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Hsl7p, the Yeast Homologue of Human JBP1, Is a Protein Methyltransferase

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The yeast protein Hsl7p is a homologue of Janus kinase binding protein 1, JBP1, a newly characterized protein methyltransferase. In this report, Hsl7p also is shown to be a methyltransferase. It can be crosslinked to [^{3}H]*S*-adenosylmethionine and exhibits *in vitro* protein methylation activity. Calf histones H2A and H4 and bovine myelin basic protein were methylated by Hsl7p, whereas histones H1, H2B, and H3 and bovine cytochrome *c* were not. We demonstrated that JBP1 can complement *Saccharomyces cerevisiae* with a disrupted *HSL7* gene as judged by a reduction of the elongated bud phenotype, and a point mutation in the JBP1 *S*-adenosylmethionine consensus binding sequence eliminated all complementation by JBP1. Therefore, we conclude the yeast protein Hsl7p is a sequence and functional homologue of JBP1. These data provide evidence for an intricate link between protein methylation and macroscopic changes in yeast morphology.

Key Words: **protein methyltransferase; signal transduction; Janus kinase; *Saccharomyces cerevisiae;* Hsl7p.**

To delineate events which occur downstream of the interferon and other receptors, a two-hybrid screen was used to identify proteins which bind to Jak2 (1). Four Jak2-binding proteins were identified. One, designated Janus kinase binding protein 1, JBP1, represents the human member of a conserved group of proteins implicated in control of the cell cycle and cell morphology (1–3). JBP1 exhibits homology to several other protein methyltransferases in the region to which AdoMet binds. In addition, we reported that JBP1 is a protein methyltransferase capable of methylating histones 2A and 4 and myelin basic protein.

The *HSL7* (histone synthetic lethal 7) gene of *Saccharomyces cerevisiae* was originally defined as a gene which is lethal when mutated in combination with histone H3 (2). In addition, Hsl7p was found to be a negative regulator of Swe1p and Ste20p function as well as a protein which associates with the septin ring during bud formation (4, 5). Disruption of *HSL7* was reported to result in cell cycle abnormalities and the production of extremely long buds (2, 4, 5). Because of the homology between Hsl7p and JBP1, we hypothesized that Hsl7p is also a protein methyltransferase. To test this hypothesis, we produced an *hsl7*Δ strain of *S. cerevisiae.* The phenotype of the *hsl7*Δ strain is characterized by elongated buds (2). Here we report that Hsl7p is a protein methyltransferase and that JBP1 can complement yeast lacking the *HSL7* gene.

MATERIALS AND METHODS

Materials. Calf thymus histones were obtained from Roche Molecular Biochemicals (Indianapolis, IN); bovine myelin basic protein, cytochrome *c*, *S*-adenosylhomocysteine from Sigma (St. Louis, MO); [^{3}H]AdoMet (specific activity 55-85 Ci/mmol) from New England Nuclear (Boston, MA); and protein A/G PLUS-agarose beads from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell growth conditions. Standard yeast genetics and transformation methods were employed (6, 7). Yeast were grown in either YEPD or synthetic media (SD/-Trp) with 2% glucose. Induction of genes under control of the *GAL1* promoter (pTKB175) was performed in SD/-Trp with 2% galactose.

Plasmid constructs. Yeast genomic DNA was obtained from Research Genetics, Inc. (Huntsville, AL). The *HSL7* gene was amplified with 5′ and 3′ primers CTGCAGTACAAAGGGTTCAGTTTG and GTCGACCAGTATATAGTATACAATGC, respectively. The amplicon digested with *Pst*I and *Sal*I, and then subcloned into plasmid pTKB175 under control of the *GAL1* promoter and containing the *TRP1* marker (8). The R368A mutant of JBP1 and wild type JBP1 cDNAs were constructed in plasmid pcDNA3 as reported (1). The JBP1 plasmids were digested with *Bam*HI and *Apa*I and subcloned into pTKB175 producing plasmids pGALJBP1-MT and pGALJBP1-

Abbreviations used: JBPl, Janus kinase binding protein 1; AdoMet, *S*-adenosyl-L-methionine; MBP, myelin basic protein.

