Genetic Analysis of Microtubule Structure: A β-Tubulin Mutation Causes the Formation of Aberrant Microtubules In Vivo and In Vitro

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Abstract. A recessive male sterile mutation ($B2t^8$) that encodes a stable variant of the testis-specific β_2-tubulin of *Drosophila* causes the assembly of aberrant microtubules both in vivo and in vitro. The $B2t^8$ mutation appears to cause defects in the formation of interprotofilament bonds. In testes from homozygous mutant males, the most commonly observed aberrant structures were sheets of protofilaments curved to form an S in cross section rather than a normal, closed microtubule. These characteristic S-shaped structures appear in the meiotic spindle, in place of axonemes in differentiating spermatids, and in cytoplasmic microtubules, including those that lie next to the nucleus during nuclear elongation. Homozygous mutant males exhibit defects in chromosome movement and cytokinesis during meiosis, flagellar elongation, and nuclear shaping, indicating that the ability to form normal closed microtubules is required for each of these events. The presence of the aberrant microtubules in three architecturally different microtubule arrays demonstrates conclusively the multifunctional nature of the β_2-tubulin gene product. Although the mutant β_2-tubulin subunit causes assembly of aberrant microtubules in vitro and in homozygous males, in the presence of wild-type β_2-tubulin in heterozygous males, the variant subunit coassembles with the wild-type subunit into functional sperm.

MICROTUBULES provide the structural basis for a variety of fundamental processes in eukaryotic cells, ranging from chromosome separation during cell division to the motility of cilia and flagella. The diversity of microtubule function is reflected in a matching diversity of form: the microtubules of the spindle are organized into an array structurally different from that of the flagellar axoneme. The mechanism by which this diversity is specified has remained one of the long-standing unanswered questions in microtubule research. Functionally and morphologically different microtubule arrays could be assembled from structurally different tubulin isoforms encoded by separate genes (Fulton and Simpson, 1976), thus reducing the problem of spatial diversity to one of differential gene expression.

The testis-specific β-tubulin gene of *Drosophila* has provided a test of the possibility that a single tubulin gene product can participate in architecturally different microtubule arrays. In addition, it offers an excellent system for genetic analysis of the role of β-tubulin structure in microtubule assembly and function. Synthesis of the testis-specific form of β-tubulin (β_2), encoded at the *B2t* locus, begins just before the onset of meiosis in *Drosophila* males. At the same time, synthesis of the major somatic β-tubulin (β_1) is decreased so that β_2 becomes the major form of β-tubulin in the adult testis (Kemphues et al., 1982). Mutations in the β_2-tubulin structural gene cause male sterility but do not affect viability or female fertility, presumably because expression of the gene is limited to the testis. Kemphues et al. (1982, 1983) isolated four recessive male-sterile mutations in the structural gene for the testis-specific β-tubulin. In the testis of flies homozygous for these mutations, designated class I alleles, both α- and β_2-tubulin are synthesized but degraded within a few hours, possibly due to the failure of the mutant β_2-tubulin subunits to form the αβ heterodimer (Kemphues et al., 1982, 1983). In the absence of β_2 and most of the α-tubulin in the testis of mutant males, there was no chromosome movement during meiosis, no axonemes were formed, little or no spermatid elongation occurred, and nuclear shaping was defective. These mutant phenotypes suggest strongly that β_2-tubulin is multifunctional and is required for at least three different kinds of microtubule arrays during spermatogenesis.

To confirm the multifunctional participation of β_2-tubulin in different kinds of microtubule arrays and to relate β_2-tubulin structure with its function in microtubule assembly, we have isolated an additional class of recessive male-sterile

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mutations in the β_2-tubulin structural gene. These class II β_2-tubulin alleles appear to encode partially functional subunits that are at least able to make the $\alpha\beta$-tubulin heterodimer (Fuller, 1986). Because in these mutants both the variant β_2-tubulin and wild-type α-tubulin are stable, the phenotype of the class II alleles reflects the presence of an aberrant β_2-tubulin subunit rather than the absence of the normal testis pools of α- and β-tubulin (Raff and Fuller, 1984).

One of the new alleles, *B2t*^{8}, encodes a variant β_2-subunit that assembles into aberrant microtubules both in vitro and in vivo. We have used the abnormal shape of the microtubules assembled in the mutant as an ultrastructural marker to demonstrate the direct participation of the mutant β_2-tubulin subunit as a structural component of the meiotic spindle, the flagellar axoneme and the microtubules involved in nuclear shaping. In addition, we have identified an aspect of β_2-tubulin primary structure required for the correct side-to-side association of protofilaments leading to the assembly of closed microtubules. Because meiosis, flagellar elongation, and nuclear shaping are all defective in this mutant, the ability to form normal, closed microtubules appears to play an important role in each of these processes.

Materials and Methods

Drosophila Strains and Genetic Characterization

Flies were raised at 25°C on standard cornmeal-molasses-agar medium. *Drosophila* visible mutations and balancers are described in Lindsley and Grell (1968). *B2t*^{8} was isolated on a *red e* background chromosome in a screen for ethyl methanesulfonate-induced alleles of the β_2-tubulin locus. It was mapped by meiotic recombination to 48.54 ± 0.16 map units, using the flanking markers *p*^{p} and *by*, in good agreement with the position of 48.49 ± 0.06 map units for the *B2t* locus determined by Kemphues et al. (1980). In that *B2t*^{8} encodes an electrophoretic variant of β_2-tubulin, and both the wild-type and variant forms are present in heterozygotes, we conclude that *B2t*^{8} is a mutation in the β_2-tubulin structural gene. The original *B2t*^{8} *red e* chromosome was used in all experiments described in this paper. This original chromosome in combination with a null mutation at the *B2t* locus (*B2t*^{8} *red e*/*B2t*^{n}) or in combination with an amorphic, class I *B2t* allele (*B2t*^{8} *red e*/*B2t*^{3}) has the same phenotype as *B2t*^{8} *red e* homozygotes when examined at the light-microscope level. *B2t*^{8} *red e*/*B2t*^{3} has also been examined at the ultrastructural level and has the same aberrant microtubule phenotype as the *B2t*^{8} *red e* homozygotes, as do homozygotes for any of three different recombinants that carry the *B2t*^{8} mutation but have crossed away parts of the original *red e* chromosome (*B2t*^{8} *cu*, *B2t*^{8} *by cu*, and *B2t*^{8} *red*). Finally, two copies of a small DNA fragment containing the wild-type β_2-tubulin gene (Raff, E. C., unpublished observations) rescues the *B2t*^{8} phenotype and restores *B2t*^{8}/*B2t*^{8} males to fertility. Taken together, these results confirm that all of the defects in spermatogenesis observed in *B2t*^{8} homozygotes are due to the mutation in the β_2-tubulin transcription unit, and not to other mutations elsewhere on the chromosome. *OreR*, *red e*, and *TM3* all behaved genetically as *B2t*$^+$ and were used for wild type as indicated in the figure legends.

