Mutations in the Essential Spindle Checkpoint Gene \textit{bub1} Cause Chromosome Missegregation and Fail to Block Apoptosis in \textit{Drosophila}

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Abstract. We have characterized the \textit{Drosophila} mitotic checkpoint control protein \textit{Bub1} and obtained mutations in the \textit{bub1} gene. \textit{Drosophila} \textit{Bub1} localizes strongly to the centromere/kinetochore of mitotic and meiotic chromosomes that have not yet reached the metaphase plate. Animals homozygous for \textit{P-element–induced, near-null mutations of bub1} die during late larval/pupal stages due to severe mitotic abnormalities indicative of a bypass of checkpoint function. These abnormalities include accelerated exit from metaphase and chromosome missegregation and fragmentation. Chromosome fragmentation possibly leads to the significantly elevated levels of apoptosis seen in mutants.

We have also investigated the relationship between \textit{Bub1} and other kinetochore components. We show that \textit{Bub1} kinase activity is not required for phosphorylation of 3F3/2 epitopes at prophase/prometaphase, but is needed for 3F3/2 dephosphorylation at metaphase. Neither 3F3/2 dephosphorylation nor loss of \textit{Bub1} from the kinetochore is a prerequisite for anaphase entry. \textit{Bub1}'s localization to the kinetochore does not depend on the products of the genes \textit{zw10}, \textit{rod}, \textit{polo}, or \textit{fizzy}, indicating that the kinetochore is constructed from several independent subassemblies.

Key words: checkpoint • kinetochore • chromosome missegregation • apoptosis

The spindle checkpoint is a surveillance mechanism that monitors the attachment of spindle microtubules to the kinetochores, thereby ensuring that the onset of anaphase is dependent on the correct completion of metaphase (reviewed by Elledge, 1996; Rudner and Murray, 1996; Wells, 1996; Nicklas, 1997; Wolf and Jackson, 1998). To date, genetic studies in yeast have identified seven genes which encode components of the spindle checkpoint: \textit{MAD1}, \textit{MAD2}, \textit{MAD3}, \textit{BUB1}, \textit{BUB2}, \textit{BUB3}, and \textit{MPS1}. Mutations disrupting these genes bypass the operation of the spindle checkpoint, leading to the initiation of anaphase in the presence of microtubule-depolymerizing drugs (Hoyt et al., 1991; Li and Murray, 1991). Homologues of \textit{MAD1}, \textit{MAD2}, \textit{BUB1}, and \textit{BUB3} from multicellular eukaryotes have been identified and establish spindle checkpoints in these organisms as well. For example, immunodepletion of \textit{MAD1} and \textit{MAD2} from \textit{Xenopus} extracts inactivates the spindle checkpoint (Chen et al., 1996, 1998).

These metazoan spindle checkpoint proteins have been shown to localize most strongly to kinetochores unattached to the spindle apparatus (Chen et al., 1996, 1998; Li and Benezra, 1996; Taylor and McKee, 1997; Taylor et al., 1998; Chan et al., 1998; Yu et al., 1999). The differential association of these molecules with attached versus unattached kinetochores is consistent with several observations implying that unattached kinetochores emit an inhibitor that delays anaphase onset (reviewed by Nicklas, 1997; Rieder and Salmon, 1998). Recent evidence indicates that the checkpoint operates by inhibiting the ability of the anaphase-promoting complex (APC)\textsuperscript{1} to ubiquitinate substrates whose degradation is a prerequisite for sister chromatid separation and other aspects of the exit from mitosis (Elledge, 1998; Hwang et al., 1998; Kim et al., 1998).

Although the function of the \textit{Bub} and \textit{Mad} proteins has been well established under conditions in which microtubule depolymerizing reagents or mutations in spindle components were employed, the importance of these proteins for normal cell division is less clear. In \textit{Saccharomyces cerevisiae}, strains carrying null mutations in most \textit{BUB} or

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\textsuperscript{1} Abbreviations used in this paper: APC, anaphase-promoting complex; TUNEL, Tdt-mediated dUTP-biotin nick end labeling.
MAD genes grow somewhat more slowly, accompanied by a weak increase in chromosome missegregation (Hoyt et al., 1991; Li and M urray, 1991; Farr and Hoyt, 1998). Similarly, Schizosaccharomyces pombe knockouts of bub1 are viable and show modest effects on the fidelity of chromosome segregation during mitosis (Bernard et al., 1998). In higher eukaryotes, tissue culture cells overexpressing presumed dominant negative versions of Bub1 exit from mitosis more quickly than usual (Taylor and MCKeon, 1997). M c roinjection of antibody against M ad2 into tissue culture cells similarly induces premature entry into anaphase (G orbsky et al., 1998). Interestingly, mutations in a human Bub1–related kinase have been detected in colorectal cancer cell lines showing chromosomal instability (Cahill et al., 1998). These mutations behave neither as null mutations nor hypomorphs, but instead generate a version of this protein that also acts in a dominant negative fashion. These results do not provide a clearcut framework for understanding how the checkpoint influences normal cell cycle progression, as we do not yet know the consequences of the absence of any checkpoint component in a developing multicellular eukaryote. To address these issues in more detail, we have begun to characterize the operation of the spindle checkpoint in Drosophila melanogaster, as we believe the combination of genetic and cytological approaches that can be employed in this organism will provide new insights into the mechanisms governing anaphase onset.

In this paper, we present a detailed phenotypic characterization of Drosophila bub1 mutants, the first mutational analysis of any component of the spindle checkpoint in any multicellular organism. We show that loss of function mutations affecting Drosophila bub1 cause severe mitotic abnormalities consistent with accelerated transit through metaphase. In addition, in partial contrast to previous findings indicating that loss of Bub1 function leads to the escape of cells from an apoptotic fate (Taylor and MCKeon, 1997), we find that mutations in bub1 generate a massive apoptotic response. We have further employed an anti-Drosophila Bub1 antibody to show that the cell cycle distribution of Bub1, including its association with unattached kinetochores, has been conserved between Drosophila and humans. The genetic and immunological agents we have generated additionally allowed us to examine several other issues, such as the role of Bub1 during meiosis, and the relationship between Bub1 kinase and other kinetochore components. These include 3F3/2 phosphoproteins and the ZW10 protein, both of which have been suggested to be intimately involved in signaling the metaphase/anaphase transition (Williams et al., 1992; Campbell and G orbsky, 1995). Our results considered together clarify the importance of the spindle checkpoint to normal cell division in higher eukaryotes.

