A search for proteins that interact genetically with histone H3 and H4 amino termini uncovers novel regulators of the Swe1 kinase in Saccharomyces cerevisiae

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In a genetic screen for second-site mutations that are lethal in combination with a deletion of the amino terminus of histone H3, we have uncovered three new gene products that regulate the Saccharomyces cerevisiae Swe1 kinase. The Swe1 protein kinase phosphorylates tyrosine residue 19 of Cdc28 and inhibits its activity. One histone synthetic-lethal gene, HSL1, encodes a putative protein kinase that has high sequence and functional homology to fission yeast cdr1/nim1, an inhibitory kinase of weel. Another gene, HSL7, is a novel negative regulator of Swe1 function. Sequences similar to Hsl7 exist in Caenorhabditis elegans and humans. In addition, we have isolated a dosage-dependent suppressor, OSS1, of hsl1 and hsl7. OSS1 is important for the transcriptional repression of SWE1 and CLN2 in G2. Mutations in HSL1 and HSL7 therefore cause hyperactivity of the Swe1 kinase, which in turn decreases mitotic Cdc28 kinase activity. Moreover, HSL5 is identical to CDC28, further suggesting that it is the decreased Cdc28 kinase activity in these hsl mutants that causes lethality in the histone mutant background. Because neither HSL1 nor HSL7 is essential in yeast, and histone transcription is unaffected by the hsl5/cdc28 mutation, it is unlikely that synthetic lethality results from reduced transcription of HSL1 and HSL7 caused by histone mutations, or from reduced histone transcription when Cdc28 kinase activity is compromised. We suggest that these cell cycle regulators function in a pathway upstream of both histones H3 and H4, thereby modulating histone function in the cell cycle.

[Key Words: Synthetic lethals; Swe1; nim1; Hsl1, Hsl7; Ossl]

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In studying the function of histones in Saccharomyces cerevisiae we have found that the highly conserved amino termini of histones H3 and H4 are nonessential for cellular viability but are important in gene regulation. Specific sequences within the H3 and H4 amino-terminal "tails" are involved in the assembly of heterochromatin-like structures at the silent mating loci [HML and HMR] and telomeres [Kayne et al. 1988; Johnson et al. 1990; Thompson et al. 1994]. This occurs through their interactions with Sir3 and Sir4 repressor proteins of the silent mating loci and telomere-adjacent regions [Hecht et al. 1995]. In addition, these histone amino termini are required for the proper activation or repression of a number of yeast genes [Durrin et al. 1991; Mann and Grunstein 1992; Roth et al. 1992; Fisher-Adams and Grunstein 1995]. Although deleting the amino terminus of H3 or H4 only causes moderate growth defects, simultaneous deletion of the H3 and H4 amino termini is lethal [Morgan et al. 1991]. This suggests that these histone domains are redundant for one or more essential functions. To identify the essential functions of the H3 and H4 amino termini, we have carried out a synthetic-lethal screen for second-site mutations that cause the yeast cell to no longer tolerate the loss of the H3 amino terminus. This type of genetic screen has been used previously to identify new components of the nuclear pore complex [for review, see Doye and Hurt 1995] and to determine a role for Cln1 and Cln2 cyclins in cytokinesis [Cvrckova et al. 1995]. Most of the hsl [for histone synthetic lethal] mutations that we have obtained have a similar phenotype, causing G2 arrest in the histone mutant background. Their identification has uncovered a number of proteins that are intimately involved in reg-

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ulating the yeast cyclin-dependent protein kinase (CDK) known as Cdc28.

In the budding yeast *S. cerevisiae*, the Cdc28 gene product associates with multiple cyclins in a stage-specific manner to drive the major cell cycle transitions (Reed 1992; Nasmyth 1993). In addition, the mitotic Cdc28 kinase is subject to inhibitory tyrosine phosphorylation, a highly conserved mechanism of CDK regulation (for review, see Morgan 1995). In the fission yeast *Schizosaccharomyces pombe*, the tyrosine residue at position 15 (Y15) of cdc2 is phosphorylated by the wee1 protein kinase in G2 (Russell and Nurse 1987b; Featherstone and Russell 1991; Parker et al. 1992). The cdc25 protein dephosphorylates this tyrosine before entry into mitosis (Russell and Nurse 1986; Dunphy and Kumagai 1991). In budding yeast, the analogous tyrosine [Y19] in Cdc28 is phosphorylated by Swel and dephosphorylated by Mih1 (Russell et al. 1989; Boorer et al. 1993). In *S. pombe*, mutations interfering with Y15 phosphorylation of cdc2 disrupt the cell cycle checkpoint function that prevents mitosis when DNA replication is incomplete or DNA damage is present (Enoch and Nurse 1990; Lundgren et al. 1991). Surprisingly, in *S. cerevisiae*, mutating Y19 of Cdc28 to the unphosphorylatable phenylalanine has no effect on this checkpoint function [Amon et al. 1991; Sorger and Murray 1992]. However, Y19 phosphorylation is part of a novel morphogenesis checkpoint delaying mitosis when bud formation is impaired (Lew and Reed 1995).

The cell cycle-specific nature of CDK tyrosine phosphorylation and its importance in various checkpoint functions suggest that the wee1/Swel kinases and the cdc25/Mih1 phosphatases themselves must be subject to regulation. In the fission yeast, the nim1 protein kinase phosphorylates the carboxy-terminal catalytic domain of wee1 and inhibits its activity [Russell and Nurse 1987a; Feilotter et al. 1991; Coleman et al. 1993; Parker et al. 1993; Wu and Russell 1993]. Whether a nim1-like protein kinase regulates Swel in *S. cerevisiae* or the wee1 kinases in any other organism is presently unknown. However, this is not the only mechanism that regulates wee1-like proteins in eukaryotes. For example, specific degradation of the human wee1 kinase in M phase may also be an important mechanism in human cells [Watanabe et al. 1995].

