The \textit{Schizosaccharomyces pombe} \textit{cdt2}+ Gene, a Target of G1-S Phase-Specific Transcription Factor Complex DSC1, Is Required for Mitotic and Meiotic DNA Replication

Shu-hei Yoshida,* Hiba Al-Amodi, † Taro Nakamura,* Christopher J. McInerny† and Chikashi Shimoda*†

*Department of Biology, Graduate School of Science, Osaka City University, Osaka 558-8585, Japan and †Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, United Kingdom

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ABSTRACT

We have defined five \textit{sev} genes by genetic analysis of \textit{Schizosaccharomyces pombe} mutants, which are defective in both proliferation and sporulation. \textit{sev1}+/\textit{cdt2}+ was transcribed during the G1-S phase of the mitotic cell cycle, as well as during the meiotic S phase. The mitotic expression of \textit{cdt2}+ was regulated by the MCB-DSC1 system. A mutant of a component of DSC1 affected \textit{cdt2}+ expression \textit{in vivo}, and a \textit{cdt2}+ promoter fragment containing MCB motifs bound DSC1 \textit{in vitro}. Cdt2 protein also accumulated in S phase and localized to the nucleus. \textit{cdt2} null mutants grew slowly at 30°C and were unable to grow at 19°C. These \textit{cdt2} mutants were also medially sensitive to hydroxyurea, camptothecin, and 4-nitroquinolinol-1-oxide and were synthetically lethal in combination with DNA replication checkpoint mutations. Flow cytometry analysis and pulsed-field gel electrophoresis revealed that S-phase progression was severely retarded in \textit{cdt2} mutants, especially at low temperatures. Under sporulation conditions, premeiotic DNA replication was impaired with meiosis I blocked. Furthermore, overexpression of \textit{suc2}+, a ribonucleotide reductase gene, fully complemented the sporulation defect of \textit{cdt2} mutants and alleviated their growth defect at 19°C. These observations suggest that \textit{cdt2}+ plays an important role in DNA replication in both the mitotic and the meiotic life cycles of fission yeast.

\textbf{Sporulation} in the fission yeast \textit{Schizosaccharomyces pombe} is an excellent model system for the study of cell differentiation (Egel 1971, 1989; Yamamoto \textit{et al}. 1997). Ascospore formation is preceded by meiosis that itself is induced by starvation. To date, many \textit{S. pombe} mutants defective solely in sporulation have been isolated. Genetic analysis of these mutants has defined sporulation-specific genes, such as \textit{spo1–spo20} (Bresch \textit{et al}. 1968; Kishida and Shimoda 1986). However, gene disruption studies of these \textit{spo} genes revealed that some of them are also essential for vegetative growth. It would appear that spore formation relies not only on specific \textit{spo} genes but also on genes required for proliferation.

Here we report the isolation and analysis of \textit{S. pombe} genes, necessary for normal growth and also required for sporulation. These genes are termed \textit{sev} (sporulation genes essential or important for vegetative growth). Among five \textit{sev} genes defined, \textit{sev1}+ is identical to the previously reported \textit{cdt2}+ (Hofmann and Beach 1994), whose function is unknown. In this article we focus on the functional analysis of \textit{sev1}+/\textit{cdt2}+.

The \textit{Cdc10}-dependent transcription 2 (\textit{cdt2}+) gene is transcriptionally regulated during the mitotic cell cycle with a peak of expression at the G1-S interval (Hofmann and Beach 1994). In eukaryotic cells, a set of genes required for DNA replication are periodically transcribed in the cell cycle, peaking at G1-S phase under control of specific transcription factors, such as the Mbp1/Swi6 complex in \textit{Saccharomyces cerevisiae} (Verma \textit{et al}. 1992; Koch \textit{et al}. 1993). In fission yeast, the equivalent transcription factor complex involved in G1-S specific transcription is named DSC1 (DNA synthesis control; also MBF). This complex is composed of the products of the \textit{cdc10}+, \textit{res1}+, \textit{res2}+, \textit{rep1}+, and \textit{rep2}+ genes (Lowndes \textit{et al}. 1992; Caligiuri and Beach 1993; Miyamoto \textit{et al}. 1994; Sugiyama \textit{et al}. 1994; Zhu \textit{et al}. 1994; Nakashima \textit{et al}. 1995). DSC1 binds to a cis-acting promoter element, called an MCB (\textit{Mit}ul cell-cycle box; ACGCCG; Lowndes \textit{et al}. 1992). \textit{cdc22}+, \textit{cdc18}+, \textit{cdt1}+, \textit{cdt2}+, and \textit{cig2}+ have been reported as target genes of DSC1 (Lowndes \textit{et al}. 1992; Kelly \textit{et al}. 1993; Hofmann and Beach 1994; Obara-Ishihara and Okayama 1994), all of which contain MCB elements in their promoters. Most of these genes are implicated in DNA replication: Cdt1 and Cdc18 are licensing factors controlling DNA replication in the cell cycle (Nishitani \textit{et al}. 2000), and Cdc22 is a regulatory subunit of ribonucleotide reductase, which catalyzes the synthesis of deoxyribonucleoside triphosphates (dNTP; Fernandez-Sarabia \textit{et al}. 1993). Here we describe evi-
dence to show that Cdt2 plays an important role in the regulation of mitotic and premeiotic S-phase progression.

