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The Spindle Pole Body of *Schizosaccharomyces pombe* Enters and Leaves the Nuclear Envelope as the Cell Cycle Proceeds

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The cycle of spindle pole body (SPB) duplication, differentiation, and segregation in *Schizosaccharomyces pombe* is different from that in some other yeasts. Like the centrosome of vertebrate cells, the SPB of *S. pombe* spends most of interphase in the cytoplasm, immediately next to the nuclear envelope. Some γ-tubulin is localized on the SPB, suggesting that it plays a role in the organization of interphase microtubules (MTs), and serial sections demonstrate that some interphase MTs end on or very near to the SPB. γ-Tubulin is also found on osmiophilic material that lies near the inner surface of the nuclear envelope, immediately adjacent to the SPB, even though there are no MTs in the interphase nucleus. Apparently, the MT initiation activities of γ-tubulin in *S. pombe* are regulated. The SPB duplicates in the cytoplasm during late G_2 phase, and the two resulting structures are connected by a darkly staining bridge until the mitotic spindle forms. As the cell enters mitosis, the nuclear envelope invaginates beside the SPB, forming a pocket of cytoplasm that accumulates dark amorphous material. The nuclear envelope then opens to form a fenestra, and the duplicated SPB settles into it. Each part of the SPB initiates intranuclear MTs, and then the two structures separate to lie in distinct fenestrae as a bipolar spindle forms. Through metaphase, the SPBs remain in their fenestrae, bound to the polar ends of spindle MTs; at about this time, a small bundle of cytoplasmic MTs forms in association with each SPB. These MTs are situated with one end near to, but not on, the SPBs, and they project into the cytoplasm at an orientation that is oblique to the spindle axis. As anaphase proceeds, the nuclear fenestrae close, and the SPBs are extruded back into the cytoplasm. These observations define new fields of enquiry about the control of SPB duplication and the dynamics of the nuclear envelope.

INTRODUCTION

The microtubules (MTs) of many cells are organized, at least in part, by a centrosome. This organelle seeds the polymerization of tubulin, defining the position of MT initiation (Wilson, 1911) and the number (Snyder and McIntosh, 1975; Kuriyama and Borisy, 1981) and polarity (Bergen *et al.*, 1980; Heidemann and McIntosh, 1980) of the MTs that form. Centrosomes also help to define the MT surface lattice (Evans *et al.*, 1985) and sometimes specify the orientation of MT growth (Cleveland, 1963; Mazia, 1984). In cycling cells the centrosome doubles during interphase and then separates so each organelle can serve as a pole for the mitotic spindle (reviewed in Sluder, 1989).

Centrosome morphology is variable across phylogeny, but the function of this organelle as the major MT organizer of the cell is strikingly consistent (reviewed in McIntosh, 1983; Mazia, 1984; Kalnins, 1991). This observation, together with the more recent realization that some form of γ-tubulin is likely to be a universal

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centrosome component (Oakley and Oakley, 1989; Oakley *et al.*, 1990; Horio *et al.*, 1991; reviewed in Raff, 1996), has encouraged the idea that centrosomes from a wide range of organisms may act by essentially the same pathway. In this context there has been increasing interest in the centrosomes of organisms like fungi, whose suitability for detailed experimentation allows the more probing work that is likely to elucidate molecular mechanisms (reviewed in Kilmartin, 1994; Snyder, 1994).

The cycle of centrosome duplication and segregation is generally correlated with the cell division cycle. In some cells, like budding yeast (Byers, 1981b) and cultured cell from vertebrates (Rattner and Phillips, 1973), centrosome duplication begins in late G_1 or at about the onset of S phase. The single centrosome that was inherited at mitosis/cytokinesis serves as a site next to which a new centrosome assembles (Cleveland, 1963; Rieder and Borisy, 1982). Later in the cycle, the duplicated centrosomes separate, and as the nuclear envelope breaks down for mitosis, they initiate the MTs that will interact with the chromosomes for mitosis. In some cells, however, the nuclear envelope never disperses, so an alternative pathway must be found to put centrosome-associated MTs from the cytoplasm in touch with the mitotic chromosomes (for a review of mitotic diversity, see Kubai, 1975). For example, in the budding yeast *Saccharomyces cerevisiae*, the centrosome, commonly called the spindle pole body (SPB), is built into an unusually large pore or "fenestra" in the nuclear envelope. This organelle initiates nuclear MTs from one surface and cytoplasmic MTs from the other. During early G_1 phase, a "half-bridge" projects from one edge of the cytoplasmic surface of the SPB immediately next to the nuclear envelope. Late in G_1 phase, a "satellite" forms at the distal end of this half-bridge, and just after "START", when the cell commits itself to another cycle of growth and division, a new SPB assembles at the satellite and settles into the nuclear envelope, immediately adjacent to the old SPB (Byers and Goetsch, 1975; Byers, 1981b; Friedman *et al.*, 1996). A little later, MTs emerge from both the nuclear- and the cytoplasmic-facing surfaces of the new SPB, so it looks just like the old one. During the course of S phase, the nuclear MTs from both SPBs attach to the kinetochores by a process that is not yet well characterized, and then SPBs turn to face each other and form a bipolar spindle that segregates both the chromosomes and the SPBs into the soon-to-be sister cells (Byers and Goetsch, 1975).

We have become interested in the division machinery of the fission yeast *Schizosaccharomyces pombe*. Cytological descriptions of mitosis in this organism have suggested that its mitotic cycle is strikingly similar to the corresponding processes in higher eukaryotes (Uzawa and Yanagida, 1992; Ding *et al.*, 1993; Funabiki *et al.*, 1993). This situation encourages the view that fission yeasts can serve as useful models for detailed analyses of cell division. While describing the MTs of the mitotic spindle, we realized that the SPB cycle of *S. pombe* is different from that in *S. cerevisiae*. Most strikingly, the SPB of *S. pombe* is cytoplasmic during interphase, entering the nuclear envelope only for the occasion of division. Herein we describe the cycle of SPB duplication, growth, and division and discuss the relationship between these processes and more universal events, like the entry of pore complexes into the nuclear envelope.

MATERIALS AND METHODS

Cell Culture

Wild-type *S. pombe*, strain 972,h^-, was obtained from the laboratory of Dr. Paul Nurse and grown as described in Moreno *et al.* (1991). When cells at a particular stage in the cell cycle were required, a culture in early logarithmic phase was synchronized by size selection, using centrifugal elutriation carried out at the Baylor College of Medicine, thanks to the generosity of Dr. Shelley Sazer. Cells in early G_2 phase were collected at 0°C, by using a standard protocol (Moreno *et al.*, 1991), and the majority of the sample was shipped to Boulder at 0°C. About 18 h later, the sample retained on ice at Baylor was transferred to 25°C, and its septation index was followed as the cells passed through the cell cycle. A symmetric peak in the septation index (mode value, 32%; width at half-height, 1 h) occurred 3.6 h after warming to 25°C, and all of the cells divided. These results were essentially identical to septation index data from cells synchronized by elutriation but never cooled, suggesting that the time spent at 0°C had no significant effect on cell viability or synchrony. In Boulder, the same preparation of cells was warmed to 32°C, and at 1.75, 2.0, 2.25, and 2.5 h after warming, samples were prepared for electron microscopy (EM).

Electron Microscopy

Cells were prepared for EM by freeze-substitution fixation after rapid freezing by one of two protocols: plunge freezing (Tanaka and Kanbe, 1986, as modified by Ding *et al.*, 1993) or high-pressure freezing (Kiss and Staehelin, 1995; McDonald *et al.*, 1996). For high-pressure freezing, cells were harvested from liquid culture by vacuum filtration onto 0.4-μm Millipore filters. Within 20 s, they were transferred to brass sample chambers and frozen with a jet of liquid N_2 at a pressure of ca. 1000 bar. Frozen samples were kept in liquid N_2 until they were chemically fixed and dehydrated by freeze substitution in acetone containing 2% OsO_4 and 0.05% uranyl acetate at −90°C for 2 to 4 d. Samples were then warmed to −20°C, held at this temperature for about 3 h, and then warmed to 0°C for 1.5 h and to room temperature for 0.5 h, whereupon they were gradually infiltrated and embedded with Epox-Araldite. Serial sections (40–60-nm thick) were cut by using a Leica Ultracut E microtome, picked up on Formvar-coated carbon-stabilized slot grids, and stained consecutively with 3% uranyl acetate in 70% methanol and Reynold's aqueous lead citrate. Sections were imaged on Kodak 4489 film in a JEOL-100C or a Philips CM10 electron microscope operating at 80–100 kV. Often, the specimen had to be tilted in the microscope to obtain optimal images of the SPBs, so the grids were generally observed with a goniometer stage.

Appropriate cells were identified in the EM by inspection of their SPBs. By using the central section of a series, the appropriate orientation for optimal viewing was identified, and then pictures of the serial sections were taken seriatim to include the entire organelle. In some cases the MTs associated with the SPBs were tracked through serial sections and their trajectories were assembled in a computer using the IMOD software package (Kremer *et al.*, 1996).

Electron Microscopic Immunocytochemistry

In preparation for immunolocalization, cells were fast-frozen in the high-pressure freezer and then freeze-substituted in 0.1% anhydrous glutaraldehyde dissolved in acetone at −90°C. Several embedding plastics were tried, but we had the best success in preserving both the antigenicity and fine structure of *S. pombe* with LR White, used according to the manufacturers instructions. Serial sections were cut with a diamond knife, picked up on nickel slot grids coated with carbon-stabilized Formvar, and used immediately for immunolabeling.

Antibodies to γ-tubulin were raised in rabbits against recombinant γ-tubulin from *Aspergillus nidulans*. The protocols for immunization and affinity purification of the antibody, as well as data describing the specificity of the antibody for *S. pombe*, have all been previously described (Horio *et al.*, 1991). Affinity-purified antibodies were diluted 1:50 with a blocking buffer containing phosphate-buffered saline, 0.02% Tween-80, 0.8% bovine serum albumin, and 0.1% fish gelatin (Amersham, Arlington Heights, IL) to minimize nonspecific binding. Sections mounted on grids were floated over night on a 20-μl drop of this solution at 4°C in a small chamber saturated with water vapor. The grids were then rinsed in buffered saline containing 0.1% Tween 80 and stained for 2 h at room temperature with goat anti-rabbit immunoglobulin labeled with 10-nm colloidal gold (British Biocell, Cardiff, United Kingdom), which had been centrifuged to remove gold aggregates and diluted 1:20 with blocking buffer. The grids were then fixed in 0.5% glutaraldehyde, stained with uranyl acetate and lead citrate, dried, and examined as described above.

RESULTS

SPBs of *S. pombe* have been recorded and analyzed by EM of 32 sets of serial sections that ran completely through the structure. More than 20 incomplete serial sets were also recorded, and more than 25 additional SPBs were identified in the microscope and scored with respect to their state of duplication and the cycle stage of the cell in which they were found. Forty-six SPBs were analyzed without serial sections for the localization of γ-tubulin.

