Mutations at the *asp* locus of *Drosophila* lead to multiple free centrosomes in syncytial embryos, but restrict centrosome duplication in larval neuroblasts

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Summary

Mutations at *abnormal spindle* result in abnormally long and wavy microtubules in the meiotic spindles of males. Some of these spindles have a single pole and take the form of unopposed hemi-spindles. Unfertilised eggs produced by homozygous *asp* females may have either no nuclei, or a small number of large nuclei, consistent with there also being an effect upon female meiosis. Such eggs also display free centrosomes and independent arrays of microtubules. Embryos that have this phenotype are also present among the progeny of fertilised homozygous *asp* females, together with embryos that undergo varying degrees of aberrant morphogenesis, developing a variety of abnormal cuticle patterns. This latter category shows asynchronous mitoses prior to cellularisation, and has abnormal arrays of spindle microtubules. Such embryos can develop large areas that are either devoid of or have a reduced number of nuclei, in which there are centrosomes that have dissociated from the mitotic spindles. Neuroblasts in the brains of homozygous *asp* larvae display a high mitotic index, and have condensed chromosomes aligned as if blocked at metaphase. Immunostaining reveals that many cells contain a single centrosome connected to the metaphase chromosomes by microtubules in a hemi-spindle-like structure.

Key words: hemi-spindles, mitosis, meiosis.

Introduction

Drosophila is a highly suitable multicellular organism for a genetic study of cell division. The embryo is initially a syncytium in which there are 13 rounds of nuclear divisions over a period of about two hours. The nuclei are first found within the interior of the embryo, but during the eighth and ninth division cycles they migrate to the cortex (Foe and Alberts, 1983; Karr and Alberts, 1986). Cellularisation occurs during cycle 14, and the cell cycle lengthens with cell division, becoming restricted to a complex series of domains in the embryo. One group of mitotic mutants can survive to adulthood as homozygotes, but the females appear sterile in that they lay eggs that cease development in early embryogenesis. Some of these are 'leaky' mutations that fail to produce a gene product of sufficient quantity or quality to meet the demands of the syncytial cleavage embryo. One such example is the mutation, *polo*, that displays abnormal spindle poles (Sunkel and Glover, 1988). Other maternal effect mutations can be in genes that appear to be specifically required in the embryo, such as *gnu*, which leads to the formation of giant nuclei (Freeman *et al.* 1986). The sensitivity of this developmental stage to the lack of mitotic products and the ease with which immunocytology can be performed in *Drosophila* embryos render this an appropiate period in which to study the alterations produced by mitotic mutants.

A second group of mutations, for example in the gene encoding cyclin A (Lehner and O'Farrell, 1989) or at *string* (Edgar and O'Farrell, 1989), show a mitotic phenotype in the embryo when the cell cycle lengthens after cellularisation, a time at which their zygotic expression is normally initiated. These two genes are the *Drosophila* homologues of the *Schizosaccharomyces pombe* genes *cdc13* and *cdc25*, respectively, and are needed for the entry into mitosis.

A final group of mutations affecting mitosis have been recognised that result in lethality in either late larval or early pupal development (Gatti *et al.* 1983; Ripoll *et al.* 1987; Glover, 1989; Gatti and Baker, 1989). The mitotic phenotype is not generally manifest until these stages, since the homozygous mutant zygote is supplied with sufficient wild-type gene product from its heterozygous mother for embryogenesis. Much of larval development involves cell growth and polyploidisation, with the exception of the imaginal and neural tissues that proliferate to form the adult structures. Many mitotic mutants thus have small imaginal discs and display mitotic abnormalities within larval neuroblast cells.

Meiosis and mitosis have many features in common

from both a cytological and a biochemical point of view. Thus most of the gene products involved in one process are likely to play a role in the other. This is borne out by many mitotic mutants of *Drosophila*, which also show corresponding meiotic abnormalities. *asp* is one such locus for which there is both cytological and genetical evidence of non-disjunction during both meiotic divisions in males bearing mutant alleles (Ripoll *et al.* 1985). Mutation in *asp* was also shown to result in other complex alterations, including sublethality, cuticular defects and high levels of non-disjunction in somatic cells (Ripoll *et al.* 1985; Gonzalez, 1986). Examination of the mitotic chromosomes in neuroblasts of homozygous *asp* larvae has suggested either that the mitotic cycle can be arrested in metaphase or that aberrant behaviour of the mitotic apparatus leads to a high frequency of aneuploid and polyploid cells. It has been previously proposed that the primary lesion produced by mutations in this locus affects the functionality of the spindle, non-disjunction being the immediate consequence and sub-lethality and morphogenic abnormalities being its final pleiotropic effects. The only evidence for the abnormal appearance of spindles comes from observations of meiotic spindles of living spermatocytes visualised by phase-contrast optics (Ripoll *et al.* 1985). These can be seen in this way because of their association with mitochondrial derivatives that also segregate to daughter cells to form the Nebenkern. In this work, we have therefore sought to extend these observations by applying immunocytological techniques to observe the spindle apparatus. This has been facilitated by isolation of additional alleles of *asp* (Gonzalez, 1986). Our observations indicate that non-disjunction in *asp* can result from the association of condensed chromosomes with hemi-spindle stuctures in both meiosis and mitosis.

Materials and methods

Isolation of new alleles of asp

New *asp* alleles were obtained by X-irradiating 2- to 4-day-old adult males with a dose of 3000 rad. These males were mated to *y; red* Sb^{63b} *asp/In(3R)TM2,* Ubx^{130} *e* females. Male offspring of genotype *y; red* Sb^{63} *asp/** (where * indicates an X-ray-treated chromosome) were individually crossed to *y; C(2)EN, c bw* females. Such females only yield viable offspring when autosomal non-disjuntion has occurred in the males (Novitski *et al.* 1981). As non-disjunction is a feature typical of *asp* homozygous males, males producing offspring in this cross were considered to be potential carriers of new alleles of *asp* (Gonzalez, 1986). A detailed report of this method and the results obtained will be presented elsewhere (Gonzalez *et al.* unpublished). The new alleles used in this work were *E3*, *L1* and *L18*.

Collection of unfertilised ASP eggs for immunostaining

In order to obtain reasonable numbers of unfertilised ASP eggs, we crossed females trans-heterozygous for different *asp* alleles with *X/O* males. These males fail to produce mature sperm, but they are known to stimulate oviposition in their partners. *X/O* males were obtained by crossing *C(1)RM,y/O* females to y/y^+Y males. 50 *X/O* males were also single mated to Oregon-R females and checked for fertility to rule out the possibility of an extra Y segregating in either of the parental stocks.

Immunostaining

Third-instar homozygous *asp* larvae, recognised by either *red* or $Tubby^+$ markers, or wild-type larvae were washed and dissected in 0.7% NaCl. Their brains were transferred onto a drop of ST (0.5 μM taxol in 0.7% NaCl) containing 3.7% formaldehyde in an Eppendorf tube and left for 30 min. The brains were then incubated in ST containing 0.3% Triton X-100, 10% fetal calf serum, for 1 h. At this stage the brains are ready for incubation with antibodies. The incubations with the first antibodies, Rb188, anti-centrosome (Whitfield *et al.* 1988) and YL 1/2, anti-tubulin (Kilmartin *et al.* 1982) were carried out overnight. Texas red-conjugated anti-rabbit IgG and fluorescein-conjugated anti-rat IgG (Jackson Labs Inc.) were used as second antibodies and were incubated with the specimen for 6 h. Antibodies were diluted in 10%FCS containing 0.3% Triton X-100 to the working concentration. The brains were washed twice for 10 min with 0.3% Triton X-100 in 0.7% NaCl after each incubation. Chromosomes were stained with propidium diiodide at a concentration of 1 $\mu g\,ml^{-1}$. Immunostaining of embryos and testes was performed as described by Freeman *et al.* (1986), and Casal *et al.* (1989), respectively.

