A Spacer Protein in the *Saccharomyces cerevisiae* Spindle Pole Body Whose Transcript Is Cell Cycle-Regulated

John V. Kilmartin, Susan L. Dyos, Douglas Kershaw, and John T. Finch

MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, England

Abstract. Monoclonal antibodies against the 110-kD component of the yeast spindle pole body (SPB) were used to clone the corresponding gene *SPC110*. *SPC110* is identical to *NUF1* (Mirzayan, C., C. S. Copeland, and M. Snyder. 1992. *J. Cell Biol.* 116:1319–1332). *SPC110/NUF1* has an MluI cell cycle box consensus sequence in its putative promoter region, and we found that the transcript was cell cycle regulated in a similar way to other MluI-regulated transcripts. Spc110p/Nuf1p has a long central region with a predicted coiled-coil structure. We expressed this region in *Escherichia coli* and showed by rotary shadowing that rods of the predicted length were pres-

ent. The 110-kD component is localized in the SPB to the gap between the central plaque and the sealed ends of the nuclear microtubules near the inner plaque (Rout, M., and J. V. Kilmartin. 1990. *J. Cell Biol.* 111:1913–1927). We found that rodlike structures bridge this gap. When truncations of *SPC110* with deletions in the coiled-coil region of the protein replaced the wild-type gene, the gap between the central plaque and the ends of the microtubules decreased in proportion to the size of the deletion. This suggests that Spc110p connects these two parts of the SPB together and that the coiled-coil domain acts as a spacer element.

THE spindle pole body (SPB)[1] of *Saccharomyces cerevisiae* is a complex cylindrical multi-layered organelle embedded in the nuclear envelope. One of the prime functions of the SPB is the initiation of microtubules, which takes place from either end of the cylindrical structure. Nuclear microtubules, which form the mitotic spindle, are initiated from an inner plaque and cytoplasmic microtubules from an outer plaque (Moens and Rapport, 1971; Peterson et al., 1972; Byers and Goetsch, 1975). The SPB is also capable of self-replication, which occurs early in G_1/S first by assembly of a satellite structure on the half-bridge attached to one side of the mother SPB, followed by the assembly of the new SPB around the satellite (Byers and Goetsch, 1974; Winey et al., 1991). SPBs also play key roles during both mating and spore wall formation during meiosis. During mating haploid SPBs fuse to form the larger diploid SPB whose size is then stably maintained during vegetative growth (Byers and Goetsch, 1975). Spore wall formation during meiosis is initiated by a differentiation of the outer plaque of the SPB (Moens and Rapport, 1971), which presumably ensures that the nucleus is entirely included within the spore.

An explanation of these processes in molecular terms is

in its very early stages, starting with a preliminary description of the molecular composition of the SPB, obtained by both biochemical and genetic approaches. The biochemical approach involved using an enriched preparation of SPBs to prepare monoclonal antibodies against three SPB components, which were then localized to different and discrete parts of the SPB structure by immuno-electron microscopy (Rout and Kilmartin, 1990; 1991). Genetic approaches have identified *KAR1*, a gene involved in nuclear fusion (Conde and Fink, 1976) and in spindle formation (Rose and Fink, 1987) which is localized to one of the two SPBs when overexpressed as a fusion protein (Vallen et al., 1992). A nuclear fusion screen has also identified *CIK1* which encodes a potential SPB component (Page and Snyder, 1992). A screen of temperature-sensitive mutants that have defects in SPB assembly (Winey et al., 1991) may identify other gene products involved in the assembly of the SPB.

In this paper we describe the use of the monoclonal antibodies against the 110-kD component of the SPB (Rout and Kilmartin, 1990) to clone the corresponding gene, which we call *SPC110* (*Spindle Pole Component*). During the course of this work, the sequence of the gene *NUF1* was published (Mirzayan et al., 1992). *NUF1* and *SPC110* are identical. Nuf1p was proposed to be a filamentous component of the yeast nucleoskeleton since by immunofluorescence it colocalized with the yeast nucleus, was insoluble to various extractions and had a long central region of heptad repeats (Mirzayan et al., 1992). We also noticed the heptad repeats

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1. *Abbreviations used in this paper*: SPB, spindle pole body; SPC, spindle pole component.

and set out to establish their role in the structure of the SPB. Firstly we expressed that part of the gene encoding most of the heptad repeats in *E. coli* and showed by EM that it forms rod-like structures of the predicted length. Secondly we prepared truncated versions of *SPC110*, reducing the length of the rod, and showed that when these genes replace the wild-type copy of *SPC110*, predictable changes in the structure of the SPB occur.

Materials and Methods

Cloning of SPC110/NUF1

Mixed supernatants from the nine mAbs against the 110-kD component of the SPB (Rout and Kilmartin, 1990) were used to screen 250,000 phage from a yeast genomic λgt11 library (Young and Davis, 1983). Positives were detected by reacting filters with affinity purified rabbit anti-mouse IgG and ^{125}I-protein A. 12 positive phage were plaque purified, and nine of these were found to be related as shown by restriction mapping with EcoRI, falling into three groups as represented by phages 4, 12, and 14 (Fig. 1 *a*). The other three phage reacted exclusively with only one mAb, 45H10, which was the only anti-110-kD mAb that gave detectable cross reactivity with another protein on whole-cell immunoblots (Rout and Kilmartin, 1990). Each of the EcoRI fragments in phage14 was subcloned into the EcoRI site of pBluescript and sequenced after digestion with ExoIII (Henikoff, 1984). Junctions between EcoRI fragments were sequenced using suitable oligos on the complete gene (using pJK21, see below). This sequence is available from EMBL under accession number X73297.

Immunofluorescence

Log phase cells were harvested by filtration and immediately washed and fixed with 3.7% formaldehyde in 0.1 M potassium phosphate, pH 6.5, containing 1 M sorbitol. Fixation was for 2 min for haploid strains and 5 min for diploid strains. Cells were then processed as described in Kilmartin and Adams (1984) except that cell walls were removed using 25 μg/ml Zymolyase 100T (Seikagaku, Tokyo, Japan) for 1 h at 30°C. Cells were stained overnight at 13°C with undiluted mAb supernatants, then washed and stained with FITC-labeled anti-mouse IgG (Amersham, Aylesbury, England). After washing cells were treated with 1 mg/ml rabbit IgG for 5 min and stained with rhodamine-labeled rabbit anti-yeast tubulin. Spheroplasts were prepared as described in Rout and Kilmartin (1990) and stained as above.

Expression of Spc110p Fragments in E. coli

An NsiI-EcoRV fragment of *SPC110* (containing bases 677-1263, using the same numbering system as in Mirzayan et al., 1992) was blunted at the NsiI site and inserted in the correct orientation into the blunted HindIII site in the polylinker of pMW172 (Way et al., 1990), a modified T7 expression vector (Studier et al., 1990), to give pJK135. The join between the blunted HindIII and NsiI sites was confirmed by DNA sequencing. This Spc110p (T7b) fragment would express residues 227-421. A longer fragment of Spc110p (T7c) containing residues 227-756 was prepared for EM. pJK135 was extended by cutting the insert with BglII (at 794) and the polylinker with StuI, then inserting a BglII-SspI (794-2268) fragment of *SPC110*. This was expressed as a soluble protein in *E. coli* and purified by chromatography of the *E. coli* lysate (Nagai and Thogersen, 1987) directly on hydroxylapatite (Calbiochem-Behring Corp., La Jolla, CA) using a gradient of 0.01 to 0.5 M potassium phosphate, pH 7.0, and then on DEAE cellulose (DE-52; Whatman, Maidstone, Kent, U.K.) using a gradient of 0 to 0.3 M NaCl in 20 mM Tris-Cl, pH 8.0, 1 mM EDTA, 1 mM DTT. All buffers contained 1 μg/ml leupeptin and a 1:5,000 dilution of the protease inhibitor cocktail solution P (Rout and Kilmartin, 1990). Proteolytic fragments were removed by two gel filtration steps using FPLC on Superose 6 (Pharmacia, Milton Keynes, U.K.) in 20 mM Tris-Cl, pH 8.0, 1 mM EDTA, 1 mM DTT. Only the leading edge of the peak was saved in each gel filtration step.