Electron and Light Microscopy

For electron microscopy, testes from recently eclosed males were dissected in Kalt and Tandler (1971) trialdehyde fixative, fixed for 2 h at room temperature in 2% glutaraldehyde, 0.1 M sodium cacodylate, pH 7.4, then rinsed, postfixed, stained, and embedded as described for *Drosophila* embryos in Mahowald et al. (1979). Cross sections were cut in the straight region of the testis. For tannic acid staining, testes were punctured many times with a sharp needle in a fixative of 1% glutaraldehyde, 1% EM grade tannic acid, 0.1 M sodium phosphate, pH 6.0, at room temperature, postfixed in 0.5% osmium tetroxide, 0.1 M sodium phosphate, pH 7.4, then rinsed, stained, and embedded as above. For light microscopy, testes embedded for EM as described above were cut in 0.25–1-μm sections and stained with 1.0% sodium borate, 0.5% toluidine blue, which had been diluted 1:1 with ethanol immediately before use.

Polyacrylamide Gel Electrophoresis

Two dimensional polyacrylamide gel electrophoresis was performed as described in Kemphues et al. (1979), but with an ampholine mixture of one part pH 3.5–10 (LKB Produkter, Bromma, Sweden) and four parts pH 5–6 (Serva Fine Biochemicals, Inc., Garden City, NY) in the first dimension and 9% acrylamide and 0.52% *N,N'*-methylene-bis-acrylamide in the second dimension.

Tubulin Assembly

Newly synthesized testis proteins were labeled with [^{35}S]methionine by incubation for 70–80 min as described by Kemphues et al. (1979). 20 labeled testes were added to an excess of unlabeled testes of the same genotype in assembly buffer (0.1 M 4-morpholine ethanesulfonate, 0.5 mM $MgCl_2$, 1 mM EGTA) supplemented with 2 mg/ml fresh GTP and 2 mg/ml fresh RNase A, and sonicated on ice until visibly disrupted. Sonicates were brought to a final volume of 0.07 μl per testis with the above buffer, sonicated again, and centrifuged at 4°C for 15 min at 100,000 *g* in a Beckman airfuge (Beckman Instruments, Inc., Fullerton, CA) to obtain a high-speed supernatant. Sonicated testes from *B2t*^{8}/*B2t*^{8} males consistently had approximately three-fourths the volume of an equivalent number of testes from heterozygotes or from wild type and so were brought to a final volume of only 0.05 μl per testis. The high speed supernatant was carried through cycles of assembly and disassembly as described by Kemphues et al. (1979) in the presence of glycerol, GTP, and RNase A. At each step, the assembly pellet was resuspended in one-half the preceding supernatant volume. The addition of 2 mg/ml RNase A was required for successful microtubule assembly from *Drosophila* testis extracts in the absence of embryo tubulin carrier. Other RNases, or lower concentrations of RNase A did not effectively stimulate assembly. The requirement for RNase could not be removed by adding histone to the assembly mixture to act as a polycation.

Results

B2t^8 Causes Defects in Microtubule Structure, Axoneme Assembly, and Flagellar Elongation

In wild-type males of *Drosophila melanogaster*, the newly formed sperm tail flagellar axoneme has a simple nine-plus-two arrangement of outer doublet and central pair microtubules. As the axoneme matures, it becomes decorated with accessory structures. A sheet of protofilaments grows out of the B subfiber of each outer doublet and curls around to form an accessory microtubule (Fig. 1, *a* and *c*). A densely staining, beaklike structure forms on the far side of each accessory tubule, giving the axoneme its characteristic pinwheel cross section (Fig. 1 *b*). Finally, the central pair and the accessory microtubules fill in with a dense central core (Fig. 1 *b*) (Kiefer, 1970; Tokuyasu, 1974*a*; see Lindsley and Tokuyasu, 1980 for a detailed review of normal spermatogenesis in *Drosophila*).

Males homozygous for the *B2t*^{8} mutation in the testis-specific β-tubulin of *Drosophila* have dramatic defects in axoneme assembly and flagellar elongation. Cross sections through the axonemal region of elongating spermatid bundles in *B2t*^{8} homozygotes reveal clusters of aberrant microtubules in place of the classic nine-plus-two axoneme (Fig. 1, *e* and *f*). Of the variety of aberrant microtubule forms observed at early stages of spermatid development, S-shaped microtubules were the most common (Fig. 1 *e*). Closed singlet microtubules were found occasionally (Fig. 1 *e*, *arrows*). At later stages, some of the aberrant microtubules become decorated with beaklike projections or dense central cores similar to the accessory structures seen in mature axo-

