The Sld5 and Psf1 proteins required for chromosomal DNA replication in *Saccharomyces cerevisiae*

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和訳（出芽酵母染色体 DNA 複製に必要な Sld5 および Psf1 タンパク質）

要旨

In *Saccharomyces cerevisiae*, chromosomal DNA replication initiates at a restricted region known as the autonomously replicating sequence (ARS). The origin recognition complex binds ARS throughout the cell cycle and the minichromosome maintenance proteins are recruited by Cdc6 from late M phase to G_1 phase to form the pre-replicative complex (pre-RC). Then, the Sld3-Cdc45 complex joins the pre-RC and ARS region is unwound in cooperation with Cdk and Cdc7/Dbf4 protein kinases. Finally, DNA polymerases are recruited to ARS and this step requires Dpb11, which forms a complex with DNA polymerase ε (Pol ε). To gain an insight into the function of Dpb11, Kamimura and coworkers isolated *sld1~6* (synthetic lethality with *dpb11-1*) mutations and cloned corresponding genes. Sld1 is identical to Dpb3, the third largest subunit of Pol ε, Sld4 is Cdc45, Sld6 is Rad53, important protein kinase in the cell cycle checkpoints, Sld2 together with Dpb11 forms a complex essential for DNA replication, and Sld3 works together with Cdc45. Although *SLD5* was already cloned, its function has not been elucidated.

In this article, I describe characterization of the*SLD5* and *PSF1*(Partner of *SLD* five) genes. The *PSF1* gene is a multicopy suppressor of four thermosensitive *sld5* mutations (*sld5-2, -8, -12* and *–13*) that are newly isolated in this study since original *sld5-1* mutation obtained by the synthetic lethal screening does not show any phenotype. The *SLD5* and *PSF1* genes are essential for cell growth and encode 34-kDa and 24-kDa proteins which

amino acid sequences are well conserved in eukaryotic cells. At the restrictive temperature, all the thermosensitive *sld5* mutant cells as well as the thermosensitive *psf1-1* mutant cells isolated also in this study arrest with a dumbbell-shape with a single nucleus with a DNA content between 1C and 2C. This is typical terminal morphology for mutants defective in DNA replication. This observation thus suggests that both the *SLD5* and *PSF1* genes are required for DNA replication.

After temperature shift up, all the thermosensitive *sld5* mutant cells divide more than once whereas *psf1-1* cells stop division in a single cell cycle. At the restrictive temperature, *psf1-1* cells released from G_1 arrest neither divide nor increase DNA content in FACS analysis while the cells released from G_2/M arrest go through mitosis and arrest with 1C DNA content in the next cell cycle. This finding suggests that Psf1 participates in the initiation step of DNA replication but not in the mitosis. Moreover, *psf1-1* cells released from S phase arrest by hydroxyurea (HU) reach to 2C DNA content later than wild type cells and then divide at the restrictive temperature. Since HU blocks late-origin firing, it is likely that Psf1 is essential for all origin-firing during S phase progression and consequently chromosome DNA replicates only from early-firing origins at the restrictive temperature in *psf1-1* cells released from HU.

The protein-level of Psf1 is roughly constant during the cell cycle although the transcript-level of *PSF1* is reported to fluctuate during the cell cycle and to peak at G_1/S phase boundary. Moreover, the Psf1 protein is localized in nucleus throughout the cell cycle, suggesting that Psf1 plays a role in the place close to chromatin DNA. As expected, in chromatin immunoprecipitation (CHIP) assay, the Psf1 and Sld5 proteins associate simultaneously with ARS1 region in S phase. It strongly suggests that both proteins

function directly for DNA replication. Furthermore, the Psf1 association with a late-firing origin is significantly delayed behind the association with early-firing origins, suggesting that Psf1 associates with replication origins immediately before firing. Besides the ARS association, in the CHIP assay, Psf1 coimmunoprecipitates with Rfa, single-stranded DNA binding protein when Psf1 associates with chromosome DNA. Because single-stranded DNA appears in unwound origins and at replication forks during DNA replication, this observation is consistent with the ARS association of Psf1.

High-copy *SLD5* and *PSF1* suppress thermosensitive growth of *psf1-1* and *sld5-12* and the *psf1-1* mutation is synthetically lethal with *sld5-12*, suggesting an interaction between Sld5 and Psf1. As suggested, Sld5 and Psf1 coimmunoprecipitate from the extracts prepared from G_1, S and G_2/M phase cells. Thus, Sld5 and Psf1 coexist in the same complex during the cell cycle. In *psf1-1* cells, however, this complex is hardly detected. Since Sld5 and Psf1 associate simultaneously with ARS1 and *psf1-1* cells are defective in DNA replication, it seems likely that the complex containing Sld5 and Psf1 associates with ARS and is required for chromosomal DNA replication.

In two-hybrid assay, Psf1 interacts with Dpb11, Dpb2 and Sld3. The Sld3-Cdc45 complex associates with ARS and this association is required for unwinding of ARS region. Dpb2 is a second largest subunit of Pol ε that forms a complex with Dpb11 and associates with ARS after unwinding of ARS. Thus, Psf1 interacts with the proteins that associate with ARS before and after its unwinding. I therefore propose that the complex containing Sld5 and Psf1 mediates between Sld3-Cdc45 and Dpb11-Pol ε complexes for recruitment of DNA polymerases to ARS.

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ABSTRACT

In *Saccharomyces cerevisiae*, Dpb11 forms a complex with DNA polymerase ε and is required for origin associations of DNA polymerases α and ε at the initiation step of DNA replication. In this article, I describe novel replication proteins, Sld5 and Psf1, which interact with Dpb11. Although Dpb11 is diverged in eukaryotic organisms, Psf1 and Sld5 are highly conserved from yeast to human cells. The Sld5 and Psf1 proteins are involved in the same complex throughout the cell cycle and this complex formation is required for chromosomal DNA replication. In chromatin immunoprecipitation assay, Sld5 and Psf1 associate with early-firing origins in early S phase and with late-firing origins in late S phase. In two hybrid assay, Psf1 interacts with an initiation protein, Sld3, and a subunit of DNA polymerase ε, Dpb2 as well as Dpb11. Therefore, it seems likely that the complex containing Sld5 and Psf1 mediates between initiation proteins and DNA polymerases to initiate DNA replication.

ABBREVIATIONS

C. elegans	*Caenorhabditis elegans*
D. melanogaster	*Drosophila melanogaster*
E. coli	*Escherichia coli*
H. sapiens	*Homo sapiens*
M. musculus	*Mus musculus*
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
X. laevis	*Xenopus laevis*
ARS	autonomously replicating sequence
bp	base pair
BRCT	Brca1 C-terminal
BSA	bovine serum albumin
C	carboxyl
CHIP	chromatin immunoprecipitation
DAPI	4', 6-diamino-2-phenylindole
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
dNTPs	deoxynucleoside triphosphates
5-FOA	5-fluoroorotic acid
HEPES	N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid
HU	hydroxyurea
IgG	immunoglobulin G
IPTG	isopropyl-β-D-thiogalactopyranoside

kb	kilo base pair
MCB	*MluI* cell cycle box
MMS	methyl methane sulfonate
MOPS	(3-N-morpholino)propanesulfonic acid
N	amino
Orc	origin recognition complex
ORF	open reading frame
PBS	phosphate bufferd saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
Pol	DNA polymerase
pre-RC	pre-replicative complex
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SV40	simian virus 40
TCA	trichloroacetic acid
UV	ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl-β-galactoside

INTRODUCTION

Eukaryotic chromosome DNA is replicated once in the S phase per cell cycle. This is mainly regulated in the initiation step of DNA replication. In *Saccharomyces cerevisiae*, chromosomal DNA replication initiates at a restricted region, the autonomously replicating sequence (ARS) (reviewed in Campbell and Newlon, 1991). ARS consists of A and two or three B elements. The A element is a well conserved 11 bp ARS consensus sequence [(A/T)TTTA(T/C)(A/G)TTT(A/T)(T/C/G)]. An origin recognition complex (Orc), comprising six subunits, binds to the ARS throughout the cell cycle (Aparicio, et al., 1997, Tanaka, et al., 1997). The Cdc6 and Mcm proteins are loaded to the ARS from M phase to G_1 phase and form the pre-replicative complex (pre-RC) with Orc. At the onset of S phase, Cdk and Cdc7 protein kinases facilitate unwinding of the ARS, followed by binding of the single-strand DNA binding protein, Rfa, to unwound ARS (Tanaka et al., 1998). Then, the three DNA polymerases, Pol α, Pol δ and Pol ε, essential for chromosomal DNA replication, are recruited to the ARS region to initiate DNA synthesis. Cdc45, which interacts with Mcm proteins, associates with the ARS region during the G_1 and S phases of the cell cycle and association of Pol α and Pol ε with the ARS is dependent on Cdc45 (Aparicio et al., 1999).

The replication proteins described above are well conserved from yeast to human cells. The Cdc45 protein of *Xenopus laevis* is also required for Pol α association with chromatin DNA (Mimura and Takisawa, 1998). Moreover,

Cdc45 and Pol α form a complex in *Xenopus* egg extracts. In *in vitro* simian virus 40 (SV40) DNA replication, T antigen unwinds the replication origin and Rfa binds the unwound single-stranded DNA. DNA primase, tightly associated with Pol α, then synthesizes an RNA primer. This RNA primer is used by Pol α to synthesize a short DNA strand, followed by elongation of the DNA strand by Pol δ and/or Pol ε (Stillman, 1994). Although the cellular counterpart of the SV40 T antigen has not been identified, it is believed that similar reactions take place during chromosomal DNA replication.

The *DPB11* gene was isolated as a multicopy suppressor of mutations in *POL2* and *DPB2*, which encode catalytic and second largest subunit of Pol ε, respectively (Araki et al., 1995). The Dpb11 protein has four copies of BRCT (Brca1 C-terminal) domain, which is important for protein-protein interaction (Bork et al., 1997, Callebaut and Mornon, 1997, Zhang et al., 1998). Dpb11 is required for DNA replication and the S phase checkpoint. During the S phase of the cell cycle, Dpb11 and Pol ε form a complex and associate with ARS. Associations of Dpb11 and Pol ε with ARS are mutually dependent and are required for the ARS association of the Pol α-primase complex (Masumoto et al., 2000).

To elucidate the Dpb11 function further, *sld* 1-6 (synthetic lethality with *dpb11-1*) mutations were isolated (Kamimura et al., 1998). *SLD1* is identical to *DPB3* encoding the third largest subunit of Pol ε (Araki et al., 1991b), *SLD4* is identical to *CDC45* required for the initiation and elongation of chromosomal DNA replication (Hopwood et al., 1996, Tercero et al., 2000, Zou et al., 1997) and

SLD6 is identical to *RAD53* required for cell cycle checkpoints (Dohrmann et al., 1999). The products of *DPB11* and *SLD2* form a complex that is involved in either the initiation or a step close to the initiation of DNA replication. *SLD2* is independently isolated as *DRC1* (DNA replication and checkpoint protein 1) (Wang and Elledge, 1999). The products of *SLD3* and *CDC45* also form a complex throughout the cell cycle. The Sld3-Cdc45 complex associates with ARSs in temporally controlled manner: it associates with early ARSs in G_1 phase and with late ARSs in late S phase. The ARS association of Sld3-Cdc45 complex is prerequisite for the ARS association of Rfa, indicative of unwinding of ARSs (Kamimura et al., in press).

So far, the Sld3-Cdc45 complex is the last component to associate with ARS before Rfa association while the Dpb11-Pol ε complex is the first component to associate with ARS after Rfa association. Although Pol ε association with ARS is dependent on Cdc45, how the Dpb11-Pol ε recognizes ARS in S phase has not been elucidated. In this article, I describe novel replication proteins, Sld5 and Psf1, which interact with both Sld3 and Dpb11. Throughout the cell cycle, the Sld5 and Psf1 proteins are involved in the same complex that is essential for DNA replication. Furthermore, both Sld5 and Psf1 associate with ARS in S phase at the same timing. Therefore, it is suggested that the complex containing Sld5 and Psf1 mediates between the Sld3-Cdc45 and Dpb11-Pol ε complexes for proper ARS association of DNA polymerases in the initiation of DNA replication.

RESULTS

The *SLD5* gene is required for DNA replication

The *SLD5* gene was cloned by Kamimura et al. (1998). DNA sequencing of the cloned gene revealed that the *SLD5* gene corresponds to YDR489w ORF (Saccharomyces Genome Database) and encodes 34-kDa protein. The amino acid sequence of Sld5 is well conserved among eukaryotic organisms (Fig. 1A).

To know whether the *SLD5* gene is essential for cell growth, one copy of *SLD5* was disrupted in a diploid strain by replacement with the *LEU2* gene. The resultant strain was sporulated and dissected. Seven of 10 tetrads examined showed two viable and two lethal spores. All viable spore clones were Leu^-, indicating that the *SLD5* gene is essential for cell growth. Microscopic observation of inviable spore clones revealed that spores germinated and formed swelling sphere cells with small buds or split sphere, some of which were lysed.

Since original *sld5-1* mutation itself is silent in any phenotype (Kamimura et al., 1998), four thermosensitive alleles of *SLD5* were isolated by plasmid shuffling method (see Materials and Methods) and their mutation sites were determined (Fig. 1A asterisks). These four mutants did not show increased sensitivity to MMS, UV and HU at permissive temperature. At the restrictive temperature, all the mutant cells arrested with a dumbbell-shape with a single nucleus with a DNA content between 1C and 2C and lost their viability (one example is shown in Fig. 4A). This result suggests that Sld5 is required for DNA replication. However, all of them divided more than once after temperature shift up. Thus, I had not characterized these mutants in detail.

Isolation of the *PSF1* gene essential for cell growth

To identify factors interacting with Sld5, a multicopy suppressor of the *sld5-12* mutation, one of four thermosensitive mutations described above, was isolated. *Sld5-12* cells grew at 25 °C but not at 30 °C. *Sld5-12* cells were transformed with a *S. cerevisiae* genomic library based on YEp24, incubated at 23 °C for a day on SD-Ura plates and incubated at 30 °C for further 2 days. From ≈20,000 Ura^+ transformants, 15 transformants grew at 30 °C. The plasmid DNA was recovered from each of them, and the DNA sequences were determined. Twelve clones were found to carry *SLD5* and the remaining 3 clones were found to carry a novel gene on chromosome IV (YDR013w) (Dardalhon et al., 2000). This novel gene was named *PSF1* (Partner of *SLD five*).

