Coordinate developmental control of the meiotic cell cycle and spermatid differentiation in *Drosophila* males

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SUMMARY

Wild-type function of four *Drosophila* genes, *spermatocyte arrest, cannonball, always early* and *meiosis I arrest,* is required both for cell-cycle progression through the G_2/M transition of meiosis I in males and for onset of spermatid differentiation. In males mutant for any one of these meiotic arrest genes, mature primary spermatocytes with partially condensed chromosomes accumulate and post-meiotic cells are lacking. The arrest in cell-cycle progression occurs prior to degradation of cyclin A protein. The block in spermatogenesis in these mutants is not simply a secondary consequence of meiotic cell-cycle arrest, as spermatid differentiation proceeds in males mutant for the cell cycle activating phosphatase *twine*. Instead, the arrest of both meiosis and spermiogenesis suggests a control point that may serve to coordinate the male meiotic cell cycle with the spermatid differentiation program. The phenotype of the *Drosophila* meiotic arrest mutants is strikingly similar to the histopathological features of meiosis I maturation arrest infertility in human males, suggesting that the control point may be conserved from flies to man.

Key words: meiotic arrest, spermatogenesis, cell cycle control, *Drosophila*, spermatid

INTRODUCTION

In metazoans, cell-cycle progression must be constrained by developmental programs to ensure that cells start, proceed through and stop the cell cycle at the correct time and place. Although the mechanisms that control cell-cycle progression in eukaryotes are being elucidated in increasing detail (Nurse, 1990), the mechanisms that regulate the core cell-cycle machinery to meet the requirements of development and differentiation are just beginning to be identified (Edgar et al., 1994; Edgar, 1995). One striking example of developmental control of the cell cycle occurs during *Drosophila* embryogenesis: the timing of mitosis in the fourteenth through the sixteenth divisions is specified by developmentally regulated transcription of *string* (Edgar and O'Farrell, 1990), a *Drosophila* homologue of the *cdc25* cell-cycle phosphatase that activates the *cdc2* kinase at the G_2/M transition (Edgar and O'Farrell, 1989; Jimenez et al., 1990).

Male gametogenesis requires correlation of both mitotic and meiotic cell cycles with a complex cellular differentiation program. During spermatogenesis in *Drosophila*, each gonial cell at the tip of the testis undergoes four rounds of synchronous mitotic divisions to produce a cyst of sixteen interconnected spermatocytes (reviewed in Fuller, 1993; Lindsley and Tokuyasu, 1980). After an extended G_2 phase during which the

spermatocytes are highly transcriptionally active and grow 25 times in volume, the sixteen mature primary spermatocytes proceed through the two meiotic divisions, followed by series of dramatic morphological changes, giving rise to a cyst of 64 elongated spermatids.

Although the meiotic divisions consistently precede spermiogenesis, prior completion of meiosis does not appear to be required for activation of the spermatid differentiation program. In *Drosophila* males, meiotic division is regulated by the cell-cycle kinase *cdc2* and its activating phosphatase *twine*, a *cdc25* homologue specific for meiosis (Alphey et al., 1992; Courtot et al., 1992). In flies mutant for either *cdc2* (Stern et al., 1993; Sigrist et al., 1995) or *twine* (White-Cooper et al., 1993; this paper) spermatocytes skip several crucial events of meiotic division, but initiate and enact spermatid differentiation. Both flagellar elongation and condensation and shaping of spermatid nuclei proceed despite failure to complete meiotic chromosome segregation and cytokinesis (Eberhart and Wasserman, 1995; White-Cooper et al., 1993), suggesting that the spermatid differentiation program is independent of meiotic cell-cycle progression.

We have identified male-sterile mutations in four autosomal *Drosophila* genes that, in contrast to *twine*, cause both arrest of cell-cycle progression at the G_2/M transition of meiosis I and failure to initiate spermatid differentiation in males. The

block in spermatogenesis characteristic of these mutants suggests a developmental control point that coordinates the meiotic cell cycle with the differentiation program of male gametogenesis. The striking similarity between the phenotype of the *Drosophila* mutants and the clinical features of the human male infertility syndrome meiosis I maturation arrest (Meyer et al., 1992; Soderstrom and Suominen, 1980) suggests that the regulatory mechanism identified by the *Drosophila* meiotic arrest mutants might be conserved and raises the possibility of a genetic basis for the human syndrome.

MATERIALS AND METHODS

Fly strains

Visible markers, balancer chromosomes and deletions are described in Lindsley and Zimm (1992), except *Df(3L)HR370+Dp(3L)pDP7* and *Df(3L)HR218* (Wohlwill and Bonner, 1991), and *Df(3L)Pc*, which is associated with a small inversion, and *Df(3L)Pc-MK* (revised by A. Carpenter, personal communication and Flybase). Note that the right breakpoint of *Df(3L)Pc-MK* extends approximately 70 Kb proximal to the right breakpoint of *Df(3L)Pc* (S. Viswanathan, unpublished). The w^+ bearing P-element insert, *Is(3)1959*, was generated in the laboratory of M. Scott and localized cytologically by D. Andrew (personal communication).

Meiotic arrest mutants

The *aly¹* allele was identified as *ms(3)ry2* in a screen for transposable element induced mutations (Cooley et al., 1988). However, genetic analysis (Fig. 2A) indicated that the *aly¹* mutation mapped to 63AB and was not associated with the marked P-element inserted at 96B in the line. The *can¹* allele was identified in a screen for male-sterile mutations generated by mobilization of a single P-element (Fuller and Scott Lab collaboration). Subsequent genetic analysis (Fig. 2B) indicated that the *can¹* mutation mapped to 67DE and was not associated with the marked P-element, which was inserted at 86C in the line. The *sa¹* allele, originally designated *VO45*, was isolated from the wild in Rome (Sandler et al., 1968) and initially characterized by D. Lindsley and R. Hardy (Lindsley and Zimm, 1992). The *mia¹* mutation was identified by J. Hackstein in a screen for EMS induced male-sterile mutants (Hackstein, 1991).

New alleles of the meiotic arrest loci were isolated by failure to complement *aly¹*, *can¹* and *sa¹* in two screens (Table 1) as follows. Isogenized *red e* males were mutagenized with a final concentration of 25 mM ethyl methanesulfonate (EMS) for 24 hours (Lewis and Bacher, 1968) and mated to *ri B₂tⁿ e/TM3* virgin females. Single *red e*/TM3* F₁ males were then crossed to either *aly¹ can¹/TM6C*, *can¹ sa¹/TM6C* or *aly¹ can¹ sa¹/TM6C* virgin females and three of their *red e*/(aly¹) can¹ (sa¹)* F2 sons tested for fertility by mating to virgin females. Altogether, 6012 EMS-treated third chromosomes were screened for *aly*, 7000 for *can* and 5447 for *sa*. P-element-induced alleles were generated using the *Birm2;Δ2-3* hybrid dysgenesis system (Robertson et al., 1988). An isogenized third chromosome marked with *red e* was crossed into the *Birm2* background and flies from the resulting stock were mated to *Sp/CyO; Δ2-3 Sb/TM6 Ubx* virgin females. *Birm2/CyO* or *Sp; red e*/Δ2-3 Sb* F₁ males were then crossed to *ri B₂tⁿ e/TM3* virgin females and single *CyO* or *Sp; red e*/TM3* F2 males resulting from this cross were mated to *aly¹ can¹ sa¹/TM6B* virgin females. For each single male, three of his *red e*/aly¹ can¹ sa¹* sons were tested for fertility by mating to virgin females. Altogether, 6295 third chromosomes were screened. For both screens, new mutants that were male sterile in *trans* to the multiple mutant tester chromosome were recovered through their *red e*/TM6C* siblings, retested for failure to complement the double or triple mutant chromosome, then tested against *aly¹*, *can¹* and *sa¹* individually to assign

them to one of the three complementation groups. All mutants isolated failed to complement the representative allele of one locus but complemented the other two.

