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Slm9, a Novel Nuclear Protein Involved in Mitotic Control in Fission Yeast

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ABSTRACT

In the fission yeast *Schizosaccharomyces pombe*, as in other eukaryotic cells, Cdc2/cyclin B complex is the key regulator of mitosis. Perhaps the most important regulation of Cdc2 is the inhibitory phosphorylation of tyrosine-15 that is catalyzed by Wee1 and Mik1. Cdc25 and Pyp3 phosphatases dephosphorylate tyrosine-15 and activate Cdc2. To isolate novel activators of Cdc2 kinase, we screened synthetic lethal mutants in a *cdc25-22* background at the permissive temperature (25°). One of the genes, *slm9*, encodes a novel protein of 807 amino acids. Slm9 is most similar to Hir2, the histone gene regulator in budding yeast. Slm9 protein level is constant and Slm9 is localized to the nucleus throughout the cell cycle. The *slm9* disruptant is delayed at the G_2-M transition as indicated by cell elongation and analysis of DNA content. Inactivation of Wee1 fully suppressed the cell elongation phenotype caused by the *slm9* mutation. The *slm9* mutant is defective in recovery from G_1 arrest after nitrogen starvation. The *slm9* mutant is also UV sensitive, showing a defect in recovery from the cell cycle arrest after UV irradiation.

EUKARYOTIC cells sense their environment and control their growth to survive in various conditions. In fission yeast *Schizosaccharomyces pombe*, checkpoints prevent mitosis when DNA is damaged or DNA replication is incomplete (Rhind and Russell 1998). When cells are starved for carbon, they stop dividing, arrest in G_2, and then enter a quiescent phase (Costello *et al.* 1986). *S. pombe* is a useful organism for the study of G_2-M control. When the entry into mitosis is inhibited, cells become elongated without septation. In fission yeast, as in other eukaryotic cells, Cdc2/cyclin B complex controls entry into mitosis. The activity of Cdc2 protein kinase is regulated in many ways. One of the most important forms of regulation is the inhibitory phosphorylation of tyrosine-15 of Cdc2 protein (Gould and Nurse 1989). Wee1 and Mik1 protein kinases phosphorylate tyrosine-15 and inactivate Cdc2 (Russell and Nurse 1987; Featherstone and Russell 1991; Lundgren *et al.* 1991; Lee *et al.* 1994). Cdc25 and Pyp3 phosphatases dephosphorylate tyrosine-15 and activate Cdc2 (Millar *et al.* 1991, 1992).

Wee1 is regulated by several proteins. Nim1/Cdr1 protein kinase phosphorylates the C-terminal catalytic domain of Wee1 and thereby inhibits Wee1 activity (Coleman *et al.* 1993; Parker *et al.* 1993; Wu and Russell 1993). Cdr2 interacts with and phosphorylates the N-terminal domain of Wee1 *in vitro* and is thought to inhibit Wee1 *in vivo* (Breeding *et al.* 1998; Kanoh and Russell 1998). Swo1 protein, which is an Hsp90 homolog in fission yeast, associates with and stabilizes Wee1 protein (Aligue *et al.* 1994). When DNA replication is blocked by hydroxyurea (HU), the protein kinase Cds1 binds to and phosphorylates Wee1 *in vitro* (Boddy *et al.* 1998). Wee1 was also proposed as a target of Chk1 kinase when DNA is damaged (O'Connell *et al.* 1997).

A mitogen-activated protein (MAP) kinase pathway in fission yeast links cell cycle control to changes in the extracellular environment that affect cell physiology. The Spc1/Sty1 MAP kinase cascade is activated by many forms of stress, such as high osmolarity, heat shock, oxidative stress, and nutrient limitation (Millar *et al.* 1995; Degols *et al.* 1996; Shiozaki and Russell 1996; Degols and Russell 1997). The *spc1* mutant is delayed at G_2-M and displays a cell elongation phenotype that is exacerbated by stress. Genetic analysis indicated that Spc1 is able to influence mitotic control independently of Cdc25 and Wee1 (Shiozaki and Russell 1995), although these findings do not exclude possible regulation of Cdc25 or Wee1 by Spc1. The available data suggest that Spc1 regulates Cdc2 activity indirectly.

In this study, we used *cdc25-22*, a temperature-sensitive mutant allele of *cdc25*, to identify novel regulators of mitosis. At the restrictive temperature of 35°, *cdc25-22* mutant cells arrest in late G_2 phase and become highly elongated (Russell and Nurse 1986). At the permissive temperature of 25°, cells are moderately elongated, because Cdc25 protein is partially inactivated and the G_2-M transition is delayed. When other mutations that show similar phenotypes, such as *nim1/cdr1*, *cdr2*, *spc1*, or *cdc13-117* (*cdc13*$^+$ encodes the major B-type cyclin in fission yeast), are combined with *cdc25-22*, the double mutant cells become highly elongated and arrest in G_2 at 25° (Young and Fantes 1987; Shiozaki and Russell 1995; Breeding *et al.* 1998; Kanoh and Russell 1998). We screened for "slm" mutations that exhibit synthetic

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lethality in a *cdc25-22* background at 25° in order to identify new genes that control the timing of the entry into mitosis (Kanoh and Russell 1998). In this article, we describe *slm9*, one of the genes identified in the screen. Genetic analyses are consistent with a model in which Slm9 regulates Cdc2 activity through Wee1. The *slm9* mutant is sensitive to UV irradiation and heat shock and is defective in recovery from nitrogen starvation. Our data suggest that Slm9 is involved in multiple signal transduction pathways that affect cell growth.

MATERIALS AND METHODS

Yeast strains and general techniques: The *S. pombe* strains used in this study are listed in Table 1. Yeast extract medium YES and synthetic minimal medium EMM2 were used for growing cells. Growth media and basic genetic and biochemical techniques for fission yeast have been described (Alfa *et al.* 1993). Immunoblot methods were performed as described (Kanoh and Russell 1998).

