GENETIC ANALYSIS OF *B2t*, THE STRUCTURAL GENE FOR A TESTIS-SPECIFIC β-TUBULIN SUBUNIT IN *DROSOPHILA MELANOGASTER*

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ABSTRACT

Genetic analysis of the *B2t* locus has resulted in the recovery of four recessive mutations in the *B2t* structural gene and a deficiency that deletes the locus. Two of the mutations were recovered as suppressors of $B2t^D$, a dominant male sterile mutation at the locus, and two were induced on wild-type chromosomes. All four mutant genes encode β_2-tubulin subunits that are synthesized at normal rates but do not accumulate. All mutants are completely male sterile as homozygotes.

TUBULIN is the major constituent of microtubules, ubiquitous components of eukaryotic cells. The molecule is a heterodimeric protein with subunits termed alpha and beta. Although tubulin and microtubules have long been the subject of extensive biochemical and structural analysis (OLMSTED and BORISY 1973; SNYDER and MCINTOSH 1976; KIRSCHNER 1978; ROBERTS and HYAMS 1979), only recently has a classical genetic approach been applied. Analysis of tubulin mutants has provided proof of the existence of multiple tubulin genes in Aspergillus (MORRIS, LAI and OAKLEY 1979) and in Drosophila (KEMPHUES *et al.* 1979) and has helped in elucidating tubulin function (OAKLEY and MORRIS 1980). In the future, genetic analysis should facilitate biochemical and structural studies of the tubulin molecule itself and may identify genes coding for proteins that interact with tubulins (MORRIS, LAI and OAKLEY 1979).

We have reported the identification of a dominant mutation ($B2t^D$) in the structural gene (*B2t*) for a testis-specific β-tubulin subunit (β_2-tubulin) in *Drosophila melanogaster* (KEMPHUES *et al.* 1979, 1980). The mutant gene, which encodes an electrophoretic variant ($\beta_2{}^D$-tubulin) of the testis-specific β-tubulin, confers sterility on male flies. However, the utility of the $B2t^D$ mutation for analysis of tubulin function *in vivo* is limited because its dominant nature results from the production of tubulin with abnormal function rather than loss of function. It is, therefore, not possible to deduce with certainty the functional role(s) of β_2-tubulin from analysis of $B2t^D$; for this, null mutations are required. Since one wild-type allele is sufficient to confer the normal phenotype for most

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loci in Drosophila (LINDSLEY and SANDLER *et al.* 1972), we expected that deletions of the *B2t* gene could be obtained and that recessive *B2t* mutations could be identified among male steriles which failed to complement those deficiencies. However, in several screens we recovered no deficiency that deleted the *B2t* locus, suggesting the presence of a haplo-insufficient site at or near *B2t* (KEMPHUES *et al.* 1980).

In this paper we report the recovery and characterization of four recessive *B2t* mutations and present an analysis of the haplo-insufficiency in the *B2t* region.

MATERIALS AND METHODS

Mutants and aberrations used are described by LINDSLEY and GRELL (1968) except for *Df(3R)Antp*$^{Ns+R17}$ and *Df(3R)dsx*$^{D+R1}$ (DUNCAN and KAUFMAN 1975). The balancer chromosomes *In(3LR)TM3, p*^{p} *Sb Ser e*^{s} and *In(3LR)CxD, D* will be abbreviated as *TM3* and *CxD*, respectively. Flies were grown at 22 or 25° in half-pint milk bottles or 8-dram vials with 60 or 10 ml, respectively, of standard cornmeal, agar, molasses culture medium supplemented with dry Baker's yeast. The X-ray source for irradiations was a Siemans Stabilipan operated at 250 kV, 125 mA, 3-mm copper filter, to give a dose rate of 585 rads/min.

Tissues and organs were dissected from anesthetized flies immersed in insect Ringer's solution (EPHRUSSI and BEADLE 1936). Proteins were isotopically labeled in the methionine-deficient tissue culture medium of WYSS and BACHMANN (1976) to which ^{35}S-methionine was added to a final concentration of 1–4 mCi/ml. After dissection, tissues were 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 and incubated for 1 hr. After incubation the tissues were washed in insect Ringer's solution, suspended in electrophoresis buffer and frozen. Levels of incorporation of ^{35}S-methionine into protein were determined by trichloroacetic acid precipitation.

Sperm were collected from 2-week-old males that had been segregated from females since eclosion. Such males accumulate mature sperm in their seminal vesicles. Seminal vesicles were disrupted in Ringer's solution in a dissecting dish. The sperm that spilled out were collected by spooling onto the tip of watchmaker's forceps. Collected spools were transferred to an Eppendorf microfuge tube containing Ringer's solution and were washed twice by 2-min centrifugations. Pelleted sperm were suspended in electrophoresis buffer and frozen.