The SPB during Interphase

A logarithmic-phase culture contains representatives of all stages of the cell cycle. Mitotic cells are easily recognized by the structure and arrangement of their spindle MTs. The cycle stage of an interphase cell can be inferred from an examination of its length, MT organization, and the structure of its septum, if any. G_1 phase in *S. pombe* occurs while the nuclei produced by the recent mitosis still inhabit the same cytoplasm. S phase occurs at about the time of septum formation, so the small cells formed by the completion of cytokinesis are in early G_2 phase (Mitchison and Nurse, 1985). Figure 1, A–D, shows the SPB of a cell that was fixed during septum formation, and Figure 1, E–G, is from a cell of median length; the two cells were, therefore, in S and G_2 phases, respectively.

From these images the SPB can be recognized as approximately an oblate ellipsoid, 90 nm thick and about 180 nm in diameter; the major axes of the ellipsoid run roughly parallel to the nearby nuclear envelope. There was only one SPB in each of these cells, demonstrating that DNA replication and part of G_2 phase precede SPB duplication.

The internal structure of a fast-frozen freeze-substituted SPB, as seen by thin-section EM, is largely amorphous, save the dark-staining line that runs parallel to the long dimension of the ellipsoid (Figure 1, C, E, and F, small arrows). This dark line is seen in sections cut at any orientation that contains the minor axis of the SPB, so it represents a lamina of darkly staining material. An appendage that stains more darkly than the SPB itself projects from one edge of the ellipsoid and runs roughly parallel to the nuclear envelope (Figure 1, C, D, and G, arrowheads). In most cells this appendage approximates a prolate ellipsoid about 130 nm long and 55 nm in diameter. In Figure 1G the structure is 150 nm long, about 25% greater than is common for interphase. This change may represent a stage in the maturation of the SPB. Two to six cytoplasmic MTs run close by the SPB (Figure 1, A, B, and E–G), and surrounding it is a ribosome-free area that helps to delineate its borders.

As first observed by McCully and Robinow (1971) and confirmed by Tanaka and Kanbe (1986), the interphase SPB of *S. pombe* lies in the cytoplasm, closely apposed to the nuclear envelope. These authors said, and we have confirmed, that the interphase SPB is always located where the surface of the nucleus lies close to the plasma membrane. Figure 1, A–D, demonstrates that the nuclear envelope is continuous beneath the entirety of the SPB at this cell cycle stage. The membranes of the envelope are much less clear in Figure 1, E–G, but this results largely from the orientation of the specimen relative to the electron beam at the time of microscopy. With a goniometer stage, one can correct this problem, but these pictures were taken to optimize the visibility of the MTs that run near the SPB.

There is finely fibrous material between the SPB and the nuclear envelope, suggesting that these two organelles may be connected during interphase. The region of the nuclear envelope that lies beside the SPB shows additional specialization, both in the narrow spacing between its inner and outer membranes and in the deposition of electron-dense material on the membranes themselves. The inner surface of the inner nuclear membrane is particularly thickened and darkly staining (Figure 1, A–C, open arrows). This is the region of the nucleus where fluorescence in situ hybridization has localized the centromeres of *S. pombe* (Funabiki *et al.*, 1993), but our electron micrographs have failed to identify structures in interphase that resemble the kinetochores seen in mitosis (Ding *et al.*, 1993).

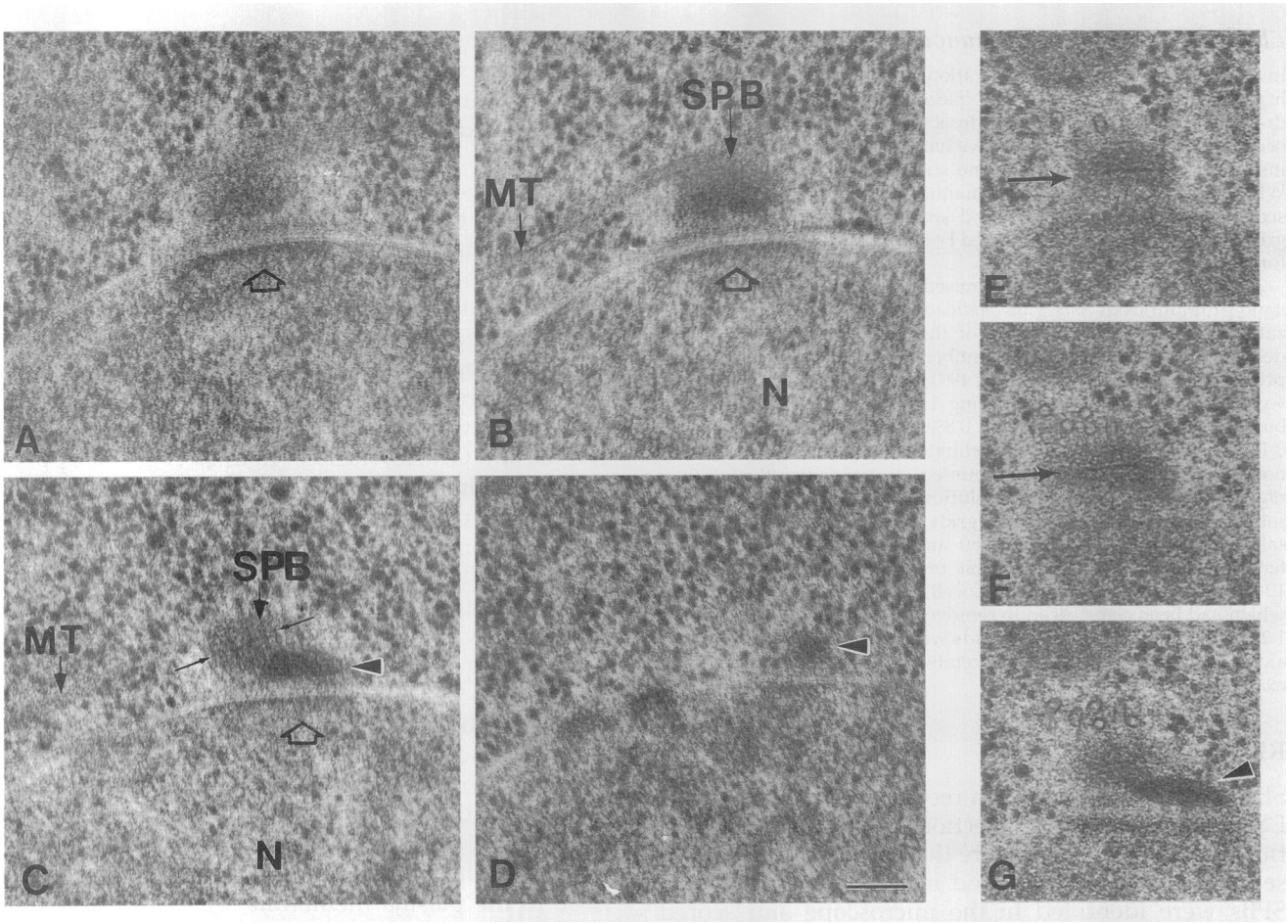

Figure 1. Serial sections through the SPBs of two interphase cells. (A–D) Micrographs from a cell in the process of septum formation (about S phase). (E–G) Micrographs from a cell of moderate length (mid-G_2 phase). The SPB is a finely granular ellipsoid (B and C, arrows) that contains a dark-staining central line (C, E, and F, small arrows). Beside the SPB is a smaller and darker ellipsoidal structure (C, D, and G, arrowheads). Electron-dense material is also seen on the inner nuclear membrane near the SPB (B and C, open arrows). The cytoplasm with its MT and the nucleoplasm (N) are clearly separated by the double membrane of the nuclear envelope (B and C). (E–G) MTs can be seen in cross-section. Comparison of the MT profiles in these sections shows that several MTs end in the immediate vicinity of the interphase SPB. Bar, 0.1 μm.

The Interphase SPB as a MT Organizing Center

Since the SPB is cytoplasmic during interphase, it is important to ask whether this organelle participates in the initiation of cytoplasmic MTs. Light microscopy of interphase cells stained with antibodies to tubulin does not reveal a radial SPB-centered arrangement of MTs (Hagan and Hyams, 1988), as might be expected for full interphase centrosome activity, but serial sections and EM show that several MTs end in the immediate vicinity of the organelle. Comparisons of Figure 1, E–G, identify three MTs that are present in Figure 1G but absent in Figure 1, F or E or both. Sections beyond Figure 1G continue to show the five MTs as they diverge from the SPB, and sections beyond Figure 1E show only two MTs, confirming that these are true MT ends that lie immediately next to the SPB. Two or three MT ends have been seen immediately next to the SPB in all four sets of serial sections that were oriented properly for accurate MT tracking, suggesting that this situation is common in *S. pombe*. Such images do not, however, distinguish between the possibilities that MTs are initiated at or are captured by the interphase SPB. Moreover, there are several interphase MTs that pass the nucleus, running approximately parallel to the axis of the cell, but that do not come near the SPB (Hagan and Hyams, 1988).

Localization of γ-Tubulin in S. pombe by Immuno-EM

If the SPB initiates interphase MTs in *S. pombe*, one might expect it to contain γ-tubulin. Studies with the light microscope have localized γ-tubulin both to the SPB and to cytoplasmic sites that form near the cell