Cloning and nucleotide sequence determination of *slm9*: The *slm9-1 cdc25-22 wee1-50* mutant strain was transformed with a *S. pombe* genomic library constructed in pDB248′ (Beach *et al.* 1982; Maeda *et al.* 1994). Transformants were grown on EMM2 plates at 25° for 7 days, and then were replica-plated onto new EMM2 plates and incubated at 35°. Three fast growing colonies were isolated among ~10,000 transformants, and they showed plasmid-dependent suppression of slow growth of the host strain at 35°. The plasmids were recovered from each transformant and were shown to be identical by restriction enzyme mapping and Southern blotting. The sequence of the insert fragment of pB29-4c was determined.

TABLE 1

***S. pombe* strains used in this study**

Strains	Genotype[a]	Source or reference
PR109	*h*$^-$	Lab stock
GL192	*h*$^+$ *cdc2-3w cdc25*::*ura4*$^+$	Lab stock
JM298	*h*$^-$ *wee1-50*	Lab stock
PR1319	*h*$^+$ *ade6-210 his7-366*	Lab stock
PR1320	*h*$^-$ *ade6-216 his7-366*	Lab stock
KS1366	*h*$^-$ *spc1*::*ura4*$^+$	Lab stock
KS1455	*h*$^-$ *rad24*::*ura4*$^+$	T. Carr
KS1686	*h*$^-$ *spc1*::*ura4*$^+$ *wee1-50*	Lab stock
OM1730	*h*$^+$ *cdc25-12myc cdc25-22*	Lab stock
LW1817	*h*$^-$ *nim1*::*ura4*$^+$	Lab stock
JK1864	*h*$^-$ *cdc25-22*	Lab stock
JK2240	*h*$^-$ *cdr2*::*ura4*$^+$	Lab stock
JK2245	*h*$^-$ *slm9-1 cdc25-22 wee1-50*	This study
JK2246	*h*$^-$ *slm9*::*ura4*$^+$	This study
JK2247	*h*$^-$ *slm9*::*ura4*$^+$ *nim1*::*ura4*$^+$	This study
JK2320	*h*$^-$ *slm9*::*ura4*$^+$ *cdc25-12myc cdc25-22*	This study
JK2353	*h*$^-$ *slm9*::*ura4*$^+$ *spc1*::*ura4*$^+$	This study
JK2360	*h*$^-$ *slm9-GFP*(*ura4*$^+$)	This study
JK2365	*h*$^-$ *slm9-HA6H*(*ura4*$^+$) *cdc25-22*	This study
JK2371	*h*$^-$ *slm9*::*ura4*$^+$ *wee1-50*	This study
JK2372	*h*$^-$ *slm9*::*ura4*$^+$ *cdc2-3w cdc25*::*ura4*$^+$	This study
JK2412	*h*$^-$ *slm9*::*ura4*$^+$ *cdr2*::*ura4*$^+$	This study

[a] All strains are *leu1-32 ura4-D18*.

Gene disruption of the *slm9* gene: One-step gene disruption of *slm9*$^+$ was carried out as follows (Rothstein 1983). A 1.1-kb *Sal*I-*Cla*I DNA fragment was cloned into pBlueScript SK (Stratagene, La Jolla, CA) and digested by *Stu*I, and then a 1.8-kb fragment of the *ura4*$^+$ cassette was inserted (Grimm *et al.* 1988). The resultant plasmid was digested by *Sal*I and *Cla*I, and the *slm9*::*ura4*$^+$ fragment was used to transform diploid cells made by crossing PR1319 with PR1320.

Chromosomal integration of *slm9-HA6H* and *slm9-GFP*: To tag genomic *slm9*$^+$ with a sequence encoding two copies of HA epitope and hexahistidine at the carboxyl terminus, the *slm9*$^+$ open reading frame (ORF) was amplified by PCR with primers jk71 (5′-TCCTCC**CCCGGG**CGATGCACATTTTTGT GCCTAAG-3′; *Sma*I site in boldface type) and jk123 (5′-AAA TAT**GCGGCCGC**ATAAAAGTGCAGATCGTCGTAAT A-3′; *Not*I site in boldface type). The PCR product was cloned into pRIP42-HA6H (Shiozaki and Russell 1997). After the *nmt1* promoter was excised from the vector, the resultant plasmid was linearized at the *Xba*I site in *slm9*$^+$ and used for transformation of wild-type (PR109) or *cdc25-22* (JK1864) strains. To tag genomic *slm9*$^+$ with a sequence encoding green fluorescent protein (GFP) at the carboxyl terminus (Chalfie *et al.* 1994), the *slm9*$^+$ ORF was amplified by PCR with primers jk94 (5′-GCGCGC**CTGCAG**TTCCCTCACCCCACAACGA-3′; *Pst*I site in boldface type) and jk123. The PCR product was cloned into pRIP-GFP. The resultant plasmid was linearized at the *Xba*I site in *slm9*$^+$ and used for transformation of wild type (PR109). Stable integration and tagging were confirmed by Southern and Western blotting. The functions of Slm9-HA6H and Slm9-GFP were confirmed by analysis of cell morphology.

