Fission yeast cut5 links nuclear chromatin and M phase regulator in the replication checkpoint control

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Fission yeast temperature-sensitive *cut5* (cell *untimely torn*) mutants are defective in initiation and/or elongation of DNA replication but allow mitosis and cell division at a restrictive temperature. We show that the cut5 protein (identical to rad4) (i) is an essential component of the replication checkpoint system but not the DNA damage checkpoint, and (ii) negatively regulates the activation of M phase kinase at mitotic entry. Even if the replication checkpoint has been activated previously, *cut5* mutations allow mitosis and cell division after shift to 36°C. Transcription of *cut5*$^+$ is not under the control of the START gene *cdc10*$^+$. The cut5 protein is enriched in the nucleus, consisting of repeating domains. An essential domain which resembles the proto-oncoprotein Ect2 has a strong negative effect on the entry into mitosis when overexpressed. Expression of the *cut5* mutant phenotype requires the function of the M phase regulator genes *cdc2*$^+$, *cdc25*$^+$ and *cdc13*$^+$. The cut5 protein forms a novel, essential link between DNA synthesis and M phase activation in the replication checkpoint control pathway.
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Introduction

Events leading to DNA replication and mitosis are ordered in the normal eukaryotic cell cycle. Replication of chromosomal DNA in S phase is followed, after traverse of the G_2 phase, by the M phase, which precedes cell division. In the fission yeast vegetative cell cycle the G_2 phase is long, whereas the G_1 phase is very short. Cell size increases during G_2. Fission yeast mutations called *cut* (cell untimely torn; Hirano *et al.*, 1986) disrupt coordination between M phase and cytokinesis, and cell division takes place in the absence of normal nuclear division. There are ~20 *cut*$^+$ genes known (Hirano *et al.*, 1986; Samejima *et al.*, 1993). DNA synthesis is not inhibited in any *cut* mutants except *cut5* (Saka and Yanagida, 1993).

Several explanations are possible as to why the major cell cycle events are not coordinated in *cut* mutants. A checkpoint control system (Hartwell and Weinert, 1989) or the feedback control (Murray, 1992) may be defective in some *cut* mutants; the *cut*$^+$ gene products could be components of a checkpoint control system. Alternatively, the loss of *cut*$^+$ gene functions may not be detected by the system so that an uncoupled cell cycle takes place. Another possibility is that the *cut* mutant phenotype is produced after mutant cells have passed a stage that commits for cell division (Hirano *et al.*, 1986); separation of small portions of the nuclear chromatin by the spindle in *top2* or certain *cut* mutant cells (Funabiki *et al.*, 1993) is apparently sufficient for triggering cytokinesis.

In *cut5* mutants, DNA synthesis is blocked whereas mitosis and cell division occur (Saka and Yanagida, 1993). The *cut5*$^+$ gene was thus postulated to be required for DNA synthesis and the restraint of mitosis until completion of the S phase. Gene cloning and sequencing indicated that the *cut5*$^+$ gene was identical to *rad4*$^+$ (Fenech *et al.*, 1991). Consistent with this, *cut5* mutants are sensitive to UV light and X-rays at the permissive temperature (Saka and Yanagida, 1993).

Phenotypes highly similar to that of *cut5* mutants at the restrictive temperature were reported recently for null mutants of the fission yeast *cdc18*$^+$ and *cdt1*$^+$ genes, both of which are essential for cell viability (Kelly *et al.*, 1993; Hofmann and Beach, 1994). Null mutations in these genes are defective in DNA replication but allow mitotic events. However, temperature-sensitive *cdc18* mutant cells are arrested in G_2 phase. The expression of both genes is cell cycle-regulated in a manner dependent on the *cdc10*$^+$ gene, the product of which is a transcriptional activator at START: the 5' region upstream of the genes contains the *Mlu*I motif known to be the binding site for the transcription factor DSC1sp, of which the *cdc10* protein is a component (Lowndes *et al.*, 1992).

Furthermore, ectopic expression of the *cdc18*$^+$ or *cdt1*$^+$ gene can complement a temperature-sensitive mutation of *cdc10* at restrictive or semi-permissive temperature. The *cdc18*$^+$ gene was proposed to be a major downstream target of the *cdc10*$^+$ start gene (Kelly *et al.*, 1993). The *cdt1*$^+$ gene is required for DNA synthesis and inhibits the initiation of premature mitotic events (Hofmann and Beach, 1994). The functions of these gene products in DNA replication, however, are unknown at the molecular level. Thus, it is important to determine the functional relationship among cdc18, cdt1 and cut5 proteins. We show that *cut5*$^+$ transcription is not regulated by *cdc10*$^+$, and the levels of *cut5*$^+$ transcript and also protein do not alter during the cell cycle, strikingly different from the transcriptional regulation of *cdc18*$^+$ and *cdt1*$^+$.

Certain fission yeast DNA repair mutants, such as *rad1*, *rad3*, *rad9*, *rad17*, *rad26* and *hus1*, lose checkpoint control for both DNA synthesis and damage (Al-Khodairy and

Carr, 1992; Enoch et al., 1992; Rowley et al., 1992; Al-Khodairy et al., 1994). However, chk1/rad27 mutants are defective only in the DNA damage checkpoint (Walworth et al., 1993; Al-Khodairy et al., 1994). Mutations related to cdc2 kinase activation in mitotic entry, such as cdc2-3w, OP-cdc25 and wee1-mik1, abolish the dependence of mitosis on completion of DNA synthesis (Enoch and Nurse, 1990; Lundgren et al., 1991). To compare the phenotype of the cut5 mutation with those mutants described above, we addressed the question of whether the checkpoint systems for DNA replication and damage are functional in cut5 mutants at the restrictive temperature.

The cut5 protein function postulated from the mutant phenotype is intriguing. It plays a positive role in replication and a negative role in cell division. We proposed previously that cut5 protein might alter its function at different stages of the cell cycle and differently mark single and twin chromatids (Saka and Yanagida, 1993). This model predicts that cut5 is a nuclear protein and alters its activity (or that of interacting proteins) before and after the completion of the S phase. We provide evidence in this paper that the cut5 protein links nuclear chromatin and M phase regulator in the replication checkpoint control system which is essential for the progression of S phase. The properties of the cut5 protein described in this report are consistent with such a role in cell cycle control. Furthermore, the cut5 protein shows structural similarity to a proto-oncoprotein Ect2, suggesting that an important functional domain for regulating cell division is conserved.

Results

Mutant chromosome DNA bands disappear in pulse field gel (PFG) electrophoresis

Previous fluorescence-activated cell sorting (FACS) analysis demonstrated that the S phase was blocked in cut5 mutant cells at the restrictive temperature (36°C); cells containing 1C DNA were greatly accumulated (Saka and Yanagida, 1993). To obtain information about the state of chromosomal DNA in cut5-blocked cells, we performed PFG electrophoresis for chromosome-sized DNA molecules.

Wild-type (WT) and mutant strains, namely cdc10-129, cut5-T401 and the double mutant cdc10−cut5, were cultured at 36°C for 0–6 h. Cells were collected, samples for PFG electrophoresis were prepared and run for 160 h at 14°C (3600 s pulse time and 50 V). Three normal chromosome DNA bands (indicated by I, II and III) were seen for wild-type at 0, 3 and 6 h (Figure 1). These bands were not seen after incubation of the cut5 mutant at 36°C for 3 h, while they were clearly observed in G_1-arrested cdc10 and the double mutant at 3 h, though not after longer incubation.