We were concerned that taxol might lead to artefactual structures, since it is known to promote nucleation and elongation of microtubules. We believe that the simultaneous incubation of the tissue preparations with taxol and formaldehyde fixes the tissue before artefacts can arise for the following reasons. First, scaling down the time that the tissue is incubated with the drug to only 30 s gives identical results. Second, real-time studies on the early embryonic cycles using fluorescently labelled tubulin (Kellog *et al.* 1988) or fluorescently labelled anti-tubulin antibodies (Warn *et al.* 1987) indicate that the images observed using a formaldehyde/taxol procedure are reasonably accurate. Finally, using these protocols we observe reproducible staining patterns in both testes and brains of several other mitotic mutants that are quite different from those described here for *asp*. In meiotic spindles of flies homozygous for *mgr* (Gonzalez *et al.* 1988), for example, the microtubules are present in spherical structures that differ from those reported in this paper for either wild-type or *asp* males (Gonzalez *et al.* unpublished data). This is also the case with the mitotic neuroblast spindles of a mutation at 87B causing 'thin', elongated bipolar spindles, that differ from either wild-type spindles, or the metaphase arrest that results from the *asp* mutation (Axton, Dombradi, Cohen and Glover, unpublished data). We have been able to visualise mitotic spindles in wild-type brains using the above procedure and omitting taxol from the buffers. This results in spindles that have similar shape to the wild-type spindles described in the results section below, but the tubulin staining is extremely fuzzy. Furthermore, whereas the use of taxol as a stabilising agent allows us to see between 10 and 20 spindles per wild-type brain, without taxol we detect one or fewer spindles per brain. We have also attempted methanol fixation in the presence of EGTA following the conditions described by Kellog *et al.* (1988) for *Drosophila* embryos. In our hands this is even less efficient at stabilising mitotic spindles in neuroblast cells, and does not permit the examination of a sufficient number of structures to draw satisfactory conclusions as to the reproducibility of meiotic defects. Nevertheless, the rare structures that have been observed correspond to those seen with formaldehyde/taxol fixation.

Results

Hemi-spindles during male meiosis in asp

abnormal spindle, asp, was so named because mutation in this gene results in mitotic and meiotic non-disjunction, and because the meiotic spindles have an abnormal appearance when observed by phase-contrast optics. We have sought to understand the nature of these spindle defects by applying recently developed cytological techniques to immunostain testis squashes.

Descriptions of meiosis in wild-type *Drosophila* males have been previously presented by Cooper (1965), Tates (1971) and Lindsley and Tokuyasu (1980). The process may be summarised as follows. After four mitotic divisions, each stem cell produces a cyst of 16 spermatocytes. These cells undergo a growth phase in which they increase in size by about 25-fold to give mature spermatocytes ready to enter meiosis. During prophase, Hoechst staining reveals

three large clouds of semi-condensed chromatin corresponding to each major bivalent. Sometimes the small fourth bivalent is also seen. The nucleolus is still visible and there is a diffuse network of tiny spots spread throughout each spermatocyte corresponding to mitochondrial DNA. The organisation of microtubules at this stage is reminiscent of the cytoskeleton in somatic cells (Fig. 1a). The most conspicuous signs of the onset of meiosis are the condensation of the chromatin at prophase and the dispersion of the nucleolus. The microtubules become organised around the three blocks of chromatin to establish the meiotic spindle (Fig. 1b). By metaphase the bivalents are very close together, so that a single spot of Hoescht-stained material can be observed, and the meiotic spindle is clearly visible (Fig. 1c). Finally, at telophase two sets of chromatin, each containing one of the homologous chromosomes, are found at the spindle poles, and the system proceeds through meiosis-II.

Stages of meiosis in *asp* mutant males are shown in Fig. 2. The staining with Hoechst permits the precise timing of the different meiotic stages in the mutant, since the chromosome cycle appears to proceed normally. Moreover, the distribution of microtubules in the interphase growing spermatocytes is indistinguishable from that in the wild-type. However, the spindle microtubules of all later stages of *asp* testes clearly depart in appearance from that observed in the wild-type. From late prophase to telophase, all of the spindle-like microtubules in *asp* testes have a long and wavy structure and are grouped into bundles (Fig. 2). In some instances, there remains a suggestion that the microtubules are clustered as two hemi-spindles (e.g. filled arrow in Fig. 2c), whereas in others only one hemi-spindle appears to be present (e.g. open arrow in Fig. 2b). Often the microtubules form unrecognisable wavy networks lacking any symmetry. We were unable to detect any allele-specific differences in this analysis of the meiosis in *asp* flies.

Free centrosomes and large nuclei in ASP embryos

Developmental defects. Chromosomes do not achieve as high a degree of condensation in male meiosis in *Drosophila* as they do in mitosis. Furthermore, the antigen Bx63, which is an excellent cytological marker for centrosomes in mitotic tissues, shows a diffuse distribution over each meiotic hemi-spindle, the significance of which is unknown (Casal *et al.* 1989). As this limits our ability to analyse the relationship between centrosomes, microtubules and chromosomes during meiosis, we chose to examine the effect of *asp* upon mitotic spindles in the syncytial embryo. All of the newly isolated alleles of *asp* show a maternal effect in which females either homozygous for a particular mutant allele or having different allelic combinations give rise to embryos (ASP embryos) that display defective mitoses. We have studied the mutant embryos produced by trans-heterozygous, rather than homozygous, females, since they show defects characteristic of *asp* and yet, for most combinations used, viability is comparable to that of wild-type flies. This permits the production of sufficient embryos for immunostaining.

Characteristic ASP embryos are displayed in Fig. 3 (a–f) together with a number of wild-type embryos at different developmental stages (g–j), stained with Hoechst to reveal DNA. The wild-type embryo in Fig. 3g is typical of the syncytial blastoderm stage, in which the nuclei are in their tenth rapid division cycle at the surface of the embryo. The cellularised embryo in Fig. 3h is undergoing gastrulation, a stage at which one begins to see restricted domains of mitotic activity. Fig. 3i shows an embryo about 7.5 h old in which germ-band shortening is beginning to take place. This follows the elongation of the embryo after gastrulation when the germ cells migrate from the posterior pole dorsally and anteriorly. Fig. 3j shows a very much later stage after germ-band shortening in which the embryo is almost mature.

ASP embryos show a wide range of phenotypic defects, which we broadly classify into three categories: those having no DNA (Fig. 3a); some in which there were a small number of large nuclei (Fig. 3b); and the remainder that showed varying degrees of multi-nuclear development (Fig. 3c–f). A similar spectrum of embryonic defects was seen with all of the allelic combinations tested (Table 1). Embryos that exhibit several rounds of mitosis frequently display phenotypic abnormalities in the syncytial stages (Fig. 3c). Instead of the uniform appearance of the wild-type syncytial blastoderm, in which parasynchronous waves of mitosis progress from the poles of the embryo (Foe and Alberts, 1983; Karr and Alberts, 1986), ASP embryos show patches of nuclei in different stages of the cell cycle intermingled with regions that appear to behave synchronously. Despite this early manifestation of abnormalities, some of these embryos reach very late stages of development, showing abnormalities that become progressively more severe. Blastoderm embryos can be frequently found that have large areas devoid of any nuclei (Fig. 3c). In some embryos, cellularisation appears to occur, and development proceeds to give embryos that seem to be in gastrulation (Fig. 3d), or in which a hint of segmentation develops (Fig. 3e). In all of these cases, however, the embryos are highly disorganised. A proportion of embryos show only amorphous staining with Hoechst and appear to be in a stage of degeneration (Fig. 3f).

Several embryos achieve high degrees of development, including cuticular segmentation, in spite of having large regions devoid of recognisable cell structure. The degree of embryonic development can be assessed by examining cuticle patterns in whole-mount preparations (van der Meer, 1977). Fig. 4 shows such preparations of ASP embryos alongside a wild-type embryo for comparison. The most prominent features of the wild-type embryo are a regular segmental array of denticle belts along its ventral surface (Fig. 4a). ASP embryos show a variety of cuticle patterns ranging from examples in which it is not possible to discern any pattern to cases in which the dorsal cuticle pattern is not seen but where various degrees of ventral cuticle patterning are present (Fig. 4b–e). Other cell cycle mutants have been described in which mitosis is arrested

Table 1. *Classes of embryos produced by mothers carrying different allelic combinations of* asp

	asp/E3	*asp*/L1	*asp*/L18	L18/E3	L1/L18
Class 1: no DNA	26%	38%	59%	35%	6%
Class 2: 2–3 big nuclei	57%	32%	11%	24%	52%
Class 3: developed	17%	30%	29%	41%	42%
Total numbers examined	70	80	71	37	66

Females heterozygous for the indicated combinations of *asp* alleles were mated to wild-type males and their eggs collected for phenotypic examination. *asp* males were not used because of their low fertility and their high degree of meiotic non-disjunction. Class 3 embryos show a variety of degrees of development ranging from arrest in blastoderm to arrest at very late stages of embryogenesis.

