

A Temperature-sensitive Mutation of the *Schizosaccharomyces pombe* Gene *nuc2⁺* That Encodes a Nuclear Scaffold-like Protein Blocks Spindle Elongation in Mitotic Anaphase

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Abstract. A temperature-sensitive mutant *nuc2-663* of the fission yeast *Schizosaccharomyces pombe* specifically blocks mitotic spindle elongation at restrictive temperature so that nuclei in arrested cells contain a short uniform spindle ($\sim 3\text{-}\mu\text{m}$ long), which runs through a metaphase plate-like structure consisting of three condensed chromosomes. In the wild-type or in the mutant cells at permissive temperature, the spindle is fully extended $\sim 15\text{-}\mu\text{m}$ long in anaphase. The *nuc2⁺* gene was cloned in a 2.4-kb genomic DNA fragment by transformation, and its complete nucleotide sequence was determined. Its coding region predicts a 665-residues internally repeating protein (76,250 mol wt). By immunoblots using anti-sera raised against *lacZ-nuc2⁺* fused proteins, a polypeptide

(designated p67; 67,000 mol wt) encoded by *nuc2⁺* is detected in the wild-type *S. pombe* extracts; the amount of p67 is greatly increased when multi-copy or high-expression plasmids carrying the *nuc2⁺* gene are introduced into the *S. pombe* cells. Cellular fractionation and Percoll gradient centrifugation combined with immunoblotting show that p67 cofractionates with nuclei and is enriched in resistant structure that is insoluble in 2 M NaCl, 25 mM lithium 3,5'-diiodosalicylate, and 1% Triton but is soluble in 8 M urea. In *nuc2* mutant cells, however, soluble p76, perhaps an unprocessed precursor, accumulates in addition to insoluble p67. The role of *nuc2⁺* gene may be to interconnect nuclear and cytoskeletal functions in chromosome separation.

IN mitosis, the diffuse interphase chromatin condenses into well defined chromosomes and a spindle apparatus, which is a bipolar fibrous structure largely composed of microtubules appears (reviewed in Inoué, 1981; Picket-Heaps et al., 1982). Metaphase chromosomes line up along the equatorial plane midway between the poles. Each chromosome consists of the paired chromatids that are joined at the region called the centromere (or the kinetochore) where kinetochore microtubules are linked. In anaphase, the paired kinetochores on each chromosome are abruptly separated, allowing each chromatid to be pulled toward a spindle pole (this stage is called anaphase A). Subsequently, the spindle fibers elongate and the two spindle poles move further apart (anaphase B). A number of hypotheses have been proposed to explain the anaphase chromosome movements based on in vivo and in vitro observations. Spindle movements are possible in lysed mitotic cells of diatom by adding ATP (Cande and Wolniak, 1978; Cande, 1982). Tubulin molecules are newly added in the midzone of two half spindles during the anaphase movements in vitro, and an ATP-dependent force-generating factor slides microtubules so as to push the two poles further apart (Masuda and Cande, 1987). Capture of the kinetochores by microtubules and subsequent microtu-

bule shortening by dynamic instability may explain the pulling of chromosomes toward the poles (Mitchison and Kirschner, 1985; Mitchison et al., 1986). A dyenin-like molecule (Pratt et al., 1980) or kinesin (Scholey et al., 1985; Vale et al., 1985), which is known to be involved in ciliary and organelle movements, may also be involved in the spindle fiber sliding.

Little is known about genetic control of spindle formation and anaphase movements. To understand the genetic control mechanisms, mutants have to be isolated. The difficulty of this approach, however, is that the defective phenotypes of the mutants are not known. We have attempted to isolate candidate mutants (reviewed in Yanagida et al., 1986) from the fission yeast *Schizosaccharomyces pombe* that is considered to be one of those organisms suitable for cell cycle analyses (Mitchison, 1970; Nurse, 1985). During *S. pombe* mitosis, chromosomes condense, cytoplasmic microtubules disappear, and spindle appears (McCully and Robinow, 1971; Toda et al., 1981; Umeson et al., 1983b; Hiraoka et al., 1984; Tanaka and Kanbe, 1986; Marks et al., 1986). Shortening of the kinetochore microtubules has not been established in this organism. Elongation of the pole-to-pole spindle fibers is the principal force for the transport of chromosomes into daughter nuclei.

Our isolation strategy for mutants that are defective in

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anaphase spindle movements was based on the phenotype of cold-sensitive (cs) mutants in α - (*nda2*) and β - tubulin (*nda3*) genes that are defective in spindle formation (Toda et al., 1984; Hiraoka et al., 1984; Yanagida et al., 1986). In these tubulin mutants, chromosomes remain condensed without separation due to the absence of a spindle. Another characteristic of these tubulin mutants is that the nucleus is displaced from the center of the cell, probably because cytoplasmic microtubules become defective. Our expectation was that mutants defective in anaphase spindle movement might show a phenotype similar to that of tubulin mutants. Thus we searched a collection of *S. pombe* ts mutants (Uemura and Yanagida, 1984) for those exhibiting an arrested phenotype similar to that of tubulin mutants.

We report here a novel temperature-sensitive (ts) mutant that apparently blocks anaphase spindle elongation. A certain phenotype of this mutant *nuc2-663* is indeed similar to that of tubulin mutants; at restrictive temperature (36°C), chromosomes condense and the nucleus is displaced. In contrast to tubulin mutants that lack spindle, however, a short spindle forms in this mutant at 36°C. We have investigated details of the mutant phenotype, and concluded that the *nuc2* gene product is essential for anaphase spindle movements. We cloned the *nuc2⁺* gene, determined its nucleotide sequence, obtained antisera against *lacZ-nuc2⁺* fused protein, and identified a polypeptide in the extracts of wild-type *S. pombe*. The *nuc2⁺* gene product is enriched in a nuclear structure insoluble in 2 M NaCl and 25 mM lithium 3,5'-diiodosalicylate (LIS).¹ In the *nuc2* mutant cells arrested at 36°C, however, a soluble gene product, larger in molecular weight than the product found in wild-type cells accumulates, suggesting that a soluble precursor might be processed to the mature *nuc2⁺* protein which incorporates into a resistant nuclear structure.

Materials and Methods

Strains and Media

Haploid strains of *Schizosaccharomyces pombe* used were *h⁻ leul*, *h⁺ leul*, *h⁻ leul nuc2-663*, and *h⁺ his2 leul nuc2-663*. Culture media for *S. pombe* were YPD (complete rich medium, 1% yeast extract, 2% polypeptone, 2% glucose; 1.7% agar was added for plates), SD (minimal medium; 0.67% yeast nitrogen base without amino acids, 2% glucose; 2% agar was added for plates), and EMM2 (minimal medium; Mitchison, 1970). *Escherichia coli* was grown in LB (0.5% yeast extract, 1% polypeptone, 1% NaCl [pH 7.5], 1.5% agar was added for plates).

Isolation of *nuc2-663* Mutant

587 ts strains (Uemura and Yanagida, 1984; Hirano et al., 1986) were individually grown at 26°C in YPD liquid medium with shaking. When the cell concentrations reached 5×10^6 /ml, the cultures were transferred to 36°C for 4 h. Cells were collected by centrifugation, washed three times with distilled water at 2°C, and chromosomes were stained by DAPI (1 μ g/ml). They were observed by an epifluorescence microscope connected with a television camera (Toda et al., 1981). Only one strain, *nuc2-663*, showed a high frequency (>80%) of condensed chromosomes. Several other strains also showed condensed chromosomes with less frequencies (<30%) and were not investigated further.

Fluorescence and Immunofluorescence Microscopy

The procedure described for DAPI staining (Toda et al., 1981) was followed. Immunofluorescence microscopy was performed by the method described

(Kilmartin and Adams, 1984; Adams and Pringle, 1984), using monoclonal anti-tubulin antibody YL1/2.

Selection Synchrony

The selection synchrony method described by Mitchison and Carter (1975) was used. Small cells of early G2 phase were selected from the top layer of a diffuse cell band after exponentially growing cells were run in sucrose gradient centrifugation. To obtain high synchrony, a small number of cells (<1% of total cells) have to be collected.

Gene Cloning by Transformation

Sau IIIA1 partial digests of *S. pombe* genomic DNA (average 10-kb long) were ligated with a shuttle vector pDB248 which contains pBR322, the *S. cerevisiae* *LEU2* gene, and 2 μ DNA (Beach and Nurse, 1980; Beach et al., 1982). This genomic DNA library was used for transformation of a host strain *h⁻ leul nuc2-663* by the lithium acetate method (Ito et al., 1983). Plates were first incubated at 26°C for 2 d and then at 36°C. A *Leu⁺ Ts⁺* transformant was obtained and the *Leu⁺* marker cosegregated with *Ts⁺*. A plasmid (designated pNC101) was recovered from the transformant.

Integration of Cloned Sequence on Chromosome

2.3-kb Hind III fragment was ligated with an integration vector YIp32, and the resulting plasmid (pNC302) was used for transformation of a host strain *h⁺ leul* by homologous recombination (Shortle et al., 1982). A *Leu⁺* transformant obtained showed a stable 2⁺:2⁻ segregation for the *Leu* marker, indicating that the plasmid was integrated on the chromosome. Tetrad analysis showed that the integrated locus was tightly linked to *nuc2*.

Nucleotide Sequence Determination, Hybridization, and RNase Mapping

Nucleotide sequence was determined by the dideoxy method using pUC plasmid (Sanger et al., 1977; Yanisch-Perron et al., 1985). RNase mapping described by Melton et al. (1984) was performed using 10 μ g of poly(A)⁺ RNA. ³²P-RNA SP6 probes were used.

