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The *Schizosaccharomyces pombe cdc5⁺* gene encodes an essential protein with homology to c-Myb

Ryoma Ohi, Dannel McCollum, Bilkis Hirani, Greg J.Den Haese, Xiaoe Zhang, John D.Burke, Kiandrea Turner and Kathleen L.Gould[1]

Department of Cell Biology, School of Medicine, Vanderbilt University, Nashville, TN 37232, USA
[1]Corresponding author

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The *Schizosaccharomyces pombe* $cdc5^+$ gene was identified in the first screen for cell division cycle mutants in this yeast. The $cdc5^+$ gene was reported to be required for nuclear division but because of its modest elongation and leaky nature at the non-permissive temperature, it was not investigated further. Here, we report the characterization of the single allele of this gene, *cdc5*-120, in more detail. The mutant arrests with a 2N DNA content and a single interphase nucleus. Further genetic analyses suggest that $cdc5^+$ gene function is essential in the G_2 phase of the cell cycle. We have cloned and sequenced the $cdc5^+$ gene. The deduced protein sequence predicts that Cdc5 is an 87 kDa protein and contains a region sharing significant homology with the DNA binding domain of the Myb family of transcription factors. Deletion mapping of the $cdc5^+$ gene has shown that the N-terminal 232 amino acids of the protein, which contain the Myb-related region, are sufficient to complement the $cdc5^{ts}$ strain. A *cdc5* null mutant was generated by homologous recombination. Haploid cells lacking $cdc5^+$ are inviable, indicating that $cdc5^+$ is an essential gene. A fusion protein consisting of bacterial glutathione *S*-transferase joined in-frame to the N-terminal 127 amino acids of the Cdc5 protein is able to bind to DNA cellulose at low salt concentrations. This evidence suggests that $cdc5^+$ might encode a transcription factor whose activity is required for cell cycle progression and growth during G_2.

Key words: cdc5/cell cycle/c-Myb/*Schizosaccharomyces pombe*

Introduction

The fission yeast *Schizosaccharomyces pombe* has proved to be a useful organism for elucidating the mechanisms which govern cell cycle progression in eukaryotic cells. A number of conditionally lethal cell division cycle (*cdc*) mutants were isolated which arrest at a specific point in the cell cycle upon incubation at a restrictive temperature (Nurse *et al.*, 1976; Nasmyth and Nurse, 1981). By studying these mutants, it has been possible to identify and characterize at the molecular level some of the genes and their respective protein products which constitute the cell cycle regulatory machinery. Genetic and biochemical analyses of the cell cycle in a wide variety of eukaryotic organisms have shown that many of the genes important for the successful traverse of the cell cycle are functionally conserved from yeast to humans.

In particular, the product of the *S.pombe* $cdc2^+$ gene, the Cdc2 protein kinase, has been the subject of intense investigation during the last few years. The $cdc2^+$ gene was identified through the first screen for *cdc* mutants in *S.pombe* (Nurse *et al.*, 1976). The $cdc2^{ts}$ strains are unique among the other *cdc* mutants in that $cdc2^+$ gene function is required both in G_1 prior to the beginning of DNA replication and in G_2 for entry into mitosis (Nurse and Bissett, 1981). Mutants allelic to $cdc2^+$ were also identified which underwent cell division at a reduced size (reviewed in MacNeill and Nurse, 1989). These findings demonstrated that $cdc2^+$ plays a central role in the regulation of the *S.pombe* cell cycle. It has since been shown that $cdc2^+$ homologs from diverse organisms are able to complement *S.pombe* $cdc2^{ts}$ mutants, and that $cdc2^+$ function is essential for entry into mitosis in all eukaryotic cells (reviewed in Nurse, 1990).

Two other *cdc* mutants identified in these original screens, $cdc13^{ts}$ and $cdc25^{ts}$, have also been studied in great detail. Both are required for the G_2/M transition. The $cdc13^+$ gene encodes a cyclin B homolog which is required for Cdc2 function (reviewed in Hunt, 1991), and the $cdc25^+$ gene encodes a protein phosphatase which activates Cdc2 (reviewed in Millar and Russell, 1991). Homologs of these genes have also been found in higher eukaryotes and detailed biochemical studies have demonstrated that the interactions between Cdc2, cyclin B and Cdc25 are fundamentally similar in all eukaryotic organisms (reviewed in Solomon, 1993).

At least five other *cdc* genes ($cdc1^+$, $cdc5^+$, $cdc6^+$, $cdc27^+$ and $cdc28^+$) are thought to be required for the G_2/M transition. Mutants defective in these genes arrest with a 2N content of DNA and have been proposed to arrest in G_2 or late S phase since they do not exhibit the characteristics of mitotic cells (Nurse *et al.*, 1976; Nasmyth and Nurse, 1981). The $cdc27^+$ gene has been cloned and sequenced. The predicted Cdc27 protein shares no homology with any of the protein sequences found within the databases and its function is unknown (Hughes *et al.*, 1992). The sequences of the $cdc1^+$, $cdc6^+$ and $cdc28^+$ genes have not been reported and the functions of their protein products are also unknown. In this report, we present a detailed characterization of the $cdc5^{ts}$ mutant. In addition, we show that the predicted Cdc5 protein sequence contains a region which bears significant sequence identity to the DNA binding domain found within Myb-related proteins. We demonstrate that $cdc5^+$ is an essential gene whose function is required during the G_2 phase of the cell cycle and that the Myb-like DNA binding domain of Cdc5 exhibits affinity for DNA when subjected to DNA–cellulose chromatography.

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Results

Phenotype of the cdc5-120 mutant

The *S.pombe* mutant strain, *cdc5*-120, was originally described as being defective for nuclear division at a restrictive temperature of 35°C (Nurse *et al.*, 1976). The *cdc5*-120 mutant was reported not to arrest cell division completely at 35 or 36°C (Nurse *et al.*, 1976; Nasmyth and Nurse, 1981). We have also found that *cdc5*-120 is leaky at 35°C (data not shown). However, when an exponentially growing culture of *cdc5*-120 cells was shifted from 25 to 37°C, cell division ceased 4.5 h following the shift to the non-permissive temperature in either rich medium or minimal medium (Figure 2A and B). On solid medium, it should be noted that *cdc5*-120 is stably arrested at 36°C as well as at 37°C, although revertant colonies begin to form 3–4 days following incubation at 36°C (data not shown).

Observation of *cdc5*-120 cells incubated at 37°C revealed that they elongated only moderately when compared with cells grown at the permissive temperature (Figure 1A). After 5 h at 37°C in rich medium, *cdc5*-120 cells elongated to an average length of 18 μm. Wild type cells grown under the same conditions reached an average length of 14.5 μm at the time of septation. Thus, *cdc5*-120 cells do not elongate to nearly the same extent as other well-characterized *cdc* mutants such as $cdc2^{ts}$ (Nurse *et al.*, 1976). Indeed, the lack of continued growth as *cdc5*-120 cells are held at the non-permissive temperature is indicative of a cell growth defect. The morphology of $cdc5^{ts}$ cells at the non-permissive temperature was also dissimilar to other well-characterized *cdc* mutants in that $cdc5^{ts}$ cells incubated at the restricitve temperature for 5 h appeared wider than wild type cells grown under the same conditions (Figure 1A). The observed increase in diameter was quantified and found to be small but reproducible. Whereas wild type cells were 4.50 μm in diameter, $cdc5^{ts}$ cells were 4.85 μm in diameter after 5 h at 37°C. The increased diameter of the $cdc5^{ts}$ mutant coupled with the increased length predicts a 1.45-fold increase in cell volume.

When stained with the DNA binding dye DAPI, *cdc5*-120 cells incubated at the restrictive temperature were observed to arrest with a single nucleus with decondensed chromosomes (Figure 1A), suggesting that these cells do not arrest in mitosis. To define further the stage of the cell cycle in which the *cdc5*-120 mutant strain arrests, *cdc5*-120 cells were subjected to flow cytometric analysis. As a control, flow cytometric analysis was also conducted on *cdc10*-129 cells which arrest in G_1 with a 1N content of DNA (Nurse *et al.*, 1976). At the permissive temperature, *cdc10*-129 cells exhibit one peak of DNA corresponding to a 2N DNA content (Figure 1B). Exponentially growing *S.pombe* cells are predominantly in the G_2 and M phases of the cell cycle and a DNA peak corresponding to a 1N DNA content is not observed (Sazer and Sherwood, 1990). After 2 and 4 h at the non-permissive temperature, a culture of *cdc10*-129 cells begins to arrest in G_1 and a 1N DNA peak was

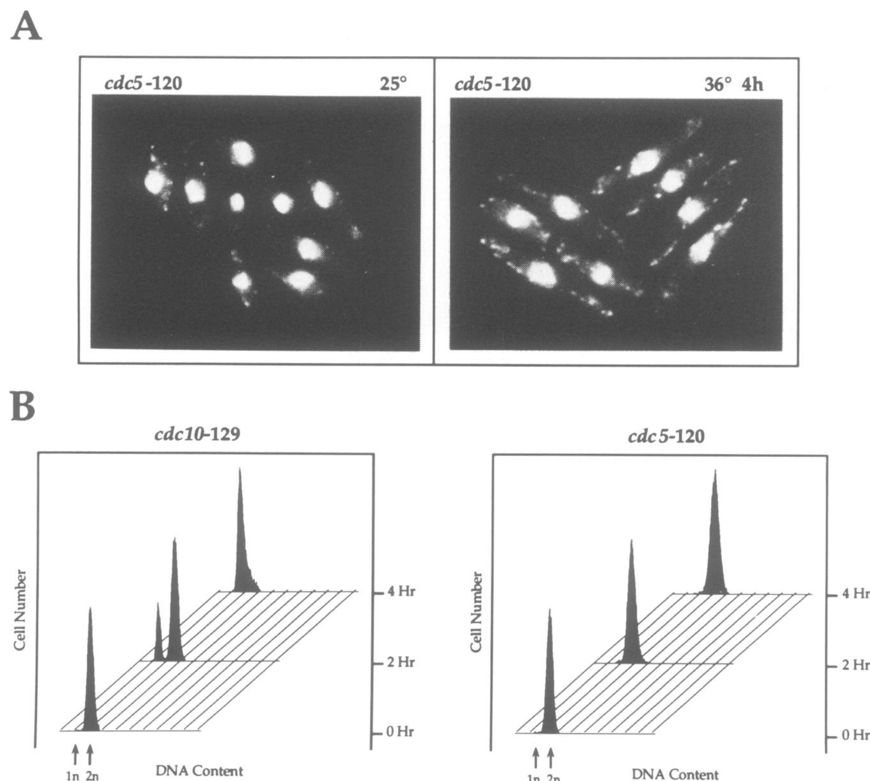

Fig. 1. Phenotypic characterization of the *cdc5*-120 mutant. (**A**) The *cdc5*-120 strain was grown to mid-exponential phase and shifted to 37°C for 4 h. Samples of cells were taken at the permissive (25°C) and restrictive (37°C) temperatures, fixed and stained with DAPI. (**B**) Cells of the genotypes *cdc10*-129 and *cdc5*-120 were grown to mid-exponential phase and shifted to 37°C. Samples of cells were taken at 0, 2 and 4 h following the temperature shift and fixed in ethanol. Cells were stained with propidium iodide and processed for flow cytometric analysis as described in Materials and methods. Linear fluorescence histograms show relative DNA content in arbitrary units on the horizontal axis and the cell number on the vertical axis. Arrows indicate the positions of 1N and 2N DNA peaks.

