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The *pelota* locus encodes a protein required for meiotic cell division: an analysis of G_2/M arrest in *Drosophila* spermatogenesis

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SUMMARY

During *Drosophila* spermatogenesis, germ cells undergo four rounds of mitosis, an extended premeiotic G_2 phase and two meiotic divisions. In males homozygous for mutations in *pelota*, the germline mitotic divisions are normal, but the cell cycle arrests prior to the first meiotic division; *pelota* males are therefore sterile. Chromosomes begin to condense in these mutants, but other meiotic processes, including nuclear envelope breakdown and spindle formation, do not occur. The arrest phenotype closely resembles that of mutations in the *Drosophila* cdc25 homolog *twine*. Although meiosis is blocked in *pelota* and *twine* homozygotes, spermatid differentiation continues. *pelota* is also required for patterning in the eye and mitotic divisions in the ovary. We have cloned the *pelota* locus and show it encodes a 44×10^3 M_r protein with yeast, plant, worm and human homologs.

Key words: cell cycle, meiosis, cdc25, chromosome condensation, *Drosophila*, *pelota*, spermatogenesis

INTRODUCTION

Meiosis plays a central role in gametogenesis, producing the haploid germ cells that become sperm and eggs. The specialized meiotic chromosome movements have been described in great detail. In contrast, the regulation of meiosis, particularly in higher eukaryotes, is poorly understood. We focus here on the initiation of meiotic divisions in *Drosophila* males, since spermatogenesis is well suited to such studies. All stages of meiosis are present in the testis, whereas in females meiosis is not completed during oogenesis. In addition, testis contents can be easily examined using light microscopy, allowing both cells undergoing meiosis and the products of meiosis to be analyzed.

Drosophila spermatogenesis begins with the generation of spermatogonia from stem cells at the tip of the testis. Each spermatogonium, encased within a cyst of two somatic cells, undergoes four rounds of mitotic division to generate 16 spermatocytes. After a premeiotic G_2 phase lasting more than 2 days, two rounds of meiotic division yield a cyst of 64 spermatids. Spermiogenesis, the differentiation of the spermatids, involves development of specialized head and tail structures, followed by sperm individualization, coiling and release into the seminal vesicle (reviewed in Fuller, 1993).

Genes that regulate the first and second meiotic divisions (meiosis I and II) in *Drosophila* spermatogenesis have recently been identified. The *twine* (*twn*) locus is required for meiosis I in males; a loss-of-function mutation in *twine* results in recessive male sterility (Alphey et al., 1992; Courtot et al., 1992). Spermatogonia and spermatocytes are produced, but no meiotic divisions occur and no mature sperm are formed. The twine protein is a homolog of the *S. pombe* phosphatase cdc25, a universal cell cycle regulator. In eukaryotes such phosphatases activate the $p34^{cdc2}$-cyclin kinase complex in meiosis and mitosis by removing inhibitory phosphates from $p34^{cdc2}$ (Dunphy and Kumagai, 1991; Gautier et al., 1991; reviewed by Coleman and Dunphy, 1994).

twine is required only for meiosis; a second *Drosophila* cdc25 homolog, *string*, regulates mitosis (Edgar and O'Farrell, 1990). The role of *string* is to control passage through the mitotic G_2/M transition. Similarly, *twine* controls the transition from the extended G_2 phase that constitutes the meiotic prophase to the onset of the first meiotic division. By analogy to the role of *string* in mitosis, this control point in meiosis is referred to as a G_2/M transition (White-Cooper et al., 1993).

The *roughex* (*rux*) gene is a dose-dependent regulator of meiosis II (Gönczy et al., 1994). In *roughex* hemizygotes, haploid germ cells attempt an extra second meiotic division, while in flies carrying two extra copies of *roughex* the second meiotic division does not occur. Lowering the gene dosage of either *cyclin A* or *twine* rescues the *roughex* meiotic phenotype, suggesting that *roughex* acts through the $p34^{cdc2}$-cyclin A kinase complex. Gönczy and colleagues propose that *roughex* functions before meiosis I to activate a target necessary for meiosis II (Gönczy et al., 1994). *roughex* also plays a role in the regulation of a mitotic cycle. In the *Drosophila* eye, *roughex* is required to inhibit cell cycle progression in and just anterior to the morphogenetic furrow (Thomas et al., 1994). Thus, *roughex* acts as a negative regulator of the cell cycle in both meiosis and mitosis.

We originally identified the *pelota* (*pelo*) locus in a P

element mutagenesis screen for male-sterile mutants (Castrillon et al., 1993). Here we analyze the *pelota* meiotic arrest point. We show that *pelota*, like *twine*, is required prior to the first meiotic division for spindle formation and nuclear envelope breakdown, but not chromosome condensation. We also show that *pelota*, like *roughex*, affects patterning in the eye. Lastly, we have isolated genomic and cDNA clones for *pelota* and demonstrate that it encodes a protein with wide evolutionary conservation.

MATERIALS AND METHODS

Drosophila strains

All crosses were performed at 25°C on yeasted cornmeal molasses agar. The *twine*^{HB5} mutation (Alphey et al., 1993; Courtot et al., 1993) and all *pelota* alleles were balanced with CyO; *rux*^{9} (Gönczy et al., 1994) was balanced with FM7c. *pelota* homozygotes are fully viable at both 18°C and 25°C, but have reduced viability in crowded conditions. Germlineless flies were generated as the progeny of females homozygous for the *oskar*^{301} mutation. Flies lacking gonads (due to transformation of the fifth abdominal segment) were the progeny of *iab4*^{166}/*iab4*^{302} trans-heterozygotes. All genetic mutations and balancers are described and referenced by Lindsley and Zimm (1992), except as noted.

Nucleic acid manipulations and analysis

Standard protocols were performed as described (Sambrook et al., 1989). Genomic DNA from *pelo*^{1} adult homozygotes was used for isolation of DNA flanking the P element insertion (Ashburner, 1989). The DNA was digested to completion with *Xba*I and *Nhe*I, circularized by self-ligation and transformed into competent XL1-Blue *E. coli*. Colonies bearing the desired plasmid were selected by kanamycin resistance. To isolate *pelota* clones, both a *Drosophila* genomic library (EMBL3 vector) provided by J. Tamkun and a testis cDNA library provided by T. Hazelrigg (Bluescript vector) were screened with probes generated in a random priming reaction (Megaprime, Amersham) from the rescued plasmid.

Sequencing was performed with a Amersham Sequenase kit; both nested deletions and directed subcloning into M13 were used to generate subclones. Sequences were assembled using Assemblylign (IBI/Kodak); homology searches used the BLASTP and TBLASTN programs (Altschul et al., 1990). A primer from the end of the P element was used to determine the transposon insertion site in the *pelo*^{1} mutation. In vitro transcription/translation reactions used the Promega TNT reticulocyte lysate system. For northern analysis, 2 μg samples of poly(A)$^{+}$ RNA were subjected to electrophoresis in 1% agarose gels containing 7% formaldehyde.