**Materials and Methods**

**Identification of Drosophila Bub1 cDNAs and Drosophila bub1 Mutants**

The ESTs LD06986 and LD18419 were identified in the Berkeley Drosophila Genome Project (BDGP) EST database when searched with the amino acid sequence of mouse Bub1 (Taylor and MCKeon, 1997), and cDNA s containing these ESTs were ordered from Genome Systems Inc. The longest of these cDNAs (that containing EST LD06986) was sequenced. By completion (Cornell University Sequencing Facility, Ithaca, NY), and was found to contain the entire amino acid coding sequence of Drosophila Bub1. The lethal P-element insertions l(2)K06109 and l(2)K03113 (gifts of D r. Todd L avery, University of California, Berkeley, CA) were identified by searching the BDGP database of sequences adjacent to P-element insertions. We have independently determined the DNA sequence of Drosophila genomic DNA flanking the P-element insertion sites in l(2)K06109 and l(2)K03113 lines, and our results are in accord with the sequences in the BDGP database.

We determined that the cytological location of the bub1 gene is polytene chromosome interval 42A 2-3 by hybridizing a probe made from the cDNA of the Drosophila gene to Drosophila polytene chromosomes as described in Williams et al. (1992). This result confirms BDGP’s localization of the P-elements causing the lethal mutations l(2)K06109 and l(2)K03113 to the same polytene chromosome bands. In further support of this position for the bub1 gene, we determined that two deletions uncovering this region of the genome, F12Rnap1 (breakpoints 41D2-41E1, 42B1-42B3), obtained from the Drosophila stock center, Bloomington, IN) and D(2R)nap2 (breakpoints 41F4-41F9, 43A1; the gift of D r. J ohn R oote, Department of Genetics, University of Cambridge, Cambridge, U.K.), failed to complement l(2)K06109 or l(2)K03113 for any of the phenotypes we have studied.

To verify that the bub1 mutant phenotype was caused by the l(2)K06109 and l(2)K03113 P-element insertions, we mobilized the P-elements in these lines by introducing FLP1 (Jacobs et al., 1992), a source of P-element transposase (Robertson et al., 1988), and selecting for loss of the white+ eye color in the next generation (G atti and G oldberg, 1991). Out of 43 white+ excision stocks generated from l(2)K06109, 21 showed complete rescue of the lethality and associated mitotic and apoptotic defects of the original bub1 mutants. For l(2)K03113, 19 out of the 37 white+ stocks obtained similarly behaved as precise excisions.

**Generation of Anti-Bub1 Antibody**

To obtain large amounts of Bub1-specific epitopes, a 1219 bp BamH1/Kpn1 fragment from LD06986 was first subcloned into the expression vector pGEX 5X-2 (Amersham) according to the manufacturer’s instructions. This affibody is a monoclonal key anti–chicken IgY (Jackson ImmunoResearch Labs) at a dilution of 1:10,000. Detection of antibody signals were performed with the ECL system (Amersham) according to the manufacturer’s instructions. This result confirms BDGP’s localiza-
ment and anaphase chromosome segregation within the mitotic domains of post-cellularization embryos (Foe, 1989); no obvious defects were seen.

To identify third instar larval homologues for bub1 mutations, chromosomes bearing both the 1(2)K06109 and 1(2)K03113 P-element insertions were re-balanced over T(2;3)SM 6a-TM 6B, a translocation between the second chromosome balancer SM 6a and the third chromosome balancer TM 6B synthesized in the laboratory of A. Garcia-Bellido (Universidad Autonoma de Madrid, Madrid, Spain). T(2;3)SM 6a-TM 6B includes the dominant larval/pupal marker Tubby, so the desired mutant animals were chosen on the basis of their non-Tubby phenotype. Oocyte stained preparations of neuroblasts from the brains of third instar larvae were obtained as described by Gatti and Goldberg (1991). Living testes from third instar larvae were observed by the techniques of Cenci et al. (1994), while fixed larval testes were analyzed by immunofluorescence as described by Williams et al. (1996). Preparation and immunolocalization analysis of Drosophila S2 tissue culture cells, and of metaphase-arrested chromosomes isolated from these cells, was as described by Basu et al. (1997); the same reference describes experimental protocols involving the 3F/2 antibody. Secondary antibodies used for immunofluorescence localization of Bub1 were TRITC or FITC conjugated Affinipure donkey anti-chicken IgY (Jackson Immunoresearch Labs), both at dilutions of 1:200.

In all figures requiring comparisons of Bub1 or 3F/2 staining between panels (Figs. 2, B and C; 3, A–I; 7, A–D; 9, A–F; and 10, A–D), the gain on the digital camera was held constant, and all images were digitally processed in the same fashion.

**Detection of Markers for Programmed Cell Death**

Labeling of apoptotic nuclei with a FITC anti-di-goxygenin conjugate was performed using the ApopTag Plus In Situ A Poptosis Detection Kit (OnCOR) according to the manufacturer’s instructions.

To follow the redistribution of phosphatidyserine (an early apoptotic marker) the annexin V-FITC kit was used (PharMingen). Brains were dissected in phosphate buffered saline (PBS); 80 mM NaHPO4, 20 mM NaH2PO4, and 100 mM NaCl, pH 7.5) and incubated for 5–10 min in binding buffer (10 mM Hapes, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl2). FITC-conjugated annexin V was added at a 1:40 dilution, and the mixture was incubated in the dark for 1 h at 25°C. The tissue was washed at room temperature in PBS and stained with propidium iodide (50 mM potassium ferricyanide, 0.15 M NaCl, and 1 mM MgCl2; 0.1 M phosphate buffer, pH 7.4; followed by incubation in FeCN solution (3 mM potassium ferrocyanide, 3 mM potassium ferricyanide, 0.15 M NaCl, and 1 mM MgCl2; in 0.01 M phosphate buffer, pH 7.2) and 0.2% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for 3 h at 37°C. Stained imaginal discs were briefly rinsed in phosphate buffer and mounted in glycerol.

