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Identification of *cut8*$^+$ and *cek1*$^+$, a Novel Protein Kinase Gene, Which Complement a Fission Yeast Mutation That Blocks Anaphase

ITARU SAMEJIMA AND MITSUHIRO YANAGIDA*

Department of Biophysics, Faculty of Science, Kyoto University, Sakyo-Ku, Kyoto 606-01, Japan

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The fission yeast *Schizosaccharomyces cerevisiae* temperature sensitivity *cut8-563* mutation causes chromosome overcondensation and short spindle formation in the absence of sister chromatid separation. The *cut8-563* mutation allows cytokinesis before the completion of anaphase, thus producing cells with a cut phenotype. The *cut8*$^+$ gene product may be required for normal progression of anaphase. Diploidization occurs at the restrictive temperature, and 60 to 70% of the cells surviving after two generations are diploid. These phenotypes are reminiscent of those of budding yeast (*Saccharomyces cerevisiae*) *ctf13* and *ctf14* (*ndc10*) mutations. The *cut8*$^+$ gene, isolated by complementation of the mutant, predicts a 262-amino-acid protein; the amino and carboxy domains are hydrophilic, while the central domain contains several hydrophobic stretches. It has a weak overall similarity to the budding yeast *DBF8* gene product. *DBF8* is an essential gene whose mutations result in delay in mitotic progression and chromosome instability. Anti-cut8 antibodies detect a 33-kDa polypeptide. Two multicopy suppressor genes for *cut8-563* are identified. They are the *cut1*$^+$ gene essential for nuclear division, and a new gene (designated *cek1*$^+$) which encodes a novel protein kinase. The *cek1*$^+$ gene product is unusually large (1,309 amino acids) and has a 112-amino-acid additional sequence in the kinase domain. The *cek1*$^+$ gene is not an essential gene. Protein phosphorylation by cek1 may facilitate the progression of anaphase through direct or indirect interaction with the cut8 protein.

DNA replication, nuclear division, and cell separation are well coordinated in a normal cell cycle. In most eukaryotic cells, DNA replication precedes nuclear division and is followed by cytokinesis. Each daughter cell will thereby receive one nucleus containing a correct set of chromosomes. A group of temperature sensitivity (ts) fission yeast mutations called *cut* (cell untimely torn [21]) disrupt such coordination in the cell cycle. At the restrictive temperature (36°C), nuclear division in these mutants is defective, but cytokinesis which bisects the nucleus at the middle of the cell follows.

Nineteen genetic loci displaying the cut phenotype have been identified (21, 43, 53). In many *cut* mutants, the first mitotic event is defective upon the shift to the restrictive temperature (18, 21, 53, 54). In the *rad4/cut5* mutant, however, the first division is normal whereas the second one becomes defective (41). This is due to a principal defect in the progression of S phase which occurs only after the first cell division (growing *Schizosaccharomyces pombe* cells are largely in G_2).

There are several possible ways to explain how the major events are not coordinated in *cut* mutants. A checkpoint control (55), which monitors the progression from mitosis to cytokinesis, might be defective in *cut* mutants. Alternatively, the loss of *cut*$^+$ gene function may not be detected by the checkpoint system. It is unknown, however, whether such a checkpoint exists in fission yeasts to delay cytokinesis when a mitotic event is disturbed. Another possibility is that the *cut*$^+$ gene product executes their function after cells have passed the stage which commits them to cytokinesis (21). For example, sister chromatid separation may trigger cytokinesis despite the fact that separation may be incomplete. In the case of DNA topoisomerase II mutant (*top2*), a small portion of chromosome DNAs (that is, the centromere DNAs) is separated by the spindle extension (12), and this highly limited sister chromatid separation may be sufficient for triggering the onset of cytokinesis. As there are approximately 20 *cut*$^+$ genes, the causes for deregulating cell cycle may well be variable.

Some of the *cut*$^+$ gene products have been identified (18, 21, 41, 53, 54). They are related to DNA replication, DNA topology, mitotic chromosome condensation and segregation, and spindle formation or spindle pole body duplication. In other mitotic mutants such as *nda2*, *nda3*, *dis1*, *dis2*, *dis3*, *nuc2*, *sds22*, *pim1*, and *mts2* (14, 22, 23, 32, 37, 38, 46, 50), cells display defective mitosis but not the cut phenotype. In some, typified by the *nuc2* mutant (22), the septum was made; the septation can occur but cytokinesis is blocked in the *nuc2* mutant. In these mutants, inhibition of mitotic events causes the block of subsequent cell division. The negative feedback control system for following postanaphase events appears to be exerted in certain mitotic mutants.

Here, we report the characterization of one of the *cut*$^+$ genes, namely, *cut8*$^+$. The *cut8-563* mutant is defective in the progression through mitotic anaphase, displaying condensed chromosomes and a partially extended spindle. The *cut8*$^+$ gene seems to be required in the normal progression of anaphase. Another phenotype of *cut8-563* is the production of diploid cells at the restrictive temperature. An increasing number of diploid cells are formed at the restrictive temperature, suggesting that the two sets of daughter chromosomes are formed once but not separated. A genomic DNA clone that derived from the *cut8*$^+$ locus and can complement *cut8-563* was isolated. Nucleotide sequencing showed that the *cut8*$^+$ gene encodes a 262-amino-acid protein. It is similar to the budding yeast *DBF8* gene product, whose mutations increase the rates of chromosome loss and disjunction (24). We also identified a multicopy suppressor gene for the *cut8-563* mutation, which encodes a novel protein kinase designated *cek1*$^+$ (cut eight suppressing kinase) with a large size (1,309 amino acids). It

* Corresponding author. Phone: 81 75 753 4205. Fax: 81 75 753 4208.

may play a positive role in completing anaphase and directly or indirectly control the *cut8*$^+$ gene product. In the Discussion, we suggest a role for cut8 protein in the progression of mitotic anaphase.

MATERIALS AND METHODS

Strains and media. Strains used were haploid and diploid wild types (16, 29), *cut8-563* (21), HM123 (*h*$^-$ *leu1*), YS106-1D (*h*$^-$ *ura4-D18*), TP4-5A (*h*$^-$ *leu1 ura4-D18 ade6-210*), and TP4-1D (*h*$^+$ *leu1 his2 ura4-D18 ade6-216*). The *ura4*$^+$ gene was deleted in *ura4-D18* (15). Diploid strains used for gene disruption were made by a cross between TP4-5A and TP4-1D. *S. pombe* was grown in YPD (complete rich medium; 1% yeast extract, 2% polypeptone, 2% glucose) or EMM2 (minimal medium [34]). Agar (1.5%) was added for plates. An SPA plate (1% glucose, 0.1% KH_2PO_4, 0.001% biotin, 0.0001% calcium pantothenate, 0.001% nicotinic acid, 0.001% meso-inositol, 3% agar) was used for sporulation. Phloxine B (10 μg/ml; Sigma) was included in YPD plates. The standard genetic procedures described for *S. pombe* by Gutz et al. (16) and Moreno et al. (36) were followed. The *Escherichia coli* strain used was MM294, unless otherwise mentioned. *E. coli* was grown in LB (0.5% yeast extract, 1% NaCl, 1% polypeptone [pH 7.5]). Agar (1.5%) was added for plates. Ampicillin (100 μg/ml) was included for plasmid maintenance.

DNA. All DNA manipulations were performed by standard procedures (42). Isolation of *S. pombe* genomic DNA and recovery of plasmid DNA from *S. pombe* cells were described by Moreno et al. (36). The nucleotide sequence was determined by the dideoxy method (44).