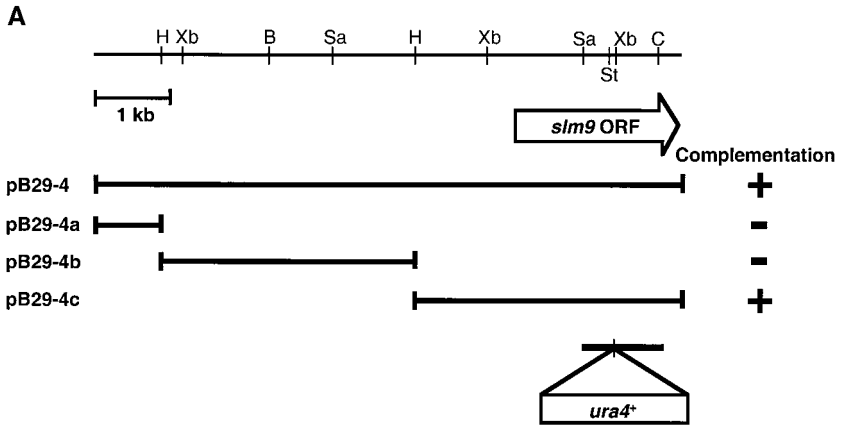
Microscopy: For the study of Slm9-GFP protein localization, cells were grown at 30° to log phase, washed with water, and observed with a Nikon Eclipse E800 microscope equipped with a Photometrics Quantix CCD camera. Images were acquired with IPLab Spectrum software (Signal Analytics Corp., Vienna, VA). For the study of Cdc25-12myc protein localization, cells were fixed with a 3.7% formaldehyde solution for 1 hr at 30° and washed with PEM buffer (100 mm PIPES, 1 mm EGTA, 1 mm $MgSO_4$, pH 6.9). The cell wall was digested with 0.5 mg/ml of Zymolyase 100T (Seikagaku, Rockville, MD) at 37° for 40 min in PEMS buffer (PEM buffer supplemented with 1 m sorbitol), followed by permeabilization with 1% Triton X-100. After washes, cells were blocked in PEMBAL buffer (PEM buffer supplemented with 1% BSA, 0.1% NaN_3, 100 mm l-lysine monohydrochloride) for 1 hr at room temperature. Cells were incubated with anti-Myc antibodies (9E10, BabCo) overnight at room temperature. After washes, cells were incubated with FITC-conjugated goat anti-mouse IgG antibodies overnight at room temperature followed by washes. Samples were suspended in PBS containing 4′,6-diamidino-2-phenylindole (DAPI). Photographs were taken as described above.

Northern blot analysis: *S. pombe* cells were harvested and lysed by vortexing with glass beads in a buffer containing Tris-HCl (pH 7.5), 0.5 m NaCl, 0.01 m EDTA, and 1% SDS. After repeated extraction with phenol-chloroform, total RNA was precipitated by ethanol. A total of 8 μg of RNA of each sample was denatured with formamide, separated by formaldehyde gel electrophoresis, and blotted to a membrane. A DNA fragment of the *H2A.1*$^+$ ORF was amplified by PCR using primers jk79 (5′-CGTCATGTCTGGAGGTAAATCTG-3′) and jk80 (5′-GACGACTGACTTTACAGCTCC-3′) and was labeled with [α-^{32}P]dCTP by the random priming method.

RESULTS

Cloning of the *slm9* gene: To identify novel mitotic genes, we screened for synthetic lethal mutations in a

cdc25-22 background at the permissive temperature of 25° (Kanoh and Russell 1998). In this article, we describe one of these genes, *slm9*. We cloned *slm9* from a *S. pombe* genomic library by complementation of the slow growth of the *slm9-1 cdc25-22 wee1-50* triple mutant at 35° (see below). A restriction enzyme map of the insert in the original clone, pB29-4, is shown in Figure 1A. The region essential for the complementation was delimited to a 4.0-kb *Hin*dIII fragment by subcloning (Figure 1A). Nucleotide sequence analysis of this fragment in pB29-4c revealed an ORF of 807 amino acids. The deduced amino acid sequence of Slm9 was compared with the databases. The ORF of the *slm9* gene was found to be identical to AL031349, an ORF in c15D4,



B

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Slm9 DPMGQFLASQSFDHTTKVWKISTFCVEKSIAKPFEQMPTGNRFLRLSWSPDGAHIASVNA 252
Hir2 DPTGQTFTVMASDRSSLVYQLNKTGTHKLLNKLTQHVQMYPLHYRLSMSPQADILPVLNS 232

Slm9 V----NEGAYVIALVQRDTWTYDLN---LVGHQGPLECATFNPYLYEDPFQKS-------- 298
Hir2 VKGVPNNATSCTALIDRNN-NYKVTKTLVTPSSNGCRVLVYSPAFYEKPNLKKGTSTRYN 291

Slm9 -IIASAAHDGCVSIWNTACARPMAVIHELSCSSFVDLQWSTSGFELYGVSLDGNLMLLQF 357
Hir2 LIATSGSTDGTLVWNTKRMKPLFNALQVSSSAINDMSWSQDGFTLFAISNDATLYTFAF 351

Slm9 EESSFG----------------EKMDTTH----------YPCDLSYFNSSRS--------- 383
Hir2 QEKDLGVALPQTEIKSLQEVNKKLPKLEEPLAEQIPKSFPENIKLEESASAAPIPNDIGR 411

Slm9 ---------KAHVNKNAAADRTTSPTQGQPESPSKSI---LLRPPPSIASS---PESKRR 428
Hir2 SAVGKKPTKKKTANNGTNGIKTIQSTSMEFNIPSYTVPRDLKRKPKEATPSNIAPGSKKQ 471

Slm9 KC---PKKFVARPPVPHPTSLYSQIRIGCPYLK----------PKLVISKSFGTLIVKNH 475
Hir2 KKELQPIDFVDTGLLL-PNVSFSRIRIATPKIRSTFKYSPINNPNLILDVKNGS---GNE 527

Slm9 NQLSLLKCTFSNLDGNDCSWFSYLPNAIVFANGTSVFWAVAIEDSSIYIYSPAGRLLLPP 535
Hir2 QRPHIVKLTSKVLDQDQVLFQDFIPKLITICTAGDTFWSFCSEDGSIYIYSDSGRKLMAP 587

Slm9 VVVAAIPCFLECCGDFLCCIVSTGLLYIWNIKNFEAIHSPVSTLPLFHSN--FSVSKIAR 593
Hir2 LVLGVSISFLEACGTYLLCITSIGFLYCWNIECKKLAFPTNIIYPLLNPSLRYSDDILTR 647

