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Cig2, a B-Type Cyclin, Promotes the Onset of S in *Schizosaccharomyces pombe*

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Cdc2, a catalytic subunit of cyclin-dependent kinases, is required for both the G_1-to-S and G_2-to-M transitions in the fission yeast *Schizosaccharomyces pombe*. Cdc13, a B-type cyclin, is required for the M-phase induction function of Cdc2. Two additional B-type cyclins, Cig1 and Cig2, have been identified in *S. pombe*, but none of the B-type cyclins are individually required for the onset of S. We report that Cdc13 is important for DNA replication in a strain lacking Cig2. Unlike Δ*cdc13* cells, double-mutant Δ*cdc13* Δ*cig2* cells are defective in undergoing multiple rounds of DNA replication. The conclusion that Cig2 promotes S is further supported by the finding that Cig2 protein and Cig2-associated kinase activity appear soon after the completion of M and peak during S, as well as the observation that S is delayed in Δ*cig2* cells as they recover from a G_1 arrest induced by nitrogen starvation. These studies indicate that Cig2 is the primary S-phase-promoting cyclin in *S. pombe* but that Cdc13 can effectively substitute for Cig2 in Δ*cig2* cells. These observations also suggest that the gradual increase in the activity of Cdc2-Cdc13 kinase can be sufficient for the correct temporal ordering of S and M phases in Δ*cig2* cells.

A key aim of cell cycle studies is to understand how orderly cell cycle progression is regulated. One productive investigative approach has been to identify fission yeast mutants that have uncoupled the DNA replication (S) and nuclear division (M) phases (32). In the fission yeast *Schizosaccharomyces pombe*, a single cyclin-dependent kinase complex, consisting of the Cdc2 catalytic subunit bound to the Cdc13 cyclin B subunit, is essential for the induction of mitosis (4, 28). Like B-type cyclins of animal cells, Cdc13 accumulates during interphase and is rapidly degraded upon exit from M. Interestingly, Δ*cdc13* cells undergo multiple rounds of S without intervening M phases (18). This leads to the formation of gigantic cells that each have a huge nucleus. Studies have also shown that high overexpression of Cdc2 and Cdc13 can force the induction of mitosis in G_1 cells (18). These observations have led to the hypothesis that the presence of Cdc2-Cdc13 complex defines a cell as being in G_2 and effectively gives the cell no choice other than to initiate M. The acquisition of the opportunity to undergo DNA replication depends on the destruction of Cdc13 that normally occurs at the end of M.

Cdc2 is required for both the G_1-to-S and G_2-to-M transitions, whereas Cdc13 is essential only for the onset of M (33). Since cyclin-dependent kinases are inactive unless bound to a cyclin, there must be at least one cyclin that is involved in promoting the onset of S in *S. pombe.* Genes encoding five additional cyclin-related proteins have been identified in *S. pombe*. Two of the genes, *mcs2*$^+$ and *pch1*$^+$, encode cyclin C-like proteins having essential functions that do not appear to be directly related to cell cycle control (14, 26). A third gene, *puc1*$^+$, encodes an unusual type of cyclin that has no ascribed function (12, 13). The remaining cyclin genes, *cig1*$^+$ and *cig2*$^+$, encode B-type cyclins (5–8, 10, 34). Mutants defective for *cig1*$^+$ or *cig2*$^+$ (*cyc17*$^+$) have no clearly defined cell cycle defects. However, Δ*cig2* mutants undergo accelerated mating when starved of nitrogen (10, 34). This finding, coupled with observation that the abundance of *cig2*$^+$ mRNA increases dramatically in nitrogen-starved cells, has led to the suggestion that Cig2 may function primarily to inhibit conjugation in situations in which the availability of nitrogen decreases below optimal levels (34). It has also been noted that the level of *cig2*$^+$ mRNA oscillates during mitotic growth, peaking near the G_1/S boundary, suggesting that Cig2 may play a role in promoting an early cell cycle event during mitotic divisions (10, 34).

Acquiring a comprehensive understanding of how the fission yeast cell cycle is ordered will be facilitated by identifying cyclins that are involved in promoting the onset of S. With this goal in mind, we have undertaken a more detailed analysis of the functions of Cig1 and Cig2 in *S. pombe*, specifically addressing the possibility that one or both B-type cyclins are involved in promoting the onset of S. These studies were prompted largely by recent findings showing that B-type cyclins are involved in promoting S in the distantly related budding yeast *Saccharomyces cerevisiae* (37, 39, 40).

MATERIALS AND METHODS

Strains, growth media, and general methods. A list of strains used in these experiments is provided in Table 1. Methods and media (YES and EMM2) used for general genetics and biochemical procedures with *S. pombe* were recently described (1, 29). YES contains yeast extract, glucose, and amino acid and nucleotide supplements as required for auxotrophic strains. EMM2 is a totally synthetic mix containing glucose, salts, minerals, and amino acid and nucleotide supplements as required for auxotrophic strains. Selection synchrony cultures were made by applying 2 liters of log-phase cells (optical density at 600 nm [OD_{600}], ~1.5) grown in YES at 30°C onto a JE-5.0 elutriation rotor (Beckman) spinning at 4,500 rpm. The smallest ~12% of the cells were collected in growth media by slowly increasing the flow rate. Cells growing in YES at 30°C were harvested (~30 OD_{600}) at 20-min intervals by filtration and washed once with ice-cold STOP buffer (50 mM NaF, 10 mM EDTA, 100 mM NaCl, 1 mM NaN_3) into a 1.5-ml microcentrifuge, and the cell pellet was then quick-frozen in a dry ice-ethanol bath. Septation index was measured by using duplicate samples of cells harvested in ice-cold water and scored with a phase contrast microscope. Approximately 300 cells were counted in each sample.