Immunoblot Analyses

Immunoblot analyses were performed by transferring the proteins electrophoretically to nitrocellulose after SDS-PAGE (Towbin et al., 1979).

Construction of *lacZ* Hybrid Plasmids

The 1.2-kb Sac I-Eco RV fragment of the *nuc2⁺* gene that contains a coding region for the COOH domain was ligated with Sma I-Sac I sites of pUC19. Resulting plasmid pTH10 was digested with Sal I, followed by partial digestion with Hind III. This procedure gave rise to three Hind III-Sal I fragments differing in length, each of which was subsequently ligated with Sal I-Hind III sites of pUC19. Two plasmids, pTH11 and pTH12, were expected to be in frame, and by nucleotide sequence determination it was shown that the *lacZ* sequence was in frame with the *nuc2⁺* gene. pTH11 and pTH12 should produce polypeptides of 317 and 353 residues, respectively, each with five *lacZ* residues in the NH₂ termini.

Purification of the Fused Protein and Preparation of Antiserum

For purification of the fused protein, the procedures described by Watt et al. (1985) were followed. *E. coli* JM109 harboring pUC19 ligated with *nuc2⁺* sequences (pTH11 and pTH12) was grown in 1 liter LB containing ampicillin (40 μ g/ml), induced by 1 mM isopropyl β -D-thiogalactopyranoside (IPTG), and incubated for 2 h. Polypeptides produced were pelleted in 0.05% sodium deoxycholate and 0.1% Triton X-100 by centrifugation and dissolved by 8 M urea. About 10 mg of *nuc2⁺* polypeptides (purity ~80%) was obtained from 1-liter cultures. Polypeptides were further purified by SDS-PAGE followed by the electroelution of protein from gel.

Rabbits were immunized subcutaneously in the back (20–50 sites) with a total of 300–500 μ g purified polypeptide emulsified with an equal volume of complete Freund's adjuvant for the primary series. A total of 300–500 μ g polypeptide in incomplete Freund's adjuvant was used for subsequent immunizations. Three to five immunizations at 2-wk intervals were required to obtain serum of high enough titer to yield positive results by immunoblot. Serum was stored in frozen form at -80°C.

1. Abbreviation used in this paper: LIS, lithium 3,5'-diiodosalicylate.

Preparation of *S. pombe* Whole Cell Extracts

A 500-ml culture of *S. pombe* HM123 (*h⁻ leu1*) containing the vector plasmid pDB248 or pNC106 with the insert of *nuc2⁺* gene was exponentially grown to 5×10^6 cells/ml in a synthetic EMM liquid medium without leucine. The transformants had been maintained in EMM (without leucine) plates and precultured in EMM (without leucine) liquid medium. The cells were harvested, washed once with chilled distilled water, and resuspended in 1 ml buffer E (20 mM Tris HCl at pH 7.6, 1 mM dithiothreitol [DTT], and 1 mM phenylmethylsulfonyl fluoride [PMSF]). An equal volume of glass beads (0.5-mm diam) was added, and the cells were broken by shaking with a vortex for 5 min at 2°C. The whole cell extracts thus prepared (~1.5 ml) were fractionated as described below. For SDS-PAGE, 10–20 μ l extracts were used for each lane.

Subcellular Fractionation

1 ml of the whole-cell extract described above was centrifuged at 5,000 rpm for 5 min, and the pellet was washed with 0.2 ml buffer E. The resulting pellet consisting of unbroken cells and cell walls was resuspended in 1.2 ml buffer E (designated P1). The mixed supernatants (total 1.2 ml; designated S1) were centrifuged at 40,000 rpm (50,000 g) for 20 min in a TL100 ultracentrifuge TLA100.2 rotor (Beckman Instruments, Inc., Palo Alto, CA). The pellet containing nuclei and other organelles was once washed in 0.2 ml buffer E under the same centrifugal condition and resuspended in 1.2 ml buffer E (designated P2). The supernatant mixed was designated S2. Under these conditions, no p67 was detected in S2 (the same result was obtained by lower centrifugation at 15,000 rpm [8,000 g] for 20 min). Each 10 μ l of the P2 suspension was mixed with 90 μ l of the following solutions (final concentrations) and incubated under the conditions indicated below: 0.1, 0.4, or 2.0 M NaCl in buffer E at 2°C for 60 min; 1% Triton X-100 or 1% NP-40 in buffer E at 26°C for 60 min; 5 mM EDTA, 5 mM EGTA, 5 M urea, or 8 M urea in buffer E at 2°C for 60 min. Then suspensions (each, 100 μ l) were centrifuged at 40,000 rpm (50,000 g) or 15,000 rpm (8,000 g) for 20 min and washed once under the same buffer conditions. Resulting pellets and supernatants were run in SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting.

Isolation of *S. pombe* Nuclei

The procedures are based on Lohr and Ide (1979) for the *S. cerevisiae* nuclei and modified for *S. pombe*. Mid or late exponentially growing cells ($0.5\text{--}2.0 \times 10^7$ /ml) in 1 liter YPD culture were collected (centrifugation by 5,000 rpm for 5 min or suction filtration using filter paper; Toyo Roshi Kaisha Ltd., Japan; GAI100), suspended in 40 ml T-buffer (0.1 M Tris HCl at pH 8.0, 0.1 M EDTA, and 0.5% 2-mercaptoethanol) and incubated at 30°C for 10 min or at 2°C for 30 min. The cells were pelleted, washed once (5,000 rpm for 5 min) in S-buffer (20 mM potassium phosphate at pH 6.5, 1 M sorbitol, 0.1 mM CaCl₂, and 1 mM PMSF), and were incubated at 30°C for 60–90 min with slow shaking in S-buffer containing 0.2 mg/ml zymolyase T100 (Kirin Brewery Co.) at the concentration of $\sim 10^9$ cells/ml. Cells were washed once in S-buffer at 3,000 rpm for 10 min and resuspended in a small volume of 15% Ficoll buffer (15% Ficoll, 20 mM potassium phosphate at pH 6.5, 0.1 mM CaCl₂, and 1 mM PMSF) at the concentration of $1\text{--}2 \times 10^9$ cells/ml. Then, the cells were homogenized with 15–20 strokes at 2°C. The extent of cell disruption and nuclear release was examined by DAPI staining during homogenization. The homogenate (5 ml for each tube) was layered on the top of a 15–40% linear gradient of Percoll buffer (1 M sorbitol, 1 mM PMSF, 0.1 mM CaCl₂, and 20 mM potassium phosphate at pH 6.5). The gradient was centrifuged at 7,000 rpm for 20–40 min in a Beckman SW27 rotor and collected into 1-ml fractions. Alternatively, the nuclear band in the middle of the gradient was collected by a syringe (cell walls and unbroken cells are pelleted at the bottom). 3 vol of 15% Ficoll buffer were added to the nuclear fraction, and the mixture was centrifuged by 13,000 rpm for 10 min. The nuclei were pelleted but Percoll remained in the supernatant under this condition. The nuclei were washed once in 15% Ficoll buffer (13,000 rpm for 10 min), resuspended in a small volume of S-buffer, and stored at -80°C .

Fractionation of Isolated Nuclei

Method A. Results are shown in Fig. 8 b. Isolated nuclei in buffer S were centrifuged at 15,000 rpm for 20 min, resuspended in 20 mM Tris HCl at pH 7.6 containing 5 mM MgCl₂, 1 mM PMSF, 10 μ g/ml DNAase I (Sigma Chemical Co., St. Louis, MO), and 10 μ g/ml RNAase A (Sigma Chemical Co.), incubated at 37°C for 20 min, and centrifuged at 40,000 rpm for 20 min. Then the nuclear pellets were treated in the following buffer conditions

in this order: (a) 20 mM Tris HCl at pH 7.6, 1 mM DTT, 1 mM PMSF containing 1% NP-40 at 26°C for 30 min; (b) the same buffer containing 0.4 M NaCl; or (c) 2 M NaCl at 2°C for 30 min. After centrifugation at 40,000 or 15,000 rpm for 20 min, resulting pellets and supernatants were run in SDS-PAGE and analyzed by immunoblots.

Method B. See Mirkovitch et al., 1984. Results are shown in Fig. 8 c. Isolated nuclei were centrifuged and the nuclear pellets were resuspended and incubated in either LIS buffer (5 mM HEPES at pH 7.4, 25 mM LIS, 2 mM EDTA, and 0.25 mM spermidine) at 25°C for 10 min or in HS buffer (20 mM Pipes at pH 6.3, 2 M NaCl, 2 mM EDTA, and 0.25 mM spermidine) at 2°C for 30 min. They were centrifuged at 15,000 rpm for 15 min, and resulting pellets were washed first by LIS or HS buffer, respectively, and then twice with PC buffer containing 20 mM Pipes at pH 6.3, 0.1 mM CaCl₂, and 1 mM PMSF. Micrococcal nuclease (8 U) was added to each and incubated at 30°C for 30 min. The reaction was terminated by addition of EDTA (final concentration; 30 mM). After centrifugation at 40,000 or 15,000 rpm for 20 min, supernatants were mixed with the previous ones and pellets were used for SDS-PAGE.

Results

Isolation of *ts nuc2-663* Showing Condensed Chromosomes

To isolate mutants defective in anaphase spindle movements, we searched a collection of 587 *ts* mutants of *S. pombe* for those showing condensed chromosomes. Cultures of *ts* mutants were individually grown at permissive temperature (26°C), then transferred to restrictive temperature (36°C) for 4 h and examined by DAPI stain for whether chromosomes of *ts* mutant cells remain condensed without separation. In the wild-type *S. pombe*, chromosomes separate after condensation (e.g., Toda et al., 1981). Therefore, if mutations specifically prevent anaphase, mutant cells should uniformly arrest, showing a terminal phenotype expected for an anaphase block; their chromosomes might remain condensed.