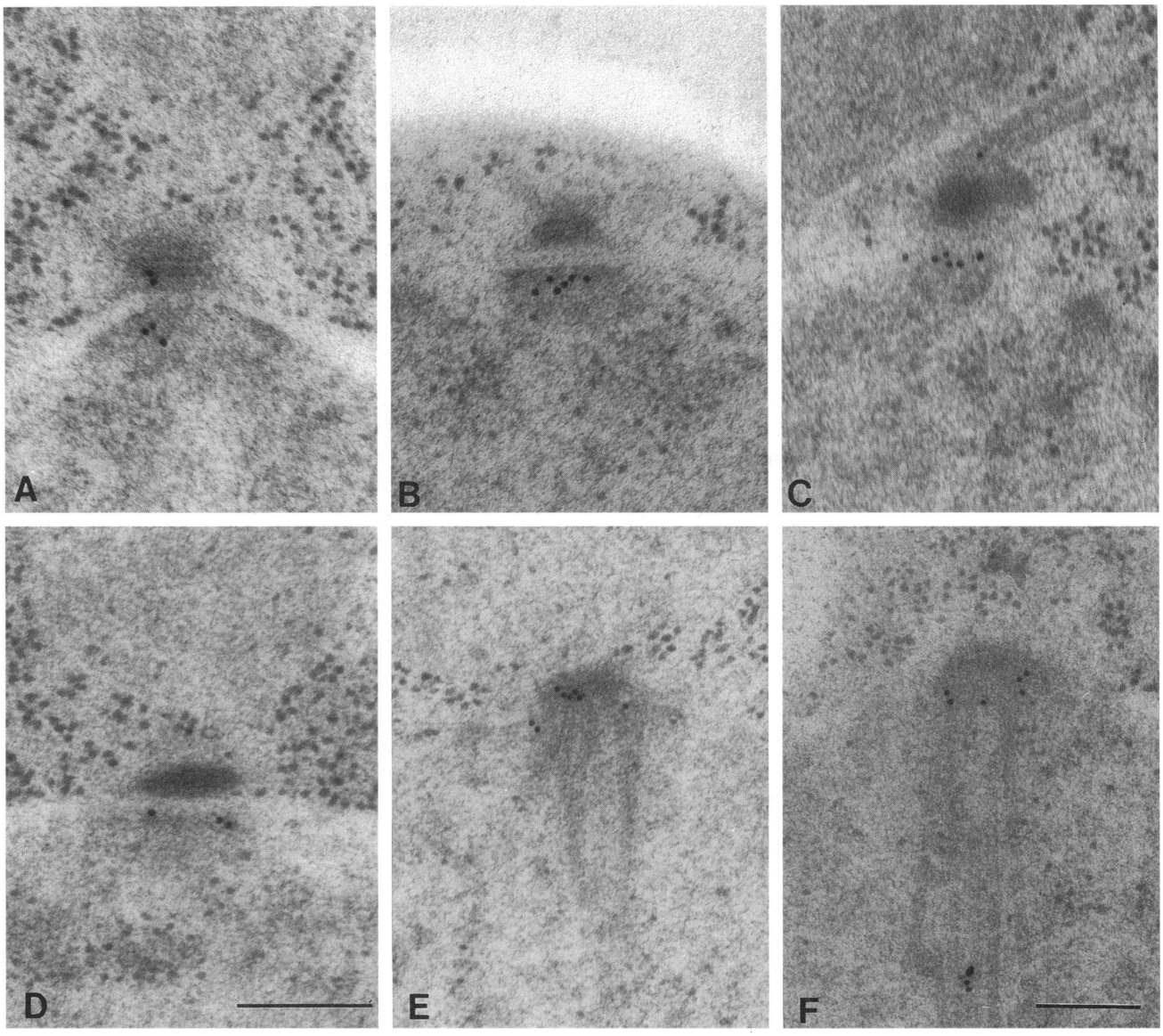

Figure 2. Images of sections reacted with affinity-purified antibodies to γ-tubulin and then with gold-labeled secondary antibodies. Interphase SPBs binds some gold, but the darkly staining material on the inner surface of the nuclear envelope opposite the SPB binds more (A–D). SPBs in mitotic cells (E and F) bind about the same amount of gold as those in interphase cells, and much of the gold is again found in the nuclear region associated with the SPB proper. Bar, 0.2 μm.

midplane at the time of septum formation (Horio *et al.*, 1991). We have used EM immunocytochemistry to examine the distribution of γ-tubulin in SPBs of *S. pombe* at higher space resolution. Images of interphase cells localized this antigen both on and near to the SPBs (Figure 2, A–D). Surprisingly, however, the majority of the gold particles were actually in the nucleus, binding to the darkly staining region immediately next to the nuclear envelope, not to the SPB itself. [A preliminary mention of this finding was reported in McDonald *et al.* (1996).] The nuclear γ-tubulin can lie beneath either the SPB itself (Figure 2, A–C) or the darkly staining appendage to the SPB (Figure 2D). On 39 samples in which a thin section included some part of an interphase SPB, there was an average of 5.4 ± 3.2 gold particles per SPB and its underlying nuclear area (mean ± SD). In the same preparations background staining was less than one gold particle per 4 μm^2 of cytoplasm and/or nucleoplasm. The largest area occupied by a SPB and its associated nuclear staining was 0.06 μm^2, meaning that at background levels of staining, we would have seen <0.014 gold particles

per SPB, rather than the 5.4 observed. Our staining is therefore at a signal-to-noise ratio of about 386, so these localizations are likely to be specific.

The gold particles are not uniformly distributed over the SPBs and their associated areas (Figure 2). In the 39 interphase cells, there were an average of 0.89 ± 0.91 particles on the SPBs (mean ± SD) and 4.49 ± 3.01 particles on the adjacent darkly staining nucleoplasm, a ratio of about 1:5. In spite of this concentration of γ-tubulin in the interphase nucleus, no MTs were detectable there, suggesting that nuclear γ-tubulin is in some way regulated. The free MT ends in the vicinity of the SPB, on the other hand, are consistent with the possibility that cytoplasmic γ-tubulin is active in MT initiation.

When a spindle forms for mitosis, some γ-tubulin is localized on the SPB, but there is also label near the SPB, commingled with the pole-proximal ends of the spindle MTs (Figure 2, E and F). In seven mitotic cells, we found an average of 4.28 ± 2.55 gold particles on or near the SPB. This is about the same level of staining as that seen during interphase, so the amount of γ-tubulin near the SPB does not change significantly as the cell goes from interphase to mitosis.

Duplication of the SPB Occurs in the Cytoplasm during Late Interphase

To understand the cell cycle behavior of SPBs in *S. pombe*, we have sought images in which this organelle was either duplicating or already duplicated. Extensive searches of preparations made from unsynchronized cultures, fixed while in logarithmic-phase growth, identified only a few examples in which interphase SPBs were already duplicated. We inferred that this step occupies only a small fraction of the cell cycle and occurs immediately before mitosis. The same conclusion is suggested by the observation that strains of *S. pombe* carrying a cold-sensitive allele of β-tubulin will arrest at restrictive temperature with only a single SPB, yet they enter mitosis within minutes of reversal to permissive temperature (Kanbe *et al.*, 1990).

Given the paucity of images elucidating SPB duplication in logarithmic-phase cultures, we examined cells that were synchronized to early G_2 phase by centrifugal elutriation and then allowed to grow for various periods before fixation by high-pressure freezing and freeze substitution. In a sample frozen after 2.0 h of growth at 32°C after elutriation synchrony, the SPBs were unduplicated. Ten such cells were scored in the EM, using complete serial sections; all of them resembled the SPBs shown in Figure 1. In the synchronized sample fixed after 2.25 h of growth, about half the SPBs were unduplicated, about one quarter were duplicated but still lying in the cytoplasm, and about one quarter were duplicated and separated and the cell was already in mitosis (N total equals 23). In the sample fixed 2.5 h after synchrony, most of the cells had already passed through mitosis, and some had begun to septate.

The SPB from a cell fixed 2.25 h after synchronization is shown in Figure 3. These serial sections demonstrate that the SPB is still localized in the cytoplasm and that the nuclear envelope is intact. The SPB now looks like two of the structures found during earlier interphase (Figure 1), though each is somewhat smaller than its earlier counterpart. The two similar parts of the duplicated SPB are connected by a bridge of darkly staining material (arrowhead) that resembles the SPB appendage of earlier interphase, except that it is thicker in the directions perpendicular to the line connecting the two parts of the duplicated organelle (note that this dark-staining region extends onto the third section of the series). Five specimens like this have been found.

Figure 3 shows many of the features described for single SPBs from earlier interphase. The structure lies outside a continuous nuclear envelope, cytoplasmic MTs pass close by, and there is darkly staining material on the inner surface of the nuclear envelope immediately opposite the entire duplicate structure. These features and the dimensions of recently duplicated SPBs have been confirmed in sections cut perpendicular to the axis that runs between the two parts of the duplicated organelle, including the observation that two to four of the MTs that pass close the SPB end in its immediate vicinity. Measurements from images in these two orientations show that the two parts of a duplicated SPB are still oblate ellipsoids, each about 90 nm thick, but they are now only 125 nm in diameter. Each SPB part is about the same volume as the other, occupying about one-half the volume of an SPB from earlier interphase (Figure 1). The bridge that connects them is now an oblate ellipsoid about 50 nm thick and 125 nm in diameter.

Two cells from the sample fixed after 2.25 h of growth after synchronization contained duplicated SPB whose two parts were each 180 nm in the dimension parallel to the line that connected them (Figure 4). The size of each SPB part in these images approximated the sizes of SPBs from earlier interphase (Figure 1) and also from mitotic cells (see below). The bridge between the two parts of these larger SPBs was more elaborate; although it contained some regions that resembled the darkly staining bridge in the smaller duplicated organelles, these bridges also contained fibrous material that somewhat resembled the internal structure of the SPB itself (Figure 4, B and C). The nuclear envelope adjacent to the large duplicated SPBs was still intact, but the darkly staining specializations on its inner surface now covered a greater area. Much of the nuclear area underlying these enlarged SPBs was darkly staining, though gaps were sometimes

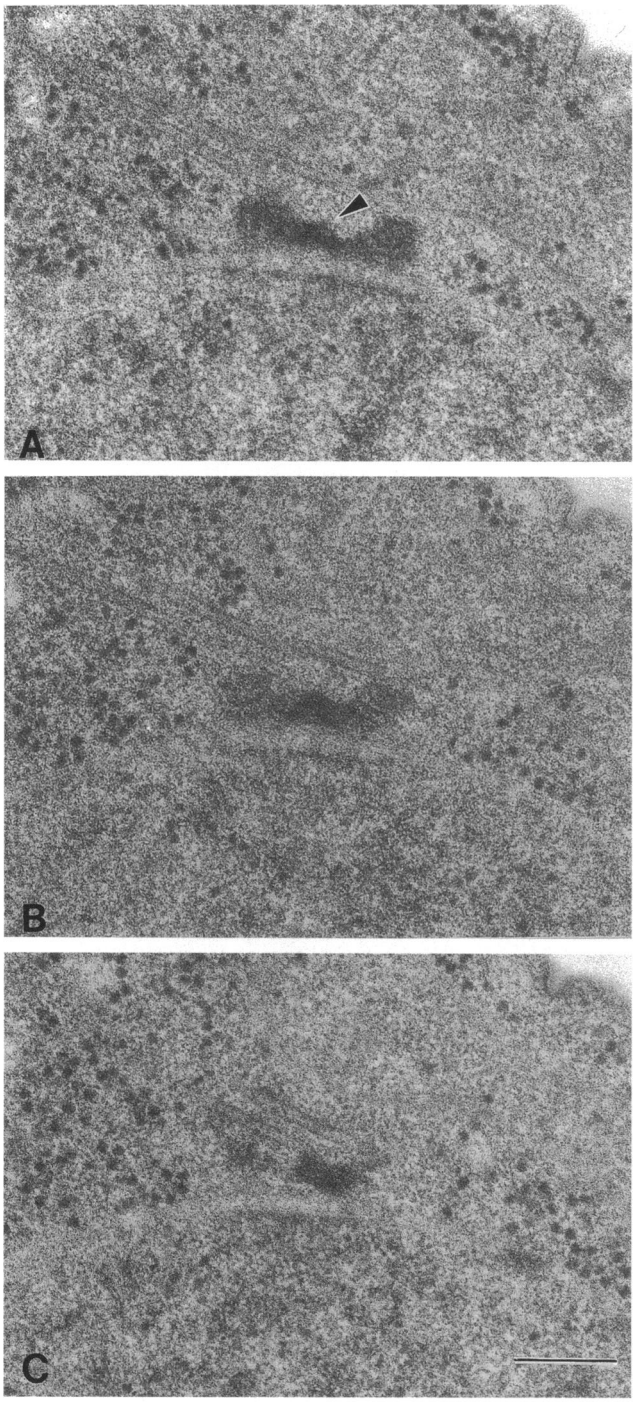

Figure 3. Three serial sections through a duplicated SPB from a cell fixed 2.25 h after synchronization by elutriation. Two small objects, each of which resembles a SPB from earlier interphase, are connected by a darkly staining ellipsoid (arrowhead) that resembles the SPB appendage. Volume estimates suggest that each part of this SPB is about half the volume of an SPB from earlier interphase. The continuity of the nuclear envelope adjacent to the SPBs, the accumulation of darkly staining material on the inner surface of the nuclear envelope, and the association of the duplicate SPB with cytoplasmic MTs are apparent. Bar, 0.2 μm.

visible (e.g., Figure 4, B and C), suggesting that the darkly staining material was arranged in a broad annulus. We interpret these larger organelles (N = 2) as SPBs fixed later in their duplication process than the ones whose parts were smaller (N = 5); the relative frequencies of the two morphologies suggest that the enlarged SPBs move rapidly to the next stage of the cell cycle.

