NDC10: A Gene Involved in Chromosome Segregation in *Saccharomyces cerevisiae*

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Abstract. A mutant, *ndc10-1*, was isolated by anti-tubulin staining of temperature-sensitive mutant banks of budding yeast. *ndc10-1* has a defect in chromosome segregation since chromosomes remain at one pole of the anaphase spindle. This produces one polyploid cell and one aploid cell, each containing a spindle pole body (SPB). *NDC10* was cloned and sequenced and is identical to *CBF2* (Jiang, W., J. Lechner, and J. Carbon. 1993. *J. Cell Biol.* 121:513–519) which is the 110-kD component of a centromere DNA binding complex (Lechner, J., and J. Carbon. 1991. *Cell.* 64:717–725). *NDC10* is an essential gene. Antibodies to Ndc10p labeled the SPB region in nearly all the cells examined including nonmitotic cells. In some cells with short spindles which may be in metaphase, staining was also observed along the spindle. The staining pattern and the phenotype of *ndc10-1* are consistent with Cbf2p/Ndc10p being a kinetochore protein, and provide in vivo evidence for its role in the attachment of chromosomes to the spindle.

THE accurate sorting and segregation of chromosomes by the mitotic apparatus during cell division is a complex process which must involve the coordinated activity of many proteins. The identification and characterization of some of these proteins is a difficult task because of their low abundance in the cell. Lower eukaryotes such as *Saccharomyces cerevisiae* offer distinct advantages for the identification of such proteins. First, cells can easily be grown in quantities sufficient for the purification or biochemical isolation of low abundance proteins. Thus Lechner and Carbon (1991) purified a complex of three proteins which bind to the centromeric DNA of *S. cerevisiae*, while we have prepared enriched spindle pole body (SPB)[1] preparations and characterized some of their components by raising mAbs against them (Rout and Kilmartin, 1990). Second, a genetic approach is possible by isolating mutants defective in various aspects of chromosome transmission and cloning the genes thus identified. In *S. cerevisiae*, various selection procedures for mutants with increased chromosome loss have been devised (Meeks-Wagner et al., 1986; Spencer et al., 1990; Hoyt et al., 1990), and have led to the identification of mitotic kinesin-like proteins (Hoyt et al., 1992; Roof et al., 1992). Cytological screens on randomly generated temperature-sensitive mutants have also been carried out on *S. cerevisiae* leading to the identification of two mutants, *ndc1-1* (Thomas and Botstein, 1986) and *mps2-1* (Winey et al., 1991*a*), where chromosomes fail to attach to one of the spindle poles. In *Schizosaccharomyces pombe* cytological screens have also identified a mitotic kinesin (Hagan and Yanagida, 1990, 1992) and two phosphatases which play a role in chromosome disjunction (Ohkura et al., 1988, 1989).

1. *Abbreviation used in this paper*: SPB, spindle pole body.

We report here the isolation of a mutant in *S. cerevisiae*, *ndc 10-1* (nuclear division cycle), which has a similar phenotype to both *ndc1-1 and mps2-1*. However, it is not linked to either mutation and has clear differences in the details of the phenotype. We also report the nucleotide sequence of *NDC10* and show that an antibody directed against Ndc10p stains the SPB region and part of the mitotic spindle, suggesting that Ndc10p is a structural component of the spindle involved in chromosome transmission. As this work was being completed we discovered that, apart from one amino acid, Ndc10p is identical to Cbf2p whose sequence is reported in the accompanying paper (Jiang et al., 1993). Cbf2p is the 110-kD component of a centromere DNA binding complex (Lechner and Carbon, 1991) which is associated with ATP-dependent minus end–directed movement on microtubules (Hyman et al., 1992). The phenotype of *ndc10-1* and of depletion of Ndc10p described in this paper provide in vivo evidence for the role of Ndc10p/Cbf2p in chromosome attachment to the spindle.

Materials and Methods

Isolation of ndc10-1

Temperature-sensitive mutants either generated by ethylmethane sulfonate (EMS) mutagenesis or taken from part of the Hartwell et al. (1973) bank (generously provided by G. R. Fink) were picked into 10 ml of YEPD and grown overnight at 23°C to ensure that the cells were in log phase, then blocked at 36°C for 4 h. Cells were fixed, permeabilized and stained with anti-tubulin and DAPI as described by Kilmartin and Adams (1984). The original isolate of *ndc10-1* was back-crossed three times to K699 (Nasmyth et al., 1990) and K700 (an isogenic MATα form of K699) to give JK418; these and other yeast strains used are shown in Table I. Each time the temperature sensitivity and the mutant phenotype cosegregated, with tetrads

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segregating 2:2. The ts banks were rescreened for further *ndc10* alleles by noncomplementation, in case the phenotype had been obscured by the presence of another ts mutation; however, new alleles were not isolated. To label SPBs with the pooled mAbs against the 90-kD SPB component (Rout and Kilmartin, 1990), cells were fixed in formaldehyde for 15–30 min only and incubated with the mAbs overnight at 12°C.

Cloning and Sequencing of NDC10

An *S. cerevisiae* genomic DNA bank in the *TRP1 CEN* vector M111 (prepared by Leslie Bell, University of Washington, Seattle, WA) was transformed into *ndc10-1* (JK418). Plasmids prepared from ts^+ transformants were transformed into TG1 *Escherichia coli*, and retested for their ability to transform *ndc10-1* to ts^+. Positive plasmids were all related by restriction mapping and the smallest insert (6.5 kb) was subcloned further to a 4.1-kb PvuII fragment able to complement *ndc10-1*. This fragment was then subcloned into the EcoRV site of pBluescript. To check that the PvuII fragment contained the actual *NDC10* locus rather than a suppressor, it was transferred to the *LEU2*-containing integrating vector pRS305 (Sikorski and Hieter, 1989). A single cut in the PvuII fragment was made with either BglII or BspEI to cause it to integrate at the homologous locus in the genome of JK418. Analysis of the resulting ts^+ transformants by Southern blotting showed that integration had occurred at a site homologous to the PvuII fragment (data not shown). When two of these ts^+ transformants were crossed to wild type (K699) and sporulated, no ts spores were found in 26 four-spored tetrads, showing that the PvuII fragment contains *NDC10* or is very tightly linked to it. The PvuII fragment was sequenced using ExoIII deletions from either end (Henikoff, 1984). All the sequence in the coding region was determined at least twice in each direction. *NDC10* is identical to *CBF2* (Jiang et al., 1993) apart from six single base changes: *NDC10* has an extra T at positions −701 and −641, an A to C change at 2320 which would change the codon for K to Q, and a C to T change at 3168 and A to C at 3351.