Fly husbandry and genetic tests

Flies were raised on standard cornmeal molasses agar medium at 25°C unless otherwise indicated. Except for the above screens, fertility tests were carried out by mating 20 single male or female flies each to three virgin female or male flies, respectively, in a vial. A fly was scored as fertile if at least 20 larvae appeared in 10 days and sterile if no larvae appeared after 10 days. Vials with fewer than 20 larvae were checked for presence of live parental flies. Those where either of the parental flies had died were not scored; otherwise, the fly was scored as semi-fertile. For genotypes designated male or female fertile, most of the scorable individual fertility tests had progeny. Genotypes were designated sterile if all of the 20 individual fertility tests had no progeny. In no cases were appreciable numbers semi-sterile. Viability tests were carried out by crossing 20 male flies to 20 female flies in a bottle and the progeny classes counted from the 9th through the 18th day at 25°C. Genetic combinations where mutant/*Df* or homozygous progeny were approximately 15-33% of the total progeny were designated viable. In all cases, the results were clearly either no progeny (lethal) or viable. *aly* and *sa* alleles were crossed to their respective deficiencies and the viability of mutant/*Df* progeny scored. Homozygous progeny were scored for *can* and *mia*.

For complementation tests, males heterozygous for a meiotic arrest mutant were crossed to heterozygous *can¹* or *mia¹* females or to females carrying deficiencies in the *aly* and *sa* regions. The doubly heterozygous sons were examined for the meiotic arrest phenotype in squashed testis preparations.

Recombination mapping was carried out in two steps. First, single recombinants between the mutant-bearing chromosome and a multiply-marked chromosome were assayed for inheritance of the meiotic arrest mutant. Once the mutation had been localized between two visible markers, additional recombinants from crossovers between those flanking markers were picked. Recombinants were tested for inheritance of the meiotic arrest phenotype by crossing to

Table 1. Isolation of additional alleles

Locus	Allele	Mutagen	Origin	Viability/ Sterility*
aly	*aly¹*	P	Cooley et al., 1988	MS, FF
	aly²	EMS	this paper	MS, FF
	aly³	EMS	this paper	lethal
	aly⁴	P	this paper	MS, FF
	aly⁵	P	this paper	MS, FF
	aly⁶	P	this paper	MS, FF
	aly⁷	P	this paper	MS, FF
	aly⁸	P	this paper	MS, FF
can	*can¹*	P	this paper	MS, FF
	can²	EMS	this paper	MS, FF
	can³	EMS	this paper	MS, FF
	can⁴	EMS	this paper	MS, FF
	can⁵	EMS	this paper	lethal
	can⁶	EMS	this paper	MS, FF
	can⁷	EMS	this paper	MS, FF
	can⁸	P	this paper	MS, FF
	can⁹	P	this paper	MS, FF
	can¹⁰	EMS	this paper	MS, FF
	can¹¹	EMS	this paper	MS, FF
mia	*mia¹*	EMS	Hackstein, 1991	MS, FF
sa	*sa¹*	spontaneous	Sandler et al., 1968	MS, FF
	sa²	EMS	this paper	MS, FF

*MS, male sterile; FF, female fertile.

a representative allele and scoring the double heterozygous progeny in squashed testis preparations.

Microscopy

Live squashed testis preparations were performed as described in Kemphues et al. (1980). For analysis of chromosome morphology, testes were dissected as above, then transferred to a drop of TB-1 buffer containing freshly diluted 10 μg/ml Hoechst 33342 (bis Benzimide) on a slide prior to squashing. Stained chromosomes were examined under fluorescence microscopy using a Zeiss compound microscope.

For immunocytochemistry, approximately 10 males of wild type and each mutant class were dissected in TB-1 buffer. Testes were fixed in 4% formaldehyde PBS for 20 minutes at room temperature, washed thrice (5 minutes each) in PBTx (PBS with 0.1% Triton X-100), incubated for 16 hours at 4°C in a 1:1000 dilution of rabbit anti-cyclin A polyclonal antibodies (gift from C. Lehner and P. O'Farrell) in PBTx with 2% normal goat serum (Vector), washed thrice (20 minutes each) in PBTx at room temperature, then incubated in a 1:500 dilution of biotinylated goat anti-rabbit antibody (Vector) in PBTx for 1 hour at room temperature, washed thrice (10 minutes each) in PBTx, treated with streptavidin HRP conjugate for 30 minutes, washed thrice (10 minutes each) in PBTx and stained with a solution containing 1 mg/ml diaminobenzidine/0.001% hydrogen peroxide. Testes were counter stained with 1 μg/ml Hoechst 33342 in PBS, mounted in 80% glycerol and examined on a Zeiss Axiophot microscope.

For immunofluorescence, samples were processed as described for immunocytochemistry up to the secondary antibody incubation stage. Testes were then incubated with a 1:200 dilution of rhodamine-conjugated goat anti-rabbit secondary antibody (Vector) for 1 hour at room temperature, washed in PBTx and counterstained with Hoechst 33342 as described above, then mounted in Polyfluor (Polysciences).

RESULTS

Function of four autosomal loci is required for entry into the first meiotic division in males and progression of spermatogenesis

Wild-type function of *always early* (*aly*), *cannonball* (*can*), *meiosis I arrest* (*mia*) and *spermatocyte arrest* (*sa*) is required both for progression of spermatogenesis through the meiotic divisions and for the onset of spermatid differentiation. In live squashed preparations of testes from wild-type adults, all stages of spermatogenesis are present (Fig. 1A). These include early spermatocytes, mature primary spermatocytes (large arrows), cells in meiotic division, round spermatids (small arrow) and elongated spermatid bundles (arrowheads). In testes from *aly, can, mia* or *sa* mutants, the early stages of spermatogenesis up through mature primary spermatocytes were present and appeared normal. However, postmeiotic stages of spermatogenesis were completely absent (Fig. 1B-E). Instead, mutant testes had an abundance of mature primary spermatocytes with large, round nuclei and prominent nucleoli.

