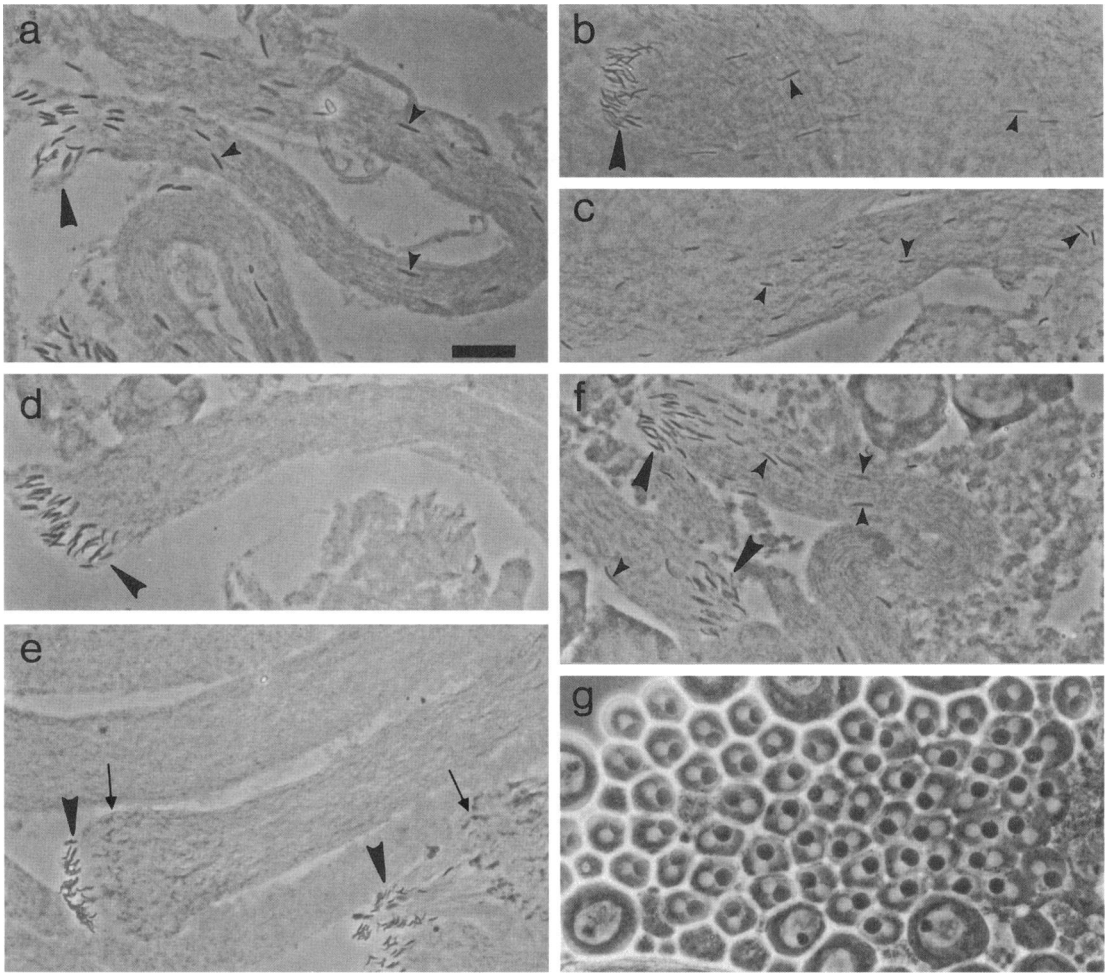

Figure 5. Spermatids in transgenic males. (a–f) Light micrographs of fixed testes stained with orcein to visualize the nuclei of developing spermatids. (a) Spermatid bundles in a male with one copy of the *α84B* gene [genotype *Df(α84B)/α84B*$^{+}$]. Males of this genotype are fertile but with reduced fecundity compared with the wild type; the total testis tubulin pool is approximately half that in wild-type males. Spermatid nuclei are normally shaped, but many spermatids are not properly aligned at the tip of the developing bundle (large arrowhead); nuclei of misaligned spermatids (small arrowheads) can be seen throughout the bundle. This phenotype is the same as in males in which the tubulin pool is reduced by decreasing the amount of β2-tubulin (Kemphues *et al.*, 1982). (b and c) Spermatid bundles in males that have two copies of the endogenous wild-type *α84B* gene and also one X chromosome insert of the p[AS-α84B] antisense construct. Expression of the antisense depresses α84B in the postmitotic germ cells, resulting in reduction of the testis tubulin pool and corresponding failure of spermatid alignment. The phenotype caused by the antisense is the same as in the heterozygous deficiency males in a: Males are fertile, but of reduced fecundity compared with the wild type; many spermatids fail to be aligned at the tip of the developing bundle (large arrowhead); and nuclei of misaligned spermatids (small arrowheads) can be seen throughout the bundles. (d and e) Spermatids in a fertile male that has one X chromosome p[AS-α84B]insert plus three copies of the wild-type *α84B* gene (two copies at the endogenous locus plus one copy of the p[α84B] transgene on the second chromosome). Spermatogenesis is wild type. Spermatids are correctly aligned at the tip of the developing bundles (large arrowheads), as in wild-type males. Thus, the misalignment of spermatids caused by expression of the antisense is rescued by an additional wild-type copy of the *α84B* gene. A syncytial bundle of spermatids is shown in d. The bundles in e are in the initial stages of individualization. The sperm heads are already individualized (large arrowheads); the region of the bundle to which the individualization process has progressed is indicated by the arrows. (f) Spermatid bundles in a sterile male that has two copies of the endogenous wild-type *α84B* gene plus four copies of the p[α85E] transgene. At this ratio of α85E:α84B, some spermatids fail to be aligned; at lower ratios, expression of α85E does not affect spermatid alignment. α85E does not affect spindles or other classes of cytoplasmic microtubules at any dose. (g) Phase-contrast micrograph of a live testis preparation showing newly formed spermatids in a male of the same genotype as in f. Spermatids are of the wild-type morphology, with a one-to-one association of each 1N nucleus (white) with a mitochondrial derivative (black), showing that during meiosis both karyokinesis and cytokinesis occurred normally.

