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The *Drosophila* gene *morula* inhibits mitotic functions in the endo cell cycle and the mitotic cell cycle

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SUMMARY

In the endo cell cycle, rounds of DNA replication occur in the absence of mitosis, giving rise to polyploid or polytene cells. We show that the *Drosophila morula* gene is essential to maintain the absence of mitosis during the endo cycle. During oogenesis in wild-type *Drosophila*, nurse cells become polyploid and do not contain cyclin B protein. Nurse cells in female-sterile alleles of *morula* begin to become polyploid but revert to a mitotic-like state, condensing the chromosomes and forming spindles. In strong, larval lethal alleles of *morula*, the polytene ring gland cells also inappropriately regress into mitosis and form spindles. In addition to its role in the endo cycle, *morula* function is necessary for dividing cells to exit mitosis. Embryonic S-M cycles and the archetypal (G_1-S-G_2-M) cell cycle are both arrested in metaphase in different *morula* mutants. These phenotypes suggest that *morula* acts to block mitosis-promoting activity in both the endo cycle and at the metaphase/anaphase transition of the mitotic cycle. Consistent with this, we found cyclin B protein to be inappropriately present in *morula* mutant nurse cells. Thus *morula* serves a dual function as a cell cycle regulator that promotes exit from mitosis and maintains the absence of mitosis during the endo cycle, possibly by activating the cyclin destruction machinery.

Key words: cell cycle, mitosis, *Drosophila*, polytenization, cyclin B, *morula*

INTRODUCTION

Progression through the cell cycle requires the inactivation of mitotic functions after M phase. In dividing cells, exit from mitosis and entry into a new cell cycle involve a shut off of mitotic activities. One mechanism by which mitotic activities are extinguished in dividing cells is the inactivation of the cdc2/cyclinB kinase by degradation of the cyclin subunit (Amon et al., 1994; King et al., 1996). In polytene cells, mitotic functions are permanently repressed, and the resulting endo cycle consists of only S phase and a gap phase. The regulation underlying the absence of mitosis in the endo cycle is not understood.

In *Drosophila*, somatic polyploidy is extensive, making this an ideal organism in which to investigate the regulation of the endo cycle. The vast majority of larval tissues are polytene, arising through endo cycles during which the replicated chromatids remain associated along their lengths. During pupation, the bristle-forming cells also grow through endo cycles (Lees and Waddington, 1942). In the adult *Drosophila*, low level polyploidy (8C-64C) is found in many tissues and high level polyploidy (>500C) is found in the nurse cells of the adult ovary (King, 1970).

Analysis of the endo cycles in the embryo suggests that polytene DNA replication results from pulses of cyclin E activity in the absence of mitotic cyclins (Knoblich et al., 1994; Sauer et al., 1995). The endo cycle initiates during the latter half of embryogenesis, after 16 mitotic divisions, in response to developmental regulation (Smith and Orr-Weaver, 1991). Polytene S phases occur in an invariant pattern in the late embryo, even in mutants in which prior cell divisions are blocked. Cyclin E, a cyclin demonstrated to regulate the onset of S phase, is transcribed in the same developmental pattern observed for BrdU incorporation (Knoblich et al., 1994). In strong alleles of *cyclin E*, DNA replication is blocked after mitosis 16 in both the endo cycles and the mitotically dividing cells of the nervous system. Several genes encoding replication factors also are transcribed during polytene S phases in the embryo; their transcription is dependent on cyclin E and the transcription factor E2F (Duronio and O'Farrell, 1994; Duronio et al., 1995; Royzman et al., 1997). Mitotic regulatory functions appear to be shut off in the endo cycle. The mitotic cyclins A and B are not expressed in the polytenizing cells, and mutations in *cdc2* do not affect the endo cycle (Lehner and O'Farrell, 1989, 1990; Stern et al., 1993; Whitfield et al., 1990).

The development of the polyploid nurse cells of the ovary has been investigated extensively (for reviews see King, 1970; Mahowald and Kambysellis, 1980; Spradling, 1993). The 15 nurse cells arise from a 16-cell cyst, with the remaining cell becoming the oocyte. Cytokinesis is incomplete in the mitotic divisions that produce the cyst, leaving these cells connected by cytoplasmic bridges. The initial rounds of DNA replication

in the nurse cells produce polytene chromosomes, but these dissociate, so that the nurse cell nuclei become polyploid and do not have visible chromosomes. In the endo cycles in the nurse cells, as in most polytene cells, DNA replication is incomplete and some genomic sequences are underreplicated. This is not the case in a weak, female-sterile, allele of *cyclin E*. In this allele, *cyclin E^{01672}*, sequences normally underrepresented appear fully present (Lilly and Spradling, 1996). Thus, in addition to being necessary for endoreplication, the levels of cyclin E protein influence the parameters of polytene DNA replication.

The absence of mitosis is the most fundamental cell cycle aspect of the endo cycle, making it crucial to identify regulators blocking mitotic functions. We examined female-sterile mutations for phenotypes suggestive of inappropriate mitosis in cells undergoing endo cycles. The strongest candidate was the female-sterile mutant *morula* (2-106.7; 60A7-16). *morula* mutations are pleiotropic (Lindsley and Zimm, 1992). The visible phenotypes of *morula*, which are temperature sensitive, include rough eyes and small bristles. The reduced bristles of *morula* have been reported to be associated with a failure of growth of the bristle-forming cells and the rough eyes with a reduction in cell division (Lees and Waddington, 1942; Waddington and Pilkington, 1943). The female sterility of *morula*, a phenotype that is not temperature sensitive, has been described as a nurse cell defect in which nurse cell chromosomes fall apart and condense to metaphase-like chromosomes before degenerating (King, 1959, 1964, 1970).

Here we present new *morula* alleles and our analysis of the *morula* phenotypes. We find that *morula* controls both the endo cycle and the mitotic cell cycle throughout *Drosophila* development. The absence of *morula* function causes a mitotic-like state. In *morula* mutants, the polyploid nurse cells and the polytene ring gland cells revert to mitosis, condensing their chromosomes and forming spindles, and cyclin B protein is present inappropriately in the nurse cells. Both the early mitotic divisions in the embryo and the larval neuroblasts arrest in metaphase in *morula* mutants. Thus *morula* is essential for inactivating mitotic functions in dividing cells and, in its absence, mitotic activities occur even in the endo cycle.

MATERIALS AND METHODS

Drosophila stocks

All stocks used in this study are listed in Table 1. Female-sterile alleles of *morula*, *mr^1* and *mr^2*, were isolated by Bridges in 1913 and 1925, respectively (Lindsley and Zimm, 1992). Alleles *mr^3*, *mr^4* and *mr^5* were selected in an EMS screen for lethal mutations that failed to complement *Df(2R)G10-BR27* (Reed, 1992). To observe the strong nurse cell phenotype different *mr^1* or *mr^2* stocks were crossed to one another. Deficiencies used to uncover *morula* alleles included *Df(2R)G10-BR27* (59F3;60A8-16), *Df(2R)or^{BR-11}* (59F6-8; 60A8-16), or *Df(2R)2651ex3* (60A7-12; 60B3-6). Detailed mapping of the *morula* locus and strategies for the synthesis and recovery of deficiencies of the *morula* region will be reported elsewhere.

The unambiguous identification of homozygous *morula* larvae was achieved by balancing all *morula* alleles over a translocation between *SM5* and *TM6B* known as *T(2;3)TSTL^{14}* (Gatti and Goldberg, 1991). Since *TM6B* carries the dominant larval/pupal marker *Tubby* (*Tb*), the use of this translocation allows larvae homozygous for any gene of interest on the second or third chromosome to be recognized by virtue of being Tb$^+$.