Immunoblotting and kinase assays. Frozen cell pellets were thawed and resuspended in ice-cold LYSIS-1 buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride [PMSF], 5 μg each of leupeptin, pepstatin, and aprotinin per ml). Chilled glass beads were added to the meniscus, and the cells were broken by vigorous shaking in a

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TABLE 1. Strain genotypes[a]

Strain	Genotype
PR109	*h*$^-$
PR1190	*ade6-M216 his7-366 h*$^-$
PR1191	*ade6-M210 his7-366 h*$^+$
JM559	*cdc13-117 h*$^-$
PR618	*cig1*::*ura4*$^+$ *ade6 h*$^-$
PR926	*cdc13-117 cig1*::*ura4*$^+$ *ade6 h*$^-$
PR930	*cdc13-117 cig1*::*ura4*$^+$ *cig2*::*LEU2 h*$^+$
PR941	*cig2*::*ura4*$^+$ *ade6-216 h*$^-$
PR1007	*cig2*::*ura4*$^+$ *ade6-216 h*$^-$
PR1009	*cdc13-117 cig2*::*ura4*$^+$ *h*$^+$
OM1378	*cdc13*::*his7*$^+$/*cdc13*$^+$
OM1390	*cdc13*::*his7*$^+$/*cdc13*$^+$ *cig1*::*ura4*$^+$/*cig1*::*ura4*$^+$
OM1391	*cdc13*::*his7*$^+$/*cdc13*$^+$ *cig2*::*ura4*$^+$/*cig2*::*ura4*$^+$
OM1392	*cdc13*::*his7*$^+$/*cdc13*$^+$ *cig1*::*ura4*$^+$/*cig1*::*ura4*$^+$ *cig2*::*LEU2*/*cig2*::*LEU2*
OM1442	*HA-cig2*$^+$ *h*$^-$
OM1464	*HA-cig2*$^+$ *cdc10-V50 h*$^-$
OM1467	*HA-cig2*$^+$ *cdc22-M45 h*$^-$

[a] All haploid strains also contained *leu1-32* and *ura4-D18*. All diploid strains were *leu1-32*/*leu1-32 ura4-D18*/*ura4-D18 ade6-M210*/*ade6-M216 his7-336*/*his7-336 h*$^+$/*h*.

vortexer for 5 min at 4°C. The cell extract was collected and then centrifuged at 14,000 × *g* in a Eppendorf microcentrifuge at 4°C for 15 min. The concentration of cell extracts was estimated by determining the OD_{280} and then normalized by adding LYSIS-1 buffer. Immunoblots were probed with either anti-HA (12CA5) monoclonal antibodies or anti-Cdc2 (9808) rabbit polyclonal antibodies (17). Immunoblots were subsequently incubated with anti-rabbit or anti-mouse immunoglobulin G antibodies, and the signals were visualized by the enhanced chemiluminescence detection system (Amersham). Kinase assays were performed with supernatants made from cells extracted with LYSIS-2 buffer (LYSIS-1 buffer containing 1% Nonidet P-40, 1 mM VnO_4, and 50 mM NaF). Samples were normalized and then immunoprecipitated with anti-Cig2 (1267) or anti-Cdc13 (SP4 or GJ6-56) antibodies. Immunocomplexes were collected with Sepharose-protein A and washed three times with LYSIS-2 buffer and then twice with KAB buffer (50 mM Tris [pH 7.4], 10 mM $MgCl_2$). Samples were resuspended in 50 μl of KAB buffer containing 1 mg of histone H1 per ml, 40 μM ATP, and 33 μCi of [γ-^{32}P]ATP and incubated for 15 min at 30°C. Reactions were terminated by adding an equal volume of sodium dodecyl sulfate (SDS) sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (12% polyacrylamide). Activities were quantified with a Molecular Dynamics PhosphorImager.

Disruption of *cdc13*$^+$ and spore germination experiments. A 3.2-kb *Bam*HI-*Sal*I fragment containing *cdc13*$^+$ cloned into pBluescript II was digested with *Eco*RI to remove a 1.4-kb fragment containing the *cdc13*$^+$ open reading frame (16). The *cdc13*$^+$ region was replaced with a 2.5-kb *Eco*RI fragment containing *his7*$^+$ derived from pEA2 (2). The resulting 4.3-kb *Bam*HI-*Sal*I fragment was used to transform the diploid strain generated by mating PR1190 and PR1191, producing *cdc13*::*his7*$^+$/*cdc13*$^+$ diploids. This strain was transformed with pUR19-*cdc13*$^+$, containing a functional copy of *cdc13*$^+$. This strain was sporulated, and viable Δ*cdc13* cells having pUR19-*cdc13*$^+$ were isolated. Such an isolate was crossed to a Δ*cig1* Δ*cig2* strain to generate mutant combinations. Diploid strains were induced to sporulate by inoculating log-phase YES cultures into liquid malt extract (ME) medium and growing them for 2 days at 30°C until stationary phase. Asci and cells were collected, resuspended in water containing 0.4% glusulase, and incubated at 37°C for 5 h. Spores were washed twice in water and stored at 4°C. The spores were resuspended in EMM2+LUA (EMM2 supplemented with leucine, uracil, and adenine) at 0.1 OD_{600} and incubated at 30°C. Samples were harvested at 17 h by centrifugation, resuspended in 3 ml of ice-cold water, and then adjusted to 70% ethanol by addition of 100% ethanol. Samples were collected and resuspended in phosphate-buffered saline prior to the addition of an equal volume of DAPI (4′,6-diamidino-2-phenylindole, concentration, 1 μg/ml).