Chromosomal DNAs in cut5 mutant cells were not like those in wild-type: they did not enter the gel at 3 h. In G_1-arrested cdc10 and the double mutant, however, chromosomal DNA entered the gel and formed the three bands at 3 h. Chromosomal DNAs in wild-type cells blocked during S phase by hydroxyurea also do not enter gel (data not shown; Hennessy et al., 1991). These results indicate that in the single cut5 mutant, a certain replication event is initiated but does not proceed further; the occur-
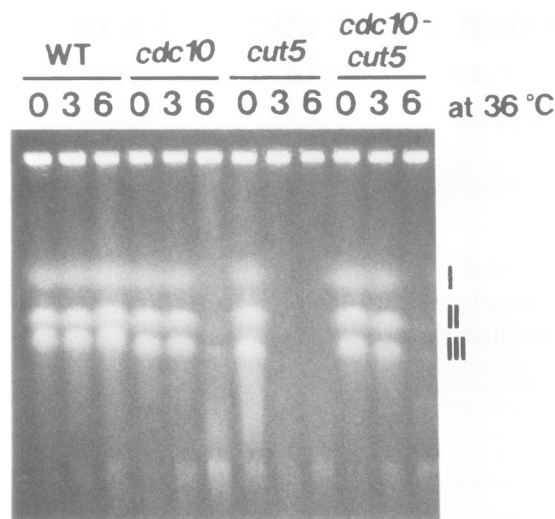
Fig. 1. PFG electrophoresis of fission yeast chromosomal DNAs. Wild-type, cdc10-129, cut5-T401 and double mutant cdc10−cut5 cells were grown at 26°C, then transferred to 36°C for 0–6 h. Portions of the culture were taken at 0, 3 and 6 h, and used for preparation of the specimens for PFG electrophoresis. The three chromosome DNA bands are indicated by I, II and III (Fan et al., 1988).

rence of this event requires the presence of cdc10+. Our previous finding that the double mutant cdc10−cut5 produces a delayed cut phenotype is consistent with this (Saka and Yanagida, 1993). DNA synthesis in the single cdc10-129 mutant is leaky, and a considerable amount of DNA is synthesized after 6 h at 36°C (Saka and Yanagida, 1993; Sazer and Nurse, 1994).

Transcript level is independent of cell cycle stage and cdc10+ gene

We next examined whether the level of cut5+ transcript was altered in cells arrested at specific stages of cell cycle. RNAs were prepared from four mutants: G_1-arrested cdc10-129, G_2-arrested cdc25-22, M phase-arrested nuc2-663 and nda3-311. These mutants were incubated at the restrictive temperature (36°C for cdc10, cdc25 and nuc2, and 20°C for nda3). RNAs were prepared from extracts of cells grown at the permissive temperature or the restrictive temperature for 3, 6 or 48 h, separated by electrophoresis and probed with the cut5+ gene (the ura4+ gene was the control probe).

As shown in Figure 2a, the amount of cut5+ transcript did not decrease in any of the mutants; the level in cdc10-129 mutant cells at 36°C was about the same as that in wild-type cells. Thus, transcription of the cut5+ gene is not reduced by the inactivation of cdc10+. In contrast, the level of cdc22+ transcript was greatly decreased in a cdc10 mutant (Fernandez-Sarabia et al., 1993). We thus conclude that transcription of the cut5+ gene is not under transcriptional control of the start gene cdc10+. Curiously, the transcript level increased in M phase-arrested nuc2 and nda3 mutant cells, which arrest at metaphase and prometaphase, respectively (Hiraoka et al., 1984; Hirano et al., 1988). The reason for this increase is not understood. The amount of cut5 protein, however, did not increase in these M phase mutant extracts (data not shown).

We then tested whether the level of cut5+ transcript fluctuated during the cell cycle. Early G_2 cells of wild-

a

b
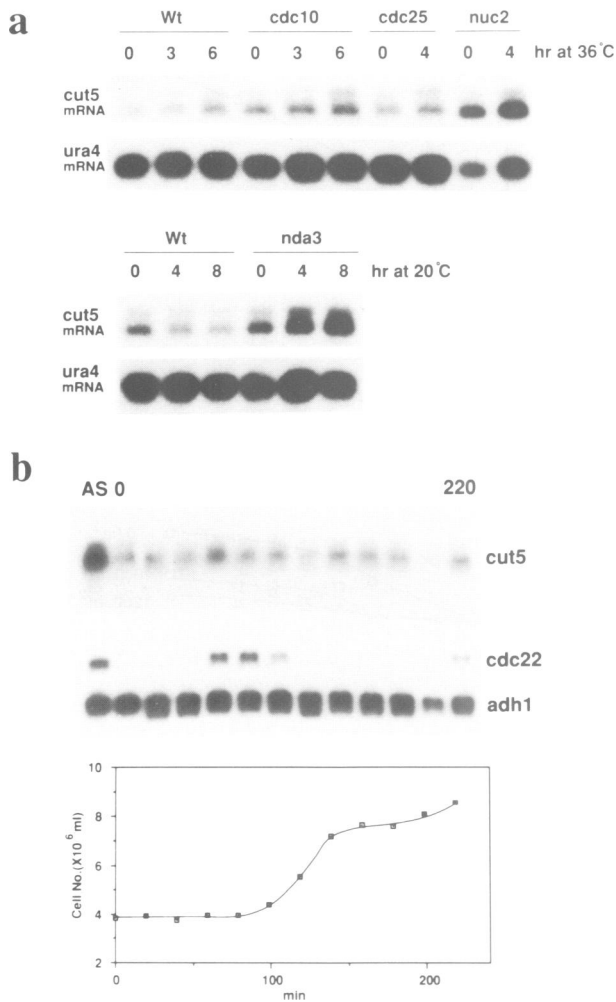
Fig. 2. Northern blot of arrested mutant and wild-type synchronous cultures. (a) Northern blotting patterns of wild-type, G_1-arrested *cdc10*, G_2-arrested *cdc25*, prophase-arrested *nda3* and metaphase-arrested *nuc2* mutant cells probed with the *cut5*$^+$ or the *ura4*$^+$ gene. Cells initially grown at permissive temperature were shifted to restrictive temperature for the times indicated, and RNA samples were prepared. The restrictive temperature for *cdc10*, *cdc25* and *nuc2* mutant cells was 36°C, while that for the *nda3* mutant was 20°C. (b) Synchronous culture of wild-type cells was prepared using an elutriator rotor. Portions of the culture were taken every 20 min from 0 to 220 min; RNA samples were prepared from extracts for Northern blotting. They were probed with the *cut5*$^+$, *cdc22*$^+$ or *adh1*$^+$ genes. AS represents hybridization of control asynchronous culture cells. The cell number change is shown at the bottom.

type were collected by an elutriator rotor (Materials and methods) and a synchronous culture was prepared. Aliquots were taken at 20 min intervals, cell number was determined and RNA samples were prepared, followed by electrophoresis (Figure 2b; Materials and methods). The probes used for Northern blotting were the *cut5*$^+$ (Fenech *et al.*, 1991; Saka and Yanagida, 1993), *cdc22*$^+$ (Gordon and Fantes, 1986; Fernandez-Sarabia *et al.*, 1993) and *adh1*$^+$ genes (Russell and Hall, 1983). The RNA sample in the first lane was prepared from an asynchronously growing culture (AS). The others were made from portions of the culture taken at 20 min intervals (0–220 min).

The level of *cut5*$^+$ transcript did not change significantly during the cell cycle, whereas the S phase-specific *cdc22*$^+$ transcript level peaked during S phase. The constitutive

a

b
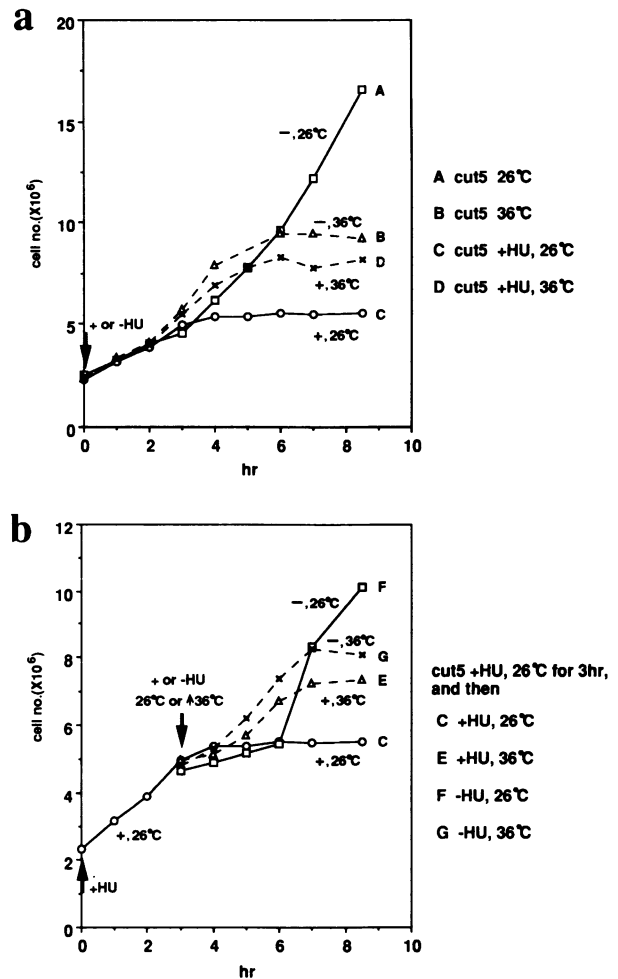
Fig. 3. Phenotype of *cut5-T401* mutant in the presence of hydroxyurea. (a) Mutant cells grown at 26°C in the absence of hydroxyurea were transferred to 36°C in the presence (×) or absence (△) of hydroxyurea, and then the cell number was counted. As a control, cells were kept at 26°C in the presence (○) or absence (□) of hydroxyurea at 26°C. (b) Mutant cells were initially treated with hydroxyurea at 26°C for 3 h, and then S phase-arrested cells were raised to 36°C in the presence (△) or absence (×) of hydroxyurea. 80% of cells showed the *cut5*-like phenotype after 2 h at 36°C. As a control, the culture was kept at 26°C in the presence (○) or absence (□) of hydroxyurea.

adh1$^+$ transcript level remained constant. These results establish that the transcription of *cut5*$^+$ is unlike that of *cdc18*$^+$ and *cdt1*$^+$ (Kelly *et al.*, 1993; Hofmann and Beach, 1994).