For the production of *mr^5* mosaics, progeny of *P[FRT 2R] mr^5/SM6a* × *P[hsFLP]*; *P[FRT 2R]/CyO* (see Table 1 for complete genotypes) were heat shocked as second and third instar larvae by immersing the culture in a 37°C water bath for 1 hour on two consecutive days (Xu and Harrison, 1994). The same method was used to induce mosaicism in *P[FRT 2R]mr^1 / P[FRT 2R][ovoD1 2R]* (see Table 1) (Chou and Perrimon, 1992, 1996).

To test for embryonic lethality, *mr^4*/*SM6a* and *mr^5*/*SM6a* stocks were outcrossed to wild type and Cy$^+$ male progeny were backcrossed to the balanced stocks. Eggs collected over a 24 hour period were aged for 48 hours and examined. No embryonic lethality of *mr^4* or *mr^5* homozygotes was observed.

For cdc2^{ts} temperature-shift experiments, *Dmcdc2^{B47}* was crossed to *Dmcdc2^{E1-24}* at 18°C (permissive temperature) (Stern et al., 1993). *Dmcdc2^{B47}*/ *Dmcdc2^{E1-24}* females were collected and maintained at the permissive temperature for 3-5 days. Ovaries were dissected, fixed and stained with DAPI 1, 3 and 5 days following shift to 29°C (restrictive temperature). *Dmcdc2^{B47}*/ *Dmcdc2^{E1-24}* maintained for 5 days at permissive temperature and balancer siblings maintained for 5 days at restrictive temperature were used as controls.

All genetic markers and special chromosomes are described in Lindsley and Zimm (1992). Standard *Drosophila* medium and culturing techniques were used (Ashburner, 1989a,b).

Suppression of the *morula* oogenesis phenotype

Homozygous *mr^1* and *mr^2* females from stocks that had been balanced for several generations were found to occasionally lay eggs that did not develop. This had been noted previously for *mr^1* (Lynch, 1919). This tendency increased dramatically in females carrying a *C(1)M3* attached-*X* chromosome. The suppression of the primary oogenesis defect of *morula* was not observed in a *C(1)M4/Y* background and is, therefore, not associated with the presence of an attached *X* or a *Y* chromosome. Even when outcrossed to males of a different *mr^1* or

Table 1. Drosophila stocks

Stock description	Source
mr^1 bs^2/In(2LR)bw^{V1}	1
b cn mr^1 bs^2/TSTL^{14}	2
mr^2/T(1;2)Bld In(2R)Cy, cn^2 Bld	1
mr^1 sp/ SM6a	2
b cn bw^D mr^2/SM6a	2
dp b cn mr^3/ TSTL^{14}	3
dp b cn mr^4/ TSTL^{14}	3
dp b cn mr^5/ TSTL^{14}	3
y w; P[ry$^+$; hs-neo; FRT]42D, P[w$^+$, πM]45F mr^5/SM6a	2
w^{1118}; P[ry$^+$; hs-neo; FRT]42D, P[w$^+$, πM]45F	1
yw; P[FRT 2R]G13 mr^1 sp/SM6a	2
P[FRT 2R]G13 P[w$^+$, ovoD1-18]2R1 P[w$^+$, ovoD1-18]2R2/ Dp(2;2) bw^D, S Ms(2)M1 bw^D/CyO	1
w^{1118} P[ry$^+$; hsFLP]1; Adv1/CyO	1
C(1)M3,y/ Dp(2;Y)CB25-67, bw; b cn bw^D mr^2	2[a]
T(Y;2)bw$^{DRev\#11}$, cn bw$^{DRev\#11}$ mr^2/ SM6a	3[b]
Df(2R)G10-BR27, cn bw/ SM6a	3[c]
Df(2R)or^{BR-11}, cn bw sp/ SM6a	3
Df(2R)2651ex3/ SM6a	3
Dmcdc2^{B47} cn bw/ SM5	4
Dmcdc2^{E1-24} b pr cn/ CyO	4

Sources: (1) Bloomington stock center; (2) Recombinant chromosomes made for this study; (3) Mutations or aberrations made for this study (Reed, 1992); (4) Christian Lehner.

[a] *Dp(2;Y)CB25-67* from T. Lyttle.

[b] Breakpoint in 59E and partial revertant of *bw^D*, X-ray induced on cn *bw^D mr^2* chromosome. Terminal duplication segregants are male sterile.

[c] Associated with *In(2LR)lt$^{G10[L]}$-BR27$^{[R]}$*, which was recovered by conversion of the autosynaptic stock *LS(2)lt^{G10}//DS(2)BR27* to its heterosynaptic form (Reed, 1992).

mr^2 stock, the *mr*-mutant female progeny of *C(1)M3,y/ Dp(2;Y)CB27-67, bw; b cn* bw^D mr^2 were prolific egg layers.

The nature of the suppression of the nurse cell phenotype is not understood, although we suspect the variable nature of the *morula* phenotype to reflect a susceptibility of mr^1 and mr^2 to genetic modifiers. It has been established that *morula* responds to modifiers. In particular, the isolation of the recessive X-linked *e(mr)*, which dramatically enhances the visible rough eye phenotype as well as causing a reduction of sex combs in homozygous mr^1 or mr^2, supports this view (Reed, 1992).

The suppression of the nurse cell phenotype clearly permits the completion of oogenesis, but a *morula* defect still occurs in the early embryonic cycles. We showed that the early division defect was due to the *morula* mutation and not other mutations in the stock. First, eggs laid by *C(1)M3,y* / *Dp(2;Y)CB27-67, bw*; *b cn* bw^D mr^2 females were uniformly wild type. This stock is homozygous for the mr^2 chromosome, has a duplication for mr^+, carries the compound *X*, but does not display the embryonic phenotype. Consequently, the metaphase arrest in early embryos does not result from a maternal-effect mutation on the *X* or second chromosomes to which *morula* is normally epistatic. Moreover, we observe the same phenotype in eggs from mr^2 homozyotes and mr^1/mr^2 transheterozygotes.

Cytology

Ovaries were dissected from 3- to 5-day-old adult females in 0.7% NaCl, fixed in 8% formaldehyde in PBS for 10-15 minutes and washed (3× 10 minutes) in PBS (Ashburner, 1989b). Subsequent methods for immunostaining, DAPI and phalloidin staining of ovaries followed Theurkauf (1994). For confocal microscopy, ovaries were stained with propidium iodide by incubating ovaries for 1 hour in PBS containing RNAase A (50 μg/ml) and propidium iodide (1 μg/ml). Following 3× 15 minutes washes in PBS, ovaries were transferred to mountant (70% glycerol, 5% n-propyl gallate in PBS).

Feulgen reagent was prepared as described (Ashburner, 1989b) and fortified immediately prior to use by mixing four parts Feulgen to one part 10% (w/v) sodium bisulfite. Ovaries were dissected and fixed as described above, washed in PBS (3× 15 minutes) and incubated in 2 N HCl at 50°C for 10-15 minutes. Following acid hydrolysis, ovaries were again washed in PBS (3× 15 minutes) and stained in Feulgen's reagent for 30 minutes at room temperature. Ovaries were rinsed in bisulfite wash solution (5 ml saturated sodium bisulfite added to 0.05 N HCl) and mounted as above.

Whole-mount immunostaining and propidium iodide staining of whole-mount larval brains was carried out as described (Gonzalez and Glover, 1993). Wild-type and mutant ring glands were dissected in PBS containing 1 μm taxol and were either left attached or dissected free of the larval brain prior to immunostaining. Taxol was omitted from all other preparations (ovary, brain and embryo). For immunostaining, ring glands were treated identically to brains.

Standard techniques were used for collecting and immunostaining embryos (Theurkauf, 1994). The collection of unfertilized eggs from wild-type and *morula* virgin females was enhanced by maintaining females with sterile males recovered from $T(Y;2)bw^{DRev\#11}$ (see Table 1). Egg collections from *morula* mutants and wild type were 0-18 hours and 0-2 hours, respectively. The chromatin of eggs/embryos was visualized using Oligreen to stain DNA as follows: RNAase-treated (1 hour room temperature; 5 μg/ml boiled RNAase A in 0.03% Triton X-100 in PBS) embryos were incubated 30 minutes in Oligreen (0.5 μg/ml Oligreen (Molecular Probes) in 0.03% Triton X-100 in PBS), after which embryos were washed (3× 5 minutes in 0.03% Triton X-100 in PBS, then 3× 5 minutes in PBS). Embryos were dehydrated through a methanol series, cleared and mounted in a 2:1 solution of benzyl benzoate: benzyl alcohol containing 50 mg/ml n-propyl gallate.