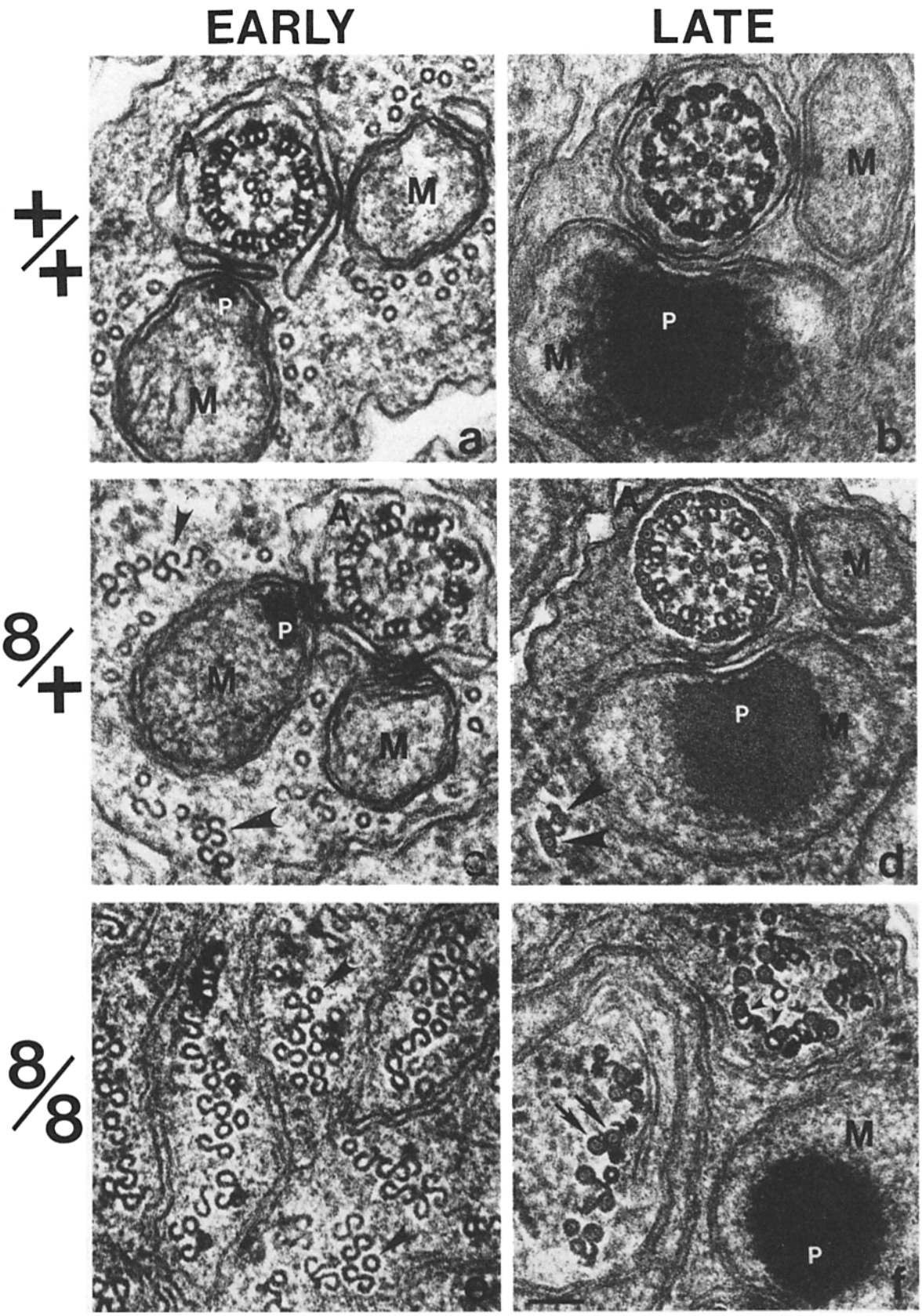

Figure 1. Axoneme structure in $B2t^8/B2t^8$, $B2t^8/+$, and wild-type males. Electron micrographs of cross sections cut through bundles of elongated spermatids. (*a*) Early and (*b*) late stages in axoneme assembly in wild-type (+/+) *Ore-R* males. (*c*) Early and (*d*) late stages in axoneme assembly in heterozygous $B2t^8/+$ males; wild type was *red e*; (*arrows*) aberrant cytoplasmic microtubules. (*e*) Early- and (*f*) late-stage spermatids in $B2t^8/B2t^8$ homozygotes; (*arrows* in *e*) closed microtubules; (*long arrows* in *f*) S-shaped microtubule with both loops filled with dense central material (see also Fig. 2 *a*); (*short arrows* in *f*) microtubules sharing a wall in doublet conformation. (*A*) axoneme; (*M*) mitochondrial derivatives; (*P*) paracrystalline material which fills the major mitochondrial derivative in late-stage spermatids. Bar, 0.1 μm.

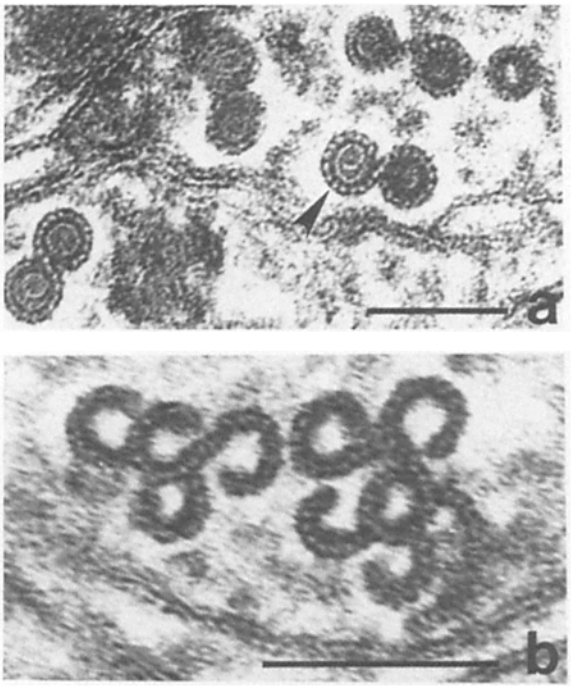

Figure 2. Protofilament arrangement in aberrant microtubules from $B2t^8$ homozygotes. Cross sections of aberrant microtubule structures in tannic-acid stained preparations of testis from $B2t^8/B2t^8$ males. (*a*) S-shaped microtubules from a late stage spermatid, decorated with dense central core material (*arrow*). (*b*) Complex aberrant microtubule structures from an early spermatid. Bar, 0.1 μm.

nemes from wild-type males (Fig. 1 *f*, *long arrows*; Fig. 2 *a*). The upper cluster of aberrant microtubules in Fig. 1 *f* resembles a broken axoneme and contains doublet microtubules (Fig. 1 *f*, *short arrows*). Such relatively organized clusters were rare and probably represent cross sections near a basal body. Representative sections from sets of serial cross sections including basal bodies revealed that basal bodies have normal morphology in $B2t^8$ males and can give rise to a ring of axonemal doublet microtubules with normal morphology. However, in the six sets of serial sections examined, the ring of outer doublet microtubules lost organization rapidly with distance and had frayed apart within 2–8 μm of the basal body. In no case were central pair microtubules observed.

Tannic acid-treated preparations clearly showed that the aberrant, S-shaped microtubules consist of a sheet of protofilaments curved in two directions to form the S-shaped cross section (Fig. 2 *a*). Because the aberrant microtubules are not completely closed, the number of protofilaments per loop was difficult to ascertain. However, each loop of the S was roughly similar in diameter to normal, closed microtubules and had 13–15 protofilaments. The S-shaped microtubules shown in Fig. 2 *a* contain dense central cores characteristic of microtubules in the axoneme region in late stage spermatids. In some images, the dense central material appeared to form a spiral structure (Fig. 2 *a*, *arrow*; see also Fig. 1 *f*). Other forms of aberrant microtubules assembled in $B2t^8$ homozygotes may be variations on the S theme. As shown in Fig. 2 *b*, curved sheets of protofilaments shaped like half of an S often appear to be added onto the side of another protofilament sheet to generate complex, composite structures.

Morphologically normal axonemes with doublet microtubules are formed in $B2t^8/+$ heterozygous males (Fig. 1, *c* and *d*). However, $B2t^8$ shows some dominant effects on the structure of cytoplasmic microtubules. During normal spermatogenesis in wild-type individuals, several cytoplasmic microtubules lie along the growing axoneme/mitochondrial derivative complex (Fig. 1 *a*). These cytoplasmic microtubules disappear during late stages of spermatid maturation (Fig. 1 *b*) and are gone before the process of individualization excludes most of the cytoplasm from mature sperm. In $B2t^8/+$ heterozygotes, aberrant microtubules appear among the morphologically normal cytoplasmic microtubules (Fig. 1 *c*, *arrows*). As spermatid maturation proceeds, these abnormal cytoplasmic microtubules often fill in with a dense central core and grow a hooked projection so that they resemble the accessory tubule complex from mature axonemes (Fig. 1 *d*, *arrows*). The decoration of the abnormal cytoplasmic tubules occurs at roughly the same time as the decoration of the accessory tubules in the maturing axoneme. Perhaps because the added structures have conferred on them stability characteristic of the microtubules of the axoneme, the aberrant cytoplasmic microtubules in $B2t^8/+$ males persist after the normal cytoplasmic microtubules disappear (Fig. 1 *d*).

