Fission Yeast Cdc24 Is a Replication Factor C- and Proliferating Cell Nuclear Antigen-Interacting Factor Essential for S-Phase Completion

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At the nonpermissive temperature the fission yeast *cdc24-M38* mutant arrests in the cell cycle with incomplete DNA replication as indicated by pulsed-field gel electrophoresis. The *cdc24*⁺ gene encodes a 501-amino-acid protein with no significant homology to any known proteins. The temperature-sensitive *cdc24* mutant is effectively rescued by *pcn1*⁺, *rfc1*⁺ (a fission yeast homologue of RFC1), and *hhl1*⁺, which encode the proliferating cell nuclear antigen (PCNA), the large subunit of replication factor C (RFC), and a casein kinase I involved in DNA damage repair, respectively. The Cdc24 protein binds PCNA and RFC1 *in vivo*, and the domains essential for Cdc24 function and for RFC1 and PCNA binding colocalize in the N-terminal two-thirds of the molecule. In addition, *cdc24*⁺ genetically interacts with the gene encoding the catalytic subunit of DNA polymerase ϵ , which is stimulated by PCNA and RFC, and with those encoding the fission yeast counterparts of Mcm2, Mcm4, and Mcm10. These results indicate that Cdc24 is an RFC- and PCNA-interacting factor required for DNA replication and might serve as a target for regulation.

Chromosomal DNA is replicated by cooperation of a number of factors and enzymes. In the budding yeast *Saccharomyces cerevisiae* they include the origin recognition complex, which is composed of the six subunits called Orc1 to Orc6 (4); Cdc6 (8); minichromosome maintenance (MCM) proteins (56); and at least three DNA polymerases. The origin recognition complex recognizes and binds origins of replication throughout the cell cycle. When cells enter S phase, MCM proteins are loaded onto the origins in a Cdc6-dependent manner (2, 53), and finally DNA polymerase α (Pol α), Pol δ , and Pol ϵ are recruited to start DNA synthesis. A recent analysis indicates that not only these polymerases but also some MCM proteins are components of replication forks (2).

The roles of Pol α , Pol δ , and other factors in DNA replication were initially elucidated by studies with a cell-free simian virus 40 (SV40) DNA replication system (reviewed in references 51 and 58). In this system, Pol α with its primase subunits synthesizes RNA primers and elongates them to short initiator DNAs, which in turn serve as primers for the leading- and lagging-strand synthesis that is catalyzed by Pol δ . Thus, during DNA replication, the polymerase used for synthesis is switched from α to δ . This switch requires two accessory proteins, replication factor C (RFC) and proliferating cell nuclear antigen (PCNA) (55). RFC binds the primer ends and thereby recruits and loads PCNA onto them (29, 54). Subsequently, Pol δ binds the complex and elongates the DNA strands with high processivity (54). RFC is composed of five related subunits,

one large and four small ones. By contrast, PCNA forms homotrimeric to act as a sliding clamp (27). This factor is essential for the high processivity of Pol δ (47, 48). These polymerases and accessory proteins are highly conserved throughout eukaryotes. The replication of chromosomal DNA, however, requires another type of DNA polymerase called Pol ϵ (3, 63), although its exact role still remains unclear. This polymerase also requires PCNA and RFC for its full activity (7, 30). Both Pol δ and Pol ϵ also play roles in damage repair DNA synthesis (41, 50).

The fission yeast *Schizosaccharomyces pombe* is another good model organism to study DNA replication and cell cycle control mechanisms. Fission yeast counterparts of many of the factors essential for chromosomal DNA replication have been identified. Orp1 and Orp2 are the counterparts of Orc1 and Orc2, respectively (28, 38). Cdc19/Nda1, Cdc21, Nda4, and Mis5 are MCM proteins (10, 15, 36, 52), and Cdc18 is the counterpart of Cdc6 (26, 42). *pol1*⁺/*swi7*⁺, *pol3*⁺/*cdc6*⁺, *cdc20*⁺, and *pcn1*⁺, encoding the catalytic subunits of Pol α , Pol δ , Pol ϵ , and PCNA, respectively, have also been identified (12, 14, 23, 46, 61).

The onset and progression of DNA replication are controlled by a variety of extra- and intracellular conditions that favor or disfavor cell proliferation. When cellular DNA is damaged, progression of DNA synthesis is temporarily halted by a cell cycle checkpoint mechanism (45) until the damage is repaired by DNA synthesis with either Pol δ or Pol ϵ (41, 50). In fission yeast, a casein kinase called Hhl1 participates in repair synthesis after damage by chemicals or irradiation (13). Cells lacking this kinase are sensitive to DNA damage due to reduced repair synthesis.