MATERIALS AND METHODS

Yeast strains, media, and genetic techniques: S. pombe strains used in this study are listed in Table 1. Complete medium (YEA) was used for routine growth. Sporulation media used were MEA and SSA for plate culture and Edinburgh minimal medium – N (EMM–N) for liquid culture. Minimal media used were MM, EMM, and SD. These standard fission yeast media have been described (EGEL and EGEL-MITANI 1974; GUTZ et al. 1974; MORENO et al. 1991). Synchronous cultures were prepared by transient temperature shifts using a cdc25-22 temperature-sensitive mutant. Cells were first cultured at cdc25-22 medium/H11002 and then released by shifting the temperature to the permissive temperature of 37°C. In meiotic experiments, a synchronous sporulation protocol using a pat1-114 mutant was adopted (IINO and YAMAMOTO 1985; NURSE 1985; BAHLER et al. 1991). Transformation was performed by the lithium acetate method (OKAZAKI et al. 1990). Conventional procedures for S. pombe genetic manipulation were followed (GUTZ et al. 1974; ALFA et al. 1993). Flow cytometry was performed as previously described (TANAKA et al. 1992; McINERNY et al. 1995).

Mutagenesis: Two S. pombe strains, C982-16C and C996-11D, were used as parents for mutagenesis. Cells (3 × 10^6) from a log-phase culture were treated with ethyl methanesulfonate (EMS) at a final concentration of 2% (v/v) for 10 min in 5 ml MM liquid medium. At the end of the treatment, 20 ml of 5% sodium thiosulfate was added, and cells were washed twice with 10 ml 5% sodium thiosulfate and then three times with 1 ml MM liquid culture. Finally, aliquots were spread on YE agar plates. Temperature-dependent growth was determined by incubation of replica plates grown at 19°C, 30°C, and 37°C. Sporulation ability was tested on MEA plates by iodine vapor method and direct observation of asci under a phase-contrast microscope.

Cloning of the sev genes: Molecular cloning of wild-type genes was conducted by phenotypic complementation of sev mutant strains transformed with the S. pombe genomic library, pTN-L1 (Nakamura et al. 2001), which contained Sau3A1 fragments constructed on a multicopy plasmid, pAL-KS (Tanaka et al. 2000). Transformants on SSA sporulation plates for 3 days were treated with iodine vapor. Positive colonies were taken and their sporulation efficiency was examined by phase-contrast microscopy. A multicopy suppressor of the cdt2-MI mutant (SY2) was isolated by selecting for cDNA clones that complemented its sporulation deficiency. An S. pombe meiotic cDNA library, pTN-RC5 (Nakamura et al. 2002), constructed on an expression vector, pREP42 (Maundrell, Foster City, CA), was used.

To show that the cloned genes were identical to the genetically defined sev genes, we constructed integration plasmids by inserting the cloned fragments into the pIL(II) integration vector (Nakamura et al. 2001). The integration plasmids were cut with appropriate restriction enzymes and then transformed into the wild-type strain TN29. The integrant strains were crossed with the respective sev mutants, and spores derived from the resultant diploids were analyzed by tetrad dissection.

Nucleotide sequence analysis of the cdt2-MI allele: The entire cdt2-MI open reading frame (ORF) was amplified by PCR, using genomic DNA of cdt2-MI as a template. The amplified DNA fragment was cloned into pGEM easy vector (Promega, Madison, WI), and its nucleotide sequence was determined using an ABI dye terminator cycle sequencing kit (PE Applied Biosystems, Foster City, CA).