The *PSF1* gene encodes a 24-kDa protein. The deduced amino acid sequence of *PSF1* is well conserved in *S. pombe*, *C. elegans*, *X. laevis*, *D. melanogaster* and human (Fig. 1B). To know whether *PSF1* is essential gene, one copy of the *PSF1* gene was disrupted in a diploid strain by replacement with the *LEU2* gene, the resultant strain was sporulated and dissected. Fifteen of 19 tetrads examined showed two viable and two lethal spores (Fig. 2). All viable spore clones were Leu^-, indicating that the *PSF1* gene is essential for cell growth. Microscopic observation of inviable spore clones revealed that spores germinated and formed swelling sphere cells with small buds, split sphere or lyzed spheres as observed in the disruptants of *SLD5*.

Psf1 is required for DNA replication

To understand the function of *PSF1*, a thermosensitive mutation, *psf1-1*, was isolated by plasmid shuffling method (see Material and Methods). The *psf1-1* mutation site was identified at position 250 in the nucleotide sequence (nucleotide 1 is A in the first ATG of the ORF) with from A to G transition, which changes the amino acid of the protein from arginine to glycine (Fig. 1B asterisk and 1C). This arginine is conserved in *S. pombe*, *C. elegans*, *X. laevis*, *D. melanogaster* and *H. sapiens* (Fig. 1B). At permissive temperature, *psf1-1* cells did not show increased sensitivity to MMS, UV and HU.

At the restrictive temperature, *psf1-1* cells arrested with a dumbbell shape with a single nucleus as observed in *sld5* thermosensitive mutants (Fig. 3). This is typical terminal morphology for mutants defective in DNA replication. To investigate whether DNA is synthesized at the restrictive temperature, *psf1-1* cells were arrested in G_1 phase with α-factor and released at 36 °C. Aliquots of cells were sampled at 20 min intervals and DNA contents were measured by FACS analysis. As shown in Fig. 4A, wild-type cells accumulated a 2C DNA content by 80 min while DNA content of *psf1-1* cells had not increased for 120 min and then gradually reached 2C. In mutant cells defective in initiation of DNA replication, DNA content does not increase for a while and then gradually increases later than wild-type cells as observed in *psf1-1* cells. On the other hand, *sld5-12* cells gradually reached a 2C DNA content, entered the second cell cycle, and arrested with a DNA content between 1C and 2C as described above.

Orc2-1 (Bell et al., 1993), *cdc45-1* (Zou et al., 1997), *dpb11-1* (Kamimura et al., 1998), *sld2-6* (Kamimura et al., 1998) and *sld3-5* (Kamimura et al., in press) cells defective in initiation of DNA replication begin to lose viability immediately

after cells start budding at the restrictive temperature. To determine the point at which the cells start losing viability, the viability and cell morphology of synchronized cells were examined. Both mutants started losing viability when cells started budding (Fig. 4B), like mutants defective in initiation of DNA replication.

To investigate whether Psf1 is involved in mitosis, *psf1-1* cells were arrested in M phase with nocodazole, incubated at 36 °C for 1 h to inactivate the Psf1-1 protein and released in fresh YPD medium at 36 °C. Aliquots of the cells were sampled and DNA content was measured by FACS analysis. DNA content of wild-type and *psf1-1* cells moved from 2C to 1C with the same kinetics (Fig. 4C). This result suggests that Psf1 is not involved in mitosis.

To examine the function of Psf1 in the other steps of DNA replication than the initiation step, I tested whether Psf1 executes any function after hydroxyurea (HU) block, because HU is an inhibitor of ribonucleotide reductase and causes replication forks to stall. Wild-type and *psf1-1* cells were arrested in G_1 phase with α-factor at 23 °C, released to HU, heat inactivated at 36 °C for 1 h and released from HU-arrest at 36 °C. Wild-type cells had reached a 2C DNA content by 60 min and entered subsequent G_1 phase at 90 min. In contrast, *psf1-1* cells were much slower, requiring 90 min to reach a 2C DNA content. At 120 min, *psf1-1* cells entered subsequent G_1 phase and accumulated a 1C DNA content (Fig. 4D). Thus, the completion of S phase in *psf1-1* cells is substantially slowed relative to wild-type control. Bulk DNA synthesis of the *cdc7* mutant cells shows similar kinetics (Bousset and Diffley, 1998). Because Cdc7 is required for all origin-firing and late origin-firing is blocked by HU (Santocanale and Diffley, 1998), chromosome DNA in *cdc7* cells after release from HU arrest is replicated

only from origins fired before HU-arrest. Thus, bulk DNA replication is slowed in *cdc7* cells (Fig. 4E). From analogy to *cdc7* cells, it is likely that Psf1 is essential for all origin-firing during S phase progression and chromosome DNA replicates only from early-firing origins in *psf1-1* cells released from HU arrest at the restrictive temperature.

The Psf1 protein exists throughout the cell cycle in nucleus

The *PSF1* gene has *MluI* cell cycle box (MCB) like sequence located in approximately 90 bp upstream from the first ATG. MCB sequence (ACGCGT) is identified as a regulatory element in genes whose transcript-level fluctuates during the cell cycle and peaked at the G_1/S phase boundary (Johnston and Lowndes, 1992). Northern blotting analysis revealed that the transcript-level of *PSF1* fluctuates during the cell cycle and peaks at G_1/S phase boundary (Cho et al., 1998).

To determine whether the protein level of Psf1 fluctuates as the transcript-level of *PSF1*, YYT69 cells, in which *6FLAG*-tagged *PSF1* replaced the *PSF1* gene by homologous recombination, were arrested in G_1 phase with α-factor and released in YPD at 23 °C. Aliquots of cells were sampled at 20 min intervals, cells were disrupted and the 6Flag-Psf1 protein was detected by western blotting using anti-FLAG antibody. The 6Flag-Psf1 protein existed at roughly constant level from 0 min to 240 min (Fig. 5), indicating that the protein-level of Psf1 does not fluctuate during the cell cycle.

Most of replication proteins are localized in nucleus throughout the cell cycle, while some of them shuttles between nucleus and cytoplasm: Cdc45 is localized in nucleus during the cell cycle (Hopwood and Dalton, 1996) while the

Mcm proteins, components of the pre-RC, are localized in nucleus from late mitosis to G_1 phase and are excluded from nucleus in S phase (Labib et al., 1999). To determine where the Psf1 protein is localized, indirect immunofluorescence microscopy was employed. Using an asynchronous cell population and anti-FLAG antibody, 6Flag-Psf1 was observed only in the nucleus, irrespective of cell morphology (Fig. 6). This observation indicates that the Psf1 protein is localized in nucleus throughout the cell cycle.

Psf1 and Sld5 associate with the ARS region in S phase

As Psf1 is a nuclear protein required for DNA replication, I examined whether the Psf1 protein associates with the ARS region *in vivo* using chromatin immunoprecipitation (CHIP) assay (Strahl-Bolsinger et al., 1997). I used PCR primers to amplify ARS1, ARS305, ARS501, and their neighboring regions (Fig. 7A). ARS1 and ARS305 fire early in S phase and ARS501 fires late in S phase (Ferguson et al., 1991).

Cells bearing *6FLAG-PSF1* or *MCM4-FLAG* were fixed with formaldehyde, disrupted, sonicated to shear chromatin DNA, and used for immunoprecipitation. Recovered DNA fraction is used for amplification of ARS1 and neighboring fragments. When I used anti-FLAG antibody, the ARS1 fragment was specifically amplified (Fig. 7B). However, any significant amplification of the ARS1 fragment was not observed when I used anti-HA antibody or cells without 6Flag-Psf1 and Mcm4-Flag. Thus, the CHIP assay is dependent on Flag-tag and Flag-specific antibody. This result also indicates that Psf1 associates with the ARS1 fragment.

Association signal of Psf1 with ARS1 is fainter than that of Mcm4 (Fig. 7B). This is probably because Psf1 associates with ARS1, transiently. Thus, I

examined ARS-association of Psf1 during the cell cycle. YYT69 cells (*6FLAG-PSF1*) were arrested in G_1 phase with α-factor and released at 16 °C to slow replication fork movement. Cells were withdrawn from the cultures every 15 min, fixed, disrupted and sonicated. The proteins and DNA were precipitated with anti-FLAG antibody and subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) and PCR reaction, respectively. 6Flag-Psf1 and Rfa1, which is a largest subunit of Rfa, were detected by western blotting using anti-FLAG and anti-Rfa antibodies. Psf1 was precipitated throughout the cell cycle and Rfa1 was precipitated from 75 min after release (Fig 7D), suggesting that Psf1 and Rfa are in close proximity in S phase. Since Rfa binds to single-stranded DNA, which appears in unwound origins and at replication forks, this finding suggests that Psf1 associates with unwound origins or replication forks. The CHIP assay revealed that Psf1 associates with early origins, ARS1 and ARS305 from 60 min after release from α-factor and 75 min with late origin, ARS501 (Fig. 7E). Psf1 also reassociated with non-ARS fragments in later period. These results are consistent with the function of Psf1 in DNA replication.

I also examined the association of Sld5 with ARS1. YYT16 cells, which has a disrupted *sld5* on chromosome and harbors YCp*FLAG-SLD5* plasmid, were synchronized and subjected to CHIP assay. As shown in Fig. 7F, Sld5 associated with ARS1 at the same timing as Psf1. This result, together with coexistence of Psf1 and Sld5 in the same complex (see later section), suggests that the complex containing Sld5 and Psf1 associates with replication origins.

Genetic interaction between *SLD5*, *PSF1* and *DPB11*

Sld5-1 was isolated as a synthetically lethal mutation with *dpb11-1*. I also examined synthetic lethality between *psf1-1*, *sld5-12* and *dpb11-1*. I crossed any combination of these mutants, resultant diploids were sporulated and dissected. Of 20 tetrads obtained from each diploid, no spore clones bearing two of these mutations were obtained. This result indicates that any pair of *psf1-1*, *sld5-12* and *dpb11-1* is lethal even at permissive temperature and suggests that Psf1 interacts with Dpb11 as well as Sld5.

A mutation occurring in one subunit of a complex is often suppressed by increased dosage of another subunit. Since high copy *PSF1* suppressed growth defect of *sld5-12* cells, we examined whether other related genes on high copy plasmid suppressed growth defect of *psf1-1* cells. *DPB11* and *SLD5* were transferred to a low- or a high-copy number plasmid (YCp or YEp plasmid respectively) and used for transformation of *psf1-1* cells. YEp*DPB11* and YEp*SLD5* restored growth of *psf1-1* cells at 33°C, whereas YCp*DPB11* and YCp*SLD5* did not (Fig. 8A). Thus, the suppression is dependent on the copy number of the genes. Similarly, the *PSF1* gene on plasmids suppressed the growth defect of *sld5-12* cells in dosage-dependent manner (Fig. 8B). I also found that *PSF1* on YEp plasmid restored the growth of *dpb11* mutant cells at the restrictive temperature (data not shown). However, when the *POL2, DPB2, SLD2, SLD3* and *SLD4* (*CDC45*) genes on YEp and YCp plasmids were introduced into *psf1-1* cells, the cells did not grow at the restrictive temperature (data not shown). These results suggest that Sld5 and Dpb11 interact specifically with Psf1.

Complex formation between Sld5 and Psf1

Genetic analyses strongly suggest that Psf1 interacts with Sld5. To determine whether Sld5 physically interacts with Psf1, co-immunoprecipitation assay was employed. Cell lysate was prepared from YYT17, which has a disrupted *psf1* on chromosome and harbors YCp*FLAG-PSF1-HA* plasmid, and the proteins were immunoprecipitated with anti-Sld5 or anti-Psf1 antibodies. The precipitates were separated by SDS-PAGE, followed by western blotting with anti-HA monoclonal antibody or anti-Sld5 antibodies. Because the Sld5 and Psf1 proteins were not precipitated with preimmune antibodies (Fig. 9A and B, lane 1), antibodies against Sld5 or Psf1 are specific to each protein. The Flag-Psf1-Ha protein was precipitated not only with anti-Psf1 antibodies but also with anti-Sld5 antibodies (Fig. 9A). Moreover, the Sld5 protein was precipitated with anti-Psf1 antibodies as well as anti-Sld5 antibodies (Fig. 9B). Thus, the Flag-Psf1-Ha and Sld5 protein co-immunoprecipitated with antibodies against one of these proteins, indicating that Psf1 and Sld5 are involved in the same complex. The complex containing Sld5 and Psf1 is hereafter described as the Sld5-Psf1 complex.

To know whether the Sld5-Psf1 complex formation is regulated during the cell cycle, cell lysates were prepared from YYT17 cells arrested in G_1, S and M phase with α-factor, HU and nocodazole, respectively. The proteins from these arrested cell lysates were immunoprecipitated with antibodies against Sld5 or Psf1. In G_1, S and M phase, Flag-Psf1-Ha was precipitated by anti-Sld5 antibodies (Fig. 9C lane3). The Sld5 protein was also precipitated with the Flag-Psf1-Ha protein by anti-Psf1 antibodies (data not shown). These results indicate that the Sld5-Psf1 complex exists throughout the cell cycle.

The *psf1-1* mutation is suppressed by high copy *SLD5* (Fig. 8A). It suggests that the Sld5-Psf1 complex formation is impaired by the *psf1-1* mutation. I thus examined whether the Sld5 and Psf1-1 proteins co-immunoprecipitate with anti-Psf1 antibodies. For immunoprecipitation assay, the wild type and mutant proteins should be precipitated by the antibodies with equal efficiency. In previous assay, I used Flag-Psf1-HA because the Psf1 protein co-migrated with light chain of rabbit IgG in SDS-PAGE. However, the tagged version of *psf1-1* could not be substituted for chromosome *PSF1* gene presumably because it is inactive. Therefore, to know the immunoprecipitation efficiency of the mutant protein, I produced the His6-tagged Psf1 and His6-tagged *Psf1-1* proteins in *Escherichia coli* and immunoprecipitated these proteins with anti-Psf1 antibodies from *E. coli* cell extract. As shown in Fig. 10A, His6-tagged Psf1-1 was precipitated with anti-Psf1 antibodies as efficient as the His6-tagged Psf1 protein. Thus, I performed immunoprecipitation assay using yeast cell lysate.