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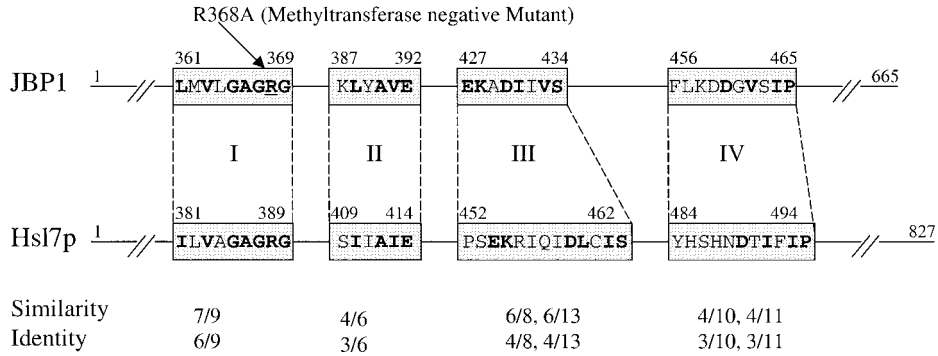


FIG. 1. Comparison of JBP1 and Hsl7p sequences. Identical and similar amino acids are in bold face. R368A indicates the location of the point mutation introduced in subdomain I of the consensus AdoMet binding site. Mutagenesis was performed as described (1). Similarity/identity numbers were calculated for each of the subdomains (I–IV). For domains III and IV, two percentages were calculated since the domains are of different sizes in HS17p and JBP1. The first set of percentages indicates similarities and identities based on the length of the *HSL7* sequence. The second set of sequences indicates similarities and identities based on the length of the JBP1 sequence.

WT, respectively. The Flag-HSL7 construct was produced by amplification of yeast genomic DNA with the 3′ primer defined above plus a 5′ primer (CGCGGATCCGCGATGGACTACAAGGACGA-CGATGACAAGATGCATAGCAACGTATTTGTTGGT) which encodes a Flag epitope (underlined bases encode Flag). The amplified DNA was digested with *Bam*HI and *Sal*I and subcloned into plasmid pTKB175 producing the yeast expression vector pGALFLAGHSL7.

Production of hsl7Δ cells. The *hsl7::URA3* disruption in pBluescript was a gift from Dr. M. Grunstein (2). This construct was transformed into haploid wild-type yeast (TKY307; Table 1) and transformants were selected with SD/-Ura medium. Disruption of *HSL7* was confirmed by Southern blotting.

Complementation assay. Cells were grown in 2% galactose overnight. An aliquot was used to reinoculate cultures which were grown to mid-log phase. The cells with and without elongated buds were then counted with a hemocytometer. At least three fields were examined for each determination. Photographs of yeast strains were made with a Zeiss Axioplan phase contrast microscope.

UV crosslinking. Crosslinking of [^{3}H]AdoMet to Hsl7p and JBP1 was performed as described (1).

In vitro methylation. Immunoprecipitated Flag-Hsl7p was used to methylate histones and myelin basic protein with minor modifications of the method previously described (1). Each reaction contained 10 μg of substrate proteins (histones, MBP, and cytochrome *c*). [^{3}H]AdoMet was added to a final concentration of 55 μCi/ml. The reaction contained 150 mM NaCl, 50 mM Tris · HCl (pH 8.0), 1% NP-40, 0.8 mM PMSF, 3 μg/ml antipain, 10 μg/ml benzamidine (10^4 kallikrein-inactivating units/ml) plus 1 μg/ml each of leupeptin, chymostatin, and pepstatin. The 50 μl reactions were incubated at 30°C for 30 min.

