

Analysis of Fission Yeast Primase Defines the Checkpoint Responses to Aberrant S Phase Initiation

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To investigate the checkpoint response to aberrant initiation, we analyzed the cell cycle checkpoint response induced by mutations of *Schizosaccharomyces pombe* DNA primase. DNA primase has two subunits, Spp1 and Spp2 (*S. pombe* primases 1 and 2). Spp1 is the catalytic subunit that synthesizes the RNA primer, which is then extended by DNA polymerase α (Pol α) to synthesize an initiation DNA structure, and this catalytic function of Pol α is a prerequisite for generating the S-M phase checkpoint. Here we show that Spp2 is required for coupling the function of Spp1 to Pol α . Thermosensitive mutations of *spp2*⁺ destabilize the Pol α -primase complex, resulting in an allele-specific S phase checkpoint defect. The mutant exhibiting a more severe checkpoint defect also has a higher extent of Pol α -primase complex instability and deficiency in the hydroxyurea-induced Cds1-mediated intra-S phase checkpoint response. However, this mutant is able to activate the Cds1 response to S phase arrest induced by temperature. These findings suggest that the Cds1 response to the S-phase arrest signal(s) induced by a initiation mutant is different from that induced by hydroxyurea. Interestingly, a *pol α s* mutant with a defective S-M phase checkpoint and an *spp2* mutant with an intact checkpoint have a similar Pol α -primase complex stability, and the Cds1 response induced by hydroxyurea or by the mutant arrests at the restrictive temperature. Thus, the Cds1-mediated intra-S phase checkpoint response induced by hydroxyurea can also be distinguished from the S-M phase checkpoint response that requires the initiation DNA synthesis by Pol α .

To maintain genomic integrity, eukaryotic cells have the checkpoint mechanisms to delay progression of the cell cycle when cells encounter perturbation of DNA replication or DNA damage (18). In fission yeast, a group of proteins, Rad1, Rad3, Rad9, Rad17, Rad26, and Hus1, known as checkpoint Rad proteins, function early in the surveillance of both the replication perturbation and DNA damage (1, 13). These checkpoint Rad proteins are thought to sense and transduce signals of aberrant replication and DNA damage to activate two downstream protein kinases, Cds1 and Chk1, to arrest the cell cycle (5–8, 40). In response to S phase arrest by hydroxyurea, *cdc* mutant arrest, or DNA damage induced during S phase, Cds1 is phosphorylated and activated (19). Cds1 activation delays the progression of S phase (termed intra-S phase checkpoint) and contributes to preventing mitosis (3, 19, 26). The Cds1 structural counterpart of budding yeast, *RAD53*, has been shown to be an essential factor for maintaining the intra-S phase checkpoint to prevent the firing of late replication origins when the progression of replication forks from early-firing origins is blocked by hydroxyurea (12, 33, 34).

Another downstream kinase, Chk1, is required to arrest mitosis when DNA is damaged in late S phase and G₂ phase and is also required to prevent mitosis when *cdc* replication mutants are used to perturb S phase (7). Chk1 is not required to prevent mitosis in response to hydroxyurea block. Following DNA damage, Chk1 protein is phosphorylated in a cell-cycle-specific manner (23), and Chk1 phosphorylation is correlated to cell cycle arrest (4). Chk1 phosphorylation allows binding of 14-3-3 proteins with Chk1 that is thought to direct Chk1 for specific substrate (9). Although Chk1 is not phosphorylated in hydroxyurea block or during early S phase perturbation (23), Chk1 is phosphorylated when S phase is blocked by hydroxy-

urea in a *cds1* Δ background (4, 19). Chk1 kinase has been shown to phosphorylate in vitro two Cdc2 kinase regulators, Wee1 kinase and Cdc25 phosphatase (15, 30, 32). Phosphorylation of Cdc25 by Chk1 allows Cdc25 to associate with 14-3-3 proteins, leading to nuclear exclusion of Cdc25 (21, 31, 42).

These findings strongly suggest that checkpoint signals generated from early-S-phase perturbation are different from those generated during ongoing or late S phase. Early-S-phase perturbation activates Cds1 kinase to maintain an intra-S phase checkpoint, while ongoing or late-S-phase perturbation results in Chk1 phosphorylation to prevent mitosis. Thus, Cds1 and Chk1 function in two distinct but mutually reinforced ways in the cell cycle surveillance mechanisms.

We are interested in defining the requirements for generating the checkpoint response to aberrant S phase initiation. To achieve this goal, we investigated the effect of mutations of DNA polymerase α (Pol α)-primase on the cell cycle events. DNA Pol α -primase, a four-subunit enzyme complex, is the principal enzyme that initiates DNA replication on both leading and lagging strands. DNA primase synthesizes an RNA primer which is then extended by Pol α to synthesize an initiation DNA structure (39, 41). DNA primase is a heterodimeric enzyme complex, consisting of a catalytic subunit that synthesizes the RNA primer, named p49 in mammalian cells and *PRII* in budding yeast, and a second subunit that has no detectable enzymatic activity, named p58 in mammalian cells and *PRIII* in budding yeast (41).

We and others have previously demonstrated that deletion or mutation of DNA Pol α results in the cells entering inappropriate mitosis (2, 11). We have previously shown that germinating spores derived from a *Schizosaccharomyces pombe* heterozygous diploid containing one copy of the *pol α* ⁺ gene with a mutation at a critical metal activator binding residue (Asp⁹⁸⁴ in region I) display an abnormal mitotic phenotype (see Fig. 2 of reference 2). In vitro studies have shown that Pol α -primase complex containing this specific Pol α mutation abolishes only the catalytic function and not the physical asso-

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TABLE 1. Strains used in this study

Strain	Genotype	Source
KG2	<i>h⁻ ade6-M216 leu1-32 ura4-D18 his3-D1</i>	K. Gould
KG23	<i>h⁻/h⁺ ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18 his3-D1/his3-D1</i>	K. Gould
KG3	<i>h⁺ ade6-M210 leu1-32 ura4-D18 his3-D1</i>	K. Gould
NW222	<i>h⁻ chk1:ep ade6-M216 leu1-32</i>	N. Walworth
DBts13	<i>h⁺ polαΔ::polats13 leu⁺ his⁺ ade6-M210 leu1-32 ura4-D18 his3-D1</i>	D. Bhaumik
ST108	<i>h⁻ spp2-8 ura4⁺ ade6-M216 leu1-32 ura4-D18 his3-D1</i>	This study
ST109	<i>h⁻ spp2-9 ura4⁺ ade6-M216 leu1-32 ura4-D18 his3-D1</i>	This study
ST118	<i>h⁻ spp2-8 ura4⁺</i>	This study
ST119	<i>h⁻ spp2-9 ura4⁺</i>	This study
ST101	<i>h⁻/h⁺ spp2⁺/spp2Δ::ura4⁺ ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18 his3-D1/his3-D1</i>	This study
ST128	<i>h⁻ spp2-8 ura4⁺ chk1:ep ade6-M216 leu1-32 his3-D1</i>	This study
ST129	<i>h⁺ spp2-9 ura4⁺ chk1:ep ade6-M216 leu1-32 his3-D1</i>	This study
ST138	<i>h⁻/h⁺ spp2⁺/spp2-8 ura4⁺ ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18 his3-D1/his3-D1</i>	This study
ST23	<i>h⁻/h⁺ ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4⁺ his3-D1/his3-D1</i>	This study
spp2-8 cds1Δ	<i>h⁻ spp2-8 cds1::ura4⁺ ade6-M216 leu1-32 ura4-D18 his3-D1</i>	This study
spp2-8 chk1Δ	<i>h⁻ spp2-8 chk1::ura4⁺ ade6-M216 leu1-32 ura4-D18 his3-D1</i>	This study
spp2-8 rad3Δ	<i>h⁺ spp2-8 rad3::ura4⁺ ade6-M210 leu1-32 ura4-D18 his3-D1</i>	This study
spp2-9 cds1Δ	<i>h⁻ spp2-9 cds1::ura4⁺ ade6-M216 leu1-32 ura4-D18 his3-D1</i>	This study
spp2-9 chk1Δ	<i>h⁻ spp2-9 chk1::ura4⁺ ade6-M216 leu1-32 ura4-D18 his3-D1</i>	This study
spp2-9 rad3Δ	<i>h⁺ spp2-9 rad3::ura4⁺ ade6-M216 leu1-32 ura4-D18 his3-D1</i>	This study

ciation of Pol α and primase, and the mutant complex is able to synthesize the RNA primer in vitro but is unable to extend the RNA primer (10). These experiments strongly suggest that the catalytic function of Pol α is required for generating the checkpoint to prevent inappropriate mitotic entry.

We isolated the fission yeast genes and cDNAs of both primase subunits, named *spp1⁺* and *spp2⁺* for *S. pombe* primases 1 and 2, respectively, and generated conditional mutants of *spp1⁺* and *spp2⁺*. In this report, we investigate the effect of *spp2* mutations on the cell cycle checkpoint response. The results of our studies indicate that Spp2 is the subunit that couples the function of Spp1 with Pol α . Mutations of *spp2* cause instability of Pol α -primase complex. Analyses of the checkpoint effector kinase response to mutations of *spp2* indicate that the requirement for Cds1 checkpoint response to an S phase initiation mutant arrest is different from that for Cds1 response to replication stall induced by the DNA synthesis inhibitor hydroxyurea. The requirements for intra-S phase Cds1 response can also be distinguished from the Pol α activity-dependent S-M phase checkpoint response.

MATERIALS AND METHODS

Yeast strains, media, and genetic, cell biological, and molecular methods. The strains used in this study were listed in Table 1. Cells were maintained either in rich medium (YE5S) or Edinburgh minimal medium with appropriate supplements as described elsewhere (25). All genetic methods were performed as described previously (17). Molecular biology techniques were performed as described by Maniatis et al. (22). Transformation of fission yeast was performed as described previously (16). Growth and viability analysis of cells were performed as described previously (2, 16). Cell extracts were prepared by glass bead disruption as described by Al-Khodairy et al. (2).