Cell lysates were prepared from YYT16 (*Δsld5::LEU2* [YCp *FLAG-SLD5*]) and YYT35 (*psf1-1 Δsld5::LEU2* [YCp *FLAG-SLD5*]) cells, and then the proteins were immunoprecipitated with anti-Sld5 or anti-Psf1 antibodies. In YYT16 cells, the Flag-Sld5 protein was precipitated with not only anti-Sld5 but also anti-Psf1 antibodies (Fig. 10B WT). On the contrary, the reduced amount of Flag-Sld5 was immunoprecipitated with anti-Psf1 antibodies from YYT35 (*psf1-1*) cell extract (Fig. 10B lane2 and 6) while almost the same amount of Flag-Sld5 was immunoprecipitated from wild type and *psf1-1* cell extracts with anti-Sld5 antibodies (Fig. 10B lane3 and 7). These results indicate that amount of the Sld5-Psf1 complex is reduced in *psf1-1* cells. The *psf1-1* mutation is suppressed

by high-copy *SLD5* but not low-copy *SLD5* (Fig. 8A). I examined whether the amount of Sld5-Psf1 complex in *psf1-1* cells is restored to wild-type level by introducing high-copy *SLD5*. Cell lysates were prepared from YYT101 (*Δsld5::LEU2* [YEp *FLAG-SLD5*]) and YYT102 (*psf1-1 Δsld5::LEU2* [YEp *FLAG-SLD5*]) cells, and then the proteins were immunoprecipitated. With anti-Sld5 and anti-Psf1 antibodies, the Flag-Sld5 protein in YYT102 cells was precipitated as efficient as those in wild-type cells (Fig. 10C). These results suggest that growth defect of *psf1-1* cells is caused by the reduced amount of the Sld5-Psf1 complex. Since *psf1-1* cells are defective in DNA replication, these results further suggest that the Sld5-Psf1 complex functions for DNA replication.

Interactions between Psf1, Slds, Dpb11 and Pol ε in two-hybrid analysis

The *dpb11-1* mutation is synthetically lethal with mutations in subunits of Pol ε, *sld* mutations and the *psf1-1* mutation, suggesting interactions between Pol ε, Slds, Psf1 and Dpb11. I therefore examined whether Psf1 interacts with Pol ε, Slds, Psf1 and Dpb11 using two-hybrid analysis. The *PSF1* and *psf1-1* ORF were fused to the LexA-binding domain of pBTM116, and the *DPB2, DPB3, DPB11, SLD2, SLD3, CDC45 (SLD4),* and *SLD5* ORF were fused to the Gal4 activation domain of pACT2. They were introduced into L40 cells, and expression of the *lacZ* gene reporter was examined. As shown in Table 1, *lacZ* expression was observed in L40 cells harboring *PSF1* and one of *DPB2*, *DPB11*, *SLD3* and *SLD5*. These results suggest that Psf1 interacts with Sld5, Dpb2, Dpb11 and Sld3. I also cloned N-terminal and C-terminal half of *DPB11* onto

pACT2 to determine which part of Dpb11 interacts with Psf1. The *LacZ* expression was observed in cells harboring *PSF1* and N-terminal half of *DPB11* but not in cells harboring *PSF1* and C-terminal half of *DPB11*, suggesting that Psf1 interacts with N-terminal half of Dpb11. Furthermore, this interaction as well as the interaction between Psf1 and Dpb11 was abolished by the *psf1-1* mutation. Thus, the *psf1-1* mutation site is important for the interaction between Dpb11 and Psf1.

DISCUSSION

The Sld5-Psf1 complex formation is involved in the DNA replication

In this article, I investigated the functions of Sld5 and Psf1. Because *sld5* mutant cells did not arrest the cell cycle immediately after temperature shift up, I characterized mostly *psf1-1* mutant cells in detail and found that Psf1 plays a role of chromosomal DNA replication. Since I showed that Psf1 and Sld5 coexist in the same complex throughout the cell cycle, I argue that Psf1 and Sld5 participate in DNA replication as components of the same complex. Several lines of evidence support this argument. First, almost all the Psf1 protein is involved in the Psf1-Sld5 complex because most of the Psf1 protein was depleted after immunoprecipitation with anti-Sld5 antibodies (my unpublished result). It suggests that mutant phenotype of *psf1-1* reflects defect of the Sld5-Psf1 complex. Second, *sld5* cells showed phenotype typical for mutants defective in DNA replication; cells arrested with a dumbbell shape with a single nucleus with a DNA content between 1C and 2C. This is similar to the phenotype of *psf1-1*. This evidence suggests that both Psf1 and Sld5 participate in the same step of DNA replication. Third, in CHIP assay, Sld5 and Psf1 associate simultaneously with ARS (Fig. 7E and F). It suggests that the Sld5-Psf1 complex associates with ARS. Fourth, I found that the *psf1-1* mutation impairs formation of the Sld5-Psf1 complex in co-immunoprecipitation assay (Fig. 10) and confers defect of DNA replication. Moreover, high copy *SLD5* restores formation of the Sld5-Psf1-1 complex and in consequence suppresses the growth defect of *psf1-*

1 cells. Similarly, high copy *PSF1* suppresses the growth defect of all *sld5* temperature sensitive mutants, suggesting that these *sld5* mutant cells are defective in DNA replication because of inefficient formation of the Sld5-Psf1 complex. These findings suggest that formation of the Sld5-Psf1 complex is required for DNA replication.

In contrast with Psf1, a fraction of Sld5 protein is not included in the Sld5-Psf1 complex because Sld5 remained in supernatant after immunoprecipitation with anti-Psf1 antibodies while apparently all the Psf1 protein precipitated (my unpublished result). Although I do not know the role of Sld5 protein that is not complexed with Psf1, it predicts that additional copies of Psf1 protein, but not Sld5 protein, increases a number of the Sld5-Psf1 complex. It might be the case for high copy suppression of *dpb11* mutants. That is, high copy *PSF1,* but not *SLD5,* suppressed the growth defect of *dpb11* mutants. This is probably because increased copy number of the Sld5-Psf1 complex restores the interaction between the Sld5-Psf1 complex and Dpb11.

Although I showed that Psf1 and Sld5 coexist in the same complex, I do not know whether the complex consists of only two protein species, Psf1 and Sld5. In two-hybrid assay, both Psf1 and Sld5 interact with Dpb11 and Psf1 interacts with Dpb2 and Sld3. A genome-wide two hybrid analysis also revealed that Sld5 interacts with not only Psf1 but also YFR043c (Uetz et al., 2000). Recently, I have isolated a novel essential gene as a multicopy suppressor of *psf1-1.* This gene corresponds to YOL146w whose deletion conferred the same morphology after the spore germinated as those observed in deletions of *PSF1* and *SLD5.* Moreover, there are strong genetic interactions between *PSF1, SLD5* and *DPB11*; any combination between *psf1-1, sld5-12* and *dpb11-1* is lethal at

permissive temperature for each of them, high copy *DPB11* suppressed thermosensitive growth of *psf1-1* cells, and high copy *PSF1* suppressed thermosensitive growth of *dpb11-1*. However, none of them except Sld5 co-immunoprecipitated with Psf1 (my unpublished results). Therefore, it is suggested that Psf1 and Sld5 form a fragile complex with other factors including Dpb11, Sld3 and Pol ε.

Psf1 in the elongation step of DNA replication

For discrimination between the initiation and elongation steps of DNA replication *in vivo*, combinations of HU and a temperature sensitive mutant have been used for a long time. At non-permissive temperature, the mutants defective only in the initiation step continue the cell cycle after release from HU arrest while the mutants defective in the elongation step do not synthesize normal DNA and consequently arrests the cell cycle by the checkpoint. Although this method is very useful, inactivation of mutant protein is crucial. I employed this method for *psf1-1* mutant cells. When *psf1-1* cells were arrested in G_1 phase with α-factor and released at the restrictive temperature, DNA content had not moved for 120 min while DNA content in wild type cells increased from 40min. This observation indicates that Psf1-1 protein in G_1 phase at the restrictive temperature is inactivated in 40 min. However, heat inactivation of HU arrested *psf1-1* cells for 1 h or 1.5 h (my unpublished result) did not give rise to immediate arrest of cell cycle. Therefore, it is likely that *psf1-1* is defective only in the initiation step of DNA replication. However, I cannot neglect the possibility that Psf1-1 at replication fork in HU arrested cells is not inactivated, unlike in G_1 phase. I also have to consider the possibility that *psf1-1* is defective only in the initiation step

although Psf1 is required for the initiation and elongation steps. To clarify this problem, further analysis is definitely required.

The CHIP assay showed that Pol ε associates first with ARS and then with its neighboring fragments (Aparicio et al, 1997; Masumoto et al., 2000). This observation suggests that Pol ε moves from ARS region to neighboring region together with replication fork progress. Psf1 association with non-ARS fragments was also observed in regions surrounding ARS. Moreover, Rfa coprecipitated with Psf1 in S phase and its level precipitating with Psf1 peaked when Psf1 associated with late-firing origin, ARS501. At that time most ARSs fire and majority of Rfa associates with single-stranded DNA at replication fork. Therefore, this evidence suggests that Psf1 associates with replication fork. However, I do not know whether Psf1 association with replication fork is required for DNA replication.

Molecular functions of the Sld5-Psf1 complex

For the initiation of DNA replication, many initiation proteins sequentially assemble on replication origins. In *S. cerevisiae,* DNA replication origins are bound by Orc throughout the cell cycle and the Mcm complex is recruited by Cdc6 from late M phase to G_1 phase to form the pre-RC complex. Sld3 and Cdc45 joins the pre-RC and then replication origins are unwound in cooperation with Cdk and Cdc7 protein kinases. Finally, DNA polymerases are recruited to origins and this step requires Dpb11 (Masumoto et al., 2000). The CHIP assay showed that Psf1 and Sld5 associate with ARS in S phase as Pol ε behaves.

This observation suggests that the Sld5-Psf1 complex associates with activated ARS (Fig. 11).

Dpb11 and Pol ε form a complex and associate with ARS. This association is prerequisite for further origin-association of Pol α-primase (Masumoto et al., 2000). However, how the Dpb11-Pol ε complex recognizes replication origins is obscure. Since Pol α forms a complex with Cdc45 in *Xenopus* extract, it is suggested that DNA polymerases are loaded onto chromatin DNA through Cdc45 (Mimura and Takisawa, 1998). On the contrary to *Xenopus* system, complex formation between Cdc45 and Pol α has not been detected in *S. cerevisiae* . Our two-hybrid assay showed that Psf1 interacts with Dpb11, Dpb2 and Sld3. Thus, it is conceivable that the Sld5-Psf1 complex mediates between Cdc45-Sld3 and Dpb11-Pol ε for ARS association of DNA polymerases. The ARS association kinetics of Psf1 and Sld5 are consistent with this idea.

Many DNA replication proteins, e.g. DNA polymerases, PCNA, Mcms and Orc, are highly conserved from yeast to human cells. However, the amino acid sequences of Dpb11, Sld2 and Sld3 do not predict their possible homologues in higher eukaryotes. It suggests that they are not required in higher eukaryotes for DNA replication or they are divergent from organism to organism. On the other hand, both Psf1 and Sld5 are highly conserved among eukaryotes (Fig. 1A and B), and *Xenopus* Sld5 and Psf1 homologues were also required for chromosomal DNA replication in *Xenopus* egg extracts (Komori and Takisawa, personal communication). Of the proteins interacting with either Psf1 or Sld5 (Table 1), only Dpb2 is well conserved from yeast to human cells. It predicts that Psf1 and Sld5 work with or close to Pol ε. Conservation of Psf1 and Sld5 among

eukaryotes further suggests that the same reaction in DNA replication takes place in different eukaryotic organisms.

Cells lacking DNA replication protein arrest with a dumbbell shape in mitotic cell cycle as well as after germination from spores. Deletion mutants of *SLD5*, *PSF1* and YOL146w, however, show swelling sphere cells with small buds, split sphere or lysed spheres after germination, suggesting that these cells have a defect of cell wall biogenesis. In contrast with germinated cells, this morphology was not observed in mitotic cell cycle when the Psf1 plasmid was lost from YYT43 (*Δpsf1*) cells by the shuffling method. Moreover, neither *psf1* nor *sld5* cells showed higher sensitivity to drugs that affect on generation of cell wall (my unpublished result). Although I cannot neglect the possibility that Psf1 and Sld5 are involved in biogenesis of cell wall in mitotic cell cycle, Psf1 and Sld5 seem to participate in biological process other than DNA replication in germination from spores.

MATERIALS and METHODS

Microorganisms

Yeast strains used in this study are listed in Table 2. *E. coli* DH5α was used for plasmid propagation.

Plasmid construction

YCp33-*DPB2*, YCp22-*DPB11* and YCp22-*POL2* were described in Araki et al. (1991a, 1992, 1995). YCp22-*SLD5* and YEp195-*SLD5* were constructed by subcloning the 1.8 kb *Nhe*I-*Nde*I *SLD5* DNA fragment into the *Xba*I-*Sma*I site of YCplac22 and YEplac195 (Gietz and Sugino, 1988), respectively. YCp22-*SLD5* was used as a template to engineer *Nde*I site at the first methionine codon and a *Xho*I site after the stop codon by PCR. The PCR product was cloned into *Nde*I–*Xho*I site of pET15b. To the *Nde*I-*Nco*I site of resultant pET15b-*SLD5*, the FLAG tag was subcloned. After inserting a 1-kb upstream fragment of *SLD5* to the *Nco*I site, the *Xba*I-*Xho*I fragment containing the whole insert of the resultant plasmid was transferred to YCplac22 (YCp22-*FLAG-SLD5*). YCp22-*PSF1* and YEp195-*PSF1* were constructed by subcloning the 1.6 kb *Kpn*I-*Eco*RI *PSF1* DNA fragment into the *Kpn*I-*Eco*RI site of YCplac22 and YEplac195 (Gietz and Sugino, 1988), respectively. pET15b-*PSF1* and pET15b-*psf1-1* were constructed as follows. YCp22-*PSF1* and YCp22-*psf1-1* were used as a template to engineer a *Nde*I site at the first methionine codon and a *Xho*I site after the stop codon by PCR. These PCR products were cloned into *Nde*I-*Xho*I site of pET15b. YCp22-*HIS6-PSF1-HA* was constructed as follows. The 1-kb upstream fragments of

PSF1 was inserted into *Nco*I site of pET15b-*PSF1*, respectively and the *Xba*I-*Xho*I fragment containing the whole insert of the resultant plasmid was transferred to YCplac22 (YCp22-*HIS6-PSF1*). The HA tag was inserted into C-terminus *Not*I site of *PSF1* of YCp22-*PSF1* engineered by PCR (YCp22-*PSF1-HA*). Upstream region of *PSF1* in YCp22-*HIS6-PSF1* and *PSF1* in YCp22-*PSF1-HA* were combined by cutting with *Nde*I and *Eco*RI and ligation. The His portion of YCp22-*HIS6-PSF1-HA* was replaced by Flag (YCp22-*FLAG-PSF1-HA*). The 5Flag tag (obtained from H. Masukata, Osaka Univ.) was inserted into *Nde*I site of YCp22-*FLAG-PSF1-HA* (YCp22-*6FLAG-PSF1-HA*). The *Hpa*II fragment of YCp22-*6FLAG-PSF1-HA* containing truncated *PSF1* was filled in and cloned to *Sma*I site of YIplac211 (YIp211-*6FLAG-PSF1*). This plasmid was digested with *Sna*BI prior to transformation. All the DNA inserts obtained by PCR were sequenced to confirm no misincorporation in PCR reaction.