A disruption of *NDC10* was prepared by the one step gene replacement method (Rothstein 1983). The 4.1-kb PvuII fragment containing *NDC10* was transferred to pBluescript cut with SacI and KpnI and blunted. This plasmid was cut at the NH_2-terminal NdeI site and the EcoRI site at 2693 close to the COOH terminus and blunted, then a blunted 2.2-kb *LEU2* fragment was ligated into this gap. A linear DNA fragment was generated by cutting with SphI and BamHI. This fragment was transformed into a diploid strain K842 (an isogenic diploid of K699 and K700) to give JK625. Southern blotting of DNA from JK625 confirmed that one copy of the *NDC10* gene had been disrupted (data not shown). JK625 was sporulated and dissected. Of all the 20 tetrads analyzed, 18 gave two viable (all Leu^-) and two inviable spores, while two gave only one viable spore (both Leu^-). Microscopic examination of the tetrads with two viable spores showed that germination of the inviable spores had occurred, producing 3–8 cells or buds before growth ceased. To check that the inviability was due to the loss of the *NDC10* locus, JK625 was transformed with an ExoIII fragment (−662–3019) subcloned into the *CEN URA3* vector pRS316 (Sikorski and Hieter, 1989). Apart from the Ndc10p open reading frame, this ExoIII fragment contains no other open reading frames longer than 50 amino acids. Sporulation of this transformed strain gave some tetrads with four viable spores, two of which were $Leu2^+$ and $Ura3^+$, showing that the spores containing the disrupted *NDC10* gene were rescued by the plasmid. These spores were dependent on the plasmid for growth since selection against the plasmid on fluoro-orotic acid medium (Boeke et al., 1984) gave no colonies.

A strain capable of over-expressing a slightly truncated Ndc10p (Ndc10pΔ1-11) was constructed by integrating a plasmid containing a ClaI fragment (11-3405) of *NDC10* under the control of *GAL* promoter (attempts to subclone an *NDC10* fragment containing the putative NH_2 terminus were unsuccessful). We assume that translation of the ClaI fragment would start at methionine 12 to produce Ndc10pΔ1-11. The blunted ClaI fragment was inserted in the correct orientation into the polylinker of YIp56X (Pelham et al., 1988), a *URA3*-based yeast integrating vector containing the *GAL* promoter. A single cut was made at the XhoI site constructed in the URA3 marker of the plasmid to direct integration to the *URA3* locus of JK625, a diploid containing *NDC10* disrupted by *LEU2*. $Leu^+Ura^+Glu^-$ spores were selected after sporulation and then dissection on YEPGal plates. These contained a disrupted *NDC10* locus rescued by the *NDC10* gene under the control of the *GAL* promoter and show that the truncated Ndc10pΔ1-11 is functional. These spores failed to produce colonies on streaking out on YEPglucose plates. One of these spores (PY60) was selected for Western blotting and examination of its phenotype in glucose medium.

EM and Flow Cytometry

For EM, log phase *ndc10-1* cells were blocked for 4 h at the restrictive temperature of 36°C, then fixed and embedded as described by Byers and Goetsch (1991) except that the cells were washed with acetone after dehydration and infiltrated with mixes of acetone and Spurrs over 2 d, neat Spurrs for 10 h, then set overnight at 70°C. For flow cytometry, wild-type cells (K699) and *ndc10-1* grown to a density of 5×10^6/ml were blocked with α factor (12.5 μg/ml) for 4 h at 23°C until all cells formed schmoos. After washing and release at 36°C, samples were removed at hourly intervals and prepared for flow cytometry (Nash et al., 1988).

Preparation of Antibodies against Ndc10p, *Immunoblots and Immunofluorescence*

NDC10 was cut with AccI at 889 and NdeI at 2116, blunted with Klenow and inserted in frame and in the correct orientation into the blunted NcoI site of a modified T7 expression vector (Way et al., 1990). The junction between the blunted NcoI and AccI sites was checked by DNA sequencing. Transformation of BlDE21 cells and induction with IPTG (Studier et al., 1990) resulted in the expression of an insoluble protein of the expected size of 47 kD. Inclusion bodies were prepared (Nagai and Thogersen, 1987) and solubilized in 8 M urea. The 47-kD protein was purified by chromatography on DEAE cellulose in 8 M urea and preparative SDS-gel electrophoresis. After elution the protein was used to immunize five rats. Sera which gave a signal on Western blots of the 47-kD protein at dilutions of 1:10,000 (two rats) were affinity purified using a column of the SDS-gel-purified 47-kD protein coupled to Sepharose beads.

Immunoblots were carried out as described in Rout and Kilmartin (1990) using a 1:10 dilution of the affinity purified anti-Ndc10p either overnight at 4°C or for 1 h at room temperature.

For immunofluorescence, cells were prepared as in Kilmartin and Adams (1984) and stained overnight at 12°C with affinity purified anti-Ndc10p (neat or diluted 1:3), followed by FITC-labeled affinity purified anti-rat IgG (absorbed against rabbit IgG) for 2 h at room temperature. To exclude any possibility of feed-through from the anti-tubulin signal, fields of cells were photographed and their position on the slide recorded on the microscope micrometer. The coverslip was prized off gently with a razor blade, the cells appeared to be tightly attached by the polylysine, and the mounting medium washed away with BSA-PBS (Kilmartin and Adams, 1984). The cells were then stained with rhodamine-labeled affinity-purified rabbit anti-yeast tubulin followed by 4',6-diamidino-2-phenylindole (DAPI). The same groups of cells were relocated using the microscope micrometer and contact prints as a guide, and photographed again.

Results

Identification and Characterization of ndc10-1

ndc10-1 was identified from a screen using anti-tubulin staining of 624 randomly generated ts mutants, 446 of which were from the ts bank used by Hartwell et al. (1973) to isolate the original *cdc* mutants. We looked for possible defects in SPB function associated with consistent abnormalities in spindle structure present in >90% of the cells at a particular cell cycle stage, and also for a different phenotype from previously described *cdc* mutants. Three such mutants were found: two of these were alleles of *mps1* (Winey et al., 1991*a*) and the other *ndc10-1* (all obtained from the Hartwell bank).

The phenotype of *ndc10-1* at the restrictive temperature is shown in Fig. 1, in which cells were labeled with anti-tubulin (Fig. 1, *a*) and the DNA stain DAPI (Fig. 1, *b*). Two abnormalities are immediately apparent: all late anaphase spindles (*arrowheads*) have DNA associated with only one pole; and presumably as a result of division of these cells, there is a substantial fraction (45%, n=398) of aploid cells (*arrows*) with apparently no nuclear DNA. Due to the density of cells in Fig. 1, *a* and *b* the aploid cells cannot be distinguished