Plasmids. A disruption vector pUS-Ura-DS, which contains an *ura4⁺* gene placed between upstream and downstream of *spp2⁺* coding sequence, was constructed as follows. First, the *ura4⁺* gene was cloned into *SalI/BamHI* sites of pBluescript SK(-) (Stratagene) to generate pBS-Ura. Then, the ~600-bp downstream region of *spp2⁺* coding sequence was amplified from genomic DNA by PCR using Spp2-5'-DS/Pst and Spp2-3'-DS/Kpn as primers and subcloned into the *PstI/KpnI* sites of pBS-Ura to create pUra-DS. Finally, the ~500-bp upstream region of *spp2⁺* coding sequence was amplified from genomic DNA by PCR using Spp2-5'-US/Xba and Spp2-3'-US/Bam as primers and subcloned into the *XbaI/BamHI* sites of pUra-DS to create pUS-Ura-DS. The coding region of *spp2⁺* cDNA was amplified from the *spp2⁺*-cDNA clone (as described below) by PCR using Spp2-5'-US/Xba and Spp2-3'-US/Bam as primers and subcloned into pET expression vector to create pET-Spp2. The oligonucleotides used for PCR were as follows: Spp2-5'-US/Xba, AGATCTAGAAGTCTTTACGATGCATTATCTAATG; Spp2-3'-US/Bam, CGCGATCCGGTGGTTAGGGAAGAGTCTA

TTTG; Spp2-5'-DS/Pst, AAACGTCAGTAAGCTAAGATACCTTTAGTTCACG; Spp2-3'-DS/Kpn, AAAGGTACCGGTGGTGTCTTATGCTTATCG; Spp2-5'-Xho, CCGCTCGAGATGTTTCAGAACGACCAAAAAGTCGAG; and Spp2-3'-Bam, AAGGATCCTTATGATTCTAACTAAGTTGAAAATATTG.

Identification and isolation of *spp2⁺* gene and cDNA. Spp2 protein was initially identified as a 54-kDa polypeptide tightly associated with DNA Pol α purified from *S. pombe* cell extract (R. Davis and T. Wang, unpublished). Mass spectrometric analysis of the 54-kDa protein identified several peptide sequences overlapping with an open reading frame in chromosome II (cosmid SPBC17D11) from the Sanger fission yeast genome database. The *spp2⁺* cDNA was isolated by screening a λ -ZAP cDNA library from *S. pombe* (S. Tan and T. Wang, unpublished results). A full-length *spp2⁺* cDNA was assembled from overlapping clones and was subcloned into pET expression vector. Homology searching through GenBank found that Spp2 shares 37, 37, and 40% sequence identity to the primase subunits p58 of humans (35) and mice (24) and of PR111 of *S. cerevisiae* (14), respectively.

Construction of *spp2Δ* strain. Heterozygous diploid strain ST101 (*spp2⁺/spp2Δ*) carrying a deletion of one copy of *spp2⁺* was constructed by one-step gene replacement. The DNA fragment, which contains a ~500-bp *spp2⁺* upstream sequence, a functional *ura4⁺* gene, and a ~600-bp *spp2⁺* downstream region, was released from pUS-Ura-DS by digesting it with *XbaI* and *KpnI* and transformed into the diploid strain KG23. The transformants were selected on minimal medium lacking uracil and screened for several rounds for the stability of the *ura4⁺* marker. Successful replacement of one copy of *spp2⁺* coding region by *ura4⁺* gene was confirmed by genomic Southern analysis. Tetrad dissection of spores derived from ST101 (*spp2⁺/spp2Δ*) resulted in two viable uracil auxotrophic spores, confirming that Spp2 is essential for cell growth. To analyze the phenotype of *spp2Δ* germinating spores, the spores isolated from ST101 (*spp2⁺/spp2Δ*) were selectively germinated in minimal medium lacking uracil. Samples were taken at indicated times to determine the DNA contents by fluorescence-activated cell sorter (FACS) analysis. A heterozygous ST23 containing only one copy of *ura4⁺* was constructed and used as a wild-type control.

Isolation of *spp2* temperature-sensitive mutants. Mutations were introduced into the *spp2⁺* gene by mutagenic PCR as described earlier (37). Briefly, *spp2⁺* cDNA was amplified from pET-Spp2 by *Taq* DNA polymerase in a buffer containing 20 mM Tris (pH 8.4), 50 mM KCl, 7 mM MgCl₂, 0.5 mM MnCl₂, 0.1% gelatin, 200 μ M dATP, 200 μ M dGTP, 1 mM dCTP, and 1 mM dTTP. The primers for PCR amplification were Spp2-3'-BamH and T7 primer (AATACG ACTCACTATAG). The PCR reaction was carried out in a 100- μ l reaction and performed with 25 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min. An *spp2* mutation library was constructed by cloning the mutagenized *spp2* cDNA into *XbaI/BamHI* sites of pUra-DS. The DNA fragments, which contain the mutagenized *spp2* cDNA, a *ura4⁺* gene, and ~600 bp of downstream of the *spp2⁺* coding region, were isolated by digesting the library with *XbaI* and *KpnI* and transformed into the haploid strain KG2. Transformants were screened by replica plating onto minimal medium lacking uracil and then tested for temperature sensitivity. Stable temperature-sensitive *ura⁺* transformants were selected and backcrossed with wild-type strain three times. Correct chromosomal integration of the *spp2* mutant was verified by genomic Southern blot analysis. All of the temperature-sensitive *spp2* mutants isolated can be rescued by pREP81 plasmid carrying *spp2⁺*.

Generation and purification of Spp2 antibody. Recombinant Spp2 protein was expressed in bacteria by using pET expression system (Novagen). The entire coding region of *spp2*⁺ was cloned into pET vector. The resulting plasmid, pET-Spp2, which contains the translation initiation codon ATG, a six-histidine tag, and the *spp2*⁺ coding region, was transformed into strain BL21(DE3). Spp2 protein expressed from BL21(DE3) harboring pET-Spp2 was purified on Ni²⁺-nitrilotriacetic acid column chromatography as described elsewhere (36). The recombinant Spp2 protein eluted from the Ni²⁺-nitrilotriacetic acid column was further purified to near homogeneity by Mono S chromatography via the SMART system (Pharmacia Biotech). Briefly, an Spp2 protein fraction isolated from the Ni²⁺-nitrilotriacetic acid column was first dialyzed against buffer containing 50 mM HEPES (pH 7.9), 0.5 mM EDTA, and 50 mM KCl and then applied to a 0.12-ml Mono S column (Pharmacia Biotech). Spp2 protein was eluted with a 1-ml linear gradient from 50 to 500 mM KCl in the same buffer. The purified recombinant Spp2 protein was used as antigen to raise antibodies against Spp2 in rabbit. The antisera were affinity purified on an Spp2 protein column prepared by immobilizing the recombinant Spp2 on *N*-hydroxysuccinimide-activated Sepharose (Pharmacia Biotech).

Cds1 kinase assay. In vitro Cds1 kinase assay was performed as described elsewhere (2, 19) with the following modifications. One milligram of total protein from cell extract was mixed with 2 μ l of affinity-purified rabbit anti-Cds1 antibody at 4°C for 2 h. Twenty microliters of protein A-agarose (as 50% slurry) was added to the mixture and incubated at 4°C for an additional hour. The immunocomplexes were precipitated and washed two times in a lysis buffer containing 150 mM HEPES (pH 7.9), 250 mM KCl, 1 mM EDTA, 6 μ M leupeptin, 2 μ M pepstatin A, 2 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride, followed by washing three times with the kinase buffer containing 10 mM HEPES (pH 7.5), 75 mM KCl, 5 mM MgCl₂, 0.5 mM EDTA, and 1 mM dithiothreitol. As a control for equal amounts of Cds1 used for the kinase assay, 3% of each sample was removed prior to immunoprecipitation and quantitated by Western blotting with anti-Cds1 antibodies. A kinase reaction was performed at 30°C for 15 min in the kinase buffer containing the Cds1 immunoprecipitate, 5 μ g of myelin basic protein (MBP), 5 μ Ci of [α -³²P]ATP, and 100 μ M ATP. Reactions were terminated by the addition of sodium dodecyl sulfate (SDS) sample buffer. After being boiled for 3 min, the samples were fractionated on an SDS-15% polyacrylamide gel. Gels were fixed in 40% methanol and 10% acetic acid and dried. The kinase activity was quantitated using an IS-1000 digital imaging system (Alpha Innotech, San Leandro, Calif.).

Immunoprecipitation of Pol α -primase complex. The Pol α -primase complex was immunoprecipitated from cell lysates by using anti-Pol α antibody (27) immobilized on protein A-agarose. One milligram of total cell extract protein was mixed with 15 μ l of the anti-Pol α antibody beads in the cell lysis buffer described above. After incubation at 4°C for 4 h, the immunocomplexes were collected and washed five times with the lysis buffer. The pellets were resuspended in 30 μ l of SDS sample loading buffer and analyzed by Western blotting using antibodies against Pol α , Spp1, and Spp2 as probes.

Flow cytometry analysis. Cells were harvested, washed in water, and fixed in 70% ethanol prior to staining with propidium iodide as described earlier (28). DNA contents were measured by using a Coulter Electronics FACS.

Cytology analysis. Cells were fixed in 70% ethanol and stained by DAPI (4',6'-diamidino-2-phenylindole) followed by calcofluor as described previously (38).

RESULTS

Characterization of cells with *spp2* mutations. To investigate the effect of *spp2*⁺ mutation on the cell cycle checkpoint response, we isolated a panel of thermosensitive mutants of *spp2*⁺. At the restrictive temperature of 36°C, the *spp2* mutants exhibited two distinct phenotypes. Two representative mutants of each phenotype, *spp2-8* and *spp2-9*, were characterized in this study. At 6 h after the shift to 36°C, *spp2-8* exhibited heterogeneous cell size, with ~60% of the cells displaying aberrant mitotic nuclear phenotype. In contrast, *spp2-9* cells had elongated cell morphology with nominal levels of cells displaying abnormal mitotic nuclear phenotype (Fig. 1A). Consistent with their phenotypes, *spp2-8* cells died more rapidly than *spp2-9* cells when mid-log-phase cultures were shifted to 36°C (Fig. 1B). At 6 h after the shift to 36°C, <2% of the *spp2-8* cells remained viable, while >30% of the *spp2-9* cells were viable (Fig. 1B). FACS profiles of these two mutants correlated with their phenotypes and their kinetics of viability loss (Fig. 1C). Wild-type and mutant cells initially had 2C DNA contents, since asynchronous *S. pombe* cultures are predominantly in the G₂ phase. The DNA content of wild-type cells remained at 2C, the peak of the FACS profile of *spp2-8*

and *spp2-9* cells shifted to about 1.5C after 3 h at 36°C and then shifted toward 2C. After 6 h, the FACS profile of *spp2-8* showed that a portion of the cells had a less than 1C DNA content and a portion of the *spp2-8* cells had a greater than 2C DNA content, which reflected the observed heterogeneous cell size and aberrant mitotic nuclear phenotype (Fig. 1A). At 6 h after the shift to 36°C, the majority of the *spp2-9* cells exhibited a greater-than-2C profile, which correlated with the observed elongated phenotype of *spp2-9* (Fig. 1A). None of the *spp2* mutants was substantially sensitive to UV or hydroxyurea at 30°C.

