Fission Yeast *cdc24*+ Encodes a Novel Replication Factor Required for Chromosome Integrity

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ABSTRACT

A mutation within the *Schizosaccharomyces pombe cdc24*+ gene was identified previously in a screen for cell division cycle mutants and the *cdc24*+ gene was determined to be essential for S phase in this yeast. We have isolated the *cdc24*+ gene by complementation of a new temperature-sensitive allele of the gene, *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 *cdc24* null mutant was generated by homologous recombination. Haploid cells lacking *cdc24*+ are inviable, indicating that *cdc24*+ is an essential gene. The transcript of *cdc24*+ is present at constant levels throughout the cell cycle. Cells lacking *cdc24*+ function show a checkpoint-dependent arrest with a 2N DNA content, indicating a block late in S phase. Arrest is accompanied by a rapid loss of viability and chromosome breakage. An *S. pombe* homolog of the replicative DNA helicase *DNA2* of *S. cerevisiae* suppresses *cdc24.* These results suggest that 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 *Schizosaccharomyces pombe* has been particularly useful as a model system for S phase regulation (reviewed in Forsburg 1996; Mac-Neill 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, 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 START of the cell cycle requires the activity of the p34*cdc2* protein kinase

(Nurse and Bissett 1981) as well as *cdc10*+, which encodes a transcription factor (Lowndes *et al.* 1992). Cdc10p activates gene expression of *cdc22*+, encoding ribonucleotide reductase (Fernandez Sarabia *et al.* 1993), and the initiator encoded by *cdc18*+ (Kelly *et al.* 1993). Based on work from a variety of systems, it is thought that the conserved Cdc18p binds to protein components of the ORC (*o*rigin *r*ecognition *c*omplex; Muzi-Falconi and Kelly 1995; Carpenter *et al.* 1996; Grallert and Nurse 1996; Leatherwood *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 *et al.* 1996; Donovan *et al.* 1997). Following the assembly of these factors, the origin fires and replication initiates, which requires additional activators including the Hsk1p/Cdc7p kinase (Sclafani and Jackson 1994; Masai *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 *et al.* 1991; Singh and Klar 1993), and polymerase epsilon (*cdc20*+; D'Urso and Nurse 1997). Elongation requires the activities of the polymerase delta gene (*pold*+/*pol3*+/ *cdc6*+; Pignede *et al.* 1991; Francesconi *et al.* 1993; Iino and Yamamoto 1997) and that of its associated regulatory subunit *cdc1*+ (MacNeill *et al.* 1996). The

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polymerase delta-associated processivity factor PCNA ($pcn1^+$; 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 ($cdc17^+$; Johnston *et al.* 1986). The Cdc18p initiator protein is degraded as replication proceeds, which provides one mechanism to ensure that each origin fires a single time per cell cycle (Jallepalli and Kelly 1996; Kominami 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*, *Δpol1*, and *Δcdc18*, bypass checkpoint activation and proceed catastrophically through mitosis (D'Urso *et al.* 1995; Kelly *et al.* 1993; Saka and Yanagida 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 Cdc$^-$ (cell division cycle) phenotype because the checkpoint system is activated succcessfully (Carr and Hoekstra 1995; Stewart and Enoch 1996). Thus, mutants with a Cdc$^-$ 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; MacNeill *et al.* 1996; Iino and Yamamoto 1997); $cdc17^+$ (DNA ligase; Johnston *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; D'Urso 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 *S. cerevisiae* 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 (Maundrell 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 × 10^6 cells/ml in YE media at 25°. Small cells in G$_2$ 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 mm 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 detailed (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', b = 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 7 Ura$^+$ colonies which could

TABLE 1

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

Strain		Genotype	Source
KGY28	h^-	*972*	Paul Nurse
KGY69	h^+	*975*	Paul Nurse
KGY205	h^+	*cdc24-M38*	Paul Nurse
KGY252	h^-	*cdc24-M44*	Paul Nurse
KGY255	h^-	*cdc24-M81*	Paul Nurse
KGY155	h^-	*cdc25-22*	Paul Nurse
KGY463	h^-	*rad1::ura4$^+$ ura4-D18 leu1-32 his3-237*	Suresh Subramani
KGY466	h^-	*cdc25-22 rad1::ura4$^+$ ura4-D18 leu1-32*	Our stock
KGY445	h^-	*cdc24-G1 ade6-M210 ura4-D18 leu1-32 his3-D1*	This study
KGY476	h^+	*cdc24-G1 ade6-M210 ura4-D18 leu1-32 his3-D1*	This study
KGY705	h^-	*cdc24-G1 rad1::ura4$^+$ ura4-D18*	This study
KGY952	h^-/h^+	*cdc24-G1/cdc24::his3$^+$ ura4-D18/ura4-D18 leu1-32/leu1-32*	This study
		his3-D1/his3-D1 ade6-M210/ade6-M216	
KGY967	h^-/h^+	*cdc24$^+$/cdc24::his3$^+$ ura4-D18/ura4-D18 leu1-32/leu1-32*	This study
		his3-D1/his3-D1 ade6-M210/ade6-M216	
KGY425	h^-	*ade6-M210 ura4-D18 leu1-32 his3-D1*	Our stock
KGY461	h^+	*ade6-M210 ura4-D18 leu1-32 his3-D1*	Our stock
KGY553	h^-	*ade6-M216 ura4-D18 leu1-32 his3-D1*	Our stock
KGY554	h^+	*ade6-M216 ura4-D18 leu1-32 his3-D1*	Our stock
FY254	h^-	*ade6-M210 ura4-D18 leu1-32 can1-1*	Our stock
FY318	h^+	*cdc25-22 ade6-M210 ura4-D18 leu1-32*	Our stock
FY370	h^+	*cdc24-M38 ura4-D18 leu1-32 can1-1*	Our stock