The SPB at Early Mitosis

To participate in mitosis, the SPBs must gain access to the chromosomes. In *S. pombe* this is accomplished by the cell's opening a fenestra in the nuclear envelope. At the onset of this process, the duplicated SPB becomes associated with an extensive array of irregular darkly staining objects that lie between it and the nuclear envelope, which appears to be invaginated (Figure 5, A–H). Cytoplasmic MTs can still be seen, passing close to the duplicated SPB (Figure 5, A–F), and the two parts of the duplicate SPB are still recognizable in the appropriate sections (Figure 5, D–G). Note that one part of the SPB is more obviously similar to SPBs of earlier interphase than the other, probably as a result of the orientation of the plane of section relative to the familiar structural features. In some sections the nuclear envelope still appears to be present (e.g., Figure 5, E, F, and H, arrowheads), but its component membranes are not obvious.

Because of the effects of orientation on the appearance of the nuclear envelope, we used the goniometer stage to reorient sections of this apparent invagination in which the nuclear envelope was not evident, trying to determine whether is was present but obscured. Figure 5, I–O, shows a series of images at 20° intervals from −60° to +60°, looking for orientations of the section shown in Figure 5D that would reveal different regions of the envelope. Although there are some suggestive images, such as those indicated by arrowheads in Figure 5, M and N, the envelope does not appear to be present at all positions around the darkly staining material.

Envelope invagination is a sufficiently unusual process that we have sought to document it in other ways. Figure 6 shows serial sections from a cell at approximately the same stage as that shown in Figure 5, but the plane of section is now parallel to the surface of the nucleus at the point of SPB proximity. Cytoplasmic MTs are obvious in Figure 6, A and B. Note also the indications of substructure within the SPB when seen from this orientation (Figure 6B, the lines drawn in the cytoplasm). Nuclear pores (arrowheads) are evident in Figure 6, B and C, as the sections cut deeper, revealing the darkly staining material that lies between the SPB and the nuclear envelope. The invaginated envelope is evident in one region of Figure 6E (arrow) but is not apparent elsewhere. Three cells with darkly staining

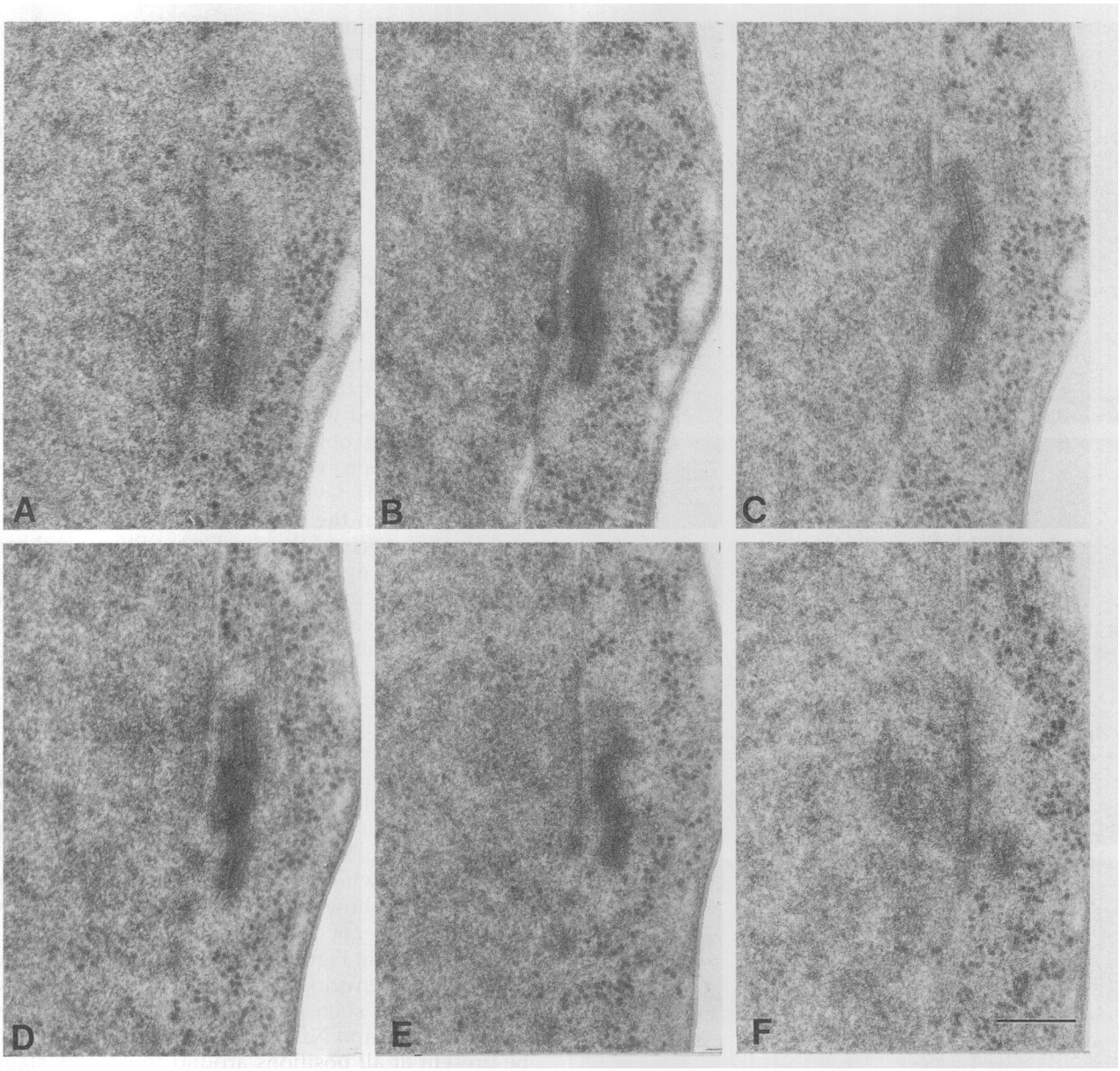

Figure 4. Six serial sections through a duplicated SPB from a cell fixed 2.25 h after synchronization by elutriation. The greater length and depth of the SPB parts, together with the greater elaboration of the bridge between them, lead us to interpret this as a stage in the SPB cycle that is later than that shown in Figure 3. Cytoplasmic MTs are still visible, but the darkly staining region of the nuclear envelope adjacent to the SPB now covers a larger area and appears to be arranged in a ring (compare Figure 3, A–E). The nucleoplasmic densities in Figure 3F may be related to condensing chromatin. Bar, 0.2 μm.

material and apparent invaginations like those in Figure 5 and 6 have been seen. We interpret the images to mean that the nuclear envelope is dispersing as it invaginates in the region adjacent to the duplicated SPB. Apparently, this is an early stage in the process by which the SPB gains access to the nucleoplasm for mitosis.

Figure 7 shows three sections from a cell in which the duplicated SPB has taken up a position within the nuclear envelope. We interpret these images as SPBs of early mitosis for several reasons: 1) The structures lie in the nuclear envelope, where the SPBs of later mitosis are found (Tanaka and Kanbe, 1986; Ding *et al.*, 1993). 2) The apparent SPBs are connected by a prolate ellipsoid that stains like an elongated version of the connection between the duplicated SPB shown in Figures 4 and 5. 3) There are cytoplasmic MTs passing the nucleus in close association with these structures (Figure 7, A–C). 4) There is a MT projecting from one of the putative SPBs directly into the nucleoplasm (Fig-

ure 7A, arrow), suggesting a very early stage in spindle formation. The fact that these putative SPBs look so different from the SPBs of interphase is due in part to the fact that this specimen was tipped to 55° with the goniometer microscope stage to orient the line of sight along the plane tangent to the nuclear envelope at this region. They also differ as a result of the accumulation of darkly staining material on their nuclear surface. Three examples of SPBs at this stage of the cycle have been found, one of which displayed more extensive but still poorly organized MTs emanating from each of the SPBs.

Figure 8 depicts an array of MTs growing from two SPBs about 1.2 μm apart. We interpret this structure as a spindle that was fixed during the process of assembly. Note that several of the MTs extend out from the SPBs at an orientation that is oblique to the pole-to-pole axis; apparently the ordered interdigitation of essentially parallel MTs that is characteristic of metaphase (Ding *et al.*, 1993) is not found in so short a spindle.

Figure 9 displays micrographs of successive serial sections that contain a SPB with MTs projecting into the nucleoplasm. A three-dimensional reconstruction from more serial sections than are shown revealed that this spindle was about 1.6 μm long and was thus likely to be in prometaphase at the time of fixation (Ding *et al.*, 1993). From Figure 9, A–F, it is clear that this SPB is at the end of a spindle and occupies a fenestra in the nuclear envelope that is slightly larger than the SPB itself (Figure 9D, arrows). When these images are combined with Figure 2 of Ding *et al.* (1993), which shows a SPB from one pole of a 2.3-μm spindle sectioned in the plane perpendicular to the spindle axis, we see that an early mitotic SPB is still an oblate ellipsoid whose major axes are about 180 nm and lie approximately parallel to the nearby nuclear envelope. The thickness of the SPB is about 50 nm, and there is a thin electron-lucent zone lying near its center. Outside this zone is a central electron-dense lamina like the one seen during interphase (Figure 9D, small arrows), giving the SPB a layered appearance. A single mitochondrion (Mi) lies near the cytoplasmic face of the SPB (Figure 9, B–E), but astral MTs are not apparent.

At the edge of the SPB there is a darkly stained structure about 90 × 60 × 60 nm (Figure 9E, arrowhead). From serial sections we know that such an appendage is found on both SPBs of a mitotic spindle. The position, size, and electron density of this structure, combined with the images in Figure 2 of Ding *et al.* (1993), suggest that these structures are related to the SPB appendages of early interphase. Since a darkly staining appendage is attached to one SPB in early interphase (Figure 1, A–D), elongates during interphase (Figure 1, E–G), connects the duplicate SPBs during late interphase (Figures 4 and 5), and then appears to break in two when the SPBs separate to become spindle poles, it has many properties of the "half-bridge" (attached to one SPB) and "bridge" (connecting two SPBs) that associate with the SPBs of *S. cerevisiae* (Byers and Goetsch, 1975). We will hereafter refer to the appendages in *S. pombe* as half-bridges or bridges, depending on whether they are attached to one or two SPBs.

The SPB at Later Mitosis

As mitosis proceeds, the structure and placement of the SPBs change. Figure 10 shows serial micrographs through a SPB from a spindle that is about 5 μm long (early anaphase B). In contrast to earlier mitosis, the SPB at this stage does not insert into the nuclear fenestra but lies just outside it (Figure 10C, arrows), even though the opening in the nuclear envelope is approximately the same width as the SPB. The length and width of the SPB are about the same as in earlier mitosis, and it still appears as a layered structure with an electron-dense central lamina (Figure 10C), but it is now ca. 80 nm thick. In Figure 10C, a slender bar extends from the SPB along the cytoplasmic surface of the nuclear envelope (the point of the V mark). In the cytoplasm next to the SPB, a mitochondrion (Mi) is still seen, and several cytoplasmic MTs are now visible (Figure 10, A–D). The SPB is again surrounded by a ribosome-free area (Figure 10, B–D). These images can be compared with Figure 3 of Ding *et al.* (1993), which displays sections cut perpendicular to the axis of a spindle 8 μm long (mid-anaphase B). Again, there is a dark staining protrusion from the SPB, suggesting that the half-bridge persists throughout mitosis.