Tubulin staining in ovaries, whole-mount larval brains and ring glands was carried out using a 1:250 dilution of anti-tubulin mouse monoclonal antibody directly conjugated to fluorescein (a gift from Wes Miyazaki, prepared by the protocol of Theurkauf et al. (1992)). For embryos, an anti-β-tubulin mouse monoclonal (1:200; Amersham) and a Texas-red-conjugated goat anti-mouse secondary (1:200; Jackson Labs) were used. Centrosomes were labeled using a rabbit polyclonal anti-CP190 antibody (1:1000, from D. Kellogg) and Donkey FITC-conjugated anti-rabbit secondary (1:250; Jackson Labs). Anti-cyclin B monoclonal F24F, provided by Pat O'Farrell, was used at a 1:5 dilution and was detected using a goat anti-mouse FITC-conjugated secondary antibody (1:200; Jackson Labs). When mutant and wild-type tissues could be distinguished from one another, as was the case for larval brain and ovary preparations, mutant and control samples were immunostained in a single Eppendorf tube.

Larval brains were dissected in 0.7% NaCl and transferred immediately to a drop of 45% glacial acetic acid on a microscope slide. After no longer than 5 seconds, the acid was replaced with a drop of stain (2% natural orcein (Gurr's 23282) in equal parts 45% acetic and 45% lactic acid). After covering with a coverslip, brains were squashed hard. A 5-10 minute hypotonic treatment in 0.5% sodium citrate prior to fixation was included when preservation of anaphase figures was not required.

A Bio-Rad MRC 600 scanning confocal microscope was used as described (Page and Orr-Weaver, 1996) in capturing confocal micrographs. Confocal images of whole eggs/embryos are projections of several optical sections such that all spindles within each egg/embryo are represented. Other confocal images represent only single focal planes.

Larval brain squashes were viewed and photographed under phase contrast using a Zeiss Axiophot microscope with a Zeiss 100×/1.30 oil immersion Plan-apochromat objective. DAPI-stained ovaries were viewed and photographed under fluorescence using 20× or 40× dry Plan Neofluar objectives. Jessop's KB14 or Pan-X black and white film was used for photography. Negatives were digitized using a Lacie Silverscanner II.

RESULTS

The polyploid nurse cells of mr^1 and mr^2 contain large inappropriate spindles and condensed metaphase-like chromosomes

In an effort to understand changes in cell cycle regulation as cells switch from the mitotic cell cycle to the endo cell cycle, we examined the female-sterile alleles of *morula* (mr^1 and mr^2). The defects observed during oogenesis are consistent with a disruption of cell cycle regulation as nurse cells exit the mitotic cell cycle and enter the endo cell cycle. Anti-tubulin and DNA staining of *morula* ovaries revealed a striking phenotype (Fig. 1B). Although the nurse cells initiated endo cycles, later egg chambers contained condensed chromosomes in place of polyploid nurse cell nuclei. These condensed chromosomes were associated with large multipolar spindles; in contrast, we never observed spindles in wild-type nurse cells at these stages (Fig. 1A). Metaphase-like chromosomes were often attached to these spindles (Fig. 1C-E). Chromosome movement was presumed to be taking place, as anaphase-like bridges were sometimes observed among the condensed chromatin and spindles (data not shown). The mutant nurse cells, having formed spindles and metaphase-like chromosomes, did not reform nuclei and showed no signs of telophase. Later stage cysts appeared collapsed and degenerated. In addition to these defects in nurse cell development, the follicle cell epithelium was sometimes observed to form a double, rather than single,

layer of cells. We did not observe the formation of spindles or condensed chromosomes in the mutant follicle cells.

The nurse cell defects in *morula* mutants are not manifest until stage 4 of egg chamber development. By stage 4, the nurse cells of wild-type egg chambers contain a DNA content of approximately 16C or 32C and their chromosomes have a slightly bundled or bulbous appearance (King, 1970). The *morula* mutant nurse cells clearly initiate polyploidization but revert to a mitotic state (Fig. 2A). In addition, we found that the initial development and organization of *morula* mutant cysts was normal (Fig. 2B,C). One oocyte and fifteen nurse cells were cytologically discernable, and FITC-phalloidin staining revealed a wild-type number and distribution of ring canals within the cyst. Ring canals were observed to degenerate shortly after the stage at which inappropriate spindles were observed in mutant cysts (Fig. 2B). The oocyte appeared diploid and cytologically normal. Consistent with our observations of normal oocyte differentiation, VASA protein localization in *morula* ovaries is normal (Lasko and Ashburner, 1990).

The *morula* nurse cell phenotype is likely germ-line dependent. We used the dominant female-sterile/FRT mosaic technique to produce homozygous mr^1 mutant germ-line clones in heterozygous mr^1/+ females (Chou and Perrimon, 1996). The mitotic recombination events to homozygose mr^1 were induced by FLP. The dominant female-sterile ovo^D was present on the copy of chromosome *2R* bearing mr^+, so only clones that were homozygous for mr^1 would lack the dominant female-sterile mutation. If *mr* were not germ-line dependent, we would have recovered mosaic females that were fertile. All of the mosaic females were sterile and, moreover, inappropriate spindles were present in their ovaries (data not shown).

The inappropriate spindles do not require centrosomes

The spindles observed in *morula* mutants could result from a primary defect in centrosome function or alternatively from a defect in cell cycle regulation. Centrioles undergo an unusual migration during *Drosophila* oogenesis. In wild-type germaria, nurse cell centrioles lose their juxtanuclear position subsequent to the formation of the 16-cell cyst; in older cysts centrioles are found clustered in the posterior region of the oocyte (Mahowald and Strassheim, 1970). Consequently, one explanation for the *mr* nurse cell phenotype was that centrosomes persisted inappropriately and that these were functional in organizing spindles even though the nuclei had become polyploid.

To address this possibility, we tested for the presence of centrosomes on the inappropriate nurse cell spindles by using anti-CP190 antibodies. This antigen is present in interphase nuclei and relocates to the centrosome during mitosis (Oegema et al., 1995). mr^2 cysts did not show any centrosomal localization of CP190 to the spindle pole regions (Fig. 3A). Dividing follicle cells served as an internal control and did show CP190 localized to the spindle pole (Fig. 3B). Thus it does not appear that centrosomes persist during polyploidization of the nurse cells in *mr* mutants.

morula is required for proper mitosis during early embryonic divisions

Because mitosis occurs inappropriately in the polyploid nurse cells of *mr* mutant ovaries, we investigated whether *morula* function was required for proper mitosis in the early embryonic divisions. These nuclear divisions take place in a syncytial cytoplasm. They are controlled by maternal pools deposited during oogenesis and occur in a S-M cycle lacking gap phases. Although mr^1 and mr^2 females are always fully sterile, it was possible to recover stocks in which the oogenesis defect was suppressed, enabling the mutant females to lay eggs that did not hatch. In these stocks, the oogenesis defect was suppressed, but the *morula* visible phenotypes were still present and we observed defects in the early embryonic cycles that resulted from the *morula* mutations (see Materials and Methods).

