Fission Yeast \textit{cdc24} Encodes a Novel Replication Factor Required for Chromosome Integrity

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ABSTRACT

A mutation within the \textit{Schizosaccharomyces pombe} \textit{cdc24} gene was identified previously in a screen for cell division cycle mutants and the \textit{cdc24} gene was determined to be essential for \textit{S} phase in this yeast. We have isolated the \textit{cdc24} gene by complementation of a new temperature-sensitive allele of the gene, \textit{cdc24-G1}. The DNA sequence predicts the presence of an open reading frame punctuated by six introns which encodes a pioneer protein of 58 kD. A \textit{cdc24} null mutant was generated by homologous recombination. Haploid cells lacking \textit{cdc24} are inviable, indicating that \textit{cdc24} is an essential gene. The transcript of \textit{cdc24} is present at constant levels throughout the cell cycle. Cells lacking \textit{cdc24} function show a checkpoint-dependent arrest with a 2N DNA content, indicating a block late in \textit{S} phase. Arrest is accompanied by a rapid loss of viability and chromosome breakage. An \textit{S. pombe} homolog of the replicative DNA helicase DNA2 of \textit{S. cerevisiae} suppresses \textit{cdc24}. These results suggest that \textit{Cdc24p} plays a role in the progression of normal DNA replication and is required to maintain genomic integrity.

EUKARYOTIC DNA replication requires a large number of gene products, most of which are highly conserved. These proteins may be broadly classified as either regulatory factors or components of the replication machinery. The interplay between these classes is essential to maintain the order of events, to ensure fidelity of replication, and to preserve the integrity of the genome during the cell cycle. However, while many elements are understood in isolation, the connections between them have become apparent only recently. Defining the network of interactions between regulators and replicators is facilitated by genetic analysis in model systems. This approach allows identification of direct and indirect interactions and helps to define pathways of interaction. The fission yeast \textit{Schizosaccharomyces pombe} has been particularly useful as a model system for \textit{S} phase regulation (reviewed in Forsburg 1996; MacNeill and Nurse 1997). Much of this work has come from the isolation of mutants defective for normal DNA replication, followed by characterization of the genes involved.

As is the case for other events in the cell cycle, \textit{S} phase in vegetative fission yeast cells requires a number of proteins that are highly conserved in all eukaryotes. It is known that the transition through \textit{START} of the cell cycle requires the activity of the p34\textit{cdc2} protein kinase (Nurse and Bissett 1981) as well as \textit{cdc10}+, which encodes a transcription factor (Lowndes \textit{et al.} 1992). \textit{Cdc10p} activates gene expression of \textit{cdc22}+, encoding ribonucleotide reductase (Fernandez Sarabia \textit{et al.} 1993), and the initiator encoded by \textit{cdc18}+ (Kelly \textit{et al.} 1993). Based on work from a variety of systems, it is thought that the conserved \textit{Cdc18p} binds to protein components of the ORC (origin recognition complex; Muzi-Falconi and Kelly 1995; Carpenter \textit{et al.} 1996; Grollert and Nurse 1996; Leatherwood \textit{et al.} 1996; Santocanale and Diffley 1996). This association is presumed to activate the origin of replication. Apparently at this point, the mini-chromosome maintenance (MCM) proteins associate with the chromatin (Romanowski \textit{et al.} 1996; Donovan \textit{et al.} 1997). Following the assembly of these factors, the origin fires and replication initiates, which requires additional activators including the \textit{Hsk1p/Cdc7p} kinase (Sclafani and Jackson 1994; Masi \textit{et al.} 1995). However, the precise events leading to origin firing remain unclear.

As in other eukaryotes, DNA synthesis in fission yeast requires multiple enzymes. Genes encoding a number of these have been identified, including the initiating DNA polymerase alpha (pol1+/+; swi7+; Damagnez \textit{et al.} 1991; Singh and Klar 1993), and polymerase epsilon (pol20+; Ursso and Nurse 1997). Elongation requires the activities of the polymerase delta gene (pol2+/+; pol3+/+; \textit{cdc6}+; Pignede \textit{et al.} 1991; Francesconi \textit{et al.} 1993; Iino and Yamamoto 1997) and that of its associated regulatory subunit \textit{cdc1}+ (MacNeill \textit{et al.} 1996). The...
polymerase delta-associated processivity factor PCNA (pct1*; Waseem et al. 1992) is required for efficient extension (reviewed in Burgers 1996). Genes encoding other conserved proteins have been cloned and include the large subunit of RPA, rad11*/rpa1*; (Parker et al. 1997) and DNA ligase (cdc17*; Johnson et al. 1986). The Cdc18p initiator protein is degraded as replication proceeds, which provides a mechanism to ensure that each origin fires a single time per cell cycle (Jalil et al. 1996; Komnami and Toda 1997).