As mitosis comes to completion, there are changes in both the morphology of the SPB and its interaction with the nuclear envelope (Figure 11). The cell shown in Figure 11 contained two daughter nuclei connected by a channel of nucleoplasm that included a remnant of the spindle, so this cell was in late anaphase or telophase at the time of its fixation. The SPB is now largely in the cytoplasm, but there is still a fenestra in the nuclear envelope, through which several MTs pass (Figure 11C, arrows). This fenestra is smaller than the ones found in earlier mitotic stages (compare Figure 11C with Figure 10C) and smaller than the SPB itself. The size of the SPB at this stage is about the same as during earlier mitosis, and it still contains a dark central lamina. Again there is an osmiophilic structure associated with the SPB that we interpret as a half-bridge (Figure 11, B and C, arrowheads). Surrounding the SPB, there is a clear area that lacks ribosomes (Figure 11, C and D). In the neighborhood of the SPB, the inner nuclear membrane is associated with darkly stained material (Figure 11D, open arrow).

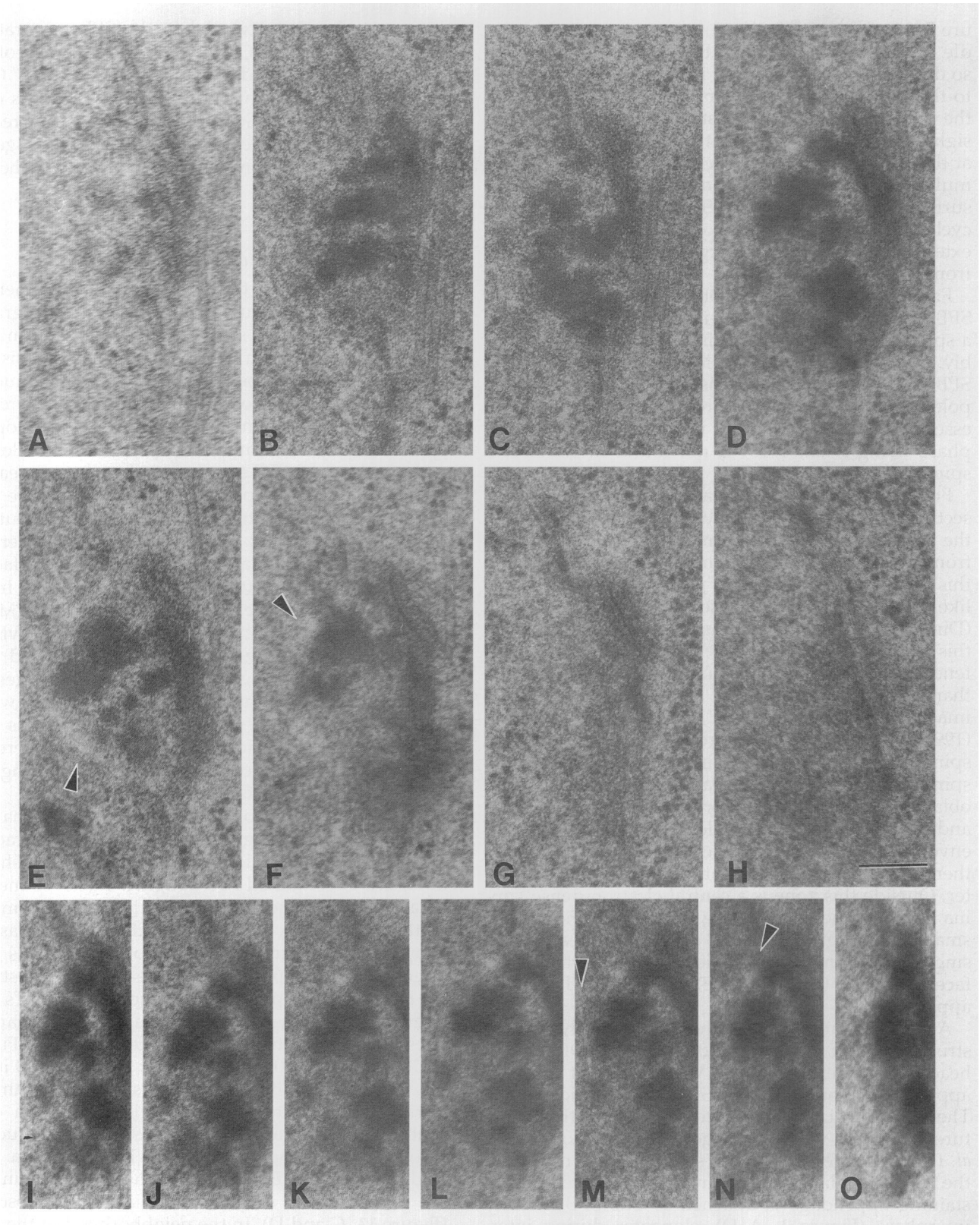

Figure 5. Eight serial sections through a duplicated SPB from a cell fixed 2.25 h after synchronization by elutriation. Cytoplasmic MTs are evident in A–F, and enlarged SPB-like structures are visible in several images in D–G. There is a striking accumulation of darkly staining

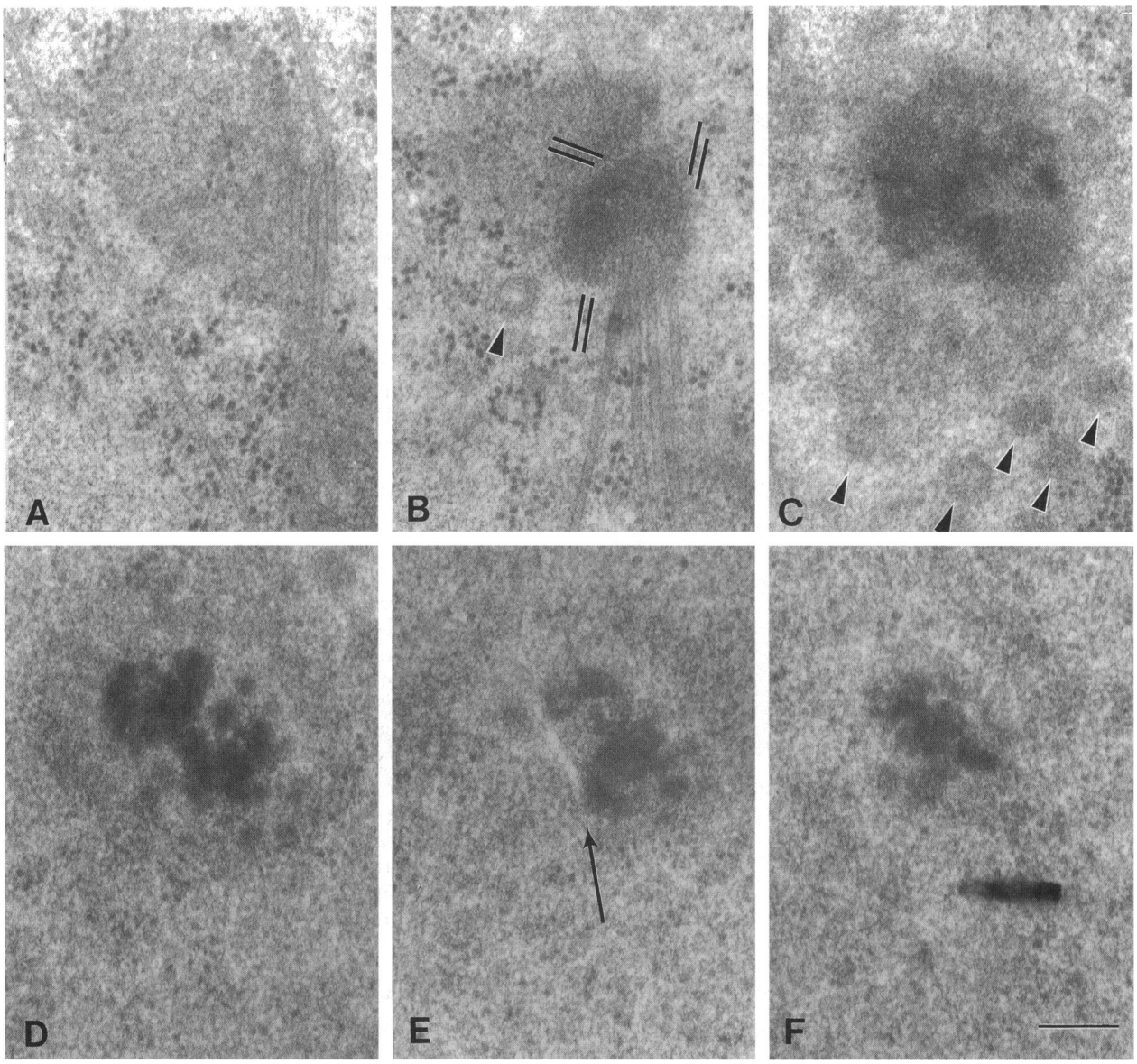

Figure 6. Six serial sections through a duplicated SPB from a cell fixed 2.25 h after synchronization by elutriation. The SPB shown here appears to be at about the same stage as that seen in Figure 5, but the orientation of the sections is perpendicular. The position of some MT ends in the vicinity of the SPB is evident in B. This plane of section includes darkly staining material that is probably a new view of the bridge between the parts of the SPB. Subtle substructure is evident in this region (B, bars). One section deeper, the face view of the SPB parts themselves reveals less-ordered and less-darkly stained domains separated by amorphous darkly stained bodies. As the sections slice into the nucleus, nuclear pores become evident (B and C, arrowheads), allowing an identification of nucleoplasm versus cytoplasm. Deeper still (D–F), the darkly staining material is prominent, and a portion of the invaginating nuclear envelope is visible (E, arrow). These images cast further doubt on the continuity of the nuclear envelope at this stage of cell cycle progression. Bar, 0.2 μm.

Figure 5 (cont). material between the SPB and the nuclear envelop, which appears to be invaginated in E and F (arrowheads). The position and continuity of the envelope is, however, questionable. (I–O) Tilt series of the section shown in D, oriented from −60° to +60° in 20° intervals. Nuclear envelope is clear in N and M, but even with these multiple orientations, the continuity of the envelope is not demonstrated. Similar results were obtained with the section shown in C. Bar, 0.2 μm.