observed (Figure 1B). After 0, 2 and 4 h of incubation at the restrictive temperature, *cdc5*-120 cells exhibited only a single DNA peak consistent with a 2N content of DNA (Figure 1B).

cdc5$^+$ function is required after the hydroxyurea block point

To elucidate in which phase of the cell cycle *cdc5*$^+$ gene activity is required, the execution point of *cdc5*-120 was sequenced with respect to the point in the cell cycle blocked by hydroxyurea (HU), an inhibitor of DNA replication. Wild type and *cdc5*-120 cells were arrested for 4 h with HU and released into rich medium at the non-permissive temperature. To ensure that the function of the *cdc5*^{ts} allele was impaired in *cdc5*-120 cells, both cultures were incubated at 37°C during the last hour of treatment with HU. Following removal of the inhibitor, wild type cells began to grow exponentially at 37°C. In contrast, *cdc5*-120 cells showed only a slight increase in cell number at the non-permissive temperature (Figure 2C). When released from a HU block at 25°C, *cdc5*^{ts} cells continued exponential growth in parallel with wild type cells (Figure 2D). These data suggest that *cdc5*-120 cells block with or after the HU block and before cell division.

cdc5^{ts} cells are not defective in DNA replication

The previous experiments demonstrated that *cdc5*$^+$ function is required after the HU block and that *cdc5*-120 cells arrested at the restrictive temperature do not contain condensed chromatin. These observations suggest that *cdc5*$^+$ is involved in either late S phase or G_2. It has been previously demonstrated that a *cdc17* (DNA ligase) *rad1* double mutant displays a greatly increased lethality at the restrictive temperature compared with the *cdc17* mutant alone (Al-Khodairy and Carr, 1992; Rowley *et al.*, 1992). This occurs because the *rad1* mutation allows cells with improperly replicated DNA to enter mitosis and die. In contrast, a double mutant between *cdc25*-22, which blocks after DNA replication in G_2, and *rad1* displays no increased lethality at the restrictive temperature (Al-Khodairy and Carr, 1992; Rowley *et al.*, 1992). In order to determine whether *cdc5*$^+$ is essential for DNA replication, we constructed a *cdc5*-120 *rad1* double mutant using a *rad1* deletion allele (Sunnerhagen *et al.*, 1990). The viability of the *cdc5*-120 *rad1::ura4*$^+$ strain was compared with *cdc5*-120 alone after incubation on plates at 36°C for 0–9 h. As a positive control, we assayed the viability of the *cdc21*-M68 mutant at the restrictive temperature when combined with the *rad1::ura4*$^+$ mutation. The *cdc21*-M68 mutant was chosen because it is thought to be defective in DNA replication although, like *cdc5*-120, it arrests with a 2N DNA content (Coxon *et al.*, 1992). Finally, a *cdc25*-22 *rad1::ura4*$^+$ strain was used as a negative control, because *cdc25*$^+$ does not play a role in S phase. As in previous experiments both the *cdc25*-22 and *cdc25*-22 *rad1::ura4*$^+$ mutant cells displayed little decrease in viability with time at 36°C (Figure 3A). However, the *cdc21*-M68 *rad1::ura4*$^+$ cells lost viability much more rapidly than *cdc21*-M68 cells alone (Figure 3B), which is consistent with *cdc21*$^+$ being involved in S phase. Both *cdc5*-120 *rad1::ura4*$^+$ and *cdc5*-120 cells showed little decrease in viability with time at the restrictive temperature (Figure 3C), suggesting that *cdc5*$^+$ is not involved in DNA replication. This is supported by the fact that both *cdc5*-120 *rad1::ura4*$^+$ (Figure 4C) and *cdc25*-22 *rad1::ura4*$^+$ combinations arrested at the restrictive temperature as elongated cells with a single nucleus, whereas *cdc21*-M68 *rad1::ura4*$^+$ cells did not elongate substantially at 36°C and appeared to enter mitosis (not shown).

cdc5$^+$ is not essential for growth in G_1

Wild type *S.pombe* cells in rich medium grow primarily during G_2 and undergo little growth in other phases of the cell cycle (reviewed in Forsburg and Nurse, 1991). Because *cdc5*-120 cells elongate only modestly at the restrictive temperature (see Figure 1A) and block in G_2 (see above), the possibility existed that *cdc5*$^+$ gene function was required primarily for growth during the cell cycle. The cell cycle arrest of *cdc5*^{ts} cells might then be a consequence of a general growth defect. To examine this possibility, we asked whether *cdc5*$^+$ function was required for growth of *cdc5*^{ts} cells at a different stage of the cell cycle. Growth during the G_1 phase of the cell cycle becomes necessary when cells re-enter the cell cycle from a G_0 state induced by nitrogen deprivation in order to achieve a critical cell size for the G_1/S transition (reviewed in Forsburg and Nurse, 1991).

Exponentially growing wild type, *cdc5*-120, *cdc11*-123 and *cdc10*-129 cells were incubated in medium lacking nitrogen for 20 h at 25°C. The four cultures were shifted to 37°C during the final hour of nitrogen starvation to inactivate the temperature-sensitive *cdc5*-120, *cdc11*-123 and *cdc10*-129 gene products. The cells were reinoculated into nitrogen-rich medium at 37°C to allow the cells to grow and re-enter the cell cycle. The *cdc11*-123 strain was chosen as a control because it is defective only for cytokinesis and not for cell growth (Nurse *et al.*, 1976). The *cdc10*-129 mutant was chosen as a control because it becomes stably arrested in G_1 at the non-permissive temperature (Nurse *et al.*, 1976). As expected, flow cytometric analyses revealed that all four strains arrested primarily in G_1 with a 1N content of DNA when starved for nitrogen (Figure 2E–H). Following the readdition of nitrogen to the media, *cdc10*-129 cells remained with a 1N DNA content, since *cdc10*$^+$ is required for the G_1/S transition (Figure 2F). Wild type, *cdc11*-123 and *cdc5*-120 cells progressed through G_1 and accumulated a 2N content of DNA with variable kinetics (Figure 2E, G and H). During the 6 h of refeeding, the overall mass of the *cdc5*-120 culture increased in parallel with wild type and *cdc11*^{ts} cells as measured by light scatter (data not shown). The average length of *cdc5*-120 cells also increased in parallel with the other two strains until 6 h following nitrogen readdition (Figure 2I). At this point, wild type cells had resumed exponential growth. These data indicate that Cdc5 is not critical for growth during the early stages of the cell cycle. Despite the parallel increase in cell length and mass during nitrogen refeeding (Figure 2I and data not shown), *cdc5*-120 cells transited the cell cycle more slowly than wild type or *cdc11*^{ts} strains (Figure 2E, G and H). Thus, while the *cdc5*-120 strain is not essential for progression through G_1, it does appear to be defective for some G_1-specific process(es) other than mass accumulation.

cdc5 cells arrest with aberrant morphology in the presence of the *cdc2*-3w mutation

Cells carrying the *cdc2*-3w mutation are defective for size control in the G_2 period of the cell cycle and enter mitosis at a reduced size as short cylindrical cells (Fantes, 1981).