We found that, among 587 strains, only one *ts* mutant designated *nuc2-663* (*nuc*; nuclear structure alteration) showed a high frequency (80–90%) of condensed chromosomes in the arrested cells (Fig. 1 b). These chromosomes do not separate even after prolonged incubation. The frequency of condensed chromosomes is ~ 100 -fold higher than that found in the wild-type cells; in vegetative wild-type culture (Toda et al., 1981), mitotic cells showing condensed chromosome domains are $<1\%$. These results suggest that the arrested stage is highly specific. At 26°C, *nuc2* cells grow normally, showing chromosomal domains by DAPI stain indistinguishable from those of the wild-type cells (Fig. 1 a). This cytological phenotype of *nuc2-663* cosegregates with *ts* lethality by tetrad analysis, and *nuc2-663* is defined as a novel genetic locus (described below). There are several other strains that show condensed chromosomes in lesser frequencies ($<30\%$), and these were not investigated further.

Incubation of asynchronous *nuc2* cells at 36°C causes the uniformly arrested phenotype (80–90%) of condensed chromosomes. Viability sharply decreases at 36°C (~ 20 and 1% after 2 and 4 h, respectively). Average cell size increases from 11.7 to 16.2 μm (Fig. 2 a). Contents of DNA, RNA, and protein in the arrested cells were measured (data not shown). The value of 2.01C was obtained (C, the amount of a genome DNA) for DNA; the DNA synthesis appears to be completed. The amounts of RNA and protein increase proportionally to the cell volume. These results are consistent with the notion that *nuc2* produces a *cdc* phenotype blocked in nuclear division (Nurse et al., 1976) except that a septum forms in the arrested cells (Fig. 1 b; see below).

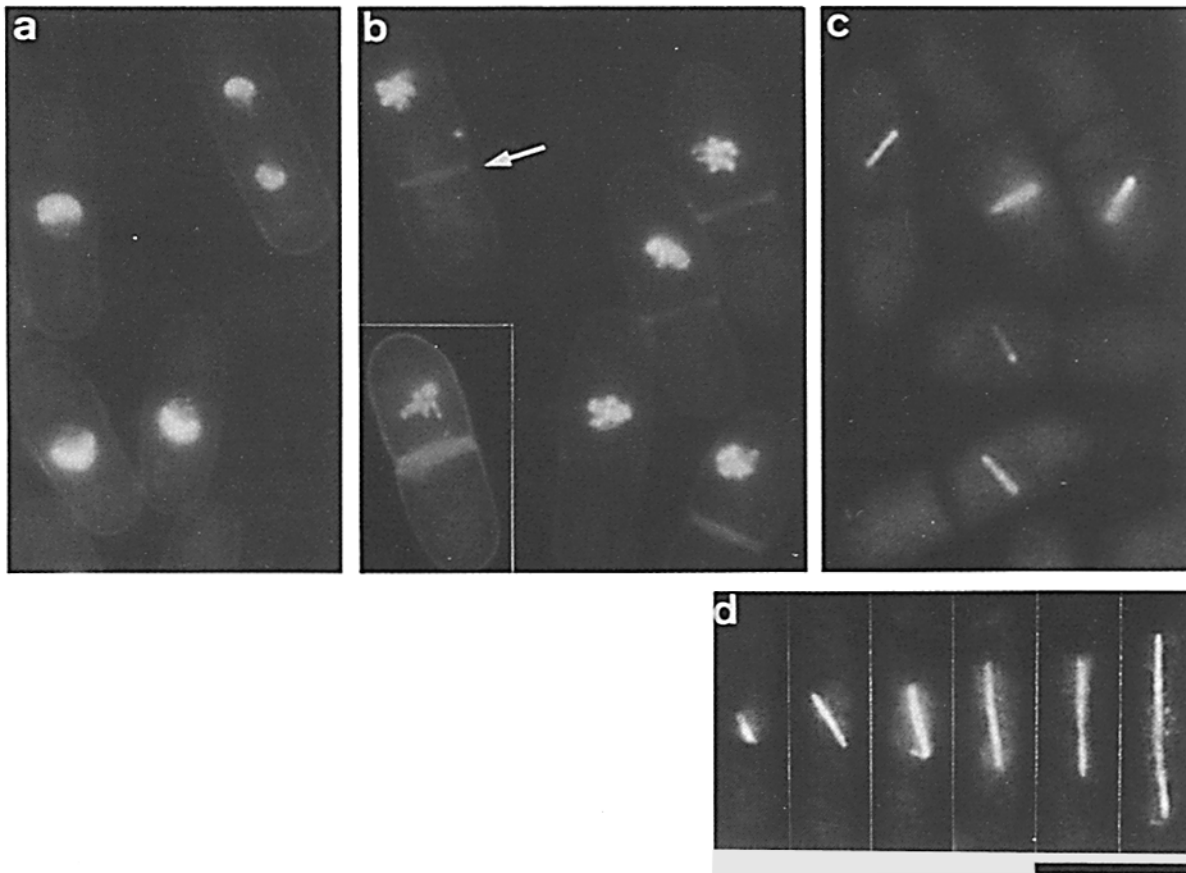


Figure 1. Fluorescence micrographs of *S. pombe nuc2* mutant cells. (a and b) Cells are stained by DAPI. (c and d) Indirect immunofluorescence micrographs by monoclonal antibody YL1/2 against yeast tubulin. (a) Exponentially growing *nuc2-663* cells at permissive temperature (26°C). Three interphase cells and one mitotic cell with dividing nucleus are shown. (b) Arrested *nuc2* cells at restrictive temperature (36°C) for 4 h are singly nucleated. Condensed chromosomes are seen, and nucleus is displaced. Septum (indicated by the arrow) forms in most cells. (c) *nuc2* cells arrested at 36°C for 4 h, showing a short spindle. (d) Wild-type *S. pombe* mitotic cells, showing spindles of various lengths. Bar, 10 μ m.

Short Spindle Forms in *nuc2-663* at 36°C

To characterize the arrested stage, we investigated whether mitotic spindle forms in *nuc2* cells at 36°C. Immunofluorescence microscopy was done using monoclonal antibody against yeast tubulin (Kilmartin and Adams, 1984; Adams and Pringle, 1984). As shown in Fig. 1 c, the *nuc2* cells at 36°C for 4 h show a remarkable spindle phenotype. A short spindle is seen in most (80–90%) of the arrested *nuc2* cells. (Because some spindles differ in focal planes, they do not appear in the figure.) These spindles are short and surprisingly uniform in length (Fig. 2 b). Their average length ($2.9 \pm 0.4 \mu$ m) is similar to the diameter of the spherical nucleus. In wild-type or *nuc2* grown at 26°C, the frequency of cells showing a spindle is $\sim 6\%$, and the spindle length varies from 0.2 to 13–15 μ m (Fig. 1 d; Hiraoka et al., 1984). Thus, the results again indicate that the arrested *nuc2* cells are highly uniform, suggesting that *nuc2* causes a specific block in the nuclear division. By electron microscopy of sections, the short spindle was seen in the spherical nucleus of the arrested *nuc2* cells (data not shown).

The axis of the short spindle is not parallel to but oblique to the cell axis. This is also true for wild-type intermediary short spindle (McCully and Robinow, 1971; Hiraoka et al.,

1984; Tanaka and Kanbe, 1986); because the fully extended spindle is parallel to the cell axis, spindle elongation should accompany the rotation of the spindle axis. Increase of spindle length by more than the diameter of the spherical nucleus causes nuclear elongation as a matter of course. (In yeasts and fungi, nuclear membranes do not dissociate during mitosis, and therefore the increase of the pole-to-pole distance beyond the size of nuclear diameter [3.0 μ m in haploid *S. pombe*] must require nuclear as well as spindle elongation.) In the wild-type anaphase stage, the nucleus of *S. pombe* becomes ellipsoidal followed by dumbbell-shaped, probably pushed by rapid spindle elongation (Tanaka and Kanbe, 1986). In the end of anaphase, the spindle axis becomes parallel to the cell axis, and becomes 4–5-fold longer than the short spindle in *nuc2* at 36°C (Fig. 1 d). Thus the *nuc2*⁺ gene product appears to become indispensable after the short spindle is assembled.

Spindle Axis Runs through the Middle of Closely Arranged Chromosomes

Condensed chromosomes formed in *nuc2* cells at 36°C are arranged closely together. In *nda3-KM3II*, which lacks a spindle, chromosomes are somewhat loosely arranged (Hira-