We examined eggs laid by homozygous mr^2 or *trans*-heterozygous mr^1/mr^2 females and observed early syncytial nuclei arrested in metaphase. The embryos usually contained between six and ten metaphase nuclei, although occasionally up to 30 were present (approximately 100 embryos were examined). The chromosomes were aligned on a metaphase plate and the spindles stained more intensely than wild type with anti-tubulin antibodies, possibly reflecting a greater density of microtubules (Fig. 4A,B). In addition, the spindles were broader than wild

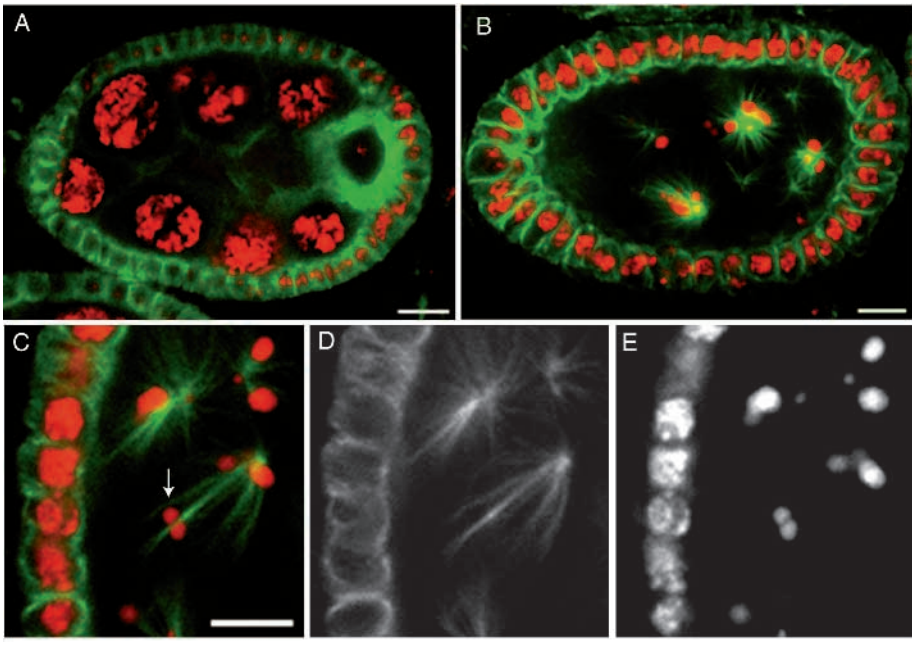

Fig. 1. The oogenesis phenotype of *morula*. Scanning confocal images of stage 4-5 egg chambers from (A) wild-type and (B) mr^2 females stained with anti-tubulin (green) and propidium iodide (red). The nurse cells of *morula* egg chambers contain condensed chromatin and spindles at a point in oogenesis that is normally associated with endopolyploidy. Condensed chromatin often appears to resemble metaphase chromosomes and associates with microtubules (arrow in C). (D) Tubulin staining; (E) DNA. Although the spindles appear monopolar in this confocal section, optical serial sectioning shows that they actually are multipolar. Bar, 10 μm.

type both at the metaphase plate and at the spindle poles. Thus, in eggs from *morula* mutant mothers, only a few divisions appear to occur followed by metaphase arrest.

Normally the three unused meiotic products from the oocyte fuse into one or two rosette structures of condensed chromosomes surrounded by a sphere of tubulin (Fig. 4A). In the fertilized embryos from *mr* females, we did not observe normal polar bodies, but consistently found metaphase spindles at the position on the dorsal surface of the embryo normally occupied by the polar bodies (Fig. 4B). In unfertilized eggs from wild-type mothers, meiosis is completed and the meiotic products form rosette structures (Fig. 4C). In unfertilized eggs from *mr* mutants, between one and four metaphase figures were present rather than the rosette structure typical of polar bodies (Fig. 4D). In some cases, the chromosomes did not appear to be as tightly aligned on the metaphase plate as in the spindles present in fertilized mutant embryos (Fig. 4D, inset). It is possible either that meiosis is not completed properly, arresting in meiotic metaphase, or that the polar bodies inappropriately assemble mitotic spindles in *mr* mutant eggs.

Lethal alleles of *morula* are mitotic mutants with metaphase arrest

The observations that *mr* mutations produced a mitotic-like state in the polyploid nurse cells, arrested the early nuclear divisions in metaphase and appeared to cause a metaphase spindle in the polar bodies, led us to examine whether *mr* affected mitosis later in development in cells undergoing an archetypic G_1-S-G_2-M cell cycle. Both mr^1 and mr^2 proved to be semilethal over non-complementing deficiencies, suggesting a general role for *mr* in cell cycle control. (The deficiencies were isolated from several screening strategies that will be reported elsewhere.) Escapers had etched tergites and very rough eyes, while most Df/mr^1 or Df/mr^2 heterozygotes died as pupae in the pharate adult stage. From this more severe phenotype, it can be assumed that mr^1 and mr^2 are not null alleles and that they are possibly hypomorphic.

Assuming that a null allele of *morula* could be selected as a lethal mutation, we performed an EMS mutagenesis screen (to be reported elsewhere) and selected lethal mutations in the *morula* region (60A7-16). Three lethal alleles were recovered that failed to complement *morula*, here referred to as mr^3, mr^4 and mr^5. The mr^3 chromosome proved to carry a second, closely linked, lethal mutation and was not used for phenotypic analysis.

The phenotype of these lethal alleles of *morula* conforms to what is now recognized as a mitotic cell cycle phenotype, the diagnostic features of which include late larval/early pupal lethality, small and poorly developed imaginal discs, and anomalous mitotic chromosome behavior as seen in larval brain neuroblast squashes (Gatti and Baker, 1989; Gatti and Goldberg, 1991). The lethal *morula* larvae often die as small third instar larvae. They may pupariate, but do not show a prolonged larval life. It is possible that the *morula* larvae that die in the third instar or as early pupae are escapers that receive a critical amount of wild-type maternal product and are able to successfully complete the embryonic divisions. We found, however, that there was no embryonic lethality associated with the lethal *morula* alleles (see Materials and Methods). This observation, together with the defects seen during the early nuclear divisions in eggs laid by mr^1 or mr^2 mutant mothers, indicate that there are maternal pools of *morula* product during early developmental stages.

All homozygous and heterozygous combinations of lethal *morula* alleles show anomalous mitotic chromosome behavior as evidenced by a metaphase block, highly overcondensed chromosomes and frequent polyploid figures in squash preparations of larval brain neuroblasts (Fig. 5). Anaphase figures were never observed in the lethal *morula* combinations, thus the primary arrest point appears to be metaphase. The presence of polyploid cells is not inconsistent with metaphase arrest, as several other late larval mutants were shown to contain both metaphase-