Our analysis of *hsl* mutants has led to the identification of three new genes (*HSL1*, *HSL7*, and *OSS1*) that regulate the Swel kinase. Hsl1 has strong sequence and functional homology to cdc1/nim1, although it is much larger in size. Hsl7 is a novel negative regulator of Swel. Oss1 is a transcriptional repressor of both *SWE1* and *CLN2*. A fourth gene, *HSL5*, is identical to *CDC28* itself. These data suggest a strong link between the cell cycle machinery and histones, which are assembled during S phase and are required for chromosomal segregation in mitosis.

**Results**

Isolation of *hsl* mutants

The highly conserved amino termini of histones H3 and H4 are functionally redundant for mitotic growth in *S. cerevisiae* because cellular viability is lost only when both of these amino-terminal tails are deleted [Morgan et al. 1991]. However, it is unclear which essential functions in yeast cells require these amino-terminal tails and which gene products interact with these amino-terminal tails directly or indirectly to carry out these functions. To address these questions, we screened for second-site mutations that prevent cellular viability when the H3 amino terminus is deleted (i.e., a synthetic-lethal screen).

The design of our genetic screen is depicted in Figure 1. In *S. cerevisiae*, there are two copies of the H3 and H4 gene pair [designated as HHT1–HHF1 and HHT2–HHF2, respectively], which are arranged such that the H3 and H4 mRNAs are transcribed divergently from each pair [Smith and Murray 1983]. To screen for mutations (*hsl*) that are lethal in combination with the deletion of the H3 amino terminus, we used the yeast strain RMY430 [Mann and Grunstein 1992]. In this strain, both chromosomal copies of the H3 and H4 gene pair are deleted, and histones are supplied from two low-copy centromeric plasmids [Fig. 1]. Plasmid A carries wild-type H3 and H4 genes under the control of the GAL1-10 promoter and has the *URA3* gene as a selectable marker. Plasmid B carries the wild-type H4 gene and a mutant H3 gene, both under the control of their native promoters, the mutation in H3 (*H3Δ-30*) is a deletion of residues 4–30. This strain grows well when GAL-driven transcription of wild-type H3 is repressed in glucose medium or when plasmid A is lost [Mann and Grunstein 1992]. Therefore, viability of this strain does not depend on plasmid A.

After mutagenesis of this strain by ethylmethanesulfonate (EMS), we searched for mutants that had been...
come dependent on plasmid A, that is, on the presence of wild-type histone H3 for viability. This was tested by two methods. Because the wild-type H3 gene on plasmid A is under the control of the GAL1-10 promoter, hsl mutants should be sensitive to growth in glucose, which represses the GAL1-10 promoter. Second, hsl represses the mutants should be sensitive to the drug 5-fluoro-orotic acid (5-FOA), which selects against cells synthesizing Ura3 (Boeke et al. 1987). After screening some 12,000 mutantized colonies by a combination of these two methods, a total of six hsl mutants were isolated and characterized. By crossing each mutant to the parental strain, we found that all were recessive hsl mutations. Of these, hsl3 was represented twice. A second round of screening of 80,000 EMS-mutagenized colonies yielded only two new hsl mutants despite the much wider scope of the second screen. This suggests that although we may not yet have saturated the screen, there are a very limited number of HSL genes. Two recessive mutants (designated hsl1-1 and hsl7-1) were found to have unusual morphological defects. Most cells are large and bear elongated buds. In the synthetic-lethal state, >90% of the cells have an undivided nucleus at the neck of the long buds, similar to a G2/M arrest [data not shown]. We also tested whether hsl1-1 and hsl7-1 mutations are lethal in the H4 amino-terminal deletion background. To this end, plasmid B was lost from these strains, which were then transformed with a similar plasmid except that it contained a wild-type H3 gene and a mutant H4 gene. The mutant H4 tested [H4Δ4-23] contained a deletion of residues 4–23, a viable mutation described previously [Kayne et al. 1988]. The resulting strains were sensitive to 5-FOA, indicating that both hsl1-1 and hsl7-1 mutants are dependent on the H4 amino terminus for viability. Therefore, these two hsl mutants require both H3 and H4 amino termini for mitotic growth.

**HSL1 encodes a nim1-like protein kinase**

The synthetic lethality phenotype of hsl1-1 in the presence of the H3 amino-terminal deletion (H3Δ4-30) was used to clone the HSL1 gene by transformation with a wild-type genomic library. A single plasmid [pE14R1] that fully complemented hsl1-1 was recovered. Partial sequences of the insert fragment in this plasmid were obtained and used to search the data bases using the BLAST [Altschul et al. 1990] service at the National Center for Biotechnology Information [NCBI]. These sequences were found to be within chromosome XI, which had been sequenced completely by the yeast genome project [Dujon et al. 1994]. Deletion analysis indicated that the complementing open reading frame (ORF) corresponds to YKL101w [GenBank accession no. Z28101; Fig. 2A]. To ascertain that this ORF corresponds to HSL1, a gapped plasmid containing a large deletion within this ORF [pHSL1-gap; Fig. 2A] was transformed into hsl1-1 and HSL1+ cells. The gap-repaired plasmids [Orr-Weaver and Szostak 1983] recovered from the hsl1-1 strain were unable to complement hsl1-1, whereas those recovered from the HSL1+ strain were fully active. We conclude that the chromosomal locus YKL101w corresponds to HSL1.