Disruption of the *SLD5* and *PSF1* genes

The *Nde*I-*Pvu*II *SLD5* fragment was cloned into the *Xba*I-*Sma*I site of pBluescript SK$^+$. The *Sal*I-*Nde*I fragment of *SLD5* was replaced by the *LEU2* fragment isolated from YDp-L (Berden et al., 1991). The resultant plasmid was cleaved with *Pvu*II and used for transformation of W303-1A/1B to disrupt the *SLD5* gene. Southern blot analysis was performed on the Leu$^+$ transformants to confirm that one copy of the endogenous *SLD5* was successfully disrupted.

The *Stu*I-*Eco*RI *PSF1* fragment was subcloned into the *Eco*RV-*Eco*RI site of pBluescript SK$^+$. The *Bgl*II fragment of *PSF1* (Fig.1C) was replaced by the *LEU2* fragment isolated from YDp-L (Berben et al., 1991). The resultant plasmid was digested with *Hin*dIII and *Pst*I, and introduced into the W303-1A/1B cell.

Southern blot analysis was performed on the Leu$^+$ transformants to confirm that one copy of the endogenous *PSF1* was successfully disrupted.

Isolation of thermosensitive mutants

YCp22-*PSF1* was used as a template to carry out the random PCR mutagenesis. Primers were located on the vector sequence. PCR was carried out using 50 ng of *Eco*RV digested plasmid DNA, 1 μl of each primers, 1X Taq Gold buffer, 0.25 mM $MnCl_2$, 0.2 mM of dNTPs and 5 Units of Taq Gold polymerase (P E, USA). PCR was performed for 11 min at 95 °C and 12 cycles for 30 sec at 95 °C, 30 sec at 50 °C, and 90 sec at 72 °C. The PCR product and *Bam*HI- and *Hin*dIII-digested YCplac22 vector were introduced into the YYT43 (*Δpsf1::LEU2* [YEp195-*PSF1*]) strain. Approximately 1500 transformants grown at 23 °C on Trp$^-$ plates were replica-plated onto two plates containing 0.1% 5-fluoroorotic acid (5-FOA) and one plate was incubated at 23 °C while another one at 37 °C. Three clones showed temperature sensitive growth. Plasmid DNA was recovered from each of them, and the DNA sequence of *PSF1* portion was determined. One clone was found to carry one point mutation, which was named *psf1-1*. The other two clones carried the same two point mutations, one of which is the same one occurring in *psf1-1* and another of which is a silent mutation. The *Kpn*I-*Eco*RI (Fig. 1C) fragment bearing *psf1-1* was cloned into YIplac211 and was used for transformation of W303-1Ab strain after digestion with *Nde*I. Ura$^+$ transformants were grown at 23 °C, spread onto 5-FOA plates, and a temperature sensitive colony in which *URA3* is popped out was selected.

For *SLD5*, PCR was carried out in 1x Taq Gold buffer, using *Eco*RV-cleaved YCp22-*SLD5* as a template and the primers on the vector. Other procedures are

the same as those described for *PSF1* except a strain harboring *Δsld5::LEU2* and YEp195-*SLD5* was used.

Synchronization of yeast cells

In order to facilitate the synchronization of cells, the *BAR1* gene was replaced with *URA3* insertion mutant allele and subsequent popping out of the *URA3* gene. Cells were grown to $5X10^6$ cells/ml, and then arrested with 30 ng of α-factor (Peptide Institute inc., Osaka, Japan) per ml at 23 °C for 3 h. α-factor was removed by centrifugation, and the cells were suspended in fresh YPD medium containing 100 μg of actinase E (Kakenseiyaku, Japan) per ml. For S or M phase arrest, cells were grown to $5X10^6$ cells/ml, and then arrested with 0.2 M hydroxyurea (Sigma, USA) or 10 μg of nocodazole (Sigma) per ml at 23 °C for 3 h.

FACS analysis

Yeast cells were fixed in 70% ethanol at −20 °C overnight. The fixed cells were washed with 50 mM Na-citrate (pH 7.5). The $4 X 10^6$ cells were suspended in 500 μl of 50 mM Na-citrate (pH 7.5) containing 250 μg of RNase A per ml at 50 °C for 1 h. Then, proteinase K (Merck, Germany) was added to 1 mg/ml and incubated at 50 °C for 1 h. They were added 500 μl of 50 mM Na-citrate (pH 7.5) containing 8 μg of propidium iodide (Nacalai tesque, Japan) per ml and sonicated. Stained cells were analyzed by FACScan (Becton-Dickinson).

Measurement of sensitivity to HU, MMS and UV

To measure HU sensitivity, cells were grown at 23 °C to 5×10^6 cells per ml in YPD medium, and HU (Sigma) was added to 0.2 M. The cells incubated for 0, 2, 4 and 6 h were spread onto YPD plates. The plates were incubated at 23 °C for 3 days and colonies were scored for viability.

To measure MMS sensitivity, cells were grown at 23 °C to 5×10^6 cells per ml in YPD medium. Ten ml of culture were harvested, and suspended in 50 mM Na-phosphate buffer (pH 7.0) and added MMS (Nacalai tesque) to 0.1%. From 0 to 120 min after addition, aliquots were withdrawn, MMS was inactivated with 10% Na-thiosulfate, and cells were spread onto YPD plates after appropriate dilution. The plates were incubated at 23 °C for 3 days and colonies were scored for viability.

To measure UV sensitivity, cells were grown at 23 °C to 5×10^6 cells per ml in YPD medium, and spread onto YPD plates. The plates were irradiated with the 0 to 120 J/m^2 dose of UV, incubated at 23 °C for 3 days and colonies were scored for viability.

Preparation of cell extracts

For western blotting, the proteins were precipitated with trichloroacetic acid (TCA, Wako, Japan) (Yeong et al., 2000). The 5×10^7cells were collected and suspended in 1 ml of ice-cold water. Then 150 μl of ice-cold YEX lysis buffer (1.85 M NaOH, 7.5% β-mercaptoethanol) was added, and suspension was kept on ice for 10 min. Then, 150 μl of 50% ice-cold TCA was added, and the suspension was left on ice for another 10 min. The proteins were precipitated by centrifugation, and the protein pellet was resuspended in 1 x gel loading buffer

(50 mM Tris-HCl (pH 6.8), 2% SDS, 0.1% BPB, 10% glycerol, 1.7% β-mercaptoethanol) and the suspension was added aliquot of 1 M Tris-HCl buffer (pH 8.8) to adjust pH to neutral.

Indirect immunofluorescence method

Cells were fixed with 3.7% formaldehyde for 1.5 h. The fixed cells were washed with 0.1 M potassium phosphate buffer (pH 7.5), spheroplasted with 5 μg/ml of zymolyase 20T (Seikagaku co., Japan), and suspended with phosphate-buffered saline (PBS). The cells were fixed onto slide glass, and incubated with 2% skim milk in PBS at room temperature for 2 h. After the cells were washed with PBS, the primary antibody was added to the cells and incubated at 4 °C overnight. After the cells were washed with PBS, the secondary antibody was added to the cells and incubated at room temperature for 2 h. Then, 50 ng/ml DAPI in mounting medium (1 mg/ml *p*-phenylenediamin, 90% glycerol) was dropped on slide glass.

For staining yeast β-tublin, the rat monoclonal antibody YOL1/34 was used at 1/2000 dilution in PBS containing 0.1% BSA and detected with Cy5-conjugated goat anti-rat secondary antibody (Jackson Immuno Resarch, USA) used at 1/1000 dilution in PBS containing 0.1% BSA. The 6Flag-Psf1 protein was detected by mouse monoclonal anti-FLAG M2 (Sigma) antibody with 1/2000 dilution in PBS containing 0.1% BSA and Cy3-conjugated goat anti-rat secondary antibody (Jackson Immuno Resarch) used at 1/1000 dilution in PBS containing 0.1% BSA.

Chromatin immunoprecipitation assay

Yeast cells (2X10^8 cells) were cross-linked with 1% formaldehyde for 20 min at 23 °C. After addition of 125 mM glycine and incubated at 23 °C for 5 min, cells were harvested and washed twice with TBS (20 mM Tris-HCl (pH 7.5), 150 mM NaCl). The cell breakage was perfomed in the 500 µl of lysis buffer (50 mM HEPES-KOH (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Sodium deoxycholate, 1 mM PMSF and 1X Complete Protease Inhibitor Cocktail [Boehringer]) with glass beads, and the cell extracts were sonicated seven times at 60 W for 15 sec each (OHTAKE WORKS, Japan). Cell lysates were clarified by centrifugation and the supernatants were immunoprecipitated with Dynabeads protein A (Dynal, Norway), which were pre-incubated with 0.5 µg of anti-FLAG M2 antibody. Precipitates were washed twice each with lysis buffer (150 mM or 500 mM NaCl, other components were same as lysis buffer), wash buffer (10 mM Tris-HCl (pH 8.0), 250 mM LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM PMSF) and TE (10 mM Tris-HCl (PH 8.0), 1 mM EDTA). DNA was eluted with elution buffer (50 mM Tris-HCl (pH8.0), 10 mM EDTA, 1% SDS) at 65 °C for 15 min. PCR was carried out in 50 µl recommended buffer containing 1/15 of the anti-FLAG M2 antibody immunoprecipitates, or 1/6000 of the cross-linked DNA samples derived from the whole cell extract using *Taq* DNA polymerase (AmpliTaq Gold, Perkin-Elmer). Three pairs of primers were used together in each PCR reaction. In PCR cycles, an initial denaturation for 10 min at 94 °C was followed by denaturation for 1 min at 95 °C, annealing for 1 min at 53 °C, extension for 2 min at 72 °C, and a final extension for 7 min at 72 °C. For ARS1 primer set; PCR reactions were performed 32 cycles. ARS 305 and 501

primer sets; PCR reactions were carried out 35 cycles. The PCR products were separated in 2.5% agarose gel.

Two-hybrid analysis

Plasmids were introduced into L40 cells, and the transformants were patched onto SD-Leu-Trp plate. When transformants were grown, cells were replicated to filter paper (Whatmann 50). The filter paper was frozen in liquid nitrogen and color was developed with Z buffer (10 mM KCl, 1 mM $MgSO_4$, Na-PO_4 pH 7.0) containing X-gal and β-mercaptoethanol.

Preparation of anti-Sld5 antibodies

BL21(DE3) cells harboring pET11d-*SLD5*/pLys-S plasmids were grown in 30 ml of LB broth containing ampicillin (200 μg/ml) and chloramphenicol (34 μg/ml) at 30 °C. When OD_{600} reached to 0.6, IPTG (0.4 mM) was added to culture for induction, and culture was further incubated at 30 °C for 1 h. The cells were harvested and suspended in sonication buffer (10% glycerol, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% NP-40, 1 mM DTT, 1 mM PMSF). The suspension was sonicated 20 times at 40 W for 5 sec (OHTAKE WORKS). Cell lysates were clarified by centrifugation for 15 min at 4 °C and the pellet was dissolved in 6 M guanidine cell lysis buffer (sonication buffer plus 6 M guanidine-HCl). The non-dissolved substances were removed, and the supernatant was added an equal volume of 100% of glycerol. The glycerol suspension was dialyzed sequentially against sonication buffer containing 75, 50, 25 and 10% glycerol. The dialyzed solution was collected and centrifuged. Since more than 80% in soluble proteins

was Sld5, the soluble proteins were injected 3 times at 2 weeks interval into rabbit.

Preparation of anti-Psf1 antibodies

BL21(DE3) cells harboring pET11d-*PSF1*/pLys-S plasmids were grown in 30 ml of LB broth containing ampicillin (200 μg/ml) and chloramphenicol (34 μg/ml) at 30 °C. When OD_{600} reached to 0.6, IPTG (0.4 mM) was added to culture, and the culture was incubated at 30 °C for 3 h. The cells were harvested and suspended in the sonication buffer. The suspension was sonicated 20 times at 40 W for 5 sec (OHTAKE WORKS). Cell lysates were clarified by centrifugation for 15 min at 4 °C and the pellet fraction was separated in 15% SDS-polyacrylamide gel. Protein band containing the His-tagged Psf1 protein were excised from SDS-PAGE, and injected 3 times at 2 weeks interval into rabbit.

Immunoprecipitation

Cells were harvested, washed once with water, and resuspended in 0.3 ml of lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.1% NP-40, 1 mM DTT, 1 mM PMSF, 1X Complete Protease Inhibitor Cocktail [Boehringer]). Cells were broken with glass beads using bead beater. Cell lysates were clarified by centrifugation for 15 min at 4 °C. The soluble protein was quantitated by a Bradford assay (Bio-Rad). Five mg of protein extracts were adsorbed against 25 μl (75% [vol/vol] slurry) of Protein G-sepharose 4 Fast Flow (Pharmacia) for 30 min at 4 °C. Then, the beads were pelleted, and the supernatant was recovered and mixed with antibodies for 2 h at 4 °C, followed by

further 2 h incubation with 25 μl of BSA masked ProteinG sepharose. The immuno-complex was recovered after the beads were washed four times with 1 ml of cold lysis buffer. The precipitated samples were boiled for 10 min in 1X gel loading buffer.