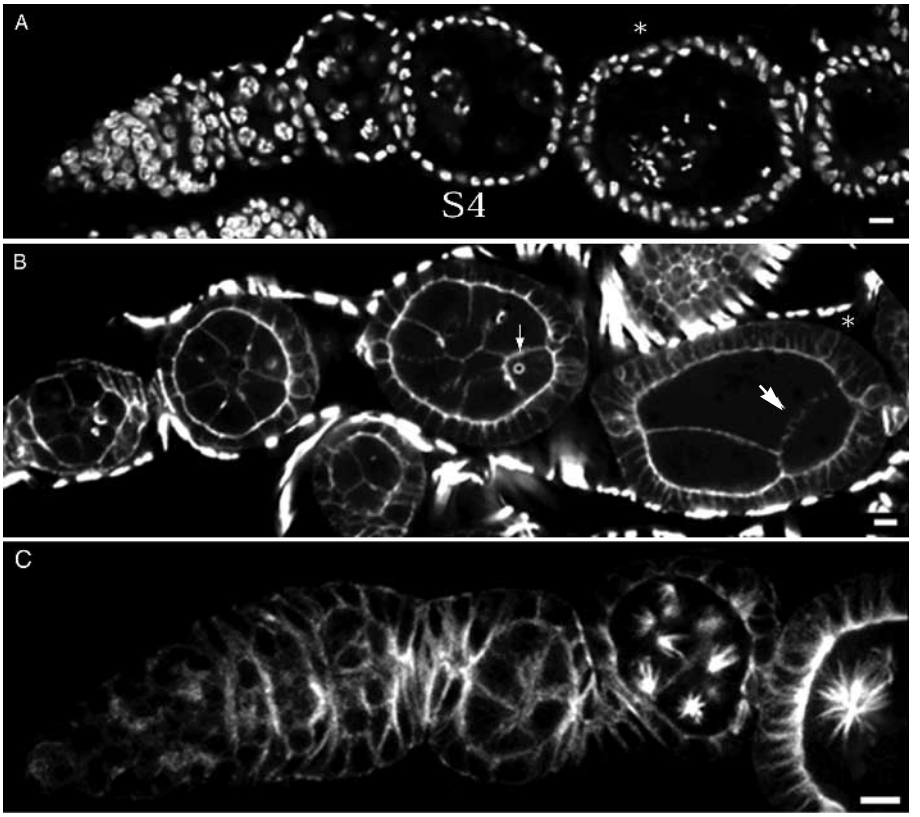

Fig. 2. The onset of the oogenesis phenotype of *morula* occurs at stage 4 (S4) and follows normal differentiation of the egg chamber. Scanning confocal images of mr^2 ovarioles stained with (A) Fuelgen, (B) FITC-phalloidin and (C) anti-tubulin mr^2 ovaries. The nuclear diameter of the nurse cells relative to the mitotically active follicle cells in A clearly shows that *morula* nurse cells are polyploid before they form spindles and inappropriate metaphase-like chromosomes. Note the double layer of follicle cells sourrounding the later cysts (asterisk in A and B). Ring canals as seen in B are formed (thin arrow) but degenerate in later stages (thick arrow). Bar, 10 μm.

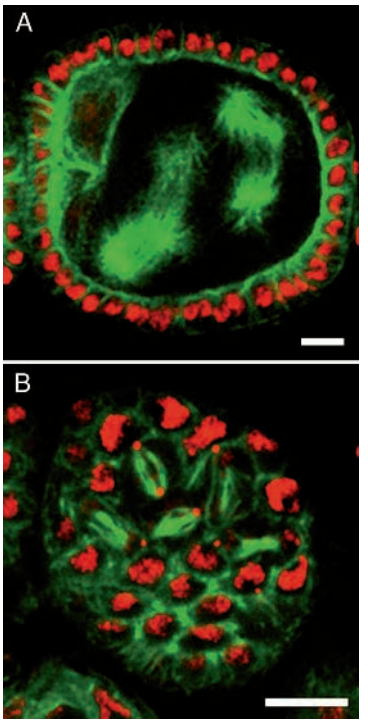

Fig. 3. The localization of CP190 centrosomal antigen does not correlate with the appearance of spindles in *morula*. Scanning confocal images of anti-CP190 (red) and anti-tubulin (green) of (A) inappropriate spindles of a mr^2 egg chamber and (B) follicle cells of a mr^2 egg chamber. The follicle cells provide a positive control for anti-CP190 staining; the antigen is nuclear during interphase but localizes to the spindle poles during follicle cell division. Bar, 10 μm.

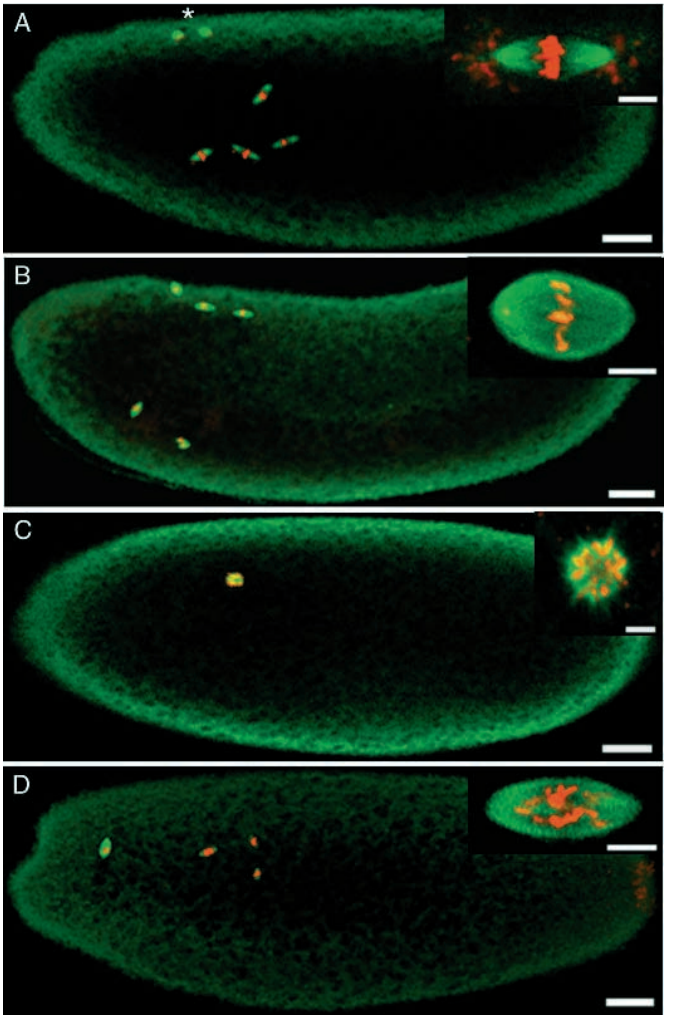

Fig. 4. *morula* females will lay eggs in certain genetic backgrounds and these show an early metaphase arrest phenotype. Polar bodies in these mutant eggs also appear as arrested metaphase figures. Scanning confocal micrographs of eggs stained with anti-tubulin (green) and Oligreen (red) laid by females that were (A) fertilized wild type, (B) fertilized mr^2, (C) unfertilized wild type and (D) mr^2 unfertilized. An example of the morphology of the metaphase spindles (A,B), polar body (C), or spindle found in place of polar body (D) is shown in the enlarged inset on each figure. The polar bodies in A are indicated by the asterick. Bars of inset, 5 μm; other bars, 50 μm. The DNA staining around the asters of the spindle in the inset of A is typical for stocks that carry *Wolbachia* endosymbionts (Kose and Karr, 1995).

arrested and polyploid cells (Gatti and Baker, 1989). As noted previously, it is likely that metaphase-arrested cells are capable of reverting to interphase and undergoing DNA replication (Gatti and Baker, 1989). Because the chromosomes of the blocked metaphases were frequently hypercondensed (Fig. 5C), it was often difficult to determine if sister chromatids had remained attached or if they had dissociated (Fig. 5E). As a consequence it was not possible to determine accurately the ploidy level of these nuclei. With this proviso in mind, metaphase figures were most frequently tetraploid or octaploid. Ploidy values higher than octaploid were not frequent. Many nuclei appeared pycnotic. Confocal microscopy of anti-tubulin- and DNA-stained mutant larval brains confirmed a metaphase arrest phenotype (Fig. 5J).

In addition to the aspects of the mutant phenotype typical of mitotic mutants, the lethal *morula* phenotype also displays necrosis of the gut just caudal to the attachment of the Malpighian tubules (data not shown). The site of the necrosis, which often appears as a dark ring visible through the larval cuticle, corresponds to the location of the hindgut imaginal ring and may reflect cell death resulting from mitotic defects. However, other imaginal rings (foregut, salivary gland) examined in mutant larvae did not appear necrotic.

Lethal alleles of *morula* also disrupt the endo cell cycle in nurse cells and at least one polytene larval tissue

To correlate the nurse cell phenotype of the viable *morula* alleles with the mitotic mutant phenotype of lethal *morula* alleles, we produced mosaics in which the germ cells of the ovary were mutant for the lethal mr^5 allele using the Flpase-FRT system (see Materials and Methods). Induction of mr^5 mosaics produced a low frequency of inappropriate spindles and metaphase-like chromosomes in stage 4 egg chambers (of twelve ovaries examined two had inappropriate spindles). The spindles observed in mr^5 mosaics were identical to those seen in mr^1 and mr^2 mutants (Fig. 6). Egg chambers with a mutant phenotype most likely occur infrequently because of a metaphase arrest occurring in the mr^5 mutant clones during the mitotic cystoblast divisions that produce the nurse cells. In

addition to the nurse cell phenotype, aspects of the visible phenotype of *morula*, namely rough eyes and reduced bristles, also were observed among the mr^5 mosaic flies. As well as demonstrating that the same mutation can disrupt the mitotic and the endo cell cycle, this result is evidence for a cell autonomous function of *morula*, further supported by our observation of patches of metaphase-arrested mitotic figures in larval brains of mr^5 mosaic larvae (data not shown).

