Spindle Pole Body Duplication in Fission Yeast Occurs at the G1/S Boundary but Maturation Is Blocked until Exit from S by an Event Downstream of Cdc10+ 

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The regulation and timing of spindle pole body (SPB) duplication and maturation in fission yeast was examined by transmission electron microscopy. When cells are arrested at G1 by nitrogen starvation, the SPB is unduplicated. On release from G1, the SPBs were duplicated after 1–2 h. In cells arrested at S by hydroxymethylurea, SPBs are duplicated but not mature. In G1 arrest/release experiments with cdc23.33 cells at the restrictive temperature, SPBs remained single, whereas in cells at the permissive temperature, SPBs were duplicated. In cdc10 mutant cells, the SPBs seem not only to be duplicated but also to undergo partial maturation, including invagination of the nuclear envelope underneath the SPB. There may be an S-phase–specific inhibitor of SPB maturation whose expression is under control of cdc10+. This model was examined by induction of overreplication of the genome by overexpression of rpm1 or cdc18p. In cdc18p-overexpressing cells, the SPBs are duplicated but not mature, suggesting that cdc18p is one component of this feedback mechanism. In contrast, cells overexpressing rpm1 have large, deformed SPBs accompanied by other features of maturation and duplication. We propose a feedback mechanism for maturation of the SPB that is coupled with exit from S to trigger morphological changes.

INTRODUCTION

The regulation and timing of spindle pole body (SPB) duplication and maturation in fission yeast was examined by transmission electron microscopy. When cells are arrested at G1 by nitrogen starvation, the SPB is unduplicated. On release from G1, the SPBs were duplicated after 1–2 h. In cells arrested at S by hydroxymethylurea, SPBs are duplicated but not mature. In G1 arrest/release experiments with cdc23.33 cells at the restrictive temperature, SPBs remained single, whereas in cells at the permissive temperature, SPBs were duplicated. In cdc10 mutant cells, the SPBs seem not only to be duplicated but also to undergo partial maturation, including invagination of the nuclear envelope underneath the SPB. There may be an S-phase–specific inhibitor of SPB maturation whose expression is under control of cdc10+. This model was examined by induction of overreplication of the genome by overexpression of rpm1 or cdc18p. In cdc18p-overexpressing cells, the SPBs are duplicated but not mature, suggesting that cdc18p is one component of this feedback mechanism. In contrast, cells overexpressing rpm1 have large, deformed SPBs accompanied by other features of maturation and duplication. We propose a feedback mechanism for maturation of the SPB that is coupled with exit from S to trigger morphological changes.

The reported cycle of SPB duplication and maturation in the fission yeast Schizosaccharomyces pombe differs from that in budding yeast and other organisms. The fission yeast SPB, a laminar body, spends most of interphase in the cytoplasm adjacent to the nuclear envelope. After the formation of a half bridge a second laminar body forms adjacent to the first. As the SPB matures, osmophilic material accumulates in a pocket in the nuclear envelope that forms as the nuclear envelope invaginates. Subsequently, as the cell enters mitosis the two laminar bodies separate as the mitotic spindle forms, giving rise to a bipolar spindle (Ding et al., 1997; Tanaka et al., 2000). The timing of SPB duplication in the fission yeast cell cycle is controversial. Although the latest reports place duplication, maturation, and separation at late G2 (Ding et al., 1997), earlier reports suggest that duplication occurs upon entry into mitosis (McCully and Robinow, 1971) or anaphase (Kanbe et al., 1990) or that it is independent of the DNA replication cycle (King et al., 1982).

We wanted to examine the timing of SPB duplication in fission yeast because it is the only organism that is reported to initiate centrosome duplication at a point in the cell cycle other than G1/S. Although we have shown in permeabilized cells that the SPB becomes competent to nucleate microtubules at G2/M (Masuda et al., 1992), it is possible that other changes in SPB structure and function, including SPB duplication, occur earlier in the cell cycle. For example, the fission yeast SPB components, alp4p and alp6p, which are homologous to the β-tubulin binding proteins, Sc. Spc97/h.GCP2 and Sc. Spc98/h.GCP3, respectively, have an essential role that is required earlier in the cell cycle than M, i.e., during G1 (Vardy and Toda, 2000). Their essential function may be associated with a step in the duplication of the SPB. Different steps in duplication and maturation of the Drosophila centrosome happen at different stages in the cell cycle (Vidwans et al., 1999). A similar cell cycle-dependent separation of SPB...
duplication and maturation may occur during the fission yeast cell cycle. To accurately place the events of fission yeast duplication and maturation in the cell cycle, we have monitored changes in SPB morphology in high-pressure fast frozen, freeze-substituted cells followed either by serial thin section analysis or tomography. Various cell cycle arrest and release techniques such as nitrogen starvation/release and hydroxyurea treatment allowed us to determine whether SPB duplication is associated with crossing the G1/S boundary. The large collection of cell cycle arrest mutants gave us further insight into the cell cycle-dependent control of SPB duplication and maturation. We find that duplication of the SPB occurs at the G1/S boundary and that maturation of the SPB occurs later in the cell cycle and requires exit from S.