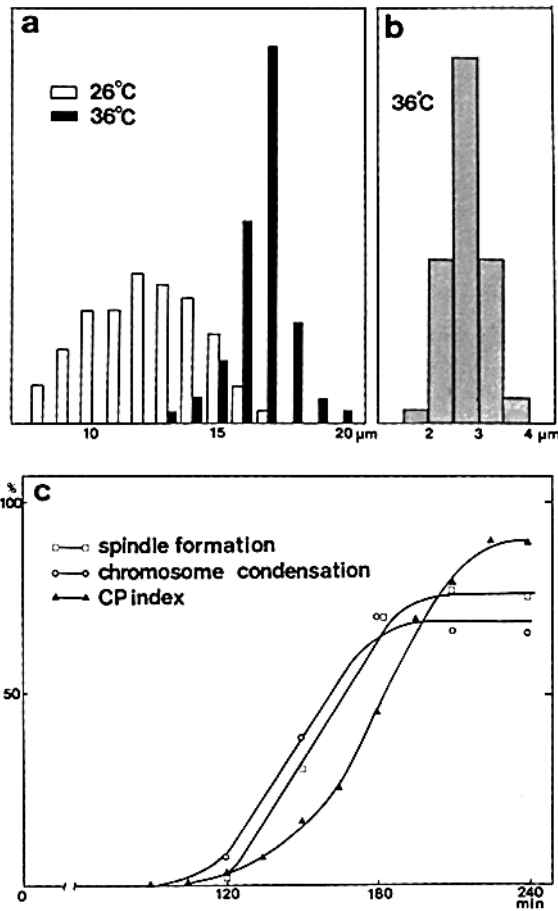


Figure 2. Cell and spindle length, and timing of mitotic events occurring in arrested *nuc2* cells. (a) Cell length of *nuc2* mutant increases when cells vegetatively grown at 26°C are transferred to restrictive condition at 26°C. (Open columns) Length of cells grown at 26°C (average length $11.7 \pm 2.2 \mu\text{m}$). (Filled columns) Length of arrested cells at 36°C for 4 h (average $16.2 \pm 1.2 \mu\text{m}$). (b) Length of spindle in arrested *nuc2* cells (36°C for 4 h) is shown (average $2.9 \pm 0.4 \mu\text{m}$). The spindle size was measured on fluorescence micrographs of cells that were judged to be flattened. (c) Selection synchrony of *nuc2* cells was done as described in Materials and Methods. Exponentially growing *nuc2* cells at 26°C were collected and run in sucrose gradient centrifugations. The small early G2 cells selected from the top of gradients were incubated at 36°C (0 min) with shaking, and aliquots were taken at a 15–30-min interval to examine the frequencies (expressed as percentage) of cells showing condensed chromosomes (circles), short spindle (rectangles), and septum (triangles). The frequency of septated cells is called CP (cell plate) index (Mitchison, 1970).

oka et al., 1984). The short spindle in *nuc2* might play a role in the clustering of chromosomes. Interestingly, the spindle axis appears to have a certain geometrical relationship to the arranged chromosomes. In Fig. 3 a, sets of fluorescence micrographs of six different *nuc2* cells by anti-tubulin (top) and by DAPI staining (middle) are shown together with illustrations of spindle and chromosome localization (bottom). Aggregates of fluorescent particles in DAPI stain indicated by the asterisks represent mitochondrial DNAs (see below).

Individual chromosomes can be seen when the spindle is viewed down along the axis (spindle seen as a dot) in im-

munofluorescence micrographs. In the side view (spindle seen as short rod), however, they are not easily distinguishable. The simplest interpretation of these images is that three rod-like chromosomes align on a plane to form a disc or a plate-like structure (which is reminiscent of the metaphase plate in higher eukaryotes), and the spindle runs through the approximate center of the plane (cartoon at the right side of Fig. 3). The angle made by the spindle and the plane is roughly perpendicular. The same relationship was found in more than 50 *nuc2* arrested cells examined. The arrested stage in *nuc2* resembles metaphase. It is not known whether a similar relationship is maintained in wild-type cells; cells at a corresponding stage are infrequent in vegetative culture.

Short Spindle Forms at Normal Timing but Its Elongation Is Apparently Blocked

To determine whether the short spindle forms at normal timing in the cell cycle, the time course of spindle appearance was examined in synchronous cultures of *nuc2* cells incubated at 36°C. *nuc2* cells asynchronously grown at 26°C were run in a sucrose gradient centrifugation (Mitchison and Carter, 1975), and those of early G2 phase cells were selected from the top of the gradients and incubated at 36°C (Materials and Methods). As shown in Fig. 2 c, frequencies of the cells containing condensed chromosomes (circles) increase after 120 min and reach the maximum ($\sim 70\%$) at 180 min. Similarly, the cells containing a short spindle (rectangles) increase and reach the maximum ($\sim 75\%$) slightly after chromosome condensation. (In different experiments, the frequencies of condensed chromosomes and short spindles reached $\sim 90\%$.) Finally the cells containing the septum (triangles) reach the maximum ($\sim 90\%$) at 240 min. (Because the nucleus is displaced, the septum forms without damaging the undivided nucleus. See below.) No cell separation, however, takes place.

Mid-points for chromosome condensation, spindle formation, and septation are 142, 155, and 177 min, respectively. Considering that the generation time of *nuc2* at 26°C, and of the wild type at 26 and 36°C are 220, 160, and 130 min, respectively, the equivalent generation time for *nuc2* at 36°C would be 180 min. Therefore, the timing for chromosome condensation and short spindle formation appears to be normal in the *nuc2* cells even at 36°C. These results support the notion that, in *nuc2* cells at 36°C, mitotic events normally initiate and lead to the formation of short spindle with condensed chromosomes by normal timing, but its elongation and concomitantly nuclear elongation are prevented.

Pleiotropic Effects of *nuc2* Mutation on Cellular Phenotypes

Other cellular phenotypes have been found in *nuc2*, in addition to those described above. (a) In $>90\%$ of *nuc2* cells at 36°C for 4 h, the nucleus was displaced from the center of the cell (Fig. 1 b). This also takes place in tubulin mutants (Toda et al., 1983; Umeson et al., 1983a; Hiraoka et al., 1984). (b) The septum formation takes place in the arrested cells but cytokinesis does not (Fig. 1 b). This is in contrast to other nuclear division arrest *cdc* (Nurse et al., 1976) or *nda* (Toda et al., 1983) mutants, in which the septum formation as well as nuclear division are inhibited. (c) Most mitochondria (can be stained by dimethylaminostyrylmethylpyri-

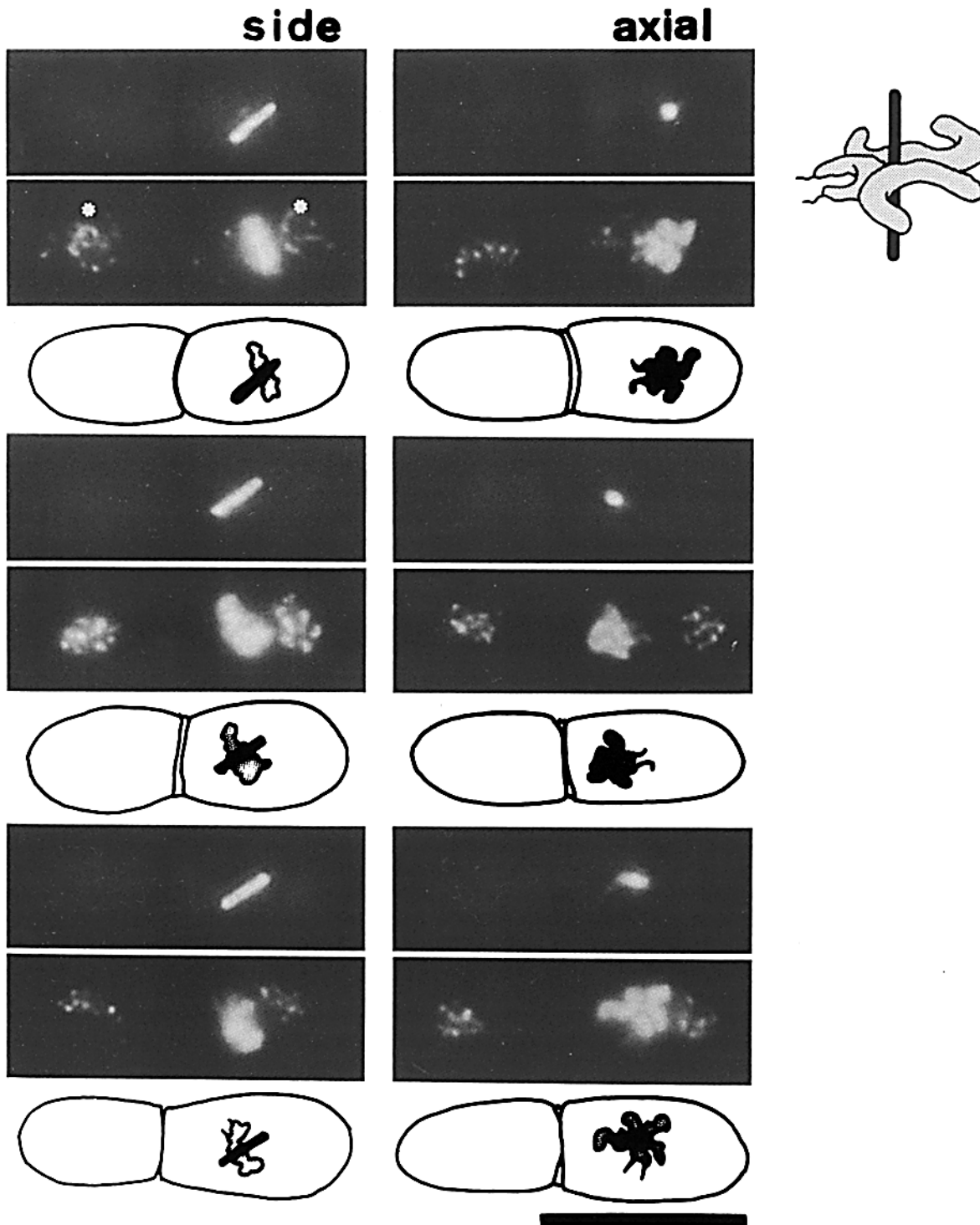


Figure 3. Spindle axis runs through the middle of closely grouped chromosomes. Six *nuc2* cells arrested at 36°C for 4 h are shown by sets of fluorescence micrographs using anti-tubulin (*top*) and DAPI stain (*middle*), and schematized combined images (*bottom*). An illustration depicting chromosomes and spindle is also shown. Aggregates of small dots (indicated by the asterisks) are mitochondrial DNAs. Bar, 10 μ m.

diniumiodine (DASPMI); Miyakawa et al., 1984) aggregate and locate at both the ends of cells (Fig. 3 *a*). In the light of our recent finding (Hirano, T., unpublished results) that mitochondrial location in the cell cycle alters in parallel to the change of cytoplasmic microtubule distribution, the phenomenon may be related to a cytoskeletal defect in *nuc2* cells.