An NH$_2$-terminal fragment of Spc110p (residues 1-215, GSTa) was expressed as a fusion protein in pGEX-3X (Smith and Johnson, 1988). An NdeI site was inserted between bases −2 and 3 by oligonucleotide mutagenesis (Kunkel et al., 1987), and an NdeI (blunted)-EcoRI fragment of *SPC110* containing bases −2 to 646 was inserted into the polylinker of PstI (blunted)-EcoRI cut pBluescript. The insert was cut out with EcoRI and BamHI and inserted in frame into BamHI-EcoRI cut pGEX-3X.

Epitope Mapping

E. coli strains carrying the expression constructs were induced with IPTG for 4 h to express the fragments GSTa, T7b, and T7c, boiled in SDS sample buffer and separated by SDS-gradient gel electrophoresis. Immunoblots were stained with undiluted mAb supernatants.

Further epitope mapping was carried out by immunofluorescence of SPBs in spheroplasts, fixed with methanol and acetone, which contained truncations of *SPC110* replacing the wild-type gene (see later in Results). All the anti-110-kD mAbs except 3D2 and 45D10 stained Δ489-710. 24A8 did not stain Δ266-303 or any of the other following longer truncations, 35A11 stained Δ266-303 but not Δ266-412 or longer truncations, 36G6 and 3D2 stained Δ266-412 and Δ216-452, but not Δ266-544 or the following two truncations, 45D10 stained Δ216-648 but not Δ216-710. 30G11 and 45H10 stained SPBs in all of these strains.

Northern Blots

Yeast cells were synchronized by α-factor release using *bar1-1* cells (Nasmyth et al., 1990). Total RNA was prepared (Nasmyth, 1985), electrophoresed in a 1% formaldehyde agarose gel (Sambrook et al., 1989), and blotted onto Hybond-N (Amersham, Amersham, U.K.). The transcript of *SPC110* is of low abundance and could only be reliably detected using a single-stranded probe (a HindIII-EcoRI fragment from the 5′ end) labeled with both ^{32}P-dATP and ^{32}P-dCTP (3,000 Ci/mmole). Both the *URA3* and *CDC9* transcripts were detected with random primed probes: a 1.2-kb HindIII fragment for *URA3* and a 2.3-kb PstI fragment for *CDC9* (Barker and Johnston, 1983).

Construction of Truncated SPC110/NUF1 Genes

λgt11 phage 14 was cut with XmnI to release a 4.8-kb fragment containing part of *TRP4* and the whole of *SPC110*, which was cloned into the EcoRV site of pBluescript to give pJK21. Then a series of ExoIII deletions of the 3.0-kb EcoRI fragment cut in the polylinker with XhoI and ApaI which extended from the EcoRI site at 878 were ligated to either the blunted EcoRI site at 643 or the blunted BglII site at 794 so that the open reading frame was maintained. Deletions ending at 1315, 1357, 1801, 1945, and 2131 were ligated to the blunted EcoRI site to make truncations called Δ216-438, Δ216-452, Δ216-600, Δ216-648, and Δ216-710. Deletions ending at 908, 1235, and a blunted NdeI fragment from 1632 were ligated to the blunted BglII site to make truncations called Δ266-303, Δ266-412, and Δ266-544. The constructs for these were made by cutting at the KpnI site in the polylinker adjacent to the start of the deletions (no other polylinker sites were present on this side, though the first G of the ApaI site is still present), the cut was blunted and the fragment released by cleavage at SalI (3382) or XbaI (3679). For the truncations Δ266-303 and Δ266-412 the ExoIII fragments were ligated into BglII (blunted)-XbaI cut pJK21, for Δ266-544 the NdeI site at −215 was removed by cutting pJK21 with PstI in the polylinker and MluI at −142, blunting and religation, and then inserting the ExoIII fragment as above. For the truncations Δ216-438, Δ216-600, Δ216-648, and Δ216-710, the EcoRI sites at −757 and −430 were removed by cutting pJK21 with ClaI in the polylinker and BstEII at −323, blunting and religation, then insertion of the ExoIII fragments into this vector cut with EcoRI and blunted then cut with XbaI in the polylinker. These truncations were removed by cutting the pBluescript polylinker with XbaI and blunting then cutting with XhoI and transferring to EagI (blunted)-XhoI cut pRS304 (Sikorski and Hieter, 1989), a pBluescript-based *TRP1* yeast integrating vector. The truncation Δ216-452 was made in a three way ligation of the ExoIII fragment with a SacI-blunted EcoRI fragment (−225/643) of pJK21 and SacI-SalI cut pRS304. The ligation of the blunted EcoRI or BglII and KpnI sites would insert TCG coding for a serine residue between the two parts of *SPC110*. A wild-type control was prepared by cutting pJK21 at 3679 with XbaI and blunting then cutting with XhoI in the polylinker and inserting this fragment into EagI (blunted)-XhoI cut pRS304. The construct for the Δ489-710 deletion was made in a three way ligation with the ExoIII fragment ending at 2131 blunted at the KpnI site and cut with SalI in the polylinker, a SacI-ScaI fragment (−225/1465) of pJK21 and SacI-SalI cut pRS304. Ligation of ScaI and blunted KpnI sites maintains the open reading frame and inserts a serine residue. All ligations involving blunted restriction enzyme sites were confirmed by sequencing.

Preparation of Yeast Strains Containing Truncated Forms of SPC110/NUF1

A near complete disruption of *SPC110/NUF1* was prepared by inserting the

LEU2 gene between the EcoRV site at −60 and an ExoIII fragment whose 5' end started at 2810. This removed all but seven COOH-terminal residues of Spc110p (note that this line in the sequence of Mirzayan et al. [1992] is incorrectly numbered, it should be 2791 rather than 2891). The construct for this was prepared by cutting pJK21 with EcoRV and SalI in the poly-linker at the 3' end, and inserting a blunted SalI-XhoI fragment of LEU2 obtained from the vector pIC19H (Marsh et al., 1984) which had a SalI-XhoI fragment of LEU2 cloned into the SalI site of the polylinker. The SmaI and BglII sites on the 3' side of this polylinker were cut and the blunted KpnI-BamHI ExoIII fragment inserted. A linear fragment was obtained by cutting with BsmI (at −433) and SnaBI (at 3053) and used to disrupt the SPC110 gene by the one step gene replacement method (Rothstein, 1983). The fragment was used to transform the diploid strain K842 to Leu⁺. All manipulations were carried out in K699 or K842, which is an isogenic diploid of K699 (Nasmyth et al., 1990). Southern blotting was used to check that the correct integration event had occurred (data not shown). Sporulation and dissection of this diploid showed that SPC110 was an essential gene, as was also found by Mirzayan et al. (1992). The lethality was rescued by transformation of the disrupted diploid with the wild-type gene in the URA3 CEN vector pRS316 (Sikorski and Hieter, 1989). After sporulation and dissection Leu⁺ Ura⁺ spores were obtained which contained the disrupted SPC110 gene rescued by the wild-type gene on the URA3 CEN plasmid. The Leu⁺ Ura⁺ progeny were transformed with the wild-type or truncated SPC110 genes in the TRP1 integrating vector pRS304 cut at the XbaI site in TRP1 so that integration was directed to the Trp1 locus. The wild-type gene on the URA3 plasmid was then removed by fluoroorotic acid selection (Boeke et al., 1987). In the absence of a rescuing SPC110 construct carried on the TRP1 vector these cells did not survive in fluoroorotic acid medium. Southern blotting was used to select survivors with only one copy of wild-type or truncated SPC110 integrated at the Trp1 locus and to establish that the correctly sized truncation was present. In addition, some strains containing truncations were prepared by transformation of the disrupted diploid with the SPC110 truncations in the URA3 CEN vector pRS316, followed by sporulation and selection of Leu⁺ Ura⁺ spores.