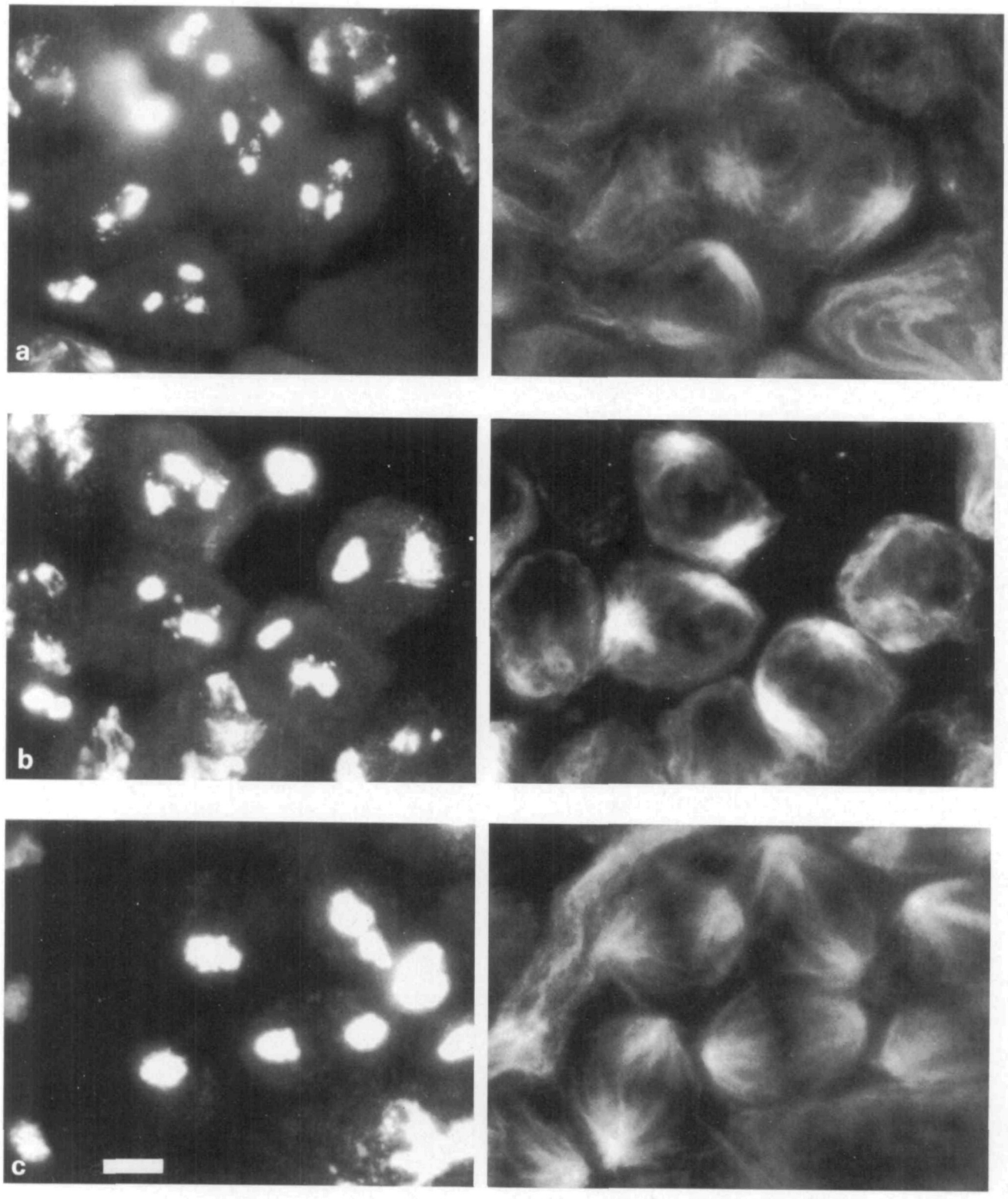

Fig. 1. Meiosis I in wild-type testis. In all panels, the respective left and right frames show the field stained with Hoechst to reveal DNA and the YL1/2 antibody to reveal tubulin by indirect immunofluorescence with fluorescein-conjugated anti-rat second antibody. Prophase (a–b) can be recognised by condensation of the three large areas of chromatin, loss of nucleolar staining and the newly forming meiotic spindle. By metaphase (c) the condensed chromosomes have moved very close together to form a single block in the center of the spindle. Anaphase figures can also be seen in this field. Bar, 5 μm.

after cellularisation. The cuticle patterns shown by embryos homozygous for mutations in *string*, for example, have a segmental pattern, but show reduced numbers of rows of denticles in each belt (Jürgens *et al.* 1984; Edgar and O'Farrell, 1989).

ASP embryos that show no mitoses. It appeared that

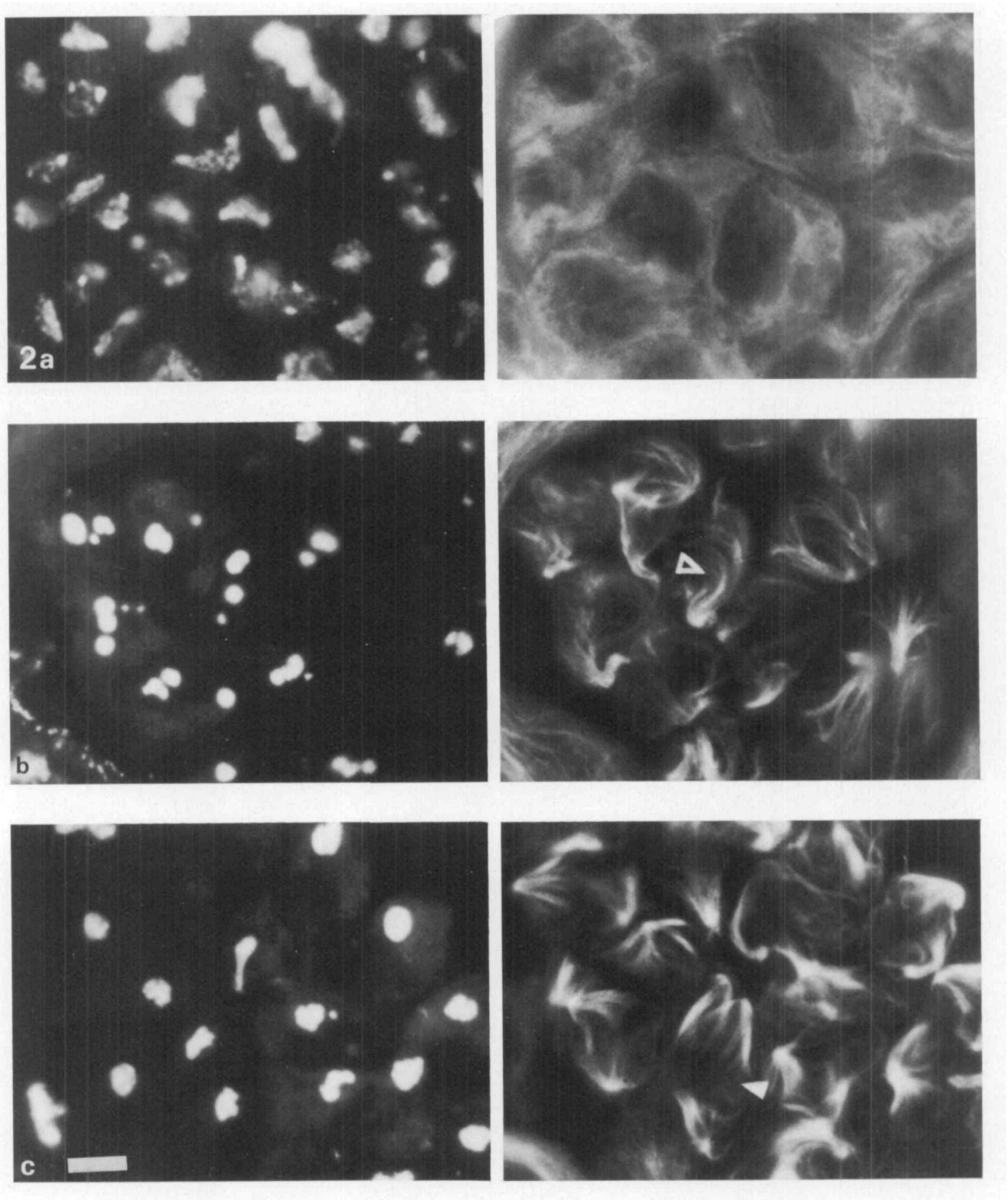

Fig. 2. Meiosis I in *asp* testis. The meiotic stages can be ascertained from the pattern of chromatin staining given by Hoechst (left hand panels). The field in a is in early prophase, and is at an earlier stage of chromosome condensation to the cells in Fig. 1a; b, in pro-metaphase; whereas c has both metaphase and anaphase figures. Microtubules are revealed by indirect immunofluorescence using the monoclonal antibody YL1/2, and fluorescein-conjugated anti-rat second antibody. The microtubular structures that form in association with metaphase figures are often hemi-spindle-like structures (open arrow, b), although examples of opposed hemi-spindles can also be seen (filled arrow, c). Bar, 5 μm.