MATERIALS AND METHODS

**Yeast Strains and Media**

The cells were cultured in rich medium (YES) for drug treatment and for temperature arrest of ts mutants. For G1 arrest, the cells were cultured in minimal medium (PM) and then starved for nitrogen and carbon sources with PM-ND (PM without ammonium chloride or dextrose) (Horie et al., 1998). The wild-type strain used was 972LΔ). For G1 arrest and release by nitrogen starvation experiments, autotroph strains were used. Cells were cultured at 25°C unless otherwise stated. Temperature-sensitive strains cdc2-33 (h–) and cdc10-50 (h+) were a kind gift of Paul Nurse (Imperial Cancer Research Fund, London, United Kingdom). The integrant strains of cdc2-33 and nmt1+ genes under nmt1+ promoter also were obtained from Paul Nurse. The strain FY1166 h/ncdc1::[ncdc1HA::LEU2]1 ura1::[DC1::LEU2]3 ade2::HIS3 used in the in situ mcm5p binding assay was a kind gift of Susan Forbush (University of Southern California).

**Electron Microscopy**

The cells were high-pressure frozen in a Bal-tec high-pressure freezer as described previously (Ding et al., 1997). The frozen cells were substituted in acetone with 2% osmium oxide and 0.1% uranyl acetate for 3 d at –80°C and then warmed to 20°C at 10°C/h. The cells were embedded in Epon 812 resin sectioned at a thickness of ~40–50 nm, and poststained in lead citrate and uranyl acetate. The pictures of SPBs were taken with either a JEOL 100CX transmission electron microscope at 80 keV, a JEOL 1200EX at 100 keV, or a Philips TECNAI 12 electron microscope at 100 keV or a Philips EM430 automated data correction system at 300 keV. The system is fully equipped with a Gatan 676 cooled slow-scan charge-coupled device camera with 2 μm/pixel at a magnification of 21,200 (1.68 nm/pixel) with a tilting angle ranging from –70° to +70° at 1.25° intervals except for a few samples for which a high tilting angle >60° was not available in one tilting direction. Each data set was processed for reconstruction by using the Priism software. The reconstructed images were displayed, analyzed, and modeled with Priism or DeltaVision software (Chen et al., 1996).

**RESULTS**

Serial Section EM Analysis Is Required to Determine Whether a SPB Is Duplicated

Our criteria for identifying SPBs at different stages of duplication were based on changes in SPB morphology and required a complete set of serial sections through the SPB to distinguish unduplicated from duplicated SPB. As shown in Figure 1, A–C, unduplicated SPBs are a single laminated body with a small well-stained appendage. This appendage was named the half bridge by Ding et al. (1997), because of the structural resemblance to the half bridges found in unduplicated SPBs of Saccharomyces cerevisiae. After duplication, the SPB is two laminated bodies interconnected by a densely stained pyramid shaped bridge (Figure 1, D–F). However, this structure does not show the morphological changes associated with maturation. During maturation, the nuclear envelope becomes invaginated, and dark staining material accumulates in the pocket that forms beneath the duplicated SPB (see Figure 5 for an example of a maturing SPB). Subsequently, the two daughter SPBs are separated by microtubules as a bipolar mitotic spindle is formed between them (Ding et al., 1997). For clarity of analysis, we have divided SPB maturation into two stages, e.g., early and late maturation. Early maturation is marked by growth in size of the lamellae bodies, invagination of the nuclear envelope, and accumulation of darkly stained material between the nuclear envelope and the SPB, whereas late maturation is marked by the physical separation of the two daughter SPBs and formation of the mitotic spindle.
Because of the contradictory literature and the difficulty in analyzing SPB morphology as it duplicates and matures, a large data set consisting of >120 complete sets of serial sections containing SPBs were obtained and analyzed for this study. When the lamellae were not clearly visible in the sections, the sections were examined with various tilting angles, and images were obtained at the best tilting angle for visualization of the lamellae. We examined complete serial sections of SPBs running from two sections outside of the boundary of the SPB and scored the SPBs as single or duplicated only when the complete sets of sections were recorded on film. Any ambiguous SPBs, including putative duplication intermediates, were scored as duplicated. In addition, three tomographic reconstructions of SPBs at different stages of duplication were made. As shown in Figure 1, the images of an unduplicated (section B) and a duplicated but immature (section D) SPB can seem similar when comparing two single sections, but they can be distinguished from each other in serial section analysis.

