Bypassing Anaphase by Fission Yeast *cut9* Mutation: Requirement of *cut9*$^+$ to Initiate Anaphase

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Abstract. A novel anaphase block phenotype was found in fission yeast temperature-sensitive *cut9* mutants. Cells enter mitosis with chromosome condensation and short spindle formation, then block anaphase, but continue to progress into postanaphase events such as degradation of the spindle, reformation of the postanaphase cytoplasmic microtubule arrays, septation, and cytokinesis. The *cut9* mutants are defective in the onset of anaphase and possibly in the restraint of postanaphase events until the completion of anaphase. The *cut9*$^+$ gene encodes a 78-kD protein containing the 10 34-amino acid repeats, tetratricopeptide repeats (TPR), and similar to budding yeast Cdc16. It is essential for viability, and the mutation sites reside in the TPR. The three genes, namely, *nuc2*$^+$, *scn1*$^+$, and *scn2*$^+$, genetically interact with *cut9*$^+$. The *nuc2*$^+$ and *cut9*$^+$ genes share an essential function to initiate anaphase. The cold-sensitive *scn1* and *scn2* mutations, defective in late anaphase, can suppress the ts phenotype of *cut9*.

THE structure of the fission yeast genome as seen by fluorescence in situ hybridization (FISH)[1] dramatically changes in structure in the nucleus in a cell cycle–dependent manner (Uzawa and Yanagida, 1992; Funabiki et al., 1993; Chikashige et al., 1994). During interphase, the centromeres of three chromosomes are situated in a cluster near the spindle pole body (SPB), whereas upon entry into prophase, they become associated with spindle microtubules, move to a metaphase arrangement in the center of the spindle, and disjunction of chromosomes by poleward movements follows (Funabiki et al., 1993). The FISH method applied to fission yeast confirmed the occurrence of anaphase A (sister centromeres move to opposite poles without the extension of the spindle). Spindle elongation in anaphase B and spindle degradation in postanaphase are analogous to those of higher eukaryotes. The fully extended spindle reaches approximately four- to fivefold the length of the metaphase spindle. Re-establishment of the cytoplasmic microtubule network in postanaphase interphase (Hagan and Hyams, 1988) appears to require a new microtubule organization center near the middle of the cell (Horio et al., 1991). Thus, the genome dynamics are apparently in concert with nuclear-cytoplasmic reorganization of the microtubule network in the fission yeast cell division cycle.

When the onset of anaphase A is blocked, one possible outcome could be the arrest of cells at metaphase. Three mutants in fission yeast, *nuc2*, *sds22*, and *mts2* display the phenotype reminiscent of metaphase arrest (Hirano et al., 1988; Ohkura and Yanagida, 1991; Stone et al., 1993; Gordon et al., 1993). The *nuc2*$^+$ gene encodes a 67-kD polypeptide that contains the 34-amino acid repeat called TPR (tetratricopeptide repeat) motif (Sikorski et al., 1990); this fits into a predicted secondary structure for the assembly of helices (Hirano et al., 1990). Genes similar to *nuc2*$^+$ are also present in budding yeast and filamentous fungi, and they are required for mitosis (Sikorski et al., 1990; reviewed in Goebl and Yanagida, 1991). The *sds22*$^+$ gene encodes a 40-kD protein that contains leucine-rich 22-amino acid repeats, and it is directly associated with type 1–like protein phosphatases dis2 or sds21 (Ohkura and Yanagida, 1991; Stone et al., 1993). Upon association with the sds22 protein, the phosphatase alters the substrate specificity. In the metaphase arrested cells, the activity of dis2 phosphatase bound to sds22 was low; the loss of type 1-like protein phosphatase paralleled the failure to initiate anaphase. The *mts2* gene codes for a subunit of proteasome that carries out ATP-dependent proteolysis (Gordon et al., 1993). These three genes are possibly functionally close or belong to the independent pathways in the onset of anaphase.

We report here the characterization of temperature-sensitive (ts) mutants in the fission yeast *cut9*$^+$ gene that appears to block the onset of anaphase. The phenotypic difference from *nuc2*, *sds22*, and *mts2* mutants is that in *cut9* mutations the cycles of spindle assembly/disassembly take place in the absence of chromosome disjunction. Such uncoupled mitosis is followed by septation and cytokinesis. Thus, the loss

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1. *Abbreviations used in this paper*: bn, cells containing normally separated nuclei; cc, condensed chromosomes; ccs, condensed chromosomes with septum; cs, cold sensitive; ct, cut phenotype; DAPI, 4′-6-diamidino-2-phenyl-indole; dns, displaced nucleus with septum; FISH, fluorescence in situ hybridization; i, interphase nucleus; ORF, open reading frame; SPB, spindle pole body; TPR, tetratricopeptide repeat; ts, temperature sensitive.

of *cut9*$^+$ gene causes a wide range of postanaphase deregulations. We identified three genes that interact with *cut9*$^+$. Cold-sensitive (cs) mutations of the two genes suppress the ts phenotype of *cut9-665*. The *cut9*$^+$ gene may play a pivotal role in the control of anaphase.

Materials and Methods

Strains, Media, and Genetic Method

Haploid and diploid fission yeast strains were used. *cut9-665*, *nuc2-663*, and other *cut* mutants were previously isolated (Hirano et al., 1986, 1988). Diploid strains were constructed from haploid strains whose genetic background includes either *ade6-210* or *ade6-216*. Culture media used were YPD (1% yeast extract, 2% polypeptone, and 2% glucose) and EMM2 (minimal medium; Mitchison, 1970). Media containing 1.6% agar were used for plating unless otherwise noted. SPA medium contained, per liter, 10 g dextrose, 1 g KH_2PO_4, 10 mg biotin, 1 mg calcium pantothenate, 10 mg nicotinic acid, 10 mg meso-inositol, and 30 g agarose, and it was used for sporulation. Standard genetic procedures for fission yeast (Gutz et al., 1974; Moreno et al., 1991) were used.

Plasmids and Yeast Transformation Procedure

Transformation of *Schizosaccharomyces pombe* was performed by the lithium method (Ito et al., 1983). An *S. pombe* genomic DNA library containing the *Saccharomyces cerevisiae LEU2* gene as the selection marker (Beach and Nurse, 1981) was used. Plasmids recovered from Ts$^+$ Leu$^+$ or Cs$^+$ Leu$^+$ transformants of *cut9* or *cut9 scnl*, respectively, were subcloned into a minimal complementable genomic DNA. For subcloning the *cut9*$^+$ gene, pCUT9-7 was partially digested by BamHI, followed by religation, while the vector used to subclone the *scnl*$^+$ gene was pDB248′. For integration of the cloned DNA on the chromosome, the integration vector pYC11 (Chikashige et al., 1989) carrying the *S. cerevisiae LEU2* gene as the marker was used. The ordered cosmid bank of *S. pombe* genome was previously described (Mizukami et al., 1993). *S. pombe* cDNA library was a gift from Dr. John Fikes (Massachusetts Institute of Technology, Cambridge, MA).

Nucleotide Sequencing

Nucleotide sequences of genomic DNA and cDNA were determined by the dideoxy method (Sanger et al., 1977). Mutation sites of *cut9* mutants were determined as follows. The mutant gene was amplified by the PCR from the mutant genomic DNA sequence. The PCR primers used were 5′-TCTCCTTACTGCTGATGAAGAGTGGGA-3′ and 5′-AGATCTGAGACATTACCTTC-3′. The resulting PCR product was used as a template for the asymmetric PCR that was carried out with one primer reduced to 1/100 concentration of the other, to generate templates for subsequent dideoxy sequencing.

Synchronous Culture Analysis

Procedures for the synchronous culture were previously described (Hirano et al., 1988, 1990; Moreno et al., 1989; Kinoshita et al., 1990). *cut9-665* cells exponentially grown at 26°C were run in a sucrose gradient or an elutriator rotor (Beckman Instruments, Inc., Fullerton, CA). Early G2 cells (2×10^8 cells) were selected by their size (average length = 7 μm) and were incubated at 36°C in rich YPD medium. Every 20 min, a portion of the culture was taken, and the cell number, cell length, and viable cell number were measured. By fluorescence microscopy of DAPI- and anti-tubulin-stained cells, the frequencies of different cell types were estimated.

Immunological Methods

A fusion protein was made using pAR3040 (Studier and Moffat, 1986): the T7 promoter was ligated to a cDNA that an NdeI site was introduced at the putative initiation codon of the *cut9*$^+$ gene. The resulting full-length polypeptide (78 kD) was purified and injected into rabbits by the procedure described in Hirano et al. (1988) and Harlow and Lane (1988). Antibodies were affinity-purified on nitrocellulose (Smith and Fisher, 1984). Immunoblotting was performed according to the method of Towbin et al. (1979).

Light Microscopy

The procedure for DAPI (4′-6-diamidino-2-phenyl-indole) staining of *S. pombe* cells described by Adachi and Yanagida (1989) was followed. Immunofluorescence microscopy using anti-tubulin antibody (TAT1, a gift from Dr. K. Gull, University of Manchester, Manchester, UK); Woods et al., 1989), and the double-aldehyde fixation method was described by Hagan and Hyams (1988). Secondary antibody used was FITC-conjugated goat anti-mouse antibody (E.Y. Lab. Inc., San Mateo, CA). Cells were fixed in culture medium containing 3.7% formaldehyde and 0.2% glutaraldehyde at a given temperature for 1 h. The fixed cells were digested with 0.5 mg/ml Zymolyase 100T (Seikagaku Corp., Tokyo) and permeabilized by 1% Triton X-100, followed by successive incubation with first and secondary antibodies, respectively.

Results

Phenotype of cut9-665

Several types of nuclear chromatin structures were observed by DAPI staining of *cut9-665* cells at 36°C in the liquid YPD cultures (Fig. 1 *A*). Observed were cells containing interphase nucleus (i), condensed chromosomes without the septum (cc), cc with the septum (ccs), displaced interphase nucleus with the septum (dns), and the cut phenotype (ct). Cells with the cc were reminiscent of those arrested at metaphase (Hirano et al., 1988; Funabiki et al., 1993). In wild-type mitosis, chromosome condensation also takes place, but the frequency of cells with condensed chromosomes and the degree of condensation are much less than those in mitotically arrested cells (Toda et al., 1981; Hiraoka et al., 1984). Septation appeared to occur in *cut9-665* when chromosomes were still condensed. The nucleus was positioned in the middle or displaced when the septum was formed. The former case led to bisection of the nucleus and the resulting cut phenotype, whereas the latter showed the interphase nucleus to be situated in one half of the septated cell (dns). These terminal phenotypes were aberrant, initially not present, and their number increased after the shift to 36°C (quantitative data described below). A control micrograph of *cut9-665* at the permissive temperature (26°C) is shown at the bottom; cells containing the interphase nucleus or normally separated nuclei (bn) are visible.

