Evidence for cell cycle-specific, spindle pole body-mediated, nuclear positioning in the fission yeast Schizosaccharomyces pombe

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SUMMARY

Specific changes in spatial order occur during cell cycle progression in fission yeast. Growth of the rod-shaped cells is highly regulated and undergoes a cell cycle and size-regulated switch from monopolar to bipolar tip extension. During both phases of growth, the interphase nucleus is maintained in a central location. Following the separation of the genome to the cell tips in mitosis, the two nuclei migrate back towards the cell equator before stopping in two new positions that will become the middle of the two new cells. Here we use simultaneous labeling of microtubules, chromatin and spindle pole bodies in wild-type and cdc mutants, to show that nuclear positioning is achieved by regulation of spindle pole body-mediated nuclear migration. We show that the number and location of nuclear positioning signals is regulated in a cell cycle-specific manner and that spindle pole body-mediated forces are likely to be responsible for maintaining correct nuclear position once the nuclei have reached the appropriate position in the cell. Accentuating the movement of the nuclei back towards the cell equator after mitosis by artificially increasing cell length shows that the spindle pole body leads the nucleus during this migration. When multiple spindle pole bodies are associated with the same or different nuclei they all go to the same point indicating that the different spindle pole bodies are responding to the same positional cue. In a septation-defective mutant cell, which contains four nuclei, the spindle pole bodies on the four different nuclei initially group as two pairs in regions that would become the middle of the new cells, were the cell able to divide. In the subsequent interphase, the nuclei aggregate as a group of four in the centre of the cell. The presence of two or three clusters of spindle pole bodies in larger cells with eight nuclei suggests that the mechanisms specifying the normally central location for multiple nuclei may be unable to operate properly as the cells get larger. Perturbation of microtubules with the microtubule poison thiabendazole prevents the spindle pole body clustering in septation mutants, demonstrating that nuclear positioning requires a functional microtubule cytoskeleton.

Key words: Schizosaccharomyces pombe, Spindle pole body, Microtubule, MTOC, Nuclear migration

INTRODUCTION

The cytoskeleton plays a key role in the establishment and maintenance of the complex patterns of organelle distribution that underlie cellular architecture (Bray, 1992). Microtubules have pivotal functions in the establishment of polarity in many processes such as embryogenesis (Sullivan and Therkauf, 1995) and the accurate positioning of the nucleus and mitotic spindle (Gönczy and Hyman, 1996; Reinsch and Karsenti, 1994; Reeve and Kelly, 1983).

A role for the microtubule cytoskeleton in nuclear positioning has been proposed in many simple eukaryotic systems such as diatoms, algae and fungi (for example, Wordeman et al., 1984; Doonan et al., 1986; Morris et al., 1995). In addition, correlative phase-contrast and electron microscopy has suggested that the microtubule-organising centre may be intimately involved in nuclear migration (e.g. Wilson and Aist, 1967; Dowding and Bakerspigel, 1954; Pickett-Heaps and Wetherbee, 1987).

The genetic tractability of the fungal systems has recently provided considerable insight into how nuclear movements can be generated at the molecular level. Movement of nuclei out of germinating spores into growing Aspergillus nidulans hyphae is a microtubule-mediated process (Oakley and Morris, 1980; Oakley and Reinhart, 1985; Meyer et al., 1988). It appears to be driven, at least in part, by a microtubule motor protein complex containing cytoplasmic dynein (Xiang et al., 1994, 1995a) and may involve a mechanism that is conserved in eukaryotes in general (Xiang et al., 1995b). Similarly, in Neurospora crassa the normal regular spacing of the nuclei along the fungal hypha is abolished by mutating the heavy chain of cytoplasmic dynein and functionally related genes (Plamann et al., 1994; Robb et al., 1995; Tinsley et al., 1996; Bruno et al., 1996). Whilst these studies clearly indicate that a motor complex is responsible for nuclear movements in filamentous ascomycetes, the key questions of how these motors generate nuclear movements and how are they regulated to establish the even distribution of the nuclei along the hyphae remain to be addressed.

Further levels of complexity have been revealed in the
budding yeast *Saccharomyces cerevisiae* by elegant experiments using drugs and multiple mutant backgrounds. Not only is the positioning of the undivided nucleus in the bud neck at the junction between the mother and daughter cells dependent upon microtubule integrity (Jacobs et al., 1988), but there is also an intimate relationship between cytoplasmic microtubules and the actin cytoskeleton that involves dynein (Li et al., 1993; Clark et al., 1994; Eshel et al., 1993; Palmer et al., 1992; Snyder et al., 1991). This interaction determines both spindle orientation and the path of elongation during mitosis and results in the tethering the spindle to one point at the base of the mother cell and to another at the tip of the bud. Similar mechanisms may underlie spindle orientation in higher systems (Gönczy and Hyman, 1996). Further evidence for a role of the actin cytoskeleton in nuclear positioning comes from studies such as those of sporulation in *Sordaria* where large actin cables appear to wind around the nuclei and are assumed to assist in the ejection of spores upon ripening (Thompson-Coffe and Zickler, 1995).

