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NDC1: A Nuclear Periphery Component Required for Yeast Spindle Pole Body Duplication

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Abstract. The spindle pole body (SPB) of *Saccharomyces cerevisiae* serves as the centrosome in this organism, undergoing duplication early in the cell cycle to generate the two poles of the mitotic spindle. The conditional lethal mutation *ndc1-1* has previously been shown to cause asymmetric segregation, wherein all the chromosomes go to one pole of the mitotic spindle (Thomas, J. H., and D. Botstein. 1986. *Cell.* 44:65–76). Examination by electron microscopy of mutant cells subjected to the nonpermissive temperature reveals a defect in SPB duplication. Although duplication is seen to occur, the nascent SPB fails to undergo insertion into the nuclear envelope. The parental SPB remains functional, organizing a monopolar spindle to which all the chromosomes are presumably attached. Order-of-function experiments reveal that the *NDC1* function is required in G1 after α-factor arrest but before the arrest caused by *cdc34.* Molecular analysis shows that the *NDC1* gene is essential and that it encodes a 656 amino acid protein (74 kD) with six or seven putative transmembrane domains. This evidence for membrane association is further supported by immunofluorescent localization of the *NDC1* product to the vicinity of the nuclear envelope. These findings suggest that the *NDC1* protein acts within the nuclear envelope to mediate insertion of the nascent SPB.

ASSEMBLY of the mitotic spindle in a eucaryotic cell is dependent on the formation of two centrosomelike organelles from which spindle microtubules emanate. The apparent duplication of centrosomal components to generate spindle poles is well described cytologically (reviewed by McIntosh, 1983; Brinkley, 1985; Sluder, 1989), but few molecular details of the underlying mechanism are known. The relevant organelle in the yeast *Saccharomyces cerevisiae* is the spindle pole body (SPB),[1] which is situated within the nuclear envelope (reviewed by Winey and Byers, 1992). As the sole microtubule organizing center in *S. cerevisiae*, the SPB forms microtubular arrays in both the cytoplasm and the nucleus. Electron microscopy of wild-type and mutant yeast strains has permitted description of the SPB duplication pathway and spindle formation (Byers, 1981*a*; Winey et al., 1991). A crucial early step in this pathway is the formation of the satellite on the outer surface of the half-bridge structure adjacent to the extant SPB. At START in G1, the satellite-bearing SPB is transformed into duplicated, side-by-side SPBs, both of which are inserted into the nuclear envelope and bear both nuclear and cytoplasmic microtubules. This G1 duplication event is thought to occur by a conservative mechanism where the satellite structure serves as the precursor for the new SPB, and the existing SPB from the previous cell cycle remains intact. Recently, Vallen et al. (1992) clearly demonstrated that a *KAR1-βGal* fusion protein is localized to only one of the newly duplicated SPBs, providing direct evidence that SPB duplication is indeed a conservative process. Separation of the two SPBs occurs later, leading to assembly of the bipolar mitotic spindle.

The various stages of SPB duplication described above for wild-type cells are also observed in *cdc*⁻ (*c*ell *d*ivision *c*ycle) mutants at their terminal arrest points. Satellite-bearing SPBs are observed in yeast cells arrested in G1 by mating pheromone or mutations in the *CDC28* gene, whereas duplicated side-by-side SPBs are observed in cells arrested later in G1 by *cdc4* and *cdc34* (Byers and Goetsch, 1974, 1975; Goebl et al., 1988). Other mutants are specifically defective in SPB duplication. Cells bearing mutations in the *CDC31* or *KAR1* (*kar*yogamy) genes fail to undergo SPB duplication altogether despite execution of other cell cycle functions. This leads to arrest as a large budded cell with G2 DNA con-

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

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tent and a single large SPB (Byers, 1981*b*; Rose and Fink, 1987). Both *CDC31* and *KAR1* are required at an early stage of SPB duplication, possibly mediating formation of the satellite. *CDC31*, at least, is not required for the transition from the satellite-bearing SPB stage to the duplicated side-by-side SPBs stage (Winey et al., 1991). Two recently identified mutants, *mps1* and *mps2* (*m*onopolar *s*pindle), identify genes whose functions are essential for this transition (Winey et al., 1991). Upon transfer to the nonpermissive temperature, *mps1-1* strains fail in SPB duplication, yielding a single large SPB of aberrant morphology. Strains containing the *mps2-1* mutation arrest with two SPBs, only one of which is functional. The defective SPB in *mps2-1* arrested cells can be detected by immunofluorescent staining of microtubules that emanate exclusively from its cytoplasmic face. Electron microscopy has shown that the defective SPB is not inserted into the nuclear envelope, but instead resides on its cytoplasmic surface. Having no access to the nucleoplasm, the defective SPB cannot act as a pole of the mitotic spindle. Despite lacking any spindle microtubules, the defective SPB is segregated away from the functional SPB by a mechanism that remains unknown.

The *ndc1-1* (*n*uclear *d*ivision *c*ycle) mutation renders yeast cold-sensitive for growth and causes several defects that are similar to those observed in *mps2* mutants, yet these mutations map to different chromosomes (Thomas and Botstein, 1986; Winey et al., 1991). When cells mutant for either gene are shifted to the nonpermissive temperature, two distinct foci of microtubule organization that are not connected by a normal spindle are detected by immunofluorescent staining of tubulin. In both cases, chromosomal DNA is associated with only one of these foci. *ndc1* mutants also exhibit asymmetric DNA segregation, all the chromosomes going to one pole of the spindle to yield a diploid and an aploid cell from an initial haploid cell transiently exposed to the nonpermissive temperature (Thomas and Botstein, 1986). It seemed possible that *ndc1* mutants have a defect in SPB duplication similar to that described above for *mps2* mutants. We report here that electron microscopic analysis of *ndc1* mutants at the nonpermissive temperature does, in fact, reveal a defect in SPB duplication that is very similar to that observed in *mps2* mutants. Using synchronized cells, we have found that *NDC1* gene activity is required during G1 for SPB duplication. Furthermore, isolation and analysis of the *NDC1* gene has shown that it encodes a 74-kD protein essential for cell viability. The predicted *NDC1* protein has six or seven stretches of hydrophobic amino acids that may constitute transmembrane domains. Consistent with the sequence data, antibody staining localizes the gene product to the nuclear periphery. These findings suggest that the *NDC1* gene product is a constituent of the nuclear envelope that is required for insertion of the nascent SPB into its normal position in the nuclear envelope.