Gene disruption: To disrupt the cdt2 allele, the whole ORF was replaced by S. pombe ura4+. A 5.3-kb BamHI-XhoI fragment containing the cdt2 ORF was inserted into the pBluescript II-KS(−) vector (Stratagene, La Jolla, CA) to yield pSY38. The
plasmid pSV38 was then subjected to PCR amplification using a pair of oligonucleotide primers, GGGAGATCT (BglII)-CGAT TTATGGAATATGA and CCCGAGATCT (BglII)-CCATATGTC CATATCAT. The amplified DNA lacking the cdt2+ ORF was digested with BglII and ligated with a BamHI fragment containing ura4+ to yield pSV38(ura4+). A 5.6-kb BamHI/ XhoI fragment containing the disrupted Δcdt2:ura4+ allele was introduced into TN29 and Ura+ transformants were selected. Correct chromosomal integration at the cdt2+ locus was verified by genomic Southern blot analysis.

Southern and Northern blot analysis: Genomic DNA was digested, separated in a 1% agarose gel, and then transferred onto nylon membranes (Biodyne B; Pall Bio-Support, New York). Total RNA was prepared as described in McNerny et al. (1995) using a Ribolyser (Hybaid), and Northern blotting was carried out using GeneScreen membrane (NEN, Life Science Products), following the manufacturer’s protocol. DNA probes (~1 kb) were labeled with [α-32P]dCTP using the random hexanucleotide labeling procedure of Feinberg and Vogelstein (1983). Equal loading of RNA in each lane was confirmed by hybridization with an αdn1+ probe. Transcripts were quantified using National Institutes of Health software.

HA-cdt2+ fusion construct: A triple hemagglutinin (HA) epitope-tagged cdt2+ was constructed as follows. First, the cdt2+ coding region was amplified by PCR using primers CCC GTGCAGC(Sal)CATGATTATAGGACATT and GGGGCGGCG CGG(Nol)ATTTTTCATGCTATCCC. The amplified DNA fragment was digested with SalI and NolI and then inserted into pSLE273 (Forsburg and Shenfield 1997) containing the nmt41 promoter and HA(3X) to yield a plasmid, pHA-cdt2C. Next the cdt2+ promoter region was amplified by PCR with primers M13 and CCCGTCGAC(XhoI)GGTATAATCATGA and then replaced with the SalI restriction fragment carrying the C-terminal and 3′ terminator region of cdt2. The resulting plasmid, pHA-cdt2PC, was then digested with Sad1 and replaced by the Sad1 fragment bearing the HA(3X)-cdt2+ introduced into the disruptant strain SY6 harboring cdt2::ura4+. A few Ura+ colonies were selected as candidates of integrants. Correct chromosomal integration of HA-cdt2+ at the cdt2+ locus was verified by genomic Southern blot analysis (data not shown). The HA-cdt2+ integrant was efficiently sporulated on MEA plates.

Western blotting: Crude cell extract was prepared from strain SY11 expressing HA-Cdt2 as described by Masai et al. (1995). Polypeptides were fractionated by SDS-PAGE on a 10% gel and then blotted onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Filters were probed with the rat anti-HA antibody 3F10 (Boehringer Mannheim) and Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes) and Alexa 546-conjugated goat anti-rabbit IgG (Molecular Probes) were used as second-antibodies. Nuclear chromatin region was stained with 4′,6-diamidino-2-phenylindole (DAPI).

RESULTS

Isolation and characterization of sev mutants: To obtain mutants defective in both vegetative growth and sporulation, two homothallic haploid strains (C982-16C and C996-11D) were mutagenized by EMS (materials and methods). Conditional lethal mutants unable to grow at 19°C (cold sensitive) or at 37°C (temperature sensitive) on YE medium were then examined for their ability to sporulate at the permissive temperature of 30°C. Mutants that produced only very few ascii were then subjected to tetrad analysis to examine whether the mutant phenotypes of defective sporulation and growth cosegregated. The isolated nonsporulating mutants defined several recessive mutations, named sev. Complementation tests of these mutants defined five genetic loci, sev1–5. Although sev2 and sev4 mutants normally complete meiosis, sev1, sev3, and sev5 mutants are defective in meiosis (data not shown).

We isolated the sev1+ and sev4+ genes by phenotypic complementation. Integration mapping demonstrated that these were genetically defined sev genes, but not multicopy suppressors. Subcloning of the plasmids, pAL(sev1) and pAL(sev4), and partial sequencing of their genomic inserts indicated that SPAC17H9.19c and SPACUNK4.07c (The S. pombe Genome Sequencing
between wild type and the mutant is not as strong; this is confirmed by the quantification (Figure 3B). This result reflects the fact that basal levels of \( cdc22^+ \) are much higher in wild-type cells than in \( cdt2^+ \).

