Isolation of a *cdc28* mutation that abrogates the dependence of S phase on completion of M phase of the budding yeast cell cycle

SANTANU KUMAR GHOSH and PRATIMA SINHA*

Department of Biochemistry, Bose Institute, Calcutta 700 054, India

Abstract

We have isolated a mutation in the budding yeast *Saccharomyces cerevisisae CDC28* gene that allows *cdc13* cells, carrying damaged DNA, to continue with the cell division cycle. While *cdc13* mutant cells are arrested as large-budded cells at the nonpermissive temperature 37°C, the *cdc13 cdc28* double mutant culture showed cells with one or more buds, most of which showed apical growth. The additional buds emerged without the intervening steps of nuclear division and cell separation. We suggest that the *cdc28* mutation abrogates a checkpoint function and allows cells with damaged or incompletely replicated DNA an entry to another round of cell cycle and bypasses the mitotic phase of the cell cycle.

[Ghosh S. K. and Sinha P. 2000 Isolation of a *cdc28* mutation that abrogates the dependence of S phase on completion of M phase of the budding yeast cell cycle. *J. Genet.* **79**, 91–96]

Introduction

Successful eukaryotic cell division requires a strict order of events, where late events require the completion of early events. For example, DNA replication must be completed faithfully before nuclear division starts. Cells unable to complete DNA replication, or carrying damaged DNA, will arrest their cell cycle till DNA replication is completed and the damage is repaired. Similarly, cells unable to complete mitosis are arrested and do not enter a new cell cycle. The control mechanisms by which this order is maintained is brought about by the checkpoint genes (reviewed in Hartwell and Weinert 1989; Elledge 1996). These genes are required for cell-cycle arrest if an early event is not successfully completed. Cells failing to get arrested, in spite of unsuccessful execution of an event, are likely to carry mutations in their checkpoint genes. Yeast *cdc13* mutants are defective in the metabolism of telomere-associated DNA and carry incompletely replicated DNA (Lin and Zakian 1996). Consequently, these cells are arrested at the G2/M boundary of the cell cycle at the nonpermissive temperature, 37°C. When a checkpoint gene responsible for this arrest was mutated in the *cdc13* mutant, the resulting double mutant continued with the cell cycle, even after a shift to the restrictive temperature, and generated cells with damaged DNA, leading to rapid cell death (Weinert *et al.* 1994). Several checkpoint genes like *MEC1*, *MEC2*, *MEC3* and *RAD9* were identified in this fashion.

Cdc28 is a serine-threonine kinase in the budding yeast *Saccharomyces cerevisiae* and is a homologue of the fission yeast *Schizosaccharomyces pombe* Cdc2 protein. This protein is required at various phases of the cell cycle and carries out a multitude of functions **(**Mendenhall and Hodge 1998). Cdc28 protein associates with G1 cyclins (Cln1, Cln2, Cln3) for passage through START (the point of commitment and entry to the cell division cycle) and to initiate budding. In association with Clb5 and Clb6 cyclins, it is required for the initiation of DNA replication, i.e. G1 to S phase transition. In association with Clb1, Clb2, Clb3 and Clb4, Cdc28 is required for mitosis. No single Cln or Clb cyclin is essential for growth—the loss of any can be functionally compensated by other cyclins of the same type. Deletions of certain combinations of

*For correspondence. E-mail: pratima@bic.boseinst.ernet.in.

Keywords. yeast; Cdc28 kinase; checkpoint; cell cycle.

cyclins may, however, be lethal to the cell. The inhibitory protein Sic1 can bind to Cdc28 and inhibits its Cdc28/Clb G2 kinase activity. In *S. pombe*, Cdc2/Cdc28 protein was shown to have a checkpoint function so that cells carrying the *cdc2-3w* allele, in presence of the DNA replication inhibitor hydroxyurea, were not arrested at S/G2/M but continued through mitosis to generate cells with fragmented or no DNA (Enoch and Nurse 1990). In *S. cerevisiae* also, a mutation in this gene abrogates the cell-cycle arrest caused by DNA damage (Li and Cai 1997). In the presence of this mutation, *cdc13* cells undergo nuclear division and complete a round of cell cycle at the nonpermissive temperature. Dahmann *et al.* (1995) have shown that when Cdc28/Clb1–Clb6 G2 kinase is transiently inhibited by the expression of the Sic1 protein, cells enter START, rebud and rereplicate DNA without mitosis. Thus B cyclins are proposed to have a dual role (Dahmann *et al.* 1995). Cdc28/Clb kinase initiates the G1 to S phase transition and, in S/G2 phase, prevents the rereplication of DNA by inhibiting the formation of pre-replicative state of chromatin.