$B2t^8/+$ males produce functional, motile sperm and are fertile when mated. Axoneme morphology was normal in individualized sperm from the seminal vesicle of heterozygous males and no aberrant cytoplasmic microtubules were observed, probably because of their exclusion with the bulk of the cytoplasm during individualization. Mature sperm isolated from the seminal vesicles of heterozygous males contain both wild-type β_2-tubulin and the basic electrophoretic variant subunit encoded by the mutant gene (Fig. 3). Thus, although the mutant tubulin subunit encoded by $B2t^8$ appears to be unable to form normal microtubules in vivo in the absence of wild-type protein, it participates in the formation of functional sperm in vivo when wild-type β_2-tubulin is present. Because individualized sperm have very little cytoplasm and the flagellar axoneme is the major tubulin-based structure in mature sperm, the variant tubulin in sperm from $B2t^8/+$ heterozygotes is most likely assembled into the axoneme.

As might be expected from the disorganized and abnormal microtubules in the axonemal region, flagellar elongation is

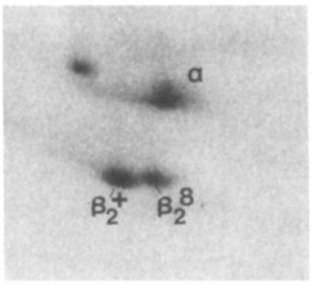

Figure 3. The mutant $B2t^8$ subunit is incorporated into functional sperm in $B2t^8/+$ heterozygotes. Mature sperm were dissected from the seminal vesicles of 20 $B2t^8/TM3$ fertile males held away from females for several days. After separation by two-dimensional gel electrophoresis, the sperm proteins were visualized by Coomassie Blue staining. Only the tubulin region of the gels is shown. The acid end of the first dimension is to the left. (α) α-Tubulin; (β_2^+) wild-type β_2-tubulin; (β_2^8) basic electrophoretic variant β_2-tubulin encoded by the $B2t^8$ mutation.

aberrant in $B2t^8$ homozygotes. During flagellar elongation in wild-type males, the spherical mitochondrial derivative in each spermatid cell unfolds, splits into two parts, and elongates along the growing axoneme. As it does so, it passes through a brief but characteristic stage during which small inclusions of cytoplasm can be observed by light microscopy within the mitochondrial mass. As flagellar elongation begins in $B2t^8$ homozygotes, the mitochondria unfold and begin to elongate, but never achieve the smooth profile characteristic of normal elongating mitochondria. The cytoplasmic inclusion stage persists well beyond its normal duration, the mitochondria usually contain irregular lumps and swirls of membrane, and the entire flagellar bundle elongates to only a fraction of its normal, wild-type length. Defective flagellar elongation and the aberrant morphology of the mitochondrial derivative in $B2t^8$ homozygotes probably result from the observed defects in the structure of the axonemal microtubules.

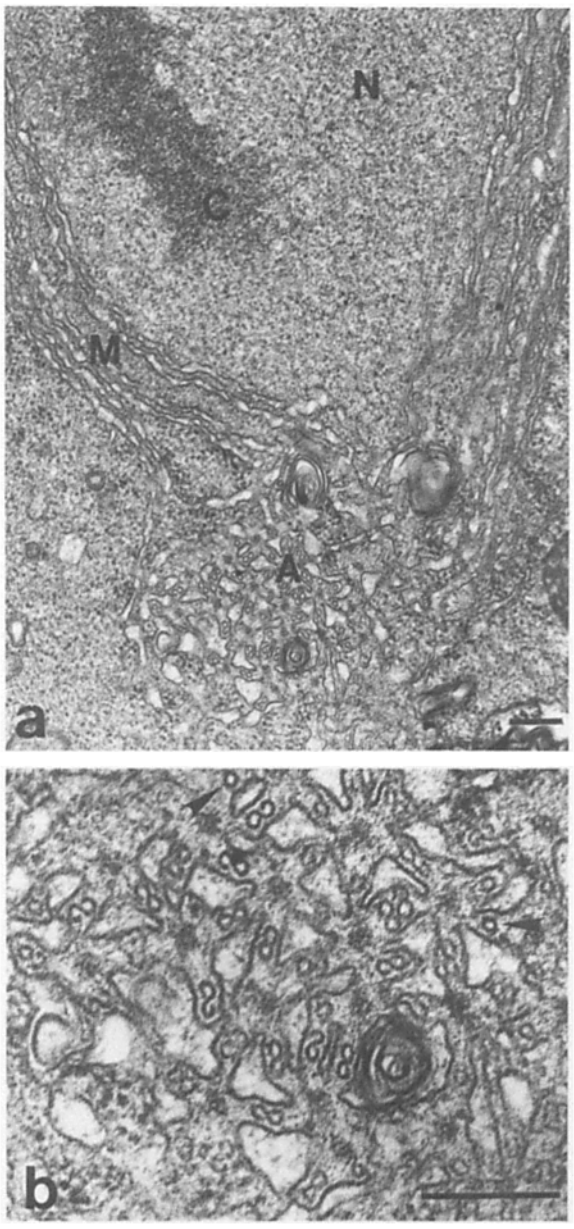

Figure 4. Aberrant microtubules in the astral region of the meiotic spindle in a $B2t^8$ homozygous male. (*a*) Longitudinal section through the meiotic spindle in a $B2t^8/B2t^8$ male. (*A*) Astral region of the spindle; (*C*) chromosome; (*M*) parafusorial membranes; (*N*) nucleus. (*b*) Higher magnification of the astral region in *a*. Note that, where discernible, the S-shaped microtubules in this field have the same handedness; (*arrows*) closed microtubules. Bars, 0.2 μm.

The Meiotic Spindle Contains Aberrant Microtubules in $B2t^8$ Homozygotes and Both Meiosis and Cytokinesis Are Defective

Ultrastructural analysis of meiotic spindles from $B2t^8$ homozygotes revealed abnormal microtubules, many of which exhibit the characteristic S-shaped cross section. Fig. 4 shows an electron micrograph of a section through the astral region of a meiotic spindle from a $B2t^8$ homozygote. In *Drosophila* the nucleus remains surrounded by a system of membranes during meiosis in normal males, and the region of the spindle pole has a characteristic array of astral membranes, among which the microtubules of the spindle aster are interspersed (Tates, 1971). Although microtubules with an apparently normal cross section (Fig. 4 *b*, *arrows*) were occasionally observed in the spindles of males homozygous for $B2t^8$, most of the microtubules in both the astral region and the central spindle were S-shaped. In $B2t^8/+$ heterozygotes, which are fertile, the meiotic spindle is composed primarily of normal-looking microtubules, although S-shaped microtubules were often observed scattered among them.

Males homozygous for the $B2t^8$ mutation have complete failure of cytokinesis after both meiosis I and meiosis II. Normally, early spermatid cells after completion of the two meiotic divisions are considerably smaller than the large primary spermatocytes (*P*) about to enter meiosis (Fig. 5 *a*). In testis from $B2t^8$ males, however, early spermatids are approximately the same size as the parent primary spermatocytes (Fig. 5 *b*).