grow at 36° 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 of *cdc24-G1* was narrowed by constructing subclones and testing them for their ability to rescue. The DNA sequence of the smallest rescuing fragment (*Eco*RV-*Hin*dIII) was then determined on both strands after subcloning into pSK(-) using Sequenase 2.0 with either custom synthesized oligonucleotides or reverse and T7 primers. A pDB20-based cDNA library (Fikes *et al.* 1990) was screened with the *Bgl*II-*Hin*dIII fragment to obtain *cdc24$^+$* cDNA clones. A single cDNA clone of ∼1.8 kb in length was identified among 300,000 cDNA clones screened. It was sequenced on both strands to confirm the presence and position of introns. To confirm the sequence of the 5′untranslated region and thus the position of the predicted ATG initiating methionine codon, 5′RACE was carried out utilizing cDNA generated from the Marathon cDNA Amplification Kit (CLONETECH, Palo Alto, CA) as template and the oligonucleotides AP-1 (supplied by the manufacturer) and RACE2 (5′-GTTTAGATTTCT CAACTAAGCGG-3′). The two resultant PCR products were sequenced 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 further upstream of nucleotide −163 (Figure 4). For sequencing of *cdc24* 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 ATTCACACGGCAGAGAG-3′). The PCR products were sequenced directly using the ThermoSequenase cycle sequencing kit (Amersham) and customized oligonucleotides.

The high copy suppressor of *cdc24-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 of *cdc24-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 (*S. pombe* Sequencing Group at the Sanger Centre at http://www.sanger.ac.uk/Projects/S_pombe/blast_server.shtml) which positioned the genomic insert to be on cosmid c16D10 (ftp://ftp.sanger.ac.uk/pub/yeast/sequences/pombe/chromosome_2/c16D10.unfinished) from chromosome *II*. Plasmids pSGP50, pSGP51, and pSGP52 contain subclones of the genomic fragment in pUR19: a 4.6-kb *Nsi*I-*Pvu*II fragment (pSGP50), a 5.4-kb *Pst*I fragment (pSGP51), and a 4.4-kb *Nsi*I-*Pvu*II fragment (pSGP52). The *Pvu*II 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 *Bam*HI fragment of the *cdc24$^+$* genomic clone was removed from pUR19 and inserted into pSK(−) at the *Bam*HI site. The two *Hpa*I fragments of *cdc24$^+$* were replaced with a 2.2-kb fragment of the *his3$^+$* gene (Ohi *et al.* 1996) to create pSK*cdc24::his3$^+$*. The deletion construct was then isolated as a *Bam*HI 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 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 the *cdc24* null mutant, the genomic *cdc24$^+$* clone in pUR19 was transformed into the heterozygous diploid

deletion strain $cdc24^+/cdc24::his3^+$ 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 ^{32}P-labeled probe corresponding to the *Bgl*II-*Hin*dIII fragment of the *cdc24* 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°. Hybridizing bands were detected with a Molecular Dynamics (Sunnyvale, CA) PhosphorImager. Genomic DNA was prepared from diploid *S. pombe* strains according to Moreno *et al.* (1991). For Southern hybridization analyses, 0.5 μg of genomic DNA was digested overnight at 37°, 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-^{32}P]dCTP-labeled probe for 16 hr at 65°. Following hybridization, the filters were washed twice at 65° for 30 min in 0.2× SSC, 0.2% SDS at 65°.