DSC1 activates G1-S transcription by binding the cis-acting promoter element MCB (Lowndes et al. 1992). The MCB DNA sequence is usually ACGCGT, although some contain only the central CGCG core thought to be essential for function (McIntosh 1993). The promoter region of \( cdt2^+ \) contains seven MCB motifs in two groups. We examined whether a DNA fragment containing these potential cis elements binds DSC1. Gel retardation assays, using cell extracts from wild-type cells, showed that a \( cdt2^+ \) promoter fragment binds DSC1. Gel retardation assays with extracts from wild-type cells, showed that a \( cdt2^+ \) promoter fragment binds DSC1, containing the three upstream MCB motifs (−314 to −453), detected a complex of low mobility (Figure 4A, lane 2). This complex was of similar size to DSC1, detected using an MCB fragment from the \( cdc22^+ \) promoter (Figure 4; Lowndes et al. 1992). This complex is likely to be DSC1 for two further reasons. Both retarded complexes disappeared when the same \( cdt2^+ \) and \( cdc22^+ \) MCB DNAs were added as cold competitors (Figure 4A, lanes 5–6 and 11–12), suggesting that the recognized complex is the same. Finally, gel retardation assays with extracts from mutants of components of DSC1, such as \( cdc10^+, \Delta res1, \Delta res2, \) and \( \Delta rep2 \), revealed loss of the retarded complex, confirming that it was DSC1 (Figure 4B).

We also examined the transcription of \( cdt2^+ \) during meiosis, because this gene is required for sporulation. A synchronous meiosis was induced in a diploid \( pat1-114 \) strain (JZ670), a temperature-sensitive repressor of meiosis. Northern blot analysis of RNA collected from such cells indicated that the level of \( cdt2^+ \) mRNA was transiently enhanced before the first meiotic division and was weakly elevated again during meiosis II (Figure 2B). This expression pattern has been observed by DNA microarray analysis (Mata et al. 2002).

**Abundance of Cdt2 in cell cycle:** We presented evidence that the expression of the \( cdt2^+ \) gene is regulated by the G1-S phase-specific transcription factor complex DSC1. We were next interested in examining the level of Cdt2 polypeptide throughout the cell cycle. The HA-tagged \( cdt2^+ \) was integrated at the \( cdt2^+ \) locus on chromosome I, so that a single copy of the fusion gene was expressed under the control of the authentic \( cdt2^+ \) promoter (Materials and Methods). The HA-\( cdt2^+ \) fusion fully complemented the \( \Delta cdt2 \) mutant (data not shown), indicating that the N-terminal fusion of the HA epitope did not affect \( cdt2^+ \) function. Synchronous cell division was induced in the integrant HA-\( cdt2^+ \) strain harboring \( cdc25-22 \) (SY11) and the HA-Cdt2 level was monitored by Western blotting using an anti-HA monoclonal antibody (3F10). The abundance of HA-Cdt2 oscillated, peaking at the beginning of the septation period (Figure 5A).

**In vivo localization of HA-Cdt2:** We studied by indirect immunofluorescence microscopy. As shown in Figure 5B, fluorescent signals of HA-Cdt2 were preferentially

**Figure 1.**—Characteristic domains of Cdt2. Cdt2 contains a potential PEST sequence (amino acid residues 21–38) and five WD40 repeats (178–208, 226–257, 285–317, 339–369, and 435–464), which are shown as solid and hatched boxes, respectively. The nonsense mutation site in the \( cdt2-M1 \) allele is indicated by an asterisk. Amino acid alignment of the five WD40 repeats with the sequence presented. Conserved and nonconserved ("x" in the consensus) amino acid residues are indicated in white letters against solid or shaded backgrounds, respectively.

