

Yeast Hsl7 (histone synthetic lethal 7) catalyses the *in vitro* formation of ω - N^G -monomethylarginine in calf thymus histone H2A

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The *HSL7* (histone synthetic lethal 7) gene in the yeast *Saccharomyces cerevisiae* encodes a protein with close sequence similarity to the mammalian PRMT5 protein, a member of the class of protein arginine methyltransferases that catalyses the formation of ω - N^G -monomethylarginine and symmetric ω - N^G,N^G -dimethylarginine residues in a number of methyl-accepting species. A full-length *HSL7* construct was expressed as a FLAG-tagged protein in *Saccharomyces cerevisiae*. We found that FLAG-tagged Hsl7 effectively catalyses the transfer of methyl groups from *S*-adenosyl-[methyl-³H]-L-methionine to calf thymus histone H2A. When the acid-hydrolysed radiolabelled protein products were separated by high-resolution cation-exchange chromatography, we were able to detect one tritiated species that co-migrated with an ω - N^G -monomethylarginine standard. No radioactivity was observed that co-migrated with either the asymmetric or symmetric dimethylated derivatives. In control experiments, no methylation of histone H2A was found with two mutant constructs

of Hsl7. Surprisingly, FLAG-Hsl7 does not appear to effectively catalyse the *in vitro* methylation of a GST (glutathione S-transferase)-GAR [glycine- and arginine-rich human fibrillar(1–148) peptide] fusion protein or bovine brain myelin basic protein, both good methyl-accepting substrates for the human homologue PRMT5. Additionally, FLAG-Hsl7 demonstrates no activity on purified calf thymus histones H1, H2B, H3 or H4. GST-Rmt1, the GST-fusion protein of the major yeast protein arginine methyltransferase, was also found to methylate calf thymus histone H2A. Although we detected Rmt1-dependent arginine methylation *in vivo* in purified yeast histones H2A, H2B, H3 and H4, we found no evidence for Hsl7-dependent methylation of endogenous yeast histones. The physiological substrates of the Hsl7 enzyme remain to be identified.

Key words: histone, histone synthetic lethal 7 (Hsl7), methyltransferase, protein arginine methylation, *Saccharomyces cerevisiae*.

INTRODUCTION

Protein arginine methylation is catalysed by a group of enzymes that modify the guanidinium side chain utilizing AdoMet (*S*-adenosylmethionine) as a methyl donor [1]. These reactions have been implicated in the regulation of signal transduction [2–4], transcription [5–7], RNA transport [8] and splicing [9]. There are four types of PRMT (protein arginine methyltransferase). Three of these have already been described in the yeast *Saccharomyces cerevisiae* (for a review, see [10]). Rmt1, a type I enzyme, catalyses the formation of MMA (ω - N^G -monomethylarginine) and ADMA (asymmetric ω - N^G,N^G -dimethylarginine) residues and is the predominant arginine methyltransferase in *S. cerevisiae* that methylates a variety of substrates including the RNA-binding proteins Npl3 and Hrp1 [11–13]. Type III methylation, in which only the MMA residue is formed, has also been detected in yeast, although no gene product capable of this type of activity has been reported [11]. Rmt2, a type IV enzyme, catalyses the formation of δ - N^G -monomethylarginine residues and has been shown to methylate the ribosomal protein L12 [14,15]. The final type of methylation (type II) results in the formation of MMA and SDMA (symmetric ω - N^G,N^G -dimethylarginine) and has not been detected to date in *S. cerevisiae* [10].

In a yeast two-hybrid screen to search for proteins that bind to the second polypeptide chain of Janus kinase 2, a mammalian protein, JBP1 (Janus kinase-binding protein 1)/PRMT5, was identified [19,20]. PRMT5 was later shown to be a type II arginine methyltransferase [20]. The apparent yeast homologue

of PRMT5, Hsl7 (histone synthetic lethal 7), was first reported to catalyse the methylation of bovine brain MBP (myelin basic protein), calf thymus histone H2A, calf thymus histone H4 [16] and subsequently GST (glutathione S-transferase)-GAR [glycine- and arginine-rich human fibrillar(1–148) peptide] [17] *in vitro*. However, another group reported that they were unable to observe any methyltransferase activity under similar conditions [18]. We thus wanted to investigate further the activity of Hsl7, particularly to determine the type of reactions that it may catalyse on arginine residues.

