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The Cloning and Characterization of the *mel-26* Gene of *Caenorhabditis elegans*

by

Michael Rhys Dow

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ABSTRACT

The *mei-1* gene of *Caenorhabditis elegans* encodes a component of the oocyte meiotic spindle. MEI-1 is essential for successful completion of meiosis, and normally observed only during meiosis. When it is allowed to persist after completion of meiosis, MEI-1 is capable of being incorporated into the mitotic spindle. The ectopic presence of MEI-1 in the mitotic spindle leads to failure of spindle rotation and migration. Previous work has identified the *mel-26* gene as the post-meiotic negative regulator of *mei-1*. This report describes the cloning and characterization of the *mel-26* gene, as well as preliminary characterization of its expression.

The *mel-26* gene has been cloned by transformation rescue of the *mel-26(ct61sb4)* loss-of-function allele. The predicted protein, MEL-26, consists of 395 amino acids and contains a BTB motif, but is otherwise novel. BTB domains have been identified in a broad array of proteins and have been proposed to play a role in protein-protein interactions. The molecular lesion present in two of the three *mel-26* alleles has been identified. The *sb45* allele is a missense allele, leading to a cysteine to tyrosine change at residue 94. The *sb4* allele is a nonsense mutation, leading to the truncation of the carboxy-terminal 75 amino acids. Consistent with the strict maternal-effect lethality of *mel-26* mutations, the

mel-26 transcript is predominantly expressed in the female germline. Immunolocalization of MEL-26 demonstrates that it is a mitosis-specific component of the centrosome. None of the *mel-26* mutations, nor the mitosis-defective *mei-1* mutation [*mei-1(ct46)*] affect the localization of MEL-26 to the centrosome. MEL-26 may not compete for centrosome localization, but it is in the right place.

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INTRODUCTION

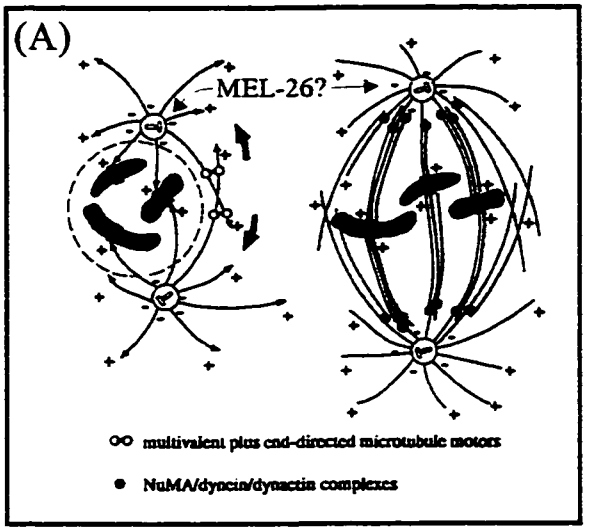
The meiotic and mitotic spindles, while superficially fairly similar, have some distinct features. The *mei-1* gene of *Caenorhabditis elegans* encodes a protein that is essential for the meiotic spindle, yet detrimental to the mitotic spindle. This project will address the nature of a regulator of the *mei-1* gene, *mel-26*, that ensures that *mei-1* activity does not interfere with mitotic spindle function. A brief overview of spindle assembly and an introduction to some of the factors known to play important roles in the spindle is presented.

Assembly of the meiotic and mitotic spindles

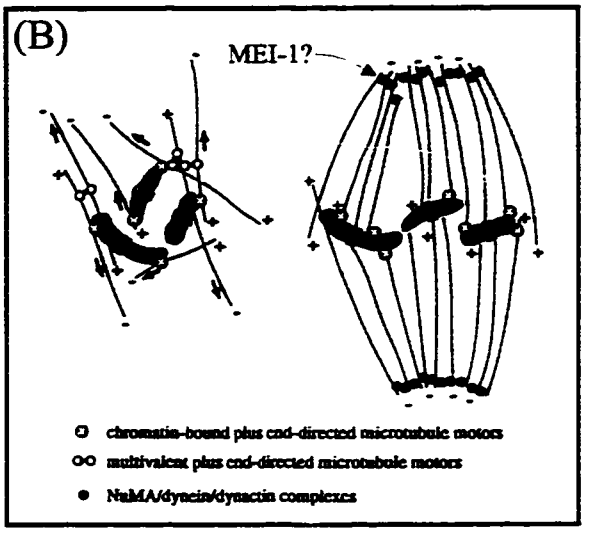
The classical view of animal spindle assembly holds that at the onset of cell division, the centrosome divides and the daughter centrosomes migrate to opposite sides of the nucleus. As the nuclear envelope breaks down, the centrosomes become competent to nucleate long arrays of microtubules, which emerge from the centrosome in all directions forming large asters (Figure 1). Some of the microtubules contact chromosomes, in particular the kinetochores located at the centromeres of the chromosomes. Others bridge the gap between the asters, and factors such as kinesin-like motor proteins cross-link the microtubules. Thus the bipolar spindle, with attached chromosomes is formed (MERDES and CLEVELAND 1997).

FIGURE 1. **Spindle assembly in presence and absence of centrosomes.** (A) When centrosomes are present, microtubules nucleate from the centrosome, pushing the centrosomes apart through the action of plus-end directed motor proteins. Following spindle assembly, the microtubules may separate from the poles, maintaining attachment through the minus-end directed activities of the NuMA-dynein-dynactin complex. (B) In the absence of centrosomes, microtubules form around the chromosomes. Various plus- and minus-end directed motors bundle the initially randomly oriented microtubules into organized spindles. Figure from MERDES and CLEVELAND (1997).

Centrosome-mediated Spindle Assembly



Centrosome-independent Spindle Assembly



This may be the mechanism that is used during formation of the vast majority of animal mitotic spindles, however it is clearly not the only mechanism. An alternative mechanism, which is likely primarily used during formation of the meiotic spindle during oogenesis has been described (HEALD *et al.* 1996; KARSENTI *et al.* 1984; MERDES and CLEVELAND 1997). This alternative spindle assembly pathway involves a more inside-out approach, likely to accommodate the absence of centrosomes [which are often donated by the sperm (SCHATTEN 1994)]. As the nuclear envelope breaks down, the chromosomes begin to induce the polymerization of microtubules around themselves (Figure 1b). Introduction of large molecules of DNA (*e.g.*, Bacteriophage T_4 or *E. coli* genomic DNA) has been shown to be sufficient to induce formation of a spindle in *Xenopus* oocytes (KARSENTI *et al.* 1984). Smaller molecules, such as pBR322 or sonicated T_4 DNA were unable to induce spindle formation and have been suggested to be incapable of recruiting factors necessary to convert the naked DNA into chromatin (KARSENTI *et al.* 1984).

Initially the meiotic microtubules form a diffuse haze around the chromosomes, then begin to coalesce into parallel arrays and finally become spindle-shaped as the ends of the arrays are bundled together. This type of spindle formation has been described in the oocytes of Ascidians

(SAWADA and SCHATTEN 1988), *Xenopus* (GARD 1992), *Drosophila* (MATTHIES *et al.* 1996) and *Caenorhabditis* (ALBERTSON and THOMSON 1993). The spindles formed via this mechanism are typically described as "barrel-shaped" and as lacking prominent asters at the spindle poles. A comparison is often made with the acentriolar mitotic spindles of many plants. If the structure of the spindle reflects the assembly mechanism, the similarity between acentriolar spindles from diverse organisms may indicate that this pathway is used in all cases.

This variant spindle assembly pathway and other differences that have been noted between various model systems and different types of cell division, prompted RIEDER *et al.* (1993) to warn against making broad claims about the nature of spindles based on observations in any particular circumstance. Unfortunately, this has not prevented some researchers from trying to do just that. It is often valuable to make comparisons between systems, though the limitations and caveats should be made apparent to the unsuspecting reader.

A recent paper (HEALD *et al.* 1996) addressed the issue of spindle assembly in "mitotic" *Xenopus* extracts and showed that spindle formation in the absence of centrosomes followed the mechanism described above for oocyte meiosis. This was used to demonstrate a common spindle assembly mechanism and that centrosomes were dispensable for mitotic spindle

assembly. Interestingly, the source of their mitotic extract was "unfertilized *Xenopus* eggs arrested in metaphase of meiosis II" (HEALD *et al.* 1996). The more appropriate conclusion might be that some meiotic factor makes the centrosomes redundant, and thus highlights the unique nature of the acentriolar meiotic spindle or its environment.

This provokes the question of what differences exist between the meiotic and mitotic spindles. This is a particularly important issue during oogenesis and the transition to embryogenesis. Prior to fertilization, the oocyte is typically arrested in some phase of meiosis, which is reinitiated upon fertilization. Immediately following the completion of meiosis, the zygote begins the rapid mitotic divisions of embryogenesis. This transition from meiotic to mitotic division occurs within a short time span in a very restricted environment. The products of meiosis and mitosis are different, the spindles are structurally unique, and they use separate assembly mechanisms. What regulatory and structural factors are necessary to cause these differences?

An Inventory of Spindle and Centrosomal Proteins

Several proteins have been identified as spindle components and some of the key factors will be briefly introduced at this point. Most known spindle components are either required for both meiotic and mitotic

spindles, or their meiotic requirement has not been determined. Very few meiosis-specific spindle components have been identified. However, differences between meiosis and mitosis may involve the regulation or deployment of some of these molecules. Activity of the *mel-26* gene ensures that MEI-1, which is capable of interacting with either the meiotic or mitotic spindles only functions in the oocyte meiotic spindles.

To understand the following description of components of the spindle and centrosome, there are several terms that should be defined. In most mitotic animal cells, a centrosome consisting of a pair of centrioles surrounded by an amorphous cloud of proteins, known as the pericentriolar material (PCM), is located adjacent to the nucleus. The centrosome is a special type of microtubule organizing center (MTOC); however several examples of non-centrosomal MTOCs are known (BRINKLEY 1985). There are both constitutive and cell-division-specific components of the centrosome. In addition to recruitment of new components, some constitutive components undergo modifications such as phosphorylation-state changes during cell division. Most oocytes and many plant cells lack a centriole-containing centrosome (PEREIRA and SCHIEBEL 1997). This raises the question of whether centrioles are entirely dispensable or whether these cells possess an alternative structure (more about this later).

Obviously, a major constituent of the spindle is tubulin; the microtubule arrays are stoichiometric heteropolymers of α- and β-tubulin. The importance of α- and β-tubulin will not be discussed further, except to mention that a maternal-specific α-tubulin isoform (TUB67C) has been identified in *Drosophila*, and that the ratio of TUB67C to the more widely expressed TUB84B and TUB84D isoforms is critical to female meiosis and early embryonic mitosis (MATTHEWS *et al.* 1993).

Several years ago, a highly divergent tubulin (γ-tubulin) was discovered to be a component of the spindle poles in virtually all organisms (OAKLEY and OAKLEY 1989; ZHENG *et al.* 1991; MASUDA and SHIBATA 1996). A number of studies (STEARNS and KIRSCHNER 1994; MASUDA and SHIBATA 1996) have suggested that γ-tubulin forms a ring that serves to nucleate α- and β-tubulin-based microtubules, though the mechanism is not clear.

In a wild-type *Drosophila* cell line, γ-tubulin has been found in association with the centrosomes throughout the cell cycle (DEBEC *et al.* 1995). On the other hand, in a mutant *Drosophila* cell line lacking centrioles, faint anti-γ-tubulin staining is found throughout the cytoplasm during interphase. Mitotic spindle formation in these cells is somewhat impaired, resulting in a large percentage of spindles having rather diffuse

poles. Faint anti-γ-tubulin staining was observed in association with the poles of most spindles, with brighter staining observed in conjunction with the better organized spindles (DEBEC *et al.* 1995).

In unfertilized *Xenopus* oocytes, γ-tubulin is found in a large (~25S) centrosome-associated particle, which seems to take the form of a ring (OAKLEY 1995). Two components of the γ-tubulin complex in *Saccharomyces cerevisiae* have been identified. The molecular activities of these proteins (Spc97p and Spc98p) have not been identified, though it is proposed that they might form the scaffold on which the γ-tubulin ring is formed (KNOP *et al.* 1997). The γ-tubulin complex is capable of independently associating with either centrosomes or microtubules (STEARNS and KIRSCHNER 1994), suggesting that it may act as an intermediate between these two structures.

Mutations in γ-tubulin prevent formation of the mitotic apparatus and behave as recessive lethals in *Aspergillus* (OAKLEY *et al.* 1990).

A widely used experimental system for studying spindle assembly employs extracts of *Xenopus* oocytes, sometimes treated with maturation-promoting factor (MPF), cytostatic factor (CSF) or calcium chloride to mimic various cell cycle stages (LOHKA and MALLER 1985; MURRAY 1991; SAWIN and MITCHISON 1991). Addition of

demembranated sperm nuclei to the extract provides centrosomes to nucleate spindle formation (SAWIN and MITCHISON 1991). While this extract is often referred to as mitotic (SAWIN and MITCHISON 1991; HEALD *et al.* 1996), it is more correctly termed a meiosis II-arrested extract. On the other hand, the oocyte also contains a pool of factors required for the mitotic divisions of embryogenesis. Introduction of centrosomes into this system makes it a hybrid between meiotic and mitotic systems, adding additional complexity to the interpretation of any results derived from this model system.

A highly conserved component of the centrosome is pericentrin, which is a component of the PCM in a wide range of organisms (DOXSEY *et al.* 1994). In addition to being present in typical centriolar MTOCs, pericentrin is found in the acentriolar MTOCs of mouse oocytes. Sequence analysis suggests that pericentrin is a large coiled-coiled protein with domains at either end that are neither helical nor coiled. Immunodepletion of pericentrin from *Xenopus* oocyte extracts indicates that it is essential for microtubule nucleation, particularly in the case of acentriolar meiotic spindles (DOXSEY *et al.* 1994). Interestingly, the localization of γ-tubulin to the centrosome is unaffected by injection of anti-pericentrin antibodies (DOXSEY *et al.* 1994).

Considerable interest over the years has been focused on a protein called NuMA, for **Nu**clear and **M**itotic **A**pparatus. During interphase, NuMA is an insoluble component of the nuclear matrix. At the onset of cell division, NuMA is phosphorylated, causing it to become soluble. Soluble NuMA binds to microtubules and may undergo an additional phosphorylation (SAREDI *et al.* 1997). When localized to the spindle, NuMA is found in association with the microtubules at the centrosome (CLEVELAND 1995). Like pericentrin, NuMA is predicted to contain a large coiled-coil region, which is thought to interact with the microtubule arrays. The globular region of NuMA interacts with dynactin and the motor protein dynein (see later) (MERDES and CLEVELAND 1997). As with pericentrin, injection of anti-NuMA antibodies has been used to analyze the function of NuMA in spindle function. NuMA appears to be necessary for both the establishment and maintenance of the mitotic spindle (YANG and SNYDER 1992).

Immunodepletion of NuMA from *Xenopus* oocyte extracts, prior to addition of sperm heads, results in the formation of radial microtubule asters around the sperm centrosomes. This indicates that NuMA is not required for the initial steps of microtubule nucleation. However, these asters did not progress to form spindles and were devoid of NuMA, pericentrin and γ-tubulin (MERDES *et al.* 1996). Subsequent addition of

NuMA induced the formation of mono-polar half-spindles and some bi-polar spindles (MERDES *et al.* 1996).

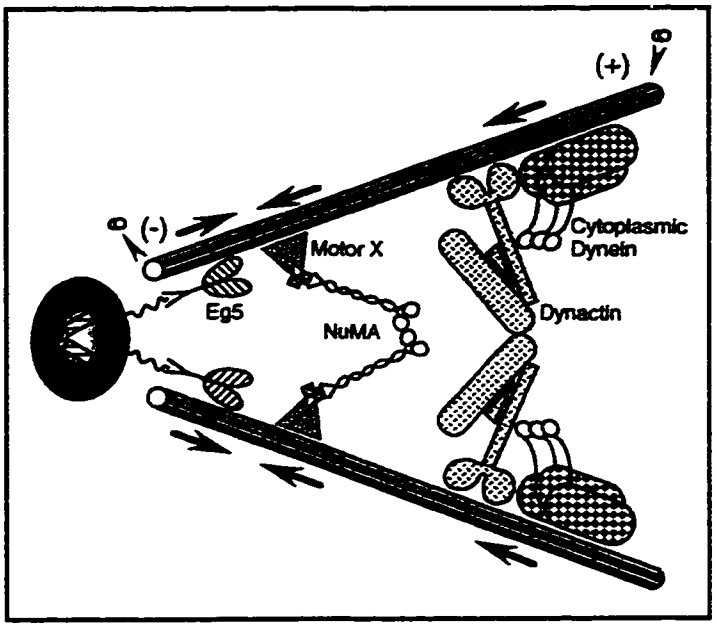
This starts to bring into question the validity of comparisons between the various experimental systems. In the absence of NuMA, sperm centrosomes are unable to recruit γ-tubulin; however in somatic cells, γ-tubulin is constitutively present in the centrosome and NuMA is recruited only at the onset of mitosis. It appears likely that different pathways are employed depending on the origin of the centrosome.

Cytoplasmic dynein is a minus-end directed microtubule-based motor complex consisting of ~10 subunits (SCHROER 1994). Several studies have implicated dynein in organization and assembly of the mitotic spindle (GAGLIO *et al.* 1996; MERDES *et al.* 1996; STEARNS 1997; GAGLIO *et al.* 1997). Injection of a monoclonal antibody specific for a cytoplasmic dynein component into tissue culture cells has been shown to disrupt mitotic spindles (GAGLIO *et al.* 1997). Within 5 minutes of antibody injection, the spindle poles lost focus and the spindles became "barrel-shaped" (GAGLIO *et al.* 1997). Addition of the dynein-specific antibody to mitotic extracts from HeLa cells, prior to spindle formation, prevented assembly of the spindle (GAGLIO *et al.* 1997), indicating that disruption of dynein affects both spindle formation and maintenance.

Dynactin is another large multi-subunit complex that appears to play a role in either regulating dynein activity or dynein targeting (ECHEVERRI *et al.* 1996). Addition of anti-dynein antibody was shown to disrupt interaction of dynactin with microtubules (GAGLIO *et al.* 1997). Like many other centrosomal proteins, at least one subunit of dynactin contains a number of coiled-coil domains (ECHEVERRI *et al.* 1996). Members of both the dynein and dynactin complexes were co-purified with NuMA, suggesting that these three proteins interact in the organization of the spindle (MERDES *et al.* 1996). Immunodepletion of the Arp1 subunit of dynactin from mitotic extracts prevents spindle formation in a manner similar to that observed when the extract is depleted of NuMA or dynein (GAGLIO *et al.* 1996).

Taken together, a model has been proposed (Figure 2) in which the NuMA-dynein-dynactin complex binds to, and cross-links, multiple microtubule strands. The minus-end directed motor activity of dynein moves the complex to the spindle poles, bundling the attached microtubules into focused poles (MERDES *et al.* 1996; GAGLIO *et al.* 1996). An additional model incorporates the observation that a large percentage of microtubules do not directly contact the centrosome, rather they end at a distance of ~1 μm from the centrosome (MASTRONARDE *et al.* 1993). It has been suggested that the NuMA-dynein-dynactin complex may provide linkages

FIGURE 2. **Model for the role of microtubule motors at the spindle pole.** GAGLIO *et al.* (1996) propose that microtubules are bundled together by two minus-end directed complexes; NuMA, in association with a hypothetical Motor X, and dynein-dynactin. The plus-end directed motor Eg5 is shown tethered to the centrosome, though this is not necessarily required. Figure from GAGLIO *et al.* (1996).


(+)
(-)
Motor X
Eg5
NuMA
Cytoplasmic
Dynein
Dynactin

between the spindle and those microtubules that do remain attached to the centrosome (GAGLIO *et al.* 1997).

In general, the activities of the various plus- and minus-end directed motors must be balanced for proper spindle assembly and function. In *Xenopus*, the minus-end directed activity of dynein is balanced, at least partially, by the plus-end directed kinesin-like protein Eg5 (GAGLIO *et al.* 1996). Several other kinesin-like or kinesin-related proteins also play roles in either spindle assembly or function. The *S. cerevisiae* genome contains a total of six kinesin family members, five of which have been identified as playing a role in spindle assembly or movement (STEARNS 1997). It is likely that there are at least as many kinesins involved in spindle function in higher eukaryotes.

The plus-end directed kinesin Eg5 is the *Xenopus* representative of the group of kinesins sometimes referred to as BimC-type, in reference to the product of the BimC gene of *A. nidulans*. Other members include Cin8p and Kip1p in *S. cerevisiae* and KLP61F in *Drosophila*. Each of these has been shown to have some role in centrosome or spindle pole body (SPB) separation (KASHINA *et al.* 1997).

A human homolog of Eg5 (HsEg5) has been identified (WHITEHEAD *et al.* 1996; BLANGY *et al.* 1995), and has been shown to play a role in movement of the centrosomes during (BLANGY *et al.* 1995) and after

mitosis (WHITEHEAD *et al.* 1996). Interaction of HsEg5 with the mitotic apparatus requires phosphorylation by $p34^{cdc2}$ at a site that appears to be conserved at least amongst BimC-type kinesins (BLANGY *et al.* 1995). The phosphorylation-dependant interaction of HsEg5 with the spindle may be mediated by dynactin. Two-hybrid and *in vitro* experiments suggest that HsEg5 interacts with the human homolog of *Drosophila* $p150^{Glued}$ (a component of dynactin), and that this interaction is enhanced by phosphorylation of HsEg5 (BLANGY *et al.* 1997). This may suggest that a megacomplex of HsEg5, NuMA, dynein and dynactin is important in the formation of the spindle poles, or may simply indicate that both dynein and HsEg5 use a component of dynactin to interact with the centrosome.

Mutations in the plus-end directed motor KLP61F from *Drosophila* cause a failure in mitotic centrosome separation. Severe alleles lead to the formation of predominantly mono-polar spindles. Weaker alleles are able to form a monastral bipolar spindle; failure of centrosome separation means that one pole lacks a proper centrosome and is unable to organize a complete spindle pole aster (WILSON *et al.* 1997).

The *S. cerevisiae* kinesins Cin8p and Kip1p are also required for spindle pole separation, though both must be eliminated to show a spindle pole separation defect. An interesting screen for genes essential in a *CIN8* mutant background yielded alleles of *KIP1* and the genes encoding dynein

heavy chain as well as dynein- and dynactin-associated proteins (GEISER *et al.* 1997). Notably, none of the genes identified encoded any additional components of the *KIP1* or *CIN8* pathways (*e.g.*, a Cin8p-specific light chain).