The cell number increased ~3.3-fold after 6 h (Fig. 1 *B*, *upper panel*; cells producing the aberrant cut phenotype were counted as two), and cell division then ceased. The percentage of cell viability measured by plating at 26°C began to decrease after 1 h, and it reached 10% after 5 h. Hence, cells were divided once or twice after the shift, and most cells had become lethal by the second division.

Frequencies of each cell type in an asynchronous culture at 36°C were measured (Fig. 1 *B*, *lower panel*). The frequency of cells containing the interphase nucleus (i) was high for the first 3 h and dropped to the level of ~20% after 6 h. The emergence of ccs cells (abbreviation of cells designated in Fig. 1 *A*) followed that of cc, suggesting that septation took place while the chromosomes were still condensed. The terminal ct and dns cells reached the levels of ~35 and ~20%, respectively at 5 h. In control wild type at 36°C or *cut9-665* at 26°C, the ct and dns cells were rarely seen.

Synchronous Culture Analysis

Synchronous culture experiments of *cut9-665* were performed three times at the restrictive temperature, and basically the same phenotypes described below were obtained.

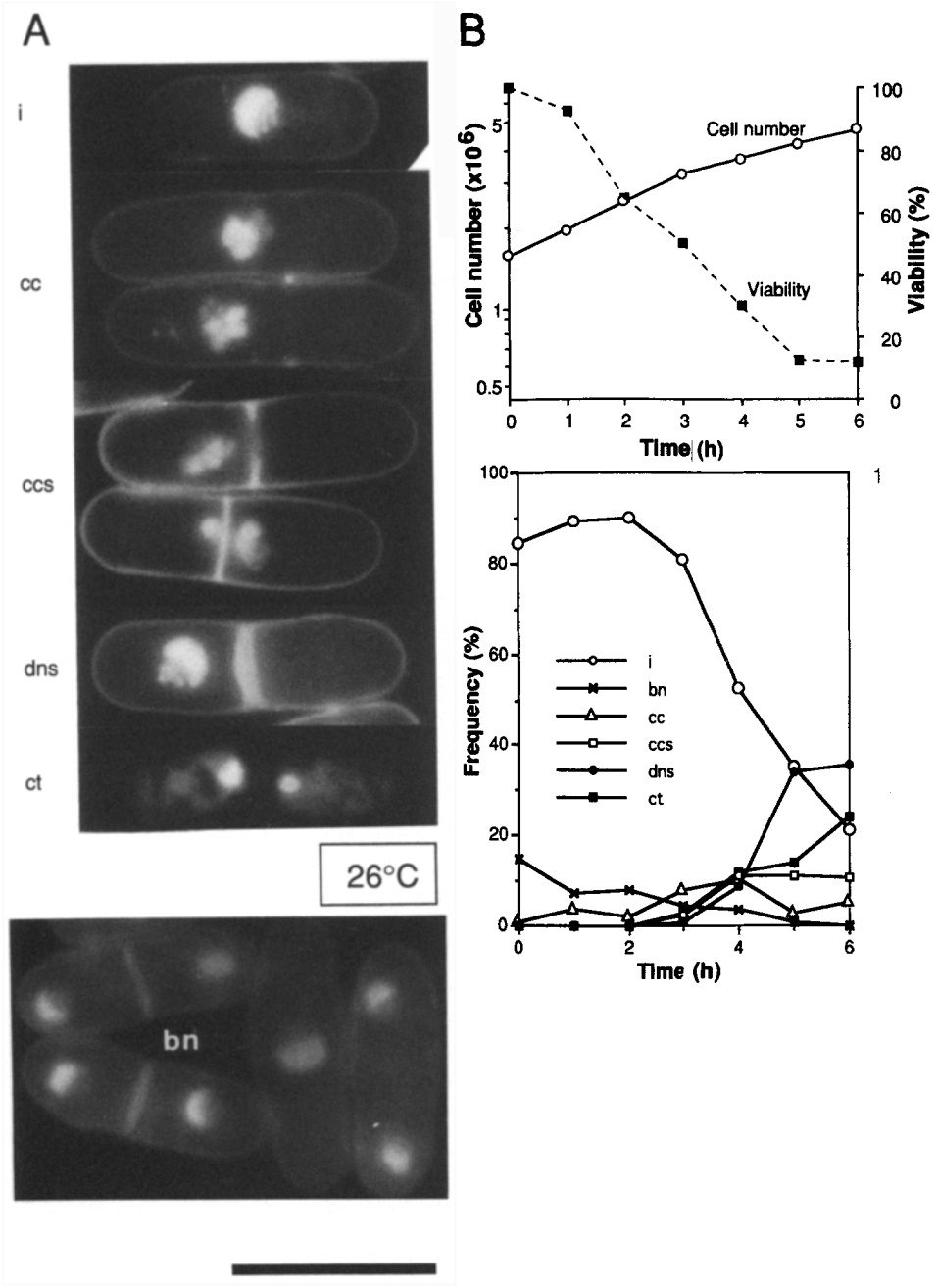


Figure 1. Phenotype of *cut9-665* cells at 36°C. (*A*) *cut9-665* incubated at 36°C and stained by DAPI. (*Top–bottom*) Cells containing the interphase nucleus (*i*), the condensed chromosomes (*cc*), condensed chromosomes with the septum (*ccs*), the displaced nucleus with the septum (*dns*), and the cut phenotype (*ct*). Below, *cut9-665* grown at 26°C. Bar, 10 μm. (*B*) *cut9-665* grown at 26°C were transferred to 36°C and incubated for 6 h. (*Top*) *Open circles*, cell number; *filled squares*, percentage of viability. (*Bottom*) The percentage of frequency of different types of cells. *Open circles*, interphase cells; *crosses*, cells showing normal divided nuclei; *open triangles*, cells showing the condensed chromosomes; *open squares*, septated cells with condensed chromosomes; *filled squares*, cells with the cut phenotype; *filled circles*, the displaced nucleus with the septum.

Cells grown at 26°C were run in an elutriator rotor (see Materials and Methods), and early G2 cells were collected and incubated at 36°C. The synchronous culture was analyzed for 5 h (equivalent to two generation times). DAPI and anti-tubulin antibody staining visualized nuclear chromatin and microtubules, respectively. Cell viability was reduced during mitosis (viability was 20 and 5% after the first and second mitoses, respectively).

The frequency of interphase cells (Fig. 2 *A*, *i*) was initially 100% but sharply decreased ~110–120 min, followed by a cycle of increase and reduction. bn were plentiful (~20%) in the first mitosis but not present at all in the second mitotic stage. In wild-type synchronous culture, the frequency of bn cells reached a high level (>50%) in both first and second mitosis (data not shown).

Cells showing condensed chromosomes (Fig. 2 *A*, *cc*, *lower panel*) peaked twice at ~100–120 and 220 min, when the number of interphase cells was low. Interestingly, these cells contained the short spindle (described below). The first mitotic peak was followed by the increase of ccs, ct, and dns cells. These three classes of aberrant mitotic cells accounted for 86% of total cells after 300 min.

Cells displaying the short spindle and condensed chromosomes became highly abundant during the first and second mitotic stages (Fig. 3, *A* and *B*). Cells showing the long spindle were much less frequent in the first mitosis and were rarely seen in the second mitosis. A control micrograph of wild-type cells showing the long spindle is shown in Fig. 3 *D*.

Cells containing the X-shaped postanaphase microtubule arrays (Fig. 3 *C*, *arrowhead*; Hagan and Hyams, 1988) then increased in parallel with the decrease of cells with the short spindle. These cells represented the ccs, ct, and dns cells.