Two-dimensional polyacrylamide gel electrophoresis was carried out as previously described (KEMPHUES *et al.* 1979) except that the gels shown in Figure 2 utilized an acrylamide to bisacrylamide ratio of 60:1. It was found that this ratio would give consistent separation of β_2-tubulin subunits in the second dimension of the gel. The gels shown in Figure 3 utilized a ratio of 37:1 and mutant and wild-type β_2-tubulins are not completely separated.

Cytological analysis was carried out by crossing males of the mutant stocks to wild-type virgin females. Salivary glands were dissected from late third instar F_1 progeny and prepared for observation by standard squash techniques. Breakpoints were determined by reference to the photographic maps of LEFEVRE (1976).

Except where specifically stated male fertility was determined by direct examination of seminal vesicles of males which had been separated from females for 4 days to 1 wk. Males with no motile sperm in either seminal vesicle were scored as sterile; males whose seminal vesicles were not distended but which contained motile sperm were scored as semisterile or weakly fertile; males whose seminal vesicles were distended with motile sperm were scored as fertile. This method does not distinguish subtle differences in fertility.

In addition, throughout the text, "female" should be taken to mean virgin female unless indicated otherwise.

RESULTS

Analysis of haplo-insufficiency near the B2t *locus*: In our initial analysis of the *B2t* locus we recovered ten deficiencies in the 85A-85E interval of the salivary

chromosome map (KEMPHUES *et al.* 1980). Oddly, the only region not included in any of the deficiencies was the small region, 85D7-11, which included the site of the *B2t* gene. The asymmetric distribution of deficiencies recovered in the screen (seven extending on either side of *p* and three apparently extending on the distal side of *by*) suggested the presence of a chromosomal site at or near *B2t* which when present in only one dose leads to inviability or infertility (haplo-insufficient site). This possibility could be tested by screening for deficiencies in this region under conditions that removed any constraints imposed by haplo-insufficiency. For this purpose, a p^p *by* chromosome containing a tandem duplication that included the *B2t* region was constructed by the method of NIX (1973). This method utilizes the fact that the dominant, haplo-insufficient phenotype of Minutes (thin bristles, slow growth, reduced fertility) is suppressed by duplication of the wild-type gene. Recognition of M^+ duplications was facilitated by incorporation of the mutation *Dfd*; *M*/+ *Dfd*/+ flies have a more severly deformed eye phenotype than do *Dfd*/+ flies. Two Minute