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° for 4 hr. For HU arrest, hydroxyurea was added to a final concentration of 15 mm for 4 hr. Each plug contained 4.8×10^7 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^s* mutants: Three temperature-sensitive alleles of the *cdc24$^+$* 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 of *cdc24* which we have designated *cdc24-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 isolated *cdc24-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 synchronized *cdc24-G1* cells following shift to the nonpermissive temperature of 36°. We found that *cdc24-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
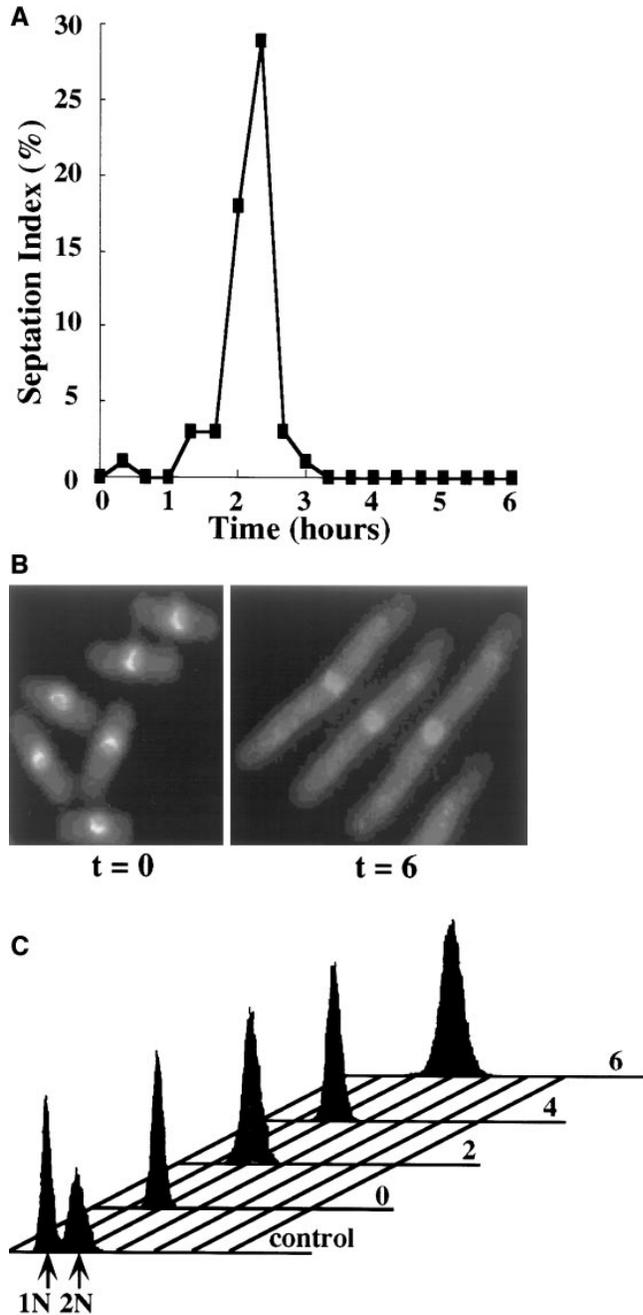
Figure 1.—Phenotypic characterization of the *cdc24-G1* mutant. The *cdc24-G1* strain was grown to midexponential phase and cells were synchronized in G$_2$ by centrifugal elutriation. The synchronized culture was shifted to 36° and the percentage of cells with a septum was determined microscopically every 20 min (A). DAPI staining was performed at 0 and 6 hr following the temperature shift (B). Samples were collected every 2 hr for 6 hr for flow cytometric analysis (C). The controls for 2N and 1N DNA contents were wild-type and nitrogen-deprived wild-type cells, respectively.

due to the continued replication of the mitochondrial genome (Sazer and Sherwood 1990). These data indicate that *cdc24-G1*, like *cdc24-M38*, arrests cell cycle progression in late S phase or G$_2$.
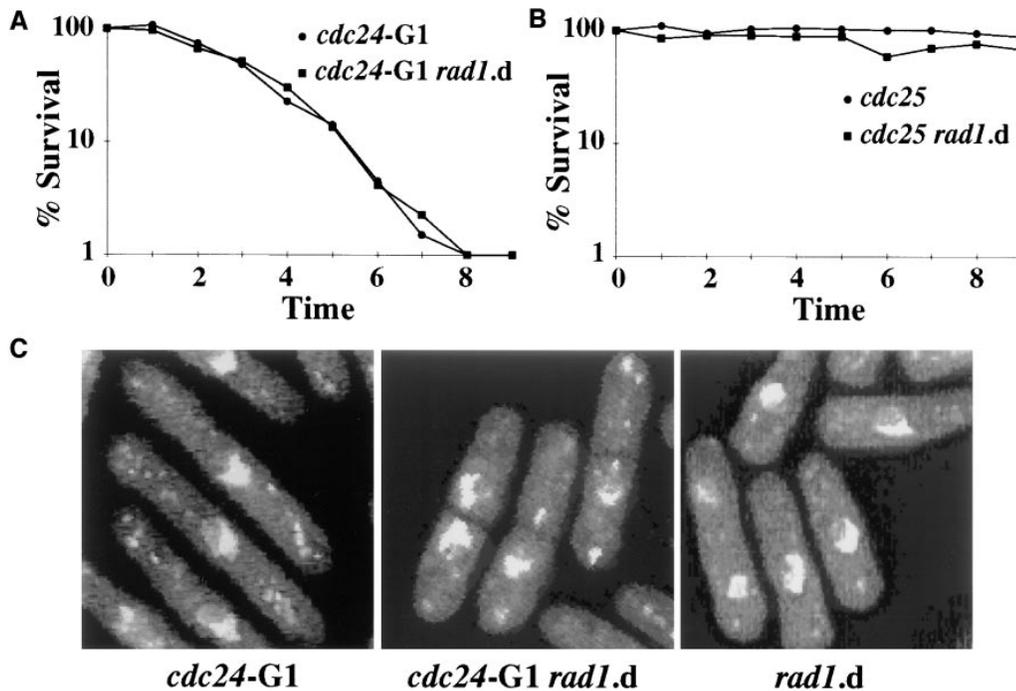
Figure 2.—Percentage survival of cdc mutants in the presence of the *rad1* null mutation at 36°. The percentage survival of (A) *cdc24-G1* and *cdc24-G1 rad1:: ura4⁺* (*rad1*.d) cells and (B) *cdc25-22* and *cdc25-22 rad1*.d cells plotted against time of incubation at 36°. (C) After incubation for 4 hr at 36° in liquid media, *cdc24-G1*, *cdc24-G1 rad1*.d, and *rad1*.d cells were fixed and stained with the DNA binding dye, DAPI.