Less is known about the relationship between the cytoskeleton and nuclear positioning in the mitotic cell cycle of the fission yeast *Schizosaccharomyces pombe*. The fission yeast interphase microtubule array resembles those of filamentous fungi such as *N. crassa* and *A. nidulans* (McKerracher and Heath, 1987; Osmani et al., 1988) and consists of a basket of microtubules extending between the cell ends (Hagan and Hyams, 1988). In the few studies of this cytoskeleton at the level of electron microscopy, interphase microtubules are generally seen as bundles of three microtubules (Hereward, 1974; Tanaka and Kanbe, 1986; Streiblova and Girbardt, 1980), suggesting that the patterns seen by immunofluorescence represent bundles rather than single microtubules. Disruption of the microtubule cytoskeleton either by mutation of tubulin genes or exposure to anti-microtubule agents can lead to branching of the normally linear rod-shaped cell and disruption of the central positioning of the nucleus (Walker, 1982; Hiraoka et al., 1984; Umesono et al., 1983). Since these manipulations also cause a mitotic arrest that can last for 1.5 generation times or more, it is unclear whether the effects on branching and nuclear positioning in these instances arise directly from the disruption of the cytoskeleton or are a secondary effect of a previous mitotic delay. However, recent data suggest that microtubules are indeed important for the establishment of cell polarity as several branched and curved mutants have altered microtubule cytoskeletons (Verde et al., 1995). One of these mutants, *ban5-3*, which is allelic to the *α* tubulin gene, *atb2* (Yaffe et al., 1996), was reported to also have defects in nuclear positioning (Verde et al., 1995).

Here we describe experiments that use antibodies that specifically recognise the spindle pole body (SPB) (Hagan and Yanagida, 1995) to characterise the basis of nuclear positioning in fission yeast. Using different genetic backgrounds to manipulate the intracellular environment, we show that interphase nuclear positioning in fission yeast is achieved by the response of the SPB to cell cycle-specific signals. Whilst the nature of this regulation of positioning remains obscure, data showing that several signals are generated in cells unable to divide for three generations are discussed in terms of fungal nuclear positioning in general. We also discuss the role of a previously unrecorded activation of the SPB at the end of anaphase in the rapid movement of the nuclei towards the cell centre after division.

**MATERIALS AND METHODS**

**Cell culture and strains**

Cell culture and maintenance was carried out according to the method of Moreno et al. (1991). The isolation of 972h+, h0 (Gutz et al., 1974) cdc7.24, cdc14.118, cdc25.22 (Nurse et al., 1976) and cdc1.206 cdc11.123 (Uzawa et al., 1990), has been described previously. cdc7.24 was a gift from Dr V. Simanis (ISREC, Lausanne). Mitotic cultures were grown in rich YPD or YES medium, with the exception of the cdc7.24 synchronous culture, which was grown in minimal EMM2 medium (Moreno et al., 1990). Mating was carried out in SSL following growth in SSL plus 5 g l−1 ammonium acetate (Egel, 1971).

**Generation of cells synchronised for cell cycle progression**

Lactose gradient synchronisation according the methodology of Mitchison (1970) was used to isolate early G2 cells for generation of cultures synchronised for cell cycle progression. A two-step synchronisation procedure was used: cells were initially synchronised on a 45 ml 10-40% lactose gradient and then immediately passed over a second (12 ml) 10-40% lactose gradient in order to achieve optimal size uniformity. Following washing, cells were inoculated into fresh medium. After removal of a 20 ml sample for incubation at 25°C to follow synchrony, the remainder of the culture was incubated at 36°C. 103 cells were harvested every 30 minutes from the 36°C culture and prepared for immunofluorescence microscopy. The cell plate index at 25°C was monitored using Calcofluor staining (Moreno et al., 1990) and scoring only those cells with an equatorial brightly staining bar as having septa. cdc14.118− cells proved to be too leaky for synchronous culture analysis as many cells septated at 36°C in rich or minimal medium. cdc7.24 synchronised cells only arrested efficiently when the experiment was conducted in minimal medium.

**Immunofluorescence microscopy**

TAT1 anti-α tubulin antibody (Woods et al., 1989) or affinity-purified Sad1 antibodies (AP9.2) (Hagan and Yanagida, 1995) were used to visualise microtubules and the SPBs, respectively. Cells were fixed with 3.6% formaldehyde and 0.2% glutaraldehyde as described in Hagan and Hyams (1988); with the exception that, at the end of the procedure, after the first wash in antibody buffer, cells were washed once in phosphate-buffered saline (PBS), resuspended in 0.5 μg ml−1 4,6-diamidino-2-phenylindole (DAPI) in PBS, pelleted and resuspended in 25 μl PBS containing 0.1% sodium azide. Fluorescein isothiocyanate (FITC) conjugated goat anti-mouse antibodies (EY labs) were used to localise TAT1 primary antibodies and CY3-conjugated goat anti-rabbit antibodies (Sigma) for AP9.2 primaries. Double staining was performed by sequential incubation in mixtures of both primary and then both secondary antibodies. False colour images were produced as described in Lange et al. (1995).

**Drug treatment**

Thiadiazole (TBZ) was prepared as a 400x stock solution in dimethyl sulphoxide (DMSO) as described by Walker (1982) and added directly to the culture to a final concentration of 50 μg ml−1. Hydroxyurea (HU) was added to the culture from a 1 M stock solution in water to a final concentration of 10 mM.