To determine the null phenotype of hsl1, most of the coding region was deleted and replaced with URA3, creating a deletion/replacement allele hsl1Δ1 [Fig. 2A]. A wild-type diploid strain was transformed with this deletion allele. Diploids heterozygous for hsl1Δ1 were identified as described in Materials and methods, and tetrad analysis was carried out. Each of the 11 complete tetrads dissected gave four viable spores, indicating that HSL1 is not essential for viability. However, microscopic examination indicated that hsl1Δ1 cells are highly elongated [Fig. 2B, left], a phenotype that is similar to, but more severe than, the original hsl1-1 cells. DNA staining with DAPI (4',6-diamidino-2-phenylindole) indicated that most cells contain DNA at the mother bud neck and so are delayed in nuclear division [Fig. 2B, right]. A second deletion [hs1Δ2] removing only the amino-terminal third portion of HSL1 causes similar morphological defects (data not shown).

The predicted Hsl1 protein is 1518 amino acids in length, and has a molecular mass of 169 kD. The protein sequence of Hsl1 was compared with sequences in the available data bases. We found that the amino-terminal portion of Hsl1 showed the highest homology [48% identical in 304 residues, see Fig. 3A] to the cdrl/nim1 protein kinase of *S. pombe* (Russell and Nurse 1987a; Feilotter et al. 1991). The region most similar between Hsl1 and Cdr1/Nim1 contains all the subdomains identified previously among serine/threonine protein kinases [Hanks et al. 1988]. It is notable, however, that the long carboxyl terminus of Hsl1 is not homologous to any known proteins and that Hsl1 is almost three times as large as cdr1/nim1.

**Hsl1 negatively regulates the Swe1 kinase**

The high sequence homology of Hsl1 with nim1 suggests that Hsl1 may carry out analogous functions in *S. cerevisiae*. In *S. pombe*, nim1 inhibits the wee1 kinase by phosphorylating the catalytic domain of wee1 [Coleman et al. 1993; Parker et al. 1993; Wu and Russell 1993]. Therefore, nim1 deletion causes a G2-delay attributable to increased wee1 protein kinase activity, which inhibits the mitotic cdc2–cyclin B kinase [Russell and Nurse 1987a; Feilotter et al. 1991]. We analyzed the hsl1Δ1 cells for DNA content by fluorescence-activated cell sorting (FACS). hsl1Δ1 cells exhibit a significant delay in G2 as indicated by the much reduced G1 peak and increased G2 peak, when compared with the isogenic wild-type strain [Fig. 3B]. If this G2-delay is attributable to a loss of inhibition of the Swe1 kinase in hsl1Δ1 cells, simultaneous deletion of the SWE1 gene should suppress this G2 delay. FACS analysis of the hsl1Δ1 swe1Δ1 cells indicates that this is the case [Fig. 3B]. Therefore, these genetic data suggest that Hsl1 is a negative regulator of the Swe1 protein kinase.

In our attempts to clone the HSL1 gene by complementing the histone synthetic-lethal phenotype of hsl1-
Cloning and disruption of HSL1.

(A) Restriction map of the insert on plasmid pE14R1, which fully complemented the synthetic lethality of hsl1-1 with H3A430. (Bold arrows) The location of a previously uncharacterized ORF, YKL101w (Dujon et al. 1994). The + and − signs indicate the complementation activity of the corresponding fragments shown at left. Digestion of this plasmid with NruI and MluI creates a gapped plasmid (pHSL1-gap), which was used to recover the mutant allele in hsl1-1 by the technique of gap repair (Orr-Weaver and Szostak 1983). Two deletion alleles are shown to indicate the regions deleted. (B) Morphological defects of hsl1Δ1 cells. hsl1Δ1 cells were grown in SD-Ura at 30°C. After staining with DAPI, cells were examined microscopically. (Left) Phase contrast microscopy (light). DNA staining with DAPI. hsl1Δ1::URA3.
the other two ORFs are not similar to any known genes in the data bases.

To determine which ORF is responsible for the hsl7 complementation activity, we used PCR to clone YBR131w and YBR133c and their immediate flanking sequences. Multiple independent PCR clones were transformed into hsl7-l. Clones containing YBR131w had full complementation activity, whereas those containing YBR133c failed to complement. To determine whether YBR133c contained the hsl7-l mutation in the genome, we used PCR to clone the region containing YBR133c using genomic DNA isolated from the hsl7-l mutant. As with hsl7-l, genomic DNA were transformed into and none of the clones derived from the hsl7-l mutant. As with hsl7-l, genomic DNA was isolated, and both fully complemented hsl7-l.

Isolation of OSS1, an extra-copy suppressor of hsl1-1 and hsl7-1

During the cloning of HSL1 and HSL7, we isolated two other genes capable of suppressing hsl1-1 and hsl7-1. One of these genes is MIH1 [data not shown], as mentioned above. The analysis of the second gene is shown in Figure 6A. Deletion analysis indicated that hsl7Δ cells are delayed in G2, and this delay was suppressed completely by swe1Δ [Fig. 4C]. Therefore, like HSL1, HSL7 is also a negative regulator of the Swe1 kinase.

A database search using the encoded amino acid sequence of Hsl7 revealed no significant similarity to known genes. However, an uncharacterized ORF in Caenorhabditis elegans [GenBank accession no. U10402, Wilson et al. 1994] showed significant homology to Hsl7 along most of its length (Fig. 5). A search against the Database for Expressed Sequence Tags [dbEST] (Boguski et al. 1994) carried out at XREFdb (Bassett et al. 1995) revealed significant similarities to the encoded amino acid sequences of two human cDNA clones [R65681, deposited to the data bases by the WashU-Merck EST program and Affy et al. 1995]. Alignment of Hsl7 with these sequences is also shown in Figure 5. The two human EST peptides are highly similar to both Hsl7 and the C. elegans ORF U10402 in two adjacent regions. Strong similarities among these sequences suggest that Hsl7 may be a conserved protein from yeast to man.
Figure 4. Cloning and analysis of HSL7.