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⁺* 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° (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° (Figure 2B), and both maintained an elongated, Cdc⁻ 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. Forsberg, 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° 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°, 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; Waseem *et al.* 1992; Maiorano *et al.* 1996; Okishio *et al.* 1996). 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* fragments was also apparent; we found this in all strains including wild type following incubation at 36° (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⁺Cdc⁺ 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 a *cdc24-G1* strain by homologous recombination. Crosses between this integrant and wild-type cells failed to segre-
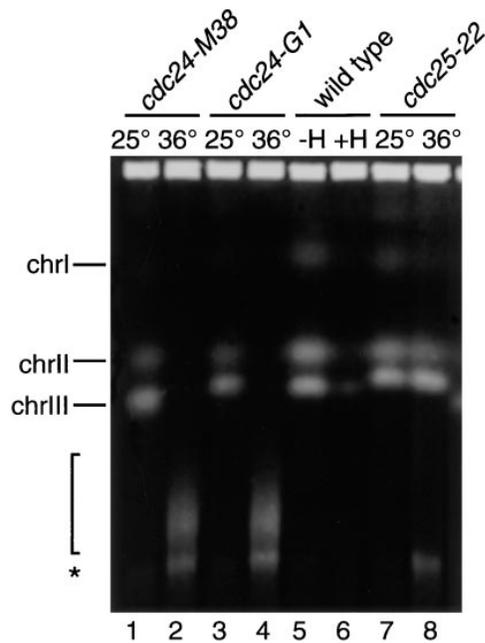
Figure 3.—Pulsed field gel analysis of chromosomes from *cdc24* mutant strains at restrictive temperature shows degraded DNA. Equal numbers of cells were prepared in agarose gel plugs from strains *cdc24-M38* (lanes 1 and 2), *cdc24-G1* (lanes 3 and 4), wild type (lanes 5 and 6), or *cdc25-22* (lanes 7 and 8). The cells were harvested after incubation at 25° (lanes 1, 3, 5, 6, 7) or 4 hr at 36° (lanes 2, 4, 8), or after 4 hr of treatment with HU to arrest S phase (lane 6). The discrete band at the bottom of the gel (*) is apparent in all strains incubated at 36° (lanes 2, 4, 8, and data not shown).

gate 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⁺* and *pki1⁺*.