Remobilization of P element to generate new alleles

The *pelo*^{1} P[*ry*$^{+}$] chromosome was brought together with a transposase source by crossing *pelo*^{1}/*CyO* ; *ry*$^{-}$/*ry*$^{-}$ flies to flies carrying the P[*ry*$^{+}$, Δ*2-3*] transposase on the third chromosome (Robertson et al., 1988). In the next generation, *ry*$^{-}$ derivatives of the *pelo*^{1} chromosome were segregated away from the P[*ry*$^{+}$, Δ*2-3*] chromosome and selected to establish lines. Molecular analysis of several fertile excisant lines demonstrated precise excision of the P element (data not shown), confirming that the transposon insertion is responsible for the *pelo*^{1} phenotype. Among the 241 *ry*$^{-}$ lines, 17 are completely blocked in meiosis, whereas in eight some aberrant meioses occur (see Results). The phenotypes of each class of mutations in *trans* to a deficiency was similar to that of homozygotes for that class.

To screen *ry*$^{-}$ lines for large deletions, genomic DNA from homozygotes was digested with *Eco*RI and *Sph*I, then subjected to Southern analysis. Southern blots were probed with a 4.5 kb *Eco*RI fragment containing *pelota* exons 5′ to the transposon insertion and a 1.5 kb *Sph*I fragment containing exons 3′ to the transposon insertion.

Germline transformation

A 15 kb *pelota* genomic sequence (G15) was cloned into the pCaSpeR4 *w*$^{+}$ P element vector (Pirrotta, 1988), then co-injected with a plasmid containing *P*[*ry*$^{+}$, Δ2-3] into *w*^{1118} embryos using standard protocols (Spradling, 1986). Genetic crosses were carried out to generate *pelo*^{1} homozygotes carrying one copy of the *P*[*w*$^{+}$, G15] third chromosome.

Analysis of live and fixed testes contents

All males analyzed were less than 24 hours old. Unfixed testes contents were examined using previously described methods (Ashburner, 1989); Hoechst 33342 (Sigma) was added to testis buffer at 0.5 μg/ml. Fluorescent studies were carried out using a Zeiss Axiophot microscope. Testes fixation and indirect immunofluorescence followed the method of Cenci et al. (1994). Tubulin staining was done using Sigma α-tubulin monoclonal T9026 clone DM1 at 1:500. The anti-lamin monoclonal antibody T47 was the generous gift of David Glover and was used neat. Cy3-conjugated donkey anti-mouse antisera from Jackson Labs was used at a 1:200 dilution. 5-bromodeoxyuridine (BrdU) labeling followed the method of Gönczy (1995); anti-BrdU monoclonal (Becton Dickinson; used at 1:10) was provided by Dennis McKearin.

Analysis of ovaries and eyes

Newly eclosed females were incubated for 2 days at 25°C in wet yeasted vials before dissection. Ovaries were removed in phosphate-buffered saline (PBS), fixed for 5 minutes in PBS containing 4% paraformaldehyde and 0.5 μg/ml Hoechst 33342, washed in PBS, then mounted in PBS containing 25% glycerol.

To visualize eye defects, adult flies were subjected to critical-point drying, mounted, then coated with palladium before analysis in the scanning electron microscope.

RESULTS

Strong *pelota* alleles block meiotic divisions but not spermiogenesis

In males homozygous for *pelo*^{1}, the cell cycle in spermatocytes arrests either just before or very early in the first meiotic cell division. The apical regions of a testis from a *pelo*^{1} homozygote appear normal, filled with visibly wild-type mitotic and growth phase cysts. The rest of the testis, which usually contains 64-cell spermatid cysts, is instead filled with 16-cell cysts (Fig. 1B). The spermatocytes attain their mature size. Furthermore, they undergo a premeiotic S phase, as evidenced by the incorporation of bromodeoxyuridine into 16-cell cysts in *pelota* testes (data not shown). However, meiotic figures, which can be easily recognized in squashed preparations of wild-type testes (Fig. 1A, open arrow), are never seen.

While meiotic divisions do not occur in *pelo*^{1} spermatocytes, other aspects of spermatogenesis continue, resulting in 4N spermatids. In wild-type testes, the round, dark nebenkern is the first major cytoplasmic structure to form after meiosis (Fig. 1A, arrowhead). It is made up of mitochondria and serves as a marker for postmeiotic cytoplasmic development (Fuller, 1993). Germline cells in *pelo*^{1} testes develop into 4N spermatids containing nebenkerne (Fig. 1B, arrowhead). As nebenkerne form the *pelo*^{1} nuclei shrink in size by one third to one half. In a small number of 4N *pelo*^{1} spermatids additional postmeiotic cytoplasmic differentiation occurs, resulting in

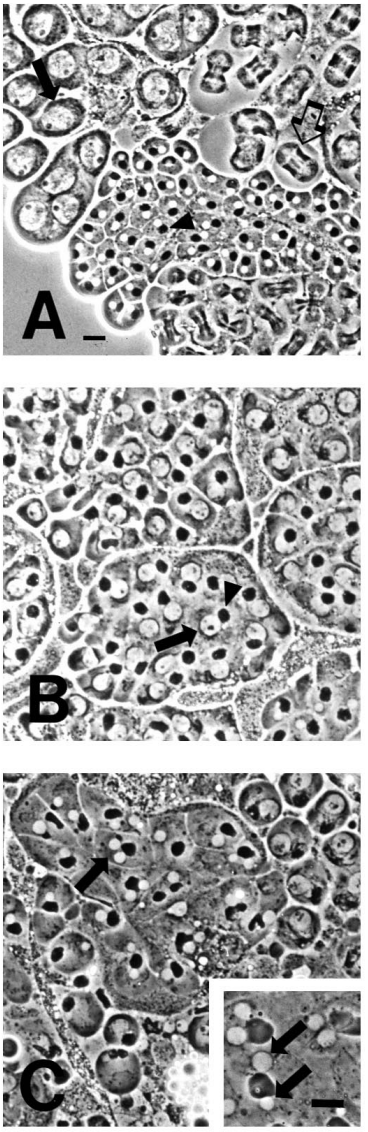


Fig. 1. Meiosis defects in *pelota* spermatogenesis. Photographs are of unfixed testis contents viewed by phase-contrast microscopy. All micrographs except insert are at the same magnification; bar represents 10 μm. (A) Wild-type testis contents. Spermatocytes (closed arrow), cells in meiosis I (open arrow) and onion stage spermatids (arrowhead) with dark nebenkerne and pale nuclei can be seen. (B) *pelo*^{1} homozygote 16-cell cysts. The 4N spermatids each contain a nucleus (arrow) and nebenkern (arrowhead). Due to their 4N chromosomal content, the spermatid nuclei are abnormally large. (C) *pelota*^{weak} homozygote cysts. In these mutant spermatids, two nuclei are often found in one cell (arrow), indicating cytokinesis has failed. In some cells, the nuclei are of different sizes (insert, arrows), a sign of unequal chromosome complements.

spermatid head and tail structures. The content of testes from flies *trans*-heterozygous for *pelo*^{1} and *Df*(2L)30A;C, a deficiency for the *pelota* region, are indistinguishable from those of *pelo*^{1} homozygotes. Thus, *pelo*^{1} is a strong allele and possibly a null.

The *pelo*^{1} testis phenotype – limited spermatid development in the absence of meiosis – closely resembles that of a mutation in the cell cycle gene *twine* (Alphey et al., 1992; Courtot et al., 1992). Although the proportion of 4N spermatids with head and tail structures is greater for *twine*^{HB5} than for *pelo*^{1}, other *pelota* alleles, generated by remobilization of the *pelo*^{1} P element (see Materials and Methods) have a phenotype identical to that of *twine*^{HB5}, the sole *twine* allele. The tails of 4N spermatids elongate significantly (Fig. 2E′, arrow) and the nuclei form wedge-shaped heads (Fig. 2E′, arrowhead). The heads are larger than in wild type, fail to cluster normally and are not attached to the tails.