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The authors present evidence that Cdt2, a protein involved in cell cycle regulation, is regulated by the G1-S phase-specific transcription factor, DSC1. They show that transcription of Cdt2 is affected by specific transcription factor DSC1, as previously reported. Cdt2 contains a potential PEST sequence and five WD40 repeats, which are conserved across different cellular proteins. The authors further analyze the function and biochemical identity of Cdt2, indicating that it is a possible target of Cdc10. They explore the expression pattern of Cdt2 through the mitotic cell cycle, with a peak at G1-S phase, and discuss the role of BASF1 in the expression of Cdt2. The authors also examine the transcription of Cdt2 during meiosis, showing that it is required for sporulation and that its expression is transiently enhanced before the first meiotic division. Finally, they present evidence for the spatial and temporal localization of HA-Cdt2 in the cell cycle, indicating oscillatory behavior, peaking at the beginning of the septation period.
detected in the nucleus of binucleate cells (probably those in the G1-S phase). The fluorescence in other phases of the cell cycle decreased to the background level of a nontagged control. From the results of Western and immunofluorescence analyses, we conclude that Cdt2 is a nuclear protein that accumulates in G1-S phase.

Δcdt2 mutants show cold-sensitive growth and deficiency in sporulation: The original sev1(Δcdt2)-M1 mutant was partially defective in vegetative growth and failed to
Consequently, \textit{cdt2-M1} is a nonsense allele, probably producing nonfunctional small peptides.

To confirm the \textit{cdt2-M1} results, we created a \textit{cdt2} null mutant in which the \textit{cdt2} ORF was completely replaced by \textit{ura4} (designated as \textit{Δcdt2}) mutant formed colonies at 30° but not at 19°, just as \textit{cdt2-M1} did (Figure 6A). The doubling time in complete medium at permissive temperature indicated that both \textit{Δcdt2} and \textit{cdt2-M1} grew more slowly than wild type (Table 2). These mutant cells were longer than wild-type cells even at the permissive temperature and elongated more remarkably at the restrictive temperature (Figure 6B). We conclude that the \textit{cdt2} gene is dispensable under favorable growth conditions, although it is essential for proliferation and survival at low incubation temperatures.

As the \textit{cdt2-M1} nonsense mutant was sporulation deficient, we next studied in more detail the meiosis and sporulation arrest phenotypes of \textit{cdt2} mutants. After 2 days incubation at 30° on sporulation medium (MEA), quantification of each transcript against \textit{adh1} (Figure 3A), nucleotide sequencing of this cold-sensitive \textit{cdt2-M1} allele revealed that it contained a single cytosine to thymine mutation, resulting in the insertion of an ochre nonsense codon at the 50th arginine residue.

Figure 3.—\textit{cdt2}+ transcription in \textit{cdc10-C4}. (A) RNA was prepared from cultures of wild type (GG1) and \textit{cdc10-C4} (GG250) grown at 25° and subjected to Northern blot with the blot consecutively hybridized with probes for \textit{cdt2}, \textit{cdc22}, and \textit{adh1}, the latter as a loading control. (B) Quantification of each transcript against \textit{adh1}. Ratio of transcripts against \textit{adh1}+ is shown.

Figure 4.—\textit{DSC1} binds to MCB motifs in the \textit{cdt2}+ promoter. (A) Gel retardation analysis using oligonucleotides containing MCB sequences from the \textit{cdt2} and \textit{cdc22} promoters, with wild-type (GG1) protein extracts. Lane F, free probe; lane P, 20 μg protein with probe. Competition reactions were performed with the same unlabeled \textit{cdt2} and \textit{cdc22} promoter DNA at 1/10 and 1/1 concentrations. Large arrow indicates DSC1, and small arrow indicates free probes. (B) Gel retardation analysis with protein extracts from wild-type and DSC1 mutant cells. Strains used for protein extraction are GG1 (wild type), GG219 (\textit{cdc10-129}), GG154 (\textit{Δres1}), GG147 (\textit{Δres2}), and GG149 (\textit{Δrep2}). The temperature-sensitive \textit{cdc10-129} mutant was cultured at 25° or 36°.
and anti-Sad1 antibody, respectively. Most of the cdt2 zygotes were still at mononucleate stage, while wild-type zygotes had completed meiosis and sporulated (Table 3). Roughly half of the Δcdt2 zygotes had one round nucleus that contained neither cytoplasmic nor spindle microtubules. The rest of the mutant zygotes were at the horse-tail stage. This observation is consistent with the previous finding that horse-tail nuclei accumulate when premeiotic DNA replication is inhibited by hydroxyurea (HU; Murakami and Nurse 1999). A proportion of mononucleate zygotes with spindle microtubules and binucleate zygotes was <10%. These results indicate that the cdt2 null mutants arrest before meiosis I.