**RESULTS**

**Nuclear movements during and after fission yeast mitosis**

During fission yeast mitosis the nuclei are separated to the cell ends at a rate of 1.6 μm per minute by an extensive anaphase B movement (McCully and Robinow, 1971; Hagan et al., 1990). When anaphase is complete, two microtubule-organis-
Fig. 1. False colour images of wild-type fission yeast cells in mitosis. Wild-type fission yeast cells were processed for immunofluorescence microscopy to visualise their SPBs (green), microtubules (red) and chromatin (blue) after growth to mid-logarithmic growth phase at 25°C. The lower panels represent a combination of the fluorescence information, which is shown in the upper panel, and the phase-contrast image of the same cell. Following spindle formation (A), the spindle undergoes an extended anaphase B (B,C) to achieve maximal nuclear separation. Thereupon two MTOCs are activated in the middle of the cell (D,E) giving rise to the PAA (F,G). As the nuclei move towards the middle of the daughter cells, they assumed a stretched profile with the SPB at the tip (F, upper nucleus).

Increasing the distance moved by nuclei after anaphase highlights the role of the SPB in nuclear migration

As spindle break down can occur before the nuclei have migrated to the cell tips (Fig. 1D,E), concrete conclusions about the role of the SPB in post-anaphase nuclear movements could not be drawn from observations in wild-type cells. Cells were therefore manipulated so as to increase the distance that the nucleus must move to accentuate nuclear movement after spindle dissolution. The cdc25.22 mutation delays mitotic commitment and concomitantly increases cell and spindle length at division (Hagan et al., 1990), which means that the nuclei have greater distances to move after anaphase to reach the correct position in the middle of the daughter cells in cdc25.22 strains grown at 26°C.

As in wild-type cells, the SPB in cdc25.22 cells always faced the cell tips during anaphase B and during the initial stages of spindle breakdown (Fig. 2A-C). PAA maturation saw more and, on average, longer, microtubules, which extended beyond the SPBs to the cell ends. This tubulin staining often appeared to kink when contacting the SPBs (Fig. 2E). In many cases, the SPBs were orientated towards the equator and the nuclei had a cone-shaped profile which formed a point at the SPB, consistent with a role for the SPBs in pulling the nucleus along microtubules (Fig. 2E,F). Once the nuclei were correctly positioned, the chromatin lost its pointed appearance and resumed the crescent shape typical of interphase (left nucleus in Fig. 2G).

Although these data are consistent with a role for the SPB in nuclear positioning following mitosis, they are qualitative rather than quantitative. Quantitative data were obtained by following cytoskeletal and nuclear morphology in a synchronous culture. The advantage of following the frequency of different characteristics in a synchronous culture lies in the fact that at the start of the experiment all of the cells are in, more or less, the same stage of the cell cycle and that all cells progress through the subsequent cell cycle with similar kinetics. Therefore, by monitoring the phenotypic changes that occur in the culture with time, we can see what course a typical cell takes (Mitchison, 1970).
Fig. 2. Increasing the distance migrated by nuclei following mitosis by mutation of the cdc25 gene shows that the SPBs lead the nuclei towards the cell equator. The figure shows a series of cells illustrating (from top to bottom) microtubules, SPBs, chromatin and the position of the chromatin within the cell. The information is summarised in cartoons at the bottom of each figure set. (A) A cell in the final stages of anaphase B just before the central MTOCs nucleate the PAA (B,C). As the PAA matures some microtubules contact the SPBs which start to move towards the cell centre (D). In cells with more complex PAA, the nuclei had a stretched appearance pointing towards the equator, with the SPBs at the apex. This suggests the SPBs are at, or extremely close to, the point of force transduction that pulls the nuclei back towards the centre of the cell (E,F). Once the nuclei were correctly located the crescent shape was resumed and the SPB positioning once again became randomised (G). cdc25.22h^− cells were grown to mid-log phase at 26°C and fixed for combined anti-tubulin and anti-Sad1 immunofluorescence microscopy. Bar, 5 μm.
The ability to synchronise cultures by size selection of smaller G2 cells was combined with the ability to synchronise mitosis further with a transient arrest at the G2/M boundary by brief inactivation of the cdc25.22 gene product (Hagan, 1988). cdc25.22 cells isolated from a lactose gradient were grown at 36°C before being returned to 24°C after 145 minutes (probably consistent with a maximum arrest at the G2/M transition of 40 minutes). Within 10 minutes of return to the permissive temperature spindles were formed with a peak of 72% (Fig. 3 open squares combined with open triangles). The wave of mitosis (Fig. 3A, open squares) was followed successively by activation of the central organising centres (Fig. 3A, open triangles) and the more extensive PAA (Fig. 3A, circles). Scoring cells with PAA as to whether either SPB was facing the centre of the cell (Fig. 3B, circles) or not (Fig. 3B, diamonds) showed that the brief period immediately after spindle breakdown, when neither SPB faces the cell centre (30-50 minutes), was followed by an interval during which the SPBs face the cell centre before a more random distribution is adopted. Since the nuclei move towards the cell centre following spindle breakdown, these data are consistent with the images in Fig. 2 and show that the SPB leads the nucleus back towards the centre of the cell immediately after spindle dissolution.

The SPB is activated as spindle microtubules depolymerise

The combination of unprecedented mitotic synchrony and the ability to detect the relative location of the SPBs and the microtubules revealed a previously unrecorded microtubule pattern. Several faint microtubules extended from the SPBs of cells in which the PAA was very immature. This staining pattern was seen in cdc25.22 cells, which were released from temporary arrest at 36°C (data not shown) as well as in the control synchronous culture maintained at 24°C (Fig. 4A-F, arrows indicate the SPBs from which the arrays extend). Re-examination of wild-type cells stained with increased concentrations of anti-tubulin antibodies revealed similar patterns (Fig. 4G-I). These microtubules were often much fainter than the strongly staining astral microtubule bundles, which contain three microtubules (Tanaka and Kanbe, 1986), raising the possibility that they may represent individual microtubules. This new pattern is consistent with activation of the SPB immediately after mitosis and is reminiscent of the cytoskeletal changes that accompany spindle dissolution in Schizosaccharomyces japonicus (Alfa and Hyams, 1990).