**Results**

**A Drosophila Homologue of Bub1**

Two Drosophila EST sequences were identified through a BLAST search of the Berkeley Drosophila Genome Project (BDGP) database with the amino acid sequence of mouse Bub1 (Taylor and Mckon, 1997). The largest of the two corresponding cDNAs was sequenced in its entirety; this sequence has been deposited in GenBank under accession number AF080399. The cDNA sequence contains an open reading frame predicting a 165-kD protein closely related to Bub1. This protein shows 24.6% identity to human Bub1, 23.8% identity to mouse Bub1, and 14.5% identity to budding yeast Bub1p; it also displays 17.2% amino acid sequence identity to the human Bub1-related protein BubR1. The size of this Drosophila protein is somewhat larger than that of previously characterized human, mouse, and yeast members of the Bub1 family, whose predicted sizes range from 117–122 kD. The COOH-terminal third of the fly protein contains the strongly conserved kinase domain characteristic of Bub1. The NH2-terminal third of the fly protein, in common with the other members of the Bub1 family, shares significant sequence similarity with the yeast checkpoint control component Mad3p (Li and Murray, 1991; Chan et al., 1998; Taylor et al., 1998). Most of the additional residues resulting in the relatively larger size of the fly protein are located in its middle third. Evidence presented below on the intracellular distribution of this Drosophila protein further substantiates its assignment as a Bub1 homologue.

**Lethal P-Element Insertions into the Drosophila bub1 Gene**

A search of the BDGP database of genomic sequences flanking P-element insertion sites with the complete sequence of the Bub1 codNA identified the lethal P-element insertions I(2)K06109 and I(2)K03113 as mutations that could potentially affect the expression of the bub1 gene. Sequence analysis performed both by ourselves and by BDGP shows that the P-elements in the two separate mutants are inserted in exactly the same position within sequences transcribed into the 5′-untranslated leader of the bub1 mRNA, 48 bp upstream of the initiator ATG.

Several lines of evidence, presented in more detail in Materials and Methods, show that the lethality and associated mitotic phenotypes (see below) of I(2)K06109 and I(2)K03113 homoygotes is due to the P-element insertions into the bub1 gene. In brief, these two independently isolated mutations are allelic to each other, and they do not complement either of two deletions [D(f2)nap1 and D(f2)nap2] that remove polytene chromosome region 42A1-3, the location to which the bub1 gene and the I(2)K06109 and I(2)K03113 P-element insertions map by in situ hybridization. In addition, precise excision of the P-element in both mutant stocks by remobilization with a source of P-element transposase resulted in complete rescue of the lethality and associated mitotic defects seen in I(2)K06109 or I(2)K03113 homozygotes, showing that the P-element alone is responsible for the phenotype of these mutants. Importantly, as discussed below, many of the mitotic phenotypes visible in the larval neuroblasts of mutant animals are precisely those that would be expected from mutations affecting the expression of a component of the spindle checkpoint in Drosophila. These observations, taken together with the Western blot and immunofluorescence data described below, argue strongly that these mutant stocks contain P-element-induced hypomorphic mutations specifically affecting the Drosophila bub1 gene.

**Drosophila Bub1 Remains at the Kinetochoore in Response to Spindle Perturbation**

In order to examine Drosophila Bub1 distribution during the cell cycle, affinity-purified antibodies were generated...
against a LacZ/Bub1 fusion protein as described in Materials and Methods. Affinity-purified IgY identifies two bands of ~165 kD (the predicted molecular mass for Drosophila Bub1) on Western blots of larval brain extracts. These bands disappear almost completely in brain extracts made from bub1 mutant brains. The antibody also recognizes a band of ~100 kD that is not Bub1-specific and that is also recognized by preimmune IgY. This cross-reacting band serves as an internal loading control.

Figure 1. Specificity of affinity-purified anti-Drosophila Bub1 antibodies. Identical amounts of Drosophila third instar larval brain extracts, from either wild-type (Oregon R) or bub1 mutant homozygotes, were loaded onto the indicated lanes of a Western blot probed with affinity-purified anti-Drosophila Bub1 antibodies (see Materials and Methods). A doublet of ~165 kD (the predicted size for Drosophila Bub1) is recognized in wild-type extracts. These two bands are almost completely absent in brain extracts made from bub1 mutant brains. The antibody also recognizes a band of ~100 kD that is not Bub1-specific and that is also recognized by preimmune IgY. This cross-reacting band serves as an internal loading control.

Drosophila Bub1 Shows a Dynamic Cell Cycle–dependent Localization Pattern

Next we used our affinity-purified anti-Bub1 antibodies to examine in detail the distribution of Bub1 during mitosis in cycling Drosophila S2 cells. Interphase cells show a generalized, diffuse nucleoplasmic staining pattern (not shown). At prophase (Fig. 3 A), Bub1 associates strongly with the kinetochore regions of the condensed chromosomes; as shown in Fig. 2, D–F, Bub1 indeed substantially colocalizes with the kinetochore marker ZW10 (Williams et al., 1992, 1994, 1996). Kinetochore staining becomes weaker at prometaphase (Fig. 3 B). At metaphase, the Bub1 signal weakens specifically for those chromosomes that have migrated to the metaphase plate (Fig. 3, C–F). Chromosomes in the same cells that have not yet reached the metaphase plate continue to show strong Bub1 staining at their kinetochores (Fig. 3, C–E). Depending on the orientation of the chromosome with respect to the spindle, one kinetochore may stain more strongly for Bub1 than the other (Fig. 3 D). Very weak kinetochore signals continue to be visible into anaphase (Fig. 3 G), but are not observed during late anaphase (Fig. 3 H) or telophase (Fig. 3 I). Some staining of the spindle midzone is detectable at late anaphase (Fig. 3 H).