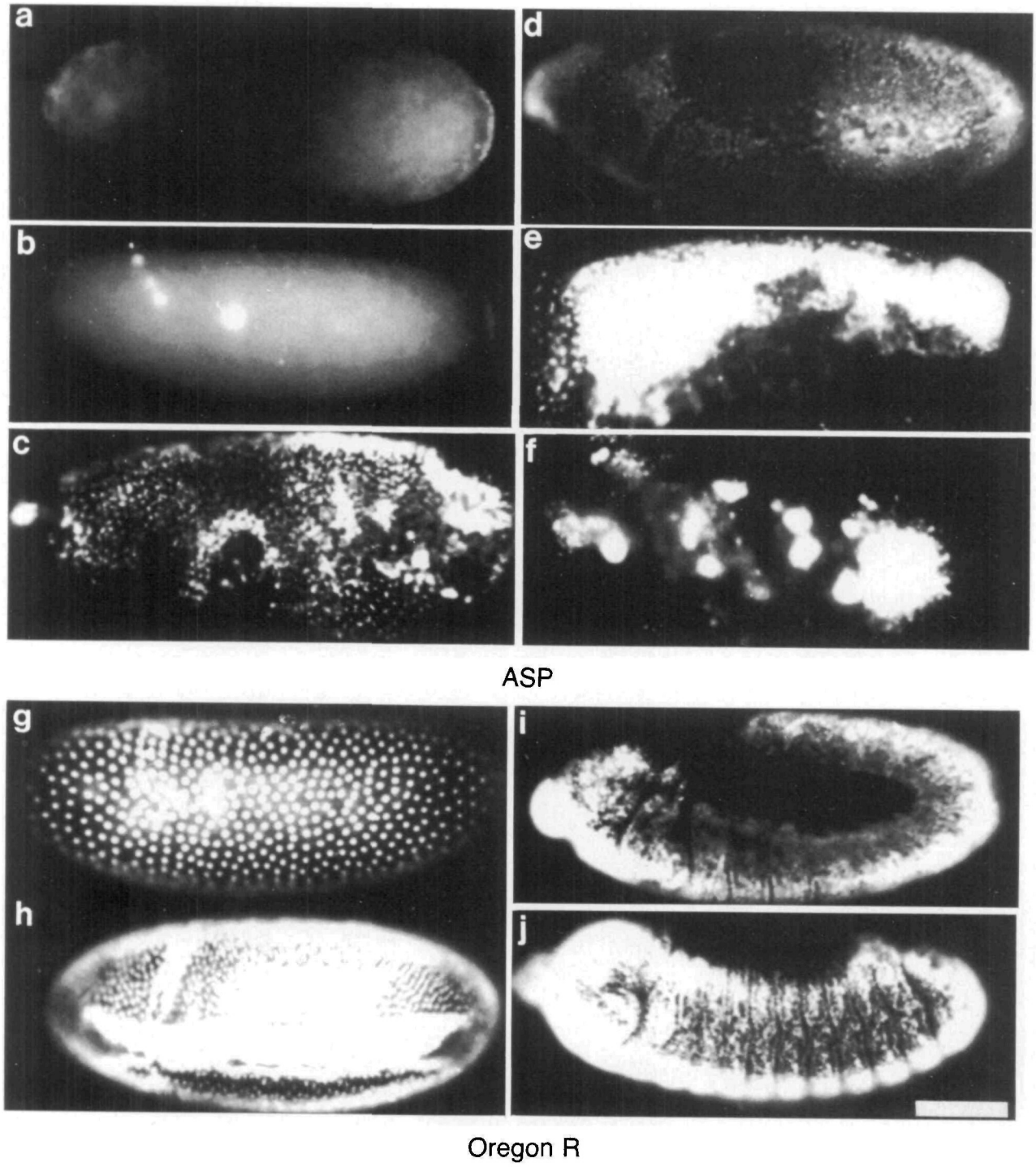

Fig. 3. a–f and g–j. Representative ASP and wild-type embryos, respectively, stained with Hoechst 33258. a and b. A class of embryos that undergo no development. The embryo in a has no nuclei, whereas that in b has a few enlarged nuclei. c. A blastoderm embryo with irregular array of nuclei; the embryos in d and e have undergone cellularisation and some degree of development, whereas the embryo in f appears to be degenerating. The wild-type embryos in g–j are in syncytial blastoderm, gastrulation, germ-band elongation, and post germ-band shortening, respectvely. Bar, 100 μm.

there were two different co-existing populations of ASP embryos produced by mutant females. In one population the embryos can undergo considerable degrees of development with evidence of many rounds of mitosis, whereas the other appeared not to undergo mitosis and either had a few (between 1 and 4) large nuclear structures (Fig. 3b) or no DNA at all (Fig. 3a). We wondered whether this latter group could be unfertilised eggs or resulted from defects occurring either very early in development or during oogenesis, and we therefore examined the phenotypes of the unfertilised eggs laid by *asp* females mated to sterile X/O males. These were indistinguishable from the category of eggs produced by fertilised *asp* females that showed no mitoses at all, having either no DNA or several large nuclei. They are quite distinct from unfertilised eggs laid by wild-type females, in which one can see up to four

smaller nuclei (three polar bodies and the female pronucleus), which often undergo fusion with each other (Doane, 1960). As *asp* is known to lead to meiotic non-disjunction in the male, one likely explanation of the observed distribution of nuclei in unfertilised ASP eggs is that non-disjunction is also occurring in the female germ line.

We have examined the distribution of microtubules and centrosomes in unfertilised eggs using the anti-tubulin antibody YL1/2 (Kilmartin *et al.* 1982), and the antibody Rb188 that recognises the Bx63 antigen associated with the *Drosophila* centrosome (Whitfield *et al.* 1988). An example of the immunostaining of two fields from ASP eggs lacking DNA is shown in Fig. 5. Two types of pattern

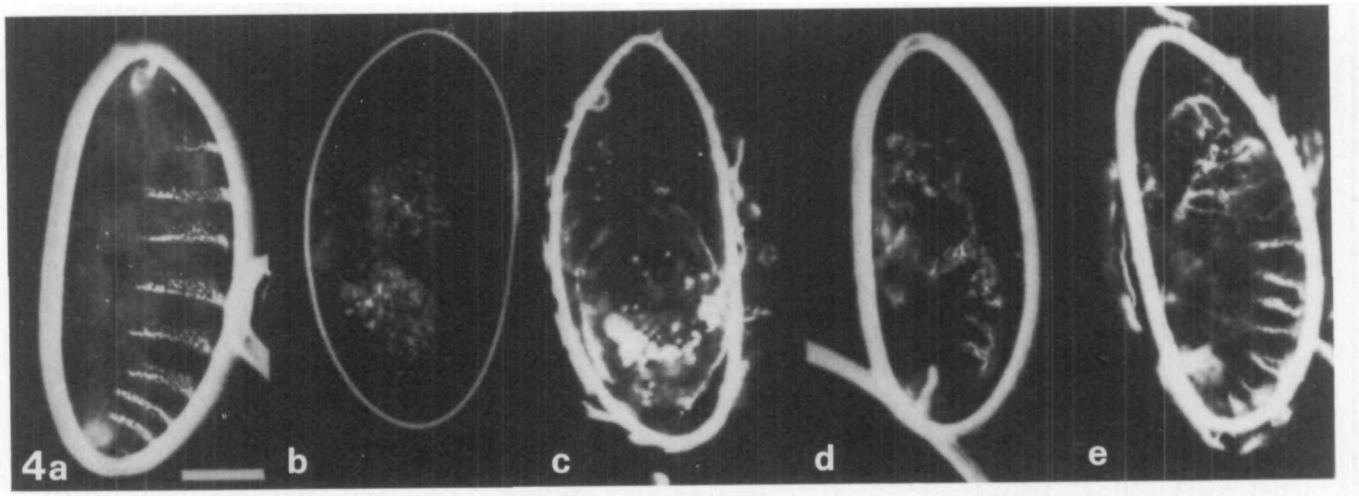

Fig. 4. Cuticle preparations from wild-type and ASP embryos a. A wild-type preparation; b–e, preparations from ASP embryos. Bar, 100 μm.