The SPB Is Duplicated in Cells Arrested at S Phase by HU

Although Ding et al. (1997) concluded that the SPB undergoes duplication and maturation at the G2/M boundary, other accounts in the literature (Vardy and Toda, 2000; Garcia et al., 2001) and our own observations of SPB morphology in log phase culture (S.U., unpublished data) suggest that SPB duplication was initiated at G1-S phase. To further study the timing of SPB duplication, we examined the morphology of SPBs in cells arrested at various stages of the cell cycle around the G1-to-S phase transition. First, we examined the SPB morphology in cells arrested at S phase by hydroxyurea. HU is an inhibitor of ribonucleotide reductase (RNR) and delays the progression of S phase by depleting the dNTP pool required for efficient DNA synthesis (Kim

Figure 1. Serial sections through two SPBs comparing the structure of an unduplicated (A–C) versus a duplicated but immature (D–F) SPB. In A–C, the nitrogen-starved cell is arrested in early G1. The SPB in this cell consists of a single laminar structure (L) and a half bridge (HBr), which lies adjacent to an intact NE. In D–F, the cdc10-arrested cell at the nonpermissive temperature is arrested at the G1/S boundary. The SPB is duplicated and has two laminar structures separated by a dark staining ellipsoid bridge (Br). In both cells, the nuclear envelope is continuous and unfenestrated and shows no signs of invagination, although dark material has accumulated on the nuclear but not the cytoplasmic face of the nuclear envelope adjacent to the SPB. Several microtubules (MT) are in proximity to the cytoplasmic face of the SPB in each cell, accompanied by mitochondria. A nuclear pore (NP) is always found near the SPB. Note the image of the unduplicated SPB in section B superficially resembles the image of the duplicated but immature SPB in section D. The size of each linear structure is similar (ranging from 70 to 90 nm) in unduplicated and duplicated SPBs. Bar, 100 nm.
The nuclear membrane had no indication of early maturation events, such as fenestration or invagination adjacent to the SPB. A bundle of cytoplasmic microtubules running parallel to the longitudinal axis of the cells were found at the cytoplasmic side of SPBs. Four to seven microtubules were usually found within the bundle; a number that matches our live data analysis for cytoplasmic microtubule behavior (Sagolla et al., 2003). Mitochondria were always associated with the microtubule bundles near the SPB and extended most of the length of the cytoplasmic bundle. This observation is consistent with genetic studies that demonstrate that microtubules are involved in mitochondrial partitioning to daughter cells during cell division (Yaffe et al., 1996). The three cells that had duplicated SPBs may be the result of an incomplete arrest by nitrogen starvation as suggested by flow cytometry (Figure 3B). This observation clearly demonstrates that the SPB is not duplicated at an early G1 arrest point.

**SPB Duplicates upon Release from Nitrogen Starvation**

To determine whether SPBs are duplicated at a stage in the cell cycle that falls between arrest by nitrogen starvation and HU arrest, we investigated the timing of SPB duplication by releasing cells from nitrogen starvation and fixing the cells shortly after release. The method of starvation and release is shown in the flow diagram in Figure 3A (Horie et al., 1998). One to 1.5 h after the addition of a nitrogen source, when cells are at the onset of S phase or in early S phase, as shown by flow cytometry (Figure 4B), the cells were fixed and the SPB morphology was examined. Where possible, only cells that had no septa were examined. The flow cytometry data suggest that these cells at 25°C have not completed DNA replication. Of the eight cells examined, five cells had duplicated SPBs (63%) in contrast to the 81% that had single SPBs in nitrogen-starved G1-arrested cells (Figure 1, A–C). Two of the three cells scored as having unduplicated SPBs contained an unknown structure associated with the half bridge, which may be a duplication intermediate (our unpublished data). None showed any signs of maturation as defined previously. Nor had any of the cells entered mitosis. This result, together with the results of the HU arrest experiment, strongly suggests that the duplication of the SPB occurs at the G1/S boundary instead of at the G2/M boundary.