The original *aly, can, mia* and *sa* mutations, which were identified in various male-sterile screens (Materials and Methods), define four separate genes based on map position and intercomplementation tests. aly^1 mapped to 3-4.4±0.9 map units (m.u.) based on recombination between the visible markers *ru* and *h*, and was localized to polytene interval 63AB based on failure to complement *Df(3L)HR370+Dp(3L)pDP7* and complementation by *Df(3L)HR218* (Fig. 2A). can^1 mapped to 3-31.0 ± 1.1 m.u. based on 57 recombinants between the
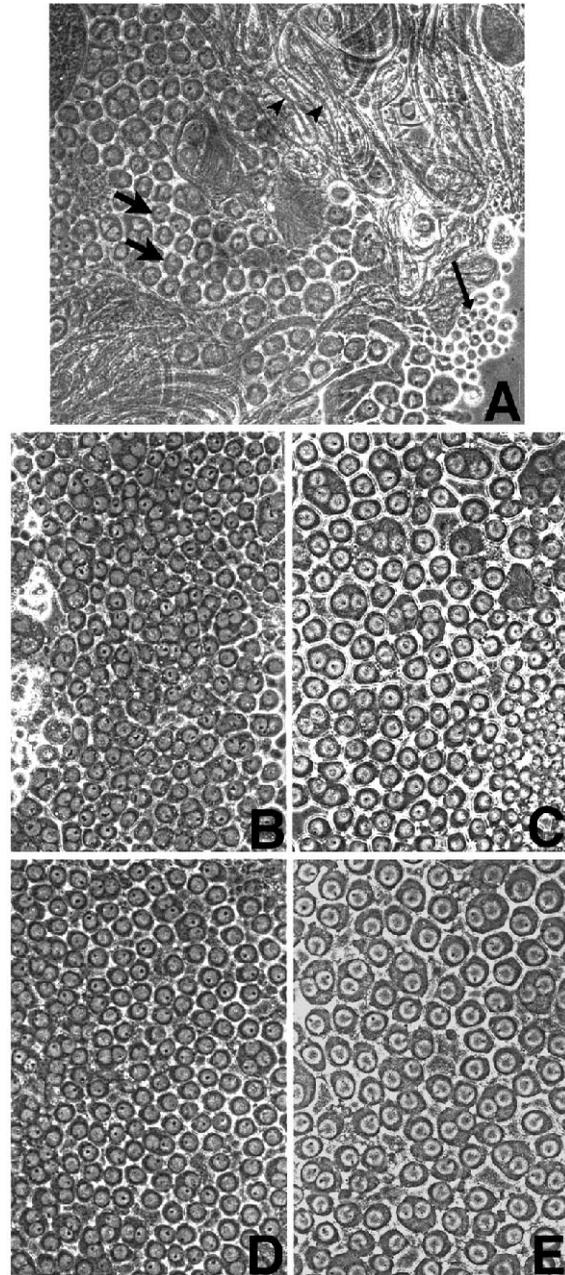

Fig. 1. Testes from *aly, can, mia* or *sa* males lack postmeiotic stages of spermatogenesis. Phase-contrast images of live squashed testis preparations. (A) Wild-type testes contain cells from all stages of spermatogenesis, including mature primary spermatocytes (large arrows), round spermatids (small arrow) and elongated spermatid bundles (arrowheads). Testes from (B) $aly^2/Df(3L)HR370$, (C) can^3/can^4, (D) $sa^1/Df(3L)Pc\text{-}cp1$ and (E) mia^1/mia^1 contain plentiful mature primary spermatocytes but lack postmeiotic stages.

visible markers *h* and *rs*. In another cross, can^1 mapped proximal to the w^+ bearing P-element insert *Is(3)1959*, located at 67C10. can^1 was complemented by *Df(3L)AC1*, *Df(3L)lxd6* and *Df(3L)vin2*, placing the locus in polytene interval 67D11-E2 (Fig. 2B). The sa^1 mutation was inseparable from ri^+ in seven recombinants in the *st p^p* interval between a sa^1 and a *st ri p^p* chromosome. sa^1 was localized to polytene interval 78C9-D2 based on its failure to complement *Df(3L)Pc-MK* and com-
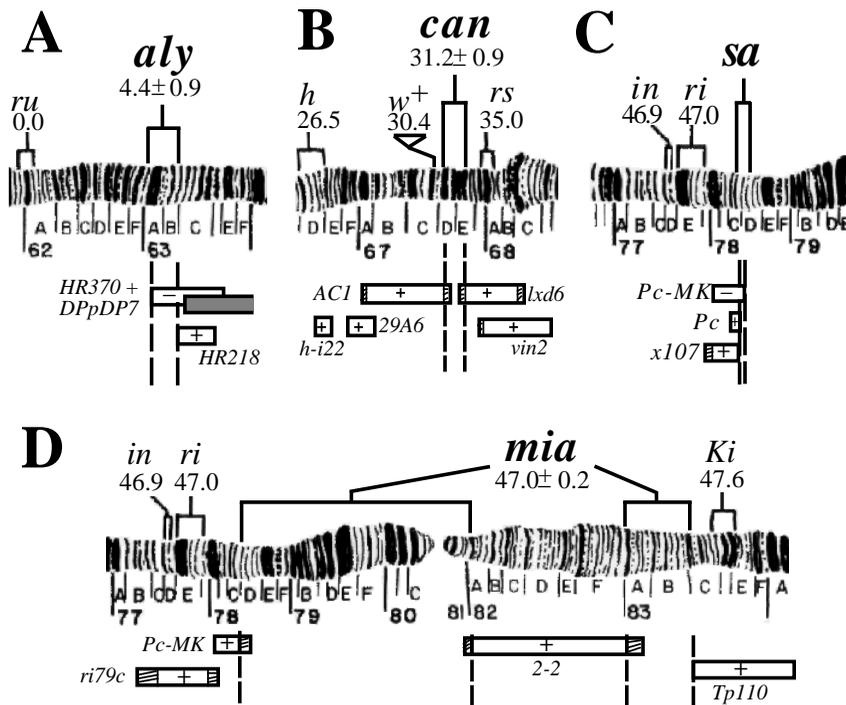
Fig. 2. Recombination and deletion maps of the meiotic arrest genes. Recombination map positions are given in numbers above the chromosome drawings, with marker loci positioned according to chromosomal region rather than genetic map. Open boxes represent regions of the genome deleted in the deficiencies, with hatched regions indicating uncertainties in the breakpoints. (+) Deficiencies that complement the mutants. (−) Deficiencies that fail to complement the mutants. Inverted triangle in B represents a P-element insertion. Polytene chromosome drawings adapted from Bridges (1935).

plementation by *Df(3L)Pc* (Fig. 2C). The *mia¹* mutation mapped to 3-47.0 ± 0.2 m.u. based on 14 recombinants between the visible markers *ri* and *Ki,* and was localized to polytene intervals 78C9-81F or 83A-C2 based on complementation by *Df(3L)ri79c, Df(3L)Pc-MK, Df(3R)2-2* and *Df(3R)Tpl10* (Fig. 2D).

Additional alleles of *aly, can* and *sa* were isolated in F₂ screens for failure to complement the original alleles after mutagenesis with EMS or P-element transposition (Table 1). The phenotype of most of the *aly, can* and *sa* alleles were similar. For both *sa* alleles and seven of the eight *aly* alleles, hemizygotes displayed the arrest phenotype in males but were viable and female fertile. *aly³* homozygotes and *aly³/Df* flies were not viable, but *aly²/aly³* flies were viable and showed the same phenotype as *aly²/aly¹* and *aly²/Df*. No deficiency for *can* is currently available. However, 10 of the 11 *can* alleles were homozygous viable and female fertile after accessary lethals on the chromosomes were removed by recombination. *can⁵* was homozygous lethal (14 recombinants between *h* and *th* tested), but was viable and female fertile when heterozygous with *can¹, can²* or *can³*. The lethality of *aly³/Df* and *can⁵* might be due to closely linked secondary lethals or rearrangements not visible in orcein-stained polytene chromosome preparations. Alternatively, *aly³* and *can⁵* might be null alleles. The single extant allele of *mia* is homozygous viable and female fertile. The triple mutant combination *aly¹ can¹ sa¹* is female fertile and male sterile, with similar effects in testis squashes as *sa¹* and *can¹*. Triple mutant *aly¹ can¹ sa¹* homozygotes are viable, although less healthy than heterozygous siblings. The similar effects of all the *aly, can* and *sa* alleles and of *aly/Df, sa/Df* and *mia/mia* on spermatogenesis suggest that, at least with respect to effects on male gametogenesis, arrest at the onset of the first meiotic division and failure to initiate spermatid differentiation is the strong loss-of-function phenotype.