Multiple SPBs within the same nucleus respond to the same signal

The involvement of the SPB in nuclear positioning immediately following anaphase led us to question whether the SPB might also be responsible for mediating nuclear positioning during interphase. We reasoned that we could determine whether the SPB responds to specific signals to determine interphase nuclear position by putting multiple SPBs in a single nucleus. If the SPB is used to direct nuclear positioning all of the SPBs would be expected to respond in concert to the nuclear positioning signal and cluster at a single point. If, on the other hand, the SPB plays no role in nuclear positioning in interphase they would be more likely to maintain independent positions.

Despite undergoing multiple S phases and forming an apparently normal spindle, cut1 mutants are unable to segregate their DNA and multiple SPBs accumulate in single nuclei (Uzawa et al., 1990). As septation continues, the nuclei in these cells are randomly cleaved by the division apparatus and the cell dies. Cell cleavage can be avoided by the introduction of additional mutations which inhibit septation and so allow a single nucleus to continue through multiple cell cycles, accumulating SPBs as it does so (Uzawa et al., 1990). Fig. 5 shows such a double mutant, cut1.206 cdc11.123. Whilst many SPBs were seen during the defective mitoses (Fig. 5G-I), only a single large dot of Sad1 staining were seen in interphase cells that had been through several abortive attempts at nuclear division (Fig. 5A-F), suggesting that all SPBs were now responding to the same signal and clustering at one point. In every case in which we could manipulate the cell cycle to increase SPB numbers.
in a single nucleus, all the SPBs appeared to respond to the same positional signal (data not shown).

Multiple SPBs on different nuclei in the same cell respond to the same signals

One caveat to the explanation for SPB clustering given above is related to our previous demonstration that the centromeres are always associated with the SPBs during interphase (Funabiki et al., 1993). If it was possible for the centromeres of one chromosome to simultaneously interact with two SPBs, the clustering of SPBs in a single nucleus could be due to centromere-mediated “cross linking”.

To test this possibility, we looked at the SPB and nuclear localisation in septation mutants in which a single cell has multiple SPBs that are each associated with a single, different, nucleus. If all of the SPBs were responding to the same signal, they would again cluster and this would be independent of any intranuclear centromere interaction.

Following the first mitosis at the restrictive temperature in any of the septation-deficient mutants, cdc14.118, cdc7.24 or cdc7.A20, the nuclei migrated back towards the cell equator (Fig. 6A-D). Thus, in the subsequent interphase the nuclei “paired up” in the centre of the cell with the SPBs at the site where the two nuclei contacted each other, often with the SPBs no longer distinguishable as separate entities. These observations showed that two SPBs associated with two separate nuclei in the same cell responded to the same nuclear positioning signal. It may be significant that these paired nuclei were invariably in a “back-to-back” configuration with their nucleoli, as determined by the morphology of the DAPI-staining region (Toda et al., 1981), facing the cell ends.

Cryptic controls over nuclear positioning revealed in septation mutants

Analysis of septation mutants undergoing a second mitosis at the restrictive temperature suggested that the signal directing nuclear positioning to the middle of the daughter cells directly after mitosis may still be present. However, the nuclei in these cells do not stay at these two positions but, instead, subsequently move to the centre of the cell, as would be appropriate for a normal interphase cell.

The morphology of the microtubules during these transitions is graphically shown in the micrographs of cdc14.118 cells in Fig. 7, whilst the false colour images of cdc7.24 cells in Fig. 8 show the locations of microtubules, SPBs and chromatin relative to the outline of the cell periphery. Following two
normal mitoses, cells contained four, round, evenly spaced nuclei (Figs 7A, 8A-C) whose nucleoli and SPBs were randomly oriented (Fig. 7B). As PAA complexity increased, the nuclei became pointed at the SPBs and the microtubules appeared to kink when contacting the SPBs (Figs 7C,D, 8D). Further development of the PAA, so that it more closely resembled an interphase cytoskeleton, resulted in two different patterns. In one, the four nuclei paired up in twos (Fig. 7E,F, 8E) whereas in the other three nuclei clustered and one remained isolated (Fig. 7G). It may be significant that, in the class with three nuclei grouped together, many cells had predominantly one or two very strong tubulin-staining filaments (probably microtubule bundles) along which all SPBs were aligned. Comparing the relative positions of the SPBs to the chromatin in Fig. 8D and E with those in Fig. 8F and G revealed a subtle difference. In Fig. 8D,E, the SPBs of each nuclear pair lie between the two "back-to-back" nuclei, whereas those in Fig. 8F,G were on the same side of the nuclei, facing the cell centre. This suggested that, in Fig. 8D and E, the direction of movement was of the two nuclei towards each other but that, in Fig. 8F and G, both nuclei of each pair were moving in concert towards the cell centre. In the final class,

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**Fig. 5.** All the SPBs in cut1.206 cdc11.123 double mutants containing multiple SPBs in one nucleus go to the same point during interphase. (A,D,G) Anti-tubulin; (B,E,H) anti-Sad1; (C,F,I) DAPI-phase contrast images. Cells were incubated for 6 hours at the restrictive temperature. Whilst multiple SPBs can be seen in the defective mitosis in G-I, all the SPBs are clustered in the two interphase cells shown in A-C and D-F. Bar, 5 μm.