Axoneme ultrastructure is shown in Figure 6. The wild-type *Drosophila* sperm tail axoneme has the "9 + 2 + 9" arrangement typical of insect sperm (Tates, 1971). Nine doublet microtubules surround a central pair of singlet microtubules; in addition, there is an outer circle of nine singlet accessory microtubules associated with the B tubule of each doublet. Each accessory microtubule is embedded in an electron-dense "eyebrow" that connects adjacent accessory microtubules around the diameter of the axoneme. A striking feature of insect sperm is that the central pair and the nine accessory microtubules each contain a filament within the lumen of the microtubule that in cross-section appears as a dot in the center of the microtubule (Fackenthal *et al.*, 1995). Figure 6, a and b, shows that morphology of the sperm tail axonemes is com-

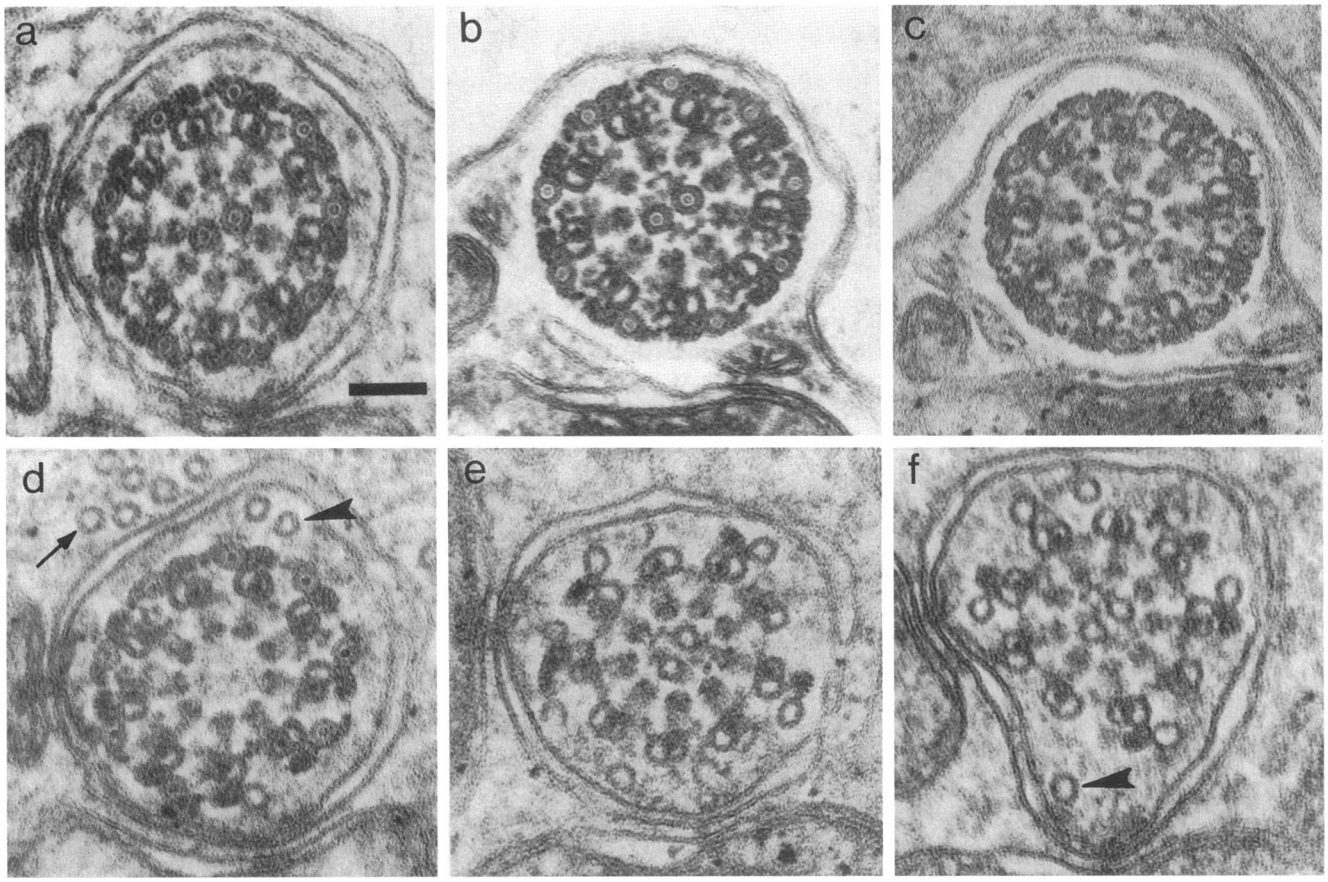

Figure 6. Expression of α85E in the postmitotic male germ cells causes defects in axoneme morphology. Each micrograph is a cross-section through the flagellum of a mature spermatid, showing the ultrastructure of the axoneme. (a and b) Wild-type *Drosophila* axoneme morphology in males that express the normal endogenous germ line α84B- and β2-tubulin isoforms, but in which the germ line tubulin pool is significantly decreased by reduction of α84B expression. Males of these genotypes are sterile because of spermatid misalignment, but axoneme morphogenesis is fully wild type. Each axoneme consists of nine doublet microtubules surrounding a central pair of microtubules plus nine outer accessory microtubules associated with the B tubule of each doublet. Each of the central pair and the nine accessory microtubules contains a luminal filament that appears in cross-section as a dot in the center of the microtubule. Each accessory microtubule is associated with an electron-dense eyebrow structure that connects adjacent accessory microtubules around the diameter of the axoneme. (a) Axoneme of normal wild-type morphology in a sterile male that has only one copy of the *α84B* gene [genotype *Df(α84B)/α84B*$^+$] and also has one X chromosome insert of the p[AS-α84B] antisense transgene. (b) Axoneme of normal wild-type morphology in a sterile male that has only one copy of the *α84B* gene and also has one autosomal insert of the p[AS-α84B] antisense transgene. (c–f) Axonemes in sterile males in which α85E is the predominant α-tubulin in the postmitotic male germ cells. All males are wild type at the β2 locus. Morphology of the axoneme doublet microtubules is normal, but the central pair and accessory microtubules are defective. (c) Axoneme in a sterile male with four copies of the p[α85E] transgene and two copies of the *α84B* gene. The accessory microtubules have luminal filaments, but the central pair does not. (d and e) Examples of axonemes in two different sterile males that have two inserts of the p[α85E] transgene, one copy of the *α84B* gene [genotype *Df(α84B)/α84B*$^+$], and one X chromosome insert of the p[AS-α84B] antisense transgene. (d) Axoneme in which the central pair is missing; eight of the accessory microtubules are nearly normal in morphology, but one does not contain a luminal filament and has an abnormal eyebrow structure. In addition, there are two ectopic singlet microtubules within the axonemal membrane (large arrowhead). Cytoplasmic microtubules are also visible in this field (arrow). (e) Axoneme in which the central pair is present but does not contain the luminal filament; some of the accessory microtubules are missing, and none of those present contains a luminal filament. In addition, the associated eyebrow structures have not been formed normally. (f) Axoneme in a sterile male that has two inserts of the p[α85E] transgene, one copy of the *α84B* gene [genotype *Df(α84B)/α84B*$^+$], and an autosomal insert of the p[AS-α84B] antisense transgene. Axoneme morphology is similar to that in e; an ectopic singlet microtubule is present within the axonemal membrane (large arrowhead).

pletely wild type in sterile males that express only the endogenous germ line tubulin isoforms, but in which the gene dose of α84B is decreased. Thus, reduction of the tubulin pool alone does not affect axoneme morphology.