RESULTS

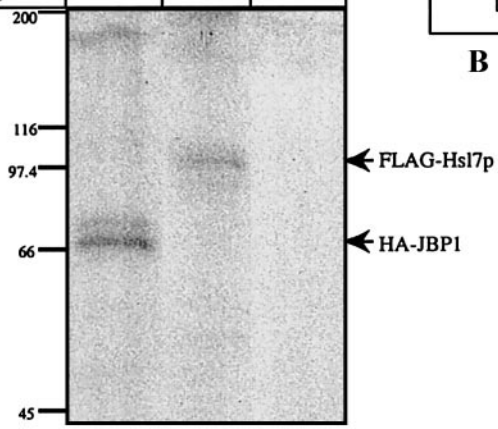
The human Jak-binding protein, JBP1, was identified in a two-hybrid screen with a 3.33-kb fragment of the Jak2 cDNA as a bait (1). When the full length JBP1 cDNA was sequenced, it was determined that JBP1 is homologous to a number of other eukaryotic protein methyltransferases, including human PRMT1 and PRMT2, rat PRMT1, and *S. cerevisiae* Hmt1p. The homology between JBP1 and Hsl7p (Fig. 1) suggests that yeast Hsl7p is also a protein methyltransferase. There are four conserved subdomains in JBP1 and Hsl7p (9). The first of these regions contains a GxGRG motif which is identical between the human and yeast proteins. This motif is known to be the site at which AdoMet is bound to the protein (10). Figure 1 shows the location of a point mutation (R368A) which was introduced in the GxGRG motif in order to produce a JBP1 without methyltransferase activity when histones or myelin basic protein are used as substrates (1).

To determine whether Hsl7p is a methyltransferase, we first asked whether Hsl7p can bind [^{3}H]AdoMet. As shown in Fig. 2, JBP1 from HeLa cells was crosslinked to [^{3}H]AdoMet (Fig. 2A, lane 1). The molecular size of this band is 72 kDa, which is the size of JBP1. When an anti-flag antibody was used to immunoprecipitate Hsl7p from *hsl7*Δ cells transformed pGALFLAGHSL7 (SPY103), a band of 97 kDa was observed at the predicted size of Flag-Hsl7p (Fig. 2A, lane 2). These results demonstrate that Hsl7p binds AdoMet, consistent with its being a methyltransferase. To examine *in vitro* methylation by Hsl7p, calf histones, bovine myelin basic protein and bovine cytochrome *c* were incubated with immunoprecipitated Hsl7p and [^{3}H]AdoMet. Figure 2B shows that H2A, H4 and myelin basic protein were methylated by Hsl7p while H1, H2B, H3 and cytochrome *c* were not. This pattern of methylation by Hsl7p is identical to that observed with human JBP1 (1), indicating that Hsl7p and JBP1 are functional as well as structural homologues. The data of Fig. 2C demonstrate that homocysteine, an inhibitor of methyltransferases that use AdoMet as the methyl donor, blocks the methylation of myelin basic protein by both JBP1 and Hsl7p.

To investigate whether JBP1 and Hsl7p are functional homologues, the *HSL7* gene was disrupted in the haploid *S. cerevisiae* strain TKY307 with an *hsl7*Δ construct which has a 1.14-kb section of the gene replaced with the *URA3* gene (2). The resultant strain, SPY101, was used

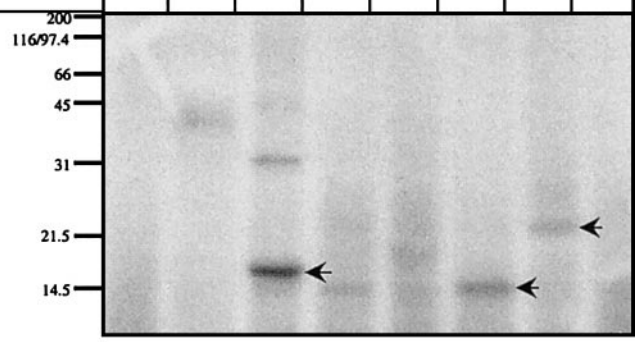
ENZYME \ LANES	1	2	3
HA-JBP1 IP	+	-	-
FLAG-Hsl7p IP	-	+	-
*hsl7*Δ(SPY101) Lysate	-	-	+