The *HSL7* gene was originally discovered in a screen for second-site mutations that are lethal in combination with a deletion of the N-terminus of histone H3 [21]. Knockouts of *HSL7* result in a G₂ delay [21], whereas overexpression of Hsl1 or Hsl7 is sufficient to override most of the G₂ delays in cells that are exposed to actin perturbations [22]. Yeast two-hybrid and co-immunoprecipitation data indicate that Hsl7 associates with both Swe1 and Hsl1 [22,23]. Hsl7 has been shown to be a negative regulator of Swe1, whose degradation is necessary in order for the cell to exit the G₂ phase [22]. Hsl1 has recently been shown to phosphorylate Hsl7 at serine residues. Localization studies show that Hsl7 is localized to the daughter side of the bud neck during mitosis [18]. It is hypothesized that Hsl7 activity is dependent on septin organization, which in turn ensures that Swe1p degradation will not begin until a bud is formed [18]. Hsl7 has also been shown to be a negative regulator of Ste20, a protein kinase in the *S. cerevisiae* filamentous growth-signalling pathway [24].

Abbreviations used: ADMA, asymmetric ω - N^G,N^G -dimethylarginine; AdoMet, *S*-adenosyl-L-methionine; GAR, glycine- and arginine-rich human fibrillar(1–148) peptide; Hsl7, histone synthetic lethal 7; MBP, myelin basic protein; MMA, ω - N^G -monomethylarginine; PRMT, protein arginine methyltransferase; SCD, synthetic complete dextrose; SDMA, symmetric ω - N^G,N^G -dimethylarginine; TBS, Tris-buffered saline.

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In the present study, we provide evidence that a FLAG-tagged Hsl7 purified from *S. cerevisiae* cells in which *RMT1* was deleted can catalyse the formation of MMA in calf thymus histone H2A. However, we did not find that FLAG-Hsl7 significantly catalysed methylation of GST-GAR, MBP or calf thymus histone H1, H2B, H3 or H4. Interestingly, we also did not find any evidence that Hsl7 could catalyse the *in vivo* methylation of endogenous yeast histones. These results suggest that the protein methyltransferase activity of this enzyme in yeast may be targeted to non-histone proteins or to only a small subfraction of histone proteins that become substrates only under certain conditions.

EXPERIMENTAL

Yeast strains

Strains CH9100-2 (*MAT α* , *prc1-407*, *prb1-1122*, *pep4-3*, *leu2*, *trp1*, *ura3-52*, *prd1/ycl57w Δ ::URA3*) and JDG9100-2 (*MAT α* , *prc1-407*, *prb1-1122*, *pep4-3*, *leu2*, *trp1*, *ura3-52*, *prd1/ycl57w Δ ::URA3*, *rmt1::LEU2*) were described previously [11]. Strains YDS2 (*MAT α* , *ade2-1*, *trp1*, *can1-100*, *leu2-3*, *112*, *his3-11*, *15*, *ura3-23*) and MAY3 (*MAT α* , *ade2-1*, *trp1*, *can1-100*, *leu2-3*, *112*, *his3-11*, *15*, *ura3-23*, *hsl7 Δ 1::URA3*) were gifts from Michael Grunstein (University of California, Los Angeles) [20].

The strain NFY1100 α (*MAT α* , *leu2-3*, *112*, *ura3-52*, *his4-149*, *trp1*, *can1*, *rmt1::LEU2*) was created by disrupting *RMT1* in the strain SEY1100 α (*MAT α* , *leu2-3*, *112*, *ura3-52*, *his4-149*, *trp1*, *can1*) using the linear pJG-RMT1::LEU2 construct as described previously [11], and the genotype was confirmed by leucine prototrophy and an *rmt1*⁻ phenotype (see below). Strains AFY4130 (*MAT α* , *ade2*, *rmt1::LEU2*) and AFY5130 (*MAT α* , *ade2*, *rmt1::LEU2*, *hsl7::URA3*) were prepared by mating strains MAY3 and NFY1100 α . The diploid cells were sporulated and tetrads were dissected. The genotypes were confirmed by (i) leucine and/or uracil prototrophy, and (ii) PCR using primers *rmt1-con1* (5'-GGTAGAAGGCGGTCTGATCTTTCCCG-3') and *rmt1-con2* (5'-GGCGAATAATCAAACCCGTAACG-3') for *RMT1* gene disruption. Strain TBM1000 where the *RMT1* gene was disrupted in the YDS2 background was made as follows. The disruption plasmid pJG-RMT1::LEU2 (10 μ g) was digested with NdeI and HindIII [11], and the entire mixture was used to transform CH9100-2 cells by the lithium acetate method [26]. The transformed cells were then selected by plating on to leucine-deficient SCD (synthetic complete dextrose) plates [26]. Positives were rescreened twice on selective plates. Genomic DNA was isolated from cells remaining after the three screens. The replacement of the wild-type *RMT1* locus by the *LEU2* disrupted version was confirmed by PCR analysis using primers RMT1-N2 (5'-TTCGTACCTTATCTTACAGAAACGC-3') and RMT1-C2 (5'-CAGTGAGTGTATGGAGCATGAGGAC-3').

