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Genetics

Mutation in a structural gene for a β-tubulin specific to testis in *Drosophila melanogaster*

(tubulin heterogeneity/microtubule assembly/spermiogenesis/axoneme formation)

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ABSTRACT **By two-dimensional gel electrophoresis of tubulins prepared from tissues of *Drosophila melanogaster* we have identified a β-tubulin subunit that is present only in the testis. Furthermore, we have isolated, as a male sterile, a third chromosome dominant mutation [*ms(3)KK^D*] in the structural gene for this β-tubulin. Males heterozygous for this mutation produce no motile spermatozoa. Beginning with meiosis, all processes in spermiogenesis are abnormal to some extent. Many microtubules (including both cytoplasmic microtubules and doublet tubules of the axoneme) show aberrant structure in cross section, and the overall morphology of the developing spermatids is disorganized. Testes from these males were shown, by two-dimensional gel electrophoresis, to contain both the normal testis-specific β-tubulin and an electrophoretic variant of this tubulin in equal amounts. Both wild-type and mutant testis-specific β-tubulins were characterized by vinblastine sulfate precipitation, coassembly with purified *Drosophila* embryo tubulin, and peptide mapping.**

Although tubulin assembly and function have been extensively studied, it has been difficult to correlate the now well-characterized biochemical properties of tubulin with its mode of function and regulation *in vivo*. Attempts to make such correlations have had to rely largely on *in vitro* assembly studies, which are subject to the criticism that these assembly conditions do not accurately reflect those in the cell.

We have chosen spermiogenesis in *Drosophila melanogaster* as a system in which to study control mechanisms that function during development and differentiation, in particular those which regulate microtubule function, because in this organism the powerful combination of biochemical and ultrastructural characterization with extensive genetic analysis is possible. Spermiogenesis in *Drosophila* is a localized differentiative process resulting in the formation of the complex microtubule-containing structure, the 9+2 axoneme of the sperm tail, a structure not formed elsewhere in the embryo or adult fly. Two lines of evidence suggested that this system might be uniquely suited for study of control of microtubule assembly *in vivo*. First, an increasing body of data suggests that microheterogeneity of tubulin subunits is a key control point for microtubule assembly; in particular, in several species the tubulins present in the 9+2 axoneme of cilia or flagella differ from those present in the cytoplasm or those which comprise the mitotic apparatus (1–8). Therefore, it might be expected in *Drosophila* that there are tubulins required for the sperm tail axoneme that are unique to spermiogenesis. Second, a number of male sterile mutations in *Drosophila* have been described that affect microtubule morphology or function (or both) during spermiogenesis without affecting viability or female fertility (9–13). Thus, it should also be possible to identify mutations affecting tubulins that function during spermiogenesis. The defects caused by variant tubulins with a specific, known function *in vivo* could then be directly correlated with changes in the biochemical properties of the altered tubulins.

We report here the initial results of such a study. We have identified a β-tubulin subunit specific to the testis and have isolated a dominant mutation in the structural gene for this tubulin.

MATERIALS AND METHODS

***D. melanogaster* Stocks and Mutant Screen.** Two wild-type *D. melanogaster* stocks were used, P2 for the embryo tubulin preparation and Oregon R for all other experiments. Descriptions of stocks used in the mutant screen can be found in Lindsley and Grell (14), except for *Df(3R)Antp^{Ns+R17}* and *Df(3R)dsx^{D+R2}*, which were described by Duncan and Kaufman (15). For the male sterile mutant screen, homozygous *red e* males were fed 25 mM ethyl methane sulfonate according to the method of Lewis and Bacher (16). These treated males were crossed to *Df(3R)Antp^{Ns+R17}/In(3LR)TM3, Sb Ser* virgin females. Progeny virgin females of the genotype *red e*/In(3LR)TM3, Sb Ser* (where * denotes chromosome treated with ethyl methane sulfonate) were mated individually to *Df(3R)dsx^{D+R2}/In(3LR)CxD* males. Progeny males of the genotype *Df(3R)dsx^{D+R2}/red e** were tested for fertility. Stocks of treated chromosomes were maintained by selecting virgin progeny of the genotypes *In(3LR)CxD/In(3LR)TM3 Sb Ser* and *red e*/In(3LR)CxD* and allowing them to interbreed. In one of these stocks a dominant male sterile mutation [*ms(3)KK^D*] was identified; this chromosome was then maintained in stock by selecting in each generation *red e*/In(3LR)CxD* virgin females and crossing them to *In(3LR)CxD/In(3LR)TM3 Sb Ser* males.