The $B2t^8$ mutation causes defects in chromosome segregation during meiosis, but not failure of sister chromatids to move apart. Early spermatids from $B2t^8$ homozygotes often contain an abnormally large mitochondrial derivative (*m*) associated with multiple nuclei (*n*) (Fig. 5 *b*). In the class I β_2-tubulin mutations, in which there appears to be no functional spindle, the chromosomes do not move apart during meiosis, there is no cytokinesis, and usually each of the resulting early spermatid cells contains a single 4N nucleus (Kemphues et al., 1982). In that early spermatid cells from $B2t^8$ homozygotes characteristically have several nuclei of various sizes, the chromosomes must separate in this mutant, although they may not segregate correctly to the poles. Because the size of the nucleus in *Drosophila* spermatids is roughly proportional to the amount of chromatin enclosed (Hardy, 1975), the variety of nuclear sizes in $B2t^8$ early spermatid cysts indicates failure of ordered chromosome segregation during meiosis. Orcein-stained squashed preparations confirm that cells in anaphase II from $B2t^8$ homozygotes contain the 4N complement of separated sister chromatids, which remain scattered across the spindle.

Aberrant Perinuclear Microtubules and Defective Nuclear Shaping in $B2t^8$ Homozygotes

During normal nuclear shaping, a band of dense cytoplasm that contains longitudinally oriented microtubules forms

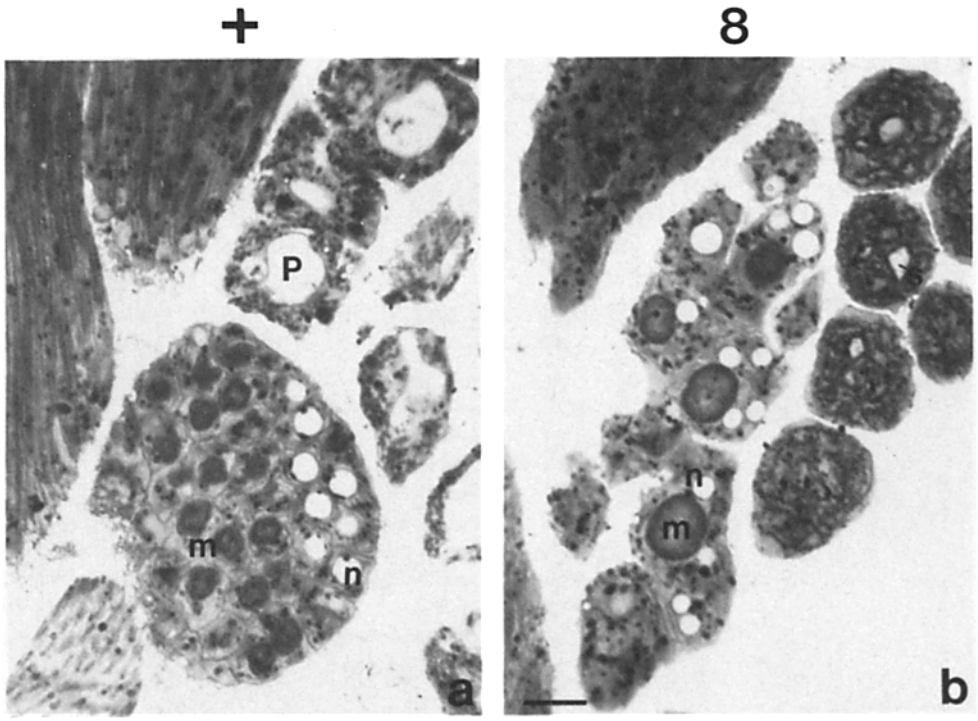

Figure 5. $B2t^8$ causes failure of cytokinesis after meiosis I and meiosis II so that early spermatid cysts contain large cells with multiple nuclei. Light micrographs of thick sections through early spermatid cysts in whole testis. (*a*) Wild type (*red e*). Cyst of 64 early spermatids. The plane of section contains the nucleus (*n*) in some cells and the mitochondrial derivative (*m*) in others. (*P*) Premeiotic primary spermatocyte. In upper left, bundles of elongating spermatids. (*b*) $B2t^8$ homozygote. Abnormally large early spermatid cells often contain a large mitochondrial derivative (*m*) associated with more than one nucleus (*n*). In upper right, aberrant spindles (*s*) in a cyst of cells undergoing meiosis II. Bar, 10 μm.

along one side of the nucleus. The side of the nucleus lying along this dense cytoplasm becomes increasingly more concave and the nucleus elongates in the direction parallel to the microtubules (Tokuyasu, 1974*b*). In $B2t^8/+$ heterozygotes, which have normal nuclear shaping and are fertile, a mixture of normal and S-shaped microtubules appear in the dense cytoplasm next to the nucleus (Fig. 6 *a*).

Orcein-stained squashed preparations of testes from $B2t^8$ homozygotes revealed that nuclear shaping is severely defective in the mutant males; late spermatid nuclei remain round