Preparation of *in vivo*-labelled yeast extracts

Strains AFY4130a and AFY5130a were labelled *in vivo* using [³H]AdoMet (*S*-adenosyl-[methyl-³H]L-methionine) (Amersham Biosciences). Cells were grown in YPD media (1% bacto-yeast extract, 2% bacto-peptone and 2% dextrose) at 30°C to early exponential phase (D_{600} of 0.5–0.7). Seven D_{600} units (12 ml) of each culture were then harvested by centrifugation at 5000 g for 5 min at 25°C and were washed twice with sterile water. The pelleted cells were resuspended in 924 μ l of YPD and incubated for 30 min at 30°C with shaking after the addition of 76 μ l of [³H]AdoMet [1 mCi/ml, 70–81 Ci/mmol, in dilute HCl/ethanol (9:1, v/v), pH 2–2.5]. Cells were then pelleted at 5000 g for 5 min at 25°C, and were washed twice with water. Labelled cell extracts were

prepared by resuspending each cell pellet in 50 μ l of lysis buffer (1% SDS and 1 mM PMSF). To each mixture, 0.2 g of baked zirconium beads (Biospec Products) were added, and then the samples were vortex-mixed for 1 min and placed on ice for 1 min. The vortex-mixing and ice incubation cycle was repeated seven times. The lysate was collected, and the beads were washed with 50 μ l of lysis buffer and the wash was combined with the lysate.

Purification of GST-GAR and GST-Rmt1

GST-GAR [23] and GST-Rmt1 [11] were overexpressed in *Escherichia coli* DH5 α cells (Life Technologies) by induction with a final concentration of 0.4 mM isopropyl β -D-thiogalactoside. The protein was then purified from extracts by binding to glutathione-Sepharose 4B beads (Amersham Biosciences) according to the manufacturer's instructions, but in the presence of 100 μ M PMSF and eluting with 30 mM glutathione, 50 mM Tris/HCl, pH 7.5, 120 mM NaCl and 2% (v/v) glycerol.

Expression and purification of wild-type and mutant FLAG-Hsl7

A yeast-*E. coli* shuttle vector containing a gene engineered to express a FLAG-tagged version of Hsl7 (pTKB-FLAG-HSL7) was a gift from Dr Sidney Pestka at Rutgers University (Piscataway, NJ, U.S.A.) [16]. Plasmids expressing a site-directed double mutant [FLAG-HSL7 (GAGRG \rightarrow GAVRV)] and a deletion mutant [FLAG-HSL7 (Δ GAGRG)] were prepared from pTKB-FLAG-HSL7 using the Stratagene QuikChange[®] II XL site-directed mutagenesis kit. The 5' primers for the double mutant and for the deletion mutant were 5'-GGTGATCCTAGTAGCCGGTGCAGTAAGAGTACCTTTAGTGG-3' and 5'-GGTGATCCTAGTAGCCGGTGCAGCTTTAGTGGATCGAAC-3' respectively. Mutagenesis was confirmed by direct DNA sequencing of both strands by Davis Sequencing.

Plasmid pTKB-FLAG-HSL7 was transformed into TBM1000 and AFY5130a using the lithium acetate method [26]. The pTKB-FLAG-HSL7 mutant and deletion constructs were also transformed into AFY5130a. Transformants were selected by plating on to tryptophan-deficient SCD plates [26]. Transformed strains were grown to a D_{600} of 1.0–2.0 in 2 litres of liquid SD (GAL)–Trp [0.17% YNB–AA/AS (yeast nitrogen base without amino acids or ammonium sulphate), 0.5% ammonium sulphate, 2% galactose, 1.3% dropout powder deficient in tryptophan]. Cells were then harvested by centrifugation at 5000 g for 5 min, washed three times with TBS (Tris-buffered saline: 50 mM Tris/HCl, pH 7.5, and 150 mM NaCl), and then resuspended in TBS to a final concentration of 2 g/ml and lysed by ten cycles of 1 min of vortex-mixing with 3 vol. of glass beads. After centrifugation at 23 300 g for 50 min at 4°C, the supernatant was then applied to an anti-FLAG affinity column as described previously [16]. Purified proteins were eluted with 5 mg/ml FLAG peptide in TBS.