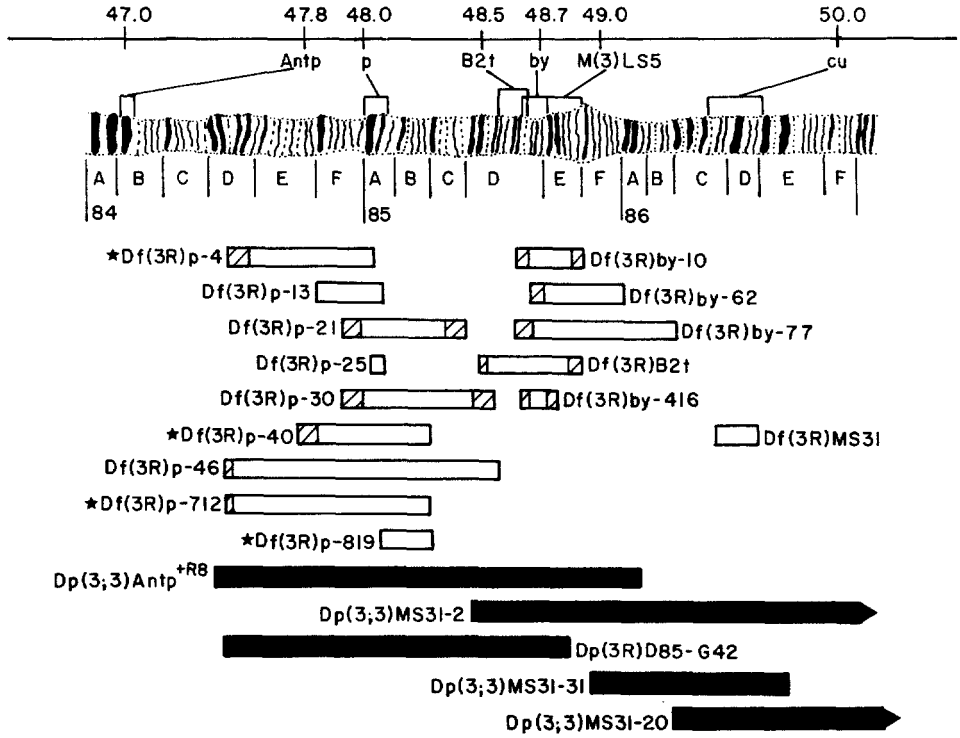

FIGURE 1.—Summary of genetic and cytogenetic analysis of the *B2t* locus. The positions of the *B2t* locus and other nearby loci on the salivary chromsome-banding pattern are shown schematically, with the corresponding recombination map above. The positions of deletions are shown by open bars and the positions of duplications by solid bars; ambiguity of breakpoint location for deletions is indicted by cross-hatching; the duplications are shown as their maximal extent. Asterisks indicate the presence of additional rearrangements, the nature of which are given in Table 1. The banding patterns are taken from BRIDGES' original maps as reproduced in LEFEVRE (1976). The band numbering in 85D counts the first two dark bands as doublets (1,2-3,4); the remaining bands are numbered singly to a total of 14 bands in the section.

TABLE 1

Summary of chromosomal aberrations

Primary aberration	Breakpoints	Other aberrations
Df(3R)p-4	84D6-10, 85A1-2	*T(2;3)34D;85A1-2*
Df(3R)p-13	84F1, 85A3	
Df(3R)p-21	84F4-6, 85C4-6	
Df(3R)p-25	85A1, 85A3	
Df(3R)p-30	84F4-6, 85D3-5	
Df(3R)p-40	84E8-9, 85B6	*In(3LR)64;90 + T(2;3)55;75*
Df(3R)p-46	84D4-6, 85D6	
Df(3R)p-712	84D4-6, 85B6	*T(2;3)25D;85B*
Df(3R)p-819	85A3, 85B6	*T(2;3)2Rhet;87C + Tp(3)64F-67F;97*
Df(3R)by-10	85D8-12, 85E7-F1	
Df(3R)by-62	85D11-14, 85F6	
Df(3R)by-77	85D8-12, 86B4	
Df(3R)B2t = Df(3R)Msc^{L}Antp^{BR}	84B1-2, 85D3-4, 85E5-7	*In(3R)84B1,2;85E5-7*
Df(3R)by-416	85D10-12, 85E1-3	
Dp(3;3)MS31-2	85D1-4, 87A5	
Dp(3;3)MS31-20	86B4, 87E1	
Dp(3;3)MS31-31	85F1-4, 86E3-5	
T(3;4)p-42	85A, 4 heterochromatin	
T(X;2;3)by-35	43D, heterochromatin, 85D	*T(X;3)4D;93E*
In(3R)p-419	84D4-6, 86A3	
In(3LR)by-36	66, 89B, 96	
In(3LR)by-44	65, *3R* heterochromatin	

loci are within five lettered salivary chromosome segments of *B2t*: *M(3)S31* at 86C,D (based on our own cytology) and a second Minute in 85E, uncovered by *Df(3R)by-10*, *Df(3R)by-62* and *Df(3R)by-77*. The 85E Minute was discovered by LINDSLEY and SANDLER *et al.* (1972), but this position was assigned to *M(3)S31* based on existing genetic data. We propose that this latter locus be named *M(3)LS5*. A *Dfd p^{p} M(3)S31* chromosome was already available and was used for the screen. Three-day-old virgin *p^{p} by* females were exposed to 4500 rads of X rays and mated to *Dfd p^{p} M(3)S31/In(3LR)TM3, y^{+} Ser e^{s}* males. F_1 progeny which were *Dfd* suppressed, *M^{+} Ser^{+}* were selected and backcrossed to the *Dfd p^{p} M(3)S31/In(3LR)TM3* stock to confirm the M^{+} phenotype. Putative duplications were maintained over *TM3*. Of 1979 flies scored, three suppressors were recovered. Salivary cytology showed that all three were tandem duplications that included the *M(3)S31* locus; the breakpoints are given in Figure 1 and Table 1. *Dp(3;3)MS31-2*, henceforth abbreviated *MS31-2*, included bands 85D7-11 in the duplicated region and was presumed to be duplicated for the *B2t* gene. The duplication of *B2t* was verified biochemically

by two-dimensional gel analysis of testes taken from *B2t*^{D}/*MS31-2* males. Densitometer scans of autoradiograms of these gels showed a 2:1 ratio of β_2- to $\beta_2{}^{D}$-tubulin.

The *MS31-2* tandem duplication was then used to recover deficiencies that might delete the *B2t* gene. Because the duplication carried the recessive markers p^p and *by* which flank the *B2t* locus at 48.0 and 48.7, respectively, it could be used to screen for X-ray-induced mutations that failed to complement one or both of the recessive markers.