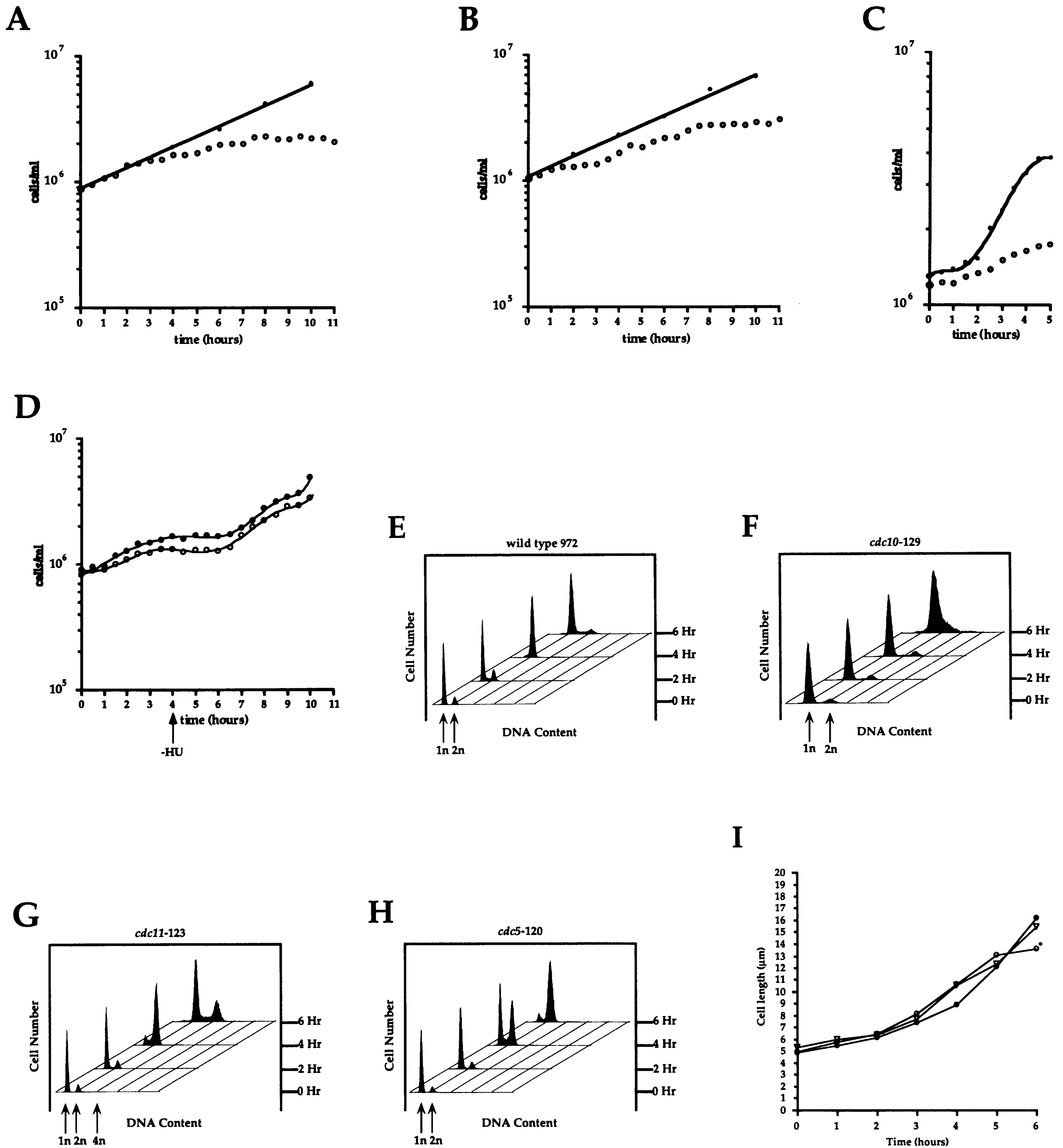

Fig. 2. Physiological analyses of the *cdc5*-120 mutant. (A) and (B) Experimental data corresponding to cells maintained at 25°C (●) were plotted on an arbitrary log scale. The real values per ml culture at 0 h are given within brackets next to the symbol key. (A) Exponentially growing *cdc5*-120 cells were incubated in yeast extract medium for 2 h at the permissive (25°C) temperature and then shifted to the restrictive (37°C) temperature for 9 h or kept at 25°C for an additional 8 h. ●, culture maintained at 25°C (1.6 × 10^6); ○, culture shifted to 37°C. (B) Exponentially growing *cdc5*-120 cells were incubated in minimal medium for 2 h at 25°C and then shifted to 37°C for 9 h or kept at 25°C for an additional 8 h. ●, culture maintained at 25°C (6.5 × 10^5); ○, culture shifted to 37°C. (C) Exponentially growing wild type and *cdc5*-120 cells were grown in yeast extract medium and treated with 12 mM HU for 4 h. During the last hour of incubation, the cells were placed at 37°C. The cells were resuspended in inhibitor-free medium at time 0 and placed at 37°C. ●, wild type; ○, *cdc5*-120. (D) Exponentially growing wild type and *cdc5*-120 cells were grown in yeast extract medium and treated with 12 mM HU for 4 h. The cells were resuspended in inhibitor-free medium and placed at 25°C. An arrow indicates the time of HU removal. ●, wild type; ○, *cdc5*-120. (E–H) Cells were starved for nitrogen for 20 h and then reinoculated into yeast extract medium prewarmed to 37°C. Samples were collected and fixed in ethanol at 0, 2, 4 and 6 h. Cells were stained with propidium iodide and subjected to flow cytometric analysis as described in Materials and methods. Linear fluorescence histograms show relative DNA content in arbitrary units on the horizontal axis and the cell number on the vertical axis. Arrows indicate the positions of 1N and 2N DNA peaks. (E) wild type; (F) *cdc10*-129; (G) *cdc11*-123; (H) *cdc5*-120. (I) The lengths of wild type, *cdc11*-123 and *cdc5*-120 cells during the nitrogen starvation and refeeding experiment were determined hourly for 6 h. The vertical axis represents cell length in micrometers and the horizontal axis represents time in hours. ○, wild type; ●, *cdc11*-123; ▽, *cdc5*-120. The asterisk indicates that only septated cells at this point were measured.

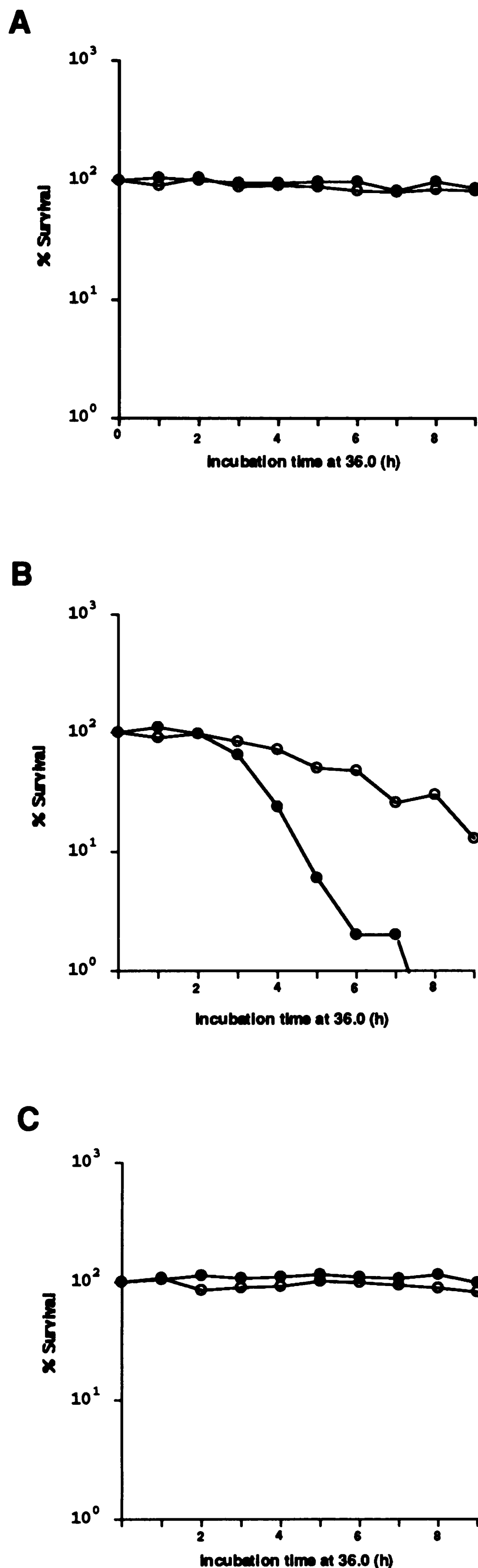

Fig. 3. Percent survival of *cdc* mutants in the presence of the *rad1* null mutation at 36°C. cdc$^-$ *rad1::ura4*$^+$ double mutants are shown in filled circles. The *cdc* mutant alone is shown in open circles. (**A**) The percent survival of *cdc25*-22 *rad1::ura4*$^+$ and *cdc25*-22 cells plotted against time of incubation at 36°C. (**B**) The percent survival of *cdc21*-M68 *rad1::ura4*$^+$ and *cdc21*-M68 cells plotted against time of incubation at 36°C. (**C**) The percent survival of *cdc5*-120 *rad1::ura4*$^+$ and *cdc5*-120 cells plotted against time of incubation at 36°C.

To address the effect of altering the normal size control over entry into mitosis on the *cdc5*^{ts} phenotype, a double mutant between *cdc5*-120 and *cdc2*-3w was constructed. Cultures of *cdc5*-120 *cdc2*-3w cells contained cells of widely varying size and shape after 5 h at 37°C, and were inviable (Figure 4D). Elongated cells as well as round or pear-shaped cells were seen. This is in contrast to the phenotypes of *cdc5*-120 (Figure 4B) and *cdc5*-120 *rad1::ura4*$^+$ (Figure 4C) cells which remained cylindrical and somewhat elongated at 37°C. DAPI staining of *cdc5*-120 *cdc2*-3w cells arrested at 37°C revealed that ~30% of these cells had entered mitosis and failed to segregate correctly their chromatin. Many of these cells displayed a cut phenotype (Hirano *et al.*, 1986; Samejima *et al.*, 1993) with a single nucleus bisected by a septum, or had a septum and only a single displaced nucleus (Figure 4E). We believe this phenotype to be numerically significant since similar abnormalities are found in only a small percentage of *cdc5*-120 or *cdc5*-120 *rad1::ura4*$^+$ cells at 37°C. Cells carrying the *cdc5*-120 mutation and a disrupted *wee1* gene displayed similar abnormalities at 37°C to those seen in the *cdc5*-120 *cdc2*-3w strain (data not shown).

Cloning of cdc5$^+$

To gain insight into the possible function of the *cdc5*$^+$ gene product, the *cdc5*$^+$ gene was cloned by complementation of the *cdc5*-120 mutant and sequenced. A *S.pombe* genomic library contained within the pUR19 vector was transformed into *cdc5*-120 *ura4*-D06 h^+ spheroplasts and uracil prototrophic transformants were selected at the restrictive temperature. From 55 000 Ura$^+$ transformants, 110 Cdc$^+$ Ura$^+$ colonies were obtained. Plasmids were recovered from 60 of these transformants and restriction mapping and Southern blot analyses indicated that they all contained the same gene. Five of these genomic clones (designated 1–5) were analyzed in greater detail (see Figure 6) after confirming that they enabled *cdc5*-120 cells to form colonies at 36°C upon retransformation. Experimental proof suggesting that these plasmids carried the *cdc5*$^+$ gene rather than a high dosage suppressor of the *cdc5*^{ts} mutant has been detailed in a later section. DNA sequencing of the smallest genomic clone (#2) revealed discontinuous open reading frames (ORFs) and suggested the presence of introns within the protein coding region of the *cdc5*$^+$ gene. To identify the protein coding region, a *S.pombe* cDNA expression library was screened using the *Apa*I–*Bst*XI restriction fragment of clone 2 as probe. Three overlapping cDNA clones of lengths 2412, ~1300 and ~600 bp were isolated. The DNA sequences of the inserts contained within genomic clone 2 and the three cDNA clones were determined on both strands.

The nucleotide sequence of the 2412 bp *cdc5*$^+$ cDNA clone and the predicted amino acid sequence are shown in Figure 5A. A comparison between the sequences of clone 2 and the longest cDNA clone revealed that the ORF of the *cdc5*$^+$ gene contained five introns (Figure 5A) of lengths 40, 66, 63, 69 and 64 bp (data not shown). Each of these intron sequences contained consensus splice donor, splice acceptor and branch point sequences known to be present in *S.pombe* introns (data not shown; Russell, 1989). An in-frame stop codon was located 6 bp upstream of the putative translation start site (Figure 5A). Additionally, no ORF was identified within 600 bp of genomic sequence located upstream of the putative translation start site (data not

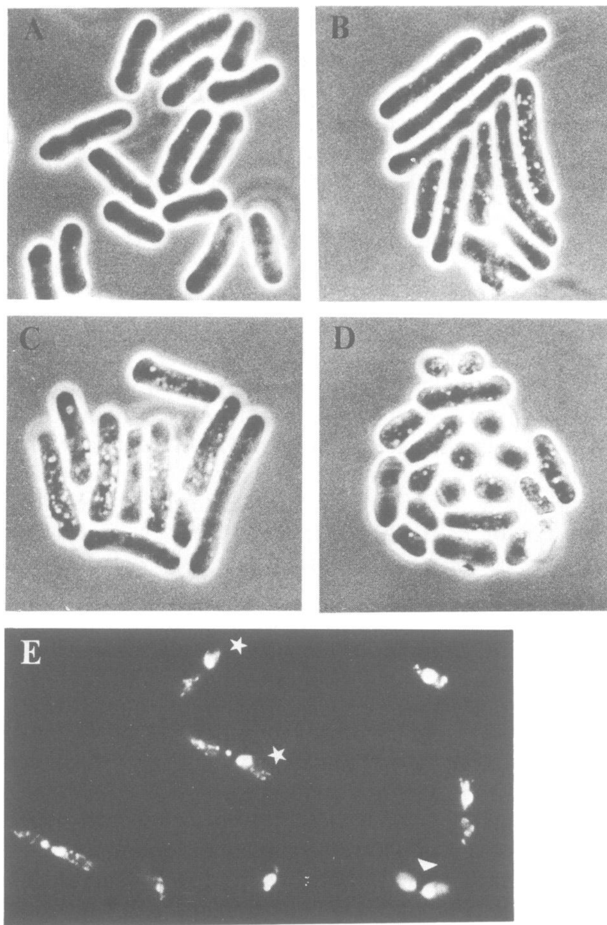

Fig. 4. Phase contrast micrographs were taken of wild type (**A**), *cdc5*-120 (**B**), *cdc5*-120 *rad1::ura4*$^+$ (**C**) or *cdc5*-120 *cdc2*-3w (**D**) cells incubated at 37°C for 5 h. (**E**) *cdc5*-120 *cdc2*-3w cells were incubated at 37°C for 5 h and then stained with DAPI. Stars indicate cells with a single displaced nucleus and a septum. A cell with a cut phenotype is indicated by a triangle.

shown). These observations suggest that the proposed translation start site for the *cdc5*$^+$ gene is most likely coinincident with the initiating methionine depicted in Figure 5. Northern blot analysis of total yeast RNA hybridized with a probe synthesized from the longest cDNA clone revealed a single message of ~2.5 kb (data not shown). These data suggest that the 2412 base pair cDNA clone is a nearly complete copy of the *cdc5*$^+$ mRNA.