Slm9 GPSIEQFFVTKQCHPVAIMSDGNAFAFIRDSSSWLRVSEGWWMIGSQYWGPL-------- 645
Hir2 AENITLCSITKKGVPIVTLSNGDGYLFDINMEIWLLVSDGWWAYGSQYWDTTNTTGLSSS 707

Slm9 --------ASESN----------EESPEGFLERCTDEETIKAGRGRFLQRLVKALMLRQ 686
Hir2 KANTDSFNGSESNINEIVSDIKNDNQSIINFLECKTNDELNKKGRIKNLQRFARTILMKE 767

Slm9 GYDNYEMEVSIRHLENRLMSSAKLDLEYDFRENLILYAKKIAEEGMKDKMDELCKELLGP 746
Hir2 GFENMEEIVTLSHLENKLLISIRLEEPEEFSKLMMVYCIRISELGYMDRLNDVFQWLYDD 827

Slm9 LRIPHNGIDTVKVGNRLWSPTIAGNNKRNLLKDIIIHTAKYRDMQRITSQYS 798
Hir2 LPISGTG----------SAFADKDFKRNLLKKIIIACGDIRQVQRVTTRYA 868
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Figure 1.—Structure of the *slm9* gene. (A) Restriction map of *slm9*$^+$. The insert DNA in the plasmid pB29-4 is shown. Restriction sites: B, *Bam*HI; C, *Cla*I; H, *Hin*dIII; Sa, *Sal*I; St, *Stu*I; Xb, *Xba*I. Restriction fragments of the insert were subcloned in pDB248′ as shown, and their ability to complement the poor growth of the *slm9 cdc25-22 wee1-50* mutant (JK2245) at 35° is indicated. The structure of the linear fragment used to disrupt the *slm9* gene is shown at the foot of the figure. (B) Comparison of amino acid sequences of Slm9 and *S. cerevisiae* Hir2. Identical amino acids between two proteins are shown in white on black, and conservative amino acids are shown in white on gray. The nucleotide sequence data of *slm9*$^+$ will appear in the GenBank/EMBL/DDBJ nucleotide sequence databases (accession no. AL031349).

a cosmid sequenced as part of the *S. pombe* genome sequencing project at the Sanger Centre (Cambridge, UK). Slm9 showed the highest similarity to Hir2, a histone gene regulator of *Saccharomyces cerevisiae* (Figure 1B; Sherwood *et al.* 1993; Spector *et al.* 1997). Slm9 and Hir2 share 30% sequence identity in a 606-amino-acid region (Figure 1B). Hir2 protein interacts with Hir1 *in vivo*, and they appear to function as transcriptional corepressors of histone genes.

Disruption of *slm9*: One-step gene disruption of *slm9* was carried out by insertion of a *S. pombe ura4*$^+$ gene cassette in diploid cells by homologous recombination (Figure 1A). Proper integration of the disruption construct was confirmed by Southern blot analysis (data not shown). The resulting heterozygous Ura$^+$ diploid cells were sporulated and most of the asci gave four viable spores, two of which were Ura$^+$. The phenotypes of the disruptant and the original *slm9-1* mutant were indistinguishable. That is, cells grew normally but were elongated compared with wild-type cells (see Figure 5). The haploid disruptant was crossed with the *slm9-1* original mutant and was found to be tightly linked. The *slm9* disruptant was crossed with the *cdc25-22* mutant. The presumptive double mutant spores germinated and showed a cdc-arrest phenotype at 25° (data not shown). These data supported the conclusion that the cloned gene was *slm9*. Overproduction of Slm9 from an *nmt1:slm9*$^+$ construct caused no obvious phenotype (data not shown).

Detection of Slm9 protein: To determine whether the level of Slm9 protein is regulated during the cell cycle, the single chromosomal copy of *slm9*$^+$ was tagged with a sequence encoding two copies of the HA epitope and six consecutive histidine residues. Cells were synchronized by a *cdc25-22* block and release protocol, and whole-cell extracts of each sample were used for the detection of Slm9-HA protein by immunoblotting. Slm9-HA protein was detected as a band of ~85 kD. Slm9 protein was present throughout the cell cycle, with no significant change in abundance and mobility (Figure 2).

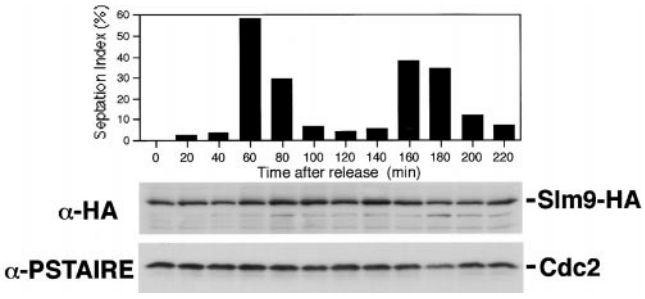

Figure 2.—The amount of Slm9 protein is constant during the cell cycle. Strain JK2365 (*h*$^-$ *slm9HA6H cdc25-22*) was grown in EMM2 medium to log phase at 25°, and cells were arrested in late G_2 phase by a temperature shift to 35.5° for 4 hr, and then released from the arrest by a temperature shift to 25°. Samples were taken every 20 min after the shift to permissive temperature. The percentage of cells with septa was determined by counting ~200 cells at each time point. Whole-cell extracts were prepared and immunoblotting was performed with anti-HA antibodies for Slm9-HA protein and with anti-PSTAIRE antibodies for Cdc2 protein (Kanoh and Russell 1998).

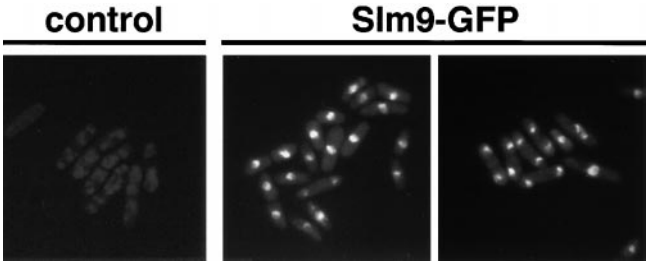

Figure 3.—Slm9-GFP is localized to the nucleus throughout the cell cycle. Wild-type (PR109) and *slm9-GFP* (JK2360) strains were grown in YES medium to log phase. Living cells in the medium were observed without fixation.