The replication machinery also interacts with elements of the checkpoint apparatus, which functions in normal cells to repress mitosis and cytokinesis until S phase is completed (reviewed in Carr and Hoekstra 1995; Stewart and Enoch 1996). Interestingly, mutants with defects in the initiation of replication including rad4* cut5*, Dpo1, and Ddc18, bypass checkpoint activation and proceed catastrophically through mitosis (Durso et al. 1995; Kelly et al. 1993; Saka and Yana-gida 1993). It thus appears that the checkpoint monitors the formation of a replication protein complex, and failure to establish such a complex bypasses the checkpoint. In contrast to the initiators, mutants with postinitiation defects arrest with a typical elongated Cdc2- (cell division cycle) phenotype because the checkpoint system is activated successfully (Carr and Hoekstra 1995; Stewart and Enoch 1996). Thus, mutants with a Cdc2- arrest phenotype usually define genes acting downstream of initiation. The S phase-inhibiting drug hydroxyurea (HU) also has been used to distinguish early from late S phase events. HU blocks the cell immediately after initiation so that the checkpoint is engaged. This entire system of replication functions must be carefully regulated to replicate DNA with high fidelity just once per cell cycle and to prevent reinitiation until replication is complete and M phase has occurred.

An early screen in fission yeast identified a number of cdc mutants required for S phase progression (Nasmyth and Nurse 1981). These included mutations affecting cdc1* and cdc6* (components of DNA polymerase delta; MacNeil et al. 1996; Iino and Yamamoto 1997); cdc17* (DNA ligase; Johnson et al. 1986); cdc18* (initiator; Kelly et al. 1993); cdc19* and cdc21* (MCM proteins; Coxon et al. 1992; Forsburg and Nurse 1994); cdc20* (polymerase epsilon; Durso and Nurse 1997); and cdc22* (ribonucleotide reductase; Fernandez Sarabia et al. 1993). Two of the genes identified, cdc23 and cdc24, have not been cloned. The original physiological characterization of cdc24 mutants suggested that this gene is required for a step after the point at which HU blocks the cell cycle but not for a chemical doubling of DNA (Nasmyth and Nurse 1981). We have further investigated the role of the cdc24* gene in S phase by cloning and characterizing the gene and its mutant alleles. Surprisingly, this novel reading frame has no similarity to other proteins in the database. Three of the mutations conferring temperature-sensitivity are clustered in a small region; two of these cause the protein to be truncated at its C terminus. However, the gene is essential for viability. cdc24 mutants arrest in a checkpoint-dependent fashion with a replicated genome, confirming that cdc24* acts during S phase after initiation. Mutant arrest is accompanied by a striking loss of viability and the appearance of large fragments of DNA in pulsed field gels. Because chromosome breakage can be caused by arrest of DNA replication, we propose that Cdc24p plays an essential role in the progression of S phase, perhaps as a novel component of the replication machinery. Consistent with this hypothesis, we isolated an S. pombe homolog of the replicative DNA helicase, DNA2, of C. albicans as a high copy suppressor of cdc24. DNA2 encodes a DNA-dependent ATPase with 3' to 5' helicase activity (Budd and Campbell 1995). DNA2 is an essential gene which has been shown to interact genetically with RAD27/RTH1, which encodes the budding yeast FEN-1 nuclease, and likely is involved in lagging strand DNA synthesis (Budd and Campbell 1995, 1997; Budd et al. 1995; Fiorentino and Crabtree 1997).

MATERIALS AND METHODS

Strains and genetic methods: S. pombe strains used in this study are listed in Table 1. Strains were constructed by tetrad analysis when necessary. Media for vegetative growth (YE; Edinburgh Minimal Medium) and for matings of S. pombe were as described (Moreno et al. 1991). For temperature-shift experiments, cultures were grown to early log phase at 25° and shifted to 36° for the indicated amounts of time. Transformations were performed by electroporation (Prentice 1992). Repression of transcription from the nmt1 promoter (Mauderli 1990) was achieved by addition of thiamine to a concentration of 2 μM. For cell synchronization, 4 liters of cells were grown to a density of ~5 × 10^8 cells/ml in YE media at 25°. Small cells in G2 were isolated using a Beckman (Fullerton, CA) JE-5.0 elutriation rotor and incubated at 25° or 36°. All temperature-shift experiments were carried out by growing cells at 25° and shifting cells to 36° while the cells were in exponential growth phase. Strain viability was determined by plating ~500 cells per YE plate. These plates were placed at 36° for 0-9 hr and then shifted back to 25° for 72 hr at which time colonies were counted on three plates for each time point. The percent viability was determined from two experiments, averaged and plotted.

Flow cytometry and microscopy: Cells were fixed in ice-cold 70% ethanol, treated with RNase A for 2 hr at 37° and 50 μM sodium citrate and stained with 2 μg/ml propidium iodide for at least 1 hr at 4° in the dark. These cells were sonicated and subjected to flow cytometry as previously described (Sazer and Sherwood 1990). To visualize nuclei, cells were fixed with formaldehyde (Moreno et al. 1991) or with glutaraldehyde (Lundgren et al. 1991) and stained with the fluorescent DNA-binding dye 4', 6-diamidino-2-phenylindole (DAPI). Cells were viewed with a Zeiss (Thornwood, NY) Axioskop photomicroscope and images were captured using a Zeiss ZVS-47DEC system.