To determine the phenotype of absence of *spp2*⁺, a heterozygous diploid of *spp2* Δ and *spp2*⁺ was constructed as described in Materials and Methods. Tetrad analysis of the diploid yielded two viable *ura4*⁻ spores, indicating that *spp2*⁺ is essential for cell viability. We have previously shown that germinating spores carrying *pol* α Δ enter mitosis with a 1C DNA content (2). Since Spp2 is a component of the Pol α -primase complex, we expect that the germinating spores carrying *spp2* Δ would display a phenotype similar to that of *pol* α Δ . Spores carrying *spp2* Δ derived from the *spp2*⁺/*spp2* Δ diploid were selected for germination in medium lacking uracil. As a control, spores carrying *spp2*⁺ derived from a *ura4-D18/ura4*⁺ diploid strain were analyzed in parallel. Spores carrying *spp2*⁺ entered S phase 8 h after inoculation, and most cells completed S phase with 2C DNA content after 10 h (Fig. 2A, 8 to 10 h). In contrast, 10 h after inoculation, spores carrying *spp2* Δ showed a delay in entering S phase, with a fraction of the cells having a 2C DNA profile similar to the wild-type spores and a fraction of the cells having 1C DNA content (Fig. 2A, 8 to 10 h). After 14 h, *spp2* Δ cells completed S phase and had a DNA content identical to that of the *spp2*⁺ cells (Fig. 2A, 14 h). The germinating *spp2* Δ spores exhibited elongated cell morphology with <3% of the cells exhibiting aberrant mitotic nuclear morphology (Fig. 2B, middle panel). This is in striking contrast to *pol* α Δ , where ~60% of *pol* α Δ germinating spores display aberrant mitotic phenotype (2).

Since *spp2-8* mutant exhibits aberrant mitotic phenotype at 36°C (Fig. 1A), it is surprising that germinating *spp2* Δ spores display an elongated phenotype. It is possible that Spp2 is a stable protein and that the residual Spp2 carried over from the original diploid has allowed the *spp2* Δ cells to initiate S phase in the absence of Spp2 transcription and translation. To test this possibility, we constructed a heterozygous diploid of *spp2*⁺ and *spp2-8* and selectively germinated the spores harboring *spp2-8* at 36°C. The *spp2-8* germinating spores had a similar FACS profile to that of the spores harboring *spp2* Δ (Fig. 2A). At 14 h following inoculation, the *spp2-8* germinating spores exhibited an elongated cell morphology with normal nuclear morphology (Fig. 2B, right panel) and not the aberrant mitotic phenotype of *spp2-8* shown in Fig. 1A. It is worth mentioning that *spp2*⁺/*spp2-8* and *spp2*⁺/*spp2-9* diploids grow normally at 36°C, indicating that both mutant alleles are recessive.

To further analyze the phenotype of *spp2* Δ germinating spores in the absence of DNA replication, we treated the germinating *spp2* Δ spores with hydroxyurea, an inhibitor of DNA synthesis, at 0 and 6 h following inoculation in selective medium lacking uracil. Under these conditions, germinating spores carrying *spp2*⁺ remained arrested with 1C DNA for up to 12 to 14 h postinoculation with elongated cell morphology. Similarly, germinating spores carrying *spp2* Δ arrested with 1C DNA content with normal nuclear morphology (data not shown). These experiments indicate that the observed elongated phenotype of *spp2* Δ is indeed due to residual wild-type Spp2 carried over from the original diploid.

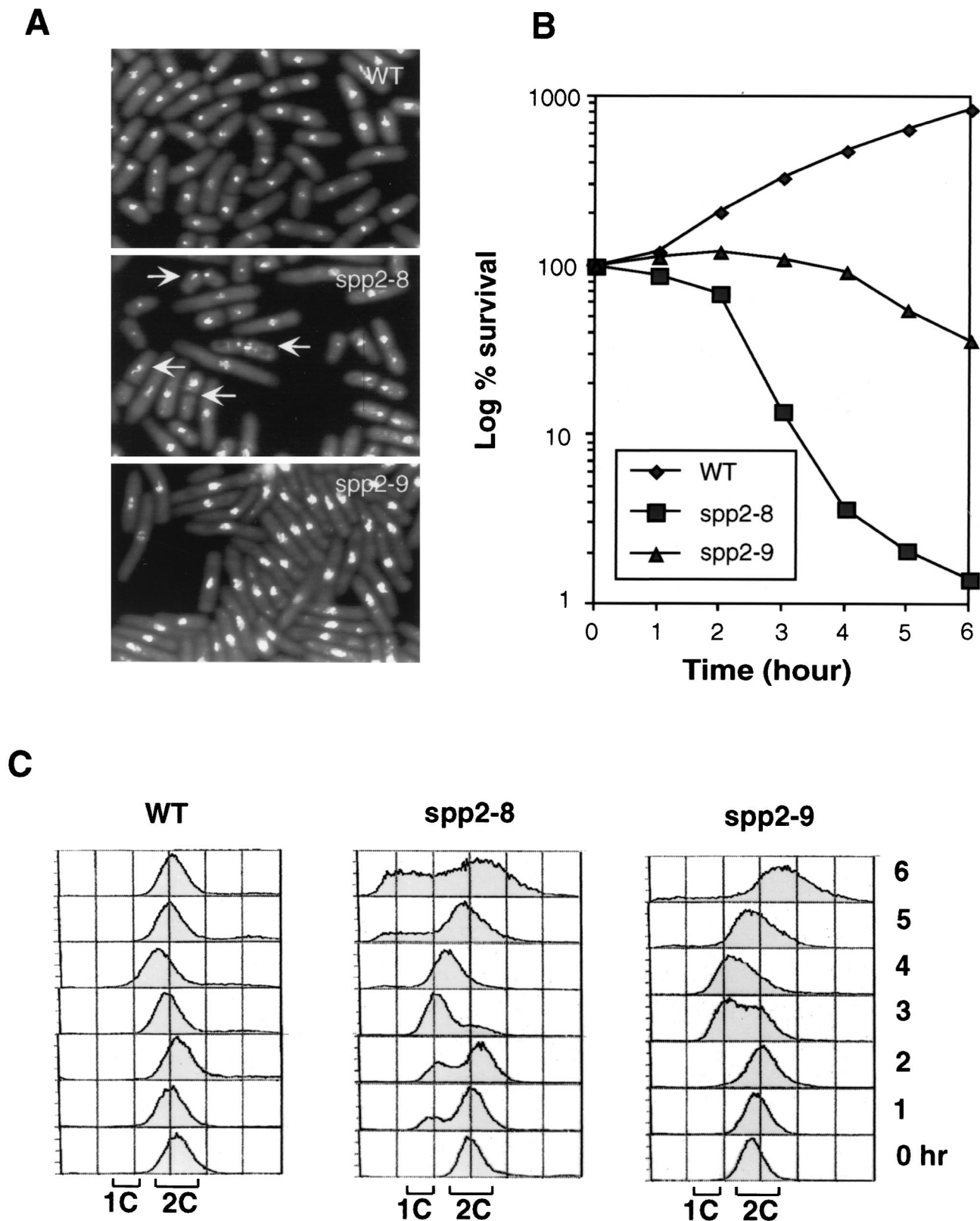


FIG. 1. Characterization of temperature-sensitive mutants of *spp2*⁺. Early-log-phase wild-type 972h⁻, ST118 (*spp2-8*), and ST119 (*spp2-9*) cells grown at 25°C were shifted to 36°C. Samples were removed at the indicated times and processed for morphological, survival, and FACS analysis. (A) Photomicrographs of wild-type and *spp2* mutant cells 6 h after the shift to 36°C. Arrows indicate *spp2-8* cells that entered inappropriate mitosis. (B) Viability of wild-type and *spp2* mutant cells. (C) FACS analysis of wild-type and *spp2* mutant cells.

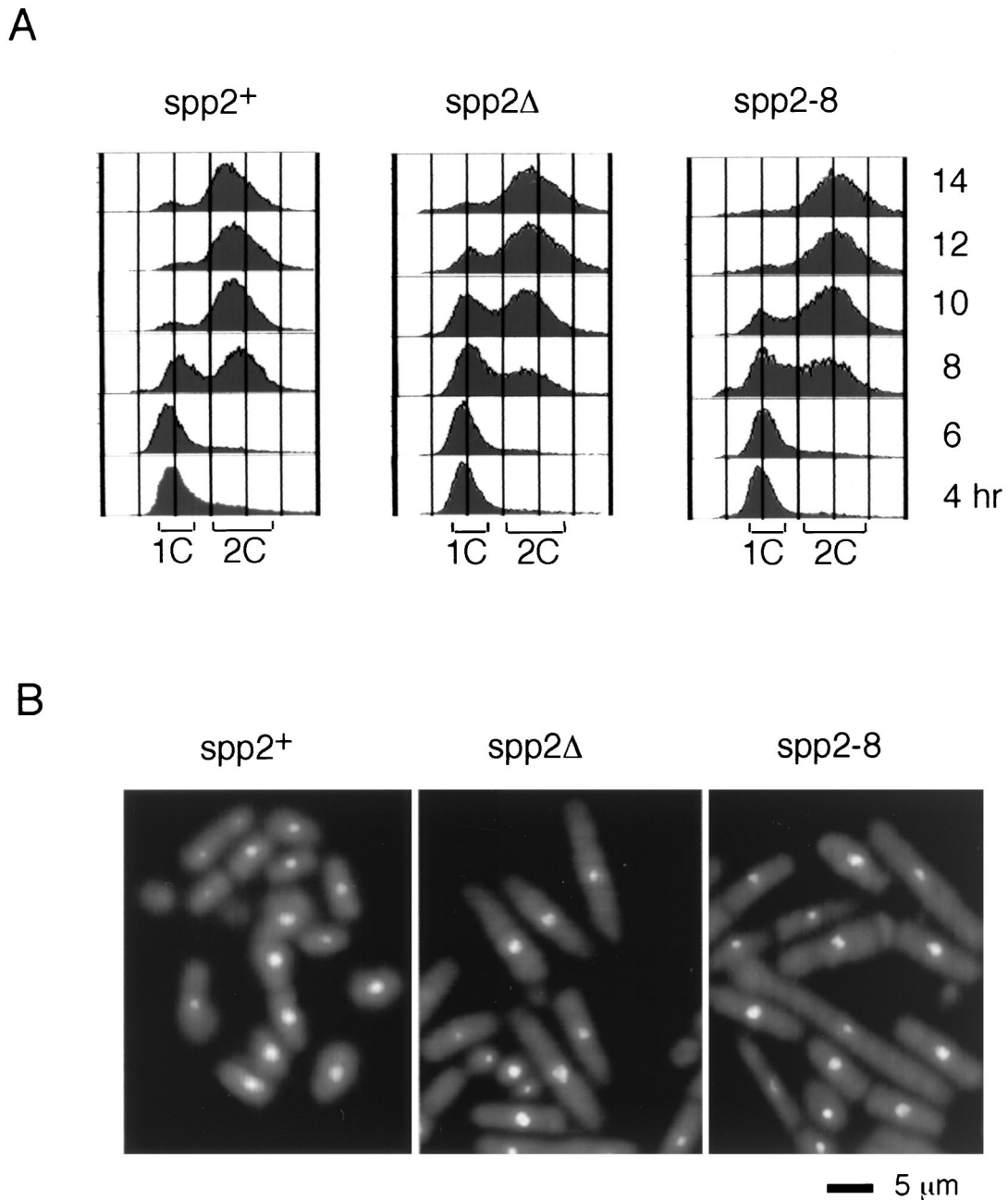
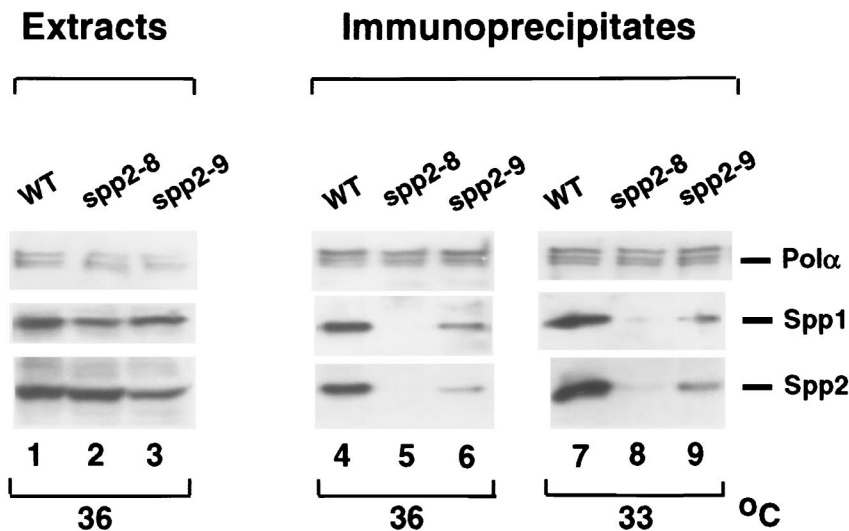