Isotopic Labeling of *Drosophila* Tissues. Proteins of *Drosophila* tissues were isotopically labeled *in vitro* with [^{35}S]methionine. The labeling medium was methionine-deficient tissue culture medium of Wyss and Bachmann (17) to which [^{35}S]methionine was added to give a concentration of 1–4 mCi/ml. Tissues and organs were dissected from anesthetized flies immersed in insect Ringer's solution (18) and placed into a drop of labeling medium in a sterile plastic culture dish (1 μl of medium per tissue or organ); the dish was placed in a moist chamber at room temperature. At the end of the incubation period (4–8 hr) the tissues were washed in insect Ringer's solution and suspended in the appropriate buffer for electrophoresis or tubulin isolation.

Gel Electrophoresis. Two-dimensional polyacrylamide gel electrophoresis was carried out with nonequilibrium pH gradients in the first dimension by the procedure of O'Farrell *et al.* (19) as modified by Waring *et al.* (20). To display the total

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protein spectrum, after incorporation of [^{35}S]methionine, we suspended tissues in 25 μl of sonication buffer (2 mM Tris·HCl/1 mM $MgCl_2$, at pH 7.4) in a 0.5-ml capped Brinkmann microcentrifuge tube and sonicated them in an ice bath for 1 min with a Branson sonicator at maximum output. The entire sonicates were then dissolved in electrophoresis buffer (2 mM Tris·HCl/1.6% sodium dodecyl sulfate/5% 2-mercaptoethanol, at pH 7.4) and frozen until use. One-dimensional sodium dodecyl sulfate/polyacrylamide gel electrophoresis was carried out by the procedure of Laemmli (21).

Vinblastine Sulfate Precipitation. Tubulin was prepared by vinblastine sulfate precipitation by the method of Olmsted *et al.* (22). After incorporation of [^{35}S]methionine, tissues were mixed with 30 unlabeled adult ovaries (to supply carrier tubulin) in 50 μl of precipitation buffer (10 mM Tris·HCl/20 mM $MgCl_2$, at pH 7.0) and sonicated. The sonicated mixture was held at 4°C for 30 min to ensure depolymerization of microtubules and then centrifuged in a Beckman airfuge at 4°C at 150,000 × *g* for 15 min. The supernatant was removed and mixed with 5 μl of a 20.9 mg/ml solution of vinblastine sulfate. After 15 min on ice, the tubulin–vinblastine crystals were pelleted by centrifugation at 100,000 × *g* for 15 min. The pellet was dissolved in electrophoresis buffer and frozen.

Purification of Embryo Tubulin and Coassembly of Testis and Embryo Tubulins. Tubulin from *Drosophila* embryos was purified by self-assembly *in vitro* by the method of Green *et al.* (23, 24) as modified by J. E. Loyd (this laboratory, unpublished data) to allow for several cycles of assembly. Embryo tubulin purified by three cycles of assembly and disassembly followed by passage over a phosphocellulose column was used as marker tubulin in gel electrophoresis experiments.

This procedure for assembly of embryo tubulin *in vitro* was also used for coassembly of [^{35}S]methionine-labeled testis tubulin with unlabeled embryo tubulin. Forty to 50 testes were incubated with [^{35}S]methionine for 4–5 hr, sonicated in 20 μl of assembly buffer (0.1 M 4-morpholineethanesulfonate/0.5 mM $MgCl_2$/1.0 mM GTP/4 M glycerol, at pH 6.6), held on ice for 30 min, and then centrifuged in the Beckman airfuge for 15 min at 100,000 × *g*. The resultant supernatant was added to 60 μl of embryo tubulin freshly prepared by one cycle of assembly and disassembly (approximately 3 mg/ml). This mixture was then carried through two more cycles of assembly and disassembly, and the final tubulin pellet was dissolved in electrophoresis buffer and frozen.