(A) Restriction map of the insert in plasmid pBB311, which fully complemented \textit{hsl7-1}. Sequence analysis indicated that the complete sequence of this fragment had been deposited to the databases by the Yeast Genome Project (accession no. X75891). Three potential ORFs are indicated. Fragments containing YBR131w and YBR133c were cloned into a low-copy yeast vector to test for complementation of \textit{hsl7-1} \( [+], \) full complementation; \(-\) no complementation. An internal deletion of the Nde fragment within YBR133c was made and marked with the \textit{URA3} gene to create the deletion allele \textit{hsl7M}.

(B) Morphology of \textit{hsl7A} cells. Strain MAY3 [\textit{hsl7A}] was grown in YEPD at 30°C, and the cells were examined microscopically after staining with DAPI. \textit{[Left]} Phase contrast microscopy; \textit{[right]} DNA staining. (C) FACS analysis. Wild-type (YDS2), \textit{hsl7A} (MAY3), and \textit{hsl7A swe1A} (MAY4) cells were grown logarithmically in YEPD at 30°C and prepared for FACS analysis.

\textit{SacI–SacII} fragment was sufficient for suppression. Sequencing of this fragment revealed that it contained only the carboxy-terminal portion of a large ORF present in the 3.5-kb \textit{EcoRI–SacI} fragment. The complete ORF encodes a 103-kD protein with no significant similarity to any known proteins but contains several potential phosphorylation sites for the Cdc28 kinase [Fig. 6B]. Later data base searches indicated that it is identical to an ORF designated YM8156.15c (accession no. Z49260), deposited to the data bases by the yeast genome project (G. Lye and C.M. Churcher; B. Barrell and M.A. Rajandream, both unpubl.). The \textit{EcoRI–SacI} region cloned by PCR using genomic DNA derived from \textit{hsl1-1} and \textit{hsl7-1} mutants can suppress both \textit{hsl1} and \textit{hsl7} to the same extent as the original clone pE148. Therefore, we conclude that this ORF represents a dosage-dependent suppressor of both \textit{hsl1-1} and \textit{hsl7-1} mutations, and was designated \textit{OSS1}, for \textit{hsl} one and seven suppressor.

An \textit{oss1} deletion allele removing most of the coding sequence in the \textit{SacI–SacII} fragment [\textit{oss1\Delta1::TRP1}, Fig. 6A] or a larger deletion allele removing most of the coding sequence in the \textit{EcoRI–SacI} fragment [\textit{oss1\Delta5::TRP1}, Fig. 6A] was readily introduced into wild-type haploid cells, indicating that \textit{OSS1} is not essential for mitotic growth. However, microscopic examination of the \textit{oss1\Delta} cells revealed that most cells have elongated buds [Fig. 6C, left], similar to those of \textit{hsl1A} and \textit{hsl7A} cells. Moreover, deleting the \textit{SWE1} gene in \textit{oss1\Delta} cells completely suppresses this abnormal morphology [Fig. 6C, right]. Therefore, \textit{OSS1} may suppress \textit{hsl1-1} and \textit{hsl7-1} by negatively regulating the \textit{Swe1} kinase.

We then asked whether \textit{OSS1} might also be an \textit{HSL} gene. We found that \textit{oss1\Delta} has a synthetic-lethal interaction with \textit{H3\Delta430} only in diploid cells. This may reflect that diploids are more sensitive to compromised CDK activity, as suggested from earlier observations that loss of Swi4, a transcription activator for \textit{Gj} cyclins, is lethal in diploid but not in haploid cells [Ogas et al. 1991].

\textit{Regulation of SWE1 and CLN2 transcription by OSS1} \textit{Oss1} does not have any homology to known kinases or phosphatases but is rich in glutamine residues, similar to those of certain known transcription factors [Mitchell and Tjian 1989]. Therefore, we asked whether \textit{OSS1} might be a negative regulator of \textit{SWE1} transcription. Because the level of Y19 phosphorylation on Cdc28 is reg-
Figure 5. Homology of Hsl7 with a C. elegans ORF (accession no. U10402) and two human EST-encoded peptides (accession nos. F12149 and R65681). Conserved residues are boxed and shaded.

It has been shown previously that G1 repression of CLN2 requires the G2 cyclins Clb1 and Clb2 (Amon et al. 1993). However, this Clb-dependent repression mechanism is not involved in the G2 repression of another GENES & DEVELOPMENT

ulated during the cell cycle (Amon et al. 1992; Sorger and Murray 1992), we wished to determine whether transcription of SWE1 may be regulated in parallel. Wild-type and isogenic oss1Δ cells were synchronized in G1 with the yeast mating pheromone α-factor, and mRNA was isolated from these cells as they were released from pheromone-induced arrest and progressed through the cell cycle. The SWE1 mRNA levels were analyzed by Northern blot hybridization. We found that the level of SWE1 transcript was strongly regulated during the cell cycle in wild-type cells showing peaks of mRNA levels at the G1/S boundary, as indicated by the emergence of small buds (Fig. 7A; data not shown). When the same RNA samples were analyzed for the G1 cyclin CLN2, it was apparent that the cell cycle profile of SWE1 mRNA was very similar to that of CLN2 (Fig. 7B), which is also maximally transcribed at the G1/S boundary (Wittenberg et al. 1990). When oss1Δ cells were analyzed in parallel, we found that the cell cycle regulation of SWE1 and CLN2 was disrupted in oss1Δ cells; activation of SWE1 and CLN2 transcription was slower by ~15 min, and their repression in G2 was delayed and incomplete (Fig. 7A,B). Therefore, OSS1 is necessary for the repression of both SWE1 and CLN2 transcription during G2 and M phases. It is notable, however, that the peak level of SWE1 and CLN2 transcription in oss1Δ cells was similar to that in wild-type cells, suggesting that OSS1 primarily regulates the timing of cell cycle-specific activation and repression of SWE1 and CLN2 transcription rather than the maximal amounts of their mRNAs.
class of G_{1}/S-specific transcripts represented by the S-phase cyclin Clb5 (Amon et al. 1993). Therefore, we asked whether transcription of CLB5 was affected by oss1Δ. Reprobing of the RNA blot with a CLB5 probe indicated that CLB5 and CLN2 are regulated in parallel in the wild-type cell cycle. However, compared with CLN2, G_{2} repression of CLB5 was much less affected by oss1Δ. Therefore, these results suggest that OSS1 is more important for the G_{2} repression of a subset of G_{1}/S-specific transcripts represented by SWE1 and CLN2.