FIG. 2. Germinating spores harboring *spp2Δ* and *spp2-8* arrested with a *cdc* phenotype. (A) FACS profiles of *spp2+* (left panel), *spp2Δ* (middle panel), and *spp2-8* (right panel) germinating spores at 36°C. (B) Phenotype of germinating spores carrying *spp2+* (left panel), *spp2Δ* (middle panel), and *spp2-8* 14 h after inoculation into selective medium. The diploid strains used for spores harboring *spp2+*, *spp2Δ*, and *spp2-8* were ST23, ST101, and ST 138, respectively.

Analysis of the stability of Pol α -primase complex in *spp2* and *polots* mutants. Since the two *spp2* mutants have a difference in their phenotype, we tested the possible protein structural differences in the mutant's Pol α -primase complex. To this end, we immunoprecipitated Pol α by anti-Pol α antibody from cell lysates of wild type and of *polots13*, *spp2-8* and *spp2-9* mutants and tested for coimmunoprecipitation of Spp1 and Spp2 proteins by Western blot using antibodies against Pol α , Spp1, and Spp2 as probes. To ensure that mutations of *spp2+* did not affect the expression of Pol α , Spp1, and Spp2 in cells, lysates from each strain grown for 4.5 h at 36°C were probed with appropriate antibodies. Wild-type and mutant cells had

comparable levels of Pol α , Spp1, and Spp2 (Fig. 3A, lanes 1 to 3). We then tested the stability of the Pol α -primase complex in these strains after incubation at semipermissive and restrictive temperatures for 4.5 h. Spp1 and Spp2 coimmunoprecipitated with Pol α from wild-type-cell extracts, indicating that the Pol α -primase complex of wild-type cells was stable at either 33 or 36°C (Fig. 3B, lanes 4 and 7). In contrast, the amounts of Spp1 and Spp2 coimmunoprecipitated with Pol α from *spp2-8* and *spp2-9* cell extracts varied substantially. Cell lysates prepared from *spp2-9* at both 33 and 36°C had reduced levels of Spp1 and Spp2 coimmunoprecipitated with Pol α (Fig. 3A, lanes 6 and 9). This indicated that at either a semipermissive or a

A



B

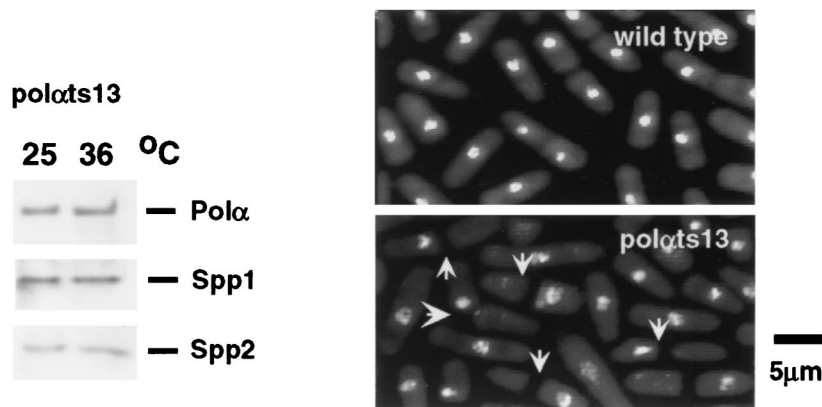


FIG. 3. Stability of Pol α -primase complex in wild-type and mutant cells. (A) Expression of Pol α , Spp1, and Spp2 in wild-type and *spp2* mutant cells. Wild-type 972h⁻, ST118 (*spp2-8*), and ST119 (*spp2-9*) cells grown at 25°C were incubated at 36°C for 4.5 h. Equal amounts of protein from cell extracts were analyzed by Western blot using antibodies against Pol α , Spp1, and Spp2 as probes (lanes 1 to 3). To test the coimmunoprecipitation of primase and Pol α , cells were grown at the indicated temperatures for 4.5 h. Pol α -primase complex was immunoprecipitated from the cell extracts by using anti-Pol α antibody and probed with appropriate antibodies as described in Materials and Methods (lanes 4 to 9). (B) Checkpoint-defective *polαts13* mutant has a stable Pol α -primase complex. (Left panel) Spp1 and Spp2 coimmunoprecipitated with Pol α in DBTs13 (*polαts13*) cells. DBTs13 (*polαts13*) cells were grown at 25 or 36°C for 4.5 h. Pol α -primase complex was immunoprecipitated from the cell extracts by using anti-Pol α antibody and probed with antibodies against Pol α , Spp1, and Spp2. (Right panel) Phenotype of wild type and DBTs13 (*polαts13*) grown at 36°C for 6 h. The arrows indicate *polαts13* cells that displayed an abnormal mitotic phenotype.

restrictive temperature, the Pol α -primase complex in *spp2-9* was slightly compromised. Mutant *spp2-8* grown at 33°C had barely detectable Spp1 and Spp2 coimmunoprecipitated with Pol α (Fig. 3B, lane 8). At 36°C, no detectable Spp1 and Spp2 coimmunoprecipitated with Pol α from *spp2-8* cell lysates when the blot was exposed for the same length of time as the immunoblots from wild-type and *spp2-9* cells (Fig. 3A, lane 5). However, with a longer exposure of the *spp2-8* blot, coimmunoprecipitation of Spp1 and Spp2 with Pol α was detectable (data not

shown). These results indicated that the Pol α -primase complex in *spp2-8* cells was severely compromised.

We observed equal proportions of Spp1 and Spp2 reproducibly being coimmunoprecipitated with Pol α from either wild-type cells or *spp2* mutants. This suggests that neither *spp2* mutant alleles affect the affinity between Spp1 and Spp2. In our separate mutational studies of *spp1*⁺, under the condition when Spp1 protein failed to coimmunoprecipitate with Pol α , Spp2 protein was still able to coimmunoprecipitate with Pol α

in all *spp1* mutants (D. Griffiths, V. Liu, P. Nurse, and T. Wang, manuscript submitted). The *spp1* mutation results indicate that Spp1 is not required for the Pol α -Spp2 complex formation and strongly suggest that Spp2 physically couples Spp1 with Pol α . The *spp1* results and the concomitant decrease of Spp1 and Spp2 coimmunoprecipitated with Pol α in *spp2* mutants shown in Fig. 3 indicate that Spp2 is the bridge protein between Spp1 and Pol α . These immunoprecipitation experiments thus indicated that thermosensitive mutations of *spp2*⁺ affected the stability of Pol α -primase complex. The Pol α -primase complex in *spp2-8* cells was severely compromised, while the complex in *spp2-9* was mildly compromised.

Finding that mutations of *spp2*⁺ affect the stability of Pol α -primase complex and *spp2-8* has a severe instability of its Pol α -primase complex, as well as an abnormal mitotic phenotype, led us to analyze the stability of the complex in the checkpoint-defective *polats13* at the restrictive temperature. Pol α was able to coimmunoprecipitate comparable amounts of Spp1 and Spp2 from the *polats13* mutant when cells were grown at either 36 or 25°C for 4.5 h (Fig. 3B, left panel). The amounts of Spp1 and Spp2 coimmunoprecipitated with Pol α in the *polats13* mutant at either 25 or 36°C, however, were lower than that of the wild type, indicating that the complex, although intact, was slightly compromised (compare Fig. 3B to 3A, lanes 4 and 6). Although the integrity of the four-subunit Pol α -primase complex was not substantially affected by mutations in *polats13*, the mutant had large fractions of the cells that entered aberrant mitosis exhibiting anucleated phenotype (Fig. 3B, right panel [2]). It is important to point out that the Pol α -primase complex of the S-M phase checkpoint-defective *polats13* had a stability similar to that of the complex of the S-M phase checkpoint-intact *spp2-9*. These indicate that the checkpoint defect of *polats13* mutant is not due to the mutant-Pol α protein's inability to associate with the primase proteins.