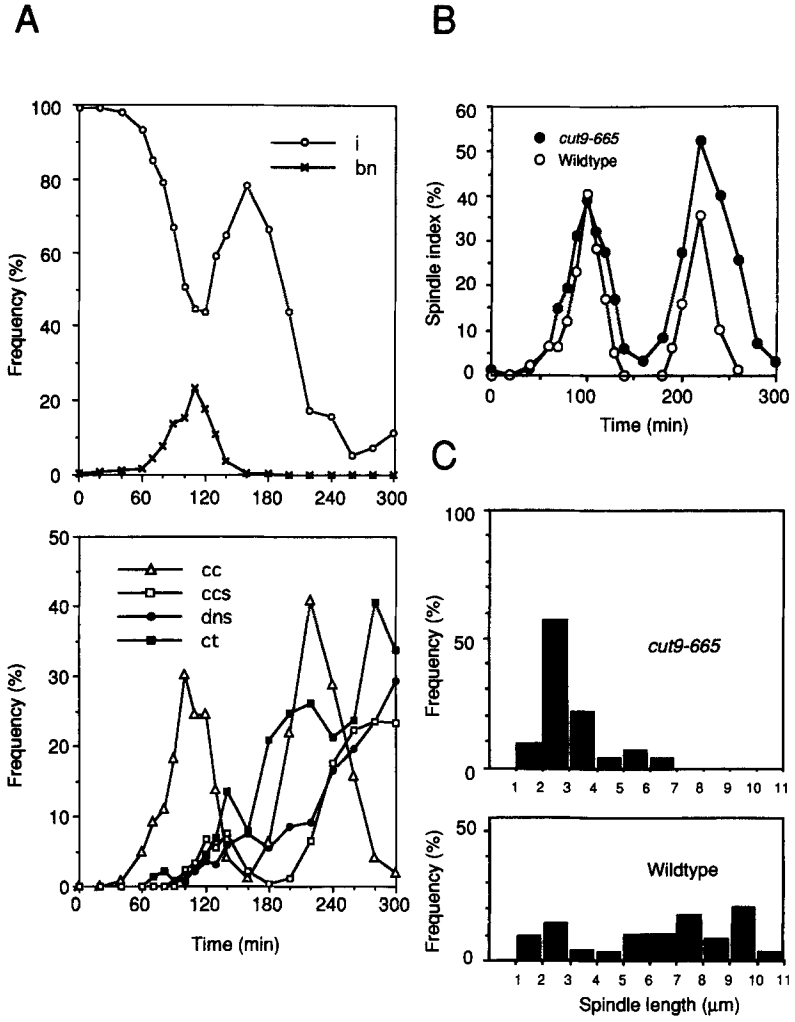


Figure 2. Synchronous culture of *cut9-665* at 36°C. *cut9-665* exponentially grown at 26°C were collected and early G2 cells were selected by elutriation, and incubated at 36°C. Aliquots of cultures were taken and examined for the following properties. Cells were fixed with glutaraldehyde and observed by DAPI staining. For immunofluorescence microscopy, anti-tubulin antibodies TAT-1 (Woods et al., 1989) were used. (*A, upper panel*) Interphase cells (*open circles*) displaying the single hemispherical nuclear chromatin region decreased when they entered mitotic stages. The crosses represent those showing the dividing nuclei (*bn*). (*Lower panel*) Cells showing the condensed chromosomes (*open triangles*). *Open squares*, septated cells with condensed chromosomes; *filled squares*, aberrant cells with the cut phenotype; *filled circles*, the displaced nucleus. (*B*) Frequencies of cells containing the spindle made in the synchronous cultures of wild-type and *cut9* mutant cells at 36°C. They reached high levels twice at ~100 and 220 min. (*C*) Spindle length was also measured in wild-type and *cut9-665* after 130 and 140 min, respectively. The average length of the spindle was shorter in *cut9* than that in wild type.

In the ccs cells, chromosomes were still condensed in spite of septation in the middle. The presence of ccs cells is a clear example of the uncoupled mitotic phenotypes in *cut9-665*: the postanaphase cytoplasmic microtubule arrays and the condensed undivided chromosomes were simultaneously present in one cell.

The dns cells often contained the cytoplasmic microtubules in one half of the cells, which contained the nucleus (Fig. 3 *C*, *arrows*). The nuclear chromatin of the dns cells appeared to be decondensed.

Frequencies of cells containing the spindle in the wild-type and *cut9* mutant were measured at 36°C (Fig. 2 *B*; data obtained were from the synchronous culture different from *A*). The timing and frequencies were nearly identical between wild-type and *cut9* mutant cells. In the second peak, the spindle index in *cut9-665* was higher than that of wild type. The cycle of spindle assembly and disassembly hence seemed to occur in *cut9-665*, whereas the progression from chromosome condensation to disjunction was blocked.

Defect in Spindle Elongation

Then, the length of the mitotic spindle was measured in wild type and *cut9-665* (Fig. 2 *C*). The spindle length in *cut9* mutant was much shorter than that of wild type; the average length in *cut9-665* was 3.0 ± 1.2 μm, whereas that in wild type was 6.3 ± 2.8 μm. Note that the spindle length of the metaphase cells is ~3 μm (Hiraoka et al., 1984; Hirano et al., 1988). The maximal wild-type spindle length at the end of anaphase B was 11–15 μm. Thus, the spindle elongation was severely inhibited in *cut9-665*, in parallel with the failure to initiate anaphase. The ts *cut9* mutation, however, allowed cells to enter postanaphase events, namely, spindle disassembly, septation, and cell division. Similar phenotypes were observed in different *cut9-2* mutant (see below).

Isolation of cut9⁺ Gene and Nucleotide Sequencing

The *cut9⁺* gene was isolated by transformation of *cut9-665* using an *S. pombe* genomic library (Materials and Methods). 10 Ts⁺ Leu⁺ transformants were obtained, and all plasmids recovered from them were overlapped and derived from a single genomic locus. Hybridization of the cloned DNA to the *S. pombe* ordered cosmid bank (Mizukami et al., 1993) showed it to be derived from a region on chromosome I, near the markers of *ade3* and *ryhl⁺*. This physical mapping data was consistent with the genetic data; *cut9-665* was crossed with *ade3* and *ryhl* strains (a gift from Dr. S. Miyake, Tohu University, Tokyo, Japan), and the map distances of 1.9 and 10.3 cM were obtained, respectively. The cloned DNA thus should be derived from the *cut9⁺* gene.

Subcloning of the *cut9⁺* gene (Fig. 4 *A*) indicated that the

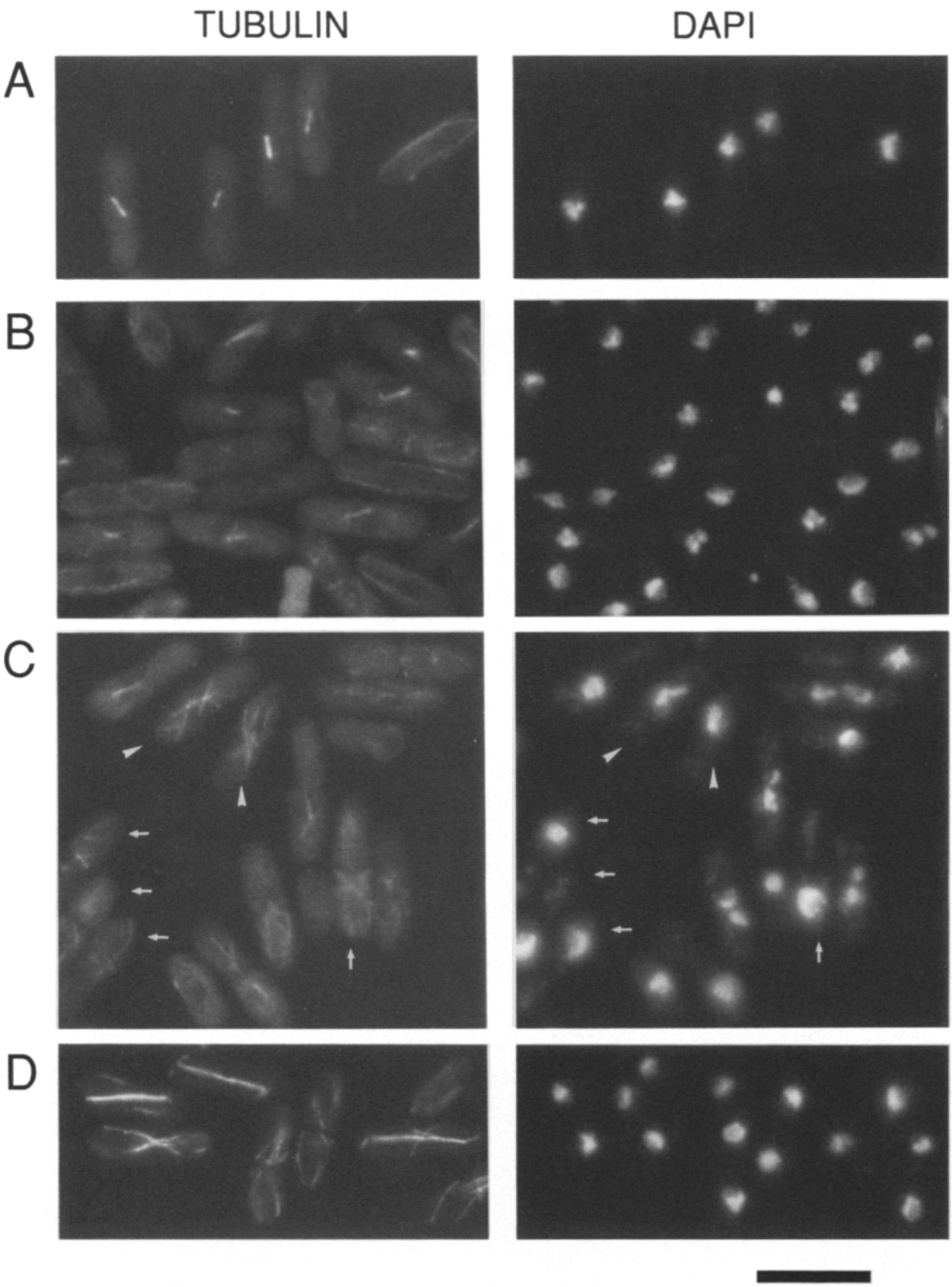
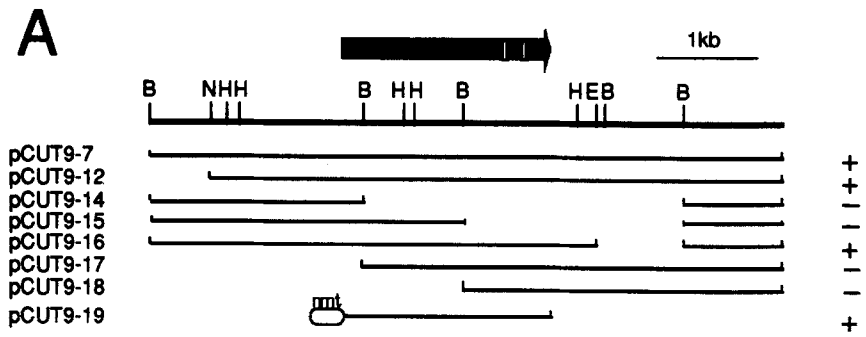


Figure 3. Anti-tubulin staining of *cut9-665* cells at 36°C. Synchronized *cut9-665* (*A–C*) or wild-type (*D*) cells were fixed and specimens for immunofluorescence microscopy were prepared using anti-tubulin antibody and DAPI. The long spindle was not found in *cut9-665*. Cells in *B* were fixed when the spindle index was maximum, while *A* and *C* were fixed 20 min before or after, respectively, *B* was fixed. Arrows and arrowheads in *C* indicate the dns and cells containing the postanaphase microtubule arrays, respectively. Cells stained by antibodies displayed only the short spindle in the synchronous culture of *cut9* at 36°C (*B*). The cells showing the long spindle that corresponded to the anaphase B stages were infrequent in *cut9-665*, while they were abundant in the wild-type control (*D*). Bar, 10 μm.