Protein production in *E. coli* and immunoprecipitation from *E. coli* extract

BL21(DE3) cells harboring pET11d/pLys-E, pET11d-*PSF1*/pLys-S or pET11d-*psf1-1*/pLys-S plasmids were grown in 30 ml of LB broth containing ampicillin (200 μg/ml) and chloramphenicol (34 μg/ml) at 30 °C. When OD_{600} reached to 0.6, IPTG (0.4 mM) was added for induction, and incubated at 30 °C for 1 h. The cells were harvested and suspended in sonication buffer. The suspension was sonicated 20 times at 40 W for 5 sec (OHTAKE WORKS). Cell lysates were clarified by centrifugation for 15 min at 4 °C. The protein extracts were adsorbed against 25 μl (75% [vol/vol] slurry) of ProteinG sepharose 4 Fast Flow (Pharmacia) for 30 min at 4 °C. Then, the beads were pelleted, and the supernatant was recovered and mixed with antibodies for 2 h at 4 °C followed by further 2 h incubation with 25 μl of BSA masked ProteinG sepharose. The immuno-complex was recovered after the beads were washed four times with 1 ml of cold sonication buffer. The precipitated samples were boiled for 5 min in 1X gel loading buffer and subjected to SDS-PAGE.

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TABLES and FIGURES

Table 1. Interaction between Psf1, Slds, Dpb11 and DNA polymerase ε*

Gal4AD	LexABD		
	PSF1	*psf1-1*	Vector
Vector			N.T.
DPB2			
DPB11			
DPB3			N.T.
SLD2			N.T.
SLD3			
CDC45			N.T
SLD5			
PSF1			N.T.
DPB11 (N)			
DPB11 (C)			N.T.

***: L40 cells harboring various combination of the pACT2 and pBTM116-*PSF1* derivatives were incubated. The transformants were assayed for β-galactosidase activity by colony color with X-gal. N.T.; not tested**