Given the defect of *morula* female-sterile alleles (mr^1 and mr^2) of disrupting endo cell cycle maintenance in nurse cells and given the hypomorphic nature of these alleles, we were anticipating a null allele of *morula* to disrupt endo cell cycles in polytene larval tissues. There was no severe disruption in the salivary glands of lethal *morula* larvae, although the glands are often small and their polytene chromosomes are thin. We used anti-tubulin staining of mutant larval tissues to search exhaustively for inappropriate spindle formation in polytene cells. There were no spindles present in the polytene cells of the salivary gland, gut, Malpighian tubules, gastric caeca, fat body or the proventriculus. However, we found a disruption of the endo cycle in the larval ring gland (Fig. 7).

In the ring glands of *morula* mutant larvae, we observed polytene cells adjacent to cells with large spindles containing more than the diploid number of condensed chromosomes (Fig. 7B,C). In wild-type development, the last mitotic division in these cells occurs during the first larval instar stage and polytenization begins prior to when the aberrant spindles were present in *morula* mutants (Aggarwal and King, 1969). Thus the mutant ring gland cells appear to initiate polytenization, but like the nurse cells, revert to a mitotic state.

Presence of cyclin B in *morula* mutant nurse cells

In *morula* mutants, there is an aberrant mitotic state in the endo cycle and a failure to exit mitosis during division. The inactivation of cdc2/cyclin B kinase normally is required for the completion of mitosis and the kinase is likely to be inactive during the endo cycle (Lehner and O'Farrell, 1990; Lilly and Spradling, 1996; Whitfield et al., 1990). Therefore one possible explanation for the observed *morula* phenotypes is that *morula* plays a role in the inactivation or the maintenance of the inactivation of the cdc2/cyclin B kinase. Cdc2/cyclin B kinase is inactivated during mitosis by cyclin B degradation (Glotzer et al., 1991). Cyclin B protein is absent in the polyploid nurse cells (Lilly and Spradling, 1996), even though the transcript is present in early egg chamber stages (Dakbt abd Gkover, 1992). To test whether *morula* could destabilize cyclin B we examined cyclin B protein levels in *morula* mutant ovaries. We compared anti-cyclin B staining in ovaries from mr^2 females and wild-type siblings (stained in the same Eppendorf tube) (Fig. 8). In the wild-type nurse cells, cyclin B protein is absent or present at levels only slightly above background (Fig. 8A). In contrast, in mr^2 mutant nurse cells, cyclin B protein is readily detectable (Fig. 8B). Note the dividing follicle cells of both wild-type and mutant egg chambers show a patchwork staining pattern reflecting local synchrony of

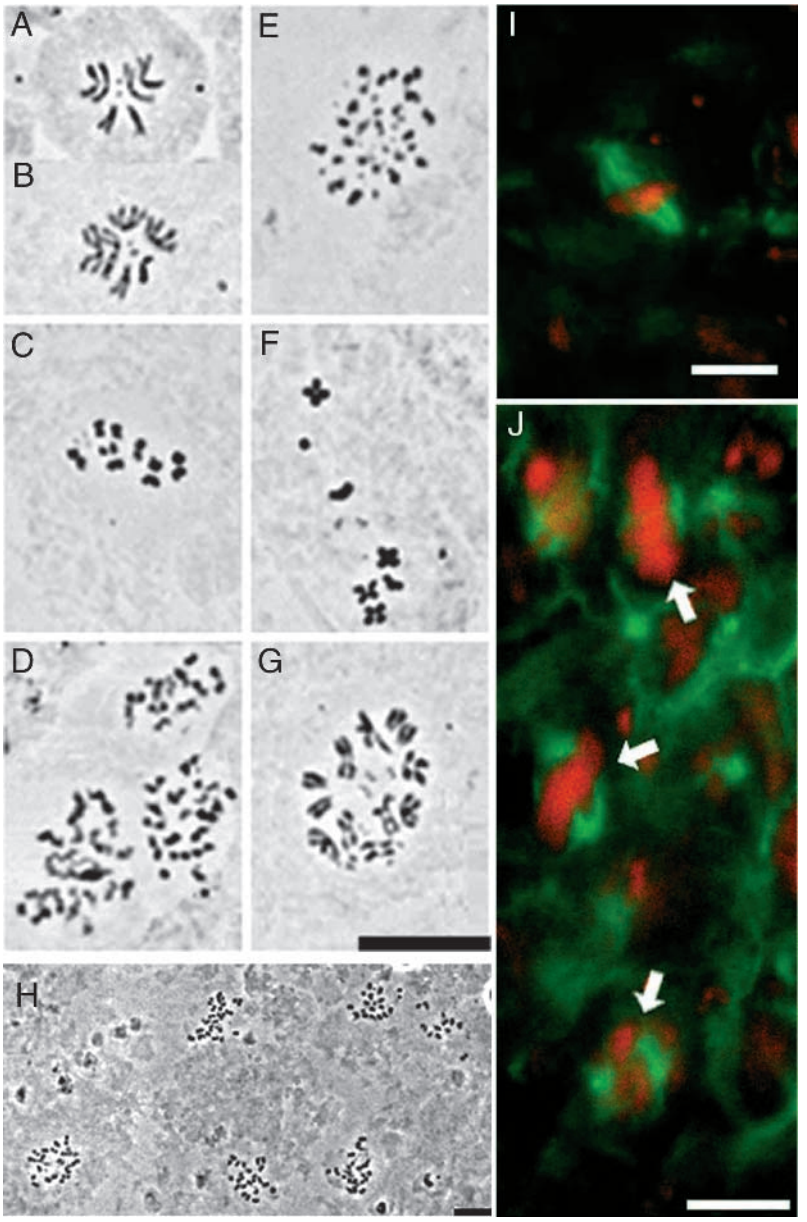

Fig. 5. Zygotic lethal mutations of *morula* exhibit late larval/early pupal lethality and are mitotic mutants showing a metaphase-arrest phenotype. Aceto-orcein-stained larval brain squashes showing mitotic figures of (A) wild-type female, (B) wild-type male and (C-H) mr^5. Chromosomal abnormalities include overcondensation (C,E,F) and polyploidy (E,G). Mitotic figures sometimes appear to be undergoing decondensation (D). (A-G) Bar in G represents 10 μm. An example of a squash showing the high mitotic index typical of lethal *morula* alleles (here mr^4/mr^5) is shown in H (bar, 10 μm). Scanning confocal images of (I) wild-type and (J) $mr^5/Df(2R)2651ex3$ larval brains stained with anti-tubulin (green) and propidium iodide (red) show spindles (arrows) of the lethal *morula* alleles to be bipolar and numerous. Bars in I and J, 5 μm.