Peptide Mapping. The procedure was that of Cleveland *et al.* (25) with only minor modifications. Tubulins labeled with [^{35}S]methionine were prepared from testes by vinblastine sulfate precipitation and mixed with sufficient embryo tubulin prepared by self-assembly *in vitro* to allow for visualization after the first electrophoresis by brief staining with Coomassie brilliant blue. A reverse 10–5% polyacrylamide gradient, stabilized with glycerol, provided optimal separation of the α from the β subunits, which were then subjected to proteolysis in the second gel. The gels displaying the peptide fragments were stained with Coomassie brilliant blue and dried, and autoradiograms were made.

RESULTS

Testis-Specific β_2-Tubulin. Initial identification of testis tubulins was made by using the criterion of comigration in two-dimensional polyacrylamide gel electrophoresis with purified *Drosophila* embryo tubulin. [^{35}S]Methionine-labeled total testis proteins were mixed with unlabeled purified embryo tubulin and electrophoresed in two dimensions. The mixture was adjusted so that only the purified embryo tubulin was visible on a stained gel. By comparing the pattern of spots on an autoradiogram of the gel with the pattern on the stained gel, we tentatively identified the tubulins from testis (Fig. 1*a*). The testis tubulins are resolved into two complexes of protein spots with electrophoretic mobilities corresponding to the α- and β-tubulin subunits. The α complex (designated α in the figures) contains at least two, but as many as five, different spots, and the β complex contains two (designated β_1 and β_2). The α complex tubulins and the β_1-tubulin from testis comigrate, respectively, with the α complex and β_1-tubulin from embryo. However, the β_2-tubulin, which is not present in the embryo, migrates slightly faster than β_1-tubulin. Comigration does not prove identity of proteins. In fact, preliminary data suggest that α-tubulins from embryos and testes differ (unpublished results).

In this paper we will restrict our discussion to the β-tubulins. The testis-specific β_2 subunit, which accounts for approximately 40% of the total testis tubulin, was demonstrated unambiguously to be a tubulin by vinblastine precipitation, coassembly with embryo tubulin, and peptide mapping. Tubulin was prepared from [^{35}S]methionine-labeled testis proteins by vinblastine sulfate precipitation and electrophoresed in two dimensions; an autoradiogram was prepared. A vinblastine sulfate precipitate from [^{35}S]methionine-labeled ovary proteins was used as a control. The resulting autoradiograms are shown in Fig. 1 *b* and *c*. Both the β_1- and β_2-tubulin subunits are present in the vinblastine sulfate precipitate prepared from testes in approximately the same ratio as in the preparation of total testis proteins (Fig. 1*a*).

Tubulin from testes was coassembled with embryo tubulin through two successive cycles of assembly and disassembly. An autoradiogram of a two-dimensional gel of the second assembly pellet is shown in Fig. 1*d*. The β_2-tubulin coassembled with embryo tubulin and is clearly visible in the autoradiogram as a thin band migrating faster than β_1-tubulin. In this gel the

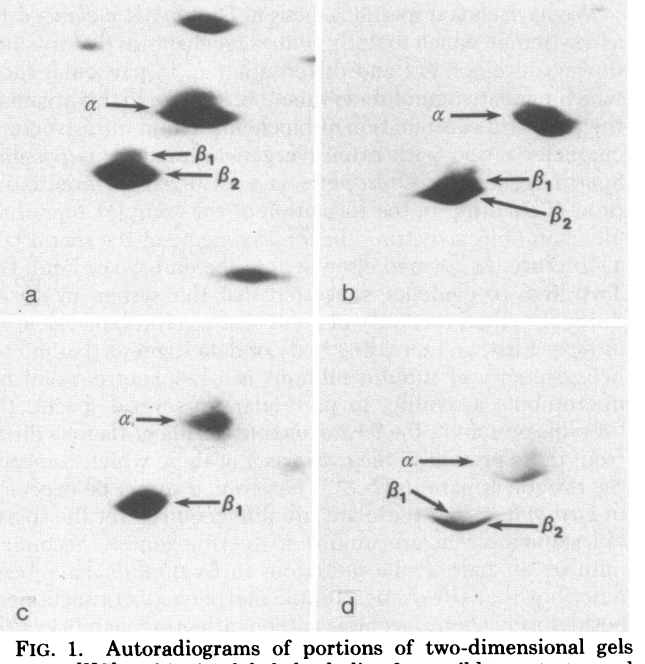

FIG. 1. Autoradiograms of portions of two-dimensional gels showing [^{35}S]methionine-labeled tubulins from wild-type testes and ovaries. (α designates the entire α-tubulin complex. See text.) (*a*) Total proteins extracted from eight adult testes showing β_1- and β_2-tubulins plus the α-tubulin complex. (*b*) Vinblastine sulfate precipitate from testes. (*c*) Vinblastine sulfate precipitate from ovaries. Note the absence of the β_2-tubulin. (*d*) Second coassembly pellet showing the testis tubulins.

resolution of the labeled β_1-tubulin subunit from testes is less distinct than in the gels shown in Fig. 1 *b* and *c* because of dilution by the large amount of embryo β_1-tubulin.