Discussion

In a search for proteins that interact with the histone H3 and H4 amino termini to mediate essential cellular functions, we have isolated and characterized seven HSL genes, mutations in which cause lethality of strains carrying a deletion at the amino terminus of H3 or H4. Five of these (HSL1, HSL3, HSL5, HSL6, and HSL7) are necessary for the G_{2}-M transition [X.-J. Ma, Q. Lu, and M. Grunstein, unpubl.]. The data presented here demonstrate that Hsl1 and Hsl7 are negative regulators of Swel, an inhibitory protein kinase that phosphorylates Cdc28 at tyrosine 19. This conclusion is based on the following. [1] All the phenotypes of hsl1 and hsl7 are suppressed completely by deletion of SWE1. [2] MIH1 was isolated as a dosage-dependent suppressor of both hsl1 and hsl7. The fact that Mih1 dephosphorylates CDC28 Y19, which Swel phosphorylates, argues strongly that hsl1 and hsl7 mutant cells have excessive Swel activity. (3) OSS1, a second dosage-dependent suppressor of hsl1 and hsl7, represses SWE1 transcription in G_{1}. (4) A mutation at Y19 (Y19F) suppresses hsl1 phenotypes completely, suggesting that HSL1 functions in the pathway leading to Y19 phosphorylation by Swel. (5) There is strong homology between Hsl1 and nim1, the inhibitory kinase of weel. These data argue that the decreased activity of Cdc28 caused by mutations in HSL1 and HSL7 results in lethality in the presence of the H3 amino-terminal deletion (H3A4-30) or the H4 amino-terminal deletion (H4Δ4-23). In support of this, we have recently found...
that \(HSL5\) is identical to \(CDC28\), and the \(hsl5\) mutation also causes cells to accumulate at the \(G_2/M\) boundary [data not shown].

We have determined that the amino-terminal deletions of \(H3\) and \(H4\) do not reduce transcription of \(CDC28\) [data not shown]. Conversely, the \(hsl5/cdc28\) mutation does not deregulate histone transcription during the cell cycle [data not shown]. In addition, because \(HSL1\), \(HSL7\), and \(OSS1\) all are nonessential genes, it is unlikely that synthetic lethality is attributable to reduced transcription of these genes in the histone amino-terminal deletions background. Therefore, it is possible that Cdc28 functions upstream of the histone H3 and H4 amino termini for the \(G_2/M\) transition. However, other possibilities exist. First, it has been shown that H4 amino-terminal mutations can cause \(G_2\) delay by activating a \(RAD9\)-dependent checkpoint that monitors genome integrity [Megee et al. 1995]. Moreover, Swel may be involved in a checkpoint that monitors bud formation, delaying cells in \(G_2\) when bud formation is impaired [Lew and Reed 1995]. Therefore, the activation of the \(RAD9\) checkpoint in combination with Swel hyperactivation attributable to \(hsl\) mutations may cause \(G_2\) arrest. Second, histone amino-terminal mutations may deregulate other genes, which in turn causes lethality in combination with a decrease in Cdc28 function. At present we are unable to distinguish among these possible models of synthetic lethality. However, the high degree of specificity of our genetic screen, as highlighted by the fact that mutations in five of the seven \(HSL\) genes that we isolated after extensive searches all result in decreased Cdc28 function, is striking. Therefore, we favor the idea that Cdc28 may function upstream of histones, thereby regulating chromosome dynamics during the cell cycle.

To date, the only regulator of \(weel\) identified and characterized so far is the \(cdrl/niml\) kinase in \(S. pombe\) [Russell and Nurse 1987a; Feilotter et al. 1991]. A prior attempt to clone the \(Drosophila niml\) homolog by complementation of a \(cdrl/niml\) mutant in fission yeast was not successful [Warbrick and Glover 1994]. It is interesting to note that Hsl1 is highly similar to \(cdrl/niml\) both in sequence and function. Because hsl1A cells grow much slower than \(hsl1\)A cells, it is likely that Hsl7, a novel protein, regulates Swel by a different mechanism. We have determined that Hsl7 does not regulate \(SWE1\) mRNA levels during the cell cycle [data not shown]. The existence of similar proteins in \(C. elegans\) and humans suggests that it may also be a highly conserved regulatory protein. The fact that both Hsl1 and Hsl7 are conserved in sequence in evolution suggests that not only is CDK function highly conserved, but the regulatory mechanisms upstream of Swel/weel are also conserved. This conservation is particularly striking in light of the fact that the budding yeast uses the Swel pathway in a different checkpoint function from other organisms [Lew and Reed 1995].
In addition, we have found that transcription of SWEl is regulated coordinately with CLN2, a Gi cyclin, during the cell cycle. The mechanism for the coordinate control is suggested from an examination of the promoter regions of both SWEl and CLN2. The SWEl promoter contains two exact copies of the sequence 5'-CGACGCG-GAAAAA-3' (Booher et al. 1993). The CGCGGAAA sequence is also present in the regulatory sequence of CLN1 and CLN2 (Ogas et al. 1991) and is highly similar (1-bp mismatch) to the consensus sequence of the Swi4, 6-dependent cell cycle box (SCB) (CACGAAA) (Koch and Nasmyth 1994). This similarity in promoter sequence among SWEl, CLN1, and CLN2 suggests that they may share regulatory mechanisms. OSS1 is important for the cell cycle periodicity of both SWEl and CLN2 transcription. Although there is also a delay in the transcriptional activation of CLB5 in ossIA cells, it remains to be determined whether this occurs indirectly because of the delayed accumulation of Cln2 or reflects a more direct role for OSS1 in CLB5 transcription.