A



ADDITIONS \ LANES	1	2	3	4	5	6	7	8
FLAG-Hsl7p IP	+	+	+	+	+	+	+	+
SUBSTRATE	-	H1	H2A	H2B	H3	H4	MBP	Cyc c
[^{3}H]AdoMet	+	+	+	+	+	+	+	+

B



ADDITIONS \ LANES	1	2	3	4	MW	5	6	7	8
HeLa-HA-JBP1 IP	+	+	+	+	-	-	-	-	-
SPY101-FLAG-Hsl7p IP	-	-	-	-	-	+	+	+	+
SUBSTRATE(MBP 10μg)	-	+	+	+	-	-	+	+	+
INHIBITOR (HOMOCYSTEINE)	-	-	1mM	3mM	-	-	-	1mM	3mM

C

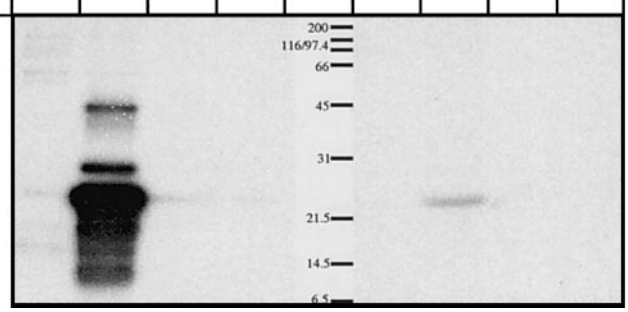


FIG. 2. (A) UV crosslinking of [^{3}H]AdoMet to Hsl7p. Details are essentially the same as those described previously (1). After UV crosslinking, the 7.5% gel was dried and exposed to Biomax MR film for 15 days at −70°C. (B) *In vitro* methylation of protein substrates by Hsl7p. Methylation reactions were done as described under Materials and Methods. Each lane contained 10 μg at substrate protein. The position of the labeled bands coincided exactly with the location of the substrate proteins or the Coomassie blue-stained gel (not shown). The dried 15% gel was exposed to Biomax MR film for 21 days at −70°C. (C) Inhibition of myelin basic protein *in vitro* methylation by homocysteine. Protein methylation reactions were conducted as described under Materials and Methods, except that homocysteine was added to the other reaction components on ice. The reactions were then incubated at 30°C for 30 min. The 15% gel was exposed to Biomax MR film for 14 days at −70°C. Protein sizes were calculated by the migration of broad range protein standards (Bio-Rad).

as a host for other constructs (Table 1). Vectors pTKB175, pGALFLAGHSL7, pGALJBP1-WT and pGALJBP1-MT were transformed into *hsl7*Δ cells (SPY101) to produce strains SPY102, SPY103, SPY104 and SPY105, respectively. These strains were grown in SD/-Trp with either 2% glucose (uninduced) or 2% galactose (induced). The morphology of these strains was similar to that reported previously: the wild type yeast have small circular or oval

TABLE 1

Genotype of Strains Used in This Study

Strain	Genotype
TKY307	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1*
SPY101	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 hsl7Δ::URA3*
SYP102	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 hsl7Δ::URA3 pTRP1*
SPY103	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 hsl7Δ::URA3 pTRP1 GALFLAG-HSL7-WT*
SPY104	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 hsl7Δ::URA3 pTRP1 GALJBP1-WT*
SPY105	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 hsl7Δ::URA3 pTRP1 GALJBP1-MT*

Note. The *hsl7* knock-out strain was generated by the homologous recombination as described under Experimental Procedures. Plasmids pGALFLAGHSL7, pGALJBP1-WT, and pGALJBP1-MT were constructed in the yeast expression vector pTKB175 having a *TRP1* marker.