In vitro labelling of MBP, GST-GAR and histones H1, H2A, H2B, H3 and H4

For chemical analysis, 10 μ g of MBP (purified from bovine brain, freeze-dried powder; Sigma product M1891), 10 μ g of histone H2A (calf thymus; Boehringer Mannheim) or 2 μ g of GST-GAR were added to 2 μ g of purified FLAG-Hsl7 enzyme preparations (see above) and incubated at 30°C for 5 h with 3 μ l of [³H]AdoMet (72.0–79.0 Ci/mmol, 13–14 μ M, 1 mCi/ml) in TBS in a final volume of 70 μ l. For analysis by gel electrophoresis, 10 μ g of histone H1, H2A, H2B, H3 or H4 (calf thymus; Boehringer Mannheim) were added to 2 μ g of purified FLAG-Hsl7 enzymes and incubated at 30°C for 5 h with 3 μ l of [³H]AdoMet in TBS in a final volume of 70 μ l. Histone H1, H2A,

H2B, H3 or H4 (10 µg) was also added to 2 µg of purified GST-Rmt1 and incubated at 30°C for 1 h with 3 µl of [³H]AdoMet in 50 mM sodium phosphate, pH 7.5, in a final volume of 30 µl.

Gel electrophoresis of *in vitro* reactions

Reactions were stopped by adding either 70 µl (HSL7 reactions) or 30 µl (RMT1 reactions) of SDS gel sample buffer (180 mM Tris/HCl, pH 6.8, 4% SDS, 0.1% 2-mercaptoethanol, 20% glycerol and 0.002% Bromophenol Blue) and heating at 100°C for 3 min. Samples were electrophoresed at 35 mA for 5 h using a Laemmli buffer system on a gel prepared with 12.6% acrylamide and 0.43% *N,N*-methylenebisacrylamide (1.5 mm thick, 10.5 cm resolving gel, 2 cm stacking gel). Gels were stained with Coomassie Brilliant Blue R-250 overnight and destained in 10% methanol and 5% ethanoic (acetic) acid for 8 h. For fluorography, gels were treated with EN³HANCE (PerkinElmer Life Sciences). Gels were dried at 70°C *in vacuo* and exposed to Kodak X-Omat AR scientific imaging film at -80°C.

Preparation and purification of ³H-methylated histones from intact yeast cells

Yeast strains CH9100-2 and JDG9100-2 were labelled *in vivo* using [³H]AdoMet. Briefly, cells were grown in YPD medium at 30°C to early exponential phase (D_{600} of 0.6). Samples of 16 D_{600} units (27 ml) of each culture were then harvested by centrifugation at 5000 g for 5 min at 25°C and were washed twice with sterile water. The pelleted cells were resuspended in 7.4 ml of YPD, and 600 µl of [³H]AdoMet (1 mCi/ml, 70–81 Ci/mmol) was added. Cells were labelled at 30°C with shaking until reaching a D_{600} of 7. Cells were then pelleted at 5000 g for 5 min at 25°C, washed twice with water and stored at -20°C overnight. Histones were then purified by a method described previously with minor changes [27]. Cells were thawed on ice, resuspended in 5 ml of 10 mM dithiothreitol and 0.1 mM Tris/HCl, pH 9.5, and were incubated with shaking at 30°C for 15 min. Cells were then spun at 2700 g for 5 min. The pellet was resuspended in 5 ml of 1.2 M sorbitol and 20 mM Hepes, pH 7.4, and re-spun as before. Zymolase (13.75 mg/g of wet weight) (ICN Biomedicals; # 32092; 20000 units of enzyme/g) was added to the pellet. The pellet was resuspended again in 5 ml of 1.2 M sorbitol and 20 mM Hepes, pH 7.4 and incubated at 30°C with shaking for 45 min. Cold 1.2 M sorbitol (10 ml), 20 mM Pipes and 1 mM MgCl₂, pH 6.8, was then added and cells were spun at 2700 g for 5 min. The pellet was resuspended in 5 ml of ice-cold NIB (0.25 M sucrose, 60 mM KCl, 15 mM NaCl, 5 mM MgCl₂, 1 mM CaCl₂, 15 mM Mes, pH 6.6, 1 mM PMSF and 0.8% Triton X-100) and incubated in ice water for 20 min. The extract was spun at 2700 g for 5 min at 4°C. The NIB wash was repeated twice. The pellet was then resuspended in 5 ml of A wash (10 mM Tris/HCl, pH 8.0, 0.5% Nonidet P40, 75 mM NaCl, 30 mM sodium butyrate and 1 mM PMSF), incubated in ice water for 15 min and spun at 2700 g for 5 min at 4°C. The wash was repeated once. The pellet was then resuspended in 5 ml of B wash (10 mM Tris/HCl, pH 8.0, 0.4 M NaCl, 30 mM sodium butyrate and 1 mM PMSF), incubated in ice water for 5 min and then spun at 2700 g for 5 min at 4°C. The B wash was repeated once. The pellet was then resuspended in 2.5 ml of B wash and spun at 2700 g for 5 min at 4°C. The pellet was resuspended in 3 vol. of cold 0.25 M HCl to extract histones. The solution was incubated in ice water for 30 min and then spun at 20800 g for 10 min at 4°C. The supernatant was removed and its volume was measured. Trichloroacetic acid was added to a final concentration of 20%, and the solution was incubated in ice water for 30 min and then spun at 20800 g for 30 min (the brake