Mutations of *spp2*⁺ affect S phase entry and progression. Since Spp2 couples the Spp1 and Pol α , we analyzed whether mutations of *spp2*⁺ could affect the cells' S phase entry. Wild-type and *spp2-8* mutant cells were synchronized in G₁ phase by nitrogen starvation for 18 h at 25°C. Cells were then released into rich medium at 36°C. FACS analysis showed that wild-type cells completed S phase within 2 to 3 h after release, whereas *spp2-8* cells remained with 1C DNA content for 2 to 3 h (Fig. 4A) and required about 6 h to reach a near-2C DNA content. Thus, when *spp2* cells were released from nitrogen starvation, mutations of *spp2* caused an approximately 3-h delay in entering S phase. As shown in Fig. 4B, this defect resulted in *spp2-8* cells losing viability after 2 h. After 6 h, <1% of *spp2-8* cells remained viable and >40% of *spp2-8* cells displayed an abnormal mitotic phenotype.

We then tested whether mutations of *spp2*⁺ could affect the progression of S phase. Wild-type and *spp2-8* cells were arrested postinitiation in early S phase by using hydroxyurea for 4 h at 25°C. Cells were then released into fresh medium at 36°C, and samples were taken every 30 min for FACS analysis. Both wild-type and *spp2-8* cells were arrested by hydroxyurea at 25°C with a 1C DNA content. After release from the hydroxyurea block, wild-type and *spp2-8* cells had nearly identical FACS profiles (Fig. 4C). We believe this observation may be due to the *S. pombe* having a short S phase. After the shift to 36°C, it requires a period of time for the temperature to have an effect on the *spp2* mutant. It is possible that the time required to remove the hydroxyurea and shift the temperature is sufficient to allow the cells to progress through S phase before the 36°C temperature exerts an effect on the Spp2 protein. To circumvent this possibility, we arrested the cells for 4 h in hydroxyurea at 25°C, shifted them to 36°C for 1 h before

release from the hydroxyurea block, and continued incubating them at 36°C. With this approach, wild-type cells completed S phase within 1 h after release from hydroxyurea, whereas *spp2-8* cells showed a 0.5- to 1-h delay of progression through S phase (Fig. 4D). Thus, the *spp2* mutant delayed S phase entry and progression at both before and after the hydroxyurea arrest point. Similar results were observed with the *spp2-9* mutant (data not shown).

***spp2* mutants require checkpoint Rad and Cds1, but not Chk1, for growth at the semipermissive temperature.** The finding that at the restrictive temperature *spp2-8* displayed aberrant mitotic phenotype and that *spp2-9* exhibited *cdc* phenotype led us to investigate the cell cycle checkpoint response induced by these two mutant alleles. We found that in the *rad1Δ*, *rad3Δ*, *rad9Δ*, *rad17Δ*, *rad26Δ*, or *hus1Δ* background, *spp2-8* and *spp2-9* were viable in liquid culture at up to 32°C and on plates at up to 33°C. This is different from the *polats* mutants, which are synthetic lethal in these checkpoint *rad* deletion backgrounds at 25°C (2). At the semipermissive temperature of 34°C on solid media, both *spp2-8* and *spp2-9* required all six checkpoint Rad proteins and Cds1 for viability but not Chk1 (Fig. 5A). To test whether the kinase activity of Cds1 is required for the viability of *spp2* mutants, *cds1*⁺ or a *cds1* kinase dead mutant, *cds1-kd*, was ectopically expressed from the *pREP1* vector in the presence of thiamine in the *spp2-8 cds1Δ* or *spp2-9 cds1Δ* double mutant. Moderate expression of *cds1*⁺ rescued the viability of the double mutants at 34°C. In contrast, the expression of kinase dead mutant of *cds1* (*cds1-kd*) was unable to rescue the double mutants at 34°C (Fig. 5B), suggesting that Cds1 kinase activity is required for the viability of *spp2* mutants at the semipermissive temperature. Thus, at the semipermissive temperature, cells with S phase perturbation due to mutations of *spp2*⁺ require the checkpoint Rad proteins and Cds1 kinase, but not Chk1 kinase, for maintaining cell viability.

Chk1 response in *spp2* mutants. Since Chk1 was not required for maintaining growth of *spp2* mutants at the semipermissive temperature, we tested how Chk1 responds to mutations of *spp2*⁺ at the restrictive temperature. As shown above, majority of the *spp2-9* cells and a fraction of the *spp2-8* cells displayed an elongated phenotype with normal nuclear morphology at the restrictive temperature (Fig. 1A). In the *chk1Δ* deletion background, both *spp2-8* and *spp2-9* cells died rapidly and exhibited a small cell size with aberrant mitotic nuclear morphology at 36°C (Fig. 6A). In contrast, *chk1Δ* cells grew normally and exhibited normal nuclear morphology. Thus, the elongated phenotype seen in *spp2-8* and *spp2-9* cells requires the function of Chk1.

We then analyzed the phosphorylation status of Chk1 protein in *spp2* mutant cells. Mutant *spp2* cells containing three hemagglutinin (HA) epitopes tagged *chk1*⁺ (40) were constructed for the detection of Chk1 phosphorylation by 12CA5 monoclonal antibody. As a control, wild-type cells containing the HA tagged *chk1*⁺ were treated with the DNA-damaging agent methyl methane sulfonate (MMS), which had been shown to induce Chk1 phosphorylation (40). As expected, Chk1 became readily phosphorylated in response to MMS treatment (Fig. 6B). In both *spp2-8* and *spp2-9* cells, only after 3 to 4.5 h of incubation at 36°C was a weak, slower-migrating Chk1 protein detectable. This is consistent with the finding that phosphorylation of Chk1 primarily responds to late S phase or G₂ phase perturbation and not early S phase perturbation (23). These results indicated that Chk1 was not required for *spp2* mutants to maintain viability at the semipermissive temperature and that mutation of *spp2*⁺ also did not substantially induce Chk1 phosphorylation at 36°C. However, at the restric-

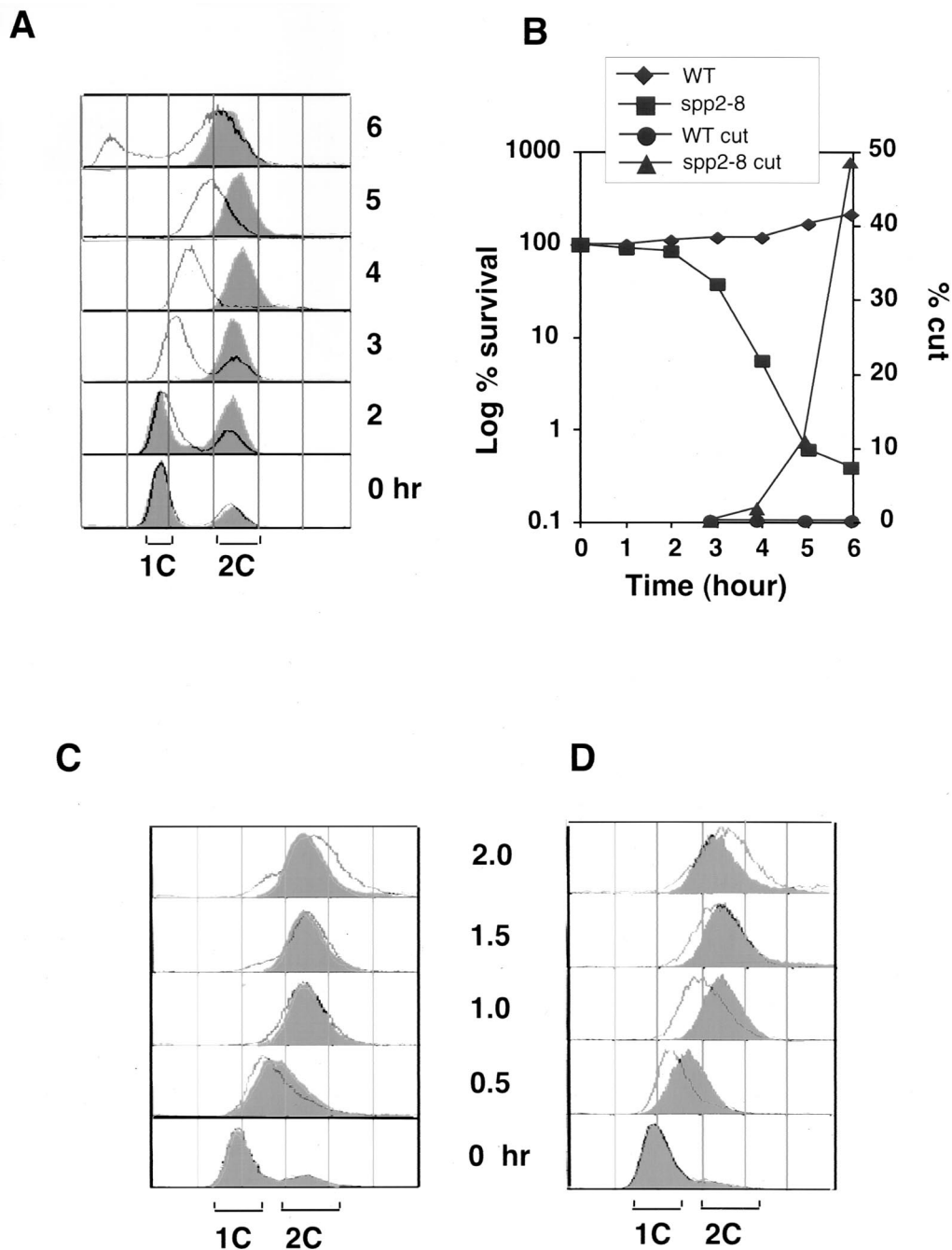


FIG. 4. *Spp2* is essential for S phase entry and progression. Wild-type 972h⁻ and mutant ST118 (*spp2-8*) cells were synchronized in G₁ phase by maintaining them in minimal medium lacking a nitrogen source for 18 h at 25°C. The cultures were then released into YES medium at 36°C and collected at the indicated times for analysis. (A) FACS analysis of wild-type and *spp2-8* cells. The wild type is shown in the overlaid histograms, and the *spp2-8* mutant is shown in the shaded histograms. (B) Viability and percentage of cells displaying an abnormal mitotic “cut” phenotype quantified by microscopic examination of DAPI-stained cells. (C) Wild-type 972h⁻ and ST118 (*spp2-8*) mutant cells were synchronized in 12 mM hydroxyurea for 4 h at 25°C and then released into YES medium at 36°C. Samples were removed after release from the hydroxyurea block at the indicated times for FACS analysis. The wild type is shown in the shaded histograms, and the *spp2-8* mutant is shown in the overlaid histograms. (D) Wild-type 972h⁻ and ST118 (*spp2-8*) mutant cells were synchronized in 12 mM hydroxyurea for 4 h at 25°C, followed by 1 h of incubation at 36°C, and then released into YES medium at 36°C. Samples were removed for FACS analysis at the indicated times after release. The wild type is shown in the shaded histograms, and the *spp2-8* mutant is shown in the overlaid histograms.

tive temperature, Chk1 did play a critical role in preventing aberrant mitotic entry of *spp2-9* cells and of a fraction of the *spp2-8* cells.