DISCUSSION

Electron micrographs of SPBs in *S. pombe* at different stages of its cell cycle show that this centrosome equivalent moves from the cytoplasm during interphase to the nuclear envelope for mitosis and back to the cytoplasm as cell division is completed. Entry and exit

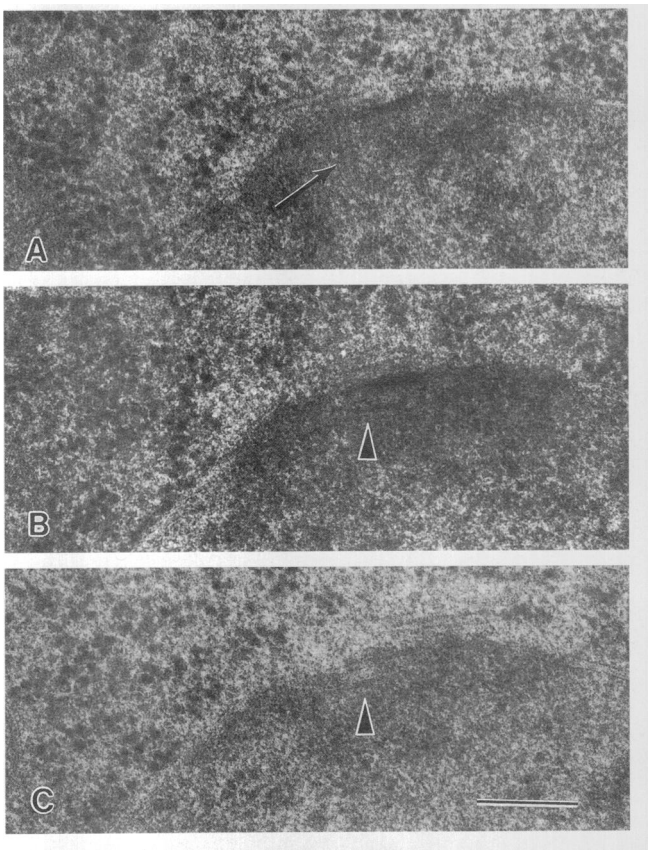

Figure 7. Three serial sections through a duplicated SPB that has entered a fenestra in the nuclear envelope. The parts of the SPB are as long as their counterparts in Figures 4 and 5, although the bridge structure that connects them is now longer and more attenuated than when it was in the cytoplasm. There are still cytoplasmic MTs associated with the SPBs, but for the first time, there is a MT on the nuclear surface of the SPB as well (A, arrow). The size of the fenestra is evident from the space between the places where the nuclear membrane bends back on itself, as it does in the vicinity of a nuclear pore. There are regions of more darkly staining material in the nucleoplasm, adjacent to the SPBs, and some darkly staining lines are visible (B and C, arrowheads). These images are reminiscent of the kinetochore-like material found in metaphase cells (Ding *et al.*, 1993). Bar, 0.2 μm.

from the nuclear envelope has not previously been detailed for any organelle, but to analyze these events in their biological context, we will begin our discussion with a treatment of SPB behavior and function.

The Life Cycle of the S. pombe SPB

The SPB of *S. pombe* resides in the cytoplasm for the large majority of interphase. Late in G_2 phase, a second SPB forms immediately beside the old one (Figures 3 and 4). The nuclear envelope then invaginates beside the duplicated structure (Figures 5 and 6) and opens to form a fenestra as the cell prepares for mitosis (Figures 5–7). The SPBs then serve as poles for the spindle that forms in the nucleus, separating as the spindle forms (Figures 7–9). When the spindle elongates, the SPBs are segregated to opposite ends of the cell (Figure 10), and as mitosis is completed, they are extruded back into the cytoplasm (Figure 11). This cycle is diagramed in Figure 12.

Although our work has demonstrated that SPB duplication occurs late in G_2 phase, the precise timing of duplication relative to genetic markers of cell cycle events remains to be determined. A single SPB has been described in a strain that carries a cold-sensitive allele of β-tubulin, when cell cycle progression is arrested at the restrictive temperature (Kanbe *et al.*, 1990), but the detail in published micrographs would not distinguish a single SPB from the duplicated but unseparated SPBs described herein. We have looked at the SPBs in seven cells of the temperature-sensitive strain *cdc25–22*, which arrests at 35°C in late G_2 immediately before the onset of mitosis (reviewed in Nurse, 1990). After 3 h at restrictive temperature, we found greatly enlarged SPBs, but it was difficult to determine whether the organelles were single or double; their structures were too different from the SPBs described herein for wild-type cells. A similar situation pertains in some mutant strains of *S. cerevisiae*, where a cell cycle arrest leads to abnormal SPB morphology (e.g., Donaldson and Kilmartin, 1996; reviewed in Winey and Byers, 1993; Kilmartin, 1994). One study has suggested that the SPB cycle in *S. pombe* is not arrested by mutations that block other aspects of cell cycle progression (King and Hyams, 1982), but further work with strains carrying diverse cell cycle mutations should contribute to our understanding of the time at which SPBs duplicate relative to markers of *S. pombe* cell cycle progression.

The SPB as a Cytoplasmic MT Organizer

All SPBs are, by definition, mitotic MT organizers, but their roles in the control of cytoplasmic MTs are more variable. MTs in *S. cerevisiae* grow from the cytoplasmic surface of each SPB and/or the bridge between them throughout the cell cycle. In *S. pombe*, however, the SPB has been thought to be unimportant for the initiation of interphase MTs (Horio *et al.*, 1991; Masuda *et al.*, 1992). Our images show that some but not all of the cytoplasmic MTs end in the immediate vicinity of an SPB during both mitosis (Figure 10) and interphase (Figure 1). These observations suggest the possibility that the SPB, or material associated with it, may contribute to the organization of cytoplasmic MTs in *S. pombe*.

Cytoplasmic MTs initiated by the SPBs of *S. cerevisiae* run approximately perpendicular to the plane of the pole body (Byers, 1981b), so the oblique or essentially parallel orientation of the cytoplasmic MTs relative to the SPBs of *S. pombe* is markedly different. If cytoplasmic MTs are

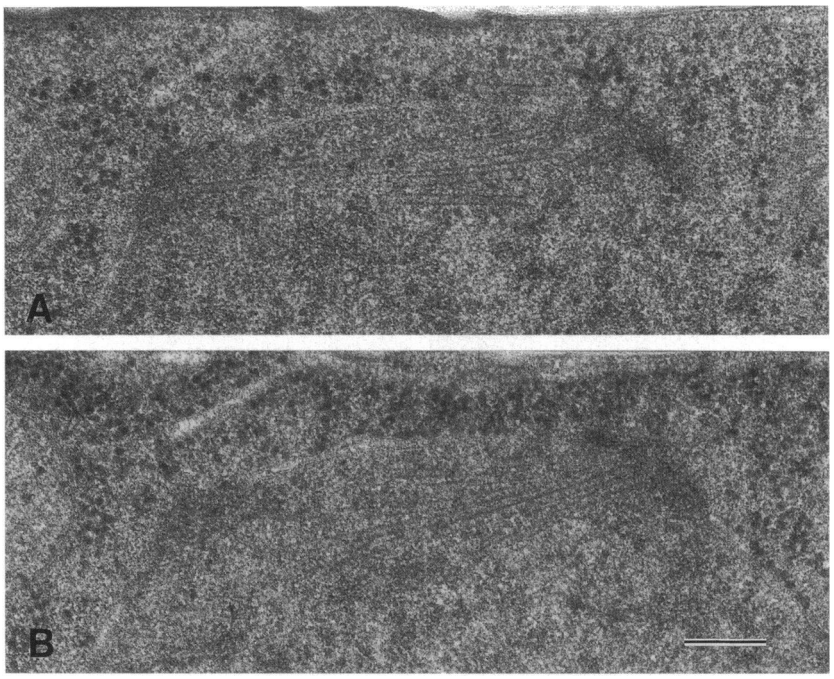

Figure 8. Two serial sections cut approximately parallel to the axis of a mitotic spindle that is only 1.2 μm long. We have been unable to track the MTs reliably in this set of longitudinal sections, but from its pole-to-pole length and its structural disorganization, this spindle appears to be earlier in mitosis than any in our previous description of *S. pombe* spindles (Ding *et al.*, 1993). We interpret this cell as in prometaphase. Only a few cytoplasmic MTs are visible at this stage. Bar, 0.2 μm.

initiated by the SPBs of fission yeasts, the process must be accomplished by SPB-associated materials that are not obvious in the EM. Moreover, interphase cells contain some cytoplasmic MTs that do not pass close to the SPB; these run approximately parallel to the long axis of the cell and are scattered apparently randomly around the cell perimeter (Hagan and Hyams, 1988). By EM these cytoskeletal elements are seen to be arranged as two to four clusters of one to four MTs each (McIntosh, West, and Morphew, unpublished results; the subject of a future contribution).

At the time of septum formation, MTs emanate from γ-tubulin-containing foci that are clearly distinct from the SPB and that are placed near the forming cleavage furrow (Horio *et al.*, 1991). The relationship between the septum-associated MTs and the cylindrically arranged elements that persist through most of interphase has not yet been established. Our data demonstrate that only a few of the cylindrically arranged MTs end in the vicinity of the SPB (Figure 1, E–G; Figure 7, A and B), so if one is going to hold that many or all of the cytoplasmic MTs are initiated by the SPB, one must postulate that once their growth has been started, at least some of them detach from their site of initiation. Such behavior has been proposed for MTs initiated by the centrosome in vertebrate cells (Vorobjev and Nadezhdina, 1987) and has recently been demonstrated directly (Keating *et al.*, 1997). Something analogous may happen during interphase in *S. pombe*, but work with living cells and studies on the structural polarity of interphase MTs will be required to test these ideas.

Nuclear γ-Tubulin Is Regulated during the Cell Cycle

The localizations of γ-tubulin shown herein suggest that most of this antigen lies within the nucleus during both mitosis and interphase. Since the interphase nuclear accumulation is very close to the SPB, this distinction was not appreciated in earlier work by light microscopy (Horio *et al.*, 1991; Masuda *et al.*, 1992), demonstrating the importance of the resolution offered by EM immunocytochemistry. There are, however, no intranuclear MTs during interphase in *S. pombe*. Our data do not say whether this lack is due to inactivity of the γ-tubulin itself, to an inability of α- and β-tubulin to reach the interphase nuclear MT initiators, or to some other factor. Studies on lysed *S. pombe* cells have suggested, however, that the MT-initiating capacity of the SPB is low during interphase and increases markedly with entry into mitosis (Masuda *et al.*, 1992), even though our data suggest that there is no change in the amount of γ-tubulin at or near the SPB as the cell enters mitosis. A similar increase in MT initiation can be accomplished by the