Cloning and DNA sequence: The cdc24-G1 strain was transformed with an S. pombe genomic DNA library in the pUR19 vector (Barbet et al. 1992) and Ura^+ colonies which could
grow at 36°C were isolated. Plasmid DNAs were recovered from these colonies and a comparison of the genomic DNA inserts by restriction mapping and Southern blotting revealed that they contained overlapping DNA sequences. The smallest clone contained a 4.8-kb insert. The region of this clone responsible for complementation ofcdc24-G1 was narrowed by constructing subclones and testing them for their ability to rescue. The DNA sequence of the smallest rescuing fragment (EcORV-HindIII) was then determined on both strands after sequencing by the manufacturer and RACE2 (5′-GGTTAGATTTCT CAACTAAGCGG-3′). The two resultant PCR products were cloned directly utilizing the ThermoSequenase cycle sequencing kit (Amersham, Arlington Heights, IL) and the oligonucleotide, RACE2. The DNA sequence of the longest PCR product indicated that the 5′ end of the mRNA began at or 1.223 nucleotides further upstream of nucleotide 163 (Figure 4). For sequencing ofcdc24 mutant alleles, genomic DNA was prepared from the relevant strains and the cdc24 coding region was amplified by PCR with the following oligonucleotides: cdc24start (5′-ACAGATGATACTAAATGG-3′) and cdc24end (5′-TGGCT ATTCACACGCGGAGAG-3′). The PCR products were sequenced directly using the ThermoSequenase cycle sequencing kit (Amersham) and customized oligonucleotides.

The high copy suppressor ofcdc24-G1 was isolated in a second transformation with the pUR19 genomic library (Barbet et al. 1992). In this case, two plasmids were isolated which rescued the growth defect ofcdc24-M38 at the nonpermissive temperature. One plasmid contained cdc24+. The second plasmid, pSGP45, contained an unrelated 10-kb insert. It was subcloned and restriction fragments were partially sequenced. The sequences were compared to the S. pombe BLAST server (http://sanger.ac.uk/Projects/S_pombe/blat server.shtml) which positioned the genomic insert to be on chromosome II. Plasmids pSGP50, pSGP51, and pSGP52 contain subclones of the genomic fragment in pUR19: a 4.6-kb NsiI-PvuII fragment (pSGP50), a 5.4-kb PstI fragment (pSGP51), and a 4.4-kb NsiI-PvuII fragment (pSGP52). The PvuII sites flank the genomic insert within the multicloning site of pUR19. cdc24-G1 was transformed with each plasmid and each strain was tested for the ability to grow at the nonpermissive temperature. The sequence alignment was performed with MegAlign (DNASTAR, Inc., Madison, WI). BESTFIT and GAP analyses were performed with the Wisconsin GCG analysis package.

**Gene deletion:** The BamHI fragment of thecdc24+ genomic clone was removed from pUR19 and inserted into pSK(−) at the BamHI site. The two HpaI fragments ofcdc24+ were replaced with a 2.2-kb fragment of the his3+ gene (Ohi et al. 1996) to create pSKcdc24::his3+. The deletion construct was then isolated as a BamHI fragment and used to transform two S. pombe diploid strains: (1) ade6-M210 ade6-M216 ura4-D18/ura4-D18 leu1-32/leu1-32 his3-D1/his3-D1 his3-D1/his3-D1 h+/h+ and (2) cdc24+/cdc24-G1 ade6-M210/ade6-M216 ura4-D18/ura4-D18 leu1-32/leu1-32 his3-D1/his3-D1 h+/h+. Stable His+ integrants were identified by replica plating to yeast extract agar five times and then back to selective conditions. Heterozygous deletion mutants were identified by Southern blot analysis and were subjected to tetrad analysis. To obtain viable haploid cells containing thecdc24 null mutant, the genomic cdc24+ clone in pUR19 was transformed into the heterozygous diploid

### TABLE 1

**S. pombe strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<tr>
<td>KGY28</td>
<td>h− 972</td>
<td>Paul Nurse</td>
</tr>
<tr>
<td>KGY69</td>
<td>h+ 975</td>
<td>Paul Nurse</td>
</tr>
<tr>
<td>KGY205</td>
<td>h+ cdc24-M38</td>
<td>Paul Nurse</td>
</tr>
<tr>
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</tr>
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<tr>
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<td>h− cdc25-22</td>
<td>Paul Nurse</td>
</tr>
<tr>
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<td>h+ rad1::ura4+ ura4-D18 leu1-32 his3-237</td>
<td>Suresh Subramani</td>
</tr>
<tr>
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</tr>
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<td>This study</td>
</tr>
<tr>
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<td>This study</td>
</tr>
<tr>
<td>KGY952</td>
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</tr>
<tr>
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deletion straincdc24+/cdc24::his3+ and His"Ura"+ transformants were selected. These diploids were sporulated and the desired His"Ura"+ haploid cells were isolated by tetrad dissection and replica-plating.

**Northern and Southern blot analysis:** Total RNA was prepared from cells which had been synchronized by centrifugal elutriation as described by Moreno et al. (1991). Approximately 20 μg of RNA of each sample was size fractionated by electrophoresis through a 1% agarose-formaldehyde gel. The RNA was transferred to GeneScreenPlus membrane (NEN Life Science Products, Boston, MA) and hybridized with a 32P-labeled probe corresponding to the BglII-HindIII fragment of thecdc24 cDNA. Hybridization was carried out for 18 hr in 50% formamide, 5× SSPE, 5× Denhardt’s solution, 2% SDS, and 100 μg hydrolyzed yeast RNA/ml. Following hybridization the filters were washed in 0.2× SSC, 0.2% SDS at 65°C. Hybridizing bands were detected with a Molecular Dynamics (Sunnyvale, CA) PhosphorImager. Genomic DNA was prepared from diploidS. pombe strains according to Moreno et al. (1991). For Southern hybridization analyses, 0.5 μg of genomic DNA was digested overnight at 37°C, size fractionated on an 0.8% agarose gel and transferred to a GeneScreenPlus membrane. The membrane was prehybridized for 30 min 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-32P]dCTP-labeled probe for 16 hr at 65°C. Following hybridization, the filters were washed twice at 65°C for 30 min in 0.2× SSC, 0.2% SDS at 65°C.