A second major family of kinesins involved in spindle assembly is often referred to as the ncd-type, in reference to the product of the *non-claret disjunctional* gene (*ncd*) in *Drosophila*. Ncd-type kinesins, such as Kar3p from *S. cerevisiae*, are minus-end directed motors. As described above, mutation of both CIN8 and KIP1 leads to failures in spindle function. These defects can be suppressed by mutation of the KAR3 gene (SAUNDERS *et al.* 1997b), emphasizing the need for balance in the activity of microtubule motors.

Mutation of KAR3 alone leads to defects in spindle formation (SAUNDERS *et al.* 1997b). Loss of KAR3 activity disrupts the order of the microtubule arrays and seems to promote the formation of longer arrays. Overexpression of KAR3, on the other hand, leads to a shortening of the spindle (SAUNDERS *et al.* 1997b). This is a phenotype observed in loss-of-function mutations in KIP1 and CIN8, again emphasizing the opposing forces that these plus-end directed motors have with respect to the minus-end directed Kar3p (SAUNDERS *et al.* 1997b).

The product of the *ncd* gene in *Drosophila* is a minus-end directed kinesin-like motor protein. Ncd is found associated with both the meiotic and mitotic spindles. During early stages of mitosis, Ncd is localized to the centrosomes and spindle poles; however as mitosis progresses, Ncd spreads throughout the entire spindle and finally localizes to the midbody region during telophase (ENDOW *et al.* 1994). Null mutations in *ncd* result in the formation of mitotic spindles that are described as "bent" or "curved" (ENDOW *et al.* 1994). Mutations in *ncd* also lead to spindle pole defects, such as splitting of the centrosomes and dissociation of the centrosomes from the poles of the mitotic spindle. During meiosis, loss of *ncd* activity leads to the formation of diffuse or multipolar meiosis I spindles. Interestingly, meiosis-specific alleles of *ncd* have been isolated, indicating that the precise role of Ncd in the meiotic spindle is distinct from that in the mitotic spindle (ENDOW *et al.* 1994). This nicely demonstrates the unique nature of the meiotic spindle.

Kinesins such as CENP-E, Nod, MCAK, MKLP1 and XKLP1 appear to play roles in attaching chromosomes to the spindle apparatus. Through these attachments, they seem to provide some measure of stability to the spindle, though for the most part they do not appear to play a primary role in spindle organization, and will not be discussed further.

The *S. cerevisiae* protein Stu2p is a component of the SPB, a structure roughly comparable to the spindle centrosome in higher eukaryotes. The *STU2* gene was identified in a screen for suppressors of a cold-sensitive β-tubulin mutation that showed defects in spindle function (WANG and HUFFAKER 1997). Localization of Stu2p to the SPB does not seem to require the presence of microtubules, though Stu2p has been shown to possess microtubule-binding activity. This has lead to the suggestion that Stu2p may tether microtubules to the SPB. Homologues of Stu2p have been identified in *S. pombe* ($p93^{dis1}$), *Xenopus* (XMAP215), human (ch-TOG), and *C. elegans* (ZYG-9, see below). All of these proteins have been found to localize to the spindle poles and microtubules during mitosis (WANG and HUFFAKER 1997).

In addition to the few well-conserved spindle components, there are a number of factors that have been, for the most part, described only in one system. For instance, the *Drosophila* gene *abnormal spindle* (*asp*) encodes a protein that has some similarity to calcium and actin binding proteins. During mitosis, Asp moves from the cytoplasm to localize to the polar regions of the spindle. As mitosis enters telophase, Asp appears to move to the central region. Mutants for *asp* show a variety of phenotypes, a prominent one being broadened or fused spindles with wavy microtubules (SAUNDERS *et al.* 1997a).

The product of another *Drosophila* gene, *twinstar* (*tsr*), is a homolog of cofilin, an actin severing protein (GUNSALUS *et al.* 1995). While cofilins are highly conserved proteins, a role in the spindle apparatus has not been previously described. In *tsr* mutants, the centrosomes divide but often fail to separate, leading to the formation of adjacent asters. There have been previous indications that the actin cytoskeleton plays a role in positioning of the centrosomes (MACK and RATTNER 1993).

The gene *centrosomin* (*cnn*), identified as a homeotic target gene in *Drosophila* encodes a component of the centrosome (LI and KAUFMAN 1996). It is obviously consistent with the importance of the homeotic genes in controlling morphogenesis that one target would be a gene important in some aspect of cell division. Cnn contains several leucine zipper motifs and kinase recognition sites, which leads to the suggestion that Cnn may be a component of a complex and may be regulated through phosphorylation (HEUER *et al.* 1995). Like many other centrosomal components, Cnn contains regions of coiled-coil sequence. Mutations in *cnn* lead to defects in spindle organization and appears to disrupt several other microtubule-dependent processes (LI and KAUFMAN 1996).

While apparently novel, the protein encoded by the *Drosophila* gene *ninein* contains a number of identifiable motifs. In addition to large stretches of coiled-coil structure, there is a potential EF hand domain, a

GTP binding site, four leucine zipper domains and a PEST region. Ninein is associated with the centrosomes in all stages of the cell cycle, however it was found in unequal abundance in newly replicated centrosomes and was not observed in all cell types (BOUCKSON-CASTAING *et al.* 1996).

The number of components known to be essential for the formation and function of the meiotic and mitotic spindles is rapidly growing. Unfortunately, much of the data remains as isolated observations, contributing little to the overall understanding of how the spindle is formed and what role the various factors play. This is due in part to the disparate experimental models, the various strengths and weaknesses of those models, and the fact that many spindle components are identified through serendipity and are often not further pursued.

Some conclusions can be made from the available data. There are two general pathways of spindle assembly. The centriole-based centrosomes, when present, serve as the nucleation site of the nascent spindle. Microtubule motors such as kinesin-like proteins or dynein mediate a controlled separation of the centrosomes through their plus- and minus-end directed activities. A bi-polar spindle then forms between the separated spindle poles, with some microtubules bridging the gap between poles, while others attach the chromosomes to the poles.

In the absence of centrioles, centrosomal components seem to be dispersed throughout the cytoplasm and must be recruited. Following breakdown of the nuclear envelope, tubulin, and presumably additional spindle components, are concentrated in the region around the chromosomes. Tubulin begins to assemble into microtubules, which are sorted into parallel arrays through the bundling actions of various motor proteins.

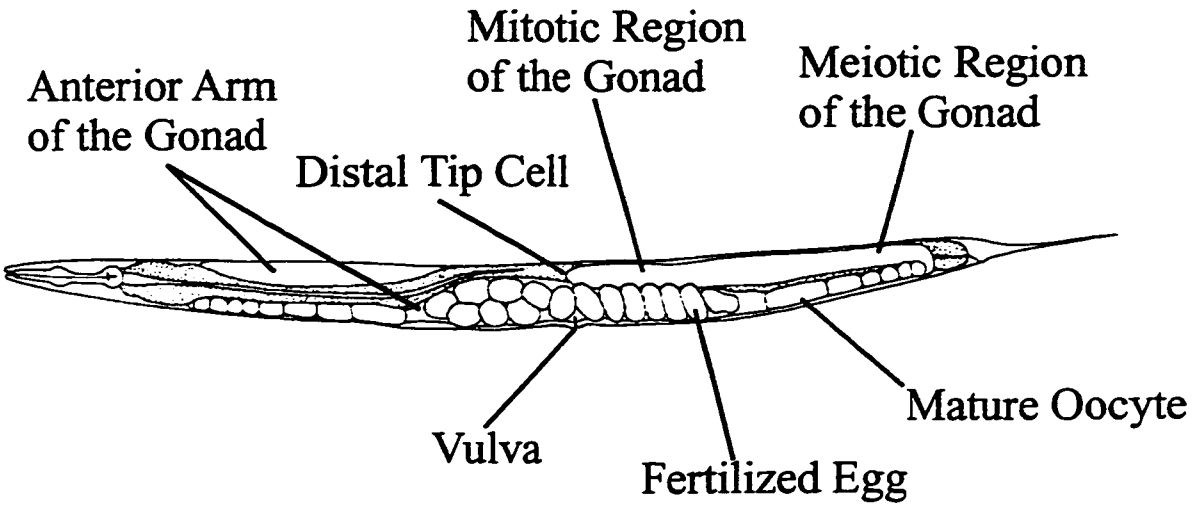
This provokes the question of what differences are responsible for the unique spindle assembly pathway used in acentriolar spindles. It can be argued that there are no additional components, rather the simple absence of organized centrosomes forces the use of a chromosome-centered assembly pathway. In this model, perhaps for greater efficiency, the centrosome remains pre-assembled around the centrioles, and when present is the preferred focus of spindle assembly. HEALD *et al.* (1996), in fact, suggest that the true function of the centrosome is to mediate the orientation of the spindle, either through cues in the cytoplasm or cell cortex and that it is entirely dispensable for spindle assembly. The suggestion the presence or absence of centrioles has no effect on the mechanism of spindle assembly, and that a common assembly mechanism is used in all spindles is argued against by three points. First, in grasshopper spermatocytes, the chromosomes are unable to nucleate

assembly of a spindle when moved some distance away from the centrosomes (ZHANG and NICKLAS 1995). Second, attachment to the cell cortex is also important for the meiotic spindle, likely as a means of ensuring that the size of the polar bodies is minimized. Finally, a spindle component essential for assembly of the oocyte meiotic spindle has been identified in *Caenorhabditis elegans* (CLARK-MAGUIRE and MAINS 1994a,b).

Meiosis in *C. elegans* oogenesis

Oogenesis in the nematode worm *C. elegans* has been well described (ALBERTSON 1984; ALBERTSON and THOMSON 1993; HIRSH *et al.* 1976). The hermaphrodite gonad is a bi-lobed syncytium (Figure 3). At the distal end of each lobe, germ nuclei are maintained in a mitotic state by the actions of the LAG-2/GLP-1 signal from the distal tip cell at the distal end of each arm of the gonad (HENDERSON *et al.* 1994). As each nucleus is forced, by proliferation, beyond the signalling range, it exits mitosis. Towards the loop region of the reflexed gonad, nuclei enter meiosis. As nuclei enter the loop region, cell membranes pinch off individual nuclei and package them into oocytes. As the oocytes progress through the gonad, they continue to advance through prophase I, arresting in diakinesis. The oocytes remain arrested in diakinesis until fertilization.

FIGURE 3. **Gravid adult hermaphrodite *Caenorhabditis elegans*.** Features of the posterior arm of the gonad are indicated. The anterior arm of the bi-lobed gonad also contains these features. Signals from the distal tip cell cause germ nuclei at the distal end of each gonad to divide by mitosis. As the nuclei pass beyond signal range, they enter meiosis. The nuclei become cellularized as they reach the bend region of the gonad. Mature oocytes pass through the spermatheca, where they are fertilized and eventually expelled through the vulva. Figure adapted from SULSTON and HORVITZ (1977).


Mitotic Region
of the Gonad
Meiotic Region
of the Gonad
Anterior Arm
of the Gonad
Distal Tip Cell
Mature Oocyte
Vulva
Fertilized Egg

The male pronucleus first appears at the posterior end of the oocyte, and is accompanied by a centriole, which immediately replicates. Upon fertilization, the oocyte nucleus, positioned at the extreme anterior end of the oocyte, completes meiosis I and II, extruding two polar bodies in the process. At the same time, the egg shell forms, effectively isolating the fertilized egg from the environment.

Following completion of oocyte meiosis, the pronuclei migrate towards each other. The female pronucleus migrates faster and further, so that the meeting takes place nearer the posterior end of the oocyte. The pronuclei meet, rotate and undergo mitosis. The asymmetric position of the nuclei leads to asymmetric positioning of the first mitotic division. The precise positioning of the spindle has been shown to be important to the subsequent development of the embryo (ALBERTSON 1984; WHITE and STROME 1996).

The time span between resumption of meiosis and the onset of the first mitotic division is on the order of 20-30 minutes. As described, this occurs within the restricted confines of the egg cytoplasm in a transcriptionally inactive cell. Given that the meiotic and mitotic spindles are a combination of differences and similarities, some mechanism must exist to maintain the differences between these two spindle types.

A Group of Genes Required for Spindle Function in *C. elegans*

A group of maternally acting genes has been identified as playing a role in the proper function of the meiotic and mitotic spindles of oogenesis and early embryogenesis in *C. elegans* (MAINS *et al.* 1990a). This pathway is centered on the *mei-1* gene, which encodes an essential component of the oocyte meiotic spindle (CLANDININ and MAINS 1993; CLARK-MAGUIRE and MAINS 1994a,b).

In wild-type *C. elegans* development, MEI-1 protein is initially found throughout the oocyte meiotic spindle, though it becomes more concentrated towards the poles as the spindle matures. During telophase, MEI-1 is found co-localized with the chromatin. In the absence of *mei-1* activity, the meiotic spindle fails to form and microtubules remain as a diffuse cloud around the condensed chromatin.

Normally, no MEI-1 is observed during the mitotic divisions of embryogenesis that follow oocyte meiosis, however a dominant gain-of-function mutation in *mei-1* has been identified, *mei-1(ct46)*, that makes MEI-1 persist beyond meiosis. If MEI-1 is allowed to persist after the completion of meiosis, it is incorporated into the mitotic spindle, where it is concentrated at the centrosomal region (CLARK-MAGUIRE and MAINS 1994a).

Screens for suppressors of *mei-1(ct46)* identified the *mei-2* gene (MAINS *et al.* 1990a). On their own, loss-of-function (*lf*) mutations in the *mei-2* gene show a similar phenotype to *mei-1(lf)* (MAINS *et al.* 1990a). Genetic analysis of these genes suggests a model in which *mei-2* acts either to activate or localize MEI-1 to the meiotic spindle. In the absence of *mei-2* activity, the meiotic spindles are not formed, and *mei-2(lf)/+* reduces *mei-1* activity sufficiently to suppress *mei-1(gf)*. In *mei-2* homozygotes, it is not clear whether MEI-1 is actually absent, or simply unable to interact with the microtubules to form a spindle. In the absence of an organized spindle, free MEI-1 may be at such a low concentration as to be invisible by indirect immunofluorescent microscopy. The *mei-2* gene has been cloned and encodes a novel protein (M. A. SRAYKO, personal communication).

Another gene that shows genetic interaction with *mei-1* alleles is *zyg-9* (MAINS *et al.* 1990a). Loss-of-function *zyg-9* alleles are reported to affect both meiosis and mitosis (MATTHEWS *et al.* 1998). Meiotic spindles are disorganized, and mitotic spindles are short and fail to rotate correctly. Alleles of *zyg-9* show enhancement of mitotic defects of *mei-1(gf)* alleles (MAINS *et al.* 1990a). Cloning of the *zyg-9* gene revealed that it shows similarity to microtubule-associated proteins, including the SPB-associated Stu2p from *S. cerevisiae* (WANG and HUFFAKER 1997). Immunolocalization of ZYG-9 reveals that it is a component of both the

meiotic and mitotic spindles. During meiosis, ZYG-9 shows a distribution similar to that of tubulin, though more concentrated at the poles. Staining of the central region of the spindle decreases as meiosis progresses. In mitosis, ZYG-9 is localized to the spindle poles, with some faint staining of the central spindle region. The centrosomal localization of ZYG-9 during mitosis is consistent with the observed interaction between *mei-1(gf)* and *zyg-9*. As mitosis concludes, ZYG-9 staining disappears from the centrosomes. The level of cytoplasmic staining decreases during mitosis, suggesting that ZYG-9 may cycle between the cytoplasm and the centrosome. ZYG-9 is found to stain the centrosome of mitotic cells in the gonad, embryo and dividing spermatocytes (MATTHEWS *et al.* 1998).

The maternal-effect lethal 26 (*mel-26*) gene has been shown to interact genetically with all of these genes (MAINS *et al.* 1990a). A model has been presented in which *mel-26* acts as a post-meiotic negative regulator of *mei-1* (CLARK-MAGUIRE and MAINS 1994a). The canonical allele of *mel-26*, *ct61*, is a dominant temperature-sensitive (*ts*) allele that acts genetically as a dominant-negative, or antimorph. At the restrictive temperature, embryos produced by *mel-26(ct61)* mothers show several defects in the mitotic spindle. The spindle is mis-oriented, shifted to the posterior region of the embryo and is smaller than wild-type. A revertant of *mel-26(ct61)* to a recessive loss-of-function allele (*ct61sb4*) has been isolated, and shows the

same phenotype, supporting the hypothesis that *ct61* is a dominant-negative allele. Recently, an additional dominant *ts* allele of *mel-26* has been isolated (MITENKO *et al.* 1997). This new allele, *sb45*, is similar to *ct61*. It acts as a dominant-negative and has spindle defects essentially indistinguishable from the spindle defects shown by *ct61* and *ct61sb4* (DOW and MAINS 1998). Immunolocalization of MEI-1 in a *mel-26(ct61)* background reveals that, as in *mei-1(ct46)*, MEI-1 is able to persist after meiosis and is incorporated into the mitotic spindle (CLARK-MAGUIRE and MAINS 1994a). This finding implicates *mel-26* in ensuring that MEI-1 is prevented from interacting with the mitotic spindle.

The complex genetic interactions between *mei-1*, *mei-2* and *mel-26* can be distilled into a fairly simple model in which MEI-1 is an essential component in the meiotic spindle, but a poison with respect to the mitotic spindle. *mei-2* gene activity is required for MEI-1 to fulfill its role in the meiotic spindle, thus *mei-2* alleles show defects in meiosis. On the other hand, *mel-26* activity is required to "inactivate" MEI-1 following meiosis. If *mel-26* is inactivated, or MEI-1 is made refractile to inactivation [*i.e.*, *mei-1(ct46)*], MEI-1 is localized to the mitotic spindle, where it disrupts the normal function of the mitotic spindle.

The mitotic defects of *mel-26* embryos can be suppressed by lowering the level of MEI-1 in the embryo, which explains the suppression of

mel-26(ct61) by *mei-1(dn)/+* and *mei-2(lf)/+* (MAINS *et al.* 1990a), both of which lead to a reduction in the activity of *mei-1*. On the other hand, *mei-1(gf)* is already ectopically persistent, so mutation of *mel-26* exacerbates the situation. The finding that *mei-1(null)* is epistatic to *mel-26(ct61)* provided confirmation of the model; in the absence of *mei-1*, *mel-26* is not needed (CLARK-MAGUIRE and MAINS 1994a).

How could *mel-26* fulfill a role as post-meiotic negative regulator of *mei-1*? Several models can be imagined; perhaps *mel-26* encodes a MEI-1-specific protease, or perhaps it complexes with MEI-1 following meiosis. Possibly MEL-26 is a component of the mitotic spindle, and when incorporated into the mitotic spindle prevents MEI-1 from interacting with the spindle. The *mei-1* transcript remains for some time following meiosis, so a role as a translational repressor could be argued. This project begins to address this issue.

MEI-1 is Similar to a Component of the Microtubule Severing Complex Katanin

Cloning of the *mei-1* gene revealed that it shows homology to members of the AAA family (ATPases Associated with different cellular Activities) (CLARK-MAGUIRE and MAINS 1994b). The AAA motif is a 230 to 250 amino acid sequence including the Walker signature sequence of

P-loop ATPases (PATEL and LATTERICH 1998). AAA family members have been identified in Archae, bacteria and eukaryotes, where they have been shown to play roles in a wide array of cellular functions. There have been suggestions over the years that the AAA motif plays some role in protein-protein interaction. In many AAA family members, the AAA-homology region extends throughout the C-terminal region of the protein. The N-terminal portion of the protein may define the cellular role of the protein; AAA family members who share a similar function show homology in the N-terminal region, while little N-terminal conservation is observed between members with no similarity of function.

One of the subunits of the microtubule severing complex katanin (MCNALLY *et al.* 1996; MCNALLY and VALE 1993) has recently been shown to be closely related to MEI-1 within the AAA domain (HARTMAN *et al.* 1998). Katanin is a heterodimeric ATP-dependent microtubule severing protein that was first identified in sea urchin eggs (VALE 1991; MCNALLY *et al.* 1996). It has been immunologically localized to the mitotic centrosomes, where it is thought to play a role in the disassembly of microtubules near their centrosomal attachment points. The p60 subunit, which contains the AAA motif, displays ATP-dependent microtubule severing activity (HARTMAN *et al.* 1998). In the presence of the p80 subunit, this activity is increased a modest 2-fold. The primary role of the

p80 subunit appears to be the localization of katanin to the centrosome, which is consistent with the presence of several WD40 repeats in the p80 sequence. WD40 domains have been implicated in mediating protein-protein interaction (KOMACHI *et al.* 1994).

Although the p60 subunit of katanin shows high sequence similarity to MEI-1 within the AAA domain, and both proteins have the potential of being localized to the centrosomes, it is unlikely that p60 and MEI-1 are homologs. If the N-terminal region of AAA family members is important in defining the cellular role of the protein, the lack of significant similarity between MEI-1 and katanin in the N-terminal region likely indicates that they do not share a function. In addition, baculovirus-expressed MEI-1 has no measurable microtubule-severing activity in an assay that was used to demonstrate severing by similarly expressed sea urchin katanin (J. J. Hartman, UCSF, personal communication).

Caenorhabditis elegans as a Model Organism to Study Spindle Function

The free-living nematode *C. elegans* provides an excellent model organism for studies of this type. *C. elegans* genetics have been well established, and coupled with the complete physical map and the nearly complete genome sequencing project, the steps from gene identification to

characterization are becoming ever easier. Following identification and genetic mapping of a gene affecting a developmental activity of interest, the correlation between the genetic and physical maps of *C. elegans* can be used to identify potential DNA sequences encoding the gene. The physical map of *C. elegans* is an ordered set of mostly cosmid clones (COULSON *et al.*, 1995). Extensive links have been made between the genetic and physical maps, either through genetic loci or methods such as RFLP or duplication endpoint mapping. Once a region of the physical map has been identified, one can first examine predicted genes in the sequence data for likely prospects. At the same time, cosmids containing the region(s) of interest can be obtained for further analysis. Techniques such as transformation rescue (MELLO *et al.* 1991), or RNA interference (GUO and KEMPHUES 1995; FIRE *et al.* 1998) can be used to identify the relevant coding sequence.

The nematode is transparent in all stages of its life cycle (SULSTON and HORVITZ 1977; SULSTON *et al.* 1983), making microscopic analysis of development relatively straightforward. This is particularly of concern for studies of the spindle; observation of meiotic and early mitotic spindles in many model organisms has proven somewhat difficult. This has undoubtedly played a role in the low abundance of studies relating to oocyte meiosis. Direct observation of living oocytes is very straightforward in *C. elegans*.