Slm9 protein is localized in the nucleus throughout the cell cycle: To examine the localization of Slm9 protein, the single chromosomal copy of *slm9*$^+$ was tagged with a sequence encoding GFP. In control wild-type cells, faint green fluorescence was observed mainly in the cytoplasm. In contrast, strong green fluorescence was observed in nuclei of cells expressing Slm9-GFP protein (Figure 3). The strength and localization of green fluorescence did not vary among cells at different stages in the cell cycle.

Slm9 does not regulate expression of *H2A.1* histone in *S. pombe*: The sequence homology between Slm9 and several regulators of histone genes suggested that Slm9 might regulate the expression of histone genes in *S.*

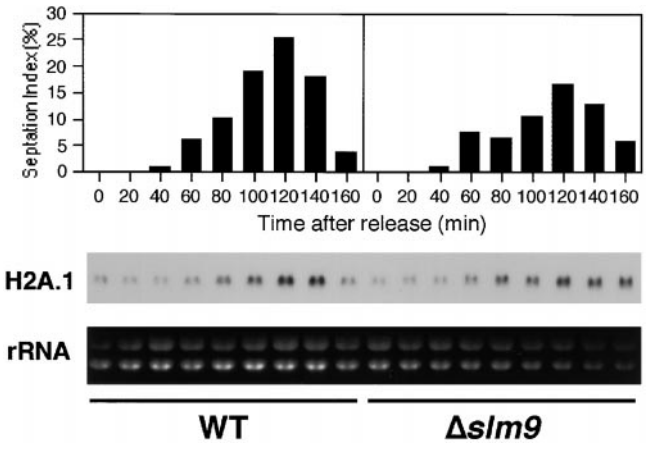

Figure 4.—Slm9 does not regulate the expression of histone H2A.1 gene in fission yeast. Wild-type (PR109) or Δ*slm9* (JK2246) cells were synchronized in early G_2 by centrifugal elutriation and reinoculated into fresh YES medium. Samples were collected every 20 min. The septation index was determined by counting ~200 cells at each point (top). The samples were analyzed by the *H2A.1* probe. Ethidium bromide staining of rRNA is shown below for the control of equal amount of loading.

TABLE 2

The Δ*slm9* mutation causes cell elongation in wild-type and Δ*cdc25 cdc2-3w* backgrounds

	Cell length at division (μm)[a]	
slm9 genotype	*cdc25*$^+$ *cdc2*$^+$	Δ*cdc25 cdc2-3w*
slm9$^+$	14.7 ± 1.0	17.8 ± 1.8
Δ*slm9*	17.3 ± 2.0	27.2 ± 4.7

[a] Standard error of mean based on measurements of 12 dividing cells grown in YES medium.

pombe. Cells were synchronized by elutriation and the abundance of mRNA of a histone gene, *H2A.1*, was examined by Northern blot analysis (Choe *et al.* 1985; Matsumoto and Yanagida 1985). Expression of *H2A.1* was increased in S phase in both wild-type and Δ*slm9* cells (Figure 4). No significant difference in the amount of *H2A.1* mRNA between the two strains was observed. Histone gene expression is thought to be coordinately regulated in fission yeast (Aves *et al.* 1985). These data suggested that Slm9 does not regulate expression of histone genes, unlike Hir2 in budding yeast (Sherwood *et al.* 1993; Spector *et al.* 1997).

Cdc25 overproduction suppresses Δ*slm9* mitotic delay phenotype: The *slm9* disruptant cells were elongated at division compared with wild-type cells. This elongation occurred in both rich YES and synthetic EMM2 media (Tables 2 and 3). These haploid cells had a 2C DNA content with a single nucleus, indicating a G_2 cell cycle delay (see Figure 6). We transformed Δ*slm9* cells with pREP3-*cdc25*. Overexpression of *cdc25*$^+$ complemented the cell elongation phenotype of Δ*slm9* (data not shown). Furthermore, the *wee1-50* mutation suppressed the synthetic lethality of the Δ*slm9 cdc25-22* strain (data not shown). These data indicate that *slm9* regulates the activity of Cdc2 directly or indirectly.

Inactivation of Wee1 fully suppresses cell elongation phenotype of Δ*slm9*: Experiments suggested that Slm9 regulates the activity of Cdc2 directly or indirectly at G_2-M. We examined whether Slm9 affects the abundance of the B-type cyclin encoded by *cdc13*$^+$, because the *cdc13-117 cdc25-22* mutant is synthetically lethal at 25° (our unpublished data). Cdc13 is required for the activation of Cdc2, whereas destruction of Cdc13 protein is required for the exit from mitosis (Yamano *et al.* 1996). The protein level of Cdc13 in the Δ*slm9* cells was similar to that in wild-type cells (data not shown). These data indicate that Cdc13 is not the primary target of Slm9.

We tested the possibility of Cdc25 as a target of Slm9. We examined the phenotype of the Δ*slm9 cdc2-3w* Δ*cdc25* strain. The *cdc2-3w* mutation activates Cdc2, thereby bypassing the requirement for Cdc25 (Fantes 1979). The Δ*slm9 cdc2-3w* Δ*cdc25* cells were elongated relative to *cdc2-3w* Δ*cdc25* cells, indicating that *slm9* regulates cell size in the absence of Cdc25 (Table 2). Cdc25 accumulates in the nucleus during late G_2 and M phase (Lopez-Girona *et al.* 1999). Deletion of *slm9*$^+$ had no obvious effect on the amount or localization of Cdc25 protein (data not shown; Figure 5). These data indicate that Cdc25 is not the primary target of Slm9.