These pleiotropic effects are not well understood in terms of the *nuc2* gene product function, but suggest that it might be involved in controlling cytoskeletal organization.

Isolation of the *nuc2*⁺ Gene by Transformation

An *S. pombe* genomic DNA library in the shuttle vector

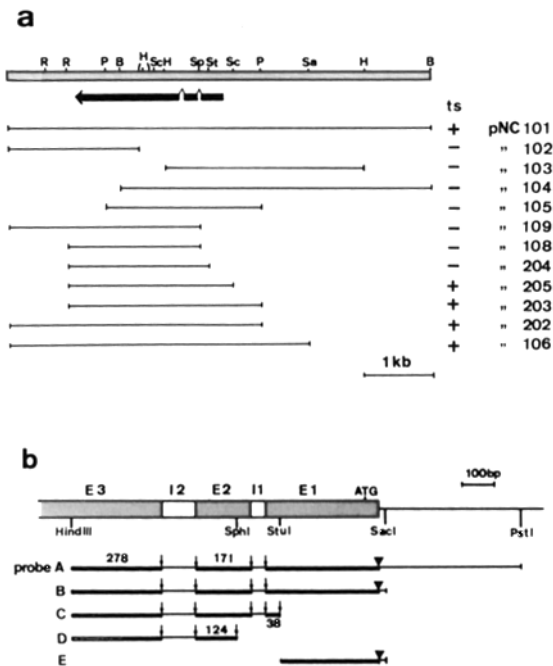


Figure 4. Cloning of the *nuc2*⁺ gene and identification of the coding region. (a) The *nuc2*⁺ gene was cloned by transformation, and various subclones were constructed (+ indicates that the clone can complement to lethality of *nuc2*). Restriction map of the inserted genomic DNA is shown. R, Eco RV; P, Pst I; B, Bam HI; H, Hind III; Sc, Sac I; Sp, Sph I; St, Stu I; Sa, Sal I. The thick arrow indicates the coding region (with two short introns) of *nuc2*⁺ gene. (b) RNase mapping experiments were done using five different probes (A-E) by the procedures described (Melton et al., 1984). Each 10 μ g of poly(A)⁺ RNA from *S. pombe* wild-type cells was hybridized with ³²P-labeled RNA probes (A-E) made by SP6 RNA polymerase, digested with RNase A and T1, and run in 6% acrylamide sequence gel. For probes A (Hind III–Pst I) and B (Hind III–Sac I), three fragments (278, 171, and 345 bp) were obtained. For probe C (Hind III–Stu I), three fragments (278, 171, and 38 bp) were obtained, while for probe D (Hind III–Sph I), two fragments (278 and 124 bp) were obtained. For probe E (Stu I–Sac I), one fragment (~310 bp) was obtained.

pDB248 (Beach and Nurse, 1981), which contains pBR322, the *S. cerevisiae* *LEU2* gene, and 2 μ DNA, was used to transform *ts nuc2 leu1*. A *Leu*⁺ *Ts*⁺ transformant was obtained, which grows normally at 36°C and can conjugate and sporulate. A plasmid (pNC101) was recovered from the transformant. A restriction map of the inserted genomic sequence in the plasmid is shown in Fig. 4a. Various subclones were constructed and were used for transformation. A minimal DNA fragment that complements *nuc2* (indicated by +) is the 2.4-kb Eco RV–Sac I fragment (pNC205). Southern and Northern hybridizations indicated, respectively, that the cloned sequence is unique in the genome and is hybridized with 2.1-kb mRNA (data not shown).

The 2.3-kb Hind III fragment (one end derived from the vector sequence) from pNC102 was ligated with an integration vector YIp32. The resulting plasmid (pNC302) was integrated onto the chromosome of a host strain *h⁺ leu1* by homologous recombination. Tetrad analyses showed that the integrated locus is tightly linked to the *nuc2* locus (PD/

NPD/TT = 11:0:0), indicating that the cloned DNA is derived from that locus. Chromosomal map position of *nuc2* has been determined by a mapping procedure described by Kishida and Shimoda (1986), and it is located at a novel locus between *ade2* and *ura2* in the left arm of chromosome I (Kohli et al., 1977; Gygax and Thuriaux, 1984). The *cut8* gene (Hirano et al., 1986) is linked to (~2 cM) but different from *nuc2*. The *nuc2* locus is unlinked to any of the tubulin genes.

Nucleotide Sequence Determination and Predicted Amino Acid Sequence

The complete nucleotide sequence of the 2,781-bp long Pst I–Eco RV fragment which complements *nuc2* was determined by the dideoxy method (Fig. 5a). There are three presumed exons (E1, E2, and E3) that are interrupted with two short introns containing consensus sequences (underlined in Fig. 6) for *S. pombe* (Toda et al., 1984; Hiraoka et al., 1984; Hindley and Phear, 1984). To confirm the presence of the two introns and to identify the initiation site for transcription, RNase mapping (Melton et al., 1984) was carried out using five different probes (A–E in Fig. 4b). Results are consistent with the presence of the two introns (II and I2), 45- and 106-bp long, at the positions of 102nd and 159th codons, respectively. If translation initiates at the 1st ATG codon, a transcription initiation site (indicated by the triangles in Fig. 4b) is at –39–41 position (see legend for Fig. 4b for details).

The presumed *nuc2*⁺ protein consists of 665 amino acid residues (calculated molecular weight is 76,300, and isoelectric point, pI, is 8.6). Predicted amino acid sequence of the *nuc2*⁺ protein is shown by single letters in Fig. 5. The hydrophathy plot (Kyte and Doolittle, 1982) indicates that the first 180 residues are relatively hydrophobic, the second 180–330 region is hydrophilic, containing a serine-rich domain (200–230), and the 330–665 residue region alternates hydrophobic and hydrophilic domains. Several repeats are found (indicated by the underlines of predicted amino acid sequence in Fig. 6). They are YKLEA, FSLQREHS, YEKS, and YKKA. Computer analyses have shown no strong sequence homology to any known protein.

Preparation of Anti-Sera against lacZ-nuc2⁺-Fused Protein

To identify the *nuc2*⁺ gene product in *S. pombe* by immunochemical methods, we constructed hybrid expression plasmids in *Escherichia coli* and raised antibodies against the *lacZ-nuc2*⁺ fused proteins. Because the NH₂ domain of *nuc2*⁺ protein contains two introns, nucleotide sequences deleting the NH₂ domain were ligated in frame with the *lacZ* region of pUC19 plasmid (Fig. 6a). Two such plasmids (pTH11 and pTH12) were constructed; pTH11 contains the 1.1-kb Hind III–Sal I fragment in frame with the region of the *lacZ* promoter and the first five residues, and is expected to produce a 317 amino acid residue polypeptide (36,900 mol wt) in *E. coli*, while pTH12 should produce a 353 residue polypeptide (41,200 mol wt).

SDS-PAGE of the extracts of *E. coli*, which is transformed with pTH11 or pTH12 and is induced for *lacZ* production, shows an additional intense polypeptide band with the expected molecular weight (Fig. 6b). These polypeptides were partially purified by the procedures described in Materials and Methods (data not shown). The protein bands in SDS-