In this paper we describe the identification of a new PCNA- and RFC-interacting factor that is essential for DNA replication in the cell cycle, namely, Cdc24, which has long been known to be required for S-phase progression (40). In concert with this biochemical interaction, *cdc24*⁺ genetically interacts with *cdc20*⁺, *cdc19*⁺, *cdc21*⁺, and *cdc23*⁺, which encode fission yeast counterparts of the catalytic subunit of Pol ϵ , Mcm2,

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FIG. 1. Properties of *cdc24-M38* cells. (A) The *cdc24-M38* mutant arrests after completion of a bulk of DNA synthesis. *h⁺ cdc24-M38* and *h⁺* wild-type cells were grown to mid-log phase at 23°C and then nitrogen starved for 24 h to be arrested in G₁. The cells were incubated at 36°C for 4 h in order for mutant Cdc24 protein to be inactivated and then reinoculated in growth medium preincubated at 36°C. Cell aliquots were taken every hour and analyzed by flow cytometry. (B) Pulsed-field gel electrophoresis of the chromosomes of the indicated strains. *h⁻ leu1-32* (wild-type [wt]), *h⁻ cdc24-M38 leu1-32* (*cdc24*), *h⁻ cdc18-K46 leu1-32* (*cdc18*), and *h⁻ cdc25-22 leu1-32* (*cdc25*) cells were grown at 23°C in PM+N supplemented with leucine to log phase, shifted up to 37°C, and incubated for the indicated times. HU, wild-type cells treated with 12 mM hydroxyurea for 4 h at 30°C. Chromosomes were separated by pulsed-field gel electrophoresis and stained with ethidium bromide. (C) Phenotypes of *cdc24* single and *cdc24 rad1* double mutants at the restrictive temperature. *h⁻ cdc24-M38 leu1-32* and *h⁻ cdc24-M38 rad1-1 leu1-32* cells were grown to mid-log phase in YEL medium at 23°C and shifted up to 36°C. Following incubation for 4 h, the cells were fixed with 70% ethanol and stained with DAPI. Arrows indicate cells that underwent aberrant mitosis. Bar, 10 μ m.

Mcm4, and Mcm10 (10, 15, 25a), which are essential for origin activation (35, 62). Interestingly, a temperature-sensitive *cdc24* mutant is rescued by *hpl1⁺*, suggesting that Cdc24 might serve as, or be closely linked to, a target for the regulation of damage repair DNA synthesis.

MATERIALS AND METHODS

Strains and media. Special strains of *S. pombe* used in this study are listed in Table 1. Media were prepared as described previously (18, 37, 44).

Libraries and vectors. An *S. pombe* cDNA library was constructed by the Okayama-Berg method (43) with the SV40-based pcD expression vector and mRNA prepared from a wild-type strain (L972) growing logarithmically in YEL medium (37). *S. pombe* genomic libraries were constructed by inserting restriction enzyme-digested wild-type DNA into the pALSK⁺ vector. The pALSK⁺ vector used for genomic DNA expression, the SV40 promoter-driven pcL, and the thiamine-repressible pREP vectors were described previously (22, 33, 39).

Temperature shift analysis. The *h⁺s cdc24-M38* and *h⁺s* wild-type cells were cultured in PM+N medium to mid-log phase at 23°C. These cells were nitrogen starved for 24 h in PM-N medium (37) and then incubated at 36°C for 4 h and reinoculated in PM+N medium (37) preincubated at 36°C. Cell aliquots were taken every hour, fixed in 70% ethanol, and analyzed by flow cytometry.

Flow cytometry. Flow cytometry was performed as described previously (9) by using the FACScan system, the CellFIT cell cycle analysis program, and the software LYSIS II (Becton Dickinson).

Pulsed-field gel electrophoresis. Chromosomes in agarose plugs were prepared as described previously (32) with slight modifications. Cells (5×10^8) were washed with CSE (20 mM citrate-phosphate [pH 5.6], 1.2 M sorbitol, 40 mM EDTA, and 150 mM β -mercaptoethanol) and resuspended in 10 ml of CSE

containing 30 mM β -mercaptoethanol and 0.3 mg of Zymolyase 100T (Seikagaku Kogyo) per ml. After digestion at 37°C for 1 h, the cells were pelleted and resuspended at 8×10^8 cells/ml in TSE (10 mM Tris-HCl [pH 7.5], 0.9 M sorbitol, 45 mM EDTA). The cell suspension was warmed to 37°C, added to an equal volume of 1% low-melting-point agarose in TSE, and dispensed in plug molds. Cells in agarose plugs were lysed first in 0.25 M EDTA–50 mM Tris-HCl (pH 7.5)–1% sodium dodecyl sulfate (SDS) for 90 min at 55°C and then in NDS (0.5 M EDTA [pH 9.5], 1% lauryl sarcosine) containing 0.5 mg of proteinase K per ml for 48 h. The plugs were stored at 4°C and equilibrated with TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) before electrophoresis. Pulsed-field gel electrophoresis was carried out in a 0.6% agarose gel with a Gene Navigator (Pharmacia) apparatus for 83 h in $0.5 \times$ TAE buffer at 50 V with a switching time of 30 min.

Isolation of the *cdc24⁺* gene and multicopy suppressors of the *cdc24* mutant. The *cdc24⁺* gene and multicopy suppressors were isolated by rescue of the temperature-sensitive *cdc24-M38* mutant as described previously (44). The *h⁻ cdc24-M38 leu1-32* cells were cultured at 23°C to mid-log phase in MB medium containing 0.015% leucine and cotransfected with the *S. pombe* cDNA library and the *Pst*I-digested transducing vector pAL19 (44). The cells were incubated on MMA plates at 23°C for 24 h and at 33°C for 4 days (44). Plasmids were recovered from transformants and confirmed for their suppression activity. Isolated cDNAs were classified by hybridization analysis and subcloned into the pcL vector for further analysis. Similarly, a *cdc24⁺* genomic DNA was isolated from the genomic DNA library by rescue of the *cdc24-M38* mutant.

DNA sequencing. DNA sequencing was performed by the dideoxy chain termination method with 373S DNA sequencer (ABI).