B

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                                                  BamHI
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Q  L  S  T  L  T  I  S  P  M  T  Y  L  A  N  N  T  S  T  D  G  S  F  L  K  E  R  N  A  Q  N  T  D  S    80

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  L  G  V  V  A  F  N  K  S  D  M  Q  T  A  I  N  H  F  Q  N  A  L  L  L  V  K  K  T  Q  S  N  E  K   513

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 P  W  A  A  T  W  A  N  L  G  H  A  Y  R  K  L  K                intron 1                          530

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      M  Y  D  A  A  I  D  A  L  N  Q  G  L  L  L  S  T  N  D  A  N  V  H  T  A  I  A  L  V  Y  L  H   562

TAAAAAAATCCCTGGTTTAGCTATTACCCACTTACACGAGGTAAGTTTATATATATATTGAAATATCTGTTTGATGTTTTATTTAACATTGATTAGAGTC  3200
 K  K  I  P  G  L  A  I  T  H  L  H  E                intron 2                          S  L   577

TGGCTATATCTCCTAATGAAATCATGGCTTCGGATTTATTGAAACGAGCACTTGAAGAAAACTCCTTAACTTCTGGTTTTCTTAATTCAAAATATGTCTT  3300
  A  I  S  P  N  E  I  M  A  S  D  L  L  K  R  A  L  E  E  N  S  L  T  S  G  F  L  N  S  K  Y  V  F   610

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 E  D  E  V  S  E  Y  M  Q  Q  S  N  L  N  T  S  D  K  S  M  S  M  E  D  Q  S  G  K  V  T  E  S  V   643

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N  D  R  T  Q  V  L  Y  A  D  S  R  S  E  M  M  M  D  D  I  E  G  N  V  S  E  Q  R  *                671