The rotations and migrations of the early spindles have been well characterized and are essentially invariant in the wild-type embryo. Mutations disrupting the stereotypical rotations are easily recognized.

In spite of the accumulating body of evidence describing the differences between meiotic and mitotic spindles, very little is known about the molecular basis of these differences. Many spindle components have been described, though the meiotic role has often not been addressed. In contrast, previous work has defined an oocyte meiosis-specific spindle component, MEI-1 (CLARK-MAGUIRE and MAINS 1994a,b). This report extends that work, describing the characterization of the gene *mel-26*, which is required to prevent interference of MEI-1 with the mitotic apparatus of the developing embryo. The *mel-26* gene has been cloned and has been shown to encode a novel component of the mitotic centrosome. Models are presented to suggest mechanisms by which MEL-26 may prevent the interaction of MEI-1 with the mitotic spindle.

MATERIALS and METHODS

Molecular Biology Methods

Unless otherwise specified, molecular biology methods such as cloning, library screening, etc. were performed using standard methods and recipes (SAMBROOK *et al.* 1989; AUSUBEL *et al.* 1991). Kits were used as suggested by the manufacturer. Unless otherwise noted, the cloning vector used was pBluescript SK^+ and the bacterial host strain was XL-1 Blue (Stratagene, La Jolla, USA). All enzymes were obtained from Boehringer Mannheim (Laval, Canada), unless otherwise indicated.

Nematode Strains and Culture Conditions

Nematode strains were maintained under standard conditions as described by BRENNER (1974) and brood analysis was conducted as described by MAINS *et al.* (1990b). Nomenclature follows that of HORVITZ *et al.* (1979). The following genes and alleles were used:

Linkage Group I: *mei-2(sb31)*, *unc-13(e1091)*, *mei-1(ct46)*, *daf-8(e1393)*, *unc-29(e1072)*, *mel-26(ct61, ct61sb4, sb45)*, *lin-11(e566)*

Linkage Group III: *glp-1(q231)*

Linkage Group IV: *fem-1(hc17)*, *fem-3(q20)*

Transformation Rescue

Plasmid DNA for injection was prepared from bacterial culture using the Wizard Miniprep kit (Promega, Madison, USA), and was eluted in dH_2O. As a transformation marker, the plasmid pRF4 containing a dominant mutant allele (*su1006*) of the *rol-6* gene was co-injected (MELLO *et al.* 1991). Transformation with pRF4 causes the worms to exhibit an easily scorable rolling (Rol) phenotype. The injection mix, containing 10 ng/μL of marker plasmid and 10-100 ng/μL of test plasmid, was prepared in dH_2O and centrifuged for 10-15 minutes immediately prior to injection.

Injections were performed as described by MELLO *et al.* (1991). The strain of worms used for injection, HR261, is heterozygous for the loss-of-function allele *mel-26(ct61sb4)*, linked to *daf-8* to mark *mel-26(ct61sb4)* in *cis* and balanced by a chromosome marked with the flanking *unc-13* and *lin-11* (DOW and MAINS 1998). This *mel-26(ct61sb4) daf-8/unc-13 lin-11* strain provides a healthy host, with essentially wild-type levels of hatching, making it quite suitable for injections. The flanking markers provide indications of undesirable cross-over events. The *daf-8* mutation is a temperature-sensitive mutation that forces entry into the alternate dauer larval stage at the restrictive temperature. Injected worms were placed at 15°C to lay eggs and were transferred to fresh plates daily. F_1 rollers were

picked to fresh plates (~5/plate) and allowed to lay eggs. Heritably transformed F_2 rollers were picked to individual plates. These worms were allowed to lay eggs, which were then upshifted to 25°C to identify the *daf-8 mel-26* individuals. Daf worms were transferred to individual plates, then allowed to emerge from dauer at 15°C to initiate a line for testing.

Rol and non-Rol progeny from each line of worms were tested for the ability of the injected DNA to rescue the *mel-26* lethality. Fourth larval stage (L4) worms were picked to individual plates at 25°C and allowed to lay eggs. Worms were transferred to new plates daily, and embryos were counted. After 1-2 days, plates were re-examined for hatched worms and percent hatching was determined. The persistence of a low level of hatching among the non-Rol worms was used to ensure that a rare double cross-over had not occurred prior to formation of the homozygous line.

Once a clone had been identified as containing DNA sufficient to rescue *mel-26(ct61sb4)*, the DNA was cut with various restriction enzymes and the digested DNA was assayed for its ability to rescue (KIM and HORVITZ 1990). This process continued until the "smallest rescuing fragment" was identified. This fragment was then subcloned and re-tested for rescue.

Library Screening

The smallest rescuing fragment was ^{32}P-labeled and used as the probe for cDNA library screening. Initially, a widely used mixed stage library in λZAP was screened (BARSTEAD and WATERSTON 1989). After 130 000 plaques were screened without success, it was concluded that the level of embryonic messages in the library was likely too low. An embryonic library, constructed from embryos containing fewer than 30 cells (SCHAUER and WOOD 1990) was screened with the same probe, and from 370 000 plaques, 3 clones were isolated. These clones were excised from the λgt11 host and subcloned into pBluescript.

Sequencing

Nested deletions of the longest cDNA clone were generated using the Erase-a-Base (Promega, Madison, USA) protocol. The resulting clones were sequenced using the ABI Prism fluorescent cycle sequencing kit. Both strands were sequenced, using the 24 nucleotide T3 and T7 primers. Individual sequences were re-assembled into a single contiguous sequence using the Genetics Computer Group (GCG, Madison, USA) suite of programs (DEVEREUX *et al.* 1984).

The rescuing genomic fragment was subcloned into three smaller fragments and deletions were generated for each fragment. The sequences of these clones were obtained as with the cDNA.

Primers were synthesized to allow the PCR amplification of the *mel-26* region from mutant worms, and additional primers were obtained to use in PCR sequencing of the amplified DNA. The positions of the 5' nucleotide [relative to the completed *mel-26* sequence (Genbank Accession #U67737)], length and direction of the primers used for amplification or sequencing are as follows: primer-17 (419, 18, forward), primer-18 (739, 18, forward), primer-19 (1060, 18, forward), primer-14 (1402, 20, forward), primer-7 (1479, 20, forward), primer-9 (1818, 21, forward), primer-8 (2019, 20, reverse), primer-22 (2108, 23, forward), primer-23 (2908, 19, reverse), primer-13 (3243, 18, reverse), primer-24 (3453, 18, forward), primer-2 (3577, 20, reverse), primer-3 (3866, 20, forward), primer-25 (4028, 20, reverse), primer-16 (4248, 19, forward), primer-12 (4318, 18, reverse), primer-15 (4846, 18, reverse).

Mutant worm DNA was obtained by placing 5-6 homozygous mutants in a PCR tube with 2.5 μL of worm lysis buffer (50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris-HCL (pH 8.3), 0.45% Tween-20, 0.45% NP-40, 0.01% gelatin, 60 μg/μL Proteinase K) (WILLIAMS *et al.* 1992). Following a 60 minute

incubation at 60°C, the temperature was increased to 95°C for 15 minutes to inactivate the Proteinase K. The entire 2.5 μL was used, without further treatment, as the template in a PCR reaction.

PCR was carried out in a total volume of 25 μL, containing the 2.5 μL of template, 25 pmol of each primer and 1U of Taq Polymerase (Boehringer Mannheim, Laval, Canada) in PCR buffer. The PCR parameters consisted of an initial 5 minute incubation at 95°C, 35 cycles of 94°C for 40 seconds, 55°C for 40 seconds and 72°C for 4 minutes, followed by a final 4 minute incubation at 72°C.

The entire PCR sample was loaded on a 1% agarose gel and the product was purified using the QiaQuick purification kit (Qiagen, Chatsworth, USA). Approximately 100 ng of the gel purified PCR product was PCR sequenced from internal primers using the ABI PRISM (Perkin Elmer, Foster City, USA) dye terminator sequencing protocol. Mutations were confirmed by sequencing an independent PCR product.

Database Comparisons

Computer manipulation and analysis were performed using the GCG suite of programs. Database searches were conducted against the NCBI database using the BLAST protocol (ALTSCHUL *et al.* 1990). Sequence

comparisons were performed using the computer program PILEUP (GCG, Madison, USA). For all programs, the default parameters were employed.

RT-PCR

Poly-A$^+$ RNA was isolated directly from mixed stage N2 worms using the Micro FastTrack 2.0 kit (Invitrogen, San Diego, CA). This RNA was non-specifically reverse transcribed using a poly-dT primer with an *Eco*RI adapter sequence (dGTC GCA GAA TTC GTC GAC $(TTT)_6$) and Superscript II reverse transcriptase (GibcoBRL, Gaithersburg, USA). The product cDNA was treated with RNase H to remove the complementary mRNA.

To test whether the *mel-26* transcript was *trans*-spliced to SL1 (ZORIO *et al.* 1994), PCR amplification of the cDNA pool was carried out using a primer complementary to SL1 and a gene-specific promoter (primer-8), using conditions suggested by the Superscript II protocol. The product of a second round of PCR using 10% of the first round product was gel purified, cloned and sequenced.

RT-PCR experiments with internal primers were conducted to address the possibility of alternative products. PCR reactions were carried out as above, except that both primers were gene-specific. The product of the second round of PCR was gel purified and sequenced as described previously.

Northern Blotting

Large scale preparations of gravid worms were isolated as previously described (CLARK-MAGUIRE and MAINS 1994b). Poly-A$^+$ RNA was purified either by isolating total RNA using the method of MEYER and CASSON (1986), then enriching for Poly-A$^+$ using the MicroFast Track Kit or by performing FastTrack purifications directly.

The RNA was separated on denaturing gels and blotted to Nylon membrane (Hybond N$^+$, Amersham, Arlington Heights, USA) according to the manufacturer's recommendations. For use as a probe, the entire *mel-26* cDNA was gel purified (QiaQuick, Qiagen, Chatsworth, USA) from its vector and $\alpha[^{32}P]$dCTP labeled using the Prime-It II (Stratagene, La Jolla, USA) random-priming labeling kit. Hybridization and washes were performed using standard stringent conditions.

Southern Blotting

Genomic DNA was isolated from large-scale preparations of gravid worms using previously described techniques (SULSTON and HODGKIN, 1988). The DNA was cut with various restriction enzymes and separated on 0.8% agarose. The DNA was blotted to nylon membrane (Hybond N$^+$, Amersham, Arlington Heights, USA) according to the manufacturer's methods. The genomic region between the *Cla*I and *Pst*I sites of the

smallest rescuing fragment was gel purified (QiaQuick) and labelled with ^{32}P (Prime-It II, Stratagene, La Jolla, USA). The blot was hybridized and washed using standard high stringency conditions.

Expression and Purification of Recombinant MEL-26

The 382 nucleotide region between the *Eco*RI and *Eco*RV sites of the *mel-26* cDNA was cloned into the pGEX-3x vector (Pharmacia, Uppsala, Sweden) generating a protein containing glutathione-S-transferase (GST) fused to the N-terminal 125 residues of MEL-26 (GST-MEL400).

In expression experiments, a 500 mL culture, in Luria broth, was started from an overnight culture and grown for 3-4 hours to an OD_{600} of ~0.3. Expression of the fusion protein was induced by addition of IPTG to 1 mM final concentration. After allowing 3-4 hours for expression, cells were harvested, extracted with acetone and suspended in 2x loading dye.

The protein sample was boiled for 10 minutes and centrifuged to remove insoluble material. The soluble fraction was loaded on a preparative SDS-PAGE gel with a 12% acrylamide resolving gel. The gel was briefly stained in Coomassie R-250. The prominent GST-MEL400 band was excised, and the gel slice was washed extensively with dH_2O.

To isolate the protein, the gel was minced and extracted several times with elution buffer (200 mM Tris-Cl (pH 7.6), 1% SDS, 100 mM DTT). The

eluates were pooled and the protein was acetone precipitated. The protein was resuspended in phosphate-buffered saline (PBS) (AUSUBEL *et al.* 1991), and the concentration and yield were determined by relative band intensities when a fraction was run on an SDS-PAGE gel next to a known concentration of bovine serum albumin.

Antibody Generation

Antisera reactive to the fusion protein were generated by the Hybridoma Facility (University of Calgary, Faculty of Medicine). Rabbit antisera were raised by intramuscular injection of 50 μg of recombinant protein in Freund's Complete adjuvant, followed by four injections of 50 μg of recombinant protein in Freund's Incomplete adjuvant at 3 week intervals. Rat antisera were raised by intraperitoneal injection of 20 μg in Freund's Complete Adjuvant, also followed by 4 injections (20 μg in Freund's Incomplete Adjuvant) at 3 week intervals. The resulting serum was prepared using standard procedures (HARLOW and LANE 1988).

Western Blotting

Large preparations of various *mel-26* strains and wild-type life stages were prepared as previously described (CLARK-MAGUIRE and MAINS 1994b). Frozen worms were ground to a fine powder in liquid nitrogen. The

resulting powder was mixed with 2x loading dye, boiled and centrifuged to remove insoluble matter.

The worm lysates were separated on SDS-PAGE gels, and the gel was Coomassie stained to judge relative loading. Based on the relative total protein levels, a gel was run and blotted to PVDF membrane. The membrane was blocked overnight at 4°C in 0.5% Tween-20, 2% skim milk, 2% BSA, 3% gelatin in PBS. Following a ~15 minute wash in 0.5% Tween-20 in PBS, the membrane was incubated with the 1° antibody, diluted 1 : 5 000 in PBS, for 60 minutes at room temperature. The membrane was washed three times with PBS+Tween and then incubated in 2° antibody, diluted 1 : 10 000 in PBS. The goat anti-rabbit secondary antibody was conjugated to horseradish peroxidase, for detection using the Enhanced Chemiluminescence (ECL) kit (Amersham, Arlington Heights, USA). Following a 60 minute incubation with the 2° antibody, the membrane was washed three times with PBS-Tween, then incubated with the detection reagents as directed by the manufacturer.

Nomarski Microscopy of *C. elegans* embryos

To observe early cell divisions and spindle orientations, embryos were cut from gravid hermaphrodites and mounted on agarose pads for microscopy (SULSTON *et al.* 1983). Microscopy was performed using a Zeiss

Axioplan microscope equipped with Nomarski optics. Embryos were viewed at a total magnification of 630x and flash photographed using Kodak TechPan film developed at 100 ASA.

Indirect Immunofluorescence Microscopy

Embryos were fixed for antibody staining using a protocol (CLARK-MAGUIRE and MAINS 1994a) based on the methods of ALBERTSON (1984) and KEMPHUES *et al.* (1986). Gravid worms were placed in a 5 μL spot of ddH_2O on polylysine-coated slides. A quartered coverslip was placed on the worms and gentle pressure was applied to force the worms to expel their embryos. The slide was immediately placed on dry ice and left for ~10 minutes. The coverslip was popped off and worms were methanol/acetone fixed by immersion in cold methanol for 15 minutes, then room temperature acetone for 2 minutes. The embryos were then re-hydrated by 2 minute incubations in 95%, 70%, 50% and 30% ethanol solutions, followed by a final soak in PBS.

Prior to staining, the embryos were blocked for 45 minutes at room temperature (25% normal donkey serum, 0.5% Tween-20 in PBS). The embryos were then incubated in 30-40 μL of primary antibody (diluted 1/50-1/200 in 0.5% Tween-20, 5% normal donkey serum in PBS) for 60 minutes at 37°C. A monoclonal antibody (4A-1) to *Drosophila* α-tubulin

(PIPERNO and FULLER 1985) was used, at a dilution of 1/50, to co-stain embryos for tubulin localization. Primary antibodies were removed by three 10 minute washes in PBS. Various 2° antibodies (Jackson Immunoresearch, West Grove, USA), including cy2-conjugated donkey anti-mouse IgG, FITC-conjugated donkey anti-mouse IgG, rhodamine-conjugated goat anti-mouse IgG, cy3-conjugated donkey anti-rabbit IgG and FITC-conjugated goat anti-rabbit IgG, were used as appropriate. Secondary antibodies were typically used at dilutions of 1/200 - 1/500. Following a 60 minute incubation at 37°C, the samples were washed 2x in PBS, chromatin was stained with 1 μg/mL 4', 6-diamidine-2'-phenylindole (DAPI) in PBS for 2 minutes, and then the slide was washed in PBS. Samples were mounted in 50% glycerol and viewed immediately.

Samples were viewed at 630x magnification on a Zeiss Axioplan microscope. Photographs were taken with Kodak TechPan film exposed at 400 ASA and developed at 100 ASA. Film negatives were scanned using a SprintScan 35^{+} slide scanner (Polaroid, Cambridge, MA, USA). The scanned images were cropped and contrast balanced using Photoshop (Adobe Systems, Mountain View, USA).

RNA Interference

RNA was *in vitro* transcribed from a linearized full-length cDNA clone of *mel-26* in pBluescript, using T3 and T7 polymerases (Boehringer-Mannheim) and standard reaction conditions. The template was removed by addition of RNase-free DNaseI (Boehringer-Mannheim). The RNA was phenol/chloroform extracted, ethanol precipitated, and resuspended in TE (pH 8). Injection solution was prepared by mixing the T3 and T7 products (FIRE *et al.* 1998) at a final RNA concentration of 100 ng/μL in RNase-free ddH_2O.

The RNA was injected into N2 worms by P.E. MAINS, using standard injection techniques. Following injection, worms were allowed to clear unaffected embryos for several hours and then transferred to individual plates to lay eggs to test for hatching.

Some embryos were examined using Nomarski optics as described previously, while others were fixed and stained for MEL-26 and tubulin as described previously.

Genetic Screen for Suppressors of *mel-26(ct61)*

To revert the dominant *ct61* mutation to a recessive loss-of-function (*lf*) allele, *unc-29 mel-26(ct61)/hT2[bli4(e937) let(h661)] I; +/hT2 III* hermaphrodites were mutagenized with ethylmethane-sulfonate (EMS)

under standard conditions (BRENNER 1974) and plated (2 worms/plate) at the permissive temperature of 15°. *hT2* is a reciprocal translocation that suppresses recombination in the desired region (EDGLEY *et al.*, 1995). The *hT2* chromosome bears a recessive larval lethal mutation [*let(h661)*], so homozygous *hT2* embryos arrest as early larvae. Plates with F_1 progeny (~200/plate) were upshifted, prior to the worms reaching adulthood, to the restrictive temperature of 25° and screened for those showing high numbers of F_2 progeny. These plates should represent suppression of the dominant *ct61* allele, either by intragenic or extragenic events.

RESULTS

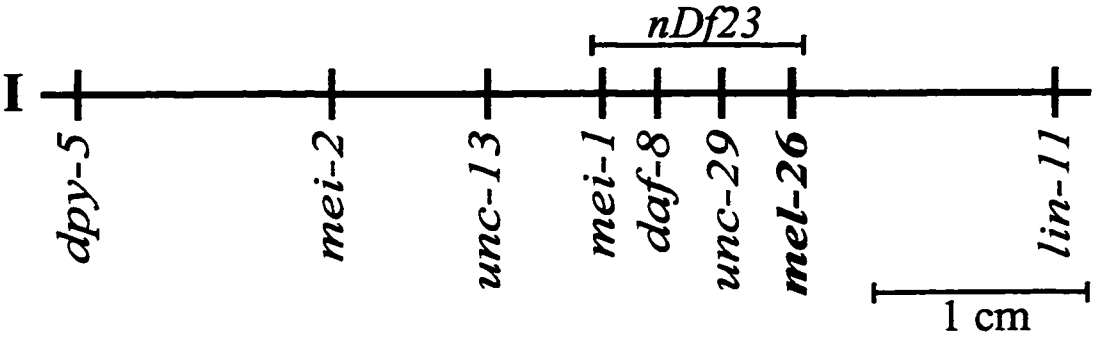
Cloning of *mel-26*

Previous experiments had mapped *mel-26(ct61)* to the interval between *unc-29* and *lin-11* (Figure 4a) (MAINS *et al.* 1990b). Based on the correlation between the genetic and physical maps in this area (Figure 4b), the cosmids D1004, W06D4, W04H6, ZK858, F25H5 and C08H1 were selected for analysis. From these, transformation rescue of the loss-of-function (*lf*) allele *mel-26(ct61sb4)* was achieved only with cosmid ZK858. In comparison to *mel-26(ct61sb4)*, which gave ~0.01% hatching at 25°C, *mel-26(ct61sb4)* transformed with ZK858 showed hatching rates ranging from 2-8% at 25°C. Likewise, injection of the 17.2 kb *Sal*I fragment, the 13.1 kb *Xho*I fragment, the 9.7 kb *Bgl*II fragment and a plasmid containing the 7.6 kb *Xho*I/*Bgl*II fragment all showed hatching levels above background. Two smaller fragments were not able to rescue the mutation (Figure 4c).

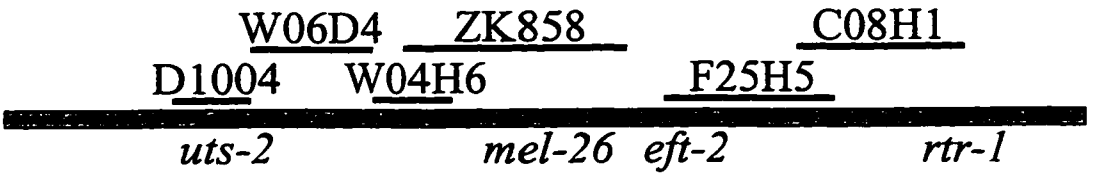
Using the 7.6 kb *Xho*I/*Bgl*II fragment as a probe, three cDNA clones were isolated from the early embryonic cDNA library of SCHAUER and WOOD (1990). Restriction mapping and hybridization experiments indicated that the clones were overlapping. The longest clone (1700 bp) was sequenced in its entirety. Approximately 5 kb of genomic sequence, including the region encoding the transcript represented by the cDNA

FIGURE 4. **Mapping and cloning of *mel-26*.** (A) The *mel-26* region of chromosome I. Genes referred to in the text are shown. The extent of the deficiency *nDf23* is indicated above the line. (B) The physical map in the *mel-26* region, located in the interval between the cloned genes *unc-29* and *lin-11*. Black lines indicate the relative positions of the cosmids injected in this study. Genes localized to the physical map in the region are shown under the thick grey line. (C) Subclones of cosmid ZK858 used to determine the location of the *mel-26* coding region. Each subclone was tested for its ability to rescue the maternal-effect lethality of *mel-26(ct61sb4)* as indicated by the "+" and "-" to the right of each.