A weaker class of *pelota* mutations, in which some meioses, albeit aberrant, occur, was also generated by mobilization of the *pelo*^{1} P element. Spermatids from weak *pelota* homozygotes commonly contain two or four nuclei in a single cell (Fig. 1C, arrow), indicating a failure of cytokinesis in one or both meiotic divisions. Variations in nuclear size, reflecting defects in chromosome segregation (Gonzalez et al., 1989), are also seen (Fig. 1C insert, arrows). Defects within a given cyst are usually heterogeneous, with some cells displaying meiotic defects while others appear wild type or are arrested before meiosis I.

In summary, during spermatogenesis, the mitotic divisions and subsequent growth phase appear normal in *pelota* mutants. The first apparent defect is a failure of meiotic cell division, resulting in the accumulation of arrested spermatocytes. The cell cycle arrest occurs early in meiosis, as no metaphase or anaphase figures can be seen. Some aspects of spermiogenesis continue, resulting in 4N spermatids with head and tail structures, a phenotype identical to *twine*^{HB5} homozygotes.

pelota is required in meiosis for spindle formation and nuclear envelope breakdown, but not for chromosome condensation

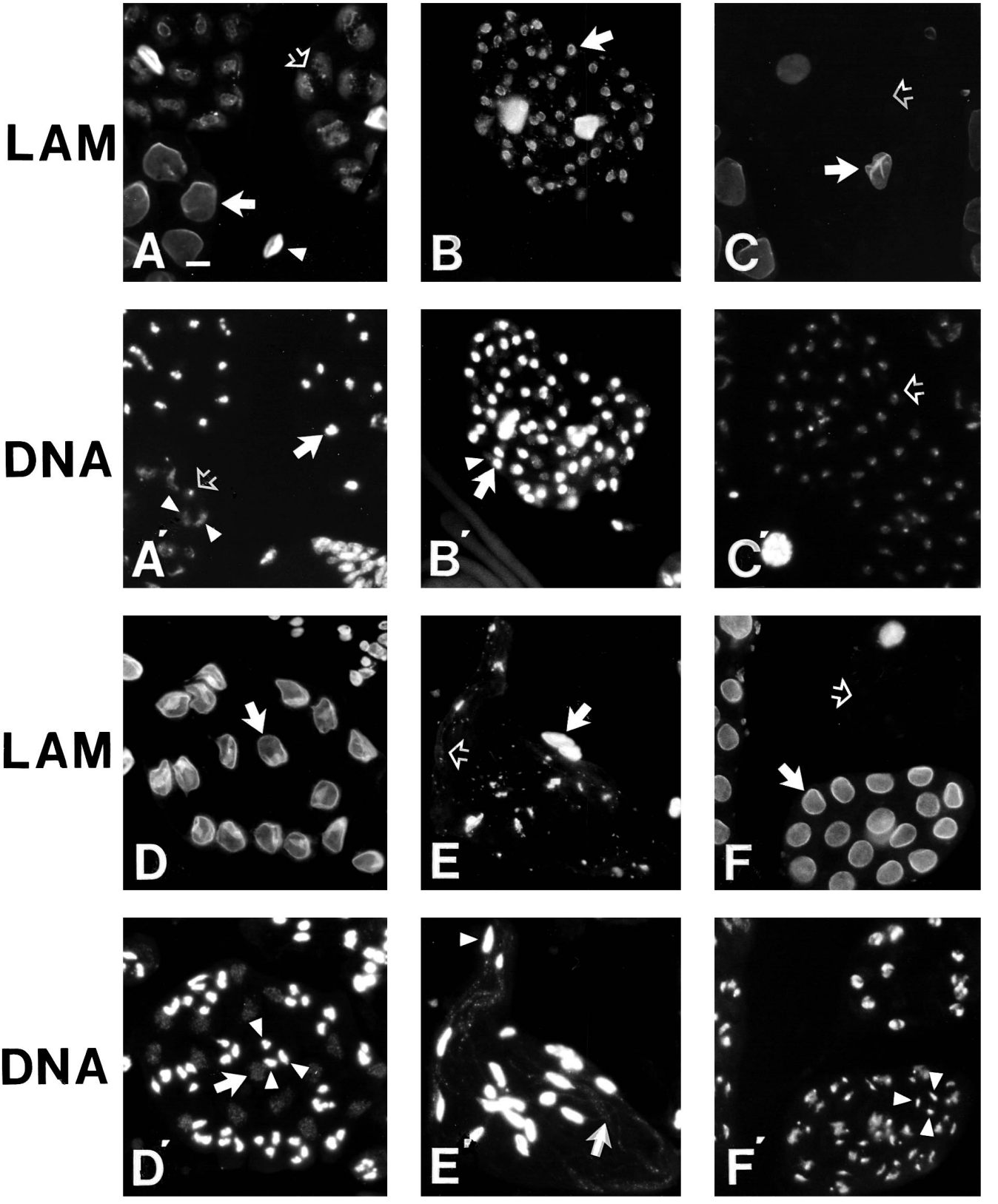
To define the arrest point of *pelota* in greater detail, we analyzed three landmark events occurring at meiosis I: chromosome condensation, spindle formation and nuclear envelope breakdown. Our analysis of the *pelota* mutant phenotype was facilitated by similar studies on *twine* (White-Cooper et al., 1993) and by a recent detailed description of chromosome and microtubule movements in spermatogenesis (Cenci et al., 1994).

During the spermatocyte growth phase leading up to meiosis, the two autosomes in each spermatocyte nucleus are visible as diffuse, mesh-like structures (Fig. 2A′, arrowheads), while the sex chromosomes are associated with the nucleolus and often appear more punctate (Fig. 2A′, open arrow; Cenci et al., 1994). Chromosome condensation occurs rapidly late in prophase; chromosomes condense on the periphery of the nucleus, compacting until they appear as dots. After the chromosomes have fully condensed they move inward to the center of the nucleus, appearing as a single mass (Fig. 2A′, solid arrow; Cenci et al., 1994). In arrested *pelota* spermatocytes, the chromosomes partially condense, but never move away from the nuclear periphery (Fig. 2D′, arrowheads; see Fig. 2D for outline of nuclear envelope). The same levels of DNA condensation are observed in arrested *twine* spermatocytes (Fig. 2F′, arrowheads; White-Cooper et al., 1993). [Differences in nuclear size between Fig. 2D and Fig. 2F are due to variability in the squashing protocol used to generate the samples.]

A second critical meiotic event is nuclear envelope breakdown. The nuclear lamins are associated with the nuclear envelope in mature spermatocytes (Fig. 2A, arrow). During

meiosis, the nuclear envelope breaks down and nuclear lamins disperse, forming diffuse clouds around the chromatin (Fig. 2A, open arrow); after meiosis the nuclear lamins associate with the reformed nuclear envelope (Fig. 2B, arrow). As spermatids continue to develop, nuclear lamin staining is lost (Fig. 2C,C′, open arrows).