Materials and Methods

Strains, Cell Culture, and Genetic Techniques

The yeast strains used in this study are listed in Table I. Yeast media and genetic techniques were as described by Hartwell (1967) and Sherman et al. (1971). The *ndc1-4* allele was isolated as an *ndc1-1* noncomplementing mutation. The *ndc1-1* allele is cold-sensitive (cs^-) for growth at 14°C. The mutant screen for *ndc1-1* noncomplementing mutations was carried out essentially as described by Stearns and Botstein (1988). A stationary phase culture of DBY1399 (Table I) was mutagenized using ethylmethane sulfonate (EMS). Mutagenized colonies were recovered on YEPD plates after 3 d at 26°C and were mated at 26°C with cells of DBY1583 and DBY1584 (Table I). Diploid products of both crosses were selected at 14°C on minimal medium (SD) supplemented with uracil. Putative noncomplementors of *ndc1-1* were identified as DBY1399 mutant colonies that mated with DBY1584 (*NDC1*) to produce diploid colonies that could grow at 14°C, but mated with DBY1583 (*ndc1-1*) to produce diploid colonies that could not grow at 14°C. These putative noncomplementors were backcrossed to $NDC1^+$ strains. For the *ndc1-4* mutation, the noncomplementing phenotype segregated as a single trait in these crosses. This phenotype also cosegregated with a recessive temperature-sensitive (ts^-, no growth at 37°C) for growth phenotype. Tetrad analysis showed that the ts^- phenotype is tightly linked to *NDC1*, suggesting that this phenotype is caused by a mutation in *NDC1*. Furthermore, the ts^- phenotype of *ndc1-4* can be complemented by a CEN plasmid that contains the wild-type *NDC1* gene. Thus, we conclude that the noncomplementation and the ts^- phenotypes are both caused by the *ndc1-4* mutation.

Table I. Yeast Strain List

Strain	Genotype
DBY1399	α, *ade2, ura3-52*
DBY1503-1	a/a, *ndc1-1/ndc1-1, his4-539/his4-539, ade2/ade2, ura3-52/ura3-52*
DBY1583	a, *ndc1-1, his4-539, ura3-52, lys2-801*
DBY1584	a, *his4-539, ura3-52, lys2-801*
DBY1826	a, *ade2, his3-Δ200, leu2-3,112, ura3-52*
DBY1826/1829	a/α, *ade2/+, his3-Δ200/his3-Δ200, leu2-3,112/leu2-3,112, lys2-801/+, trp1-1/+, ura3-52/ura3-52*
Dndc1-4	a/α, *ndc1-4/ndc1-4, his4-539/his4-539, ura3-52/ura3-52*
Dndc/34	a/α, *ndc1-1/ndc1-1, cdc34-2/cdc34-2, ade2/+, his3/+, lys2/+, ura3-52/ura3-52*
MAY98	a, *ndc1::LEU2, ura3-52, leu2-3,112,* (pMA1011 = *NDC1* − *URA3* − *CEN*)

Yeast cells were arrested in G1 with α-factor (7–10 μM) produced by custom peptide synthesis using F-MOC chemistry on a peptide synthesizer (model 488, Applied Biosystems Inc., Foster City, CA). The efficiency of a given arrest was monitored by determining the budding index (proportion of budded cells in a sample of 200 cells) of briefly sonicated aliquots. Arrests were considered adequate when 95% of the cells were unbudded, and the arrest was later confirmed by flow cytometry (see below) to show that the population was predominantly comprised of cells with G1 DNA content. Cells arrested by treatment with α-factor or by the *cdc34-2* mutation at the nonpermissive temperature (36°C) were released from these blocks by rinsing twice in growth medium equilibrated to the appropriate temperature for the experiment. In these experiments, entry into and progression through the cell cycle were monitored by budding index and flow cytometry.

Isolation and Characterization of the NDC1 Gene

Yeast strain DBY1583 (*ndc1-1*, Table II) was transformed with plasmid DNA from a genomic library in a *URA3*-CEN vector (Rose et al., 1987). Cells transformed to uracil prototrophy were obtained at 30°C and replica transferred to minimal media (SD) minus uracil plates at 11° and 14°C. The plasmid DNA from three cold-resistant transformants was transferred to *E. coli* for further analysis. All three were found to contain overlapping regions of chromosomal DNA (see Results). The region encoding the *NDC1* complementing activity was identified by subcloning smaller fragments into yeast vectors and reintroduction into DBY1583. In addition, the bacterial transposon γδ was used to create disruptions of a plasmid-carried *NDC1* gene using the protocol of Guyer (1978). The DNA comprising nucleotides −300 to +2258 (Fig. 3) was sequenced on both strands using sequential overlapping clones produced by the method of Dale et al. (1985). The resulting M13 clones were sequenced with a kit designed for this purpose (Amersham Corp., Arlington Heights, IL) following the instructions provided by the supplier. Analysis of the DNA sequence of the *NDC1* gene and its derived amino acid sequence was carried out using programs in the EuGene

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Table II. Spindle and SPB Morphologies in ndc1-1 Strains after Release from Various G1 Arrests

G1 arrest (SPB state)*	Release temperature‡	Spindles§ WT	Spindles§ ndc	SPB‖ WT	SPB‖ ndc
α-factor¶ (satellite-bearing SPB)	25°C	162	0	18	0
	15°C	0	204	1	47
*cdc34*** (duplicated side-by-side SPBs)	25°C	112	0	10	0
	15°C	118	5	10	0

* SPB morphology in arrested cells was confirmed by electron microscopy.
‡ 25°C is the permissive temperature for *ndc1-1*, and this release was for 1.5 h.; 14°C is the nonpermissive temperature for *ndc1-1*, and this release was for 6 h. The duration of the releases is the time necessary for the cells to have completed budding and to have completed S-phase.
§ Spindle morphology determined by immunofluorescent staining of microtubules as shown in Fig. 1: *WT* means normal, and *ndc* represents the cytology in *ndc1* mutants.
‖ SPB morphology determined by electron microscopy. Fig. 2 *B* shows a normal mitotic spindle which is denoted by *WT*, and *ndc* represents the phenotype shown for *ndc1* mutants in Fig. 2, *A* and *C–G*.
¶ Strain: DBY1503-1, Table I.
** Strain: Dndc/34, Table I.

software package from the Molecular Biology Information Resource at the Department of Cell Biology, Baylor College of Medicine (Waco, TX).