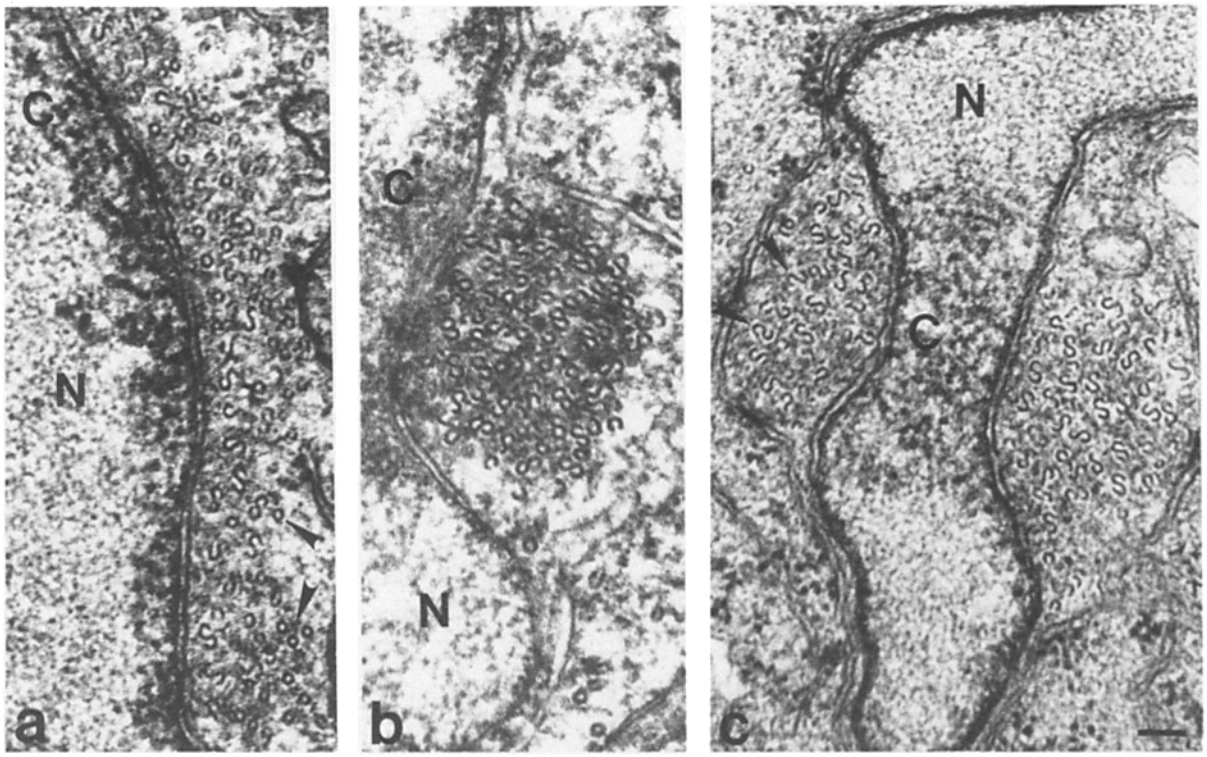

Figure 6. Defective perinuclear microtubules in $B2t^8$ elongating spermatids. Electron micrographs of microtubules in the region of dense cytoplasm next to the nucleus (*N*) during nuclear shaping. (*C*) Chromatin condensing along the inner surface of the nuclear membrane. (*a*) $B2t^8/+$ (*red e*) heterozygote; (*arrows*) normal microtubules. (*b* and *c*) $B2t^8/B2t^8$ homozygotes; the nucleus in *c* is highly abnormal in shape. Note that although most S-shaped microtubules in *c* are right-handed, a few (*arrows*) show opposite orientation. Bar, 0.1 μm.

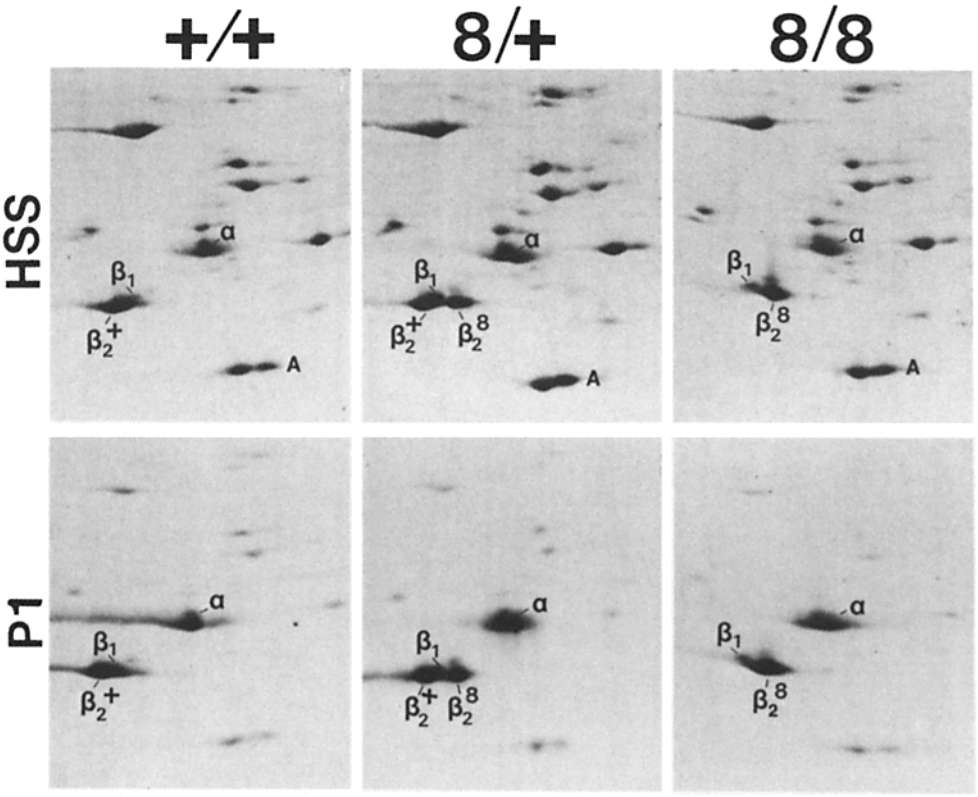

Figure 7. In vitro assembly of testis tubulin from wild-type, heterozygous, and homozygous $B2t^8$ males. Autoradiograms of the tubulin region of two-dimensional gels. (*HSS*) High-speed supernatants from testis extracts; (*Pl*) pellets after one round of microtubule assembly in vitro. Starting samples contained 160–175 unlabeled testes as carrier and half of the first assembly pellet was used as gel sample. The assembly pellets were also enriched for α- and β-tubulin in the corresponding Coomassie blue-stained gels (not shown). Wild-type β_2-tubulin migrates below and to the acidic (left) side of β_1, the major somatic tubulin, which is synthesized at low levels in adult testis (Kemphues et al., 1982). $B2t^8$ encodes an electrophoretic variant subunit which migrates below and to the basic (right) side of β_1-tubulin. (α) α-Tubulin; (A) actin. Genotypes: (+/+) *red e/red e*; (8/+) $B2t^8$/*TM3*; (8/8) $B2t^8/B2t^8$.

or only partially shaped and are scattered along the spermatid bundle instead of clustered at one end. In $B2t^8$ homozygotes most of the perinuclear microtubules are abnormal and many show the characteristic S-shaped cross section (Fig. 6, *b* and *c*). Nuclei sometimes became concave on two sides and displayed more than one cluster of microtubules (Fig. 6 *c*). Nuclei with bundles of dense cytoplasm along two sides were often observed by light microscopy in squashes of testis from $B2t^8$ homozygotes.

$B2t^8$ Tubulin Assembles into Aberrant Microtubules In Vitro As Well As in Vivo

$B2t^8$ tubulin assembles in vitro in both the presence and the absence of wild-type β_2-tubulin subunits. Testis extracts from wild-type, heterozygous, and homozygous $B2t^8$ males were carried in parallel through rounds of temperature-dependent microtubule assembly/disassembly. Fig. 7 shows two-dimensional gels of the starting material for the assembly reactions (*HSS*) and the proteins found in the pellet after the first round of microtubule assembly (*Pl*). In all cases, α- and β-tubulin were enriched in the assembly pellets (compare with actin). In the presence of wild-type β_2-tubulin in extracts from heterozygotes, the $B2t^8$ subunit was able to assemble in vitro as efficiently as the wild-type subunit. Wild-type and mutant β_2 protein were present in roughly the same ratio in the high speed supernatant and the first assembly pellet (Fig. 7, *center panels*). The $B2t^8$ subunit also assembled in vitro in the absence of wild-type β_2-tubulin in testis extracts from homozygous mutant males (Fig. 7, *right panels*). In all genotypes, the testis extracts contained a small amount of the general somatic β-tubulin, β_1. This low level of β_1-tubulin might have facilitated the assembly of the mutant $B2t^8$ subunits in the extract from homozygous mutant males. However, assembly did not result in a net enrichment of β_1-tubulin: β_1- and β_2-tubulin are present in roughly the same ratio in the high speed supernatant and the assembly pellet (Fig. 8, *right panels*).