**The Duplication of SPB Is Dependent on cdc2p Kinase at the G1/S Boundary**

To investigate the role of cdc2p kinase during SPB duplication, a nitrogen starvation arrest/release experiment was carried out with a temperature-sensitive allele of cdc2p gene. Wild-type cdc2p kinase activity is required both for entrance into S and into M. Normally, if a log phase population of cdc2p33 cells were shifted to the nonpermissive temperature, almost all cells arrest at the G2/M boundary because most cells in a log phase population are in G2 (King and Hyams, 1982). In this experiment, cells were arrested at G1 by nitrogen starvation and then released from arrest by addition of the nitrogen source. The population of nitrogen-starved, G1-arrested cdc2p33 cells was divided into two cultures, and one-half was shifted up to 35°C, the nonpermissive temperature (Figure 4A, diagram). Ten minutes later, the G1 arrest was released by addition of a prewarmed nitrogen source to both cultures. The cells were fixed 1–1.5 h after the addition of the nitrogen source (Figure 4B, bar), as in the nitrogen arrest/release experiment. At the nonpermissive temperature, 83% (10/12) of cells had a single, unduplicated SPB. In contrast, 13% of the cells (2/16 cells) at the permissive temperature (25°C) had unduplicated SPBs. These results clearly demonstrate that the duplication of the SPB occurs at the

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and Huberman, 2001). Figure 2, A and B, shows SPBs from wild-type cells treated with HU for 3.5 h at 25°C. All 11 SPBs scored were duplicated according to our criteria. It is striking that the most of the SPBs found in HU-treated cells are uniform in shape and length as shown in Figure 2. SPBs contained a pair of lamellar bodies; one was smaller and at an angle relative to the nuclear envelope, and the other larger and slightly more extended. They are interconnected by a pyramid-shaped bridge that is on top of a continuous nuclear envelope. The nuclear envelope beneath the SPB was straight and closely oppressed to the SPB. Electron-dense material was found in the nucleus adjacent to the dense material was found in the nucleus adjacent to the centromere cluster is located (Tanaka and Kanbe, 1986; Nishimoto et al., 1992; Uzawa and Yanagida, 1992; Funabiki et al., 1993; Kniola et al., 2001). Because all SPB scored had a duplicated SPB, we conclude that the SPB duplication is completed before or during S phase. Moreover, because 100% of the cells have duplicated SPBs, it is not possible that SPB duplication in HU-arrested cells is actually a later cell cycle event unaffected by HU treatment because the cell population is variable in the length of time spent arrested in S. If the HU-treated cells had progressed further in the cell cycle with respect to SPB duplication, then only a fraction of the cells would have had duplicated SPBs.

As shown by flow cytometry (Figure 2C), cells fixed after 3.5 h in HU for electron microscopy were arrested in early S phase with unreplicated DNA. At the beginning of S phase, the mcm4p/mcm6p complex is bound to chromatin and is then displaced from chromatin as DNA replication occurs and becomes soluble in the nucleus (Kearsey et al., 2000). As shown in Figure 2D by using an in situ binding assay for chromatin bound mcm4p, HA-tagged mcm4p is retained in the nucleus after high-detergent treatment in the HU-treated cells. This result, consistent with that shown previously by Kearsey et al. (2000), confirms that SPB duplication can occur in cells blocked in DNA replication during early S phase. By way of contrast in the wild-type, non-HU-treated population where most cells are in G2, the only cells observed that have retained mcm4p after detergent treatment are binucleate cells undergoing septation that are at the G1/S boundary.

**The SPB Is Not Duplicated in Cells Arrested at G1 Phase by Nitrogen Starvation**

To further study the timing of SPB duplication, we examined the morphology of SPBs in cells arrested at various stages of the cell cycle around the G1/S boundary. G1 arrest by nitrogen starvation is before cdc2 arrest and clearly is preStart. For an unknown reason, the conventional method for G1 arrest (nitrogen starvation for 16 h or more) yielded very poor quality specimens for EM. An alternative protocol developed by Shimoda (Horie et al., 1998) yielded cells that had well preserved ultrastructure. By this method, cells in log phase are arrested at G2 by starving with both nitrogen and carbon sources (Figure 3A). On addition of a carbon source, the cells undergo two rapid successive cell divisions and then arrest at G1. Although many cells in this population are multiply septated and daughters have not separated (Figure 3B), only cells lacking a septum were used for EM. Flow cytometry of the arrested population combined together with a septation index suggested that >66% of the cells were arrested in G1 by this nitrogen starvation protocol (Figure 3B). A single laminated structure with two layers and a half bridge (Figure 1, A–C) was found in most of the cells examined (81%; 13/16), demonstrating that the SPB is not duplicated in cells arrested at G1. The nuclear membrane running under the SPB seems to be straight and continuous.
G1/S boundary in fission yeast and is downstream of G1/S cdc2p kinase activity. As is shown by the FACS analysis of the cdc2.33 populations of cells (Figure 4B), at 25°C the cells progressed through the cell cycle with kinetics similar to wild-type cells at the same temperature, whereas at 35°C after being released from nitrogen starvation the cdc2.33 cells remain arrested at the G1/S boundary with unreplicated DNA.