Definitive characterization of testis β_2-tubulin was obtained by peptide mapping after specific limited proteolysis by the method of Cleveland *et al.* (25). Fig. 2 shows a comparison of the peptides obtained from tubulins prepared from embryos by assembly *in vitro* (Fig. 2 lanes a and b) and from [^{35}S]methionine-labeled testes by vinblastine sulfate precipitation (Fig. 2 lane c).

Although α- and β-tubulins are well resolved in the first gel, β_1- and β_2-tubulins are not as well resolved, and the β-tubulin slice was cut such that it included both testis β species. Although the labeled testis peptides include those from both the β_1- and β_2-tubulin subunits, those from β_2 by far predominate because the β_2 subunit is present in at least 4-fold excess over the β_1 subunit in the testis. Whereas the peptide patterns from the β subunits of tubulin from embryos and testes are clearly homologous, there are distinct differences (indicated in the figure by arrows), demonstrating that β_2-tubulin is indeed a bona fide β-tubulin but distinct from β_1. For comparison and to illustrate the sensitivity of this technique, we include the peptide pattern obtained under these conditions from testis α-tubulins.

Finally, the specificity of the β_2-tubulin subunit to testis, initially indicated by its absence in embryos and in ovaries, was confirmed by the following experiment: brains, salivary glands, and imaginal discs from third instar larvae were incubated with [^{35}S]methionine; tubulins were then prepared by vinblastine sulfate precipitation and subjected to electrophoresis with the gel system of Laemmli (21). None of these tissues synthesized the β_2-tubulin subunit. β_2-Tubulin was synthesized, however, in larval testes. These results alone do not entirely eliminate the possibility that β_2-tubulin may occur in some other tissue; to do that would require a systematic examination of all larval and adult tissues. However, these results, taken together with those presented below, strongly support the hypothesis that β_2-tubulin is indeed specific to spermiogenesis.

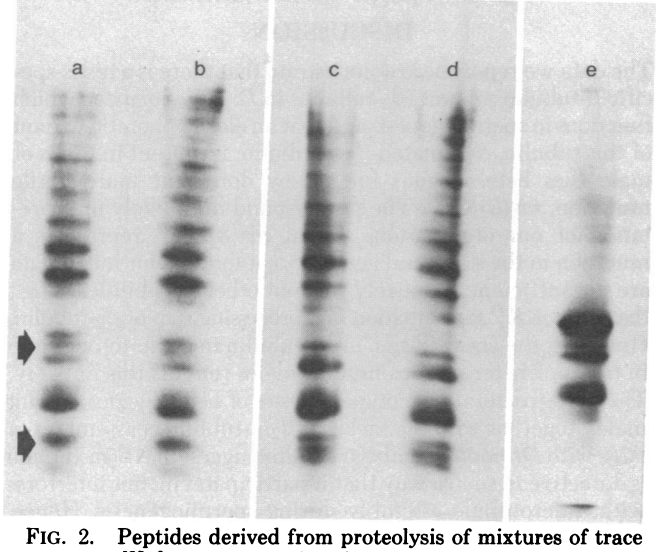

FIG. 2. Peptides derived from proteolysis of mixtures of trace amounts of [^{35}S]methionine-labeled testis tubulins and unlabeled embryo tubulins. Protease *Staphylococcus aureus* was used at a concentration of 1.25 μg/ml. (Lanes a and c) Labeled wild-type testis β_1- and β_2-tubulins with embryo β_1-tubulin carrier: lane a, Coomassie blue staining pattern of the peptides derived from the embryo β_1-tubulin; lane c, autoradiogram showing the peptides derived from the labeled testis tubulins. Arrows indicate regions of the gel in which differences between embryo and testis tubulins are apparent. (Lanes b and d) Labeled β_1-, β_2-, and β_2m-tubulins from *ms(3)KK^D* heterozygotes with embryo tubulin carrier: lane b, Coomassie blue staining pattern of the embryo β_1-tubulin peptides; lane d, autoradiogram showing the labeled peptides from testis β-tubulins. (Lane e) For comparison, the pattern of labeled peptides derived from the α-tubulin complex of testis; the stained pattern from the embryo α-tubulin complex was identical.