Similar intracellular protein distributions have already been documented by us for the Drosophila mitotic checkpoint control component Bub3 (Basu et al., 1998a), and have also been observed for human Bub1 and BubR1 (Chan et al., 1998; Jablonski et al., 1998). A previous report for mouse Bub1 failed to detect its association with kinetochores during metaphase or subsequent stages of mitosis (Taylor and McKeon, 1997); it is not clear whether this represents a true difference between the mouse and the human or Drosophila patterns of Bub1 distribution, or is instead the result of lower signal intensities obtained with the monoclonal anti–mouse Bub1 antibody employed in that study.

Mitotic Defects in bub1 Mutants

To determine the developmental stage at which bub1 mutants arrest their development, we rebalanced the (l(2)K06109- or l(2)K03113-bearing chromosomes over a balancer chromosome bearing the dominant marker Tubby, whose effects are visible in larvae and pupae. In these rebalanced stocks, ~30% of the third instar larvae were non-Tubby, in line with Mendelian expectations that bub1 homozygotes would constitute one-third of the animals that hatch from embryos. Many pupae were also non-Tubby, but these constituted a slightly smaller percentage...
(approximately 22%) of the total pupae. These results indicate that the lethality caused by the two bub1 mutations occurs mainly during the pupal stages, with most mutant homozygotes surviving through the third larval instar. Gatti and Baker (1989) have previously argued that animals homozygous for mutations in genes controlling essential cell cycle functions in Drosophila should survive to third instar larval stages or to the larval–pupal transition, because cell divisions prior to these stages could be supported by maternally supplied components contributed by their heterozygous mothers. This expectation has been borne out by many subsequent investigations of cell cycle mutants in flies (e.g., Williams et al., 1992). Indeed, we have detected no mitotic abnormalities in any of >1,000 post-cellularization divisions from a total of 22 homozygous mutant embryos observed at high resolution (see Materials and Methods and Discussion). Thus, we analyzed squashed preparations of neuroblasts taken from the brains of homozygous bub1 mutant third instar larvae to define the functional role of Bub1 in Drosophila cell divisions.

We initially observed that bub1 mutants possess the hallmark of a defect in the spindle checkpoint: that is, a failure to maintain sister chromatid cohesion when the spindle is disrupted. In wild-type brains incubated with colchicine for 1 h, sister chromatids remain attached in 98% of all mitotic figures (Fig. 4A and Table 1), revealing activity of the spindle checkpoint. Similar values have been obtained in previous experiments by our laboratory (Williams et al., 1992) and by others (Gonzales et al., 1991). Under identical conditions, sister chromatids remain attached in only 32% of mitotic figures in bub1 brains, indicating that the spindle checkpoint has often been bypassed (Fig. 4B and C, and Table 1). In fact, the frequency of neuroblasts with separated sister chromatids in bub1 mutant brains is essentially unaffected by colchicine treatment, in stark contrast with wild-type.

Table 1 details quantitative measurements of various mitotic parameters in squashed preparations of third instar larval brains that provide an overview of the phenotype associated with the l(2)K03113 mutation. In brains untreated with colchicine, the percentage of bub1 mutant mitotic cells with separated sister chromatids is much higher, and the percentage of mitotic cells in prophase or prometaphase is much lower, than in wild-type. Relative to wild-type controls, the brains of bub1 homozygotes show a threefold reduction in the mitotic index, operationally defined as the number of mitotic figures per optic field, with every optic field in the brain being scored. More limited data sets obtained through observations of l(2)K06109 homozygotes or of l(2)K06109/l(2)K03113 trans-heterozygotes yielded almost identical results (not shown).

In all particulars, the brains of larvae heterozygous for either bub1 mutation with either of two deletions removing the bub1 locus displayed phenotypes qualitatively identical to those seen in bub1 homozygotes. However, there is some slight quantitative variation in mitotic parameters between these deletion heterozygotes and the mutation homozygotes (Table 1); we do not know whether these effects are due to the activity of the bub1 gene or due to background effects. Based on these genetic criteria, the
two bub1 mutations behave as very strong hypomorphs that are probably nearly but not completely null alleles.

A significant proportion of the anaphase figures in bub1 mutant brains are aberrant (61–84%, depending upon genotype; Fig. 4, D–I). Three major kinds of abnormalities are seen at high frequency. First, in many neuroblast anaphases, chromatin bridges extend between the two separating groups of chromosomes (Fig. 4, E–F). In other anaphase figures, lagging chromatids remain at the position of the metaphase plate while the other chromosomes have migrated to positions near the poles (Fig. 4 G). Finally, we observe extensive chromosome fragmentation in many mutant anaphases (Fig. 4, H–I). We believe that these anaphase aberrations explain the observation that many of the cells in colchicine-treated mutant brains appear to be aneuploid (Fig. 4, B and C). These aneuploid cells could be produced by the maldistribution of intact chromosomes during anaphase of a previous cell generation. However, we suspect that many of the chromatids seen in mitotic cells like those depicted in Fig. 4, B and C may actually be chromosome fragments, resulting in an overestimate of the degree of aneuploidy.

Absence of Bub1 Leads to Apoptosis in Drosophila Larval Brains

A striking feature of bub1 mutant brains examined with DNA staining is the occurrence of extremely high frequencies of pycnotic nuclei with highly condensed chromatin. These nuclei are strongly positive when labeled by Tdt-mediated dUTP-biotin nick end labeling (TUNEL)-based techniques (Fig. 5, A–F; see Materials and Methods). Because the TUNEL procedure detects chromosome damage (normally induced in the pathway for apoptosis), the TUNEL signals could reflect either the occurrence of bona fide programmed cell death, or alternatively simply the chromosome fragmentation that occurs during anaphase in bub1 mutant cells. To discriminate between these possibilities, we asked whether mutant nuclei showed elevated expression of two apoptotic events independent of chromosome breakage. The first of these markers was the redistribution of phosphatidylserine, which early in apoptosis rapidly moves from the internal face of the plasma membrane to the outside of the membrane; this redistribution was detected by use of FITC-
conjugated Annexin V, a protein with very strong affinity for the serine in phosphatidylserine (Martin et al., 1995; see Materials and Methods). The second marker was a β-galactosidase reporter for reaper, a gene whose expression is needed to activate programmed cell death in Drosophila (White et al., 1994). Use of both markers verifies that mitotic cells in bub1 mutants undergo vastly elevated levels of apoptosis (Figs. 5, G–I, and 6). Levels of apoptotic nuclei are similar in l(2)K06109 or l(2)K03113 homozygotes as well as in trans-heterozygotes for either of the two alleles with deletions of the region (not shown).