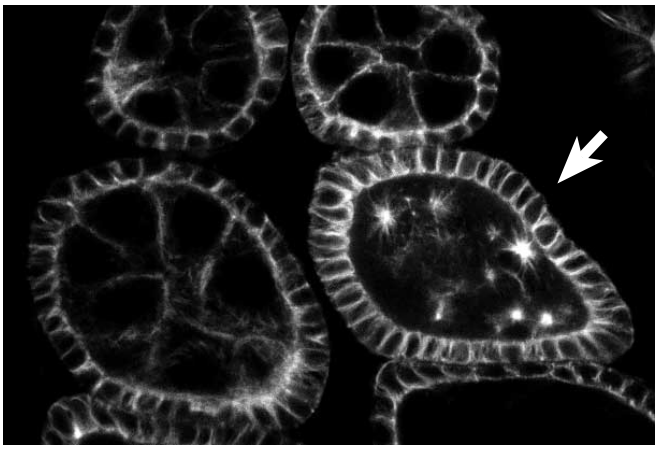

Fig. 6. Mitotic spindles in mr^5 mosaic ovarioles. Homozygous mutant clones of the lethal mr^5 allele were induced in the ovary using the FLP recombinase system. Staining with anti-tubulin antibody reveals the inappropriate presence of mitotic spindles in the mutant egg chamber on the right (arrow). A heterozygous, wild-type, egg chamber of the same stage is shown on the left.

mitotic divisions. In this experiment, the intensity of the follicle cell staining serves as an internal reference. Comparing the cytoplasmic staining in the nurse cells to the patchwork pattern of the follicle cells, it can be observed that the nurse cell staining of mr^2 nurse cells matches the bright follicle cell staining. In contrast, the nurse cells of the sibling control more closely resemble the weakly staining follicle cells. Thus, in *mr* mutant nurse cells, cyclin B protein is present at inappropriately high levels, suggesting that cdc2 kinase activity is also high. Because the follicle cells are actively dividing during the relevant stages of oogenesis, it was not possible to directly assay nurse cell kinase activity in ovary extracts.

If high cyclin B levels and associated cdc2 kinase activity disrupt the endo cycle by inducing mitotic functions in *morula* mutants, then cdc2 activity must normally not be required during polyploidization of the nurse cells. The endo cell cycle of the larval polytene cells does not require cdc2 activity (Stern et al., 1993). We tested whether nurse cell development normally requires cdc2 by using a temperature-sensitive allele to eliminate activity during oogenesis. By crossing the temperature-sensitive mutation $Dmcdc2^{E1\text{-}24}$ to the $Dmcdc2^{B47}$ null allele (see Materials and Methods), adult females were recovered that ceased to lay eggs at the restrictive temperature. Upon shifting to restrictive temperature, dividing cells in the ovary gradually disappeared over a period of 5 days (Fig. 9). In contrast, nurse cell nuclei persisted. Since the overall size of nurse cell nuclei increased with time spent at restrictive temperature, the temperature shift did not arrest nurse cell growth (Fig. 9C-E). Using nurse cell growth to indicate endo cell cycle activity, we conclude that wild-type nurse cell endo cycles do not require cdc2. This is consistent with the proposal that the inappropriate mitotic state in *morula* nurse cells results from misregulation of cdc2/cyclin B kinase.

DISCUSSION

As cells make the transition from the mitotic cell cycle to the endo cycle, they must inactivate and maintain the inactivation of mitotic functions. Although mitotic regulators have been identified, *morula* is the first example of a gene that blocks mitotic activities in the endo cycle. Both the female-sterile and the lethal alleles of *morula* cause the appearance of mitotic spindles and condensed chromosomes in tissues undergoing the endo cell cycle. In nurse cells, we found this phenotype to be correlated with inappropriately high levels of cyclin B. *morula* is also needed for the metaphase-anaphase transition

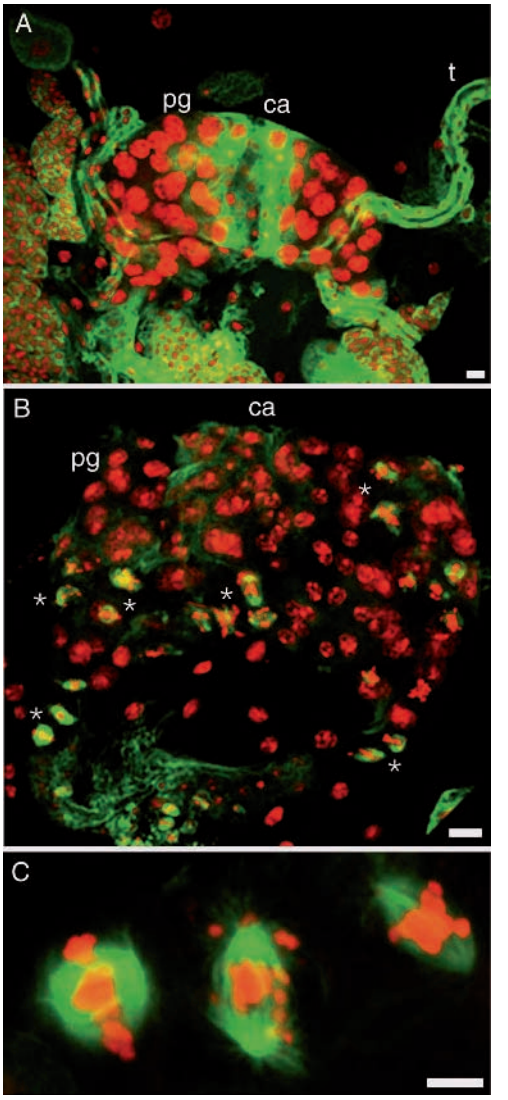

Fig. 7. Ring gland and associated tissue of (A) wild-type and (B)mr^5 larvae stained with anti-tubulin (green) and propidium iodide (red). The ring gland consists of four cell types (Aggarwal and King, 1969; King et al., 1966): (1) prothoracic gland cells, roughly 60 cells that are visibly polytene, (pg); (2) the corpus allatum cells, 20 in number, not visibly polytene but reported to have 32C DNA content, (ca); (3) corpus cardiacum cells (not marked); and (4) tracheal cells (t). (B) In ring glands of third instar larvae of *morula* lethals, inappropriate spindles and chromosomes form in the prothoracic gland cells, indicated by asterisks. Some, but not all prothoracic gland cells of the mutant are clearly disrupted and contain large metaphase-arrested spindles. (C) Enlargement of inapproriate spindles and chromosomes. Bar in A,B, 10 μm; bar in C, 5 μm

during mitosis. Thus this gene plays a critical role in inactivating mitotic functions in several types of cell cycles.

One explanation for the defects in *morula* mutants is the stabilization of microtubules. While this accounts for the presence of spindles in the nurse cells and the ring gland as well as the mitotic block in embryos and larval neuroblasts, it does not explain chromosome condensation in the mutant polyploid nurse cells and polytene ring gland. Although artificial stabilization of the microtubules in the nurse cells by taxol treatment can promote microtubule arrays, it does not lead to chromosome condensation as seen in *morula* mutants (J. Robinson and T. Hays, personal communication). It is also possible that the primary defect in *morula* is inappropriate chromosome condensation. Condensed meiotic chromosomes are capable of assembling spindles in the oocyte, so it is not impossible for chromosomes of the mutant polyploid, polytene and mitotic tissues to have similar properties. This hypothesis, however, does not easily account for the presence of cyclin B protein in the mutant nurse cells because it requires cyclin B protein levels to be downstream of chromosome condensation. This contradicts the causal order of events in mitosis.

We favor a model in which *morula* controls cell cycle regulators. The dual role of *morula* in the endo and mitotic cell cycles is explained by a common function in inactivating mitosis-promoting activity. In this model, *morula* function is required in the mitotic cycle for the metaphase-anaphase transition and in the endo cycle for maintaining an inactive state of mitosis-promoting activity. The most simple possibility is that *morula* acts, directly or indirectly, to inactivate cdc2/cyclin B kinase. This is consistent with the observation that diploid mitotic cells can become polytene in *cdc2* mutants (Hayashi, 1996). There are several reasons, however, why *morula* cannot solely inactivate cdc2/cyclin B kinase. One known mechanism for the inactivation of cdc2/cyclin B kinase is the regulated degradation of cyclin B protein. In *S. cerevisiae* and *Xenopus* in vitro extracts, nondegradable forms of cyclin B result in an anaphase arrest, rather than a metaphase arrest as observed in *morula* mutants (Holloway et al., 1993; Surana et al., 1993). The *Drosophila* FIZZY protein is needed for the degradation of cyclin A and B at the metaphase/ anaphase transition (Dawson et al., 1993, 1995; Sigrist et al., 1995). We examined the nurse cell morphology at the non-permissive temperature in fzy^6 mutant females. Despite the fact that the females failed to produce eggs, the nurse cells did not contain the mitotic spindles seen in *morula* mutants (B. Reed, unpublished observations).