The SPB Is Duplicated and Undergoes Early Maturation in cdc10-arrested Cells

To further characterize regulation of SPB duplication timing, we investigated the morphology of SPBs in G1-arrested cells with the cdc10.50 ts- mutation, which arrested at a step in the cell cycle downstream from cdc2p kinase but earlier than HU arrest. cdc10p is a component of (MLIL binding factor transcription complex, which regulates the transcription of genes required for S phase, including the large subunit of RNR and cdc18p (Tanaka and Okayama, 2000). cdc10p activity is regulated through phosphorylation and is a downstream target of cdc2p/G1 cyclin complex. The execution point of the cdc10+ gene is commonly used to define Start in the fission yeast cell cycle (Nurse and Bissett, 1981).

As shown by flow cytometry, cdc10 cells after 3 h at the nonpermissive temperature are arrested with a 1C DNA content (Figure 5B). mcm4p is not retained in the nucleus in permeabilized cells at the nonpermissive temperature (Figure 2D), presumably because it cannot be loaded onto chromosomes in the absence of cdc18p expression (Kearsey et al., 2000). Ogawa et al. (1999) have previously demonstrated that the mcm4p/mcm6p complex is not bound to chromosomes in the absence of cdc10p. Cells were fixed for EM after 3.5 h at the nonpermissive temperature, at a time when all cells remain arrested at the G1/S boundary with 1C DNA content. The SPBs were found to be duplicated in cdc10-arrested cells as shown in Figure 1, D–F and Figure 5, A–L, and the Supplemental Movie. Two laminated bodies were found in 97% (26/27 cells) of the SPBs examined, demonstrating that they have already undergone duplication. This result shows that SPB duplication has happened before the initiation of S phase. The SPB scored as “single” in the cdc10-arrested culture had an appendage at the tip of half bridge, indicating that it is actually a duplication intermediate. Although to be consistent we scored this SPB as single, this unusual mor-

![Figure 2](image-url)
Asynchronous Culture
85% G2, 15% M
Starvation with Carbon/Nitrogen source (2hr)
100% G2
Addition of Carbon Source (6hr)
2 Mitosis → arrest at G1
(81% Single SPBs)
Addition of Nitrogen Source (1~2hr)
Initiation of DNA Replication
(37% Single SPBs)

Figure 3. (A) Flow diagram shows the method used for G1 arrest in this study (Horie et al., 1998). The cells cultured in rich medium were transferred to a synthetic medium lacking both carbon source and nitrogen source (PM-ND). The cells were cultured for 2 h to allow them to arrest in G2 phase followed by addition of carbon source (2% dextrose final). After two rapid sequential divisions, cells arrest at G1. For arrest/release experiments, nitrogen source (ammonium chloride, 1% final) was added back. The percentage of unduplicated versus duplicated SPBs is shown in bold in parentheses. (B) Schematic DNA histogram of cells arrested in G1 as determined by flow cytometry. The square represents the cells where the cell cycle phase is indicated. The highest peak represents cells with 2C DNA content, including G1 cells with incomplete separation after septation, and G2 cells. The percentage of cells that have not separated is 33%. Therefore, 66% of the cells were in G1. The lesser, left-hand peak (33%) is the cells that have 1C DNA content and have undergone septation and separation. The phase micrographs show three cells from the 2C peak; the top cell has 2C DNA, and the middle and bottom binucleate cells have 1C nuclear DNA content. The lower two cells have formed septa (arrow), and the bottom cell shows partial separation of the two daughters. The bar to the right of the histograms indicates times when cells were fixed for electron microscopy.

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already have initiated maturation during the induction period for cdc18p-OE. If the frequency of SPBs with early stage maturation in the cdc18p-OE population (10%) reflects the execution point for this process, then early stage maturation must be initiated very close to the boundary of the G2/M transition. This estimate is consistent with the previous observation that the maturation does not start until the later stages of G2 (Ding et al., 1997). All of the SPBs observed in cdc18p-OE cells seemed normal in terms of size and morphology, in contrast to the SPBs found in rum1p-OE cells (described below). The observation that most of the SPBs show no signs of maturation demonstrates that the block of early maturation during S phase is dependent on either the cdc18p or an S-phase event such as formation of replication forks.