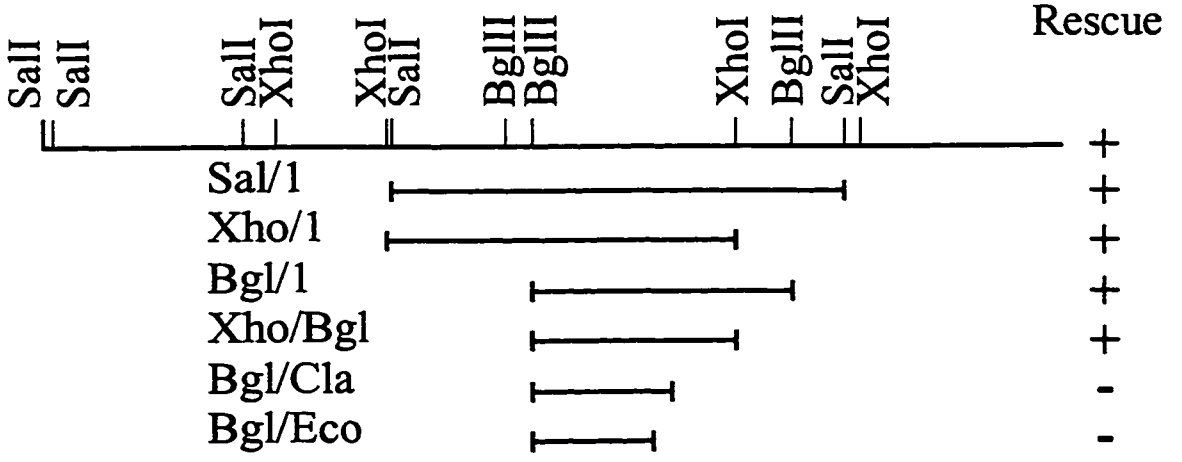
(A)


nDf23
I
dpy-5
mei-2
unc-13
mei-1
daf-8
unc-29
mel-26
lin-11
1 cm

(B)


W06D4
ZK858
C08H1
D1004
W04H6
F25H5
uts-2
mel-26
eft-2
rtr-1

(C)


SalI
SalI
SalI
XhoI
XhoI
SalI
BglII
BglII
XhoI
BglII
SalI
XhoI
Rescue
Sal/1
Xho/1
Bgl/1
Xho/Bgl
Bgl/Cla
Bgl/Eco
+
+
+
+
+
-
-

clone, was also sequenced (Figure 5). Cosmid ZK858 has since been sequenced in its entirety by the *C. elegans* Genome Sequencing Project and deposited in GenBank (Accession #Z79759). The region around ORF ZK858.4 is identical to the sequence of *mel-26*, as represented in Figure 5.

Sequence Analysis

Comparison of the cDNA and genomic sequences revealed that the transcript is derived from 6 exons spread over approximately 3.2 kb (Figure 6a). As is typical for *C. elegans*, the gene is compact; two introns are less than 50 bp and the longest is less than 1 kb. The splice donor and acceptor sequences generally match the *C. elegans* consensus [GTRART and TTTCAG respectively (KRAUSE, 1995)] (Figure 6b). Computer predictions of the splicing of ZK858.4, carried out by the Genome Sequencing Consortium using the program Genefinder do not completely match the observed splice pattern of *mel-26*. The Genefinder program, presumably biased towards larger exons, predicts earlier splice acceptor sites in the fourth and fifth introns. RT-PCR experiments, and sequencing of *mel-26* cDNAs (data not shown) suggest that these predicted splice acceptor sequences are not used.

Sequence database comparisons revealed that the *C. elegans* EST cm01e12 (WATERSTON *et al.* 1992) represents the *mel-26* gene. The first 10

FIGURE 5. **Sequence of *mel-26*.** The genomic sequence surrounding the *mel-26* gene is shown in plain text. Sequence corresponding to the cDNA is indicated by capital letters, while the inferred protein sequence is indicated in bold text by the single letter code positioned below the first position of each codon. The codons altered in the *sb45* (the first grey box) and *sb4* (the second grey box) alleles are indicated. The putative polyadenylation signal is underlined. This sequence (Genbank Accession #U67737) is identical to the region surrounding the ORF ZK858.4 in Genbank Accession #Z79759, which represents sequence data obtained by the *C. elegans* Genome Sequencing Project.

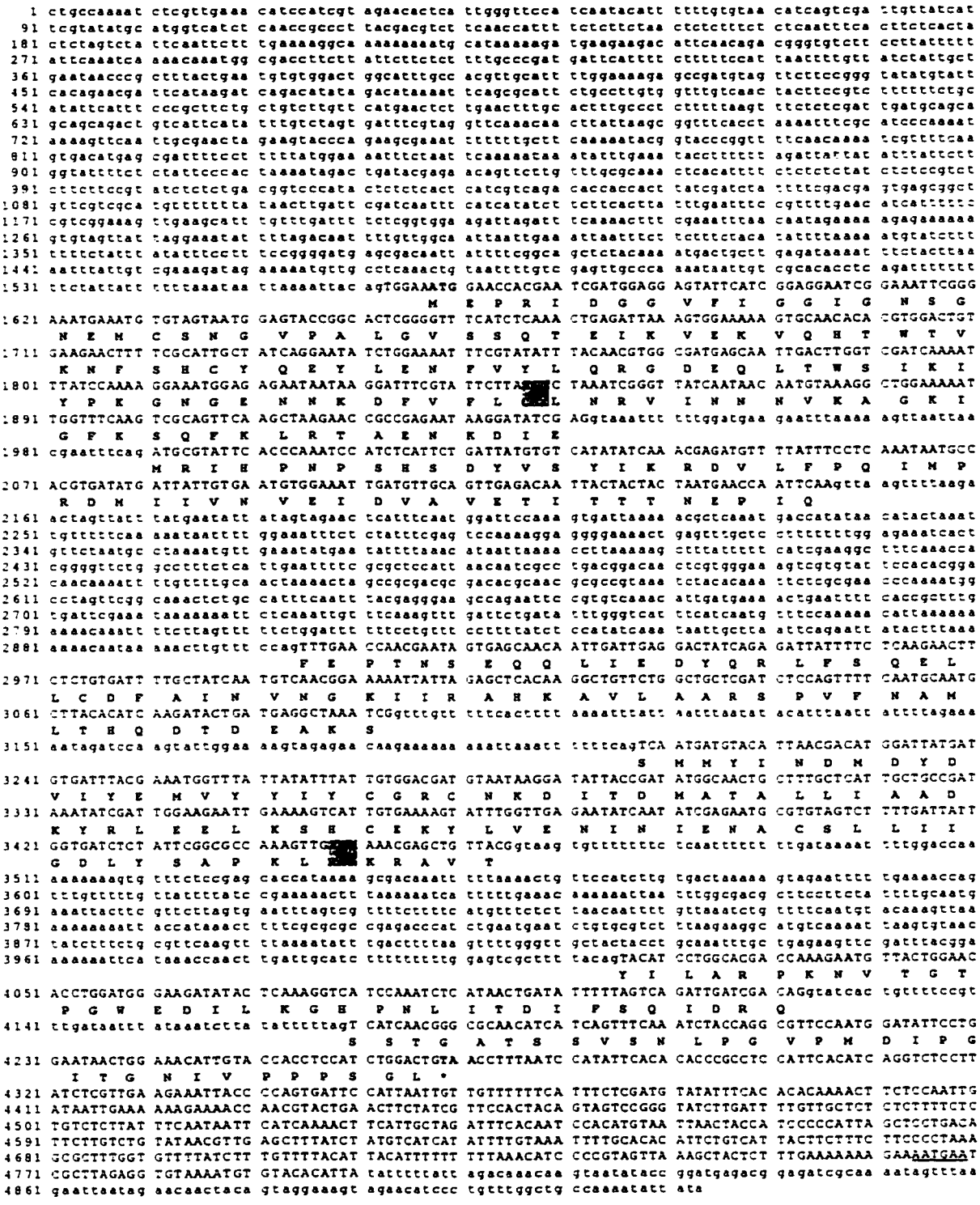
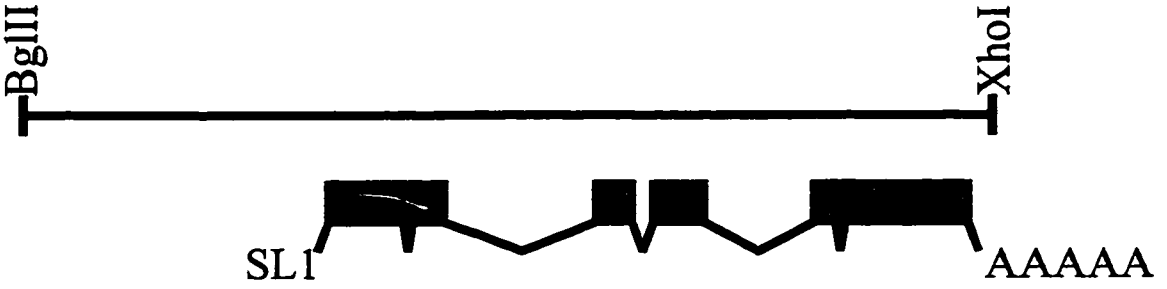


FIGURE 6. **Molecular characterization of *mel-26*.** (A) The intron/exon structure of *mel-26* is shown below the line representing the rescuing fragment, and indicates the position of the gene within the fragment. (B) The consensus splice donors and acceptors of *C. elegans* are shown in comparison to those found within *mel-26*. A consensus for the *mel-26* gene is also shown for comparison. (C) The 5' end of the *mel-26* cDNA #1 is compared with the EST cm01e12 and the sequences of the *trans*-splice leader sequences SL1 and SL2. The underlined sequence represents the *Eco*RI site of the adapter used in the cDNA library. The match between cm01e12 and SL1 suggests that *mel-26* can be *trans*-spliced to SL1.

(A)



(B)

C. elegans consensus	**NAG**	**GTRART**	**A/TTTTCAG RNN**
			ATTaCAG tGG
	GAG	GTAAAT	ATTTCAG ATG
	CAa	GTAAGT	TTTcCAG tTT
	TcG	GTttGT	TTTTCAG tCA
	AcG	GTAAGT	TTTaCAG tAC
	CAG	GTAtca	TTTTtAG tCA
mel-26 consensus	**NAG**	**GTAAGT**	**TTTTCAG TYR**

(C)

```
SL2       GGTTTTAACCCAGTTACTCAAG
SL1       GGTTTAATTACCCAAGTTTGAG
                     ||||||||||
cm01e12              CAAGTTTGAGTGGAAATGG
                              ||||||||||
cDNA 1                 GGAATTCCGTGGAAATGG
```

nucleotides of cm01e12 (CAAGTTTCAG) match the 3' end of the *trans*-splice leader SL1 (GGTTTAATTACCCAAGTTTCAG) (Figure 6c), which is spliced onto the 5' end of many *C. elegans* mRNAs (ZORIO *et al.* 1994). None of the cDNAs isolated in the library screen contained this sequence. To confirm that *mel-26* was *trans*-spliced, RT-PCR was conducted using a primer pair consisting of one primer that was specific for SL1 and another specific for a region in the second exon of *mel-26* (primer-8; see Materials and Methods). A PCR product of appropriate size was obtained. This DNA fragment was cloned, sequenced, and shown to represent the product of *trans*-splicing SL1 to the 5' end of the *mel-26* sequence (data not shown).

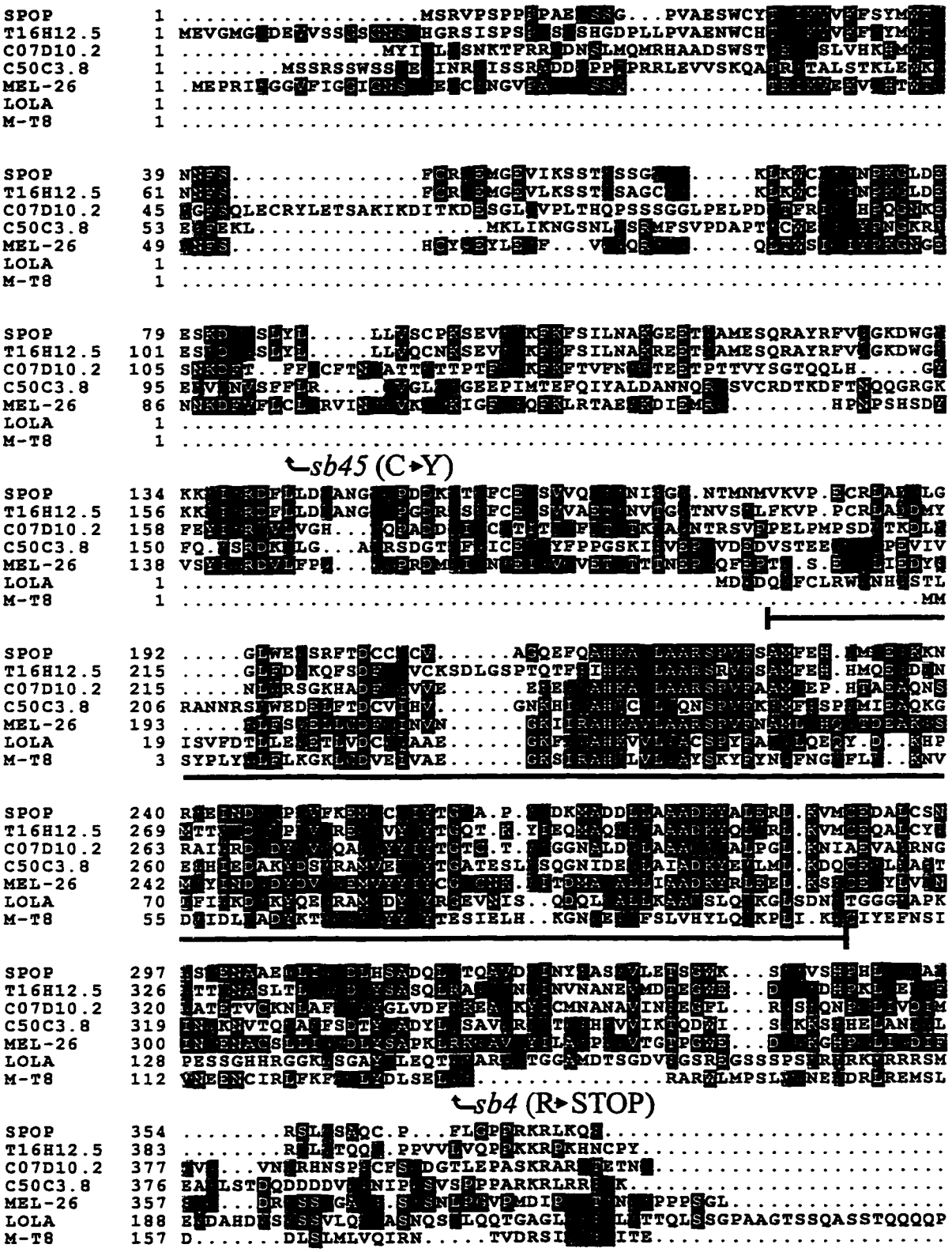
A poly-A^+ addition sequence was not identified in the longest cDNA, so the 3' ends of the remaining cDNA clones were sequenced to determine the point of polyadenylation. The apparent polyadenylation site in the two clones is different, occurring at positions 4772 and 4800 in the genomic sequence (Figure 5). The AAUGAA sequence starting at position 4764 is a likely candidate for the polyadenylation signal; the *C. elegans* consensus is AAUAAA, with 13% having a G at the fourth position (KRAUSE, 1995).

In silico translation of the *mel-26* cDNA suggests that the product, MEL-26, would be 395 amino acids in length. Database searches using the BLAST set of programs (ALTSCHUL *et al.* 1990) revealed that MEL-26 shows full-length similarity to three predicted *C. elegans* open reading frames

(ORFs) and SPOP (speckle-type **POZ** protein) from human (Figure 7) (NAGAI *et al.* 1997). The *C. elegans* sequences C50C3.8 (*III*), T16H12.5 (*III*) and C07D10.2 (*II*) are products of the *C. elegans* Genome Sequencing Project and have not been characterized. Pairwise comparisons between these proteins reveal that they show 25-33% identity (39-47% similarity) to MEL-26 over their entire length. While the sequence showing the highest similarity to MEL-26 is SPOP (33% identical), it is likely that SPOP is actually the human homolog of T16H12.5, as the two proteins share 62.7% identity overall (NAGAI *et al.* 1997).

MEL-26, the *C. elegans* ORFs and SPOP all contain a region of similarity to a previously identified motif known as either the ***bric à brac***, ***tramtrack*** and ***Broad-Complex*** (BTB) motif (ZOLLMAN *et al.* 1994) or the **p**oxvirus and **z**inc finger (POZ) motif (BARDWELL and TREISMAN 1994). The BTB/POZ domain is a ~115 amino acid region predicted to be alpha helical in nature (ALBAGLI *et al.* 1995). Within the BTB/POZ domain of MEL-26, proteins showing high similarity to MEL-26 include *longitudinals lacking* (Lola) from *Drosophila* (GINIGER *et al.* 1994) and the product of the T8 gene of Myxoma virus (MT-8) (UPTON *et al.* 1990). These proteins show 33-34% identity (42-47% similarity) to MEL-26 within the BTB region, but share little or no similarity outside of it. Database searches using MEL-26

FIGURE 7. **Alignment and comparison of the inferred protein sequence of MEL-26 and related proteins.** Alignment of the sequences was performed using the Genetics Computer Group (Madison, USA) PILEUP program; the freeware program BOXSHADE was used to illustrate the sequence similarity. Black and grey shading indicate regions of identity and similarity, respectively, to the MEL-26 sequence. The BTB region is underlined. The positions, and codon changes, of *sb45* and *sb4* are indicated.


sb45 (C→Y)
sb4 (R→STOP)

sequences outside of the BTB domain identified only SPOP and the *C. elegans* ORFs.

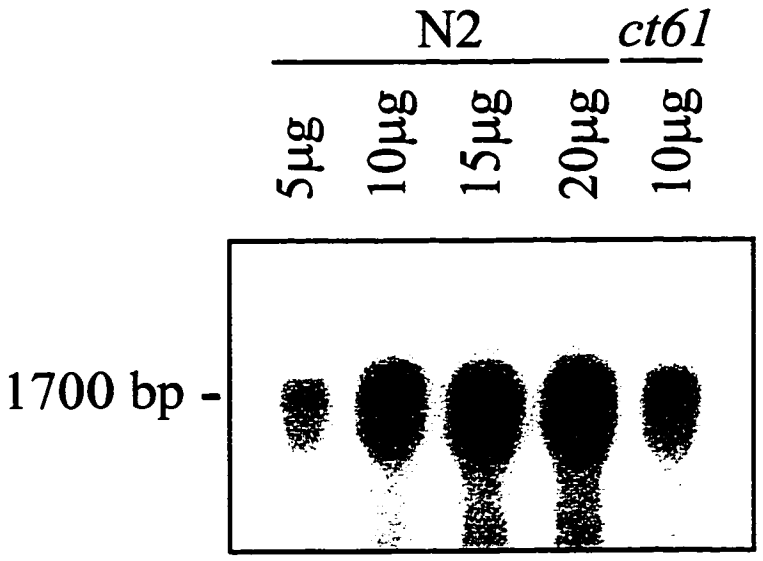
Identification of *mel-26* Mutant Sequences

To identify the molecular lesion present in each allele, 6-8 homozygous mutant worms were used as a source of template in a PCR amplification. The PCR product was gel purified and directly sequenced using various primers. Sequencing of worms homozygous for the recessive allele *ct61sb4* revealed the presence of a stop codon at amino acid position 320 (AGA→TGA), which was not present in either wild-type or *mel-26(ct61)* (Figure 5 and 7). In the dominant-negative *sb45* allele, a G to A transition in codon 94 (TGT→TAT) predicts a cysteine to tyrosine change. No molecular change has been identified in *mel-26(ct61)*, though all coding sequence, introns and ~1000 bp of promoter have been sequenced.

Efforts to Identify the *ct61* Lesion

In addition to sequencing the entire *mel-26* gene region, Northern and Southern blot experiments were performed to try to identify any molecular difference in *mel-26(ct61)*. A Northern blot comparing the size and abundance of the *mel-26* message in *mel-26(ct61)* revealed no apparent difference between *ct61* and wild-type (Figure 8). Likewise, a Southern blot to address the possibility of a larger-scale genomic rearrangement in the

FIGURE 8. **Northern blot analysis of *mel-26(ct61)* and wild-type RNA.** The size and abundance of *mel-26* transcript in increasing amounts of wild-type poly-A^+ RNA is compared to that found in 10 μg of poly-A^+ RNA from *mel-26(ct61)* animals. The probe was synthesized from the full-length *mel-26* cDNA.


N2
ct61
5µg
10µg
15µg
20µg
10µg
1700 bp -

area did not reveal any change in *ct61*, relative to wild-type (Figure 9). The nature of the *ct61* lesion remains unknown. While the Northern blot indicates that the abundance of the message is unchanged in a *ct61* background, changes in the timing and location of *mel-26* transcription can not be ruled out (though such changes might be expected to change the total abundance). The presence of an extra band in Western blots of *ct61* worm lysates (see later) further confuses matters.

Tissue Localization of *mel-26* Expression

Previous genetic analysis of *mel-26* mutations indicated a strict maternal requirement for gene activity, suggesting that it is required only in the female germline. The *mel-26(ct61)* temperature-sensitive period begins at the one-cell stage and extends to the onset of gastrulation (approximately the 28 cell stage, 2 hours post-fertilization) (MAINS *et al.* 1990b). Northern blot analysis was conducted (by FRANCES ALLEN) to determine the relative abundance of *mel-26* transcript in various tissues using strains bearing mutations affecting development of the male, female or both germlines. A single transcript of 1700 nt, as expected from the sequence, was enriched in the female germline and embryos (Figure 10). The mutation *glp-1(q231)* disrupts mitotic proliferation of the germline; the few germ cells that are produced (~15) develop into sperm cells (AUSTIN

FIGURE 9. **Southern blot analysis of *mel-26(ct61)* and wild-type DNA.** Genomic DNA purified from wild-type and *mel-26(ct61)* animals was digested with the restriction enzymes indicated above the autoradiograph. At the bottom of the figure, the position of the sequence used to generate the probe, indicated by a thick black line, is shown relative to the *mel-26* coding region, indicated by the grey boxes. The DNA fragments predicted to hybridize to the probe are shown in thin black lines.