PstI
CTGCAGTGTATT CAGGATATTCTTGGGACTTGGAAATTCATAA AACTA AACTTCACTCTAGTACTTTTACGTCAGTGTCCGCTTTCCACAAGACTGCGT -383
AATAATGGATCTAAATTGGGAGACATTGAACGACTTGAATTTTTTTTATTAAACATGTATCATCGCATTGTTT TAGACCGTGATGCCTCTTTGCCTGT -283
CTCCCTTAATGCTTGGATCGAGTTCGATTAGTAGTGTTTTTTATTCTCTGATAATTTACCCAAACTCTTTTGGATTTATCAGAGTTTACACA AACTCTA -183
TACACCATCCAGATGATTTACCTATTTTTGTTTATATGTTTTTTTACTAAAATGCTACATTGCTTTATAAGATTTAACATATCTTAAACGATGGAGCTCA -83
AACAATAACAACCACCTGTTTGTACCCACATGTTTTGTTGACATTA AACTCCCATCGTTTCCAAA AACTTTAATAGATTTGTCATGACAGATCGATTGAAA 18
H T D R L K 6
TGTTTAATATGGTATTGCATTGATAATCAGAATTATGATAATTTCAATTTTTTATT CACAACGTTTACATGCAATTGAAGATTCAAACGAGAGTTTGTATC 118
C L I W Y C I D N Q N Y D N S I F Y S E R L H A I E D S N E S L Y L 40
TTTTGGCATATTGCGATTTCCTAAACCTCGATTACAATTTGTATACGACTTATTAGATAGAGTAATTAGTCATGTTCTTGCACATACTTATTGGCAAG 218
L A Y S H F L N L D Y N I V Y D L L D R V I S H V P C T Y L F A R 73
GACCAGCCTTATTTTAGGCAGATATAAACAAGGAATAAGTCTGTGGAGGCCTGTCGATCGAATTGGCGCTCCATTAGCCAAAACAGTATGTTGGTGATT 318
T S L I L G R Y K Q G I S A V E A C R S N W R S I Q P N I 102
SacI
ATTTCGGAAAACCTCTAACTAAGTTATCTAGTAAATGACTCAATTAGCAGTCTGGACATCCAGATGCCTTTCGATGCTTGATGTTTGGGTA CTACTGT 418
H D S I S S R G H P D A S C H L D V L G T M Y 125
ATAAAAAGGCAGGGTTCCTCAAAAAAGCTACAGATTGTTTTGTAGAAGCTGCTCCATTAACCCATATAATTTCTCTGCTTTCCAGAAATTA AACTGCAAT 518
K K A G F L K K A T D C F V E A V S I N P Y N F S A F Q N L T A I 158
AGGTATGTGACAGATGTTTTGTTGTGAATTAAGTAAATCACTTTTGTCAAAAAAAAATACTTATTTTCGTTTATTCTATAATTGTGCTAAATATATTTTG 618
C
CTGAATAGCGCTGCCACTCGATGCTAATAATGTATTGTTATTCCACCCTACCTTACGGCAATGAAGGGTTTTGAAAAATCTCAAACGAATGCTACAGCT 718
V P L D A N N V F V I P P Y L T A M K G F E K S Q T N A T A 189
TCGGTACGAGAACCCTTTTTGAAGAAAAGTAAAGACTTCTGCTCATCTTCCAACAAGTTTTCGGTTTCTGAATCGATAGCAAAATGTTATTCAA AACT 818
S V P E P S F L K K S K E S S S S S S N K F S V S E S I A N S Y S N S 223
CATCCATTTGACGATTTACTAAGTGTGTTGATAGGGTTGACGCTTCTGAGCTTCCAGGAAGTGAAGGAAACGACATCAAAGCTTGA AATTACAATCTCA 918
S I S A F T K W F D R V D A S E L P G S E K E R H Q S L K L Q S Q 256
ATCTCAGACTAGCAAAAACCTTTTGGCTTCAATGATGCTCAAAAAGCTGATTTCAACAATAGGGATACGCTTTAAAATCCCACTTTGTGGAACCTAGA 1018
S Q T S K N L L A F N D A Q K A D S N N R D T S L K S H F V E P R 289
ACCCAAGCATTAAAGACCAGGAGCTCTTTAAATATAAATACGCGAAGCGAGAAGTTCTAAAAGAGGAGAGCACACCTCAAAGCTTCCGCGAAGAGG 1118
T Q A L R P G A R L T Y K L R E A R S S K R G E S T P Q S P R E E D 323
ACAATAATTTGATGGAATTAAGTATTTCGGTAAAGGGTCTTACCTGCTGCGCCACTATAAGTACGAGAGGCTTTAAATGTTTCAAAGCTTGGC 1218
N N L M E L L K L F G K G V Y L L A Q Y K L R E A L N C F Q S L P 356
CATCGAACAGCAAAAACACCTTTTCTTCTGCAAAAGCTTGGAAATACCTACTTTGAAGTGGTTGATTACGAAAAATCTGAAGAAGTGTTTCAA AAAATTA 1318
I T C G Q N T P F V L A K L G I T Y F E L V D Y E K S E E V F Q K L 389
AGGGACTTGTCCGCTTACAGTGTCAAAGATATGAAGTCTTTTCAACTGCACTTTGGCATTGCAAAAGCTGTTCCTTTATCTTACCTTGGCCATGAAA 1418
R D L S P S R V K D H E V F S T A L W H L Q K S V P L S Y L A H E T 423
CITTCGAAA AACTCCTTATTCCCCAGAATCATGGTGCATTCTTGCTAATTGTTTCTCACTTCAACGTAACACTCGCAGGCATTA AATATGATTAATAG 1518
L E T N P Y S P E S W C I L A N W F S L Q R E H S Q A L K C I N R 456
AGCTATTCAATTTGATCCA AACTTTTGAATATGCTTATACGCTTCAAGGGCAGGAGCATTCTGCAACGAAGAATACGAAAAATCGAAAACATCTTTCCGC 1618
A I Q L D P T F E Y A Y T L Q Q H E H S A N E E Y E K S K T S F R 489
AAAGCAATTAGAGTAAATGTTTCGACATTACAATGCATGGTATGGCCTGGGAATGCTTTATTTAAAAACAGGGCGAAATGATCAAGCAGACTTTCATTTTC 1718
K A I R V N V R H Y N A W Y G L G M V Y L K T G R N D Q A D F H F Q 523
AACGTGCTGCAGAGATCAATCCTAACA AACTGTACTCATCACTTGTATTGGTATGATTTACGAACGCTGCAAAAGATTACAAAAAAGCACTTGAATTTTA 1818
R A A E I N P N N S V L I T C I G M I Y E R C K D Y K K A L D F Y 556
TGATCGGGCATGTA AACTGGATGAAAAGTCTTCGCTTCCAGGTTCAAGAAAAGCCAAAGTCTTATTTTATTACATGATCAGATAAAGCACTCGTTGAA 1918
D R A C K L D E K S S L A R F K K A K V L I L L H D H D K A L V E 589
TTGGAACAATTA AAGGCAATTCGCCAGATGAAGCAAATGTTCAATTTTACTCGGCAAAATTTTCAAGCAAATTCGGAAAAA AATTTAGCCTTAAAGC 2018
L E Q L K A I A P D E A N V H F L L G K I F K Q M R K K N L A L K H 623
ACTTCACTATAGCATGGA AACTTAGACGGCAAGGCTACGCATATTATTAAGGAATCGATTGA AATCTGGATATTCCCGAAGAAA AATTTGTTAACTGAAAC 2118
F T I A W N L D G K A T H I I K E S I E N L D I P E N L L T E T 656
AGGTGAAAATTTATAGGAATCTGAAA AACTTAAGAAATATGCTTGTATTTTAGGGATGCCAAAAAATGTGCAATGAATAAGTTAATGAGTTAGAATGTT 2218
G E I Y R N L E T stop 665
TACGCTTTCATTTCTGTTAGGTTAAAAAATGATGATTTTCGTTTAGAAGTTATATATTA AAAATAAAGGTATAATAGATATC **EcoRV** 2299

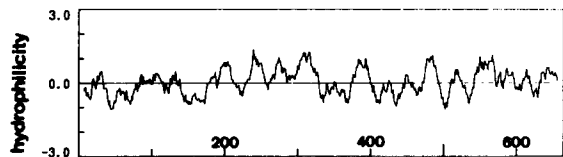


Figure 5. Nucleotide and predicted amino acid sequences of the *nuc2⁺* gene. A, 2,781-bp long nucleotide sequence of Pst I-Eco RV fragment is shown with predicted amino acids. Single amino acid designations are used for predicted *nuc2⁺* polypeptide. Two introns are underlined with the consensus sequences (double underlined; Hiraoka et al., 1984; Hindley and Phears, 1984). Underlines for amino acid sequences indicate repeating elements. Serine clusters (see text) are shown by dots. Some restriction sites are also shown. Asterisks indicate the sites for transcription initiation deduced by RNase mapping. Hydrophilicity is expressed in plus values. Hydropathy plot was made according to Kyte and Doolittle (1982) with a window of 15 residues.

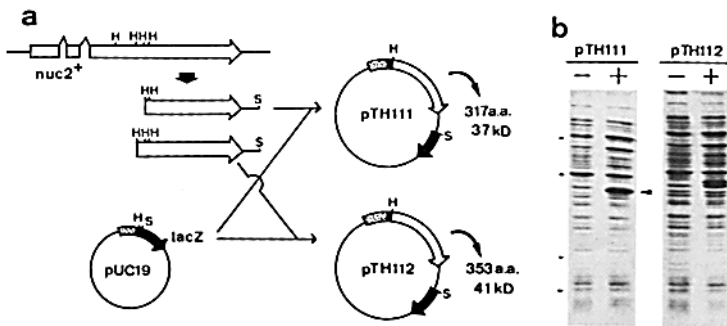


Figure 6. Construction of hybrid expression plasmids to produce *nuc2⁺-lacZ* fusion polypeptides in *E. coli*. (a) Plasmid constructions: pUC19 was ligated with a 1.1- or a 1.2-kb long *Sal* I–*Hind* III fragment. The resulting plasmids, pTH111 and pTH112, respectively, should produce 317 and 353 residues fusion polypeptides. (b) SDS-PAGE of *E. coli* extracts bearing plasmids pTH111 or pTH112. Polypeptides with expected molecular weights are produced under the condition of expressed *lacZ* gene (indicated by +).

PAGE were electroeluted, and ~2 mg of each purified protein was injected into rabbits subcutaneously with an interval of 2 wk. Immunoblots showed that each antiserum produced antibodies against the fused protein (data not shown). Antibodies obtained in these antisera were affinity-purified by the fused proteins.

Detection of p67 in *S. pombe* by Immunoblots

To prove that the immunoblot band corresponds to the gene product of *nuc2⁺*, we constructed various genetically engineered *S. pombe* strains in which the amount or molecular weight of the *nuc2⁺* polypeptide is specifically altered so that the band intensity and mobility in immunoblots should change according to the strains used. As shown in Fig. 7, a polypeptide (67,000 mol wt; hereafter designated p67) is detected using antiserum against the 41-kD *nuc2⁺-lacZ* fusion polypeptide. Extract of the strain (pDB248) bearing multicopy vector pDB248 (without insert) shows a faint p67 band (the same faint band was obtained for wild-type extract without the vector). Extract of the strain (pNC106) bearing pNC106 with *nuc2⁺* gene insert, however, shows an intense p67 band (about 20-fold increase in intensity).