Variant β_2-Tubulin in Testes of Male Flies Heterozygous for a New Dominant Male Sterile Mutation. A dominant male sterile mutation [*ms(3)KK^D*] was identified in a screen for mutations induced by ethyl methane sulfonate in the third chromosome. In this mutant stock, males heterozygous for the mutation are sterile, while heterozygous females are fertile. As shown in Fig. 3*a*, two-dimensional gel analysis of [^{35}S]methionine-labeled proteins from testes of heterozygous [*ms(3)-KK^D)/In(3LR)CxD*] males revealed the presence of a new protein spot (β_2m-tubulin), which migrated to a slightly more basic region of the pH gradient gel than the tubulin from testes of wild-type flies and migrated slightly faster than wild-type β_2-tubulin in the second-dimension gel. Autoradiograms of gels of *ms(3)KK^D/In(3LR)CxD* and wild-type testis proteins were compared by using a densitometer to measure spot intensity. In testes of *ms(3)KK^D* heterozygotes, the amount of [^{35}S]methionine incorporated into β_2m-tubulin was equal to that incorporated into β_2-tubulin, but incorporation of [^{35}S]methionine into each was only one-half of that incorporated into β_2-tubulin in wild-type testes. Thus, β_2m-tubulin was identified as an electrophoretic variant of testis-specific β_2-tubulin induced by ethyl methane sulfonate. This was confirmed by two control experiments. First, as shown in Fig. 3*b*, two-dimensional gel electrophoresis of [^{35}S]methionine-labeled total proteins showed that the variant β_2m-tubulin is not present in ovaries from females heterozygous for the mutation. Second, the variant β_2m-tubulin was not present in testes of males of similar genetic background but carrying an untreated third chromosome; that is, testes of *red e/In(3LR)CxD* males contained β_1- and β_2-tubulin in the same propositions as in wild-type males, but did not contain β_2m-tubulin.

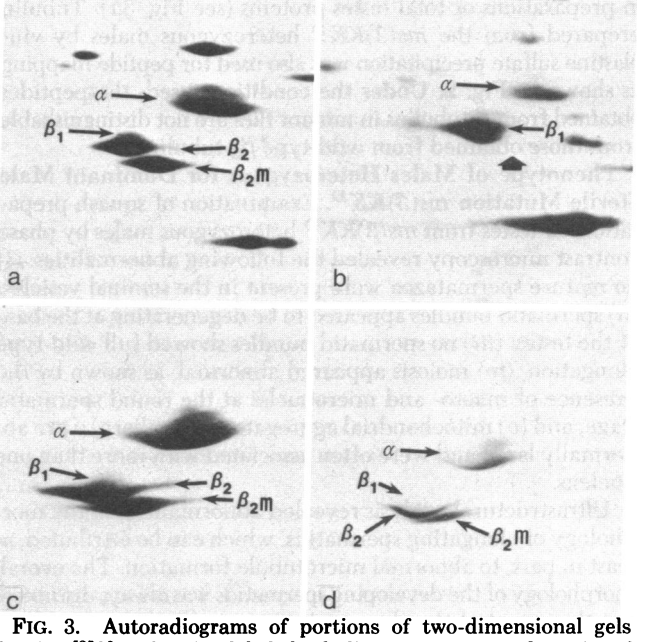

FIG. 3. Autoradiograms of portions of two-dimensional gels showing [^{35}S]methionine-labeled tubulins from testes and ovaries of flies heterozygous for *ms(3)KK^D*. (*a*) Total proteins extracted from eight mutant testes showing the β_2m-tubulin variant. (*b*) Total proteins extracted from two mutant ovaries. Note the absence of the β_2m-tubulin variant (heavy arrow). (*c*) Vinblastine sulfate precipitate from mutant testes. (*d*) Second coassembly pellet from mutant testes.