Table 2. *S.cerevisiae* strains used in this study

Strain	Genotype	Source
W303-1A	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein
W303-1B	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein
W303-1A/1B	*MAT***a**/α diploid, cross of W303-1A and 1B.	R. Rothstein
W303-1Ab	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 Δbar1*	Y. Kamimura
CB018	*MAT***a** *pep4Δ::HIS3 prb1::hisG pre1::hisG ade2 ura3 his3 trp1 leu2 can1*	E. W. Jones
L40	*MAT***a** *his3-Δ200 trp1-901 leu2-3,112 ade2 LYS2::(lexAop)*$_4$*-HIS3 URA3::(lexAop)*$_8$*-lacZ*	Bartel and Fields 1995
YYT43	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 Δpsf1::LEU2* [YEp195-PSF1]	This study
YYT12	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 Δsld5::LEU2* [YEp195-SLD5]	This study
YYT17	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 Δbar1 Δpsf1::LEU2* [YCp22-FLAG-PSF1-HA]	This study
YYT16	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 Δbar1 Δsld5::LEU2* [YCp22-FLAG-SLD5]	This study
YYT35	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 Δbar1 psf1-1 Δsld5::LEU2* [YCp22-FLAG-SLD5]	This study
YYT30	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 Δbar1 sld5-12*	This study
YYT47	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 Δbar1 psf1-1*	This study
YYT69	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 Δbar1 6FLAG-PSF1*	This study
YYT101	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 Δbar1 Δsld5::LEU2* [YEp195-FLAG-SLD5]	This study
YYT102	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 Δbar1 psf1-1 Δsld5::LEU2* [YEp195-FLAG-SLD5]	This study
Mcm4-FLAG	*MAT***a** *ade1 his2 leu2-3,112 trp1-1 ura3Δns Δbar1 MCM4-FLAG* (15Dau background)	S. Tanaka

```
                      S21P(Sld5-8)
                          *
S.c.    1: MDINIDDILAELDKETTAVDSTKITQGSSSHTHRDANTIVGSSLDLNDKTQIYVSPQQDF   60
C.e.    1: -------------------------------MAVTDSATTFLDFDDDEYDEMTTEEEVL   28
D.m.    1: --------------------MSDVEDVPEEQLEIDVSDGAGLEDEDDDDMEQITAQKVL   39
M.m.    1: -------------------------------MTEVLDLHGQDSDGGSEEMVLTEAELI   27
S.p.    1: -------------------------------------MEWDADDLLIEPTTEVENDY   20

    S66P(sld5-8) W67R(sld5-12)
              **
S.c.   61: SDIMKSWKNERGSPELLEYPHQLMKRLLNRISMQSQLLENISMGFLDMQNASNANPPMPN  120
C.e.   29: RKMTATWQNELGAECLLETQMEIVEILLDQLQGMEENIGK-----------------QTD   71
D.m.   40: EIIETAWINEMGAPEILESQTDMLELMVSQVAHMEEQMRD-----------------LD   91
M.m.   28: EKLEQAWMNEKFAPELIESKAEIVECVMEQLEHMEENLRR-----------------AK   69
S.p.   21: EDLCTQWVNERMAEDLLEFAEEIVSRVLDRIEAQRETLQLAIG--------------TSS   66
                                K150E(sld5-2)
                                    *
S.c.  121: ESKLPLLCMETELERLKFVIRSYIRCRLSKIDKESLYLRQLNEDENSLISLTDLLSKDEI  180
C.e.   72: KMQLRISVHRVEEQRIG--------CRLQKIESNPHDAIDQHKKRK-EEGKSDLLSESEM  122
D.m.   92: KNDFRAVVHSMELERVRYIMASYLRCRLQKIETETQHILNQEESR---EPDDKRESPEET  148
M.m.   70: KGDLKVSIHRMEMERIRYVLSSYLRCRLMKIEKEFPHILEKEKVRS--EGEPSSLSPEEF  128
S.p.   67: ATSYRSVLMQTELERVKEVLRSYMRTRINKIDKYAQYIQSHPNLL-------LYLSSPER  119

S.c.  181: KYHDTHSLIWLKIVNDSILK----------------------------------------  200
C.e.  123: RFAEEYALAESNLFQKTVEGTADGFLVENFFFSLKNCKISLNATWQFLQFFFQNFKKKKF  182
D.m.  149: RFAQEFASNVDEYFHKVAT-----------------------------------------  162
M.m.  129: VFAKEYMDHTETHFKNVAI-----------------------------------------  147
S.p.  120: QYLLRHQQTVHRHYMDSFLR----------------------------------------  149

S.c.  201: ----------------YMPEELQAINDTEGSVN---MIDEPDWNKFVEIHVNGPPDGKWN  241
C.e.  183: QNSQKKTFFFKISKKKKFPKNSQFSSEFMPAALKKMPVPRGDHDDVMVYAKVTSDDVGNV  242
D.m.  168: ---------------QYMPNQQRGEAEQR--------IVTENLMSHVELKANV--AVPAV  202
M.m.  148: ---------------KHMPPNLQKVDLLR-A------VPKPDLDSYVELRMKE--RQENI  183
S.p.  150: ----------------EVPAKMNKLDDLVGN---LSMVASPDMDTAVECVVNES------  194
                                                        L293P(sld5-13)
                                                               *
S.c.  242: EDELLQENEFGKPCYTVTIPDLKEEVELTIGSIYVMPYEVIRDLLRDDKVALI     294 aa
C.e.  243: AIPDWQDLNG------------EVILEMEPESCHLIPFESVHQLVEDGNIQLM     283 aa
D.m.  203: IVGVDDEE-----------------VDMAAGSQHIIPYQLVADLIQNNQAQLI     238 aa
M.m.  184: LVEPEADEQR------------DYVIDLEVGSQHLIRYKTIAPLVASGAVQLI     224 aa
S.p.  195: -VEENFRVSE------------NEYITLDKGDVLILRYSVISDYLRLGVVSLI     234 aa
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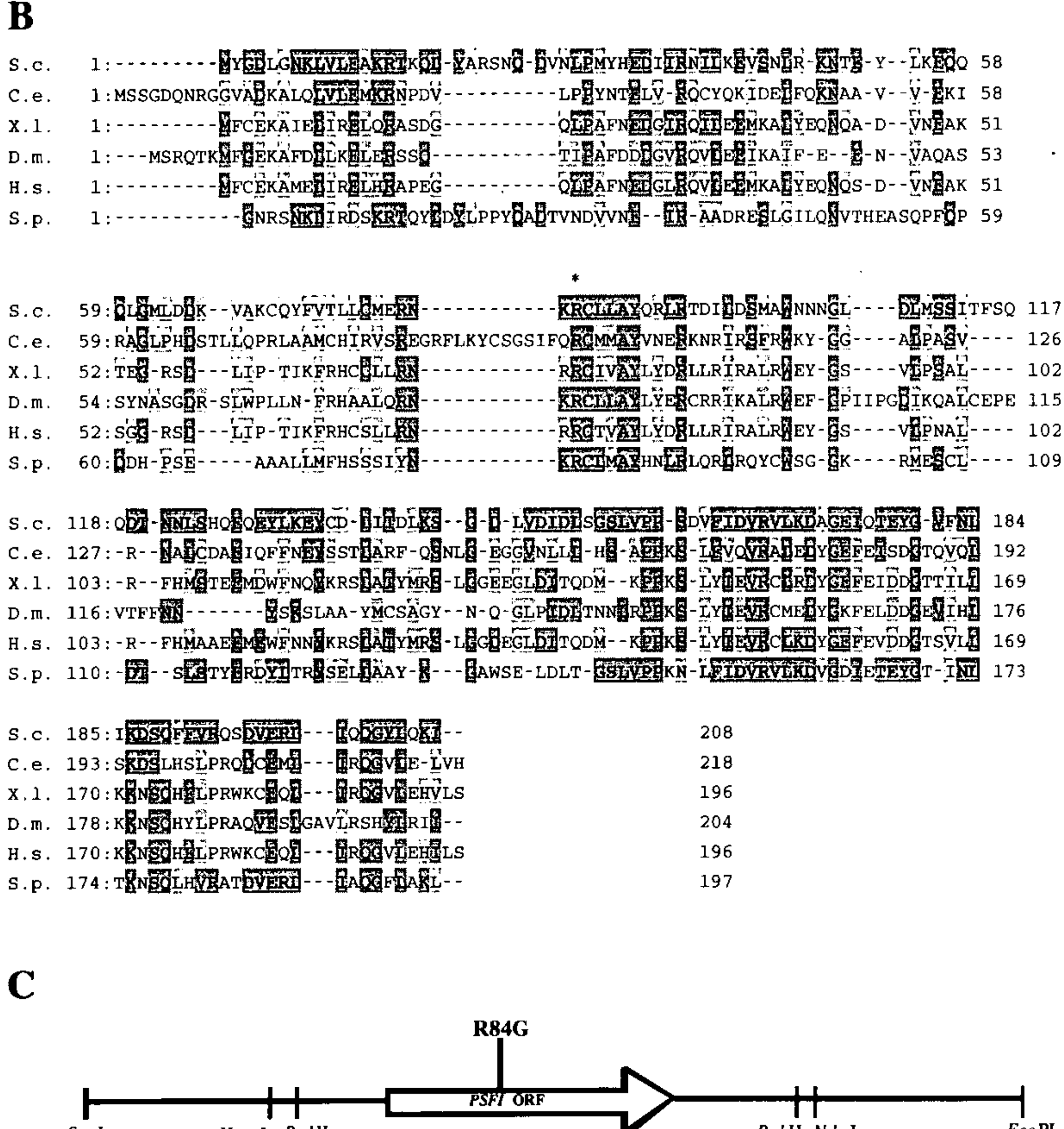


Figure 1. Alignment of Sld5, Psf1 and their possible homologues.
Identical amino acids are shown with dark shadow and similarities with light shadow. A) Amino acid sequence alignment of Sld5 (S.c.) and its possible homologues in *C. elegans* (C.e.), *S. pombe* (S.p.), *D. melanogaster* (D.m.), *M. musculus* (M.m.). The *sld5* mutation sites are shown asterisks with the amino acid change. B) Amino acid sequence alignment of Psf1 (S.c.) and its possible homologues in *C. elegans* (C.e.), *X. laevis* (X.l.), *S. pombe* (S.p.), *D. melanogaster* (D.m.), *H. sapiens* (H.s.). C) Location of a mutation site in the *PSF1* gene. In the *psf1-1* allele, Gly replaces the Arg at amino acid 84.