was left off). The pellet was washed in 1 ml of cold acidic acetone (0.5% HCl/acetone) and spun for 5 min at 20800 g. The pellet was washed in 1 ml of cold acetone and spun again at 20800 g for 5 min. The pellet was air-dried, and purified histones were resuspended in 30 µl of 10 mM Tris/HCl, pH 8.0. Histones were stored at -20°C.

Purified histones were electrophoresed at 35 mA for 7 h using the SDS Laemmli buffer system on a gel prepared with 15% acrylamide and 0.52% *N,N*-methylenebisacrylamide (1.5 mm thick, 10.5 cm resolving gel, 2 cm stacking gel). Gels were stained with Coomassie Blue R-250 overnight, and destained with 10% methanol and 5% ethanoic acid for 8 h. The Coomassie band corresponding to histones H2A, H2B, H3 and H4 were excised from the gel for further analysis.

Chemical analysis of ³H-methylated species

The *in vitro* reactions were mixed with 11.1 µg (11.1 mg/ml) of BSA as a carrier protein. Both the *in vitro* reactions and *in vivo*-labelled extracts were mixed with an equal volume of 25% (w/v) trichloroacetic acid (71 µl for the *in vitro* reactions and 101 µl for the *in vivo*-labelled extracts) in a 6 mm × 50 mm glass vial and incubated at room temperature (24°C) for 30 min. The precipitated protein was then centrifuged at 1000 g for 30 min at 25°C and the supernatant was drawn off and discarded. Pellets were washed once with ice-cold acetone and allowed to air dry. Acid hydrolysis was then carried out on these reactions in a Waters Pico-Tag vapour-phase apparatus *in vacuo* for 20 h at 110°C using 200 µl of 6 M HCl.

For purified labelled histones, the Coomassie Blue bands corresponding to histones H2A, H2B, H3 and H4 were excised and added to a 6 mm × 50 mm glass vial and dried by vacuum centrifugation. A 100 µl volume of 6 M HCl was added to the vial, and the sample was hydrolysed *in vacuo* for 20 h at 110°C. After hydrolysis, residual HCl was removed from the glass vial by vacuum centrifugation.

Hydrolysed samples were resuspended in 50 µl of water and mixed with 1.0 µmol of each of the standards MMA (Sigma product M7033; acetate salt) and ADMA (Sigma product D4268; hydrochloride) for amino acid analysis by column chromatography. A 500 µl volume of citrate dilution buffer (0.2 M Na⁺, pH 2.2) was added to the hydrolysed samples before loading on to a cation-exchange column (Beckman AA-15 sulphonated polystyrene beads; 0.9 cm inner diameter × 11 cm column height) equilibrated and eluted with sodium citrate buffer (0.35 M Na⁺, pH 5.27) at 1 ml/min at 55°C.

RESULTS AND DISCUSSION

Conflicting results have been reported for the enzymatic activity of the yeast Hsl7 protein. It was first shown that a FLAG-tagged Hsl7 protein purified from wild-type *S. cerevisiae* could methylate mammalian MBP and histones H2A and H4 [16]. Subsequently, it was reported that a GST-Hsl7 fusion protein expressed in *E. coli* could methylate the GST-GAR fusion protein containing the N-terminal region of fibrillarlin [17]. However, in a study where Hsl7-myc and GST-Hsl7 purified from *E. coli* and *S. cerevisiae* were analysed, no methyltransferase activity was found [18]. Thus we found it important to revisit this issue.