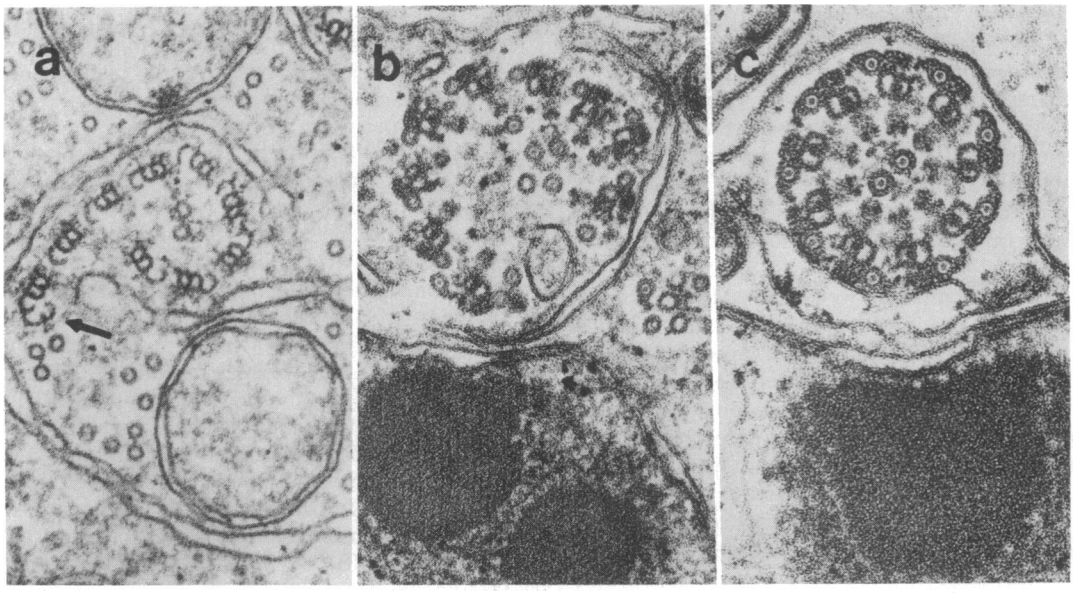

FIG. 4. Cross sections of axonemes from $ms(3)KK^D$ heterozygous and wild-type testes. (*a*) Early stage in development of mutant axoneme showing abnormal configuration of doublet tubules. Note the aberrant accessory tubule precursor (arrow). (×7200.) (*b*) Mutant axoneme at later stage showing general disorganization of axoneme and presence of multiple central tubules. (×8800.) (*c*) Late-stage wild-type axoneme. (×11,300.)

Vinblastine precipitation, coassembly with embryo tubulin, and peptide mapping were used to unequivocally demonstrate that β_2m-tubulin is an electrophoretic variant of β_2-tubulin. Testes from $ms(3)KK^D$ heterozygous flies were incubated with [^{35}S]methionine. Tubulin was then prepared by vinblastine sulfate precipitation and by coassembly with embryo tubulin and electrophoresed in the two-dimensional gel system. Autoradiograms of the resulting gels are shown in Fig. 3 *c* and *d* (compare to Fig. 1 *b* and *d*). In both cases both β_2- and β_2m-tubulin were present in tubulin prepared from testes of mutant flies in approximately the same ratio as they appeared in preparations of total testes proteins (see Fig. 3*a*). Tubulin prepared from the $ms(3)KK^D$ heterozygous males by vinblastine sulfate precipitation was also used for peptide mapping as shown in Fig. 2. Under the conditions used, the peptides obtained from β-tubulins in mutant flies are not distinguishable from those obtained from wild-type β_2-tubulin.

Phenotype of Males Heterozygous for Dominant Male Sterile Mutation $ms(3)KK^D$. Examination of squash preparations of testes from $ms(3)KK^D$ heterozygous males by phase contrast microscopy revealed the following abnormalities: (*i*) no mature spermatazoa were present in the seminal vesicles; (*ii*) spermatid bundles appeared to be degenerating at the base of the testes; (*iii*) no spermatid bundles showed full wild-type elongation; (*iv*) meiosis appeared abnormal, as shown by the presence of macro- and micronuclei at the round spermatid stage; and (*v*) mitochondrial aggregates (nebenkern) were abnormally large and were often associated with more than one nucleus.