**Pulsed field gels:** Cells were prepared in agarose plugs as described (Kelly et al. 1993). Wild-type cells (FY254), cdc25-22 (FY318), cdc24-M38 (FY370), or cdc24-G1 (KGY445) were grown in EMM and shifted where indicated to 36°C for 4 hr. For HU arrest, hydroxyurea was added to a final concentration of 15 mm for 4 hr. Each plug contained 4.8×107 cells as determined by counting the cultures with a hemocytometer. Pulsed field gel electrophoresis was carried out in 0.6% high-gelling-temperature agarose (Seakem) in a CHEF apparatus from C.B.S. Scientific (Delmar, CA). Electrophoresis was for 72 hr in 0.5× TAE buffer at 50 V with a switch time of 30 min.

**RESULTS**

**Analysis of cdc24" mutants:** Three temperature-sensitive alleles of thecdc24+ gene were isolated in a screen designed to identify genes essential for DNA replication (Nasmyth and Nurse 1981). We independently identified a temperature-sensitive allele ofcdc24 which we have designatedcdc24-G1 in a screen for new cdc mutants using N-ethyl nitroso urea (ENU) rather than nitrosoguanidine as a mutagen (D. McCollum and K. L. Gould, unpublished results). The previously isolatedcdc24-M38 allele was shown to double its DNA content at the nonpermissive temperature although the replicated DNA was not intact (Nasmyth and Nurse 1981). We examined the DNA content and nuclear morphology of synchronizedcdc24-G1 cells following shift to the nonpermissive temperature of 36°C. We found thatcdc24-G1 arrested in the first cell cycle after shift to the nonpermissive temperature (Figure 1A) with a single interphase nucleus, an elongated morphology (Figure 1B), and a 2N DNA content (Figure 1C). The increase in DNA content at the later time points is most likely due to the continued replication of the mitochondrial genome (Sazer and Sherwood 1990). These data indicate thatcdc24-G1, likecdc24-M38, arrests cell cycle progression in late S phase or G2.
cdc24 Encodes a Novel Replication Factor

We looked for interactions of cdc24 mutations with other mutations affecting S phase in S. pombe. There was no evidence for any synthetic interactions or reciprocal suppression events when cdc24-M38 or cdc24-G1 alleles were combined with mutants in cdc1 (MacNeill et al. 1996), cdc27 (Hughes et al. 1992; MacNeill et al. 1996), cdc18 (Kelly et al. 1993), cdc19 (Forsburg and Nurse 1994), or DNA polymerase delta (Francesconi et al. 1993).

The absence of cdc24 + function triggers the replication checkpoint control: To confirm that cdc24 + function is required for the completion of S phase and to determine whether the absence of cdc24 + gene function triggers the replication checkpoint control, we examined the behavior of a cdc24-G1 rad1::ura4 + double mutant strain. The rad1 mutation bypasses the replication checkpoint, and allows cells with improperly replicated DNA to enter mitosis. Thus, if cdc24-G1 were defective in completing DNA replication, the double mutant strain should proceed into mitosis and display the “cut” phenotype typical of mutants with defective checkpoints. Both the cdc24-G1 and the cdc24-G1 rad1::ura4 + mutant strains lost viability rapidly upon incubation at 36 °C (Figure 2A). However, the cdc24-G1 rad1::ura4 + strain displayed a very different phenotype from that of cdc24-G1. DAPI staining showed that whereas the single mutant cells remained in interphase, the double mutant cells entered mitosis and accumulated cells with a cut phenotype (Figure 2C). These data indicate that cdc24-G1 cells do indeed have a defect in DNA replication. As negative controls, the viability of the cdc25-22 and cdc25-22 rad1::ura4 + mutant strains were monitored in parallel. Since cdc25 + does not play a role in DNA replication, neither of these strains lost viability upon incubation at 36 °C (Figure 2B), and both maintained an elongated, Cdc25 morphology (data not shown). We also noted that the few cdc24-G1 cells that survived incubation at the restrictive temperature suffered increased rates of chromosome loss and mitotic recombination relative to wild type (data not shown), suggesting that DNA metabolism had been severely disrupted.