In this study we have used *S. cerevisiae cdc13* cells to identify more checkpoint genes required for G2/M arrest in response to DNA damage. Apart from the previously identified mutants like *mec1, mec3, rad17* and *rad24*, we have also isolated a novel *cdc28* mutant that allowed *cdc13* cells a premature entry to START from S phase, without mitosis and cytokinesis. In this respect, it is different from the previously identified mutation in this gene (Li and Cai 1997), which causes *cdc13* cells to enter START after undergoing nuclear division and cytokinesis.

Materials and methods

***Media*:** Yeast rich medium (YEPD) and minimal medium have been described earlier (Maiti and Sinha 1992). Two per cent raffinose was used for the YEP (raffinose) plates and 2% galactose + 2% raffinose was used for the YEP (galactose + raffinose) plates.

***Strains and plasmids*:** The *Escherichia coli* strain XL1-B was used for all plasmid preparations and manipulations. Yeast strains used were: US389 (MAT*a ura3 his3 trp1 leu2 bar1::LEU2 CDC13*), US369 (MAT*a ura3 his3 trp1 leu2 bar1::LEU2 cdc13*), US369-7 (MAT*a ura3 his3 trp1 leu2 bar1::LEU2 cdc13 cdc28-7C*), US369-7C (MAT*a ura3 trp1 leu2 his3 cdc13 cdc28-7C*) and US118 (MAT*a ura3 his3 leu2 trp1 cdc13*). US369-7C was generated by crossing US369-7 with US118. All the US yeast strains were obtained from Dr U. Surana, Institute of Molecular and Cell Biology, Singapore. The yeast strains US389 and US369 had a cassette carrying the HO box in between *GAL* promoter and a reporter gene (*lacZ*), integrated into their genome at the *ho* locus. The HO box allows transcription from the *GAL* promoter only when the cell passes through START, an entry point of the cell cycle (Nasmyth 1983). Plasmid YEp13 has been described in Broach *et al.* (1979). Plasmid pUS212, which carries *CDC28* genomic clone in YEp13, was also obtained from Dr U. Surana. Plasmid *CDC13*-YIplac211 contains a genomic clone of *CDC13* (*Saccharomyces* Genome Database, Stanford University, Stanford, USA. URL: http://genome-www.stanford.edu) in the integrative plasmid YIplac211 (Gietz and Sugino 1988). The plasmid was digested with *Xho*I for creating genomic integrations of *CDC13*.

***Strategy for isolation of checkpoint mutants*:** Normally, *cdc13* cells halt at the G2/M phase of the cell cycle at the nonpermissive temperature, 37°C. US369 was chosen to isolate checkpoint mutations which would allow cells to enter another round of cell cycle. Such mutant cells would express the *lacZ* gene when incubated on galactose plates at 37°C, owing to the presence of the HO box. *cdc13* mutant colonies, not containing such mutations, would not synthesize *b*-galactosidase owing to the G2/M arrest. This strategy was used to isolate checkpoint mutations in *cdc13* background. US369 strain was mutagenized according to Sikorski and Boeke (1991) by using the mutagen ethylmethanesulphonate to 30% survival. After mutagenesis the cells were plated for single colonies on YEP (raffinose) medium at 23°C. The colonies were then lifted on a nylon filter by replica plating and incubated on prewarmed YEP (raffinose) plates at 37°C for 6 hours. After this, the filter was placed on prewarmed YEP (galactose + raffinose) for 6 hours for induction of the GAL promoter. Thereafter, the colonies on the filter were assayed for blue colour (see below) indicative of the synthesis of *b*-galactosidase. Cells on the nylon filter were lysed by rapid freeze–thaw method by putting the filter in liquid nitrogen for about 30 seconds. The filter was then placed at 30°C on a Whatman No. 1 filter soaked in the assay buffer containing 25 µl of 33 mg/ml X-gal and 1.8 ml of Z buffer (60 mM $Na_2HPO_4.7H_2O$, 40 mM $NaH_2PO_4.7H_2O$, 10 mM KCl, 1 mM $MgSO_4$, 5 mM *b*-mercaptoethanol). Under these conditions US389 develops colour within half an hour. Mutant colonies showing colour within an hour of incubation were used for further studies. Out of about 8000 colonies tested, 20 showed a reproducible production of blue colour.