**Duplication and Early Maturation of SPB Can Occur without Preceding M Phase**

We further investigated the requirement of previous cell cycle events for the initiation of SPB duplication. rum1p is an inhibitor of the mitotic form of cdc2p kinase and prevents mitosis from occurring prematurely. Overexpression of rum1p directs cells to go through G1, S, and G2 and reenter G1 before Start without going through M, and as a result cells end up with a higher DNA content (Figure 6A, diagram). As shown in Figure 6B, these cells have a 4C or greater DNA content and are abnormally long because mitosis and cytokinesis has not occurred. In cells overexpressing rum1p, overreplication of the genome is dependent on cdc2p kinase and the G1 cyclins pcl1p, cig1p, and partially cig2p and requires activation of the res2p–cdc10p complex. (Fisher and Nurse, 1995; Martín-Castellanos and Moreno, 1996; Tanaka and Okayama, 2000). Rum1p-OE affects pre-Start events, whereas cdc18p-OE affects post-Start events but only with rum1p-OE does the cell actually leave S and enter G2. Analysis of SPB maturation in cells overexpressing rum1p allowed us to determine whether maturation requires a cdc10p-dependent passage through Start and exit from S.

The SPBs in cells (13 cells examined) overexpressing rum1p having gone through the equivalent of two or more S phases (Figure 6B) and have a morphology consistent not only with duplication but also with multiple early maturations (Figure 8). In the example shown in Figure 8, A–D, two pairs of laminated structures, one member of the pair more than twofold larger than the other in length, can be distinguished in the serial sections. The consistent size difference between the two lamellae bodies in each pair, observed in all cells examined, may reflect the difference between new and old SPBs as described by Grillert et al. (2004). However, SPB maturation, as determined by extent of membrane invagination and deposition of darkly staining material, was more variable in the cell population. For example, in a second cell (Figure 8, E–I), although the SPB has a similar morphology to that observed in Figure 8, A–D, several invaginations in the nuclear envelope are present beneath this complex structure. However, the morphology is also consistent with two rounds of duplication of the laminated structure, and several early maturations, because the laminated structures have not physically moved far apart, and the nuclear envelope (NE) seems to be intact. As shown by examining serial sections (our unpublished data), the lamellar bodies are discrete structures and the laminations in the two structures are at different angles with respect to each other. These results demonstrate that duplication of SPB and partial maturation of SPBs can occur even in the absence of a previous M phase, provided that cells enter S phase in a cdc2-dependent manner and leave S (Figure 6A, diagram). Also, these observations demonstrate that SPB duplication can occur in the absence of late-stage maturation or separation of the SPBs by mitosis.

**DISCUSSION**

In most organisms investigated to date, centrosomes are duplicated during S phase in parallel with the replication of the genomic DNA, and the process is initiated near the G1/S...
boundary. However, it has previously been reported that in fission yeast SPB duplication occurs at the G2/M boundary (Ding et al., 1997). We have investigated the cell cycle-dependent regulation and timing of SPB duplication in fission yeast by monitoring the morphology of SPBs in cells during cell cycle arrest/release experiments and summarize our results in Figure 9. We show that duplication of the SPB is initiated near the G1/S boundary and is under the regulation of cdc2p kinase but is independent of cdc10+. SPB maturation occurs in two phases; early maturation leading to NE invagination and deposition of material under the SPB requires exit from S, whereas late maturation, leading to fenestration of the NE and SPB separation as the spindle forms requires entrance into M. Expression of cdc18p is sufficient for inhibition of early maturation and highly likely to be required for it, too. We suggest that in S. pombe cells there is a feedback inhibition mechanism downstream from cdc10+ that delays early SPB maturation until S phase is complete (Figure 9B, model). To test this model we analyzed SPB duplication in cells with prolonged or repeated S phases (Figure 6A). Provided that cells never left S, maturation was inhibited. However, cells that repeatedly left and reentered S, even if they never transited M, underwent both duplication and maturation, although maturation was not complete. Our observation suggests that once the two major events of S phase, DNA replication and SPB duplication, are triggered by cdc2p/G1 cyclin complexes, they are independent of each other. A feedback control of SPB maturation may serve as a mechanism to coordinate these two independent pathways and prevent accidental spindle formation during S phase, an event which leads to catastrophic cell death.