Gene disruption. A null mutant of the *cdc24⁺* gene was constructed as follows. The internal 1.9-kb *Bgl*II-*Hind*III fragment containing 97% of the *cdc24⁺* coding region was replaced with the 1.8-kb *Hind*III-excised *ura4⁺* gene. The 3.9-kb *Bam*HI-*Sal*I fragment containing the disrupted *cdc24* gene was transfected into

TABLE 1. Special strains used in this study

Strain	Genotype
H159-18A	<i>h⁻ cdc19-P1 cdc24-M38 leu1-32</i>
H185-131	<i>h⁻ cdc20-M10 cdc24-M38 leu1-32</i>
H154-12A	<i>h⁻ cdc21-M68 cdc24-M38 leu1-32</i>
H152-21	<i>h⁻ cdc23-M36 cdc24-M38 leu1-32</i>
DH3-9	<i>h⁺/h⁻ cdc24⁺/cdc24::ura4⁺ ade6-M210/ade6-M216 ura4-D18/ura4-D18 leu1-32/leu1-32</i>
DH3TS-9	<i>h⁺/h⁻ cdc24-M38/cdc24::ura4⁺ ade6-M210/ade6-M216 ura4-D18/ura4-D18 leu1-32/leu1-32</i>
U1-2	<i>h⁺/h⁻ ade6-M210/ade6-M216 ura4⁺/ura4-D18 leu1-32/leu1-32</i>

TABLE 2. Ability of multicopy suppressors to rescue *cdc24-M38* cells^a

Plasmid	% Suppression ^b
pcL-cdc24	41.9
pcL-rfc1	33.1
pcL-pcn1	28.8
pcL-hhp1	24.1
pcL-X	<0.1

^a *h⁻ cdc24-M38 leu1-32* cells were transfected with the indicated plasmids, incubated at 23°C for 24 h and selected at 32.5°C for 5 days.

^b Ratio of the number of colonies formed at 32.5°C to the number formed at 23°C.

FIG. 2. Suppression of the temperature sensitivity of the *cdc24*-M38 mutant by *cdc24*⁺, *rfc1*⁺, *pcn1*⁺, and *hhp1*⁺. *h*⁻ *cdc24*-M38 *leu1*-32 cells carrying the indicated plasmid were streaked on SD plates and incubated at the indicated temperatures for 4 days. Initially isolated 5'-truncated *rfc1*⁺ was used.

a diploid strain (*h*⁻/*h*⁺ *leu1*-32/*leu1*-32 *ura4*-D18/*ura*-D18 *ade6*-M210/*ade6*-M216). Eleven stable transformants were obtained, and successful disruptants were identified by Southern blot analysis with the *Sal*I-*Bam*HI 1.4-kb fragment as a probe.

Analysis of germinating spores. Spores were isolated by treatment with helicase (31, 37). Diploid strains were sporulated on MEA plates for 7 days (37). Spores formed were washed with water and incubated at 30°C for 18 h in 40 ml of water containing 0.1 ml of helicase (IBF). After being washed with water three times, the spores were cultured in PM medium containing adenine and leucine at 30 or 35°C to induce preferential germination of Δ *cdc24* (*ura*⁺) cells. Germinating cells were collected every 2 h and fixed with 70% ethanol, followed by 4',6-diamidino-2-phenylindole (DAPI) staining and fluorescence-activated cell sorter analysis.

Construction of epitope-tagged genes. The entire *cdc24*⁺ open reading frame (ORF) sequence was amplified by PCR with primers containing an *Nde*I (for the 5' primer [5' GCGCCATATGGATTTCAGGTCTG 3']) or *Bam*HI (for the 3' primer [5' GGCCGGATCCTATTCACACGGCAGAGAG 3']) restriction enzyme recognition site and a FLAG epitope-coding sequence (for 5' FLAG tagging, 5' GCGCCATATGGACTACAAGGACGACGATGACAAGATGGA TTTCCAGGTCTGAT 3'; for 3' FLAG tagging, 5' GGCCGGATCCTCAC TTGTCATCGTCGTCCTTGTAGTCTTCACACGGCAGAGAGTTT 3') just before and after the *cdc24*⁺ ORF, respectively. Amplified fragments were digested with *Nde*I and *Bam*HI and inserted between the *Nde*I and *Bam*HI sites of the pREP41-X vector. *cdc24*⁺ gene deletion mutants were constructed by PCR with the pREP41-*cdc24*-FLAG or pREP41-FLAG-*cdc24* plasmids as templates. The primers (5' GGCCGGATCCTACAATACTGGAACAAAGCTAG 3', 5' GGCCGGATCCTAGCTAATGCATAGCAAATCTC 3', 5' GCGCCATATGC AATATGCTGCATCAAAAA 3', and 5' GCGCCATATGCCAATCAATCAT TCTTCTGA 3') were designed and used to obtain N-terminally deleted or C-terminally deleted, FLAG-tagged *cdc24* sequences, which were then digested with *Nde*I and *Bam*HI and inserted into the pREP41 vector. Consequently, N-terminally deleted *cdc24*⁺ sequences were translated from the ATG codon at *Nde*I restriction enzyme recognition sites. The FW253AA mutant, which con-

tains alanine and alanine substitutions of phenylalanine and tryptophan at amino acid residues 253 and 254, was constructed by site-directed mutagenesis (TTT TGG to GCT GCG) with PCR.