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**Fig. 6.** "Back-to-back" nuclear positioning in cdc14.118 mutants incubated at the restrictive temperature for 3 hours to inhibit cytokinesis. (A-D) Anti-tubulin, anti-Sad1, DAPI and DAPI phase-contrast images, respectively, of the same cdc14.118 cells after the first mitosis at the restrictive temperature of 36°C. As in wild-type and cdc25.22 cells, the SPB appeared to lead the nucleus along the microtubules of the PAA back towards the cell equator immediately following mitosis (cell on right). Once an interphase microtubule array had been established, a typical "back-to-back" configuration was achieved as the two nucleoli faced the cell tips and the SPBs were tightly associated into one brightly staining dot in the cell centre. Cells were grown in rich YPD medium to early log phase and then shifted from the permissive to the restrictive temperature. Bar 5 μm.
Fig. 7. Complex patterns of nuclear positioning are seen following the second mitosis at the restrictive temperature in the septation mutant cdc14.118. As in Fig. 2, each panel is composed of separate images to show from top to bottom: microtubules, SPBs, chromatin and the relationship of the chromatin to the cell outlines. Following a double mitosis (A), a PAA was initiated at the cell centre (B). As the PAA developed, the nuclei began to take on a pointed appearance with SPBs at the tips of the points (C) which, in cells with more mature PAAs (D), seemed to be pulling them to two common points (E,F). As the microtubules returned to less complex, more strongly staining patterns (G), some cells are seen with three nuclei clustered and one free. Interestingly, in this situation the SPBs are virtually always on the same microtubule (in addition to the microtubule in the focal plane in G there are others in different focal planes). As many cells show four nuclei clustered in the cell centre (H,I), this is assumed to be the terminal phenotype (see Fig. 9). Cells were grown to early-log phase in rich YPD medium at the permissive temperature prior to incubation at the restrictive temperature for 4½ hours. Bar, 5 μm.
which predominated in the culture with prolonged incubation, and thus is presumably the final state of cells containing four nuclei, all four SPBs clustered in the cell centre (Figs 7H, I, 8H).

**Synchronous culture analysis shows that nuclei move successively to two different locations in a cell cycle-dependent manner**

To determine whether there was a temporal order to the phenotypic classes described above, these classes were quantified in a synchronous culture of *cdc7.24* at the restrictive temperature of 36°C (Fig. 9). The synchrony of the culture was reflected in successive mitotic peaks (Fig. 9A). The frequency of first and second cycle phenotypes are shown separately in Fig. 9B and C, respectively.

As expected, the first mitotic peak (110 minutes) was immediately followed by a peak in cells in which the nuclei were post-mitotic but not in a “back-to-back” configuration (Fig. 9B circles). Subsequently cells with “back-to-back” interphase nuclei peaked at 160 minutes (Fig. 9B, triangles).

In the early stages of the second cycle, there were peaks both of cells in which there were no apparent SPB/SPB clustering (Fig. 9C, squares) and those in which SPBs grouped as one or two off centre pairs (Fig. 9C, diamonds). These peaks were followed by a peak of cells in which all four nuclei were clustered in the cell centre (Fig. 9C, circles). The progressive order of peaks in Fig. 9C indicated that, in the individual members of the population, the phenotypes with all four nuclei were initially separate, then paired up off centre before all clustering in the middle of the cell. The greater area defined by the peak representing all four nuclei centrally located indicates that this stage persists for longer than the others.

The curves defined by scoring cells with three and four clustered nuclei were symmetrical showing that they represent transient states with no particular delay at any stage. In contrast, the curve defined by scoring paired nuclei declined much more slowly indicating either that central clustering was slow or that a subpopulation did not make the transition from paired nuclei to central clustering. To differentiate between these possibilities, a delay to cell cycle progression beyond the fourth nuclei stage was imposed to determine whether this delay would allow sufficient time for all four nuclei to cluster in the middle, which would be consistent with the first possibility. Because S phase immediately follows spindle dissolution in logarithmically growing cultures, the addition of the DNA synthesis inhibitor hydroxyurea (HU) after the first wave of mitosis will not affect the ability of the cells to execute the second mitosis (because the DNA replication for this division is already complete). It will, however, block further cell cycle progression into a third mitosis due to checkpoint-dependent inhibition of mitosis (Carr, 1995).

The culture incubated at 36°C in Fig. 9 was split after the first mitosis (150 minutes) and HU added to one half (Fig. 9D). The resulting extension of interphase led to fewer cells with paired nuclei but it did not result in a complete loss of the phenotypic class. This shows that some cells in the actively cycling population scored for Fig. 9C did not implement the second stage of nuclear positioning to move all nuclei to the cell centre.

The inability of some cells to cluster all four nuclei after the second mitosis anticipates the patterns seen following the next, third, mitosis as the nuclei aggregate in two or three clumps (Fig. 10), indicating that at this stage cells were no longer able to establish a single “nuclear positioning zone” in the centre of the cell.

Data from these experiments showed that, in the majority of cells following a second mitosis in a single cell, the nuclei initially moved to two non-central locations before all grouping in the cell centre. This suggests that the SPBs were responding to cell cycle-regulated nuclear positioning signals.

**Nuclear clustering in septation mutants is disrupted by anti-microtubule agents**

As the preceding staining patterns suggest that microtubules are used as the “rails” along which the SPBs move nuclei, the effect of disrupting microtubule integrity on nuclear positioning was examined. The data in Figs 7, 8 and 9 suggested that the most sensitive test would be the ability of the four nuclei to cluster following a second mitosis in a septation mutant. A synchronised culture of *cdc7.24* cells was therefore treated with the anti-microtubule drug thiabendazole (TBZ) at the restrictive temperature (Walker, 1982).