To interpret our studies properly, it is necessary to make a distinction between the unduplicated and duplicated SPB and the subsequent changes in morphology as it matures. As described previously (Ding et al., 1997), the morphology of the fission yeast SPB immediately after duplication consists of two laminated structures connected by an ellipsoid bridge. Because this structure sits on the outside of the NE it is difficult to distinguish it from unduplicated SPBs and may have been misidentified in cells grown in log phase (Ding et al., 1997; Kniola et al., 2001). To avoid this problem, in all experiments only complete serial sections running through the SPB were scored, and in several experiments tomo-graphic reconstructions were made to confirm our interpretation of the serial sectioned SPBs. Our sample size was also large—in all, 123 SPBs were reconstructed in this study.

Even though cdc10+ was originally used to define Start in S. pombe (Nurse and Bissett, 1981), as we show in our results, some events down stream from the G1/S transition occur in

Figure 5. (A) 3D tomographic reconstruction of a SPB in a cdc10-arrested cell. The 3D volume of the tomographic reconstruction is shown as consecutive two-dimensional projections (A–L). Each projection is 16 slices, which is the equivalent of an image of a serial section 27 nm in thickness. The SPB shows invagination of the NE, accumulation of dark material in the membrane pocket (D), microtubule (MT) in the cytoplasm associated with SPB, and expansion in overall size of the SPB (L, laminar structure). The invaginated nuclear envelope is continuous and is not fenestrated. Bar, 100 nm. A movie of the whole volume can be found in Supplemental Data. (B) Schematic DNA histograms of cells after switching cdc10 cells to the nonpermissive temperature (35°C) at time 0, as determined by flow cytometry. The first peak represents cells with 1C DNA content; the second peak cells with 2C DNA content. At zero time, almost all cells had 2C DNA content, but by 3 h almost all cells had 1C DNA content. Cells were fixed for electron microscopy after 3.5 h as indicated by the *.
cdc10-arrested cells. For this discussion, rather than defining Start as the cdc10 execution point, we will define Start as being under cdc2p kinase control and cdc10p as downstream from that event. This is in agreement with the definition of START in budding yeast and many other organisms (Nurse and Bissett, 1981).

cdc2p Kinase Control of SPB Duplication at the G1/S Boundary

Using nitrogen starvation to arrest cells in G1, and then releasing them from the block by using a ts− allele of cdc2p, we demonstrate that SPB duplication in S. pombe, like in S. cerevisiae and mammalian cells, is under control of cdc2p kinase and occurs at the G1/S boundary. It is likely that they are under a direct control and in a pathway independent of cdc10+ because duplication does not require cdc10+, which is immediately downstream of cdc2+ (Labib et al., 1995). There are three G1 cyclins in fission yeast, namely, cig1p, cig2p, and puc1p (Fisher and Nurse, 1995; Martín-Castellanos et al., 1996; Martín-Castellanos et al., 2000; Tanaka and Okayama, 2000). In addition to these characterized cyclins, there are other putative G1 cyclins in the genome database (S. pombe genome database, Sanger Institute, Cambridge, United Kingdom). One or more of these G1 CDK complexes may be responsible for controlling SPB duplication. Our conclusions placing the timing of duplication at the G1/S boundary are also supported by evidence for SPB duplication in HU-treated cells and cdc10 ts− cells at the nonpermissive temperature. After HU treatment, cells enter S but proceed very slowly if all to replicate their DNA. The cdc10 mutants cells never start DNA replication. Under both conditions, the SPBs are duplicated. These experiments show that SPB duplication has been uncoupled from DNA replication and is consistent with SPB duplication happening at the G1/S boundary. In mammalian cells, it has been demonstrated that centrosome duplication occurs at the G1/S boundary and is under control of cdk2/cyclin E complex, or in some cases cyclin A (Hinchcliffe et al., 1999; Lacey et al., 1999; Matsumoto et al., 1999; Meraldi et al., 1999). Similarly in S. cerevisiae, SPB duplication is regulated by Cdc28 and the three G1 cyclins Cln1, 2, and 3 (Haase et al., 2001). Thus, the timing of centrosome duplication at the G1/S boundary seems to be an evolutionary conserved event.