Replication checkpoint system is disrupted by the inactivation of cut5

Mitosis and cell division take place in *cut5* mutant cells at 36°C without the completion of DNA replication (Saka and Yanagida, 1993). The next question was whether the same phenotype would occur when the replication checkpoint had been first activated by hydroxyurea and then the *cut5*$^+$ gene product was inactivated at 36°C. In wild-type cells, hydroxyurea causes the block of S phase and activates the replication checkpoint so that the subsequent M phase is blocked (Enoch and Nurse, 1990). Blocked cells elongate without nuclear division.

In the first experiment, *cut5-T401* cells grown at 26°C

were shifted to 36°C with (+) or without (−) 15 mM hydroxyurea (Figure 3a). In the control culture at 26°C, mutant cells divided once in the presence of hydroxyurea, and then arrested with 1C DNA content and elongated. At 36°C, however, cells underwent further cell divisions in the absence or presence of hydroxyurea. Light microscopic inspection showed the *cut* phenotype. The cell number increases with and without hydroxyurea at 36°C after 8.5 h, respectively, were 3.3- and 3.9-fold in comparison with that at 0 h. In contrast, the cell number increase with and without hydroxyurea at 26°C after 8.5 h, respectively, were 2.4- and 6.6-fold in comparison with that at 0 h.

In a second experiment (Figure 3b), *cut5* mutant cells were cultured at 26°C for 3 h in the presence of hydroxyurea to activate the replication checkpoint and then transferred to 36°C with or without the drug to inactivate the cut5 protein. Mutant cells continued to divide under these conditions, indicating that the replication checkpoint system once activated became disrupted by the transfer to 36°C. In contrast, mutant cells maintained at 26°C ceased to divide after 3 h if the checkpoint system was maintained by the presence of hydroxyurea. If the drug was removed from the culture, cells divided again after 7 h, demonstrating that the effect of hydroxyurea on the block of cell division was reversible. The above results strongly suggest that *cut5* protein is an essential component of the replication checkpoint, and its loss leads to inactivation of the replication checkpoint system.

DNA damage checkpoint is maintained in the absence of cut5 function

At 26°C, both *rad4* and *cut5* mutants are moderately sensitive to UV and X-ray irradiation (Duck *et al.*, 1976; Al-Khodairy and Carr, 1992; Rowley *et al.*, 1992; Saka and Yanagida, 1993). We examined whether UV irradiation of *cut5* mutant cells at 36°C delays cell division. Wild-type haploid 972 and *cut5-T401* mutant strains were irradiated in liquid culture with UV light and then incubated at 36°C (Figure 4a, upper panel). Survival was 36% for wild-type and 22% for *cut5* treated with a UV dose of 1800 J/m². In wild-type, cell division was delayed by UV irradiation due to activation of the DNA damage checkpoint system (Al-Khodairy and Carr, 1992; Rowley *et al.*, 1992). In *cut5* mutant cells, cell division also did not occur at 36°C (Figure 4a, lower panel). Even at lower UV doses (600–1200 J/m²), cell division of the mutant was severely inhibited at 36°C.

A similar experiment was performed using a DNA-damaging drug, 4-nitroquinoline-1-oxide (4NQO). *cut5-T401* cells were cultured in the presence of 1 µg/ml 4NQO at 26 or 36°C. Portions of the cultures were taken at time intervals and cell number was measured (Figure 4b). Mutant cells did not divide at either 26 or 36°C when 4NQO was added. These results demonstrate that DNA damage blocks cell division in a *cut5* mutant at both 26 and 36°C, suggesting that the DNA damage checkpoint system is functional in the absence of *cut5* function. We conclude that the *cut5+* gene is not required for the DNA damage checkpoint.

Protein domains and similarity of N-terminus to an oncoprotein

The amino acid sequence of the cut5 protein resembles that of human XRCC1 (Thompson *et al.*, 1990), which is
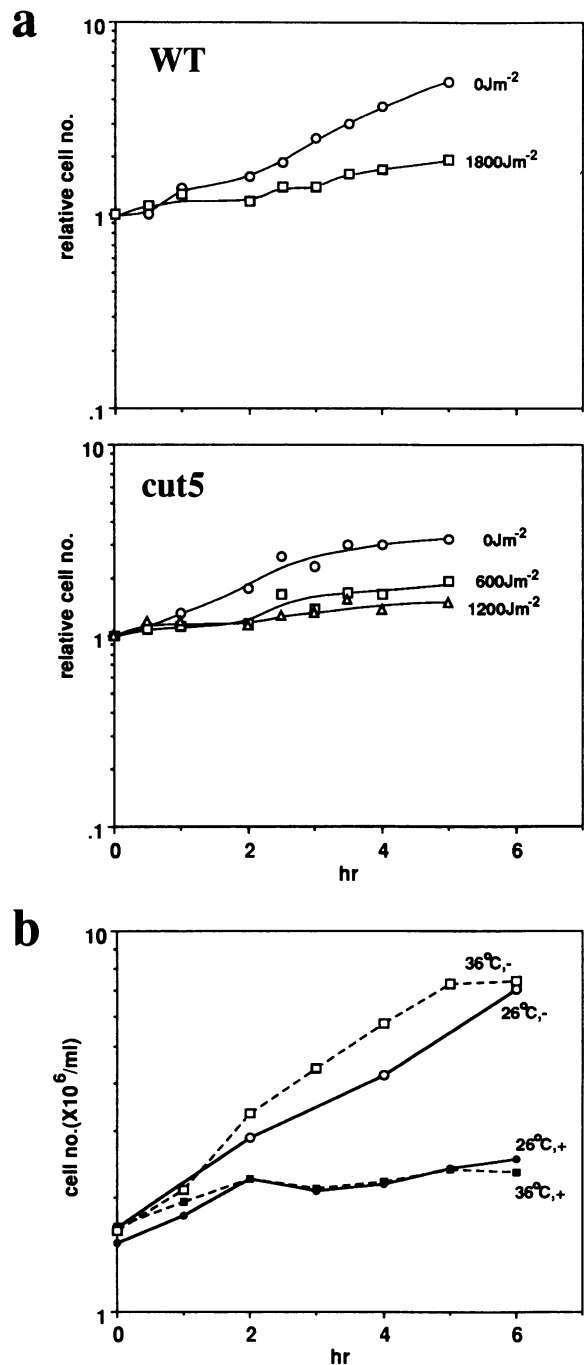
Fig. 4. Block of cell division after UV irradiation and 4NQO treatment. (a) Wild-type and *cut5-T401* cells were grown at 26°C in YPD medium, and the cell suspensions were irradiated with UV light (254 nm at room temperature, followed by the transfer to 36°C in YPD medium). The cell number of the culture was counted. Top panel, wild-type cells. Data obtained after UV treatment with 0 and 1800 J/m² are shown. Bottom panel, *cut5-T401* mutant cells treated with 0, 600 and 1200 J/m² UV rays. (b) *cut5-T401* cells were cultured at 26 or 36°C in the presence (1 µg/ml) or absence of 4NQO. (○) 26°C, no drug; (□) 36°C, no drug; (●) 26°C, with drug; (■) 36°C, with drug.