**cdt2 mutants are sensitive to HU, 4NQO, and CPT:**

As the cdt2+ gene is expressed at the G1-S phase under the control of DSC1, this suggests that Cdt2 protein may have a role in DNA replication, or S-phase progression. Generally, DNA replication mutants in fission yeast are supersensitive to inhibitors of DNA synthesis, and to DNA-damaging agents. To see if this was also the case for cdt2+, Δcdt2, cdt2-M1, and Δrad3 cells were incubated at 30° on YE plates containing various concentrations of HU, a potent inhibitor of ribonucleotide reductase, camptothecin (CPT), a DNA topoisomerase poison, or 4-nitroquinoline-1-oxide (4NQO), a base-modifying agent. As shown in Figure 7, the viability of both cdt2 mutants was considerably reduced in a dose-dependent manner relative to wild type, although a loss of viability of Δrad3 was more conspicuous. It is concluded that cdt2 mutants are medially sensitive to these drugs.

**cdt2 mutants are defective in DNA replication:**

To examine whether Cdt2 is involved in the normal progression of S phase, flow cytometry and PFGE analyses were carried out with synchronously dividing mitotic cultures of both wild-type and mutant strains. cdt2+ Δcdt2-22 (SY8) and cdt2-M1 Δcdt2-22 (SY9) strains were transiently arrested in G2 at 37° for 4 hr before shifting the cultures to 19°, which is the permissive temperature for Δcdt2-22, but the restrictive temperature for cdt2-M1. At 40-min intervals, cell samples were taken and prepared for flow cytometry and PFGE. In the wild-type culture the frequency of septated cells (corresponding to the G1-S phase) fluctuated periodically after temperature shift, with two peaks around 180 and 450 min (Figure
had a 2C DNA content, with those completing S phase. The cdt2 mutant is defective in the initiation and/or progression of S phase and then appeared again as they finished DNA replication (Figure 8B, bottom). These data indicate that although cdt2-M1 mutant cells are able to initiate DNA replication, the progression of DNA replication is strongly retarded.

Finally, we explored premeiotic DNA replication with propidium-iodide-stained cdt2 mutant cells by flow cytometry. Homothallic haploid strains harboring cdt2, cdt2-M1, Δspo3, or Δspo15 alleles were incubated on the MEA sporulation medium. Haploid cells conjugated to form zygotes, which then underwent meiosis. After 24 hr of incubation, zygotic cells containing 4C DNA content appeared, indicating that these cells completed premeiotic DNA replication (Figure 9). Because wild-type zygotes formed spores, which gave abnormally intense signals, the 4C peak gradually decreased. Δspo3 and Δspo15 mutants are defective in spore formation, although their premeiotic DNA synthesis and meiotic divisions progress normally (Iremoto et al. 2000; Nakamura et al. 2002). These mutant zygotes, which were not converted to asci, exhibited a discrete 4C peak. In the cdt2 mutant cultures, however, the 4C peak was absent even after 72 hr of incubation. These observations strongly suggest that premeiotic DNA synthesis did not occur in the cdt2 mutants.

**cdt2-M1 shows synthetic lethality with DNA replication checkpoint mutants:** Completion of DNA replication and DNA damage repair is monitored by a checkpoint mechanism in which several checkpoint rad genes are involved (reviewed by Caspari and Carr 1999). When DNA is unreplicated or damaged, the cell cycle is blocked by this mechanism. The significance of this checkpoint for cell survival is evident because exposure of the checkpoint mutants to HU causes lethality (Al-Khodairy and Carr 1992). Our PFGE data indicated that replication intermediates accumulated in cdt2 mutant cells incubated at 19°C. These intermediates may be detected by the DNA replication checkpoint, resulting in cell-cycle arrest. If the checkpoint genes were impaired by mutation, cdt2 mutants would be predicted to be lethal. To test this possibility, the cdt2-M1 mutant was crossed with Δrad1, Δrad3, Δrad17, Δrad26, or Δcls1 checkpoint mutants. The heterozygous diploids were then sporulated and tetrads were dissected. Among segregants, no double mutants were found, indicating that the cdt2-M1 mutant was synthetically lethal in combination with all the DNA replication checkpoint mutations tested (data...
Role of Cdt2 in DNA Replication

TABLE 3

Terminal morphology of cd2 mutants in sporulation

<table>
<thead>
<tr>
<th>No. of nuclei</th>
<th>Morphology of zygote</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Wild type</td>
</tr>
<tr>
<td>1</td>
<td>Horse-tail</td>
<td>0.4</td>
</tr>
<tr>
<td>1</td>
<td>Round</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>Round</td>
<td>5.7</td>
</tr>
<tr>
<td>1</td>
<td>Round</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Round</td>
<td>1.7</td>
</tr>
<tr>
<td>3 or 4</td>
<td>Round</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>Four-spored asci</td>
<td>89.1</td>
</tr>
</tbody>
</table>

Cells were incubated on MEA sporulation medium at 30°C for 2 days. Strains C996-11D, SY3, and SY6 were used.