***DAPI staining*:** Cells were stained with the DNA staining dye DAPI (4′,6-diamidino-2-phenylindole) to study nuclear morphology. This was done according to the method described by Kikuchi *et al.* (1988). Stained cells were illuminated at wavelength of 340–360 nm and viewed using a Leitz fluorescence microscope, and the cells were photographed using a Wild MPS 45 camera.

***Cell morphology*:** Cells were grown in appropriate liquid medium at 23°C (permissive) up to mid-log phase and then the culture was transferred to 37°C (restrictive) for

6 hours. The cells were sonicated for 5 seconds at 50 watts using a Braunsonic 1510 sonicator. The morphology of the cells was observed through a Leitz fluorescence microscope and the cells were photographed using a Wild MPS 45 camera. Cells were considered large-budded when the diameter of the bud was at least 80% of mother cell diameter.

***Flow cytometry*:** This was done according to the protocol described by Lim *et al.* (1996). Flow cytometry was done in a FACSCalibur (Becton Dickinson).

All other methods have been described earlier (Maiti and Sinha 1992).

Results

Isolation of checkpoint mutations which allow cdc13 cells to reenter START

Checkpoint mutations were isolated using the strategy described in Materials and methods. The presence of these mutations lets *cdc13* cells reenter START, thus activating transcription through the *GAL* promoter to produce *b*-galactosidase. Presence of this enzyme was assayed in the mutagenized colonies and those that showed blue colour were selected for further studies. One such mutant clone, US369-7, was analysed further. Figure 1 shows that *b*-galactosidase is synthesized in the wild-type strain US389 and in the double mutant US369-7 cells, but not in US369 (*cdc13*) cells.

Cell morphology of US369-7 cells

Wild-type cells growing at 37°C show cells in all phases of the cell cycle. Thus, there are single G1 cells, cells with small buds in S phase, and cells with larger-sized buds undergoing mitosis and cytokinesis (figure 2a).

Figure 1. Expression of *b*-galactosidase in US389 (wild type), US369 (*cdc13*) and US369-7 (*cdc13 cdc28*) cells carrying GAL – HO box – *lacZ* cassette. US389 and US369-7 cells turned blue, indicating the synthesis of *b*-galactosidase.