Translation of the putative coding region of *cdc5*$^+$ predicts a 757 amino acid polypeptide of molecular weight 87 kDa. Comparison of the predicted protein sequence with those contained within the SwissProt database revealed that the protein product of the isolated gene is most related to human c-Myb and the family of c-Myb-related proteins. The sequence similarity is restricted to the DNA binding N-terminal region of c-Myb (Figure 5B) which is characterized by three imperfect repeats (R1, R2, R3) of 51–52 amino acids (reviewed in Lüscher and Eisenmen, 1990). It is significant to note that *cdc5*$^+$ encodes only two of the three Myb repeats. They are located in the N-terminal domain of Cdc5 between amino acid residues 4 and 105. Figure 5B shows the similarities of the Myb-related region of Cdc5 with the human c-*myb* protein and c-Myb-related proteins from a variety of organisms. The derived amino acid sequence of the c-Myb-related region of Cdc5 shares 33% identity with regions R2 and R3 of human c-*myb*. It shares 25, 33 and 30% identity with the c-Myb-related regions found in *Saccharomyces cerevisiae* transcription factors *REB1* and *BAS1*, and C1 of *Zea mays*, respectively. The remaining 651 amino acids of the polypeptide share no significant homology with other proteins contained within the SwissProt database.

To ascertain the genomic location of the *cdc5*$^+$ gene, the full-length cDNA clone was hybridized to bacteriophage and cosmid clones covering the entire *S.pombe* genome (a gift from Dr E.Maier; Maier *et al.*, 1992, 1993; Hoheisel *et al.*, 1993). The *cdc5*$^+$ gene was found to be located within cosmid 14d8 on the *Not*I (H) fragment of chromosome 1 <30 kb away from the *rhp51* gene (see Hoheisel *et al.*, 1993) (data not shown).

Minimal complementing region of cdc5$^+$

To define the minimal region of *cdc5*$^+$ required to complement the *cdc5*^{ts} strain, a series of fragments with 5′- and 3′-terminal deletions of the *cdc5*$^+$ gene were tested for their ability to promote colony formation of the *cdc5*-120 strain at 36°C (Figure 6). Deletion mutants at the 5′ end of the genomic clone were generated to determine whether or not specific sequences located upstream of the proposed coding region were required for complementation. The ability of these constructs to complement *cdc5*-120 suggests that sequences >186 bp upstream of the putative initiation ATG codon do not contain promoter or enhancer elements that are essential for the gene's expression. Deletion mutants at the 3′ end of the *cdc5*$^+$ genomic clone showed that only the N-terminal 232 amino acids of the proposed protein are required for complementation of the *cdc5*-120 mutant strain. As expected, deletion constructs which removed both the putative promoter region and the c-*myb*-like DNA binding domain abolished complementing activity (Figure 6). The minimal genomic *cdc5*$^+$ fragment required to rescue growth of *cdc5*-120 cells at the restrictive temperature on solid medium was found to lie between the *Xmn*I and *Pfl*MI restriction sites (Figure 6).

Despite the ability of most *cdc5*$^+$ genomic fragments to rescue the *cdc5*^{ts} mutant on agar plates, there were noticeable differences in the time and efficiency of colony formation following transformation. To quantitate some of these differences, the doubling times of some of the transformant strains in liquid medium at 37°C were measured. Transformant strains carrying genomic deletion mutants lacking both sequences upstream of the translation start site and in the C-terminal third of the predicted Cdc5 polypeptide displayed longer generation times than strains carrying full-length genomic clones (Figure 6). However, truncation of either 5′ untranslated sequences or up to one-third of the C-terminal protein coding sequences individually did not significantly affect strain generation times (Figure 6). Strains dependent on severly truncated forms of the gene displayed long generation times. Indeed, the *cdc5*-120 transformant strain carrying the minimal rescuing fragment (*Xmn*I–*Pfl*MI) showed extremely poor growth in liquid medium at the restrictive temperature (Figure 6).

Disruption of cdc5$^+$

To understand further the role of *cdc5*$^+$ gene function, a null allele of the gene was constructed by the one-step gene disruption method. The entire 2.0 kb *Apa*I–*Bgl*II coding region fragment of *cdc5*$^+$ was excised and replaced with

A

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                                                                                                      Ssp I
-41                                                                    GCTTTCTGCGAATTGTCAGGGTTTTGGCGAATATTTAGAAA
                ▼                ▼                ▼                                   Apa I
1    ATGGTTGTTTTAAAAGGAGGTGCCTGGAAAAATACAGAGGATGAAATCTTAAAGGCTGCTGTCAGTAAATACGGTAAAAACCAATGGGCCCGCATAAGTTCATTGCTGGTTCGCAAAACT
1     M   V   V   L   K   G   G   A   W   K   N   T   E   D   E   I   L   K   A   A   V   S   K   Y   G   K   N   Q   W   A   R   I   S   S   L   L   V   R   K   T
                                          Cla I           ▼
121  CCTAAACAATGTAAAGCTCGTTGGTATGAATGGATCGATCCAAGCATTAAAAAGACTGAATGGAGTCGTGAAGAGGACGAAAAATTATTACACTTAGCTAAGTTACTTCCTACACAGTGG
41    P   K   Q   C   K   A   R   W   Y   E   W   I   D   P   S   I   K   K   T   E   W   S   R   E   E   D   E   K   L   L   H   L   A   K   L   L   P   T   Q   W
                                                                                                                   Bcl I
241  CGGACGATTGCTCCAATTGTAGGTCGGACGGCCACTCAATGTTTAGAACGCTATCAAAAACTTCTTGATGATTTGGAAGCGAAAGAGAACGAGCAGTTGGGATTGATCAGTGGAGAAGGT
81    R   T   I   A   P   I   V   G   R   T   A   T   Q   C   L   E   R   Y   Q   K   L   L   D   D   L   E   A   K   E   N   E   Q   L   G   L   I   S   G   E   G
        Pvu II       Hpa I                                                                                BstX I
361  GCAGAAGCAGCTGCGCCTGTTAACGATCCAAATTCCCGGCTTCGTTTTGGCGAAGCAGAGCCCAATTTAGAAACCCTACCTGCACTCCCTGATGCCATTGACATGGATGAAGACGAAAAA
121   A   E   A   A   A   P   V   N   D   P   N   S   R   L   R   F   G   E   A   E   P   N   L   E   T   L   P   A   L   P   D   A   I   D   M   D   E   D   E   K

481  GAGATGTTGAGTGAAGCACGTGCACGTTTAGCAAACACACAAGGAAAGAAAGCCAAACGAAAGGATAGAGAAAAGCAGCTTGAATTAACTAGAAGATTGTCACATCTCCAAAAGCGAAGA
161   E   M   L   S   E   A   R   A   R   L   A   N   T   Q   G   K   K   A   K   R   K   D   R   E   K   Q   L   E   L   T   R   R   L   S   H   L   Q   K   R   R
        Pvu II       ▼                                                                             PflMI
601  GAATTGAAGGCAGCTGGAATCAATATAAAGCTCTTCCGTCGAAAGAAGAATGAAATGGATTATAACGCTTCTATTCCATTTGAAAAAAAGCCAGCGATTGGTTTTTATGATACCTCAGAA
201   E   L   K   A   A   G   I   N   I   K   L   F   R   R   K   K   N   E   M   D   Y   N   A   S   I   P   F   E   K   K   P   A   I   G   F   Y   D   T   S   E
                                          AvaII
721  GAGGACAGGCAAAATTTTCGGGAAAAGCGAGAAGCGGACCAGAAGATAATTGAAAATGGAATACGGAATAATGAAATGGAATCCGAAGGTCGAAAATTTGGTCATTTTGAAAAGCCAAAA
241   E   D   R   Q   N   F   R   E   K   R   E   A   D   Q   K   I   I   E   N   G   I   R   N   N   E   M   E   S   E   G   R   K   F   G   H   F   E   K   P   K

841  CCTATTGATAGAGTAAAAAAGCCCAACAAAGATGCCCAAGAAGAAAAAATGCGACGTCTTGCCGAGGCTGAGCAGATGAGCAAGAGGCGGAAGTTGAATTTACCTAGTCCTACTGTGTCT
281   P   I   D   R   V   K   K   P   N   K   D   A   Q   E   E   K   M   R   R   L   A   E   A   E   Q   M   S   K   R   R   K   L   N   L   P   S   P   T   V   S
                                                      Pvu I                                Sca I
961  CAAGATGAACTGGATAAAGTAGTGAAACTAGGCTTTGCAGGCGATCGTGCTCGCGCTATGACTGATACGACTCCAGATGCTAATTACAGTACTAATTTGTTAGGAAAGTATACACAAATT
321   Q   D   E   L   D   K   V   V   K   L   G   F   A   G   D   R   A   R   A   M   T   D   T   T   P   D   A   N   Y   S   T   N   L   L   G   K   Y   T   Q   I

1081 GAACGAGCTACTCCATTGAGAACACCAATTAGTGGTGAACTTGAGGGAAGAGAGGATTCAGTTACAATTGAAGTTAGAAATCAATTGATGAGGAACCGCGAACAATCTTCTCTCTTGGGT
361   E   R   A   T   P   L   R   T   P   I   S   G   E   L   E   G   R   E   D   S   V   T   I   E   V   R   N   Q   L   M   R   N   R   E   Q   S   S   L   L   G

1201 CAGGAAAGTATACCTTTACAACCTGGTGGTACTGGTTACACAGGAGTTACTCCCAGTCATGCAGCTAATGGCTCAGCTTTGGCAGCACCTCAAGCAACTCCTTTTAGAACACCACGTGAC
401   Q   E   S   I   P   L   Q   P   G   G   T   G   Y   T   G   V   T   P   S   H   A   A   N   G   S   A   L   A   A   P   Q   A   T   P   F   R   T   P   R   D
            Pvu II
1321 ACATTTTCTATTAATGCAGCTGCGGAGCGAGCAGGACGATTAGCAAGTGAAAGAGAAAATAAAATTCGATTGAAGGCTTTGCGAGAATTGCTTGCAAAACTACCTAAACCTAAAAACGAC
441   T   F   S   I   N   A   A   A   E   R   A   G   R   L   A   S   E   R   E   N   K   I   R   L   K   A   L   R   E   L   L   A   K   L   P   K   P   K   N   D