To examine the morphology of the structures assembled in vitro, pellets from the second round of a similar assembly experiment were embedded and sectioned for electron microscopy (Fig. 8). The microtubules assembled in vitro from homozygous mutant males and from heterozygotes were clearly abnormal. In both cases, cross sections showing closed microtubules were rare and a variety of aberrant structures were observed, some of which resembled the S-shaped microtubules or complex joined sheets of protofilaments observed in the mutant in vivo (Fig. 2). The assembly pellet from heterozygous males appeared to contain slightly longer or more rigid structures than the assembly pellet from the homozygous mutant testes (Fig. 8, *longitudinal sections*). Thus, the mutant $B2t^8$ subunit appears to be at least partially dominant in in vitro assembly experiments under our conditions, causing the assembly of aberrant microtubules in mixtures of mutant and wild-type β_2-tubulin protein.

Discussion

Aberrant microtubules assembled in homozygous $B2t^8$ males provide a convenient ultrastructural tag with which to localize the mutant gene product in the major microtubule arrays

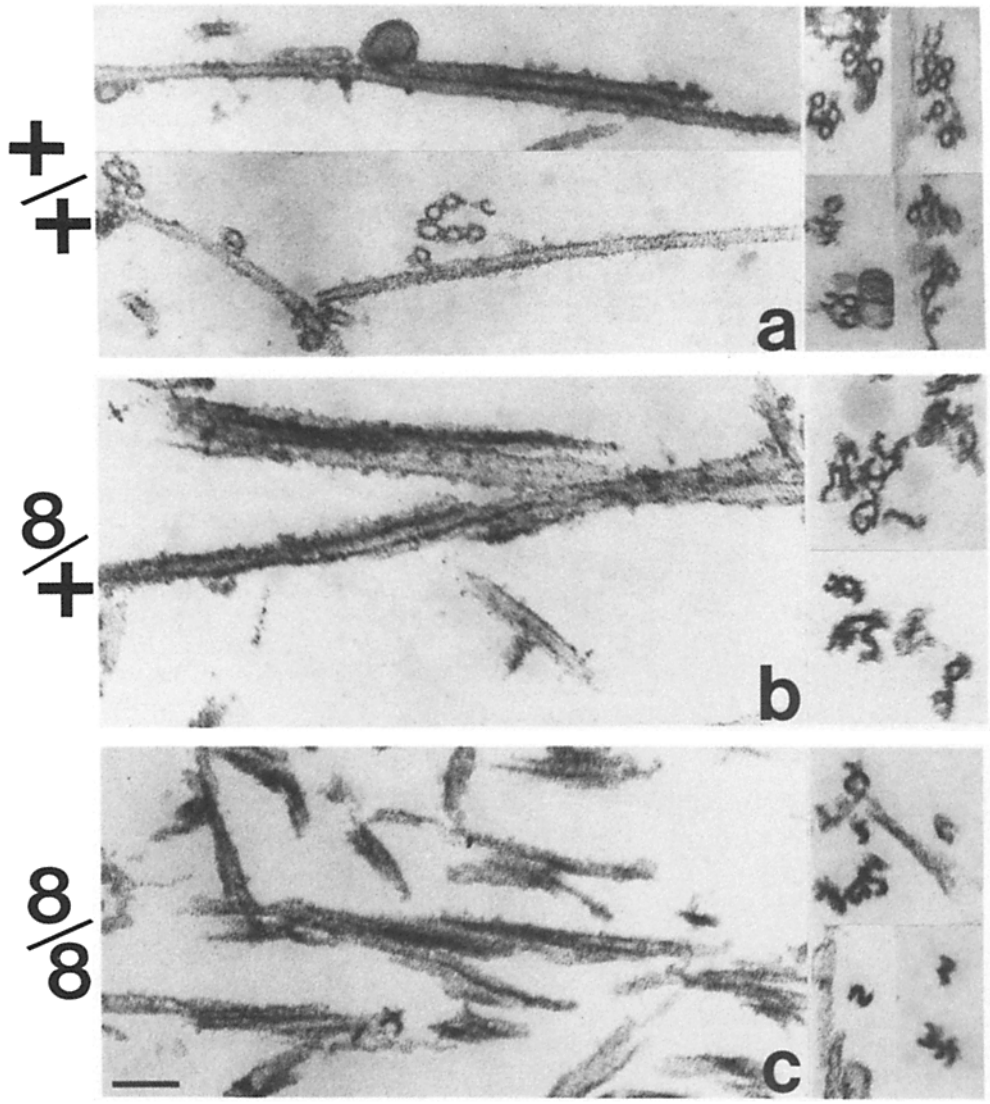

Figure 8. Morphology of structures assembled in vitro from testis from wildtype, heterozygous, and homozygous mutant males. Electron micrographs of sections cut through pellets from the second round of in vitro assembly. (*A*) Wild type (*red e*); (*b*) heterozygous (*B2t*^{8}/*TM3*); (*c*) homozygous (*B2t*^{8}/*B2t*^{8}). Starting samples contained (*a*) 609, (*b*) 560, and (*c*) 989 unlabeled carrier testes of the respective genotype. At the final polymerization step, each sample was divided so that the pellet from part of the sample could be fixed and embedded for electron microscopy whereas the pellet prepared in parallel was resuspended and analyzed by two-dimensional polyacrylamide gel electrophoresis. The second assembly pellet from the heterozygous males (*b*) contained approximately equal amounts of mutant and wild-type β_2-tubulin subunits. The major components of the second assembly pellet from the homozygous mutant males were α- and *B2t*^{8} tubulin (not shown). Assembly conditions included glycerol and 2 mg/ml RNase A. Bar, 0.1 μm.

involved in sperm cell morphogenesis. The presence of the characteristic aberrant microtubules in the meiotic spindle, in place of the sperm tail flagellar axoneme, and among the perinuclear microtubules during nuclear shaping in *B2t*^{8} homozygotes demonstrates clearly that the product of the β_2-tubulin gene is a major structural component of at least three morphologically and functionally different microtubule arrays. Failure of meiosis, cytokinesis, flagellar elongation, and nuclear shaping in *B2t*^{8}/*B2t*^{8} males indicates that the ability of the β_2-tubulin subunit to assemble into normal, closed microtubules is a prerequisite for each of these functions.

Defects in nuclear shaping in *B2t*^{8} homozygotes indicate that the perinuclear microtubules are required for nuclear shaping and confirm the direct role of β_2-tubulin in the process. The complete failure of cytokinesis after meiosis I and II in *B2t*^{8} homozygous males may reflect defects in the size or rigidity of the spindle asters due to their abnormal microtubules. Classical experiments on embryos from marine invertebrates have shown that the position of the cleavage furrow is determined by the mitotic spindle asters (reviewed in Rappaport, 1975). α-Tubulin is synthesized and accumulates in normal amounts in testes from *B2t*^{8} homozygotes, so defects in microtubule function in this mutant cannot be ascribed to lack of the normal pools of α-tubulin.