As shown in Figure 6, c–f, the dominant sterility caused by α85E results from specific defects in axoneme morphogenesis. In α85E-expressing males, the overall architecture of the axoneme is normal and the doublet microtubules are always formed correctly.

Table 3. α85E-Tubulin causes defects in morphogenesis of the axoneme central pair and accessory microtubules

α-Tubulin genes in test males						No. of axonemes scored (no. of males)	Axonemes with central microtubules (%)	Central pair microtubules with luminal filaments (%)	Axonemes with accessory microtubules (%)	Accessory microtubules with luminal filaments (%)
A. Males that express only α84B in the germ line (*α84B* gene dose <2)										
						368 (17)	100	99	100	99.9
B. Males that express both α85E and α84B in the germ line										
	p[α85E]	p[AS-α84B]	α84B$^+$	Total *α84B* gene dose	Ratio α85E:α84B					
a:	2	—	2	2	1	162 (4)	96	86	100	100
b:	4	—	2	2	2	124 (5)	81	43	96	99
c:	2	—	1	1	2	119 (7)	98	26	61	55
d:	3	—	1	1	3	29 (2)	90	13	17	10
e:	4	—	1	1	4	25 (2)	76	5	24	5
f:	2	1(X)	2	<2	>1	250 (8)	89	35	72	81
g:	2	1(A)	2	<2	>1	49 (5)	92	11	37	15
h:	2	1(X)	1	<1	>2	66 (3)	97	11	30	21
i:	2	1(A)	1	<1	>2	50 (5)	92	2	16	10

Axoneme morphology was scored in electron micrographs of cross-sections of mature spermatids. Morphology of the axoneme doublet microtubules was normal in all males examined. A, Axoneme morphology in males that express only the endogenous germ line isoform α84B. Data shown are pooled for the genotype classes in which the functional *α84B* gene dose was decreased relative to wild type using different combinations of the deletion chromosome *Df(α84B)* and the p[AS-α84B] antisense transgene. (Genotypes include those shown in Table 1, experiments b, h–k, and Table 2, A, experiments d–f.) Reduction of the tubulin pool does not significantly disrupt axoneme morphogenesis. Defects in central pair or accessory microtubules were observed in only six axonemes, four of which were in a single male. B, Axoneme morphology in males that express both α85E and α84B in the postmitotic male germ cells. The α-tubulin genes expressed in the male germ line are designated as in Table 1; all males were wild type at the β2 locus. Many axonemes had missing or defective central and accessory microtubules; the severity of the phenotype increases with an increasing ratio of α85E:α84B in the tubulin pool.

However, the morphology of the singlet microtubules of the axoneme, the central pair and accessory microtubules, is abnormal. The most consistent defect in α85E-expressing males is the absence of the luminal filament that is normally present in the central pair and accessory microtubules. Also, in some cases, one or more of the central pair or accessory microtubules fails to be formed at all, and frequently the eyebrow structure associated with each accessory microtubule is either abnormal or absent. In addition to defects in central pair and accessory microtubules, we also observed that some axonemes contain ectopic singlet microtubules within the axonemal membrane. Such microtubules are never present in wild-type axonemes; their morphology does not allow us to distinguish whether these microtubules are aberrant accessory microtubules or misplaced cytoplasmic microtubules. In α85E-expressing males, about 30% of the accessory microtubules that lack luminal filaments also lack the associated eyebrow structure, whereas correctly formed accessory microtubules only rarely lack the associated structures. We found a few rare examples of eyebrows present in the absence of the accessory microtubule, suggesting that assembly of the accessory microtubules and construction of the associated structures are separable processes but that they are normally linked.

The examples shown in Figure 6, c–f, illustrate the range of defects in males that express different ratios of α85E and α84B. We quantified axoneme phenotypes in control males (Table 3A) and males that express α85E (Table 3B) by scoring the morphology of the central pair and accessory microtubules in electron micrographs of cysts of mature spermatids in testes from several males of each genotype. In males that express α85E, the number of defective axonemes and the severity of the defects are proportional to the ratio of α85E to α84B in the tubulin pool.