The nuclear envelope does not break down in arrested *pelota* spermatocytes. In strong *pelota* alleles, heavy nuclear staining



is seen in spermatocytes whose condensed chromosomes indicate they have just arrested in late meiotic prophase. Nuclear lamin staining is still present (Fig. 2D, arrow) after the nebenkerne form (Fig. 2D′, arrow). In *pelota* 4N spermatids that develop shaped head structures and elongate tails, lamin staining becomes fragmented and disappears (Fig. 2E, open arrow). The fate of nuclear lamins in *twine* resembles that in *pelota*: the nuclear envelope remains intact at the initial arrest (Fig. 2F, solid arrow), the nuclear lamins are then degraded in conjunction with sperm head and tail formation (Fig. 2F, open arrow).

Formation of the spindle is a third event central to meiotic cell division. During the spermatocyte growth phase, microtubules form a diffuse cytoplasmic network; this network persists until the cells mature. Late in meiotic prophase the centrosomes separate and move towards the poles (Fig. 3A, arrows); after they reach the poles the cytoplasmic tubulin network breaks down and a spindle is formed (Fig. 3B, arrow). In arrested *pelota* spermatocytes, the microtubules remain in the cytoplasm (Fig. 3C). The centrosomes separate (Fig. 3C, arrows), but don't complete their migration and nucleate no significant asters; a spindle is never observed. An identical phenotype is reported for *twine* mutants (White-Cooper et al., 1993).

Our studies indicate that spermatogenesis in *pelota* and *twine* homozygotes is normal until late meiotic prophase, at which point the cell cycle arrests. No spindles form in these mutants and the nuclear lamins do not disperse or degrade until significant spermatid differentiation has occurred. Not all meiotic events are blocked, however, since some chromosome condensation is seen. Cytoplasmic structures that in wild type form after meiosis – such as nebenkerne, shaped heads and elongated tails – develop in these 4N spermatids.

Fig. 2. Nuclear envelope breakdown and chromosome condensation in spermatogenesis. All photographs are of fixed testes contents. The images are paired, showing staining with either antibodies to nuclear lamin (A,B,C,D,E,F) or the DNA-binding dye Hoechst (A′,B′,C′,D′,E′,F′) for the same region. Magnification is the same for all panels; bar represents 10 μm. (A,A′) Wild-type cysts. The nuclear envelope (shown in A) is intact in mature spermatocytes (closed arrow). After nuclear envelope breakdown in meiosis I, nuclear lamins form clouds around the condensed meiotic chromosomes (open arrow). The cells in the upper left corner are in meiotic interphase. In the lower right corner are comet-stage spermatids – named for their elongated tails – in which the nuclear lamins have been degraded. The somatic cyst cell nucleus associated with the spermatids provides a positive control for staining (arrowhead). The autosomes in the mature spermatocytes (shown in A′) are diffuse (arrowheads), while the sex chromosomes are more punctate (open arrow). In the meiosis I cells, some chromosomes are aligned on the metaphase plate (closed arrow) while others have begun to separate in anaphase. (B,B′) Part of a wild-type onion stage spermatid cyst. The nuclear envelope (shown in B) has reformed in these post-meiotic cells (arrow). The two large, bright nuclei are those of the somatic cyst cells. Spermatid nuclei with condensed DNA (shown in B′, arrow) and nebenkerne (arrowhead) can be seen. The nebenkerne stain is due to mitochondrial DNA. (C,C′) Wild-type early comet stage spermatid cyst. The nuclear lamins in these spermatids (shown in C) have been degraded as the heads mature (open arrow); staining of the somatic cyst cell persists (arrow). (D,D′) *pelo*^{1} homozygote cyst of onion stage 4N spermatids. The nuclear envelopes (shown in D) are intact in these cells. The chromosomes in these arrested cells (shown in D′) are partially condensed (arrowheads); nebenkerne have formed (arrow). (E,E′) *pelo*^{2} homozygote cyst of late comet-stage 4N spermatids. The nuclear envelopes (shown in E) of the somatic cyst cells are still intact (arrow), while those of the 4N spermatids are fragmented or gone (open arrow). The characteristic wedge-shaped sperm heads (shown in E′) have begun to form (arrowhead); these 4N heads are much larger than those in wild type. Mitochondria associated with spermatid tails allow these elongated structures to be visualized (arrow). (F,F′) *twine* homozygote cysts. At the initial spermatocyte arrest point in *twine* the nuclear envelope (shown in F) is still intact (closed arrow); as the spermatid heads continue to develop the nuclear lamins are degraded (open arrow). The partially condensed chromosomes (shown in F′) in the *twine*-arrested spermatocytes indicate they are in late prophase (arrowheads). Differences in nuclear size between D and F are due to variability in the squashing protocol used to generate the samples.

pelota is active outside the testis

In flies homozygous for strong *pelota* alleles, the eyes are often rough, with disordered ommatidial arrays and bristles (Fig. 4, compare A and B). In addition, the eyes of *pelota* homozygotes are up to 30% smaller than those of heterozygous siblings. The severity of the eye defects varies between flies and some homozygotes have eyes that appear wild type. These results demonstrate that *pelota* is required for *Drosophila* eye development.

Defects that we observed in females homozygous for strong *pelota* mutations suggest a role for *pelota* in mitotic growth. Such females are only weakly fertile – fewer than half lay eggs. The ovaries of *pelota* homozygotes are very small. The mitotic zone of the germarium appears disorganized and often contains degenerating cells. Later stages of oogenesis are also affected, as degenerating egg chambers of all stages are observed (data not shown). These defects, like those in the eye, are variable.

Cloning and germline rescue of *pelota*

Plasmid sequences in the P element used to generate the *pelo*^{1} allele allowed cloning of genomic DNA adjacent to the element's insertion site (Mlodzik and Hiromi, 1992). We used this approach to recover a 1.2 kb genomic insert. This fragment was used to probe a genomic phage library and overlapping genomic phage clones were isolated. Restriction enzyme analysis defined 20 kb of DNA surrounding the P element insertion (Fig. 5A).

The 15 kb genomic insert of phage λG-3 (Fig. 5E) was subcloned into the germline transformation vector pCaSpeR4 (Pirrotta, 1988) and the resulting plasmid DNA was used to transform w^{1118} flies. A transgenic line containing the sequences inserted on the third chromosome rescues all *pelota* phenotypes.

Genomic probes from λG-3 detect transcripts of 0.5, 2.0 and 2.1 kb. The 0.5 and 2.1 kb transcripts are fully contained within λG-3, while the 2.0 kb transcript is also detected by probes outside λG-3. The P element is inserted into the center of the 2.1 kb transcript (Fig. 5). Whereas in *pelo*^{1} the 0.5 kb transcript is of wild-type size, the 2.1 kb trancript appears truncated (see below). We therefore believe that the 2.1 kb transcript represents *pelota*.