Further deletions of the smallest genomic clone indicated that the DNA responsible for rescue of *cdc24-G1* was contained within the *Eco*RV-*Hin*dIII 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 *Bam*HI-*Hin*dIII 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⁺* 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⁺* function would result in a cell cycle defect similar to that of the temperature-sensitive mutations, a null allele of *cdc24⁺* was constructed by the one-step gene disruption method. Two internal *Hpa*I fragments within the *cdc24⁺* coding region were replaced with the

```
-270                                                                         GATATCTGT
-261  TTTTAAATGAATTAAATATTATATTTGGTTATATGCTGTTTATTGTGTCTTCTTACTTAAAATTTCGATTGTGTGATATGAAATAACAA
-174  AAATAATGACAGCAAACAAGTTTTTAAATTGTAAAAATTTTATAAGTATTGTTCAATATCATTAGGCGTAACAGTATAAAAACAGAT
-87   GATACTAAATGGAGTATTTAGGCAGCTGTCAATTATCTATATTCTATCAACTACTAATCTTAGAGCAGTAGTAAATTTAATGATTAT
                     BglII
1     ATGGATTTTCCAGGTCTGATTAAAAAAATAAAAGAAGATCTAGAAAACCGCTTAGTTGAGAAATCTAAACAACCAGCATTCACTGAT
1      M  D  F  P  G  L  I  K  K  I  K  E  D  L  E  N  R  L  V  E  K  S  K  Q  P  A  F  T  D

88    TACTGGTTAATAAACAAAACTATCAGCTTGTTAAAGGAGTATCCCTCAGGGTTTAGCTATCCCATTTTAATTAACGAGATTGAATTA
30     Y  W  L  I  N  K  T  I  S  L  L  K  E  Y  P  S  G  F  S  Y  P  I  L  I  N  E  I  E  L
                                                  HpaI
175   GCGTGCTCGGAGAGAAATAAATTGGACAATGTTTTTTCTTCTTTGGAATGGTTAACGAAATCAGATGCAGTTATTTATTTGGAAGAC
59     A  C  S  E  R  N  K  L  D  N  V  F  S  S  L  E  W  L  T  K  S  D  A  V  I  Y  L  E  D

262   CAGTTTCATTTTCATTGCTATGATTATTATTATACCCCAATACCGTCTTTGAAGCTGAAAGATAGTGAGGAGAATACGTGCGAAATG
88     Q  F  H  F  H  C  Y  D  Y  Y  Y  T  P  I  P  S  L  K  L  K  D  S  E  E  N  T  C  E  M

349   ATTTTGCATTCCAGTTTTTATTTACTCTTCCATGAACCATTTAACATTTTGAAAGGTTCAACTTTACGCTTTTCTCAGTCACGCATC
117    I  L  H  S  S  F  Y  L  L  F  H  E  P  F  N  I  L  K  G  S  T  L  R  F  S  Q  S  R  I

436   CATCAAAAATGGTATTTACGAAAGCAAATACGTTTCCTTCCTACAACTAGCTTTGTTCCAGTATTGCAATATGCTGCATCAAAAAGT
146    H  Q  K  W  Y  L  R  K  Q  I  R  F  L  P  T  T  S  F  V  P  V  L  Q  Y  A  A  S  K  S
                                EcoRI
523   TTTGTGAAATCAATGGTTGAATCAACTGAACATGAATTCTTTCAAATACGTGATATTACTATAAGCAAAAGTGATCAAGGTGTGAAA
175    F  V  K  S  M  V  E  S  T  E  H  E  F  F  Q  I  R  D  I  T  I  S  K  S  D  Q  G  V  K

610   CGAATTACTGTGGAATTGGGTCGCATCATTTCTTTAGACAAAAAAGGTGCTTTTCTTATAGACAACAATACCAATTTTATACATTTC
204    R  I  T  V  E  L  G  R  I  I  S  L  D  K  K  G  A  F  L  I  D  N  N  T  N  F  I  H  F

697   TCAGGGACTTACCATGATTTCCCTAAGCTGCTAGACAAGCAACAATACCTACTTATTCCATTTTGGAGTACCGTTTGGGAAGGCAGT
233    S  G  T  Y  H  D  F  P  K  L  L  D  K  Q  Q  Y  L  L  I  P  F  W  S  T  V  W  E  G  S

784   ACCAACTGGCAGGATGGAATGATCGGTATTTTGGTAATTTTAAGTGAGAATGACTTTGAAGAAAAAAAATGCGACTCCTGGCAAGTG
262    T  N  W  Q  D  G  M  I  G  I  L  V  I  L  S  E  N  D  F  E  E  K  K  C  D  S  W  Q  V

871   GGACGCTTAGCTGCCGTTAATTCAAATTTTCTTGTACTGGATAACAATGGTCAGCGAGTGAAGATATTGTATTCCAATTCTCAAAAG
291    G  R  L  A  A  V  N  S  N  F  L  V  L  D  N  N  G  Q  R  V  K  I  L  Y  S  N  S  Q  K

958   CATTATTTCAAGGATACCTATATTGGAGATTTGCTATGCATTAGCCCAATCAATCATTCTTCTGACAATCTAAGTGATTATTCATGC
320    H  Y  F  K  D  T  Y  I  G  D  L  L  C  I  S  P  I  N  H  S  S  D  N  L  S  D  Y  S  C
                            ↓
1045  GTTAGTGACACAACGACTGAAATTCTGTCAAACTTGGAGAACCCGTTATCCTCAAACTTTTTGAGAAATGATTTATGCAAGTTCTCC
349    V  S  D  T  T  T  E  I  L  S  N  L  E  N  P  L  S  S  N  F  L  R  N  D  L  C  K  F  S
                                                                         HpaI
1132  AACCTTTTTTCAGGTGTTTCTGGGCATTTAAAAAATTCAACCGTTAACTTTGGTGATCACCGGATTTGTAGCGAATTTAACCCGCCAA
378    N  L  F  S  G  V  S  G  H  L  K  I  Q  P  L  T  L  V  I  T  G  F  V  A  N  L  T  R  Q

1219  TCTGATTTAAATACCAGTTTGGTCTTGCCGGAAGTAAATCCATTAACAGCAAAAATGGAGGTAATTGACTGTTTAAGGAATAGTTTT
407    S  D  L  N  T  S  L  V  L  P  E  V  N  P  L  T  A  K  M  E  V  I  D  C  L  R  N  S  F

1306  TGGGCAGCTTTACGACCGAATTGTGTTTCATATTTTGCGTCTATGATTTCCGGCTTTAAAGAATTATTCCAAAAAGAAATGGAATTT
436    W  A  A  L  R  P  N  C  V  S  Y  F  A  S  M  I  S  G  F  K  E  L  F  Q  K  E  M  E  F

1393  CCAATATACCTGGATTTGAAGTCGTACAAGCTCGGATTCCATTGGTGTGATATTTATGTGGACTCAGAACACAACTGCCATATACAA
465    P  I  Y  L  D  L  K  S  Y  K  L  G  F  H  W  C  D  I  Y  V  D  S  E  H  N  C  H  I  Q

1480  AAAATAAACTCTCTGCCGTGTGAATAGCCATCGCCATTATCTGAATAATTATGTCTAACAAATAAAAATCAAACATTGAATGTTTGA
494    K  I  N  S  L  P  C  E  *

1567  ATGAAAAGATGTACAGTTCATTCAATCATTCTCCGTTTTAAAAGCTTAGTTTGCTTAATTTACTAATATACTACAGTTTAAAAGGCT
```