It has been shown previously that Gi repression of CLN2 requires mitotic cyclins, especially Clbl and Clb2 (Amon et al. 1993). However, it is unclear how Clbs repress CLN2 transcription and whether additional repressors are also required. Whether OSS1 is part of the Clb-dependent repression mechanism remains to be determined. However, the presence of multiple potential phosphorylation sites for Cdc28 within OSS1 would suggest that the activity of OSS1 may be subject to regulation by the Cdc28 protein kinase. Preliminary experiments indicated that some of these potential phosphorylation sites are critical for the suppressor activity of OSS1 (X. Ma and M. Grunstein, unpubl.). Therefore, it will be interesting to determine whether OSS1 activity is dependent on Cdc28 and mitotic cyclins.

Our results indicate that compromised mitotic Cdc28 activity, caused by nonlethal mutations in HSL1, HSL7, and CDC28, results in lethality of cells carrying viable H3 or H4 amino-terminal deletions. These cell cycle regulators are all important for the Gi/M transition. Significantly, histone H3 and H4 amino-terminal deletions also cause cells to accumulate in Gi/M (Kayne et al. 1988, Morgan et al. 1991), suggesting that the histone amino termini have important roles in allowing cells to progress through Gi. One possible role may be in replication-coupled nucleosome assembly during S phase (Kaufman and Botchan 1994; Kaufman et al. 1995). A close temporal link between DNA synthesis and nucleosome assembly is reflected by the fact that histone synthesis takes place in S phase (Hereford et al. 1981; Cross and Smith 1988). We have demonstrated recently that the amino-termini of H3 and H4 are required for nucleosome assembly in a redundant fashion both in vivo and in vitro (Ling et al. 1996). Because both H3 and H4 amino termini are phosphorylated in eukaryotic cells (Van Holde 1989), it is possible that a cascade of protein kinases controlled by Cdc28 may lead to the phosphorylation of histones and regulate their assembly into nucleosomes.

A second role for the H3 and H4 amino termini may be in the process of mitotic chromosome condensation (Guacci et al. 1994), which is triggered by the activation of mitotic CDKs (for review, see Yanagida 1995). A function for the H3 amino terminus during chromosome condensation has been suggested by earlier observations that phosphorylation of the serine residue at position 10 (S10) accompanies chromosome condensation (Gurley et al. 1978). However, the fact that the H3 mutant carrying a deletion of residues 4–30 including S10 is viable suggests that S10 phosphorylation alone is not essential for mitosis. Rather, the structure and phosphorylation state of other histone amino termini may also contribute to chromosome condensation. Consistent with this, removal of the amino-terminal tails of all four core histones by trypsin digestion prevents salt-induced condensation of polyinucleosomes in vitro (Garcia Ramirez et al. 1992). Moreover, deletion of the H4 amino terminus in vivo not only causes a delay of cell cycle progression in Gi but increased sensitivity of chromatin to micrococcal nuclease, suggesting a decondensed state (Kayne et al. 1988).

In conclusion, the ultimate result of a cell-division cycle is the accurate duplication and segregation of chromosomes. Our data have not only uncovered new regulators of Cdc28 but argue that the functional interactions between Cdc28 and histones are essential for the progression through the Gi/M transition. The mechanism by which Cdc28 may regulate nucleosome assembly or chromosomal condensation is a mystery at present. The discovery that Nap1, a nucleosome assembly protein conserved throughout evolution, is associated with Clb2 in S. cerevisiae may provide one missing link between the cell cycle and chromatin assembly (Kellogg et al. 1995; Kellogg and Murray 1995). How the CDK complex interacts with such proteins to coordinate nucleosome assembly and chromosomal condensation with other cell cycle events remains to be determined.

Materials and methods

Strains and media
Strains used in this study are listed in Table 1. Standard yeast growth media were used, and standard genetic techniques were followed (Ausubel et al. 1987). Rich medium contains YEP (1% yeast extract, 2% peptone) plus 2% glucose (YPEP) or 2% galactose (YEPG). Synthetic complete media (SD or SG, containing 2% glucose or galactose, respectively) lacking uracil or specified amino acids were prepared as described (Ausubel et al. 1987). 5-FOA was added to synthetic selective media at the concentration of 1 gram/liter. Yeast transformations were carried out as described (Gietz and Schiestl 1991).