Recovery from G_1 arrest experiments. Strains were grown to log phase in YES at 25°C, washed twice with EMM2-NH_4 (EMM2 lacking nitrogen), and then resuspended in the same medium at 0.25 OD_{600}. Cells were incubated for 22 h at 25°C, during which cell growth ceased at ~2.0 OD_{600}. The cells were harvested and resuspended in YES prewarmed at 36 or 37°C at ~0.5 OD_{600}. Samples were taken at regular intervals and prepared for flow cytometry analysis as described previously (1). The samples were finally resuspended in 50 mM sodium citrate buffer containing 10 μg of propidium iodide per ml (1) and analyzed with a Becton Dickinson FACSort flow cytometer.

Construction of *HA-cig2*$^+$. Genomic *cig2*$^+$ was amplified from genomic DNA by PCR with the primers CIG2.1 (CTGGA GCAAC AATAG AATAA TTCGG ACC) and CIG2.2 (CTCGA GAGGA TTAAG CATCA ACACG G). The 1.7-kb fragment was cloned into pCRII (Invitrogen). A ~140-bp fragment containing three tandem copies of the *HA* epitope was amplified from plasmid pGTEP1 by PCR with the primers NDEI-HA-5′ (CCATA TGGCT TACCC ATACG ATGTT CCTGA C) and NDEI-HA-3′ (GCATA TGGGG CCCTC CAGCG TAATC TGGAA CGTCG TATGG ATAGG) and cloned into pCRII. The epitope tag fragment of pCRII-HA was liberated by digestion with *Nde*I and cloned into the unique *Nde*I site located at the initiator ATG codon of *cig2*$^+$ in the pCRII-cig2 plasmid described above. DNA sequencing was used to confirm the orientation of the insert. The resulting ~1.8-kb *HA-cig2*$^+$ fragment was liberated by digestion with *Xho*I and used to replace *cig2*::*ura4*$^+$ in strain PR1007. Gene replacement was confirmed by Southern and immunoblotting analysis. The ~1.8-kb *HA-cig2*$^+$ *Xho*I fragment was also cloned into the *Sal*I site of pREP4 to generate pREP4–HA-cig2 (25).

Generation of anti-Cig2 antibodies. A 1.7-kb *Nde*I-*Not*I fragment containing *cig2*$^+$ was isolated from pCRII-cig2, cloned into pET20b (Novagen), and transformed into BL21-DE3 bacteria. Upon induction of expression, the ~49-kDa Cig2 protein was found in the inclusion bodies. Inclusion bodies were isolated and used to immunize rabbit 1267.

RESULTS

Cig2 protein abundance and Cig2-associated kinase activity peak shortly after M. Earlier studies showed that the abundance of *cig2*$^+$ mRNA oscillates during the cell cycle, peaking near the G_1-to-S transition (10, 34). A series of experiments were carried out to determine whether this oscillatory pattern was reflected in a cell cycle periodicity of the levels of Cig2 protein and Cig2-associated kinase activity. Analysis of Cig2

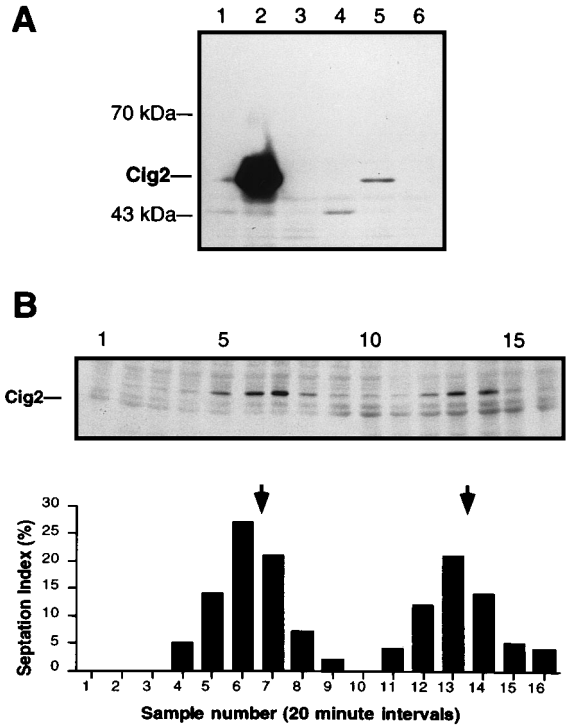

FIG. 1. The level of Cig2 protein peaks after M. (A) Immunoblot with the anti-HA epitope monoclonal antibody 12CA5. A ~50-kDa protein is specifically detected in cells that produce epitope-tagged Cig2 at wild-type levels (lane 5) or at high levels (lane 2). Lanes: 1, wild-type cells (PR109) transformed with pREP4-HA-cig2 grown under repressing conditions (+ thiamine); 2, wild-type cells (PR109) transformed with pREP4-HA-cig2 grown under inducing conditions (without thiamine); 3, Δ*cig2* cells (*cig2*::*ura4*$^+$, strain PR1007); 4, wild-type cells (PR109) expressing pREP4-cig2 grown under inducing conditions (without thiamine); 5, *HA-cig2*$^+$ cells (strain OM1442); 6, wild-type cells (PR109). (B) Anti-HA immunoblot of samples taken from a synchronous culture of *HA-cig2*$^+$ cells (OM1442). The level of Cig2 protein undergoes a large oscillation, being most abundant in samples 7 and 13 to 14, indicated by arrows in the septation index plot (lower panel). The peak levels of Cig2 protein occurs after the peak of septation index.