One to 4-day-old *red e* males were treated with 4000 rads of X rays and mated to females of the genotype *MS31-2/TM3*. Males were removed after 4 days to ensure that all mutations recovered were independent events (CHANDLEY and BATEMAN 1962). F_1 *red e/MS31-2* progeny that showed either pink or blistery phenotypes were collected and backcrossed to *MS31-2/TM3* flies. Individual backcross progeny males carrying each new allele that was transmitted (*p** or *by* red e/MS31-2*) were crossed to *MS31-2/TM3* females. Because the putative deficiencies might be dominant steriles, stocks were maintained by selecting *p** or *by* red e/MS31-2* males and *MS31-2/TM3* females. A screen of 6730 chromosomes resulted in the recovery of two deletions of pink (*p-712, p-819*) and one deletion of blistery (*by-416*). Unfortunately, none of these uncovered the *B2t* locus. Consequently, a deficiency that included *B2t* was constructed by another method. Females heterozygous for two overlapping inversions produce chromosomes that are deleted or duplicated for the regions of nonoverlap by recombination in the region of the inversions held in common (STURTEVANT and BEADLE 1936).

Two inversions were available that were well suited for creating the desired deficiency: *In(3R)Antp*^{B}, broken at 84B1,2 and 85E and *In(3R)Msc*, broken at 84B1,2 and 85C. *In(3R)Antp*^{B}, p^p/*In(3R)Msc* females were mated to *MS31-2*, p^p *by/TM3* males. Because the *by* gene lies in the region of nonoverlap, the exchange-generated deletion chromosome is expected to express the blistery phenotype over *MS31-2*, p^p *by*. Of 5725 progeny screened, four males were found that expressed the *by* phenotype. Subsequent salivary cytology of the progeny of these males showed that all four chromosomes recovered were, in fact, deficient for chromosomal region 85C-85E. This construct, *Df(3R)Msc*^{L} *Antp*^{BR}, will be referred to here as *Df(3R)B2t*. It is maintained by selecting and crossing *Df(3R)B2t/MS31-2* males and *MS31-2/TM3* females. It should be noted that this chromosome is also deleted for a portion of 84B1,2. However, we know from other studies (DUNCAN and KAUFMAN 1975) that such a deletion has no effect on viability or fertility when heterozygous.

To test the viability of deficiency heterozygotes lacking the tandem duplication, *MS31-2*, p^p *by*/*Df(3R)B2t*, p^+ *by*$^-$ males were crossed to p^p *by* females. Flies heterozygous for the deficiency could be identified as the p^+ *by M* [due to the deletion of *M(3)LS5*] progeny. Heterozygotes for the deficiency were present in the expected ratio. Two-dimensional gels of testis proteins extracted from *Df(3R)B2t/B2t*^{D} flies showed only the $\beta_2{}^{D}$-tubulin subunit, providing biochemical verification that the *B2t* gene was deleted. Fertility was checked by collecting *Df(3R)B2t/p*^{P} *by* males and females and mating them in groups of 50

with wild-type males or females for 5 days. No large scale numerical analysis of fertility was performed, but it was clear from inspection that the fertility of both males and females heterozygous for the *B2t* deficiency was drastically reduced. For example, in one experiment performed shortly after the isolation of the deficiency, 50 males gave a total of 17 progeny, whereas 50 females gave 24 progeny in 5 days of laying. Male fertility was also assayed by direct examination of seminal vesicles of the deficiency-bearing males. Of 148 flies examined, none had motile sperm in their seminal vesicles. When the fertility was monitored after the deficiency had been maintained in stock for a year, female fertility had increased somewhat, with more than 100 progeny per bottle of 50 females, whereas male fertility remained the same. The initial sterility of females and the subsequent increase in fertility were similar to the behavior of deletions of *by* which also included *M(3)LS5* (K. J. KEMPHUES, unpublished observation); this suggests that the female sterility is due to the deletion of the Minute or of some other locus uncovered by all four deficiencies.