Preparation of Enriched SPBs

The nuclei preparation used in the SPB enrichment procedure developed for S. uvarum (Rout and Kilmartin, 1990) was modified slightly to handle S. cerevisiae strains. Cells were digested with twice the amount of glusulase (DuPont Corp., Boston, MA), and spheroplasts were resuspended very gently after digestion, spheroplast lysis was carried out at low Polytron (Kinematica, Luzern, Switzerland) speeds to minimize nuclear lysis. After nuclear extraction the SPBs were separated on a sucrose gradient (Rout and Kilmartin, 1990) and those banding at the 2.0/2.25 M layers were used for EM. The extent of SPB enrichment was not as good as in S. uvarum.

Electron Microscopy

SPBs prepared as described above were pelleted and embedded in hard Spurr's resin, and sections between 40–50 nm were cut. SPBs sectioned close to the perpendicular were selected on the basis of the appearance of the central plaque and the intermediate lines. Distances between the sealed ends of the nuclear microtubules and the nearest edge of the central plaque were measured relative to the average microtubule diameter on that section, which was set at 25 nm (Moens and Rapport, 1971). Rotary shadowing essentially followed the method of Tyler and Branton (1980). Equal volumes of 50% glycerol and sample (20 μg/ml) in 500 mM NaCl, 20 mM Tris-Cl, pH 8.0, and 1 mM DTT were sprayed onto freshly cleaved mica, vacuum dried for 2 h, and rotary shadowed with platinum at an angle of 6°.

Results

Cloning of SPC110 with mAbs Against the 110-kD Component of the SPB

The pooled mAbs against the 110-kD component of the SPB (Rout and Kilmartin, 1990) were used to screen a λgt11 expression bank to clone the corresponding gene. Three related classes of positive phage were found (Fig. 1 a) with different sized inserts, the largest of which was in phage 14. This insert was sequenced and a long open reading frame encoding a gene product of 112 kD was found which we called
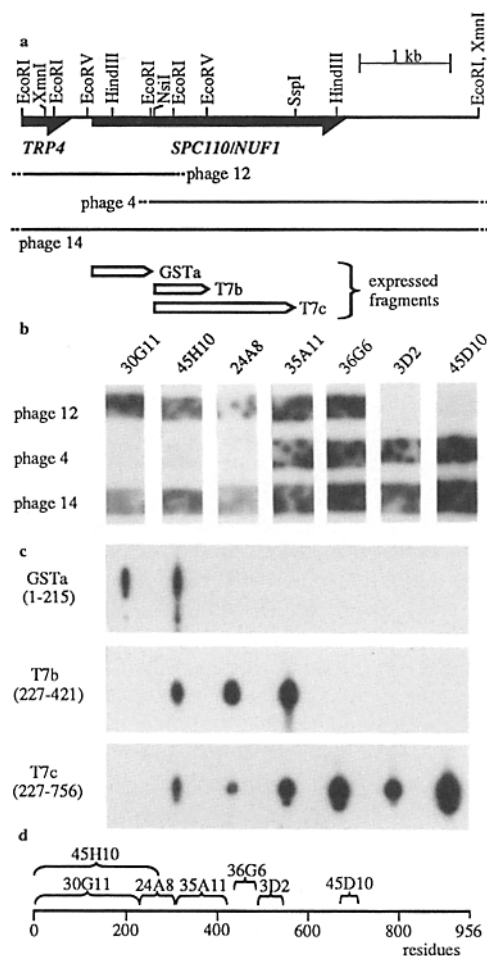

Figure 1. (a) Restriction map of SPC110/NUF1 and part of TRP4, diagram of the inserts of λgt11 phage isolated by screening an expression bank with mAbs against the 110-kD component of the SPB, and diagram of fragments expressed in E. coli. Other sites for EcoRV, NsiI, and SspI are present. (b) Immunoblots of the individual anti-110–kD mAbs with nitrocellulose filters containing E. coli lysates induced by each of the three phage. (c) Immunoblots of the anti-110–kD mAbs with fragments of SPC110 expressed in E. coli. These were: GSTa, a fusion of residues 1-215 with glutathione S-transferase, which had an apparent molecular weight of 51 kD compared with the expected molecular weight of 52 kD; T7b and T7c, using the T7 vector to express residues 227-421 and 227-756, which had apparent molecular weights of 26.5 and 65 kD compared to the expected molecular weights of 23.8 and 64 kD. (d) Tentative epitope map of the anti-110–kD mAbs based on the reactivity of the mAbs with the phage in b, the expressed fragments in c, and also the immunofluorescent staining of yeast strains expressing truncations of SPC110 (see Materials and Methods).

SPC110. To check that the correct gene had been cloned we used the isolated phage and expressed fragments of SPC110 to define the epitopes of the anti-110–kD mAbs. Screening expression banks with mAbs poses special problems since the epitope or part of the epitope for a particular mAb can be a short piece of sequence and thus present on unrelated proteins (Wehland et al., 1984), thus it was important to show that there were multiple epitopes in the encoded protein.

When the reactivity of the individual anti-110–kD mAbs was tested against these different phage, it was found that, as expected, all the mAbs reacted with phage 14 containing the complete insert (Fig. 1 b). However, there were clear

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differences in the reactivity with the phage containing incomplete inserts showing that these mAbs defined at least three different epitopes. This was confirmed and extended by testing the reactivity of the anti-110-kD mAbs against three expressed fragments of *SPC110:* GSTa containing residues 1-215 and T7b and T7c containing residues 227-421 and 227-756, respectively. These immunoblots (Fig. 1 *c*) suggested six different epitopes, since there are differences between 30G11, 45H10, and 24A8 and also between 35A11 and 36G6. Immunofluorescent staining of SPBs in yeast spheroplasts fixed with acetone and methanol was used to confirm and further specify the epitopes (see Materials and Methods). These spheroplasts contained deletions of the wild-type *SPC110* rescued by various truncations of *SPC110.* We assume that antigen accessibility is not affected in these strains. All these results suggest that the nine anti-110-kD mAbs specify seven different epitopes, which could be tentatively arranged as in Fig. 1 *d.* We were not able to distinguish the epitopes of 30G11 from 48G4 and 24A8 from 23G3. 45H10 may have a repeating epitope (possibly LESKL at residues 138-142 and 393-397) since it reacts with both the nonoverlapping constructs GSTa and T7b. 36G6 probably has another epitope in the NH_2-terminal region which is conformationally dependent since it reacts with phage 12 (which should encode up to around residue 293) but not T7b or GSTa.