Landmark events of the G₂/M transition for meiosis I in wild type

To pinpoint the mutant arrest point, we first established a time line of the cytological events of the wild-type G₂/M transition of meiosis I, based on a series of still images of unfixed cells (Fig. 3). At the end of the post-S growth phase, wild-type mature primary spermatocytes go through a G₂/M cell-cycle transition, during which a number of morphological events mark onset of the first meiotic division. Mature primary spermatocytes in unfixed squashed preparations have large, blocky nuclei and prominent nucleoli (Fig. 3A). The bivalents are already paired (Cooper, 1965) and appeared as three discrete structures next to the nuclear membrane in living cells viewed by fluorescence microscopy (Fig. 3A′, diagrammed in Fig. 4A). At the initiation of the G₂/M transition of meiosis I, the nuclei became round, the nucleoli gradually became pale and smaller (Tates, 1971; Figs 3B, 4B). Additionally, an organized, aster-like array of cytoplasmic components became visible at one side of each nucleus and the bivalents began to condense. The two autosomal bivalents often underwent condensation at different rates, resulting in one oblong and one rounded bivalent in the same nucleus. The sex bivalent, which is associated with the nucleolus, stained less brightly and appeared punctate at this stage when visualized by Hoechst staining (Figs 3B, 3B′, 4B). At the next landmark stage, the nucleoli had broken down completely, a characteristic refractile body and array of small particles appeared in the nucleus, and two distinct astral arrays of cytoplasmic components marking the meiotic spindle poles could be seen separating to opposite sides of the nucleus (Cenci et al., 1994; Figs 3C, 4C). The bivalents continued condensation and often began to move inward away from the nuclear membrane, and the sex bivalent again became brightly staining at this stage (Figs 3C′, 4C). By prometaphase, the two asters were at opposite poles and bivalents were fully condensed and had moved inward from the nuclear membrane
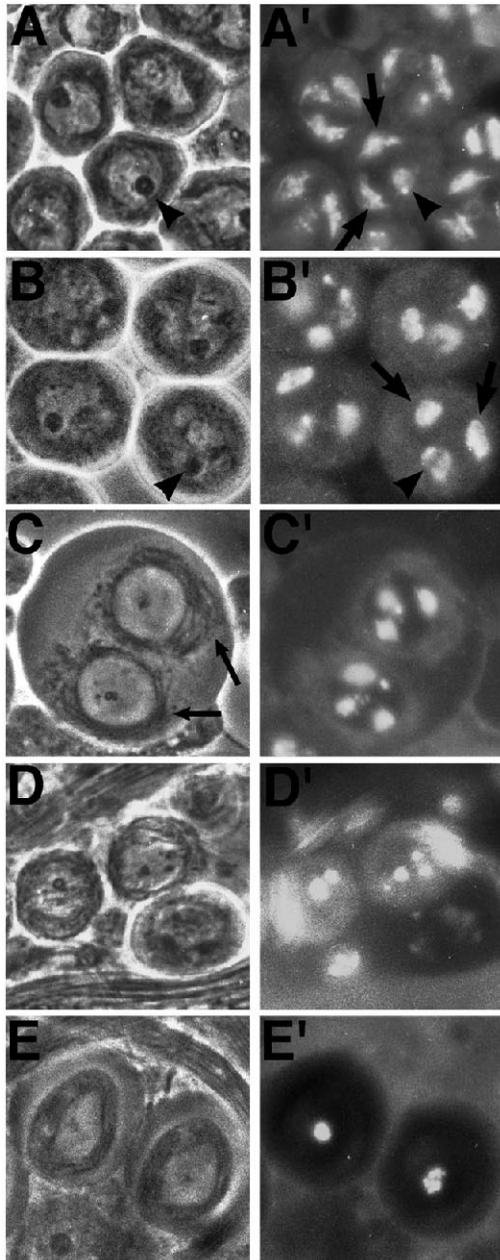
Fig. 3. Phase-contrast (left) and Hoechst (right) images of wild-type spermatocytes during the G_2/M transition. (A,A′) Mature primary spermatocytes with irregularly shaped nucleus, dark nucleolus (arrowhead in A) and paired meiotic bivalents (arrows and arrowhead in A′). The nucleolus is associated with the sex bivalent (arrowheads in A′,B′). (B,B′) Start of the G_2/M transition. Nucleus rounds up and bivalents initiate condensation. (C,C′) Nucleolus breaks down and the partially condensed chromosomes move inward. Asters (small arrows) separating to opposite poles. (D,D′) Prometaphase. Bivalents fully condensed. (E,E′) Metaphase. Nucleus spindle shaped. Bivalents aligned at the metaphase plate.

(Figs 3D, 3D′, 4D). The nucleus then became oval shaped and the astral membranes and mitochondria assumed the characteristic distribution around its perimeter. The bivalents often briefly aligned at a metaphase plate before the onset of anaphase (Figs 3E, 3E′, 4E).
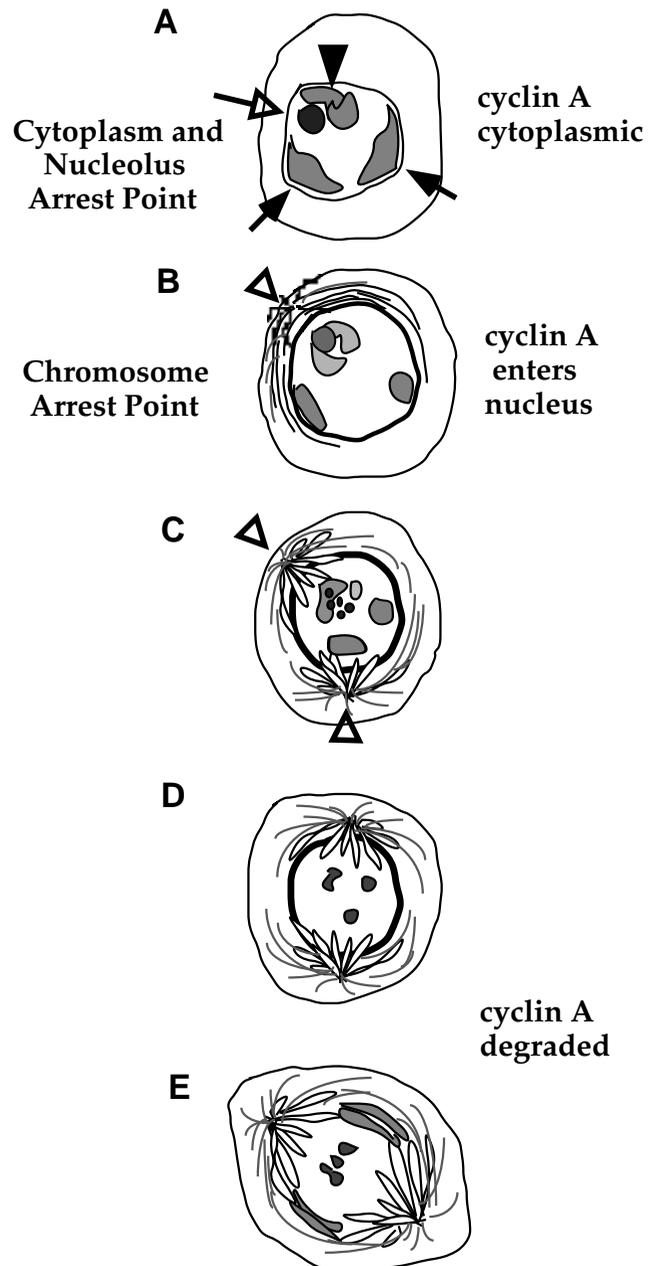
Fig. 4. Time line of the G_2/M transition for meiosis I in wild-type testis. Diagram representing superimposed phase-contrast and Hoechst images. (A) Mature primary spermatocytes with two autosomal bivalents (arrows) and a sex bivalent (arrowhead). The prominent nucleolus is dark (open arrow). Cyclin A protein is cytoplasmic. (B) Nucleus becomes round, bivalents initiate condensation and an aster-like array of cytoplasmic components (open arrowhead) becomes visible in phase contrast. Nucleolus gradually becomes paler and smaller. Cyclin A protein enters nucleus. Degree of chromosomal condensation is similar to that in mutant arrested spermatocytes. However, in the mutant arrested cells, neither nascent asters nor fading nucleolus were observed. (C) Nucleolus breaks down, asters (open arrowheads) separate, bivalents condense further and move away from nuclear membrane. Characteristic small nuclear refractile body appears. (D) Prometaphase. Bivalents fully condensed and asters at opposite poles of the nucleus. (E) Metaphase. Cyclin A protein has been degraded.