Overexpression of cd2 causes a cell-cycle delay: As cd2 mutants are defective in S-phase progression, we next examined whether overexpression of wild-type cd2 affected cell-cycle progression. When cd2 was overexpressed using the strong nmt1 promoter in wild-type cells, growth was delayed (data not shown). Cells containing overexpressed cd2 exhibited a discrete 1C peak, as shown by flow cytometry (data not shown), indicating that overexpression of cd2 causes cell-cycle delay in G1 or in early S phase. These observations are consistent with the suggestion that Cdt2 has a role in G1-S progression.

cd2 genetically interacts with suc22: To understand how Cdt2 functions in DNA replication, we isolated multicopy suppressors of the cd2-1 allele. An S. pombe cDNA library was introduced into cd2-1 cells and several Spo transformants were isolated. Sequencing of isolated clones defined two genes: One was cd2 itself, whereas the other clone contained suc22, which encodes the small subunit of ribonucleotide reductase (Fernandez-Sarabia et al. 1993). Overexpression of suc22 in cd2-1 cells completely suppressed the sporulation deficiency while only partly rescuing the cold sensitivity (Figure 10). S. pombe ribonucleotide reductase is composed of Suc22 (the catalytic subunit) and Cdc22 (the regulatory subunit). We found that overexpression of cd2 did not complement the sporulation deficiency of cd2-1 cells (data not shown).

FIGURE 7.—HU, 4NQO, and CPT sensitivity of cd2 mutants. Wild-type (C996-11D), cd2-M1 (SY3), Δcd2 (SY6), and Δrad3 (SY12) cells were spread onto YE plates containing various concentrations of HU, 4NQO, or CPT. After 5 days of incubation at 30°C, colony numbers were counted. Viability was calculated as a percentage of control cells that were not treated with drugs. Viability of Δrad3 was <0.1% on YE plates containing 2 mM HU, 0.05 μM 4NQO, or 4 μM CPT.

DISCUSSION

Cell differentiation is promoted by a set of genes that are not required for proliferation. Sporulation is an important differentiation process in unicellular microorganisms such as budding and fission yeasts. Genetic studies on sporulation-deficient mutants of S. pombe have defined at least 20 spo genes. Recently, we found that spo14 and spo20 encode essential components of a general protein secretion pathway, both of which are essential for proliferative growth (Nakase et al. 2001;
Figure 8.—Flow cytometric and PFGE analyses of DNA replication in synchronous culture. cdt2+ cdc25-22 (SY8) and cdt2-M1 cdc25-22 (SY9) cultures were transiently incubated at 37°C for 4 hr before shifting to 19°C to restart the cell cycle from G2 phase. At 40-min intervals, samples were taken for seption index (A), flow cytometry (B), and PFGE (C). “P” in C indicates the sample plug.

In the present study we describe a genetic screen for sev genes that are required for both sporulation and vegetative growth. We defined five sev loci, two of which have been cloned and their products identified. It appears that these sev gene products have roles in a wide range of cellular processes. Further genome-wide screening of sev genes may shed light on the problem of how growth-indispensable and sporulation-specific genes share functions for cell differentiation.

The sev1+ gene is identical to cdt2+, which has previously been identified as a target of the transcription factor DSC1 (Hofmann and Beach 1994). In the present study, we provide direct and indirect evidence that Cdt2 is involved in DNA replication, like other targets of DSC1: (1) cdt2+ is transcribed preferentially at the G1-S boundary and Cdt2 protein accumulates in S phase; (2) cdt2+ expression is affected by cdc10-C4, a mutant of a component of DSC1, in vivo, and a cdt2+ promoter fragment binds to DSC1 in vitro; (3) cdt2-M1 mutant cells prevent an increase in DNA content after M phase; (4) S-phase progression is inhibited in cdt2 mutant; (5) transcription of cdt2+ is stimulated in the sexual cycle before meiosis I; (6) premeiotic DNA replication is inhibited and meiosis arrests before first division in cdt2 mutants; (7) cdt2 and DNA replication checkpoint mutation are synthetically lethal; (8) cdt2 mutant cells are sensitive to HU; (9) cdt2 mutations are suppressed by overexpression of suc22+, which encodes the small subunit of ribonucleotide reductase; and (10) overexpression of cdt2+ inhibits the G1-to-S-phase transition. Combined, these observations suggest that Cdt2 has an important regulatory role relating DNA replication during S phase of both the mitotic and the meiotic life cycles in fission yeast.