For hemagglutinin (HA) tagging, *pcn1*⁺ and *rfc1*⁺ were amplified by PCR with primers (5' TCTAGAGCGGCCGCTATGCTTGAAGCTAGATT 3', 5' GGC CGGATCCCTACTCCTCATCCTCTCA 3', 5' CGCGCATATGGCAAAGT CCCGACTTG 3', and 5' GGCCTCTAGAGCGGCCGCTAGCTGTCTTTTT CTGGATC 3') containing an appropriate restriction enzyme recognition site. After digestion with the corresponding restriction enzyme, DNA was inserted into the N-terminal or C-terminal three-HA-epitope-containing version of the pREP42 and pREP2 vectors, respectively. The initially isolated 5'-truncated form of *rfc1*⁺ was used for construction (the expected translation start site is marked with an asterisk in Fig. 4). All of the sequences amplified by PCR were confirmed by DNA sequencing. Several of these tagged genes were digested with *Nde*I and *Bam*HI, purified, and subcloned into the weakest version of pREP81/82 vectors.

Protein extraction and immunoprecipitation. The *h*⁻ *ura4*-D18 *leu1*-32 cells were transformed with FLAG-tagged and HA-tagged plasmids. The cells transformed with the two distinct plasmids were first grown to mid-log phase at 30°C in PM medium containing 5 μ g of thiamine per ml and then cultured for 16 h in thiamine-free PM medium to induce the expression of epitope-tagged proteins. Cell extracts were made with glass beads and H buffer as described previously (5). The tagged proteins were immunoprecipitated from the lysates with the anti-FLAG monoclonal antibody M2 covalently attached to agarose beads (IBI) (2.5 mg of protein in 1 ml of H buffer) pretreated with bovine serum albumin. Immunoprecipitates were washed four times with H buffer containing 0.15 M NaCl and 2.5 mg of bovine serum albumin per ml and two times with H buffer containing 0.15 M NaCl.

Immunoblot analysis. Crude cell extracts (50 or 100 μ g of protein) and immunoprecipitates from 5 times more crude cell extracts were separated by SDS-10% polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride filter. Epitope-tagged proteins were detected with 1:300-diluted M2

FIG. 3. Analysis of the *cdc24*⁺ gene and *cdc24* null cells. (A) Restriction map of *cdc24*⁺. The *cdc24*⁺ ORF and its cDNA are shown. This gene contains six introns. The *Bgl*II-*Hind*III region of *cdc24*⁺ was replaced with a *ura4*⁺ gene cassette for generating *cdc24* null cells. (B) Predicted amino acid sequence of the protein encoded by *cdc24*⁺. The entire amino acid sequence is shown in single-letter code. The N-terminal two-thirds of the sequence, essential for Cdc24 function, is boxed, and a sequence similar to the PCNA binding motif is underlined. (C) Terminal phenotype of Δ *cdc24* cells. *cdc24*⁺/ Δ *putative-cdc24* diploid cells were tetrad dissected on YEA plates and incubated at 30°C for 4 days. Cells that germinated from a single Δ *putative-cdc24* spore on a YEA plate were photographed. Bar, 10 μ m. (D) DAPI staining of germinating Δ *cdc24* cells. Δ *putative-cdc24* (*ura*⁺) spores derived from Δ *putative-cdc24*/*cdc24*⁺ cells were preferentially germinated in minimal medium lacking uracil. Germinating cells cultured for 18 h were fixed with 70% ethanol, stained with DAPI, and photographed under the microscope. Bar, 10 μ m. (E) *cdc24*-M38/ Δ *putative-cdc24* diploid cells are temperature sensitive. *cdc24*-M38/ Δ *putative-cdc24* and *cdc24*⁺/ Δ *putative-cdc24* diploid strains (DH3TS-9 and DH3-9) were streaked on SD plates and incubated for 3 days at the indicated temperatures. (F) Flow cytometric analysis of Δ *cdc24* cells after spore germination. Spores derived from DH3-9, DH3TS-9, and a *ura4*⁺/*ura4*-D18 control strain (U1-2) were germinated in minimal medium lacking uracil at 30°C (DH3-9 and U1-2) or at 35°C (DH3TS-9). Germinating cells were collected every 2 h, fixed with 70% ethanol, and analyzed by flow cytometry. The positions of the 1C and 2C DNA peaks are indicated.

B

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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 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 A C
61  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 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 I L H S
121 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 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 F V K S M V
181 E S T E H E F F Q I   R D I T I S K S D Q   G V K 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 S G T Y H D F P
241 K L L D K Q Q Y L L   I P F W S T V W E G   S 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   G R L A A V N S N F
301 L V L D N N G Q R V   K I L Y S N S Q K 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 V S   D T T E I L S N L
361 E N P L S S N F L R   N D L C K F S 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 S D L N   T S L V L P E V N P
421 L T A K M E V I D C   L R N S F 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 P I Y L D L   K S Y K L G F H W C
481 D I Y V D S E H N C   H I Q K I N S L P C   E
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anti-FLAG antibody (IBI) or 1:1,000-diluted 12CA5 anti-HA antibody (Boehringer Mannheim) and enhanced chemiluminescence (Amersham).

Analysis of temperature sensitivity of *S. pombe* mutants. The *h⁻cdc20cdc24leu1-32*, *h⁻cdc23cdc24leu1-32*, *h⁻cdc19cdc24leu1-32*, and *h⁻cdc21cdc24leu1-32* double mutants were constructed by crossing corresponding single mutants followed by tetrad analysis at 23°C. The double mutants were streaked on YEA plates and incubated for 3 days at the indicated temperatures.