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TCTGTAATTTTTTTTACCAAGCTCCCACCTTGCGAGCCAAATTCGTGCTTTGATTGTTTCTGGAGAAATCTCCGTTTCTTCAGTTTTAATACATTGCTTA  3900
TACATAGAAGCGGCAGAATTC                                                                              3921
        EcoRI
```

Figure 4. Isolation of the *cut9*$^{+}$ gene. (*A*) Plasmids that complemented *cut9-665*. + indicates complementation. The coding region with the putative initiation codon that was ligated with the nmt1 promoter (pCUT9-19) complemented *cut9-665*. *B*, BamHI; *E*, EcoRI; *H*, HindIII; *N*, Nhe I. (*B*) Nucleotide and predicted amino acid sequences of the *cut9*$^{+}$ gene. Two putative introns (intron 1, 56 bp, and intron 2, 53 bp) are present in the COOH domain. The DNA data base accession number is D31844. The consensus sequences for fission yeast introns are underlined. The presence of these two introns was verified by PCR cloning of cDNA sequences and subsequent sequencing. The predicted coding region contains 671 amino acids (calculated mol wt = 75,913 and pI = 5.49). The initiating Met was presumed to locate farthest upstream.

complementable sequence resided in a 4-kb long NheI-EcoRI present in common with pCUT9-12 and pCUT9-16. This was proved by complementation with pCUT9-19, which contained the putative coding region downstream of the nmt1 promoter.

Nucleotide sequence of the EcoRI-NheI fragment was determined (Fig. 4 *B*). One coding region was found with two putative introns in the COOH-terminal domain; the consensus sequences for splicing in fission yeast are underlined. To verify existence of the introns, the PCR method was used to isolate clones covering the introns from a cDNA bank (a gift of Dr. John Fikes; Fikes et al., 1990), and sequenced. cDNA clones thus obtained completely lacked the putative intron sequences, indicating that splicing actually took place. The presumed cut9 protein thus contains 671 amino acids (calculated mol wt = 75,900 and pI = 5.49; Fig. 4 *B*).

cut9 and Budding Yeast CDC16 Are Similar

Database search indicated that the presumed cut9 protein was similar to the *S. cerevisiae CDC16* gene product (Fig. 5 *A*, identical residues are indicated by asterisks). These two proteins are 40% identical in the central region (80–600 residues in cut9 protein), but are nonhomologous in the NH_2- and COOH-terminal domains. The CDC16 gene product (Icho and Wickner, 1987) was found to contain TPR repeat motifs (Sikorski et al., 1990; boxed in Fig. 5 *A*), which are present in a number of cell division cycle genes (Sikorski et al., 1990; reviewed in Goebl and Yanagida, 1991). Similarity between CDC16 and cut9 proteins was not restricted to within the repeats, but it extended to other regions (Fig. 5 *A*). Both cut9 and CDC16 proteins had 10 TPR repeats with the spacer sequences between the second and third, and between the third and fourth TPR repeat. Hydropathy plots (Kyte and Doolittle, 1982) of cut9 and CDC16 (Fig. 5 *B*) indicated that the NH_2 and COOH termini were commonly rich in hydrophilic residues. In spite of this overall similarity, pTIC21, a plasmid carrying the *CDC16* gene (Icho and Wickner, 1987), did not complement *cut9-665*.

Terminal Domains Are Not Required for Complementation

To establish the protein domains essential for complementation, terminally truncated *cut9* genes were used for transformation of *cut9-665* (Fig. 5 *C*). The *S. pombe*–inducible promoter nmt1 (Maundrell, 1990) was ligated with *cut9* gene truncated at 31st and 56th codons. Resulting plasmids complemented *cut9-665* in the presence or the absence of thiamine, indicating that the NH_2-terminal 56 residues were dispensable for complementation. COOH-terminal truncated plasmids were also made, showing that some 60 COOH-terminal residues were nonessential for complementation. These nonessential regions for complementation were nonhomologous to CDC16.

The cut9⁺ Is Essential for Viability

To disrupt the *cut9⁺* gene by one-step gene replacement (Rothstein, 1983), a plasmid pCUT9-50 containing the *S. pombe ura4⁺* gene that substituted for the 1-kb BamHI fragment within the *cut9⁺* gene was made, linearized by HgiAI and introduced into a Ura⁻ diploid (Fig. 6 *A*). Genomic Southern hybridization of stable Ura⁺ transformed diploids indicated that one of the two *cut9⁺* genes was disrupted: two expected HindIII bands newly appeared at positions of 1.2 and 1.3 kb (Fig. 6 *B*, lane *2*), in comparison with non-transformed diploid (Fig. 6 *B*, lane *1*). The Ura⁺ heterozygous diploid transformants were dissected by tetrad; only two viable colonies were formed and all were Ura⁻. The *cut9⁺* gene, hence, was essential for viability. The *cut9* null spores were germinated, but either did not divide or divided once at most. Preliminary analysis of germinated cells in the medium lacking uracil indicated that they displayed the short spindle with condensed chromosomes, terminal cut, and nuclear-displaced phenotypes.

Mutation Sites in the Repeat Region

Mutation sites in two *cut9* strains (Hirano et al., 1986; Samejima et al., 1993) were determined. First, for locating approximate positions of mutation sites, four truncated *cut9* genes (pCUT9-14, pCUT9-15, pCUT9-17, and pCUT9-18, Fig. 4 *A*) were integrated onto the chromosome of either *cut9-665* or *cut9-T98* strain by homologous recombination. pCUT9-17 and pCUT9-18 gave rise to stable Ts⁺ transformants for both strains, but the others did not, suggesting that the mutation sites were in the COOH-terminal 245 amino acids. This region of mutant genomic DNAs was amplified by the PCR method and sequenced directly. Surprisingly, the same 535th codon was altered from G to A in both *cut9-665* and *cut9-T98* strains (these two strains were independently isolated in Kyoto and Cold Spring Harbor Laboratory). The amino acid substitution from wild-type Ala to mutant Thr took place in the middle of the ninth TPR repeat. This Ala residue is highly conserved in all the TPR repeats so far identified, and locates at the base of the knob for postulated snap helix (Hirano et al., 1990). Consistently, the phenotype of *cut9-T98* was indistinguishable from that of *cut9-665*.

The fission yeast *nuc2⁺* gene also contains the TPR repeats, and its ts *nuc2-663* mutation was reported to be the substitution (from Gly to Asp) at the 504th residue in the 7th TPR repeat (Hirano et al., 1990). This essential Gly is highly conserved among other TPR proteins, and in cut9 protein, it resided in the 6th repeat (412 th Gly). To see whether the substitution mutant of *cut9-Asp⁴¹²* also causes the ts phenotype in *cut9*, plasmid carrying *cut9-Asp⁴¹²* was made and integrated onto the chromosome of Ura⁺ heterozygous diploid (described above) by gene replacement using the FOA (fluoro-orotic acid) method (Boeke et al., 1984). Ura⁻ diploids thus obtained were tetrad dissected. Only two Ura⁻-viable spores were obtained at any temperature. Hence, *cut9-Asp⁴¹²* seemed to be lethal at any temperature. From these results, the repeat regions of cut9 protein are essential for the execution of its function in cell cycle control.