Figure 4.—Nucleotide sequence of the *cdc24*⁺ gene and deduced primary structure of the encoded protein. The nucleotide sequence is numbered starting with the first base of the proposed ATG initiation codon. The ORF is interrupted by six introns in the gene whose positions are indicated by arrowheads. The amino acid sequence is in single letter code underneath the DNA sequence. An asterisk denotes the position of the stop codon.

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-*Hin*dIII 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 *Bgl*II fragment. Two hybridizing bands were

TABLE 2

Identification of *cdc24* alleles

Mutant allele	G1	M38	M44	M81
Wild-type sequence	GTA Splice donor 5th intron	AGA Arg370	TCA Ser358	TCA Ser136
Mutant sequence	ATA Splice donor?	TGA Stop	CCA Pro358	CCA Pro136

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 Cdc− 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° accumulating many elongated and cell cycle-arrested cells in the culture (data not shown). When spores from this diploid were incubated at 36° 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
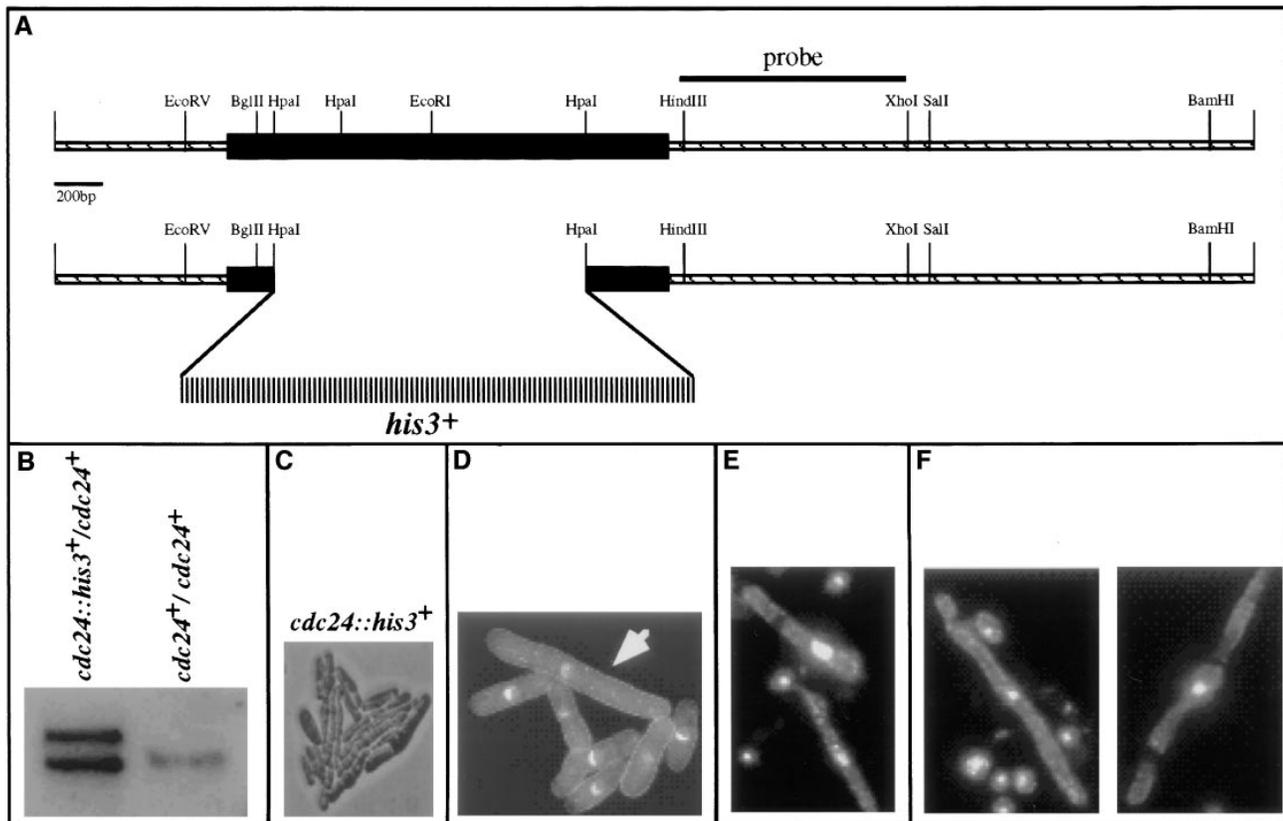
Figure 5.—Deletion of the *cdc24+* gene. (A) Restriction map of the *cdc24+* gene. Relevant restriction sites are indicated. The *cdc24* coding region is represented by the solid black box. The construction of pSK(−)*cdc24::his3+* is shown below the map. The *Hpa*I fragments were removed and replaced with the *his3+* gene. The *Hin*dIII-*Xho*I 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 *Bgl*II 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°. (D) Phenotype of *cdc24+* loss. A haploid *cdc24* deletion strain carrying the multicopy pUR19*cdc24+* plasmid was grown to midexponential phase in nonselective medium to allow plasmid loss. Cells were fixed in formaldehyde and stained with DAPI.