An important observation is that even in males in which the α85E isoform constituted greater than 80% of the total α-tubulin pool, an axoneme was assembled for each spermatid. This demonstrates that although α85E lacks the functional capacity to generate the correct architecture of the axoneme central pair and accessory microtubules, it can nonetheless provide the functions necessary to initiate axoneme assembly and to generate the basic features of the axoneme suprastructure. Even at the highest levels of α85E that we examined, the axonemal singlet microtubules are the only categories of microtubules in which we observed defects. We

Table 4. α85E-Tubulin can provide partial rescue of defective α84B-tubulin function in spermatogenesis

	α-Tubulin genes in test males			
	No. of copies of p[α85E]	*α84B* Alleles	Fertility phenotype (fraction of males that produce sperm)	
a:	0	$\alpha84B^{5}/\alpha84B^{nc33}$	Sterile	(0/74)
b:	1	$\alpha84B^{5}/\alpha84B^{nc33}$	V. W. fertile	(21/82)
c:	2	$\alpha84B^{5}/\alpha84B^{nc33}$	Sterile	(0/15)
d:	0	$\alpha84B^{2}/\alpha84B^{6}$	Sterile	(0/29)
e:	1	$\alpha84B^{2}/\alpha84B^{6}$	V. W. fertile	(4/20)
f:	0	$\alpha84B^{m}/\alpha84B^{+}$	Fertile to fertile, R. F.	(35/37)
g:	1	$\alpha84B^{m}/\alpha84B^{+}$	Fertile, R. F.	(56/63)
h:	2	$\alpha84B^{m}/\alpha84B^{+}$	Sterile	(0/25)
i:	0	p[α84B]; $\alpha84B^{m}/\alpha84B^{m'}$	Fertile	(30/30)
j:	0	p[α84B]/p[α84B]; $\alpha84B^{m}/\alpha84B^{m'}$	Fertile	(36/36)
k:	0	p[α84B]/p[α84B]; $\alpha84B^{+}/\alpha84B^{+}$	Fertile	(52/52)

α-Tubulin genes expressed in the male germ line for each genotype class tested: the first column shows the gene dose of p[α85E]; the second column shows the genotype at the endogenous *α84B*-tubulin locus, and for males in experiments i–k, the number of copies of the p[α84B] transgene generated by Matthews *et al.* (1993). All males were wild type for β2-tubulin. Fertility phenotype and sperm production were determined as described in MATERIALS AND METHODS. Experiments a–h: one copy of p[α85E] partially rescues the fertility of males that carry viable but male-sterile allele combinations of mutations in the *α84B* gene, but two copies of p[α85E] cause sterility in any background with compromised α84B function. In experiments f–h, data for $\alpha84B^{m}/\alpha84B^{+}$ are pooled data for males heterozygous for recessive *α84B* mutations, where $\alpha84B^{m}$ represents $\alpha84B^{5}$, $\alpha84B^{nc33}$, $\alpha84B^{2}$, or $\alpha84B^{6}$ and the endogenous wild-type α84B-tubulin gene is indicated by $\alpha84B^{+}$. Experiments i and j: control experiments to show that fertility of viable but male-sterile allele combinations of mutations in the *α84B* gene is fully rescued by p[α84B]. Data for $\alpha84B^{m}/\alpha84B^{m'}$ are pooled data for males of genotype $\alpha84B^{5}/\alpha84B^{nc33}$, $\alpha84B^{nc33}/Df[\alpha84B]$, or $\alpha84B^{5}/Df[\alpha84B]$. One copy of p[α84B] is sufficient to rescue both viability and fertility of animals carrying the lethal allele combination $\alpha84B^{5}/Df[\alpha84B]$. Experiment k: control experiment to show that the dominant sterility caused by increasing the dose of α85E is not simply the consequence of an increase in total α-tubulin gene dose. Fertility data for males with four copies of the wild-type *α84B* gene are the same as those shown in Table 2.

never observed defects in the structure of the axoneme doublet microtubules or in cytoplasmic microtubules. All of the differentiative functions mediated by cytoplasmic microtubules proceed normally in males that express α85E at any level we have examined, including meiosis (Figure 5g), mitochondrial elongation, and nuclear shaping. Figure 7 shows the morphologically normal array of nuclear shaping microtubules in a sterile α85E-expressing male in which the α85E isoform constitutes greater than 80% of the total α-tubulin pool in the postmitotic germ cells. In males of this genotype, nuclear shaping and other cytoplasmic microtubule functions are wild type, but there are defects in the central pair or accessory microtubules in all axonemes (Table 3, experiment i).

Taken together, the phenotypes of spermatogenesis in males that express α85E in the germ line show that α85E is similar to α84B in overall assembly capacity but is not functionally equivalent to α84B. Consequently, even very closely related tubulins have acquired specialized functional properties. In particular, α85E lacks the ability to support tissue-specific functions unique to the male germ line.

α85E Can Provide Sufficient α-Tubulin Function to Allow Production of Some Functional Sperm by Males in Which Endogenous α84B Tubulin Function Is Defective

To further distinguish between the microtubule assembly capacities of the α85E and α84B isoforms, we examined the effect of α85E expression on spermatogenesis in males that carry viable but male sterile combinations of mutations in the *α84B* gene (see MATERIALS AND METHODS for a description of the mutations). The variant forms of α84B expressed by the mutant males can support all microtubule functions necessary for embryonic and pupal development, as well as for viability of the adult fly, but cannot support male fertility. Thus, we could use these genetic backgrounds to define the ability of α85E to support microtubule functions specifically required for spermatogenesis. These experiments are shown in Table 4 and Figure 8.