The *pelota* transcript was present in all developmental stages examined, but was most abundant in 0-2 hour old embryos and adults (Fig. 6B, lanes 1 and 7). The larval transcript appeared larger in some, but not all, northern blot experiments (Fig. 6B; data not shown). The 2.1 kb transcript was present in the germlineless progeny of *oskar*^{301} flies (Fig. 6A, lane 3) and in

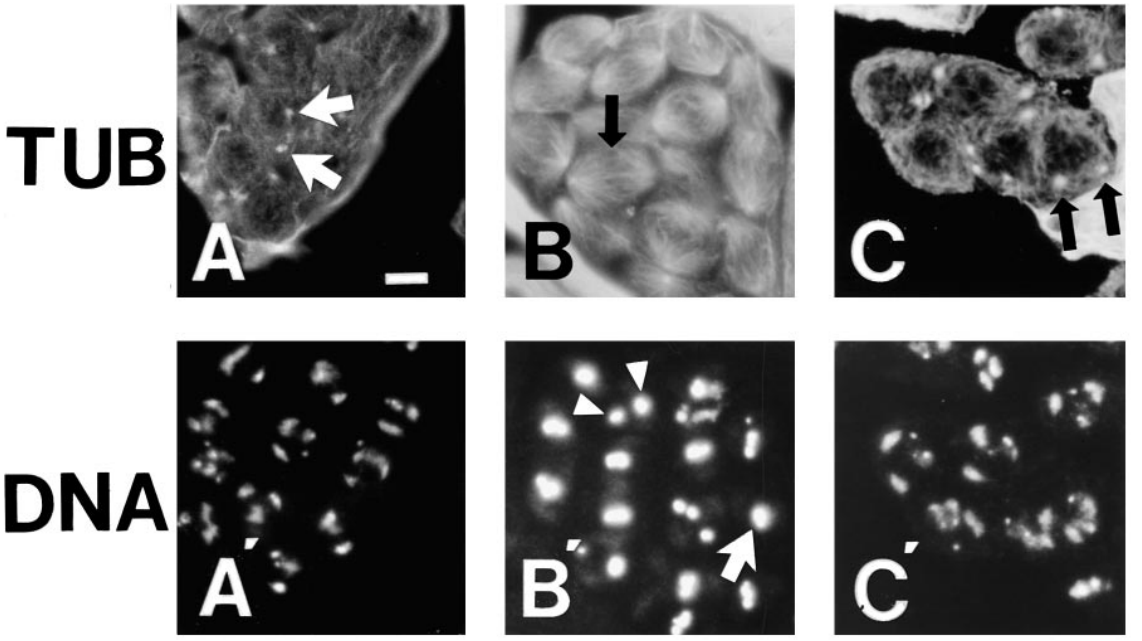


Fig. 3. Spindle formation and chromosome condensation in spermatogenesis. All pictures are of fixed testes contents. The images are paired and show staining with either antibodies to tubulin (A,B,C) or the DNA-binding dye Hoechst (A′,B′,C′) for the same region. All micrographs are at the same magnification; bar represents 10 μm. (A,A′) Wild-type spermatocytes in late meiotic prophase. Tubulin (shown in A) still forms a cytoplasmic network, but the centrosomes have separated (arrows). The chromosomes of these spermatocytes (shown in A′) have condensed significantly, but have not yet moved away from the nuclear periphery. (B,B′) Wild-type spermatocytes in metaphase and anaphase of meiosis I. The spindles of these spermatocytes (shown in B) are fully formed (arrow). The chromosomes of these cells (shown in B′) are still at the metaphase plate (arrow) or just beginning to separate (arrowheads). (C,C′) Arrested spermatocytes from *pelo*^{1} homozygote. The tubulin network in these cells (shown in C) resembles that of the mature spermatocytes. The centrosomes have separated but do not nucleate asters (arrows). The chromosomes of the arrested cells (shown in C′) have partially condensed.

iab^4 flies that lack a gonad (data not shown). Thus the *pelota* transcript is not restricted to the germline or gonad. In *pelo*^{1} adults both genomic and cDNA probes detected only a 1.1 kb transcript, presumably a truncation of the 2.1 kb transcript due to insertion of the transposon (Fig. 6A, lane 4).

Molecular analysis of *pelota* revertants

All revertant lines were analyzed to determine which, if any, contained deletions of *pelota* exons. This analysis revealed that, in revertant lines *pelo*^{2} and *pelo*^{3}, exons 3′ of the P element insertion were deleted (Fig. 5D). Both of these pelota alleles fall in the first revertant class, with a phenotype identical to *twine*. In the other revertant lines of the first class imprecise excision left several kilobases of P element sequences at the insertion site. The weak pelota alleles of the second class also contain residual P element sequences.

pelota cDNA isolation and sequence analysis

A cDNA for the 2.1 kb *pelota* transcript was isolated from an adult testis library kindly provided by T. Hazelrigg. Sequencing of the 2028 bp cDNA revealed a single long open reading frame (ORF) following sequences that match the *Drosophila* translational start consensus sequence (Cavener, 1987). The encoded protein is 395 amino acids in length and has a predicted M_r of 44×10^3 (Fig. 7). When the 2.1 kb cDNA was used in an in vitro transcription/translation reaction, a protein with an electrophoretic mobility corresponding to an apparent M_r of 49×10^3 was generated (data not shown), confirming the first methionine as the translational start site.

Sequencing of genomic DNA surrounding the P element insertion site in *pelo*^{1} revealed that the transposon resides in an intron; the insertion site lies 572 nucleotides 5′ of an exon which begins at amino acid 319. Northern analysis with probes made from 3′ *pelota* exons do not detect the 1.1 kb shortened transcript (data not shown). Thus the highly conserved coding region following amino acid 319 (see below) is almost certainly absent in *pelo*^{1}.

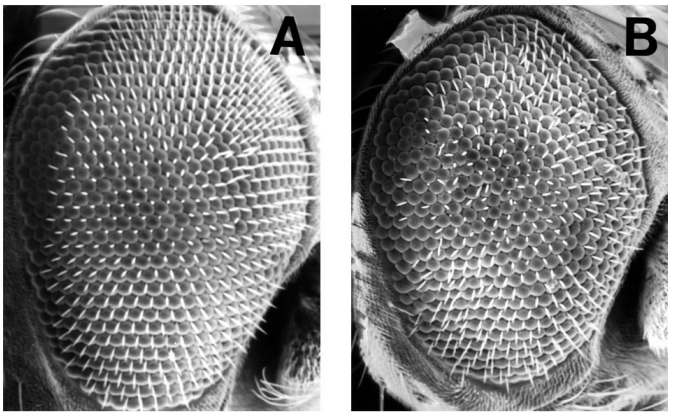


Fig. 4. *pelota* eye defects. The images are scanning electron micrographs of equal magnification. (A) *pelo*^{1}/CyO. Note the well-ordered ommatidia and bristles. (B) *pelo*^{1} homozygote. The eye is substantially smaller, with rough and disordered ommatidia and bristles. The *pelo*^{1} eye phenotype is variable.

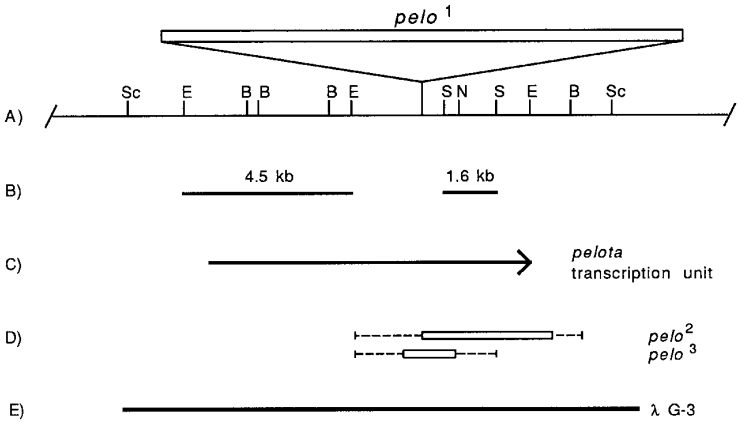


Fig. 5. Schematic diagram of the *pelota* region. (A) Restriction enzyme map of the *pelota* region (*Sac*I (Sc), *Eco*RI (E), *Bam*HI (B), *Sph*I (S), *Nhe*I (N)). The *pelo*[1] P element is drawn to scale. (B) The 4.5 kb *Eco*RI fragment and 1.6 kb *Sph*I fragment used to analyze the revertant lines. (C) Genomic extent of the *pelota* transcription unit, as determined by northern analysis and hybridization of the *pelota* cDNA to genomic DNA. The direction of transcription was determined by hybridization of 5′ and 3′ cDNA fragments to genomic DNA. Intron/exon boundaries of *pelota* are not all known, but at least two introns must exist based on the cDNA hybridization pattern. (D) Genomic deletions in revertant alleles. Boxes represent sequences known to be deleted. Error bars represent fragments which are polymorphic in revertant lines; a portion of these fragments are deleted. (E) The 15 kb region contained in the λ G-3 phage (G15) used to construct the *pelota* transgene.