1441 TATGAACTAATGGAGCCTCGATTTGCGGACGAAACTGATGTTGAAGCTACTGTTGGAGTGCTTGAAGAAGACGCAACAGACCGAGAACGTCGTATCCAAGAACGTATTGCAGAAAAAGAA
481   Y   E   L   M   E   P   R   F   A   D   E   T   D   V   E   A   T   V   G   V   L   E   E   D   A   T   D   R   E   R   R   I   Q   E   R   I   A   E   K   E
                                                                                     BstX I
1561 AGGTTGGCAAAAGCGAGGAGGTCACAGGTTATTCAAAGAGATTTGATTCGACCATCTGTAACACAACCAGAAAAATGGAAGCGTTCACTTGAAAATGAAGATCCGACTGCAAATGTTTTA
521   R   L   A   K   A   R   R   S   Q   V   I   Q   R   D   L   I   R   P   S   V   T   Q   P   E   K   W   K   R   S   L   E   N   E   D   P   T   A   N   V   L

1681 TTGAAAGAAATGATTGCTTTGATTTCATCGGACGCCATTAATTATCCGTTTGGAAATTCAAAAGTGAAAGGAACGGCTAACAAAGTGCCTGATTTGTCAAATGAAGAAATTGAGAGGTGT
561   L   K   E   M   I   A   L   I   S   S   D   A   I   N   Y   P   F   G   N   S   K   V   K   G   T   A   N   K   V   P   D   L   S   N   E   E   I   E   R   C

1801 AGATTGTTACTAAAGAAAGAGATAGGGCAACTAGAAAGTGACGATTATATCCAATTCGAGAAGGAATTTTTGGAAACGTACAGTGCACTTCACAACACCTCAAGTTTGTTGCCAGGCTTG
601   R   L   L   L   K   K   E   I   G   Q   L   E   S   D   D   Y   I   Q   F   E   K   E   F   L   E   T   Y   S   A   L   H   N   T   S   S   L   L   P   G   L

1921 GTAATCTACGAGGAAGACGATGAAGACGTTGAAGCCGCTGAAAAGTTTTATACGAACGACATTCAACGAGATTTAGCTAAGAAAGCATTGGAATGTAACAAGTTGGAAAATCGGGTTTAT
641   V   I   Y   E   E   D   D   E   D   V   E   A   A   E   K   F   Y   T   N   D   I   Q   R   D   L   A   K   K   A   L   E   C   N   K   L   E   N   R   V   Y
          Bgl II Nde I                                     Sph I
2041 GATTTGGTTAGATCTTCATATGAGCAACGTAATTTTTTGATAAAGAAAATCTCGCATGCTTGGAAGGCTTTGCAAACAGAAAGGAAAAATTTAACGTGCTACGAATTTCTATACAATCAG
681   D   L   V   R   S   S   Y   E   Q   R   N   F   L   I   K   K   I   S   H   A   W   K   A   L   Q   T   E   R   K   N   L   T   C   Y   E   F   L   Y   N   Q
                                     Pvu II
2161 GAGCGATTAGCTCTACCTAATAGGCTTGAAGCAGCTGAAATAGAGCTAAGCAAAATGCAACAAATCGAGGCGTATGCTCAACAAGATTATGCTAGGGTTACTGGACAAAATTAAACTTCA
721   E   R   L   A   L   P   N   R   L   E   A   A   E   I   E   L   S   K   M   Q   Q   I   E   A   Y   A   Q   Q   D   Y   A   R   V   T   G   Q   N   *

2281 CTTCACTTTGTTTTAAATAATTGTCATAGCTTATCATTCATAGTGTATATTATCATGCCTGAAATAAAACGAAAGTTTTATAAAAAGAT(A)n
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B

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            (4) L K G G A W K N T E D E I L K A A V S K Y G K N Q W A R I S S L L V       R K T P K Q C K A R W Y E W I D P S    S. pombe cdc5
                L I K G P W T K E E D Q R V I E L V Q K Y G P K R W S V I A K H L K       G R I G K Q C R E R W H N H L N P E    H. sap. c-myb
Repeat 1        L K K G K W T Q E E D E Q L L K A Y E E H G P   H W L S I S M D I P       G R T E D Q C A K R Y I E V L G P G    S. cer. BAS1
                V K R G A W T S K E D D A L A A Y V K A H G E G K W R E V P Q K A G       R R C G K S C R L R W L N Y L R P N    Z. mays C1
                Y K K G L W T V E E D N I L M D Y V L N H G T G Q W N R I V R K T G L     K R C G K S C R L R W M N Y L S P N    A. thal. RL1
                E Q R G K W T A E E E Q E L A K L C A E K   E G Q W A E I G K T L         G R M P E D C R D R W R N Y V K C G    S. cer. REB1