Cds1 response in *spp2* and *polα* mutants. Since Cds1 is required for both *spp2-8* and *spp2-9* cells to maintain viability at the semipermissive temperature (Fig. 5) and since Cds1

kinase is activated in thermosensitive *polα* mutants (2, 19), we analyzed the Cds1 response to S phase arrest by *spp2* mutants and compared it to that of the *polα13* mutant. Cds1 protein immunoprecipitated from cell extracts was assayed for kinase activity in vitro using MBP as the substrate (Fig. 7). The Cds1 kinase in *spp2-8*, *spp2-9*, and *polα13* was activated three- to

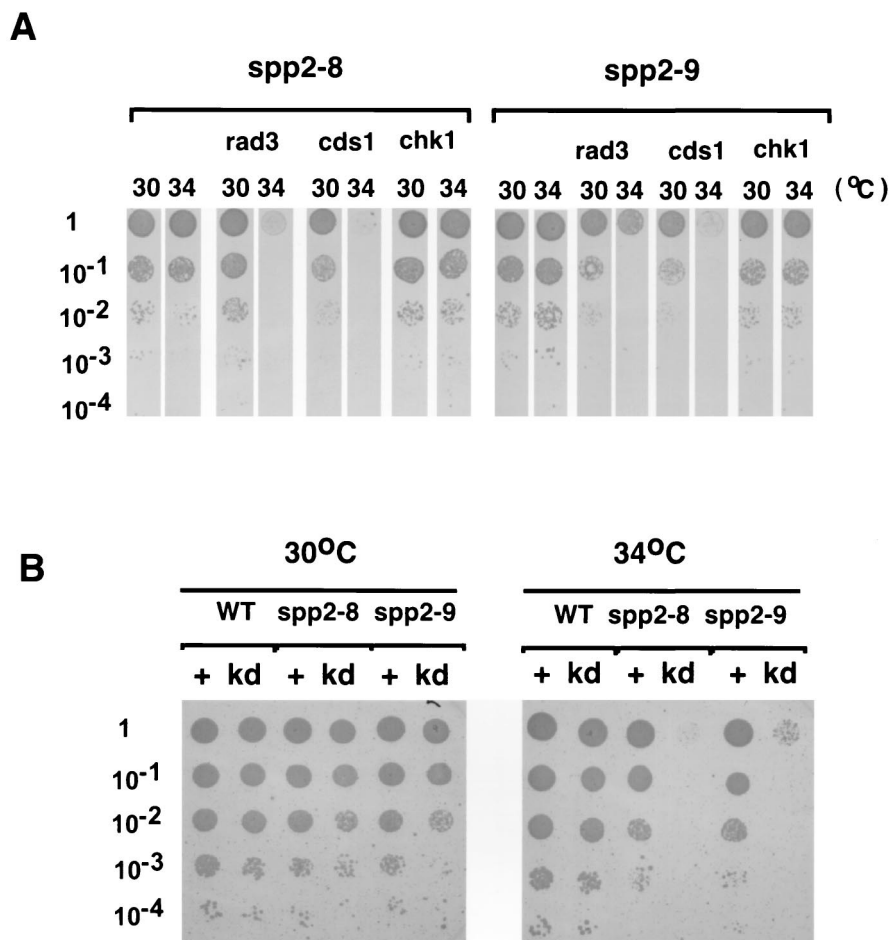


FIG. 5. *spp2* mutants require checkpoint Rads and Cds1, but not Chk1, for growth at the semipermissive temperature. (A) Serial dilutions of *spp2-8* and *spp2-9* mutants and the *spp2-8* and *spp2-9* alleles in *rad3*, *cds1*, and *chk1* deletion background were spotted on YES plates in duplicate. One set of plates was incubated at 30°C, and the other set was incubated at 34°C. (B) Ectopic expression of Cds1, but not a kinase-dead Cds1 mutant, rescues the growth of *spp2 cds1Δ* double mutants at 34°C. *pREP1* plasmid, carrying either *cds1*⁺ or kinase-dead *cds1* mutant, was introduced into *spp2 cds1Δ* double mutants in minimal medium lacking leucine. Thiamine was included in the medium to reduce the expression of Cds1 since the overexpression of Cds1 is toxic to the cells. Serial dilutions of transformants were spotted on the selective medium in duplicates and incubated independently at 30 and 34°C. The transformants of *cds1*⁺ and kinase-dead *cds1* mutant are indicated as “+” and “kd,” respectively. Cell density, 10⁴ cells per spot.

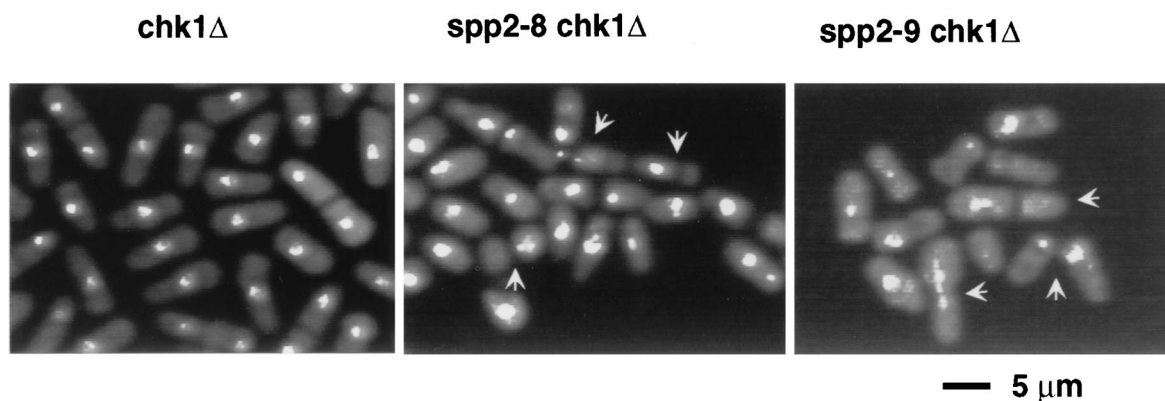
fourfold over the wild-type level after incubation of the cells at 36°C for 4.5 h (Fig. 7, lanes 1 to 4). The kinase activity measured under these conditions was Cds1 specific because the phosphorylation of MBP was not detected in *cds1Δ* cells (Fig. 7, lane 5). It is important to mention that the Pol α -primase complex is extremely unstable in *spp2-8* cells, whereas the complex is only mildly compromised in *spp2-9* and *polats13* cells (Fig. 3). Nonetheless, the Cds1 kinase was activated to a similar extent when all three mutants were arrested in early S phase at the restrictive temperature. These results suggest that early S phase arrest by these initiation mutants, regardless of the extent of their Pol α -primase complex instability, generates a signal to moderately activate Cds1 kinase.

Cds1 kinase is highly activated when S phase is arrested by hydroxyurea (19). We therefore examined the hydroxyurea-induced Cds1 response in *spp2* and *polα* mutants. Mid-log-phase cells grown at 25°C were shifted to 36°C for 1 h and then incubated for an additional 3.5 h at 36°C in 12 mM hydroxyurea. As expected, Cds1 kinase was activated >20-fold by hydroxyurea in wild-type cells (Fig. 7, compare lane 6 to lane 1). The hydroxyurea-induced Cds1 kinase activity in *spp2-9* cells was about 65 to 70% of the wild-type level (Fig. 7, lane 8),

whereas the hydroxyurea-induced Cds1 kinase activity in *spp2-8* cells was only about 25% of the wild-type level (Fig. 7, lane 7). Therefore, although the Cds1 kinase can be activated to a similar extent in *spp2-8* and *spp2-9* mutants when they are arrested by temperature, there is a striking difference in their hydroxyurea-induced Cds1 kinase activation at 36°C. Thus, at the restrictive temperature, the Cds1 response to signals generated from S phase arrest induced by an initiation mutant is different from that induced by hydroxyurea arrest.

As shown in this study and elsewhere (2), at 36°C *polats13* cells exhibit heterogeneous cell morphology with >40% of the cells displaying abnormal nuclear phenotype; however, the Pol α -primase complex in *polats13* was only slightly compromised (Fig. 3B). We therefore tested the hydroxyurea-induced Cds1 response in the *polats13* mutant. Surprisingly, the Cds1 kinase was activated to approximately 75% of the wild-type level (Fig. 7, lane 9), which was comparable to the levels of the checkpoint-proficient mutant *spp2-9*. Thus, the abnormal mitotic phenotype of *polats13* is due to a checkpoint defect that can be distinguished from the hydroxyurea-induced Cds1-mediated checkpoint pathway.

A



B

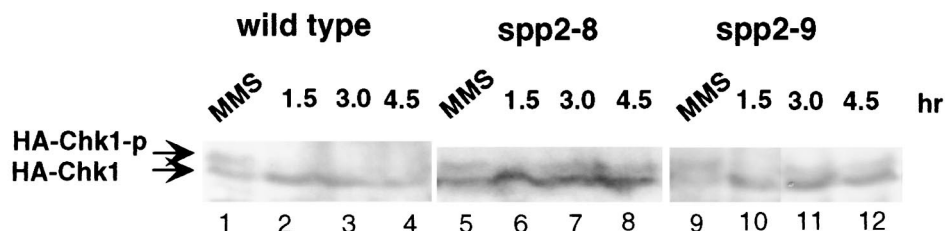


FIG. 6. Chk1 response to *spp2* mutation at 36°C. (A) DAPI staining of *chk1Δ*, *spp2-8 chk1Δ*, and *spp2-9 chk1Δ* cells after incubation at 36°C for 6 h. (B) Chk1 phosphorylation induced by mutation of *spp2* at 36°C. Mid-log-phase cells grown at 25°C were shifted to 36°C. At the indicated times, cells were collected and lysed. A portion (30 μg) of cell lysates was fractionated on an SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and probed with mouse anti-HA monoclonal antibody (12CA5) as described previously (2). As a control, each strain was treated with 0.2% MMS at 25°C for 1 h. The HA-tagged *chk1*⁺ strains used for wild-type, *spp2-8*, and *spp2-9* cells were NW222, ST128, and ST129, respectively. The phosphorylated and unphosphorylated HA-Chk1 proteins are indicated as HA-Chk1-p and HA-Chk1, respectively.