The wild-type stages from the onset of G_2/M transition to metaphase were relatively rare, suggesting that these phases of cell cycle proceed rapidly. In careful examination of 49 testes, we observed an average of 0.9 cysts of 16 cells each at the G_2/M transition of meiosis I out of an estimated total of 150 cysts per wild-type testis (Lindsley and Tokuyasu, 1980). Among the cysts of spermatocytes at the transition phase that we examined by Hoechst stain of live squashes, 38% were at stage B (Figs 3B, 4B), 27% were at stage C (Figs 3C, 4C), 21% were in prometaphase (Figs 3D, 4D) and 14% were at metaphase (Figs 3E, 4E). Wild-type spermatocytes within the same cyst often spanned more than one stage in the G_2/M transition due to a wave of developmental asynchrony across the cyst. This developmental asynchrony helped us order the stages. For example, within a single cyst, the nucleoli might be completely broken down (stage C) in some cells while other cells in the same cyst still retained pale nucleoli and less condensed bivalents (stage B).

aly, can, mia or *sa* spermatocytes arrest in the G_2/M transition of meiosis I with partial chromosome condensation

In *aly, can, mia* or *sa* males, the progression of spermatogenesis appears to be blocked during the G_2/M transition of meiosis I, so that what is a transient and rare stage in wild type becomes the predominant cell type in the mutant testis. The effect of each of the mutations was similar. In testes from males mutant for *aly, can, mia* or *sa*, the early stages of spermatogenesis appeared normal. However, large numbers of spermatocytes at the early stage of the G_2/M transition accumulate (average of 206 cells per testis, $n=40$ testes). Spermatocytes in the mutants arrested prior to full chromosome condensation, spindle pole organization and nucleolar breakdown (Fig. 5). Typically, the arrested cells had rounded nuclei and partially condensed chromosomes as in wild-type stage B (Figs 3B, 4B). The partially condensed chromosomes in the mutants were typically round, but usually remained next to the nuclear membrane and never became fully condensed. The nucleolus remained dark and prominent in the mutant cells, resembling the nucleolus of wild-type primary spermatocytes at stage A (Figs 3A, 4A). Especially in *can*, arrested spermatocyte nuclei

often contained an array of small particles resembling those in wild-type stage C. The sex bivalent was usually pale in Hoechst, with several bright punctate dots as in stage B of wild type. Immunofluorescence staining with antibodies against tubulin showed that accumulating spermatocytes in the mutants did not have organized spindle asters (data not shown). In contrast, in wild-type spermatocytes with chromosomes at the same degree of condensation, the nucleoli had already initiated breakdown and cytoplasmic astral arrays of microtubules were visible. Thus, in the mutants, the cytoplasm and the nucleolus of the accumulated spermatocytes appear to arrest at the mature primary spermatocyte stage, while the chromosomes appear to initiate condensation. The mutant spermatocytes failed to progress to subsequent stages of meiosis. Instead, they accumulated and then eventually degenerated at the base of the testis.

The appearance of the chromosomes in the arrested *aly* spermatocytes was subtly different from the chromosomes in the arrested *can, mia* and *sa* spermatocytes, which resembled wild-type stage B chromosomes. For several different *aly* allelic combinations examined, the chromosomes appeared less well condensed and their morphology less discrete than in *can, mia, sa* or wild type (Fig. 5), suggesting that *aly* mutations might affect events of chromosome condensation as well as progression of spermatogenesis.

To test whether the arrested cells that accumulate in *aly* testes are still functional intermediates in spermatogenesis, we took advantage of the temperature sensitivity of the aly^1 allele. Testes from aly^1/aly^1 or aly^1/Df males raised at 27°C showed the phenotype described above. In aly^1 males raised at 18°C, however, spermatocytes completed meiosis and underwent abnormal spermatid differentiation. When males raised at 27°C were shifted to 18°C, the G_2/M spermatocytes that had accumulated at the non-permissive temperature did not synchronously proceed through meiosis. No cells undergoing meiotic divisions or spermatid differentiation were observed in aly^1/aly^1 or aly^1/Df mutant testes 48 hours after the temperature shift. At 75 and 96 hours after the shift, at most one cyst of early spermatid cells per testis was observed. Most of these early spermatid cells that progressed beyond the arrest point appeared abnormal. The delayed timing and
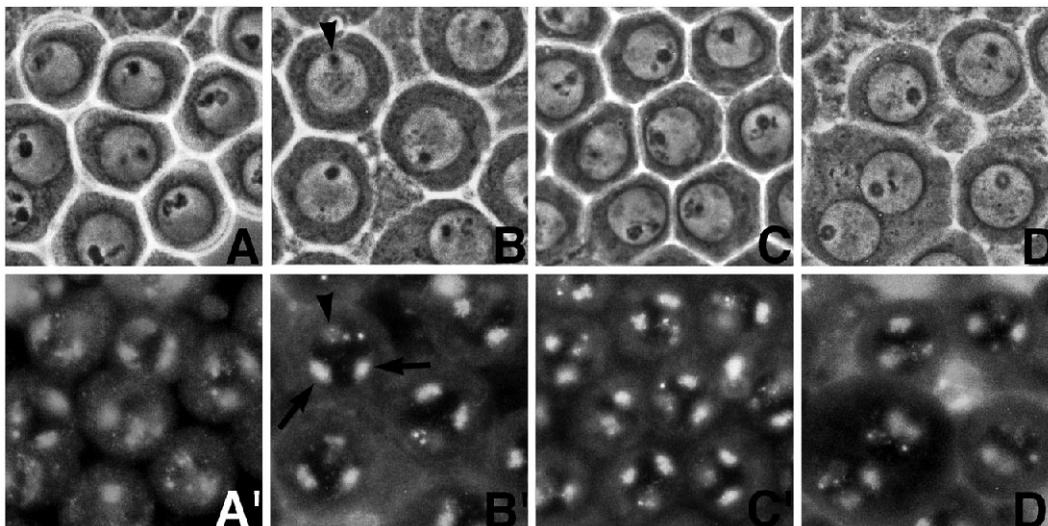