           (56) I K K T E W S R E E D E K L L H L A K L L P   T Q W R T I A P I A P I V G R T A T Q C L E R Y Q K L L D D L    S. pombe cdc5
                V K K T S W T E E E D R I I Y Q A H K R L G   N R W A E I A K L L P       G R T D N A I K N H W N S T M R R K    H. sap. c-myb
Repeat 2        G R L R E W T L E E D L N L I S K V K A Y G   T K W R K I S S E M E       F R P S L T C R N R W R K I I T M V    S. cer. BAS1
                I R R G N I S Y D E E D L I I R L H R L L G   N R W S L I A G R L P       G R T D N E I K N Y W N S T L G R R    Z. mays C1
                V N K G N F T E Q E E D L I I R L H K L L G   N R W S L I A K R V P       G R T D N Q V K N Y W N T H L S K K    A. thal. RL1
                R A S N R W S V E E E L L . . .                                                                                  S. cer. REB1
```

Fig. 5. Nucleotide sequence of the *cdc5*$^{+}$ gene and deduced primary structure of the encoded protein. (**A**) The nucleotide sequence is numbered starting with the first base of the ATG initiation codon. The ORF is interrupted by five introns in the gene whose positions are indicated with arrowheads. The amino acid sequence is in single letter code underneath the DNA sequence. An asterisk denotes the position of the stop codon. Consensus polyadenylation signals are underlined. (**B**) Amino acid sequence alignment of Myb-related repeats. The predicted amino acid sequence of the N-terminus of Cdc5 is compared with human c-Myb (Majello *et al.*, 1986; Slamon *et al.*, 1986), *S.cerevisiae* BAS1 (Tice-Baldwin *et al.*, 1989), *Zea mays* C1 (Paz-Ares *et al.*, 1987), *Arabidopsis thaliana* GL1 (Oppenheimer *et al.*, 1991) and *S.cerevisiae* REB1 (Ju *et al.*, 1990). The two repeating motifs of ~50 amino acids are grouped. The characteristic tryptophans, critical cysteine and other amino acids which are identical in four or more proteins are in bold type. The numbers in parentheses represent residue numbers of Cdc5 which begin the line.

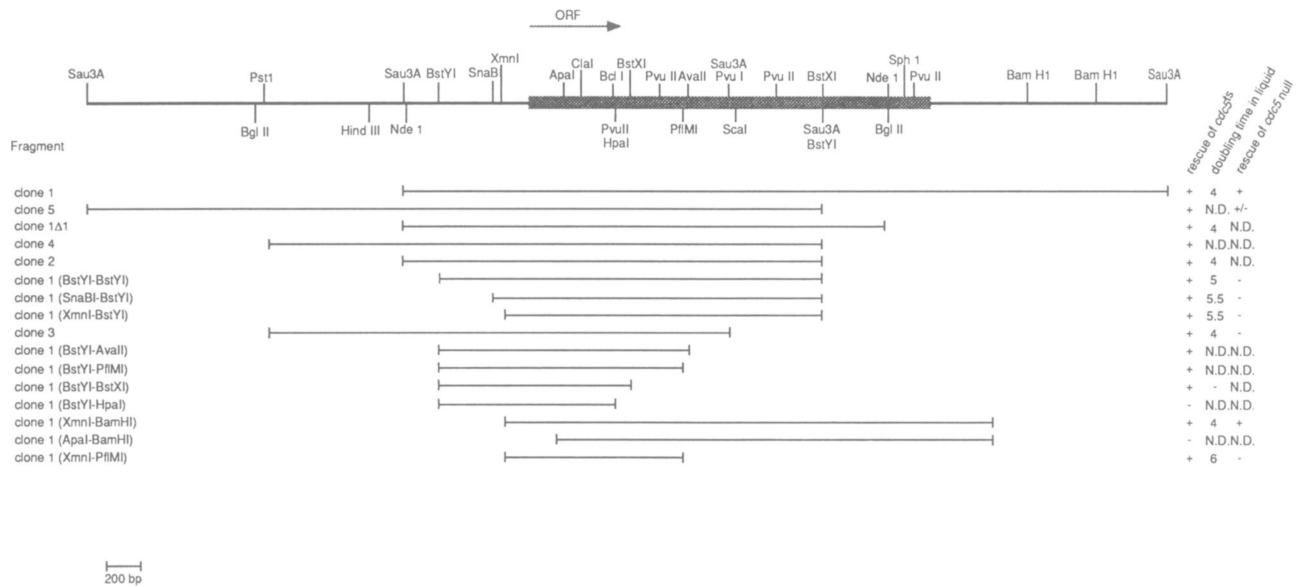

Fig. 6. Restriction map and deletion analysis of the *cdc5*$^+$ gene. The restriction map of the *cdc5*$^+$ gene locus was compiled from five genomic clones. Relevant restricition enzyme cleavage sites are indicated. The protein coding region is boxed. The direction of transcription is indicated with an arrow. A '+' indicates the ability of the plasmids to complement the mutants on solid media. A '−' indicates that the clone did not support growth of the mutants. N.D., not done. Doubling times of *cdc5*^{ts} transformants grown in liquid medium at 37°C are listed in hours.

the *ura4*$^+$ gene (Figure 7A). The resulting *cdc5::ura4*$^+$ fragment was subcloned into the yeast expression vector pIRT2 and was shown to be incapable of complementing the *cdc5*^{ts} mutant (data not shown).

A uracil auxotrophic diploid strain was transformed with the *cdc5::ura4*$^+$ fragment. Southern blot analysis of genomic DNA from 12 stable Ura$^+$ integrants digested with *Hin*dIII revealed that a single transformant appeared to be of the heterozygous genotype *cdc5*$^+$/*cdc5::ura4*$^+$. Genomic DNA from this single positive disruptant was subjected to further analysis to show unequivocally that one copy of the *cdc5*$^+$ gene had been successfully replaced by the *cdc5::ura4*$^+$ fragment. The *cdc5*$^+$ gene is released on a single restriction fragment when *S.pombe* genomic DNA is digested with either *Bam*HI or *Pst*I. Since the restriction enzyme *Eco*RV cuts within the *ura4*$^+$ coding region, but not within *Bam*HI or *Pst*I fragments containing the *cdc5*$^+$ gene, it was used to show that the diploid strain was indeed heterozygous for the *cdc5* null allele (Figure 7B). The probe that was used in the Southern analyses corresponded to the *Nde*I−*Xmn*I restriction fragment located upstream of the *cdc5*$^+$ coding region (Figure 7B).

In order to visualize the phenotype of the *cdc5* null mutant, the *cdc5::ura4*$^+$/*cdc5*$^+$ diploid was sporulated. When plated onto minimal medium lacking uracil, these spores failed to form colonies, indicating that *cdc5*$^+$ is an essential gene. Haploid spores carrying the null mutation germinated, but died as elongated cells. To obtain a more precise characterization of the terminal phenotype displayed by the *cdc5* null mutant, spores were germinated in liquid medium lacking uracil and observed microscopically. DAPI staining revealed that three phenotypes predominanted during the 20 h period in which the germination and growth of spores was observed: (i) elongated cells with a single circular or half-moon shaped nucleus, (ii) elongated cells with a single nucleus stretched along the length of the cell and (iii) elongated cells with two nuclei and a single septum (Figure 7C). A small percentage of cells displayed other varied phenotypes. Flow cytometric analysis of germinated spores indicated that the null mutant, like the *cdc5*^{ts} mutant, arrested with a 2N DNA content (data not shown).

The phenotype of a *cdc5* null mutant was also examined in a different manner. Cdc5 was expressed from a multicopy plasmid in a haploid strain containing a deletion of the genomic copy of *cdc5*$^+$. These cells were grown in rich medium to allow for plasmid loss and the phenotypes were assessed by DAPI staining (Figure 7D). The majority of cells which had lost the plasmid were elongated and contained a single nucleus with no septum, although a small portion of arrested cells did contain a single septum. These phenotypes were very similar to those observed in germinating *cdc5::ura4*$^+$ spores.

As described above, the full-length Cdc5 protein was able to rescue growth of the *cdc5* null mutant (Figure 6) when expressed from a multicopy plasmid. In contrast, C-terminal truncated forms of the protein, which were able to rescue growth of the *cdc5*^{ts} strain, were unable to rescue growth of the *cdc5* null mutant (Figure 6).

As proof that the cloned gene was in fact the *cdc5*$^+$ gene and not a suppressor, the diploid strain *cdc5::ura4*$^+$/*cdc5*-120 h^+/h^- was constructed. This strain is viable at the permissive temperature on minimal medium lacking uracil, but is Cdc$^-$ at 36°C. A diploid strain of the genotype *cdc5::ura4*$^+$/*cdc5*$^+$, in contrast, is able to form colonies at both 25 and 36°C (Figure 8). This result is possible only if the cloned gene is the wild type allele of *cdc5*$^+$. Therefore, we conclude that we have cloned the *cdc5*$^+$ gene.

DNA binding activity of the cdc5$^+$ gene product

Since *cdc5*$^+$ encodes a protein that shares significant homology to known DNA binding transcription factors, it was of interest to test whether the *cdc5*$^+$ gene product had DNA binding activity. The N-terminal domain of Cdc5 was fused in-frame with bacterial glutathione *S*-transferase (GST), and was subsequently purified from bacterial cells using

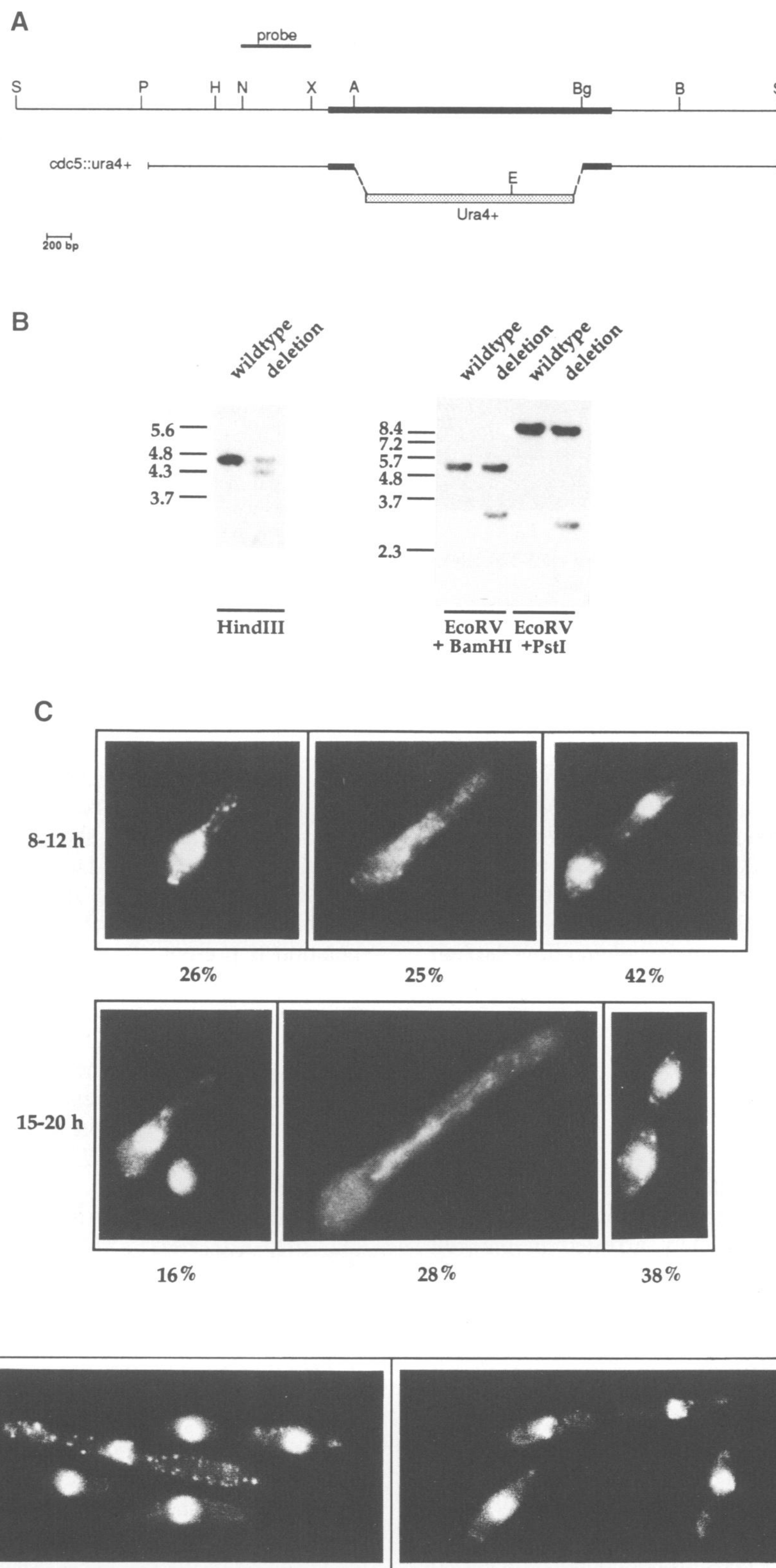

Fig. 7. Deletion of the *cdc5*$^+$ gene. **(A)** Restriction map of the *cdc5*$^+$ gene. Relevant restriction sites are indicated; *Sau*3A (S), *Pst*I (P), *Hin*dIII (H), *Nde*I (N), *Xmn*I (X), *Apa*I (A), *Bam*HI (B) and *Bgl*II (Bg). The construction of pTZ19R*cdc5::ura4* is shown below the map. The 2.0 kb *Apa*I–*Bgl*II fragment was removed and replaced with the *ura4* gene. The *Nde*I–*Xmn*I fragment used to probe Southern blots in panel B is indicated above the map. **(B)** Southern blot analysis of chromosomal *cdc5* deletion. Genomic DNAs from a wild type diploid and a heterozygous *cdc5::ura4* diploid were digested with *Hin*dIII, *Eco*RV and *Bam*HI, or *Eco*RV and *Pst*I, and then resolved on a 0.7% agarose gel. DNAs were blotted to GeneScreen plus and hybridized with the probe fragment indicated in panel A. Bands were visualized by autoradiography. Exposure time was 3 days at −70°C. **(C)** Phenotype of the *cdc5* null mutant. Spores from the *cdc5::ura4*$^+$/*cdc5*$^+$ diploid were germinated in minimal medium lacking uracil. Samples were fixed in formaldehyde at 8–12 h (early germination period) and 15–20 h (late germination period), and stained with DAPI. The relative percentages of cells with each phenotype are given. **(D)** Phenotype of Cdc5 loss. A haploid *cdc5* deletion strain carrying a Cdc5-expressing multipcopy plasmid was grown to mid-exponential phase in non-selective medium to allow plasmid loss. Cells were fixed in formaldehyde and stained with DAPI.

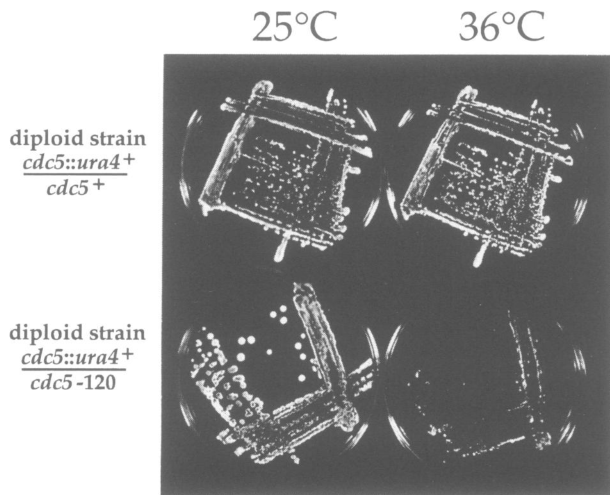

Fig. 8. Complementation of the *cdc5* null allele by the *cdc5*-120 allele. The heterozygous diploid strains, *cdc5::ura4*$^{+}$/*cdc5*$^{+}$ and *cdc5::ura4*$^{+}$/*cdc5*-120 were grown at 25°C and then replica plated and incubated at the permissive (25°C) and restrictive (36°C) temperatures.

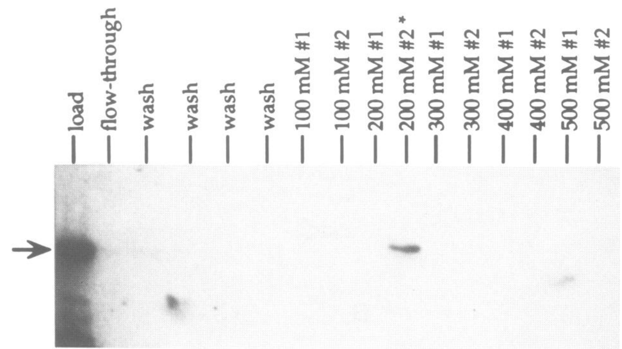

Fig. 9. DNA binding activity of Cdc5. The first 127 amino acids of Cdc5 were fused in-frame to the bacterial GST enzyme. The fusion protein was expressed in bacteria and purified as described in Materials and methods. Ten μg of the fusion protein were loaded onto a DNA–cellulose column. The column was washed and proteins were eluted with a salt step gradient as described in Materials and methods. One-thirtieth of each flow through, wash and eluate sample was resolved on an SDS–polyacrylamide gel along with 10 μg of the fusion protein, and then transferred to immobilon-P. The fusion protein was detected with antibodies to GST followed by [^{125}I]protein A, and the bands were visualized by autoradiography. The exposure time was 16 h. Ten μg of the fusion protein were in the lane designated 'load'. The asterisk depicts the lane in which the majority of the fusion protein was detected.

immobilized glutathione. The purified fusion protein was subjected to chromatography on DNA–cellulose (Figure 9). At 40 mM NaCl, traces of the fusion protein were detectable in the column flow through and in the wash fractions. Subsequent washing of the column with 100 mM NaCl did not elute a detectable amount of Cdc5 fusion protein. However, upon addition of 200 mM NaCl, the majority of the fusion protein was released from the column. Affinity-purified GST alone showed no specific DNA binding activity. It was detected primarily in the column flow through and traces were also observed in the 40 mM wash fractions (data not shown). From these data, we conclude that the N-terminal 127 amino acids of Cdc5 are capable of binding DNA at low salt concentrations.

Discussion