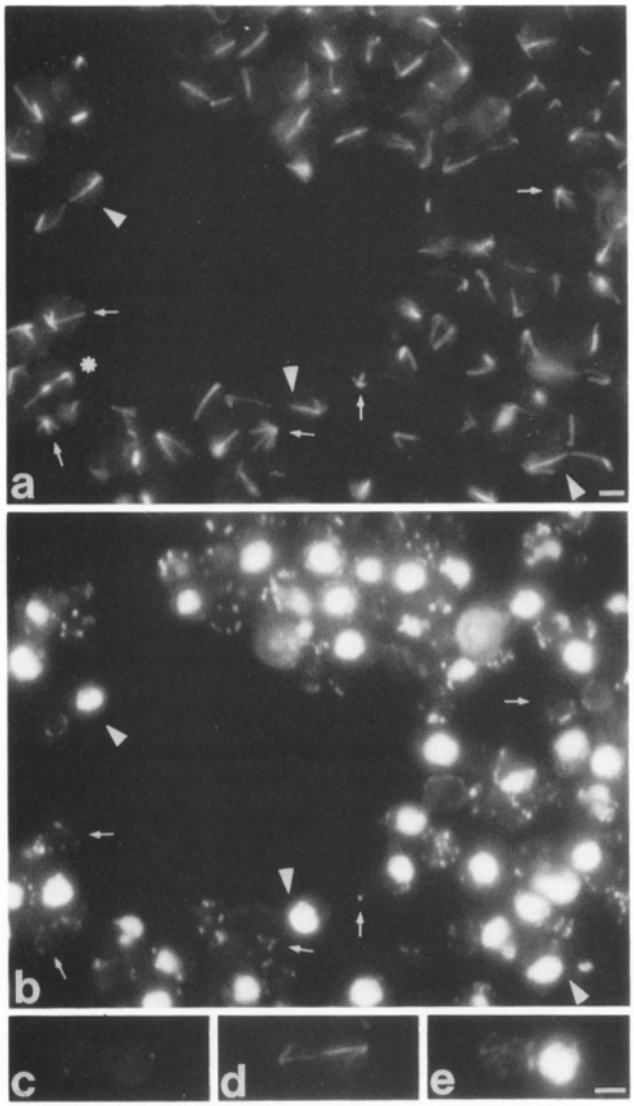

Figure 1. Immunofluorescence of *ndc10-1* blocked at the restrictive temperature 36°C for 4 h. Cells were stained with anti-tubulin (*a* and *d*), DAPI (*b* and *e*) and pooled mAbs against the 90-kD component of the SPB (*c*). In *a* and *b*, arrowheads indicate anaphase spindles where nuclear DNA has failed to segregate, while arrows show aploid cells containing microtubule asters apparently lacking nuclear DNA. The asterisk shows a possible early anaphase spindle with abnormal morphology (see Discussion). The gaps in the anti-tubulin staining in the middle of anaphase spindles are due to decreased permeability in the neck region (see Fig. 3 *c* in Kilmartin and Adams, 1984). Bar, 2.5 μm.

from large buds, however other views (not shown) and flow cytometry analyses (see Fig. 3) show that the aploid cells are separate suggesting that cell division continues in the mutant. There also appear to be abnormalities in the structure of the anaphase spindles. The late anaphase spindles are asymmetrically stained with anti-tubulin, suggesting different numbers of microtubules at each pole. Also, the early anaphase spindles appear to have abnormal morphology (Fig. 1 *a, asterisk*), while normal early anaphase spindles containing a parallel bundle of microtubules (similar to that in Fig. 6 *g* of Kilmartin and Adams, 1984) were only rarely observed. The anti-tubulin staining pattern of the mutant indicates that SPBs are segregated normally since astral arrays are present in the aploid cells and at either end of the spindle in budded cells. This was confirmed in a triple labelling experiment (Fig. 1, *c, d,* and *e*) using pooled mAbs to a 90-kD component of the SPB (Rout and Kilmartin, 1990), which showed that this SPB antigen clearly segregates into the aploid cell.

The phenotype of *ndc10-1* has some similarities to that of the cs mutant *ndc1-1* (Thomas and Botstein, 1986) and the ts mutant *mps2-1* (Winey et al., 1991*a*) in that DNA remains at one pole and aploid cells apparently containing SPBs accumulate. However, *ndc10-1* is not linked to *ndc1-1* since a cross between the two mutants produces a substantial proportion of wild-type spores (PD:NPD:T=2:3:7). *ndc10-1* is also unlikely to be allelic to *mps2-1* because it complements that mutant (data not shown). In addition, there is a clear difference in the anti-tubulin staining between *ndc10-1* and both *ndc1-1* and *mps2-1* in that *ndc10-1* appears to have anaphase spindles, whereas *ndc1-1* and *mps2-1* appear to lack spindle microtubules connecting the two poles at anaphase (Winey et al., 1991*a,b*). There are also differences in the distribution of the nuclear DNA between *ndc10-1* and *ndc1-1*. In *ndc1-1*, nuclear DNA is transmitted equally to either the mother (marked by α-factor treatment) or the bud (Thomas and Botstein, 1986), whereas in *ndc10-1* cells in late anaphase, nuclear DNA remains in 96% of the schmooed mother cells (data not shown). Recently we discovered that Kopski, K., and T. Huffaker (personal communication) had isolated another allele of *ndc10* in a similar screen of a different ts bank. It too shows a detachment of chromosomes from the spindle poles.

The phenotype of *ndc10-1* observed by immunofluorescence was confirmed by EM. Fig. 2 shows an *ndc10-1* cell in anaphase. It has a large nucleus outlined by dense nuclear pores presumably in the mother cell, with a thin sheath of nuclear membrane extending into the bud with an apparently

Table I. Yeast Strains

Strain	Relevant genotype	Source
K699	*a ura3 leu2-3,112 GAL⁺*	Nasmyth et al. (1990)
K700	*α ura3 leu2-3,112 GAL⁺*	Nasmyth et al. (1990)
K842	*a/α diploid ura3 leu2-3,112 GAL⁺*	Nasmyth et al. (1990)
DBY1583	*a ndc1-1*	Thomas and Botstein (1986)
Wx228-14d	*a mps2-1*	Winey et al. (1991*a*)
JK418	*a ura3 leu2-3,112 trp1 ndc10-1*	This paper
JK625	*a/α diploid ura3 leu2-3,112 NDC10/ndc10Δ1::LEU2*	This paper
PY60	*a ura3 leu2-3,112 ndc10Δ1::LEU2 URA3::GAL-NDC10*	This paper