Nucleotide sequence accession number. The DDBJ/EMBL/GenBank accession number for *cdc24⁺* is AB015436.

RESULTS

The *cdc24-M38* mutant arrests with incomplete DNA synthesis. The *cdc24-M38* mutant was initially isolated as a *cdc* (cell division cycle) mutant (40). This mutant was judged to arrest in S phase at the nonpermissive temperature based on transition point analysis (40). We investigated the arrest point by other methods. When *cdc24-M38* mutant cells were arrested in G₁ by nitrogen starvation, shifted to the nonpermissive temperature, and released to start cell cycling by the addition of a nitrogen source, they entered S phase without any significant delay, just like wild-type cells, and arrested with a 2C DNA content as shown by flow cytometric analysis (Fig. 1A). To characterize this arrest point further, chromosomal DNA was analyzed by pulsed-field gel electrophoresis, in which only completely replicated chromosomes are allowed to enter the gel (19). As shown in Fig. 1B, unlike those of wild-type and *cdc25* (G₂ mutant) cells, but just like those of the cells arrested in S by a *cdc18* mutation or hydroxyurea block, chromosomes of *cdc24-M38* cells incubated at 36°C failed to enter the gel. These results show that despite having a 2C DNA content, *cdc24-M38* cells arrested without completion of DNA replication. This was confirmed by another experiment. Generally, S-phase mutants enter premature mitosis upon a shift to the restrictive temperature if they also carry a checkpoint *rad* mutation (1, 49). The *cdc24* mutant behaved similarly. When cultured for 4 h at the nonpermissive temperature of 36°C, *cdc24-M38 rad1-1* double mutant cells prematurely entered mitosis as indicated by the production of anucleate cells and cells with the "cut" phenotype (Fig. 1C) (20). Thus, we concluded that the *cdc24⁺* gene product is required for completion of DNA replication.

Isolation of the *cdc24⁺* gene. An *S. pombe* cDNA library was screened for clones that could suppress the temperature sensitivity of *cdc24-M38* cells. After extensive screening, four distinct cDNA clones were isolated, all of which had the ability to rescue the mutant (Table 2), but the extent to which the clones rescued the mutant phenotype varied. One clone was found to rescue the mutant even at 36°C (Fig. 2), and the rescued cells were nearly identical to wild-type cells in length (data not shown). Therefore, this clone was characterized first, and it was found to be the *cdc24⁺* gene itself (see below). Genomic DNA clones of this gene were also isolated by phenotypic complementation of *cdc24-M38* cells and were found to contain an ORF with six introns and capable of encoding a 501-amino-acid protein with an estimated molecular mass of 58 kDa (Fig. 3A and B); a FLAG-tagged protein migrated as an approximately 60-kDa protein in SDS gel electrophoresis (see below). An amino acid homology search of the DNA databases revealed that the predicted protein has no significant homology to any known proteins. However, the predicted Cdc24 protein does contain a sequence similar to the PCNA binding motif (QXXLXXFF) found in the p21 cyclin-dependent kinase inhibitor, Fen1 nuclease, and DNA ligase I (25, 60) (Fig. 3B; underlined). This sequence is located within the region essential for Cdc24 function (highlighted in Fig. 3B; see Fig. 5B and Table 3).

To identify the authenticity of this clone as *cdc24⁺*, cells

lacking the putative *cdc24⁺* gene (here designated *putative-cdc24*) were constructed by one-step gene replacement with a *ura4⁺* gene cassette followed by transfection into an appropriate diploid strain (Fig. 3A). Tetrad analysis of *cdc24⁺/Δputative-cdc24* diploid cells demonstrated that two viable and two inviable spores were present in each ascus and that all viable spores were uracil auxotrophic. Microscopic observation revealed that spores with *putative-cdc24* deleted germinated but, after three or four divisions, arrested with cell elongation and one nucleus (Fig. 3C and D). The growth inability of the disruptant was effectively rescued by the putative *cdc24⁺* gene or its cDNA, showing that this phenotype was indeed generated by the deletion of this gene. To obtain definitive evidence for the authenticity of the isolated gene as *cdc24⁺*, *Δputative-cdc24/cdc24-M38* diploid cells were constructed by conjugation of the plasmid-rescued *Δputative-cdc24* cells with the *cdc24-M38* mutant followed by selection of diploid cells that had lost the plasmid. The diploid cells were still temperature sensitive for growth (Fig. 3E) and failed to yield any haploid spores that could grow at 36°C (data not shown). These results led us to conclude that the cloned gene was indeed *cdc24⁺* itself.

Cdc24 is dispensable for bulk DNA synthesis. As shown in Fig. 1A and B, *cdc24⁺* was required for the completion of DNA synthesis, although *cdc24-M38* cells released from G₁ arrest did not have an obvious delay in DNA synthesis at the nonpermissive temperature. We therefore determined whether *cdc24⁺* is required for bulk DNA synthesis by examining the S-phase progression of the *Δcdc24* spores germinated in medium lacking uracil. For this experiment, spores generated from the *Δcdc24/cdc24⁺* and *Δcdc24/cdc24-M38* diploid cells were used. As shown in Fig. 3F, there was no obvious delay in DNA synthesis in *Δcdc24* cells even when they were derived from *Δcdc24/cdc24-M38* cells. Thus, *cdc24⁺* seems to be dispensable for bulk DNA synthesis.