A protein-protein BLAST search of the GenBank[®] NR database with the Hsl7 sequence found the best matches to known proteins to be mammalian PRMT5 in mice (27.7% over 678 residues), human (27.7% over 678 residues) and rats (27.9% over 678 residues). Examination of the alignment of PRMT5 and Hsl7 in the methyltransferase motif regions reveals common sequences

Motif II			
Hsl17	⁴⁵⁹ IDLCIS	ELLGSFGCNLSPECL	
PRMT5	⁴⁴⁹ ADIIVS	ELLGSFADNELSPECL	
PRMT1	¹⁴⁶ VDIIIS	EWMGYCLFYESMLNTV	
PRMT2	²⁰⁵ VDVLVS	EWMGTCLLFEFMIESI	
PRMT3	³⁰⁴ VDVVIS	EWMGYFLIFESMLDSV	
PRMT4	²⁵¹ VDIIIS	EPMGYMLFNERMLESY	
PRMT6	¹⁴⁹ VDIVS	EWMGYGLLHESMLSSV	
PRMT7	¹³⁸ ANILVT	ELFDTELI GEGALPSY	
PRMT8	¹⁷⁹ VDIIIS	EWMGYCLFYESMLNTV	
		*	*

Figure 1 Amino acid sequence alignment of yeast Hsl17 with mammalian arginine methyltransferases in the motif II/post motif II region

Sequences are compared for motif II and the adjoining residues that have been shown to be important for the catalytic activity of each enzyme. Glutamate residues making up the 'double E' loop are indicated by asterisks. Residues in bold distinguish Hsl17 and PRMT5 from the other enzymes (see text).

after motif II that are not shared with mammalian PRMT1, 2, 3, 4, 6, 7 and 8, or yeast Rmt1 and Rmt2 (Figure 1). Residues in this sequence have been predicted to be useful in determining the type of methylation that each enzyme catalyses [20]. Here we see a highly conserved motif II (residues 459–464 of Hsl17) and the invariant 'double E' residues 465 and 474 that are important in AdoMet binding. At Hsl17 positions 471, 472 and 475–477, the sequences of Hsl17 and PRMT5 can be distinguished from all the other enzymes. At positions 471 and 472, a Gly-Cys (Hsl17) or an Ala-Asp (PRMT5) is replaced by a Leu-(Phe/Leu/Ile) sequence in all other enzymes. At positions 475–477, a Leu-Ser-Pro sequence in Hsl17 and PRMT5 is replaced by a Xaa-(Met/Ala)-(Leu/Ile) sequence in all other enzymes. It has been suggested that the side chain at 476 is a factor in determining whether an enzyme will catalyse the formation of ADMA or SDMA residues [20]. It is thus reasonable to predict that Hsl17 would have a similar activity as PRMT5.

To determine whether the *HSL7* gene indeed encodes an active methyltransferase, we used strains of *S. cerevisiae* in which the gene for the major arginine methyltransferase, *RMT1*, was deleted to ensure that there would be no contribution from Rmt1 activity. Deletion strains that had either *RMT1* (AFY4130a) or both *RMT1* and *HSL7* (AFY5130a) genes knocked out were labelled with [³H]AdoMet and then lysed. Proteins in the lysate were precipitated, acid-hydrolysed to free amino acids and chromatographed on a high-resolution cation-exchange column. We observed the same pattern of radioactivity in the strain in which only *RMT1* was deleted (Figure 2A) and in the strain in which both *RMT1* and *HSL7* were deleted (Figure 2B). In both cases, a small peak of radioactive protein was eluted in the position expected for [³H]MMA [11,20,25]; an additional unidentified peak was found to elute approx. 4 min earlier than the standard of ADMA. These results indicate that either Hsl17 does not, in fact, encode a methyltransferase or its endogenous methylated products are present in too low an abundance to be observed above the background radioactivity. In a control experiment, we analysed a strain deleted for *HSL7*, but containing the intact *RMT1* gene, and observed the expected major radioactive peak corresponding to ADMA (Figure 2C) [11,25]. Similar results were found for the wild-type strain as for the *HSL7*-deletion strain (results not shown).