pelota homologs in yeast, worms, humans and plants

We compared the pelota protein sequence to protein sequences in the NCBI database using the BLASTP program (Altshul et al., 1990). This search resulted in two significant matches: *S. cerevisiae* DOM34 and *C. elegans* R74.6. Dom34 was described by Lalo and colleagues in their analysis of sequences duplicated between yeast chromosomes 3 and 14 (Lalo et al., 1993) and is 33% identical to pelota with only one gap (Fig. 8). R74.6 was identified in the *C. elegans* genome sequencing effort; it is 58% identical to pelota, with no gaps and an identically positioned start methionine (Fig. 8). All three proteins feature a potential nuclear localization sequence in the N terminal half (Fig. 8, asterisks), as well as an acidic domain at the carboxyl terminus.

Searches of nucleotide databases translated in six frames using the PBLASTN program revealed three cDNA expressed sequence tags (ESTs) encoding predicted proteins with high identity to pelota. Two *H. sapiens* polypeptide sequences, 110 and 93 amino acids in length, and a 96 amino acid *A. thaliana* polypeptide are 70%, 53% and 55% identical to pelota, respectively (Fig. 8). The two human sequences are non-overlapping, but may represent portions of a single pelota homolog, since one contains the conserved nuclear localization signal and the other the carboxyl terminal acidic domain.

DISCUSSION

Control points in spermatogenesis

Spermatogenesis in *pelota* homozygotes appears wild type until spermatocytes mature; at this point, the cell cycle arrests, blocking the meiotic divisions. Spermatid differentiation continues in the absence of meiosis, however, resulting in 4N spermatids with head and tail structures. The fraction of cysts containing elongated spermatids is comparable in wild-type, *twine* and most strong *pelota* alleles; fewer such cysts are seen in *pelo*[1] testes. The reason for this difference is not known. Nevertheless, the continued differentiation of 4N spermatids after meiotic failure suggests that *pelota* is required only for meiotic cell division and not for other aspects of spermatogenesis.

The *Drosophila* cdc25 homolog *twine* has a spermatogenesis phenotype identical to that of *pelota*. Three X-linked male-sterile mutations, *ms(1)413*, *ms(1)682* and *ms(1)RD11*, which are probably allelic to one another, have a phenotype similar to *pelota* and *twine* when analyzed by phase contrast microscopy, but have not been analyzed by immunocytochemistry (Lifschytz and Hareven, 1977). Together with *twn*[HB5] and the strong *pelota* alleles, these mutations define a control point in spermatogenesis specific for meiotic cell cycle regulation.

A more global spermatogenesis control point, required for both meiotic cell division and spermatid differentiation, has also been identified (Fuller, 1993). Males homozygous for mutations in *spermatocyte arrest* (*sa*), *always early* (*aly*), *meiosis I arrest* (*mia*) and *cannonball* (*can*) arrest at this earlier control point. This global block results in testes filled with 16-cell cysts of arrested spermatocytes which do not undergo further differentiation.

Analysis of the meiotic arrest in *pelota* and *twine*

In arrested *pelota* and *twine* spermatocytes no spindles form

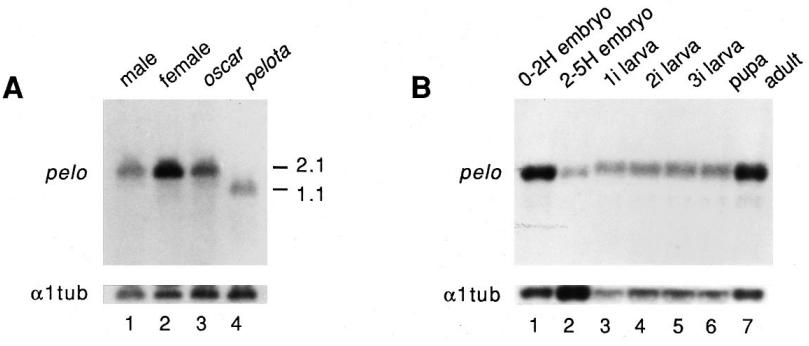


Fig. 6. Northern analysis of *pelota* region. Autoradiograph of northern blot probed with *pelota* cDNA, then stripped and reprobed with α1 tubulin to control loading. Each lane was loaded with 2 μg of poly(A)$^+$ RNA. The *pelota* transcript sizes are indicated in kilobases to the right of the figure in A. (A) Lane 1, wild-type males; lane 2, wild-type females; lane 3, progeny of *oscar*[301] females – these flies lack a germline; lane 4, *pelo*[1] homozygotes. (B) All samples from wild-type flies. Lane 1, embryos 0-2 hours old; lane 2, embryos 2-5 hours old; lane 3, first instar larvae; lane 4, second instar larvae; lane 5, third instar larvae; lane 6, pupae; lane 7, adult females.

and the nuclear envelope remains intact, yet the chromosomes partially condense. Thus these genes are required for meiotic spindle formation and nuclear envelope breakdown, but not for chromosome condensation. It was previously reported that *twine* was not required for nuclear envelope breakdown in meiosis (White-Cooper et al., 1993). We show here that in both *pelota* and *twine* the nuclear lamins remain associated with a spermatocyte nuclear envelope at the initial cell cycle arrest and are only degraded later, as the spermatid heads develop. The nuclear lamins are also degraded in wild-type spermatids as they mature; the loss of lamin staining in *pelota* and *twine* mutants is therefore probably due to the onset of the developmental program for shaping the sperm head.

It is possible that the meiotic failure observed in *pelota* and *twine* testes results from premature activation of spermatid differentiation programs that then block meiotic division. Such a model was advanced to explain a similar phenotype observed in the *Drosophila hydei ms(3)HB366 heterochron* mutation (Hackstein, 1991; Fuller, 1993). This explanation seems less plausible for *pelota* and *twine*, since we see in these mutants significant numbers of spermatocytes that have arrested in the meiotic prophase, but have not yet formed postmeiotic structures. The biochemical evidence demonstrating that cdc25 activates the cell cycle apparatus by dephosphorylating $p34^{cdc2}$ (Dunphy and Kumagai, 1991; Gautier et al., 1991) also supports an active role for *twine* in the initiation of meiotic cell divisions. It therefore seems likely that meiotic cell cycle arrest is the primary defect in *pelota* and *twine*, while continued spermatid development is due to the parallel nature of differentiation programs in spermatogenesis.