**Table I. Quantification of Mitotic Parameters of Ore-R and bub1 Mutant Neuroblasts after Colchicine Treatment**

| Genotype          | Time in colchicine (min) | Number of optic fields* | Number of cells in mitosis | Number of cells in prophase + prometaphase | Frequency of prophase + prometaphase | Number of cells with SCS‡ | Frequency of mitotic cells with SCS | Mitotic index | Ratio of prophase + prometaphase/ anaphase |
|-------------------|--------------------------|-------------------------|---------------------------|-------------------------------------------|------------------------------------|--------------------------|-------------------------------------|--------------|--------------------------------------------|
| Wild-type         | 0                        | 992                     | 1,766                     | 1,279                                     | 0.72                               | 487                      | 0.28                                | 1.78         | 2.63                                       |
|                   | 30                       | 754                     | 2,029                     | 1,754                                     | 0.86                               | 275                      | 0.14                                | 2.70         | 6.38                                       |
|                   | 60                       | 554                     | 3,988                     | 3,892                                     | 0.98                               | 96                       | 0.02                                | 7.20         | 40.54                                      |
| l(2)K02113        | 0                        | 1,325                   | 802                       | 169                                       | 0.21                               | 633                      | 0.79                                | 0.61         | 0.27                                       |
| l(2)K03113        | 30                       | 787                     | 437                       | 142                                       | 0.32                               | 295                      | 0.68                                | 0.56         | 0.48                                       |
|                   | 60                       | 639                     | 339                       | 109                                       | 0.32                               | 230                      | 0.68                                | 0.53         | 0.47                                       |
| Df[2R]nap2        | 0                        | 952                     | 305                       | 154                                       | 0.50                               | 160                      | 0.52                                | 0.32         | 0.96                                       |
|                   | 30                       | 383                     | 136                       | 56                                        | 0.41                               | 80                       | 0.59                                | 0.36         | 0.70                                       |
|                   | 60                       | 436                     | 113                       | 50                                        | 0.44                               | 63                       | 0.56                                | 0.26         | 0.79                                       |
| Df[2R]nap1        | 0                        | 614                     | 131                       | 38                                        | 0.29                               | 93                       | 0.71                                | 0.21         | 0.41                                       |
|                   | 30                       | 352                     | 117                       | 48                                        | 0.41                               | 69                       | 0.59                                | 0.33         | 0.69                                       |
|                   | 60                       | 575                     | 142                       | 61                                        | 0.43                               | 81                       | 0.57                                | 0.25         | 0.75                                       |

*aFive brains were observed in each case.
‡SCS, sister chromatid separation.
Bub1 Is Not Required for Phosphorylation of 3F3/2 Kinetochoore Epitopes

Dephosphorylation of 3F3/2 epitopes is associated with the metaphase-anaphase transition (Campbell and Gorbsky, 1995). Microinjection of anti-3F3/2 antibodies into cultured cells blocks 3F3/2 dephosphorylation and delays anaphase onset, implying that dephosphorylation of 3F3/2 epitopes may be a prerequisite for entry into anaphase (Campbell and Gorbsky, 1995). The Bub1 kinase has been suggested as a candidate 3F3/2 kinase, both because of its function in the spindle checkpoint and because its intracellular distribution shows similarities with that of 3F3/2 epitopes (Chan et al., 1998). In order to examine these questions in more detail, we asked whether bub1 mutations would affect the distribution of 3F3/2 epitopes. As shown in Fig. 7, A and B, 3F3/2 signals are present at the kinetochores in bub1 prophase/prometaphase and metaphase figures at levels comparable to that of wild-type brains (see Bousbaa et al., 1997 for a description of 3F3/2 staining in wild-type Drosophila neuroblasts). This result demonstrates that Bub1 kinase does not contribute significantly to 3F3/2 kinase activity in vivo.

Interestingly, 3F3/2 staining continues to be detectable at the kinetochore at significant levels in many anaphase
figures from bub1 mutant brains (Fig. 7 D). In wild-type Drosophila neuroblasts, 3F3/2 phosphoepitopes at the kinetochore are completely lost by the start of anaphase (Fig. 7 C; Bousbaa et al., 1997). This observation indicates that dephosphorylation of kinetochore-associated 3F3/2 phosphoepitopes is not essential for entry into anaphase, at least in a bub1 mutant background.

**Mutations in Genes Encoding Several Other Kinetochore Components Do Not Disrupt the Association of Bub1 with the Kinetochore**

To establish the possible relationship between bub1 and other genes known to influence the fidelity of cell division in Drosophila, we explored the effects of mutations in these genes on the intracellular distribution of Bub1. We have focused on genes encoding other proteins that localize to the kinetochore, as the results of this analysis would further our understanding of kinetochore assembly.

Mutations in zw10 and rough deal disrupt the segregation of chromosomes during anaphase of mitosis and meiosis. Intriguingly, mutations in both genes cause precocious sister chromatid separation in colchicine treated larval neuroblasts, indicating a bypass of the spindle checkpoint (Smith et al., 1985; Karess and Glover, 1989; Williams et al., 1992). Both the ZW10 and Rod proteins are associated with the kinetochore during prophase/prometaphase of mitosis and both meiotic divisions (Williams et al., 1992; Williams and Goldberg, 1994; Scarrerou, F., and R. Karess, personal communication). We found that mutations in zw10 or rod do not affect the localization of Bub1 to the kinetochore (Fig. 8, A and E). Interestingly, in these mutant cells Bub1 continues to be associated with the kinetochores of precociously separated sister chromatids (Fig. 8 A), indicating that sister chromatid separation does not require the loss of Bub1 from the kinetochore. Similar results were observed when precocious sister chromatid separation was induced in wild-type
colchicine-arrested neuroblasts subjected to prolonged hypotonic shock (data not shown). Conversely, bub1 mutations do not block the association of ZW10 with the kinetochore (Fig. 8 B).