Isolation of hisl mutants
The strain RMY430 was transformed with pRM102 (Mann and Grunstein 1992). The resulting strain was grown in SG - Ura - Trp to mid-log phase. A suspension of these cells was incubated with 3% EMS for 1 hr at 30°C. Surviving cells (10%–20% of the starting cells) were grown on SG - Ura - Trp plates for 3 days at 30°C. Colonies (~12,000) were replica-plated onto SD - Ura - Trp or SG - Trp + 5-FOA for 2 days at 30°C. Colonies on the master plates and the replica plates were compared, and
Table 1. Strains used in this study

| Strain   | Genotype                                         | Origin/reference               |
|----------|--------------------------------------------------|--------------------------------|
| RMY430   | MATa ade2-101 his3-Δ201 lys2-801 trp1-Δ901 ura3-52 hht1 hhf1::LEU2 hht2hh2::HIS3 plus pRM430 | Mann and Grunstein [1992]      |
| YDS2     | MATa ade2-1 trp1 can1-100 leu2-3,112 his3-11,15 ura3-23 | David Shore (Columbia University, New York, NY) |
| YDS3     | MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-23 | this study                     |
| YDS23    | MATa/a ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-23 | this study                     |
| MAY1     | MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-23 hs1Δ1::URA3 | this study                     |
| MAY2     | MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-23 hs1Δ1::URA3 swe1Δ::LEU2 | this study                     |
| MAY3     | MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-23 hs1Δ1::URA3  | this study                     |
| MAY4     | MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-23 hs1Δ1::URA3 swe1Δ::LEU2 | this study                     |
| MAY5     | MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-23 os1Δ1::TRP1 | this study                     |
| MAY6     | MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-23 os1Δ1::TRP1 swe1Δ::LEU2 | this study                     |
| MAY7     | MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-23 os1Δ5::TRP1 | this study                     |
| PS692    | MATa ura3-52 leu2 trp1-1 CDC28::TRP1               | Sorger and Murray [1992]       |
| PS694    | MATa ura3-52 leu2 trp1-1 cdc28-F::TRP1             | Sorger and Murray [1992]       |

those failing to grow on the glucose and 5-FOA plates were identified as candidate hsl mutants whose viability became dependent on the GAL-controlled wild-type H3 gene present on pRM102 (plasmid A, see Fig. 1). To eliminate those mutants that depend on plasmid A because of inviable mutations on the H3 or H4 genes on pRM430 (plasmid B), all candidates were grown nonselectively on YEPG to lose plasmid B. These strains were retransformed with plasmid B or pRM200, which carries both wild-type H3 and H4 with their native promoters (Mann and Grunstein 1992). The resulting transformants were re-tested for 5-FOA sensitivity. Mutants that remained sensitive to 5-FOA with the newly introduced plasmid B, but became 5-FOA resistant when containing pRM200, were selected as final hsl mutants.

To determine for dominance of the hsl mutations, the collection of hsl mutants were crossed to the parental strain of the opposite mating type (MATa). The resulting diploids were tested for histone synthetic lethality on 5-FOA plates. Recessive hsl mutations were indicated by the 5-FOA resistance of heterozygous diploids.

Cloning of HSL1, HSL7, and OSS1

Wild-type genomic libraries were constructed by partial digestion of genomic DNA with Sph3AI or a combination of BamHl and BglIII, and ligating fragments of 8–12 kb into plRS200 (Sikorski and Hieter 1989) digested with BamHl and BglIII. A genomic library on plRS200 purchased from American Type Culture Collection (Rockville, MD) was also used in some of the cloning experiments.

For the purpose of transformation with the TRP1-marked genomic libraries, pRM430 (plasmid B) in hsl mutants was exchanged for pMX430. pMX430 was constructed by cloning the EcoRI-Sall fragment containing the H3 and H4 genes from pRM430 into the vector pRS317, a LYS2-marked centromeric vector (Sikorski and Hieter 1989). Mutants were grown in SC – Ura – Lys medium and transformed with genomic library DNA. Transformants were replica-plated onto SD – Lys – Trp + 5-FOA plates. Colonies from 5-FOA plates were regrown in SD – Lys – Trp medium, and total DNA extracted. Plasmids were recovered after transformation of total yeast DNA into Escherichia coli (DH5α).

For the hsl1-1 mutant, a total of four plasmids were found to rescue the hsl phenotype. One clone (pE14R1) fully rescued this mutant, and three other plasmids (pE147, pE148, and pE149) only allowed slow growth on 5-FOA plates. pE147 and pE149 had overlapping inserts based on restriction analyses. The overlapping region was partially sequenced and found to contain the MHI1 gene, which was responsible for the rescue activity. Deletion analysis of pE148 was performed, and the 3.5-kb EcoRI–Sall fragment containing full activity was sequenced completely using the Erase-a-Base system (Promega, Madison, WI) and synthetic oligonucleotides. Partial sequencing of the clone pE14R1 indicated that it contained an 8-kb fragment sequenced completely by the S. cerevisiae sequencing project (Dujon et al. 1994). Deletion analysis using restriction sites was carried out to determine the gene responsible for hsl1 complementation.

To determine whether pE14R1 contained the HSL1 gene as opposed to a copy-dependent suppressor of hsl1-1, the gap-repair technique (Orr-Weaver and Szostak 1983) was used to show that the corresponding chromosomal region in hsl1-1 mutant was incapable of complementing hsl1. As control, similarly gap-repaired plasmids from HSL1 wild-type strain were fully active in hsl1-1 complementation. Therefore, the pE14R1 contained the HSL1 gene. In contrast, the gene present in pE148, capable of rescuing hsl1-1 and hsl1-1, was phenotypically wild type in either the hsl1-1 or the hsl1-1 mutant. This was determined as follows: Genomic DNAs derived from hsl1-1, hsl1-1, and HSL1 strains were used to amplify the 3.5-kb EcoRI–Sall region by PCR, and the resulting PCR products were cloned into pRS200. The primers used are 5' primer, AGATCTGAATTCTATCATCGAAGCGACCG, and 3' primer, AGCAGTCATTGGCAGTATCATCCCTGACGG.

For the purpose of transformation with the TRP1-marked genomic libraries, pRM430 (plasmid B) in hsl mutants was exchanged for pMX430. pMX430 was constructed by cloning the EcoRI–Sall fragment containing the H3 and H4 genes from pRM430 into the vector pRS317, a LYS2-marked centromeric vector (Sikorski and Hieter 1989). Mutants were grown in SC – Ura – Lys medium and transformed with genomic library DNA. Transformants were replica-plated onto SD – Lys – Trp + 5-FOA plates. Colonies from 5-FOA plates were regrown in SD – Lys – Trp medium, and total DNA extracted. Plasmids were recovered after transformation of total yeast DNA into Escherichia coli (DH5α).