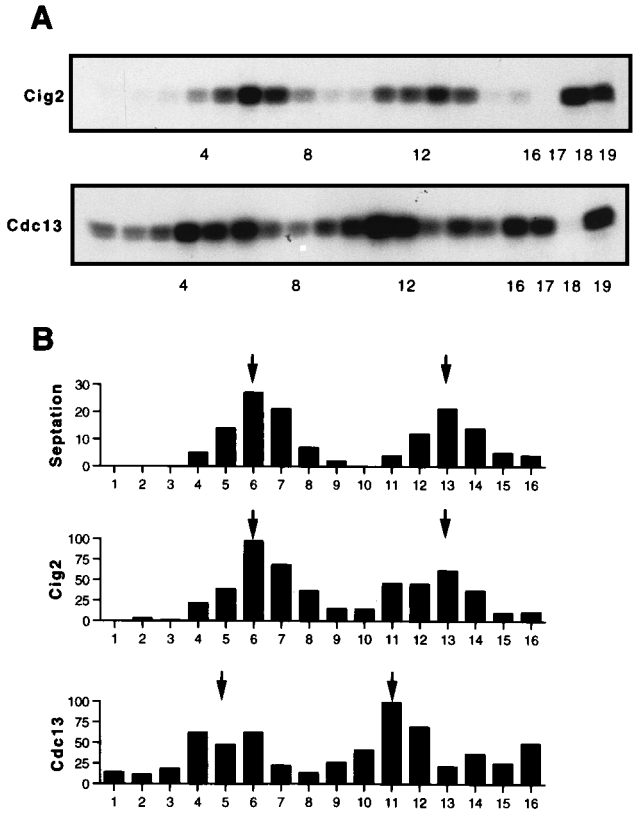

FIG. 2. The level of Cig2-associated kinase activity is highest immediately after M phase. (A) The levels of kinase activity associated with Cig2 (upper panel) and Cdc13 (lower panel) were assayed in samples taken from a synchronous culture of OM1442 cells produced by centrifugal elutriation. Assays were conducted with anti-Cig2 and anti-Cdc13 immunocomplexes with histone H1 as a substrate. Lanes 1 to 16 are from the synchronous culture; lane 17 contains Δ*cig2* cells (*cig2*::*ura4*$^+$, strain PR1007); lane 18 contains Δ*cdc13* cells derived from sporulation of a *cdc13*::*his7*$^+$/*cdc13*$^+$ strain (OM1378) in EMM2 lacking histidine; and lane 19 contains wild-type cells (PR109). As expected, Cig2-associated kinase activity was absent in the Δ*cig2* cells (lane 17) and Cdc13-associated activity was absent in the Δ*cdc13* cells (lane 18). (B) Quantification of septation index and kinase activity data. Cdc13-associated kinase activity peaks shortly before the peak of septation index, whereas the peak of Cig2-associated kinase activity coincides with the peak of septation index. In log-phase cultures grown in EMM2, septated cells are in G_1 and S (1).

protein was facilitated by constructing a strain in which genomic *cig2*$^+$ expressed a protein having three copies of the influenza virus hemagglutin protein (HA) epitope at the N terminus. In this strain, Cig2 was detected as a ~50-kDa protein by SDS-PAGE (Fig. 1A). A synchronous culture of this strain was made by centrifugal elutriation. Immunoblotting showed that the level of Cig2 protein underwent a dramatic oscillation during each cell cycle (Fig. 1B) whereas the abundance of control proteins such as Cdc2 remained constant (27). The peak Cig2 samples (samples 6 and 7 and samples 13 and 14) occurred at or immediately after the peak in septation index (samples 6 and 13). The peak of septation index corresponds to the G_1 and S phases, since in *S. pombe* cells septation occurs immediately after the completion of M phase and cells remain septated through G_1 and S (1). The level of Cig2 protein was very low in samples 3 and 4 and samples 10 and 11, which would be expected to have the peak fractions of late G_2 and early M cells (1).

Samples from the same experiment were used to measure the protein kinase activity of anti-Cig2 immunocomplexes. There was a large oscillation in Cig2-associated kinase activity during the cell cycle (Fig. 2). The peak of activity coincided very closely with the peak of septation index (samples 6 and 13). For the purpose of comparison, we also measured the level of Cdc13-associated kinase activity in the same experiment. As expected, Cdc13-associated kinase activity was highest in the fractions immediately prior to the peaks in septation index.

The appearance of Cig2 protein is dependent on Cdc10. The above findings suggested that Cig2 protein is produced and active shortly after the completion of mitosis, during G_1 and/or S phase. In *S. pombe*, the Cdc10 transcription factor is required for the expression of several key genes involved in DNA replication, including *cdc18*$^+$, *cdc22*$^+$, and *cdt1*$^+$ (3, 9, 20, 21, 24, 36). Immunoblotting experiments were carried out to determine whether the appearance of Cig2 protein was dependent on Cdc10 function. These studies utilized the *cdc10-V50* allele (19). Cig2 was undetectable in cells arrested in G_1 with the *cdc10-V50* mutation (Fig. 3, lane 4). In contrast, in cells that were arrested in S phase with hydroxyurea, the level of Cig2 protein increased ~10-fold above the level seen in asynchronous cultures (lanes 1 and 2). The abundance of Cig2 was also very high in cells that were arrested in S with the *cdc22-M45* mutation (lane 7). The level of Cig2 was moderately increased in *cdc22-M45* cells that were grown at the permissive temperature (lane 6). This may reflect the fact that these cells are partially defective in progression through S. Interestingly, the level of Cig2 was not elevated in *cdc10-V50* cells that were grown at the permissive temperature in the presence of hydroxyurea (lane 5). This suggests that Cdc10 has a very important role in regulating the abundance of Cig2, the simplest possibility being that Cdc10 regulates the periodic transcription of *cig2*$^+$. As mentioned above, this proposition is consistent with previous studies that have shown that the abundance of *cig2*$^+$ mRNA oscillates during the cell cycle, peaking near the G_1-to-S transition (10, 34).