Synchronised *cdc7.24* cells were inoculated into minimal medium at 36°C to inhibit septation and the culture was split into three aliquots after the first mitotic peak (210 minutes). TBZ was added to a final concentration of 50 μg ml⁻¹ to one aliquot, DMSO (the solvent for TBZ) to another and the third was left as an untreated control (Fig. 11C, B and A, respectively). The profile of the DMSO-treated aliquots mimicked the untreated sample: the frequency of cells with one or two nuclei decreased whilst those with four and then eight increased, indicating that DMSO had no effect upon cell cycle progression (Fig. 11A, B). In contrast, in the TBZ-treated culture, the decline in cells with one and two nuclei and the rise of those with four nuclei was much less pronounced, and there was no commitment to a third mitosis (Fig. 11C) showing that cell cycle progression was severely compromised by drug treatment.

Interphase microtubule cytoskeletons were largely intact despite the drug treatment, although they were considerably more disorganised (data not shown). Fig. 11E shows the relative frequencies of nuclear positioning profiles in TBZ-treated cells containing an interphase cytoskeleton and four nuclei at 480 minutes. In stark contrast to the predominance of cells with all four SPBs clustered and the low frequency of cells with all four SPBs separate in controls (Fig. 11D), the majority of interphase cells with four nuclei in the TBZ-treated culture (40%) showed no apparent SPB clustering at all, and it was the minority that positioned all four SPBs together (Fig. 11E).

These data indicate that microtubule integrity is required for nuclear positioning throughout interphase in fission yeast.

**Central MTOCs are not observed following meiosis I**

The possibility that the SPB may be responsible for directing nuclear migration following mitosis suggests that the function of the PAA may be to ensure that an interphase cytoskeleton is re-established sufficiently rapidly to orchestrate correct nuclear positioning before cytokinesis, thus preventing inappropriate partitioning or cleavage of the nucleus by the septum. If this were the case the central microtubule organisers might not be expected to be required after a nuclear division that is
not succeeded by cytokinesis or post-anaphase nuclear migration: the first meiotic division.

When homothallic cells are starved for a source of nitrogen, they initially undergo a series of mitotic divisions that result in a reduction in cell size, then they conjugate and undergo meiosis. Fig. 12 shows two zygotes at successive stages at the end of meiosis I. In contrast to cells after the completion of mitotic anaphase, no MTOCs were seen, rather microtubules appeared to nucleate at random in the cytoplasm. The lack of a PAA and nuclear migration after this nuclear division is consistent with the primary role for the PAA being to ensure that the nuclei are not cleaved by the incipient septum rather than being required for the final stages of nuclear division per se.

DISCUSSION

A well-established strength of genetic systems is the ability to
Fig. 9. Progressive appearance of phenotypes in a synchronous cdc7.A20 culture grown at the restrictive temperature shows that nuclei pair up in two non-equatorial positions prior to clustering equatorially. Data from a single culture are presented in three separate panels each containing different information.

(A) Successive waves of mitoses at the restrictive temperature. Cells were scored for being in their first (■), second (○) or third (●) mitosis within a single cell and the results plotted against time. The synchrony and sequential appearance of the phenotypes is apparent. n>220 for each time point. (B) First cycle characteristics. The following phenotypes are indicated: ■, interphase; ○, first mitotic spindle; ●, post-first mitosis but not “back-to-back”; ▲, “back-to-back”. Clearly individuals within the population were progressing from interphase into mitosis and then the nuclei took a little while to pair up finally as “back-to-back” nuclei in the centre of the cell. (C) Second cycle characteristics. n>250 for each time point. The following phenotypes are indicated: ■, 4 nuclei all SPBs separate; ○, cells containing one or two off centre paired SPBs; ▲, cells containing three SPBs in one cluster and one free; ●, cells containing all four SPBs clustered together in the centre of the cell. As the frequency of the “four SPBs separate” and “cells containing one or two pairs” rose almost immediately after the second mitotic peak to simultaneously peak around 210 minutes, these two classes predominate immediately after the second mitosis and were transitory rather than terminal phenotypes. The terminal phenotype following the second mitosis appeared to be four SPBs all clustered in the cell centre as its peak was later and higher. Whilst the “three SPBs clustered and one free” and “all four nuclei centrally located” phenotypic classes rose and fell with similar kinetics, the peak of cells with paired nuclei (●) declines more gradually than it rises. n>290 for each time point (for t=210, n=664). (D) The addition of hydroxyurea shows the inability of some cells to reposition their nuclei centrally. The culture in A was split into two and HU was added to a final concentration of 10 mM at t=150 minutes to provide the data for D. The symbols are the same as in C with the exception that those for ‘none clustered’ and paired are exchanged. The data show a much greater accumulation of cells with four centrally located nuclei, but more significantly, the frequency of cells with pairs of nuclei does not decline to zero, rather, it appears to decay slowly suggesting that not all cells go on to locate their nuclei centrally. Cells were grown to mid-log phase in YES medium before selection of small G2-phase cells on lactose gradients and washing in and inoculation into fresh YES at 36°C at the beginning of the experiment. n>330 for each time point.