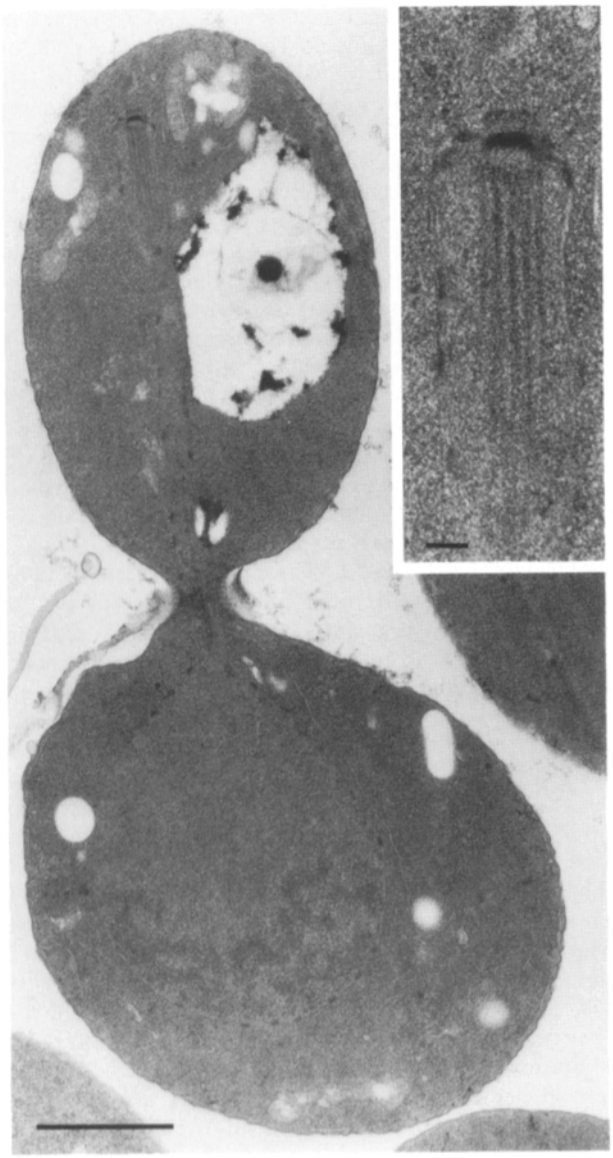

Figure 2. Electron micrograph of a thin section of an *ndc10-1* cell blocked at the restrictive temperature 36°C for 4 h. This micrograph shows a large nucleus in the lower half of the cell with a thin isthmus of nuclear envelope delineated by dense nuclear pores, extending into the upper half of the cell and ending with an SPB containing nuclear microtubules. (*Inset*) A higher magnification of the SPB region of the cell. Bars: 1 μm; (*inset*) 0.1 μm.

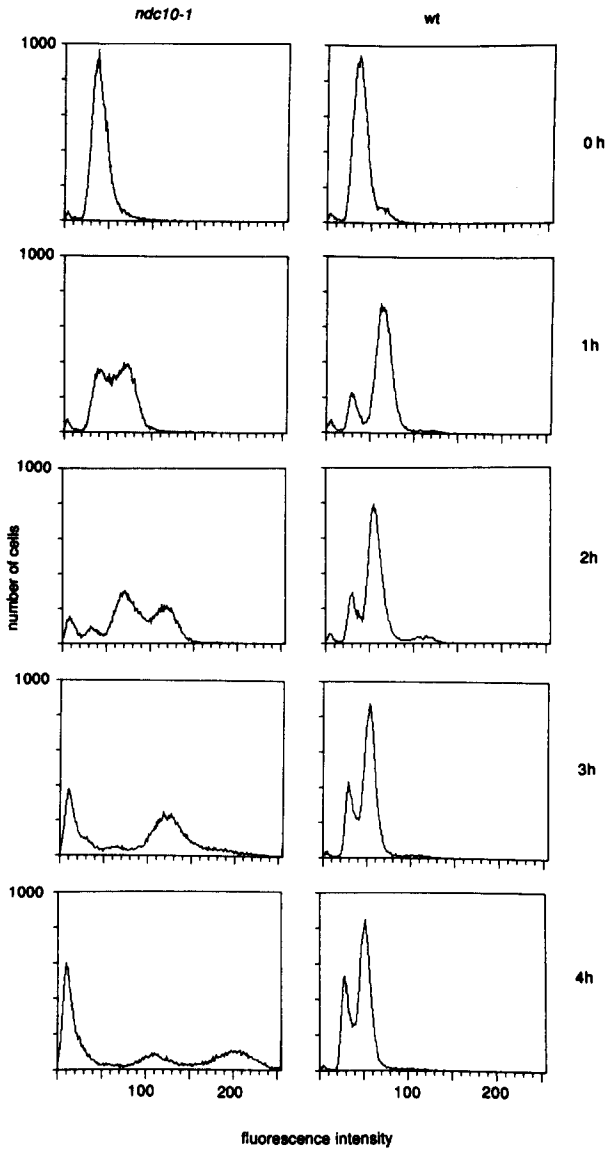

Figure 3. Flow cytometry analysis of *ndc10-1* and wild-type cells synchronized with α-factor at the permissive temperature 23°C then released at the restrictive temperature 36°C.

normal SPB at its apex. This SPB has clear nuclear microtubules attached, although we cannot determine whether this cell has an intact anaphase spindle because such long spindles are difficult to follow in longitudinal thin sections. We have observed a similar phenotype, that is a narrow rod of nuclear envelope containing microtubules and ending in an SPB, in at least 11 other cells. The small area of the rod-shaped nucleus extending into the bud suggests that there is little or no nuclear DNA associated with the SPB at the apex of the rod. The phenotype of *ndc10-1* as studied by EM is thus in agreement with that found by immunofluorescence.

A flow cytometry assay (Fig. 3) using cells blocked with α-factor and released at the restrictive temperature showed that DNA replication and cytokinesis continue in this mutant producing polyploid as well as aploid cells, with the ploidy rising to around octoploid after 4 h. As expected from this accumulation of aploid and increasingly polyploid cells, the *ndc10-1* mutation is lethal: the viability of unsynchronized cells dropped to 10% after 4 h at 36°C. Immunofluorescence of cells fixed at the 2-, 3-, and 4-h time points gave similar phenotypes to those seen in Fig. 1.

Isolation and Sequencing of NDC10

NDC10 was isolated from an *S. cerevisiae* genomic DNA bank in a *CEN* vector which was used to transform *ndc10-1* to ts$^+$. The plasmid with the smallest insert was subcloned further to a 4-kb PvuII fragment which complemented the mutation. This fragment was able to direct the *LEU2* gene to the *ndc10* locus (see Materials and Methods), confirming that it contained the actual *NDC10* gene rather than a suppressor. The PvuII fragment was sequenced and found to contain a single long open reading frame (Fig. 4) predicting

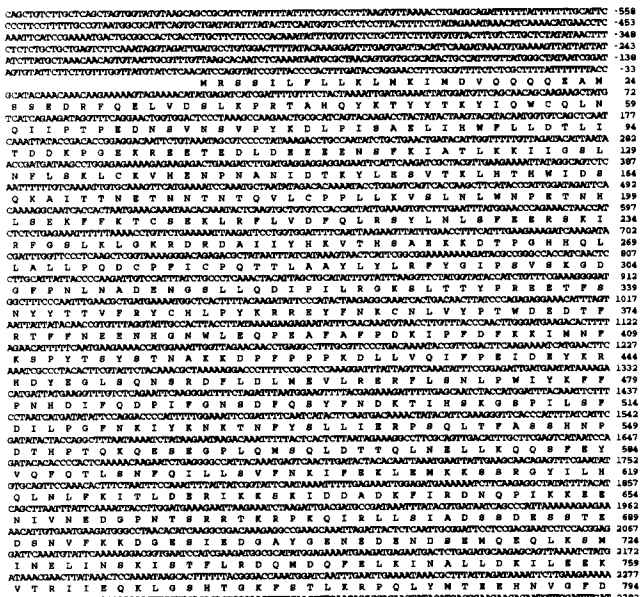

Figure 4. Nucleotide sequence of the PvuII fragment containing *NDC10*, and the predicted amino acid sequence of the open reading frame. These sequence data are available from EMBL under accession number X69300.

a polypeptide of 956 amino acids. Fragments which lacked sequences in this open reading frame on either side of the BglII site at 322 or the XbaI site at 1,372 failed to complement *ndc10-1*. No significant homology was found between Ndc10p and other protein sequences in the SWISSPROT database (release 22) using the FASTA search program (Pearson and Lipman, 1988). An almost complete disruption of the open reading frame of the *NDC10* gene leaving only 58 residues of the COOH terminus showed that the gene is essential (see Materials and Methods).