During *Drosophila* spermatogenesis several developmental processes commonly function in parallel (Lifschytz and Hareven, 1977). For instance, the mitochondrially derived nebenkern can elongate in the absence of a functional flagellar axoneme (Hoyle and Raff, 1990); while, in *no mitochondrial derivative*, the spermatid's axoneme can elongate even if the mitochondrial structures it associates with do not (Fuller, 1993). These parallel pathways are the likely basis for the ability of flies with mutations in *pelota*, *twine*, or other genes required for meiotic divisions to nonetheless proceed with spermatid differentiation. In $\beta 2$ tubulin mutants, for example, the meiotic spindle does not form, but meiotic chromosome condensation and differentiation of 4N spermatids still occurs (Fuller, 1993).

Meiotic chromosome condensation may not be regulated by $p34^{cdc2}$-cyclin

The germline cell cycle appears to arrest during the meiotic G_2/M transition in *pelota* and *twine* male homozygotes. The absence of spindle formation and nuclear envelope breakdown in arrested spermatocytes supports this hypothesis. Less consistent with an arrest during the G_2/M transition is the existence of partially condensed spermatocyte chromosomes in these mutants (our results; White-Cooper et al., 1993). However,

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   1  GCCACGAGGTGAATGTCACTTGGCCGCCGATTCTACTGACCACCACCATTGCCACACCATCCTGCCGATTCGATCAGCGGTGTGAACTTA
  91  GCAACTGCGTGAACCCAATGACAACGATGCACCGCCGCCTTTGTGAACGGAGGGAATTACTGGAATCTGGGTGCAAGTGACAAGCGGCTA
 181  GAGGAATAAACCACGCTGGTATAAAGGCAGTGCTGCGACGAAACAAATACCAAATCTGACCAGCAACGAGACGGGATGAAGCTGCTGGGC
   1                                                                                  M  K  L  L  G

 271  AAATACGTGGACAAGGGCATGCAGGGAAATGTGACCCTGGTGCCGGAGGAGTCCGAGGATATGTGGCACGCCTACAATTTGATTGCGAAA
   6   K  Y  V  D  K  G  M  Q  G  N  V  T  L  V  P  E  E  S  E  D  M  W  H  A  Y  N  L  I  A  K

 361  GGCGACAGCGTGCGCAGCACCACTATTCGCAAGGTGCAGAATGAAACGGCTACTGGCTCCTCCACCAGCAGCCGGGTGCGCACCACACTA
  36   G  D  S  V  R  S  T  T  I  R  K  V  Q  N  E  T  A  T  G  S  S  T  S  S  R  V  R  T  T  L

 451  ACCATCGCAGTGGAGAGCATAGATTTTGATACGCAGGCTTGTGTCGTCCGCCTTAAAGGCAGAAACATCGAGGAGAACCAGTATGTCAAG
  66   T  I  A  V  E  S  I  D  F  D  T  Q  A  C  V  V  R  L  K  G  R  N  I  E  E  N  Q  Y  V  K

 541  ATGGGCGCCTATCACACCCTCGACCTGGAGCTGAATCGCAAGTTCGAGCTGCGTAAGCCAGAATGGGACACCATTGCCCTGGAGCGCATC
  96   M  G  A  Y  H  T  L  D  L  E  L  N  R  K  F  E  L  R  K  P  E  W  D  T  I  A  L  E  R  I

 631  GAAATGGCCTGCGATCCCACACAGTCTGCGGATGTGGCCGCCGTTGTGATGCAGGAGGGATTAGCACACGTCTGCCTGATCACCGCCAGT
 126   E  M  A  C  D  P  T  Q  S  A  D  V  A  A  V  V  M  Q  E  G  L  A  H  V  C  L  I  T  A  S

 721  ATGACCCTGGTGCGGAGCAAGATAGAGGTCTCCATACCGCGCAAGCGCAAGGGCAGTGTCCAGCAACACGAGAAGGGACTGGCCAAGTTC
 156   M  T  L  V  R  S  K  I  E  V  S  I  P  R  K  R  K  G  S  V  Q  Q  H  E  K  G  L  A  K  F

 811  TACGAGCAGGTTATGCAGAGCATCCTGCGGCACGTCGACTTCGATGTGGTCAAGTGTGTGTTGATTGCGTCGCCGGGATTCGTGCGCGAT
 186   Y  E  Q  V  M  Q  S  I  L  R  H  V  D  F  D  V  V  K  C  V  L  I  A  S  P  G  F  V  R  D

 901  CAGTTCTACGACTACATGTTCCAGCAGGCGGTCAAAATGGATTACAAGCTGCTGCTGGACAACAAGAGCAAGTTTATGCTGGTGCACGCC
 216   Q  F  Y  D  Y  M  F  Q  Q  A  V  K  M  D  Y  K  L  L  L  D  N  K  S  K  F  M  L  V  H  A

 991  TCCTCGGGATTTAAGCACTCCTTAAGAGAAATTCTACAGGATCCCGCCGTGCTGGCCAAGATGTCTGACACCAAGGCAGCTGGCGAGGTC
 246   S  S  G  F  K  H  S  L  R  E  I  L  Q  D  P  A  V  L  A  K  M  S  D  T  K  A  A  G  E  V

1081  AAGGCCCTGGAACAATTCTACATGATGCTGCAATGTGAGCCCGCCAAGGCCTTCTATGGAAAGAAACATGTCCTCCAAGCCGCCGAGTCG
 276   K  A  L  E  Q  F  Y  M  M  L  Q  C  E  P  A  K  A  F  Y  G  K  K  H  V  L  Q  A  A  E  S

1171  CAGGCCATCGAAACGCTGCTGATCTCGGACAACCTCTTTAGATGCCAAGACGTTAGTCTGAGAAAGGAATATGTCAATCTGGTCGAATCC
 306   Q  A  I  E  T  L  L  I  S  D  N  L  F  R  C  Q  D  V  S  L  R  K  E  Y  V  N  L  V  E  S

1261  ATTCGCGATGCTGGGGGAGAAGTCAAAATCTTTTCGAGCATGCACATCTCAGGAGAACAACTTGCTCAACTAACTGGAATTGCAGCCCTC
 336   I  R  D  A  G  G  E  V  K  I  F  S  S  M  H  I  S  G  E  Q  L  A  Q  L  T  G  I  A  A  L

1351  CTGCGCTTCCCGATGCCCGAACTGGAGGACAGCGACGATGATGATGATGAGGATGGAGCGGCAGGCGGGGCGGCAGATAGCGATAGCGAC
 366   L  R  F  P  M  P  E  L  E  D  S  D  D  D  D  D  E  D  G  A  A  G  G  A  A  D  S  D  S  D

1441  TAGGATCATAAATAGGAATAGCTTCATGGAATCTTGGCATTTGATAACGGAAAATTTTGACTTAGGAACTTAACTAGCGATCGATATACA
1531  AGTAGACTAATAGGAACCACGACAGACTTGGACGTCAGGGCATATACAATATACATACATAGTGCTAATTCAATACAATATACACACAAA
1621  TATCAGCTTAAGTTCACCCGTGGCTGGGCCTACAAATTCAACATTTTAGATTCTTTGTGTTCAAGTAAATGGTTAGGTTTCAATTATTGC
1711  TTCATATGAGTTGCGTTAACTATATTCGGCAAACTAAATTTCTTTCCGGCGCACTTAAATTCATTGTTCATTCATTTGTAAAATGGAAAA
1801  CGGAGTTTGCAAAATGATTACAACCGGCACATAATATAGTTATTTATCCAGTTTCGTTGTTTAAACCCGGATTTTATGCATCTTCAGAAC
1891  GCTCTTAATGTTGCTTAACTTTATAAGATTTGTAATTATCATTTTATGCTGTAAACATAACCCTTTGTTAAACCCTACTAGTTGATACAG
1981  AATGTAAGTAATTAACCATGCTGTAAATATATAAATGTAAATAATGCA 2028
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Fig. 7. *pelota* cDNA sequence with predicted protein sequence (Genbank U27197).