The mitotic mutation polo also affects mitotic fidelity and leads to chromosome missegregation and spindle abnormalities (Sunkel and Glover, 1988). The polo gene product is a protein kinase which shows a dynamic, cell cycle–dependent localization with several components of the mitotic apparatus, including the kinetochores (Logarinho and Sunkel, 1998). However, mutations in polo do not affect the distribution of Bub1 (Fig. 8 C), and the Polo protein kinase is localized normally to the kinetochores in a bub1 mutant background (Fig. 8 D).

Mutations in the Drosophila gene fizzy lead to metaphase arrest (Sigrist et al., 1995), and Fizzy/Cdc20/Slp1/p55CDC has been shown to be required to mediate the Bub/Mad-dependent inactivation of the APC (for review see Townsley and Ruderman, 1998). p55CDC, the mammalian homolog of Fizzy, has recently been shown to be concentrated at kinetochores from late prophase to telophase (Kallio et al., 1998). Because the action of the Fizzy protein is thought to be downstream of Bub1, we predicted that mutations in fizzy would not affect the ability of Bub1 to localize to the kinetochores. Fig. 8 F shows that this is indeed the case.

Role of Bub1 in Drosophila Spermatogenesis

The existence of a spindle checkpoint in meiotically dividing Drosophila spermatocytes is currently uncertain. The presence of univalents (chromosomes without pairing partners) does not prevent primary spermatocytes from entering anaphase. Furthermore, although mei-S332 or ord mutations cause sister chromatids to separate during the first meiotic division, chromosomes in mutant secondary spermatocytes still undergo obvious anaphase poleward movements (Goldstein, 1980; Lin and Church, 1982; Kerrebrock et al., 1992; Miyazaki and Orr-Weaver, 1992). If a spindle checkpoint were active, it should have prevented anaphase onset under either of these conditions because chromosomes could not be subject to the bipolar tension needed to deactivate the checkpoint (Nicklas et al., 1995; Nicklas, 1997). Finally, testes treated with colchicine contain many spermatids with polyploid nuclei, showing that spermatocytes with aberrant spindles do not arrest in metaphase and instead progress through meiosis and differentiate into spermatids (our unpublished results).

To explore the apparent absence or weakness of the spindle checkpoint in meiotic Drosophila spermatocytes, we examined the distribution of Bub1 during spermatogenesis using techniques we have previously developed (Williams et al., 1996). Bub1 localizes to the kinetochores of bivalents in primary spermatocytes during prometaphase.
...as shown in Fig. 9 A. The kinetochore association of Bub1 decreases significantly as the bivalents align at the metaphase plate (Fig. 9 B) and becomes undetectable at anaphase (Fig. 9 C), although some nuclear and spindle staining above background is visible during these cell cycle stages. This dynamic localization pattern is repeated during the second meiotic division (Fig. 9, D–F). Thus, the pattern of Bub1 distribution during both meiotic divisions parallels that seen during mitosis.

Is the association of Bub1 with the kinetochores during male meiosis in Drosophila responsive to tension? To answer this question, we analyzed the distribution of Bub1 in primary spermatocytes containing univalents: the attached XY (X’Y’) and the compound 4th [C(4)RM], which are never subject to bipolar tension as they can attach only to a single pole (Ault and Lin, 1984; Ault and Nicklas, 1989). Fig. 10 A shows that the intensity of Bub1 staining is comparable between univalents and bivalents at prometaphase I. However, once the bivalents align at the metaphase plate, the intensity of Bub1 staining on their kinetochores decreases drastically, while the univalents in the same cell retain strong Bub1 signals (Fig. 10, B–D). Thus, the spindle checkpoint component Bub1 is not only properly localized during male meiosis, but it is also capable of discriminating between the presence or absence of bipolar tension at kinetochores. Tension has also been recently implicated in regulating the localization of Mad2 at the kinetochores in maize spermatocytes (Yu et al., 1999).

Finally, we have examined larval testes from bub1 mutants for evidence of mitotic and meiotic defects. These testes are significantly smaller than wild-type testes, suggesting that mitotic proliferation of the germline has been substantially suppressed or that many mutant germline cells are directed into an apoptotic fate as seen in neuroblasts. A thorough analysis of these data is presented elsewhere (Fuller, 1993). It is possible that the testicular defects are due to chromosome missegregation, although this has not been directly demonstrated. However, it has been hypothesized that bub1 mutations strongly affect meiosis as well. Living testes from bub1 mutants examined by phase contrast optical microscopy show meiotic figures with severe spindle abnormalities at metaphase and anaphase, and multiple nuclei of variable volume at telophase (not shown). Oogenesis stage spermatids from bub1 mutant testes contain abnormal numbers of nuclei of variable size (including micronuclei) associated with a single Nebenkern of normal size (Fig. 9, G and H). A similar phenotype has been observed in Drosophila neuroblasts (Rodin, 1993), suggesting that the bub1 mutations affect meiotic chromosome segregation.

**Discussion**

**A Drosophila Homologue of Bub1**

We report in this paper the identification and molecular analysis of a Drosophila protein closely related to the spindle checkpoint component Bub1. Several lines of evidence...
support the assignment of this protein as the Drosophila Bub1 homologue. First, its primary sequence has been conserved across the phylogenetic spectrum, and is more similar to human and mouse Bub1 than to the related human BubR1 protein kinase (Taylor and McKeon, 1997; Chan et al., 1998; Taylor et al., 1998). Second, we show that the cell cycle distribution of the fly protein is essentially the same as that previously reported for human Bub1. Both proteins associate strongly with the kinetochores of chromosomes unattached to the spindle prior to anaphase onset of normal mitosis, and with all the kinetochores in cells treated with microtubule depolymerizing drugs. Reduced amounts of both proteins are also found at the kinetochores of chromosomes either at the metaphase plate or being pulled toward the poles at anaphase (Figs. 2 and 3; Chan et al., 1998; Jablonski et al., 1998). Third, near null mutations in the gene encoding this Drosophila protein cause phenotypes indicating an abrogation of the spindle checkpoint. Finally, we have previously shown that these same mutations abolish the ability of another checkpoint component, Drosophila Bub3, to localize to the kinetochores (Basu et al., 1998a). This latter finding fits well with a wealth of data substantiating an intimate relationship between Bub1 and Bub3 (Roberts et al., 1994; Taylor et al., 1998; Farr and Hoyt, 1998). Taken together, we believe that these observations in Drosophila provide strong evidence for the conservation of Bub1 function throughout evolution.