In this study, we have characterized the *cdc5*$^{+}$ gene of *S.pombe*. Our results confirm the initial observation that the *cdc5*-120 mutant is not completely arrested in the cell division cycle at 35 or 36°C in liquid culture (Nurse *et al.*, 1976; Nasmyth and Nurse, 1981). However, we have found that *cdc5*-120 cells block tightly when incubated in either yeast extract or minimal medium at 37°C (Figure 1A and B). This has allowed a further analysis of the *cdc5*^{ts} phenotype and the *cdc5*$^{+}$ gene function.

An asynchronous culture of *cdc5*-120 cells arrests with a 2N DNA content as measured by flow cytometry (this study), in agreement with previous results obtained by direct DNA measurements (Nurse *et al.*, 1976). DAPI staining revealed that arrested *cdc5*^{ts} cells do not contain condensed chromosomes, a feature characteristic of *S.pombe* cells as they enter metaphase. When subjected to treatment with HU, a well-known inhibitor of DNA replication, *cdc5*-120 cells, like wild type cells, accumulated in S phase with little increase in cell number (data not shown). Upon concurrent removal of the inhibitor and placement of the mutant cells at the restrictive temperature, cell number measurements indicated that the cells did not proceed to divide. These data indicated that *cdc5*$^{+}$ gene function is required either in late S phase or in G_2. To understand more accurately in which phase of the cell cycle *cdc5*$^{+}$ gene function is required, the viability of a *cdc5*-120 *rad1::ura4*$^{+}$ double mutant was determined. It has been shown previously that a mutant defective in *cdc17*$^{+}$, a DNA ligase whose function is involved in the completion of S phase, displays an increased lethality at the restrictive temperature when the *rad1* null mutation is present. Therefore, if *cdc5*$^{+}$ gene function is required during late S phase for completion of DNA replication, the double mutant may have shown a decreased viablity when grown at 25°C following incubation at the restrictive temperature when compared with the *cdc5*-120 single mutant alone. The results presented here suggest that *cdc5*$^{+}$ executes its function following the completion of S phase as the *cdc5*-120 *rad1::ura4*$^{+}$ double mutant displays no increase in lethality.

It is of further interest to note that although both *cdc5*-120 and *cdc5*-120 *rad1::ura4*$^{+}$ cells arrested at 37°C as elongated cylindrical cells with a single nucleus, the presence of the *cdc2*-3w mutation caused *cdc5*-120 cells to become aberrantly shaped. Many of these cells displayed a round or pear-shaped morphology. A significant percentage of *cdc5*-120 *cdc2*-3w cells appeared to enter mitosis and were unable to segregate their chromatin properly. These abnormalities are significant in light of the fact that *cdc2*-3w mutant cells are defective in both a size control checkpoint as well as the checkpoint control for unreplicated DNA (Enoch and Nurse, 1990). Both of these checkpoints act in the G_2 phase of the cell cycle. Since *cdc5*-120 *rad1::ura4*$^{+}$ cells arrested with a similar phenotype to *cdc5*-120 cells, it seems unlikely that the abnormalities seen in the *cdc5*-120 *cdc2*-3w strain were due to the lack of a replication checkpoint in these cells. This conclusion is supported by the fact that *wee1* cells are defective for the size control but not the DNA replication checkpoint in G_2 (Enoch and Nurse, 1990), and we found that *cdc5*-120 *wee1::ura4*$^{+}$ cells displayed similar abnormalities to *cdc5*-120 *cdc2*-3w cells at the restrictive temperature. A possible explanation for these results is that inactivation of Cdc5 triggers a

checkpoint control mechanism that causes cells to arrest in G_2. The *cdc2*-3w mutant may be defective for this checkpoint. Therefore, *cdc5*-120 *cdc2*-3w cells at 37°C attempt to progress through the cell cycle in the absence of *cdc5*$^+$ function, and this leads to a variety of phenotypic abnormalities.

A haploid cell containing a null allele of the *cdc5*$^+$ gene was found to be inviable. Analysis of spores generated from a diploid strain heterozygous for the *cdc5*$^+$ null allele revealed that they germinated, but elongated and arrested in the first cell cycle with three predominant phenotypes. Two-thirds of a population of *cdc5* null mutants elongated and contained a single nucleus, although a portion of these cells contained chromatin that was dispersed and stretched along the length of the cell. The other third contained two nuclei and a single septum. This result indicates that *cdc5*$^+$ is an essential gene whose function is required for completion of the cell division cycle.

Results described previously (Nurse *et al.*, 1976) and in this report raised the possibility that the *cdc5*-120 mutant is defective for general growth processes at the restrictive temperature. Indeed, at 37°C *cdc5*-120 cells elongate only modestly and cell mass increases as well as cell cycle progression stops (Nasmyth and Nurse, 1981; this study). Although a defect in cell mass accumulation is indicated, results presented here suggest that the primary defect of the *cdc5*-120 strain lies in cell cycle progression. First, *cdc5*-120 cells arrest with 1.4 times the volume of wild type cells on the verge of septation. These cells would be predicted to contain sufficient material to form two daughter cells. Second, *cdc5*-120 cells blocked in S phase with HU do not divide when reinoculated into inhibitor-free medium at the restrictive temperature. Cells arrested at the HU block point also become elongated. If *cdc5*$^+$ function was required primarily for growth, *cdc5*^{ts} cells would be expected to arrest in the second, not the first, cell cycle after release from the HU block. Third, *cdc5*-120 cells subjected to nitrogen starvation followed by reinoculation into rich medium at the restrictive temperature increased in length with the same kinetics as wild type cells. However, flow cytometric analysis of these cells showed that cell cycle progression was slowed. This indicates that *cdc5*^{ts} cells were not defective for growth during the early stages of the cell cycle and that Cdc5 might be important, although not essential, for cell cycle progression through G_1 as well as G_2. Finally, spores containing the *cdc5* null mutation germinated and elongated. If Cdc5 was crucial for cell growth, spores would not be expected to germinate or to grow. Therefore, we propose that *cdc5*$^+$ gene function is required primarily for *S.pombe* cell cycle progression during G_2. A role for Cdc5 in additional cell processes is certainly possible, especially since Cdc5 is likely to function as a transcription factor (see below) which might have several target genes.

To gain a better understanding of the possible function of the *cdc5*$^+$ gene product, the *cdc5*$^+$ gene and corresponding cDNAs were cloned and sequenced. The protein product predicted from the nucleotide sequence of *cdc5*$^+$ is 87 kDa in molecular mass and comprises 757 amino acids. Although the C-terminal 650 amino acids do not bear significant sequence similarity to any proteins within the databases, the first 100 amino acids share striking identity with the DNA binding domain of the Myb family of transcription factors. This domain in Myb-related proteins comprises up to three imperfect repeats of ~50 amino acids each (reviewed in Lüscher and Eisenman, 1990) which have been proposed to form a helix–turn–helix motif (Ogata *et al.*, 1992). Three tryptophan residues 18 or 19 amino acids apart are characteristic of each repeat and are required for Myb DNA binding activity (Saikumar *et al.*, 1990; Frampton *et al.*, 1991). The *cdc5*$^+$ gene product contains two such repeats, although a tyrosine is found in the position where the sixth tryptophan residue is expected (see Figure 5B). Other conserved residues in the Myb-related repeats are important for high affinity DNA binding activity (Saikumar *et al.*, 1990; Frampton *et al.*, 1991). In particular, a cysteine residue has been shown to be critical for both c-Myb and v-Myb DNA binding activity (Grasser *et al.*, 1992; Guehmann *et al.*, 1992). The *cdc5*$^+$ gene product contains all of these critical residues (see Figure 5).

The deletion mapping of *cdc5*$^+$ presented in this study indicates that the C-terminal two-thirds of Cdc5 is not required for complementation of the *cdc5*^{ts} mutant. In contrast, the entire *cdc5*$^+$ protein coding region is required to complement the *cdc5* null mutant. A possible explanation for this result is that the active form of Cdc5 is a homodimer. This assumes that the protein product of the *cdc5*-120 allele is impaired in its ability to bind DNA at 37°C and that the C-terminal region of Cdc5 is involved in regulation. Thus, a dimer formed between the temperature-sensitive protein product of the *cdc5*-120 allele and a Cdc5 molecule truncated at the C-terminus would have both functional DNA binding and regulatory domains. A more refined analysis of the Cdc5 polypeptide will be needed to identify accurately functional domains within the protein.

By analogy to the Myb family of proteins, it is possible that *cdc5*$^+$ encodes a transcription factor. To test this hypothesis, we have shown that Cdc5 has affinity for DNA. A GST–Cdc5 fusion protein containing the putative DNA binding domain of Cdc5 is retained by DNA–cellulose at low salt concentrations. This evidence supports the notion that Cdc5 could be functioning as a sequence-specific DNA binding protein. Other transcription factors that have roles in cell cycle progression have been isolated in the yeasts *S.pombe* and *S.cerevisiae*. The products of the fission yeast genes *cdc10*$^+$ (reviewed in Johnston and Lowndes, 1992) and *sct1*$^+$/*res1*$^+$ (Tanaka *et al.*, 1992; Caligiuri and Beach, 1993) are transcription factors which act in concert during the G_1 phase of the cell cycle. The genes *SWI4* and *SWI6* of *S.cerevisiae* encode transcription factors that resemble *sct1*$^+$/*res1*$^+$ and *cdc10*$^+$ and also act in G_1 (reviewed in Johnston and Lowndes, 1992; Caligiuri and Beach, 1993). To date, the product of the *cdc5*$^+$ gene is the first transcription factor reported to have a role in the G_2 phase of the cell cycle and it will therefore be very interesting to determine the nature of target gene(s) for Cdc5.

Materials and methods

Strains, growth media and genetic methods

The yeast strains used in this study are listed in Table I. Media used to grow *S.pombe* cells and general genetic manipulations of *S.pombe* have been described (Moreno *et al.*, 1991). Transformations were performed by the lithium acetate and spheroplast procedures (Moreno *et al.*, 1991) or by electroporation (Prentice, 1991).

Physiological analysis