Cig2 is required for rereplication in a Δ*cdc13* background. The findings described above showed that Cig2 is most abundant and active during S phase. On the basis of these observations, we developed the hypothesis that Cig2 is normally involved in promoting the onset of S but that in the absence of Cig2, this function can be carried out by Cdc13. This idea was tested by determining whether Cig2 was essential for rereplication in Δ*cdc13* cells in a spore germination experiment (18). In these experiments Δ*cdc13*/*cdc13*$^+$ diploid cells were induced to undergo meiosis and then germinated in media which supported the growth of only Δ*cdc13* cells (see Materials and

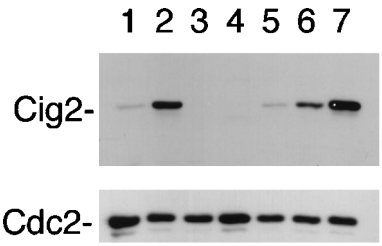

FIG. 3. Cig2 protein is absent in cells arrested in G_1 and highly abundant in cells arrested in S. Cells having an epitope tagged copy of *cig2*$^+$ (*HA-cig2*$^+$) were analyzed by immunoblotting. Samples are as follows: 1, asynchronous wild-type cells (OM1442) incubated at 30°C; 2, wild-type cells arrested in S phase by treatment with hydroxyurea for 4 h at 30°C; 3, *cdc10-V50* cells (OM1464) grown at the permissive temperature of 25°C; 4, *cdc10-V50* cells arrested in G_1 by incubation at 36°C for 4 h; 5, *cdc10-V50* cells arrested in S by treatment with hydroxyurea for 4 h at 25°C; 6, *cdc22-M45* cells grown at the permissive temperature of 25°C; 7, *cdc22-M45* cells (OM1467) arrested in S phase by incubation at 36°C for 4 h. A duplicate immunoblot was also probed with anti-Cdc2 antibodies, showing that the abundance of Cdc2 varied less than twofold.