The chances of an unrelated protein containing all of these seven different epitopes are remote, strongly indicating that the insert in phage 14 encodes the gene product for the 110-kD component of the SPB. Furthermore, as shown later on in Results, when the wild-type *SPC110* gene was replaced by truncated versions, immunoblots of enriched SPB preparations from these strains showed that the 110-kD band stained with these mAbs disappeared and was replaced by a lower molecular weight band of the appropriate size (see Fig. 7 *b*).

Immunofluorescent Staining Pattern with Antibodies against Spc110p/Nuf1p

While this work was in progress, the sequence of *NUF1* was published (Mirzayan et al., 1992), it is identical to *SPC110*, apart from the very first eight bases of the 5' noncoding sequence. Our antibodies to Spc110p clearly identify it as an SPB component (Rout and Kilmartin, 1990), yet antibodies to the identical protein Nuf1p stain the yeast nucleus (Mirzayan et al., 1992). We believe that the differences between the two staining patterns are probably due to the different fixation conditions used. Mirzayan et al. (1992) used formaldehyde for fixation, whereas we used methanol and acetone on spheroplasts since the binding of mAbs to SPBs was very susceptible to formaldehyde fixation (Rout and Kilmartin, 1990). We have re-examined the staining patterns of the different mAbs in cells after brief formaldehyde fixation (column labeled intact cells in Fig. 2) and found three types of staining pattern. The first consists of SPB staining and relatively weak nuclear staining given by mAbs 24A8, 30G11, and 35A11 (Fig. 2 *A*), the second is SPB staining with relatively stronger nuclear staining given by mAbs 3D2 and 36G6 (not shown), and the third is nuclear staining only given by mAb 45D10 (Fig. 2 *B*). This latter staining pattern is similar to that obtained by Mirzayan et al. (1992), the intensity of staining seen with 45D10 is weaker because only a single epitope is binding to the first antibody. All the mAbs

gave only SPB staining in spheroplasts fixed with methanol and acetone (Fig. 2, *A* and *B*) as was found previously (Rout and Kilmartin, 1990). The three mAbs (3D2, 36G6, and 45D10) which give the stronger nuclear staining pattern in formaldehyde fixed cells appear to have distinct epitopes (Fig. 1) making it very unlikely that they are staining an unrelated protein. We attempted to confirm this by using the same three mAbs to stain formaldehyde fixed cells containing a truncation of *SPC110* (Δ216-710 described later; see Fig. 7 *a*) in which the epitopes for these three mAbs would be absent. No nuclear staining was observed in these cells, however a clear conclusion could not be drawn since other truncations gave very variable nuclear staining with the same three mAbs, for example Δ216-438 gave stronger nuclear staining than wild-type cells while Δ216-452 and Δ489-710 gave SPB staining but undetectable nuclear staining (data not shown).

The intensity of both SPB and nuclear staining was highest in cells given a brief period (2-5 min) of formaldehyde fixation and declined in intensity until at 30 min (10 min for spheroplasts) both SPB and nuclear staining were absent. We assume that the lack of SPB staining after prolonged formaldehyde fixation is due to cross-linking of the densely packed proteins in and around the organelle, thereby blocking access to antibodies (Rout and Kilmartin, 1990). This may be a general phenomenon, since others have found that immunofluorescent staining of components of the yeast nuclear pore complex, parts of which are densely packed, also show a variable and sometimes very high susceptibility to formaldehyde fixation (Wente et al., 1992). In the case of the SPB, both mAbs and polyclonal antibodies behave similarly since SPB staining by affinity-purified polyclonal antibodies to fragment T7b shows the same susceptibility to formaldehyde fixation (data not shown). The absence of SPB staining by 45D10 in formaldehyde fixed intact cells is presumably caused by the uniquely high susceptibility of its epitope to formaldehyde fixation. Thus brief fixation of spheroplasts with formaldehyde completely removes SPB staining by 45D10 (Fig. 2 *B*). This susceptibility to formaldehyde fixation probably accounts for why Mirzayan et al. (1992) failed to observe SPB staining with their anti-Nuf1p antibodies. In conclusion, it seems likely that Spc110p/Nuf1p is present both in the SPB and in the nucleus, and that its appearance is fixation dependent.

Some Features of the SPC110/NUF1 Sequence

The most striking feature of the *SPC110/NUF1* sequence is the central domain of heptad repeats (Mirzayan et al., 1992). There are some other interesting features of the sequence which were not commented upon by Mirzayan et al. (1992). Firstly, on the 5' side of the gene, from position −759 to −58, we found an exact match (Fig. 3) with the *TRP4* sequence (Furter et al., 1986). This indicates that the gene maps close to *TRP4* on chromosome IV. The cell cycle gene *CDC40* is tightly linked to *TRP4* (Kassir et al., 1985), but *SPC110* is unlikely to be *CDC40* because their restriction maps are quite different (Kassir et al., 1985). Secondly, there is an exact fit to an MluI cell cycle box consensus sequence ACGCGTNA (Johnston et al., 1991; McIntosh et al., 1991) at positions −144 to −137 in the promoter region, assuming that translation starts at the first ATG of the open reading frame. This position is within the range of about −100 to
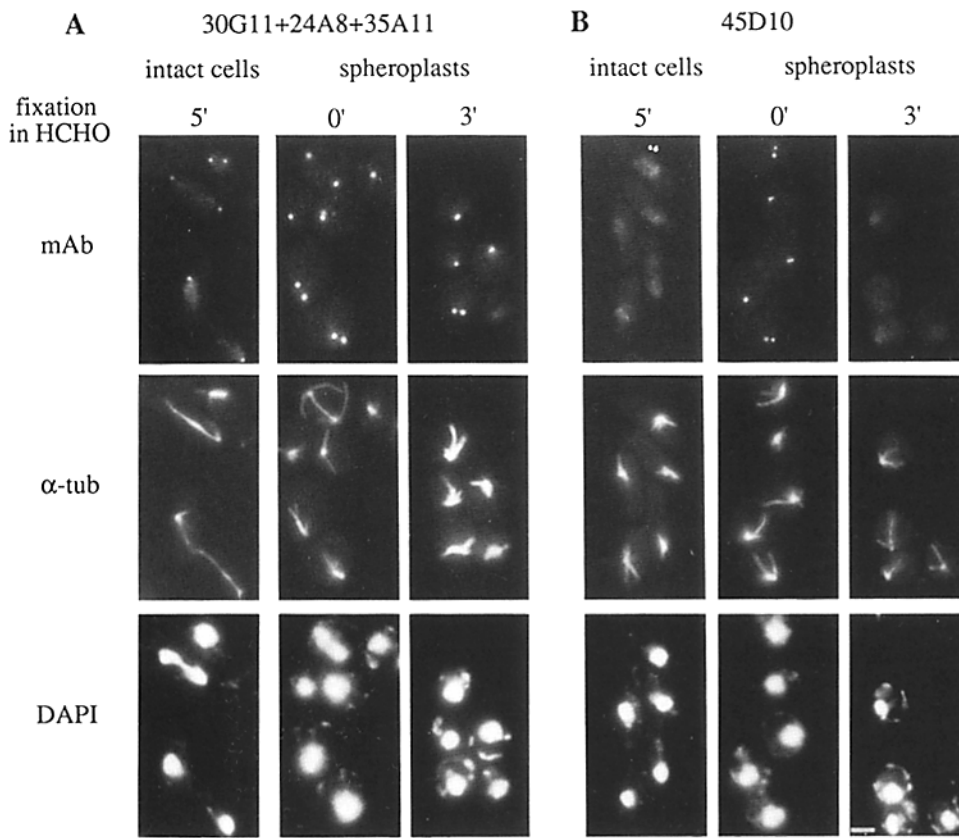
A 30G11+24A8+35A11