Three other strains constructed carry plasmid pEVP11, pTH202, or pTH201. pEVP11 is an expression vector containing the promoter sequence for *S. pombe* alcohol dehydrogenase (ADH) gene (Russell and Hall, 1983). pTH202 is made by ligating the full length of the *nuc2⁺* gene with ADH promoter in pEVP11 while, in pTH201, COOH domain of the *nuc2⁺* gene (expected to produce a polypeptide of 39,500 mol wt) was ligated with pEVP11. As shown in Fig. 7, the amount of p67 increases due to the ADH promoter, and the molecular weight of *nuc2⁺* gene product decreases according to the reduced size of the coding frame in pTH201. The same blotting pattern was obtained using another antiserum against the 37-kD *lacZ-nuc2⁺* fusion polypeptide. From these results we concluded that the *nuc2⁺* gene encodes for p67, although its molecular weight estimated in SDS-PAGE is significantly less than that calculated from the nucleotide sequence (see below).

p67 Is Enriched in the Nucleus and Is Insoluble

To understand the *nuc2⁺* gene function, it is essential to determine the cellular localization of p67. For this purpose, we prepared whole-cell extracts of wild-type *S. pombe* by disruption of the cells with glass beads, and estimated by immunoblots the amount of p67 in various fractions (described in Materials and Methods). As shown in Fig. 8 a, p67 is insoluble under most solution conditions examined, although

Coomassie Brilliant Blue staining shows many of other polypeptides become soluble. p67 remains in pellets of the extracts treated with 0.1, 0.4, and 2.0 M NaCl, 1% Triton, or 1% NP-40. Chelating agents (EDTA and EGTA) do not improve its solubility. On the other hand, 5 M urea partly and 8 M urea completely solubilizes p67. A similar result was obtained using the high gene dosage extract (prepared from a strain bearing multicopy plasmid pNC106), indicating that solubility properties of p67 do not significantly alter with an increase in the amount of p67.

To test whether p67 may locate in the nucleus, we isolated nuclei of wild-type cells by Percoll gradient centrifugation

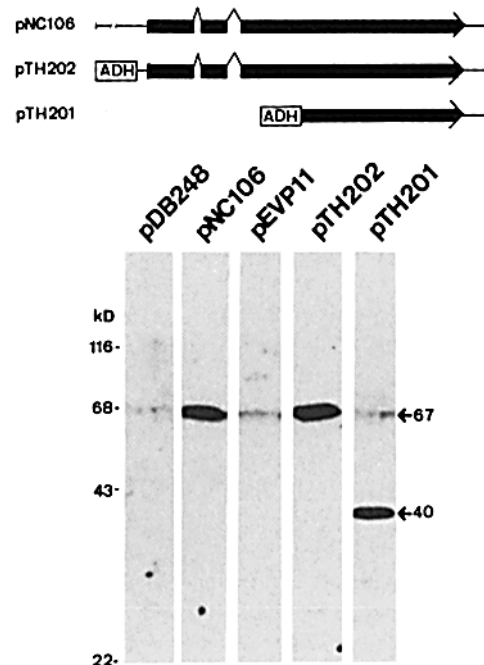


Figure 7. Detection of p67 in *S. pombe* extracts by immunoblots. Whole cell extracts of the following *S. pombe* strains were prepared and their immunoblots using anti-*lacZ-nuc2⁺* antibody are shown. pDB248, a strain carrying multicopy vector pDB248 without insert; pNC106, with insert of *nuc2⁺* gene; pEVP11, expression vector having the *S. pombe* ADH promoter (Russell and Hall, 1983); pTH202, with fully completable *nuc2⁺* gene; pTH201, with a part of the coding region encoding COOH domain (see text). Faint bands are observed at the position of 67,000 mol wt for pDB248, pEVP11, and also pTH201, corresponding to p67 made by the genomic *nuc2⁺* gene. Intense bands at 67,000 and 40,000 mol wt are produced by pNC106, pTH202, and pTH201 plasmids, respectively. Molecular weights determined by standards are also shown.

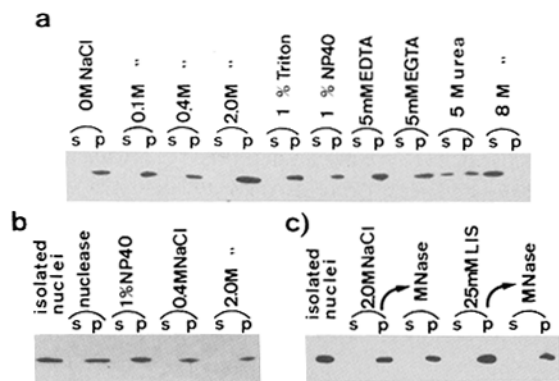


Figure 8. Fractionation of p67. (a) Whole-cell extracts of *S. pombe* were prepared, treated under different solution conditions, and centrifuged at 40,000 rpm for 20 min (Materials and Methods). Pellets (p) and supernatants (s) were run in SDS-PAGE. Immunoblot patterns using anti-*lacZ-nuc2⁺* antibody are shown. (b and c) Nuclei were isolated by Percoll gradient centrifugations (Materials and Methods), and treated with nucleases, 1% NP-40, 0.4 M NaCl, and 2.0 M NaCl. Supernatants and pellets were run in SDS-PAGE, and their immunoblots are shown in b. Alternatively, isolated nuclei were treated with 2.0 M NaCl or 25 mM LIS followed by or without the treatment by micrococcal nuclease. Supernatants and pellets were run in SDS-PAGE and their immunoblots are shown in c.

(Materials and Methods). *S. pombe* cell extracts (wild-type cells containing multicopy vector pDB248 without insert and pNC106 with *nuc2⁺* gene) were prepared by zymolyase digestion followed with homogenization; the extracts were overlaid and run on a 15–40% linear Percoll gradient (Fig. 9). Nuclei made a sharp band in the middle of gradient. They were morphologically intact and free from membraneous materials, judging by phase-contrast and DAPI-stained fluorescence microscopy. The nuclear fractions contained DNA topoisomerase II and histones (data not shown). Soluble components, mitochondria, and other small organelles including disrupted nuclear materials remained in the top or the upper part of gradient. Cell envelopes and nondisrupted cells were pelleted in the gradients. Each fraction was run in SDS-PAGE, blotted, and examined for the presence of p67 by anti-*lacZ-nuc2⁺* antibody. p67 was present in the nuclear band as well as in the upper band. The amount of p67 appeared to be more in the nuclear fraction. Consistently, in high *nuc2⁺* gene-dosage extract (pNC106), a greater part of p67 also was found in the nuclear band. Thus we conclude that p67 cofractionates with nuclei although smaller fraction may locate in non-nuclear organelles.

We investigated in which nuclear subfraction p67 is present. Isolated nuclei were fractionated (Materials and Methods), and each fraction was run in SDS-PAGE gel electrophoresis, blotted, and examined by anti-*lacZ-nuc2⁺* antibody (Fig. 8 b). Consistent with the results obtained by whole-cell extracts, p67 is present in the nuclear substructure that is resistant to 2 M NaCl and 1% NP-40. Nuclease treatment does not affect the solubility of p67. As shown in Fig. 8 c, p67 was also insoluble in 25 mM LIS (followed by or without nuclease digestion) that dissolved most of the nuclear polypeptides stained by Coomassie Brilliant Blue (data not shown). LIS is known to dissolve most nuclear components except those called nuclear scaffold (Mirkovitch et al.,

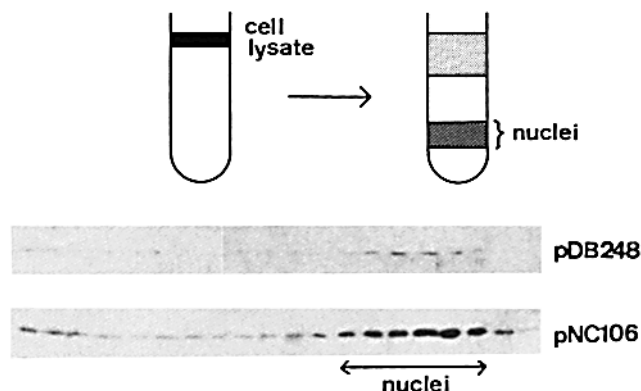


Figure 9. Distribution of p67 in Percoll gradient centrifugation. Two *S. pombe* strains (*h⁻ leu1*) carrying either multicopy vector pDB248 without insert or pNC106 with *nuc2⁺* gene were grown at 30°C in EMM2 and cells were digested with zymolyase followed by homogenization (Materials and Methods). Extracts were centrifuged on a 15–40% linear Percoll gradient. Fractions were run in SDS-PAGE and immunoblots done using anti-*lacZ-nuc2⁺* antibody.

1984), lamina and nuclear pore complex (Aaronson and Blobel, 1975; Franke et al., 1981), and nuclear matrix (Berezney and Coffey, 1974; Comings and Okada, 1976) in the higher eukaryotic nucleus.

Soluble Antigen in *nuc2* Mutant Cells

We investigated the state of the *nuc2* gene product in the *nuc2* mutant cells arrested at 36°C and unexpectedly found that anti-*lacZ-nuc2⁺* antibody detects a soluble protein in *nuc2* mutant extracts. As shown in Fig. 10, extracts prepared from *nuc2* mutant cells grown at 26°C show only insoluble p67 in immunoblots, but for extracts prepared from *nuc2* cells arrested at 36°C for 4 h, an additional band (designated at p76) is found at 76,000 mol wt. Furthermore, p76 remains in the

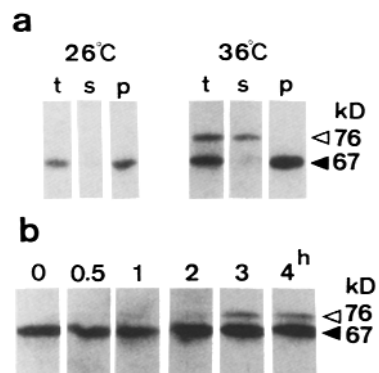


Figure 10. Accumulation of soluble antigen in arrested *nuc2* mutant cells. (a) Whole-cell extracts of *nuc2* mutant cells either grown at 26°C or arrested at 36°C for 4 h were prepared, centrifuged at 40,000 rpm for 20 min, run in SDS-PAGE, and electrotransferred to nitrocellulose membranes. Affinity-purified anti-*lacZ-nuc2⁺* antibody was used to detect the gene product of *nuc2* in mutant extracts. *t*, whole extract; *s*, supernatant; *p*, pellet. Soluble antigen at 76,000 mol wt is found in extracts of arrested mutant cells. (b) Whole extracts of *nuc2* mutant cells transferred from 26 to 36°C were prepared at 0, 0.5, 1, 2, 3, and 4 h. Immunoblot patterns using anti-*lacZ-nuc2⁺* antibody are shown.

supernatants after centrifugation at 40,000 rpm for 20 min. This soluble p76 accumulates upon transfer of *nuc2* mutant cultures to a nonpermissive condition. The amount of p76 increases by incubating the *nuc2* cells at 36°C (Fig. 10 b). It should be noted that p76 has the molecular weight expected from the nucleotide sequence of the cloned *nuc2⁺* gene (described above) and therefore might be a soluble precursor for processed insoluble p67.