buds (Fig. 3A) whereas the *hsl7*Δ cells have elongated buds (Fig. 3B). Complementation of the *hsl7*Δ cells with the *HSL7* expression vector yielded cells with a normal phenotype (Fig. 3C). In addition, complementation of the *hsl7*Δ cells with the JBP1-WT expression vector produced cells which were nearly normal (Fig. 3D), but complementation with the mutant JBP1-MT did not (Fig. 3E). Quantitation of the number of cells expressing the elongated bud phenotype in each strain, is shown in Fig. 4. Wild type cells (TKY307) were found to have no elongated buds, whereas 15% of the *hsl7*Δ cells expressed elongated buds (SPY101, Fig. 3B). When *hsl7*Δ cells were transformed with pGALFLAGHSL7 (SPY103, Fig. 3C), the wild type phenotype was completely restored whereas the vector alone had no effect (SPY102, Fig. 4). To determine the extent JBP1 complements its Hsl7p homologue, the *hsl7*Δ cells were transformed with the cDNA for human JBP1 expressed under control of the yeast GAL1 promoter. In the presence of 2% galactose, the percentage of cells with elongated buds was reduced by approximately 70% (SPY104, Fig. 4) compared to *hsl7*Δ cells grown in galactose (SPY101, Fig. 4), or compared to *hsl7*Δ+JBP1-WT cells (SPY104) grown in glucose (data not shown). Since Hsl7p and JBP1 may each perform a number of cellular functions, it was important to determine whether the observed phenotypic complementation is due to protein methyltransferase activity of JBP1 or to some other function. JBP1 was therefore mutated (R368A) at the GxGRG motif and the vector expressing the mutant JBP1 was transformed into *hsl7*Δ cells (SPY101). As shown in Fig. 4, *hsl7*Δ+JBP1-MT (SPY105) cells have as many elongated buds as does the *hsl7*Δ strain. This demonstrates that JBP1 is a functional homologue of Hsl7p and that the protein methyltransferase activity is required for complementation.

DISCUSSION

Hsl7p is crucial for many functions in yeast. Disruption of *HSL7* affects cell morphology (2, 4, 5), cell cycle progression (2) and sensitivity to chemicals, including calcium, caffeine, calcofluor white, vanadate, and verapamil (11). The fact that Hsl7p is a protein methyltransferase and that the mutant JBP1-MT does not complement *hsl7*Δ indicates that some of the phenotypic effects of Hsl7p and JBP1 are produced by methylation of target proteins. In our *in vitro* methylation assays we used histones and myelin basic protein as methyl group acceptors, however, the identity of the *in vivo* substrates for Hsl7p remains to be determined.

A number of methyltransferases have been identified in yeast: mRNA cap (12), rRNA (13), isoprenylcysteine (14) and tRNA (15) methyltransferases; two protein methyltransferases in *S. cerevisiae:* Rmt1p (also referred to as Hmt1p or Odp1p; see 16, 17) and Rmt2p (9). Rmt2p was discovered during a search for yeast proteins containing conserved AdoMet binding motifs (9), it methylates the δ-nitrogen atom of arginine residues, but its *in vivo* substrate proteins are not known. Rmt1p, on the other hand, is an arginine methyltransferase which methylates a number of yeast proteins such as Np13p and Hrp1p, which are hnRNPs (18, 19) and poly(A)+ RNA binding proteins (16). *In vitro*