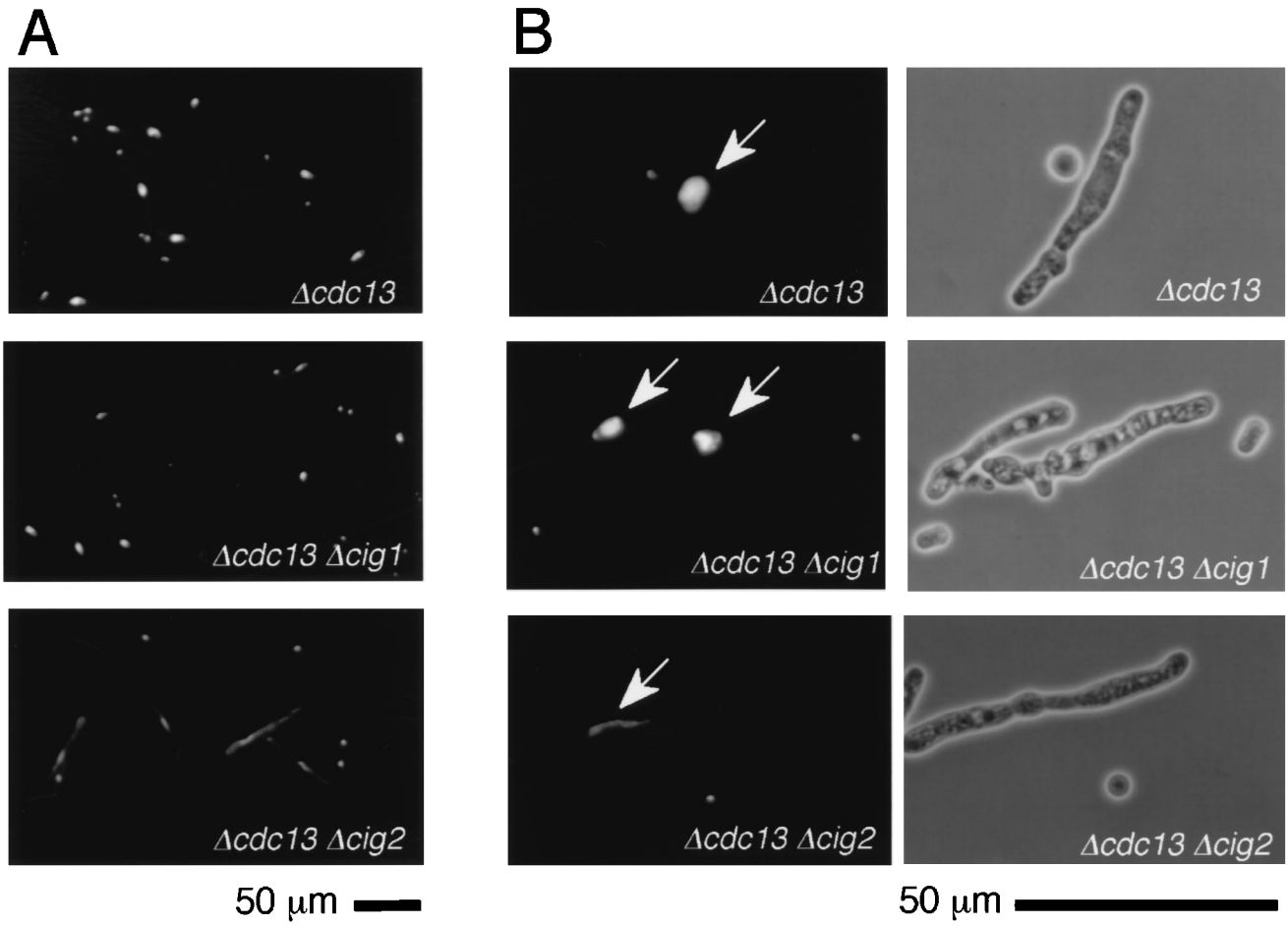

FIG. 4. Cig2 is required for rereplication in a Δ*cdc13* background. OM1390 (Δ*cdc13*/*cdc13*$^+$), OM1391 (Δ*cdc13*/*cdc13*$^+$ Δ*cig1*/Δ*cig1*), and OM1392 (Δ*cdc13*/*cdc13*$^+$ Δ*cig2*/Δ*cig2*) diploids were induced to sporulate, and spores were collected and germinated in medium that supported growth only of cells having the Δ*cdc13* mutation. (A) DAPI-stained cells 17 h after germination. Rereplicating cells are identified by the large and intensely staining nuclei in the upper two panels. The size and intensity of the DAPI staining increased with time in Δ*cdc13* and Δ*cdc13* Δ*cig1* cells. (B) Enlarged images of cells visualized with DAPI (left panels) or phase-contrast optics (right panels).

Methods). As expected, approximately half of the spores germinated to form huge cells, examples of which are shown in Fig. 4. Rereplication was scored by staining with the DNA stain DAPI followed by microscopic observation. Approximately 66 to 90% of the Δ*cdc13* spores germinated to form huge cells having a rereplication phenotype (Fig. 4; Table 2). An almost identical result was obtained with Δ*cdc13* Δ*cig1* spores, approximately 65 to 88% of which were scored as rereplication positive (Table 2). In contrast, Δ*cdc13* Δ*cig2* spores exhibited no sign of rereplication, although they also germinated and grew to a gigantic size (Fig. 4; Table 2). We also attempted to carry out a spore germination experiment with a Δ*cdc13*/*cdc13*$^+$ Δ*cig1*/Δ*cig1* Δ*cig2*/Δ*cig2* diploid but found that the germination frequency was highly depressed, a result that perhaps suggests that these cyclins have overlapping roles in meiosis.

TABLE 2. Frequency of DNA rereplication in cell cycle-arrested cells derived from spores of the indicated genotypes

Expt no.	Frequency (%) of genotype (no. with genotype/total no.):		
	Δ*cdc13*	Δ*cdc13* Δ*cig1*	Δ*cdc13* Δ*cig2*
1	90 (450/500)	88 (440/500)	0 (0/500)
2	66 (330/500)	65 (325/500)	0 (0/500)

***cdc13-117* Δ*cig2* cells are defective in initiating S.** The rereplication study strongly suggested that either Cig2 or Cdc13 is sufficient to promote the onset of S. However, these findings did not exclude the possibility that Cig2 and Cdc13 share an essential post-S activity that must be completed before Δ*cdc13* cells can reset from G_2 to G_1. To resolve this question, we determined whether G_1 cells that were impaired for Cig2 and Cdc13 activity were defective in initiating S phase. These studies were carried out with temperature-sensitive *cdc13-117* cells that also had Δ*cig1* and/or Δ*cig2* mutations. G_1 populations of these cells were generated by nitrogen starvation. Reentry into the cell cycle was initiated by resuspending cells in growth medium containing nitrogen. These cultures were simultaneously shifted to the restrictive temperature of 36°C. The onset of S was monitored by the disappearance of the 1C DNA (1 complement of DNA) peak as measured by flow cytometry (see Materials and Methods). As shown in Fig. 5A, *cdc13-117* and *cdc13-117* Δ*cig1* cells underwent S with the same kinetics, with the level of the 1C fraction being reduced by ~75% within 2 h. In contrast, exit from G_1 was delayed by ~90 min in the *cdc13-117* Δ*cig2* cells (Fig. 5A). The same delay was observed in the *cdc13-117* Δ*cig1* Δ*cig2* cells (Fig. 5A), suggesting that Cig1 plays little or no role in promoting the onset of S in these circumstances. We suspected that the ability of *cdc13-117* Δ*cig1* Δ*cig2* cells to eventually undergo S was due to the resid-