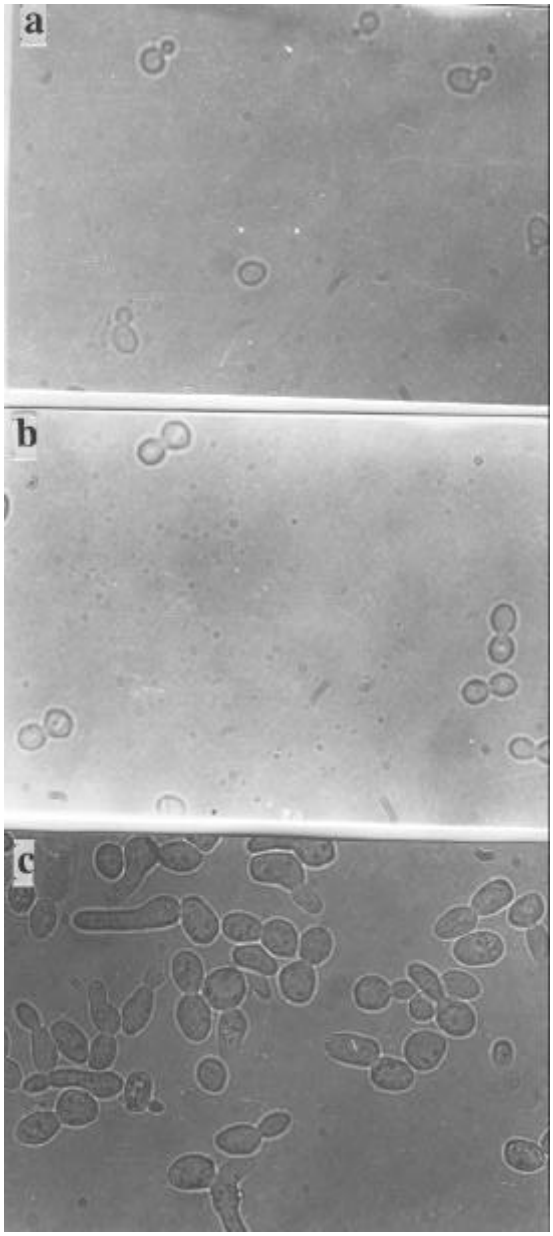

Figure 2. Morphology of US389 (wild type), US369 (*cdc13*) and US369-7 (*cdc13 cdc28*) cells. The cells were cultured for 6 hours at 37°C. (a) US389, the wild-type strain, shows cells in all phases of the cell cycle. (b) US369 (*cdc13*) shows large-budded morphology. (c) US369-7 (*cdc13 cdc28-7C*) cells display more than one bud. The additional bud could be at the distal end of the daughter, giving a chain like appearance, or close to the mother-bud neck giving rise to two apical projections from the same site.

When *cdc13* mutant cells are incubated at 37°C and the culture viewed after 6 hours, most of the cells are seen to be large-budded (figure 2b). The G1 cell throws out a bud, replicates its DNA but gets arrested at G2/M phase of the cycle in response to replication defects caused by the *cdc13* mutation. However, the bud continues to enlarge giving a dumbbell morphology; such a cell is known as the large-budded cell. Cells that have crossed

the *cdc13* execution point at the time of temperature shift would undergo cytokinesis and the daughter cells will again ultimately get arrested at G2/M owing to the *cdc13* mutation. When these cells are streaked for single colonies at 37°C and incubated overnight, the colonies consist of one or two large-budded cells, depending on whether the parent cell of the colony was a single cell or a budded cell which had already entered mitosis at the time of temperature shift.

When US369-7 cells were cultured for 6 hours at 37°C, most of them (93%) were found to be budded (figure 2c). About 31% of the budded population had two or more buds. Among cells with single large buds, 75% had the buds growing like apical projections from the mother cell and only 25% showed symmetric growth like the original *cdc13* mutant. The emergence of the second bud is indicative of passage of US369-7 cells through START, without cytokinesis.

DNA of these cells was stained with DAPI. Wild-type cells invariably showed nuclear division when buds reached a diameter about 60–70% that of the mother (figure 3). *cdc13* cells showed a single nucleus at the neck between the mother and daughter cell (figure 4a). US369-7 cells also showed presence of a single nucleus, mostly at the neck between the mother and original daughter, even though there were multiple buds (figure 4b). This indicates that entry to another round of cell cycle was occurring without mitosis and cytokinesis. When US369-7 cells were streaked for single colonies at 37°C and observed after overnight incubation, microcolonies consisting of 4–10 cells could be observed, suggesting a repeated budding pattern.

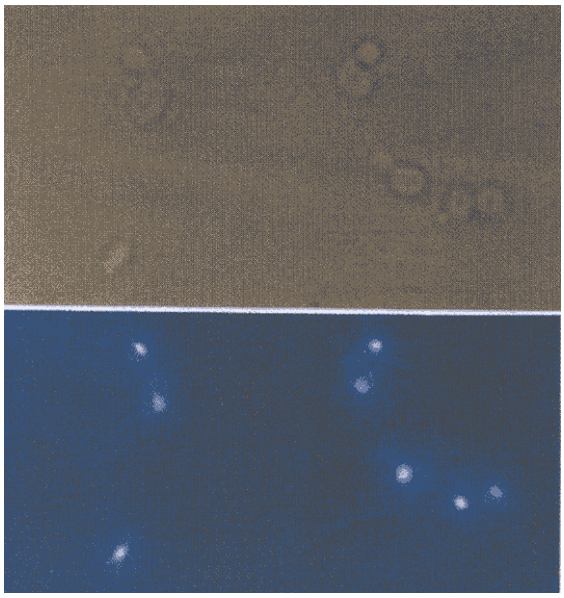

Figure 3. Nuclear morphology of DAPI-stained US389 (wild type) cells after 6 hours of incubation at 37°C. Nuclear division can be seen in the large-budded cells.