A

B
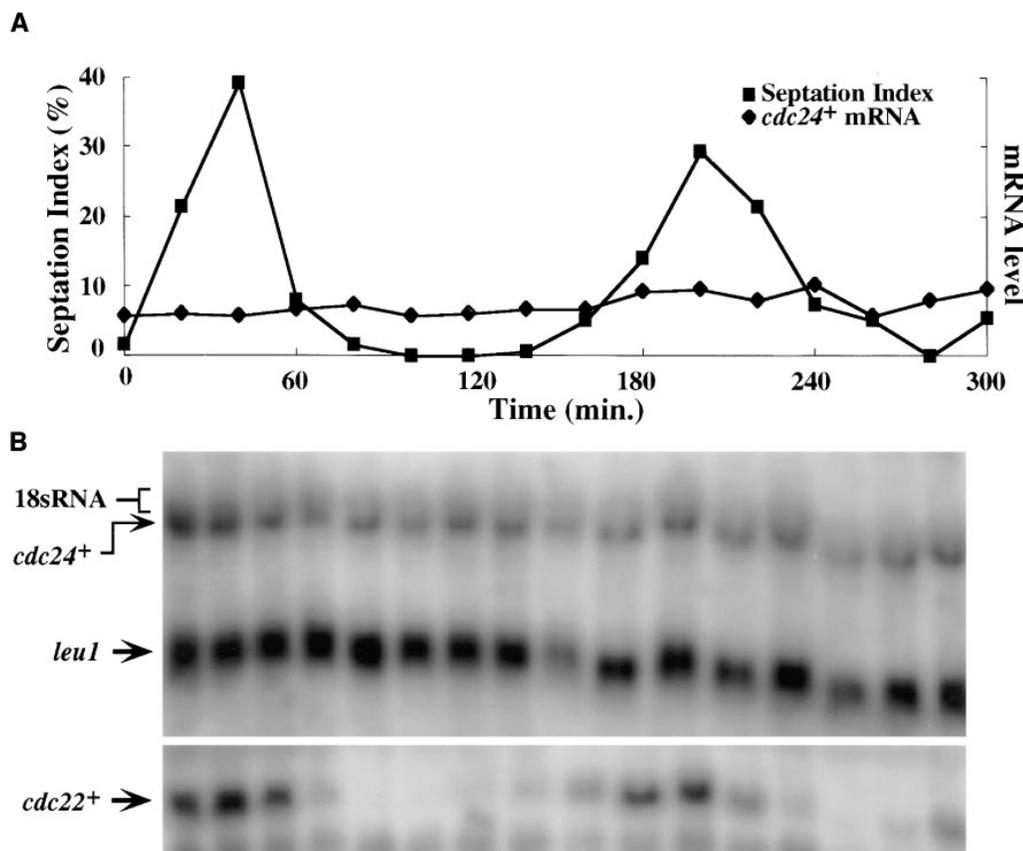
Figure 6.—Level of *cdc 24+* transcript through the cell cycle. Wild-type 972 cells were grown to midexponential phase, small cells in early G₂ were isolated by centrifugal elutriation, and the synchronized culture was incubated at 32° through two cell divisions. (A) The septation index of the culture 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 an 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 *Nsi*I-*Pvu*II 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 on 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

```
        1 kb
              E     PE        B N   N      P   P          rescue?
pSGP45  |----|--|------------|-|---|------|---|--------         +
pSGP50                        |-----------|-------------        +
pSGP51                    |--------------------|                -
pSGP52  |----------------------|                                -
```