B 45D10

intact cells   spheroplasts   intact cells   spheroplasts

fixation in HCHO   5'   0'   3'   5'   0'   3'

mAb

α-tub

DAPI

Figure 2. Immunofluorescent staining of a mixture of anti-110–kD mAbs (*30G11, 24A8,* and *35A11*) which give SPB staining and relatively weaker nuclear staining (*A*), and one mAb (*45D10*) which gives either nuclear or SPB staining depending on the fixation conditions (*B*). Cells (*S. uvarum* NCYC 74) were triple labeled with the mAb (*top*), rabbit anti-yeast tubulin (*middle*), and DAPI (*bottom*). All the cells shown were fixed in methanol and acetone. In addition the intact cells were fixed beforehand for 5 min with formaldehyde (*left* column in *A* and *B*), or spheroplasts were fixed for 3 min beforehand with formaldehyde (*right* column in *A* and *B*). Bar, 2.5 μm.

about −250 occupied by other MluI cell cycle boxes (Johnson et al., 1991; McIntosh et al., 1991). To test whether this sequence might be causing cell cycle-regulated transcription of *SPC110,* we carried out Northern blots to identify the transcript and check its regulation in cells synchronized with α-factor (Fig. 4). A single band was obtained in Northern blots which migrated just ahead of the 25S ribosomal RNA band (3.4 kb), suggesting a size of about 3 kb (data not shown). This is close to the correct size required to transcribe the proposed open reading frame of 2,935 bases. Cells synchronized with α-factor show cell cycle regulation of the transcript (Fig. 4) whose phase is close to that of another MluI-regulated gene *CDC9* (White et al., 1986).

Structure of the Central Region of Spc110p

When the sequence of Spc110p/Nuf1p was compared to other proteins in the data base using the FASTA program (Pearson and Lipman, 1988) the similarity of its central region to other proteins with coiled-coil domains was clear. Mirzayan

et al. (1992) used such an alignment with the *Dictyostelium discoideum* myosin heavy chain to predict eleven regions of coiled-coil between residues 165 and 791. There were nine insertions of varying length and one single amino acid deletion separating the coiled–coil regions, though they suggested that since there were no prolines in this region, it might be a single long coiled–coil. We found that it was possible to line up residues 168 (starting at position d) to 773 with the coiled–coil periodicity of 28 residue repeats using four stutters of four residues (after residues 420, 480, 512, and 544), three of one residue (after residues 223, 604, and 661) and one single residue deletion (after residue 717). These stutters and deletion are commonly found in other coiled–coil proteins (Cohen and Parry, 1990), suggesting that the entire region between 168 and 773 is a single coiled–coil. This was tested by examining the structure of a fragment of Spc110p containing residues 227-756 which was expressed in *E. coli* (Fig. 5 *a*). We used the T7 vector (Studier et al., 1990) because only a few extra amino acids would be added to the NH₂- and COOH-termini (MGS-MVSL to the N terminus, PLV to the COOH-terminus). In collaboration with Ian Fearnley (MRC Laboratory of Mo-

TRP4:
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I  K  A  E  E  S  I  H  S  G  N    365
TTAAGGCAGAAGAAAGCATACATTCTGGTAATG  -265
A  L  R  S  L  E  H  F  I  D  S    376
CATTACGTTCTTTAGAACACTTTATAGATAGTG  -232
V  S  S  L                         380
TGAGCTCCTTGTAGACATTATTTCATATGTTAT  -199
ACTTAAACTAGATAGAGACGTTTTATTTGTACT  -166
                 MluI
AAGCTGAACATTACCCTAATTACGCGTTATAAG  -133
TTTTAATCCTTCATTTTGAAGGAAAACAAATAA  -100
ACAAAAAGTTGATGTGGAACAAATACAATGTGC   -67
GCGGATATCTTAAGCAACTTCGTTAGTTACAGT   -34
CCACACAAAGCGAAAAAGGGCTGATAAACACTC    -1
SPC110/NUF1:
M  D  E  A  S  H  L  P  N  G  S     11
ATGGACGAAGCGTCACATCTCCCAAATGGGAGC   33
L  K  N  M  E  F  T  P  V  G  F     22
TTGAAGAACATGGAATTTACGCCTGTAGGATTT   66
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Figure 3. Some features of the *SPC110/NUF1* sequence showing the nearby *TRP4* gene and the MluI cell cycle box consensus sequence. These sequence data are available from EMBL under accession number X73297.
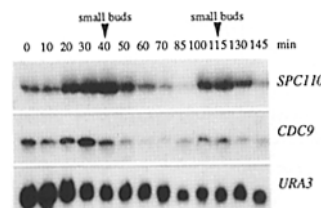

small buds   small buds

0 10 20 30 40 50 60 70 85 100 115 130 145   min

SPC110

CDC9

URA3

Figure 4. Northern blots of total RNA isolated from cells synchronized with α-factor using probes against *SPC110* (*top*), *CDC9* (*middle*), and *URA3* (*bottom*). Arrowheads indicate when small buds appear.

Figure 6. Thin section of a preparation of DEAE–dextran extracted SPBs (Rout and Kilmartin, 1990) showing rodlike structures (arrowheads) connecting the central and inner plaques. Bar, 0.1 μm.
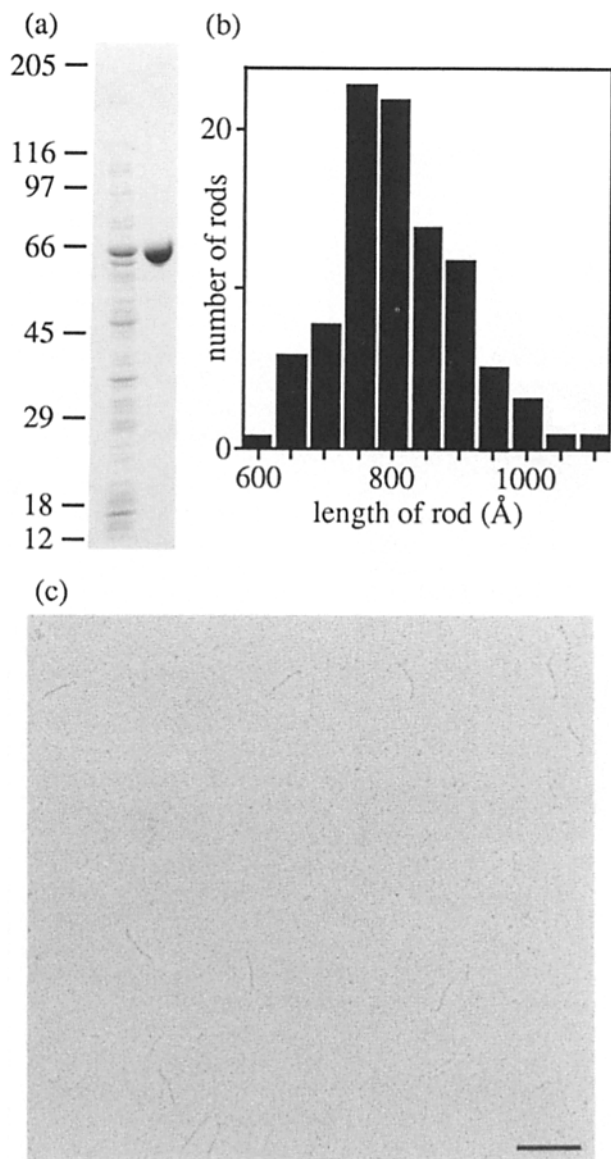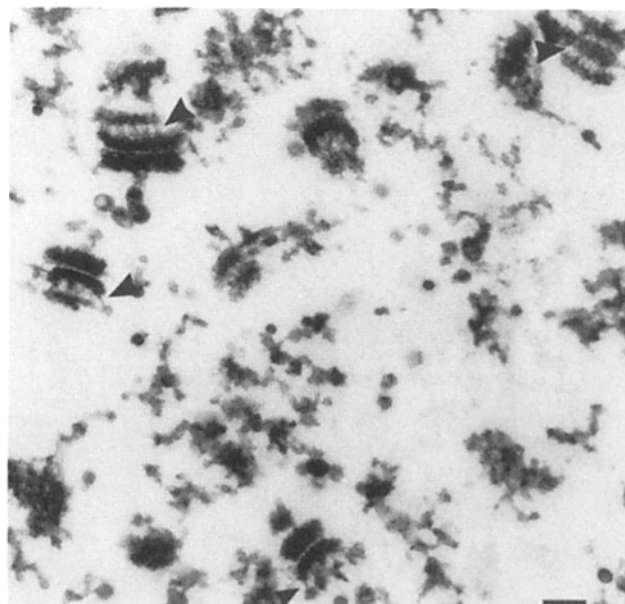
Figure 5. Expression and EM of fragment T7c (residues 227-756) containing most of the predicted coiled–coil region of Spc110p (see Fig. 7 a). (a) SDS gradient gel of a lysate of E. coli cells expressing T7c (left lane) and purified T7c (right lane). (b) Histogram of the distribution of rod lengths observed by rotary shadowing. The average rod length was 783 ± 94 Å. (c) Micrograph of rotary shadowed T7c rods. Bar, 0.1 μm.