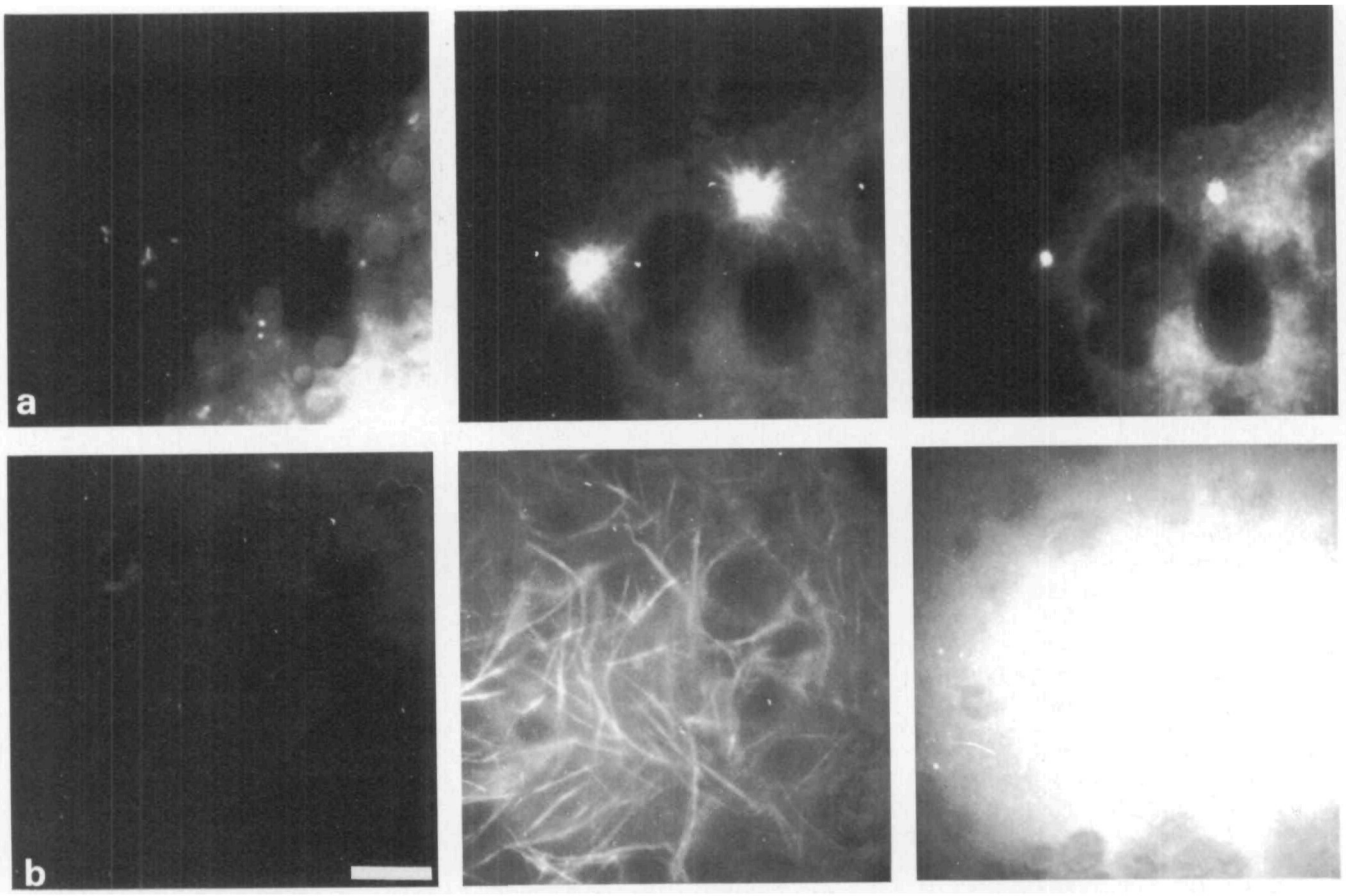

Fig. 5. Two fields from ASP embryos containing no nuclei stained (left to right) with Hoechst to show DNA, with YL1/2 and fluorescein-conjugated anti-rat IgG to reveal microtubules, and with RB188 antibody and rhodamine-conjugated anti-rabbit IgG to show centrosomes. In field a, there is no DNA but there are two centrosomes nucleating asters of microtubules. In field b, there are irregular arrays of microtubules associated neither with DNA nor centrosomes. Bar, 5 μm.

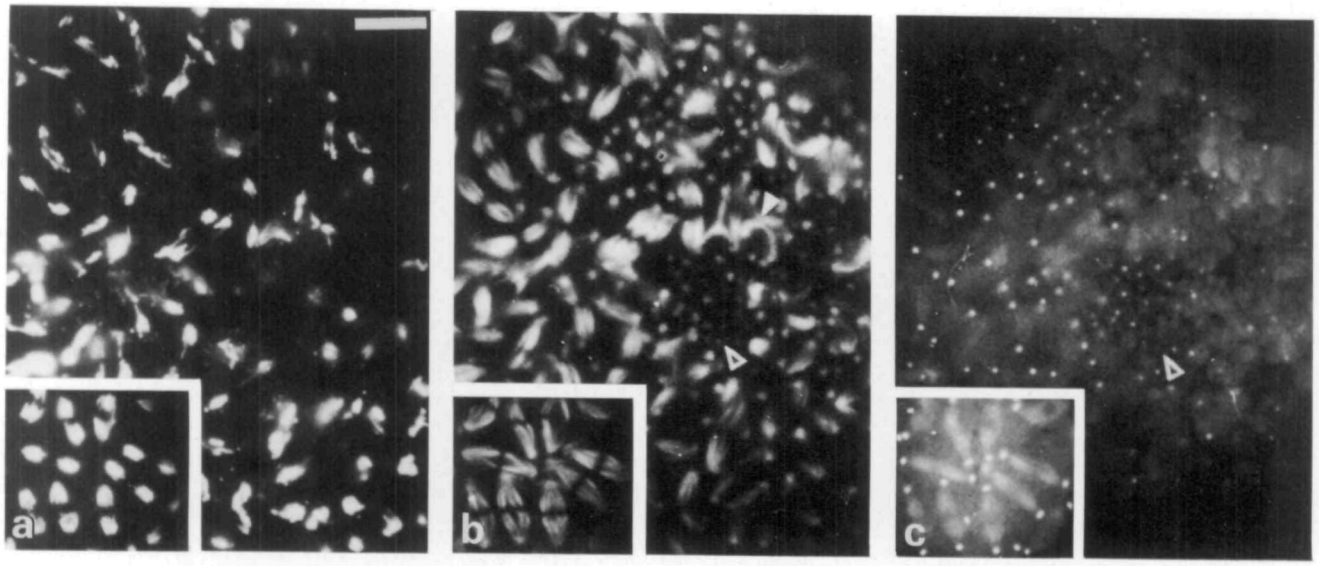

Fig. 6. A typical field illustrating abnormalities seen in the multinuclear class of ASP embryos at syncytial blastoderm. a,b,c. Stained with Hoechst to show DNA, YL1/2 to show tubulin, and RB188 to show centrosomes as in the legend to Fig. 5. The filled arrow (b) indicates long wavy microtubules; and the open arrows, free centrosomes (c), which nucleate asters of microtubules (b) not associated with nuclei. The insets show a field from a wild-type embryo of corresponding stage. Bar, 10 μm.

are seen. There may be either a small number of centrosomes nucleating large asters of microtubules (Fig. 5a), or networks of microtubules in toothpick-like structures scattered throughout the embryo (Fig. 5b). These patterns are seen in all ASP eggs having either no DNA or a small number of giant nuclei. This is in contrast to unfertilised wild-type eggs, which never develop free centrosomes, but are arrested in their development. We have occasionally observed toothpick-like arrays of microtubules in old unfertilised wild-type eggs, whereas most ASP eggs contain such arrays.