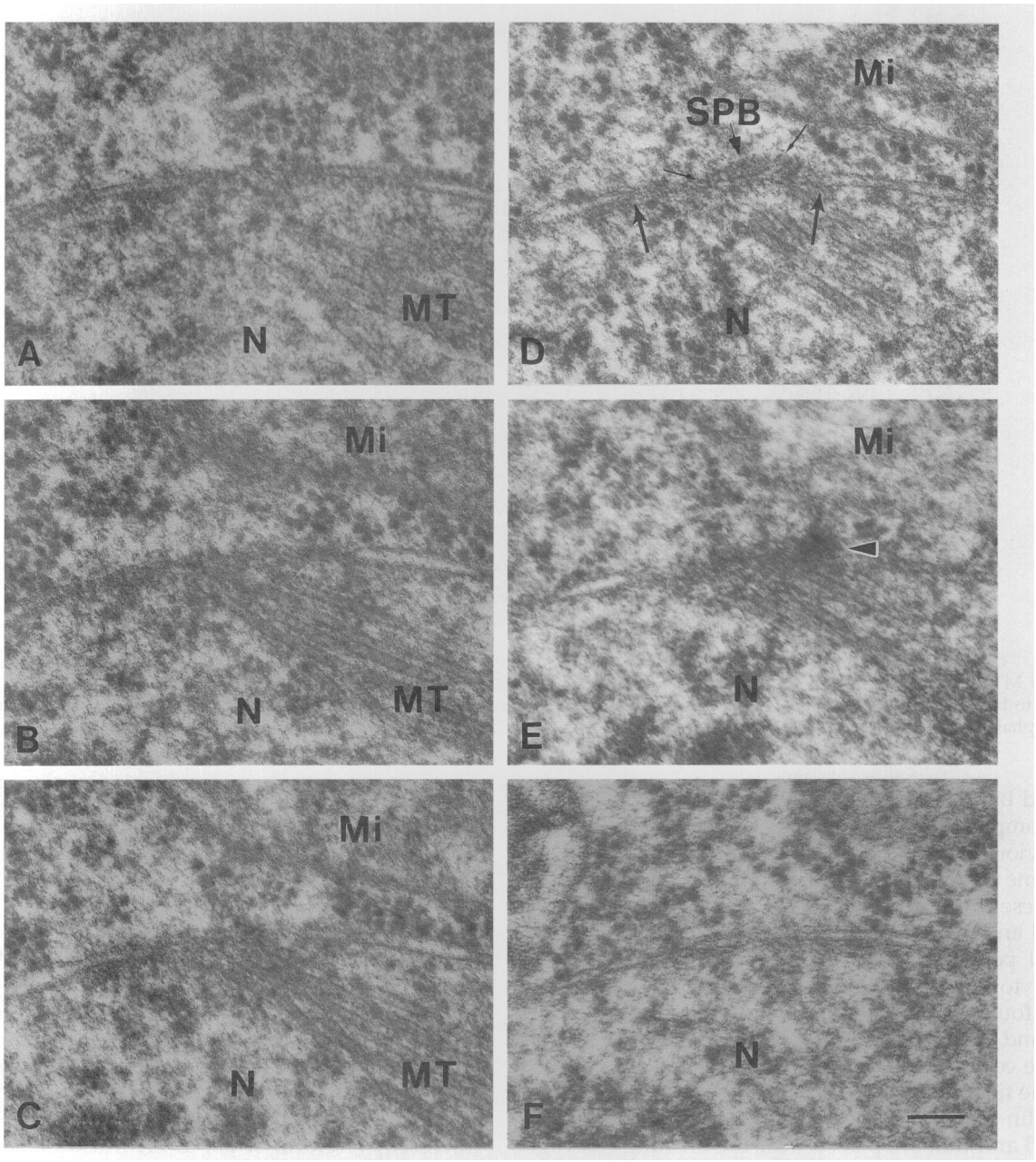

Figure 9. Serial sections through a SPB that is associated with an early mitotic spindle (~1.6-μm long). The SPB is located in a nuclear fenestra, and nuclear MTs are numerous. The large arrows in D indicate the places where the nuclear membrane reflects back. The small arrows in D indicate the dark central lamina of the SPB and the electron-lucent zone beneath it. A structure resembling a half-bridge is shown in E (arrowhead). No cytoplasmic MTs are visible at this stage. Mi, mitochondrion; N, nucleus. Bar, 0.1 μm.

phosphorylation of interphase SPBs with mitotic (but not interphase) extracts from *Xenopus* eggs. Thus, these results suggest that there is a cell cycle-dependent regulation of γ-tubulin activity in *S. pombe*.

Structure of the SPB and Its Associated Half-Bridge

In all stages of the fission cell cycle, the SPB of *S. pombe* is approximately an oblate ellipsoid with an internal dark-staining lamina. In the early stages of mitosis, it also contains an electron-lucent region (Figures 8–11). An internal layered structure was not reported in the SPB of *Schizosaccharomyces octosporus* (Heath, 1981), but McCully and Robinow (1971) described layers and other structural features in the SPB of *S. pombe* that were somewhat different from the ones described herein. The discrepancies between these accounts are

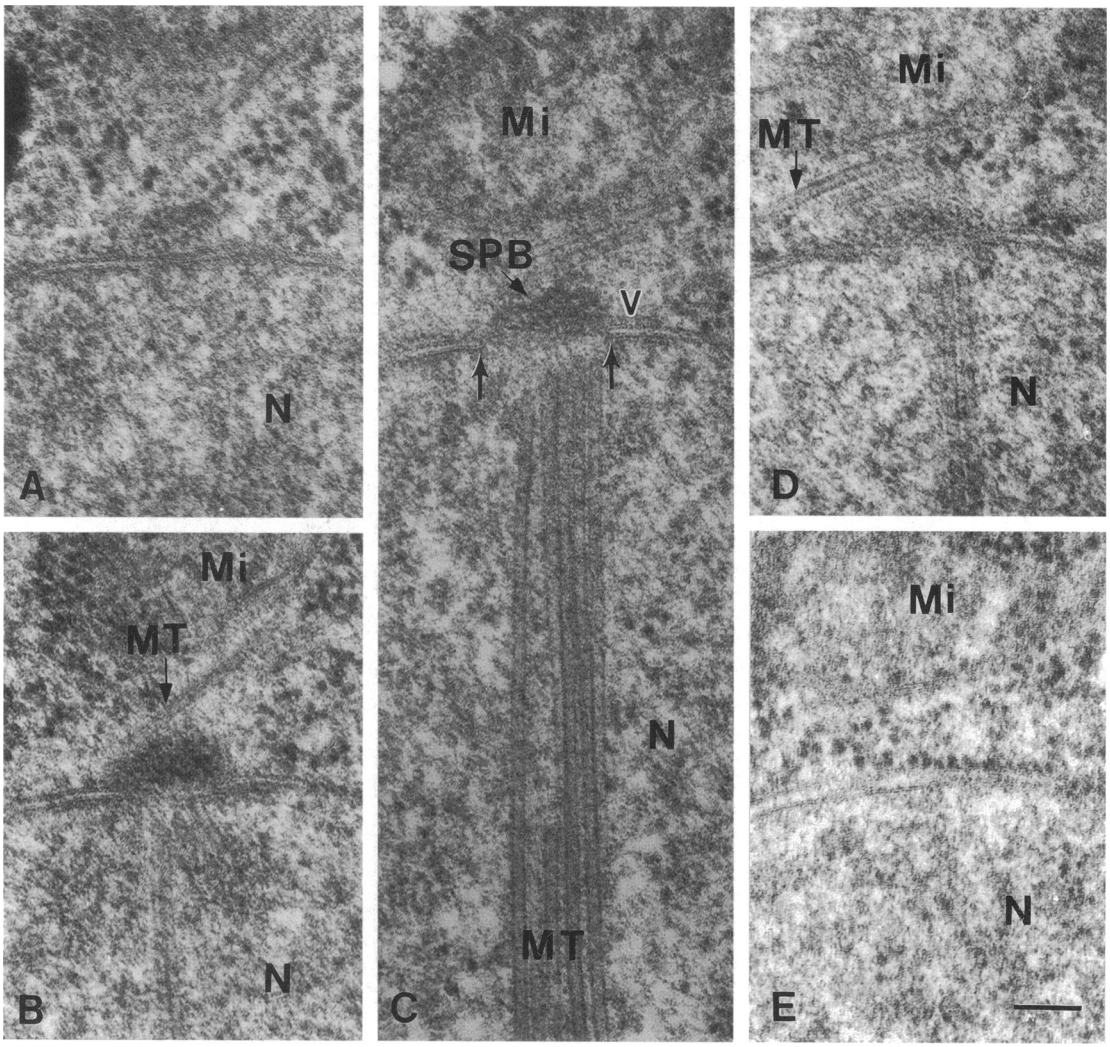

Figure 10. Serial sections through a SPB associated with an anaphase spindle. The nuclear envelope at the edge of the fenestra is indicated by arrows in C. A bar structure, which extends from the SPB onto the cytoplasmic surface of the nuclear envelope, is indicated by V (C). Cytoplasmic MTs are now visible, running at an orientation oblique to the spindle axis (B-D, MT). Mi, mitochondrion; N, nucleus. Bar, 0.1 μm.

likely to be due to differences in the fixation procedures used. We think that the rapid cessation of molecular movement that accompanies high-pressure or plunge freezing and the demonstrated efficacy of freeze substitution fixation for preserving cellular ultrastructure (Howard and Aist, 1979; Heath and Rethoret, 1982; Nicolas, 1991) make it likely that our images are a reasonable approximation to the structures found in vivo.

The SPBs described herein are also quite different from the trilaminar structures that comprise the SPBs of the budding yeast *S. cerevisiae* (Byers and Goetsch, 1975; Byers, 1981a; Donaldson and Kilmartin, 1996). Some of this difference is again probably due to fixation procedures, but some may be a result of significant differences between the analogous organelles in these two yeasts. Further work with molecular markers will be required to determine the extent and significance of the real structural differences between SPBs of related microorganisms.

The reproducible changes in size shown by the SPB as it duplicates are intriguing. The volumes of each part of the newly duplicated SPB are very close to one-half of the volume of a SPB from earlier interphase. Subsequently, the volume of each part increases as the cell prepares for mitosis. Such behavior is consistent with a model in which the material of the first SPB is divided between the two parts of the SPB as the second part appears. Since the new part forms at the distal end of the preexisting bridge, we propose