Discussion

We isolated an *S. pombe* ts mutant *nuc2-663* that blocks mitosis specifically at a step in anaphase spindle elongation. The *nuc2⁺* gene product appears to become essential for spindle elongation after microtubules assemble into the short spindle. We have identified the product of *nuc2⁺* gene in *S. pombe* extracts by immunochemical analyses using antisera raised against the *lacZ-nuc2⁺* fusion proteins. Experimental results suggest that the direct product (p76) of the *nuc2⁺* gene is soluble and processed to p67, which is insoluble and enriched in a nuclear fraction. The results pose a question of how the *nuc2⁺* gene product participates in nuclear spindle movements that separate chromosomes. We suppose that the *nuc2⁺* gene product located in the stable nuclear structure interacts with spindle apparatus, chromosomes, or nuclear envelope, and interconnect nuclear and cytoskeletal functions in mitosis.

Similarity in Defective Phenotypes between *nuc2* and Tubulin Mutants

The phenotype of *nuc2* is similar to that of α 1- (*nda2*) and β - (*nda3*) tubulin mutants (Toda et al., 1984; Hiraoka et al., 1984; Yanagida, 1987). First, spindle assembly or movement is defective in these mutants. In the tubulin mutants, the spindle is absent, and in *nuc2* the spindle fails to elongate. Second, chromosomes condense in the arrested cells. This would be a consequence of spindle defects. Third, the nucleus is displaced from the center of the cell. Immunofluorescence microscopy has shown that cytoplasmic microtubules are not present in *nuc2* cells arrested at 36°C for 4 h but is present in those grown at 26°C (data not shown). The nuclear displacement in *nuc2* cells might be due to cytoskeletal defect as in tubulin mutants or due to prolonged dissolution of cytoplasmic microtubules by mitotic arrest. Fourth, cytokinesis is inhibited in both tubulin and *nuc2* mutants.

Among 587 ts mutants tested in the present study, only *nuc2-663* shows a high frequency of condensed chromosomes. Nuclear displacement also is found only in this mutant. In a collection of 980 cs mutants, on the other hand, more than 20 strains exhibit high frequencies of condensed chromosomes (Adachi, Y., and H. Ohkura, unpublished results). About half of them turn out to be α 1-tubulin mutant (*nda2*); nuclear displacement is again present in all of these *nda2* mutants. None of the remaining strains, however, is *nuc2*. They do not show nuclear displacement and genetically differ from *nuc2*. Thus, among more than 1,500 ts and cs mutants, only *nuc2* and tubulin mutants show the combined phenotype of chromosome condensation and nuclear displacement. This circumstantial evidence suggests that *nuc2* may be related to a microtubular function. Failure in an attempt to construct a double mutant between ts *nuc2* and cs *nda3* is consistent with this notion.

Metaphase Plate-like Chromosome Alignment in Arrested Cells

Chromosomes in the arrested *nuc2* cells are closely arranged. They align and form a plate-like structure. In tubulin mutants, chromosomes are positioned less closely at restrictive temperature (Umesono et al., 1983b; Hiraoka et al., 1984). Upon transfer to permissive temperature, however, the spindle forms and dispersed chromosomes become tightly clustered before separation (Hiraoka et al., 1984), suggesting that the clustering of condensed chromosomes might be a prerequisite for chromosome separation. Also, the clustering occurs in the presence of the short spindle. It is intriguing that the spindle and chromosomes show such higher and regular ordered structure in a lower eukaryotic nucleus. This geometrical relationship between spindle and chromosomes is reminiscent of the metaphase plate in higher eukaryotes. If the metaphase plate formation in higher eukaryotes were required for concerted sister chromatid separation, then similar events might take place in *S. pombe*.

Protein Product of *nuc2⁺* and Its Nuclear Localization

The length of *nuc2* mRNA (~2.1 kb) probed with 2.4-kb Sac I-Eco RV genomic DNA fragment which complements *nuc2* ts lethality is consistent with the nucleotide sequence determination of the fragment that predicts a coding region consisting of three exons (total 1,995 bp) and two introns (total 151 bp). RNase mapping experiments confirmed the presence of two introns located at the sites expected from nucleotide sequence data. Predicted *nuc2⁺* polypeptide is 76,300 mol wt, but anti-*lacZ-nuc2⁺* antibody detects a smaller polypeptide (p67) in the wild-type *S. pombe* extract. This apparent discrepancy in molecular weight can be reconciled if we assume that the *nuc2⁺* gene product undergoes processing. The larger polypeptide (p76) accumulated only in mutant extracts at restrictive temperature has the molecular weight expected from the nucleotide sequence determination and is perhaps the precursor form of p67, although definitive evidence requires pulse-chase radiolabeling experiments (Davis and Blobel, 1986).

In Percoll gradient centrifugation, a greater part of p67 cosediments with nuclei. The remaining p67, which sediments slowly, is also insoluble in 2 M NaCl, 25 mM LIS, and 1% Triton, and indistinguishable from that cosedimenting with nuclei; it might be a component of other organella or be derived from nuclei broken during preparation. The amount of p67 seems to be low. We are not able to identify the protein band of p67 by Coomassie Brilliant Blue staining even in the LIS insoluble nuclear fractions derived from cells of high *nuc2⁺* gene dosage.

Subnuclear localization of p67 remains unknown. This is due to our failure in immunofluorescence microscopy using anti-*lacZ-nuc2⁺* antibodies, which did not show any significant fluorescent stain in nuclear structures including the spindle or spindle pole body. The amount of p67 may be very low. Alternatively, epitopes recognized by the antibodies are not exposed on the surface in specimens for immunofluorescence microscopy. Thus even a possibility that p67 may locate at or bind to the outer nuclear periphery has not been excluded. The insoluble nature of p67 in various solution conditions, however, indicates that p67 resembles a component in the nuclear scaffold (Mirkovitch et al., 1984),

or matrix (Berezney and Coffey, 1974, 1977; Comings and Okada, 1976), lamina and nuclear pore complex (Aaronson and Blobel, 1975; Franke et al., 1981) of higher eukaryotic nuclei. Although such structures may exist in yeast nuclei (Potashkin et al., 1984), their characteristics have not been firmly established. If the insoluble structure containing the *nuc2⁺* gene product represents the nuclear scaffold, *nuc2-663* would become the first mutant for such nuclear structure. If p67 does not have a relation to the nuclear skeleton, it could still be at least a part of the resistant nuclear structure.

Possible Roles of *nuc2⁺* in Mitotic Anaphase

The *nuc2⁺* gene product is essential for anaphase spindle movements. Then, how does it play its role? There seem to be three possibilities. First, p67 in a nuclear scaffold-like structure may directly or indirectly interact with microtubules or the spindle apparatus. This hypothesis is consistent with the result that the phenotype of *nuc2-663* is similar to that of tubulin mutants and that p67 preferentially locates in nucleus. The *nuc2* gene product is not required for the initiation of spindle assembly but becomes essential after the length of assembled spindle reaches the size of the spherical nucleus. Masuda and Cande (1987) demonstrated that anaphase B spindle in diatom extends by, in the presence of ATP, an ATP-consuming, vanadate-sensitive factor, preceded by incorporation of new tubulin molecules in the spindle midzone. The product of the *nuc2⁺* gene located in the resistant nuclear structure may be involved in the addition of external tubulin molecules to the spindle apparatus or the force-generating system for spindle elongation.

Second, p67 may interact with a chromosomal domain such as the centromere. In the *nuc2* mutant cells, pole-to-pole microtubules can assemble into the short spindle but do not elongate possibly due to the failure in the shortening of pole to chromosome distance. (In this hypothesis, anaphase B takes place only after the completion of anaphase A). That is, p67 may be a kinetochore protein that plays a role in the separation of sister kinetochores in anaphase A. Alternatively, p67 might be required for association of kinetochore microtubules with centromeres. Certain kinetochore proteins are known to behave as nuclear scaffold proteins (Earnshaw et al., 1984). Third, the product of *nuc2⁺* might only indirectly relate to spindle elongation and be directly involved in the elongation of nuclear membranes. In most lower eukaryotes, nuclear membranes remain during mitosis and therefore, spindle elongation should accompany nuclear membrane elongation. If the nuclear membrane is defective in elongating, the spindle may not be able to elongate although the spindle itself is normal. This might be consistent with the requirement of *nuc2* gene product after the spindle reaches the size of the nucleus. It is unlikely, however, that p67 is a membrane component; p67 is neither soluble in 1% Triton nor 1% NP-40 but soluble in 8 M urea. Predicted amino acid sequence of the *nuc2* gene product does not contain hydrophobic membrane-binding domain. At the present, the above three hypotheses are equally possible. Future work is necessary to identify the role of *nuc2* gene product in anaphase spindle movement in *S. pombe*.

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