Fluorescence microscopy. The procedure described for 4′,6-diamidino-2-phenylindole (DAPI) staining by Adachi and Yanagida (1) was followed. Immunofluorescence microscopy was performed by the method described by Hagan and Hyams (17). The anti-tubulin monoclonal antibody, TAT1 (56), was used as a primary antibody for microtubule staining.

Cloning of the *cut8*$^+$ gene. The *cut8*$^+$ gene was cloned, taking advantage of the fact that the *cut8* mutation has a tight linkage to *nuc2*. An *S. pombe* genomic DNA library was screened by Southern hybridization, using the *nuc2*$^+$ gene as a probe. The *S. pombe* cosmid library used here has a *Saccharomyces cerevisiae LEU2* gene as a selection marker. Cosmid clones were transferred onto a nitrocellulose membrane and probed with a 2.2-kb *Pst*I fragment of pNC106 (22) encompassing the *nuc2*$^+$ gene. Hybridization was performed under high-stringency conditions (65°C in 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–0.3% sodium dodecyl sulfate [SDS], followed by a wash in 2× SSC–0.5% SDS and three successive washes in 0.2× SSC–0.5% SDS). Three of 5,000 clones were positive. The positive clones were used for transformation of the *cut8* mutant by the lithium acetate method (25).

Mutation site determination. The mutant gene was amplified by PCR from the mutant genomic DNA sequence. The PCR primers used were 5′-GTCCGACTAGTGGTGCC-3′ and 5′-TTGCTAGCTTTCATAAG-3′. The resulting PCR product was used as a template for the asymmetric PCR that was carried out with one primer reduced to a 1/100 concentration of the other, in order to generate templates for subsequent dideoxy sequencing.

Preparation of antibodies. Plasmid pCUT8-23 was made for expression of the *cut8*$^+$ gene product in bacteria. An *Nde*I site was introduced at the initiation methionine codon. The 1.0-kb *Nde*I-*Bgl*II fragment that contained the entire coding region of *cut8*$^+$ was ligated with the *Nde*I and *Bam*HI sites of pAR3040 (47), which contained the bacteriophage T7 gene 10 promoter, an *Nde*I, site and the terminator for T7 RNA polymerase. The resulting plasmid, designated pCUT8-23, should produce the *cut8*$^+$ gene product, a 262-amino-acid polypeptide. The plasmid was introduced in *E. coli* BL21(DE3), which contained the T7 RNA polymerase gene under the control of the *lac* UV5 promoter. For purification of the fusion protein, the procedures described by Marston et al. (31) were followed. *E. coli* BL21 (DE3) harboring pCUT8-23 was grown in LB medium containing ampicillin (100 μg/ml), induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and incubated for 2 to 4 h. Cells were pelleted and lysed. The cell lysate was fractionated, and the inclusion body was purified. The inclusion body was subjected to SDS gel electrophoresis. The gel band containing the 33-kDa cut8 polypeptide was excised, and the polypeptide was electroeluted from the gel. Rabbits were immunized by a procedure described elsewhere (20). Antisera were affinity purified with the antigen immobilized on a nitrocellulose filter (45). 12CA5, an anti-hemagglutinin antigen (HA) tag monoclonal antibody was purchased (BAbCO, Berkeley, Calif.) (11). *S. pombe* cell extracts were prepared as described by Hirano et al. (22). Briefly, cells were broken by glass beads in buffer (20 mM Tris [pH 7.5], 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride), and then cell debris was removed by centrifugation at 5,000 rpm for 5 min. The whole-cell extract thus prepared was run on SDS-polyacrylamide gel electrophoresis (PAGE) and then electrophoretically transferred to a nitrocellulose filter. The filter was then used for immunoblotting (51).

Cloning of multicopy suppressor genes. A genomic *S. pombe* library was constructed in pDB248. *Sau3A*I partial digests of the genomic DNA (average length, 10 kb) were ligated with a shuttle vector, pDB248, which contains pBR322, the *S. cerevisiae LEU2* gene, and 2μm DNA (4,5). This genomic DNA library was used for transformation of a *cut8* mutant strain by the lithium acetate method (25). A Leu$^+$ Ts$^+$ transformant was obtained, and the Leu$^+$ marker cosegregated with Ts$^+$. A plasmid (designated pS81) was recovered from the transformant.

Mapping of the *cek1*$^+$ gene. Chromosomal location of the *cek1*$^+$ gene was determined by a physical mapping method. Southern blot analysis with an ordered cosmid library (35) was probed with a segment of pS81. pS81 was mapped on a contig on chromosome III nearby *ade6*.

FACS. Fluorescence-activated cell sorter (FACS) analysis was performed by the method of Costello et al. (8). Cells fixed by ethanol were treated with RNase prior to being stained with propidium iodine. Fluorescence was measured by a Beckton Dickinson FACScan apparatus.

Gene disruption of *cek1*$^+$. The 4-kb *Bgl*II fragment from *cek1*$^+$ DNA was replaced with the 1.8-kb *ura4*$^+$ gene, and a 4.8-kb fragment from this construct was used to transform a haploid uracil-auxotrophic strain. Eleven transformants screened had a stable Ura$^+$ phenotype, indicating that the disrupted gene had been stably integrated. All the strains analyzed by Southern hybridization were found to carry the disrupted *cek1* gene.

Nucleotide sequence accession number. The nucleotide sequences of the insert in pCUT8-15 and the *cek1*$^+$ gene product have been submitted to the EMBL database under accession numbers D31772 and D31773, respectively.

RESULTS

Cell inviability of *cut8-563*. The *cut8-563* mutant was inviable at 36°C, while small and normal-size colonies, respectively,