Recovery of recessive mutations at the B2t *locus—dominant suppression of* $B2t^D$*-induced sterility*: Although the dominant sterility associated with the *B2t* deletion eliminated the possibility of using it in screens for recessive mutations at the locus, two recessive mutations were obtained using another approach. The presence of β_2^D-tubulin in mature sperm and the ability of the altered protein to assemble *in vitro* indicated that the dominant male sterility induced by β_2^D-tubulin was probably due to the improper functioning of the assembled variant protein rather than failure to assemble *in vivo* (KEMPHUES *et al.* 1979, 1980). Assuming that one dose of wild-type *B2t* is sufficient for fertility [an assumption made prior to the discovery of the sterility associated with *Df(3R)B2t*], it seemed reasonable that a mutation that prevented the β_2^D-tubulin subunit from participating in normal tubulin functions could act to suppress the dominant sterility. Two types of suppression can be imagined: *cis*-acting suppression due to mutation of a site within or closely linked to the $B2t^D$ allele or *trans*-acting suppression due to mutation at a second locus. A *cis*-acting suppressor of dominant sterility could be expected to behave as a recessive male-sterile at the *B2t* locus and could be used in screens for simple recessives at the locus. Two screens for dominant suppressors of $B2t^D$-induced dominant male sterility were undertaken. For the first screen, 3- to 6-day-old *st* $B2t^D$ *e* females [obtained by crossing *Dp(3;3)Antp*$^{73b+R8}$*/st* $B2t^D$ *e* semifertile males to *st* $B2t^D$ *e/TM3* females (KEMPHUES *et al.* 1980)] were treated with EMS according to the method of LEWIS and BACHER (1968) and mated to *CxD/TM3* males (ten each per bottle). F_1 progeny were collected and transferred to fresh bottles in groups of 50 of each sex. Putative suppressors were identified in those bottles containing progeny that were phenotypically scarlet and ebony. Progeny females were mated to *CxD/TM3* males to recover the putative suppressor chromosome. Stocks of recovered suppressors were constructed by collecting *st* $B2t^{D*}$ *e/TM3* males and backcrossing them to *CxD/TM3* females (only those males carrying the suppressor would be fertile) and interbreeding the *st* $B2t^{D*}$ *e/CxD* or *TM3* progeny. This screen yielded one suppressor (designated $B2t^{D+R1}$) out of 5751

F_1 males tested. The second screen was similar but was designed to counteract the possibility that dominant sterility might result from a null mutant at the *B2t* locus. Females of the genotype *st* $B2t^D$ *by* were treated with EMS and mated to *MS31-2/TM3* males. F_1 *MS31-2/st* $B2t^D$* *by* males were collected and crossed to *MS31-2/TM3* females. Any progeny from this cross were considered carriers of putative suppressors of $B2t^D$ and were maintained in stock by selecting *MS31-2/st* $B2t^D$* *by* males and crossing them to *MS31-2/TM3* females. This screen yielded one dominant suppressor ($B2t^{D+R2}$) out of 8873 F_1 males tested. Unlike *Dp(3;3)Antp*$^{73b+R8}$, *MS31-2* does not partially suppress the dominant sterility of $B2t^D$; therefore, it was not difficult to detect suppression in this genetic background.

Analysis of $B2t^{D+R1}$ *and* $B2t^{D+R2}$*—basis for suppression*: When heterozygous with a wild-type chromosome, the suppressors $B2t^{D+R1}$ and $B2t^{D+R2}$ are male fertile or semisterile, but as homozygotes the suppressors are male-sterile and female-fertile. Both segregate as third chromosome mutations, and $B2t^{D+R1}$ has been mapped to 48.5 ± 0.1, at or near the *B2t* locus (48.5). Both are *cis*-acting in that $B2t^D/B2t^{D+R1}$ or $B2t^{D+R2}$ is sterile. Thus, at least with respect to male fertility, both behave as recessive mutations at the $B2t^D$ locus. The suppression of $B2t^D$ dominance is likely the result of a second mutation in or near the β_2-tubulin structural gene that prevents the abnormal $B2t^D$ product from participating in normal tubulin function. Such a mutation could act by eliminating the $B2t^D$ gene product or by altering its properties.

To distinguish between these possibilities, four testes each were dissected from $B2t^{D+R1}/B2t^+$ and $B2t^{D+R2}/B2t^+$ flies and incubated in organ culture in the presence of ^{35}S-methionine for 1 hr. After washing in fresh culture medium, ten unlabeled testes of like genotype were added, and proteins were extracted and separated by two-dimensional gel electrophoresis. Gels were stained with Coomassie blue and dried, and autoradiograms were prepared. The results of this experiment are shown in Figure 2. The autoradiograms, which show the proteins being synthesized during the short-term labeling period, indicate that both suppressors synthesize a β_2-tubulin subunit with the same electrophoretic mobility as the β_2^D-tubulin subunit from which they were derived. (In comigration experiments not shown, the new mutant tubulins were inseparable from β_2^D-tubulin in this gel system.) Comparison of the autoradiograms with the stained gels, which show the total testis protein pools, reveals that, although the mutant β_2-tubulins are synthesized at rates comparable to wild-type β_2-tubulin in the same testes, they are not represented at an equivalent level in the total protein pool. Pulse-chase experiments have demonstrated that the mutant β_2-tubulins are subject to degradation in the testes (KEMPHUES *et al.* 1982).