Fig. 5. Phase-contrast (top) and Hoechst (bottom) images of arrested cells in the meiotic arrest mutant testes. Mutant spermatocytes are blocked at a stage where the bivalents (arrows and arrowhead in B′) have begun to condense but the nucleolus (arrowhead in B) is still intact. (A,A′) *aly²/Df(3L)HR370*; (B,B′) *can³/can⁴*; (C,C′) *sa¹/Df(3L)Pc-cp1*; (D,D′) *mia¹/mia¹*.
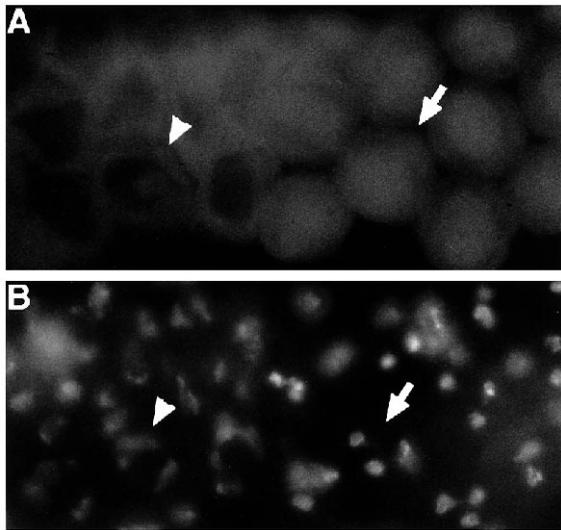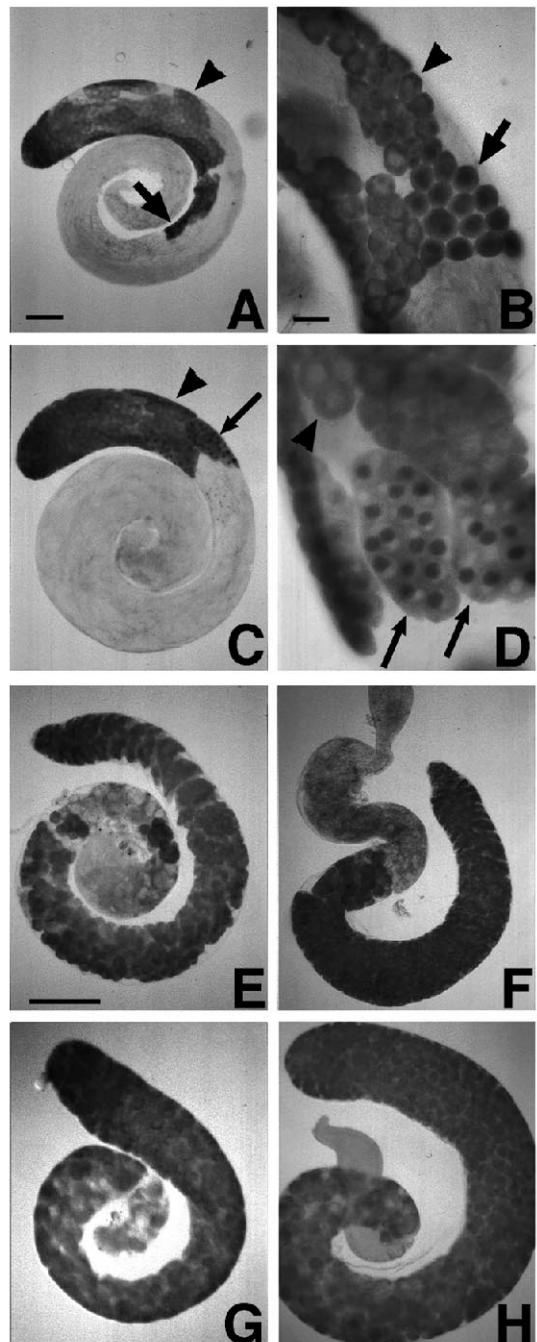
Fig. 6. Cyclin A protein localization in wild-type spermatocytes at
G$_2$/M transition. Whole-mount testes stained with (A) cyclin A
antibody and (B) Hoechst. Left half, mature primary spermatocytes
with cytoplasmic cyclin A protein and crescent-shaped chromosomes
(arrowheads). Right half, spermatocytes undergoing G$_2$/M transition
of meiosis I. Cyclin A protein has entered the nucleus and
chromosomes are partially condensed and next to the nuclear
membrane as in stage B (large arrows).

aly, can, mia and sa primary spermatocytes arrest cell-cycle progression prior to the degradation of cyclin A protein

The subcellular localization of cyclin A protein changes during
the meiotic cell cycle. In wild-type testes, cyclin A protein was
located largely in the cytoplasm in spermatocytes from early
stages until the onset of the G$_2$/M transition, prior to chromo-
some condensation (Gönczy et al., 1994; Figs 6, 7A,B arrow-
heads). Much of the cyclin A protein moved into the nucleus
early in the G$_2$/M transition as the chromosomes underwent
condensation (arrows) and remained there through
prometaphase. Cyclin A protein was abruptly degraded just
prior to metaphase I and was not detected in subsequent stages
of meiosis (Fig. 7A,B). In wild type, fewer than one cyst per

small number suggest that the spermatids were most likely
derived from spermatocytes that had not yet reached the
arrest point at the time of the temperature shift. Later
spermatid stages with elongated flagella were never observed
in mutant testes even up to 168 hours (7 days) after the tem-
perature shift. Thus, the cells that accumulate in *aly^1* testes
at non-permissive temperature do not appear to be normal,
functional intermediates in spermatogenesis. This could be
directly due to the *aly* lesion. Alternatively, the arrested cells
may become incompetent to proceed through meiosis due to
turnover of message stores during the arrested state. In wild
type, transcription is largely shut down by the time sperma-
tocytes enter the first meiotic division (Gould-Somero and
Holland, 1974; Olivieri and Olivieri, 1965).

Fig. 7. Cyclin A protein localization in wild-type, *twine* and meiotic
arrest mutant testes. Whole-mount testes immunocytochemically
stained with cyclin A antibody. (A,C,E,G,H) Whole testis with apical
tip toward upper left or (F) upper right. (B,D) High magnification
views of cysts in meiosis I (arrow in B) or the round spermatid stage
(arrow in D). (A,B) Wild type. Cyclin A protein is cytoplasmic in
mature primary spermatocytes (arrowheads) and enters the nucleus
during the initiation of G$_2$/M transition of meiosis I (large arrows).
Cyclin A protein is abruptly degraded before metaphase I so that
spermatocytes at later stages contain no detectable cyclin A protein.
(C,D) *twine* mutant testes. Cyclin A protein is initially cytoplasmic
in large primary spermatocytes (arrowheads) and enters the nucleus
in later spermatocytes as in wild type. However, unlike wild type,
cyclin A protein remains nuclear up to the round spermatid stage
(small arrows), then is subsequently degraded. In *aly^2/Df(3L)HR370*
(E), *can^3/can^4* (F), *mia^1/mia^1* (G) and *sa^1/Df(3L)Pc-cp1* (H) testes,
cyclin A protein persists until the cells degenerate at the base of the
testes. Bar in A,C and E-H, 100 μm; in B,D, 25 μm.

testis (7 spermatocyte cysts in 19 testes) had cyclin A in the nucleus. The rapid disappearance of cyclin A protein between prometaphase and metaphase provides a biochemical indicator of cell-cycle progression.