The *SalI/XhoI* fragment containing the *LEU2* gene from YEp13 (Broach et al., 1979) was inserted into the *SalI* site in the *NDC1* open reading frame to create *ndc1::LEU2*. Linear DNA containing this allele was used to transform an *NDC1*$^+$/*NDC1*$^+$ diploid strain containing pMA1011, a CEN plasmid that carries *NDC1* and *URA3*. Transformants were tested for the ability to grow on 5-fluoro-orotic acid (5-FOA, Boeke et al., 1984) medium with and without leucine. Those that could segregate 5-FOA-resistant clones only when leucine was present presumably had the *ndc1::LEU2* allele integrated into the plasmid and were discarded. Those that could segregate 5-FOA-resistant clones regardless of the presence or absence of leucine presumably had the *ndc1::LEU2* allele integrated into the chromosomal genome. Three members of this latter class were sporulated and dissected. All Leu$^+$ spores obtained were also Ura$^+$ and could not segregate 5-FOA resistant clones at any temperature tested, thus indicating that the *NDC1* gene is essential for mitotic viability.

A 2-μm based plasmid carrying the *NDC1* gene (pRB1239) was constructed by inserting the *NDC1*-containing *HindIII/BglII* fragment into pRB307, a 2 μm derivative of YIp5 (Broach et al., 1979).

Antibody Production

Plasmid pCC119 was constructed by inserting a 2.5-kb *SspI/HindIII* DNA fragment that contains most of the *NDC1* gene into the *SmaI/HindIII* sites of the *Trp*E fusion protein vector pATH10 (Koerner et al., 1991). This construction created an in-frame fusion between *Trp*E and *NDC1*. The *Trp*E-*NDC1* fusion protein was partially purified as an insoluble protein from *E. coli* cells harboring pCC119 (Koerner et al., 1991) and was further purified on a preparative SDS-polyacrylamide gel for use as antigen in injections of rabbits. Immune sera were adsorbed three times against heat-treated total *E. coli* extract prepared from cells carrying pATH10. The *E. coli*-depleted sera were then used for affinity-purification of anti-Ndc1p antibodies by adsorption against partially purified (as above) *Trp*E-*NDC1* fusion protein that was immobilized on a nitrocellulose membrane (Pringle et al., 1989).

For immunoblotting experiments, whole cell lysates were prepared from yeast cells (DBY1826, Table I) carrying the control plasmid pRB307 or the high-copy number *NDC1*- containing plasmid pRB1239. Proteins were transferred from an SDS-polyacrylamide gel to a nitrocellulose filter (Schleicher and Schuell, Keene, NH) as described by Burnette (1981). To detect the *NDC1* gene product (Ndc1p) on the nitrocellulose filter, affinity-purified anti-Ndc1p antibodies were used as primary antibodies that were subsequently detected by ^{125}I-protein A (New England Nuclear, Cambridge, MA).

Cytological Techniques

Cytological experiments were carried out using diploid strains because their larger SPBs and spindles are easier to visualize. Immunofluorescent staining of microtubules was carried out as described by Kilmartin and Adams (1984) as modified by Jacobs et al. (1988) using the rat mAb YOL1/34 (anti-α-tubulin) and FITC-conjugated goat anti-rat antibodies (Accurate Chemical and Scientific Corp., Westbury, NY). DNA was stained with DAPI (1.0 μg/ml: Sigma Chemical Co., St. Louis, MO). For the subcellular localization of Ndc1p, formaldehyde-fixed yeast spheroplasts (DBY1826/1829 carrying pRB1239, Table I) were stained first with affinity-purified rabbit anti-Ndc1p antibodies (Pringle et al., 1989), then with affinity-purified goat anti-rabbit IgG (Zymed Laboratories, Inc., South San Francisco, CA), and finally with FITC-conjugated affinity-purified rabbit anti-goat IgG (Organon Teknika Corp., West Chester, PA). Stained cells were viewed with a Nikon Microphot FX fluorescence microscope and photographed with Kodak Kodachrome 200 Professional film, or viewed with a Zeiss Axioskop fluorescence microscope and photographed with Kodak Type 2415 Technical Pan hypersensitized film (Lumicon, Livermore, CA).

Yeast cells were prepared for flow cytometry by the method of Hutter and Eipel (1979) using the DNA stain propidium iodide (Sigma Chemical Co., St. Louis, MO). Stained cells were analyzed on a Becton Dickinson FACSCAN® flow cytometer using the CELLFIT and LYSYS software packages to obtain and analyze data. Yeast cells were prepared for electron microscopy by procedures described by Byers and Goetsch (1974, 1975) and serial thin sections were viewed on a Philips EM300 electron microscope.

Results

The NDC1 Gene Is Required for Spindle Pole Body Duplication in G1

Strains mutant for *NDC1*, when shifted to the nonpermissive temperature (15°C for *ndc1-1*, Thomas and Botstein, 1986; or 37°C for *ndc1-4*, see Materials and Methods), segregate all their chromosomal DNA to one spindle pole, yielding one cell with twice the original ploidy and another cell that is aploid. Similar phenotypes have also been observed as a result of the monopolar mitosis of other mutants that are defective in SPB duplication (reviewed by Winey and Byers, 1992). Fluorescence microscopy of *ndc1-1* cells subjected to the nonpermissive temperature and stained with antibodies

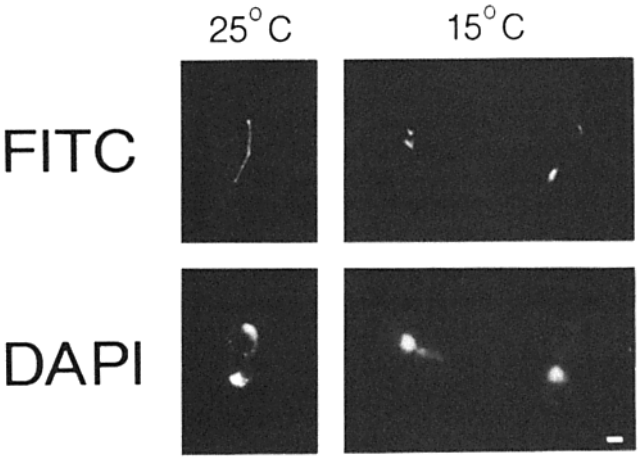

Figure 1. Immunofluorescent staining of *ndc1-1* containing strain DBY1503-1 (Table I, see Materials and Methods). A late mitotic cell at the permissive temperature (25°C) with a long spindle (*FITC*) and separated DNA (*DAPI*) is shown. At the nonpermissive temperature (15°C), two distinct foci of microtubules which do not appear to be connected by a mitotic spindle (FITC) are observed, and the DNA is not separated (DAPI).