The checkpoint mutation in US369-7 is in the CDC28 gene

To examine the phenotype of the checkpoint mutation alone, the *CDC13* gene was integrated in US369-7 cells. US369-7/*CDC13* cells were unable to grow at 37°C. An examination of microcolonies formed on plates after overnight incubation at 37°C showed the same repeated pattern of budding, except that the number of buds was even higher than in US369-7 cells. More than 90% of the multiply budded cells had a single nucleus, not necessarily at the neck (not shown). Thus it was clear that this mutation was causing an exit from the cycle in the absence of mitosis and cytokinesis. It is known that Cdc28/Cln G1 kinase leads to apical growth of buds and that Cdc28/Clb1–2 G2 kinase is required to switch this apical growth to isotropic growth (Lew and Reed 1993). If the G2 kinase is inactivated, the buds become elongated and switch from isotropic to apical growth.

To test if the checkpoint mutation in US369-7 was a mutation in the *CDC28* gene, this strain was transformed with the plasmid pUS212 carrying the *CDC28* gene. The transformed cells were found to revert to *cdc13* phenotype—US369-7/*CDC28* transformants were arrested at 37°C with the large-bud morphology. The complementation of the checkpoint phenotype by the *CDC28* gene suggested that the checkpoint mutation was a *cdc28* mutational allele. This was confirmed by checking for complementation with *cdc28-IN*, a well-characterized recessive mutation in the *CDC28* gene (Piggott *et al*. 1982). It was found that the diploid *cdc28* (this study) × *cdc28-1N* failed to grow at 37°C. The failure of two recessive mutations to complement each other places both in the same complementation group and, therefore, places the checkpoint mutation of US369-7 in the *CDC28* gene. We call this allele *cdc28-7C*; C implies a defect in the checkpoint function of this gene.

Flow cytometry analysis of the mutant cells

The repeated budding pattern exhibited by US369-7 cells suggests that these cells would progressively increase their DNA content. To determine the DNA content of US369-7 cells flow cytometry analysis was done. Figure 5A shows the population of cells with G1 (haploid) or G2 (diploid) DNA content in wild-type (US389), *cdc13* (US369) and *cdc13 cdc28-7C* (US369-7) cultures at 23°C (top row) and after 4 hours of growth at 37°C (bottom row). At 23°C all the three growing cultures showed characteristic distribution of cells with G1 and G2 DNA, suggesting that cells in all phases of the cell cycle were present in the three cultures. At 37°C, however, only the wild-type cells showed this distribution. US369 cells were arrested, as expected, with G2 DNA content. US369-7 cells showed a DNA distribution that varied more, and cells were present that had higher DNA content than US369 cells. Figure 5B shows that when *CDC13*

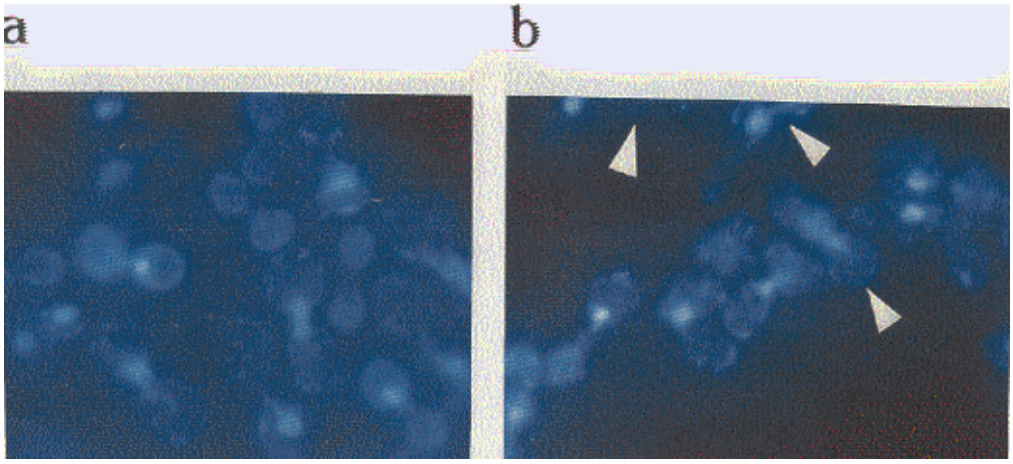

Figure 4. Nuclear morphology of DAPI-stained US369 (*cdc13*) and US369-7 (*cdc13 cdc28*) cells after 6 hours incubation at 37°C. The large-budded cells of *cdc13* (a) and the multibudded cells of *cdc13 cdc28* (b) carry a single nucleus at the neck between the mother and daughter cells, suggesting a block of mitosis. Multiply budded cells of US369-7 are shown by arrowheads.