We examined whether the Δ*slm9* cell elongation phenotype was suppressed by the temperature-sensitive *wee1-50* mutation. The Δ*slm9 wee1-50* cells were slightly elongated at permissive temperature of 20° and became the same size as *wee1-50* cells at the restrictive temperature of 35° (Table 3). In contrast, Δ*spc1 wee1-50* cells were longer than *wee1-50* cells at both temperatures (Table 3). Thus, inactivation of Wee1 fully suppressed the cell elongation phenotype of Δ*slm9*. These data suggested that Slm9 might regulate Wee1.

Slm9 influences mitotic entry independently of Nim1/Cdr1 and Cdr2: The data described above suggested that Slm9 might regulate Wee1 by controlling

TABLE 3

Inactivation of Wee1 fully suppresses the Δ*slm9* cell elongation phenotype

	Cell length at division (μm)[a]		
Genotype	*wee1*$^+$	*wee1-50* (20°)	*wee1-50* (35°)
Wild type	13.5 ± 1.0	12.1 ± 1.0	6.4 ± 0.9
Δ*slm9*	17.6 ± 2.5	14.8 ± 2.4	6.2 ± 0.9
Δ*spc1*	19.4 ± 1.4	18.1 ± 1.7	8.7 ± 1.0

[a] Standard error of mean based on measurements of 12 dividing cells grown in EMM medium.

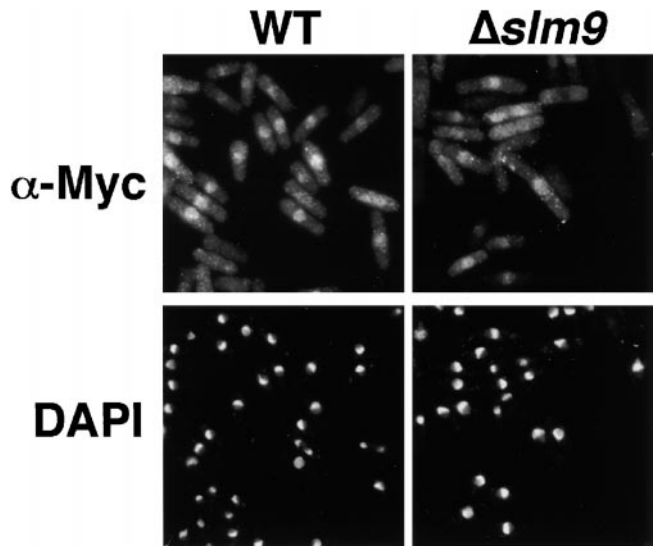

Figure 5.—Cellular localization of Cdc25 is unaffected by Δ*slm9* mutation. Strains OM1730 (*cdc25-12myc*) and JK2320 (Δ*slm9 cdc25-12myc*) were grown in YES medium, stained with anti-Myc antibodies, and processed for indirect immunofluorescence microscopy.

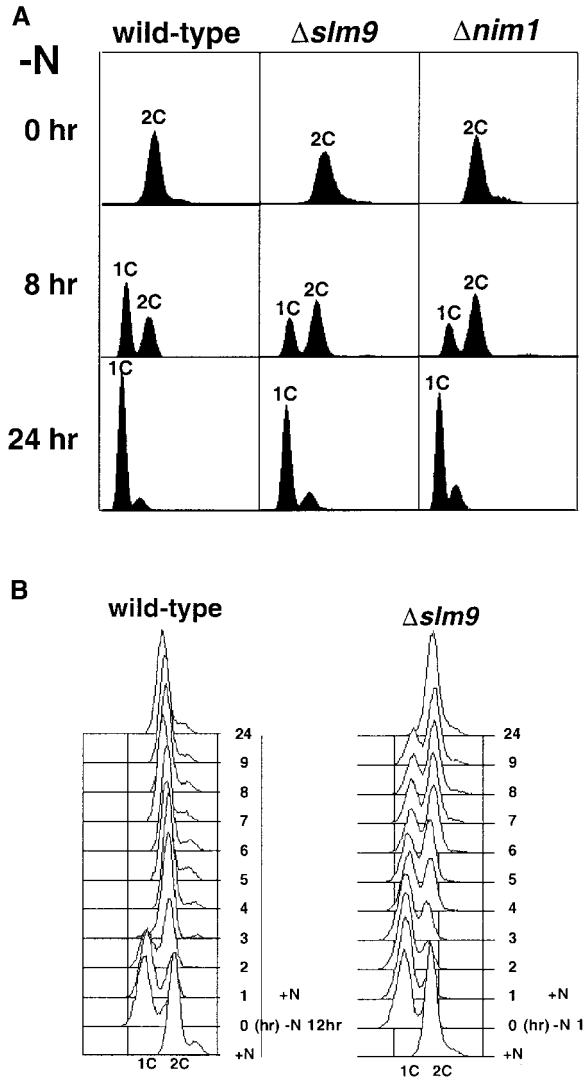

Figure 6.—The *slm9* disruptant cells have a defect in recovery from nitrogen starvation. (A) Δ*slm9* cells arrest in G_1 after nitrogen starvation. Flow cytometric analyses of DNA content of wild-type (PR109), Δ*slm9* (JK2246), or Δ*nim1* (LW1817) cells. Cells were grown in EMM2 medium to log phase and then shifted to nitrogen-free EMM2 medium at 30°. Cells were harvested at indicated intervals after nitrogen starvation and subjected to flow cytometry. (B) The Δ*slm9* cells cannot restart the cell cycle normally after arresting in G_1. Cells of wild-type (PR109) or Δ*slm9* (JK2246) strains were grown in EMM2 medium to log phase, shifted to nitrogen-free EMM2 medium, and incubated for 12 hr at 30°. Cells were then shifted to EMM2 medium with nitrogen and harvested at indicated intervals after adding nitrogen back to the medium. Each sample was subjected to flow cytometry.

Nim1/Cdr1 or Cdr2 activity. To test this possibility, the phenotypes of Δ*slm9* Δ*nim1* and Δ*slm9* Δ*cdr2* strains were examined. The Δ*slm9* Δ*nim1* and Δ*slm9* Δ*cdr2* double mutants were longer than either single mutant (data not shown). This finding indicated that Slm9 acts independently of Nim1 or Cdr2.