We first tested the effect of α85E expression on the fertility of males homozygous for $\alpha84B^{nc33}$, a recessive male-sterile mutation in the *α84B* gene (Raff and Fuller, 1984; Hays *et al.*, 1989) and of males that carried this allele in combination with a deletion of the *α84B* gene or with $\alpha84B^{5}$, a recessive lethal hypomorphic

allele isolated by Matthews *et al.* (1987). Expression of α85E in the germ line partially restored fertility of males carrying the heteroallelic combination $\alpha 84B^{nc33}/\alpha 84B^{5}$ (Table 4, experiments a and b). About one-quarter of $\alpha 84B^{nc33}/\alpha 84B^{5}$ males carrying one copy of p[α85E] produced some sperm and gave some progeny in mating tests, albeit far fewer progeny than wild-type males. $\alpha 84B^{nc33}/\alpha 84B^{5}$ was the only mutant combination in the $\alpha 84B^{nc33}$ series that could be rescued by the α85E isoform; p[α85E] did not restore the ability to produce sperm to males in which the $\alpha 84B^{nc33}$ protein was the only other α-tubulin available in the pool (e.g., in either $\alpha 84B^{nc33}$ homozygotes or hemizygotes). Since α85E can provide partial rescue for $\alpha 84B^{nc33}/\alpha 84B^{5}$ males but not for $\alpha 84B^{nc33}$ homozygotes or hemizygotes, we conclude that the variant protein encoded by $\alpha 84B^{5}$ provides some residual activity that neither α85E nor the $\alpha 84B^{nc33}$ variant can provide. Rescue of male fertility by α85E is not specific to the $\alpha 84B^{nc33}/\alpha 84B^{5}$ allele combination, however. α85E also partially rescued fertility of $\alpha 84B^{2}/\alpha 84B^{6}$ males (Table 4, experiments d and e), a viable male-sterile combination of two other recessive lethal *α84B* mutations isolated by Matthews *et al.* (1987).

Although one copy of p[α85E] gave only minimal rescue of fertility of $\alpha 84B^{nc33}/\alpha 84B^{5}$ males, additional copies did not improve male fertility further but instead were deleterious. Thus, $\alpha 84B^{nc33}/\alpha 84B^{5}$ males carrying two copies of p[α85E] were completely sterile (Table 4, experiment c). We also tested the effect of expression of α85E in males that were heterozygous for one of the *α84B* mutations. In all cases, one copy of p[α85E] in a heterozygous α84B mutant background slightly decreased fecundity, while two copies resulted in complete sterility (Table 4, experiments f–h). These results are consistent with the data shown above demonstrating that increasing the dose of α85E in a wild-type α84B background results in dominant sterility, attributable to the failure of α85E to support correct morphogenesis of the axoneme central pair and accessory microtubules. To make sure that the deleterious effect of two copies versus one copy of p[α85E] was not simply a consequence of increasing the total number of α-tubulin genes, we examined rescue of mutants with copies of the wild-type α84B supplied on a transgene. Viability and male fertility of all of the *α84B* allele combinations were rescued by either one or two copies of the wild-type *α84B* gene (Table 4, experiments i and j). In addition, males with four copies of the wild-type gene exhibited wild-type fertility (Table 4, experiment k). Thus, at least at the gene copy numbers we have examined, overexpression of α-tubulin per se is not deleterious.

Examination of the phenotype of $\alpha 84B^{nc33}/\alpha 84B^{5}$ males in the presence and absence of p[α85E] at the light microscope and ultrastructural level showed

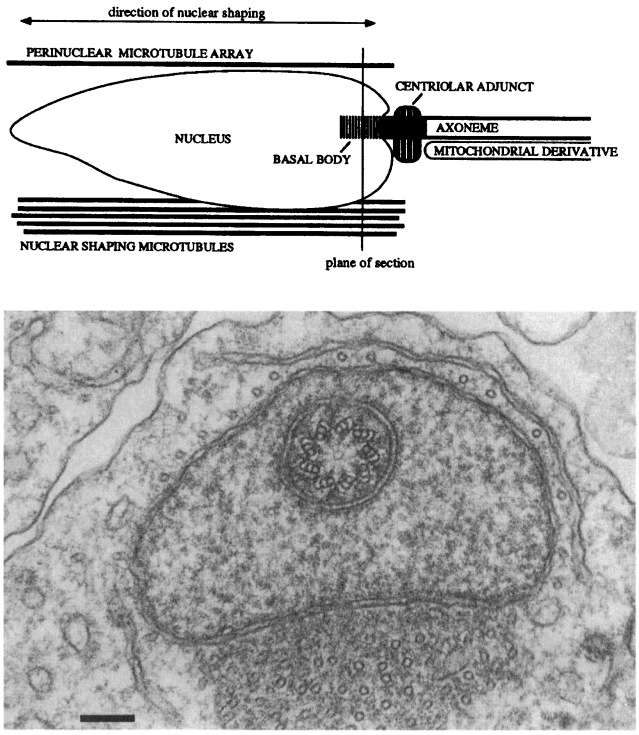

Figure 7. Microtubules that mediate shaping of the spermatid nucleus function normally when α85E is the major α-tubulin subunit in the tubulin pool. Top, diagram of the process of nuclear shaping that indicates the position of the electron micrograph cross-section (bottom). Note that the sperm basal body pushes into the nucleus at the proximal end, so that in cross-section the basal body appears to be enclosed by the double nuclear membrane; the distal end of the basal body is associated with a dense region of cytoplasm, the centriolar adjunct. The cross-section shows the nuclear shaping microtubules in a sterile male that has two inserts of the p[α85E] transgene, one copy of the *α84B* gene [$Df(\alpha 84B)/\alpha 84B^{+}$], and one autosomal insert of the p[AS-α84B] antisense transgene.

that α85E could provide function for several aspects of spermatogenic microtubule function, particularly axoneme assembly. In spermatogenesis in $\alpha 84B^{nc33}/\alpha 84B^{5}$ mutant males, the mitochondrial-associated microtubules function normally, and there are only occasional defects in meiotic spindle function; thus, the combination of the $\alpha 84B^{nc33}$ and $\alpha 84B^{5}$ variant proteins supports classes of microtubules that are similar to those in many other cell types. The processes that fail in $\alpha 84B^{nc33}/\alpha 84B^{5}$ mutant males are axoneme assembly, nuclear shaping, and spermatid alignment, i.e., microtubule-mediated functions that are unique to spermatogenesis. Expression of α85E rescues axoneme assembly and ameliorates but does not completely rescue spermatid alignment and nuclear shaping. Since spermatids are misaligned in the rescued males, many mature spermatids fail to be individualized, resulting in production of only small amounts of sperm. Figure 8 shows axoneme