lecular Biology, Cambridge) we checked that this was the probable composition of the molecule by accurate measurement of its molecular weight by electro-spray mass spectrometry (Skehel et al., 1991). This gave a mass of 63,695 ± 4, compared to the calculated molecular weight 63,689 (assuming that the NH₂-terminal methionine is removed), indicating that the deduced sequence is probably correct. Examination of the Spc110p fragment by rotary shadowing (Fig. 5 c) shows rods of an average length of 783 ± 94 Å (Fig. 5 b). This is in reasonable agreement with the expected length of 795 Å assuming 1.5 Å/residue as in other coiled–coil proteins (Fraser and MacRae, 1973).

Spc110p Is a Spacer Protein between the Central and Inner Plaques of the SPB

Since Spc110p probably contains a central rod region and is localized in the SPB between the nuclear side of the central plaque and the inner plaque (Rout and Kilmartin, 1990), is there any evidence for rods in this part of the SPB? Examination of SPBs extracted with DEAE–dextran (Rout and Kilmartin, 1990) showed fibrous material connecting the central and inner plaques (arrowheads in Fig. 6), which might be aggregates of Spc110p rods. These results suggest a model whereby Spc110p connects the two plaques with the central rod region possibly acting as a linker or spacer protein. We tested this by preparing a series of deletions of the central rod region of Spc110p (Fig. 7 a). Three removed about half the rod, two from the NH₂-terminal half (Δ216-438 and Δ216-452), and one from the COOH-terminal end (Δ489-710). Another deletion removed about three quarters of the rod from the NH₂-terminal end (Δ216-600), and the last removed most of the rod (Δ216-710). These constructs were used to replace the wild-type SPC110 gene either by plasmid shuffling (Boeke et al., 1987), or by transformation of a diploid containing a single deleted SPC110 followed by sporulation (see Materials and Methods). All these strains containing truncated SPC110 genes appeared to grow at close to normal rates, except Δ216-710 which grew more slowly. Further deletions into the COOH terminus (data not shown) produced either a severe growth phenotype (Δ489-809) or were lethal (Δ489-824). Immunoblots of enriched SPB preparations from each of the strains with normal growth rates using the anti-110–kD mAbs showed that the apparent molecular weight of the antigen had decreased to approximately the correct size (Fig. 7 b). Thin sections of SPBs isolated from strains containing truncations of the rod region of Spc110p were examined and the distance measured between the nuclear edge of the central plaque and the sealed ends of the nuclear microtubules (Byers et al., 1978), which
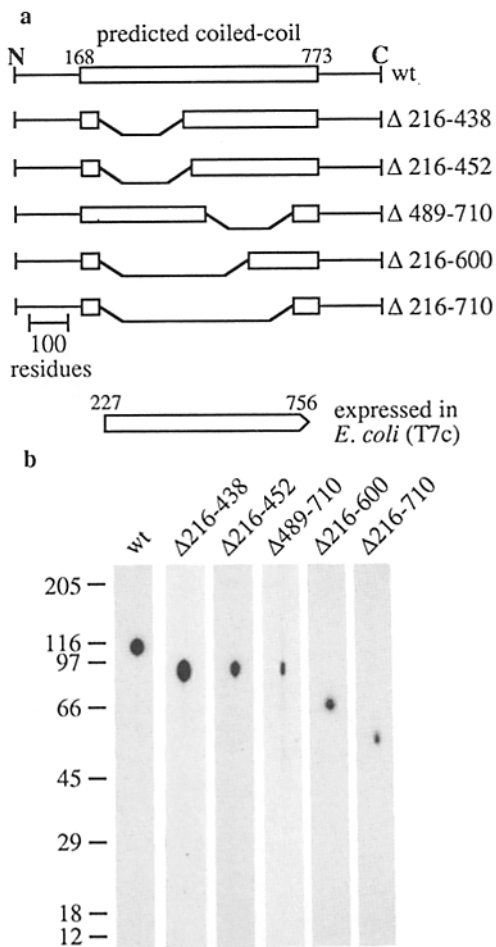
Figure 7. (a) Diagram of the deletions of the coiled–coil region of Spc110p which were used to replace the wild-type gene. At the bottom is shown the fragment of the coiled-coil region of Spc110p expressed in E. coli (T7c). (b) Immunoblots of strains with these deletions stained with the pooled anti-110–kD mAbs. The signals from Δ216-438, Δ216-452, Δ489-710, Δ216-600, and Δ216-710 gave apparent molecular weights of 90, 92, 90, 69, and 53 kD compared to the expected molecular weights of 86, 84, 86, 65, and 54 kD, respectively.

are near the somewhat ill-defined inner plaque position (Rout and Kilmartin, 1990). Most measurements were taken from integrated truncations, though indistinguishable results were obtained from truncations on CEN plasmids. Examples of the different classes of SPBs are shown in Fig. 8. Small fields are also shown to indicate that the distances between the nuclear edge of the central plaque and the microtubule sealed ends (Fig. 8 b, arrowheads) are consistent within each field (Fig. 8 b). These distances are plotted for a larger number of SPBs in Fig. 9. It can be seen that as the length of the central coiled-coil rod of Spc110p is decreased, the distance between the central plaque and the microtubule also decreases, suggesting that the rod acts a spacer element in the SPB.