Synthetic Lethality between nuc2 and cut9

To examine whether the *cut9⁺* and *nuc2⁺* genes had overlapping function, a cross was made between *nuc2-663* and *cut9-665* to study the double mutant phenotype. Because *nuc2-663* was sterile (Hirano et al., 1988), mutant carrying plasmid pNC106 (Hirano et al., 1988) was used for crossing. Tetrads thus obtained were dissected. The double-mutant *nuc2-cut9* did not form a colony at 26°C. Spores of the double mutant were germinated and divided only two or three

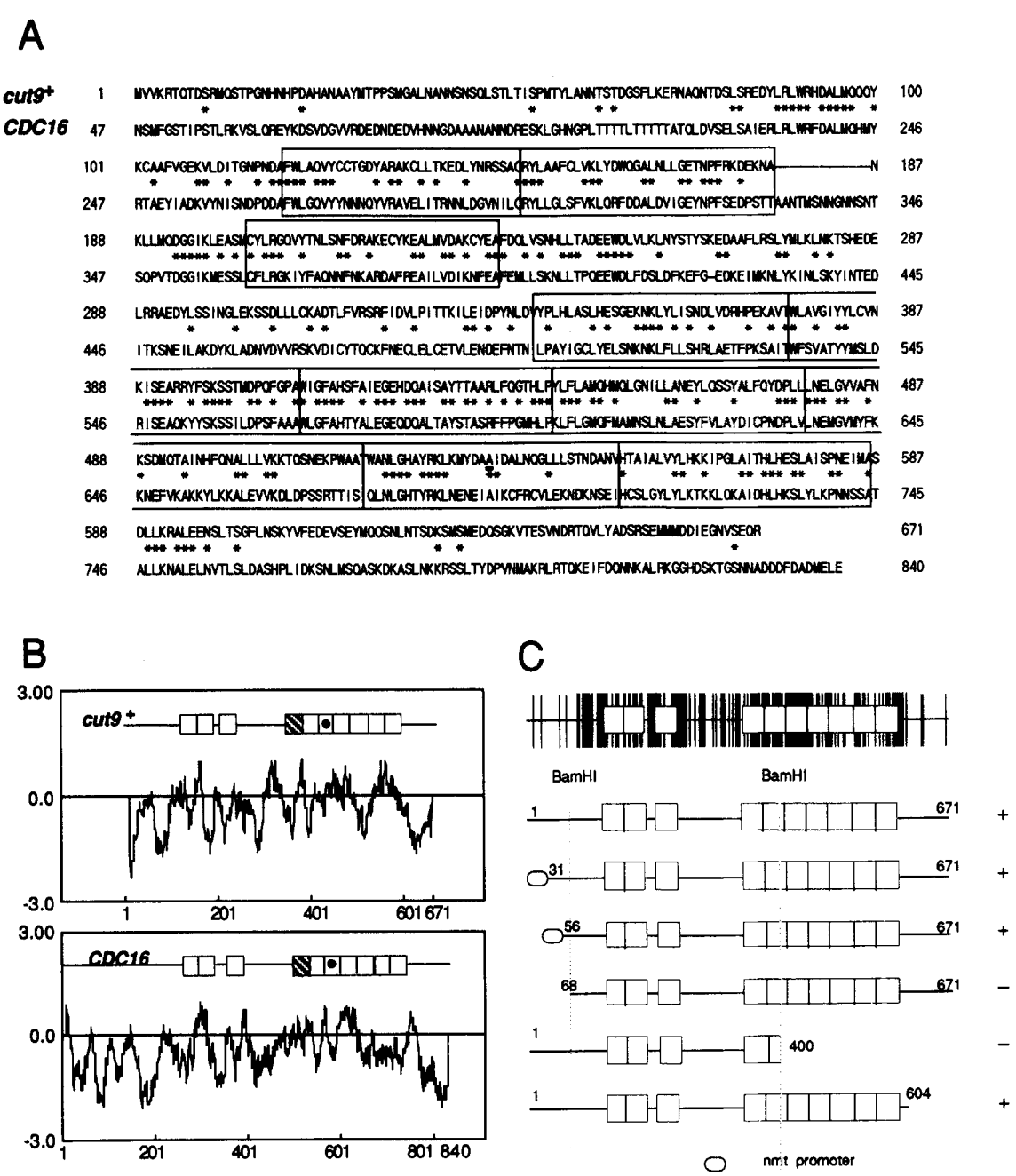


Figure 5. Similarity to CDC16 and region essential for complementation. (*A*) Comparison of cut9 protein with budding yeast CDC16. Identical amino acids are indicated by asterisks. The TPR repeats are boxed. The mutated amino acid is indicated by the underline. (*B*) Hydropathy plots (Kyte and Doolittle, 1982) for cut9 and CDC16 proteins. The TPR repeats are boxed. Filled dots indicate the essential glycine. (*C*) Subclones tested for complementation. A vertical line indicates a position of residues identical to CDC16.

times at 26°C. Combination of the two mutations thus caused synthetic lethality. The *cut9*$^+$ gene shares an essential function with the *nuc2*$^+$ gene for viability.

Identification of the Gene Product

Rabbit antisera were raised against a cut9 fusion protein made in *Escherichia coli* (see Materials and Methods). Affinity-purified antibodies were prepared and used for immunoblot of fission yeast cell extracts. Wild-type cells carrying the vector plasmid produced the 78-kD band (Fig. 7, lane *1*), which was not detected by preimmune serum (data not shown). This band increased in intensity in cells containing multicopy plasmid pCUT9-7 coding for the full-length cut9 protein (lane *2*). In cells containing the COOH-truncated plasmid, pCUT9-15, a 50-kD polypeptide with the expected molecular mass was obtained in addition to the wild-type band (lane *3*). We concluded that the 78-kD band obtained in cell extracts represented cut9 polypeptide.

Cold-sensitive Suppressor Mutations of cut9-665

To obtain suppressor mutants for *cut9-665*, spontaneous reversion mutants of *cut9-665* were isolated by plating cells

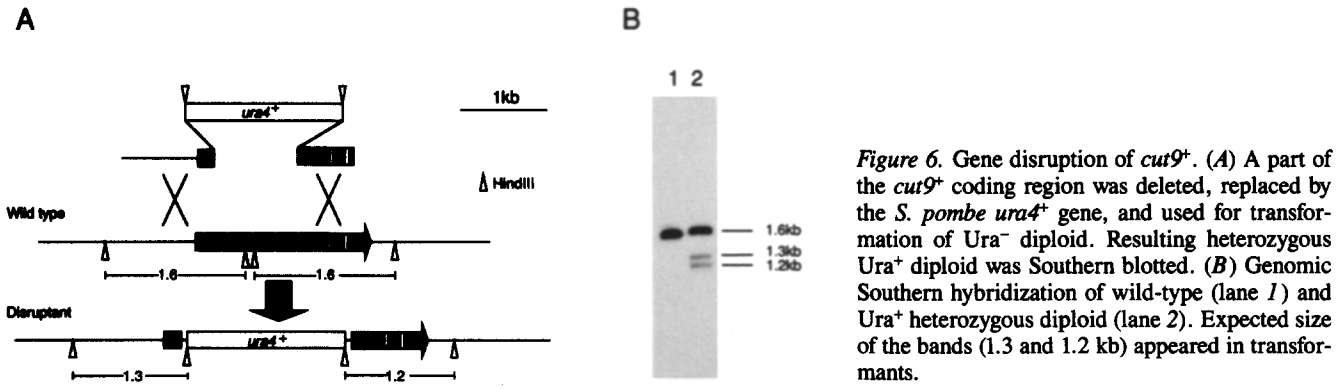


Figure 6. Gene disruption of *cut9*$^+$. (*A*) A part of the *cut9*$^+$ coding region was deleted, replaced by the *S. pombe ura4*$^+$ gene, and used for transformation of Ura$^-$ diploid. Resulting heterozygous Ura$^+$ diploid was Southern blotted. (*B*) Genomic Southern hybridization of wild-type (lane *1*) and Ura$^+$ heterozygous diploid (lane *2*). Expected size of the bands (1.3 and 1.2 kb) appeared in transformants.

on the YPD plates at 36°C. 1.6 × 10^9 cells were plated, and Ts$^+$ colonies isolated were replated at 22° and 36°C. 16 Cs$^-$ Ts$^+$ revertants that did not form colonies at 22°C were obtained and analyzed. By tetrad analysis, Ts$^+$ suppression and Cs$^-$ phenotype were found to cosegregate. Cs$^+$ and Cs$^-$ phenotypes segregated at 2:2, indicating that a single mutation was responsible for suppression of *cut9-665*. Pairwise crosses among these suppressor strains and testing cold sensitivity indicated that they fell into only two complementation groups, designated *scn1* and *scn2* (suppressor for *cut9*). They were unlinked to the *cut9-665* locus, and they mapped different genetic loci. Tetrad dissection indicated that *scn2* was linked to *cut9* in the chromosome I, whereas *scn1* was unlinked (the cloned *scn1*$^+$ located in the 900-kb NotI fragment).

Phenotypes of scn1 and scn2 Mutants

Growth phenotype of single *scn* segregants (obtained by crossing *cut9-scn1* or *cut9-scn2* with wild type) was examined by plating them at 36°C and 22°C on YPD (Fig. 8 *A*). The *scn* mutant segregants showed cs phenotype; no colony was formed at 22°C. As expected, crossing of the single *scn* segregants with *cut9-665* yielded the Ts$^+$ double mutant. Suppression of *cut9* null by *scn1* mutation, however, did not take place, indicating that the *scn1*$^+$ gene did not substitute for the function of *cut9*$^+$.

The cs phenotype of *scn* mutations seemed to be semidominant, either in the presence or absence of *cut9-665* mutation; heterozygous diploids (*scn1*/+ or *scn2*/+) were leaky Cs$^-$ and produced tiny colonies at 22°C (Fig. 8 *B*).

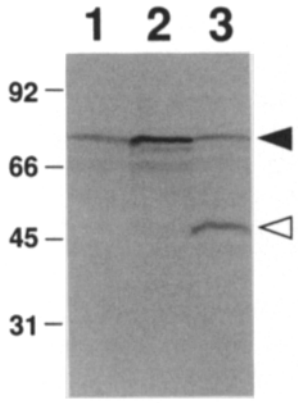


Figure 7. Identification of the *cut9*$^+$ gene product. Affinity-purified antibodies against cut9 fusion protein made in *Escherichia coli* were used for immunoblotting of the fission yeast cell extracts. A 78-kD polypeptide band produced in wild-type cells (lane *1*) increased the intensity in cells containing multicopy plasmid pCUT9-7 (lane *2*). In cells containing the COOH-truncated plasmid pCUT9-15, a 50-kD polypeptide with the expected molecular weight was produced in addition to the wild-type band (lane *3*).

Although single *scn* mutants did not produce visible colonies after incubation at 22°C for 7 d, microcolonies that formed were visible under a microscope (Fig. 8 *C*). Approximately 20–100 cells were seen, suggesting that several cell divisions could occur at 22°C.

These two *scn* mutations interact genetically. Tetrad analysis of the cross between *scn1* and *scn2* indicated that the double mutant was lethal. As shown below, the cloned *scn1*$^+$ gene complemented *scn2* mutant.

We found that the double mutations between *nuc2* and *scn1* or *scn2* were synthetic lethal. *nuc2-663* (which is sterile) carrying plasmid with the *nuc2*$^+$ gene was crossed with *scn1* or *scn2* mutant, and the zygotes were dissected. The double mutant spores did not form colony at 26°C (the permissive temperature for these mutant strains).

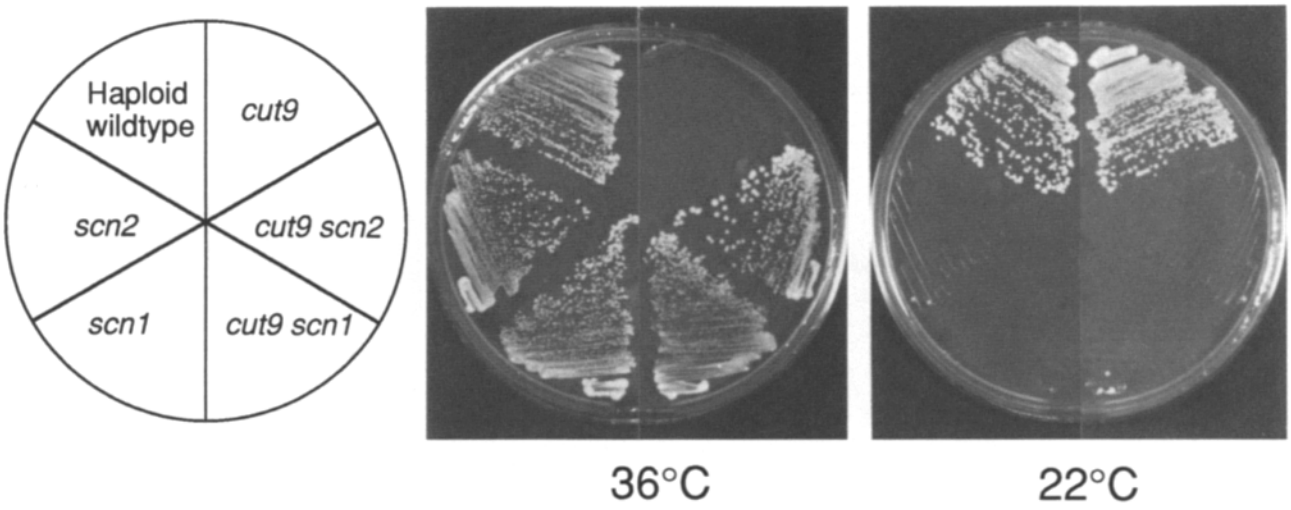
Anaphase Defect in scn1 and scn2 Mutants

Cytological phenotype of single *scn1* and *scn2* mutants at 20°C was examined. Cells grown in the liquid YPD culture at 33°C were transferred to 20°C, and they were observed by DAPI staining after glutaraldehyde fixation. Both *scn1* and *scn2* mutant cells showed similar phenotypes. Cell division arrest occurred only after 30 h (the generation time of wild type at 20°C is 5–6 h). Normal-looking cells were abundant initially (5 h). However, a fraction of cells (~5%) contained two nuclei separated by a distance shorter than that of fully elongated anaphase spindle (Fig. 9 *A*). Such cells defective in late anaphase increased to 20–30% of cell populations after 20 h, some of which were with the septum, resulting in cell death. In wild-type culture under the same conditions, the frequency of cells in late anaphase was <1%.

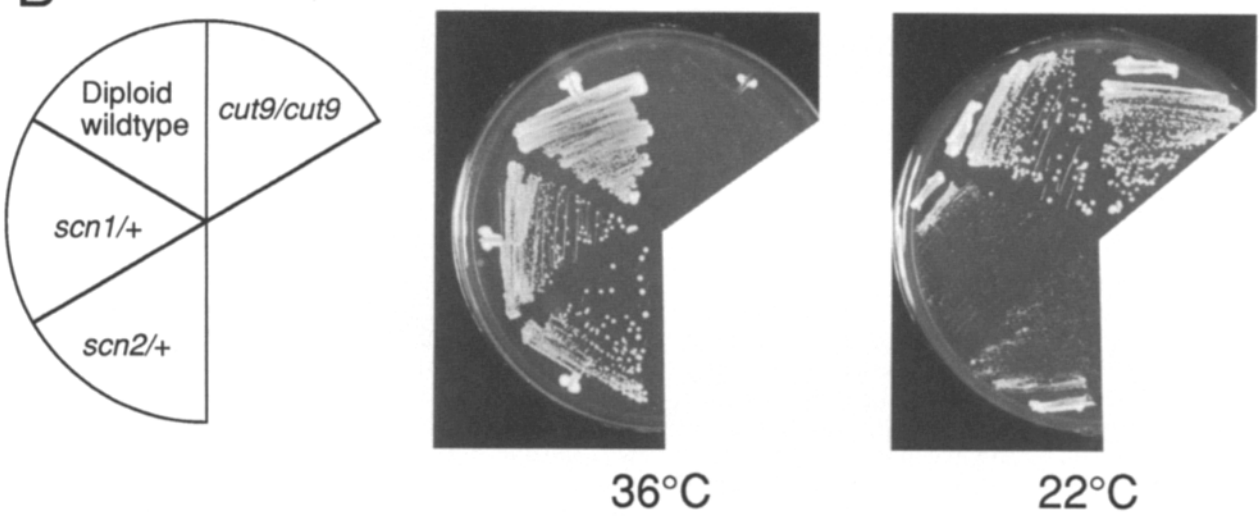
Another characteristic of *scn* mutants was that chromosome decondensation in the end of anaphase did not normally occur. The DAPI-stained nuclear chromatin region was significantly more condensed than that of wild-type nuclei in the same anaphase stage. A chromosome-like structure was often clearly recognizable at the end of anaphase B in *scn* mutant cells (also see Fig. 9 *B*). This was not seen in the wild-type anaphase cells.