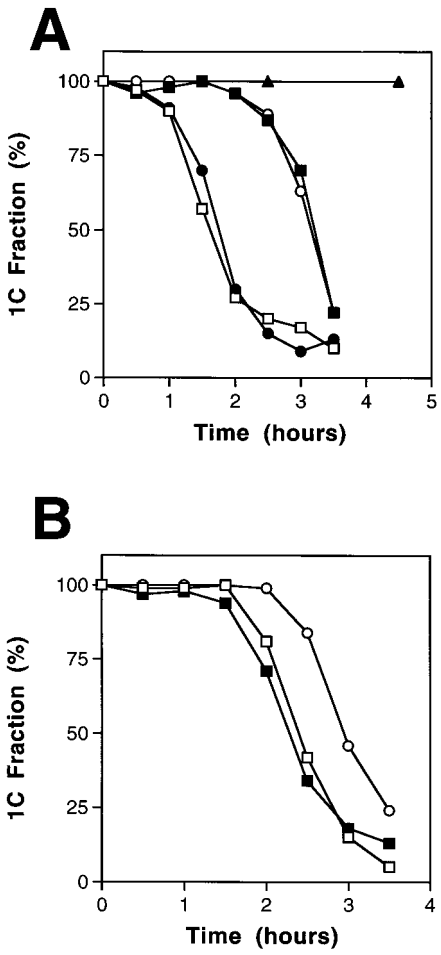

FIG. 5. Cig2 and Cdc13 share an essential role in promoting the onset of S. (A) Various *cdc13-117* strains having Δ*cig1* and/or Δ*cig2* mutations were arrested in G_1 by nitrogen starvation at the permissive temperature of 29°C and then resuspended in EMM2 at the restrictive temperature of 36°C (except as noted). Cells were collected for flow cytometry analysis at regular intervals. The onset of S, as measured by the decrease of the 1C DNA peak, was delayed by ~90 min in strains having the Δ*cig2* mutation in a *cdc13-117* background. Triple-mutant cells (*cdc13-117* Δ*cig1* Δ*cig2*) failed to enter G_1 when incubated at 37°C. Strains were JM559 (*cdc13-117*) (●), PR926 (*cdc13-117* Δ*cig1*) (□), PR1009 (*cdc13-117* Δ*cig2*) (■), and PR930 (*cdc13-117* Δ*cig1* Δ*cig2*) (○). In a separate experiment, strain PR930 was reinoculated into EMM2 at 37°C (▲). For the sake of clarity, the flow cytometry data of a wild-type control (PR109) at 37°C are not plotted. In this experiment, the fraction of 1C wild-type cells was ~10% at 2.5 h and ~2% at 4.5 h. (B) Wild-type (PR109) (□), Δ*cig1* (PR618) (■), and Δ*cig2* (PR941) (○) cells were arrested in G_1 by nitrogen starvation and then resuspended in EMM2. Cells were collected at regular intervals for flow cytometry analysis. The onset of S was delayed by ~30 min in the Δ*cig2* cells.

ual temperature-insensitive activity of the *cdc13-117* gene product (16). Evidence in favor of this interpretation was provided by finding that the G_1-to-S delay of *cdc13-117* Δ*cig1* Δ*cig2* cells was greatly extended by incubating cells at 37°C as opposed to 36°C (Fig. 5A). These data strongly suggested that failure of Δ*cdc13* Δ*cig2* cells to undergo rereplication was due to a defect in the initiation of S.

The onset of S is delayed in Δ*cig2* cells. Previous flow cytometry studies have failed to reveal a defect in the initiation of S in Δ*cig2* cells (7, 10, 34). However, in log-phase cultures, S phase is normally completed prior to final cytokinesis; therefore, a moderate G_1-to-S delay would not be detected by this approach. In an effort to address this problem, we asked whether the onset of S was delayed in Δ*cig2* cells that resumed cell cycle progression following a G_1 arrest induced by nitrogen starvation. These studies revealed that the onset of S was delayed by ~30 min in Δ*cig2* cells compared with wild-type or Δ*cig1* cells (Fig. 5B).

DISCUSSION

Studies carried out with fission yeast *cdc2*^{ts} mutants were the first to establish that cyclin-dependent kinases are required for onset of S and M phases (33). The subsequent demonstration that Cdc2 kinase activity was rate limiting for the induction of M (15, 38), and presumably also for S, presented a paradox concerning a fundamental question of cell cycle control. How is it possible to ensure that S and M phases occur in the correct order if a single cyclin-dependent kinase is responsible for promoting both events? The discovery, first with *S. cerevisiae* and then later with animal cells, that Cdc2 homologs associated with different cyclins during different phases of the cell cycle appeared to provide a simple answer to this question (23). Cyclins that associate with Cdc2 during G_1 would be capable only of promoting early cell cycle events such as the onset of S, whereas the G_2-specific cyclins would be capable only of promoting the G_2-to-M transition. Thus, specificity could be determined by restricting substrate interactions, either by directly modulating substrate preference or by changing intracellular localization. Indeed, there are a number of studies which indicate that different Cdk-cyclin complexes have different substrate preferences and localization (31, 35). Thus, functional specificity of different cyclins, combined with a mechanism for providing for oscillatory waves of the appearance of S- and M-phase-promoting cyclins, would be sufficient to ensure that S and M were correctly ordered in the cell cycle.

An impediment to testing this model in *S. pombe* has been the difficulty in identifying cyclins that are essential for the onset of S, even though a total of six genes encoding cyclin-related proteins have been described in *S. pombe*. This situation has now been improved by our findings, which strongly indicate that Cig2 is the primary S-phase-promoting cyclin in *S. pombe*. Cig2 has several properties expected of an S-phase cyclin. The onset of S is delayed in Δ*cig2* cells. Moreover, in synchronous cell cultures, Cig2 mRNA and protein are most abundant and Cig2-associated kinase is most active shortly after the exit from M, the period in the cell cycle that corresponds to G_1 and S in *S. pombe* (10, 34). Indeed, Cig2 abundance rises to very high levels in cells arrested in S. In many key respects, Cig2 is similar to the Clb5 and Clb6 B-type cyclins in *S. cerevisiae* (37, 39, 40). The onset of S is delayed in a Δ*clb5* Δ*clb6* double mutant, and either Clb5 or Clb6 is essential for the initiation of S in a mutant that lacks Clb1, Clb2, Clb3, and Clb4. Like Cig2, the budding yeast Clb5 and Clb6 proteins are most abundant and their associated kinase activity is most active during late G_1 and S phases. As is the case in *S. cerevisiae*, the role of S-phase cyclins is obscured because M-phase-promoting cyclins are effective at promoting S in mutants lacking the S-phase cyclins. These observations indicate that a role for B-type cyclins in promoting S phase may be a common feature of lower eukaryotes.