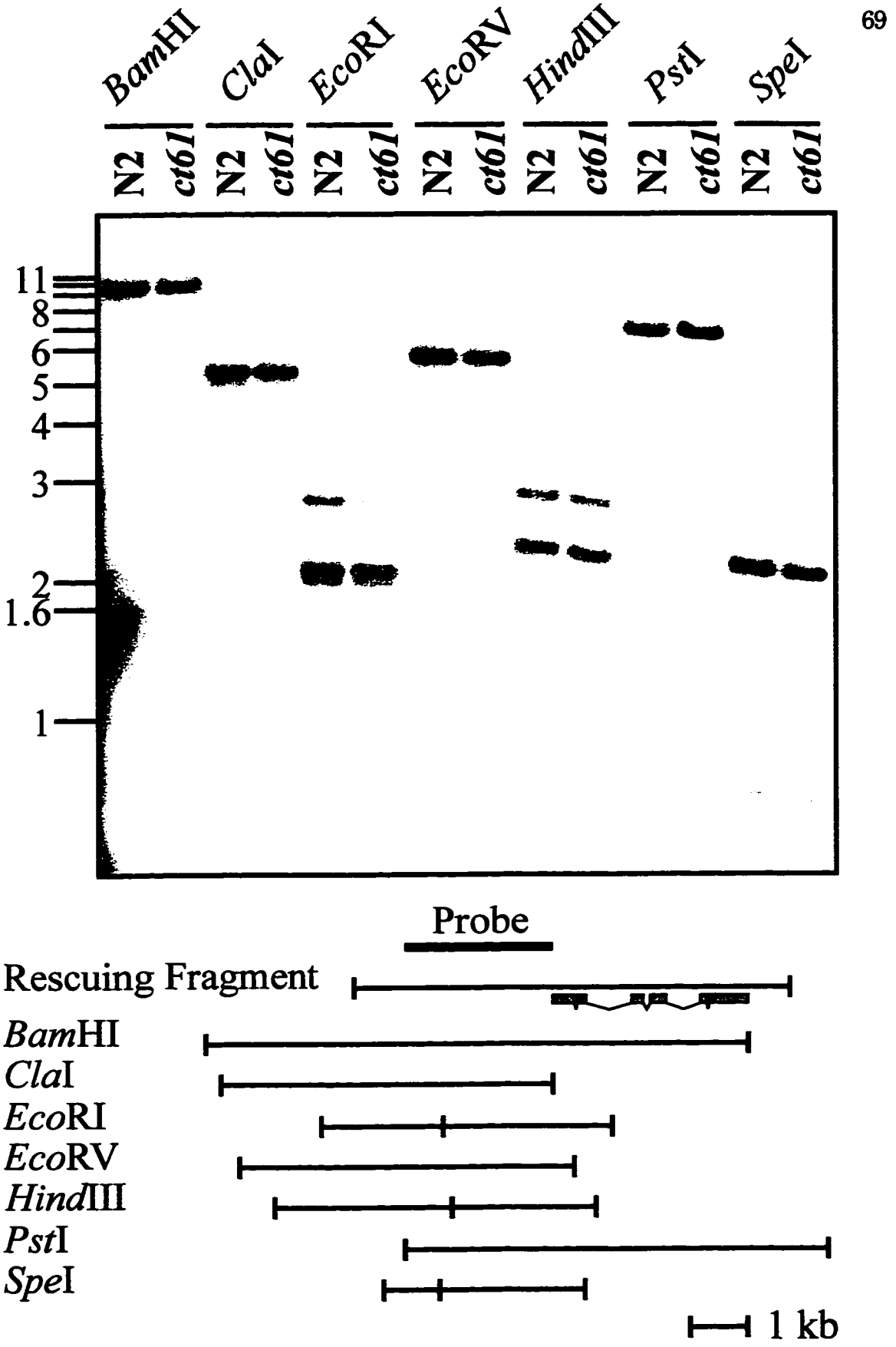
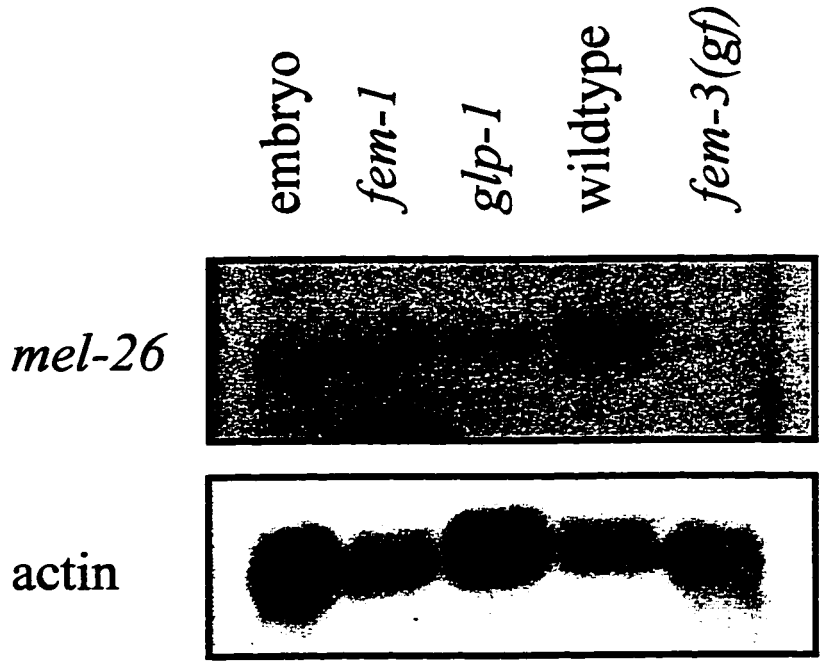

BamHI
ClaI
EcoRI
EcoRV
HindIII
PstI
SpeI
N2
ct61
11
8
6
5
4
3
2
1.6
1
Probe
Rescuing Fragment
BamHI
ClaI
EcoRI
EcoRV
HindIII
PstI
SpeI
1 kb

FIGURE 10. **Northern blot analysis of *mel-26* to determine expression in germ tissues.** The abundance of *mel-26* message in poly-A$^+$ RNA from wild-type embryos and gravid hermaphrodites is compared to samples obtained from adult hermaphrodites mutant for *fem-1* (lacking sperm and fertilized eggs), *fem-3(gf)* (lacking oocytes and fertilized embryos) and *glp-1* (lacking both male and female germline). The blot was probed with the *mel-26* full-length cDNA, and again with actin to show relative loading levels.


embryo
fem-1
glp-1
wildtype
fem-3(gf)
mel-26
actin

and KIMBLE 1987). Even though there was more mRNA in the *glp-1* sample, it is clear that the relative level of *mel-26* was lower than in wild-type gravid hermaphrodites. Germlines of *fem-1(lf)* worms are female, with oocytes but no sperm, while *fem-3(gf)* show the opposite phenotype. Comparisons of the relative abundance of the *mel-26* message in these strains indicated that *mel-26* mRNA is expressed in the developing oocytes. While a high level of *mel-26* mRNA accumulated in *fem-1(lf)* animals, no *mel-26* message was evident in the *fem-3(gf)* sample. The message is present in fertilized embryos, though the abundance of the message may be decreasing, relative to gravid hermaphrodites.

Analysis of MEL-26 by Western Blotting

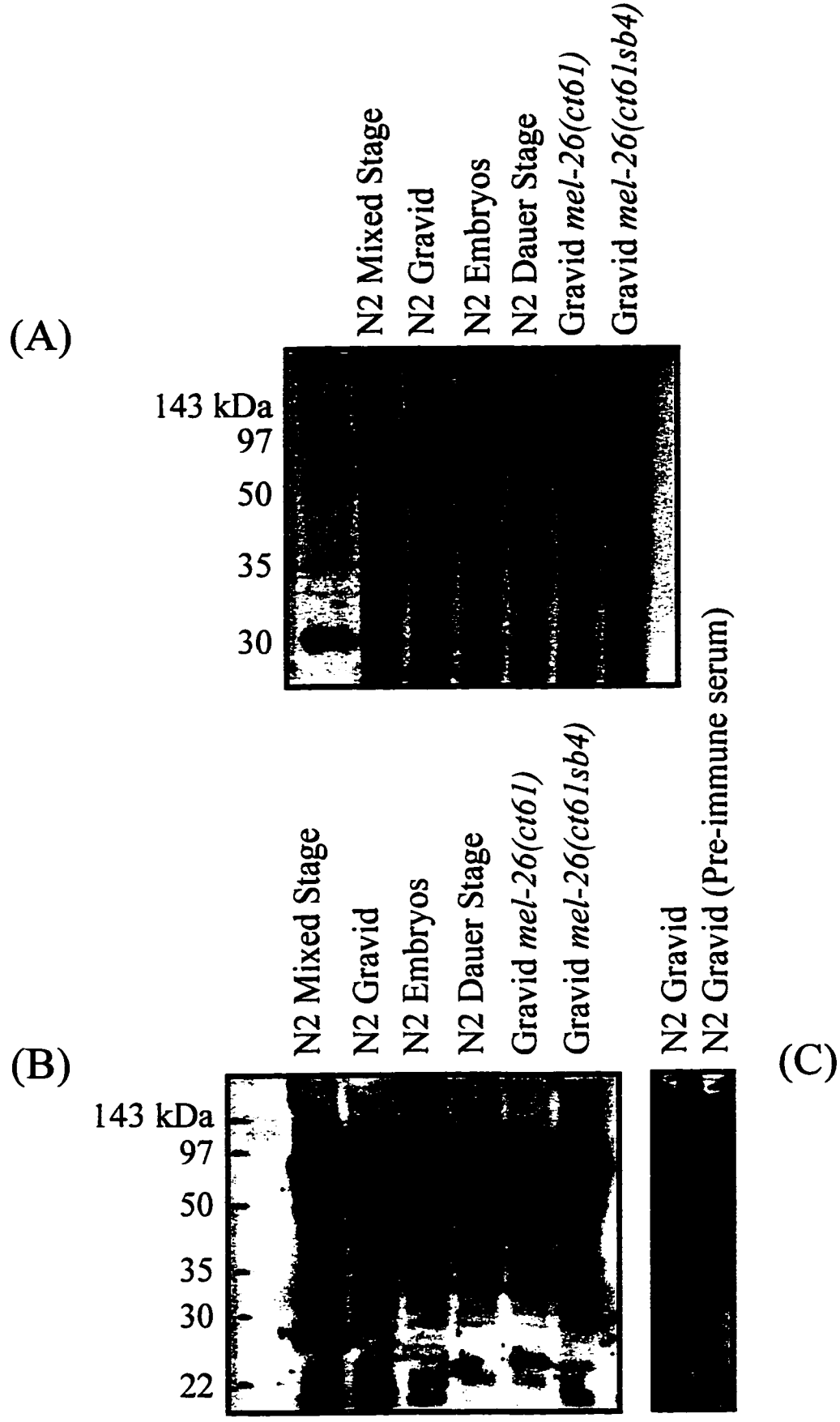
The MEL-26 antibody used in this study was one of three polyclonal rabbit antisera raised against a fusion of glutathione-S-transferase (GST) to the N-terminal 125 amino acids of MEL-26. A second fusion protein was also prepared, with the same portion of MEL-26 fused to an N-terminal 6xHIS tag. This HIS-tagged protein was used to affinity purify the anti-MEL-26 antibody both on a nickel-NTA-agarose column and from Western blot strips. Neither method of affinity purification was successful, and all results presented here were obtained with the crude antiserum.

Western blots of total worm lysates reveal that the polyclonal anti-MEL-26 antibody from rabbits reacts to a large number of *C. elegans* proteins (Figure 11b). Some of these bands are evident in the pre-immune serum (Figure 11c), indicating that the reactivity is unrelated to MEL-26.

While the molecular nature of the *ct61* allele is unknown, the *ct61sb4* allele is a nonsense mutation, which is predicted to yield a protein truncated at approximately 35 kDa, as opposed to the full-length 44.5 kDa size. A prominent band at approximately 50 kDa is unique to the post-immunization serum, however this band seems unaffected by mutations in *mel-26*. Lysates of *mel-26(ct61)* worms consistently show bands at approximately 47 and 25 kDa, which are absent in wild-type and *mel-26(ct61sb4)* lysates (Figure 11b). In several experiments, a prominent band at approximately 35 kDa was absent in *mel-26(ct61sb4)* lysate, however this band is clearly present in Figure 11b.

In addition to *mel-26* mutants, lysates were prepared from staged populations to address the relative abundance of MEL-26 in embryos. A staged population of gravid worms or embryos would be predicted to contain a greater representation of embryonic products, while mixed stage or dauer worms, as a divergent larval stage, would be predicted to contain essentially no embryonic products. The relative intensities of the 50 kDa and 35 kDa bands are not affected in a manner consistent with representing

FIGURE 11. **Western blot analysis of MEL-26.** (A) Coomassie-stained SDS-PAGE gel showing relative loading of total worm extracts. Samples were prepared from wild-type hermaphrodites of mixed stages, staged gravid hermaphrodites, embryos, dauer larvae and gravid hermaphrodites of *mel-26(ct61)* and *mel-26(ct61sb4)*, raised at the restrictive temperature. (B) Western blot corresponding to the gel shown in (A), using anti-MEL-26 as a probe. (C) Sequential Western blot strips probed with anti-MEL-26 and pre-immune serum.


(A)
143 kDa
97
50
35
30
N2 Mixed Stage
N2 Gravid
N2 Embryos
N2 Dauer Stage
Gravid mel-26(ct61)
Gravid mel-26(ct61sb4)
(B)
143 kDa
97
50
35
30
22
N2 Mixed Stage
N2 Gravid
N2 Embryos
N2 Dauer Stage
Gravid mel-26(ct61)
Gravid mel-26(ct61sb4)
(C)
N2 Gravid
N2 Gravid (Pre-immune serum)

an embryonically expressed protein. For instance, while the 50 kDa band is reduced in dauer lysates relative to gravid or mixed staged lysates, it is also in lower abundance in embryos. The 35 kDa band may actually be in greater abundance in the lysate prepared from dauer larvae.

There are several possibilities for these results. One is that neither of these bands represents MEL-26. Almost certainly, one of them does not. Based on the predicted molecular weight of MEL-26, the most likely band is the 50 kDa one. Unfortunately, the predicted molecular weights of the three *C. elegans* ORFs that are similar to MEL-26 are also approximately 50 kDa. If any of these proteins is constitutively expressed at a moderate to high level, cross-reactivity with them may prevent detection of MEL-26. Clearly another explanation is that the antisera are not suitable for use in Western blot experiments.

Microscopic Analysis of *mel-26* Embryos

The effects of *mel-26* mutations on mitosis were observed directly by cutting embryos from gravid hermaphrodites. The embryos were mounted for microscopy on agarose pads and viewed using Nomarski optics. In wild-type embryos, the female pronucleus at the anterior of the embryo migrates towards the male pronucleus in the posterior. After meeting, the pronuclei migrate back towards the centre. With the centrosomes on

opposite sides of the pair of pronuclei on the dorsal-ventral axis, the centrosome-nuclear complex then rotates 90° to form the first mitotic spindle on the anterior-posterior axis in a position shifted slightly to the posterior end (Figure 12). This final rotation appears to be due to an interaction between one of the centrosomes and some factor at the anterior end of the embryo (HYMAN 1989).

In embryos produced by *mel-26* mutant hermaphrodites, both the joint nuclear migration back towards the embryo centre and the rotation frequently fail (Figure 12). This phenotype is observed for all *mel-26* alleles; the dominant-negative *ct61* and *sb45* alleles, as well as the loss-of-function *sb4* allele. The resulting spindle is typically oriented in a dorsal-ventral direction, is located in the extreme posterior of the embryo and is often smaller than wild-type. It is presently unclear whether the reduced spindle size is a primary or secondary effect; that is, could the reduced size be due to the reduced space imposed by the incorrect orientation of the spindle, or due to shorter microtubules? An identical phenotype was observed in embryos produced by hermaphrodites that had been injected with *mel-26* RNA [RNAi experiments (GUO and KEMPHUES 1995; FIRE *et al.* 1998)].

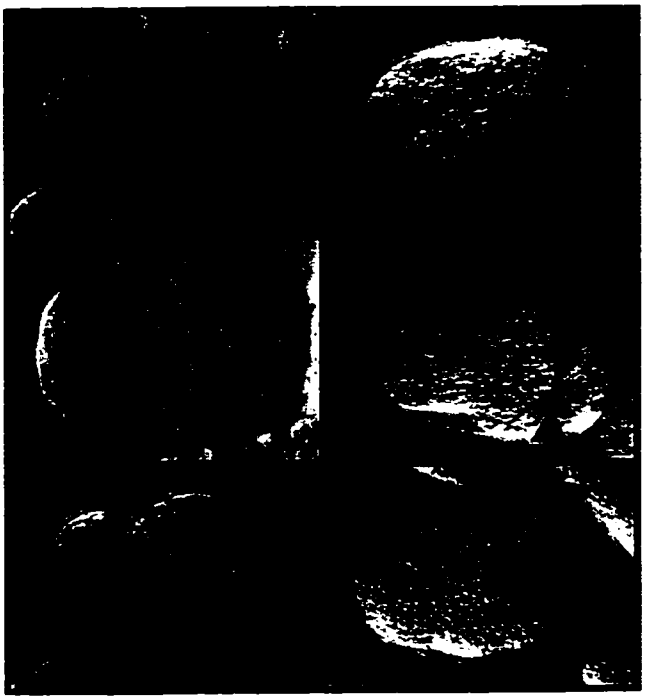
Due to the incorrect location and orientation of the mitotic spindle in *mel-26* mutant embryos, the products of the first cleavage are generally aberrant. Cytokinesis typically fails, likely due to the improper localization

FIGURE 12. **Nomarski photomicrographs showing the first mitotic spindle in *C. elegans* embryos.** In wild-type embryos the first mitotic spindle is oriented in an anterior-posterior position, shifted slightly posterior of centre. The *mei-1* gain-of-function allele *ct46*, all three *mel-26* alleles, and *mel-26* RNA interference all cause the first mitotic spindle to form in a dorsal-ventral orientation, positioned at the extreme posterior of the embryo. All embryos are shown with anterior to the left, and are approximately 50 μm in length.

Wildtype

mei-1(gf)

mel-26(RNAi)



mel-26(sb45)

mel-26(ct61)

mel-26(ct61sb4)

of the spindle and centrosomes. In wild-type embryos, in which the first mitotic spindle is located on the anterior-posterior axis, shifted slightly to the posterior, the first cleavage furrow forms midway between the asters. This leads to the formation of a larger anterior and smaller posterior daughter cell. In *mel-26* embryos, in addition to a dorsal-ventral cleavage in the anterior, which will form a cytoplast, an anterior-posterior cleavage furrow at the posterior end of the embryo occurs midway between the aberrantly oriented spindle poles. The resulting cleavages are frequently incomplete, and eventually regress, leading to the formation of multinucleate cells. The stereotypical positions of the early blastomeres are rapidly disrupted and little order is observed in *mel-26* embryos. This is the same phenotype that is observed in *mei-1(gf)* embryos.

Localization of MEL-26, MEI-1 and tubulin in *mel-26* mutant embryos

In wild-type embryos, MEI-1 staining is confined to the meiotic spindle. Previous experiments showed that MEI-1 is also localized to the mitotic spindle in embryos produced by *mei-1(ct46)* or *mel-26(ct61)* hermaphrodites (CLARK-MAGUIRE and MAINS 1994a). Likewise, *mel-26(ct61sb4)* and *mel-26(sb45)* also show localization of MEI-1 to the mitotic spindle. Indirect immunofluorescent staining of MEI-1 and tubulin

show that when localized to the mitotic spindle, MEI-1 is concentrated at the poles, with some weaker staining along the microtubules (Figure 13).

Indirect immunofluorescent staining of MEL-26 reveals that it is confined to the mitotic centrosome (Figure 14). There is no MEL-26 observed in association with the meiotic spindle. MEL-26 is first observed in association with the asters forming around the male pronucleus. During the subsequent mitotic division, the intensity of MEL-26 staining appears to be highest during metaphase-anaphase. As mitosis concludes, MEL-26 staining disappears and is not seen again until the centrosomes begin to separate and migrate around the nucleus in preparation for the next round of mitosis. The temperature-sensitive period for *mel-26(ct61)* extends for two hours post-fertilization and MEL-26 is found localized to the spindle poles of embryos that have undergone several divisicns. MATTHEWS *et al.* (1998) made a similar observation with the *zyg-9* gene and its product.

None of the *mel-26* mutations, nor *mei-1(ct46)* has any effect on this expression pattern (Figure 15). The expression pattern of MEL-26 in *mel-26(sb45)* embryos was difficult to predict given the gain-of-functicn nature of the allele. The premature stop codon in the *sb4* allele could either lead to rapid mRNA degradation (PULAK and ANDERSON 1993), thus lowering protein levels, or could simply produce normal levels of truncated protein. As the molecular nature of the *ct61* allele is unknown, no

FIGURE 13. **Localization of MEI-1 in *mel-26* mutant embryos.** Anti-tubulin and anti-MEI-1 staining of mitotic embryos demonstrate that in wild-type, MEI-1 is absent from mitotic poles, but it is localized to the centrosomes in *mel-26(ct61)*, *mel-26(sb45)* and *mel-26(ct61sb4)* mutant embryos. All embryos are shown with anterior to the left, and are approximately 50 μm in length. The *mel-26(ct61sb4)* embryo contains two nuclei (following a failure in cytokinesis); all other embryos are undergoing first mitosis.