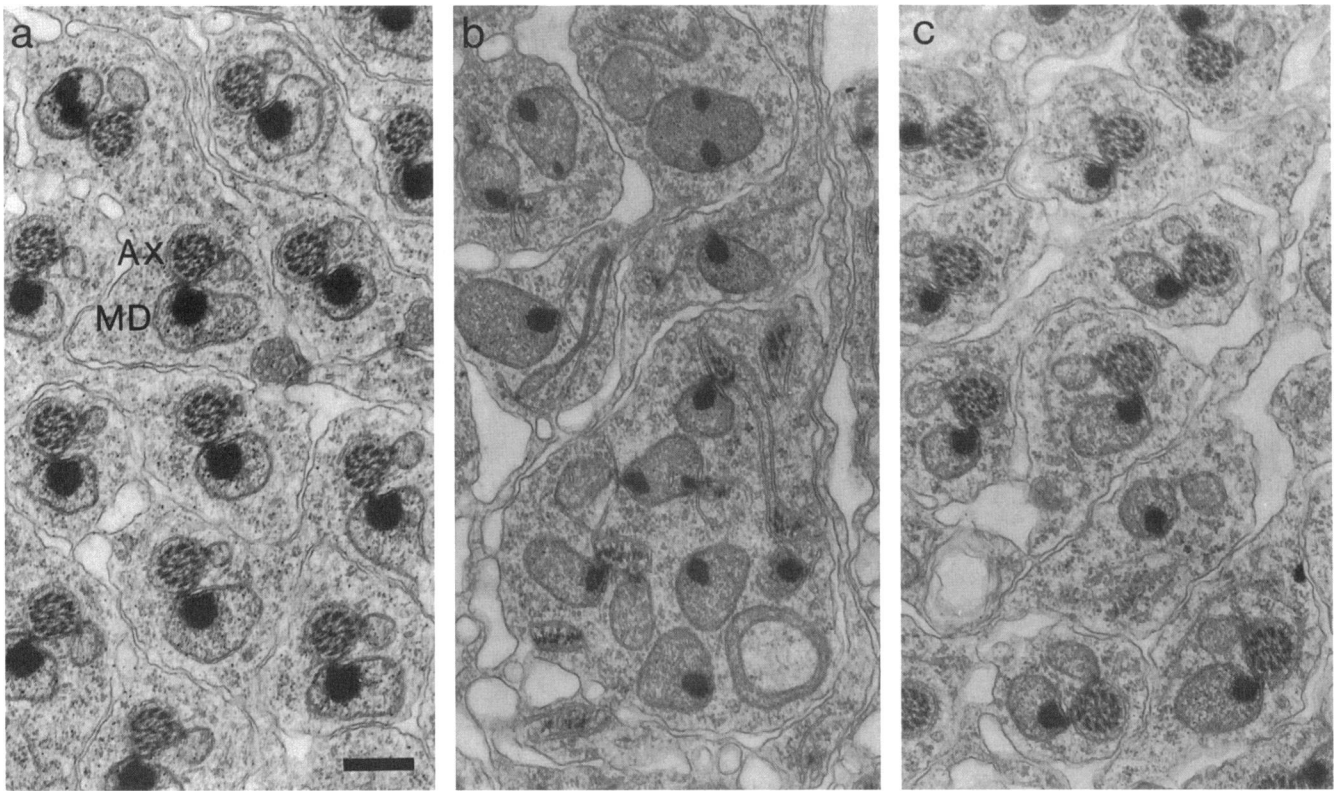

Figure 8. α85E can partially rescue the sterility of males that are defective in α84B function. (a) Electron micrograph showing a cross-section through the sperm tails of developing spermatids in a wild-type male. Each sperm tail has an axoneme (AX) associated with a mitochondrial derivative, which at this stage of development is composed of a major component (MD) containing electron-dense material and a minor component associated with the axoneme at right angle to the major component (Tates, 1971). (b) Cross-section of developing spermatids of a similar developmental stage in a sterile male of genotype $\alpha 84B^{nc33}/\alpha 84B^{5}$. No complete axonemes are formed. Many spermatids do not have an axoneme; others have partial or fragmented axonemes. (c) Cross-section of developing spermatids of a similar developmental stage in a weakly fertile male that is genotype $\alpha 84B^{nc33}/\alpha 84B^{5}$ at the endogenous *α84B* locus but also has one copy of the p[α85E] transgene. Most spermatids have complete axonemes. (In the field shown, one spermatid does not have an axoneme.)

assembly and organization in fertile wild-type males (Figure 8a), in sterile $\alpha 84B^{nc33}/\alpha 84B^{5}$ males (Figure 8b), and in rescued $\alpha 84B^{nc33}/\alpha 84B^{5}$ males that also have one copy of p[α85E] (Figure 8c). The mutant α-tubulins cannot support significant axoneme assembly, but axonemes are assembled when α85E is present. In the rescued males, many axonemes are normal in morphology, but some axonemes exhibit central pair defects typical of the dominant α85E phenotype described above. Thus, in any background in which it is tested, α85E fails to support correct morphogenesis of the singlet microtubules of the axoneme. We conclude that the defects in axoneme morphogenesis contribute to the low fecundity of the rescued males and account for the fact that rescue is not improved by adding more copies of p[α85E].

We do not know the precise basis for α85E rescue of $\alpha 84B^{2}/\alpha 84B^{6}$ sterility, but rescue appears to reflect the ability of α85E to function in male germ line-specific microtubules other than axonemes. Like sterile $\alpha 84B^{nc33}/\alpha 84B^{5}$ males, sterile $\alpha 84B^{2}/\alpha 84B^{6}$ males are defective in head shaping and spermatid alignment; however, axonemes are assembled in sterile $\alpha 84B^{2}/\alpha 84B^{6}$ males.