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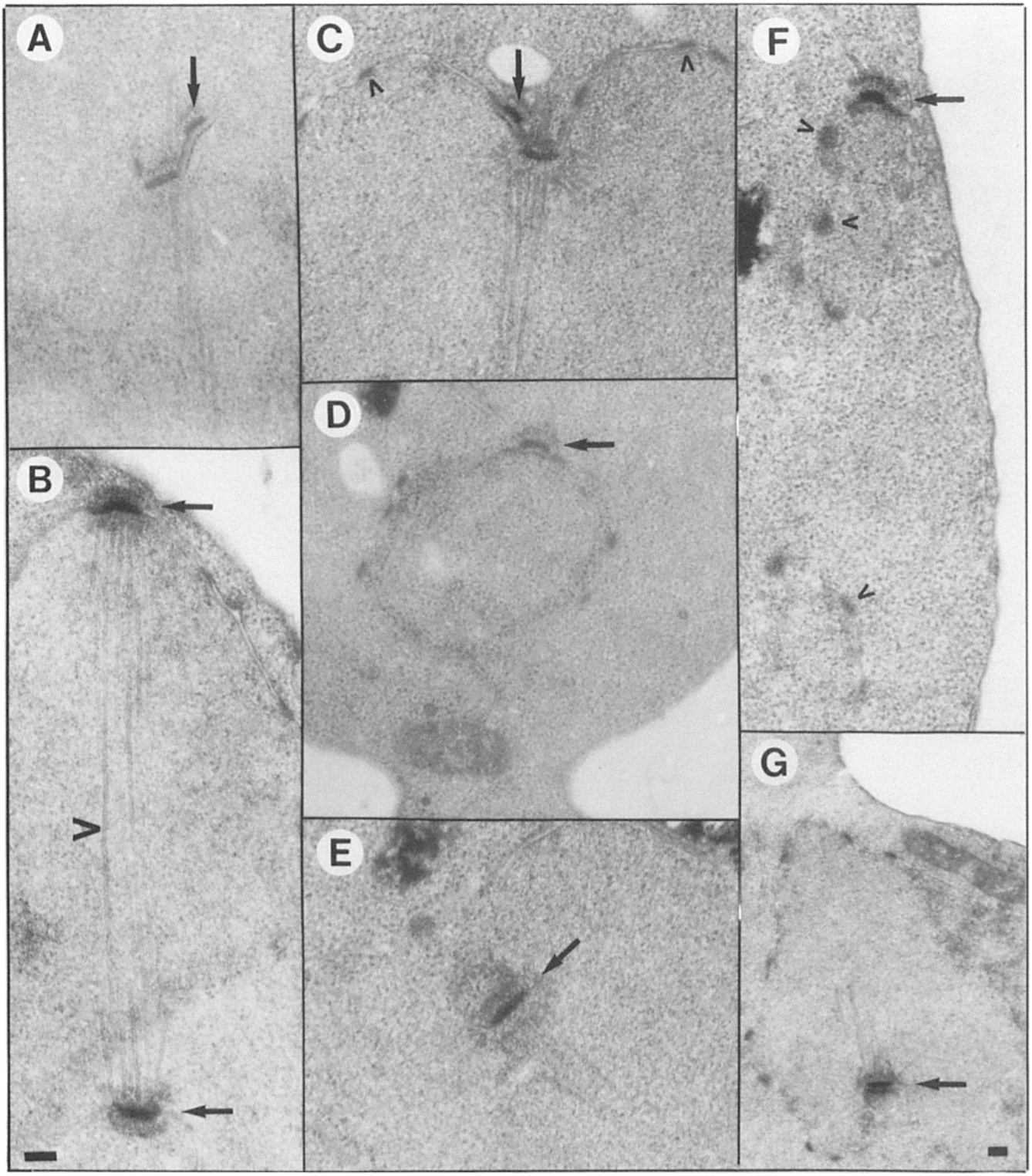

Figure 2. Electron microscopic analysis of *ndcl-1* (DBY1503-1) and *ndcl-4* (Dndcl-4) containing strains (Table I, see Materials and Methods). *B* shows a normal mitotic spindle observed in DBY1503-1 at the permissive temperature. The arrows indicate SPBs, large carets highlight spindle microtubules, and nuclear pores are indicated by small carets in other panels. Defective SPBs (*arrows*) adjacent to the functional SPB found in DBY1503-1 at the nonpermissive temperature (14°C) are shown in *A* and *C*. A representative separated monopolar spindle (*E*) and defective SPB (*D*) found in DBY1503-1 at the nonpermissive temperature (14°C) is shown. Identical morphologies of a monopolar spindle (*G*) and defective SPB (*F*) are observed in a *ndcl-4* mutant cell at its nonpermissive temperature of 36°C. These morphologies have been observed in no less than 15 serially sectioned nuclei of each mutant. Bars, 0.1 μm; and *A*, *C–F* are at the same magnification as *B*.