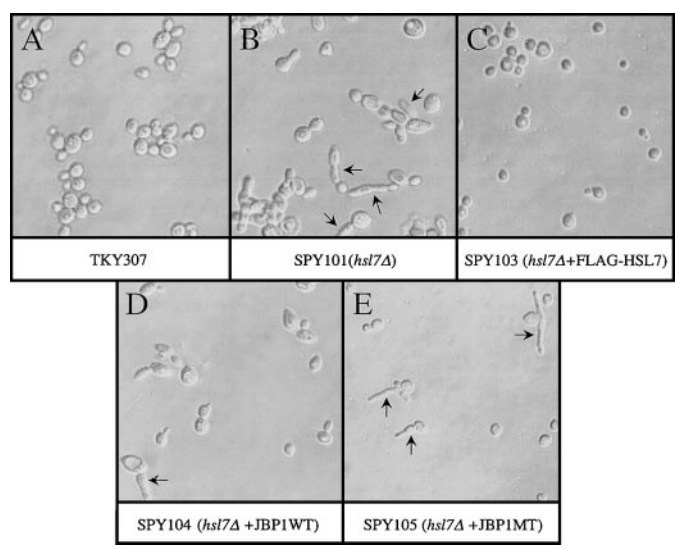


FIG. 3. Morphological characteristics of different yeast strains used in this study. A, wild type (TKY307) yeast; B, yeast with a disrupted *HSL7* gene (SPY101); C, *hsl7*Δ yeast transformed with pGALFLAGHSL7 (SPY103); D, *hsl7*Δ yeast expressing JBP1 (SPY104); E, *hs17*Δ yeast expressing JBP1-MT (SPY105). Arrows indicate cells with elongated buds. All cells were grown in media containing 2% galactose to induce the gene under the control of the *GAL1* promoter. The elongated bud phenotype was never observed in wild type yeast or in *hs17*Δ yeast transformed with pGALFLAGHSL7 (SPY103). In the *hsl7*Δ strain, 15 to 20% of the yeast have elongated buds. Complementation with JBP1 reduces the elongated bud phenotype significantly but not completely.

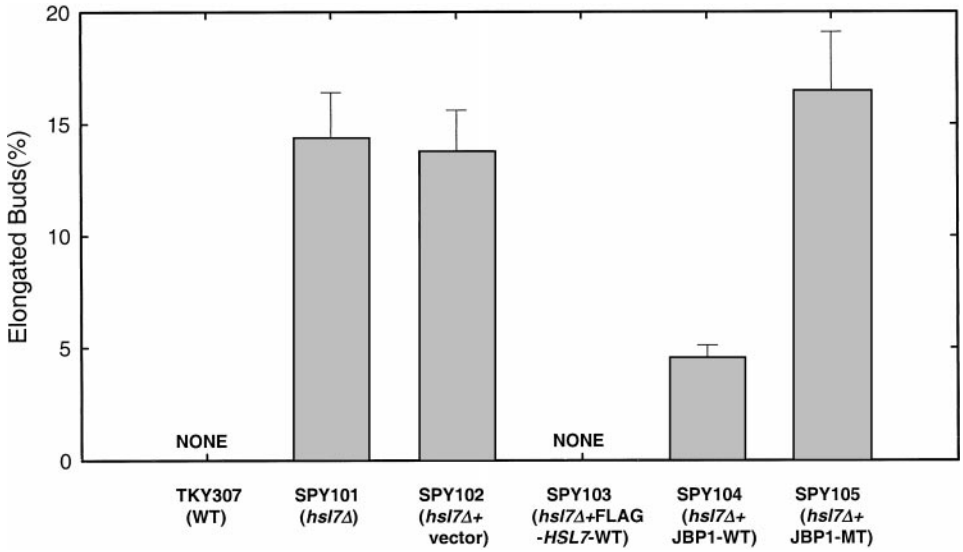


FIG. 4. Effect of *HSL7* gene disruption and complementation on elongated bud phenotype in yeast. Cells were grown in 2% galactose to induce the gene under the control of the *GAL1* promoter. At least three different fields were counted for each determination. JBP1-WT refers to the wild-type JBP1 cDNA; JBP1-MT indicates the mutated (R368A) JBP1 cDNA. Values are ±SEM.

Rmt1p methylates mammalian hnRNP A1, cytochrome *c*, histones and myoglobin, but not myelin basic protein. Clearly, Hsl7p exhibits different substrate specificity *in vitro* than Rmt1p. Hsl7p methylates myelin basic protein whereas Rmt1p does not; Rmt1p methylates cytochrome *c* whereas Hsl7p does not. These differences imply that Hsl7p and Rmt1p play distinct cellular roles.