Slm9 role in recovery from nitrogen starvation: To investigate whether Δ*slm9* cells have a defect in monitor-

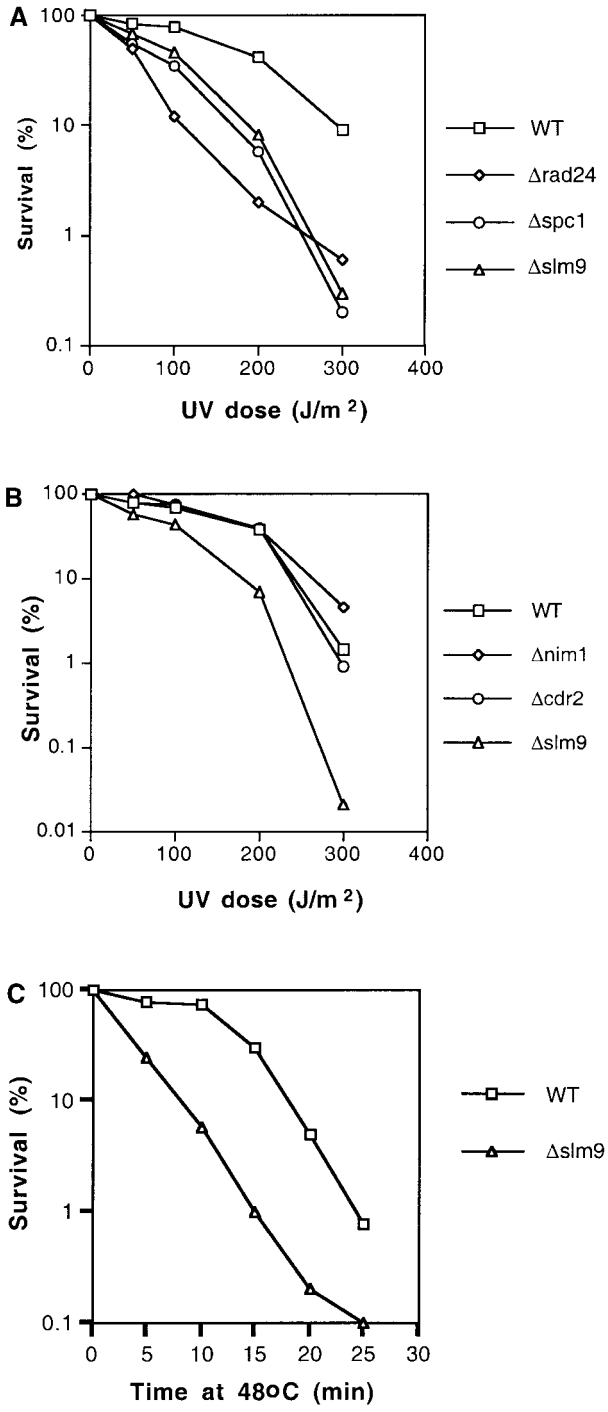

Figure 7.—The *slm9* disruptant is UV and heat-shock sensitive. (A) Wild-type (PR109), Δ*slm9* (JK2246), Δ*spc1* (KS1366), or Δ*rad24* (KS1455) strains were grown to log phase, plated on YES medium, and then exposed to the indicated doses of UV irradiation at 254 nm using Bio-Rad Genelinker (Richmond, CA). Survival was measured by counting the number of colonies formed on YES plates. (B) Wild-type (PR109), Δ*slm9* (JK2246), Δ*nim1* (LW1817), or Δ*cdr2* (JK2240) strains were exposed to UV irradiation. (C) The *slm9* disruptant is sensitive to heat shock. Wild-type (PR109) or Δ*slm9* (JK2246) strains were grown to the log phase in YES medium at 25° and were shifted to 48°. After each interval, an aliquot was taken and diluted with ice-cold YES medium and then plated onto YES plates. The numbers of colonies were counted after 6 days of incubation at 25°.

ing nutritional conditions, as proposed for some other mutants that show a G_2 delay (Young and Fantes 1987; Shiozaki and Russell 1996; Wu and Russell 1997; Breeding *et al.* 1998; Kanoh and Russell 1998), we examined the response of Δ*slm9* cells to nitrogen starvation. Arrest of Δ*slm9* cells with a 1C DNA content was delayed in response to nitrogen starvation, but most of the cells eventually arrested in G_1 phase after 24 hr of starvation (Figure 6A). When nitrogen was added back to the medium, wild-type cells resumed cell cycle progression by entering S phase. The first round of DNA synthesis was completed by 4 hr (Figure 6B). In contrast, the first round of DNA replication was substantially delayed in Δ*slm9* cells. A large fraction of cells remained with a 1C DNA content even 9 hr after addition of nitrogen (Figure 6B). These data suggested that Slm9 is required for the proper recovery from nitrogen starvation.

UV sensitivity of Δ*slm9* cells: Figure 6 showed that the Δ*slm9* mutant has a defect in recovery from G_1 arrest after nitrogen starvation, a finding which suggested that Δ*slm9* cells might have a similar defect in recovery from other forms of stress. We tested the sensitivity of the Δ*slm9* mutant to UV and HU treatment. The Δ*slm9* mutant was not abnormally sensitive to HU (data not shown). On the other hand, Δ*slm9* cells were sensitive to UV treatment (Figure 7A). The sensitivity of the *slm9* mutant was similar to that of the Δ*spc1* mutant, which is sensitive to various forms of stress, such as high osmolarity, heat shock, oxidative stress, and nutritional limitation. Next, we examined whether other mutants that show a G_2 delay were also sensitive to UV. The Δ*nim1* and Δ*cdr2* mutants were not sensitive to UV (Figure 7B). At 24 hr postirradiation (200 J/m^2), most of the Δ*slm9* cells were elongated without a septum (data not shown). These cells appeared to be arrested in interphase (data not shown). Furthermore, deletion of *rad3*$^+$, which encodes an essential component of both DNA damage and DNA replication checkpoint (Enoch and Nurse 1990; Bentley *et al.* 1996), did not fully suppress the cell elongation phenotype of the Δ*slm9* mutant (data not shown). This observation indicated that the G_2 delay caused by the Δ*slm9* mutation was not due to a defect in DNA damage checkpoint. These data suggested that Slm9 is required for recovery from cell cycle arrest after UV irradiation.