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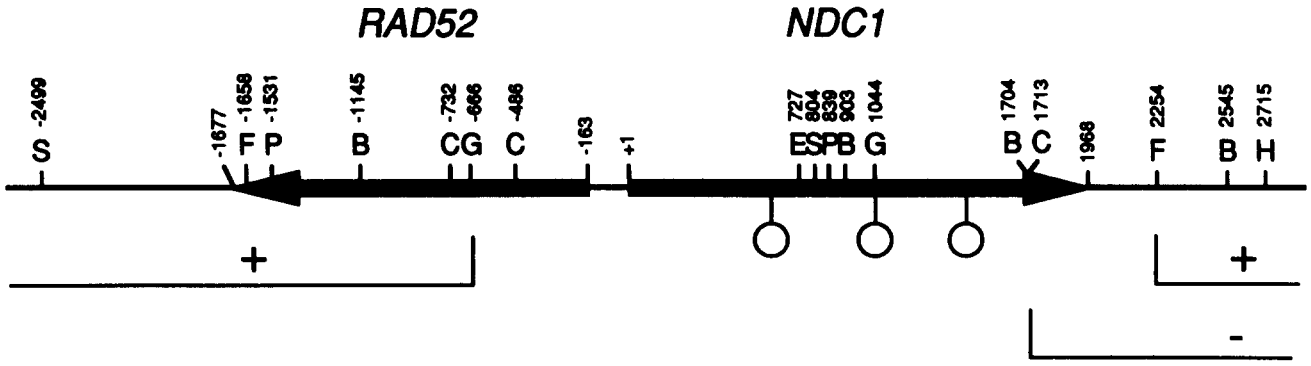

Figure 3. Restriction map of the *NDC1* locus. Nucleotide positions are numbered with the predicted initiation codon of *NDC1* at position +1. Arrows indicate the extent and the direction of the *NDC1* and *RAD52* open reading frames. The *RAD52* initiation codon (position −163) is the furthest upstream in frame ATG, although this position may not be utilized in vivo (see Adzuma et al., 1984). The brackets below the line labeled (+) indicate the extent of deletions that do not affect *NDC1*-complementing activity. The bracket labeled (−) signifies a deletion that destroys *NDC1*-complementing activity. The open circles indicate the positions of transposon γδ insertions that destroy *NDC1*-complementing activity. Between this work and Adzuma et al. (1984), the DNA sequence of the entire region between *SalI* (−2499) and *SphI* (2254) has been determined. Restriction enzyme sites: S, *SalI*; F, *SphI*; P, *PstI*; B, *BamHI*; C, *ClaI*; G, *BglII*; E, *EcoRI*; H, *HindIII*.

specific for tubulin shows that the large budded cells contain two microtubule organizing centers, but the associated microtubules do not appear to form a normal mitotic spindle (Fig. 1). Electron microscopic observation clarifies the nature of the defect in *ndcl* mutants at nonpermissive conditions. Examination of serial thin-sections reveals that each large budded cell contains one SPB of normal appearance and a second one that is defective (Fig. 2). The normal SPB bears microtubules on both its nuclear and cytoplasmic faces, whereas the defective SPB has microtubules only on the cytoplasmic face. In most cells, the defective SPB undergoes separation from the functional SPB and is often located at the end of a thin projection of the nuclear envelope (Fig. 2 *F*).

The structural defect observed in *ndcl* mutants suggests a specific role for the gene during SPB duplication. To assess the nature of this requirement, we synchronized *ndcl-1* cells at two different points in the G1 phase of the cell cycle before release at either permissive or nonpermissive temperatures. One type of G1 arrest was achieved by treatment with α-factor, which blocks progression at the stage with a satellite-bearing SPB (Byers and Goetsch, 1975). The other G1 arrest used was caused by the *cdc34-2* mutation, wherein mutants transferred to the nonpermissive temperature are blocked at a stage with duplicated side-by-side SPBs (Goebl et al., 1988). An α-factor sensitive diploid strain homozygous for *ndcl-1* (a/a, DBY1503-1, Table I) was arrested with α-factor at 25°C, then released from this arrest at 14°C to inactivate the *ndcl-1* gene product or at 25°C (permissive condition). As expected, electron microscopy confirmed that these α-factor arrested cells contain satellite-bearing SPBs. Cells released from this arrest were monitored for entry into the cell cycle using light microscopy to assay bud formation and flow cytometry to observe entry into and completion of DNA synthesis. Cells released at 25°C budded and completed DNA synthesis in about 90 min. Examination by indirect immunofluorescent staining of microtubules showed that these cells contained normal mitotic spindles (as in Fig. 1, see Table II). Cells released at 14°C, on the other hand, exhibited the *ndcl* phenotype described earlier (Table II). These observations were confirmed by electron microscopic analysis of the same cultures. Cells released at 25°C were found to contain normal spindles and SPBs (as in Fig. 2 *B*; Table II), whereas those released at 14°C contained the characteristic monopolar spindle and the defective SPB (as in Fig. 2, *A*, *C–G*; Table II). Among the cells released at 14°C and viewed by electron microscopy, only one cell of 48 examined was found to contain a normal mitotic spindle. The vast majority of cells (47/48) had uniformly suffered the defect in SPB duplication. We conclude that the *NDC1* gene function is required at a stage of SPB duplication that follows the satellite-bearing SPB stage.

In a second experiment, the requirement for *NDC1* function after completion of SPB duplication was tested. A strain (Dndc/34, Table I) doubly mutant for *CDC34* and *NDC1* was brought to the *cdc34* arrest by incubation at 36°C. Cells arrested in this manner contain duplicated side-by-side SPBs, a phenotype that was confirmed by electron microscopy. The cells were then released from this arrest by transfer either to a temperature permissive for both mutations (25°C) or to the nonpermissive temperature for only the *ndcl-1* mutation (14°C). Cell cycle progression upon release from the *cdc34* block was again monitored by flow cytometry, and spindle structure was monitored both by immunofluorescent staining of microtubules and by electron microscopy. Release of these cells from the *cdc34* block at either temperature led to the formation of normal mitotic spindles (Table II), demonstrating that the *NDC1* function is not required either for separation of the SPBs or the subsequent formation of the mitotic spindle.