Immunofluorescence microscopy using anti-tubulin antibodies indicated that the spindle length in *scn1* mutant cells was indeed short in comparison with that of wild type (Fig. 9 *B*). The average separation of such dividing nuclei was 6 μm, roughly half the length of the wild-type fully elongated spindle. We concluded that *scn1* and *scn2* mutants were defective in the progression of late anaphase, that is, in spindle elongation and chromosome decondensation.

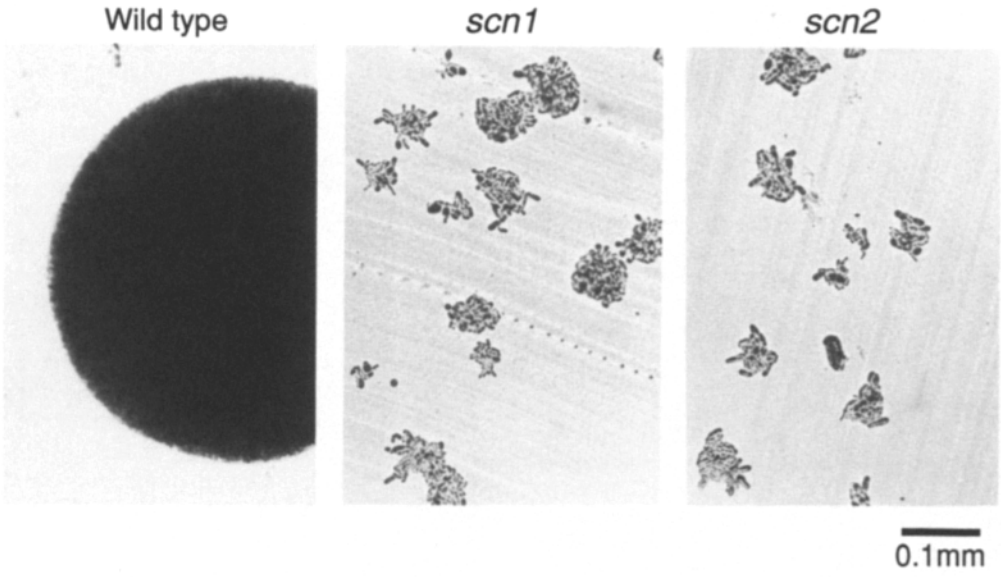
A


Haploid wildtype
cut9
scn2
cut9 scn2
scn1
cut9 scn1
36°C
22°C

B


Diploid wildtype
cut9/cut9
scn1/+
scn2/+
36°C
22°C

C


Wild type
scn1
scn2
0.1mm

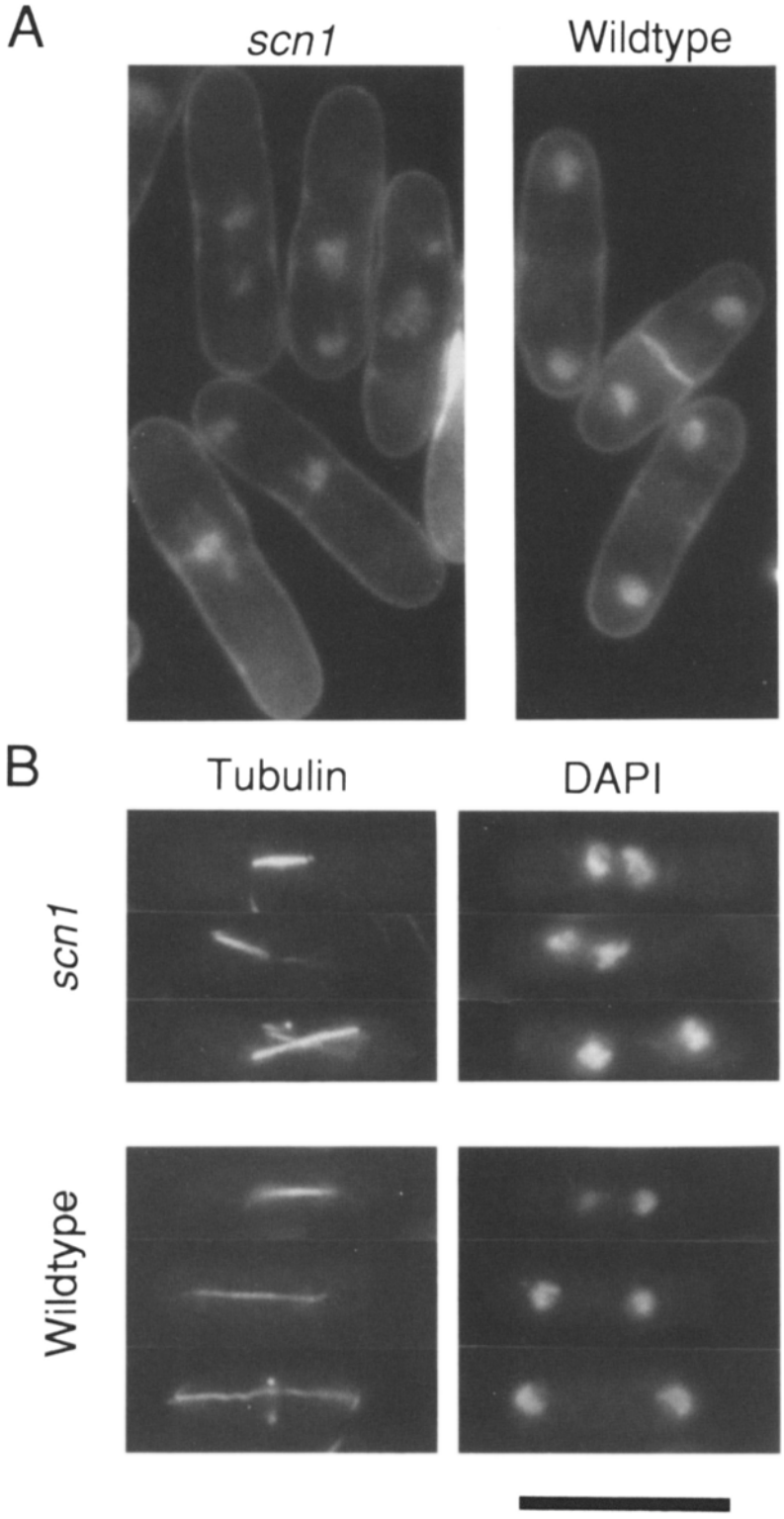


Figure 9. Phenotype of *scn1* and *scn2* at 20°C. (*A*, *left panel*) *scn1* mutant cells were incubated at 20°C for 5 h before fixation and DAPI staining. (Right panel) Wild-type cell. (*B*) Immunofluorescence microscopy of *scn1* cells incubated at 20°C for 5 h using anti-tubulin. Cells were fixed with glutaraldehyde. The spindle was shorter in *scn1* than in wild type; maximal length of spindle in *scn1* mutant cells was ~6 μm, in contrast to 10–12-μm long wild-type anaphase B spindle. Bar, 10 μm.

Figure 8. Growth properties of *scn1* and *scn2*. (*A*) *scn1* and *scn2* mutants were plated on YPD at 36°C and 22°C. The double mutants *scn1 cut9* and *scn2 cut9* are also shown. (*B*) Heterozygous diploids *scn1*/+ and *scn2*/+ were plated on YPD at 36°C and 22°C. The wild-type diploid and the double mutant diploid cut9/*cut9* are also shown. (*C*) Plates were incubated at 22°C for 5 d, and colonies on each plate were photographed under a microscope.

Revertants for scn Mutants

Further evidence for interaction between *cut9*$^+$ and *scn*$^+$ genes came from analysis of Cs$^+$ suppressors for *scn* mutants. Cs$^+$ revertants were obtained by plating a strain, *h*$^-$ *scn2 cut9*$^+$ at 22°C. A number (240) of Cs$^+$ colonies were obtained from 3.6×10^8 cells by spontaneous mutations. 32 revertants were analyzed, and all were Cs$^+$ Ts$^-$; no colony was formed at 36°C. Pairwise crossing indicated that Ts$^-$ mutations made two complementation groups. By genetic crossing, the locus for one complementation group was found to be tightly linked to *cut9*. Furthermore, the Ts$^-$ phenotype of a strain in this group was complemented by a plasmid carrying the *cut9*$^+$ gene, confirming that a *cut9* mutation (designated *cut9-2*) could suppress the Cs$^-$ phenotype of *scn2*. The cytological phenotype of *cut9-2* at 36°C is similar to that of *cut9-665*. By integration of the plasmids described above, which had a truncated gene, the mutation site in *cut9-2* was found to reside in the COOH-terminal region (data not shown). The genetic locus of the other complementation group remains to be determined.

Isolation of the scn1$^+$ *Gene*

An *S. pombe* genomic DNA library was used to isolate plasmids that suppressed the cs phenotype of *scn1 cut9*. Plasmids recovered from six transformants were all identical. A part of their DNA (pSi211) was integrated onto the chromosome by homologous recombination, and was confirmed by Southern blot analysis (data not shown). Stable Leu$^+$ transformants were crossed with *scn1*, and tetrads were dissected. Leu$^+$ and Cs$^-$ were tightly linked (PD/NPD/TT = 16:0:0) and, hence, the cloned sequence should be derived from the *scn1*$^+$ gene. The minimal complementable clone was a 1.4-kb SpeI fragment (Fig. 10 *A*, pSi214). This DNA clone did complement the *scn2* mutant and the *scn1* mutant, demonstrating a further functional relationship between the *scn1*$^+$ and *scn2*$^+$ genes. The *scn2*$^+$ gene has not been isolated.

Nucleotide sequencing of the 1.4-kb SpeI fragment showed that it contained a 1,164-bp region encoding a 387-amino acid protein (Fig. 10 *B*). Database search indicated that an open reading frame (ORF) (313 amino acids; EMBL accession number M88172) with unknown function in *S. cerevisiae* was similar to scn1 (30% identity in the NH_2 domain), as shown in Fig. 10 *C*.

The *scn1*$^+$ gene was not essential for viability. A plasmid containing the *S. pombe ura4*$^+$ gene that replaced the 1.4-kb SpeI fragment encompassing the *scn1*$^+$ was constructed, and linearized plasmid was introduced into a Ura$^-$ diploid. Genomic Southern hybridization of stable Ura$^+$ transformants showed that the one of *scn1*$^+$ gene was disrupted. Heterozygous diploids obtained were sporulated, and all four spores were viable. Hence the *scn1*$^+$ gene was nonessential for viability. The null mutant was not cold sensitive, and it did not suppress ts of *cut9-665*. Thus, the phenotype of cs *scn1* missense mutants was not caused by a loss of function, consistent with its dominant property in heterozygous diploid.

Discussion

We report here the characterization of fission yeast ts mutant *cut9-665* and identification of the *cut9*$^+$ and related genes. A principal defect in *cut9-665* is the absence of chromosome disjunction and spindle elongation. Mutant cells enter mitosis, and the short metaphase spindle forms with condensed chromosomes, but neither sister chromatid separation in anaphase A nor spindle elongation in anaphase B takes place (schematized in Fig. 11 *A*). In other *cut* mutants (Uemura and Yanagida, 1986; Hirano et al., 1986; Uzawa et al., 1990; Saka and Yanagida, 1993), the spindle made is elongated, accompanied by abnormal chromosome disjunction. Hence, *cut9-665* is unique among *cut* mutants studied so far in regard to the absence of spindle elongation after metaphase.

Another main defect is the occurrence of postanaphase events in the absence of anaphase. The X-shaped cytoplasmic microtubule arrays characteristic of postanaphase cells (Hagan and Hyams, 1988) were observed after spindle degradation, followed by septation and/or cytokinesis. Synchronous culture analysis indicated that chromosomes were still condensed when the spindle was degraded, and then mutant cells were septated and divided. The *cut9* mutant, hence, is defective in coupling between the onset of anaphase and the restraint of postanaphase events until the completion of anaphase. In other words, the anaphase is apparently bypassed in *cut9-665* mutant cells. This might be caused by the "leakiness" in the ts mutant, but both ts and deletion mutants showed similar phenotypes so that the apparent bypass phenotype is not specific for a single allele.

In other fission yeast, mitotic mutants such as *nda3* (the *nda3*$^+$ gene encodes β-tubulin), which enters mitosis with chromosome condensation and high H1 kinase, but is blocked from forming the spindle; both septation and cell division are inhibited (Hiraoka et al., 1984; Moreno et al., 1989; Kanbe et al., 1990), suggesting that dependence of postanaphase events on the completion of previous mitotic events is maintained. In *dis1*, *dis2*, and *dis3* mutants (*dis2*$^+$ encodes a type 1–like protein phosphatase), the spindle elongates without sister chromatid separation, but subsequent cell division does not occur (Ohkura et al., 1988 and 1989). In *sds22* mutant (*sds22*$^+$ encodes a regulator for dis2 phosphatase), which forms the metaphase spindle with condensed chromosomes, cytokinesis does not take place although the septum forms after the nucleus is displaced from the middle (Ohkura and Yanagida, 1991; Stone et al., 1993). In these mutants, inhibition of mitotic events causes the block of subsequent cell division. These mitotic mutant phenotypes suggested the presence of a negative feedback control system (Weinert and Hartwell, 1988; Murray, 1992; Murray and Hunt, 1993; Sheldrick and Carr, 1993) for the progression of anaphase in fission yeast. If the initiation of anaphase is blocked or delayed, such a restraint system for postanaphase events might be exerted.