The low levels of mutant protein in the suppressor strains suggested that perhaps suppression of $B2t^D$ male sterility is a direct consequence of reduced amounts of the mutant protein and is not due to an alteration in its functional properties. One means of determining whether the altered β_2-tubulins retain any functional capacity for assembly into microtubules is to assay for their presence in functional motile sperm. Figure 3 shows portions of two-dimen-

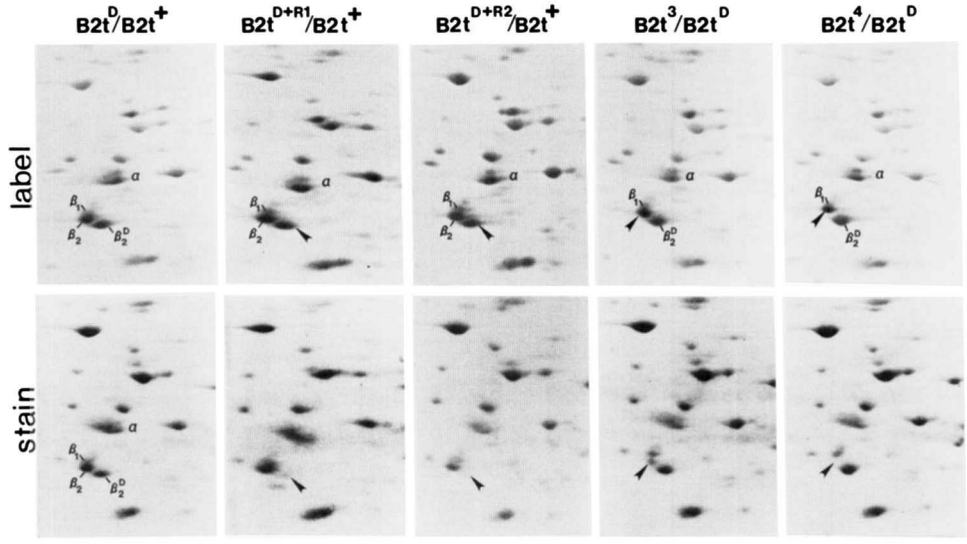

FIGURE 2.—Tubulin synthesis patterns and total tubulin pools in testes of males expressing mutations in the gene for β_2-tubulin. The top panels are portions of autoradiograms of two-dimensional gels displaying the tubulins synthesized in testes of males of the indicated genotypes. The bottom panels show portions of the same gels stained with Coomassie blue to reveal the total stable testis protein pools. The positions of the mutant β_2-tubulin is indicated by the arrow.

sional gels stained with silver (MERRIL *et al.* 1981); they display proteins present in mature motile sperm dissected from seminal vesicles of wild-type males (Figure 3a) and males that express mutations in the β_2-tubulin gene. Figure 3b shows that $\beta_2{}^D$-tubulin is incorporated into motile sperm in males heterozygous for $B2t^D$ and $Dp(3;3)Antp^{73b+R8}$, a tandem duplication that includes the $B2t^+$ locus and that partially rescues the dominant sterility associated with $B2t^D$ (KEMPHUES *et al.* 1980). The ratio of normal β_2-tubulin to the variant $\beta_2{}^D$-tubulin is approximately 2:1, as is found in whole testes of males of this genotype (KEMPHUES *et al.* 1980). In $B2t^{D+R1}/B2t^+$ males, as shown in Figure 3c, the variant protein encoded by $B2t^{D+R1}$ is also incorporated into motile sperm, in a ratio to normal β_2-tubulin that reflects its lower abundance in the total testis tubulin pool (see Figure 2).

A comparison of Figure 3c and the stain pattern in Figure 2 for the same genotype ($B2t^{D+R1}/B2t^+$) shows an apparently greater amount of mutant protein present in sperm than whole testes. This may indicate that once polymerized into microtubules the abnormal protein is stabilized and made unavailable to the degradative process. However, since the two gels were stained by two very different techniques we cannot be certain that the observation is not a result of this latter fact.

Two-dimensional gels of sperm proteins from $B2t^{D+R2}/B2t^+$ showed only the wild-type subunit; however, this result is equivocal because the level of stable mutant β_2-tubulin is so low that we cannot detect it in whole testes (see Figure 2). Thus, the mutant protein, even if present in sperm, would probably not be detected on our gels.