The behavior of cyclin A protein in the mutant testes supports the conclusion that spermatocytes arrest cell-cycle progression at the G_2/M transition of meiosis I. In *aly, can, mia* or *sa* mutants, cyclin A protein was mainly cytoplasmic in growing primary spermatocytes, as in wild type. However, cyclin A protein persisted in the arrested mutant spermatocytes until the cells themselves degenerated (Fig. 7E-H). The persistence of cyclin A protein in the meiotic arrest mutants is consistent with cell-cycle arrest prior to metaphase I and the activation of the machinery for cell-cycle-dependent destruction of cyclin A protein

aly, can, mia and *sa* affect male meiotic cell-cycle progression and spermatogenesis at a more global level than the *cdc25* homologue *twine*

Although *aly, can, mia* and *sa* mutants block meiotic cell-cycle progression, the phenotypes of meiotic arrest mutants are strikingly different from the phenotype of mutants in the meiosis-specific *cdc25* homologue *twine*. Male germ cells in *twine* mutants skipped certain aspects of meiosis, but nevertheless proceeded through the spermatid differentiation program (Alphey et al., 1992; Courtot et al., 1992; White-Cooper et al., 1993). In *twine* primary spermatocytes entering the first meiotic division, we observed bivalents with partially condensed chromosomes (Fig. 8B′, large arrows) similar to those found in the wild-type spermatocytes at stage B of the G_2/M transition (Fig. 3B′). However, we did not detect fully condensed chromosomes in *twine* males. As described previously, chromosome segregation and cytokinesis failed to occur (Alphey et al., 1992) and meiotic spindles did not form in the *twine* spermatocytes (data not shown; White-Cooper et al., 1993). In contrast to *aly, can, mia* and *sa* mutants, spermatocytes in *twine* males did not arrest in meiosis (average of 2.3 meiotic cysts per testis, *n*=20 testes). Nucleolar breakdown occurred (Fig. 8B, arrowhead) and the cells proceeded to the

round spermatid stage and mitochondrial aggregates formed in *twine* males (Fig. 8C,C′,D,D′, small arrows; Courtot et al., 1992). Eventually, based on Hoechst staining, the bivalents decondensed and assumed a thin crescent shape closely apposed to the nuclear membrane (Fig. 8D′).

In *twine* males, cyclin A protein became nuclear at a similar stage as in wild type (Fig. 7C, small arrow). However, degradation of cyclin A protein was delayed in *twine* males compared to wild type. Germ cells at the round spermatid stage commonly had nuclear cyclin A protein in *twine* males (Fig. 7D, small arrows). In 14 homozygous *twine* testes examined, cyclin A protein was nuclear in three spermatocyte and 70 spermatid cysts. In contrast, cyclin A protein was never observed in spermatids in wild type. Cyclin A protein eventually was degraded in *twine* males, although the disappearance appeared gradual rather than abrupt as in wild type. We occasionally observed cysts of 16 *twine* spermatids with very low levels of residual nuclear cyclin A protein (note faint dots in clear region of testes, Fig. 7C). We also occasionally observed *twine* spermatid cysts where some cells had nuclear cyclin A protein while others did not.

Comparison of the phenotypes suggested that the meiotic arrest mutants arrest spermatogenesis at a more global point than *twine*. Consistent with this hypothesis, the phenotype of *twine; sa¹* and *twine; can³* double mutants was similar to the *sa¹/Df* and *can³/can⁴* phenotype. *twine; sa¹* and *twine; can³* spermatocytes arrested at the G_2/M transition stage, with partially condensed chromosomes and intact nucleoli. Cells did not progress beyond this arrest point (average of 346 cells per testis, *n*=15 testes) and the testes were devoid of postmeiotic spermatid stages.

DISCUSSION

Mutations in four *Drosophila* genes block both the meiotic cell cycle and progression to spermatid differentiation in the male germ line

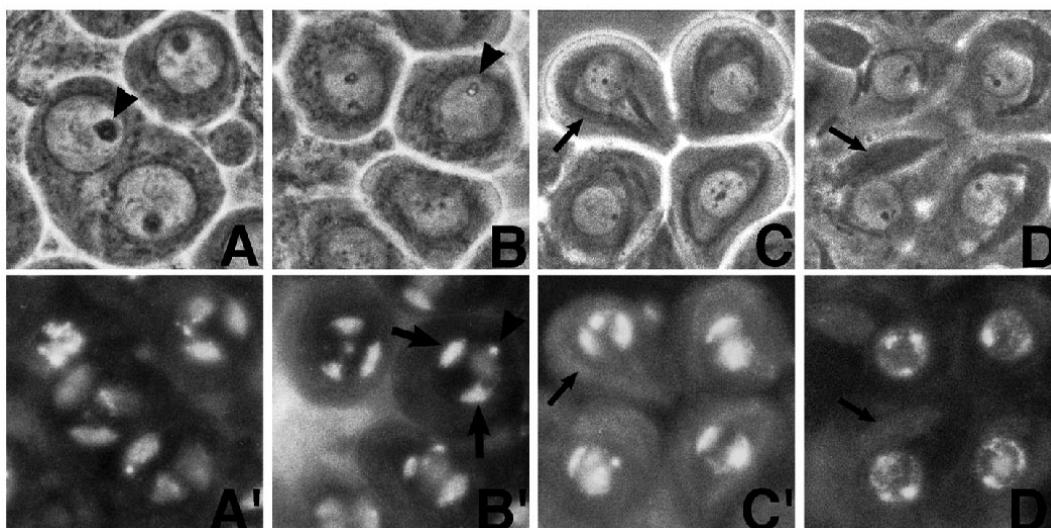We have identified four autosomal genes in *Drosophila* that

Fig. 8. Phase-contrast (top) and Hoechst (bottom) images of G_2/M and early spermatid cells in *twine* testes. (A,A′) Mature primary spermatocytes with prominent nucleoli (arrowhead) and crescent-shaped autosomal bivalents. (B,B′) G_2/M spermatocytes with partially condensed bivalents (arrows and arrowhead in B′) as in wild-type stage B, but with nucleoli broken down (arrowhead in B) as in wild-type stage C. (C,C′) 4N early spermatid cells. Mitochondrial derivatives start to form (small arrows). (D,D′) Spermatid cells with mitochondrial derivatives formed (small arrows). Thin crescent-shaped chromosomes at the periphery of nucleus are characteristic of early spermatids in wild type.
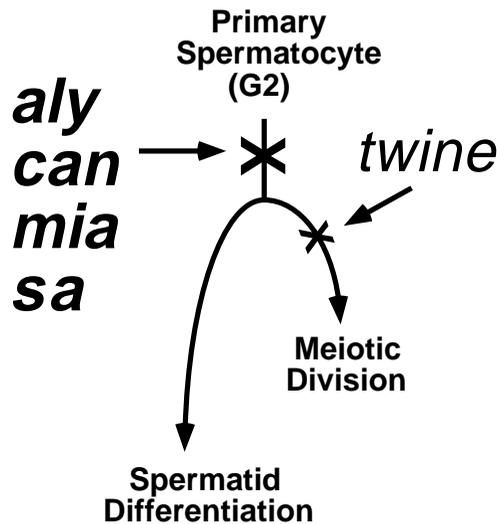
**Primary
Spermatocyte
(G2)**

*aly
can
mia
sa*

twine

**Meiotic
Division**

**Spermatid
Differentiation**

Fig. 9. Model for a branched pathway regulating male meiosis and spermiogenesis. Spermatogenesis appears to proceed by a branched pathway, with formation and differentiation of spermatids (left arm) independent of completion of the meiotic cell cycle (right arm). Mutations in *aly, can, mia* and *sa* cause arrest of both meiotic cell-cycle progression and spermatid differentiation, suggesting that these genes act upstream of the branch point, affecting spermatogenesis at a more global level than the cell-cycle control gene *twine*.

are required for progression of the spermatogenesis program through meiotic division and the onset of spermatid differentiation. Mutations in any one of these genes results in cell-cycle arrest in the G_2/M transition of meiosis I and accumulation of large numbers of primary spermatocytes with partially condensed meiotic chromosomes. In contrast to the effect of mutations in the cell-cycle phosphatase *twine* on male meiosis, spermatids neither form nor differentiate in the meiotic arrest mutants. The effects of these mutants on both cell-cycle progression and spermatid differentiation suggest that these two events may be regulated by a common developmental control point in the spermatogenesis program.