Localization of Ndc10p

An antibody against the *NDC10* gene product was prepared by expressing an AccI/NdeI fragment in *E. coli* and injecting this into rats. Antibodies were affinity purified from two rats and their specificity checked by Western blots of whole cell extracts. A weak signal was obtained from extracts of *S. uvarum* and wild-type cells (Fig. 5), which had an apparent molecular mass of 108 kD, close to the expected size of 112 kD. Since the signal from wild-type cells was low, we further checked the specificity of the anti-Ndc10p antibodies on a strain overexpressing Ndc10p under the inducible *GAL* promoter. Although this was missing the first 11 amino acids (assuming translation starts at the next ATG), Ndc10pΔ1-11 was functional since it rescued a deletion of *NDC10* in galactose medium (see Materials and Methods). Western blotting of this strain with affinity-purified anti-Ndc10p gave a clearer and more intense band of similar mobility (Fig. 5), confirming the specificity of the antibody.

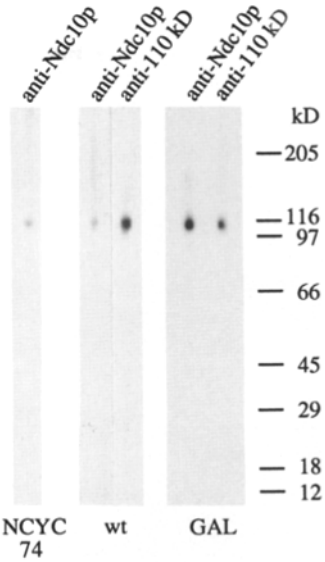

Figure 5. Immunoblots of yeast cell extracts with anti-Ndc10p. *S. uvarum* (NCYC 74), wild type (K699) or cells overexpressing Ndc10p under the *GAL* promoter (PY60) were immunoblotted with affinity-purified anti-Ndc10p or with pooled mAbs against the 110-kD component of the SPB as a loading control.

The affinity-purified antibodies were used to stain fixed yeast cells and, as with other spindle antigens (Rout and Kilmartin, 1990), the staining intensity decreased with increasing time of formaldehyde fixation. Fixation for 30 min seemed to give the best balance between staining intensity and spindle preservation. Even under these conditions the signal from Ndc10p was weak. To ensure that in double labeling experiments there was no feed-through from the much brighter anti-tubulin staining, cells stained with anti-Ndc10p were photographed, then stained with anti-tubulin, relocated and photographed again.

The anti-Ndc10p antibodies gave a staining pattern of low intensity consisting of either one or two sometimes fuzzy dots localized to the region of the SPB (Fig. 6, *a* and *d*). In some nonmitotic cells, the staining appeared as a cluster of dots (Fig. 6 *a, small arrowhead*). There was also some diffuse nuclear staining in some of the cells. In cells with short spindles, presumably in metaphase, the staining pattern was either two dots close to the SPB region (Fig. 6 *a, arrows*) or somewhat diffuse or sometimes granular staining along the spindle (Fig. 6, *a* and *d, large arrowheads*). In the short spindles, the distance between the dots and the length of the diffuse staining was less than the spindle length. This is similar to what was found with an anti–80-kD mAb which labels the spindle near the SPB (Rout and Kilmartin, 1990), suggesting that, like the 80-kD antigen, the anti-Ndc10p staining is associated with the spindle rather than with the SPB. The anti-Ndc10p antibodies stained close to the SPB in nearly all the cells examined including nonmitotic cells, though the intensity of staining in these cells was variable. In anaphase or late anaphase/telophase spindles, there was often weak staining along the spindle.

Phenotype of Cells Depleted of Ndc10pΔ1-11

The phenotype of Ndc10p depletion was compared with the phenotype in the ts mutant *ndc10-1* using the yeast strain PY60. In PY60 a slightly truncated version of Ndc10p (Ndc10pΔ1-11) whose expression is under the control of the inducible GAL promoter is able to rescue the deleted *NDC10* gene. In galactose medium Ndc10pΔ1-11 is highly expressed, while in glucose where the *GAL* promoter is switched off, the