dna2+

B

pUR19   pURcdc24

pSGP50   pSGP45

C

```
Sp LNEDQITALKRCHAAEHMSLILGMPGTGKTTTTSSILRSI 821
Sc LNLNQKEAIKDVMRAEDYALILGMPGTGKTTVIAEIIKIL 1091

Sp LAKKKKILLTSFTHLAVDNILIKIKGCDSTIVRLGSPHKI 861
Sc VSEGKRVLLTSYTHSAVDNILIKLRNTNISIMRLGMKHKV 1131

Sp HPLVKEFCLTEGTTFDDLASLKHFY---EDPQIVACSSLG 898
Sc HPDTQKY----VPNYASVKSYNDYLSKINSTSVVATTCLG 1167

Sp VYQZVIESDGNEQTTNNISSIFNKRKFDYCIIDEASQIPL 938
Sc INDILFT---------------LNEKDFDYVILDEASQISM 1193

Sp HICLGPIQLAEKFVLVGDHYQLPPLVKNSRTSKDGISLSI 978
Sc HVALGPIRYGNRHIMVGDHYQLPPLVKNDAARLGGIEESI 1233

Sp FKLLSEKHPEAVTTIRLQYRMNEDINSLSSELIYGGNIVQ 1018
Sc FKTFCEKHPESVAELILTLQYRMCGDIVTLSNFLIYDNKLKQ 1273

Sp GSKTISQKKIILEKAH-LSDGLPDSSSELHWVNKLINFSH 1057
Sc GNNEVFAQSLFELFMPEALSRYRNESANSKQWLEDILEETR 1313

Sp SVIFFNTDDILGV-FSKTNNILENFHTEAFIIEQAMSSFLE 1096
Sc KVVFLNYDNCPDIIFQSEKDNITNHGEAELTLQCVEGMLL 1353

Sp RGVKQSSIGIISIMKSQVELISRNLKS--FTEIFINTVDR 1134
Sc SGVPCEDIGVMTLYRAQLRLIKKIFNKNVYDGLEIIIADQ 1393

Sp YQGRDKDITLLISFVRSNSKNLVSIEPIIYTTANHIFRLEN 1174
Sc FQGRDKKCIIIISMVRRNSQ--LNGGALL----KEIRRVN- 1426

Sp CYAIGIVVALSRAKVRCIMFGSLSTLSSSNIVSHLLKLLE 1214
Sc -------VAMTRAKSKLIIIGSKSTIGGSVPEIKSFVNLLE 1459

Sp KNKWIFTLNENDIAT-KFDENSSPIKDCSQV-ATTNNAKV 1252
Sc ERNWVYLMCKDALYKYKFPDRSNALDEARKGCGKRTGAKP 1499

Sp IIRKNQRFFNSDNLCEKAIIPQLFF              1277
Sc ITSKSK--FVSDKPIIKEIIQEYES              1522
```

Figure 7.—(A) Analysis of *cdc24* suppressor. Restriction map of the genomic fragment that complements *cdc24*[ts] growth defect at nonpermissive temperature. pSGP45 is the pUR19 genomic library plasmid which rescues growth of *cdc24* at 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 *Nsi*I-*Pvu*II fragment, a 5.4-kb *Pst*I fragment, and a 4.4-kb *Nsi*I-*Pvu*II fragment, respectively. These plasmids were tested for the ability to rescue *cdc24*[ts] growth at the nonpermissive temperature: plus (+) indicates complementation, minus (−) indicates no complementation. E, *Eco*RI; B, *Bst*XI; N, *Nsi*I; P, *Pst*I. The black arrow

mic fragment of pSGP50 encodes 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 *dna2*+. Thus, the suppressor of the *cdc24*[ts] growth defect at the nonpermissive temperature is a putative DNA helicase.

DISCUSSION

In this report, we have described the cloning and characterization of the fission yeast *cdc24*+ gene. Previously, *cdc24* was shown to be required for normal S phase progression, and execution point analysis suggested that *cdc24*+ functions after the HU arrest point in early S phase (Nasmyth and Nurse 1981). Further, it was shown that *cdc24* mutants accumulate broken DNA. Our results support and extend the original analysis.

We have cloned and sequenced the *cdc24*+ gene. 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-sensitive *cdc24* alleles were determined. On the basis of genetic crosses, it was concluded previously that *cdc24-M38* and *cdc24-M44* were 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 within *cdc24-M38* introduces a premature stop codon at posi-

corresponds to the rescuing ORF which is homologous to ScDna2p. (B) The fission yeast Dna2p homolog rescues growth of *cdc24* at the nonpermissive temperature. *cdc24-G1* strains carrying the empty vector (pUR19), the genomic *cdc24*+ (pURcdc24), the original high-copy suppressor (pSGP45), or a 4.6-kb fragment of the suppressor (pSGP50), were streaked out on agar plates and incubated at 36° for 72 hr before photography. (C) Alignment of C-terminal third of SpDna2p and ScDna2p. In the alignment, the top line is the fission yeast Dna2p homolog, SpDna2p (labeled Sp, residues 782–1277) and the bottom line is ScDna2p (labeled Sc, 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-M44* 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/Poldp; 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; Δ*pcn1* cells go through at least one or two divisions before arresting, suggesting that this protein is sufficiently abundant and stable to be packaged 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 *pcn1* (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; Palitti 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 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 dna2ᵗˢ* 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 *cdc24ᵗˢ* 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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Communicating editor: P. G. Young