Maintenance of the hydroxyurea-induced Cds1 kinase in *spp2* and *polα* mutants. We then tested the ability of the mutants to maintain the hydroxyurea-induced Cds1 kinase activation. Wild-type and mutant cells were first incubated in

hydroxyurea at 25°C for 3.5 h to activate the Cds1 kinase. Cells were then shifted to 36°C with fresh hydroxyurea added and further incubated at 36°C. At the indicated times, cell samples were taken for analysis of the Cds1 kinase activity (Fig. 8A),

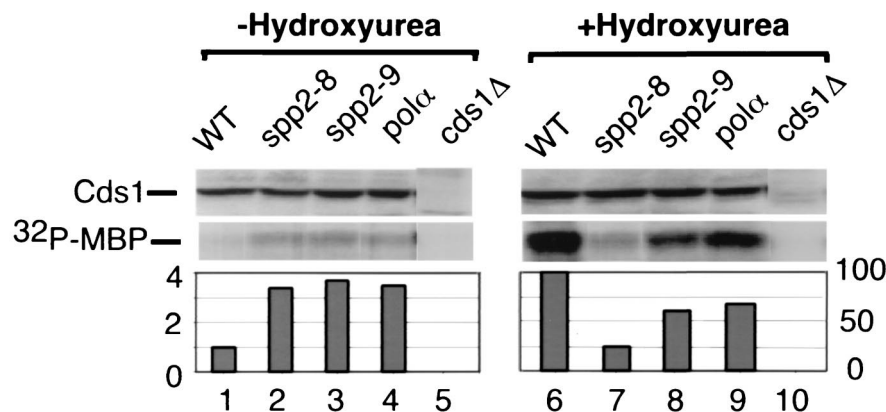


FIG. 7. Cds1 response to *spp2* mutations. Cds1 kinase activity of wild-type 972h⁻, ST118 (*spp2-8*), and ST119 (*spp2-9*) in the absence or presence of 12 mM hydroxyurea at 36°C. Mid-log-phase cells grown at 25°C were shift to 36°C for 1 h, and incubation continued at 36°C for 3.5 h in the absence (-Hydroxyurea) or presence (+Hydroxyurea) of 12 mM hydroxyurea. Cds1 kinase was purified from cell extracts and assayed for kinase activity as described in Materials and Methods. The relative amounts of Cds1 proteins used in the kinase assay were estimated by Western blot (upper panel, Cds1). The kinase activity was measured by phosphorylation of MBP (lower panel, ³²P-MBP) and quantified by PhosphorImager analysis.

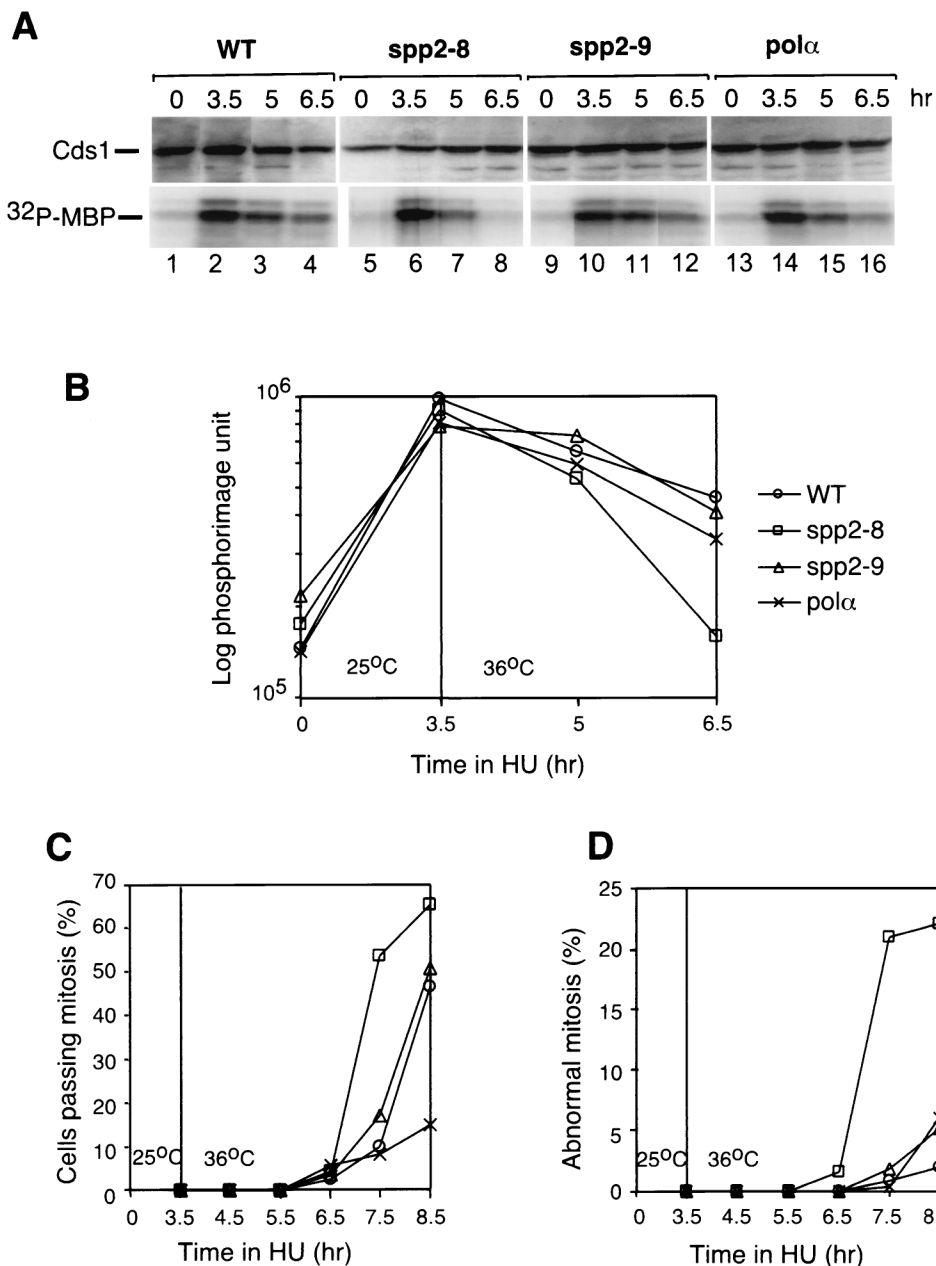


FIG. 8. Maintenance of hydroxyurea-induced Cds1 kinase activity. Cds1 activation was induced in wild-type and mutant cells by 12 mM hydroxyurea at 25°C for 3.5 h. The cells were then shifted to 36°C with 12 mM hydroxyurea added. At the indicated times, samples were removed and analyzed for Cds1 kinase activity (A and B), the percentage of cells passing mitosis (C), and the percentage of cells entering into aberrant mitosis described as the cut phenotype (D). The relative amounts of Cds1 proteins used in the kinase assay were estimated by Western blot (A, upper panel, Cds1). The kinase activity was measured by phosphorylation of MBP (A, lower panel, ³²P-MBP) and was quantified by PhosphorImager analysis (B).

the percentage of cells passing mitosis (Fig. 8B), and the percentage of cells entering inappropriate mitosis (Fig. 8C). After incubation of the cells for 3.5 h in hydroxyurea at 25°C, Cds1 kinase was activated in *spp2-8*, *spp2-9*, and *polats13* cells to 80 to 90% of the wild-type cells levels (Fig. 8A, 3.5 h, compare lanes 6, 10, and 14 to lane 2; quantified in the lower graph). After 1.5 h at 36°C (Fig. 8A, 5-h time point), the Cds1 kinase activity in wild-type cells decreased to approximately 75 to 80% of the original level (Fig. 8A, 5-h time point, lane 3 [quantified in the lower graph]). After 3 h at 36°C (Fig. 8A, 6.5-h time point), the hydroxyurea-induced Cds1 kinase activity established at the permissive temperature in wild-type cells de-

creased to approximately 60% of the original level (Fig. 8A, 6.5-h time point, lane 4 [quantified in the lower graph]). The level of Cds1 kinase activity in *spp2-8* after 1.5 h at 36°C decreased to 50% of its original levels that had been established at 25°C (Fig. 8A, 5-h time point, compare lane 7 to lane 6 [quantified in the lower graph]), whereas the Cds1 kinase activity in *spp2-9* was maintained at its original level established at 25°C (Fig. 8A, compare lanes 10 and 11). The Cds1 kinase activity in *polats13* after 1.5 h at 36°C decreased to approximately 75% of the levels established at 25°C (Fig. 8A, compare lanes 14 and 15 [quantified in the lower graph]). Interestingly, after 3 h at 36°C (Fig. 8A, 6.5-h time points), the

Cds1 kinase activity in *spp2-8* was maintained at 15% of its original levels established at 25°C, while both *spp2-9* and *polats13* decreased to approximately 40% of the levels established at 25°C (Fig. 8A, lanes 8, 12, and 16 [lower quantification graph]).

The observed hydroxyurea-induced Cds1 response in these mutants was also consistent with their kinetics of passing mitosis (Fig. 8B) and the appearance of abnormal mitotic phenotype (Fig. 8C). At 4 h after the shift to 36°C (7.5-h time point of Fig. 8B and C), >50% of the *spp2-8* cells passed mitosis and >20% of the *spp2-8* cells displayed an abnormal mitotic phenotype. At the same time point, <20% of the wild-type, *spp2-9*, and *polats13* cells passed mitosis and ~2% of the cells exhibited an abnormal mitotic phenotype. Interestingly, after the shift to 36°C for 5 h (8.5-h time point of Fig. 8B and C), <15% of the *polats13* cells entered mitosis, while >45% of the wild-type and *spp2-9* cells entered mitosis, suggesting that *polats13* and hydroxyurea arrest the cell cycle at close proximity.

Together, these results indicated that both the induction and the maintenance of the hydroxyurea-induced Cds1 response were severely compromised in *spp2-8* cells and moderately compromised in *spp2-9* and *polats13* cells. It is important to note that *spp2-8* which has the most unstable Pol α -primase complex, also has the lowest ability to induce and maintain the hydroxyurea-induced Cds1 response.

DISCUSSION

The goal of this study was to determine the checkpoint response(s) to aberrant initiation of S phase. Here, we analyzed the mutational effects of the DNA primase gene *spp2*⁺ encoding the primase subunit, Spp2, on the cell cycle checkpoint response and compared to that of *pol α* mutation. Four main points emerged from our studies. First, Spp2 is required for coupling RNA primer synthesis by Spp1 to initiation DNA synthesis by Pol α , suggesting that Spp2 is the bridge protein between Spp1 and Pol α . Second, mutations of *spp2*⁺ destabilize the mutant's Pol α -primase complex. The *spp2* mutant that has a severely compromised Pol α -primase complex also exhibits the aberrant mitotic phenotype at the restrictive temperature, suggesting a correlation between the stability of the complex and the aberrant mitotic phenotype. Third, the *spp2* mutant that has the most severely compromised Pol α -primase complex also has the lowest ability to activate and maintain the hydroxyurea-induced Cds1 response; however, this mutant is able to activate the Cds1 response induced by its temperature arrest. Fourth, a checkpoint defective *polats* mutant has an intact Cds1 response in either the presence or the absence of hydroxyurea and a similar Pol α -primase complex stability as a checkpoint intact *spp2* mutant.