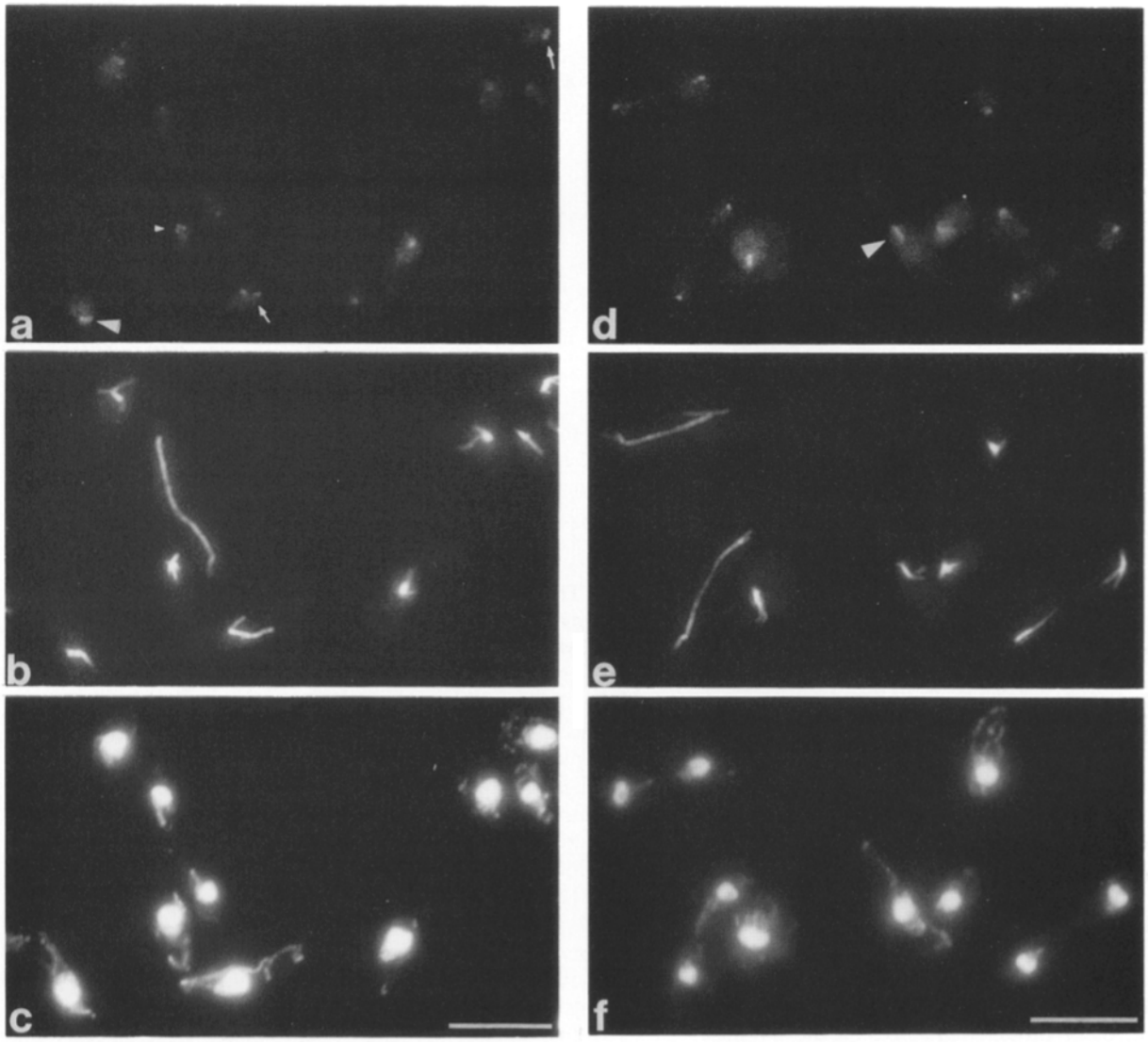

Figure 6. Immunofluorescent staining of yeast cells (*S. uvarum*, NCYC 74). Cells were stained with affinity purified anti-Ndc10p (*a* and *d*), anti-tubulin (*b* and *e*) and DAPI (*c* and *f*). The cells in *a* and *d* were photographed before anti-tubulin staining, one of the cells in the lower center of *a* and on the righthand side of *d* was washed away during subsequent staining procedures. Cells with large arrowheads in *a* and *d* have anti-Ndc10p staining along short presumably metaphase spindles, while the arrowed cells in *a* have staining associated with the poles of such a spindle. The cell with a small arrowhead in *a* shows a small cluster of dots associated with the microtubule aster (see Discussion). Bar, 10 μm.

cells are depleted of Ndc10pΔ1-11. Thus the phenotype of a null mutation can be observed and the specificity of the anti-Ndc10p antibodies checked since the staining pattern should disappear. Only anaphase or late anaphase cells were examined since these gave the brightest anti-Ndc10p staining and were the easiest to classify because of their large size and avoidance of superposition effects between the DAPI and antibody staining.

PY60 cells continued to divide for up to 17 h in glucose, after which the cells stopped dividing but continued to increase in size with a decrease in viability (see Fig. 7 *a*). At early time points, the cells showed bright overall nuclear staining with anti-Ndc10p, presumably caused mainly by overexpression of the protein. This gradually decreased in intensity between 7 and 11 h, until staining was confined to the SPB region (Fig. 7 *b*). The slow transition was due to the large variation in the amount of Ndc10p in individual cells, which would lead to a gradual expression of the phenotype. At 15 h, anti-Ndc10p staining became undetectable (Fig. 7 *b*), confirming the specificity of the antibody. At the same time the first abnormalities appeared, including an accumulation of large-budded cells containing short spindles and a decrease in the compactness of DAPI staining. The abnormalities in anaphase at this time point consisted of either unequal DAPI staining at the two poles with the staining at one pole 2–3 times more intense than at the other, or DAPI staining along the anaphase spindle rather than at the poles (Fig. 7 *c, asterisked cell* is a combination of these phenotypes). We assume that for the particular cell at 15 h in Fig. 7 *b*, since some chromosome segregation occurred, a small number of copies of Ndc10p undetectable by immunofluorescence can exert partial function. At later time points, the proportion of *ndc10-1*–like anaphase spindles containing nuclear DNA at one pole gradually increased (Fig. 7 *a*), rising

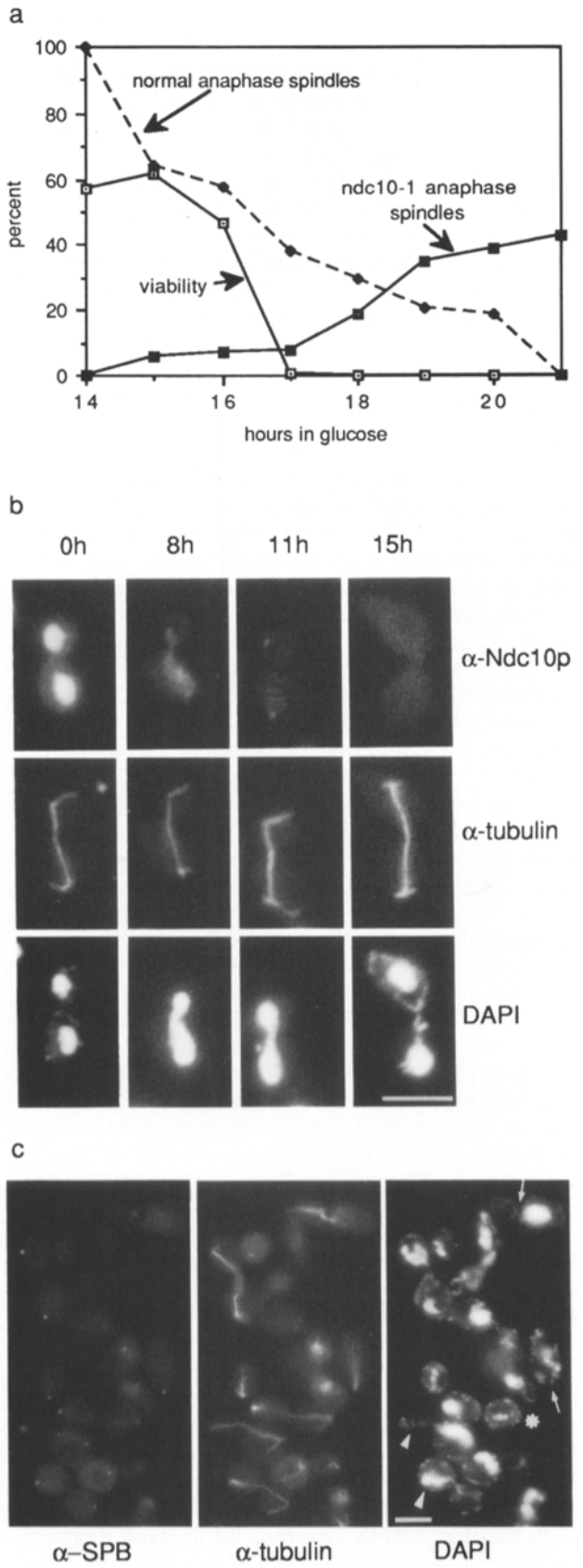

Figure 7. Analysis of PY60 cells depleted of Ndc10p by repressing the *GAL* promoter in glucose medium. (*a*) Graph showing viability, percent normal anaphase spindles and percent *ndc10-1*–like anaphase spindles as PY60 cells are depleted of Ndc10pΔ1-11 in glucose. *ndc10-1*–like anaphase spindles have DNA associated with only one pole, while the rest of the abnormal anaphase spindles have either DNA spread along their length or at earlier time points, unequal distribution of DNA at the two poles. (*b*) Anti-Ndc10p, anti-tubulin and DAPI staining of cells during Ndc10pΔ1-11 depletion. Cells stained with anti-Ndc10p at 11 and 15 h were photographed before staining with anti-tubulin. (*c*) Cells after 19 h of depletion stained with pooled mAbs to the 90-kD component of the SPB, anti-tubulin and DAPI. Cells with arrowheads have *ndc10-1* anaphase spindles, while the cell with an asterisk has both an unequal distribution of DNA and DNA spread along the spindle. Arrows indicate dissociation of the bulk of DAPI staining from the SPB. Bars, 5 μm.

to ~40% of the total, the rest being spindles with the DAPI staining spread along their length. After 21 h no anaphase spindles were present. Fig. 7 *c* shows a group of cells at 19 h, two with *ndc10-1*–like anaphase spindles (*arrowheads*), one showing unequal distribution of chromosomes (*asterisk*), and two cells on the extreme middle and top right showing apparent dissociation of chromosomes from the SPB (*arrows*). These results are consistent with the phenotype obtained with *ndc10-1* in that they show a detachment of chromosomes from the spindle pole.