required for repair of DNA damage induced by X-rays (Fenech *et al.*, 1991; Saka and Yanagida, 1993). Examination of the sequence alignments revealed a duplication in the N-terminal domain (Lehmann, 1993): two stretches (amino acids 1–58 and 96–153) were similar
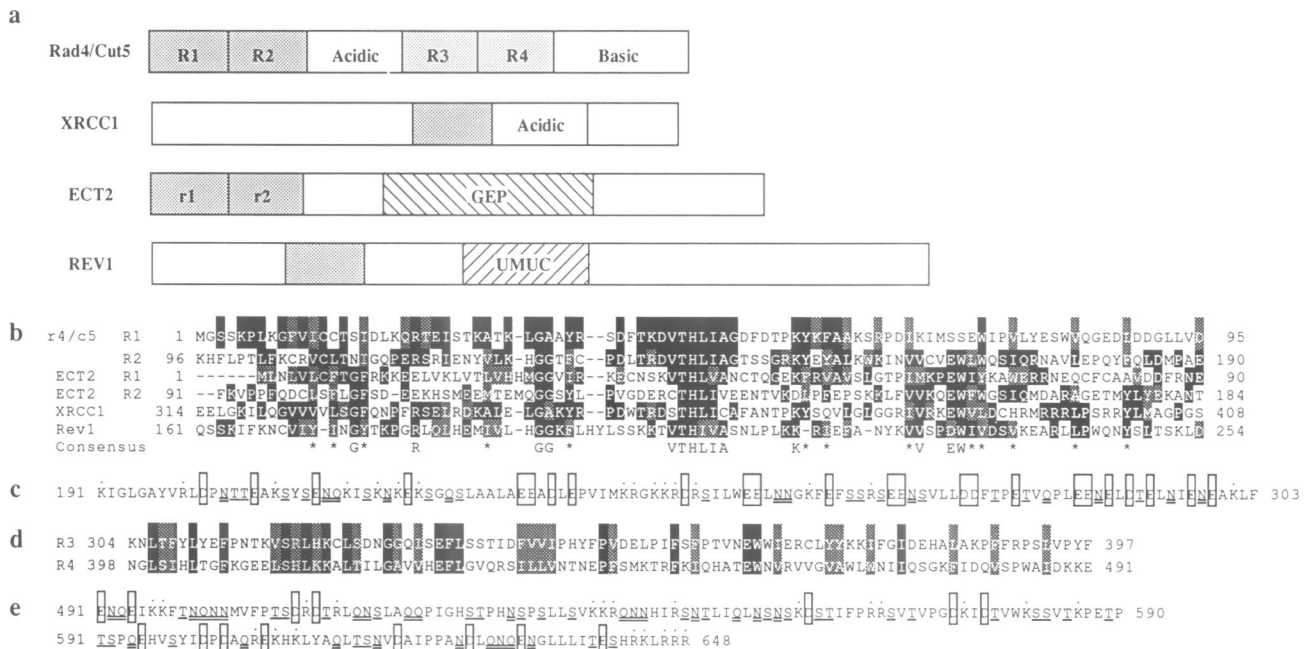
Fig. 5. Domain structure of cut5 protein. (a) Cut5 protein consists of four domains (N-terminal repeats, central acidic domain, central repeats and C-terminal basic region). The sequences similar to the N-terminus were found in human DNA repair protein XRCC1, the oncoprotein Ect2 and the budding yeast Rev1 protein. Similar regions are indicated by the hatched boxes. Ect2 contained the central region with the sequence similarity to GTP-exchanging factor (GEP). Rev1 contained the domain similar to bacterial DNA repair protein UmuC. (b) Amino acid sequence alignment among similar regions of cut5 (r4/c5), Ect2, XRCC1 and Rev1 proteins. Identical amino acids are indicated by white characters. Similar amino acids are shown by hatched boxes. The consensus sequence modified from that reported by Lehmann (1993) is shown at the bottom. Asterisks indicate hydrophobic amino acids. (c) Sequence of the central, acidic hydrophilic region of cut5 protein. Basic and acidic residues are indicated, respectively, by the dot and the box. The single and double underlines are, respectively, S/T and N/Q. (d) The R3/R4 sequences of cut5 protein are compared. Identical amino acids are shown by white characters. Similar amino acids are indicated by hatched boxes. (e) The C-terminal basic hydrophilic sequence of the cut5 protein is shown. The symbols are the same as described in (c).

to amino acids 314–371 of XRCC1 (41 and 33% sequence identity, respectively).

By database search, we found that two other proteins, namely an oncogene product Ect2 (Miki *et al.*, 1993) and the budding yeast DNA repair protein Rev1 (Larimer *et al.*, 1989), were significantly similar to the N-terminal region of the cut5 protein (Figure 5a and b). By close comparison with Ect2 and Rev1, the N-terminal domain of cut5 protein was found to be tandemly duplicated (R1, amino acids 1–95, and R2, 96–190). This ~100 amino acid unit was also found to be duplicated in Ect2. Only one unit, however, existed in XRCC1 and Rev1. The consensus sequence deduced from comparison between the four proteins is indicated at the bottom of Figure 5b ('*' indicates hydrophobic residues). The most conserved stretch in the consensus sequence is VTHLIA.

The N-terminal domain of *cut5* is essential for complementation of temperature-sensitive *cut5* mutants (Saka and Yanagida, 1993). The N-terminal domain of Ect2 is important for regulating the transforming activity (Miki *et al.*, 1993); truncating the N-terminus increases dramatically the transforming efficiency, suggesting that the N-terminus has a negative effect on cell division. The central domain of Ect2 contains a sequence similar to those of CDC24 or dbl, known to be regulators for small G proteins (Bender and Pringle, 1989; Diekmann *et al.*, 1991; Hart *et al.*, 1991). The N-terminal domain of Rev1, which shows similarity to cut5, spans the region in which the *rev1* mutant sites lie, and is thus essential for function; the central domain contains a sequence similar to the bacterial DNA repair protein UmuC (Larimer *et al.*, 1989).

The amino acid stretch 191–300 is highly hydrophilic and acidic (Figure 5c), with eight net negative charges (24 E+D and 16 K+R) and abundant neutral hydrophilic residues (26 S/T + Q/N). Clustered basic residues KRGKKRDR in the region may represent a nuclear localizing signal (NLS). We noted two other weak ~100 amino acid long repeats between amino acids 304 and 397 (indicated by R3), and 398 and 491 (R4) (Figure 5d). The repeat unit partly resembled the N-terminal R1 and R2 repeats. GG/A--EW might be a functional motif.

The C-terminal domain (491–648) was hydrophilic and basic (Figure 5e). The highly basic C-terminal sequence RKLRRR might serve as another NLS.

We conclude from the sequence analysis that the cut5 protein is a repeating protein consisting of four domains, namely the N-terminal repeat region followed by a central acidic hydrophilic region, and the other repeat region followed by the C-terminal basic hydrophilic domain. Each region consisted of 100–160 amino acids.