Abnormal mitoses in ASP embryos. The ability of *asp* females to produce embryos that undergo some development has enabled us to examine the mutant mitotic spindles. A typical field from an ASP embryo at syncytial blastoderm is shown in Fig. 6. The majority of nuclei in this field are in an anaphase-like state, enabling a comparison to be made with the anaphase spindles from a wild-type embryo at a similar stage (Fig. 6 inset). The most striking features of the ASP embryos are that the dividing nuclei are not uniformly distributed at the cortex as in the wild-type and, furthermore, the divisions appear asynchronous. Staining of the chromatin with Hoechst (Fig. 6a) reveals abnormal segregation of condensed chromosomes by comparison with wild-type. Second, the structure of many spindles is clearly abnormal: there are some in which the microtubules form long sweeping curves (Fig. 6b, filled arrow), and others that intermingle in complex networks. These abnormal spindle microtubules are associated with an aberrant distribution of chromatids on the anaphase spindles. We were always able to observe centrosomes at the poles of the abnormal spindles, although sometimes this necessitated looking at different focal planes (Fig. 6c). However, there are regions devoid of chromatin, in which there are free centrosomes that have dissociated from nuclei. These can be seen either as dots, by staining with the Rb188 antibody, or by the asters of microtubules that they nucleate (Fig. 6b,c, open arrows).

Hemi-spindles in larval neuroblasts

Homozygous *asp* embryos from heterozygous parents survive embryogenesis, presumably utilising the wild-type *asp* gene product from their mothers. As with many other mitotic mutations, this permits development through the larval stages, in which many tissues become polyploid. The exceptions are the developing central nervous system and the imaginal tissues. Many of the stained chromosomes in squashed preparations of neuroblasts of larvae homozygous for *asp* are in clusters that typify metaphase-arrested cells (Ripoll *et al.* 1985). Orcein-stained squashed preparations do not permit visualisation of the mitotic spindle, which would in any event be disrupted by the mechanical squashing of the preparation. We therefore sought to extend these observations by developing an immunological staining technique for whole-mount preparations of larval brain material. Examination of these preparations by confocal microscopy permits the spatial arrangement of mitotic chromosomes to be examined with respect to spindle microtubules and centrosomes.

Figs 7 and 8 show fields of cells from wild-type and *asp* brains examined in this way with a variety of antibodies and dyes (see figure legends for details). The examples of confocal micrographs from wild-type neuroblasts show mitotic spindles stained to reveal centrosomes and microtubules (Fig. 7a and b, respectively); or to give an image of chromatin in one channel of the instrument, and microtubules in the other channel (Fig. 7c and d, respectively). Very few mitotic figures can be seen per wild-type brain. They are invariably bipolar and optical sectioning with the confocal microscope reveals two centrosomes, one at each spindle pole.

We have examined mitotic figures in neuroblasts of larvae homozygous for either the asp^1 or the asp^{E3} alleles. No allele-specific differences were found. In contrast to wild-type, brains from homozygous *asp* larvae show a high frequency of mitotic figures arrested in metaphase. Some cells have a polyploid complement of over-condensed chromosomes as described previously (Ripoll *et al.* 1985).

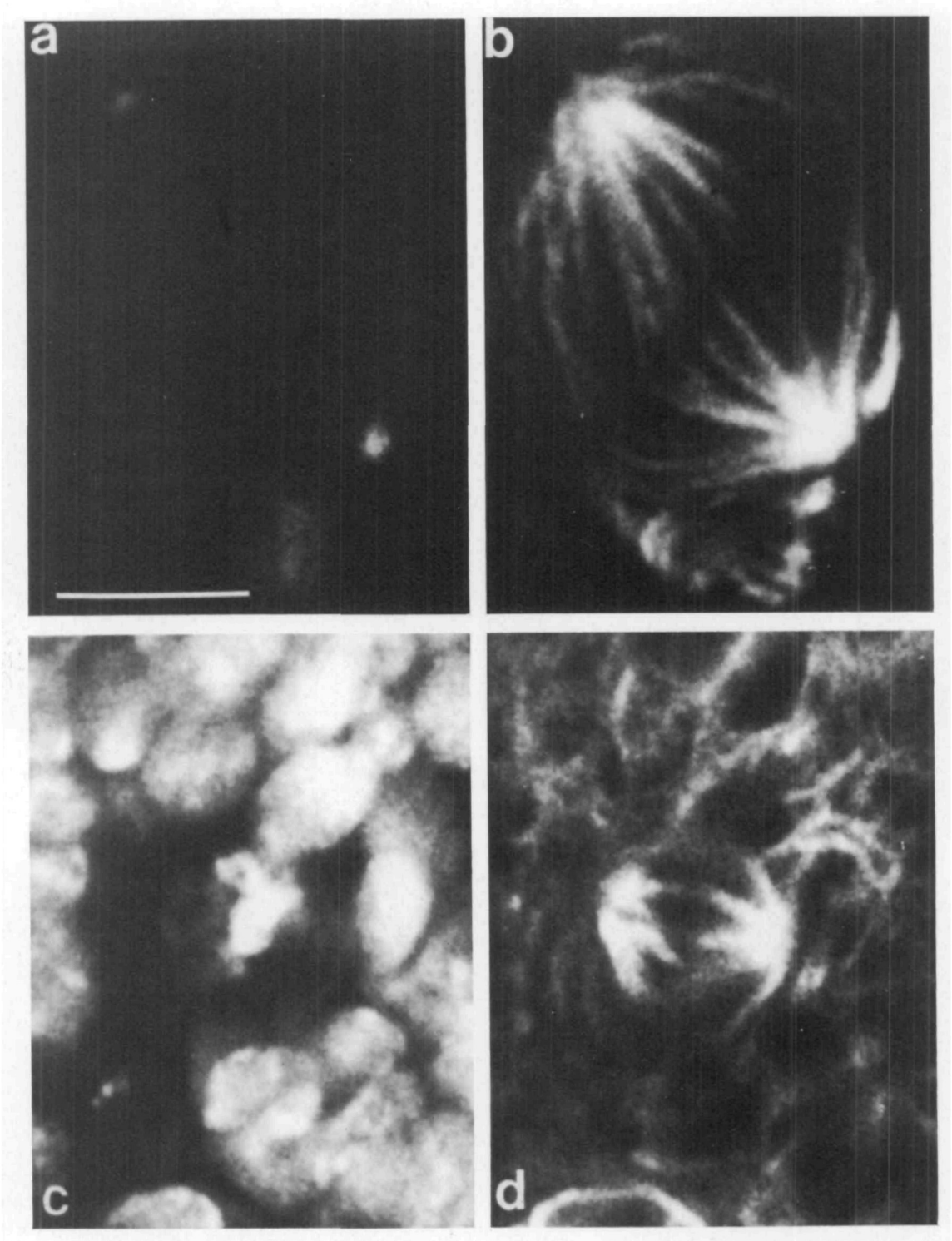

Fig. 7. Mitotic spindles in wild-type neuroblasts. In the upper confocal micrograph, centrosomes have been revealed by staining with the rabbit antibody RB188 followed by fluorescein-conjugated anti-rabbit IgG (a), and the spindle microtubules with the rat monoclonal antibody YL1/2 followed by Texas-red-conjugated anti-rat IgG. In the lower field propidium di-iodide is used to reveal DNA in the left panel (c) and tubulin is stained with the YL1/2 antibody in the right-hand panel (d). Bar, 4 μm.