Discussion

Phenotype of ndc10-1

In this paper we describe the isolation of a novel ts mutant *ndc10-1*, which has a defect in chromosome segregation. The sequence of *NDC10* is identical to that of *CBF2* (Jiang et al., 1993) which encodes the 110-kD component of a centromere DNA binding complex CBF3 (Lechner and Carbon, 1991). Moreover, an enriched preparation of CBF3 is capable of minus-end–directed movement on microtubules (Hyman et al., 1992), suggesting that *CBF2/NDC10* may be part of the yeast kinetochore, although other kinetochore activities that can associate with microtubules may also be present (Hyman et al., 1992). Although there are a number of possible explanations for the phenotype observed in *ndc10-1* and Ndc10p-depleted cells, in view of the identity of *NDC10* and *CBF2*, it seems reasonable to confine the discussion of the mutant phenotype mainly to that of a defect in kinetochore function. Clearly the phenotypes are consistent with that interpretation and give direct in vivo evidence that Ndc10p/Cbf2p has a role in chromosome attachment to the spindle.

Since *CBF2/NDC10* probably encodes a yeast kinetochore protein, it is interesting to look at the phenotype of *ndc10-1* further since it may indicate the consequences of kinetochore disruption. Flow cytometry analysis indicates that DNA replication continues at the restrictive temperature in the ts mutant. In both synchronized and unsynchronized cells at early stages of mitosis, normal-looking short spindles with the characteristic dumbbell shape (Kilmartin and Adams, 1984) form, indicating the presence of chromosomal microtubules close to the poles (Peterson and Ris, 1976). The morphology of spindles that are apparently in the metaphase/anaphase transition or in anaphase appears somewhat variable. A small proportion of these spindles have a parallel bundle of microtubules connecting the two poles as in wild-type cells (Kilmartin and Adams, 1984). In others there are only a few microtubules between the two poles,

each with a bundle of short, apparently nuclear microtubules attached (see Fig. 1, *a, asterisk*). Late anaphase spindles have a consistent morphology: few or no chromosomes are attached to the SPB that enters the bud and the anti-tubulin staining appears to be asymmetrical, with the more intensely stained half associated with the chromosomes (Fig. 1). The *dis* mutants of *S. pombe* (Ohkura et al., 1988) also show the same phenomenon, although in this case the defect is thought to be one of chromosomal nondisjunction. Since the chromosomal microtubules are very short and closely attached to the poles at late anaphase (Peterson and Ris, 1976), the asymmetry in the anti-tubulin staining in both mutants could be due to the stabilization of the spindle microtubules by the environment around the chromosomes.

In the ts mutant the replicated DNA remains in the mother cell after mitosis and cell division. It is not clear why this happens, but it might be caused by a defect in chromosome attachment to the newly replicated SPB which Vallen et al. (1991) have shown to be preferentially segregated to the bud, while the pre-existing SPB would retain chromosomes in the mother. This would require the pre-existing kinetochores to remain active and preferentially associate with the mother SPB. In addition disjunction should not occur since there would now be two sets of chromatids, due to DNA replication continuing normally, but only one set of active kinetochores. However a preferential association of pre-existing kinetochores with the mother SPB seems unlikely since Neff and Burke (1991) have shown that pre-existing and newly replicated chromatids are randomly segregated, suggesting that the pre-existing kinetochores would be shared between the two SPBs, as indeed was also shown by Heath and Rethoret (1981) from EM of *Saprolegnia*. An alternative and possibly more likely explanation could be based on the findings that in wild-type cells the nuclear DNA remains in the mother prior to anaphase (Palmer et al., 1989), and that multiple copy circular *ARS* plasmids lacking segregation elements also remain in the mother after mitosis (Murray and Szostak, 1983). These suggest the possibility of a different mechanism for retention of DNA in the mother cell, so that if all the kinetochores were inactive in the ts mutant, the DNA would remain in the mother cell. This proposal could also explain the difference in phenotype in the Ndc10p-depleted cells where unequal segregation is seen initially. Here, unattached chromosomes would remain in the mother while attached chromosomes would segregate between mother and bud.

Recently, Saunders and Hoyt (1992) proposed that there is a balance of forces in the budding yeast metaphase spindle. An elongating force operated by the mitotic kinesins Kip1p and Cin8p is balanced partly by Kar3p and possibly also kinetochore microtubules linked to paired but unseparated chromatids. The *ndc10-1* mutant might be useful in a further test of this model, provided the microtubule-kinetochore linkage is absent during metaphase when the proposed elongating force due to the kinesins is operating. The abnormalities in the early anaphase spindles of *ndc10-1* may be consistent with this model. It is possible that the elongating force could be dominant in the mutant so that the dumbbell metaphase spindle would begin to slide apart prematurely, leading to the observed morphology of two poles each with short microtubules attached and connected by a few pole to pole microtubules. However, a more careful analysis of the phenotype would be necessary before reaching a definite conclusion.

Clearly both *ndc10-1* and the depleted cells are able to bypass any potential checkpoint system (Hartwell and Weinert, 1989) monitoring chromosome attachment to the spindle, since cells enter anaphase with chromosome detached from the spindle. It is possible that a checkpoint might be simply overwhelmed in the mutants or that Ndc10p function is normally carried out with high reliability so no checkpoint is needed. There did however appear to be a delay in entering anaphase, particularly in the depleted cells, leading to an accumulation of large budded cells with short spindles, which is similar to the phenotype found in cells with centromere DNA mutations (Spencer and Hieter, 1992).

Localization of Ndc10p

The immunofluorescence pattern seen with anti-Ndc10p shows staining of the SPB region in nearly all cells examined, suggesting that localization to the SPB region is relatively cell cycle independent; in addition, there was fainter staining along short, presumably metaphase, spindles. Since Ndc10p is identical to Cbf2p (Jiang et al., 1993), a potential yeast kinetochore protein (Hyman et al., 1992), then does this observed staining pattern reflect kinetochore position during the cell cycle? To answer this question a number of points need to be considered.