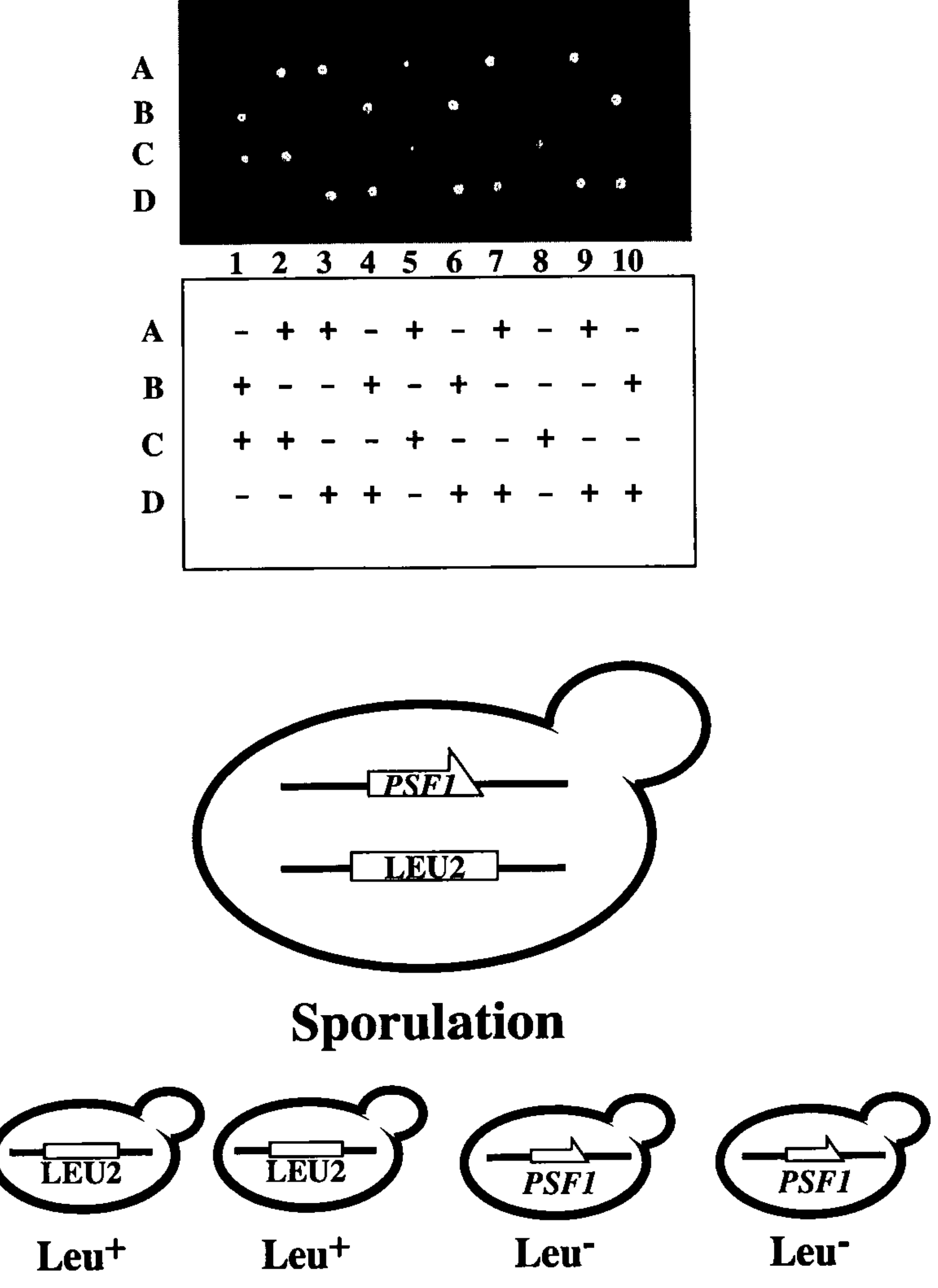


Figure 2. The *PSF1* gene is essential for cell growth.
Germinated spores microdissected from tetrads of the *PSF1* / Δ*psf1* diploid. Two spores from each tetrad grew, and all viable spores were Leu-. Viable spores are shown +, non viable spores are shown -. Disruption of the *PSF1* gene and dissected spores were illustrated in bottom half.

DAPI

Phase-contrast

Figure 3. Terminal morphology of *psf1-1* cells.
The *psf1-1* cells grown at 23 °C were shifted to 36 °C and further incubated for 3 h. The cells were stained with 4', 6-diamino-2-phenylindole (DAPI) and observed by epifluorescent microscopy.

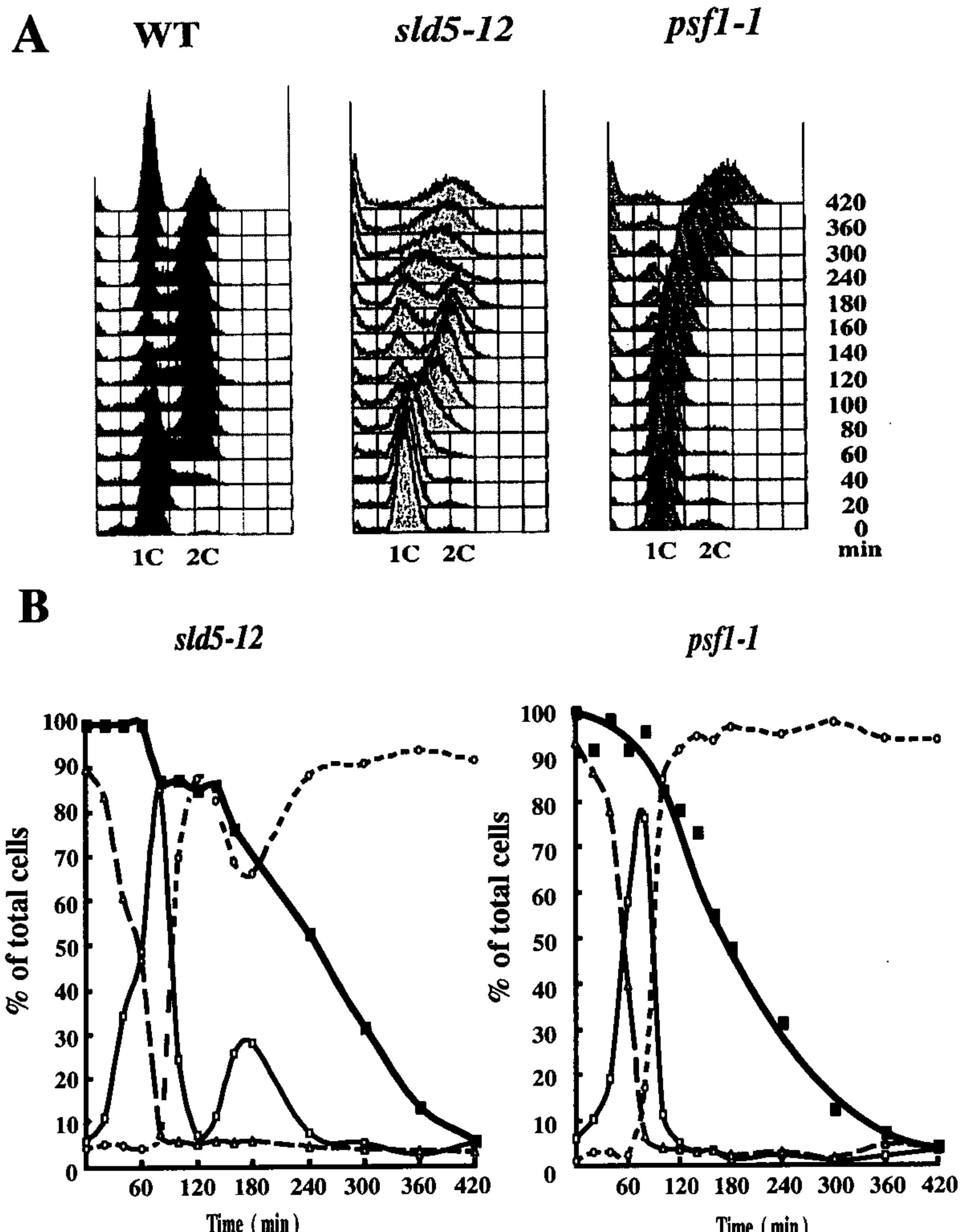


Figure 4. *Psf1-1* cells are defective in chromosomal DNA replication.

A) FACS analysis of cells released from G1 phase arrest. Wild-type (WT), *sld5-12* and *psf1-1* cells were synchronized with α-factor at 23 °C and released from α-factor at 36 °C. At the indicated times, aliquots were treated with propidium iodide and DNA content was measured by FACScan. 1C and 2C indicate DNA content of G1 and G2/M cells. B) Viability and cell morphology of *sld5-12* and *psf1-1* mutant cells. Portions of the same samples incubated at 36 °C described in A), were used to determine cell number and cell morphology. The cells were spread onto YPD plates to measure viability. Symbols: ■, cell viability; △, cells without bud; □, cells with small bud; O, cells with large bud.

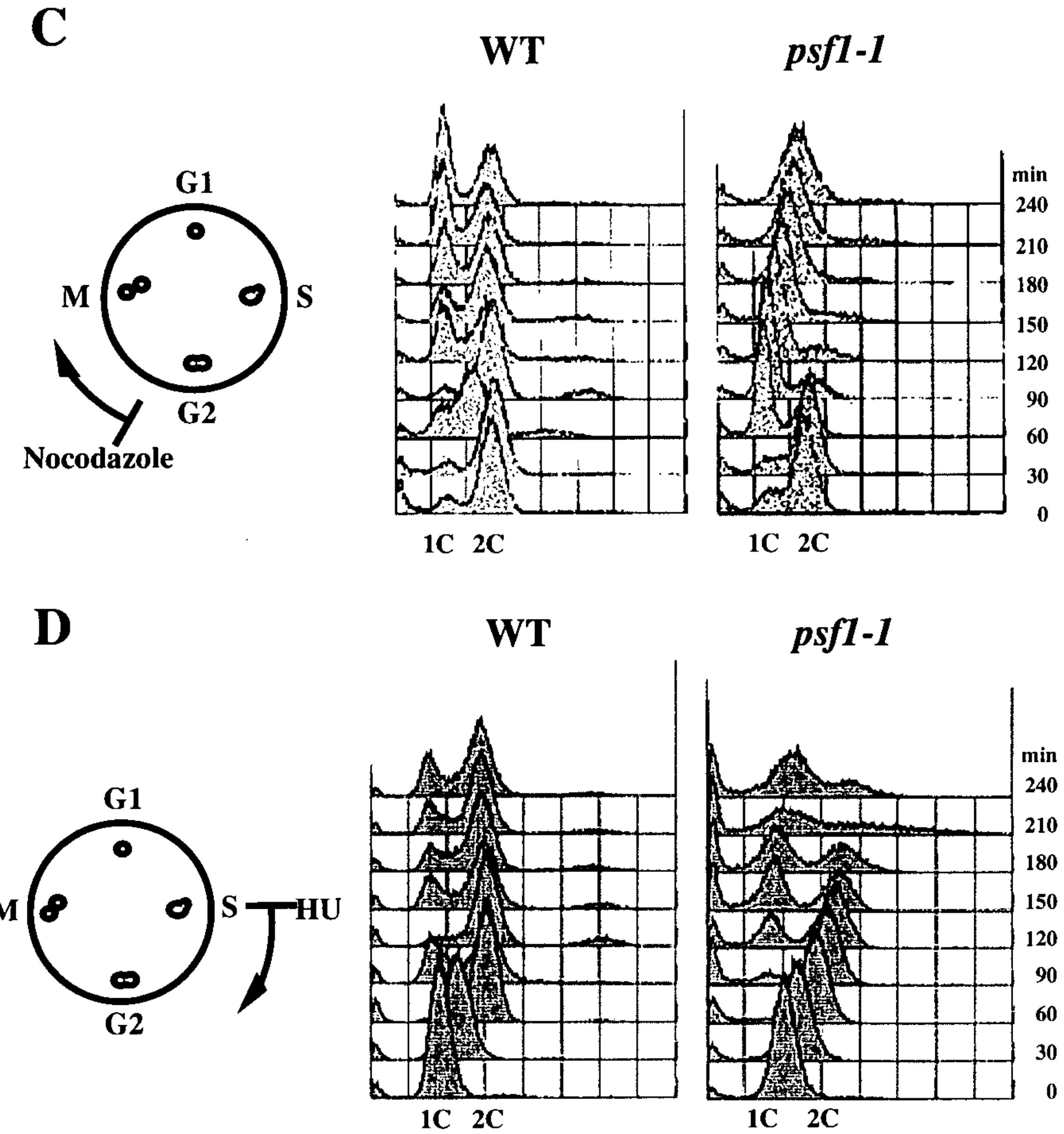


Figure 4. continue

C) FACS analysis of cells released from M phase arrest. Wild-type and *psf1-1* cells were synchronized with α-factor at 23 °C, released to YPD containing nocodazole at 23 °C and incubated for 1 h, followed by 1 h incubation at 36 °C. Nocodazole was washed out and cells were suspended in fresh YPD at 36 °C. D) FACS analysis of cells released from S phase arrest. Cells were synchronized with α-factor at 23 °C, released to YPD containing HU at 23 °C and incubated for 1 h, followed by 1 h incubation at 36 °C. HU was washed out and cells were suspended in fresh YPD at 36 °C.

E Wild-type

Mutants

Early orign

HU arrest

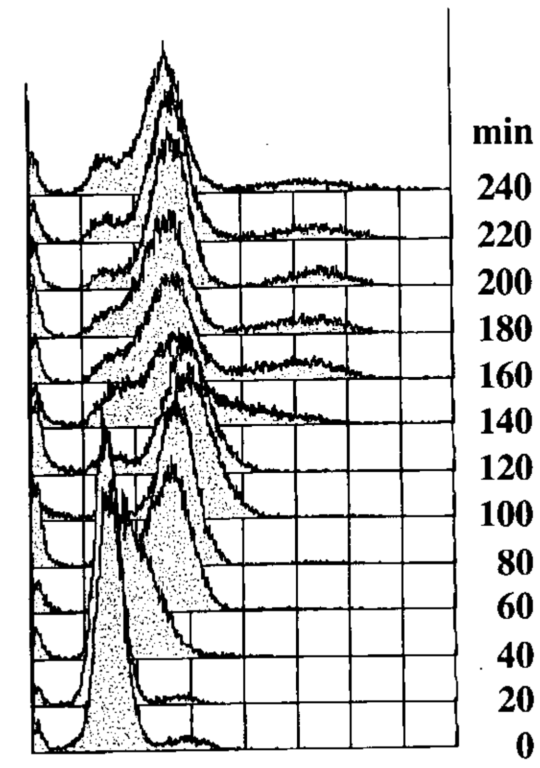
Early orign

release from HU at the restrictive temperature

Late origin

Figure 4. continue

E) Illustration of the DNA replication from HU arrested. In HU arrest cells, early origin is fired. When these cells are released from HU, late origin is fired in wild-type cells. However, late origin firing is not occurred in mutant cells. Consequently, DNA replication in wild-type cells completes faster than that in mutant cells.



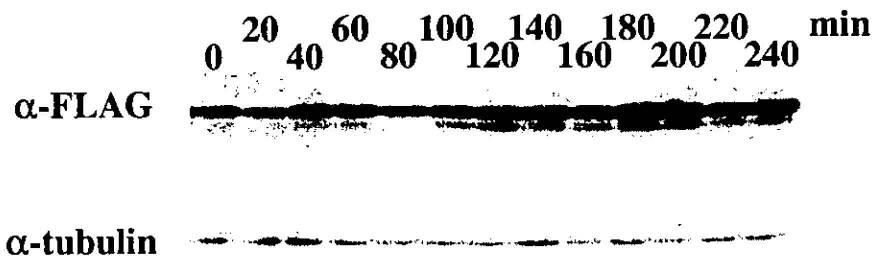


Figure 5. The Psf1 protein exists throughout the cell cycle.
YYT69 cells (*6FLAG-PSF1*) were synchronized with α-factor at 23 °C and released from α-factor arrest at 23 °C. At the indicated times, aliquots were treated with propidium iodide and their DNA content was measured by FACScan. The cell extracts were subjected to SDS-PAGE, followed by western blotting with anti-FLAG M2 and anti-tubulin antibodies.

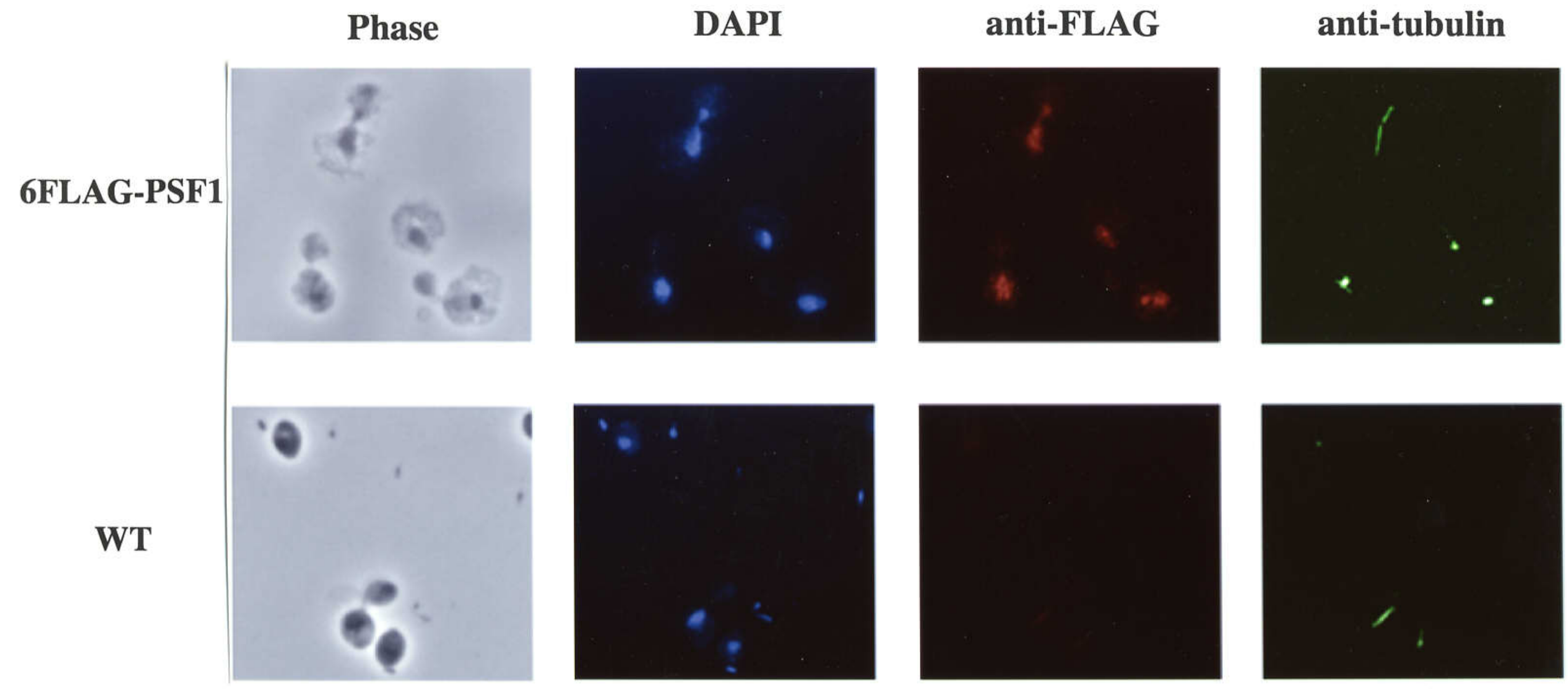
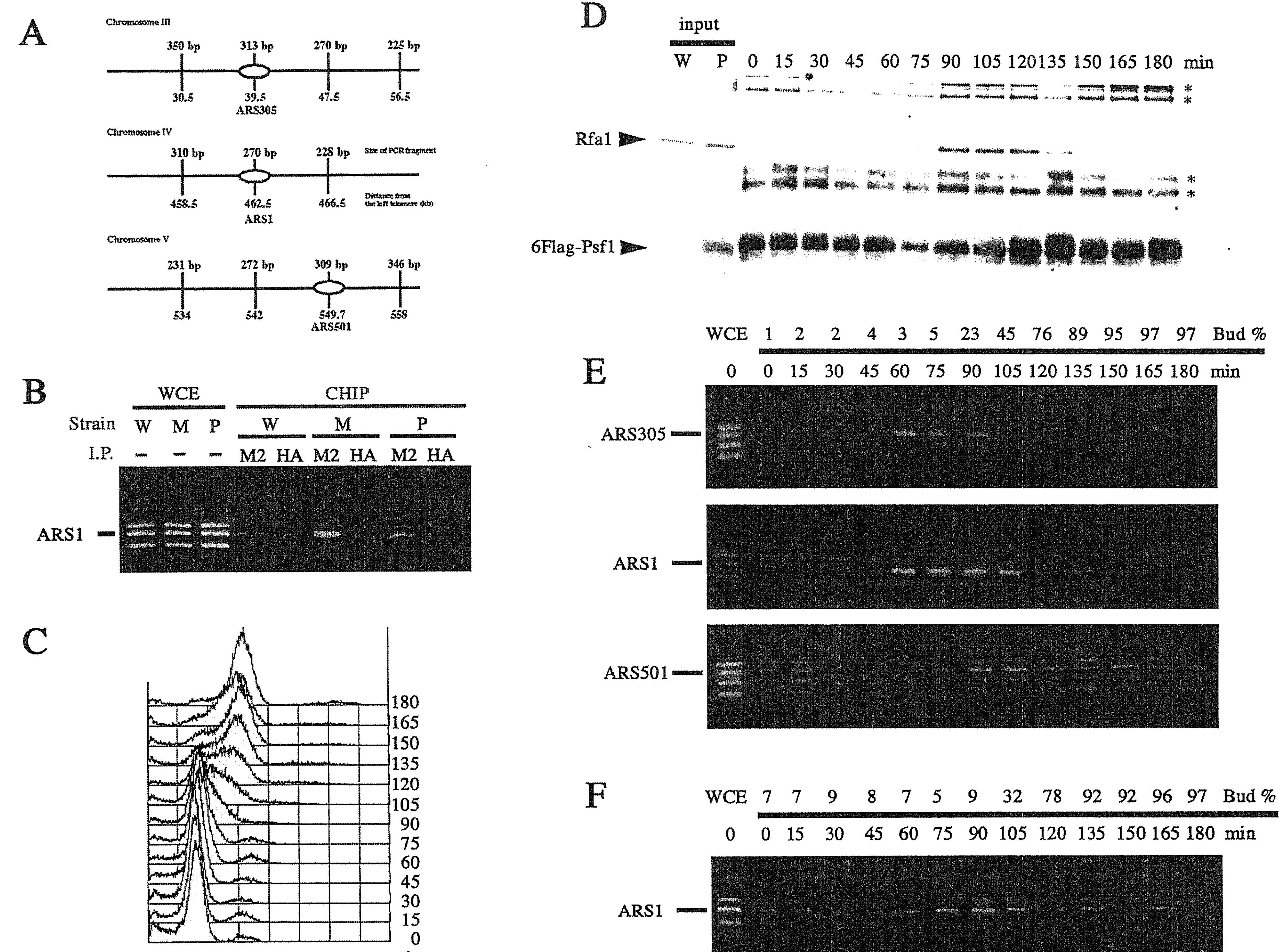


Figure 6. The Psf1 protein is localized in the nucleus throughout the cell cycle.

Wild-type or *6FLAG-PSF1* cells were fixed. DNA was visualized with DAPI and the 6Flag-Psf1 proteins were visualized with anti-FLAG M2 antibody. Microtubules were visualized with anti-tubulin antibodies.

Figure 7. Association of Psf1 with ARS regions.

A) Genomic intervals near or at ARSs amplified by PCR primers. B) Association of Psf1 and Mcm4 with ARS regions in CHIP assay. Asynchronous cells of wild-type (W), Mcm4-FLAG (M) and YYT69 (P) (*6FLAG-PSF1*) strain were fixed with formaldehyde. The cell lysate was sonicated and used for immunoprecipitation with anti-FLAG M2 or anti-HA (12CA5) antibody. PCR was performed with ARS1 primer set. C) The DNA content of synchronized cells used for the CHIP assay was measured by FACS analysis. D) Western blotting analysis of CHIP sample. Chromatin immunoprecipitates were suspended in gel loading buffer and incubated at 95 °C for 30 min. The samples were analyzed by western blotting with anti-FLAG M2 antibody and anti-Rfa antibodies. Asterisks indicate cross-reacting bands. E) Association of Flag-Psf1 with ARS1, ARS305 or ARS501 region. YYT69 cells were arrested in G_1 phase with α-factor and released in YPD medium at 16 °C. Cells were withdrawn from the culture every 15 min and fixed with formaldehyde. The cell lysate was sonicated and used for immunoprecipitation. PCR was performed either on immunoprecipitates derived from the same number of cells at each time point or on the 0 min chromatin fraction from the whole cells extract (WCE). The percentage of budded cells is also shown. F) Association of Flag-Sld5 with ARS1 region. YYT16 cells (*Δsld5* [YCp*FLAG-SLD5*]) were arrested in G_1 phase with α-factor and released in YPD medium at 16 °C. Cells were withdrawn from the culture every 15 min and fixed with formaldehyde. The chromatin fraction was sonicated and used for immunoprecipitation. PCR was performed either on immunoprecipitates derived from the same number of cells at each time point or on the 0 min chromatin fraction from the whole cells extract (WCE). The percentage of budded cells is also shown.