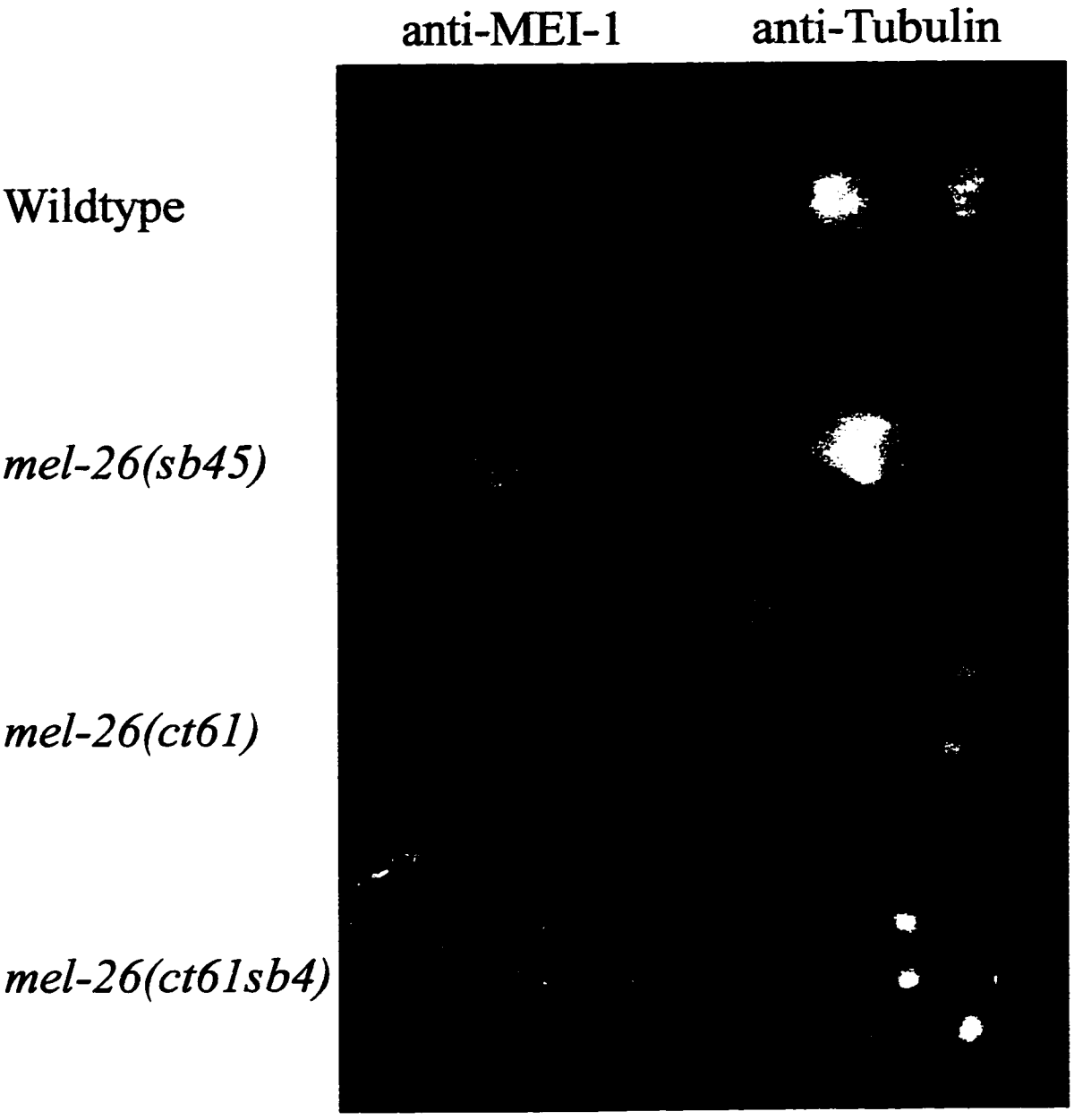

anti-MEI-1
anti-Tubulin
Wildtype
mel-26(sb45)
mel-26(ct61)
mel-26(ct61sb4)

FIGURE 14. **Localization of MEL-26 in wild-type embryos.** Anti-tubulin, anti-MEL-26 and DNA staining with DAPI demonstrate that MEL-26 is concentrated at the mitotic spindle poles. Centrosomal staining is not observed in the pre-immune serum from the anti-MEL-26 serum host. MEL-26 is not observed in association with the meiotic spindle (the position of which is shown with white triangles), but is observed in prophase, metaphase and anaphase of mitosis. Black triangles point out faint MEL-26 staining. All embryos are shown with anterior to the left, and are approximately 50 μm in length.

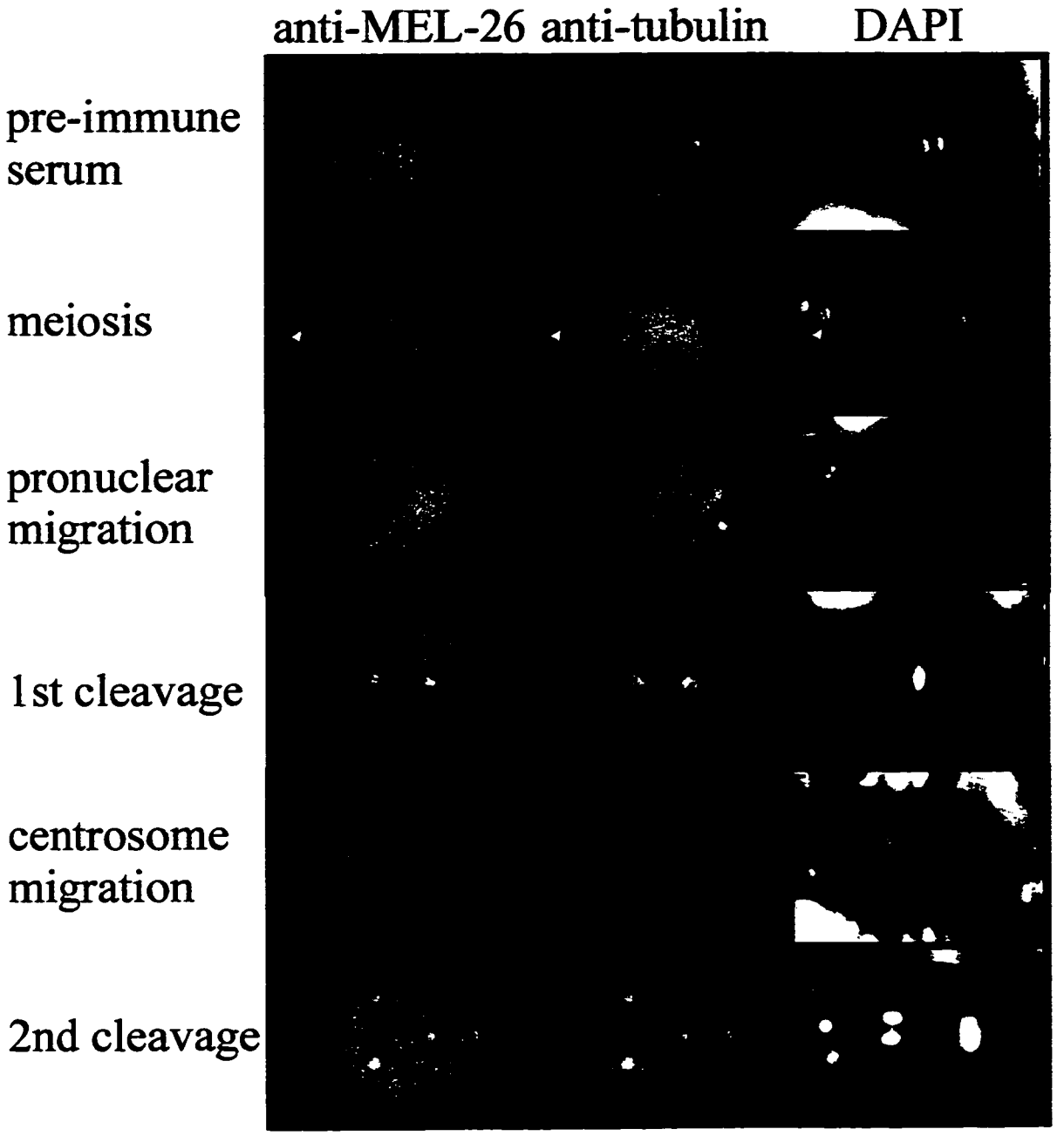
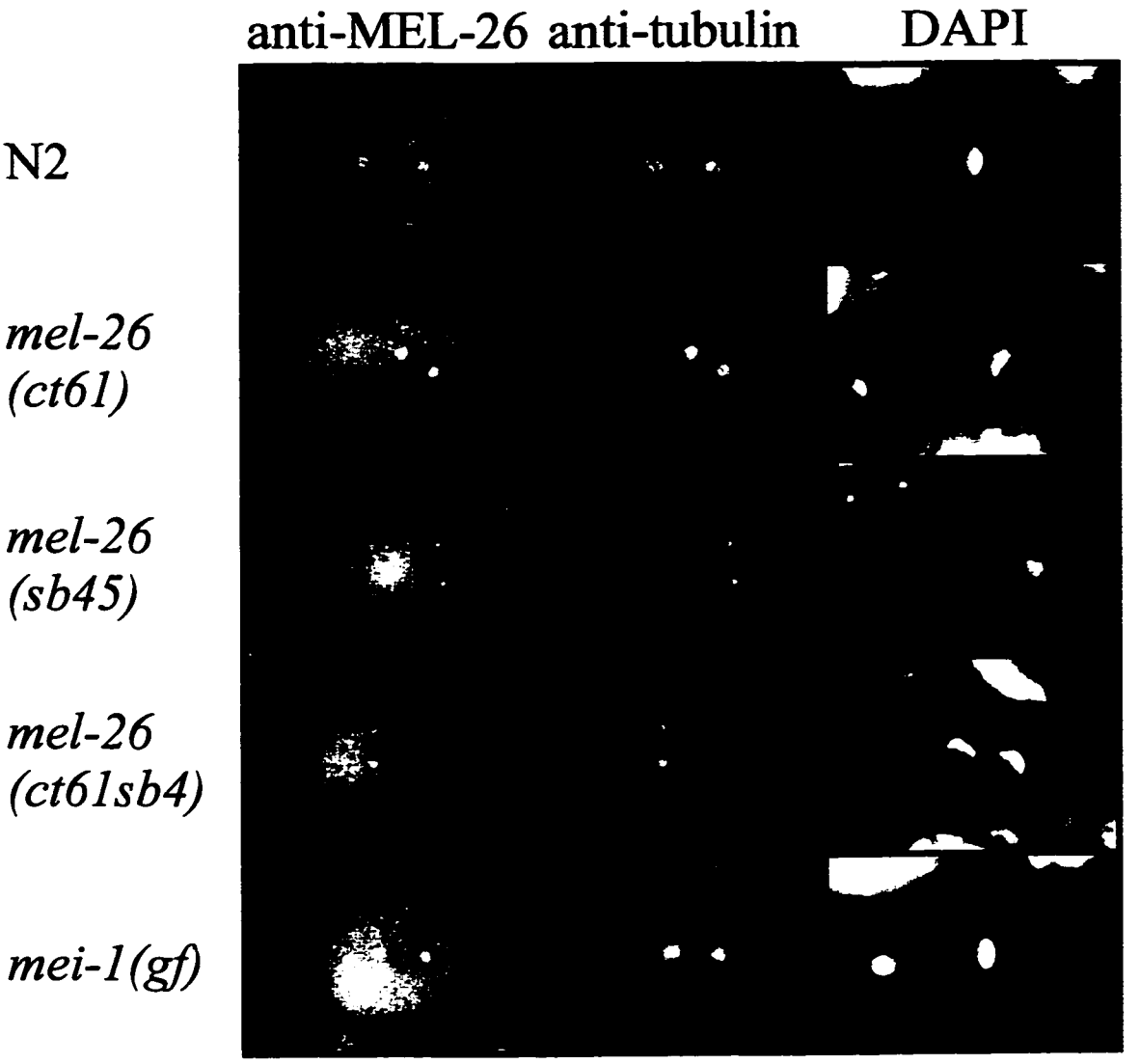

anti-MEL-26 anti-tubulin DAPI
pre-immune serum
meiosis
pronuclear migration
1st cleavage
centrosome migration
2nd cleavage

FIGURE 15. **MEL-26 localization in meiotic and mitotic mutant embryos.** Anti-tubulin, anti-MEL-26 and DNA staining with DAPI demonstrate that MEL-26 localization is not disrupted by any of the three *mel-26* alleles or gain-of-function mutations in *mei-1*. The intensity of anti-MEL-26 staining is lower in some embryos, but may reflect the abundance of microtubules at the spindle poles, which seems to be reduced in both *mei-1* and *mel-26* mitotic mutant embryos. All embryos are shown with anterior to the left, and are approximately 50 μm in length.


anti-MEL-26 anti-tubulin DAPI
N2
mel-26 (ct61)
mel-26 (sb45)
mel-26 (ct61sb4)
mei-1(gf)

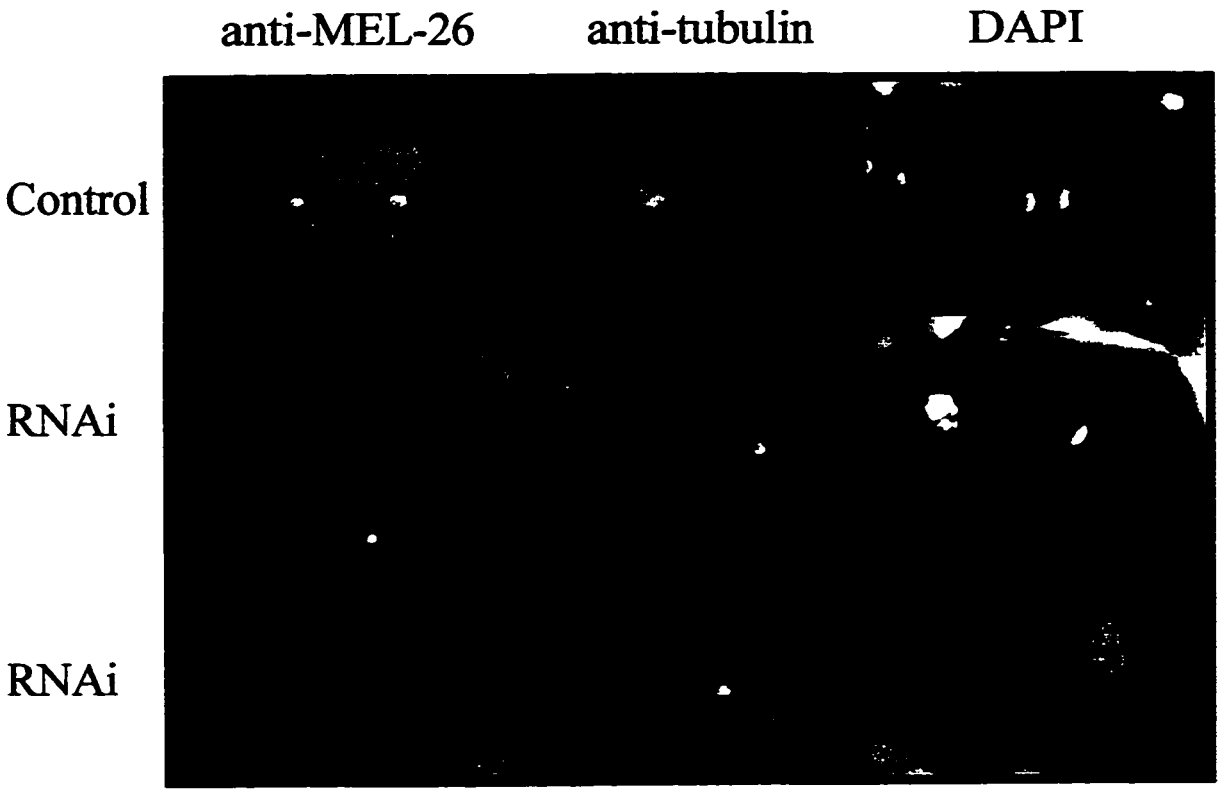
predictions regarding its effect on protein expression were possible. It would appear that the portion of MEL-26 that is removed in *ct61sb4* is not required for localization to the centrosome. The specificity of the centrosomal staining is indicated by the lack of staining with anti-MEL-26 in embryos produced by wild-type hermaphrodites subjected to RNAi experiments (Figure 16).

The localization of MEL-26 to the mitotic centrosome argues against the simple model of MEL-26 leading to the rapid turnover of MEI-1. The observation of both MEI-1 and MEL-26 localization to the mitotic spindle poles in embryos produced by hermaphrodites bearing *mei-1(ct46)* or any of the *mel-26* mutations argues that MEL-26 does not inhibit MEI-1 mitotic interference by simple steric mechanisms.

A Genetic Screen for Suppressors of *mel-26(ct61)*

Genetic analysis of a gene is complicated when using dominant alleles, such as the canonical allele of *mel-26*, *ct61*. The revertant allele, *sb4*, is recessive, though the temperature-sensitive nature of this allele suggests that some of the gain-of-function nature of *ct61* may persist. To aid in the genetic analysis of *mel-26*, a screen was conducted to identify revertants of *mel-26(ct61)* to a simple loss-of-function phenotype. An additional purpose

FIGURE 16. ***mel-26(RNAi)* eliminates anti-MEL-26 centrosomal staining.** Disruption of *mel-26* activity by RNA interference generates a mitotic-defective phenotype similar to that observed with all three *mel-26* alleles; the mitotic spindles are misplaced and misoriented and the abundance of tubulin in the spindle is reduced. The absence of anti-MEL-26 staining at the centrosomes indicates that the centrosomal staining is specific for MEL-26. All embryos are shown with anterior to the left, and are approximately 50 μm in length.


anti-MEL-26
anti-tubulin
DAPI
Control
RNAi
RNAi

was the potential to identify interacting genes that might be isolated as dominant extragenic suppressors.

From a screen of approximately 5000 *unc-29 mel-26(ct61)/hT2* F1 animals, 10 suppressors were isolated. Of these, seven failed to complement *mel-26(ct61sb4),* suggesting that they represented alleles of *mel-26.* Subsequent microscopic analysis, however, showed that all of these strains showed meiotic defects, indicative of *mei-1* and *mei-2* mutations. The strongest suppressor isolated was genetically separated from *mel-26(ct61)* and complementation testing demonstrated that it was an allele of *mei-1* (P. E. MAINS, personal communication).

Due to the complexity of the *mei-1* locus and the genetic interactions between alleles of *mei-1* and *mel-26,* as well as their proximity on the genetic map, genetic characterization of the suppressor alleles was not straightforward. However, we can explain the apparent non-complementation with *sb4* as follows. Dominant-negative alleles of *mei-1* are able to dominantly suppress *mel-26(ct61).* Double heterozygotes (*mei-1(dn) mel-26(ct61)*/+ +) show higher hatching rates than *mel-26(ct61)* heterozygotes, and therefore appear as the expected intragenic revertants (*i.e., mel-26(lf)*/+). In addition, homozygous *mei-1(dn) mel-26(ct61)* worms produce dead embryos due to meiotic defects; *mel-26(lf)* would also be predicted to produce dead embryos, though the defect would likely be

mitotic. Thus, the *mei-1(dn) mel-26(ct61)* combinations behaved as a recessive loss-of-function maternal-effect lethal (Mel) mutation, and were therefore tested for complementation with *mel-26* using the *sb4* allele. Unfortunately, *mel-26(ct61)/mel-26(ct61sb4)* itself shows a much stronger Mel phenotype than *mel-26(ct61)/+*. Since the *ct61* allele was still present in the revertant alleles, the actual genotype of the complementation test strain was *mei-1(dn) mel-26(ct61)/+ mel-26(ct61sb4)*, which naturally showed failure to complement.

As an additional complication, *mei-1* appears to mutate to dominant-negative alleles that can suppress *mel-26* at a high (1/700) frequency (CLARK-MAGUIRE and MAINS 1994b). At the same time, previous reversion experiments with *mel-26(ct61)* have suggested that *ct61* reverts to a loss-of-function phenotype (*e.g., sb4*) at a low frequency (P. E. MAINS, unpublished; DOW and MAINS 1998), though this may reflect the indications that *ct61* is not a simple coding-sequence mutation.

DISCUSSION

While superficially similar, the processes of mitosis and meiosis have many differences. For instance, while chromosome segregation in both mitosis and meiosis utilizes a tubulin-based spindle, it is becoming clear that the spindles can be assembled via unique pathways and may contain distinct components. The transition from oogenesis to embryogenesis presents an interesting regulatory requirement. Typically, the oocyte is arrested in some phase of meiosis at the time of fertilization. Entry of the sperm triggers resumption of meiosis. Following completion of meiosis and disassembly of the meiotic spindle, the cell must support the assembly of a mitotic spindle. The same cytoplasm must be able to support both a meiotic and a mitotic spindle, often within the space of 20-30 minutes.

Many of the proteins previously described (see Introduction) are thought to play a role in the functioning of both meiotic and mitotic spindles. Some, such as the *Drosophila* minus-end directed motor, Ncd, appear to have similar, though perhaps slightly different roles in the acentriolar meiotic spindle and early cleavage spindles, as compared to later mitotic spindles. Others, such as the *C. elegans* oocyte meiotic-specific spindle component MEI-1, must function specifically in one type of spindle. It is therefore critical that the cell be able to ensure that components of the

meiotic spindle are not allowed to interfere with assembly and function of the mitotic spindles of embryogenesis. This work describes the characterization of a gene, *mel-26*, that plays a critical role in that type of regulation.

MEL-26 is a Post-Meiotic Negative Regulator of MEI-1

The *mei-1* gene encodes an essential component of the oocyte meiotic spindle (CLARK-MAGUIRE and MAINS 1994a). In the absence of *mei-1* activity, the spindle fails to form, with a cloud of tubulin remaining around the chromatin. In wild-type embryos, MEI-1 is found localized to the meiotic spindle. Following completion of meiosis, MEI-1 is not observed in the embryo.

A gain-of-function mutation (*ct46*) has been identified in the *mei-1* gene that causes MEI-1 to persist following meiosis (CLANDININ and MAINS 1993; CLARK-MAGUIRE and MAINS 1994a). In *mei-1(ct46)* embryos, meiosis and MEI-1 localization during meiosis appear normal. During the subsequent mitotic divisions, however, MEI-1 is found localized to the poles of the mitotic spindle. The presence of MEI-1 in the mitotic spindle is detrimental to either spindle assembly or function. In wild-type embryos, when the pronuclei meet, the sperm-provided centrosomes are located dorsal and ventral to the joined pronuclei. The centrosomes and pronuclei

rotate onto the anterior-posterior axis to form the spindle on the anterior-posterior axis, shifted slightly posterior of center. Presence of MEI-1 in the mitotic spindle is associated with failure of this centrosomal rotation and the first mitotic spindle is formed in a dorsal-ventral direction, at the extreme posterior of the embryo (Figure 12). The spindle is also reduced in size and often bent.

Genetic analysis of *mei-1* has defined a regulatory pathway (CLARK-MAGUIRE and MAINS 1994a,b). The *mei-2* gene acts as a positive regulator, ensuring that *mei-1* activity is high enough, or properly localized, to support assembly of the meiotic spindle. The *mel-26* gene, on the other hand, acts as a post-meiotic negative regulator, ensuring that MEI-1 is not incorporated into the mitotic spindle. Several additional genes have been identified that show various genetic interactions with these three.

Three alleles of *mel-26* have been identified. The canonical allele, *ct61*, is a dominant, temperature-sensitive mutation that behaves genetically as a dominant negative. A revertant of this allele, *sb4*, converts *ct61* to a recessive temperature-sensitive loss-of-function allele. Genetic analysis of *ct61sb4* suggests that it represents a genetic null at the *mel-26* locus (DOW and MAINS 1998). The final allele (*sb45*), isolated independently (MITENKO *et al.* 1997), is similar to *ct61*. It is a dominant,

temperature-sensitive allele, which results in a loss-of-function (*i.e.*, a dominant-negative).