Samples for cell number determinations were collected at 30 min intervals, fixed in 0.12 M NaCl, 3% formaldehyde and cell number determined using a Coulter Counter. The phase of the cell cycle in which the *cdc5*-120 mutant

Table I. A list of yeast strains and their genotypes

Genotype	Source
cdc5-120 h^+	P.Nurse
cdc5-120 *ura4*-D06 h^+	P.Nurse
cdc5-120 *ura4*-D06 *leu1*-32 h^+	P.Nurse
cdc5-120 *rad1::ura4*$^+$ *ura4*-D06 h^+	This study
cdc5::ura4$^+$/*cdc5*$^+$ *ade6*-704/*ade6*-704 *ura4*-294/*ura4*-294 h^+/h^-	This study
cdc25-22 *ura4*-D18 h^-	P.Nurse
cdc25-22 *ura4*-D18 h^+	P.Nurse
cdc25-22 *rad1::ura4*$^+$ *ura4*-D18 h^+	This study
cdc10-129 h^+	P.Nurse
cdc21-M68 h^+	P.Nurse
cdc21-M68 *rad1::ura4*$^+$ h^+	This study
rad1::ura4$^+$ *his3*-237 *leu1*-32 *ura4*-D18	S.Subramani
cdc2-3w *leu1*-32	P.Nurse
wee1::ura4$^+$ *ura4*-D18 *leu1*-32 h^-	P.Nurse
972 h^-	P.Nurse
ade6-M210 *leu1*-32 *ura4*-D18 h^+	P.Nurse
ade6-704/*ade6*-704 *ura4*-294/*ura4*-294 h^+/h^-	P.Nurse

arrests was sequenced with respect to the HU block point essentially as described by Nasmyth and Nurse (1981). The mutant strain *cdc5*-120 h^+ was incubated in yeast extract medium containing 12 mM HU for 3 h at 25°C. Under these conditions, cell division is no longer observed as HU inhibits DNA replication (Nasmyth and Nurse, 1981). The culture was then shifted to 37°C for 1 h to inactivate *cdc5*^{ts} gene function. The HU was then removed from the culture by pelleting the cells and resuspending them in fresh yeast extract medium that had been prewarmed to 37°C. For nitrogen starvation, cells were grown in minimal medium to a density of 4×10^6 cells/ml, washed three times in minimal medium lacking a nitrogen source, and then inoculated into the nitrogen-deprived medium for 19 h at 25°C and 1 h at 37°C to inactivate temperature-sensitive mutant proteins. Cells were then pelleted and reinoculated into yeast extract medium that had been prewarmed to 37°C. Samples were collected and subjected to flow cytometric analysis as described below.

Flow cytometry and microscopy

Cells for flow cytometric analysis were fixed in ice-cold 70% ethanol, treated with RNase A for 2 h at 37°C in 50 mM sodium citrate and stained with 2 µg/ml propidium iodide for at least 1 h at 4°C in the dark. These cells were sonicated and subjected to flow cytometry as previously detailed (Sazer and Sherwood, 1990). To visualize nuclei, cells and spores were fixed with formaldehyde and stained with the fluorescent DNA binding dye DAPI as previously described (Moreno *et al.*, 1991), and photographed using a Zeiss Axioscope photomicroscope and Kodak Ektachrome 400 film. To determine cell length and width, cells were measured microscopically in liquid medium using phase optics and a ×100 objective with an eyepiece drum micrometer. For cell length determinations, 16, 20 or 100 cells were measured for each sample. For cell width determinations, 20 cells were measured for each sample. The standard error of each mean was <5%.

Viability counts

The viability of *cdc5*-120 *rad1::ura4*$^+$; *cdc21*-M68 *rad1::ura4*$^+$ and *cdc25*-22 *rad1::ura4*$^+$ strains was determined by plating ~500 cells per YE plate. These plates were placed at 36°C for 0–9 h and then shifted back to 25°C for 72 h at which time, colonies were counted.

Cloning and DNA sequence

Spheroplasts prepared from *cdc5*-120 *ura4*-D06 h^+ cells were transformed with a *S.pombe* genomic DNA library in which fragments from a partial *Sau*3A digest were inserted into pUR19 (a gift from Dr A.M.Carr; Barbet *et al.*, 1992). Transformants were plated at the restrictive temperature on minimal plates lacking uracil to select for cells that could form colonies under these conditions. Plasmid DNAs were recovered from Ura$^+$ Cdc$^+$ transformants and were introduced into *cdc5*-120 *ura4*-D06 h^+ spheroplasts to confirm rescue.

A pDB20 based cDNA library (Fikes *et al.*, 1990) was screened with the *Apa*I–*Bst*XI fragment of genomic clone 2 (see Figure 6) to obtain *cdc5*$^+$ cDNA clones. Two clones corresponded to the 5′ and 3′ halves of the gene and overlapped with a third clone which was full-length. Restriction fragments of genomic clone 2 and the three cDNA clones were subcloned into pTZ19R and pTZ18R and single strands were generated. The single strands were sequenced using Sequenase 2.0 (United States Biochemical, Cleveland, OH) with either custom synthesized oligonucleotides (Operon, Alameda, CA) or the M13 reverse primer. The predicted amino acid sequence was used to search the SwissProt protein database for homologous sequences by utilizing the FASTDB program (Brutlag *et al.*, 1990). Plasmids used in delineating the minimal rescuing fragment of *cdc5*$^+$ were contructed by digesting either clone 1 or 2 with the desired restriction enzymes (see Figure 6) and ligating the resulting fragments into the yeast expression vector pIRT2 (Moreno *et al.*, 1991).

Gene disruption

To create a *cdc5*$^+$ genomic clone with sufficient flanking sequences at both the 5′ and 3′ ends of the protein coding region to allow efficient homologous recombination to occur between the clone and the genomic copy of *cdc5*$^+$, the inserts of two *cdc5*$^+$ genomic clones (clones 1 and 4) were joined (see Figures 6 and 7). The insert of clone 1 was excised as a 4.5 kb *Pst*I–*Sac*I fragment and subcloned into pTZ19R to form the plasmid pTZ19R53. This plasmid was linearized with *Pst*I, blunt-ended, digested with *Apa*I and the vector fragment from this digest was purified. Clone 4 (see Figure 6) was linearized with *Eco*RI, blunt-ended and cut with *Apa*I. The resulting 1.8 kb insert fragment was subsequently cloned into the prepared pTZ19R53 vector to form the plasmid pTZ19R553.

The DNA construct used for the *cdc5*$^+$ gene deletion was produced by replacing the 2.0 kb *Apa*I–*Bgl*II *cdc5*$^+$ protein coding region contained within pTZ19R553 with a 1.8 kb *Hin*dIII fragment containing the *S.pombe* selectable marker, *ura4*$^+$. The resulting plasmid, designated pTZ19R*cdc5::ura4*$^+$, was cut with *Sac*I and the 6.1 kb *cdc5::ura4*$^+$ insert was used to transform the diploid *S.pombe* strain *ade6*-704/*ade6*-704 *ura4*-294/*ura4*-294 h^+/h^+. Stable Ura$^+$ integrants were isolated by replica plating to yeast extract agar five times and then back to selective conditions. A heterozygous deletion mutant was identified by Southern blot analysis (described below). A sporulation competent diploid strain (*cdc5::ura4*$^+$/*cdc5*$^+$ *ade6*-704/*ade6*-704 *ura4*-294/*ura4*-294 h^{90}/h^+) arose spontaneously from the parental h^+/h^+ disruptant strain, and was detected by replica plating colonies to sporulation medium. Colonies which contained spores were identified by exposure to iodine vapors as previously described (Moreno *et al.*, 1991).

To obtain viable haploid cells containing the *cdc5* null mutant, the genomic *cdc5*$^+$ clone in the plasmid, pIRT2, was transformed into the heterozygous diploid deletion strain (*cdc5::ura4*$^+$/*cdc5*$^+$ *ade6*-704/*ade6*-704 *leu1*-32/*leu1*-32 *ura4*-294/*ura4*-294 h^-/h^+) and Leu$^+$ transformants were selected. These diploids were sporulated and the desired Leu$^+$ Ura$^+$ haploid cells were isolated.

Southern blot analysis

Genomic DNA was prepared from diploid *S.pombe* strains by procedures detailed previously (Moreno *et al.*, 1991). For Southern hybridization analyses, 0.5 µg of genomic DNA was digested overnight at 37°C, size fractionated on a 0.8% agarose gel and transferred to a Gene Screen Plus membrane. The membrane was prehybridized for 1 h in hybridization buffer (5 × Denhardt's solution, 0.5% SDS, 5 × SSPE and 100 µg/ml hydrolyzed yeast RNA), and then incubated with a random primed [α-^{32}P]dCTP-labelled probe for 16 h at 65°C. Following hybridization, the filters were washed twice at 65°C for 30 min in 0.1 × SSPE, 0.1% SDS at 65°C.

Bacterial expression and purification of cdc5 protein

Oligonucleotide primers were used to amplify the 2.4 kb *cdc5*$^+$ cDNA described above by PCR. Primer 1 (5′-GTAT*GGATCC*ATGGTTGTTT-TA-3′) was complementary to the 5′ end of the cDNA and primer 2 (5′-ACAAA*GGATCC*TGAAGTTTA-3′) was complementary to the 3′ end of the cDNA. Both primers were designed to add *Bam*HI restriction sites to the ends of the coding region during amplification. The amplified cDNAs were blunt-ended and subcloned into pTZ19R linearized with *Sma*I to form the plasmid pTZ19R5cDNA. The insert of pTZ19R5cDNA was then sequenced on one strand using a single-stranded template as described above to ensure that no mutations had been introduced by PCR. The *Bam*HI–*Hpa*I fragment corresponding to the N-terminal third of Cdc5 was excised from pTZ19R5cDNA and ligated downstream of the IPTG-inducible promoter at the *Bam*HI site in the bacterial expression vector pGEX2T (Smith and Johnson, 1988). This contruct produced an inframe fusion protein with GST. The Cdc5 fusion protein was expressed from the plasmid, designated pGEX2T5ΔHpaI, in the *Escherichia coli* strain BL21DE3 and purified by affinity chromatography on immobilized glutathione as described (Smith and Johnson, 1988).

DNA – cellulose chromatography and Western blotting

The procedure for DNA–cellulose chromatography was modified from Klempnauer and Sippel (1986). A total of 10 μg of purified GST–Cdc5ΔHpaI fusion protein in 0.5 ml of 10 mM HEPES (pH 8.0) and 1 mM EDTA were loaded onto a native calf thymus DNA–cellulose column (bed volume was equal to ~1 ml; Sigma Chemical Co., St Louis, MO). Equilibration of the column and adjustment of the flow rate were as described (Klempnauer and Sippel, 1986). The column was then washed with 4 ml of ice-cold equilibration buffer containing 10 mM HEPES (pH 8.0) and 1 mM EDTA. Elution of protein bound to the column was accomplished by sequentially adding two column volumes each of 100, 200, 300, 400 and 500 mM NaCl in 10 mM HEPES (pH 8.0), 1 mM EDTA.

Samples of the flowthrough, wash and eluates were analyzed by immunoblotting. A 15 μl aliquot of each fraction was boiled in 1 × SDS gel sample buffer and resolved on a 10% SDS–polyacrylamide gel. Proteins were then transferred to immobilon-P (Millipore, Bedford, MA) and incubated with a 500-fold dilution of antibodies which recognize the GST component of the Cdc5 fusion protein. These antibodies were produced against a GST fusion protein in which bacterial GST was linked to a vertebrate homeodomain-containing protein (a gift from Dr C.V.E.Wright). Detection of immunocomplexes was accomplished with [^{125}I]protein A and results were visualized by autoradiography.

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