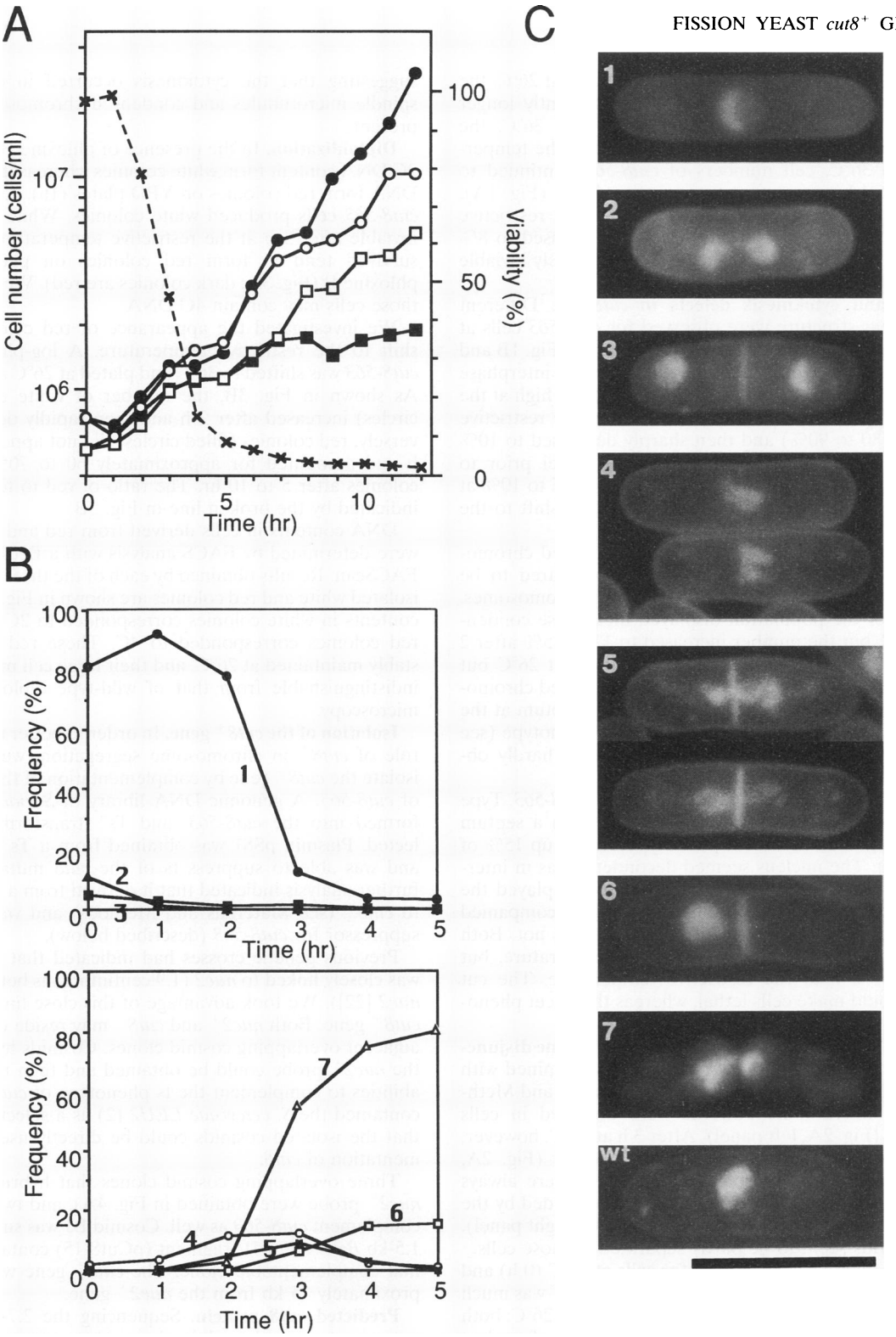


FIG. 1. *cut8-563* mutant phenotype. (A) Cell number increase at 36°C. *cut8-563* and wild-type cells were grown in YPD medium at 26°C, and then a portion was transferred to 36°C and the cell number of the cultures was measured at 1-h intervals. Cells producing the cut phenotype were counted as two. Open squares, *cut8-563* at 26°C; filled squares, *cut8-563* at 36°C; open circles, wild type at 26°C; filled circles, wild type at 36°C. To estimate the viable cell number after the shift to 36°C, the cultures were plated at 26°C and the numbers of colonies were counted (broken line). (B and C) Time course appearance of different cell types after temperature shift to 36°C. *cut8-563* cells were first grown at 26°C and then shifted to 36°C. Aliquots of the cultures were taken at 1-h intervals and were then DAPI stained. Seven different types of cells (C, numbers 1 to 7) were scored: 1, interphase cells containing a single nucleus (filled circles in panel B); 2, mitotic cells containing two nuclei (filled triangles in panel B); 3, postanaphase cells containing two nuclei and septum (filled squares in panel B); 4, mitotic cells containing condensed chromosomes (open circles in panel B); 5, cells containing condensed (often extended) chromosomes with septum (crosses in panel B); 6, cells containing a single decondensed nucleus with septum (open squares in panel B); 7, cells showing the cut phenotype (open triangles in panel B); wt, wild-type cell with condensed chromosomes. Bar, 10 μm.

formed at 30 and 26°C. In rich YPD liquid culture at 26°C, the generation time of *cut8-563* (3.5 h) was significantly longer than that of the wild type (2.5 h) (Fig. 1A). At 36°C, the generation time of the wild type was 2.1 h. After the temperature shift to 36°C, cell numbers of *cut8-563* continued to increase up to 4 h, and then cells ceased to divide (Fig. 1A); cells divided once or twice after the shift to the restrictive temperature. Correspondingly, cell viability decreased to 8% by 5 h. These results suggested that cells were largely inviable after the second division.

Anaphase and cytokinesis defects in *cut8-563*. Different types of nuclear structure were observed for *cut8-563* cells at 36°C in the liquid YPD cultures by DAPI staining (Fig. 1B and C). The frequency of type 1 cells containing a single interphase nucleus (Fig. 1B, curve 1, and Fig. 1C, panel 1) was high at the permissive temperature and for the first 2 h at the restrictive temperature (80 to 90%) and then sharply decreased to 10% after 3 h. The type 2 cells containing two nuclei prior to septation and the type 3 cells after septation were 5 to 10% at 26°C and then dropped to 1 to 4% 1 h after the shift to the restrictive temperature.

Two other type of cells (4 and 5) with condensed chromosomes were seen. Chromosomes in type 4 appeared to be overcondensed, reminiscent of the metaphase chromosomes. Two percent of the population displayed metaphase condensation at 26°C, but the number increased to 12 to 15% after 2 to 3 h at 36°C. Type 5 cells were not observed at 26°C but increased to 10% after 3 h at 36°C. These condensed chromosomes were often extended and bisected by the septum at the middle. These structures may precede the cut phenotype (see below). Normal anaphase structures hence were hardly observed in *cut8-563* at 36°C after 2 h.

Types 6 and 7 cells accumulated terminally in *cut8-563*. Type 6 cells, containing a single displaced nucleus with a septum (hereafter designated the uncut phenotype), made up 15% of the population. The nucleus seemed decondensed as in interphase nuclei. Eighty percent of the population displayed the cut phenotype (type 7). The cut phenotype was accompanied by cell division, whereas the uncut phenotype was not. Both types were not observed at the permissive temperature, but appeared after 2 h at the restrictive temperature. The cut phenotype should make cells lethal, whereas the uncut phenotype might not.

Short spindle formation and failure of chromosome disjunction. Microtubule distribution in *cut8-563* was examined with monoclonal anti-tubulin antibody TAT1 (Materials and Methods). Normal microtubule patterns were observed in cells grown at 26°C (Fig. 2A, left panel). After 3 h at 36°C, however, more cells displayed the short metaphase spindle (Fig. 2A, upper right panel). Chromosomes in such cells were always condensed. In some cells, chromosomes were extended by the spindle but clearly fail to separate (Fig. 2A, lower right panel). Sister chromatids seem to be partly separated in those cells.

The spindle length was measured for cells at 26°C (0 h) and at 36°C for 3 h (Fig. 2B). The average length at 36°C was much shorter (3.5 ± 1.7 μm) than those (4.5 ± 2.4 μm) at 26°C; both short metaphase and long anaphase spindles were found at 26°C, whereas short metaphase spindle predominated at 36°C. These results demonstrated that the mitotic spindle was formed in *cut8-563* but was not fully extended. Whether the failure in anaphase progression was due to the defect in spindle elongation or in the high frequency of condensed chromosomes or both was not determined. Furthermore, cells displaying the X-shape postanaphase microtubule arrays, which were seen prior to normal cytokinesis (indicated by the arrow in Fig. 2A) (17), were infrequent in the *cut8-563* mutant at 36°C, suggesting that the cytokinesis occurred in *cut8-563* while spindle microtubules and condensed chromosomes were still present.

Diploidization. In the presence of phloxine B, haploids with 2C DNA content form white colonies whereas diploids with 4C DNA form red colonies on YPD plates (16). At 26°C, haploid *cut8-563* cells produced white colonies. While most cells are inviable after 5 h at the restrictive temperature, the few that survived tend to form red colonies on plates containing phloxine B (Fig. 3A; dark colonies are red). We suspected that those cells may contain 4C DNA.