The mitotic spindle defects of *mei-1(ct46)* and any of the *mel-26* alleles are essentially indistinguishable. In addition, MEI-1 is found ectopically localized to the mitotic spindle in *mel-26* mutants, suggesting that *mel-26* activity is required to prevent MEI-1 from interfering with the mitotic spindle. In the absence of *mei-1* activity, while meiosis is abnormal, an apparently normal mitotic spindle is formed. In a *mei-1(null) mel-26* double mutant, mitotic spindles are also normal, indicating that *mel-26(+)* activity is only required when *mei-1(+)* is present.

To help understand the regulation of *mei-1* activity, and possibly address one mechanism used to ensure meiosis-specific gene activity, this project was initiated to clone and characterize the *mel-26* gene.

The *mel-26* Gene Encodes a BTB-containing Protein

The *mel-26* gene was cloned by transformation rescue of the recessive maternal-effect lethality of *mel-26(ct61sb4)*. One coding region and its cDNA were identified in the minimal rescuing fragment. Northern blot analysis of its transcript indicates that it is most highly expressed in the female germline, consistent with the genetic analysis of *mel-26*, which indicated only maternal gene function. The predicted MEL-26 protein is a member of

a family of related *C. elegans* proteins. MEL-26 shows full-length similarity to the predicted products of three ORFs identified by the *C. elegans* Genome Sequencing Project. These ORFs, T16H12.5, C07D10.2 and C50C3.8 are not located near any genes known to interact with *mel-26* or *mei-1*.

The recently identified human speckle-type POZ protein (SPOP) also shows full-length similarity to MEL-26 (Figure 7). SPOP was isolated by virtue of its speckled pattern of nuclear expression as determined by staining with human autoantibodies. Immunolocalization studies indicated that SPOP was co-localized with a splicing factor and it was suggested that it may interact with spliceosomes via interaction with its POZ domain (see below) (NAGAI *et al.* 1997). Although BLAST comparisons with MEL-26 reveal that SPOP is the most closely related protein identified so far (33% identical overall), it is more likely that SPOP and the ORF T16H12.5 are homologs as they show large blocks of identity and are nearly 63% identical overall (with 91% identity in the N-terminus) (NAGAI *et al.* 1997).

MEL-26, the three *C. elegans* ORFs and SPOP all contain a region of similarity to a motif known as both the BTB (ZOLLMAN *et al.* 1994) and POZ (BARDWELL and TREISMAN 1994) domain. The BTB domain was first identified as a motif in the *Drosophila* zinc finger proteins encoded by the ***bric à brac***, ***tramtrack*** and ***Broad-Complex*** genes. Subsequent PCR

screening identified six additional *Drosophila* genes encoding proteins containing BTB domains (ZOLLMAN *et al.* 1994). The POZ domain was identified in poxvirus and zinc finger proteins and was shown to affect the DNA-binding of at least three different zinc finger proteins (BARDWELL and TREISMAN 1994). Analysis of the cellular activities of several BTB/POZ domain-containing proteins indicated that the domain was common to DNA- and actin-binding proteins (ALBAGLI *et al.* 1995). Secondary structure predictions suggest that the BTB/POZ domain forms four helices separated by regions of β-sheet (BARDWELL and TREISMAN 1994). None of MEL-26, the three *C. elegans* ORFs nor SPOP are zinc finger proteins. In both initial reports (ZOLLMAN *et al.* 1994; BARDWELL and TREISMAN 1994), the domain was implicated in mediating protein-protein interactions. Given the large number of proteins that are known to localize to the centrosome, protein-protein interactions are likely very important to the function of the centrosome.

Within the BTB/POZ region, MEL-26 shows similarity to several known BTB/POZ domain proteins, including the proteins encoded by the *longitudinals lacking* (*lola*) gene of *Drosophila* and the MT-8 gene of *Myxoma* virus (Figure 7). None of the proteins identified as being similar to MEL-26 has any obvious connection to the spindle or centrosome.

Despite fairly extensive efforts, we have been unable to identify a molecular change in the *ct61* allele. The entire coding region, all introns and approximately 1 kb of promoter have been completely sequenced. Southern blot analysis of the region does not indicate the presence of any genomic rearrangments in the *mel-26* region, and a Northern blot indicates that there is no change in the size or abundance of the *mel-26* transcript in a *ct61* background. Centrosomal localization of MEL-26 is normal in *ct61* embryos, confirming that the protein is present and at least partially functional. The presence of two extra bands in the Western blots represents the only molecular indication of the *ct61* mutation. Given that the entire gene has been sequenced, this finding is simply contradictory, and bears further investigation.

There are at least three possible explanations for the failure to identify a *ct61* lesion. First, it is formally possible that there are two closely linked loci that interact genetically [*i.e., mel-26(ct61)* and *sup(sb4)*] and that we have cloned *sup(sb4)*, but not *mel-26*. Since *sb45* results in a sequence change in the same gene as *sb4*, it would therefore be an allele of *sup(sb4)* rather than *mel-26*. This scenario is unlikely; *ct61* and *sb45* are very similar to one another in that both are dominant temperature-sensitive maternal-effect lethal mutations and both result in similar mitotic defects. Furthermore, both mutations respond in the same fashion when in *trans* to

either *ct61sb4* or a deficiency of the region: both *ct61* and *sb45* enhance *mei-1(ct46)* and both are suppressed by *mei-1* suppressors. In addition, the failure to identify any crossovers between *ct61* and either *sb4* or *sb45* suggests that the maximum genetic distance between these mutations is 0.03 cM (DOW and MAINS 1998). Based on the estimates of BARNES *et al.* (1997), this genetic distance would correspond to a region of approximately 45 kb or less. Examination of the *C. elegans* Genome Sequencing Project's sequence data does not reveal any genome duplications or candidate interacting genes within at least 45 kb of *mel-26*.

The second possible explanation for the inability to identify the *ct61* lesion is that the *ct61* allele is due to a promoter mutation, 5' of the region that has been sequenced. A precedent exists in *C. elegans* for dominant *ts* promoter mutations (PERRY *et al.* 1994); however, these mutations were hypermorphic, whereas *ct61* is antimorphic.

The third possibility is that there is an additional exon, which may be used in a rare class of transcripts, that has not been identified. Though the results of the Northern blot experiments would make this unlikely, it is possible that a small change in the transcript size would not be detected.

It is notable that *ct61sb4* was found at the relatively low frequency of 1/12 000 mutagenized chromosomes. The forward mutation rate after standard EMS mutagenesis in *C. elegans* is usually in the range of 1/1000 to

1/5000 (ANDERSON and BRENNER 1984; BRENNER 1974; PARK and HORVITZ 1986; ROGALSKI and RIDDLE 1988). The two antimorphic alleles, *ct61* and *sb45*, were found among 24 000 mutagenized chromosomes (MAINS *et al.* 1990b; MITENKO *et al.* 1997), the same frequency as *sb4*. This may indicate that *mel-26* has as many EMS-mutable sites disposing it to mutate to antimorphic as to null alleles. Alternatively, this may be supporting the indications that *ct61* is not a simple coding sequence mutation, and that only a limited set of mutations can convert *ct61* to a recessive loss-of-function allele.

Two of the alleles of *mel-26*, *ct61* and *sb45*, act as antimorphs. Antimorphs, also called dominant-negatives (MULLER 1932; HERSKOWITZ 1987), are alleles that are not only deficient in their wild-type function, but also interfere with any wild-type alleles present. One simple model to explain antimorphic alleles is that the wild-type product of the gene is a component of a multimeric complex and that the mutant allele encodes a protein that, when incorporated into such a complex, "poisons" the entire complex. This suggests that MEL-26 may be a component of such a complex, interacting via the BTB domain. The strong loss-of-function allele, *ct61sb4*, which was isolated as a suppressor of *ct61*, is a nonsense mutation, resulting in the loss of 75 amino acids from the C-terminal end of the protein. This could suggest that this 75 amino acid region (which does not include the

BTB domain) is perhaps also important in the formation or function of the putative multimeric complex. The observation that the *ct61sb4* allele does not disrupt MEL-26 localization to the centrosome might argue against this.

MEL-26 is a Component of the Mitotic Centrosome

Analysis of MEL-26 expression by indirect immunofluorescence reveals that MEL-26 is a component of the mitotic centrosome. MEL-26 was not found in association with the meiotic spindle microtubules. Following completion of meiosis, MEL-26 localized to the centrosomes, which are contributed by the sperm. Staining of the centrosome with MEL-26 antibody is most prominent during mitotic divisions and is either much less abundant, or entirely absent, during interphase. The mitotic localization of MEL-26 appears to be confined to the centrosomes, with no other spindle staining evident. After the completion of mitosis, MEL-26 does not appear to localize to the centrosome. Unfortunately, with the high level of background staining, it is difficult to state conclusively that MEL-26 is strictly a component of the mitotic centrosomes, or rather found throughout the cell cycle. However, it is clear that the abundance of MEL-26 in non-mitotic centrosomes is greatly reduced (Figure 14).

The centrosomal localization of MEL-26 is not prevented by *mei-1(ct46)* or any of the *mel-26* alleles, though in some cases the abundance

of MEL-26 at the centrosome may be diminished slightly (Figure 15). Neither of the sequenced alleles are molecular nulls, so this result was not entirely unexpected. The finding that the *sb4* allele does not affect MEL-26 localization suggests that the carboxy-terminal 75 amino acids, absent due to the premature stop codon, are not necessary for the interaction of MEL-26 with the centrosome. Beyond that, it is difficult to make any conclusions about *mel-26(ct61sb4)*, as the molecular nature of the *ct61* mutation is still unknown. The *sb45* allele, a single amino acid substitution in the N-terminal third of the protein, implicates at least that portion of the amino-terminal region in function rather than localization. Neither of the sequenced alleles affected the BTB region; considering the suggested role of the BTB region in protein-protein interaction, it is likely that this domain is important in the interaction between MEL-26 and the centrosome.

All three *mel-26* mutations, *mei-1(ct46)* and *mel-26(RNAi)* all lead to reduced prominence of the microtubule asters in the mitotic spindle. Whether this represents a primary or secondary defect is uncertain (see below).

Possible Models for the Function of MEL-26

It is not clear how MEL-26 prevents the interference of MEI-1 in the proper functioning of the mitotic spindle. A better understanding of the

exact role of MEI-1 in the meiotic spindle might help clarify the role of MEL-26. What sort of role might MEI-1 have in the meiotic spindle?

In the absence of *mei-1* activity, the meiotic spindle fails to form, and a cloud of tubulin is observed surrounding the chromosomes. Therefore, one could argue that MEI-1 either helps assemble or maintain the stability of the spindle. Given that we know that spindle assembly in mitosis and meiosis can occur via different mechanisms, the presence of a meiosis-specific assembly factor is anticipated.

Alternatively, the similarity of MEI-1 to the microtubule-severing protein katanin might suggest that MEI-1 is a microtubule severing protein. Though katanin is actually a component of the mitotic spindle, it could be argued that a meiosis-specific microtubule severing activity could be important to maintain the small size of the meiotic spindle and reduce the amount of cytoplasm lost in polar body extrusion. However, loss of a microtubule-severing activity would likely result in larger spindles, not dissolution of the spindle altogether.

In the absence of MEL-26 activity, when MEI-1 is allowed to localize to the mitotic spindle, the abundance of tubulin, particularly in the spindle asters, is greatly reduced. Once again, this suggests that MEI-1 may be capable of severing microtubules. The defects observed in the mitotic spindle, in the presence of MEI-1, can largely be explained if one assumes

that MEI-1 causes shortening of microtubules. The rotation and positioning of the spindle seem to be a function of the long astral microtubules, which seem to be absent from the mitotic spindle when MEI-1 is present. The similarity of the *mei-1(ct46)* and *mel-26* mutant phenotypes to the effects of the microtubule inhibitor nocodazole on the first mitotic spindle (STROME and WOOD 1983) argues for this model.

With the available data, it is difficult to make any conclusions about the true role of MEI-1 in the meiotic spindle and what activity it imposes on the mitotic spindle. Given that, it is not possible to be certain as to the mechanism of MEL-26 function.

The mitotic localization of MEI-1 in *mel-26* mutants provides support for the argument that *mel-26* is the post-meiotic negative regulator of *mei-1*. Analysis of the predicted MEI-1 sequence reveals that the region surrounding the *mei-1(ct46)* lesion yields a significant PEST score (RECHSTEINER and ROGERS 1996), and that the *ct46* change reduces that score dramatically (P.E. MAINS, personal communication). PEST scores refer to a calculation based on the relative abundance of proline (P), glutamic acid (E), serine (S) and threonine (T) in a protein sequence, and protein domains showing high PEST scores have been implicated in affecting protein turnover (RECHSTEINER and ROGERS 1996). This provides a simple model for the mechanism of action of MEL-26; perhaps

MEL-26 leads to the rapid turnover of MEI-1 following completion of meiosis.

The lack of any protease characteristics, based on sequence analysis, and the specific localization of MEL-26 to the mitotic centrosome lends no support to a model in which MEL-26 acts as part of a MEI-1-specific protease. Similarly, MEL-26 could have been proposed to fulfill its role by binding to MEI-1, sequestering it in the cytoplasm following completion of meiosis. Since MEI-1 is not a component of the mitotic spindle in wild-type embryos, and MEL-26 is, this model is also unlikely.

Another possible model is that MEL-26 prevents the interaction of MEI-1 with the mitotic centrosome by simple steric means. The ability of both MEI-1 and MEL-26 to bind to the mitotic spindle poles has been shown in embryos from *mei-1(ct46)* and any of the three *mel-26* mutant alleles, suggesting that this may not be the case. It should be noted that these proteins are all mutant, though *mei-1(ct46)* and *mel-26(sb45)* are simple point mutations and may not disrupt the tertiary structure.

Future Directions

Perhaps additional experiments could help clarify the issue. Both MEI-1 and MEL-26 contain motifs described as protein-protein interaction domains. It would be interesting to determine what sort of partners these

two proteins interact with. The classical means to address this issue employs techniques such as co-immunoprecipitations or the yeast two-hybrid system. In addition to the antibodies to MEI-1 and MEL-26, an antibody to ZYG-9, which is a potential protein partner for either MEI-1 or MEL-26, is now available (MATTHEWS *et al.* 1998) and could be used in these experiments.

An alternative method would be to perform additional genetic screens. Genes identified as extragenic suppressors of the dominant *mei-1* and *mel-26* alleles may define some of their partner proteins.

While the screen employed in this project was not fruitful, the lessons we have learned could be used to improve future screens. One simple modification would be to include a *mei-1(null)* allele on the chromosome containing *mel-26(ct61).* This would eliminate the *mei-1* gene on that chromosome as a target for mutagenesis, and would make the wild-type allele on the balancer chromosome essential, thus eliminating it as a target.

Another approach might be to perform a screen similar to the one conducted in this study, but with the *sb45* allele of *mel-26.* This screen would be likely to isolate the same type of *mei-1(dn)* alleles as the original screen, however as this is now anticipated, the potential suppressors could be first screened visually for meiotic defects.

The molecular nature of the *ct61* allele remains a perplexing problem. With the Western blots of total worm lysates providing the only (weak) indication of a molecular change due to the *ct61* allele, it is increasingly important to refine the Western blot results. Possibly alternate antibody purification techniques, or if necessary another antisera altogether, would yield a clean Western blot. With a more interpretable Western, the effects of *ct61* on MEL-26 could be more clearly distinguished, ultimately leading to the elucidation of the molecular nature of the *ct61* allele.

The newest tool in the *C. elegans* researcher's repertoire is the technique of RNA interference (GUO and KEMPHUES 1995; FIRE *et al.* 1998). With the rapidly approaching completion of the *C. elegans* Genome Sequencing Project, a number of predicted genes of interest have been identified. RNAi screens for predicted open reading frames affecting formation of the first mitotic spindle could be conducted fairly easily. Obvious targets for RNAi experiments are the three *C. elegans* ORFs that show similarity to MEL-26. While other organisms also contain several proteins containing a BTB domain, the *C. elegans* proteins appear to show a full-length similarity not demonstrated in other systems. The cellular function of the other *C. elegans* proteins should be addressed; perhaps RNAi of these genes would reveal mitotic or even meiotic, phenotypes.

Genetic analysis and general characterization of other genes with the same mitotic phenotype should also be continued. An independent screen for maternal-effect lethal mutations (MITENKO *et al.* 1997) yielded a mutation in the *mel-45* gene that causes a similar mitotic defect to *mel-26*. A neomorphic allele of the *unc-116* gene, which encodes a classical kinesin heavy chain, also shows a similar mitotic defect (PATEL *et al.* 1993).

Final Word

While the precise cellular activity of MEL-26 is not clear, this work does begin to extend our understanding, by defining some of the components responsible for the differences between the acentriolar oocyte meiotic spindle and the centriole-containing spindles of the embryonic mitotic divisions. Many avenues of experimentation are available, using the *mel-26* gene as a starting point.

BIBLIOGRAPHY

ALBAGLI, O., P. DHORDAIN, C. DEWEINDT, G. LECOCQ and D. LEPRINCE, 1995 The BTB/POZ domain: A new protein-protein interaction motif common to DNA- and actin-binding proteins. Cell Growth and Differentiation **6**: 1193-1198.

ALBERTSON, D.G., 1984 Formation of the first cleavage spindle in nematode embryos. Dev. Biol **101**: 61-72.

ALBERTSON, D.G. and J.N. THOMSON, 1993 Segregation of holocentric chromosomes at meiosis in the nematode, *Caenorhabditis elegans*. Chromosome Res. **1**: 15-26.

ALTSCHUL, S.F., W. GISH, W. MILLER, E.W. MYERS and D.J. LIPMAN, 1990 Basic local alignment search tool. J. Mol. Biol **215**: 403-410.

ANDERSON, P. and S. BRENNER, 1984 A selection for myosin heavy chain mutants in the nematode *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. U. S. A. **81**: 4470-4474.

AUSTIN, J. and J. KIMBLE, 1987 *glp-1* is required in the germ line for regulation of the decision between mitosis and meiosis in *C. elegans*. Cell **51**: 589-599.

AUSUBEL, F.M., R. BRENT, R.E. KINGSTON, D.D. MOORE, J.G. SEIDMAN *et al.*, 1991 *Current Protocols in Molecular Biology*, John Wiley and Sons, Inc., New York.

BARDWELL, V.J. and R. TREISMAN, 1994 The POZ domain: A conserved protein-protein interaction motif. Genes Dev. **8**: 1664-1677.

BARNES, T.M., Y. KOHARA, A. COULSON and S. HEKIMI, 1997 Meiotic recombination, noncoding DNA and genomic organization in *Caenorhabditis elegans*. Genetics **141**: 159-179.

BARSTEAD, R.J. and R.H. WATERSTON, 1989 The basal component of the nematode dense-body is vinculin. J. Biol Chem. **264**: 10177-10185.

BLANGY, A., H.A. LANE, P. D'HERIN, M. HARPER, M. KRESS *et al.*, 1995 Phosphorylation by $p34^{cdc2}$ regulates spindle association of human Eg5, a kinesin-related motor essential for bipolar spindle formation *in vivo*. Cell **83:** 1159-1169.

BLANGY, A., L. ARNAUD and E.A. NIGG, 1997 Phosphorylation by $p34^{cdc2}$ protein kinase regulates binding of the kinesin-related motor HsEg5 to the dynactin subunit p150. J. Biol Chem. **272:** 19418-19424.

BOUCKSON-CASTAING, V., M. MOUDJOU, D.J. FERGUSON, S. MUCKLOW, Y. BELKAID *et al.*, 1996 Molecular characterisation of *ninein*, a new coiled-coil protein of the centrosome. J. Cell Sci. **109:** 179-190.

BRENNER, S., 1974 The genetics of *Caenorhabditis elegans*. Genetics **77:** 71-94.

BRINKLEY, B.R., 1985 Microtubule organizing centers. Annu. Rev. Cell Biol 1: 145-172.

CLANDININ, T.R. and P.E. MAINS, 1993 Genetic studies of *mei-1* gene activity during the transition from meiosis to mitosis in *Caenorhabditis elegans*. Genetics **134:** 199-210.

CLARK-MAGUIRE, S. and P.E. MAINS, 1994a Localization of the *mei-1* gene product of *Caenorhabditis elegans*, a meiotic-specific spindle component. J. Cell Biol **126:** 199-209.

CLARK-MAGUIRE, S. and P.E. MAINS, 1994b *mei-1*, a gene required for meiotic spindle formation in *Caenorhabditis elegans*, is a member of a family of ATPases. Genetics **136:** 533-546.

CLEVELAND, D.W., 1995 NuMA: a protein involved in nuclear structure, spindle assembly, and nuclear re-formation. Trends Cell Biol **5:** 60-64.

COULSON, A., C. HUYNH, Y. KOZONO and R. SHOWNKEEN. 1995 The Physical Map of the *Caenorhabditis elegans* Genome, pp.533-550 in *Caenorhabditis elegans*: Modern Biological Analysis of an Organism. (Ed. Epstein, H.F. and D.C. Shakes) Academic Press, Inc.,San Diego.

DEBEC, A., C. DETRAVES, C. MONTMORY, G. GERAUD and M. WRIGHT, 1995 Polar organization of γ-tubulin in acentriolar mitotic spindles of *Drosophila melanogaster* cells. J. Cell Sci. **108:** 2645-2653.

DEVEREUX, J., P. HAEBERLI and O. SMITHIES, 1984 A comprehensive set of sequence analysis programs for the VAX. Nucleic. Acids Res. **12:** 387-395.

DOW, M.R. and P.E. MAINS, 1998 Genetic and molecular characterization of the *Caenorhabditis elegans* gene, *mel-26,* a post-meiotic negative regulator of MEI-1, a meiotic-specific spindle component. Genetics (In Press)

DOXSEY, S.J., P. STEIN, L. EVANS, P.D. CALARCO and M. KIRSCHNER, 1994 Pericentrin, a highly conserved centrosome protein involved in microtubule organization. Cell **76:** 639-650.

ECHEVERRI, C.J., B.M. PASCHAL, K.T. VAUGHAN and R.B. VALLEE, 1996 Molecular characterization of the 50-kD subunit of dynactin reveals function for the complex in chromosome alignment and spindle organization during mitosis. J. Cell Biol **132:** 617-633.