Effects of the B2t^{8} *Mutation on Microtubule Structure and Assembly*

B2t^{8} encodes a partially functional β_2-tubulin subunit, able at least to form the αβ heterodimer and protofilaments. However, side-to-side interprotofilament bonds are occasionally abnormal in the presence of the mutant subunit. The variety of abnormal microtubule structures observed in *B2t*^{8} homozygotes is reminiscent of the structures assembled in vitro from tubulin that has been nicked by partial proteolysis (Mandelkow et al., 1985), or from normal mammalian brain tubulin under certain marginal assembly conditions (Burton and Himes, 1978; Mandelkow and Mandelkow, 1979). Image analysis of the S-shaped microtubules asembled in vitro in these experiments indicated that the protofilaments in the two halves of the S had the same longitudinal polarity (Mandelkow and Mandelkow, 1979). Formally, the structure is analogous to that which would arise if a single

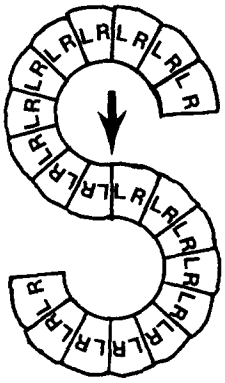

Figure 9. Model for the arrangement of protofilaments in S-shaped microtubules. Each protofilament in a microtubule can be considered to have an inner and an outer face and a left (*L*) and a right (*R*) side. In a normal microtubule, adjacent protofilaments are joined by an *RL* bond. According to the model, in S-shaped microtubules, most of the bonds between adjacent protofilaments are normal, but at the point of inflection (*arrow*) adjacent protofilaments are joined by an abnormal *LL* bond. As a result, the protofilament sheet curves back to form the lower half of the S. Model and drawing based on Burton and Himes (1978) and Mandelkow and Mandelkow (1979).

microtubule were split longitudinally in half and the two halves shifted slightly and rejoined in the S configuration. As a result, the two protofilaments at the point of inflection of the S must be joined by an abnormal *LL* or *RR* bond, instead of the *LR* bond between neighboring protofilaments characteristic of a normal microtubule (Fig. 9).

If the mutant $B2t^8$ subunit tends to assemble with unusual interprotofilament bonds, why do most of the aberrant structures show a largely normal curvature? The most common aberrant form in vivo, the S-shaped microtubule, appears to be constructed with a single abnormal interprotofilament bond located at the point of inflection (Fig. 9). If the assembly of a microtubule is initiated by the lateral interaction of protofilaments (Kirschner et al., 1975) and this nucleation event is kinetically limiting (Bordas et al., 1983), it is quite likely that the physical properties of the lateral association of the first two protofilaments are different from those for the addition of subsequent protofilaments to the sheet. In this case, the first interprotofilament bonds formed in the mutant could tend to be abnormal—resulting in the point of inflection of the S—whereas subsequent side-to-side interactions between neighboring protofilaments may be largely normal—resulting in two halves of the S with normal curvature but facing in opposite directions.

Burton and Himes (1978) found that assembly of S-shaped microtubules and other complex forms in vitro occurred at pH values slightly below the value optimal for the assembly of normal, closed microtubules. The assembly of abnormal microtubules in vivo in $B2t^8$ could result from an increase in the pH optimum for morphologically normal protofilament alignment. The $B2t^8$ mutation causes a shift of two charge units in the basic direction in the electrophoretic mobility of the β_2-tubulin subunit. DNA sequence analysis has revealed that the $B2t^8$ mutation is a single G → A base change resulting in the substitution of the basic amino acid lysine for glutamic acid (Rudolph, J. E., M. Kimble, H. D. Hoyle, M. A. Subler, and E. C. Raff, manuscript submitted for publication).

The S-shaped microtubules in cross sections through the axoneme region of elongating spermatid bundles from $B2t^8$ homozygotes always appeared to have the same orientation (S vs. reverse S). Occasionally we observed S-shaped microtubules with reverse handedness among the cytoplasmic (Fig. 1 *c*) or the perinuclear microtubules (Fig. 8 *c*). Thus, the polarity of aberrant cytoplasmic microtubules may not be so tightly constrained as the microtubules in the axonemal region. Many of the complex aberrant microtubule structures observed in testis from $B2t^8/B2t^8$ or $B2t^8/+$ males appear to be collections of C-shaped hooks joined to the wall of S-shaped microtubules (Fig. 2 *b*). These structures resemble the tubulin hooks assembled on to microtubules in vitro and used to assay the polarity of microtubules assembled in vivo by McIntosh and co-workers (Heidemann and McIntosh, 1980; Euteneuer and McIntosh, 1980, 1981; McIntosh and Euteneuer, 1984). If the S-shaped microtubules formed in vivo in $B2t^8$ males follow the same structural rules as the hook-decorated microtubules studied by McIntosh et al., then the S-shaped microtubules in spermatid cross-sections that have the S (clockwise) rather than the reversed S (counterclockwise) orientation would be viewed from the plus end towards the minus (or basal body) end (Euteneuer and McIntosh, 1981).

Although $B2t^8/+$ males are fertile, semidominant effects of the mutation are clearly visible at the ultrastructural level. A small but significant number of aberrant, S-shaped microtubules appear in the mitotic spindle, among the cytoplasmic microtubules of elongating spermatids and among the perinuclear microtubules in $B2t^8/+$ heterozygotes. Despite these occasional aberrant microtubules, meiosis, flagellar elongation and nuclear shaping proceed in heterozygotes from the stock.

In the presence of equal amounts of wild-type β_2-tubulin in $B2t^8/+$ heterozygotes, the $B2t^8$ mutant subunit appears to participate in the assembly of morphologically normal, functional flagellar axonemes in vivo. If microtubules are made of a mixture of mutant and wild-type β_2-tubulin subunits in heterozygous males, it should be possible to titrate the effects of the mutation by altering the dosage of the β_2-tubulin gene. Flies homozygous for $B2t^8$ but carrying a single copy of the wild-type gene for β_2-tubulin introduced into the genome by transformation (8/8/+) are still sterile, but the mutant phenotype is much less extreme than in $B2t^8/B2t^8$ males. Males homozygous for $B2t^8$ but carrying two copies of the transformed piece of DNA (8/8/+/+) are fertile (Raff, E. C., unpublished experiments). Thus, the effect of the mutant $B2t^8$ subunit on microtubule function in vivo depends on the ratio of mutant to wild-type subunits produced in the cell. The result that the effects of aberrant tubulin subunits on microtubule assembly and architecture in vivo can be moderated by the presence of wild-type tubulin urges caution in the interpretation of experiments in which heterologous or altered tubulin subunits have little affect on microtubule function when introduced into cells with an existing pool of endogenous tubulin. Rescue of the $B2t^8$ phenotype by the wild-type gene confirms that the defects in microtubule assembly and function observed in $B2t^8$ homozygotes are due to the mutation in the β_2-tubulin transcription unit.

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