We investigated the appearance of red colonies after the shift to the restrictive temperature. A log-phase culture of *cut8-563* was shifted to 36°C and plated at 26°C at 1-h intervals. As shown in Fig. 3B, the number of white colonies (open circles) increased after 2 h and then rapidly decreased. Conversely, red colonies (filled circles) did not appear until after 3 h and accounted for approximately 60 to 70% of the total colonies after 5 to 10 hr. The ratio of red to total colonies is indicated by the broken line in Fig. 3B.

DNA contents in cells derived from red and white colonies were determined by FACS analysis with a Beckton-Dickinson FACScan. Results obtained by each of the three independently isolated white and red colonies are shown in Fig. 3C. The DNA contents in white colonies corresponded to 2C while those in red colonies corresponded to 4C. These red colonies were stably maintained at 26°C, and their large cell morphology was indistinguishable from that of wild-type diploid under light microscopy.

Isolation of the *cut8*$^+$ gene. In order to better understand the role of *cut8*$^+$ in chromosome segregation, we attempted to isolate the *cut8*$^+$ gene by complementation of the ts phenotype of *cut8-563*. A genomic DNA library of *S. pombe* was transformed into the *cut8-563*, and Ts$^+$ transformants were selected. Plasmid pS81 was obtained from a Ts$^+$ transformant and was able to suppress ts of the *cut8* mutants. However, further analysis indicated that it derived from a locus unlinked to *cut8*$^+$ (see Materials and Methods) and was a multicopy suppressor for *cut8-563* (described below).

Previous genetic crosses had indicated that the *cut8* locus was closely linked to *nuc2* (1.9 centimorgans between *cut8* and *nuc2* [22]). We took advantage of this close linkage to isolate *cut8*$^+$ gene. Both *nuc2*$^+$ and *cut8*$^+$ may reside on the same or adjacent overlapping cosmid clones. Cosmids hybridizing with the *nuc2*$^+$ probe could be obtained and then tested for their abilities to complement the ts phenotype of *cut8*. The cosmid contained the *S. cerevisiae LEU2* (2) as a selection marker so that the isolated cosmids could be directly used for complementation of *cut8*.

Three overlapping cosmid clones that hybridized with the *nuc2*$^+$ probe were obtained in Fig. 4A), and two were able to complement *cut8-563* as well. Cosmid B1 was subcloned, and a 1.5-kb *Bgl*II-*Hin*dIII fragment (pCut8-15) contained the minimal complementable clone. The *cut8*$^+$ gene was located approximately 15 kb from the *nuc2*$^+$ gene.

Predicted cut8 protein. Sequencing the 2.7-kb *Bgl*II fragment containing the minimal complementary clone revealed an open reading frame (ORF) of 786 bp (262 amino acids; calculated molecular weight, 30,260 [Fig. 4B]). No other reading frame was present. A search in the EMBL and GenBank databases did not yield any known protein with significant homology. A weak similarity to the sequence of the *S. cerevisiae DBF8* gene product, however, was found (12a, 24). The *dbf8* mutant is one of the cell division cycle mutants previously identified (DBF stands for dumbbell former [27]). Twenty-five percent of the residues were identical over 208

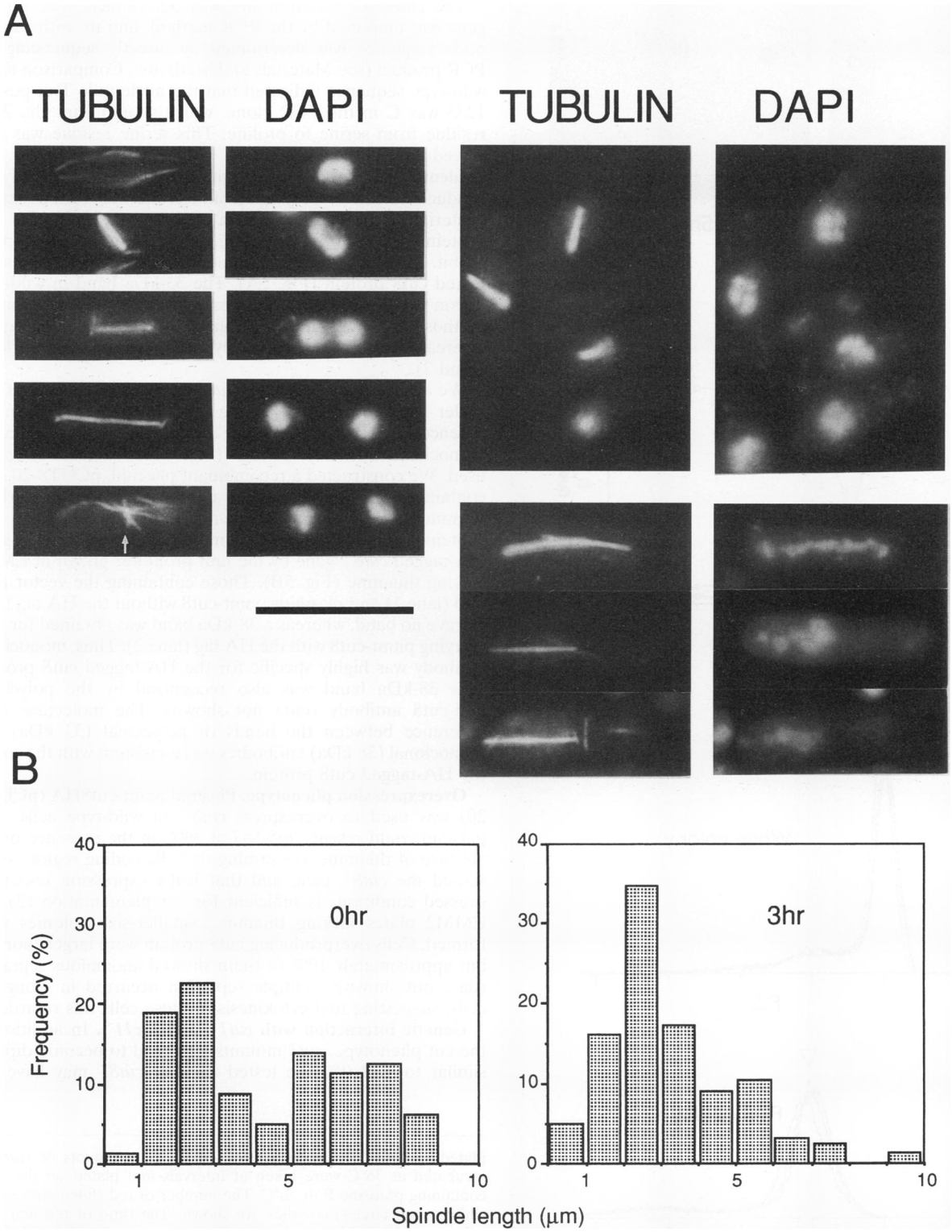


FIG. 2. Short spindle formed in *cut8-563* at 36°C. (A) *cut8-563* cells were grown exponentially at 26°C and then incubated at 36°C for 3 h. Cells were collected, fixed, and stained by monoclonal anti-tubulin antibody TAT1 (56). DAPI was used for DNA staining. Left panels, *cut8-563* cells before the shift to 36°C (from top to bottom): interphase cell, mitotic cell with the short spindle, anaphase cell with elongating spindle, anaphase cell with fully extended spindle, and postanaphase cells with the X-shape microtubule array. Right panels, *cut8-563* cells at 36°C for 3 h. Top, cells with the short spindle and condensed chromosomes: bottom, cells with the elongated spindle and the extended chromosomes. Bar, 10 μm. (B) Spindle size distribution in *cut8-563* cells at 36°C. Spindle length of *cut8-563* cells incubated at 36°C for 0 and 3 h was measured.