The *cut9*$^+$ gene product is a potential element in such a control system. The presence of the *cut9*$^+$ gene might ensure restraint of the onset of septation and cytokinesis until the completion of anaphase. In *cut9* mutants at restrictive temperature, the dependence between anaphase and postanaphase is abolished, possibly because of the disruption of the control system; the cut9 protein is required for the onset of anaphase, but also possibly for surveillance of anaphase progression. An analogous situation has been found in ts *cut5* mutants that are defective in the S phase and the restraint of mitosis and cell division until the completion of S phase (Saka and Yanagida, 1993). The *cut5*$^+$ gene product

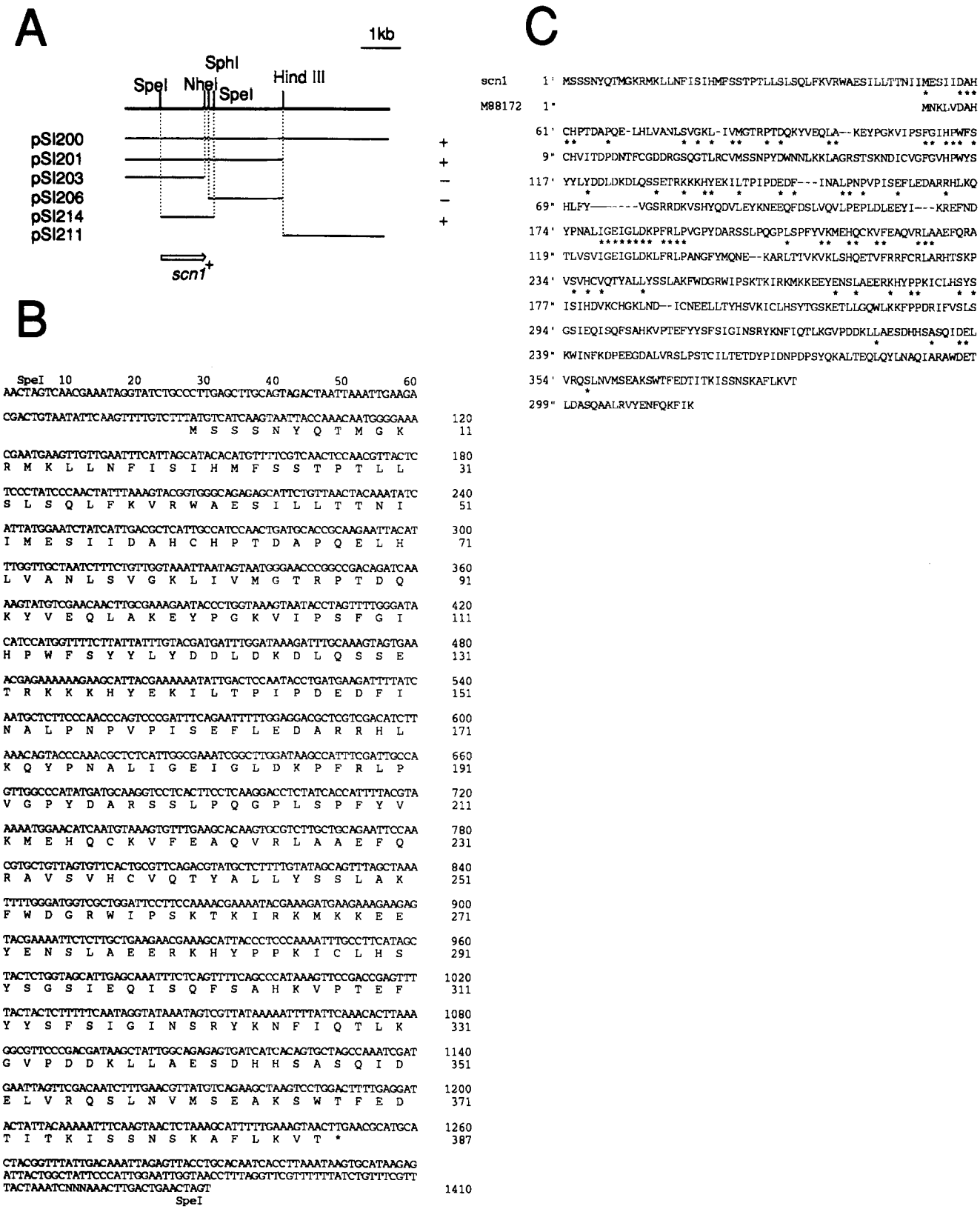


Figure 10. Isolation of the *scn1*$^+$ gene. (*A*) An *S. pombe* genomic DNA library was used to isolate plasmids that suppressed the cs phenotype of *scn1 cut9* mutant. By subcloning, the minimal complementable clone was 1.4 kb SpeI (pSi214). This DNA clone complemented not only single *scn1*, but also *scn2* mutant. (*B*) Nucleotide sequencing of the 1.4-kb SpeI fragment showed that it contained a 1,164-bp ORF encoding a 387-amino acid protein. The DNA database accession number is D31845. (*C*) Database search indicated that an ORF (313 amino acids; EMBL No. M88172) with unknown function in *S. cerevisiae* was similar to scn1 (30% identity in the NH_2 domain).

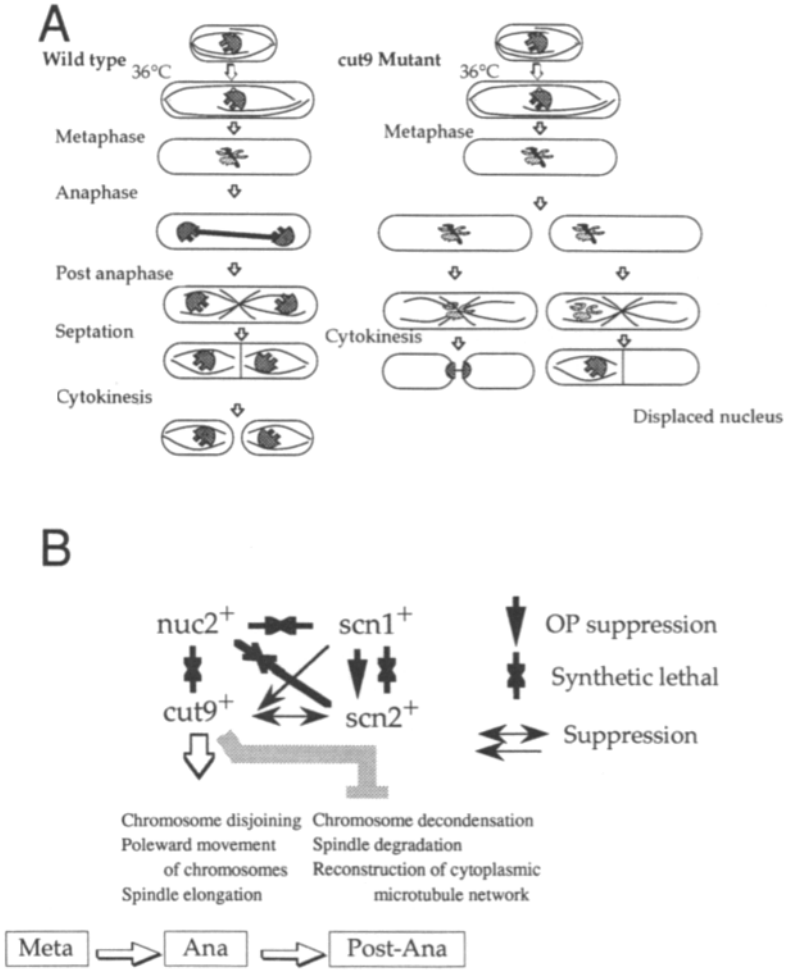


Figure 11. Phenotype of *cut9-665* mutant and the role of *cut9*$^+$ gene in anaphase. (*A*) The phenotype of *cut9-665* mutant at 36°C is schematized. (*B*) The *cut9*$^+$ gene is required for the onset of anaphase. It is also possibly required for restraining postanaphase until the completion of anaphase. Alternatively, anaphase is physically blocked by the loss of function in the spindle, the spindle poles or chromosomes, although the signal for anaphase may normally turn on and off. Relationships among *cut9*$^+$ and related genes are schematized. Meta, Ana, and Post-ana represent, respectively, metaphase, anaphase, and postanaphase. OP suppression indicates the suppression by an elevated gene dosage. *nuc2-cut9*, *scn1-scn2*, *nuc2-scn1*, and *nuc2-scn2* double mutants are synthetic lethal, whereas ts *cut9* is suppressed by cs mutations in *scn1* and *scn2*. Cs *scn2* is suppressed by chromosomal mutations in *cut9*. The cloned *scn1*$^+$ gene can suppress cs *scn2* mutation as a multicopy suppressor.

seems to be an essential component for the replication checkpoint (Saka et al., 1994).

Another possibility is that anaphase was physically blocked in *cut9* mutants, although the signal for the onset of anaphase was triggered. This physical block might be caused by the absence of structurally essential proteins in the spindle, the spindle pole bodies, or mitotic chromosomes. In *cut9* mutant cells, regulators required for the progression of anaphase may normally turn on and off, but the actural physical mechanism for chromosome disjunction is completely shut off.

In budding yeast, the genes (*BUB* and *MAD*) that are required for mitotic arrest in response to loss of microtubule function have been identified (Hoyt et al., 1991; Li and Murray, 1991). These gene products are thought to maintain a high level of M phase kinase activity upon the removal of functional microtubules. A possible role of cut9 protein would be to lead to the reduced level of M phase kinase activity during anaphase and restrain the activation of postanaphase (G1/S) kinase until the completion of anaphase. In fission yeast, the G1 cyclin gene has not been isolated.

Identification of proteins that interact with cut9 is a promising approach to understand the role of cut9 in cell cycle control. We found that *cut9*$^+$ genetically interacts with three genes, namely, *nuc2*$^+$, *scn1*$^+$, and *scn2*$^+$ (Fig. 11 *B*). The products of the *cut9*$^+$ and *nuc2*$^+$ genes, containing the TPR repeats, may share an essential function for the onset of anaphase. Their mutant phenotype is similar. The difference is that the postanaphase deregulation in *nuc2* mutant (Hirano et al., 1988) is not as extensive as in *cut9-665*. All the mutation sites in *cut9* and *nuc2* locate in the TPR repeat regions that may serve as domains for inter- or intramolecular interactions (Hirano et al., 1990; Sikorski et al., 1990; Goebl and Yanagida, 1991). A variety of defects in mitosis, signal transduction, transcription, RNA splicing, and transport are reported for mutations in the TPR-containing genes. However, the actual TPR motif function is hardly understood (Goebl and Yanagida, 1991). The cut9 protein might directly or indirectly interact with the nuc2 protein.

Recent reports indicated that anaphase (sister chromatid separation) is not initiated by the inactivation of M phase kinase, but rather by ubiquitin-mediated proteolysis (Holloway et al., 1993), and that the execution of anaphase and the destruction of M phase kinase appears to take place independent of one another in budding yeast (Surana et al., 1993). Hence, the cut9 protein function might be implicated in proteolysis or other events distinct from the inactivation of M phase kinase activity. It is of interest to examine the possible interaction between cut9 and proteasome or protein phosphatase (Gordon et al., 1993; Stone et al., 1993). The level of various M phase-related activities, such as H1 kinase, phosphatase, and cyclin proteolysis should be investigated in *cut9* mutant cells.

The relationships of the genes studied in the present study are illustrated in Fig. 11 *B*. Three kinds of genetic interac-

tions, namely, synthetic lethal, suppressions by chromosomal mutation, or overproduction, are present among the four genes. The cs *scn2* mutation is suppressed by ts *cut9-2* so that the suppression between *cut9* and *scn2* is bilateral. As genes similar to *cut9*$^+$, *nuc2*$^+$ and *scn1*$^+$ have been found in distant organisms, interactions among these genes might be evolutionarily conserved. Cold-sensitive *scn1* and *scn2* mutations caused accumulation of the late anaphase cells at restrictive temperature, suggesting that the completion of anaphase was defective in *scn* mutant. The cut9 and nuc2 proteins might play a pivotal role in the control of anaphase, interacting directly or indirectly with the two scn proteins.

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