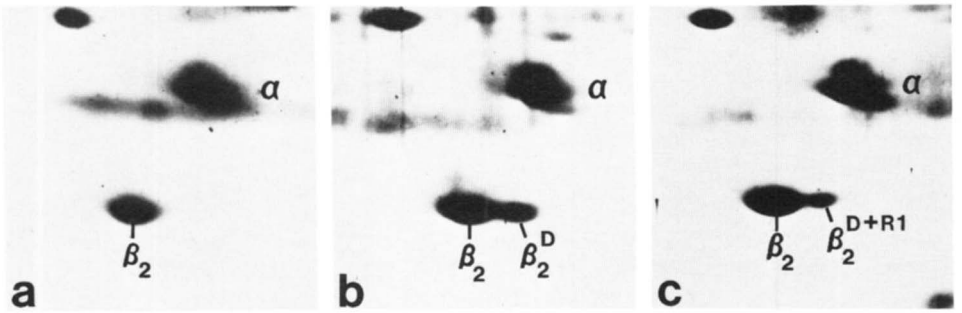

FIGURE 3.—Portions of two-dimensional gels stained with silver showing tubulin subunits present in motile sperm of wild-type males (a), *B2tD*/*Dp(3;3)Antp*$^{73b+R8}$ males (b) and *B2t*$^{D+R1}$/*B2t*$^{+}$ males (c).

In summary, the mutations *B2t*$^{D+R1}$ and *B2t*$^{D+R2}$ are *cis*-acting suppressors of *B2t*^{D}-induced dominant male sterility. Both appear to be recessive male-sterile mutations at the *B2t* locus. Both synthesize a β_2-tubulin with electrophoretic properties identical with the product of the parent *B2t*^{D} chromosome. The β_2-tubulin produced by the suppressors are synthesized at normal rates but are thereafter rapidly degraded. As a result, the mutant tubulins contribute very little to the total tubulin pools within the testis. The mutant β_2-tubulin produced by *B2t*$^{D+R1}$ is capable of incorporating into motile sperm. Our interpretation is that *B2t*$^{D+R1}$ and *B2t*$^{D+R2}$ are the result of missense mutations in the *B2t*^{D} structural gene. The mutations confer instability on the protein but, at least in the case of *B2t*$^{D+R1}$, do not entirely eliminate its capacity to be incorporated into microtubules. However, it is not known whether the reduced amounts of the mutant β_2-tubulins account entirely for the suppression or whether altered behavior of the mutant proteins is also involved.

Isolation of B2t^{3} *and* B2t^{4}: Because *B2t*$^{D+R1}$ and *B2t*$^{D+R2}$ were derived from the dominant mutation *B2t*^{D}, analysis of these mutations is complicated by the possibility that both may possess residual antimorphic or neomorphic characteristics, leading to phenotypes that would not necessarily reflect the true function of β_2-tubulin. Therefore, we utilized *B2t*$^{D+R1}$ to obtain recessive mutations derived from wild-type *B2t* genes. Recessive mutations were obtained by two screens. In the first, 149 existing third chromosome male-sterile mutations were screened to determine whether any failed to complement *B2t*$^{D+R1}$. All of these mutations were induced with EMS and consisted of 91 from D. LINDSLEY, six from R. LEWIS and 52 that we had isolated in earlier screens. One mutation, *B2t*^{3}, was identified among the tested steriles. The mutant chromosome was isolated by R. LEWIS as a homozygous lethal which, when heterozygous for the deletion *Df(3R)Antp*$^{Ns+R17}$, was male-sterile but female-fertile. Recombination analysis revealed that the male sterility mapped to 48.7 ± 0.3 and could be separated from the lethality. *B2t*^{3} homozygotes are male-sterile and female-sterile. *B2t*^{3}/*B2t*$^{+}$ males are fertile or semisterile. The failure of *B2t*^{3} (in salivary region 85) to complement *Df(3R)Antp*$^{Ns+R17}$ (in salivary region 84) could be explained in two ways: either the deficiency chromosome carried a *B2t* mutation or the double heterozygote was sterile

as a result of synthetic sterility. Recombination analysis demonstrated that the *Df(3R)Antp*$^{Ns+R17}$ chromosome was not mutant at *B2t*. Behavior of deficiencies that overlapped or were nested within *Df(3R)Antp*$^{Ns+R17}$ as double heterozygotes with *B2t*^{3} suggest that no single locus or even subregion of 84 is responsible for the failure to complement (E. C. RAFF, unpublished observations).