NDC1 Encodes an Essential, Hydrophobic Protein

The *NDC1* gene was isolated by complementation of the *ndcl-1* cold-sensitive phenotype. Plasmids that conferred on *ndcl-1* strain DBY1583 the ability to grow at 11°C were isolated from a centromere vector-based genomic library (Rose et al., 1987). Restriction enzyme sites were mapped in the genomic DNA inserts from three such plasmids, and restriction fragments common to all three plasmids were identified. Tight genetic linkage between *NDC1* and the DNA damage repair gene *RAD52* had been reported (Thomas and Bot-

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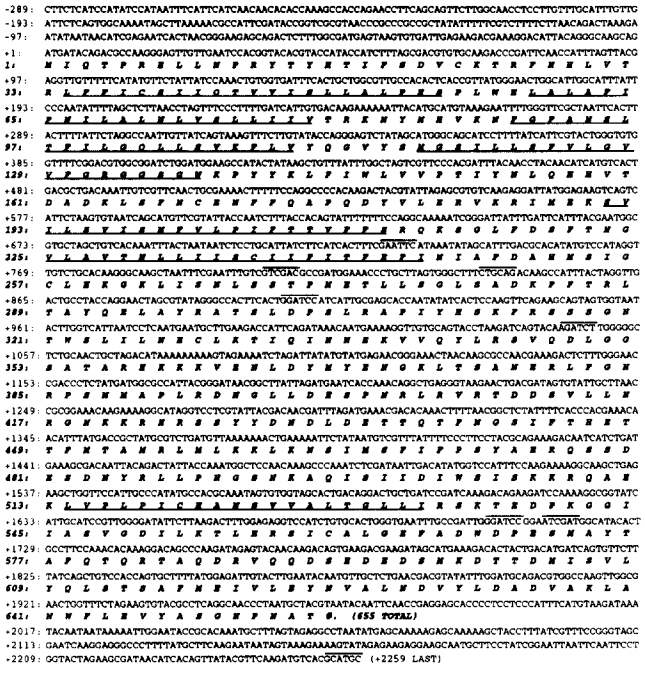

Figure 4. The DNA sequence of the *NDC1*-containing region and inferred amino acid sequence. The underscored regions of the amino acid sequence are putative transmembrane domains (see Table III). The overscored segments of the DNA sequence are restriction enzyme recognition sites (reading 5′ to 3′): *EcoRI*, *SalI*, *PstI*, *BamHI*, *BglII*, *BamHI*, *ClaI*, and *SphI*. These sequence data are available from EMBL/GenBank/DDBJ under accession number X70281 (Id. SCNDC1A).

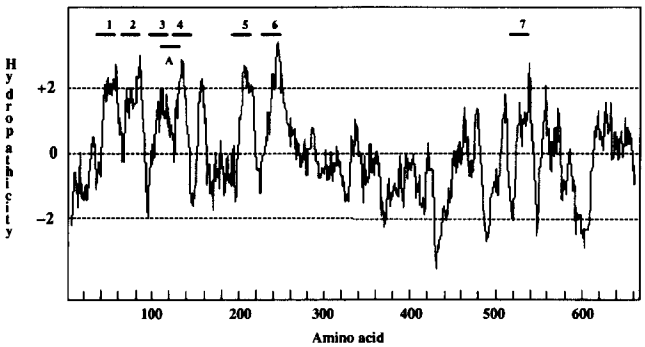

Figure 5. Kyte-Doolittle hydropathy plot of the *NDC1* encoded protein, where the average hydrophobicity of each amino acid was calculated using a window of nine amino acids. The regions with positive values are more hydrophobic. The positions of the seven possible transmembrane domains are shown as bars above the plot. Also shown is the position of the alternate (*A*) transmembrane domain, which spans putative transmembrane domains 3 and 4. Detailed information on these domains is presented in Table III, which uses the same nomenclature.

stein, 1986), so the observation of common restriction fragments between the *ndc1*-suppressing plasmids and YEp13-[*RAD52*] (Schild et al., 1983) was not unexpected. Therefore, the DNA insert in these plasmids corresponds to the *RAD52* and *NDC1* loci on chromosome XIII (Mortimer et al., 1989). The *ndc1*-complementing activity was further localized within the isolated genomic DNA by subcloning and by bacterial transposon $\gamma\delta$ mutagenesis (Fig. 3). These experiments and DNA sequencing of this region revealed that the reading frames of the *NDC1* gene and the adjacent *RAD52* gene lie in opposite orientation with proximal 5′ ends (Fig. 3). The 5′ end of the then unidentified *NDC1* gene had been detected and partially characterized by Adzuma et al. (1984) in their analysis of the *RAD52* locus. As shown in that study, the transcription of *NDC1* initiates ~45 bp upstream of the predicted *NDC1* start codon.

The *NDC1* gene was disrupted to determine the effect of a null allele on viability. Haploid strains were constructed (i.e., MAY98, Table I) that carried wild-type alleles of *NDC1* and *URA3* on a plasmid and the *ndc1*::*LEU2* disruption and *ura3-52* alleles at their respective chromosomal loci. At temperatures ranging from 11–37°C, these strains were unable to segregate cells resistant to 5-fluoro-orotic acid, a compound that inhibits growth of *URA3*$^+$ cells (Boeke et al., 1984). This result indicated that these strains required the continued presence of the *NDC1*-containing plasmid for viability and therefore that the *NDC1* gene is essential for mitotic viability.

The derived 656 amino acid sequence (predicted molecular weight of 74 kD, see Fig. 4) of the *NDC1* gene was compared to all amino acid sequences in GenBank (Release 74.0), and no protein with significant similarity was detected. A hydropathy plot for the *NDC1* amino acid sequence is shown in Fig. 5, and stretches of amino acids that appear hydrophobic according to the algorithm of Kyte and Doolittle (1982) are indicated. These regions were also examined by the algorithm of Eisenberg et al. (1984) as shown in Table III. Six or seven regions of the predicted *NDC1* protein are of sufficient length and hydrophobicity to be membrane spanning segments. The mean hydrophobic moment of each putative transmembrane segment (Table III) suggests that these regions could interact with each other, or interact with the transmembrane domains of other integral membrane protein(s) (Eisenberg et al., 1984).

Table III. Characteristics of Predicted Transmembrane Domains of the NDC1 Protein

Trans-membrane Segment*	Kyte-Doolittle‡		Eisenberg§		
	Amino acids	<h>	Amino acids	<H>	<μ_H>
1	34–52	2.3	34–54	0.65	0.23
2	61–79	2.4	59–79	0.79	0.23
3	92–110	1.5	90–110	0.55	0.17
4	117–135	1.4	117–137	0.55	0.30
5	192–210	2.4	191–211	0.73	0.22
6	225–243	2.3	225–245	0.71	0.18
7	514–532	1.6	513–533	0.51	0.23
Alternate‖	112–130	1.7	110–130	0.67	0.26

* These predicted transmembrane segments are underscored in Fig. 4, and are shown in Fig. 5.