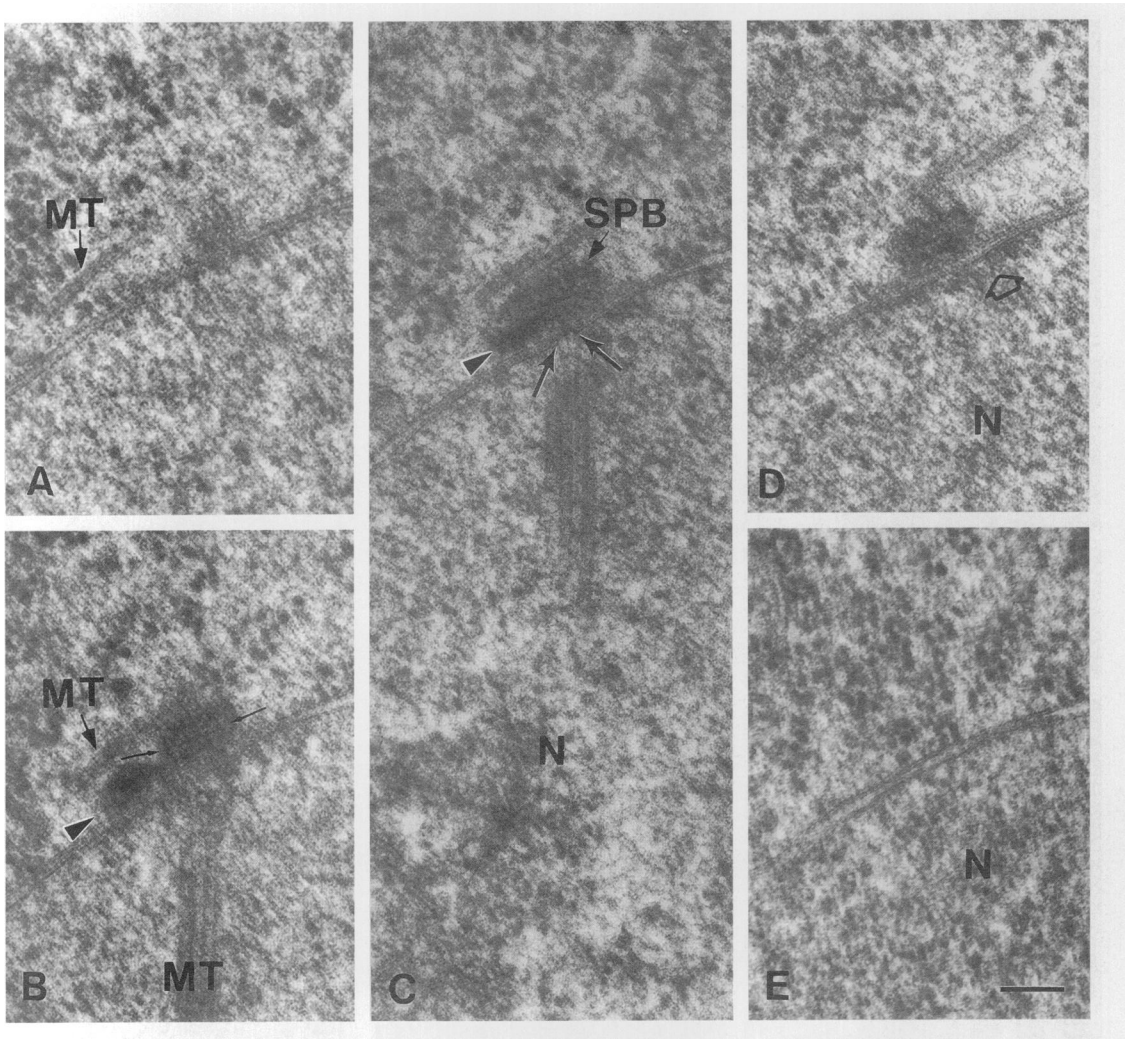

Figure 11. Five serial sections through a SPB associated with a mitotic spindle in late anaphase or telophase. The arrows in C indicate the edges of the fenestra in the nuclear envelope. Arrowheads in B and C indicate a half-bridge. Small arrows (B) mark the dark central lamina of the SPB itself, and cytoplasmic MTs are visible in A–D. Electron-dense material bound to the inner nuclear membrane is indicated by an open arrow (D). N, nucleus. Bar, 0.1 μm.

that there is an equilibrium between material from the old SPB, a soluble pool of SPB subunits, and a site that initiates the formation of a new SPB at the distal tip of the bridge. This idea of a labile SPB is consistent with the striking changes in the SPB of *S. cerevisiae* that are seen when cell cycle progression is arrested (Byers, 1981a) and with the fact that SPBs can fuse as karyogamy is completed (Byers, 1981b).

The structure of the half-bridge or bridge associated with the SPB of *S. pombe* is more variable than that of the SPB itself. It appears to elongate during interphase, to become more compact as the second SPB forms in late G_2 phase, and then to develop complex internal structure as the cell prepares for mitosis. After entry of the SPBs into the nuclear fenestra, the bridge appears thinned and extended relative to its interphase counterpart, perhaps in anticipation of its breaking as the two SPBs separate to serve as spindle poles. The dimensions of the half-bridges during mitosis are rather variable, perhaps because the bridge does not always break evenly in two, but by early in the subsequent interphase, half-bridge sizes have again become consistent. The functions of the half-bridge and bridge are at present only matters for speculation, but the formation of the new SPB part at the distal end of the elongated half-bridge suggests roles in both initiation and positioning of SPB formation, roles much like those postulated for the bridge in *S. cerevisiae*.

Interaction of the SPB with the Nuclear Envelope

The nuclear envelope immediately adjacent to the SPB shows some intriguing specializations. A thickening

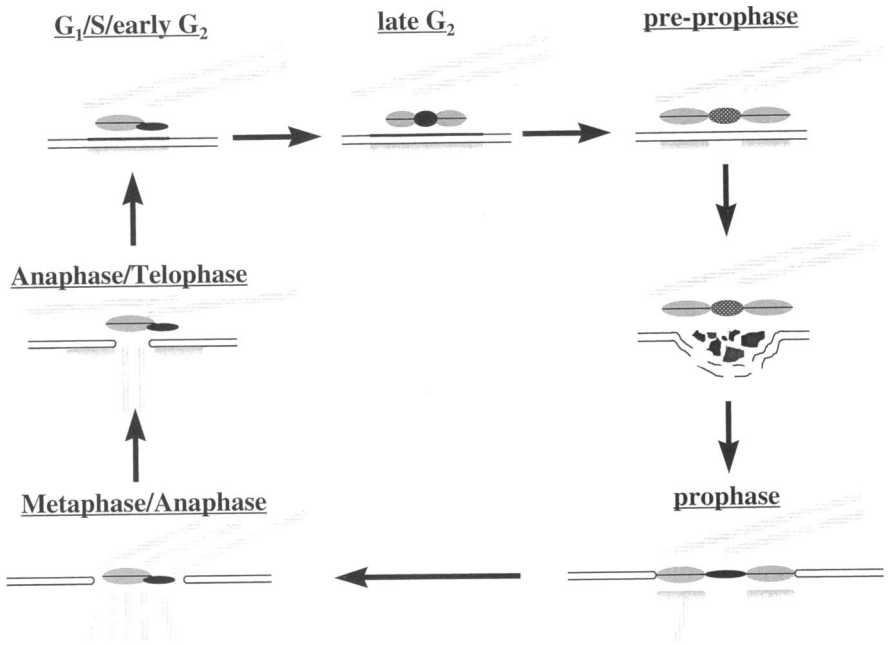

Figure 12. Diagram summarizing the SPB cycle and its relationship to the nuclear envelope during the *S. pombe* cell cycle. The SPB (shaded ellipse with line) is associated with an appendage (small solid ellipse) and lies close to a specialization on the nuclear envelope. Late in G_2 phase, the SPB duplicates to form two similar but smaller structures connected by a bridge that appears to derive from the appendage. The components of the duplicated structure then elongate, and in their vicinity, the nuclear envelope invaginates, perforates, and then receives the SPB for mitosis. The two halves of the structure separate as the spindle forms, so each occupies its own fenestra, and then as mitosis proceeds, the fenestrae close and extrude the SPB back into the cytoplasm for the next interphase.

of the inner nuclear membrane and an association of electron-dense material were previously noted for the SPB in two species of *Schizosaccharomyces* (McCully and Robinow, 1971; Heath, 1981). King and Hyams (1982) interpreted these densities as parts of the SPB itself, but the fact that the membranes of the interphase nuclear envelope run continuously through this region casts doubt on their view. Nonetheless, the persistent association of the SPB with the nuclear envelope, the material seen between the SPB and the envelope, and the localization of γ-tubulin to the dark-staining region of the interphase nucleoplasm that underlies the SPB and its bridge or half-bridge suggest that there is a complex of molecular associations that defines a structure including both the SPB and a portion of the nucleus. More detailed work with immunolocalizations using additional antibodies should help to identify the components of this complex and its role in MT dynamics.

Our work shows that the portion of the nuclear envelope that is associated with the SPB undergoes a series of cell cycle-dependent changes. Late in interphase, after the SPB has duplicated, this part of the envelope invaginates and opens to admit the duplicate SPB. The existence of a special mitotic opening of the nuclear envelope was previously reported for a different species of *Schizosaccharomyces* by Heath (1981), but this observation was disputed (King and Hyams, 1982). Our observations show that the nuclear envelope of *S. pombe* does form a fenestra to admit the SPB for mitosis. From prophase through midanaphase, the location of the SPB in fission yeast is very similar to that seen in budding yeast (Byers and Goetsch, 1975), but later in mitosis, the SPBs of *S. pombe* are extruded back into the cytoplasm. It is interesting to consider the role that nuclear fenestrae may play in facilitating the nuclear entry of proteins, like tubulin, that are necessary for mitosis.

The image of the duplicated SPB just after it has entered the nuclear envelope is particularly interesting, because this stage is the point in the cell cycle at which the SPB cycle in *S. pombe* and the analogous cycle in *S. cerevisiae* become similar. At this stage, the duplicated SPB occupies a single fenestra, and the initiation of nuclear MTs has begun. The nuclear surface of the SPBs is now associated with a particularly great accumulation of darkly staining material (Figure 7). This material may be a residue from the darkly stained material seen between the SPB and the nuclear envelope in Figures 5 and 6, or it may be the centromeric heterochromatin that has been shown by in situ hybridization to lie near the SPBs during interphase

(Funabiki *et al.*, 1993). One would expect such chromatin now to be condensing for mitosis, and this may give it a special quality of staining. The several short darkly staining lines that lie on the nucleoplasmic side of the SPBs (Figure 7, arrowheads) do resemble the mitotic kinetochores previously identified by EM in reconstructions of metaphase and early anaphase spindles (Ding *et al.*, 1993).

The SPB is not the only structure that resides in the nuclear envelop and somehow must be inserted into this double membrane structure; nuclear pore complexes (NPCs) too are added. The pathway for NPC incorporation into the nuclear envelop has not yet been described, but there are indications that the process may resemble the insertion mechanism for SPBs that is described above. The product of the *NDC1* gene localizes to spots on the nuclear envelope of *S. cerevisiae*, suggestive of an association with nuclear pores (Winey *et al.*, 1993). When a strain carrying a cold-sensitive allele of this gene is grown at restrictive temperature, the nascent SPB fails to insert properly into the nuclear envelope. The predicted sequence of Ndc1p includes six or seven putative membrane-spanning domains, consistent with the possibility that it mediates the insertion of a nascent SPB into the nuclear envelope. Given its localization to nuclear pores, it may serve a parallel role in the insertion or binding of NPCs and SPBs (Winey *et al.*, 1993). Because the SPB of *S. pombe* is also inserted into the nuclear envelope in preparation for mitosis, it is plausible that a gene like *NDC1* exists in *S. pombe* too. A candidate for this role, *cut11*$^{+}$, is now under investigation in our laboratory.

A second indication that NPCs are incorporated into the nuclear envelope by a process that resembles SPB incorporation is found in budding yeasts that carry a deletion of *NUP116*. At 36°C, this strain forms nuclear pores whose transport is inhibited by a blister of nuclear envelope that remains on the cytoplasmic side of the NPC (Wente and Blobel, 1993). We propose that NPCs are normally inserted from the nuclear side by an envelope out-pocketing, analogous to the envelope in-pocketing described herein for SPBs; in cells that are *nup116-null*, the process of removing the evaginated envelope is presumably blocked.

After the mitotic separation of the duplicate SPBs, each of the two fenestrae is somewhat larger than the SPB itself (Figure 9D). A little later in mitosis, the fenestrae become about the same size as the SPBs (Figure 10C). These results suggest that in early mitosis there are structures that anchor the SPB to the nuclear envelope and hold it in the right location. The material that lies between the SPB and the edge of the outer nuclear membrane (Figure 9D) and the bar-like structure (Figure 10C) may play roles in this anchoring process.

This study has helped to characterize the behavior of SPBs during the cell cycle of *S. pombe* and will thereby provide baseline information against which to compare the behavior of mutant strains in which the SPB cycle is perturbed. Our study will also serve as a starting point from which to characterize the processes by which structures can enter openings in the nuclear envelope. We anticipate that the processes that are dramatically seen during fenestra formation for SPB incorporation may resemble the more subtle events that allow NPCs to join an existing envelope and contribute to nuclear–cytoplasmic transport.

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