Ultrastructural analysis revealed abnormalities in the morphology of elongating spermatids, which can be attributed, at least in part, to abnormal microtubule formation. The overall morphology of the developing spermatids was always disrupted, but the extent of the abnormalities varied in individual flies. Cross sections of elongating spermatids from testes of $ms(3)KK^D$ heterozygous males, which demonstrate the most consistently observed abnormalities, are shown in Fig. 4 *a* and *b*. Mutant axonemes at early (Fig. 4*a*) and later (Fig. 4*b*) stages of differentiation show a variety of defects: general disorganization of the axoneme, abnormal accessory tubules, additional central tubules, and abnormal mitochondrial derivatives. Fig. 4*c* shows a cross section through a wild-type spermatid for comparison. The derangement of the mutant axoneme illustrated in Fig. 4*a* appears to be due to a defect in assembly of the axoneme rather than to degeneration because differentiation proceeds to a later stage (Fig. 4*b*) and because degeneration of axoneme in *D. melanogaster* testes, as described by Kiefer (26), follows a consistent sequence not in evidence here.

DISCUSSION

The data we report here demonstrate that there is a testis-specific β-tubulin subunit (β_2-tubulin) in *D. melanogaster* which functions in spermiogenesis and that an electrophoretic variant of this tubulin, designated β_2m-tubulin, is present in testes of male flies heterozygous for a new dominant male sterile mutation, $ms(3)KK^D$. The simplest and most likely interpretation of our observations is that $ms(3)KK^D$ represents a mutation in the structural gene for β_2-tubulin. Our initial data are not sufficient to entirely rule out other possibilities—e.g., that $ms(3)KK^D$ is a mutation in a processing step of β_2-tubulin. However, the coexpression of β_2-tubulin in a one-to-one ratio in testes of heterozygous mutant males renders this unlikely. The ultrastructural phenotype of testes of heterozygous mutant males, together with the fact that β_2m-tubulin coassembles *in vitro* with *Drosophila* embryo tubulin, suggest that β_2m-tubulin is defective in such a way that it participates in, but interferes with, microtubule assembly during spermiogenesis. Hence, completion of normal spermatid differentiation is precluded. The range of morphological defects we observed in testes of individual male flies heterozygous for $ms(3)KK^D$ suggests that the altered β_2m-tubulin interferes in at least two processes of spermatid development: meiosis and formation of the sperm tail axoneme. If the altered tubulin simply failed to assemble, one would expect production of sperm of normal morphology but reduced numbers assembled from the half complement of normal β_2-tubulin present in the testes of the heterozygous mutant males. At present we do not know whether the points at which abnormal microtubule assembly occurs in testes of mutant males accurately reflect the processes in which β_2-tubulin normally functions or whether β_2m-tubulin may interfere at points in which β_2-tubulin is not normally functional.

Our findings are consistent with reports describing tubulin heterogeneity in other organisms. For example, tubulins specific to the axoneme of cilia or flagella have been observed in *Naegleria* (6–8) and in the sea urchin (1–5). We have observed testis-specific tubulins in the Mexican axolotl and other amphibians (27). In addition, microheterogeneity of cytoplasmic tubulins, particularly in the α subunit, has been observed in vertebrate brain by workers in a number of laboratories (reviewed in ref. 28). Finally, a recent study by Sheir-Neiss *et al.* (29) demonstrated extensive heterogeneity in both the α and β subunits of cytoplasmic tubulins in the fungus *Aspergillus nidulans* similar to that which we have observed in *Drosophila* tissues. These authors were also the first to identify electrophoretic variants of tubulin in mutants selected for resistance to antimitotic drugs. They found that a number of *Aspergillus* mutants with altered sensitivity to benzimidazole antimitotic drugs have β-tubulin subunits with altered electrophoretic mobility. All of the above-mentioned observations of tubulin heterogeneity, including the data reported here on spermiogenesis in *Drosophila*, lend strong support to the hypothesis that control of the assembly of functionally different microtubules is governed, at least in part, by the availability of functionally different tubulin subunits. Spermiogenesis in *Drosophila* appears to be a system uniquely suited both to correlate the role of functionally different tubulins *in vivo* with their biochemical properties *in vitro* and to identify the regulatory mechanisms that control the time and localization of their synthesis.

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