Since we did not detect any evidence for Hsl17 methyltransferase activity *in vivo*, we next decided to see whether it had methyltransferase activity *in vitro*. A plasmid encoding a fusion construct of a FLAG tag and the full-length coding sequence of Hsl17

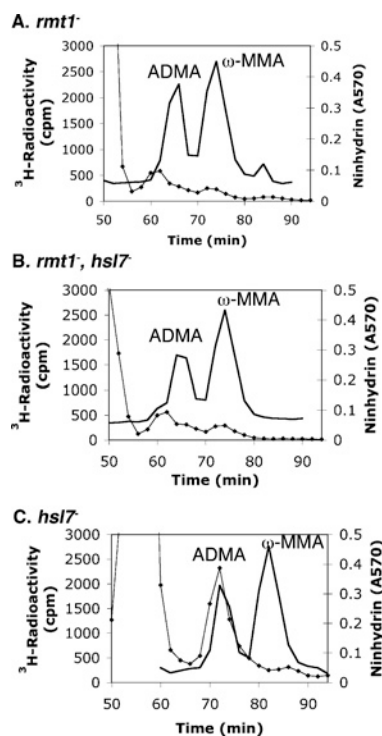


Figure 2 Disruption of yeast *HSL7* does not lead to a decrease in protein arginine methylation *in vivo*

Strains AFY4130a (*rmt1*) (A), AFY5130a (*rmt1, hsl7*) (B) and MAY3 (*hsl7*) (C) were labelled *in vivo* with [³H]AdoMet, and extracts were acid-hydrolysed as described in the Experimental section. Samples were resuspended in water and mixed with the standards MMA and ADMA and chromatographed on a high-resolution cation-exchange column as described in the Experimental section. [³H]-radioactivity (◆) was determined by counting a 200 μl aliquot of every fraction diluted with 400 μl of water in 5 ml of fluor (Safety Solve; Research Products International) three times for 3 min. The unlabelled amino acid standards were analysed using a ninhydrin assay with 100 μl aliquots of every other fraction (solid line) [11].

was expressed in a *S. cerevisiae* strain with a disrupted *RMT1* gene. The FLAG-tagged protein was purified by affinity chromatography, as described in the Experimental section, and was characterized as a single polypeptide band of the appropriate size on SDS gel electrophoresis (Figure 3). Mass spectrometric analysis of tryptic peptides of the polypeptide band confirmed its identification as Hsl17 (results not shown).

For methyl-accepting substrates, we tested calf thymus histone H2A, bovine brain MBP and GST-GAR, known substrates of PRMT5, the mammalian Hsl17 homologue [20]. These substrates, as well as calf thymus histone H4, have been shown previously to be methylated by Hsl17 *in vitro* [16,17]. FLAG-Hsl17 was thus incubated with GST-GAR, MBP or histone H2A in the presence of [³H]AdoMet. Protein was precipitated with trichloroacetic acid, acid-hydrolysed to their amino acid components, and fractionated by high-resolution amino acid cation-exchange chromatography along with standards of unlabelled ADMA and MMA. Very little PRMT activity was observed when GST-GAR or MBP was used as a methyl-accepting substrate (Figures 4A and 4B). However, a large amount of activity was seen when histone H2A was used as a substrate, with a radiolabelled product eluting just ahead of the MMA standard (Figure 4C). The substitution of the three tritium atoms for three hydrogen atoms on the methyl group of the [³H]-labelled amino acid has been found to give it a slight shift in the elution position; the peak of radioactivity observed in Figure 4(C) elutes in the position expected for MMA [11,20,25]. We

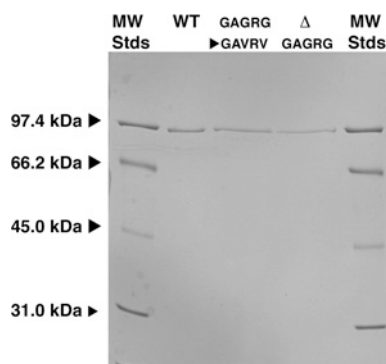


Figure 3 Gel electrophoresis of purified FLAG-tagged Hsl7 proteins expressed in yeast

Wild-type protein expressed in strain TMB1000 or mutant and deletion proteins expressed in strain AFY5130a were denatured in SDS and electrophoresed at 35 mA for 5 h using a Laemmli buffer system on a gel prepared with 8% acrylamide and 0.28% *N,N*-methylenebisacrylamide (1.5 mm thick, 10.5 cm resolving gel, 2 cm stacking gel). The gel was stained with Coomassie Brilliant Blue R-250 for 1 h and destained overnight in 10% methanol and 5% ethanoic acid. The position of marker proteins (Bio-Rad low-molecular-mass standards; MW Stds) electrophoresed in parallel lanes are shown by the arrows on the left (rabbit muscle phosphorylase, 97.4 kDa; BSA, 66.2 kDa; hen's-egg ovalbumin, 45.0 kDa; bovine carbonic anhydrase, 31 kDa). The FLAG-Hsl7 wild-type (WT) and mutant proteins migrated to approx. 95 kDa.