In the second screen, male flies homozygous for *red* and *e* were treated as described before with EMS and mated to *CxD/TM3* females. F_1 *red e*/CxD* or *rea e*/TM3* females were mated individually with three to five *st B2t*$^{D+R1}$ *e/TM3* or *st B2t*$^{D+R1}$ *e/CxD* males, respectively. Two to ten *st B2t*$^{D+R1}$ *e/red e** male progeny were tested for fertility by mating to virgin females. Sibling *red e*/CxD* or *TM3* males and females were allowed to interbreed to establish a stock. From 1028 EMS-treated chromosomes screened, one mutation (*B2t*^{4}) was isolated. *B2t*^{4} homozygotes are male-sterile but female-fertile. *B2t*^{4}/*B2t*$^{+}$ is semisterile. The mutation was mapped by recombination to 48.8 ± 0.3. *B2t*^{4} is male-sterile in heterozygous combination with all other *B2t* mutations. Indeed, all interallelic combinations are male-sterile.

Biochemical characterization of B2t^{3} *and* B2t^{4}: Biochemical analysis was performed to determine whether males that express these mutations synthesize a β_2-tubulin protein, whether that protein differs from wild type in its electrophoretic mobility and whether that protein, like those produced by *B2t*$^{D+R1}$ and *B2t*$^{D+R2}$, is unstable. Testis proteins from *B2t*^{3} or *B2t*^{4}/*B2t*^{D} males were analyzed by two-dimensional gel electrophoresis as described before. As shown in Figure 2, this analysis demonstrated that testes from both mutants synthesize a β_2-tubulin subunit that is identical in mobility to wild-type β_2-tubulin; however, like the β_2-tubulin produced by the suppressor mutants, it is unstable, so that extremely low levels of the variant β_2-tubulin are present in the total testis tubulin pools.

DISCUSSION

We have isolated four recessive mutations in the *B2t* locus structural gene and have synthesized a deficiency that deletes the locus; this acts as a dominant male-sterile. In addition, a number of chromosomal rearrangements in the environs of the *B2t* locus were recovered.

Two of the recessive mutations, *B2t*$^{D+R1}$ and *B2t*$^{D+R2}$, were identified as suppressors of the *B2t*^{D} dominant male sterility. Two others, *B2t*^{3} and *B2t*^{4}, were induced on wild-type chromosomes and were recovered in screens for male-sterile mutations that failed to complement *B2t*$^{D+R1}$. All four are male-sterile and female-fertile as homozygotes and fail to complement for male fertility; however, heterozygous males can be semisterile.

It is striking that all four of the recessive mutations synthesize a β_2-tubulin protein which is unstable. The instability of the β_2-tubulin protein within the testis is most likely due to the action of the normal intracellular machinery which recognizes abnormal proteins and degrades them. Such intracellular protein degradation systems have been demonstrated to be a part of normal "housekeeping" in bacterial and mammalian cells (GOLDBERG and DICE 1974;

GOLDBERG and ST. JOHN 1976), and it is reasonable to assume that similar systems operate in Drosophila.

None of the *B2t* alleles we have thus far recovered are null for synthesis of protein. There are two possible explanations for the absence of null mutations. First, our sample size may be too small. Alternatively, there may be some constraint on the recovery of null mutations. It is possible that β_2-tubulin is required in very small amounts for some vital function. A more likely constraint is suggested by the sterility associated with *Df(3R)B2t*: null mutations, like the deficiency, may act as dominant steriles and, hence, would not have been recovered in most of our screens. However, it is by no means certain that the male sterility of *Df(3R)B2t* is solely due to deletion of the *B2t* locus. In fact, several pieces of evidence suggest that the dominant sterility of this deficiency is the result of the combination of two or more lesions. *Df(3R)B2t* affects fertility of both males and females; presumably deletion of some locus or loci other than *B2t* acts to reduce fertility in females. Because Minutes generally reduce fertility in both sexes (SCHULTZ 1929), the deletion of *M(3)LS5* is likely to be at least partly responsible for the observed sterility. The more severe sterility in males could be due to the additional effect of deleting *B2t*. Males heterozygous for both $B2t^{D+R1}$ and *Df(3R)by-10* are either completely sterile or only weakly fertile (K. J. KEMPHUES, unpublished observations), supporting the notion of synthetic sterility in males. Synthetic sterility may also account for the perplexing failure of $Df(3R)Antp^{Ns+R17}$ to complement *B2t* mutations. Because $Df(3R)Antp^{Ns+R17}/+$ males are semisterile (T. C. KAUFMAN, unpublished observations), it seems possible that the combined stress imposed by the possession of one copy of the *B2t* locus could lead to complete sterility.

The sum of this circumstantial evidence argues that a deletion or a null mutation at the *B2t* locus might not of necessity lead to dominant sterility. However, such a lesion in combination with a variety of other normally cryptic deficiencies in male fertility can lead to dominant sterility and, hence, decrease the chances of recovering such a null at the *B2t* locus.

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