The multicopy suppressors of *cdc24-M38* are *rfc1⁺*, *pcn1⁺*, and *hhp1⁺*. The remainder of the multicopy suppressors were next characterized by DNA hybridization, DNA sequencing, and DNA database searching. Two of them were *pcn1⁺*, which encodes PCNA (61), and *hhp1⁺*, which encodes a casein kinase I involved in DNA repair (13). The third gene was capable of encoding a protein homologous with the large subunit of RFC identified in other organisms but was truncated at the 5' region (Fig. 4). This gene was recently sequenced by the *S. pombe* Sequencing Project at Sanger Center and was contained in the *S. pombe* chromosome II cosmid clone c23E6 (EMBL accession number AL023287). Amino acid alignments with the budding yeast and human counterparts (6, 21) were made. The amino acid sequences are particularly conserved in the RFC homology boxes (11). Based on such structural similarity, we tentatively assigned this gene as a fission yeast homologue of RFC1, encoding the large subunit of RFC, and named it *rfc1⁺*. The amino acid identity between *rfc1⁺* and homologues from other species ranged from 30 to 40%. Further characterization is required to conclude that this gene product is a large subunit of RFC in fission yeast.

Previous work has shown that PCNA and RFC1 interact functionally and physically (16, 34, 57) and act as accessory proteins for Pol δ and Pol ε. On the other hand, the Hhp1 casein kinase I plays a role in DNA repair by promoting damage repair DNA synthesis (13). The abilities of these suppressors to rescue *cdc24-M38* varied as indicated by percent suppression at the minimum restriction temperature (Table 2) and the maximum temperatures that allowed rescued cells to grow. *rfc1⁺* (5' truncated) was able to rescue the mutant at up to 35°C, whereas *pcn1⁺* and *hhp1⁺* rescued at up to 34°C (Fig. 2).

FIG. 4. Predicted amino acid sequence of *rfc1*⁺ and alignment with homologous proteins. The predicted amino acid sequence of *S. pombe* Rfc1 (spRFC1) is shown in single-letter code and aligned with those of the large subunits of RFC of budding yeast (scRFC1) and human (hsRFC1). Amino acids identical to those in *S. pombe* Rfc1 are boxed. The ligase homology domain and the RFC homology boxes (I to VIII) are indicated. The first methionine of the product of the initially cloned truncated gene is indicated by asterisk.

Cdc24 binds Pcn1 and Rfc1 in vivo via its N-terminal two-thirds. The relatively strong suppression of *cdc24-M38* by *pcn1⁺* and *rfc1⁺* suggests that Cdc24 might cooperate with these factors for S-phase progression or completion. To gain insight into the function of Cdc24, we examined possible physical interactions between Cdc24 and PCNA or the large subunit of RFC. To this end, Cdc24, Rfc1 (5' truncated), and Pcn1 were tagged with the FLAG or HA epitope, inserted into the repressible, relatively weak pREP expression vectors, and expressed in wild-type cells. Lysates were prepared from the cells expressing each combination of the genes, immunoprecipitated with anti-FLAG antibody, and analyzed by gel electrophoresis and immunoblotting with anti-FLAG or anti-HA antibodies. As shown in Fig. 5A, Rfc1 and Pcn1 coprecipitated with Cdc24. A rough estimation by densitometric reads of the bands of coprecipitated Rfc1-HA and HA-pcn1 indicated that approximately 10 to 30% and 5 to 20% of these molecules were coprecipitated with Cdc24, respectively. Negative controls expressing an empty vector with no insert gave no coprecipitation, eliminating possible artifacts caused by antibody cross-reactions. To confirm these results, all of these tagged genes were inserted into the weakest version of the pREP vectors pREP81/82 (10-fold weaker than pREP41/42) and similarly analyzed. As shown in Fig. 5A (right panel), tagged Pcn1 and Rfc1 coprecipitated with Cdc24 with efficiencies similar to those obtained with expression from the stronger promoters. These results indicate that Cdc24 forms complexes with Rfc1 and Pcn1 in vivo.

To determine the region of the Cdc24 molecule responsible for binding to Rfc1 and Pcn1, the same tagging and coprecipitation assay was performed for variously truncated Cdc24 molecules (Fig. 5B). In addition, we constructed and tested *cdc24⁺* with two point mutations in the sequence similar to the PCNA binding motif (QXXLXXFF) found in the p21 cyclin-dependent kinase inhibitor and Fen1 nuclease (60). This mutant, named FW253AA, contains alanine substitutions for phenylalanine and tryptophan at amino acid residues 253 and 254. Such substitutions have been shown to inactivate the ability of the p21-derived peptide to bind PCNA (59). This double mutant and the four deletion mutants corresponding to the N-terminal one-third, N-terminal two-thirds, C-terminal two-thirds, and C-terminal one-third were tagged with FLAG, inserted into the relatively weak pREP41 inducible vector, and expressed in wild-type cells, together with HA-tagged Rfc1 or Pcn1. As shown in Fig. 5C, when the N-terminal one-third or two-thirds of Cdc24 was expressed, significant amounts of both Pcn1 and Rfc1 were coprecipitated. In addition, the C-terminal two-thirds of Cdc24 coprecipitated Pcn1 and Rfc1, but to lesser extents, suggesting that the Pcn1 and Rfc1 binding ability is likely to be contained in the N-terminal two-thirds of the molecule. The region of Cdc24 required for binding to Pcn1 and Rfc1 is also the region required for its essential function in

vivo. Among the *cdc24* deletion mutants, only the full-length and N-terminal two-thirds of *cdc24⁺* were able to rescue the *cdc24-M38* mutant at up to 36°C, although the rescuing ability of the N-terminal two-thirds was significantly lower at this temperature (Table 3). The sequence similar to a PCNA binding motif that is contained in this region, however, did not seem to significantly contribute to the Rfc1 and Pcn1 binding activity. The mutant FW253AA coprecipitated with amounts of Pcn1 and Rfc1 similar to that of the mutant Cdc24 protein and was able to rescue *cdc24-M38* at up to 36°C, although its ability to do so was slightly reduced (Fig. 5D; Table 3).