p74 is susceptible to proteolysis

Rabbit antiserum (designated CFc) was made against a fusion protein consisting of the central domain of the cut5 protein (see Materials and methods). Immunoblotting of wild-type extracts carrying the vector plasmid using preimmune serum (Figure 6a, left panel, lane 1) showed no band. Using affinity-purified CFc antibodies, however, wild-type carrying the vector gave four bands at the positions of 74, 61, 49 and 38 kDa (lane 2), whereas wild-type carrying multicopy plasmid with the *cut5*+ gene
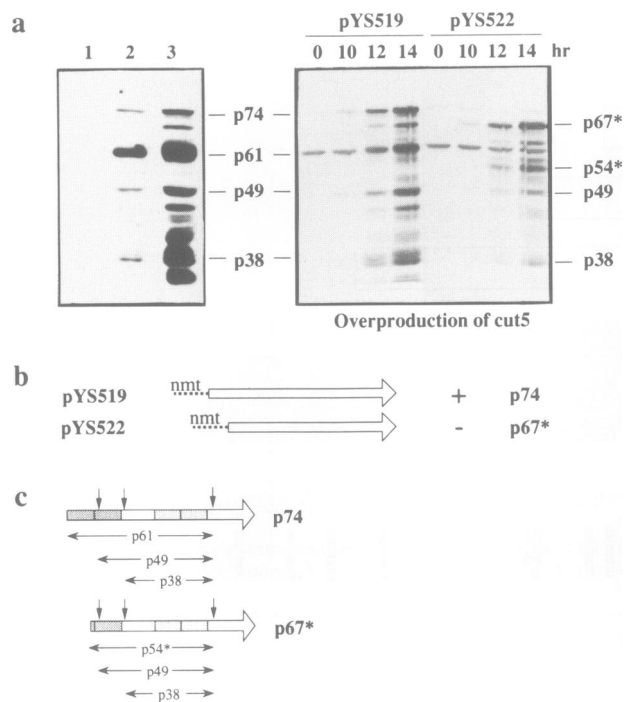
Fig. 6. Protease sensitivity of cut5. (a) Left panel: immunoblot of wild-type cells carrying vector (lanes 1 and 2) or plasmid pYS514 (lane 3) was performed by using affinity-purified CFc antibodies. No band was obtained by preimmune serum (lane 1), but multiple protein bands (the major ones at p74, p61, p49 and p38 in lane 2) were obtained with immune serum. More bands were obtained if extracts of cells carrying plasmid with the *cut5*+ gene were used (lane 3). Right panel: wild-type cells carrying plasmids pYS519 or pYS522 that overproduced the full-length or the N-terminal truncated protein, respectively, after the depletion of thiamin from the growth medium, were immunoblotted using affinity-purified CFc antibodies. Cells were taken from the cultures at 0, 10, 12 and 14 h after the removal of thiamin. For full-length protein, bands at p74, p61, p49 and p38 increased in intensity, whereas for N-truncated protein the bands at p67, p54, p49 and p38 increased in intensity. (b) The inserts in pYS519 and pYS522 are depicted; the coding region was ligated with the nmt1 promoter. (+) indicates complementation of temperature-sensitive *cut5* mutant. (c) Schematic representation of the probable cleavage sites (indicated by the arrows) in the cut5 protein.

produced more bands (lane 3). The 74 kDa band was the expected molecular weight for the cut5 protein (Saka and Yanagida, 1993), but the intensity of the 61 kDa band was always much higher than that of the 74 kDa band.

To ensure that these protein bands were derived from the same gene, we constructed two plasmids with the inducible promoter for overproduction (Figure 6b). pYS519 contains the wild-type gene downstream of the nmt1 promoter (Maundrell, 1990), while pYS522 contains the N-terminal truncated gene downstream of the nmt1 promoter. In the absence of thiamin, the promoter is on and overproduction starts after 10 h (Maundrell, 1990).

Immunoblot using affinity-purified CFc showed enhancement of the band intensity for the 74, 61, 49 and 38 kDa bands in cells carrying plasmid pYS519 after 10 h (Figure 6a, right panel). Many more additional bands were seen after 14 h. Cells carrying plasmid pYS522 with the N-terminally truncated gene (Figure 6b), however, did not increase the band intensity of the 74 and 61 kDa bands. Instead, new bands of 67 and 54 kDa appeared. In addition, the intensity of 49 and 38 kDa bands was

enhanced. The 67 and 54 kDa bands, respectively, corresponded to the 74 and 61 kDa bands that had lost the N-terminal 7 kDa. We thus concluded that native p74 was degraded into polypeptides with different molecular weights by proteolysis. The temperature-sensitive mutant phenotype was complemented by pYS519 (both in the absence and presence of thiamin) but not by pYS522, indicating that the N-terminal 7 kDa region is essential for the function.

These results show that the cut5 protein is highly susceptible to proteolysis. Protein cleavage was presumed to take place in the C-terminus to produce p61 (indicated by the arrows in Figure 6c). p49 and p38 were produced by subsequent proteolysis in the N-domain. Note that these presumed cleavage sites correspond to the boundaries between the domains described above.
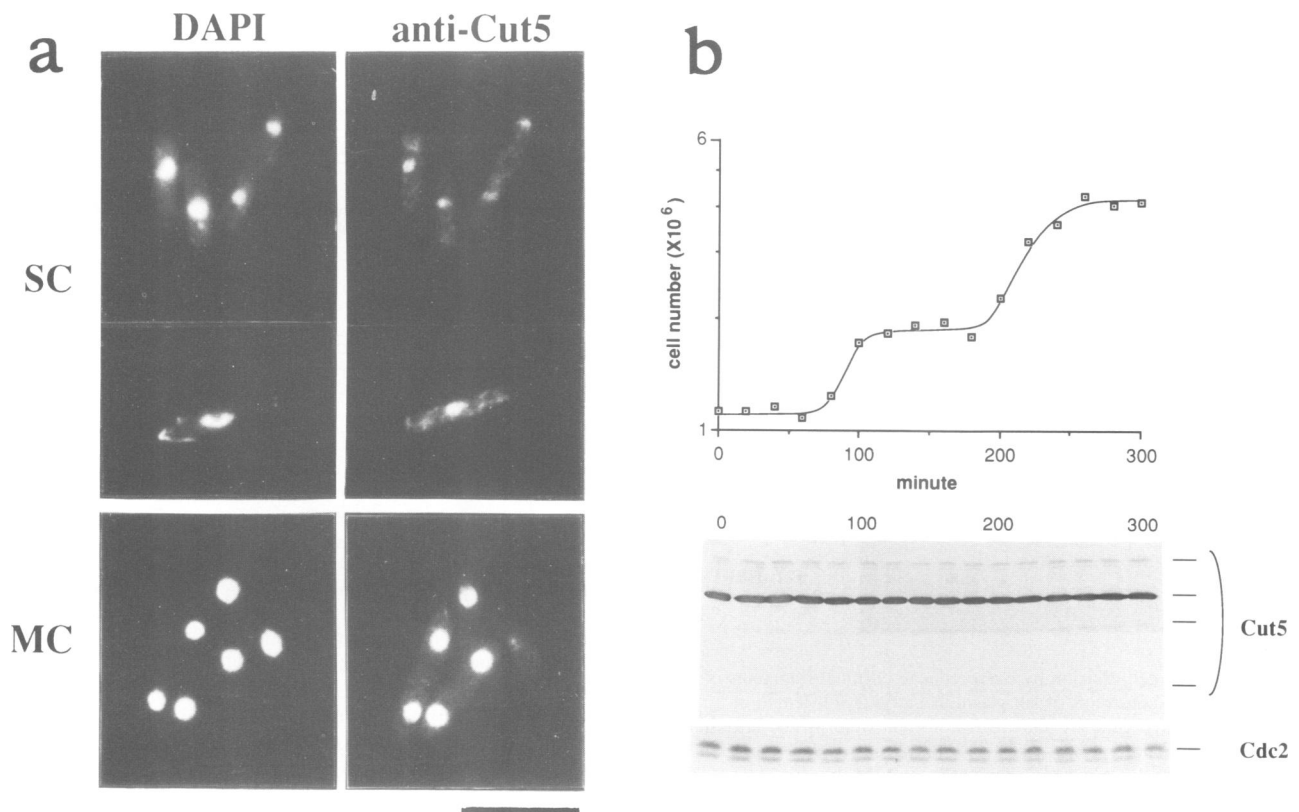
Nuclear localization

Immunolocalization of cut5 protein was performed using affinity-purified antibodies CFc (see Materials and methods). Immunofluorescence was enriched in the nucleus of wild-type cells (SC in Figure 7a). Judging from comparison with the area stained by DAPI, relatively more fluorescence was detected in the non-chromosomal domain than in the chromatin region. Relatively weak immunofluorescence was also found in the cytoplasm. Similar immunofluorescence patterns were observed when anti-dis2 phosphatase antibodies were employed (Ohkura *et al.*, 1989). No significant change in localization and intensity was observed at different cell cycle stages. When cells carrying multicopy plasmid with the *cut5*+ gene were examined, the entire nuclear immunofluorescence was enhanced (bottom panel labelled MC). Immunofluorescence was negligible when preimmune serum was employed (data not shown).