First, is the sensitivity of the immunofluorescence method sufficient to detect yeast kinetochore proteins? There may only be a minimum of 32 copies of a particular kinetochore protein per spindle pole of a diploid yeast strain, assuming one copy per chromosome (Lechner and Carbon, 1991). Work in other systems suggests that such a copy number should be detectable if the molecules are in close proximity: for example myosin minifilaments containing 8 molecules can be detected by immunofluorescent staining with a mAb against the myosin head (Yonemura and Pollard, 1992), while around 50 molecules of a fluorescent cyanine dye were detected when bound to low density lipoprotein on the surface of a cell (Barak and Webb, 1981). In our case 32 copies of protein would have around a thousand molecules of FITC bound assuming threefold amplification by both first and second antibodies and with four moles of FITC per mole of second antibody. Thus both examples indicate that the fluorescent method has sufficient sensitivity, provided the molecules are in close proximity. However it may not detect functionally important molecules of Ndc10p present in low copy number.

Second, since the staining pattern was of low intensity it is important to establish that the antibodies used were specific to Ndc10p. Although the antibodies gave only weak staining of Ndc10p on immunoblots of whole wild-type cell extracts, due presumably to the very low copy number (Lechner and Carbon, 1991), they gave a strong specific signal close to the predicted size of 111 kD on immunoblots of cells overexpressing a slightly truncated Ndc10p. When Ndc10p was depleted, by switching off the *GAL* promoter, the staining of the SPB region and of short spindles disappeared at about the same time that the phenotype appeared, providing further evidence that the staining pattern observed is due to the presence of Ndc10p.

Third, since yeast kinetochores are not visible in whole

cells by conventional EM, then their position during the cell cycle can only be inferred from indirect experiments in yeast and from work on other fungi where kinetochores are visible. In *S. cerevisiae* Peterson and Ris (1976) mapped the position of discontinuous, probably chromosomal, microtubules. In *Saprolegnia* kinetochore position was mapped by EM throughout the cell cycle (Heath, 1980*a*; Heath and Rethoret, 1981), and in *S. pombe* preliminary experiments have mapped centromere position using DNA probes (Uzawa and Yanagida, 1992; Takahashi et al., 1992). If these three sets of results are combined, then some tentative conclusions can be drawn about kinetochore position during the cell cycle in *S. cerevisiae*. Since *S. cerevisiae*, like *Saprolegnia*, has nuclear microtubules attached to the SPB during interphase, then, as in *Saprolegnia* (Heath, 1980*a*), kinetochores may also be attached to these microtubules in interphase *S. cerevisiae* as proposed by Murray and Szostak (1985). During SPB replication, assuming *S. cerevisiae* is like *Saprolegnia* (Heath and Rethoret, 1981), the pre-existing kinetochores would be shared between the two SPBs and remain closely attached to them, a short spindle would form, and shortly after this kinetochore replication could take place. During metaphase there is some disagreement about the position of the kinetochores. Peterson and Ris (1976) concluded that there was a metaphase plate in *S. cerevisiae*; however, other Ascomycetes lack such an ordered array of chromatids, with kinetochore pairing occurring instead at various points along the metaphase spindle (Aist and Williams, 1972; Heath, 1980*a*). It is possible that the preparative technique used by Peterson and Ris (1976), swelling and lysis of spheroplasts during fixation to displace obscuring chromatin away from the spindle, may have removed paired chromosomes from the outside of the spindle and preferentially retained chromatin in the central core. This might give the appearance of an apparent metaphase plate. During spindle elongation at anaphase, paired kinetochores would divide and move towards the two poles becoming closely associated with the SPBs at later stages of anaphase. Note that this proposed behavior of fungal kinetochores, that is close association with the SPB except during metaphase, when pairing occurs along the spindle, is different from mammalian kinetochores. These are distributed through the nucleoplasm in interphase and form an ordered array in the center of the metaphase spindle (Moroi et al., 1980).

The immunofluorescence staining pattern observed with anti-Ndc10p antibodies is broadly but not completely in agreement with these proposed fungal kinetochore movements. In cells containing a single microtubule aster, which are presumably in interphase, staining is found close to the SPB. Sometimes the staining has a hole in the center or occurs as several spots close to the SPB, which we assume may reflect clusters of kinetochores joined to different bundles of nuclear microtubules. There are however other explanations for the association of Ndc10p with the SPB region. Dynein, another minus-end-directed microtubule motor (Schroer et al., 1989), is also present at spindle poles (Pfarr et al., 1990; Steuer et al., 1990), and can produce artificial asters in *Xenopus* extracts by binding microtubules and sliding towards their minus ends (Verde et al., 1991). Thus surplus Ndc10p not bound to kinetochores might still bind to microtubules and move toward the minus end which is probably at the SPB (Yamamoto et al., 1990), and thereby produce the observed staining pattern. That such surplus Ndc10p is present is indicated by the nuclear staining seen in some cells (Fig. 6, *a* and *d*); possibly this fraction of Ndc10p is dephosphorylated and thus has low centromere DNA binding activity (Lechner and Carbon, 1991).

Cells with short spindles have one of two staining patterns. In the first the signal is concentrated close to the SPBs, presumably reflecting the situation either before kinetochore pairing, or after pairing in longer spindles which are just entering anaphase. In the second staining pattern the signal can be somewhat more diffuse and is spread along the spindle though still closely associated with it. This pattern may reflect chromosome pairing via kinetochore microtubules occurring at various points along the spindle. Alternatively, this staining pattern would also be consistent with a suggestion by Koshland (1992) that the CBF3-associated motor might have a function in prometaphase chromosome sorting rather than anaphase A. Thus the motor would bind chromatids laterally to prometaphase spindle microtubules until by some dynamic process metaphase pairing was achieved, when each sister chromatid had bound to a microtubule of opposite polarity.

Anaphase and late anaphase spindles have staining closely associated with the SPBs, and somewhat surprisingly, since all the kinetochores should be by the pole at this point, there is weak staining associated with such spindles. We have no explanation for this result; however, since the protein is part of CBF3, a fraction with microtubule motor activity (Hyman et al., 1992), this staining may reflect surplus active CBF3 associated with microtubules. A mammalian kinetochore antigen, CENP-E (Yen et al., 1991, 1992), also shows an unexpected distribution during mitosis. It is associated with the kinetochores at prometaphase and metaphase, but transfers to the midzone of the spindle at anaphase and then to the midbody after telophase. Thus, the immunofluorescent staining pattern of Ndc10p is for the most part consistent with the proposed pattern of kinetochore localization, but there are some inconsistencies and other possible interpretations of the staining pattern. Some of these problems may be resolved by study of a yeast with more favorable chromosome morphology, for example *Lipomyces lipofer* (Robinow, 1961).

In conclusion, we have isolated a nuclear division cycle gene *ndc10-1* whose phenotype is a detachment of chromosomes from the spindle while DNA replication, anaphase and cytokinesis continue. *NDC10* is an essential gene whose gene product is localized to the SPB region during the cell cycle and along some short, presumably metaphase, spindles. The phenotype of both *ndc10-1* and of cells depleted of Ndc10p and the localization of Ndc10p are consistent with its identity to *CBF2* (Jiang et al., 1993), a component of a yeast centromere DNA-binding complex (Lechner and Carbon, 1991).

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