Fig. 10. Nuclei cluster in either two or three groups after the third mitosis at the restrictive temperature in cdc7.A20 cells. Two representative fields of cells from the culture used in Fig. 9 after 390 minutes at the restrictive temperature. The representation of the signals as false colour images is the same as that for Fig. 8. (A-C) One cell with nuclei clustered in two groups and another in three, whilst both cells in D-F have two clusters. As in Fig. 8, the impression given by the relative location of the SPB and chromatin signals is that the SPBs are actively involved in the generation and maintenance of the clusters. The relative frequency of the cells were scored as to whether there was no obvious grouping of the interphase nuclei, they were found in two separate clusters or they were grouped in three distinct clusters. At 360 minutes; these frequencies were 32:53:15; respectively, while at 390 minutes the relative ratios were 22:55:23.
alter cellular environments to test hypotheses. Here we have exploited mutations in a number of cell division cycle genes in order to reveal the otherwise cryptic role of the SPB in positioning the interphase nucleus to different locations at different cell cycle stages. The SPB uses microtubule tracks to position the interphase nucleus.

Our initial observations in wild-type cells and those dividing at an increased size show that the nucleus is preceded in its movements around the cell by the SPB. We conclude from experiments in which nuclear positioning is disrupted following treatment with microtubule depolymerising drugs that the SPB is migrating along microtubule tracks. This conclusion is consistent with defects in nuclear positioning arising from overproduction of an SPB component and mutation of the ab2+ gene (Hagan and Yanagida, 1995; Verde et al., 1995; Yaffe et al., 1996). Because the centromeres are always associated with the interphase SPB (Funabiki et al., 1993), the genome follows the SPB in its migrations and thus nuclear positioning is achieved.

SPB-mediated movement could be achieved either via motors, or via static non-motor attachment to moving microtubules (Morris et al., 1995). Support for the existence of SPB-bound motor proteins comes from the localisation of known motor proteins to, or near to, SPBs in both budding and fission yeasts (Page et al., 1994; Yeh et al., 1995; Hagan and Yanagida, 1992). Their presence is also strongly suggested by the SPB-led nuclear migration in fission yeast during karyogamy and meiotic prophase (Chikashige et al., 1994).

The nuclear profiles after the second mitosis in septation mutants (Fig. 8D) suggest that the SPBs initially move towards two non-central points even though their eventual destination is the cell centre. This seems to be the case even if they started out close to the cell centre immediately after division and...
suggests active movement to these two points. If one assumes that all of the single microtubules of the early PAA (Horio et al., 1981) extend from the cell equator with the same polarity, then activating a unidirectional motor would result in simultaneous migration of all four nuclei either towards, or away from, the MTOCs of the PAA. As this is not observed, the SPBs must be capable of moving in either direction along the microtubules of the PAA.

There are two ways in which the SPB might use microtubule motors to generate such movement. Firstly, the SPB could possess both plus-end and minus-end directed motors or a single motor with regulatable directionality of motility (Euteneuer et al., 1988). A second, less attractive, possibility is that the SPB may contain only one unidirectional motor. Such an SPB could still move towards and away from the cell centre but only if the PAA consisted of anti-parallel microtubule bundles, which is not consistent with the one electron micrograph of the PAA published to date (Horio et al., 1981).

It is also possible that there are no motor proteins at the SPB and that the SPB is just a tethering point to attach the nucleus to moving microtubules which are being pulled around the cell by either plasma membrane-bound motors, or motors generating microtubule/microtubule sliding within the cytoplasmic bundles (Plamann et al., 1994; Morris et al., 1995). In this case, the SPB must be able to attach and detach depending on where it is relative to a positioning signal. In other words, it must be able to attach to the moving microtubule if the nucleus is in the wrong place, and detach when correctly positioned. It might not be necessary (or desirable) for the SPB to release completely from the microtubule, however, if the attachment was via a regulatable ratchet it would allow alternating tight binding, when the SPB would be pulled along by the MT, with loose binding, where the microtubule slides past the SPB whilst maintaining long-term attachment. As ratcheting has been described for dynein (Vale et al., 1989), this model can be viewed as a variation on the SPB-bound motor model.

Of these two possibilities, bi-directional, motor-mediated SPB motility seems most likely to account for the tight SPB clustering that we report in fission yeast septation mutants.

**Increasing the number of SPBs in a cell reveals cryptic controls**

One of our key observations demonstrating the fidelity of SPB-mediated nuclear positioning was the finding that multiple SPBs on one nucleus, or single SPBs on different nuclei within a single cell aggregate at the same point/s. This showed that the different SPBs responded to common signals.

Immediately following the second mitosis in septation mutants, the signals for nuclear localisation ensure that the four nuclei were positioned in two regions roughly a third of the way from either tip (Fig. 13). Later, all four nuclei moved to the centre of the cell. This suggests that instead of being an irreversible commitment to keep the two points defined as the cell mid-points prior to cytokinesis, this signal is turned off and a subsequent signal tuned on to define a single central point as the cell cycle progresses. These data strongly suggest that the similar positioning of nuclei in wild-type cells does not arise from a default maintenance of the two post-mitotic positions after cytokinesis but that there is a switch to a central location at some point in early G2 phase of the cell cycle after cytokinesis is completed.