The cdt2+ gene encodes a nuclear protein containing 490 amino acid residues. No known proteins have a high degree of sequence similarity with Cdt2. Cdt2 con-
Role of Cdt2 in DNA Replication

cated protein of only 49 amino acids. The \textit{cdt2-M1} mutant and \Delta\textit{cdt2} cells lacking the entire ORF are able to grow at 30°C with an extended doubling time, indicating that Cdt2 is not absolutely required for DNA replication. This growth defect is more severe at low incubation temperatures. We speculate that Cdt2 functions to assist the formation of protein complex required for DNA replication. Higher incubation temperature may facilitate interaction between protein components without Cdt2. The PEST sequence has been often found in unstable proteins. HA-Cdt2, expressed from its native chromosomal locus, is almost exclusively present in binucleate cells and scarcely detected in mononucleate cells. This observation suggests that HA-Cdt2 accumulates during S phase and decreases in G2 phase, implying that Cdt2 is unstable. The PEST sequence present in Cdt2 may contribute to such protein instability.

Ribonucleotide reductase is an essential enzyme required for the synthesis of dNTPs, and thus its activity is indispensable for DNA replication and DNA repair (Jordan and Reichard 1998). From yeasts to mammals, this enzyme is composed of two subunits: a small catalytic subunit and a large regulatory subunit. In fission yeast, \textit{cdc22} and \textit{suc22} encode a large and a small subunit of ribonucleotide reductase, respectively (Fernandez-Sarabia et al. 1993). We found that \textit{cdt2} mutations are rescued by \textit{suc22} when overexpressed, but not by \textit{cdc22}. Furthermore, \textit{cdt2} mutants are sensitive to HU, a potent inhibitor of ribonucleotide reductase. These findings imply that Cdt2 regulates the activity of Suc22 during the G1-S phase. This hypothesis could be tested by measuring dNTP levels in the \textit{cdt2} mutants. We note that the requirement for Cdt2 during sporulation is higher than that for cell growth, because \textit{cdt2} mutants display sporulation defects even at the permissive temperature for growth. The suppression of \textit{cdt2} defects by \textit{suc22} is more remarkable in recovery of sporulation than suppression of cold-sensitive mitotic growth. Presumably, these results suggest a closer relationship be-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure9}
\caption{Premeiotic DNA replication in \textit{cdt2} mutants. Wild-type (C996-11D), \textit{cdt2-M1} (SY3), \Delta\textit{cdt2} (SY6), \Delta\textit{spo3} (MKD3), and \Delta\textit{spo15} (SI51) mutants were incubated on MEA medium for 2 days. Cells were collected and subjected to flow cytometry. 1C, G1-arrested vegetative cells; 2C, zygotes before premeiotic S phase; and 4C, zygotes after premeiotic S phase. Note that no discrete 4C peak was detected in \textit{cdt2} mutants.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure10}
\caption{Suppression of \textit{cdt2} mutants by \textit{suc22}. (A) Growth defect of \textit{cdt2} mutant partially rescued by a \textit{suc22}-carrying plasmid. \Delta\textit{cdt2} (SY6) transformed with pREP41, pREP41 (\textit{suc22}), or pAL (\textit{sev1})1 was incubated in liquid EMM medium without thiamine. pAL (\textit{sev1})1 carries the \textit{cdt2} gene. Growth was measured by optical density at 530 nm (OD\textsubscript{530}). Mean OD\textsubscript{530} values of three independent cultures run in parallel are presented. Differences in OD\textsubscript{530} at 60 hr between different plasmids are statistically significant. (B) Sporulation defect in \textit{cdt2} mutants was completely recovered by \textit{suc22}. Strain SY6 (\Delta\textit{cdt2}) transformed with pREP41, pREP41 (\textit{suc22}), or pAL (\textit{sev1})1 was incubated on thiamine-free SSA sporulation medium at 30°C for 2 days. Frequency of asci was counted under a phase-contrast microscope.}
\end{figure}
between Cdt2 and Suc22 in premeiotic S phase than in the mitotic cell cycle.

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LITERATURE CITED


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