cdc24 mutants suffer chromosome breakage: Pulsed field gels of wild-type fission yeast cells separate all three chromosomes, unless the cells are arrested in S phase by HU treatment. The lack of separation after HU treatment is thought to reflect unresolved replication intermediates that remain in the well, and is typical not only of chromosomes from HU-treated cells, but also those of S phase mutants including PCNA, cdc17, cdc18, cdc19, and cdc21 (D. T. Liang, S. G. Pasion and S. L. Forsburg, unpublished results; Waseem et al. 1992; Kelly et al. 1993; Maiorano et al. 1996; Okishio et al. 1996). We analyzed the effect of cdc24 mutations on chromosome structure (Figure 3). Chromosomes from cdc24-M38 and cdc24-G1 mutant cells incubated at the permissive temperature of 25 °C entered the gel properly (compare lanes 1 and 3 to wild type in lane 5). After incubation of cells for 4 hr at the restrictive temperature of 36 °C, the chromosomes from the cdc24 strains were no longer visible in the gel (lanes 2 and 4). This failure to migrate normally is typical of cells arrested in S phase by mutation or by HU treatment (lane 6). Interestingly however, a broad band of fragmented DNA was visible below chromosome III in the cdc24 mutant strains but is not observed in other S phase mutants (D. T. Liang and S. L. Forsburg, unpublished results; Kelly et al. 1993).
By comparison to several different DNA markers, we estimated that this band ranged from 0.3 to 1 Mb in size. A discrete band below the cdc24 fragment was also apparent; we found this in all strains including wild type following incubation at 36°C (marked by * in Figure 3; S. G. Paison and S. L. Forsburg, unpublished results). Previous analysis suggested that cdc24 mutants accumulate fragmented DNA approximately 1/20 the size of intact chromosomes, or approximately 0.25 Mb, based on alkaline sucrose gradient analysis (Nasmyth and Nurse 1981). Our results are consistent with this finding, suggesting that the chromosomes in cdc24 mutants were not blocked from entering the gel but are actually degraded.

**Cloning and sequence of cdc24** and its mutant alleles:

An S. pombe genomic library constructed in the pUR19 vector was used to isolate the cdc24 gene by complementation of the cdc24-G1 mutation. Seven Ura<sup>+</sup>Cdc<sup>+</sup> colonies were obtained from the library transformation and plasmids were recovered from each of them. Restriction mapping and Southern blot analyses indicated that they all contained the same gene. The smallest genomic clone was linearized and integrated into the genome of an cdc24-G1 strain by homologous recombination. Crosses between this integrant and wild-type cells failed to segregate temperature-sensitive cdc24-G1 progeny indicating that the cloned gene and the cdc24 locus are identical or tightly linked. A probe derived from the cloned gene hybridized to ICRFc60F1108 derived from chromosome I in an ordered S. pombe genomic library (Hoheisel et al. 1993). Hybridization to various overlapping cosmids placed the gene between swi4<sup>+</sup> and pki1<sup>+</sup>.

Further deletions of the smallest genomic clone indicated that the DNA responsible for rescue of cdc24-G1 was contained within the EcoRV-HindIII fragment. DNA sequencing of this fragment revealed discontinuous open reading frames (ORFs) and suggested the presence of introns within the protein coding region of the cdc24 gene. To identify the protein coding region, an S. pombe cDNA library was screened using the BamHI-HindIII fragment of the genomic clone. Two identical cDNA clones of ~1.8 kb were isolated and the DNA sequences of the inserts were determined. An alignment of the cDNA sequence with that of the genomic clone indicated that the ORF comprised 1503 nucleotides separated by 6 introns (Figure 4). Each intron contained splice donor, splice acceptor, and branch point sequences indicative of S. pombe introns (Zhang and Marr 1994). A 5'RACE procedure was employed to confirm that the isolated cDNA contained all of the predicted ORF (see materials and methods). Further evidence that the initiating methionine was as proposed was that the cDNA, when placed downstream of the nmt1 promoter, was able to rescue growth of the cdc24-G1 mutant strain (data not shown).

Translation of the putative ORF of cdc24<sup>+</sup> predicted a 501 amino acid polypeptide of molecular mass 58 kD (Figure 4). Comparison of the predicted protein sequence with those contained within the current databases revealed that Cdc24p is a pioneer protein.

To determine the positions of mutations within the cdc24 gene in the cdc24-G1, -M38, -M44, and -M81 mutant strains, the coding regions were amplified by the polymerase chain reaction. The PCR products were sequenced directly and in each case single base substitutions were found (Table 2). Interestingly, three of the mutations (in -G1, -M38, and -M44) were located very close to one another. The mutation within cdc24-M38 introduced a premature stop codon at amino acid 370. The mutation within cdc24-G1 changed the splice donor site within intron 5. This change would presumably abolish splicing of this intron and would then result in a truncation of the protein at amino acid 356 and the addition of 14 amino acids encoded by the intron; a stop codon lies in frame with the coding region at the end of intron 5 (data not shown).

**Deletion of cdc24**:

To determine whether complete loss of cdc24<sup>+</sup> function would result in a cell cycle defect similar to that of the temperature-sensitive mutations, a null allele of cdc24<sup>+</sup> was constructed by the one-step gene disruption method. Two internal HpaI fragments within the cdc24<sup>+</sup> coding region were replaced with the
his3+ gene (Figure 5A). The resulting cdc24::his3+ fragment was transformed into a histidine auxotrophic diploid strain and stable His+ transformants were isolated. Southern blot analyses were performed on genomic DNA isolated from a putative heterozygous diploid to confirm that the desired recombination events had occurred. The Xho-I-HindIII restriction fragment located downstream of the cdc24+ coding region was used as a probe (Figure 5A). The cdc24+ gene is present on an ~4.8-kb BglII fragment. Two hybridizing bands were
detected in the diploid heterozygous for the cdc24 null allele (Figure 5B). The lower band corresponded to the wild-type gene locus, and the upper band corresponded in size to the predicted addition of 860 bp to the cdc24 gene locus, had the null allele correctly replaced one copy of the wild-type gene.