**Microtubules, SPBs, the PAA and nuclear positioning in fission yeast**

The temporal coupling of nuclear division and cytokinesis means that it is important to establish the correct nuclear positioning quickly after mitosis to avoid fatal random cleavage of the nucleus by the septum (the “cut” phenotype; Hirano et al., 1986), or the generation of binucleate and anucleate cells. Thus it may be significant that two MTOCs are specifically activated at the end of nuclear division in mitosis but not in meiosis. Analysis of the cell cycle mutant wee1.50 has established that PAA formation is not required for cytokinesis (Hagan and Hyams, 1988) indicating that the difference in the microtubule organisation after mitotic and meiotic divisions must be for some other function, perhaps nuclear positioning. Nuclear positioning in the mitotic cycle is not as important as in the mitotic cycle because the positional requirements following meiosis are that the nucleus is encapsulated within the spore wall rather than positioned to avoid cleavage by a septum. Spore encapsulation is guaranteed by the initiation of the spore wall from the outer face of the SPB during metaphase of meiosis II (Hirata and Tanaka, 1982), thus the nucleus itself contains the inherent information to ensure packaging into a spore; nuclear position within meiotic cells is therefore largely irrelevant.

It is clear both from analysis of meiosis and the regrowth of microtubules after reversible depolymerisation by cold in the mitotic cell cycle (our unpublished observations and cited

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**Fig. 13.** A cartoon summarising nuclear positioning in cytokinesis mutants. The SPBs are represented as dots on the nuclei. The temporal order of the images is from left to right. The two arrows indicate the location of the two proposed nuclear positioning signals which occur sequentially following the end of mitosis. This signal pattern is highly reminiscent of that which must occur in wild-type cells.
by Verde et al., 1995) that an apparently normal interphase cytoskeleton can be re-established by virtually random microtubule nucleation throughout the cytoplasm. The function of the PAA may therefore be to increase the fidelity of re-establishing a cytoskeleton throughout the cell and so aiding rapid nuclear positioning and hence survival in the mitotic cell cycle. The microtubules of the PAA may scan the cytoplasm to find SPBs in a way similar to the scanning of the nucleoplasm for kinetochores by the microtubules of the prometaphase centrosome (Kirschner and Mitchison, 1986).

The coincidence of the activation of microtubule nucleation of both the PAA and the cytoplasmic face of the SPB directly after mitosis maximally enhances the fidelity of nuclear positioning at the end of anaphase B. Following the breakdown of the mitotic spindle, the SPB needs to interact with PAA microtubules to migrate to its correct location, but it is on the wrong side of the nucleus. It is therefore faced with a problem similar to that faced for microtubule capture by kinetochores (Nicklas and Ward, 1994): the bulk of the nucleus will sterically block PAA microtubules from reaching the SPB. The microtubules polymerised from the SPB at the end of mitosis extend beyond the chromatin and can thus interact with microtubules extending from the PAA. If we assume that such contacts enable microtubule/microtubule sliding, the SPB would be dragged, or pushed round to face the PAA enabling further or stronger interactions of PAA microtubules directly with the SPB.

A requirement for rapid and accurate nuclear positioning directly after mitosis is not so obvious under logarithmic growth in liquid culture where the cell is sufficiently large that the nuclei could wander a considerable distance from the middle of the daughter cells without producing a “cut” or binucleate cell. In contrast, nutrient limitation, which is often encountered in the centre of a colony and in the natural environment, leads to considerable reduction in cell size (Nasmith, 1979) and thus a significant increase in the likelihood of cleavage of either nucleus by the septum if nuclear positioning immediately following mitosis is not ensured.

The possible nature of the nuclear positioning signal

The nature of the signal responsible for directing SPB movement remains obscure and awaits the identification of mutants defective in nuclear positioning but it is likely to be generated in response to morphogenic or ion gradients (Harold, 1990; Nurse, 1994). The fact that overexpression of a fission yeast protein phosphatase 1B homologue results in aberrant SPB positioning suggests that protein phosphorylation cycles are involved (Yoshida et al., 1994). The continual rocking motion of nuclei in time-lapse sequences of mitotic cells suggests that the SPB may use such cycles to constantly assess its position (Hagan et al., 1990; Hagan, 1988).

A clue as to the identity of the positioning signal may come from studies of the \textit{cdc11}\textsuperscript{+} gene because, in the \textit{cdc11.136} mutant, the nuclei do not always cluster centrally (Hagan and Hyams, 1988). In contrast, nuclear clustering has been seen in all other septum mutants studied to date: \textit{cdc7}, \textit{cdc14}, \textit{plo1} and \textit{skp1} (Hagan and Hyams, 1988; Ohkura et al., 1995; Pylte et al., 1996). This difference may be significant especially since the MTOCs of the PAA in \textit{cdc11.136} mutants are similarly unrestricted to the central zone.

Implications for nuclear positioning in fungi in general

There is a striking similarity between the distribution of clusters of nuclei in the \textit{cdc7} cells containing eight nuclei and the regular distribution of clusters of groups of nuclei along the hyphae of the \textit{nuDF7} \textit{Aspergillus nidulans} mutant grown at the permissive temperature. This may suggest that similar underlying nuclear positioning mechanisms may underlie nuclear positioning in unicellular and coenocytic fungi (Woklow et al., 1996). Indeed, it has long been suggested, based on correlative phase-contrast and electron microscopic images, that the SPB is intimately involved in nuclear migration. The resistance of \textit{Nectaria haematococa} interphase nuclei to optical tweezers-generated pulling forces has been interpreted as indicating positional anchoring by the SPB (Berns et al., 1992). Thus SPB-mediated positioning of nuclei is an attractive mechanism not only for bulk nuclear migration but also for determining the accurate nuclear positioning in fungi in general. Given the sensitivity of the clustering assay in fission yeast and the organism’s established genetics, the way is open for genetic analysis of this complex phenomenon.

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