Discussion

Cell Cycle Regulation of the SPC110/NUF1 Transcript

We have cloned the gene encoding the 110-kD component of

the SPB (Rout and Kilmartin, 1990), SPC110, and found that it is identical to NUF1 (Mirzayan et al., 1992). Upstream from the coding sequence there is an MluI cell cycle box consensus sequence ACGCGTNA within the appropriate part of the putative promoter region (Johnston et al., 1991; McIntosh et al., 1991). This sequence is probably causing the cell cycle regulation of the SPC110NUF1 transcript (Fig. 4); indeed the sequence ACGCGTTAA, which is a close match to the sequence ACGCGTTAT in SPC110, was sufficient to cause cell cycle regulation of a reporter gene (McIntosh et al., 1991). So far all the genes which are cell cycle regulated by this sequence are involved in DNA synthesis (Johnston et al., 1991). However, it seems reasonable for a structural component of the SPB to make use of the same system, since the SPB is assembled after the G_1/S boundary during S phase (Byers, 1981). There are some ts mutants, such as cdc4, where S phase is not initiated but SPBs are still assembled (Byers and Goetsch, 1974). However in this mutant, despite the absence of S phase, the MluI-regulated DNA synthesis genes are still transcriptionally regulated (White et al., 1987).

The nuclear staining pattern given by antibodies to Spc110p/Nuf1p in formaldehyde fixed cells suggests that either there is surplus product in the nucleus or the protein may play a dual role both in the SPB and as a filamentous component of the nuclear skeleton as proposed by Mirzayan et al. (1992). If the nuclear staining represents some surplus product, then why is it necessary to regulate the transcript? This question seems to be a general problem with other MluI-regulated genes. Thus, although the CDC9 (DNA ligase) transcript is relatively tightly regulated, DNA ligase is a very stable enzyme which persists throughout the cell cycle and there is only a transient twofold increase in the specific activity during S phase (Johnson et al., 1986; White et al., 1986). This might provide some selective advantage, by providing extra protein at the time it is needed.

The cell cycle–regulated control of the SPC110 transcript seems unlikely to be essential for the control of the assembly of the SPB. This is because in SWI6-deleted cells the MluI system is almost inoperative and transcripts are constitutively synthesized (Dirick et al., 1992; Lowndes et al., 1992), yet growth and therefore presumably normal assembly of the SPB continues.

Role of Spc110p in the Morphology of the SPB

The deduced amino acid sequence of Spc110p suggests a coiled-coil structure for about 60 kD of the central part of the protein, and when this part of the protein was expressed in E. coli we found by rotary shadowing that rods of about the predicted length were present. Spc110p is localized in the SPB to a space between the central plaque and the inner nuclear plaque (Rout and Kilmartin, 1990) near where the sealed ends (Byers et al., 1978) of the nuclear microtubules are situated. EM of thin sections of the SPB show that there are filamentous structures which bridge the gap between the two plaques in DEAE–dextran extracted SPBs (Fig. 6), and in the gap between the central plaque and the microtubule sealed ends in whole SPBs (Fig. 8). This gap is between 500 and up to 700 Å when measured from thin sections of SPBs (Moens and Rapport, 1971; Peterson et al., 1972; Byers and Goetsch, 1974, 1975). Thus it would be possible for the
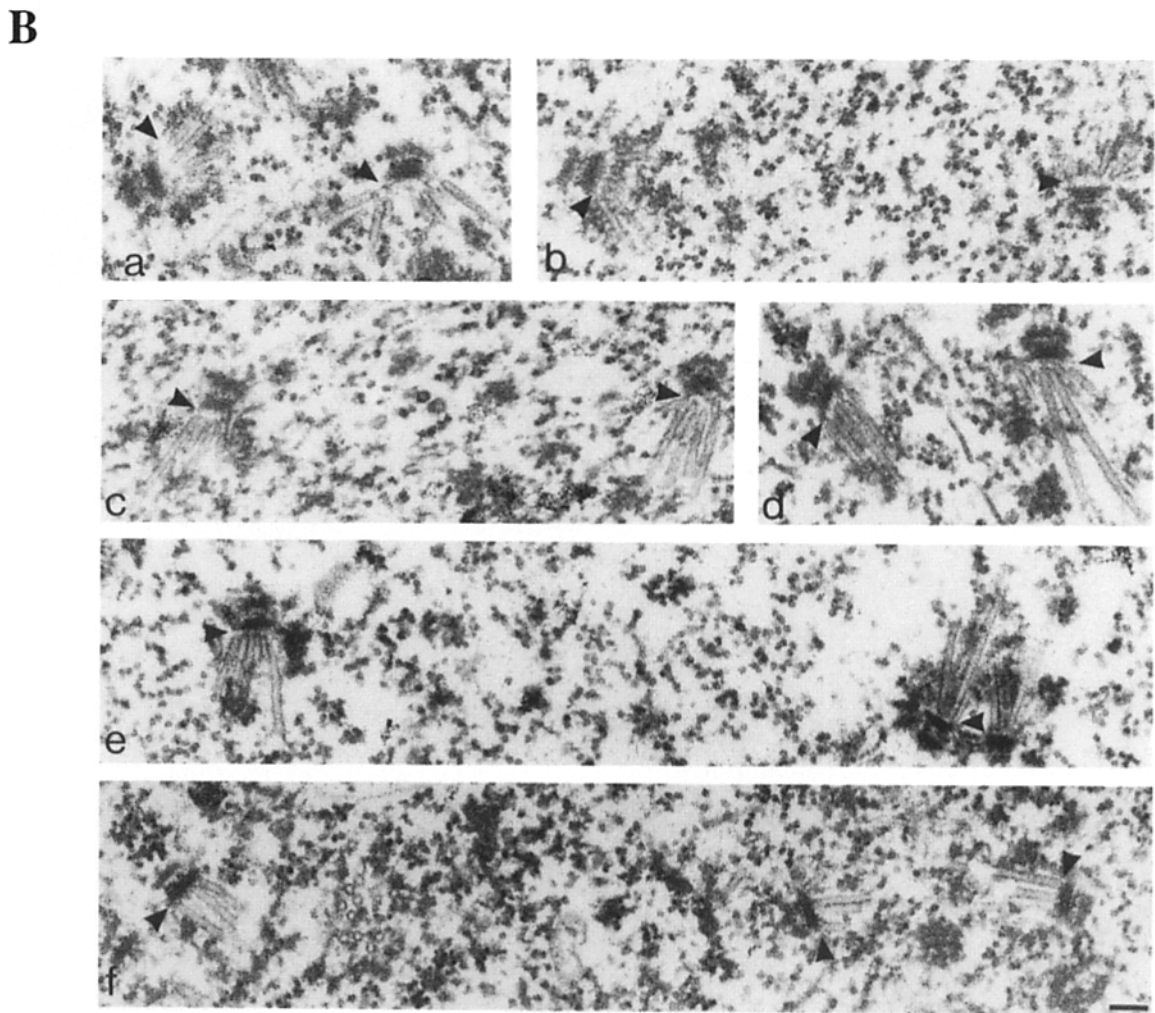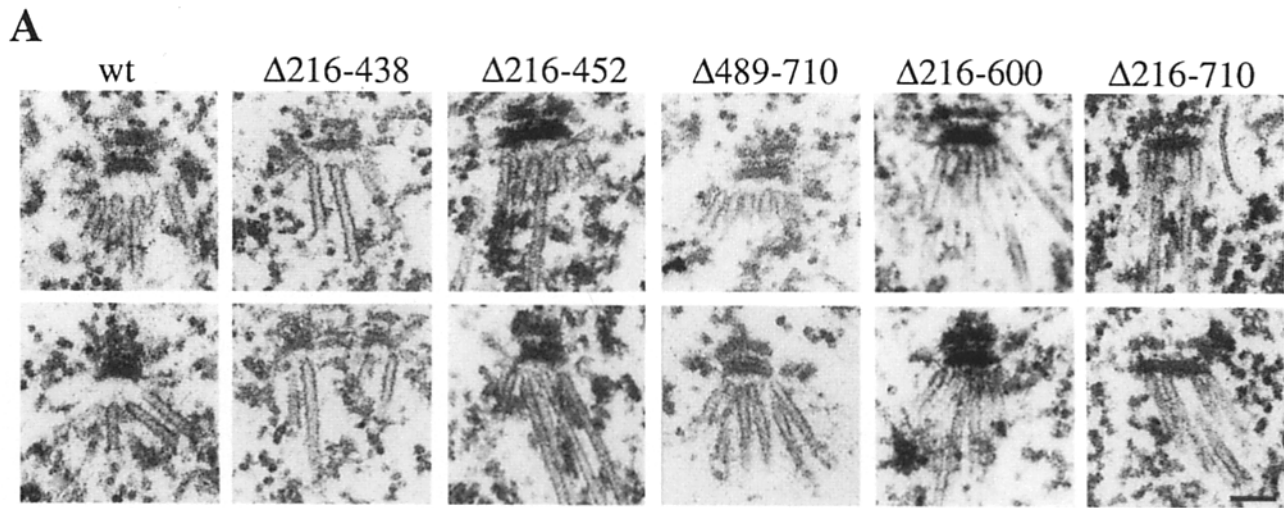
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A