**Bub1 Is an Essential Checkpoint Component Required for Proper Cell Cycle Progression and Chromosome Segregation**

This paper describes the first genetic dissection of the function of a spindle checkpoint protein in a multicellular eukaryote. In *S. cerevisiae*, bub and mad genes are nonessential in the absence of microtubule depolymerizing agents, though the growth of mutant cells is slowed (Hoyt et al., 1991; Li and Murray, 1991; Roberts et al., 1994). In *S. pombe*, bub1 null mutants are viable, though some abnormalities in chromosome segregation are observable during mitosis (Bernard et al., 1998). In marked contrast,
loss of bub1 function in Drosophila leads to lethality at the larval/pupal transition. Lethality at this stage has been observed for many mutations affecting essential cell cycle components, presumably because maternally supplied stores of protein obtained from a nonmutant mother are exhausted by this point in development (Gatti and Baker, 1989). Examination of neuroblasts dissected from dying third instar bub1 homozygous mutant larvae has thus allowed us to define how loss of checkpoint function affects cell division in a multicellular organism. In the description below, we cannot exclude the possibility that some aspects of the phenotype we report are indirect consequences of problems encountered in earlier cell divisions. However, it should be noted that all embryonic divisions appear to be normal, and essentially all bub1 mutant animals hatch into larvae that survive until the third instar. As there is very little cell division in the larval brain before the third instar (Ito and Hotta, 1992), the number of cell divisions that could take place between the exhaustion of maternal stores of Bub1 protein and the time of analysis is limited. Moreover, we note that these phenotypes are quite specific to bub1 mutants, and have not been observed in our analysis of many other mitotic mutants in Drosophila.

As shown in Fig. 4, B and C, treatment of bub1 mutant neuroblasts with colchicine causes precocious sister chromatid separation, instead of the prometaphase arrest with attached sister chromatids typical of wild-type neuroblasts (Fig. 4 A; Gonzalez et al., 1991). This phenotype is a predictable property of mutations affecting the operation of the spindle checkpoint, as it indicates that bub1 mutant neuroblasts attempt to enter anaphase despite the absence of a functional spindle.

More interesting are the effects of bub1 mutations on normal cell division in neuroblasts that have not been treated with microtubule depolymerizing drugs. Our observations suggest that bub1 mutant neuroblasts enter anaphase prematurely even in these untreated cells. In particular, the ratio of metaphase figures to anaphase figures is decreased 5–10-fold in bub1 brains relative to wild-type brains (Table I). This result is consistent with studies showing that microinjection of Mad2 antibodies into mammalian cells causes premature sister chromatid separation and entry into anaphase (Gorbsky et al., 1998). Interestingly, loss of Bub1 in Drosophila generates a sharp decrease in mitotic index (Table I). This finding could be explained by an accelerated transit through mitosis as has been suggested for mammalian cell cultures expressing dominant negative forms of mouse Bub1 (Taylor and Mckean, 1997). However, it is also possible that the lowered mitotic index reflects the assumption of an apoptotic fate by many neuroblasts in the brain (see below).

A high proportion of anaphases in untreated bub1 mutant brains show a variety of aberrations, including extensive chromatin bridging (Fig. 4, E and F), lagging chromo-
A striking feature of Drosophila bub1 mutants is the occurrence of significantly elevated frequencies of apoptotic nuclei in larval brains (Figs. 5 and 6). This result was unexpected, as expression of a dominant negative form of mouse Bub1 has been reported to reduce the frequency of apoptotic nuclei in nocodazole-treated cells (Taylor and Mckeen, 1997), implying that loss of checkpoint function prevents the apoptotic response. The reasons for the apparent dichotomy between our results in Drosophila and those from mouse tissue culture cells are not clear. These effects could be organism or cell type–specific, or the differences could reflect unusual consequences of the dominant negative forms of Bub1 utilized in the mouse study.

A strong possibility for the high level of apoptotic cells seen in bub1 mutant brains emerges from our findings that the chromosomes in many mutant anaphase figures are extensively fragmented (Fig. 4, H and I). It has been well documented that chromosome breakage in Drosophila is normally a cell lethal event preventing entry into the next round of mitosis (Gatti, 1979; Baker et al., 1982). We have examined the larval brains of a number of new, relatively uncharacterized mitotic mutants that cause massive chromosome fragmentation, and these uniformly have high levels of apoptotic cells (our unpublished results). Moreover, Ahmed and Golic (1999) have recently demonstrated that the induction of chromosome breakage with the FLP/FRT system is also associated with apoptosis. A apoptosis (or in fact any aspect of the bub1 mutant phenotype) cannot be an indirect consequence of aneuploidy, because brains from zw10 and rod mutants, which have many aneuploid cells (Karess and Glover, 1989; Williams et al., 1992), do not show the massive apoptotic response (or any of the cell cycle defects) generated by bub1 mutants (data not shown). Regardless of the mechanism underlying the induction of apoptosis in bub1 mutant brains, it is clear that loss of spindle checkpoint function does not prevent a cell’s entry into the apoptotic pathway.

**Bub1 Is Not a Significant Source of the Kinase Activity Responsible for Phosphorylating 3F3/2 Epitopes**

In yeast, Bub1 acts as a kinase that can phosphorylate both itself and Bub3 (Roberts et al., 1994). Because Bub1’s cell cycle distribution parallels that of 3F3/2 phosphoepitopes that appear to be intimately involved in the metaphase–anaphase transition (Campbell and Gorbsky, 1995), Bub1 has been suggested as a possible source of the kinase activity that generates these phosphoepitopes (Nicklas, 1997; Chan et al., 1998). Our results show that this is not the case. As shown in Fig. 7, 3F3/2 epitopes are strongly phosphorylated in a bub1 mutant, showing that Bub1 cannot be a significant source of 3F3/2 kinase activity in vivo. In addition, we have previously demonstrated that Bub3 fails to associate with the kinetochores in bub1 mutants (Basu et al., 1998a), ruling out Bub3 as a major 3F3/2 phosphoepitope.