Mutations that result in a uniform block of the progression of spermatogenesis are rare among male-sterile mutants in *Drosophila melanogaster*. Of 806 male-sterile mutants isolated in various screens (Castrillon et al., 1993; Hackstein, 1991; Lindsley and Lifschytz, 1972; McKearin and Spradling, 1990; E. Y. Xu and C.-I. Wu, personal communication; this paper), only six mutations caused a complete block of the spermatogenesis program just prior to meiotic division (Hackstein, 1991; E. Y. Xu and C.-I. Wu, personal communication; this paper). For most male-sterile mutants, spermatogenesis proceeds despite defects in one or even several morphogenetic events, leading to production of aberrant spermatids (Fuller, 1993), suggesting that the majority of events in spermatogenesis proceed by independent pathways (Lifschytz, 1987; Fuller, 1993). In this context, mutations that block spermatogenesis completely instead of producing abnormal spermatids are likely to identify key regulatory genes and/or coordination points. So far the screens for male-sterile mutants, including meiotic arrest mutants, have not yet reached saturation. Only one allele of each of the four meiotic arrest loci that we describe was isolated in general screens for male-sterile mutations.

aly, can, mia and *sa* mutations block spermatogenesis but mutant females are fertile. The apparent sex specificity of these meiotic arrest mutants suggests that the mechanisms that regulate progression of the meiotic cell cycle differ between the sexes. In *Drosophila* oogenesis, meiosis is arrested in metaphase I (Mahowald and Kambysellis, 1980; Theurkauf and Hawley, 1992) and resumes in response to a signal received during passage through the oviduct (Doane, 1960). In males, in contrast, there is no meiotic arrest. Perhaps the machinery for meiotic arrest also exists in males, but is normally cryptic because a signal for progression through meiosis and completion of spermatogenesis is provided intrinsically by the meiotic arrest genes.

Progression through the meiotic cell cycle and the onset of the spermatid differentiation program occur via separate pathways

The cellular events of meiotic division in *Drosophila* males can be divided into two categories: *twine*-dependent and *twine*-independent. Chromosome segregation and cytokinesis depend on *twine* function (Alphey et al., 1992), so are regulated by the core cell-cycle machinery, while breakdown of the nucleolus and the onset of spermatid differentiation are independent of *twine* function. Early round spermatids form despite the continued presence of nuclear cyclin A protein and failure to complete meiosis in *twine* males. Therefore, both breakdown of the nucleolus and progression through spermatid differentiation appear to be regulated by developmental timing mechanisms, not the core cell-cycle machinery.

Persistence of nuclear cyclin A protein into spermatid stages in *twine* suggests that abrupt degradation of cyclin A protein in meiosis I is governed by similar mechanisms as in mitosis. Degradation of cyclin A protein in mitosis occurs via a ubiquitin-mediated pathway (Glotzer et al., 1991; Sudakin et al., 1995) triggered by the active cdc2-cyclin B complex (Luca et al., 1991). In *twine* male meiosis, the cdc2-cyclin B complex presumably remains inactive due to inhibitory phosphorylation. Cyclin A protein does enter the nucleus and chromosome condensation initiates in *twine*, suggesting these events are independent of cdc2-cyclin B activation. Although *twine* spermatocytes never complete meiosis, cyclin A protein eventually is degraded in spermatids, possibly due to normal protein turnover. In contrast, cyclin A protein persisits in arrested *aly, can, mia* and *sa* spermatocytes until the cells themselves degenerate. The arrested spermatocytes could degenerate before normal protein turnover depletes cyclin A. However, the high numbers per testis argue that the cells remain in the arrested state for quite some time. Cyclin A message normally disappears at or before onset of meiotic division in wild type (H. White-Cooper, personal communication), possibly to ensure absence of cyclin A protein during meiosis II. Thus, alternatively, persistence of cyclin A protein in the meiotic arrest mutants could be due to two factors: failure to activate the cell-cycle programmed, ubiquitin-dependent protein degradation, plus failure to enact a developmentally programmed disappearance of cyclin A message.

Independent meiotic cell-cycle and spermatid differentiation programs may be coordinated through a control point in mature spermatocytes

Mutations in *aly, can, mia* and *sa* block both the *twine*-

dependent and *twine*-independent events of meiosis, as well as spermatid formation and differentiation. This global arrest phenotype strongly suggests that the meiotic cell-cycle and spermatid differentiation programs, although separable in *twine* mutants, may be coordinately regulated by a developmental control point acting in primary spermatocytes. Wild-type function of *aly, can, mia* and *sa* could be required to proceed through a control point before the meiotic cell-cycle and the spermatid differentiation programs become independent (Fig. 9). Alternatively, the meiotic arrest genes could be required for two different functions to allow progression along two independent pathways. The phenotype of *twine;sa¹* and *twine;can³* double mutants is consistent with either alternative. In the double mutants, spermatocytes do not progress beyond the arrest point identified in the meiotic arrest mutants, as the nucleoli remain prominent and spermatid differentiation fails to occur. Meiotic chromosomes appear to arrest condensation at a similar point in *twine* as in the meiotic arrest mutants, raising the possibility that activation of either *cdc25* or *cdc2* in meiosis I could require wild-type function of *aly, can, mia* and *sa*.

Role of the meiotic arrest genes in regulation of the G₂/M cell-cycle transition

The similar phenotype and arrest point suggest that the four meiotic arrest genes might act either together or in a sequential pathway to specify cell-cycle and developmental progression in spermatogenesis. The meiotic arrest genes could regulate cell-cycle progression at any of several levels. The function of these genes could directly or indirectly regulate the activity of core cell-cycle components such as *cdc2, twine* or *cyclin B*, either at the level of expression or by post-translational modification.

One or more of the meiotic arrest genes could affect cell-cycle and developmental progression indirectly, via a checkpoint mechanism. In this case, failure of the meiotic arrest gene function could send a signal that blocks both meiotic cell-cycle progression and spermatid differentiation. Such meiotic cell-cycle checkpoints have been identified in *Saccharomyces cerevisiae*, where chromosomal lesions caused by mutation in *cdc13* elicit a *RAD9*-dependent checkpoint that arrests meiotic progression (Weber and Byers, 1992). Mutations in *ZIP1*, a yeast gene encoding a component of synapsed meiotic chromosomes and mutations in *DMC1*, a yeast gene encoding a protein involved in chromosome metabolism during meiotic prophase, also cause meiotic arrest and failure of sporulation by a checkpoint mechanism (Sym et al., 1993: Bishop et al., 1992). Because the *aly* meiotic chromosomes appeared abnormal, the *aly* locus is a good candidate to encode such an early function in *Drosophila* males.

The regulatory control point at the G₂/M transition of male meiosis I is likely to be conserved

The characteristic spermatogenesis defects in *aly, can, mia* and *sa* mutants are strikingly similar to the clinical manifestation of human meiosis I maturation arrest male infertility. Meiosis I maturation arrest is the most common cause of azoospermia, the complete absence of sperm from the seminal fluid, in human idiopathic male infertility (Colgan et al., 1980; Meyer et al., 1992; Wong et al., 1973). In the clinical description of meiosis I maturation arrest, the early stages of spermatogen-

esis typically are abundant and appear normal, and mature primary spermatocytes are present. However, all postmeiotic stages of spermatogenesis are absent and spermatocytes with abnormal, partially condensed chromatin accumulate in the testis (Soderstrom and Suominen, 1980). This striking phenotypic similarity strongly suggests that the control point in spermatogenesis that we have identified may be conserved from flies to man. If so, the meiosis I maturation arrest syndrome in humans may have a genetic basis, with human homologues of the *Drosophila aly, can, mia* and *sa* genes playing important roles in regulating progression of spermatogenesis.

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