‡ The average hydropathy (<h>) of 19 amino acid segments was calculated using the hydropathy index of Kyte and Doolittle (1982).

§ The mean hydrophicity (<H>) was calculated across 21 amino acids using the hydrophobicity index of Eisenberg et al. (1984). The mean hydrophobic moment (<μ_H>) for these 21 amino acid segments was calculated by using the μ_H for each amino acid in the algorithm of Eisenberg et al. (1984).

‖ An alternate transmembrane segment can be identified that would replace transmembrane segments 3 and 4, yielding a model with six transmembrane segments.

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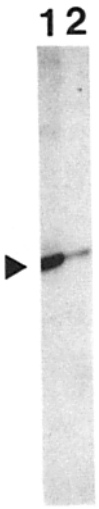

Figure 6. Specificity of affinity-purified anti-Ndclp antibodies. Roughly equal amounts of yeast whole cell lysates prepared from DBY1826 carrying the control plasmid pRB307 (lane *2*) or the high copy number *NDC1* plasmid pRB1239 (lane *1*) were used for immunoblotting with affinity-purified anti-Ndclp antibodies. The arrowhead shows the detected Ndclp. With longer exposure of the autoradiogram, protein species of much higher apparent molecular weights are also detected in cells containing pRB1239.

The NDC1 Gene Product Localizes to the Vicinity of the Nuclear Envelope

To better understand the cause of the cytological defect seen in *ndc1* mutants, we determined the localization of the *NDC1* gene product (Ndclp). Affinity-purified anti-Ndclp antibody used in an immunoblotting experiment recognized a single protein from a wild-type yeast extract (Fig. 6, lane *2*). This protein, which migrates on SDS-polyacrylamide gels with an apparent molecular weight of about 62 kD, is present in greater abundance in yeast cells that carry the *NDC1* gene on a high copy number plasmid (Fig. 6, lane *1*).

Monospecific affinity-purified anti-Ndclp antibodies were used to determine the subcellular localization of Ndclp by indirect immunofluorescence of yeast cells. In initial tests using FITC-conjugated goat anti-rabbit IgG as secondary antibody, very weak Ndclp staining that appeared to be perinuclear was detected in some cells. This staining pattern was more readily observed in cells carrying the *NDC1* gene on a high copy-number plasmid. As an added improvement, an extra antibody amplification step was added to the immunofluorescence procedure (see Materials and Methods). This latter modification yielded more consistent Ndclp staining, especially in cells carrying the *NDC1* gene on a high copy number plasmid.

The characteristic pattern of Ndclp staining seen in cells carrying the *NDC1* 2-μm plasmid is shown in Fig. 7. Here, as in other cells, the immunofluorescent staining generally overlaps the boundary of a phase dark area, which is known from DAPI staining to be occupied by the nucleus. The intensity of this staining varies between cells, perhaps because of variation in copy number of the *NDC1* plasmid. The perinuclear nature of Ndclp staining was especially obvious when different focal planes of stained cells were examined. In a small proportion of cells, Ndclp staining could be seen to extend outward from the nuclear periphery (Fig. 7 *E*), perhaps representing either the ER or simply a protruding portion of the nucleus. The distribution of Ndclp staining is therefore quite similar to the staining pattern seen for yeast nuclear pore components (Davis and Fink, 1990; Nehrbass et al., 1990; Wente et al., 1992), consistent with the possibility that Ndclp is a constituent of the nuclear envelope, although localization to the ER is also possible.

Discussion

We have shown here that the *NDC1* gene encodes an essential, 656 amino acid protein with a calculated molecular weight of 74 kD. Found within the sequence are several stretches of hydrophobic amino acids that could be trans-

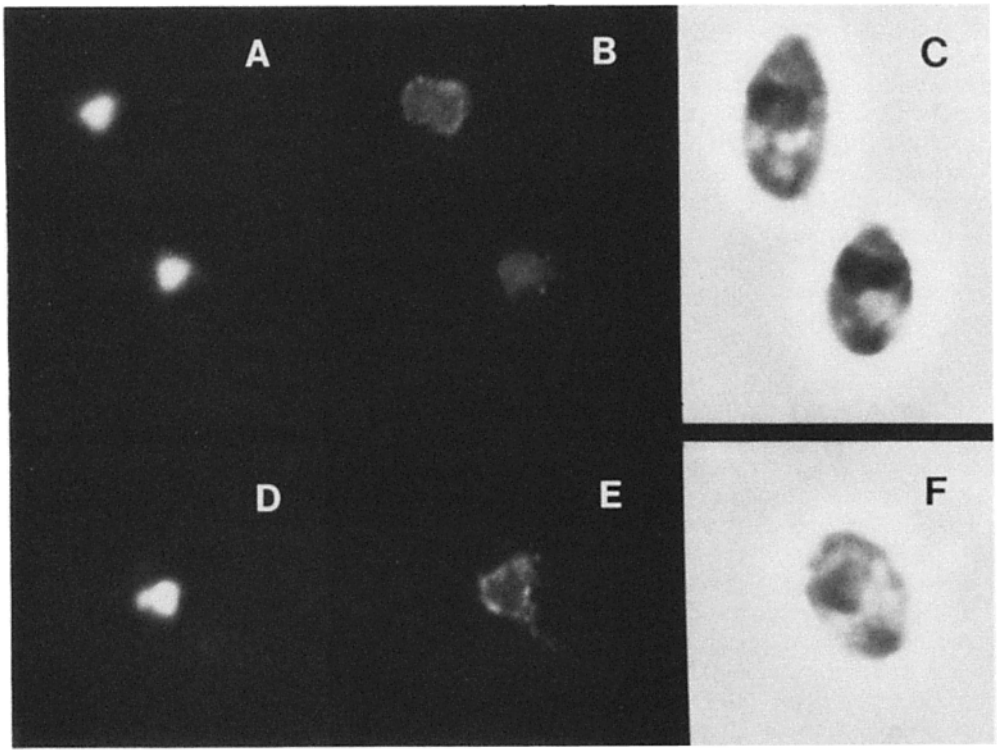

Figure 7. Localization of Ndclp in yeast cells carrying the high copy-number *NDC1*-plasmid pRB1239. Fluorescence images of cells stained with DAPI (*A* and *D*) and affinity-purified anti-Ndclp antibodies (*B* and *E*) and phase contrast images (*C* and *F*) of the same cells are shown.