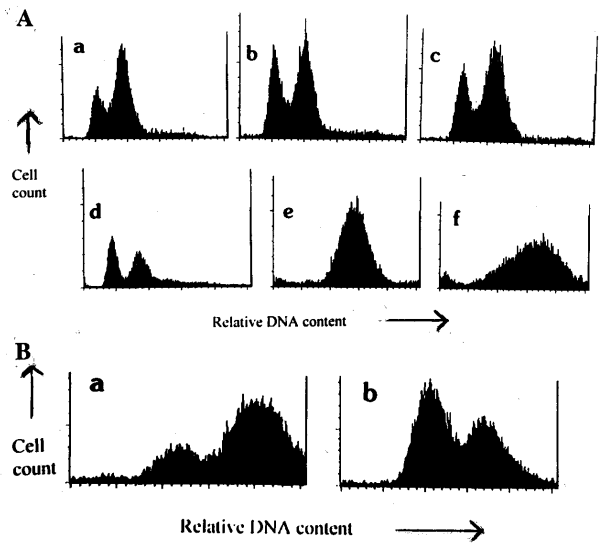

Figure 5. Flow cytometry analysis of mutant and wild-type cells. (A) Flow cytometry analysis of wild-type (US389), *cdc13* (US369) and *cdc13 cdc28-7C* (US369-7C) cells at 23°C (respectively a, b, c) and 37°C (respectively d, e, f). The cells were harvested in their exponential phase of growth in YEPD and were processed for analysis of their DNA content by staining with propidium iodide. The X-axis shows fluorescence caused by propidium iodide, a measure of DNA content, and the Y-axis represents relative number of cells. Cells in G1 phase are represented by the first peak while the second peak corresponds to cells in the G2 phase. (B) *cdc28-7C* cells have an average DNA content that is lower than 2N. Flow cytometry analysis of (a) US369-7C (*cdc13 cdc28-7C*) and (b) US369-7C/*CDC13* cells. The average DNA content of cells in (a) is higher than that in (b).

was integrated in US369-7, the average DNA content of these cells became lower than that of US369-7 (*cdc13 cdc28-7C*) and US369 (*cdc13*) cells. This suggests that *cdc28-7C* cells were exiting from the cycle before G2, perhaps at an earlier time point in the S phase.

Discussion

We have isolated a *cdc28* mutant of *S. cerevisiae* that abolishes the dependence of S phase on completion of M phase of the cell cycle. Thus *cdc13 cdc28-7C* cells rebud without mitosis and accumulate DNA content that is higher than 2*N*, the DNA content of *cdc13* mutants alone (figure 5A, e & f). This suggests that *cdc13 cdc28-7C* cells exit from S phase and reenter another round of the cell cycle to reinitiate DNA synthesis and increase their DNA content. Since the cells are able to initiate bud formation, it can be inferred that this mutation does not alter the START-specific G1 kinase activity of this gene. This suggests that the Cdc28/Clb G2 kinase was affected. In this *cdc28-7C* is different from the previously identified mutation *cdc28-5M* in the *CDC28* gene, which abolishes the dependence of M phase on the completion of S phase (Li and Cai 1997).

Dahmann *et al.* (1995) have shown that when the G2 kinase is transiently inhibited by synthesis of the Sic1 protein, cells reenter START, initiate bud formation, and rereplicate their DNA without mitosis. This was consistent with the phenotypes of *sim1* and *sim2* mutations isolated by them. In the absence of Clb1, Clb2, Clb3 and Clb4 cyclins, under nonpermissive conditions these mutants displayed the same phenotypes as the cells transiently depleted in Cdc28/Clb G2 kinase activity. The mutations were shown to affect the remaining Cdc28/Clb5–Clb6 kinse activity. The authors propose that, in the absence of all the B cyclins, START-specific transcription is activated. Cdc28–Cln kinase gets acti-

vated, initiates bud formation, and the cells reduplicate their DNA without nuclear division. It is not clear how the cells reenter S phase in the absence of the Clb5 and Clb6 cyclins. The authors suggest that when START-specific transcription is activated transcription of *CLB5* and *CLB6* might also be activated. Enough protein may be synthesized to drive G1 to S phase transition. Alternatively, it is proposed that Cdc28/Clb5 kinase activity may be low enough to allow rebudding but may be high enough for causing G1 to S phase transition.