If Bub1 does not phosphorylate 3F3/2 phosphoepitopes, what kinase(s) can supply such an activity? A recent report indicates that ERK and MKK (extracellular signal–regulated protein kinase and mitogen-activated protein kinase kinase) localize to the kinetochores and can phosphorylate 3F3/2 phosphoepitopes (Shapiro et al., 1998). It is not clear whether this activity is direct or indirect; in any event, our results indicate that Bub1 does not participate in the same 3F3/2 phosphorylation pathway.

We were surprised to find that 3F3/2 epitopes at the kinetochores remain phosphorylated in anaphase figures from bub1 mutants (Fig. 7 D). In contrast, 3F3/2 phosphoepitopes at the kinetochores are normally lost completely at the start of anaphase (Fig. 7 C; Bousbaa et al., 1997). The implications of this result are twofold. First, dephosphorylation of kinetochore-associated 3F3/2 epitopes is not required for the metaphase/anaphase transition, at least in a bub1 mutant background. One possibility is that 3F3/2 dephosphorylation is not as commonly suggested (Campbell and Gorbsky, 1995; Nicklas et al., 1995) as part of the signaling pathway for anaphase onset, but is instead a downstream response to the signal. A second implication of our observation is that Bub1 kinase activity is required, presumably indirectly, for the dephosphorylation of 3F3/2 epitopes at the metaphase–anaphase transition. A possible explanation for the continued phosphorylation of kinetochore–associated 3F3/2 epitopes is that the accelerated transit through mitosis in bub1 mutants may not allow enough time for action of the relevant phosphatase(s).

**Separable Pathways in the Construction of Kinetochores**

We show in this paper that the localization of Bub1 to the kinetochore is not abolished by mutations in several genes.
encoding other kinetochore components, nor do mutations in bub1 affect the association of ZW10 or Polo proteins with the kinetochore. Combined with previous observations from our laboratories, these findings suggest that the kinetochore may be assembled in at least two independent pathways. In one pathway, interaction between Bub1 and Bub3 is required for the kinetochore targeting of either protein (Roberts et al., 1994; Basu et al., 1998; Taylor et al., 1998). In a second subassembly, ZW10 and Rod proteins form a complex needed for the recruitment of the microtubule motor dynein to the kinetochore (Starr et al., 1998). The fact that polo mutations do not disrupt the kinetochore localization of Bub1, Bub3, or ZW10 (Fig. 8 C and our unpublished observations) suggests either a third independent pathway or that the kinetochore binding of Polo protein is subsequent to the recruitment of one of the two subassemblies.

In colchicine-treated larval neuroblasts from zw10 mutants where the sister chromatids have separated prematurely, high levels of Bub1 protein remain at the kinetochores (Fig. 8 A). This phenomenon is not restricted to a zw10 mutant background, as prolonged treatment of wild-type larval neuroblasts with hypotonic solution after colchicine incubation also generates precocious sister chromatid separation with continued strong Bub1 staining at the kinetochores (not shown). These observations indicate that it is possible to initiate anaphase despite the presence of the Bub1 "wait-anaphase" signal at the kinetochores. It is thus conceivable that the relative loss of Bub1 from kinetochores at metaphase and anaphase (Figs. 3, C–E, and 9, B and C) is not normally a prerequisite for anaphase onset.

**Does the Spindle Checkpoint Function in Drosophila Meiosis?**

Although the existence of a tension-dependent "wait-anaphase" checkpoint in meiotic grasshopper spermatocytes has been well established (Nicklas et al., 1995; Nicklas, 1997), several observations suggest that such a checkpoint may not play a major role in Drosophila meiotic spermatogenesis. The presence of univalents (chromosomes without a pairing partner) does not obviously affect meiosis. The presence of univalents (chromosomes without a pairing partner) does not obviously affect mei-1998). The fact that crotubule motor dynein to the kinetochore (Starr et al., 1998). In a second subassembly, ZW10 and Rod proteins form a complex needed for the recruitment of the microtubule motor dynein to the kinetochore (Starr et al., 1998). The fact that polo mutations do not disrupt the kinetochore localization of Bub1, Bub3, or ZW10 (Fig. 8 C and our unpublished observations) suggests either a third independent pathway or that the kinetochore binding of Polo protein is subsequent to the recruitment of one of the two subassemblies.

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Nevertheless, Drosophila Bub1 and Bub3 both associate strongly with the kinetochores of primary spermatocytes before metaphase of both meiotic divisions (Figs. 9 A and 10 A; Basu et al., 1998a), and we have recently been able to observe kinetochore staining with antibodies against Xenopus Mad2 (Chen et al., 1996) in prometaphase primary spermatocytes (our unpublished observations). Bub1 responds differentially to the presence and absence of tension across chromosomes during meiosis (Fig. 10, B–D) exactly as would be predicted were it acting as part of a functional spindle checkpoint. In addition, bub1 mutations have a dramatic effect on Drosophila spermatogenesis. Though it is difficult to distinguish aberrations introduced during mitotic germ line cell proliferation from those occurring during meiosis, the appearance of disrupted meiotic spindles (not shown) and of multiple nuclei of uneven volume within "onion-stage" spermatids (Fig. 9, G and H) are suggestive of defects specifically affecting meiosis.

On the basis of these observations, we believe that a spindle checkpoint does exist in Drosophila meiotic spermatocytes, but that it operates with significantly reduced efficiency or according to different signals. The reasons underlying this apparent inefficiency remain unclear, but very likely involve part of the checkpoint pathway downstream of Bub1. One prediction of this viewpoint is that conditions that should enable the checkpoint would delay, but not completely block, cell cycle progression past the metaphase of either meiotic division. It will thus be of importance in the near future to verify this prediction through real-time observations of male meiosis in cultured spermatocytes.

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