EDGLEY, M.L., D.L. BAILLIE, D.L. RIDDLE and A.M. ROSE. 1995 Genetic Balancers, pp.147-184 in *Caenorhabditis elegans*: Modern Biological Analysis of an Organism. (Ed. Epstein, H.F. and D.C. Shakes) Academic Press, Inc.,San Diego.

ENDOW, S.A., R. CHANDRA, D.J. KOMMA, A.H. YAMAMOTO and E.D. SALMON, 1994 Mutants of the Drosophila ncd microtubule motor protein cause centrosomal and spindle pole defects in mitosis. J. Cell Sci. **107:** 859-867.

FIRE, A., S. XU, M.K. MONTGOMERY, S.A. KOSTAS, S.E. DRIVER *et al.,* 1998 Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans.* Nature **391:** 806-811.

GAGLIO, T., A. SAREDI, J.B. BINGHAM, M.J. HASBANI, S.R. GILL *et al.,* 1996 Opposing motor activities are required for the organization of the mammalian mitotic spindle pole. J. Cell Biol **135:** 399-414.

GAGLIO, T., M.A. DIONNE and D.A. COMPTON, 1997 Mitotic spindle poles are organized by structural and motor proteins in addition to centrosomes. J. Cell Biol **138:** 1055-1066.

GARD, D., 1992 Microtubule organization during maturation of *Xenopus* oocytes: Assembly and rotation of the meiotic spindles. Dev. Biol **151:** 516-530.

GEISER, J.R., E.J. SCHOTT, T.J. KINGSBURY, N.B. COLE, L.J. TOTIS *et al.*, 1997 *Saccharomyces cerevisiae* genes required in the absence of the *CIN8*-encoded spindle motor act in functionally diverse mitotic pathways. Mol. Biol Cell **8:** 1035-1050.

GINIGER, E., K. TIETJE, L.Y. JAN and Y.N. JAN, 1994 *lola* encodes a putative transcription factor required for axon growth and guidance in *Drosophila*. Develop. **120:** 1385-1398.

GUNSALUS, K.C., S. BONACCORSI, E. WILLIAMS, F. VERNI, M. GATTI *et al.*, 1995 Mutations in *twinstar*, a *Drosophila* gene encoding a cofilin/ADF homologue, result in defects in centrosome migration and cytokinesis. J. Cell Biol **131:** 1243-1259.

GUO, S. and K.J. KEMPHUES, 1995 *par-1*, a gene required for establishing polarity in *C. elegans* embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. Cell **81:** 611-620.

HARLOW, E. and D. LANE (Editors), 1988 *Antibodies: A Laboratory Manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y..

HARTMAN, J.J., J. MAHR, K. MCNALLY, K. OKAWA, A. IWAMATSU *et al.*, 1998 Katanin, a microtubule-severing protein, is a novel AAA ATPase that targets to the centrosome using a WD40-containing subunit. Cell **93:** 277-287.

HEALD, R., R. TOURNEBIZE, T. BLANK, R. SANDALTZOPOULOS, P. BECKER *et al.*, 1996 Self-organization of microtubules into bipolar spindles around artificial chromosomes in *Xenopus* egg extracts. Nature **382:** 420-425.

HENDERSON, S.T., D. GAO, E.J. LAMBIE and J. KIMBLE, 1994 *lag-2* may encode a signaling ligand for the GLP-1 and LIN-12 receptors of *C. elegans*. Develop. **120:** 2913-2924.

HERSKOWITZ, I., 1987 Functional inactivation of genes by dominant negative mutations. Nature **329:** 219-222.

HEUER, J.G., K. LI and T.C. KAUFMAN, 1995 The *Drosophila* homeotic target gene centrosomin (*cnn*) encodes a novel centrosomal protein with leucine zippers and maps to a genomic region required for midgut morphogenesis. Develop. **121:** 3861-3876.

HIRSH, D., D. OPPENHEIM and M. KLASS, 1976 Development of the reproductive system of *Caenorhabditis elegans*. Dev. Biol **49:** 200-219.

HORVITZ, H.R., S. BRENNER, J. HODGKIN and R.K. HERMAN, 1979 A uniform genetic nomenclature for the nematode *Caenorhabditis elegans*. Mol. Gen. Genet. **175:** 129-133.

HYMAN, A.A., 1989 Centrosome movement in the early divisions of *Caenorhabditis elegans*: A cortical site determining centrosome position. J. Cell Biol **109:** 1185-1193.

KARSENTI, E., J. NEWPORT and M. KIRSCHNER, 1984 Respective roles of centrosomes and chromatin in the conversion of microtubule arrays from interphase to metaphase. J. Cell Biol **99:** 47s-54s.

KASHINA, A.S., G.C. ROGERS and J.M. SCHOLEY, 1997 The bimC family of kinesins: essential bipolar mitotic motors driving centrosome separation. Biochim Biophys Acta **1357:** 257-271.

KEMPHUES, K.J., N. WOLF, W.B. WOOD and D. HIRSH, 1986 Two loci required for cytoplasmic organization in early embyros of *Caenorhabditis elegans*. Dev. Biol **113:** 449-460.

KIM, S.K. and H.R. HORVITZ, 1990 The *Caenorhabditis elegans* gene *lin-10* is broadly expressed while required specifically for the determination of vulval cell fates. Genes Dev. **4:** 357-371.

KNOP, M., G. PEREIRA, S. GEISSLER, K. GREIN and E. SCHIEBEL, 1997 The spindle pole body component Spc97p interacts with the γ- tubulin of *Saccharomyces cerevisiae* and functions in microtubule organization and spindle pole body duplication. EMBO J. **16:** 1550-1564.

KOMACHI, K., M.J. REDD and A.D. JOHNSON, 1994 The WD repeats of Tup1 interact with the homeo domain protein alpha 2. Genes Dev. **8:** 2857-2867.

KRAUSE, M. 1995 Transcription and Translation, pp.483-512 in *Caenorhabditis elegans*: Modern Biological Analysis of an Organism. (Ed. Epstein, H.F. and D.C. Shakes) Academic Press, Inc.,San Diego.

LI, K. and T.C. KAUFMAN, 1996 The homeotic target gene *centrosomin* encodes an essential centrosomal component. Cell **85:** 585-596.

LOHKA, M.J. and J.L. MALLER, 1985 Induction of nuclear envelope breakdown, chromosome condensation, and spindle formation in cell-free extracts.. J. Cell Biol **101:** 518-523.

MACK, G. and J.B. RATTNER, 1993 Centrosome repositioning immediately following karyokinesis and prior to cytokinesis. Cell Motility & the Cytoskeleton **26:** 239-247.

MAINS, P.E., K.J. KEMPHUES, S.A. SPRUNGER, I.A. SULSTON and W.B. WOOD, 1990a Mutations affecting the meiotic and mitotic divisions of the early *Caenorhabditis elegans* embryo. Genetics **126:** 593-605.

MAINS, P.E., I.A. SULSTON and W.B. WOOD, 1990b Dominant maternal-effect mutations causing embryonic lethality in *Caenorhabditis elegans*. Genetics **125:** 351-369.

MASTRONARDE, D.N., K.L. MCDONALD, R. DING and J.R. MCINTOSH, 1993 Interpolar spindle microtubules in PTK cells. J. Cell Biol **123:** 1475-1489.

MASUDA, H. and T. SHIBATA, 1996 Role of γ-tubulin in mitosis-specific microtubule nucleation from the *Schizosaccharomyces pombe* spindle pole body. J. Cell Sci. **109:** 165-177.

MATTHEWS, K.A., D. REES and T.C. KAUFMAN, 1993 A functionally specialized α-tubulin is required for oocyte meiosis and cleavage mitoses in *Drosophila*. Develop. **117:** 977-991.

MATTHEWS, L.R., P. CARTER, D. THIERRY-MIEG and K. KEMPHUES, 1998 ZYG-9, a *Caenorhabditis elegans* protein required for microtubule

organization and function, is a component of meiotic and mitotic spindle poles. J. Cell Biol **141:** 1159-1168.

MATTHIES, H.J.G., H.B. MCDONALD, L.S.B. GOLDSTEIN and W.E. THEURKAUF, 1996 Anastral meiotic spindle morphogenesis: Role of the non-claret disjunctional kinesin-like protein. J. Cell Biol **134:** 455-464.

MCNALLY, F.J., K. OKAWA, A. IWAMATSU and R.D. VALE, 1996 Katanin, the microtubule-severing ATPase, is concentrated at centrosomes. J. Cell Sci. **109:** 561-567.

MCNALLY, F.J. and R.D. VALE, 1993 Identification of Katanin, an ATPase that severs and disassembles stable microtubules. Cell **75:** 419-429.

MELLO, C.C., J.M. KRAMER, D. STINCHCOMB and V. AMBROS, 1991 Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. EMBO J. **10:** 3959-3970.

MERDES, A., K. RAMYAR, J.D. VECHIO and D.W. CLEVELAND, 1996 A complex of NuMA and cytoplasmic dynein is essential for mitotic spindle assembly. Cell **87:** 447-458.

MERDES, A. and D.W. CLEVELAND, 1997 Pathways of spindle pole formation: Different mechanisms; conserved components. J. Cell Biol **138:** 953-956.

MEYER, B.J. and L.P. CASSON, 1986 *C. elegans* compensates for the difference in X chromosome dosage between the sexes by regulating transcript levels. Cell **47:** 871-881.

MITENKO, N.L., J.R. EISNER, J.R. SWISTON and P.E. MAINS, 1997 A limited number of *Caenorhabditis elegans* genes are readily mutable to dominant, temperature-sensitive maternal-effect embryonic lethality. Genetics **147:** 1665-1674.

MULLER, H.J., 1932 Further studies on the nature and causes of gene mutations. Proc. 6th Int. Congr. Genet. **1:** 213-255.

MURRAY, A.W., 1991 Cell cycle extracts. Methods in Cell Biology **36:** 581-605.

NAGAI, Y., T. KOJIMA, Y. MURO, T. HACHIYA, Y. NISHIZAWA *et al.*, 1997 Identification of a novel nuclear speckle-type protein, SPOP. FEBS Lett **418:** 23-26.

OAKLEY, B.R., C.E. OAKLEY, Y. YOON and M.K. JUNG, 1990 Gamma-tubulin is a component of the spindle pole body that is essential for microtubule function in *Aspergillus nidulans*. Cell **61:** 1289-1301.

OAKLEY, B.R., 1995 A nice ring to the centrosome. Nature **378:** 555-556.

OAKLEY, C.E. and B.R. OAKLEY, 1989 Identification of γ-tubulin, a new member of the tubulin superfamily encoded by *mipA* gene of *Aspergillus nidulans*. Nature **338:** 662-664.

PARK, E.C. and H.R. HORVITZ, 1986 Mutations with dominant effects on the behaviour and morphology of the nematode *Caenorhabditis elegans*. Genetics **113:** 821-852.

PATEL, N., D. THIERRY-MIEG and J.R. MANCILLAS, 1993 Cloning by insertional mutagenesis of a cDNA encoding *Caenorhabditis elegans* kinesin heavy chain. Proc. Natl. Acad. Sci. U. S. A. **90:** 9181-9185.

PATEL, S. and M. LATTERICH, 1998 The AAA team: Related ATPases with diverse functions. Trends Cell Biol **8:** 65-71.

PEREIRA, G. and E. SCHIEBEL, 1997 Centrosome-microtubule nucleation. J. Cell Sci. **110:** 295-300.

PERRY, M.D., C. TRENT, B. ROBERTSON, C. CHAMBLIN and W.B. WOOD, 1994 Sequenced alleles of the *Caenorhabditis elegans* sex-determination gene *her-1* include a novel class of conditional promoter mutations. Genetics **138:** 317-327.

PIPERNO, G. and M.T. FULLER, 1985 Monoclonal antibodies specific for an acetylated form of α-tubulin recognize the antigen in cilia and flagella from a variety of organisms. J. Cell Biol. **101:** 2085-2094.

PULAK, R. and P. ANDERSON, 1993 mRNA surveillance by the *Caenorhabditis elegans smg* genes. Genes Dev. **7:** 1885-1897.

RECHSTEINER, M. and S.W. ROGERS, 1996 PEST sequences and regulation by proteolysis. Trends Biochem. Sci. **21:** 267-271.

RIEDER, C.L., J.G. AULT, U. EICHENLAUB-RITTER and G. SLUDER. 1993 Morphogenesis of the mitotic and meiotic spindle: Conclusions obtained from one system are not necessarily applicable to the other, pp.183-197 in Chromosome Segregation and Aneuploidy. (Ed. Vig, B.K.) Springer-Verlag,Berlin.

ROGALSKI, T.M. and D.L. RIDDLE, 1988 A *Caenorhabditis elegans* RNA polymerase II gene, *ama-1 IV*, and nearby essential genes. Genetics **118:** 61-74.

SAMBROOK, J., E.F. FRITSCH, and T. MANIATIS (Editors), 1989 *Molecular cloning: A laboratory manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y..

SAREDI, A., L. HOWARD and D.A. COMPTON, 1997 Phosphorylation regulates the assembly of NuMA in a mammalian mitotic extract. J. Cell Sci. **110:** 1287-1297.

SAUNDERS, R.D.C., M. DO CARMO AVIDES, T. HOWARD, C. GONZALEZ and D.M. GLOVER, 1997a The *Drosophila* gene *abnormal spindle* encodes a novel microtubule-associated protein that associates with the polar regions of the mitotic spindle. J. Cell Biol **137:** 881-890.

SAUNDERS, W., V. LENGYEL and M.A. HOYT, 1997b Mitotic spindle function in *Saccharomyces cerevisiae* requires a balance between different types of kinesin-related motors. Mol. Biol Cell **8:** 1025-1033.

SAWADA, T. and G. SCHATTEN, 1988 Microtubules in Ascidian eggs during meiosis, fertilization, and mitosis. Cell Motil. Cytoskeleton **9:** 219-230.

SAWIN, K.E. and T.J. MITCHISON, 1991 Mitotic spindle assembly by two different pathways *in vitro*. J. Cell Biol **112:** 925-940.

SCHATTEN, G., 1994 The centrosome and its mode of inheritance: the reduction of the centrosome during gametogenesis and its restoration during fertilization. Dev. Biol **165:** 299-335.

SCHAUER, I.E. and W.B. WOOD, 1990 Early *C. elegans* embryos are transcriptionally active. Develop. **110:** 1303-1317.

SCHROER, T.A., 1994 Structure, function and regulation of cytoplasmic dynein. Curr. Opin. Cell Biol **6:** 69-73.

STEARNS, T., 1997 Motoring to the finish: kinesin and dynein work together to orient the yeast mitotic spindle. J. Cell Biol **138:** 957-960.

STEARNS, T. and M. KIRSCHNER, 1994 *In vitro* reconstitution of centrosome assembly and function: The central role of γ-tubulin. Cell **76:** 623-637.

STROME, S. and W.B. WOOD, 1983 Generation of asymmetry and segregation of germ-line granules in early *C. elegans* embryos. Cell **35:** 15-25.

SULSTON, J. and J. HODGKIN. 1988 Methods, pp.587-606 in The nematode *Caenorhabditis elegans.* (Ed. Wood, W.B.) Cold spring Harbor Laboratory,Cold Spring Harbor, N.Y..

SULSTON, J.E., E. SCHIERENBERG, J.G. WHITE and J.N. THOMSON, 1983 The embryonic cell lineage of the nematode *Caenorhabditis elegans.* Dev. Biol **100:** 64-119.

SULSTON, J.E. and H.R. HORVITZ, 1977 Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans.* Dev. Biol **56:** 110-156.

UPTON, C., J.L. MACEN, D.S. WISHART and G. MCFADDEN, 1990 Myxoma virus and malignant rabbit fibroma virus encode a serpin-like protein important for virus virulence. Virology **179:** 618-631.

VALE, R.D., 1991 Severing of stable microtubules by a mitotically activated protein in Xenopus egg extracts. Cell **64:** 827-839.

WANG, P.J. and T.C. HUFFAKER, 1997 Stu2p: A microtubule-binding protein that is an essential component of the yeast spindle pole body. J. Cell Biol **139:** 1271-1280.

WATERSTON, R., C. MARTIN, M. CRAXTON, C. HUYNH, A. COULSON *et al.*, 1992 A survey of expressed genes in *Caenorhabditis elegans*. Nat. Genet. 1: 114-123.

WHITE, J. and S. STROME, 1996 Cleavage plane specification in *C. elegans*: how to divide the spoils. Cell 84: 195-198.

WHITEHEAD, C.M., R.J. WINKFEIN and J.B. RATTNER, 1996 The relationship of HsEg5 and the actin cytoskeleton to centrosome separation. Cell Motility & the Cytoskeleton 35: 298-308.

WILLIAMS, B.D., B. SCHRANK, C. HUYNH, R. SHOWNKEEN and R.H. WATERSTON, 1992 A genetic mapping system in *Caenorhabditis elegans* based on polymorphic sequence-tagged sites. Genetics 131: 609-624.

WILSON, P.G., M.T. FULLER and G.G. BORISY, 1997 Monastral bipolar spindles: implications for dynamic centrosome organization. J. Cell Sci. 110: 451-464.

YANG, C.H. and M. SNYDER, 1992 The nuclear-mitotic apparatus protein is important in the establishment and maintenance of the bipolar mitotic spindle apparatus. Mol. Biol Cell 3: 1259-1267.

ZHANG, D. and R.B. NICKLAS, 1995 The impact of chromosomes and centrosomes on spindle assembly as observed in living cells. J. Cell Biol 129: 1287-1300.

ZHENG, Y., M.K. JUNG and B.R. OAKLEY, 1991 γ-tubulin is present in *Drosophila melanogaster* and *Homo sapiens* and is associated with the centrosome. Cell 65: 817-823.

ZOLLMAN, S., D. GODT, G.G. PRIVE, J.L. COUDERC and F.A. LASKI, 1994 The BTB domain, found primarily in zinc finger proteins, defines an evolutionarily conserved family that includes several developmentally regulated genes in *Drosophila*. Proc. Natl. Acad. Sci. U. S. A. 91: 10717-10721.

ZORIO, D.A.R., N.N. CHENG, T. BLUMENTHAL and J. SPIETH, 1994 Operons as a common form of chromosomal organization in *C. elegans*. Nature 372: 270-272.

IMAGE EVALUATION
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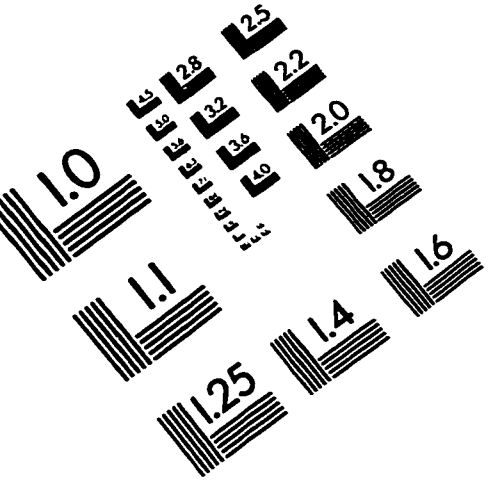

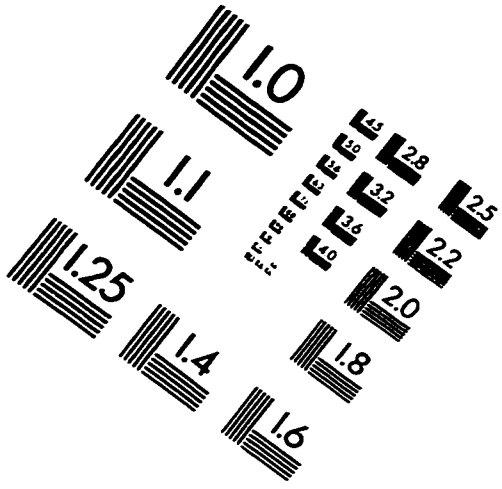

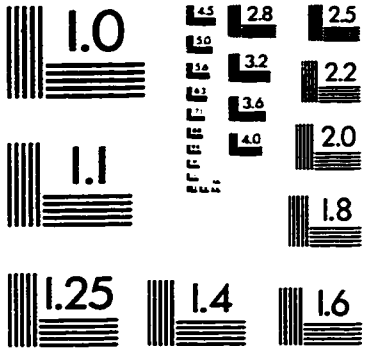

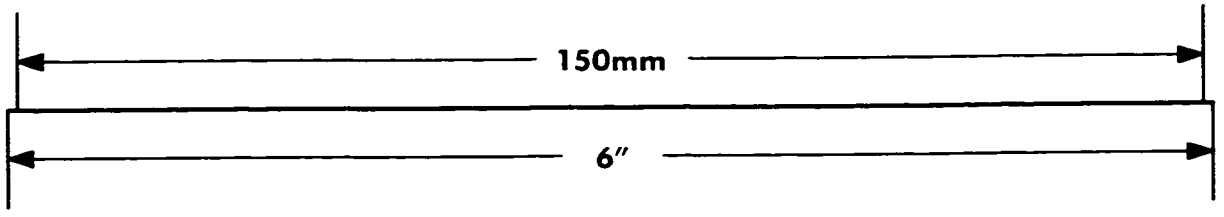

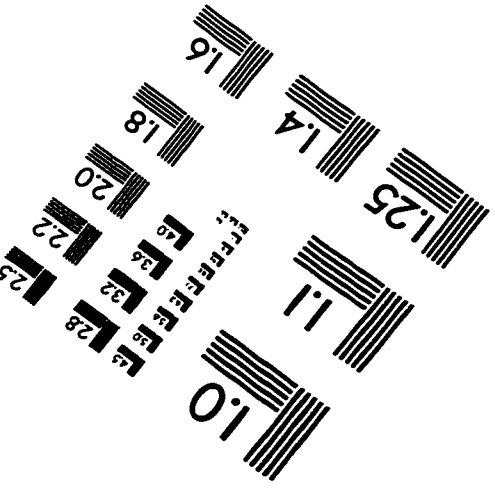

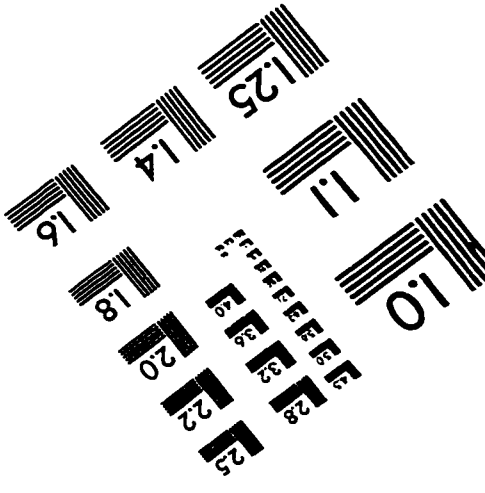

APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989