To investigate the phenotype of the cdc24 null mutant, the cdc24+/cdc24::his3+ diploid was induced to sporulate and tetrads were dissected. In each tetrad, only two spores produced colonies and all colonies were His-. Thus, Cdc24p is essential for viability. Microscopic examination revealed that the presumed His+ spores had germinated and undergone two to four cell divisions before arresting cell division (for example, see Figure 5C). In each microcolony, elongated cells with a typical Cdc24p morphology were observed although not all cells appeared to adopt this terminal morphology.

The phenotype of a cdc24 null mutant was examined more closely by two other means, plasmid loss and spore germination. In the first approach, a multicopy plasmid containing the cdc24 gene was passed through the heterozygous diploid, and haploid cells bearing the null allele of cdc24 and the plasmid were isolated. After a period of growth without maintaining selection for the plasmid, some of the cells, presumably those which had lost the plasmid, arrested cell division with an elongated morphology and a single nucleus, indicative of an interphase arrest (Figure 5D). In the second approach, a diploid with the genotype cdc24-G1/cdc24::his3 was constructed by the one-step gene replacement procedure and its genotype confirmed by Southern blot analysis (data not shown). Unlike the cdc24+/cdc24::his3 diploid, this temperature-sensitive diploid strain grew very poorly at 25°C accumulating many elongated and cell cycle-arrested cells in the culture (data not shown). When spores from this diploid were incubated at 36°C following tetrad dissection, microscopic examination revealed that the presumed His+ spores germinated, elongated, but failed to undergo cell division (data not shown). Spores isolated from this diploid were induced

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**Figure 5.** Deletion of the cdc24+ gene. (A) Restriction map of the cdc24+ gene. Relevant restriction sites are indicated. The cdc24 coding region is represented by the solid black box. The construction of pSK(-)cdc24::his3+ is shown below the map. The HpaI fragments were removed and replaced with the his3+ gene. The HindIII-XhoI fragment used to probe the Southern blot in panel B is indicated above the map. (B) Southern blot analysis of chromosomal cdc24 deletion. Genomic DNAs from a wild-type diploid and a heterozygous cdc24::his3+ diploid were digested with BglII and then resolved on an 0.8% agarose gel. DNAs were blotted to membrane and hybridized with the probe fragment indicated in panel A. Bands were visualized by autoradiography. (C) Phenotype of the cdc24 null mutant. A representative microcolony which contains the cdc24 null allele that arose following tetrad dissection of a cdc24::his3+/cdc24+ diploid strain was photographed after 3 days of incubation at 32°C. (D) Phenotype of cdc24+ loss. A haploid cdc24 deletion strain carrying the multicopy pUR19cdc24+ plasmid was grown to midexponential phase in nonselective medium to allow plasmid loss. Cells were fixed in formaldehyde and stained with DAPI.
cdc24 Encodes a Novel Replication Factor

Figure 6.—Level of cdc24 transcript through the cell cycle. Wild-type 972 cells were grown to midexponential phase, small cells in early G2 were isolated by centrifugal elutriation, and the synchronized culture was incubated at 32° through two cell divisions. (A) The septation index of the culture was determined at 20 min intervals and the relative level of cdc24 transcript determined from B is also plotted. (B) Northern analysis of total RNA (20 μg per lane) prepared from the same experiment as in A and at the same time points as in A. The same blot was hybridized with a cdc24 DNA fragment, a leu1 DNA fragment as a loading control and a cdc22 DNA fragment as a control for cell cycle oscillations in mRNA abundance. mRNA levels were quantified with a Molecular Dynamics PhosphorImager and the level of cdc24 mRNA relative to that of leu1 mRNA is plotted in A.

to germinate in liquid media by incubating them in the absence of histidine at 36°. By 14 hr, elongated and cell cycle-arrested cells began to accumulate (Figure 5E); longer cells were observed at the 24 hr time point (Figure 5F). Septa were frequently observed in the undivided cells (Figure 5, E and F). These results support the hypothesis that cdc24 is essential for DNA replication.

cdc24 RNA levels do not change during the cell cycle: Since some genes required for S phase in S. pombe are expressed periodically through the cell cycle (Lowndes et al. 1992; Kelly et al. 1993), we examined the level of cdc24 mRNA in a synchronous culture. Wild-type S. pombe cells were separated on the basis of size by centrifugal elutriation and small cells in G2 were isolated and incubated at 32°. The synchrony of the culture was determined by monitoring the percentage of septating cells microscopically (Figure 6A). Total RNA was prepared from cells every 20 min for two cell cycles and the amount of cdc24 mRNA was determined by Northern blotting. There was a single detectable cdc24 mRNA that migrated just faster than the 18S ribosomal RNA at ~1.6 kb (Figure 6B, top panel). It did not oscillate in abundance through the cell cycle. The Northern blot was also probed with a fragment of leu1 whose transcription is not periodic in the cell cycle to provide an RNA loading control. The blot was then probed with a fragment of the cell cycle-regulated cdc22 gene to provide evidence that cell cycle oscillations in transcript abundance would have been detected had they occurred (Figure 6B, bottom panel).