The phenotype of *cdc28-7C* cells mimics that of the cells in which B cyclins are transiently inhibited or that of *sim1* and *sim2* mutants. It is possible that this allele has caused a loss of Cdc28/Clb G2 kinase activity so that it is not sufficient for preventing rebudding. However, the residual activity may be sufficient to initiate DNA replication. This notion is consistent with the proposal of Dahmann *et al.* (1995) that lower levels of Cdc28/Clb kinase activity may be required for G1 to S phase transition than the levels required for rebudding. *In vitro* assays for this Cdc28/Clb mutant kinase activity, using various purified cyclins, could help in determining the specific roles of various cyclins in giving rise to this phenotype. DNA sequencing of the *cdc28-7C* mutation will help in studying the structure–function relation of the Cdc28 protein.

Acknowledgements

We are grateful to Dr Uttam Surana, Institute of Molecular and Cell Biology, Singapore, in whose laboratory the mutants were isolated, for the gift of strains and plasmids and for discussions. The laboratory assistance of Asraf Ali Molla is gratefully acknowledged. S.K.G. was supported by a Council of Scientific and Industrial Research fellowship (9/15(175)/96 EMR-II).

References

Broach J. R., Strathern J. N. and Hicks J. B. 1979 Transformation in yeast: development of a hybrid cloning vector and isolation of the *CAN1* gene. *Gene* **8**, 121–133.

Dahmann C., Diffley J. F. X. and Nasmyth K. A. 1995 S-phase-promoting cyclin-dependent kinases prevent rereplication by inhibiting the transition of replication origins to a pre-replicative stage. *Curr. Biol.* **5**, 1257–1269.

Elledge S. J. 1996 Cell cycle checkpoints: preventing an identity crisis. *Science* **274**, 1664–1672.

Enoch T. and Nurse P. 1990 Mutation of fission yeast cell cycle control genes abolishes dependence of mitosis on DNA replication. *Cell* **60**, 665–673.

Gietz R. D. and Sugino A. 1988 New yeast–*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base-pair restriction sites. *Gene* **74**, 527–534.

Hartwell L. H. and Weinert T. A. 1989 Checkpoints: controls that ensure the order of the cell cycle events. *Science* **246**, 629–634.

Kikuchi Y., Simatake H. and Kikuchi A. 1988 A yeast gene required for the G1-to-S transition encodes a protein containing an A-kinase target site and an ATPase domain. *EMBO J.* **17**, 1175–1182.

Lew D. J. and Reed S. I. 1993 Morphogenesis in the yeast cell cycle: regulation by cdc28 and cyclins. *J. Cell Biol.* **120**, 1305–1320.

Li X. and Cai M. 1997 Inactivation of the cyclin-dependent kinase Cdc28 abrogates cell cycle arrest induced by DNA damage and disassembly of mitotic spindles in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **17**, 2723–3734.

Lim H. H., Goh P. Y. and Surana U. 1996 Spindle pole body separation in *Saccharomyces cerevisiae* requires dephosphorylation of the tyrosine 19 residue of Cdc28. *Mol. Cell. Biol.* **16**, 6385–6397.

Lin J.-J. and Zakian V. A. 1996 The *Saccharomyces CDC13* protein is a single-strand TG_{1-3} telomeric DNA-binding protein *in vitro* that affects telomere behaviour *in vivo*. *Proc. Natl. Acad. Sci. USA* **93**, 13760–13765.

Maiti A. K. and Sinha P. 1992 The *mcm2* mutation of yeast affects replication, rather than segregation or amplification of the two micron plasmid. *J. Mol. Biol.* **224**, 545–558.

Mendenhall M. D. and Hodge A. E. 1998 Regulation of Cdc28 cyclin dependent protein kinase activity during the cell cycle of the yeast *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **62**, 1191–1243.

Nasmyth K. 1983 Molecular analysis of a cell lineage. *Nature* **302**, 670–676.

Piggott J. R., Rai R. and Carter B. L. A. 1982 A bifunctional gene product involved in two phases of the yeast cell cycle. *Nature* **298**, 391–393.

Sikorski R. S. and Boeke J. D. 1991 *In vitro* mutagenesis and plasmid shuffling: from cloned gene to mutant yeast. *Methods Enzymol.* **194**, 302–318.

Weinert T. A., Kiser G. L. and Hartwell L. H. 1994 Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair. *Genes Dev.* **8**, 652–665.

Received 29 November 2000