The phenotypic complementation assays indicate that JBP1 does not completely rescue *hsl7*Δ cells (SPY104). Differences in the yeast lysate proteins methylated by JBP1 and Hsl7p (J.-H. Lee, J. R. Cook, and S. Pestka, unpublished results) could account for this. The R368A mutation of JBP1 which did not restore normal morphology demonstrated that complementation in *hsl7*Δ yeast absolutely requires methyltransferase activity. Although we have illustrated only four conserved regions in JBP1 and Hsl7p (Fig. 1), JBP1 and Hsl7p share extensive homology in other regions as well (1).

The *S. pombe* homologue of Hsl7p is skb1, a protein which is known to interact with the kinase Shk1 (3). A *S. pombe* skb1 deletion mutant exhibits altered morphology where the wild type cells are more elongated than the mutants and over-expression of skb1 results in hyper-elongated cells. JBP1 was shown to functionally complement skb1 in terms of cell length while Hsl7p did not (20). Nevertheless, the roles of skb1 and Hsl7p *S. pombe* and *S. cerevisiae*, respectively, are likely to be similar. For example, Skb1 and Hsl7p are involved in the ras signaling pathway in *S. pombe* (3) and in *S. cerevisiae* (4); and deletion of both genes produces cells with growth abnormalities (2, 3).

In *S. cerevisiae*, Hsl7p is a functional component of the MAP kinase pathway where it was shown to compete with Cdc42p for binding to the amino-terminal half of Ste20p (4). Ste20p is a member of the $p65^{PAK}$ protein kinase family and is involved in several yeast signal transduction pathways. In the haploid mating pathway, Ste20p is a kinase downstream of the Ste12p and Ste3p receptors which bind α-factor and a-factor, respectively (21). Ste20p, Ste11p, Ste7p, and Ste12p are also required for the switch from an axial to a bipolar mode of budding which results in invasive growth (21, 22). In diploid cells, nitrogen starvation produces a filamentous type of growth which is mediated through Ste20p and the MAP kinase pathway (4, 23); Ras2p also enhances this pathway (24). Hsl7p contributes to all of these phenotypes via Ste20p (4). Hsl7p also inhibits the Swe1p kinase that phosphorylates Cdc28, thereby producing changes in the cell cycle. In the Swelp/Cdc28p morphogenesis checkpoint pathway, Swelp and Hsllp both associate with Hsl7p (5). Hsl7p localizes to septin rings formed at the bud necks of dividing cells where it forms a complex with Hsl1p, Swe1p and the septins and is involved in the Cdc28-mediated G2/M cell cycle transition. McMillan *et al.* (25) reported that Hsl1p can phosphorylate Hsl7p. While the levels of Hsl7p appear to be relatively constant during the cell cycle, Hsl1p expression is cell cycle-dependent and so is its phosphorylation of Hsl7p. Ultimately Hsl7p and Hsl1p interact to promote the degradation of Swe1p, possibly by polyubiquitination (25). Thus, Hsl7p is a functional component of both the Swelp/Cdc28p morphogenesis checkpoint and MAP kinase pathways and may serve as a link between these two pathways.

Although methylation of proteins such as the histones was recognized decades ago, a clear function for histone methylation has not been delineated. Recently, the methyltransferase CARM1 was reported to methylate histones H2A and H3 *in vitro* and enhance the transcription of nuclear receptors, suggesting that it activates transcription through histone methylation (26). The homologue of Hsl7p, JBP1, interacts with all the Janus kinases (Jak1, Jak2, Jak3, and Tyk2), kinases required for signal transduction of interferons, cytokines and growth factors (1). As described above, Hsl7p is intrinsically involved in at least two pathways: the Swe1p/Cdc28p morphogenesis checkpoint; and Ras signaling in the MAP kinase pathway. Furthermore, because Hsl7p methylates histones, Hsl7p could be involved in chromatin remodeling and may contribute to the "histone code" that can control downstream events (27). Our data presented in this report provide evidence that there is an intricate link between protein methylation and yeast morphogenesis and provide a biochemical basis for understanding the mechanism by which Hsl7p modulates these many diverse actions.

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