A homolog of S. cerevisiae DNA2 suppresses cdc24 mutants: In our attempts to clone cdc24, a plasmid, pSGP45, carrying a second gene within a 10-kb fragment was isolated (Figure 7A). pSGP45 was able to suppress the growth defect of both cdc24-M38 and cdc24-G1. In order to define the smallest fragment of pSGP45 able to suppress the growth defect of cdc24, we subcloned restriction fragments into pUR19 and tested for rescue of the cdc24-G1 strain. pSGP50, pSGP51, and pSGP52 contained overlapping fragments spanning the genomic fragment in pSGP45. cdc24 strains carrying cdc24 or the suppressor in pSGP45 or pSGP50, which contains a 4.6-kb NsiI-PvuII fragment of pSGP45, were able to form colonies at the nonpermissive temperature (Figure 7, A and B).

Partial sequencing of internal restriction fragments and analysis of the S. pombe BLAST Server revealed that the pSGP45 genomic fragment contained two ORFs. Our subcloning analysis (Figure 7, A and B) showed that one of these ORFs is sufficient to suppress cdc24. BLAST analysis of this ORF identified this gene to have significant homology with S. cerevisiae DNA2, a replicative DNA helicase (Budd and Campbell 1995; Budd et al. 1995). The S. pombe gene present on the 4.6-kb geno-
A 1247 amino acid, 141-kD deduced protein with 56% similarity and 33% identity to budding yeast ScDna2p over the length of the proteins (Altschul et al. 1990). Alignment of the C-terminal third of each protein highlights this homology (Figure 7C). Analysis of the deduced sequence reveals the presence of several conserved segments I, II, V, and VI, characteristic of DNA helicases (Gorbalenya et al. 1989; Budd and Campbell 1995), which are indicated in Figure 7C. Segments I and II comprise the nucleotide-binding motif. Based on this homology, this gene probably encodes the S. pombe homolog of ScDna2p, and will be referred to as dna21. Thus, the suppressor of thecdc24growth defect at the nonpermissive temperature is a putative DNA helicase.

**DISCUSSION**

In this report, we have described the cloning and characterization of the fission yeastcdc24gene. Previously,cdc24was shown to be required for normal S phase progression, and execution point analysis suggested thatcdc24functions after the HU arrest point in early S phase (Nasmyth and Nurse 1981). Further, it was shown thatcdc24mutants accumulate broken DNA. Our results support and extend the original analysis.

We have cloned and sequenced thecdc24gene. The gene maps to chromosome I between swi4+ and pki1+. Its reading frame, interrupted by several introns, encodes a novel protein of predicted molecular weight 58 kD. FASTA and BLAST searches against GenBank or expressed sequence tag (EST) databases fail to identify any homology to known proteins; Cdc24p is thus a pioneer. In an attempt to identify critical domains of the protein, the mutations within four temperature-sensitivecdc24alleles were determined. On the basis of genetic crosses, it was concluded previously thatcdc24-M38andcdc24-M44were homoallelic (Nasmyth and Nurse 1981). We have found by sequence analysis, however, that although the mutations occur very close to one another, they are separate alleles. The mutation withincdc24-M38introduces a premature stop codon at position 178 corresponding to the rescuing ORF which is homologous to ScDna2p. (B) The fission yeastDna2phomolog rescues growth ofcdc24ats the nonpermissive temperature.

![Figure 7](image)

**Figure 7.** (A) Analysis ofcdc24suppressor. Restriction map of the genomic fragment that complementscdc24growth defect at nonpermissive temperature. pSGP45 is the pUR19 genomic library plasmid which rescues growth ofcdc24at 36° and contains a 10-kb genomic fragment. Plasmids pSGP50, pSGP51, and pSGP52, are pUR19 containing sub-clones of the genomic fragment: a 4.6-kb NsiI-PvuII fragment, a 5.4-kb PstI fragment, and a 4.4-kb NsiI-PvuII fragment, respectively. These plasmids were tested for the ability to rescuecdc24growth at the nonpermissive temperature: plus (+) indicates complementation, minus (−) indicates no complementation. E, EcoRI; B, BstXI; N, NsiI; P, PstI. The black arrow corresponds to the rescuing ORF which is homologous to ScDna2p. (B) The fission yeastDna2phomolog rescues growth ofcdc24ats the nonpermissive temperature.cdc24-G1 strains carrying the empty vector (pUR19), the genomiccdc24(pURdc24), the original high-copy suppressor (pSGP45), or a 4.6-kb fragment of the suppressor (pSGP50), or a 4.4-kb PvuII fragment, were streaked out on agar plates and incubated at 36° for 72 hr before photography. (C) Alignment of C-terminal third ofSpDna2p andScDna2p. In the alignment, the top line is the fission yeastDna2phomolog,SpDna2p(labeledSp, residues 782-1277) and the bottom line isScDna2p(labeledSc, residues 1052-1522, GenBank accession number U00027). The boxed residues indicate identical residues. The numbers on the right refer to the amino acid residue at the end of each line. The bars indicate helicase motifs I, II, V, and VI (Gorbalenya et al. 1989; Budd and Campbell 1995).
tion 370 whereas the mutation within cdc24-M 44 alters S358 to P358. Interestingly, the mutation within cdc24-G1 alters the first base within an intron, a base critical to the function of a splice donor site. This, presumably, would result in the production of a truncated protein also; in this case, the truncation would occur at residue 356. These data suggest that the C terminus of the protein is not essential for its function. Further, the temperature-sensitivity of the strains producing the truncated proteins indicates that either the C terminus stabilizes the rest of the protein or that Cdc24p is part of a protein complex in which the C terminus of Cdc24p plays a stabilizing role.