Protein level during the cell cycle

The level of cut5 protein during the cell cycle was examined. A synchronous culture of wild-type cells was prepared by elutriation, and early G_2 cells were cultured in YPD medium. Portions of the culture were taken at 20 min intervals, their cell number was counted (Figure 7b, upper panel) and samples for immunoblotting were prepared (lower panel). Multiple protein bands seen in asynchronous cell extracts were also found throughout the cell cycle without significant change in the level of protein band intensity (bottom panel; the major four bands are indicated by the short lines). The band intensity of cdc2 protein used as a control was constant. Thus, there is no cell cycle-dependent fluctuation in the level of cut5 protein product.

cut5 N-terminal overexpression inhibits cell division

To examine the effects of overproduction of parts of the cut5 protein on cell division, we made a series of N- and C-terminal truncations of the gene (Figure 8, upper panel). They were ligated downstream of the nmt promoter in pREP1. Cut5 overproduction by plasmids pYS519, 522, 526 and 527 in the absence of thiamin was verified by immunoblot (data not shown). Wild-type cells carrying pYS519, 522, 526 or 527 in the absence of thiamin formed colonies (lower panel). In liquid culture, normal cells were

Fig. 7. Localization and level of cut5 protein. (**a**) Immunofluorescence microscopy of wild-type cells (SC) using affinity-purified CFc antibodies. Immunofluorescence was enriched in the nucleus. Cells carrying plasmid with the *cut5*⁺ gene showed stronger fluorescence in the nucleus (MC). Bar indicates 10 μm. (**b**) Synchronous culture of wild-type cells was prepared as described in Materials and methods. Aliquots of the culture were taken every 20 min. The cell number was counted and cell extracts were prepared for immunoblot using affinity-purified CFc and anti-cdc2 (anti-PSTAIRE) antibodies.

mostly found with the occasional appearance of cells with the *cut* phenotype. These results suggested that overproduction of full-length or N- or C-deleted protein was not significantly inhibitory to cell division. Wild-type cells carrying pYS525 and pYS528, however, did not form colonies in the absence of thiamin (lower panel). Thus overproduction of the N- or C-terminal fragment blocks cell division.

In liquid culture of wild-type cells carrying pYS525, cell division was blocked after 10 h and FACS analysis showed that these arrested cells contained 2C DNA (data not shown). The arrested cells contained a single nucleus, suggesting that cells were blocked in G₂ phase. Neither condensed chromosomes nor aberrant nuclear morphology were found in cells overproducing the cut5 protein.

Cell division does not occur in double mutants of cut5 with cdc2 or cdc25

In an attempt to search for genes which interact with the *cut5*⁺, a number of *Schizosaccharomyces pombe* mutant strains were crossed with *cut5-T401*; the phenotypes of resulting double mutants were determined (Table I). Interestingly, the double mutants constructed by crossing with temperature-sensitive *cdc2*, *cdc13* or *cdc25* mutants produced the G2-blocked cdc phenotype, i.e. cell elongation with a single nucleus. The temperature-sensitive *cdc2*, *cdc13* and *cdc25* mutations (Nurse, 1990) are epistatic to the *cut5* mutation, indicating that expression of the

unrestrained cell division phenotype of the *cut5* mutant is dependent on the presence of these genes.

We then examined how the double mutant *cdc25−cut5* responded to hydroxyurea treatment. The double mutant cells were first cultured in the presence of hydroxyurea for 3 h at 26°C to activate the replication checkpoint, then shifted to 36°C in the presence of drug (Figure 9, arrows), inactivating both *cdc25* and *cut5* proteins. Cell division of the double mutant ceased as in wild-type and the single *cdc25* mutant, whereas single *cut5* cells continue to divide (Figure 9a). Double mutant cells displaying the *cut* phenotype were hardly observed. Similar results were obtained by using the double mutants *cut5−cdc2* and *cut5−cdc13* (Figure 9b). The replication checkpoint is expected to be inactivated at 36°C in the double mutant due to the loss of *cut5*⁺ function (Figure 3b). However, cell division did not take place, indicating that the downstream target of wild-type *cut5* protein for restraining mitosis may be the M phase regulators, i.e. the components required for the activation of *cdc2* kinase.

Discussion

Loss of replication checkpoint control in cut5 mutant

Two lines of evidence indicate that *cut5* mutant cells are defective in DNA replication at 36°C. First, the amount of DNA in mutant cells at 36°C becomes ~1C (Saka and
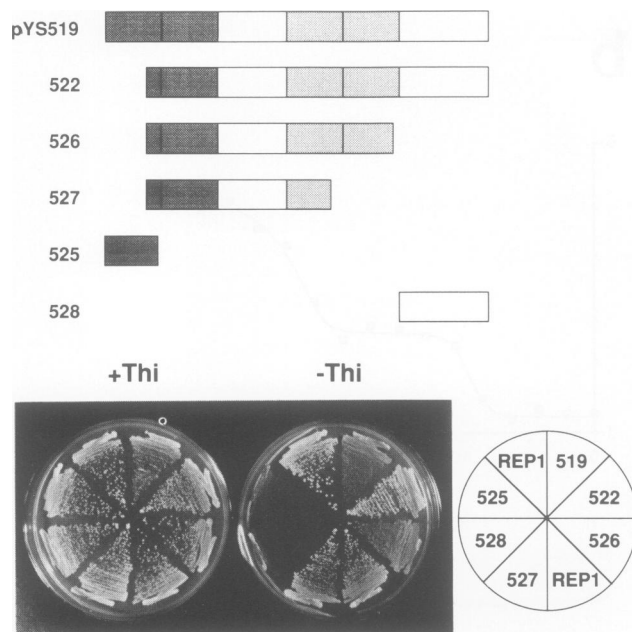
Fig. 8. Block of cell division by overproduced terminal fragments of cut5. Top panel: constructs of six plasmids carrying full-length or variously truncated gene are shown. All of them were ligated into the pREP1 vector. Wild-type was transformed with one of these plasmids and transformants were plated in the absence of thiamin for overproduction. Bottom panel: colony formation of wild-type cells carrying each of the six plasmids above at 33°C in the absence (−Thi) or presence (+Thi) of thiamin.
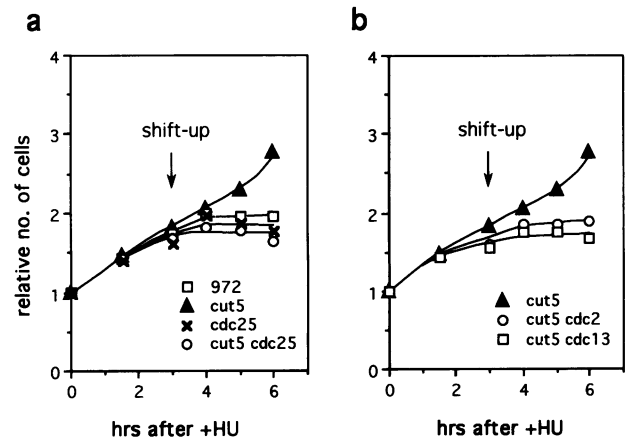

Fig. 9. Cell division phenotype of the double mutant *cdc25*−*cut5*. (a) Wild-type 972, single *cut5-T401* and *cdc25-22* and the double mutant *cut5*−*cdc25* were cultured in the presence of hydroxyurea (HU) for 3 h at 26°C and then shifted to 36°C in the continued presence of hydroxyurea. The cell number of each culture was measured. (b) A similar experiment but using the double mutants *cut5*−*cdc2* and *cut5*−*cdc13*. Although cell division immediately started in *cut5* mutant cells after the shift, cell division was inhibited in the double mutants as in single *cdc25* mutant and wild-type cells.

Table I. Crosses between *cut5-T401* and strains listed below

Strains	Phenotype of double mutant at:		References[a]
	26°C	36°C	
cdc2-33	normal	cdc2-like	1, 2
cdc10-129	normal	cdc10/cut5 mix[b]	1–3
cdc13-117	normal	cdc13-like	1, 4
cdc17-K42	normal	cut5-like	5
cdc18-K46	normal	cut5-like	5, 6
cdc21-M68	normal	cut5-like	5, 7
cdc25-22	normal	cdc25-like	8
wee1-50	normal	cut5-like	9, 10
wee1::ura4⁺	wee	cut5-like	9
cdc2-1w	wee	cut5-like	9, 10
cdc2-3w	wee	cut5-like	9–11
3X[wee1⁺]	slightly elongated	cut5-like	9
rad1-1	normal	cut5-like	12, 13

[a]1, Nurse *et al.* (1976); 2, Nurse and Bissett (1981); 3, Lowndes *et al.* (1992); 4, Hagan *et al.* (1988); 5, Nasmyth and Nurse (1981); 6, Kelly *et al.* (1993); 7, Coxon *et al.* (1992); 8, Russell and Nurse (1986); 9, Russell and Nurse (1987); 10, Nurse (1975); 11, Enoch and Nurse (1990); 12, Rowley *et al.* (1992); 13, Al-Khodairy and Carr (1992).
[b]Timing for entry into mitosis is delayed compared with single *cut5* mutant (Saka and Yanagida, 1993).