Cdc24 genetically interacts with Pol ϵ and MCM proteins. The suppression and coprecipitation assays indicated that Cdc24 functionally and physically interacts with RFC and PCNA, both of which are required for full activities of Pol δ and Pol ϵ . If RFC and PCNA were physiological targets of Cdc24 action, genetic interaction between *cdc24⁺* and at least one of the genes encoding these polymerases might be expected. Indeed, the *cdc24-M38* mutant combined with *cdc20-M10*, a temperature-sensitive mutation in the catalytic subunit of Pol ϵ , displayed a 0.5 to 1°C drop in the restrictive temperature from the lower temperature for each single mutant (Fig. 6). However, contrary to expectation, double mutants containing *cdc24-M38* and the temperature-sensitive *cdc6-23*, *cdc1-7*, or *cdc27-K3* gene, which encode the catalytic and noncatalytic subunits of Pol δ (23, 31, 64), did not show a detectable drop in the restrictive temperature (data not shown). This result suggests that there is a genetic interaction at least between *cdc24⁺* and *cdc20⁺*, albeit a marginal one.

Interestingly, genetic interactions were also observed with some MCM proteins. *cdc19⁺*, *cdc21⁺*, and *cdc23⁺* encode fission yeast counterparts of Mcm2, Mcm4, and Mcm10, respectively (10, 15, 25a, 35). Double mutants with these genes exhibited 1 to 2°C reductions in the restrictive temperature (Fig. 6). These MCM proteins are essential for the activation of replication origins (35, 62). Moreover, like DNA polymerases, some MCM proteins are also components of replication forks (2). Given these facts, our results suggest that a role of Cdc24 might be to cooperate with PCNA and RFC to activate at least Pol ϵ .

DISCUSSION

All of the genetic and biochemical data presented indicate that Cdc24 plays an essential role in DNA synthesis, a role likely achieved through cooperation with PCNA and RFC. Three lines of evidence suggest that PCNA and RFC are likely to be critical targets for the action of Cdc24. First, expression of *pcn1⁺* and *rfc1⁺* efficiently rescued the *cdc24-M38* mutant. Second, Cdc24 physically interacted with PCNA and the large subunit of RFC as shown by coimmunoprecipitation. Third, the regions responsible for the physical interaction with PCNA

FIG. 5. Cdc24 protein interacts with Pcn1 and Rfc1 in vivo. (A) Left panel, HA-tagged Rfc1 (5'-truncated form) or HA-tagged Pcn1 was coexpressed with or without FLAG-tagged Cdc24 in wild type (*h⁻ ura4-D18 leu1-32*) cells under the control of the *nmr1*-repressible promoters (pREP41-X, pREP41-cdc24-FLAG, pREP2-rfc1-HA, and pREP42-HA-pcn1). Extracts were prepared from exponentially growing cells after a 16-h induction and immunoprecipitated (IP) with anti-FLAG (α -FLAG) antibody. Immunoprecipitates and crude lysates were analyzed by immunoblotting (IB) with anti-FLAG and/or anti-HA antibody. Numbers on the right indicate molecular masses in kilodaltons. Right panel, the same experiment was performed except that all of the tagged proteins were expressed from the weakest version (pREP81/82) of the *nmr1*-repressible promoters (pREP81-X, pREP81-cdc24-FLAG, pREP82-rfc1-HA, and pREP82-HA-pcn1). (B) Schematic illustration of the structures of Cdc24 mutants. Amino acid residues contained in mutant proteins are indicated in parentheses. Four deletion mutants (N 1/3, N 2/3, C 2/3, and C 1/3) and one point mutant (FW253AA) were constructed. Asterisks indicate the positions of point mutations. (C) Cdc24 binds Pcn1 and Rfc1 via its N-terminal two-thirds. Four Cdc24 deletion mutant proteins were coexpressed in *S. pombe* wild-type cells together with HA-tagged Pcn1 or Rfc1 (5'-truncated form) under the control of the *nmr1*-repressible promoter. Cdc24 mutant proteins, Rfc1-HA, and HA-Pcn1 were expressed from the pREP41, pREP2, and pREP42 vectors, respectively. Extracts were prepared from exponentially growing cells after a 16-h induction and immunoprecipitated with anti-FLAG antibody. Immunoprecipitates were analyzed by immunoblotting with anti-FLAG or anti-HA antibody. Immunoblotting with anti-HA antibody against lysates shows the level of HA-tagged Rfc1 or HA-tagged Pcn1 proteins in the cells. (D) Cdc24(FW253AA) binds Pcn1 and Rfc1. The ability of Cdc24(FW253AA) protein to bind Pcn1 and Rfc1 was analyzed as described for panel C.