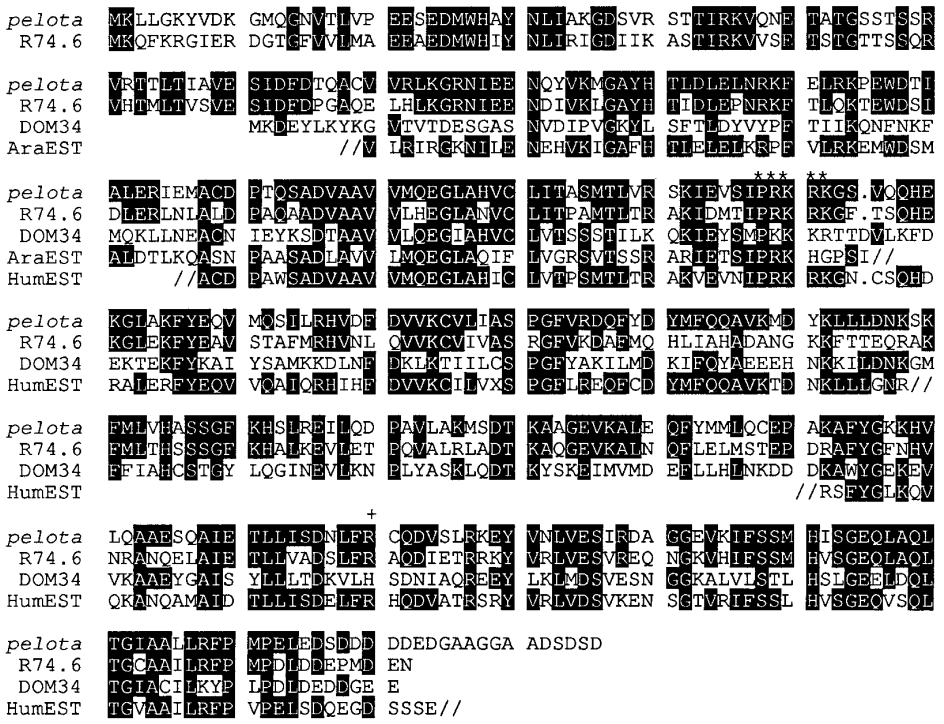

Fig. 8. Alignment of full-length pelota, *S. cerevisiae* Dom34 (p33309) and *C. elegans* R74.6 (z36238) protein sequences, as well as predicted ORFs from expressed sequence tags (EST) in *Arabidopsis* (T20628) and Human (T30453, HUMGS01758). Slash marks indicate the end of the EST sequences. Boxed amino acids indicate identity to the pelota sequence; dots represent gaps generated in the alignment. Over the region of shown, pelota is 34% identical to Dom34, 58% identical to R74.6, 54% identical to T20628, 70% identical to T30453 and 55% identical to HUMGS01758. The asterisks over the *pelota* sequence PRKRK identify a potential nuclear localization sequence; the + over the R at position 319 represents the first amino acid of the exon 3′ of the *pelo*[1] P element insertion.

chromosomes also condense in *sa*, *aly*, *mia* and *can* arrested spermatocytes (Fuller, 1993). Thus chromosome condensation is a common feature in spermatocytes arresting prior to meiotic divisions, suggesting that in *Drosophila* meiosis at least some chromosome condensation is independent of $p34^{cdc2}$-cyclin activation during the G_2/M transition.

There is precedent in other organisms for the initiation of meiosis prior to the onset of the meiotic cell cycle. In *S. cerevisiae*, for example, conditional mutations in Cdc28 ($p34^{cdc2}$) and several other Start genes cause meiotic arrest only after homolog pairing and recombination (Shuster and Byers, 1989). In *Drosophila* males no recombination occurs. The pairing of autosomes in meiosis is mediated by a poorly characterized mechanism, which may involve general homology between chromosomes (McKee et al., 1993). Perhaps these meiotic specializations require changes in the regulation of chromosome condensation prior to cell division, resulting in the partial chromosome condensation observed during the G_2/M transition.

It is possible that the initial meiotic chromosome condensation is mediated by a cyclin-dependent kinase activated before the G_2/M transition. However, such a kinase must not require cdc25 phosphatase for activation, since no *string* message is present in prophase spermatocytes (Alphey et al., 1992; Courtot et al., 1992) and chromosome condensation occurs in mutants lacking *twine* phosphatase (our results; White-Cooper et al., 1993). Alternatively, a kinase that is not cyclin-dependent may regulate the initial meiotic chromosome condensation. In this regard it is worth noting that chromosome condensation is maintained during the meiotic interphase in frog and starfish oocytes in the absence of $p34^{cdc2}$-cyclin kinase activity (for discussion, see Hunt and Murray, 1993).

Analysis of meiotic defects in weak *pelota* alleles

In weak *pelota* alleles the cell cycle sometimes proceeds through the meiotic G_2/M transition, but the meiotic cell divisions that occur are often aberrant, with defects in chromosome segregation and cytokinesis. It is possible that *pelota* plays separate roles in the initiation of meiotic divisions and in specific meiotic processes such as spindle formation and cytokinesis. We favor another possibility, that the chromosome segregation and cytokinesis defects in weak *pelota* alleles are linked to the initiation of meiotic divisions. Mutations in other genes regulating G_2/M also cause specific defects in cell division. In *Drosophila cyclin B* mutants, for example, embryonic cells transit from G_2 to M, but form defective spindles, resulting in improper segregation of chromosomes (Knoblich and Lehner, 1993).

Conserved roles for *pelota*

Northern analysis of *pelota* expression suggests the gene is active in processes other than spermatogenesis, for *pelota* mRNA is found throughout development and is not restricted to the germline in adults. Embryos 0-2 hours old contain the highest level of *pelota* mRNA. Rapid waves of mitotic division take place in these early embryos, so *pelota* may be required for mitotic as well as meiotic divisions. Defects that we observe in the ovary and the eye also suggest that *pelota* plays a role in mitosis.

The *pelota* gene has yeast, plant, worm and human homologs. A canonical nuclear localization sequence is present in at least four of these five proteins. Perhaps *pelota* and its homologs are involved in $p34^{cdc2}$ kinase function in the nucleus. Alternatively, *pelota* may function outside the $p34^{cdc2}$ signaling pathway and cause arrest at meiotic G_2/M by a checkpoint mechanism.

DOM34, an *S. cerevisiae* protein with substantial sequence similarity to pelota, functions in both mitotic and meiotic divisions in yeast (JoAnne Engebrecht and Luther Davis, personal communication). In *dom34* mutants the mitotic cell cycle is slowed. Meiotic divisions, in contrast, are too rapid

and fewer spores are produced than in wild type. The yeast mutants also fail to segregate chromosomes properly – a phenotype similar to that of weak *pelota* mutants. Introduction of the wild-type *pelota* gene into a *dom34* mutant provides substantial rescue of the *dom34* growth defects (C. G. E., S. A. W., L. Davis and J. Engebrecht, unpublished observations). Thus, DOM34 and pelota are homologs and encode a conserved activity for the regulation of cell division.

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