| wt | Δ216-438 | Δ216-452 | Δ489-710 | Δ216-600 | Δ216-710 |

B

Figure 8. (*A*) Pairs of individual isolated SPBs containing deletions of the coiled–coil region of Spc110p. (*B*) Small fields of isolated SPBs, (*a*) wild-type rescue, (*b*) Δ216-438, (*c*) Δ216-452, (*d*) Δ489-710, (*e*) Δ216-600, and (*f*) Δ216-710. Arrowheads show the decreasing gap between the sealed ends of the microtubules and the central plaque as more of the rod region of Spc110p is removed. Bars, 0.1 μm.

Spc110p rod, which can span up to around 800 Å (Fig. 5), to bridge this gap, and thereby act as a linker and possibly also a spacer element between the central plaque and the sealed ends of the microtubules.

We tested this proposal by preparing a series of deletions

of the rod domain of Spc110p, which were able to replace the wild-type Spc110p with little change in growth rate at 30°C. When the distance between the central plaque and the sealed ends of the microtubules was measured in these strains there was a reduction in the size of the gap, which was
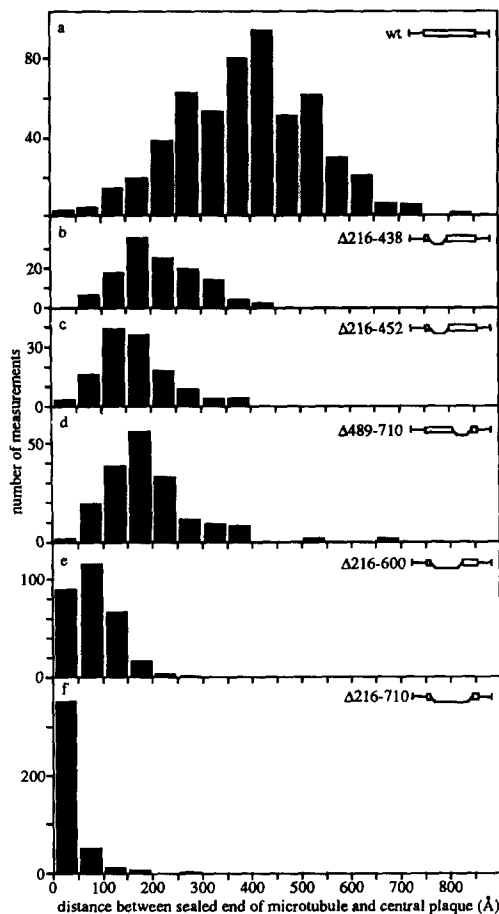
Figure 9. Histogram of measurements of the distance between the sealed ends of the nuclear microtubules and the nearest edge of the central plaque. Distances were measured without knowing the identity of the sample and using the average microtubule diameter on the section as an internal standard. SPBs were isolated from strains containing deletions of the coiled-coil region of Spc110p. (*a*) wild-type rescue, (*b*) Δ216-438, (*c*) Δ216-452, (*d*) Δ489-710, (*e*) Δ216-600, and (*f*) Δ216-710.

proportional to the length of the deletion (Figs. 8 and 9). Quantitatively there was not an exact correlation between the length of rod removed, calculated for an α-helical rod at 1.5 Å/residue, and the decrease in the gap between the central plaque and the microtubule ends. This might be because other proteins bind to the rod domain of Spc110p, which might modify its structure in some way such that deletion of that part of the rod would not lead to an exact corresponding decrease in the gap. Clearly also measurements of the distances from thin sections would not give the most accurate values. In addition to these deletions, we also attempted to increase the length of the Spc110p rod by inserting repeats of blocks of heptads in phase with the endogenous heptads. These constructs were able to replace the wild-type Spc110p and some SPBs from these strains did have larger gaps (data not shown), but these SPBs were not frequent enough to allow classification of an unidentified micrograph, unlike the SPBs in the deletion series where unidentified micrographs were readily classified. It might be that as the gap becomes wider then the variability in the measured distances from thin sections increases, as is seen with the wild-type SPBs (Fig. 9 *a*).

These results suggest that Spc110p has an essential role in the structure of the SPB by linking the central plaque to the inner plaque area which contains the sealed ends of the nuclear microtubules. The rod domain of Spc110p acts as a non-essential spacer element between these two parts of the structure. A possible function of the rod domain might be to add flexibility to the SPB structure. This would facilitate the re-orientation of microtubules from the side-by-side array after SPB replication to the anti-parallel array after spindle formation. There are however some puzzling features of Spc110p which may indicate a more complex function, in particular the extra protein present in the nucleus (Mirzayan et al., 1992). Nuclear fractionation indicates that this is associated with the SPB (Rout and Kilmartin, 1990), and Coomassie Blue staining of the 110-kD band (assuming that this is all due to Spc110p) in both enriched and DEAE–dextran extracted SPBs indicates that Spc110p is present at a higher stoichiometry than other SPB components. One possibility might be that Spc110p is both an integral component of the SPB and part of a filamentous system attached to the SPB. However both immuno-EM of enriched SPBs with anti-110-kD mAbs and negative staining of isolated SPBs (Rout and Kilmartin, 1990) showed no evidence for such a filamentous system attached to the SPB. Perhaps the generation of ts mutants in *SPC110/NUF1* would indicate whether the simple proposal for Spc110p function presented in this paper, that is as a linker protein in the SPB with some surplus protein in the nucleus, is correct, or whether the role of Spc110p is more complex with a function both in the SPB and in the nucleus.

A similar experiment to that described in this paper was carried out on the λ phage tail protein gpH. This protein has a potential α-helical domain which acts as a template to specify phage tail length; thus a series of deletions and one small duplication in the corresponding gene *H* caused proportionate changes in the length of the phage tail (Katsura and Hendrix, 1984; Katsura, 1987). Two recently isolated genes, SCP1 in rats (Meuwissen et al., 1992) and *ZIP1* in *S. cerevisiae* (Sym et al., 1993) may also be candidates for length determining molecules. Both have long central domains predicted to encode coiled-coil regions as in *SPC110/ NUF1*, and localize to a filamentous region of the synaptonemal complex. In the case of *ZIP1*, it would be interesting to test whether deletions of the potential coiled-coil domain would cause predictable alterations in the structure of the synaptonemal complex, similar to those described in this paper for the yeast SPB.

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