Cyclin B and cyclin A proteins are targeted for degradation during mitosis by the cyclosome or anaphase-promoting complex (APC) (Irniger et al., 1995; King et al., 1995; Sudakin et al., 1995). Interestingly, there are other substrates of the APC whose degradation is necessary for the metaphase/anaphase transition (Cohen-Fix et al., 1996; Funabiki et al., 1996). Consequently, it is possible that *morula* controls the activity of the

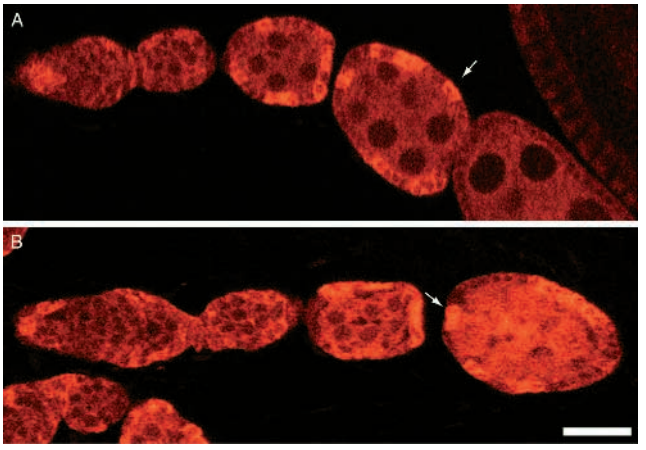

Fig. 8. Anti-cyclin B stained (A) wild-type and (B) mr^2 ovarioles. Samples were stained in the same Eppendorf tube. Confocal images are cross sections through the interior of the egg chambers and were scanned in only one plane of focus. The patchwork pattern of anti-cyclin staining in the follicle cells reflects localized sychrony of mitosis in this dividing population of cells. The arrows indicate follicle cells with high levels of cyclin B staining. This patchwork pattern serves as an internal reference for the nurse cell cytoplasmic staining which is elevated in *morula*. Bar, 25 μm.

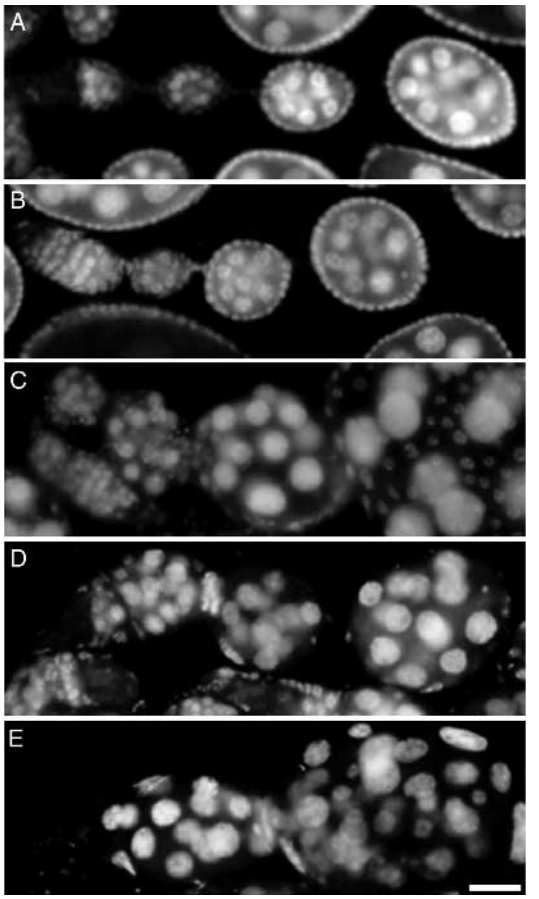

Fig. 9. The growth of nurse cell nuclei does not show a requirement for cdc2 kinase activity. (A,C-E) DAPI-stained ovaries of $Dmcdc2^{B47}/Dmcdc2^{E1\text{-}24}$, (B) wild-type sibling control. (A) Mutant control maintained at permissive temperature (18°C) for 5 days, (B) wild-type control maintained at the restrictive temperature for 5 days. (C-E) mutants shifted from permissive to restrictive temperature and fixed following 1 days, 3 days and 5 days, respectively. Note the gradual disappearance of follicle cells as well as early egg chambers and the persistence and growth of polyploid nurse cell nuclei through (C-E). Bar, 25 μm.

APC. In mitotic cells in *morula* mutants, a failure to activate the APC would cause a metaphase arrest. In polyploid or polytene cells in *morula* mutants, a failure to maintain active APC could lead to the stabilization of a set of mitotic proteins, including cyclin B, producing an inappropriate mitotic state. A model in which active APC blocks mitosis in the endo cycle is consistent with the observation that one of the APC subunits, *bimE*, plays a role in inhibiting the onset of mitosis in *Aspergillus* (James et al., 1995; Osmani et al., 1988, 1991; Peters et al., 1996; Zachariae et al., 1996).

Some, but not all, of the tissues in the endo cell cycle are affected by *morula* mutations. The presence of the mutant phenotype in the nurse cells and larval ring gland can be explained by *morula* function being particularly critical during the early rounds of the endo cycle when there may be residual levels of mitotic functions. Aggarwal and King (1969) report that the prothoracic cells of the ring gland initiate the endo cycle late in larval development, between 46 and 97 hours (Aggarwal and King, 1969). The onset of endo cycles in this tissue is thus very late in development, as most polytene larval tissues initiate endo cycles during embryogenesis (Smith and Orr-Weaver, 1991). The depletion of maternal *morula* in the ring gland may render it less able to maintain inactivation of mitotic functions during the endo cycle than other tissues that polytenize earlier in development. Only some of the ring gland cells contain mitotic spindles and this may reflect a difference in perdurance of maternal *morula* product between different cells. At the same developmental times that the ring gland is affected in *morula* mutants, other polytene tissues maintain a proper endo cycle. This indicates that *morula* function is particularly critical at the transition between the mitotic and endo cycles and during early rounds of polytenization.

In *morula* mutants, the nurse cells begin to become polyploid but revert to mitosis at stage 4. The appearance of the defect at this stage is striking because mitotic-like features appear at stage 4 during normal nurse cell development (Ribbert, 1979; Spradling, 1993). Prior to stage 4, the nurse cell chromosomes are polytene. At stage 4, they become bulbous and the bundles of sister chromatids dissociate, leaving five distinct blobs. This separation of the polytene chromosomes appears to involve chromatin condensation and it may represent a partial mitotic state, requiring some mitotic activities. Thus the stage 4 nurse cells may be vulnerable to regressing into mitosis, making *morula* function particularly critical at this developmental stage. Moreover, stage 4 nurse cells may require maximal levels of *morula* activity and this may explain why this tissue is affected by hypomorphic alleles. Other tissues in the adult that initiate endo cycles, such as the follicle cells, presumably are not as vulnerable to reverting to mitosis and, consequently, are not affected by leaky *morula* alleles. In contrast, the small bristles in viable *morula* alleles suggests that these polyploid cells do require *morula* (Lees and Waddington, 1942).

Identifying the protein encoded by *morula* is likely to provide insights into its cell cycle targets. An intriguing question is how the *morula* product serves to maintain mitotic functions in an inactive state in the endo cycle yet cyclically inactivates them in dividing cells. This suggests that the *morula* gene itself is subject to differential regulatory mechanisms in distinct types of cell cycles. The *morula* gene provides the opportunity to understand how one regulatory activity participates in different cell cycles to provide modulation of the cell cycle.

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