Based on these findings, we propose a model of how different types of perturbation of S phase initiation could induce different checkpoint responses: an unstable initiation complex or a stalled initiation generates a signal to moderately activate the Cds1 kinase to stabilize and/or recover the initiation complex to prevent replication fork collapse and accumulation of mutations (Fig. 9A). Following an early S phase stall caused by hydroxyurea, a stable and intact Pol α -primase complex is required to signal high levels of Cds1 kinase activation to prevent progression of the early replication fork and premature initiation of the late-firing replicons (Fig. 9B). Once DNA replication is initiated, the synthesis of an initiation DNA structure by Pol α is required for generating the S phase checkpoint to prevent inappropriate mitotic entry (Fig. 9C).

A moderately activated Cds1 kinase is required to maintain an unstable Pol α -primase complex. Cds1 was originally iden-

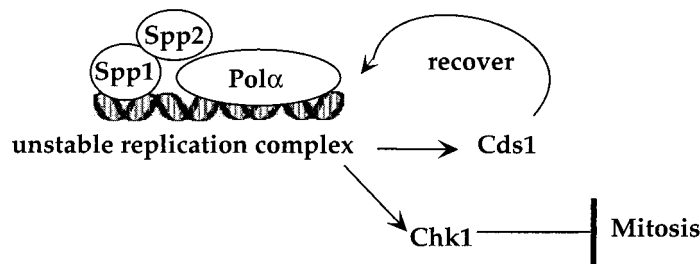
tified as a high-copy suppressor of a temperature-sensitive *pol α* mutant, *swi7-H4* (26). The Cds1 structural counterpart in budding yeast, *RAD53*, has been reported to modulate phosphorylation of the B subunit of Pol α -primase complex (29). We showed in this study that mutations of *spp2* destabilize the Pol α -primase complex (Fig. 3) and that Cds1 kinase is moderately activated in the *spp2* and *pol α* mutants at the restrictive temperature (Fig. 7). We have previously shown that deletion of Cds1 decreases the semipermissive temperature of several thermosensitive mutant alleles of *pol α* and exacerbates the mutator phenotypes of these mutants (20). Thus, mutations of either *spp2* or *pol α* require the function of Cds1 kinase at both the semipermissive and the restrictive temperatures. It is not yet clear whether Cds1 directly or indirectly interacts with the Pol α -primase complex and what the substrates of Cds1 kinase are. The results of this study and in combination with our previous studies (20) suggest that an unstable or stalled Pol α -primase complex of *pol α* or *spp2* mutants requires a moderate levels of Cds1 kinase activation to stabilize and/or to prevent the formation of unrecoverable replication complex, replication fork collapse, and/or the accumulation of mutations (Fig. 9A).

It is important to mention that both *spp2-8* and *polats13* display a mixed phenotype. The elongated cells seen in the *spp2* population (Fig. 1A) and *polats13* mutants (Fig. 3B and reference 2) can be attributed to the Chk1 response to prevent aberrant mitotic entry of these initiation mutants (Fig. 6A). Chk1 is not significantly phosphorylated during early-S-phase arrest by *spp2* mutants (Fig. 6B). It is possible that the unstable initiation complex in *spp2-8* and/or the stalled initiation complex in *polats13* are not sufficient to activate Chk1 to a level that can fully prevent inappropriate mitotic entry, thus resulting in a mixed phenotype.

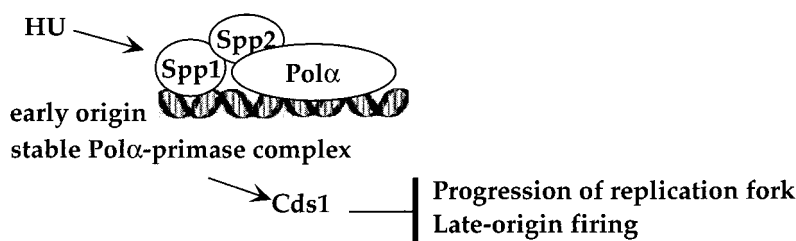
A stable Pol α -primase complex is required for activation of the Cds1-mediated intra-S phase checkpoint. We propose that following an early S phase stall caused by hydroxyurea, a stable and intact Pol α -primase complex is required to signal the activation of high levels of Cds1 kinase to prevent the premature initiation of the late-firing replicons. In budding yeast, it has been shown that, following hydroxyurea-induced arrest, the initiation complexes of the late-firing replicons remain in an initiation-competent state for a long period of time (12, 33). In fission yeast, after hydroxyurea-induced arrest, the Cds1-mediated checkpoint response is also thought to prevent the initiation of late-firing replicons, and/or stabilize existing replicons (19). This process is termed intra-S phase checkpoint.

We found that at the restrictive temperature, the hydroxyurea-induced Cds1 kinase of *spp2-8* was only activated at 25% of the wild-type cell's level (Fig. 7). After 3 h at 36°C, *spp2-8* was unable to maintain the hydroxyurea-induced Cds1 kinase established at 25°C (Fig. 8A). As shown in Fig. 8B and in our previous mutational studies of *pol α* (2), hydroxyurea arrests the S phase at point close to the arrest point of *polats* mutant, causing a stalled replication. It is not yet clear at the molecular level how Cds1 kinase inhibits the progression of the replication fork of the early-firing replicons and prevents initiation of the late-firing replicons following hydroxyurea-induced arrest. It is possible that a stable Pol α -primase complex in the replication complex is required to generate the signal for high levels of Cds1 kinase activation to prevent progression of the early-firing replication fork and initiation of late-firing replicons. The rationale for this hypothesis is our finding that *spp2-8* with a severely compromised Pol α -primase complex also has the lowest ability to activate and maintain the hydroxyurea-induced Cds1 kinase (Fig. 3, 7, and 8). The inability of *spp2-8* to activate and to maintain the hydroxyurea-induced Cds1 ki-

A. Cds1 response induced by initiation mutants



B. Cds1 response induced by hydroxyurea (HU)



C. Requirements for the S-phase checkpoint response

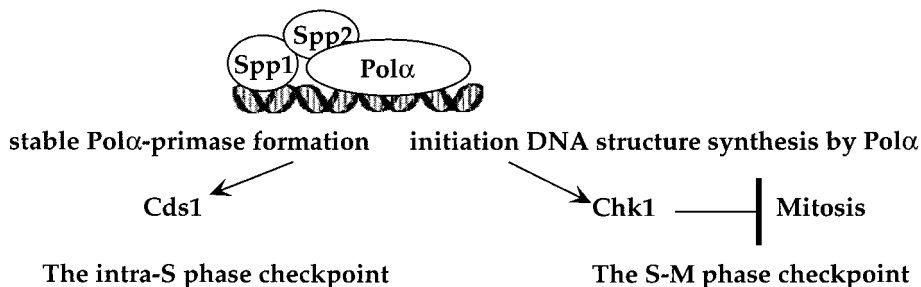


FIG. 9. Model illustrating the proposed Cds1 responses to the aberrant initiation of S phase. The proposed Cds1 response to unstable replication complex (A), a stalled replication fork (B), and the requirement for cells to generate a signal (C) for the intra-S phase checkpoint and the S-M phase checkpoint as described in the Discussion.

nase activity may cause the cell to be unable to prevent the progression of early-firing replication fork and/or premature firing of the late replicons. Thus, *spp2-8* has a higher percentage of cells entering abnormal mitosis than does *spp2-9* (Fig. 8B and C). *spp2-9* and *polats13* both have mildly compromised Polα-primase complex. The mildly compromised complex might be sufficient to generate a signal to activate sufficient levels of Cds1 to prevent the firing of late replicons. The activated Cds1 kinase in *spp2-9* and *polats13*, however, is insufficient to fully prevent inappropriate mitotic entry as the wild-type cells after 3.5 h at 36°C (Fig. 8A). Thus, *spp2-9* and *polats13*, despite having the wild-type-like percentage of cells passing mitosis and mitotic phenotype after 5 h in 36°C (Fig. 8B and C, 8.5-h time point), these mutants eventually die. These results suggest that, upon hydroxyurea-induced arrest, a functional Spp2 coupling Spp1 and Polα to establish a stable Polα-primase complex is required to generate a signal for high levels of Cds1 activation for the intra-S phase checkpoint (Fig. 9B).

As shown in Fig. 7, in all of the mutants, the levels of Cds1 kinase activity induced by hydroxyurea are much higher than the levels induced by *spp2* or *polats* mutant arrest (Fig. 7). It is not clear whether, following hydroxyurea-induced arrest or early S-phase arrest by *spp2* and *polats*, Cds1 proteins are being phosphorylated at the same or different sites, thus being activated at different levels. However, our results suggest that mutations of *spp2*⁺ cause an unstable initiation complex, resulting in an aberrant initiation that induces a moderate level of Cds1 response to recover from the perturbation. Upon hydroxyurea inhibition, a stable Polα-primase complex is required to induce higher levels of Cds1 kinase for the intra-S phase checkpoint response. Thus, Cds1 response(s) to the early-S-phase arrest signals induced by a replication initiation mutant is different from that induced by hydroxyurea.

The requirement for the S-M phase checkpoint can be distinguished from the requirement for the intra-S phase checkpoint. We showed in this study that at the restrictive temper-

ature, *polats* mutant exhibits an aberrant mitotic phenotype but has an intact but mildly compromised Pol α -primase complex (Fig. 3B). Importantly, the checkpoint-defective *polats13* and the checkpoint-intact *spp2-9* at 36°C have similar Pol α -primase complex stabilities and similar abilities to activate and maintain the hydroxyurea-induced Cds1 kinase. These suggest that the S-M phase checkpoint defect of *polats* mutant most likely is not due to an unstable Pol α -primase complex or to inability to activate and maintain Cds1 kinase activity. This also suggests that Cds1 activation does not play a major role in preventing the inappropriate mitotic entry of *polats13*. We have previously shown that the catalytic activity of Pol α to synthesize an initiation DNA structure is required for generating the replication checkpoint to prevent inappropriate mitosis during S phase (2). Thus, we propose that, upon S phase initiation, the requirement for generating the replication checkpoint to prevent inappropriate mitotic entry is the initiation DNA synthesis of Pol α (Fig. 9C) and that this requirement is distinguished from the requirements for generating the hydroxyurea-induced Cds1-mediated intra-S phase checkpoint response.

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