The number of centrosomes did not increase with polyploidisation, however, and in most cases such cells had only two centrosomes (never more) at the poles of the metaphase spindle (Fig. 8a and b). The majority of cells were apparently arrested at metaphase with a wild-type complement of chromosomes. The bipolar spindles associated with these cells have somewhat irregular arrays of microtubules (Fig. 8c and d) in comparison with the wild-type. Between 10 and 20% of cells in which a spindle-like structure can be seen contain a wild-type complement of condensed chromosomes arranged around a single centrosome (Fig. 8c and d). When stained with anti-tubulin antibody such cells show large unidirectional arrays of microtubules nucleated by the single centrosome (Fig. 8e

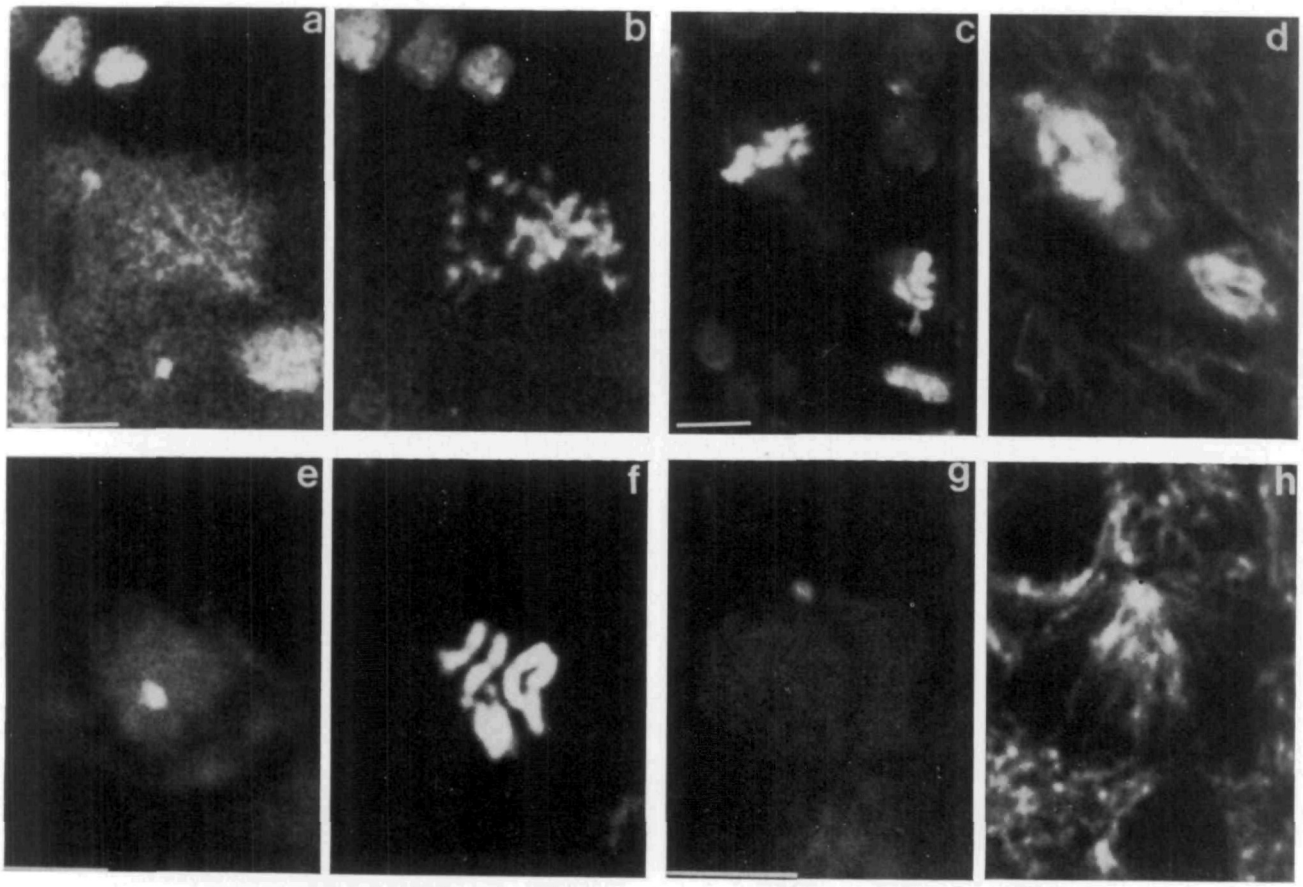

Fig. 8. Mitotic spindles of neuroblasts from homozygous *asp* larvae. a,b. Bipolar mitosis in a polyploid cell in which centrosomes have been labeled by staining with the rabbit antibody RB188 followed by fluorescein-conjugated anti-rabbit IgG (a), and the condensed chromosomes labeled with propidium di-iodide (b). c,d. Two cells with bipolar spindles in which DNA is labeled with propidium di-iodide, and microtubules with Yl1/2 antibody, respectively. e,f and g,h. Two cells with monopolar spindles in which the centrosomes are labelled with RB188 (e and g). The cell on the left is stained to reveal DNA with propidium di-iodide (f), and the one on the right with YL1/2 to reveal microtubules (h). Bars: 3 μm (a–d); 4 μm (e–h).

and f). It is apparent when staining for chromatin and microtubules that this structure is a hemi-spindle, in which microtubules are attached to the centrosome at one end, and condensed chromosomes at the other, with microtubules appearing to pass through the kitochore regions to terminate shortly beyond the chromosomes (Fig. 9).

Discussion

Mutation in *asp* has effects upon meiotic and mitotic divisions. Cytological and genetic evidence indicates non-disjunction during both meiotic divisions in males bearing mutant alleles and phase-contrast microscopy indicates that meiotic spindles of mutant males are abnormal (Ripoll *et al.* 1985). We have found that these spindles contain abnormal, long, wavy arrays of microtubules, which often form complex networks, and sometimes exist as hemi-spindles. Such structures would account for the high degree of meiotic non-disjunction. The interphase microtubules in growing-stage cells during spermatogenesis, are indistinguishable from the same stage in wild-type, suggesting that in this lineage, the *asp* gene product is required specifically for spindle formation.

The infertility of *asp* females precludes a genetic analysis of non-disjunction in female meiosis, which in addition is less tractable to cytological analysis. As the unfertilised eggs of homozygous *asp* females can either have no nuclei or a small number of large nuclei, we infer that the mutations also lead to non-disjunction in the female germline. These eggs are quite distinct from those non-inseminated eggs laid by wild-type females in which one sees the four haploid products of female meiosis that may sometimes be fused together. The appearance of these large nuclei in ASP eggs is somewhat reminiscent of the giant nuclei seen in both fertilised and unfertilised eggs of females homozygous for the mutation *gnu* (Freeman *et al.* 1986; Freeman and Glover, 1987), although the nuclei in GNU eggs become much larger. It has previously been suggested that the GNU phenotype results from an uncoupling of the block to DNA synthesis that is normally imposed after the completion of female meiosis (Freeman and Glover, 1987). It is possible that the specific mechanisms that control post-meiotic DNA synthesis are in some way coupled to the meiotic process itself. If so, one might expect there to be a set of genes that when mutated give rise to phenotypes that resemble *gnu* to some degree, which play a role in the correct completion of meiosis and consequently the initiation of zygotic development.

The unfertilised ASP eggs contain both free centrosomes that nucleate microtubules, and networks of short microtubules that are not nucleated by centrosomes. The maternally provided centrosome-associated Bx63 antigen, identified by the antibody used to stain centrosomes in this