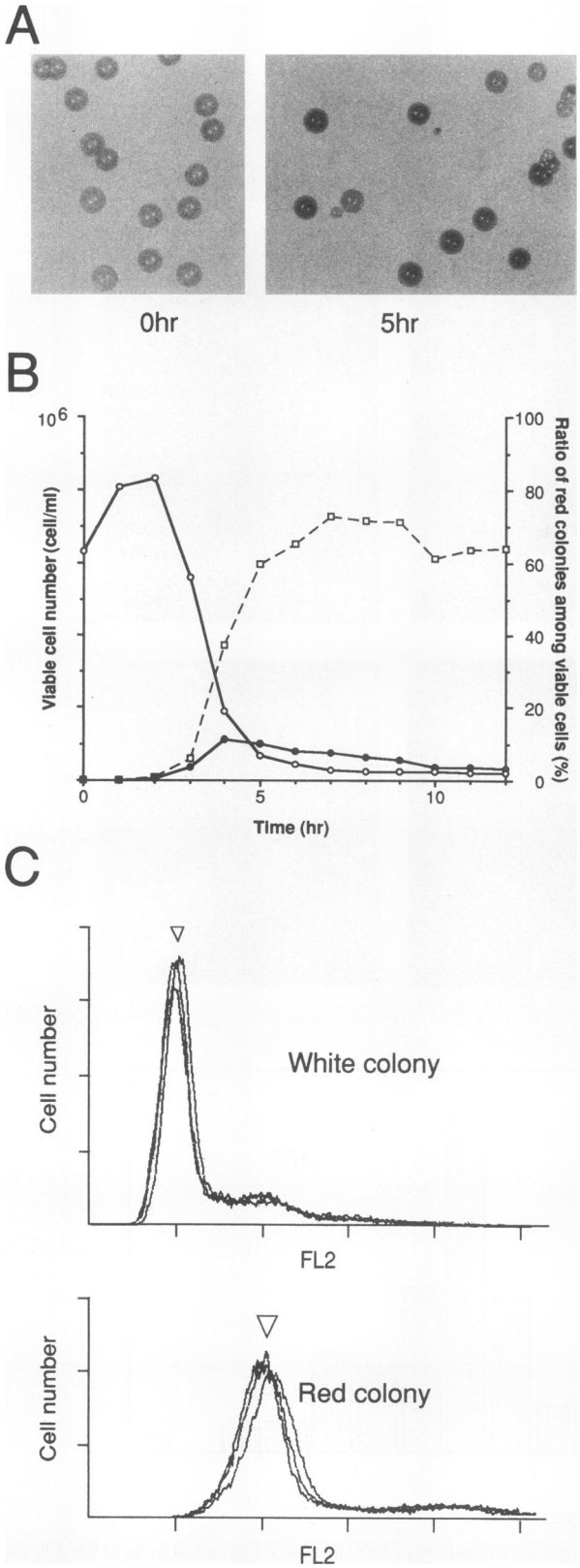


FIG. 3. Diploidization of *cut8-563* at 36°C. (A) Cells of *cut8-563* grown at 26°C were incubated at 36°C for 5 h and then plated at 26°C on the rich YPD medium containing phloxine B. Red colonies were produced (5 h), whereas no red colony was formed if the cells were plated without incubation at 36°C (0 h). (B) Aliquots of *cut8-563* incubated at 36°C were taken at intervals and plated on the YPD containing phloxine B at 26°C. The number of red (filled circles) and white (open circles) colonies are shown. The ratio of red and total colonies indicated by the broken line increased after 3 h. (C) DNA contents of cells derived from each of the three red and white colonies were estimated by FACScan. The DNA contents of red colonies were identical to that of diploid cells. The peak positions for the wild-type haploids and diploids are indicated by the small and large arrowheads, respectively. Units for cell number and FL2 (fluorescent light intensity to DNA content) are arbitrary.

amino acids (Fig. 4C). Similarity was highest in the amino half of the protein.

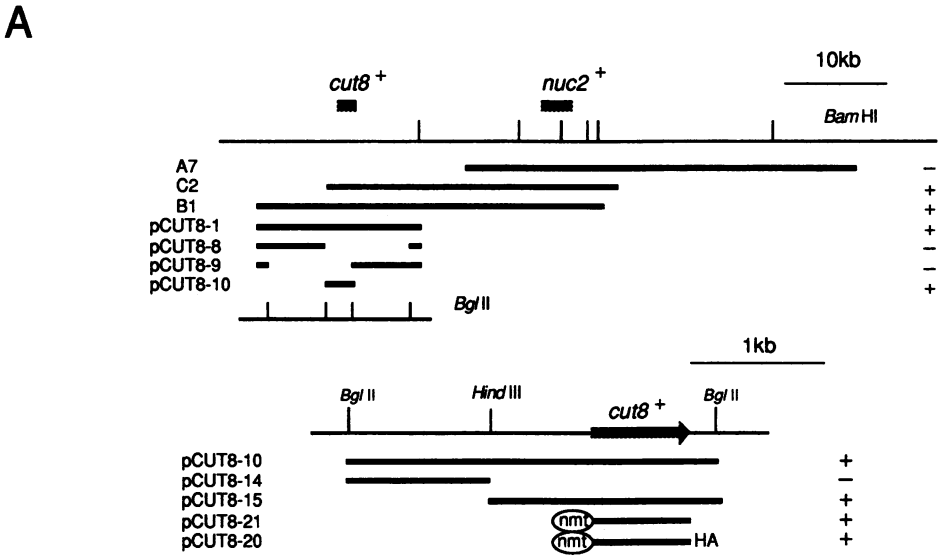
The *cut8-563* mutation site was determined. The mutant gene was amplified by the PCR method, and its entire nucleotide sequence was determined by directly sequencing the PCR product (see Materials and Methods). Comparison to the wild-type sequence indicated that the nucleotide T at postion 1283 was C in the PCR clone, which should alter the 201st residue from serine to proline. This serine residue was conserved in the DBF8 protein.

Identification of p33^{cut8} by immunoblotting. To identify the product of *cut8*$^+$, polyclonal antiserum was raised against bacterially expressed full-length cut8 protein. A 33-kDa cut8 protein was produced in *E. coli*, purified, and injected into a rabbit. Immunoblot with polyclonal anti-cut8 antibodies detected cut8 protein (Fig. 5A). The 33-kDa band in wild-type fission yeast cells (lane 3) increased the band intensity (lane 4) in those carrying multicopy plasmids with the *cut8*$^+$ gene, whereas it was not recognized by the preimmune serum (lanes 1 and 2).

We also overexpressed the *cut8*$^+$ gene by placing cut8 ORF under the control of *S. pombe* promoter nmt1 (33). In the absence of thiamine, the *cut8*$^+$ gene was overexpressed. A monoclonal antibody, 12CA5 (11), which detects HA, was used. We constructed a recombinant plasmid, pCUT8-20, that contained the *cut8*$^+$ gene and a HA tag (52) at the carboxy terminus. pCUT8-20 rescued *cut8-563* so that the tagged cut8 protein was functional. We examined cells overexpressing the HA-tagged *cut8*$^+$ gene by the nmt promoter grown in EMM2 lacking thiamine (Fig. 5B). Those containing the vector plasmid (lane 1) and plasmids pnmt-cut8 without the HA tag (lane 3) gave no band, whereas a 38-kDa band was obtained for cells carrying pnmt-cut8 with the HA tag (lane 2). Thus, monoclonal antibody was highly specific for the HA-tagged cut8 protein. This 38-kDa band was also recognized by the polyclonal anti-cut8 antibody (data not shown). The molecular mass difference between the bands of polyclonal (33 kDa) and monoclonal (38 kDa) antibodies was consistent with the size of the HA-tagged cut8 protein.