Although these findings are illuminating about how the onset of S phase is regulated in *S. pombe*, they also present a problem by reviving the paradox of how a single cyclin-dependent kinase can properly regulate the order of S and M phases. A Δ*cig2* cell has no difficulty in bringing about the onset of S and M in the proper order, yet the onset of both of these events depends on Cdc2-Cdc13 kinase. The paradox is deepened by the realization that in Δ*cig2* cells, Cdc13 promotes entry into S

phase in G_1 cells and then presumably represses the initiation of DNA replication during G_2. It has been argued that the presence of the Cdc2-Cdc13 complex defines a cell as being in G_2, with no option other than to initiate M as the next step in the cell cycle (18). This view of cell cycle control must be modified, since the presence of the Cdc2-Cdc13 complex not only is compatible with the onset of DNA replication but also is actually required for the initiation of S in Δ*cig2* cells.

In Δ*cig2* cells, how does Cdc2-Cdc13 kinase first promote S early in the cell cycle and then prevent the reinitiation of S during G_2? Two general models seem reasonable. One model supposes that there is an intrinsic functional difference between the Cdc2-Cdc13 complexes in G_1 and G_2 cells. This is a derivation of the model described above, except that something other than binding to different cyclins modulates Cdc2 substrate specificity or cellular localization during the cell cycle. The functional difference between Cdc2-Cdc13 kinases in G_1 and G_2 phases could be caused by posttranslational modification of the kinase complex or by interaction with a regulatory subunit. Interaction with a regulatory subunit might, for example, prevent the Cdc2-Cdc13 complex from promoting M and inhibiting S during G_1 but allow the complex to initiate S. Initiation of S would relieve the regulation by the regulatory subunit, perhaps by degradation or phosphorylation of the regulatory protein. A possible Cdc2-Cdc13 regulatory subunit that may have some of these properties is Rum1 (30). Overproduction of Rum1 causes a phenotype that is similar to the Δ*cdc13* phenotype; cells appear to undergo multiple rounds of DNA replication without intervening phases of nuclear division. Perhaps Rum1 blocks the G_2- and M-phase activities of Cdc2-Cdc13 without blocking the S-phase-induction activity. Determining the effect of Rum1 overproduction in a Δ*cig2* strain will be an important test of this possibility. However, the Δ*rum1* mutation does not change the order of S and M; in fact, the mitotic growth properties of Δ*rum1* cells appear normal (30). For this reason, Rum1 cannot account for all predictions of this model, although it is possible that another protein shares redundant functions with Rum1.

A second model assumes that there is no critical qualitative difference between the G_1 and G_2 forms of Cdc2-Cdc13. Instead, it proposes that two factors work together to ensure that S is followed by M and that M is followed by S. One factor may be that the onset of S requires significantly less Cdc2 kinase activity than the onset of M does. This could be accomplished by a number of different mechanisms; perhaps substrates which must be phosphorylated to induce the onset of S require a lower degree of phosphorylation than those that are involved in promoting M. The second factor may be that the same Cdc2-catalyzed event that initiates S phase also renders the cell incapable of initiating new rounds of S prior to the completion of M. For example, one could imagine that a Cdc2-catalyzed activation of the DNA replication machinery bound to replication origins also causes the disassociation of an essential component of the origin-binding complex. Reassociation of the complex in a state that is competent to initiate a new round of replication would be prevented until Cdc2 kinase activity was decreased after exit from M. Such a model is consistent with recent studies showing that Cdc2 directly interacts in vivo with a putative origin-binding protein, Orp2, that is required for DNA replication (22). This model is attractive because it requires only the periodic increase of cyclin B-associated kinase activity during interphase that is interrupted by the punctuated destruction of cyclin B upon exit from mitosis. Other embellishments of the processes regulating Cdc2 activity, such as the addition of multiple cyclins that are expressed at different points in the cell cycle, the posttranslational regulation of Cdc2-Cdc13 by phosphorylation of Cdc2 on tyrosine 15 (15), and perhaps the binding of inhibitor proteins, serve to bring a greater degree of control to the cell cycle.

Finally, it should be stated that these studies have not addressed whether Cdc13, Cig1, or Cig2 are involved in executing Start, which is defined as the point at which cells commit to undergoing a mitotic cycle as opposed to initiating conjugation or meiosis (11). In *S. cerevisiae*, execution of Start requires G_1 cyclins encoded by *CLN1*, *CLN2*, and *CLN3*. The three *CLN* gene have semiequivalent functions, since mutants lacking any two of the *CLN* genes are viable. Interestingly, a strain lacking all three *CLN* genes is rescued by constitutive expression of *CLB5*, indicating that the essential shared function of the Cln cyclins may be only to activate the transcription factors that are required for the periodic expression of *CLB5* and *CLB6* (40). Puc1 is a candidate G_1 cyclin in *S. pombe*, although Δ*puc1* causes no phenotype in cells grown in nitrogen-rich medium (12, 13). However, the Δ*puc1* mutation does accelerate G_1 arrest in nitrogen-starved cells. This may suggest that Puc1 has a role in executing Start, presumably in collaboration with other, as yet undiscovered, fission yeast G_1 cyclins.

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