Yanagida, 1993). Secondly, chromosomal DNA bands in PFG electrophoresis disappear in *cut5* mutant cells at 36°C after 3 h. The failure of the DNA to enter the gel suggests that a replication event such as opening of origins might be initiated in *cut5* mutant cells, although little or no bulk DNA synthesis takes place. This is possibly due to the leakiness of *cut5* mutant, and the cut5 protein may

be involved in the initiation and/or elongation of DNA replication.

Normally, mitosis is dependent on the completion of DNA replication, so that if replication is blocked, subsequent mitosis is also blocked. This dependency system is thought to be mediated by a checkpoint control or negative feedback control (Osmani *et al.*, 1988; Hartwell and Weinert, 1989; Enoch and Nurse, 1990, 1991; Enoch *et al.*, 1992; Murray, 1992; Sheldrick and Carr, 1993). This system is disrupted in *cut5* mutants at 36°C, as shown by the occurrence of mitosis (albeit aberrant) despite the absence of DNA replication (Saka and Yanagida, 1993).

Two types of hypothesis would explain this. According to the first, the failure to undergo DNA replication in *cut5* mutants is not detected by the mitotic checkpoint, in contrast to the consequences of hydroxyurea treatment, which are detected. Alternatively, cut5 may itself be part of the checkpoint mechanism, and the defect in *cut5* mutants has two consequences: the process of DNA replication is impaired and the signal that normally activates the mitotic checkpoint is blocked. To distinguish between these possibilities, we first activated the checkpoint with hydroxyurea in *cut5* cells at 26°C and then shifted the cells to 36°C. Under these conditions the cells entered mitosis and divided. This indicates that even though the checkpoint system had been activated previously, the effect of this was reversed when the *cut5* protein was inactivated. We conclude that the loss of cut5 function is not ignored by the checkpoint system, but rather causes loss of checkpoint function, i.e. cut5 is itself likely to be part of the DNA replication checkpoint system. It may be worth noting that the loss of cut5 causes premature mitosis; the duration of time for the aberrant second cell division was only 80 min rather than 140 min for the normal first division in the synchronous culture (Saka and Yanagida, 1993).

Loss of the replication checkpoint control has also been found in the mutants *cdc2-3w*, overproduced *cdc25⁺* (OP-*cdc25*) and *wee1-mik1* (Enoch and Nurse, 1990;

Lundgren *et al.*, 1991; Enoch *et al.*, 1992). These mutations in M phase regulators can prematurely cause mitosis, regardless of the completion of S phase. These regulators are the 'receivers' of information about whether DNA synthesis is blocked or completed. Thus the cut5 protein may form a link between the replication machinery and the mitotic activators (or inhibitors) for cdc2 kinase in the cellular signal transduction pathway that couples mitosis to the completion of DNA replication. The cut5 protein located in the nucleus, probably as a chromatin protein, may send a negative signal to the activation of cdc2 kinase. It is unknown how the cut5 protein interacts with the replication machinery. It may cooperate with the initiation complex and monitor the progression of replication (schematized in Figure 10). Alternatively, it may be activated after the completion of mitosis and can promote the initiation of replication. Another possibility is that the cut5 protein is the receiver of the initiation signal. It may interact with the products of $cdc18^+$ and $cdt1^+$ which are under direct transcriptional control of the START gene $cdc10^+$.

Analysis of the effect of UV irradiation indicates that the checkpoint control for DNA damage was maintained in the cut5 mutant at both 26 and 36°C. This is strikingly different from the phenotypes of mutants such as *rad1*, *rad3*, *rad9*, *rad17* and *rad26*, which are involved in the checkpoint control systems for both replication and DNA damage (Al-Khodairy and Carr, 1992; Rowley *et al.*, 1992; Sheldrick and Carr, 1993). When DNA synthesis is inhibited or DNA damage occurs, mitosis is no more restrained in these mutants.

The $chk1^+/rad27^+$ gene, which encodes a protein kinase, appears to have a role complementary to that of cut5. It is required for the radiation damage checkpoint pathway and restrains the activation of cdc2 kinase upon DNA damage, but not for the replication checkpoint control (Walworth *et al.*, 1993). Note that none of the checkpoint rad^+ genes nor $chk1^+/rad27^+$ is essential for viability. In contrast, the $cut5^+$ gene is essential for viability and acts specifically in the replication checkpoint control. Thus, it seems to differ from these previously known genes involved in the replication and/or damage checkpoint controls. The $cut5^+$ gene is the first example which is essential for viability and specifically involved in replication checkpoint control.

No transcriptional regulation by the START gene $cdc10^+$

We show that the transcript level of the $cut5^+$ gene remains approximately constant throughout the cell cycle and is not under the control of the START gene $cdc10^+$. This is in sharp contrast to $cdc18^+$ and $cdt1^+$ (Kelly *et al.*, 1993; Hofmann and Beach, 1994), whose transcript levels peak during S phase and are directly regulated by $cdc10^+$. Although the phenotypes of *cdc18*, *cdt1* null mutants and temperature-sensitive cut5 are similar, their transcriptional regulation is different. Ectopic expression of the $cdc18^+$ and $cdt1^+$ genes suppresses a temperature-sensitive *cdc10* mutant (Kelly *et al.*, 1993; Hofmann and Beach, 1994), suggesting that these genes are the major transcriptional targets of $cdc10^+$; the nmt promoter-directed overproduction of $cut5^+$, however, did not rescue a *cdc10* mutant (unpublished results).
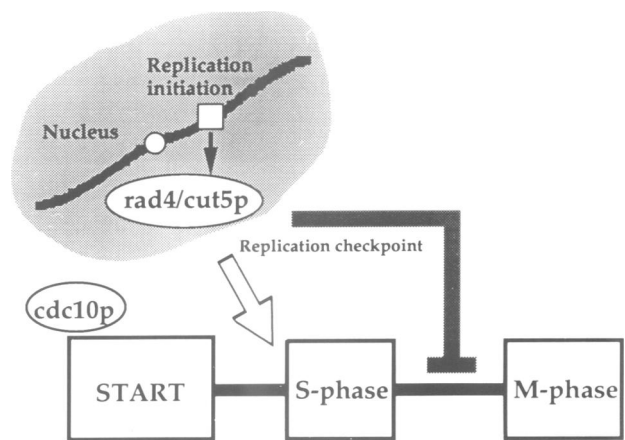
Fig. 10. The proposed relationship of cut5 protein with replication and M phase regulation in replication checkpoint control. See text.

Expression of the cut5 phenotype, however, is apparently not completely independent of $cdc10^+$. Cell division in the double mutant *cut5—cdc10* is delayed compared with that of the single cut5 mutant. The chromosome-sized DNA bands remain in *cut5—cdc10* cells after 3 h at 36°C but not in the single cut5 mutant. A hypothesis to explain these results is that the cut5 protein might be post-translationally regulated by the product of a gene under $cdc10^+$ control. This is consistent with the failure to find any effect of $cdc10^+$ on transcription of $cut5^+$, and no change in protein or RNA level in the synchronous culture. A different but related explanation is that the $cdc10^+$ gene function is more remotely related to cut5 protein; multiple proteins may intervene between cdc10 and cut5 protein functions. In both cases, the cut5 phenotype is dependent upon the $cdc10^+$ START function. The *cdc10* mutant used in the previous and present studies is somewhat leaky (Saka and Yanagida, 1993; Sazer and Nurse, 1994), so that certain replication or mitotic events occur in a delayed fashion. Indeed, chromosomal DNA from *cdc10* or *cut5—cdc10* cells arrested for 6 h is unable to enter the PFG (Figure 1), indicating that initiation has occurred. If the cell cycle could be strictly blocked at START in a cut5 mutant, subsequent cell cycle events, such as entry into S phase, entry into aberrant mitosis and development of *cut* phenotype, might not take place.

N-terminal domain of cut5 is similar to Ect2 and negatively affects cell division

The cut5 protein consists of four domains: two repeated domains and two hydrophilic domains. These domains appear to have different functions. The N-terminal 190 amino acid region consisting of tandemly arranged R1 and R2 units is essential for complementation of $cut5^{ts}$. Overproduction of full-length protein or of central domains did not significantly affect cell growth, but overexpression of the N-terminal region severely inhibited cell division. The functional importance of the N-terminal region is further supported by the fact that the mutation sites of $cut5^{ts}$ alleles are located in the N-terminal region (our unpublished result).