Overexpression phenotype. Plasmid pnmt-cut8HA (pCUT8-20) was used to overexpress *cut8*$^+$ in wild-type cells. This plasmid could rescue *cut8-563* at 36°C in the presence or the absence of thiamine, confirming that the coding region represented the *cut8*$^+$ gene and that leaky expression under repressed conditions is sufficient for complementation (3). On EMM2 plates lacking thiamine, smaller-size colonies were formed. Cells overproducing cut8 protein were largely normal, but approximately 10% of them showed anomalous septation (data not shown); multiple septation occurred in elongated cells, suggesting that cytokinesis in those cells was retarded.

Genetic interaction with *cut1*$^+$ and *cdc11*$^+$. In addition to the cut phenotype, *cut1* mutants also tend to become diploid, similar to *cut8* (9). We tested whether *cut8*$^+$ may have any



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HindIII 10        20        30        40        50        60        70        80        90        100       110       120
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 A  D  F  D  E  D  I  S  K  R  A  R  Q  L  P  V  G  E  Q  L  P  L  S  R  L  L  Q  Y  S  D  K  Q  Q  L  F  T  I  L  L  Q    53

ATGTGTAGAAAAGCATCCTGATCTCGCACGTGATATACGGGGCATATTACCGGCGCCGTCCATGGATACTTGTGTTGAAACGTTGCGTAAACTTTTAATTAACTTGAATGACTCATTTCC   960
 C  V  E  K  H  P  D  L  A  R  D  I  R  G  I  L  P  A  P  S  M  D  T  C  V  E  T  L  R  K  L  L  I  N  L  N  D  S  F  P    93
                                Eco RI
CTATGGTGGTGACAAGAGGGGTGATTACGCATTTAATAGAATTCGTGAAAAGTACATGGCTGTCTTACATGCGCTTAATGATATGGTTCCTTGTTATTTACCTCCATACTCTACTTGTTT  1080
 Y  G  G  D  K  R  G  D  Y  A  F  N  R  I  R  E  K  Y  M  A  V  L  H  A  L  N  D  M  V  P  C  Y  L  P  P  Y  S  T  C  F   133
                                                   Eco RI
TGAGAAAAATATTACTTTTCTTGATGCCGCTACAAACGTTGTCCACGAACTGCCAGAATTCCATAATCCCAACCATAATGTTTATAAAAGTCAAGCGTACTATGAATTAACGGGCGCGTG  1200
 E  K  N  I  T  F  L  D  A  A  T  N  V  V  H  E  L  P  E  F  H  N  P  N  H  N  V  Y  K  S  Q  A  Y  Y  E  L  T  G  A  W   173

GCTTGTTGTTTTAAGACAACTTGAGGATCGGCCTGTAGTCCCTTTACTACCTTTGGAAGAGTTGGAAGAGCACAATAAAACATCACAAAACAGAATGGAAGAGGCATTAAATTACCTTAA  1320
 L  V  V  L  R  Q  L  E  D  R  P  V  V  P  L  L  P  L  E  E  L  E  E  H  N  K  T  S  Q  N  R  M  E  E  A  L  N  Y  L  K   213

GCAATTACAAAAAAATGAGCCACTTGTACATGAAAGAAGTCATACTTTTCAACAAACAAATCCACAAAACAATTTTCATCGTCATACTAATAGTATGAACATTGGGAATGACAATGGAAT  1440
 Q  L  Q  K  N  E  P  L  V  H  E  R  S  H  T  F  Q  Q  T  N  P  Q  N  N  F  H  R  H  T  N  S  M  N  I  G  N  D  N  G  M   253

GGGTTGGCACAGTATGCATCAATATATTTAAAAACTAGAACAGAAGGCCTATAAGGAAACATATAATGGCTAATAGGAAAGCGGTTTGCACAGTAACTGTGTTATGAATTTGAGGAACAA  1560
 G  W  H  S  M  H  Q  Y  I  *                                                                                                 262

CCTTGGCACCACTAGTCGGACGCCCAAAAGACGATGAAACGCGTGCAAGTCCTATTTGATTTCCACTGTTTCCAGCGAACATAGATGGACTCGTAGCTGTTGATTGTCTTGTGTTATTAA  1680
TTACATTAGCGGTAGATCT                                                                                                          1699
          Bgl II
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C

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cut8      1'  METLSYSQIKKRKADFDEDISKRARQLPVGEQLPLSRLLQYSDKQQLFTILLQCVEKHPD
Dbf8     46"  EEEELPRNKNVMKYGGVSKRRPQPGSSIRGQPPPQEGMELMNKNQLQQLLVDLMTKHPE

          61'  LARDI--RGLLPAPSMDTCVETLRKLLINLNDSFPY----GGDKRGDYAFNKIREKYMAV
         106"  IQQSVHTRVLGLDFSIQKGLDMLKQKSEAVYQSIPYMRSYESNKLDDYAEVEMKPQILEF

         115'  LHANDMVPCYLPPYSTCFEKNITPLCAAINVVHELPEHNPNPNNVYKSQAYYELTGAWL
         166"  NNCEVDFILDNIPRRLENLHASLKPICHELVIKLRREELASNVYYDKCIEQLSHVWC

         175'  VVLRQLEDRPVVP---------LLPLEEEEEHNKTSQNRMEEALNYLKQLQKNEPLVHERS
         226"  TLIEHVARDRIILLADNSSVWKSHMTRLQVYNEPSNGLLRRPLQLFKSLDMGSPSAASSS