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Multiple clones derived from each strain background were tested for rescue activity of hsl1-1 and hsl1-1, and all clones derived from both the mutants and the wild type were identical in complementation activity. Therefore, we conclude that the 3.5-kb EcoRI–Sall region contained a dosage-dependent suppressor, which was designated as OSS1.

Complementation cloning of hsl7-1 yielded two complementing plasmids. pBB311 fully complemented hsl7-1, whereas...
p311 only allowed slow growth of the *hsl7* mutant on a 5-FOA plate. Restriction and sequence analyses indicated that p311 overlapped with PE148 in the *OSS1* region. The insert in pBB311 was sequenced from both ends and found to contain an 8-kb region completely sequenced by the yeast genome project (Feldmann et al. 1994). Regions containing YBY133c and YBR131w were cloned individually into pRS200 after PCR amplification using wild-type genomic DNA as template. The primers for cloning YBR133c were 5’ primer, CTTGAGCTCTGGTGGCATGAG (the *SacI* site is underlined), and 3’ primer, GACTTGTTGATCCATAGGCT (the *KpnI* site is underlined). The primers used for PCR YBR131w were 5’ primer, GTTAGAATGACGCTCGCCCGAAA (the *SacI* site is underlined), and 3’ primer, ATTTCTATGGACCCCAATGCTAACG (the *SalI* site is underlined). Unique restriction enzyme sites, as indicated above, were engineered into each primer to facilitate cloning. To recover the mutant allele in the *hsl7-l* mutant, the same primers used to amplify YBR133c by PCR, as described above, were used to amplify and clone the corresponding region from the *hsl7-l* genome.

**Construction of null alleles of hsl1l, hsl7l, and oss1 and swel**

To construct plasmids for one-step gene disruption of *HSL1*, the 6-kb *KpnI–SacI* fragment from PE141R1 was cloned into pBlueScript SK. The resulting plasmid was digested with *NruI* and *MluI* (*hsl1A-l*) or *NruI* and *Stul* (*hsl1A-2*) to remove *HSL1*-coding sequences, which were replaced by the *URA3* gene isolated from Yep24.

The *SacI–KpnI* fragment containing *HSL7* obtained by PCR using primers described in the last section was subcloned into pBlueScript SK. The internal *NarI* fragment was deleted from the insert and replaced with the *URA3* gene.

The *oss1A-l* allele was constructed as follows: The 1.3-kb *SacI–SacII* fragment was subcloned into pBlueScript SK, and resulting plasmid was cut with *HindIII* to delete the *OSS1*-coding sequence, which was replaced by *TRP1*. The *oss1A-5* allele was constructed as follows: The 3.5-kb *EcoRI–SacII* fragment was subcloned into pBlueScript, the resulting plasmid was cut with *Eco47III* and *Stul*, and the *OSS1*-coding sequence was replaced with the *TRF* gene.

A *swel* disruption plasmid *swelA::LEU2* was kindly provided by R. Boorer (Boorer et al. 1993). Plasmids containing disruption alleles described above were cut to generate the gene disruption fragments, which were used to transform haploid and diploid cells. Correct gene replacements were confirmed by Southern blotting and PCR analysis.

**Cell culture synchronization, RNA isolation, and Northern analysis**

Exponentially growing cultures (OD<sub>600</sub> of 0.2–0.3) of appropriate strains were incubated with two doses of a-factor (5 µg/ml) in YPD to arrest cells in G1, for a total of 160 min. After washing the cells with water to remove a-factor, the cells were returned to growth in fresh YPD at 30°C. Portions of the culture were taken at 15-min intervals, and total RNA isolated as described (Cross and Tinkelenberg 1991). RNA was separated on a formaldehyde-agarose gel and transferred onto Nytran Plus membrane (Schleicher & Schuell). The RNA blot was hybridized with probes labeled with <sup>32</sup>P by the random prime labeling technique (Feinberg and Vogelstein 1984). The probes for *SWE1*, *CLB5*, and *CDC28* were obtained by amplifying the coding region of the respective genes by PCR. The *CLN2* probe was a gift from Steve Reed (Scripps Research Institute, La Jolla, CA).

**FACS analysis**

Approximately 1x10<sup>7</sup> cells from exponentially growing cultures were sonicated to disrupt cell aggregates and fixed with 70% ethanol overnight at 4°C. The fixed cells were washed with 50 mM sodium citrate (pH 7) and resuspended in the same buffer containing RNase A at 10 µg/ml. RNase digestion was carried out for 3 hr at 37°C. The resulting cells were stained with propidium iodide (50 µg/ml) in 180 mM Tris at pH 7.5, 190 mM NaCl, and 70 mM MgCl<sub>2</sub> overnight. The cells were analyzed on Beckton-Dickson FACScan. Ten thousand events were analyzed for each sample.

**DNA sequencing and sequence analysis**

All sequencing reactions were carried out with double-stranded plasmid DNA as template using Sequenase version 2.0 (U.S. Biochemical-Amersham) according to the manufacturer’s recommendations. The Erase-a-Base system (Promega) was used to create sequential deletions for sequencing *OSS1*. Synthetic oligonucleotides were also used to obtain the complete sequence of *OSS1*.

DNA sequences were analyzed by DNA Strider and the Wisconsin GCG package (Madison, WI). Homology searches were carried out using the BLAST (Altschul et al. 1990) service at NCBI.

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*Genes Dev.* 1996, 10: Access the most recent version at doi:10.1101/gad.10.11.1327

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