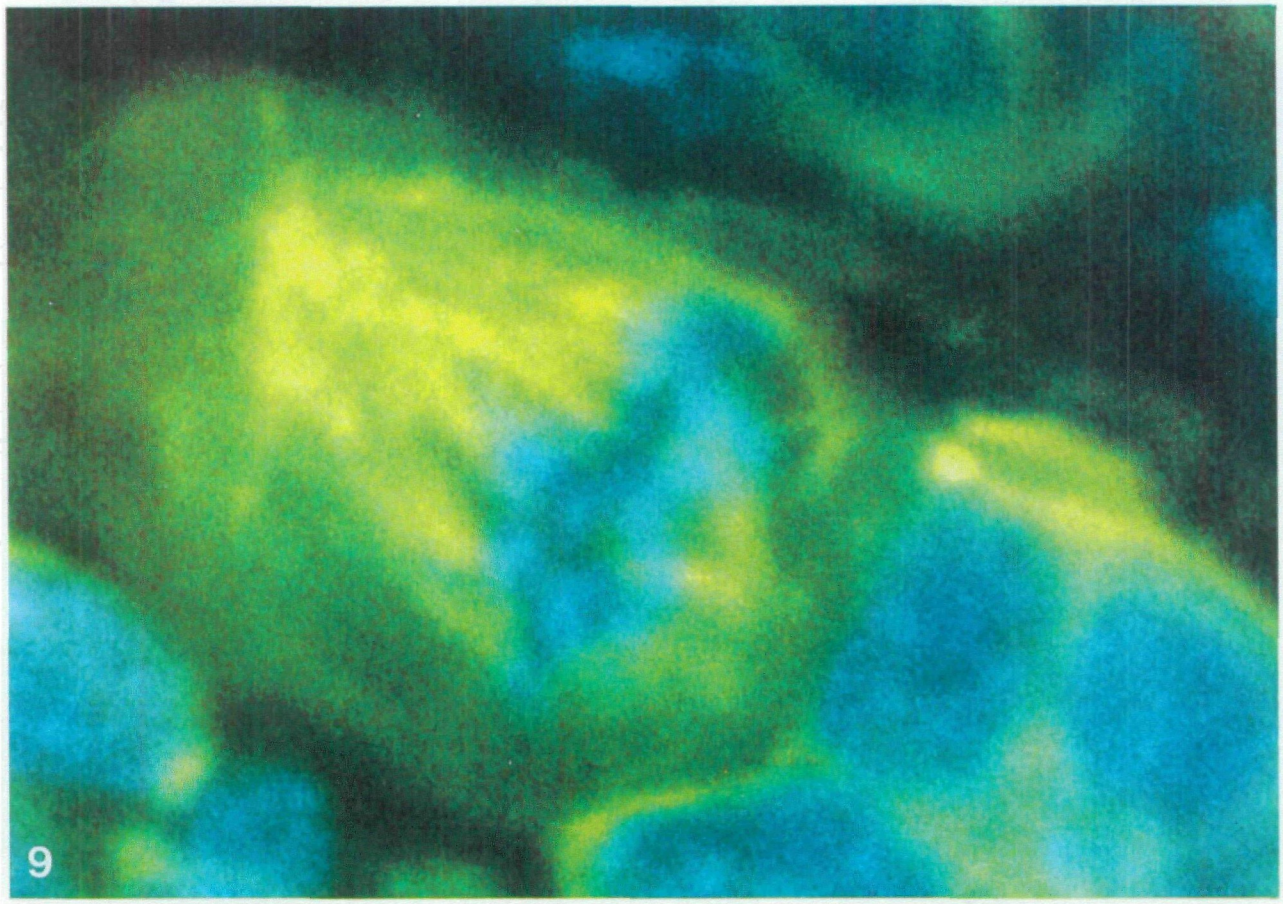

Fig. 9. Hemi-spindle in an ASP neuroblast. DNA and centrosomes have been labeled with propidium di-iodide, and RB188 followed by Texas-red-conjugated anti-rabbit IgG, respectively (blue), and microtubules by YL1/2 and fluorescein-conjugated anti-rat IgG (yellow). The images from the two channels of the confocal microscope have been merged and false colour added.

work, is normally sequestered into centrosomes only as they replicate (Frasch *et al.* 1985; Whitfield *et al.* 1988). Similarly, the assembly of maternally provided tubulin monomers into microtubules is normally nucleated by the centrosomes upon commencement of mitotic divisions. Extensive replication of centrosomes occurs in unfertilised eggs laid by females homozygous for the mutation *gnu*. The finding of free centrosomes in unfertilised ASP eggs could be another similarity between mutations at the two loci, pointing towards their involvement in a common path of events that synchronises mitotic development of the zygote following the completion of meiosis and fertilisation.

A high proportion of fertilised eggs develop to late stages of embryogenesis, but inevitably show some mitotic defects during the syncytial stages. Unlike wild-type embryos, their mitotic divisions are asynchronous, and the structure of the mitotic spindles is highly abnormal. The networks of wavy spindle microtubules are reminiscent of those of the mutant meiotic spindles. It is, however, difficult to make such comparisons because the meiotic spindles are much larger than the mitotic spindles of the cleavage embryo. The abnormal spindles in ASP embryos are associated with chromatin that appears not to be segregating correctly. This is likely to result in the dissociation of free centrosomes from the mitotic apparatus, as is observed in wild-type embryos that have been injected with the DNA polymerase inhibitor, aphidicolin (Raff and Glover, 1988, 1989). Free centrosomes that undergo independent rounds of replication have been seen when the mitotic apparatus is disrupted by other mitotic mutants (Freeman *et al.* 1986; Glover, 1989; Glover *et al.* 1989). It seems that, in these cases, several aspects of mitosis can continue to cycle independently of nuclear division: in aphidicolin-treated embryos DNA replication is blocked and as a consequence chromosomes do not replicate and segregate; whereas in GNU embryos, nuclear division does not occur and DNA synthesis continues. There are therefore a variety of situations arising in embryos that result in centrosomes becoming uncoupled from an aberrant mitotic apparatus on which segregation cannot occur.

ASP embryos can undergo a considerable degree of development following cellularisation, resulting in a variety of abnormal cuticular patterns. Other mutations have also been described in which mitosis is arrested after cellularisation and in which considerable further development then occurs. The zygotic activity of the locus *string*, for example, is required for cell proliferation in the embryo following cellularisation. In *string* embryos, cell division is uniformly arrested, and most morphogenetic aspects of development proceed reasonably normally, giving embryos with a reduced cell number (Edgar and O'Farrell, 1989). This has led to the suggestion of a degree of independence between morphogenesis and cell proliferation. The mitotic defects in ASP embryos occur before cellularisation, and can result in large areas of the embryo being devoid of regular arrays of nuclei. It is presumably this effect of depopulating random areas of the blastoderm embryo of nuclei that results in the wide range of developmental phenotypes.

The effect of *asp* on the behaviour of centrosomes in larval neuroblasts is in marked contrast to that seen in syncytial embryos. In the syncytial ASP embryo, centrosomes can dissociate from abnormal spindles and seem likely to undergo autonomous replication of the kind found in GNU embryos or aphidicolin-injected embryos (Freeman *et al.* 1986; Raff and Glover, 1988). In neuroblasts of homozygous *asp* larvae, on the other hand, the mutation appears to prevent or delay the replication of centrosomes. Thus, in the metaphase-arrested neuroblasts, there is often only a single centrosome, and never more than two. To explain the differences between the embryonic and larval phenotypes, we suggest that the *asp* mutation does not affect the structure and function of the centrosome directly, and that the phenotypes are the indirect consequences of an affect of the mutation on some other component of the mitotic spindle. The consequences of the mutation would reflect basic differences in the cell cycles at these developmental stages. The cycle in syncytial embryos is 'abbreviated' to comprise rapidly alternating S and M phases. Furthermore, one can inhibit S phase with the DNA polymerase inhibitor aphidicolin, and still permit many mitotic activities to cycle (Raff and Glover, 1988). The cell cycles of *Drosophila* neuroblasts seem to be similar to those in mammalian cells and yeast, for example, in that progression through S is required before mitosis and cytokinesis occur, and in which there are distinct sequential G_1, S, G_2 and M phases. It would appear that the centrosome cycle of neuroblasts is much more tightly coupled to the rest of the mitotic cycle than it is in the syncytial embryo.

The structure of the mitotic apparatus in metaphase-arrested *asp* neuroblasts is striking. The cells frequently have a wild-type metaphase complement of aligned condensed chromosomes, which are connected to a single centrosome on what appears to be a hemi-spindle (Fig. 8). Such a structure could not permit the segregation of chromatids into daughter cells and so allows one to postulate a mechanism whereby cells can become initially tetraploid in ASP brains. It may be that when the phenotype first develops, centrosome replication is delayed, and that subsequently it can occur but only to a limited extent. This might account for those polyploid cells that have highly condensed chromosomes and contain two centrosomes. Such cells never contain any more centrosomes, however, and the high degree of chromatin condensation suggests that they have been arrested at metaphase for some time.

The *Drosophila* mutation *mgr* results in polyploid cells, in which the mitotic chromosomes in conventional chromosome squashes appear to be arranged as if around a monopolar spindle (Gonzalez *et al.* 1988). The condensed chromosomes of these structures are arranged in circles with their centromeres inward and arms pointing outwards. This phenotype, which is not seen in preparations treated with microtubule-destabilising drugs, has been interpreted as suggesting that the chromosomes are under traction to a single spindle pole. Such circular arrangements are not seen in conventional cytological preparations of *asp* neuroblasts, or in the double mutant *asp mgr* (Gonzalez *et al.* 1988). We therefore speculate that the structure we now describe in *asp* neuroblasts might be static, whereas the putative monopolar spindles in *mgr* would be somewhat dynamic. It should now be possible to clarify the structure of the *mgr* and *polo* spindles by applying the new immunostaining techniques described in this paper. Ultimately, a thorough understanding of each of these loci will require the molecular characterisation of their gene products, and to this end we are in the process of cloning these genes.

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