TABLE 3. Ability of *cdc24*⁺ deletion mutants to rescue *cdc24-M38* cells^a

Plasmid	% Suppression ^b at:	
	33°C	36°C
pREP41- <i>cdc24</i> -FLAG	50.0	39.4
pREP41-FLAG- <i>cdc24</i> (1-167)	<0.1	<0.1
pREP41-FLAG- <i>cdc24</i> (1-334)	18.6	0.7
pREP41- <i>cdc24</i> (168-501)-FLAG	<0.1	<0.1
pREP41- <i>cdc24</i> (335-501)-FLAG	<0.1	<0.1
pREP41- <i>cdc24</i> (FW253AA)-FLAG	39.2	16.9
pREP41-X	<0.1	<0.1

^a *h⁻ cdc24-M38 leu1-32* cells were transfected with the indicated plasmids, incubated at 23°C for 24 h, and selected for 4 days at 33 or 36°C.

^b Ratio of the number of colonies formed at the nonpermissive temperature to the number formed at the permissive temperature.

and Rfc1 and for *cdc24*⁺ function are located within the N-terminal two-thirds of the molecule. These lines of evidence allow us to propose that Cdc24 is a factor acting closely with RFC and PCNA at a certain stage of DNA replication.

The motif responsible for PCNA and Rfc1 binding is unclear at present. The N-terminal two-thirds contains a sequence similar to the PCNA binding motif (QXXLXXFF) that was found in the p21 cyclin-dependent kinase inhibitor, Fen1 nuclease, and DNA ligase I (25, 60). However, this motif does not significantly contribute to Cdc24's ability to bind PCNA and Rfc1. The same mutations in the motif that inactivate p21's ability to bind PCNA failed to impair Cdc24 binding to PCNA and Rfc1 or to impair Cdc24 function.

Cells with *cdc24*⁺ deleted or inactivated completed bulk DNA synthesis with no significant delay in S phase (Fig. 3F) and arrested cell cycling without completion of chromosomal replication (Fig. 1B). However, this result does not necessarily

exclude the possibility that Cdc24 is still required for bulk DNA synthesis, because residual temperature-sensitive Cdc24 protein might be sufficient for completion of bulk DNA synthesis. Beside this, three possibilities seem to be suggested by this result. First, PCNA and RFC are the only targets for Cdc24, but during bulk DNA synthesis, they are regulated by a molecule similar to Cdc24. Second, PCNA and RFC require Cdc24 for their activity only at a certain stage late in DNA synthesis. Third, PCNA and RFC are not the critical targets for Cdc24. The genetic interaction of Cdc24 with Pol ε tends to support the first two possibilities, and the lack of any report of copurification of a Cdc24-like protein with PCNA or RFC (47, 64) or even with Pol δ (64) is consistent with the functional interaction of Cdc24 with these replication factors only at a certain stage of S phase.

PCNA and RFC are required for full activity of both Pol ε and Pol δ. We detected a genetic interaction of Cdc24 with the Pol ε catalytic subunit but not with catalytic and small subunits of Pol δ. The reason for this is unclear. The Pol δ mutant alleles tested might not have been the appropriate ones to reveal interaction, or, alternatively, Cdc24 might preferentially function with Pol ε. Interestingly, *cdc24*⁺ also showed genetic interactions with the fission yeast counterparts of *MCM2*, *MCM4*, and *MCM10* (Fig. 6). The corresponding MCM proteins are essential for the activation of replication origins (35, 62). Recent findings show that some MCM proteins have intrinsic DNA helicase activity (24) and, like Pol α, δ, and ε, are also components of replication forks (2). Therefore, such seemingly peculiar genetic interactions seem to be consistent with the suggested function of Cdc24. However, because the genetic interactions between *cdc24-M38* and these DNA replication gene mutations are modest, they might be reflections of additive effects by indirect interactions.

Cdc24 might serve as a target for regulation. *hnp1*⁺, encoding a casein kinase I involved in damage repair DNA synthesis,

FIG. 6. Temperature sensitivities of *cdc20 cdc24*, *cdc23 cdc24*, *cdc19 cdc24*, and *cdc21 cdc24* double mutants compared to those of their parent single mutants. Double mutants (H185-131, H152-21, H159-18A, and H154-12A) were obtained by genetic crossing followed by tetrad analysis and were maintained at 23°C. The cells were streaked on YEA plates and incubated for 3 days at the indicated temperatures.

was isolated as a multicopy suppressor of the *cdc24-M38* mutant. Although much work needs to be done to reach a definite conclusion, this fact is certainly consistent with the possibility that Cdc24 might serve as, or be closely linked to, a target for the regulation of damage repair DNA synthesis.

The early characterization of *cdc24-M38* mutant cells revealed the production of fragmented DNA (1/20 the size of chromosomes) by alkaline sucrose gradient centrifugation (40). We could not detect broken chromosomes by pulsed-field gel electrophoresis under our experimental conditions. Fragmented DNA may be produced by impaired DNA replication fork movement caused by inactivation of Cdc24 protein.

Most recently, cloning of the *cdc24⁺* gene and characterization of *cdc24-M38* and a new mutant allele of this gene were reported, while this paper was in preparation (17). The isolation of the replicative DNA helicase gene *dna2⁺* as a multicopy suppressor of a *cdc24* mutant (17) is also consistent with our conclusion that Cdc24 is a PCNA- and RFC-interacting factor.

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