         227'  HTFQQTNPQNNFHRHTNSMNIGNDNGMGWHSMHQYI
         286"  TLSLQESIIYHHDTMTANENNNNSGSAATDSPF
```

FIG. 4. Isolation and nucleotide sequencing of the *cut8*$^+$ gene. (A) Three cosmids, A7, C2, and B1, hybridized with the *nuc2*$^+$ probe, were used for complementation of the *cut8-563* mutation. Two cosmids, C2 and B1, complemented *cut8-563* (indicated by +). Subsequent subcloning yielded plasmids pCUT8-1 and pCUT8-15 which complemented *cut8-563*. pCUT8-20 and pCUT8-21 were constructed by ligating the putative initiation codon with the *S. pombe* inducible promoter of *nmt1*. The termination codon of *cut8*$^+$ in pCUT8-20 was ligated with HA. Both plasmids complemented *cut8-563* in the presence or the absence of thiamine. (B) Nucleotide sequence of the insert in pCUT8-15 was determined. A 786-bp ORF coding for 262 amino acids is shown. Some restriction sites are indicated. (C) Comparison of the cut8 amino acid sequence with that of budding yeast Dbf8 (24). Identical amino acids are designated with boxes. The budding yeast Dbf8 sequence was kindly provided by C. Holm.

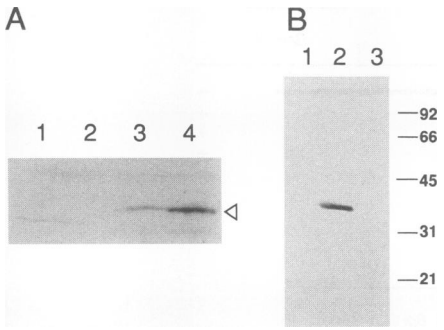

FIG. 5. Identification of *cut8*$^+$ gene product. (A) Extracts of *S. pombe* wild-type cells carrying the vector plasmid (lanes 1 and 3) or the multicopy plasmid with the *cut8*$^+$ gene (lanes 2 and 4) were prepared, run in SDS-PAGE, and immunoblotted by preimmune serum (lanes 1 and 2) or affinity-purified polyclonal anti-cut8 antibodies (lanes 3 and 4). The 33-kDa band which increased its band intensity by the multicopy plasmid is indicated by the arrowhead. The bands above and below the 33-kDa protein are cross-reacting bands. (B) Extracts of *S. pombe* wild-type cells carrying the vector plasmid (lane 1), plasmid with the HA-tagged *cut8*$^+$ gene (lane 2), and plasmid with the *cut8*$^+$ gene without the tag (lane 3) were prepared, run in SDS-PAGE, and immunoblotted with monoclonal antibody against the HA. The 38-kDa band was obtained in lane 2. No band was detected in the other two extracts.

interactions with *cut1*$^+$ or other *cut*$^+$ genes. The previously isolated *cut*$^+$ genes were used for transformation of *cut8-563*. Among the five *cut*$^+$ genes examined (*cut1*$^+$, *cut2*$^+$, *cut5*$^+$, *cut7*$^+$, and *cut9*$^+$), only *cut1*$^+$ was able to suppress *cut8-563*. Since the cloned *cut8*$^+$ gene could not rescue any *cut1* mutant alleles, suppression was not bilateral. The phenotype of *cut1* mutant is partly similar to that of *cut8-563* in regard to spindle formation and elongation (54).

In the presence of the *cdc11* mutation which blocks septum formation, *cut1* mutants undergo rounds of spindle pole body duplication and DNA replication, producing giant cells with polyploid nuclei and multiple spindle pole bodies (9, 54). To examine whether *cut8* mutant cells exhibit a similar phenotype, the double mutant *cut8-cdc11* was constructed by genetic crosses. Unexpectedly, septation and cytokinesis took place in the double mutant, producing the cut phenotype (data not shown); the phenotype of *cut8-563* was epistatic to that of *cdc11*. Although it was not understood how the cut phenotype of the double mutant *cut8-cdc11* was produced, the modes of interactions between *cut1*$^+$ and *cdc11*$^+$ and between *cut8*$^+$ with *cdc11*$^+$ were clearly different.

Novel protein kinase cek1 as a multicopy suppressor. Multicopy plasmid pS81 rescued *cut8-563* mutation. Subcloning revealed that the 4.5-kb *Xba*I-*Bgl*II fragment was able to suppress the ts phenotype of *cut8* (Fig. 6A). Its entire nucleotide sequence was determined, and a large ORF coding for a 1,309-amino-acid protein was found (Fig. 6B).

The central domain of the ORF was similar to a number of protein kinases. This suppressor gene was designated *cek1*$^+$ (cut eight suppressing kinase). Eleven kinase domains are conserved (Fig. 6C). No kinase showed strong similarity to the kinase domain of cek1. The highest similarity (30 to 40%) in the kinase region was found for protein kinases C and A. The kinase consensus sequence spans from residue 585 to 987, with an interruption of a 112-amino-acid intervening sequence between domains VII and VIII. A similarly positioned insert was described for the *S. cerevisiae* CDC7 kinase (19), but overall similarity between cek1 and CDC7 was low. The amino acid sequence outside the kinase domain was not similar to those of any known proteins. Two hydrophobic regions were found in the amino domain (indicated by arrowheads in Fig. 6D).

cek1 kinase is unusual not only because of its large size but also of its potential role in exiting mitosis. In this regard, cek1 is similar to dsk1 kinase (48). Sequence similarity between the two kinases, however, was not high. Interestingly, both kinases contained the insertions in the kinase coding regions. The insertion sequence of dsk1 protein signals cell cycle-dependent nuclear localization (48).

***cek1*$^+$ is nonessential for viability.** The *cek1* null strain was constructed by one-step gene disruption (40). Almost all of the coding region was replaced by the *ura4*$^+$ gene (see Materials and Methods). Plasmid pS820 with the *ura4*$^+$ replacement was linearized, transformed into a Ura$^-$ haploid, and selected on medium lacking uracil. Subsequent genomic Southern analysis reveals that *ura4*$^+$ replaces *cek1*$^+$ and *ura*$^+$ segregates as a single gene. Haploid cells lacking the *cek1*$^+$ gene produced normal colonies at 22, 26, 30, 33, and 36°C. Hence, *cek1*$^+$ is nonessential for viability.

DISCUSSION

In this paper, we describe the characterization of the *cut8-563* mutant phenotype and identification of the *cut8*$^+$ and suppressor *cek1*$^+$ genes. Although there are approximately 20 genetic loci that generate the cut phenotype (21, 43, 53), the *cut8-563* phenotype is unique with regard to the behavior of chromosomes and the spindle at the restrictive temperature. The predicted amino acid sequences of *cut8*$^+$ and *cek1*$^+$ do not resemble any known *cut*$^+$ gene products. cek1 is the first protein kinase related to the function of *cut*$^+$ genes. The product of *cut8*$^+$ is similar to that of the budding yeast *DBF8* gene, which is essential for viability (24). Temperature sensitivity *dbf8* mutations cause the increased rates of chromosome loss and nondisjunction even at the permissive temperature.