The ability of α85E to rescue male fertility in different α84B-defective backgrounds confirms the conclusion drawn from previous experiments that α85E differs from α84B in a restricted set of assembly properties required for axoneme morphogenesis.

α85E and β3 Isoforms Do Not Form a Functionally Specialized Heterodimer

Although not completely overlapping, the complex developmental patterns of expression of α85E and β3 are strikingly similar and both isoforms appear to be primarily used in one kind of cellular context, i.e., in cells that undergo extensive shape changes (Kimble *et al.*, 1989; Matthews *et al.*, 1990). α85E is always coexpressed with α84B and never makes up more than a small percentage of the total α-tubulin pool; in the cell

types in which it is most prevalent, α85E may be a maximum of half the total cellular α-tubulin pool (Matthews *et al.*, 1989). When α85E and β3 are coexpressed, there will likely be a mixed isoform population in which both α85E-β3 and α84B-β3 pairs are possible. There may also be β-tubulin diversity in the possible heterodimer combinations, since in some cell types, the β1 isoform may be present along with β3 (Kimble *et al.*, 1990; Dettman *et al.*, 1996).

The shared context of utilization raised the possibility that α85E-β3 heterodimer pairs might possess microtubule assembly properties that differ from heterodimers in which they are paired with other partners. If α85E-β3 is a "better" dimer than α85E-β2 or α84B-β3 pairs, then we would expect the dominant phenotypes to be alleviated. Alternatively, if the α85E-β3 heterodimer has specialized properties required in cells undergoing rearrangements of the microtubule cytoskeleton, it might be worse at supporting male germ line-specific microtubule functions. In this case we would predict that coexpression of α85E and β3 would result in a unique set of specific phenotypes (i.e., analogous to allele-specific genetic interactions).

Our data do not support the model that the α85E-β3 heterodimer has unique properties. We compared the phenotype of spermatogenesis in males in which β3 is the only β-tubulin expressed in the postmitotic germ cells when α84B was the only available α-tubulin or when both α85E and α84B were present at equal amounts, the situation that approximates the maximal levels of α85E in its normal sites of expression. The phenotypes were exactly the same: whether or not α85E was present, mitochondrial derivative elongation proceeded, but no other microtubule-mediated functions occurred (our unpublished data). Thus, the inability of β3 to assemble multiple classes of microtubules is not rescued by the availability of α85E as a partner. As a second test, we compared microtubule function in spermatogenesis in males that express both α85E and β3 with that in males that express both α85E and a mutant form of β2-tubulin. As shown in Table 5, we found that α85E and β3 do not exhibit a specific genetic interaction; rather, expression of α85E in the male germ line with any β-tubulin variant causes additive effects on spermatogenesis. That is, male fertility is compromised in any situation in which the germ line tubulin pool contains both α85E and a β-tubulin variant. Thus, our results suggest that the α85E-β3 combination is not a "preferred" or specialized heterodimer pair. We conclude that if they possess specialized functional properties required for their normal sites of expression, α85E and β3 must act independently to modulate the assembly or function of the microtubule structures of which they are components. These results are also consistent with the model that the microtubule assembly properties of a given isoform pool reflect the individual properties of the constituent isoforms (for discussion, see Raff, 1994).

DISCUSSION

Previous studies that demonstrated isoform specialization in *Drosophila* compared divergent pairs of isoforms (e.g., β3 versus β2, and α67C versus α84B). The possibility remained that similar isoforms are functionally equivalent. In this study, we found that even though α85E and α84B are very similar in sequence, they are functionally distinct. Our tests of α85E func-

Table 5. α85E-Tubulin and β-tubulin variants result in additive effects on spermatogenesis

Tubulin genes in test males (all males are wild type for α84B-tubulin)

	No. of copies of p[α85E]	β-Tubulins	Fertility phenotype (fraction of males that produce sperm)
a:	1	$B2t^{+}/B2t^{+}$	Fertile (229/232)
b:	0	$B2t^{m}/B2t^{+}$	Fertile (51/51)
c:	0	p[β3*], $B2t^{+}/B2t^{+}$	Fertile (70/74)
d:	1	$B2t^{m}/B2t^{+}$	Sterile (0/77)
e:	1	p[β3*], $B2t^{+}/B2t^{+}$	Sterile (1/111)

α- and β-tubulin genes expressed in the male germ line are shown for each genotype class tested. Fertility phenotype and sperm production were determined as described in MATERIALS AND METHODS. Male fertility is not compromised in males that carry one copy of p[α85E] in an otherwise wild-type background (experiment a), in males heterozygous for recessive male-sterile mutations in the β2 gene (experiment b), or in males that express β3 in the male germ cells from the p[β3*] transgene described in MATERIALS AND METHODS. However, males that carry both one copy of p[α85E] plus any of the β-tubulin variants tested are sterile (experiments d and e). Fertility data for males with one copy of p[α85E] in an otherwise wild-type background are the same as shown in Table 2. $B2t^{m}/B2t^{+}$ represents pooled data for males that are heterozygous for recessive mutations in the β2-tubulin gene: $B2t^{m}$ represents $B2t^{6}$, $B2t^{7}$, or $B2t^{8}$, recessive male-sterile alleles that encode stable β2 variants defective in different aspects of microtubule function in spermatogenesis (Fuller *et al.*, 1987, 1988; Fackenthal *et al.*, 1995); the wild-type β2-tubulin gene is indicated by $B2t^{+}$.

tion in spermatogenesis show that α85E can provide function in the male germ line for "generic" microtubule arrays such as spindles and cytoskeletal microtubules similar to those in other cells and to those in which α85E normally functions, but α85E lacks a subset of the functional properties possessed by the α84B isoform necessary for specific features of microtubule assembly unique to the male germ cells. Our data thus reveal that even small changes in tubulin structure may have important consequences for regulation of the microtubule cytoskeleton and suggest that in complex metazoans, tubulin isoform specialization may be the general case. Moreover, we would suggest that specialization of tubulin function may occur not only in isoforms within the tubulin gene family in a given species but also in the homologous isoforms in tubulin families in related species. For example, we have found that the *Drosophila* testis-specific β2-tubulin isoform cannot be replaced by the β2 homologue from another insect, even though the two isoforms perform very similar repertoires of functions in vivo (Raff *et al.*, 1997).