SPB Maturation Occurs in Two Phases, Probably under Different Cell Cycle Control

SPB maturation involves different events in fission yeast than in budding yeast and is not completed until the G2/M boundary. In budding yeast, SPB maturation, defined morphologically as SPB separation, is dependent on the S phase and M phase cyclins. Maturation is completed in S, and a small central spindle is present in S phase budding yeast cells (Haase et al., 2001). In fission yeast, there is no spindle formation in S-phase cells, and there must be a mechanism, either
Figure 8. SPBs in two cells after rum1p overexpression. In A–D (the sections are not a continuous series), the SPB has undergone duplication and maturation. The nuclear envelope has invaginated, and several dense masses of dark staining material (D) have accumulated in the pockets in the NE beneath the SPB. Only one of the two pairs of laminated structures (labeled L1 and L2) is displayed, and it is accompanied by two discrete invaginations in the nuclear envelope beneath the SPB. The small one is comparable in size to ones found in wild-type, partially matured SPB (measured 80 nm) and lies at an oblique angle relative to its large partner. The larger ones were 230 nm in length. In serial sections E–I, part of another SPB in serial section is shown. In this cell, the nuclear envelope invagination is larger and the whole SPB is within it. The SPB consists of four laminated structures; two pairs, one large and one small, each labeled as L1 through L4. Careful examination of serial sections revealed that these laminated structures are not continuous but are separate structures. Two large laminated structures were connected by a bridge (Br), which has also grown in size (180 nm) compared with the ones found in other duplicated SPBs (70 nm). Bar, 100 nm.
We suggest that late stage maturation is under a different cell cycle regulation than early phase maturation. In log growth phase cells, early and late phase maturation events seem to occur in rapid succession; early phase events were seldom observed in these cultures (Ding et al., 1997). However, in the cell cycle arrest/release experiments described here, maturation when observed was incomplete and seemed not to progress beyond the early stage. Using log phase cells, Ding et al., 1997 demonstrated that maturation occurred in G2. We concur and think that both stages of maturation occur after leaving S, but late stage maturation requires crossing the G2/M boundary for its completion. Because the cells used in our experiments were never allowed to cross the G2/M boundary, the conditions for late stage maturation were not met in our arrest/release experiments. In cells overexpressing rum1p, the cell in question cross the G1/S boundary, initiate DNA replication in a cdc10δ-dependent manner, exit into G2, and then reenter G1 without ever going through M (Moreno and Nurse, 1994; Nishitani and Nurse, 1995). rum1p-overexpressing cells show early stage maturation. In contrast, cdc18p-overexpressing cells bypass a requirement for cdc2p/G1 cyclins and res1p-cdc10p to initiate DNA replication and never leave S. Although cdc18p-overexpressing cells undergo SPB duplication, SPB maturation is not initiated. This is consistent with a model that exit from S is required for the early stage maturation to occur and that late stage maturation requires entrance into M (Figure 9B).

S Phase-dependent Feedback Inhibition of Early SPB Maturation

We invoke a feedback inhibition mechanism downstream of cdc10δ to block the early phase of maturation until completion of S, i.e., completion of DNA synthesis. Although it is possible that early stage maturation is under positive cell cycle and only G2 is permissive for early stage maturation to occur, this simple model is contradicted by our observations of SPB duplication and early stage maturation in cdc10δ-inactive cells. When cells initiated SPB duplication in the absence of normal cdc10δ, they also underwent early stage maturation, even though these cells never entered S or G2. Maturation was blocked in cells overexpressing cdc18p and cdc2p/mitotic cyclin complex is active under these conditions (Labib et al., 1995; Tanaka and Okayama, 2000). The absence of SPB maturation in HU-treated cells, conditions that place these cells downstream of a requirement for cdc10δ. The simplest explanation of these results is that during S-phase early stage maturation is blocked by a feedback inhibition mechanism that is cdc10δ-dependent. These results also demonstrate that early stage maturation cannot be under control of the cdc2p/mitotic cyclin complex because only the cdc2p/S phase cyclin complex is active under these conditions (Labib et al., 1995; Tanaka and Okayama, 2000). The absence of SPB maturation in HU-treated cells and in cells overexpressing cdc18p suggests that the mechanism of feedback inhibition is coupled to an event involved in DNA synthesis, for example, the formation of replication forks or that it requires cdc18p itself.

We propose that cdc18p is the protein blocking the early maturation of duplicated SPBs until the completion of the S phase. Cdc18p has been shown to be required for maintenance of the replication fork and it activates the S phase DNA replication checkpoint pathway (Murakami et al., 2002). The presence of replication forks maintained by cdc18p could send signals to the cell to inhibit early phase SPB maturation and thus coordinate SPB duplication/early maturation with DNA replication. This model is consistent with our observation of early stage maturation in cells overexpressing rum1p, because rum1p OE triggers entrance into
S upstream of cdc10+1, but these cells subsequently exit from S. In the future, it will be important to identify other proteins involved in the SPB duplication/maturation checkpoint pathway and to show whether it shares some common components with the DNA replication checkpoint pathway.

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