A cellular defect initially recognized in the *cut8-563* mutant after the shift to 36°C was the formation of overcondensed chromosomes and short spindles. In some cells, chromosomes were abnormally extended by the spindle which did not fully elongate. This anaphase defect occurred 2 to 3 h after the shift to the restrictive temperature, when most cells had divided once. The full inactivation of the *cut8-563* protein may require the duration of a certain time at 36°C. Alternatively, cells which reside mostly in G_2 may have to pass through the G_1/S phase at 36°C to produce the mutant phenotype, which leads to the 2-h delay. Analysis with a synchronous culture as well as the release from the G_1 arrest by nitrogen starvation will be necessary to determine the likelihood of either of these possibilities.

In the budding yeast *dbf8* mutant, cells are arrested with a short spindle (24). Both *cut8* and *dbf8* mutants are defective in sister chromatid separation and accompany a short spindle. The carboxy domain of the Dbf8 protein is essential at the restrictive temperature. This is consistent with our finding that the putative mutation site of *cut8-563* resides in the carboxy-terminal domain. Minichromosomes introduced to *cut8-563* were not unstable. The difference from *dbf8* mutants which lose minichromosomes at high loss rates is possibly due to the allele-specific phenotype of *cut8-563*. The *cut8*$^+$ gene may not be a functional homolog of the budding yeast *DBF8* gene, but they are probably functionally similar proteins belonging to the same gene family.

It remained to be determined whether the *cut8*$^+$ gene was essential for viability. The phenotype of the *cut8-563* mutant

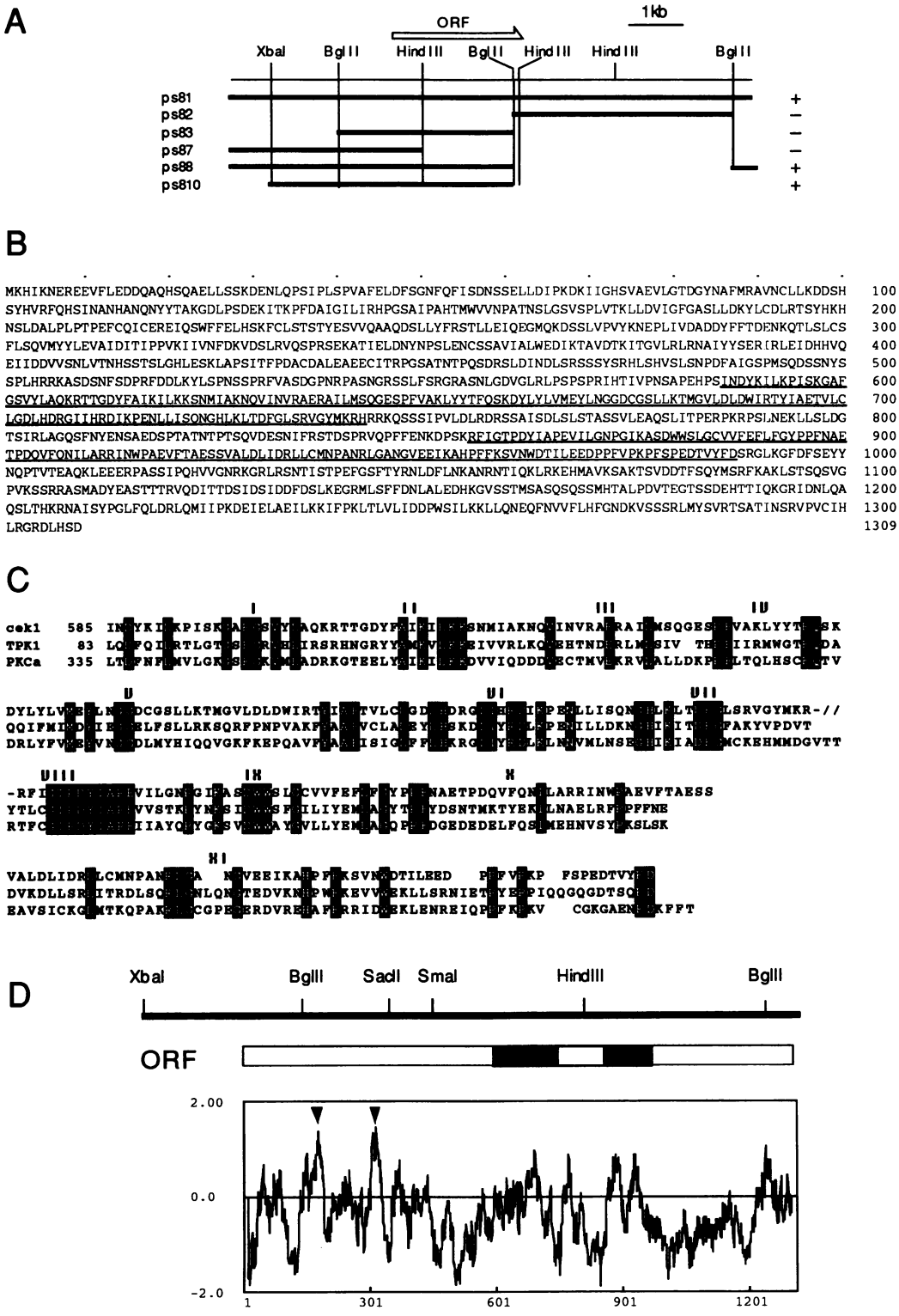

FIG. 6. Isolation and nucleotide sequencing of the *cek1*$^+$ gene. (A) Subcloning of the *cek1*$^+$ gene. +, suppression of *cut8-563*. The coding region and its direction of transcription are indicated by the arrow. Some restriction sites are indicated. (B) Predicted amino acid sequences of the *cek1*$^+$ gene product. The coding region contains 1,309 amino acids. (C) Comparison of the cek1 kinase domain with those of other kinases, TPK1 (7, 30, 49) and protein kinase Ca (PKCa) (39). Identical amino acids are boxed. Eleven kinase domains are numbered. (D) Schematic representation of cek1 protein with the hydropathy plot. Hydropathy was calculated by the method of Kyte and Doolittle (28). The arrowheads indicate the hydrophobic stretches. The hatched regions represent the kinase domains.

hence has to be cautiously interpreted since the phenotype of the *cut8* null is not known. Gene disruption by two different constructs has not been successful. The *S. cerevisiae DBF8* gene is an essential gene for viability (24). The recessive *cut8-563* mutation (21) causes the decrease in cell viability at the restrictive temperature. This result suggests that aberrant anaphase or subsequent cytokinesis as a result of the loss of *cut8*$^+$ would be lethal. Not all the cells became inviable, however; approximately 10% of cells were still viable after 5 h at 36°C. We suspected that the viable cells represented the uncut phenotype containing the single nucleus with the septum. Interestingly, many of these viable cells were diploid and were stably maintained and homozygous for *cut8-563*. We hypothesize that these cells displaced nuclei resulting from a missorting of chromosomes to one end of the spindle when only one nucleus receive all the chromosomes. Thus, the cells contain twice the amount of DNA. Fission yeast diploidization mutants *dip* were isolated by a procedure with a brief heat shock (6), and some were allelic to *cut1* and *cut7* (43). Hence, these *cut*$^+$ genes may be required for the stable maintenance of haploid cells. Improper diploidization and the appearance of the cut phenotype could have been brought about by a common defect.

The phenotypes of *cut8-563* resemble those of budding yeast mutants *ctf13* and *ctf14* (identical to *ndc10*/*cbf2*), defective in the genes that encode essential components of the kinetochore (10, 13, 26). *ctf13* mutants missegregate chromosomes and transiently arrest during mitosis with a short spindle. Polyploid nuclei are frequently found in the mother cells, probably because of the impaired interaction between chromosomes and microtubules. In *cut8-563* mutant cells, the interaction between condensed mitotic chromosomes and kinetochore microtubules may also be defective.

Since a high dosage of the *cek1*$^+$ gene can overcome the defect in *cut8* mutation, cek1 kinase may affect directly or indirectly *cut8*$^+$ gene function. One of the features of cek1 kinase is its 112-amino-acid intervening sequence between the 7th and 8th kinase domains. This sequence is rich in serine/threonine, proline, and charged amino acids, and the amino acid composition was similar to that found in dsk1 kinase (48). The dsk1 kinase was isolated as a multicopy suppressor for *dis1* mutants which were defective in mitotic anaphase (37) and appears to function as a positive element for mitotic exit (48). dsk1 kinase is activated in metaphase-arrested mutant cells and enriched in the nucleus. The intervening sequence of dsk1 kinase seems to be responsible for cell cycle-dependent nuclear localization. The cek1 sequence may have a similar role. The cut8 protein may be phosphorylated by cek1 kinase, and upon phosphorylation, it might be activated. Alternatively, cut8 might interact with cek1 as a positive modulator. Another possibility is that overproduced cek1 kinase might bypass the *cut8*$^+$ function.

The *cut8*$^+$ gene appears to be required for the normal progression of anaphase. It is apparently also implicated in restraining cytokinesis until a certain stage in anaphase. Multiple septa made in *cut8*$^+$-overexpressed cells might be consistent with a hypothesis that overproduced cut8 protein retarded cytokinesis. The *cut8*$^+$ gene possibly plays a dual role in regulating anaphase and cytokinesis. It might have a role in checkpoint control for the progression of anaphase; postanaphase events are restrained by the *cut8*$^+$ gene until the completion of anaphase. We think, however, that a primary role of the *cut8*$^+$ gene would more likely reside in anaphase progression. We speculate that partial separation of chromosomes in *cut8* mutant cells may be sufficient to trigger cytokinesis. Similar phenotypes were found in *cut1* and *top2* mutants (21, 54). The mode of interaction between *cut8*$^+$ and *cut1*$^+$ and between *cut8*$^+$ and *cek1*$^+$ is the subject of a future investigation.

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