In addition to differential tubulin gene expression, the diversity within cellular tubulin pools is increased by diverse posttranslational modifications of both α- and β-tubulins. Tubulin modifications constitute a major riddle because, despite their evolutionary conservation and prevalence in diverse cell types, the cellular role has not been defined for any modification. Our data suggest that the differences in the functional properties of α85E and α84B in the male germ cells may be related to differences in how the two isoforms are modified. Thus *Drosophila* spermatogenesis provides a system in which elucidation of the role of posttranslational tubulin modifications may be accessible to genetic analysis.

Many lines of evidence suggest that assembly of the motile axoneme imposes stringent structural constraints on tubulins (Silflow, 1991; Raff, 1994). We have previously defined axoneme-specific requirements for β-tubulin structure (Fackenthal *et al.*, 1993, 1995; Hoyle *et al.*, 1995). The data we report herein demonstrate that different subsets of axoneme microtubules have separable requirements for α- and β-tubulin structure. We found that α85E lacks a specific subset of properties required for axoneme assembly, but our data suggest that for other microtubule arrays, α85E may be interchangeable with α84B. Our results do not distinguish between the possibilities that α85E has unique assembly properties required in the cells in which it functions, or that because α85E is not utilized in axonemes, it has undergone sufficient sequence drift that its functional capacity has become limited relative to that of α84B.

When the α85E isoform is ectopically expressed in the male germ cells, axonemes are assembled and the doublet microtubules have normal morphology but assembly of the central pair and accessory microtubules is defective and these microtubules often lack luminal filaments (Figure 6). In contrast, when the β3 isoform is coexpressed with the endogenous germ line β2-tubulin, assembly of the central pair and accessory microtubules occurs normally, but the doublet microtubules acquire the morphological characteristics of the singlet axoneme microtubules (Hoyle and Raff, 1990; Hoyle *et al.*, 1995). Thus, expression of α85E or β3 in the male germ cells results in precisely reciprocal effects on assembly of axoneme microtubules. We have examined axoneme structure in males in which both α85E and β3 are expressed and observed that few doublets acquire ectopic luminal filaments when α85E is the major α-tubulin in the spermatids, even when β3 is also present. Thus, disruption of morphogenesis of the central pair and accessory microtubules caused by α85E is dominant to the morphological defects in the doublet microtubules caused by β3.

The recessive β2 mutation $B2t^6$ causes the same phenotype as β3, and the $B2t^6$ protein and β3 have common structural features (Fuller *et al.*, 1988; Fackenthal *et al.*, 1995). We previously showed that in $B2t^6$ males, the severity of defects in doublet microtubules depends on the size of the germ line tubulin pool; i.e., on tubulin concentration (Fackenthal *et al.*, 1995). Based on this observation, we proposed a kinetic model for axoneme assembly in which we hypothesized that β3 or the $B2t^6$ protein caused mis-specification of doublet microtubule morphology by interfering at the initiation step of doublet microtubule assembly. Consistent with this model, in addition to causing defects in morphology of the doublets, both β3 and the $B2t^6$ protein cause production of spermatids in which the axoneme is missing (Hoyle *et al.*, 1995; Fackenthal *et al.*, 1995). This contrasts with α85E, which does not inhibit axoneme assembly per se. The observation that the axoneme defects caused by α85E are epistatic to the defects caused by β3 suggests that there is a necessary physical interaction between α-tubulin and some component required for singlet microtubule assembly, perhaps a component of the luminal filaments or of the "machinery" responsible for adding luminal filaments to the central pair and accessory microtubules. Thus, even when β3 is present and the kinetics of doublet microtubule assembly are perturbed, luminal filaments cannot be added to any microtubule when α85E is present.

The defects in axoneme ultrastructure caused by α85E also reveal other key features about how the axoneme is put together. The spokes and linkers associated with the doublets are normal in α85E-expressing males, consistent with our previous conclusion that each doublet microtubule/spoke-linker complex forms an independent architectural module (Hoyle *et al.*, 1995). However, α85E disrupts formation of the eyebrow structures associated with the accessory mi-

crotubules, suggesting that the accessory microtubule complex also constitutes an assembly module. Furthermore, the central pair and accessory microtubules are differentially affected by α85E; thus, the dominant α85E phenotype distinguishes between requirements for assembly of these two classes of axoneme microtubules. In α85E-expressing males, the central microtubules are usually assembled, even though in most cases the luminal filament is not added. Thus, α85E does not significantly interfere with initiation of central pair assembly. In contrast, the accessory microtubules often fail to be assembled at all. This suggests that α85E isoform disrupts the novel initiation mechanism for accessory microtubules, which are directly nucleated from the B-tubule of the adjacent doublet (Tates, 1971; Raff *et al.*, 1997).

In normal development, the α85E and β3 isoforms are expressed together in many cells undergoing cell shape changes. The common cellular context for expression of these isoforms raised the possibility that the α85E-β3 heterodimer pair might have unique properties. Our data suggest that this is not the case. We conclude that if the use of these isoforms reflects cellular requirements for specialized functional properties, each isoform must act individually to modulate the assembly properties of the tubulin pool in which it is present. Alternatively, expression of β3 and α85E might serve to boost the tubulin pool in cells with high demands for tubulin. We suspect the most likely case is that expression of these isoforms serves both kinds of roles in different cells. Consistent with this idea, we have found that the β3 isoform is essential in some of the cell types in which it is expressed but is dispensable in others (Dettman *et al.*, 1996).

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