The amino acid sequence of the N-terminal domain is significantly similar to that of the proto-oncogene product Ect2 and the budding yeast DNA repair protein Rev1

(Larimer *et al.*, 1989; Miki *et al.*, 1993). Ect2 was isolated as a cDNA which was able to induce transformed phenotype if the N-terminal region was truncated; the N-terminal domain has a strong negative effect on the transforming efficiency. The central sequence of Ect2 resembles the oncoprotein dbl (Diekmann *et al.*, 1991; Hart *et al.*, 1991) and budding yeast Cdc24 (Bender and Pringle, 1989), known to be the regulators of small GTP binding proteins. The N-terminal regions of cut5 and Ect2 proteins may bind to similar protein(s).

The cut5 protein is enriched in the nucleus. The localization pattern was similar to that of type I-like protein phosphatase dis2 and DNA topoisomerase II (Ohkura *et al.*, 1989; Shiozaki and Yanagida, 1992). The *cut5* protein may be bound to chromatin. It has two putative NLSs. The C-terminal domain of cut5 protein is basic so that this domain may bind directly to DNA. Alternatively, the acidic central domain possibly binds to the basic chromatin proteins.

Relationship between cut5 protein and cell cycle regulators

We propose that the cut5 protein is an essential component for DNA replication and also the checkpoint control system which couples S and M phases. It is required for the initiation and/or elongation of DNA replication. Unlike the genes such as $cdc18^+$ and $cdt1^+$, it is not directly regulated by $cdc10^+$-dependent transcription. Unlike the genes such as $rad1^+$, it is not required for the DNA damage checkpoint control. The *cut5* mutant phenotype is similar to $cdc2$-$3w$, OP$cdc25^+$ and $wee1$-$mik1$ mutants with respect to the defect in the replication checkpoint and premature mitosis, but different in that *cut5* is defective in DNA synthesis. The *cut5* phenotypes are produced in the absence of hydroxyurea, differing from those hydroxyurea-dependent phenotypes displayed in mutants such as $rad1$ and $hus1$.

The cut5 protein may directly or indirectly interact with chromatin proteins to form the complex required for the initiation and/or progression of DNA synthesis. In *cut5* mutant cells the complex is defective or even absent so that there is no DNA replication. In addition, the replication checkpoint system, which recognizes the complex or a DNA structure made by the complex and sends a negative signal to M phase kinase for restraining mitosis, is not functional. The cut5 protein may form a link between nuclear chromatin for replication and the M phase regulatory system. This hypothesis adds a novel component in the checkpoint control pathway postulated by previous studies (Enoch and Nurse, 1990, 1991; Lundgren *et al.*, 1991; Enoch *et al.*, 1992; Sheldrick and Carr, 1993).

Materials and methods

Media, strains and plasmids

Haploid *S.pombe* wild-type *972 h⁻*, *975 h⁺*, temperature-sensitive *cut5-580* and *cut5-T401* mutant strains were used (Hirano *et al.*, 1986; Saka and Yanagida, 1993; Samejima *et al.*, 1993). Other strains used are listed in Table I. Transformation of *S.pombe* was performed by the lithium method (Ito *et al.*, 1983). The genotype of the host strain used for transformation of plasmid nmtOP-cut5 was *h⁻ leu1* or *h⁻ leu1 cut5-T401*. Cells were grown to a density of 3×10^6 or 1×10^7 cells/ml at 26°C in rich YPD medium (1% yeast extract, 2% polypeptone, 2% glucose). The synthetic EMM2 medium was as described (Mitchison, 1970). 2% agar was added for plating. Viability was estimated by plating

cut5 cells at 26°C. The cell number was counted by a Micro cell counter (Sysmex).

UV irradiation and 4NQO treatment

The procedures for UV irradiation described previously (Saka and Yanagida, 1993) were employed with a modification. Log phase cells of *S.pombe* cells in 2.5 ml YPD on a 10×14 cm plate were irradiated in a Stratalinker (Stratagene). The stock solution of 4NQO (50 mg/ml) kept at −20°C in acetone was finally diluted to 1 µg/ml in the culture media.

Synchronous culture and RNA preparation

Haploid wild-type strain 972 was grown in EMM2 at 32°C to a cell density of 4.5×10^6/ml; a sample was taken for RNA preparation and synchronized using a Beckman JE-6 rotor. Samples were taken at 20 min intervals for cell number estimation on a Coulter electronic particle counter and for preparation of RNA for Northern blot analysis. Total RNA was prepared using a hot phenol protocol, as described by Aves *et al.* (1985). 10 µg samples of each RNA sample were separated on a MOPS−formaldehyde gel and transferred to Genescreen, according to the manufacturer's instructions. The blot was hybridized consecutively with $cut5^+$ (2.7 kb *Hin*dIII fragment), $cdc22^+$ (equimolar amounts of the *Hin*dIII inserts of pCDC22-1; Gordon and Fantes, 1986) and $adh1^+$ (1.1 kb *Eco*RI fragment from the *S.pombe* $adh1^+$ gene; Russell and Hall, 1983). $ura4^+$(1.8 kb *Hin*dIII fragment) of *S.pombe* was also used as a control probe. Fragments were radioactively labelled using the random priming method in the presence of $[^{32}P]dCTP$.

PFG electrophoresis

The procedures described for separating individual chromosomes of *S.pombe* (Fan *et al.*, 1988; Chikashige *et al.*, 1989) were followed. *cut5-T401* and wild-type strains were first grown at 26°C and then transferred to 36°C. Cells collected were treated for sample preparation and separated by PFG electrophoresis (0.6% agar) at 14°C with a pulse time of 3600 s at 50 V.

Construction of plasmids for overexpression

pREP1 (Maundrell, 1990) contained the inducible nmt1 promoter. It was restricted with *Nde*I and *Bam*HI and treated with alkaline phosphatase. pREP1 was ligated with full-length (Saka and Yanagida, 1993) or truncated $cut5^+$ genes; resulting plasmids expressed the entire coding region or parts of the $rad4^+/cut5^+$ gene in the absence of thiamin. Transformants containing these plasmids were grown at 33°C in synthetic EMM2 (+thiamin, final concentration 2 µM) medium and then transferred to the same medium lacking thiamin at 33°C. Aliquots of the culture were taken at 0, 8, 10, 12, 14, 16 and 18 h.

Preparation of antibodies and immunochemical methods

Fusion protein was made using pGEX-KG (Guan and Dixon, 1991). The 1.1 kb *Bgl*II−*Nco*I fragment of $cut5^+$ was cloned into pGEX-KG. GST fusion protein was made in *Escherichia coli* MM294. Insoluble fusion protein was purified according to the procedures of Watt *et al.* (1985). Resulting polypeptide was injected into rabbits by the procedures described previously (Hirano *et al.*, 1988). Antigen was injected at 1 month intervals after the second injection, and sera were taken 10–14 days after injection. Antibodies were affinity-purified, first by incubating 10-fold diluted serum with the nitrocellulose membrane blotted with GST in PBS/5% skimmed milk solution overnight, and then by incubating with membrane blotted with fusion protein antigen. Cells were disrupted by glass beads and total protein extract was prepared in buffer containing 25 mM Tris (pH 7.5), 15 mM EGTA, 15 mM EDTA, 0.1% NP-40, 60 mM β-glycerophosphate, 15 mM *p*-nitrophenylphosphate, 1 mM DTT, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml pepstatin A, 100 µg/ml TPCK, 0.1 mM NaF and 0.5 mM Na_3VO_4. Immunoblotting was performed according to Towbin *et al.* (1979). The second antibody used was HRP-labelled protein A (1/200 dilution); it was detected by the ECL Western blotting system (Amersham).

Fluorescence microscopy

DAPI staining of fission yeast cells was performed as described (Adachi and Yanagida, 1989). For immunofluorescence microscopy, the procedure for preparing and fixing cells described by Hagan and Hyams (1988) was followed. Cells were fixed by methanol; anti-rabbit IgG antibody Cy3 (1/500 dilution; Chemicon International Inc.) was used as the second antibody. Fluorescence was observed by a Zeiss Axiophot with a 100 W light source (HBO 100W/2).

Acknowledgements

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