The *slm9* mutant is sensitive to heat shock: As the *slm9-1 cdc25-22 wee1-50* mutant grew slowly at 35°, we examined whether Δ*slm9* cells were sensitive to heat shock. Log phase cells were grown in YES medium at 25° and were shifted to 48°. After the heat treatment, survival of wild-type and Δ*slm9* cells was examined. The sensitivity of Δ*slm9* cells was higher than wild type (Figure 7C). After 15 min of incubation at 48°, only 0.9% of the Δ*slm9* cells survived, whereas 31.3% of the wild-type cells were viable. These data suggested that Slm9 is important for survival of heat shock.

DISCUSSION

We have described the cloning and initial analysis of *slm9*$^+$, a novel gene that appears to be involved in regulation of the onset of mitosis in fission yeast. The *slm9* disruptant cells are moderately elongated with a 2C DNA content and the Δ*slm9 cdc25-22* mutant is synthetically lethal when Cdc25 is partially inactivated at the permissive temperature. The effect of Δ*slm9* mutation could be seen in the absence of Cdc25, and the cell elongation phenotype of the Δ*slm9* mutant was fully suppressed by inactivation of Wee1. These findings are most consistent with a model in which Slm9 regulates the onset of mitosis by affecting Wee1 function. The double mutants, Δ*slm9* Δ*nim1* and Δ*slm9* Δ*cdr2*, have an additive effect on cell length. Therefore, Slm9 appears to influence Wee1 function in a different manner from Nim1 or Cdr2.

The amino acid sequence of Slm9 protein is similar to that of corepressors of histone gene transcription in budding yeast. *S. cerevisiae* Hir1 and Hir2 are thought to interact indirectly with DNA because they have no obvious DNA-binding motifs (Sherwood *et al.* 1993; Spector *et al.* 1997). Thus, it is thought that Hir1 and Hir2 are targeted to specific histone gene promoters by association with other DNA-binding proteins. Slm9 protein is localized in the nucleus throughout the cell cycle, but it does not bind to DNA *in vitro* (our unpublished data). These observations suggest that Slm9 might regulate the gene transcription in a manner similar to Hir1 and Hir2, although expression of the histone gene *H2A.1* appears normal in Δ*slm9* cells. A molecular understanding of the functions of Hir1 and Hir2 is currently lacking; however, progress has been made recently with the demonstration that Hir1 and Hir2 form a complex *in vivo* with components of the Swi/Snf complex of proteins that regulate transcription of a subset of genes in budding yeast (Dimova *et al.* 1999).

Although our data suggest that Slm9 might regulate Wee1 function, the Δ*slm9* mutation did not cause a significant change in the abundance of Wee1 protein (data not shown). Thus, Slm9 might be involved in regulation of genes which affect Wee1 function. Wee1 protein is predominantly localized to the nucleus (Wu *et al.* 1996). It is possible that Slm9 forms a complex with Wee1 and other proteins to regulate Wee1 activity. Identification of Slm9-binding proteins would be helpful to clarify the putative signal cascade in which Slm9 participates.

We found that the Δ*slm9* mutant is sensitive to heat shock and UV irradiation. The terminal phenotype of the Δ*slm9* mutant after UV irradiation was almost the same as the Δ*spc1* mutant (Degols and Russell 1997), a finding which suggests that Slm9 and Spc1 might share some functions *in vivo.* However, it is clear that they do not function completely in the same pathway, because there are several differences between Δ*slm9* and Δ*spc1*

mutations. First, the cell elongation phenotype of the Δ*spc1* mutant is not fully suppressed by inactivation of Wee1. Second, the Δ*spc1* mutant cells are more elongated in synthetic EMM2 medium than in complete YES medium (Shiozaki and Russell 1995), while the cell length of the Δ*slm9* mutant is not significantly affected by nutritional condition (Tables 2 and 3). Third, the Δ*spc1* mutant cannot arrest in G_1 after nitrogen starvation (Shiozaki and Russell 1996). On the other hand, the Δ*slm9* mutant can arrest in G_1, but cannot normally recover from the arrest after addition of nitrogen to the medium. Fourth, the Δ*spc1* mutant is highly sensitive to high osmolarity (Shiozaki and Russell 1995), while the Δ*slm9* mutant is not sensitive to high osmolarity (our unpublished data).

In summary, Slm9 appears to be important for many cellular functions: regulation of mitosis, recovery from G_1 arrest caused by nitrogen starvation, heat shock, and UV irradiation. Genetic studies are consistent with a model in which Slm9 regulates Wee1 function, although this regulation appears not to involve any of the proteins that are known or suspected to regulate Wee1. The other phenotypes caused by *slm9* mutations are not easily explained by regulation of Wee1, nor do there appear to be mutants that share the same spectrum of phenotypes. Although the Slm9 sequence is most similar to Hir2 protein in budding yeast, the *slm9* and *hir2* phenotypes have little in common. Hir2 appears to be a transcriptional corepressor in budding yeast (Sherwood *et al.* 1993; Spector *et al.* 1997), but very little is known about the molecular function of Hir2 protein. The studies described in this report lay the foundation for future analyses of an important but poorly understood class of proteins represented by Slm9 and Hir2. A fission yeast gene (accession no. P87314) that encodes a protein with 27% (197/722) identity to Slm9 has been discovered recently in the fission yeast genome sequence project. Future studies will be aimed at determining if Slm9 and this new protein have functional overlap.

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