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membrane domains as defined by the methods of both Kyte and Doolittle (1982) and Eisenberg et al. (1984). The suggested association of the protein with a membrane is further supported by the localization of the *NDC1* gene product by immunofluorescence microscopy. Ndc1p is found in greatest concentration in the immediate vicinity of the nuclear envelope, where it presumably performs its role in SPB duplication. This localization should be considered tentative because Ndc1p could only be reliably detected when overexpressed from a 2-μm plasmid. The localization of Ndc1p to the entire nuclear envelope and perhaps the ER is thought to be accurate, but may be a result of over-expression.

Other than displaying the overall pattern of hydrophobicity typical of integral membrane proteins, the *NDC1* gene product appears not to share striking homology with any known class of membrane proteins. Several yeast proteins, encoded by the *NPS1, NUP1, NUP49, NUP100,* and *NUP116* genes (Hurt, 1988; Nehrbass et al., 1990; Davis and Fink, 1990; Wente et al., 1992), have been previously localized to the nuclear envelope, and are thought to be peripheral membrane components of the nuclear pore complex. The putative *NDC1* protein bears no resemblance to the amino acid sequences of any of these gene products, nor to any component of the nuclear pore complex of mammalian cells for which sequence data is available (reviewed by Miller et al., 1991), or to members of the large family of receptor molecules that also possess seven transmembrane domains (reviewed by Dohlman et al., 1991).

The specific type of failure in SPB duplication leading to formation of a monopolar spindle and a defective SPB, as seen here for *ndc1* mutants, had previously been observed as part of the characterization of an *mps2* mutant (Winey et al., 1991). That analysis suggested that *mps2-1* identified a new step in SPB duplication on the basis of its novel phenotype and the results of order-of-function experiments. It was evident from that work that the *MPS2*-dependent step in SPB duplication occurs at a late stage of the process, but probably before duplication is complete. However, no methods were available to test this viewpoint adequately. It is now apparent from the very similar phenotypes observed for *mps2* and *ndc1* mutants at their respective nonpermissive temperatures that both genes may be required for the same step in SPB duplication. We have been able to show that *NDC1*, like *MPS2*, is required after release from α-factor arrest. The timing of the putative *MPS2/NDC1* dependent step relative to the point of *cdc34* arrest was uniquely accessible in the case of *ndc1-1* because it is a cold-sensitive mutation. Successful completion of the SPB duplication cycle when doubly mutant cells arrested at the *cdc34* step were transferred to the nonpermissive temperature for *ndc1-1* demonstrated that *NDC1* is not required after SPB duplication has occurred for the SPBs to undergo separation and participate in formation of the mitotic spindle. The present experiments clearly show that *NDC1* is required for the G1 transition from satellite-bearing SPB to side-by-side duplicated SPBs and render it likely that *MPS2* is required in the same process.

We report here that execution of the *NDC1* gene function occurs in G1, while Thomas and Botstein (1986) reported an execution point in G2. This discrepancy is resolved by examining the definition of *NDC1* gene function execution in these two studies. In this study, the failed execution of *NDC1* function in *ndc1-1* strains has been defined by direct analysis of SPB duplication using immunofluorescence and electron microscopy. In contrast, Thomas and Botstein (1986) used diploidization (endomitosis) of *ndc1-1* haploid strains as the signal that *NDC1* had failed to function and inferred the *NDC1* execution point on this basis. We now understand that this increase-in-ploidy assay may have revealed the execution point for endomitosis, but did not effectively report the execution point of *NDC1* for SPB duplication. The endomitotic event should require not only the formation of a monopolar spindle, but also the completion of DNA synthesis, so that two sets of chromosomes would be present. The proposed dependence of endomitosis on DNA synthesis would limit the execution point for diploid formation to a point subsequent to S phase, but would not similarly constrain the execution point for SPB duplication. Our results are consistent with the model that *NDC1* function is executed in G1, but endomitosis resulting from failure of *NDC1* function does not occur until after DNA synthesis is complete.

The phenotype observed in *ndc1* and *mps2* mutants suggests that SPB duplication is a conservative process, the preexisting SPB remaining unaltered while the other is a nascent structure. In the defective processes caused by these mutations, the existing SPB evidently serves as the sole functional spindle pole while the nascent SPB is defective and plays no role in the monopolar spindle. This idea is further supported by the analysis of a *KAR1-LacZ* fusion protein, which is localized to the SPB of an α-factor arrested cell, but is associated with only one SPB later in the cell cycle when two SPBs are present (Vallen et al., 1992). When expression of the *KAR1-βGal* fusion gene construct in a *ndc1-1* mutant at the nonpermissive temperature was examined, the fusion protein was found to be localized to what is now known to be the defective SPB. Vallen et al. (1992) suggest that the *KAR1* gene product is localized to the nascent SPB, and the defective SPB in *ndc1-1* strains is the remnant of the nascent SPB. This interpretation is consistent with the results we have presented here.

We have demonstrated that the yeast *NDC1* gene is essential and is required for the duplication of SPBs during the G1 phase of the cell cycle. Furthermore, the *NDC1* gene is shown to encode a hydrophobic protein that is localized to the vicinity of the nuclear envelope. We propose that the *NDC1* protein is an integral membrane protein located within the nuclear envelope where its functions include the insertion of the nascent SPB into the envelope. The *NDC1* protein might play a structural role in the nuclear envelope, such as providing a site of insertion for the SPB. Alternatively, the *NDC1* gene product might be involved in signaling across the nuclear envelope to coordinate activities, such as SPB duplication, that may involve functions of both the cytoplasm and the nucleus. Further analysis of the *NDC1* and *MPS2* genes should yield insight to the mechanism of this step in SPB duplication.

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