A
Chromosome III
350 bp
313 bp
270 bp
225 bp
30.5
39.5
47.5
56.5
ARS305
Chromosome IV
310 bp
270 bp
228 bp
Size of PCR fragment
458.5
462.5
466.5
Distance from the left telomere (kb)
ARS1
Chromosome V
231 bp
272 bp
309 bp
346 bp
534
542
549.7
558
ARS501
B
WCE
CHIP
Strain W M P
W M P
I.P. – – –
M2 HA M2 HA M2 HA
ARS1
C
180
165
150
135
120
105
90
75
60
45
30
15
0
min
1C 2C
D
input
W P 0 15 30 45 60 75 90 105 120 135 150 165 180 min
Rfa1
6Flag-Psf1
*
*
*
*
E
WCE 1 2 2 4 3 5 23 45 76 89 95 97 97 Bud %
0 0 15 30 45 60 75 90 105 120 135 150 165 180 min
ARS305
ARS1
ARS501
F
WCE 7 7 9 8 7 5 9 32 78 92 92 96 97 Bud %
0 0 15 30 45 60 75 90 105 120 135 150 165 180 min
ARS1

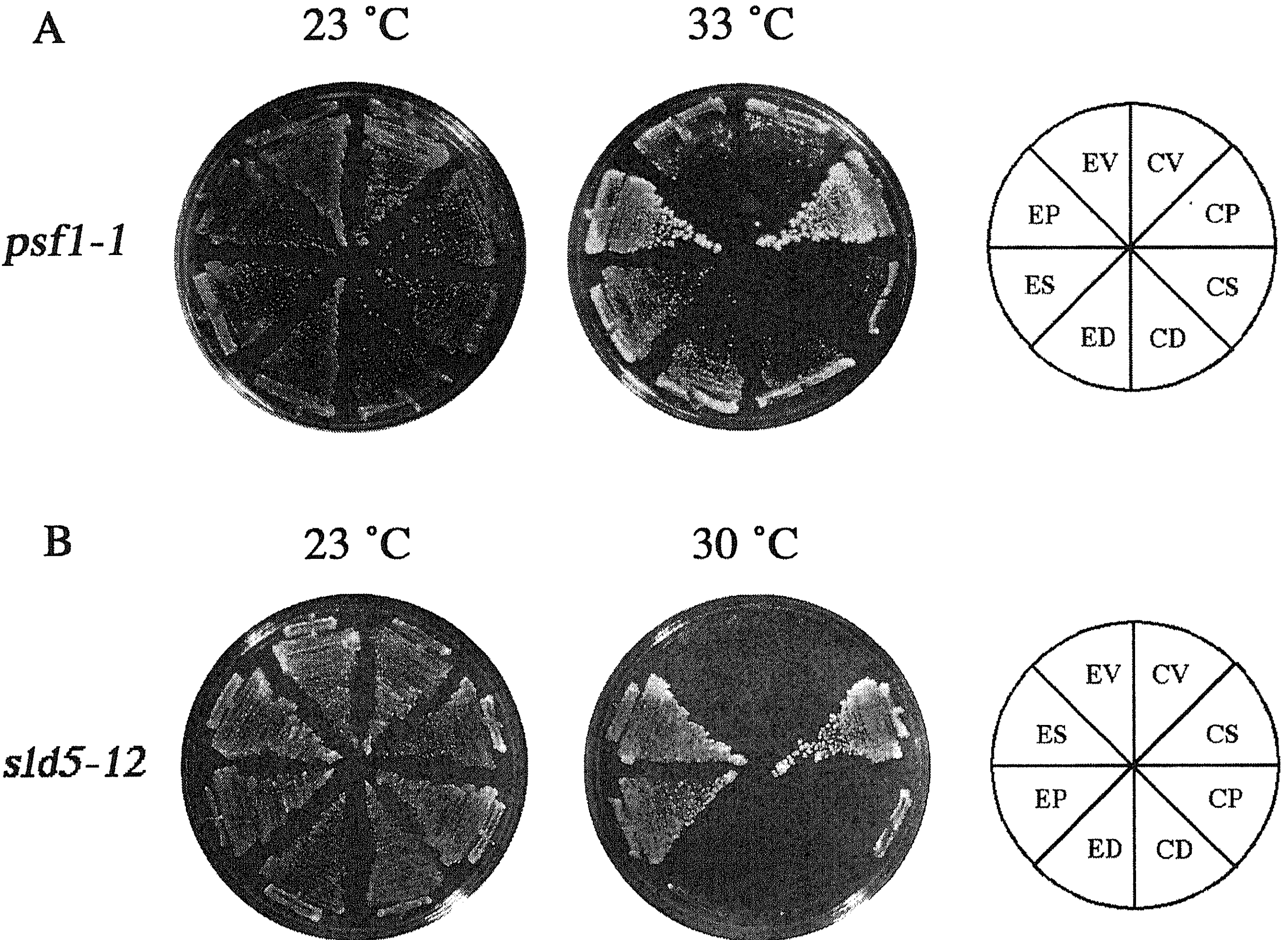


Figure 8. Suppression of temperature-sensitive growth of *psf1-1*.

A) *Psf1-1* cells harboring YCplac22 (CV), YCp22-*PSF1* (CP), YCp22-*SLD5* (CS), YCp22-*DPB11* (CD), YEplac195 (EV), YEp195-*PSF1* (EP), YEp195-*SLD5* (ES) and YEp195-*DPB11* (ED) were streaked onto YPD plates and incubated at the indicated temperature for 2 days. B) *Sld5-12* cells harboring YCplac22 (CV), YCp22-*SLD5* (CS), YCp22-*PSF1* (CP), YCp22-*DPB11* (CD), YEplac195 (EV), YEp195-*SLD5* (ES), YEp195-*PSF1* (EP) and YEp195-*DPB11* (ED) were streaked onto YPD plates and incubated at the indicated temperature for 3 days.

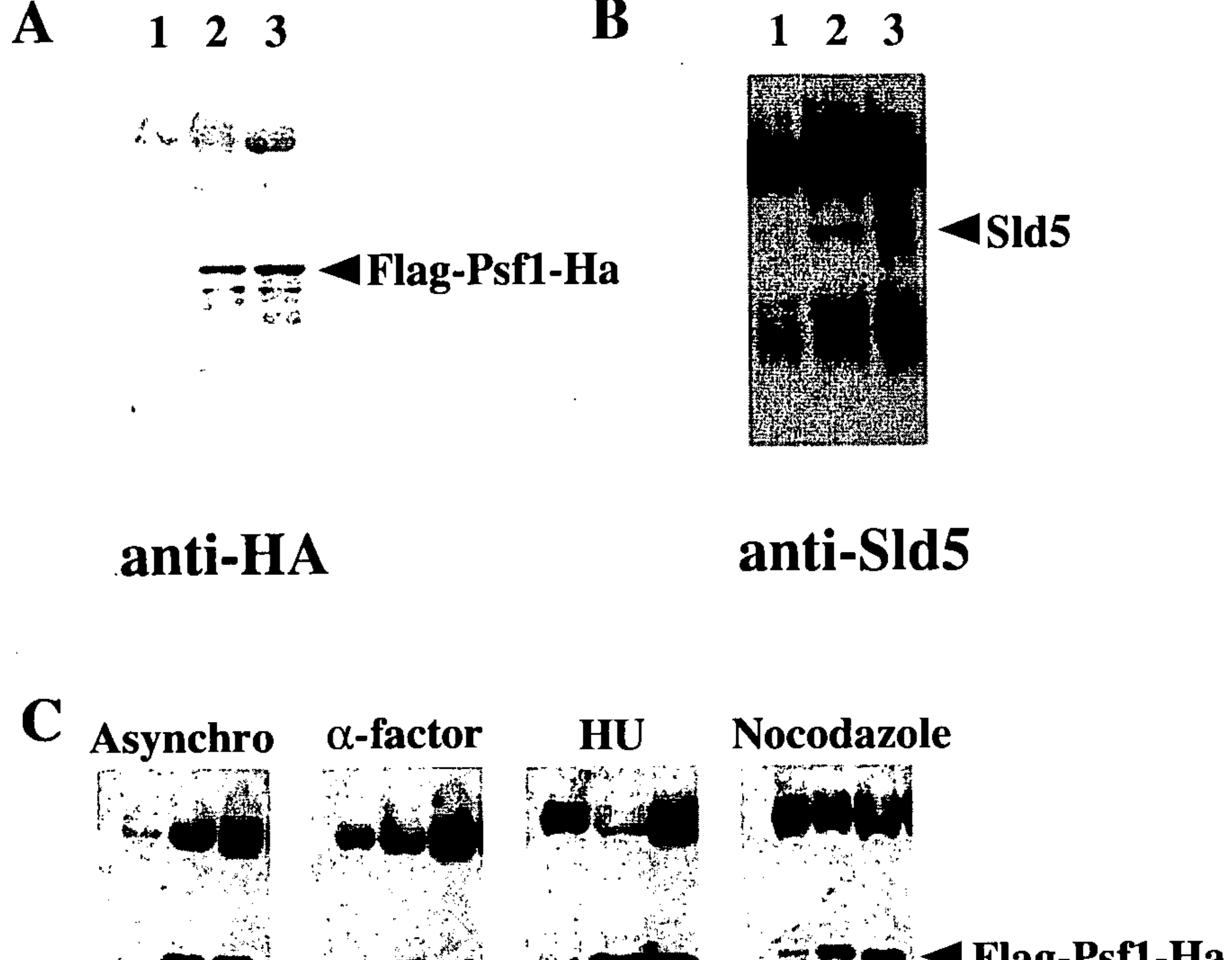
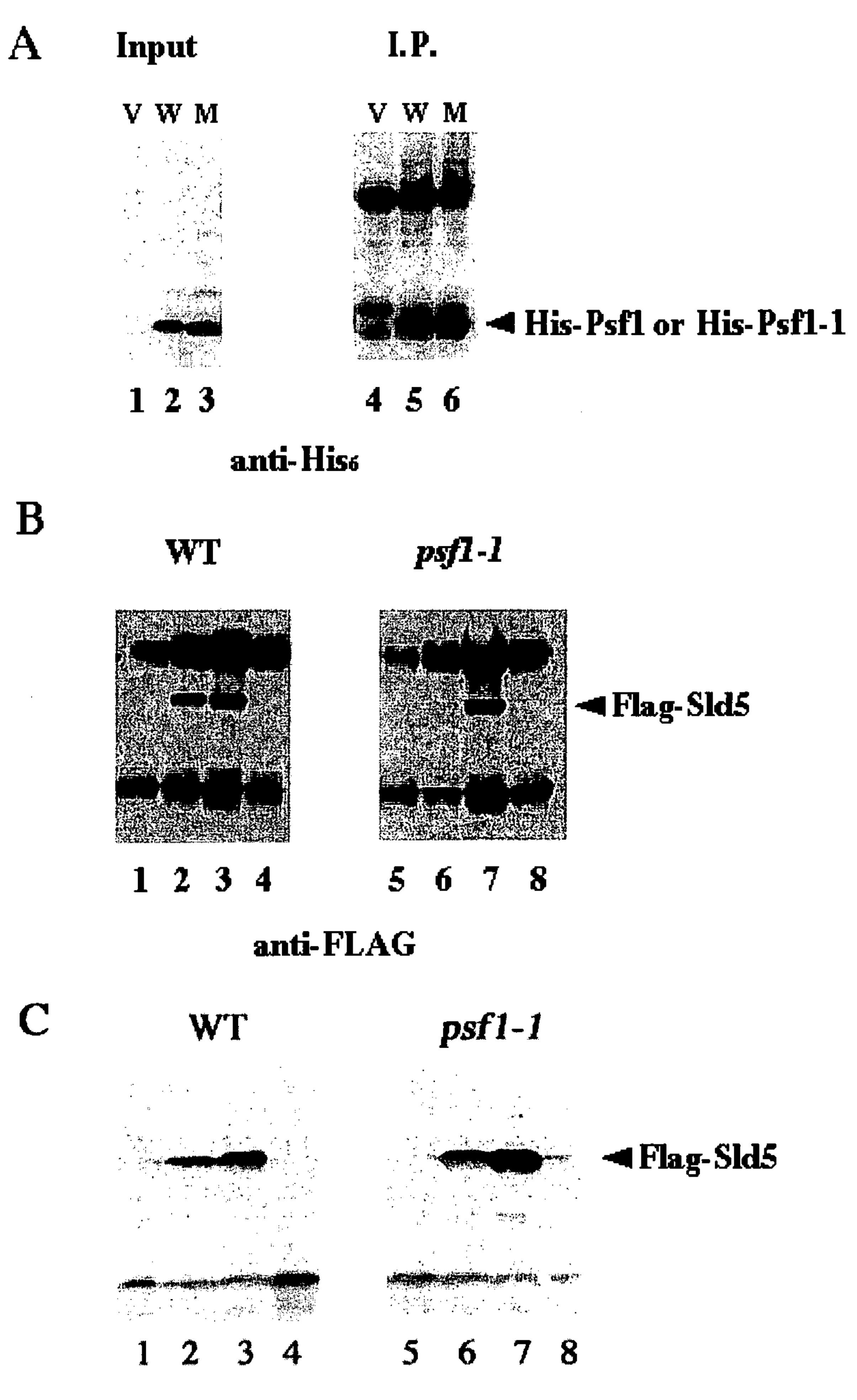


Figure 9. Co-immunoprecipitation of Sld5 and Psf1.

Cell lysate was prepared from *PSF1* disrupted cells harboring YCp22-*FLAG-PSF1-HA* plasmid and the proteins were precipitated with preimmune (lane 1), anti-Sld5 (lane 2) or anti-Psf1 (lane 3) antibodies. The precipitates were analyzed by western blotting probed with anti-HA 16B12 (A) or anti-Sld5 antibodies (B). C) Cells harboring *PSF1* disruption on chromosome and YCp22-*FLAG-PSF1-HA* plasmid were arrested with α-factor, HU and nocodazole. The cell lysates were prepared from these arrested cells and the proteins were precipitated with preimmune (lane 1), anti-Psf1 (lane 2) or anti-Sld5 (lane 3) antibodies. The precipitates were analyzed by western blotting probed with anti-HA 16B12 antibody. Note that lane 3 in nocodazole-arrested cells was distorted during electrophoresis.

Figure 10. Reduced interaction between Sld5 and Psf1-1 protein.

A) *E. coli* cells producing His6-tagged Psf1 (W), His6-tagged *Psf1-1* (M) or mock (V) were sonicated, and the soluble fraction was mixed with anti-Psf1 antibodies and Protein G sepharose. The precipitated proteins were analyzed by western blotting with anti-His6 monoclonal antibody. B) Yeast cell lysate was prepared from wild-type cells or *psf1-1* cells bearing *SLD5* disruption on chromosome and YCp22-*FLAG-SLD5* plasmid and the proteins were precipitated with anti-Psf1 preimmune (lane 1 and 5), anti-Psf1 (lane 2 and 6), anti-Sld5 (lane 3 and 7) and anti-Sld5 preimmune (lane 4 and 8) antibodies. The precipitates were separated in 12% SDS-PAGE and analyzed by western blotting probed with anti-FLAG M5 monoclonal antibody. C) Yeast cell lysate was prepared from wild-type cells or *psf1-1* cells bearing *SLD5* disruption on chromosome and YEp195-*FLAG-SLD5* plasmid and the proteins were precipitated with anti-Psf1 preimmune (lane 1 and 5), anti-Psf1 (lane 2 and 6), anti-Sld5 (lane 3 and 7) and anti-Sld5 preimmune (lane 4 and 8) antibodies.


A
Input
I.P.
V W M
V W M
His-Psf1 or His-Psf1-1
1 2 3
4 5 6
anti-His6
B
WT
psf1-1
Flag-Sld5
1 2 3 4
5 6 7 8
anti-FLAG
C
WT
psf1-1
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1 2 3 4
5 6 7 8
anti-FLAG

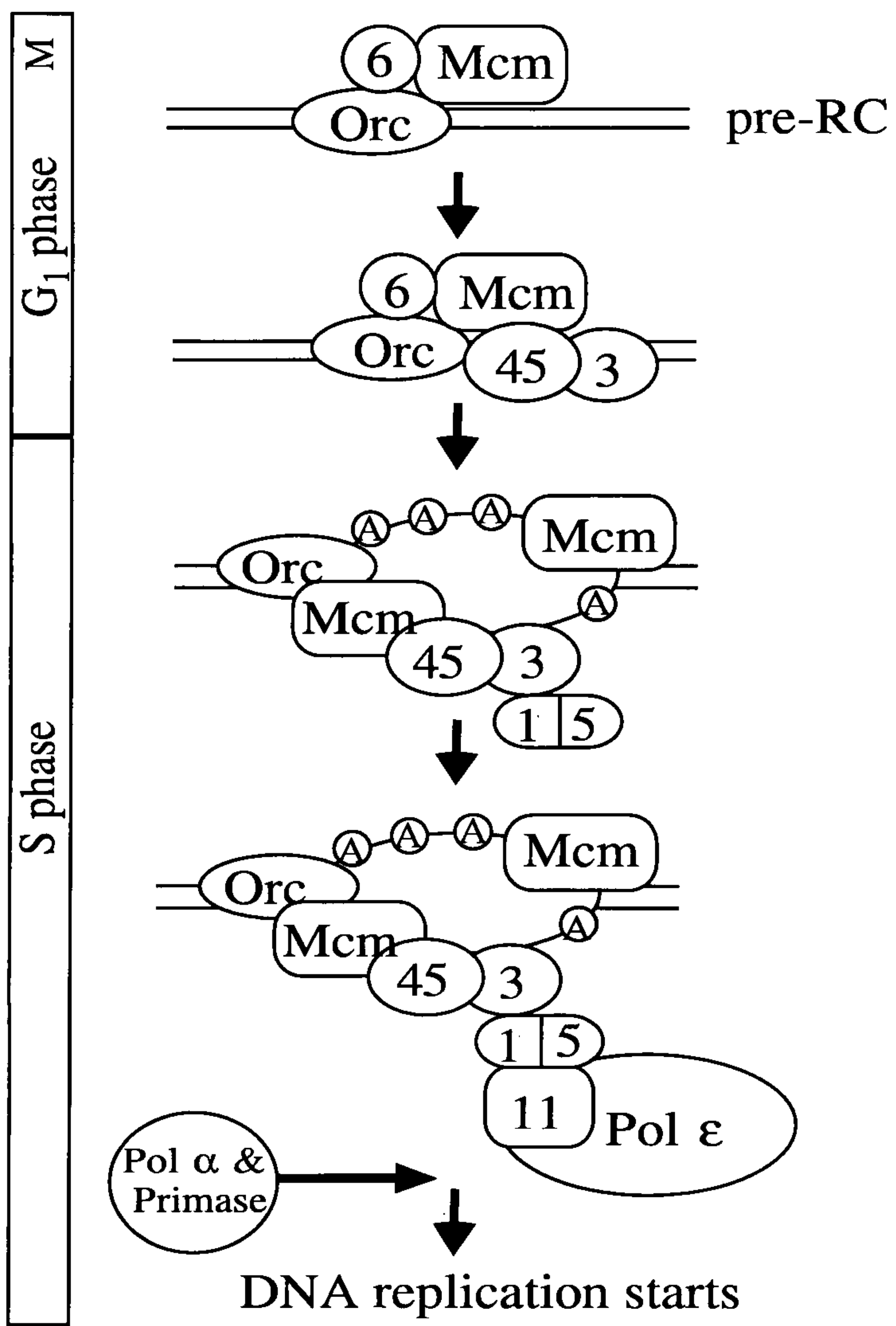

Figure 11. Model for the initiation step of DNA replication in *S. cerevisiae*.
The Orc binds to ARS throughout the cell cycle. From late M to G1 phase, the Mcm proteins are loaded by Cdc6 onto ARS to form the pre-RC. Then, Cdc45 (45) and Sld3 (3) join the pre-RC in G1 or S phase. ARS region is unwound in cooperation with Cdk and Cdc7/Dbf4 protein kinases and Rfa (A) binds to unwound DNA. The Sld5 (5) - Psf1 (1) complex mediates between the Cdc45-Sld3 and the Dpb11 (11) – Pol ε complexes for proper association of DNA polymerases with ARS, and finally DNA replication starts.