We confirm that cdc24+ is required in S phase. Although cdc24 temperature-sensitive mutant cells block the cell cycle with a 2N DNA content, their arrest depends upon an intact replication checkpoint, demonstrating that the Cdc− arrest results from damaged DNA or incomplete DNA replication. We note that a number of temperature-sensitive S phase mutants show a similar arrest phenotype, including strains with mutations in components of DNA polymerase delta (Cdc1p, Cdc6p; Francesconi et al. 1993, 1995; MacNeill et al. 1996; Iino and Yamamoto 1997); in DNA ligase (Cdc17p; Carr and Hoekstra 1995; Johnston et al. 1986); or in MCM proteins, Cdc19p and Cdc21p (Coxon et al. 1992; Forsburg and Nurse 1994; Maiorano et al. 1996).

Interestingly, cells with a deletion of cdc24+ are able to go through several rounds of division before they die. This also has been seen for mutants lacking the replication processivity factor PCNA; Δpob1 cells go through at least one or two divisions before arresting, suggesting that this protein is sufficient and abundant and capable of being in the null spores (Waseem et al. 1992). Thus, we conclude that the Cdc24p protein is similarly a stable molecule and is packaged in spores in sufficient amounts to allow several cell cycles.

In contrast, inactivation of cdc24+ by a temperature-sensitive mutation leads to a number of striking phenotypes. First, cells undergo S phase arrest in the first cell cycle. Second, cells suffer serious loss of viability, suggesting the occurrence of DNA damage from which they cannot recover. This is not typical of all S phase mutants; for example, DNA polymerase delta mutants can maintain viability and recover upon return to the permissive temperature (D. T. Liang and S. L. Forsburg, unpublished results). Third, and most dramatic, the cdc24 mutant cells undergo chromosome breakage at the restrictive temperature. Our results using pulsed field gel analysis agree with previous observations that the fragments of DNA are on the order of 0.2 to 1 Mb in size. This phenotype is not seen for cells with mutations in other S phase genes including cdc17, cdc18, cdc19, cdc21, or pob1 (D. T. Liang and S. L. Forsburg, unpublished results; Waseem et al. 1992; Kelly et al. 1993; Maiorano et al. 1996).

The chromosome breakage we observed explains why cdc24 cells lose viability rapidly at the restrictive temperature. It appears to be a consequence of passage through S phase. What could cause such an effect? Replication-coupled double-stranded breaks have been observed for cells treated with topoisomerase inhibitors such as camptothecin (reviewed in D’Incalci 1993; Palotti 1993), possibly as a consequence of blocking replication complex passage (Bierne and Michel 1994). Interestingly, mutation of a replication-associated helicase in Escherichia coli is also accompanied by chromosome breakage (Michel et al. 1997). We suggest that Cdc24p is a novel replication factor, perhaps associated with the elongation complex. When cdc24+ is inactivated, the moving replication fork might stall, and the DNA thus becomes prone to breakage. In this respect, it is particularly interesting that the S. pombe homolog of S. cerevisiae DNA2 was isolated as a high copy suppressor of cdc24. DNA2 encodes an essential replicative DNA helicase which has been shown to interact genetically and biochemically with the yeast FEN-1 nuclease and has been proposed to have a role in lagging strand DNA synthesis, possibly in Okazaki fragment maturation (Budd and Campbell 1995, 1997; Fiorentino and Crabtree 1997). Several S. cerevisiae dna2+ mutants have been characterized; at the nonpermissive temperature they arrest with a large budded morphology and a single nucleus (Kuo et al. 1983; Budd and Campbell 1995; Fiorentino and Crabtree 1997). By flow cytometry, these mutants have replicated their DNA and arrest with a 2C DNA content (Fiorentino and Crabtree 1997). However, metabolic labeling studies performed with the dna2-1 strain indicate that only low molecular weight DNA fragments (less than 10 kb) were synthesized at the nonpermissive temperature (Budd and Campbell 1993, 1995) likely reflecting the inability to process Okazaki fragments. PFGE analysis of cdc24 revealed the accumulation of low molecular weight DNAs in cells grown at the nonpermissive temperature but these fragments were much larger than Okazaki fragments.

At this time, the mechanism whereby dna2+ suppresses cdc24 mutants is not known. Dna2p might interact directly with Cdc24p or might be involved in a parallel or related mechanism. Nevertheless, the ability of dna2+, a DNA helicase proposed to be involved in lagging strand synthesis, to suppress the cdc24+ growth defect is consistent with cdc24 having a role in late S phase, perhaps in processing late replication intermediates. It will be of considerable interest to determine whether cdc24+ represents a conserved function, or plays a role unique to fission yeast.

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