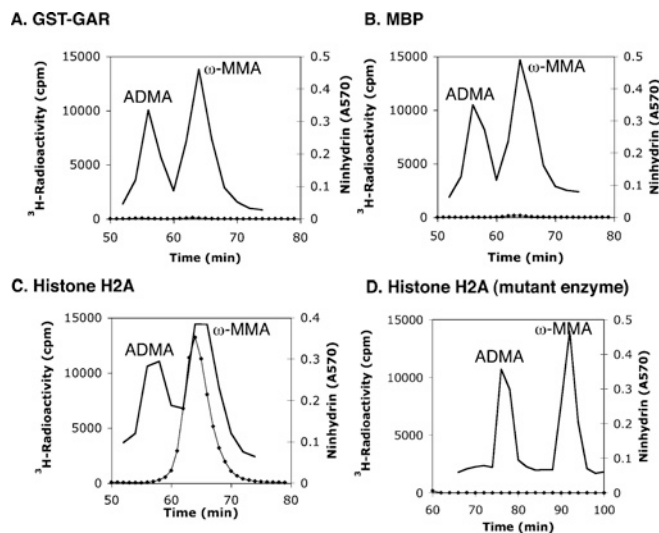


Figure 4 FLAG-Hsl7 catalyses the formation of MMA in calf thymus histone H2A

Substrates were incubated with [³H]AdoMet and enzyme, and the reaction mixture was precipitated with trichloroacetic acid, acid-hydrolysed and analysed by amino acid analysis as described in the Experimental section. ³H-radioactivity (◆) and ninhydrin colour (solid lines) were determined as described in the legend to Figure 2. (A) Analysis of GST-GAR labelled *in vitro* with FLAG-Hsl7. (B) Analysis of bovine MBP labelled with FLAG-Hsl7. (C) Analysis of calf thymus histone H2A labelled with FLAG-Hsl7. (D) Analysis of histone H2A labelled *in vitro* with the double mutant FLAG-Hsl7 GAGRG → GAVRV enzyme.

observed little or no label in the expected elution position of either the ADMA standard (included in Figure 4C) or of SDMA that elutes between the positions of the MMA and ADMA standards [20]. We cannot, however, exclude the possibility of the formation of a small amount of the SDMA residue.

To ensure that the activity detected with histone H2A was in fact due to Hsl7, we prepared two plasmids with site-directed muta-

tions in residues of conserved motif I of Hsl7. One construct resulted in a change from GAGRG to GAVRV, the other in a deletion of these five residues. The mutant and deletion FLAG-tagged proteins were purified as for the wild-type species and characterized by SDS/polyacrylamide gel electrophoresis (Figure 3). A single band of the appropriate size was observed for each protein. We incubated each of these proteins with [³H]AdoMet and histone H2A. No methylated products were seen with either the double mutant enzyme (Figure 4D) or with the deletion enzyme (results not shown). These results show clearly that the methylation of histone H2A seen in Figure 4(C) was due to the activity of the Hsl7 gene product and not a contaminant from the yeast cells. These results also suggest that Hsl7 may be a type III PRMT that is only capable of monomethylation of the ω-nitrogen atom. Alternatively, it may be capable of catalysing a second methylation reaction on other substrates or under other conditions.

In initial control experiments, we characterized the *in vitro* activity of a FLAG-tagged Hsl7 preparation purified from yeast cells containing the intact *RMT1* gene for the major PRMT. Here, we did find significant activity that generated MMA residues with MBP and GST-GAR substrates (results not shown). These results are consistent with the previous demonstration of the methylation of the polypeptide bands of MBP using a similarly purified preparation of FLAG-Hsl7 [16]. However, the near absence of methylation of MBP or GST-GAR using the FLAG-Hsl7 enzyme purified from cells lacking the *Rmt1* methyltransferase (Figures 4A and 4B) does suggest that *Rmt1* activity can contribute to the formation of methylated products seen with FLAG-Hsl7 purified from non-mutant cells.

We next wanted to see whether FLAG-Hsl7 purified from *rmt1*⁻ yeast cells could methylate other histones or if it is specific for histone H2A. FLAG-Hsl7 was incubated with calf thymus histone H1, H2A, H2B, H3 or H4 in the presence of [³H]AdoMet. Reaction mixtures were then analysed by electrophoresis on an SDS gel. As shown in Figure 5(A), FLAG-Hsl7 effectively methylated histone H2A. However, no methylation of histone H1, H2B or H3 was detected. There was some methylation seen with the histone H4 preparation. However, upon close inspection, the radiolabelled band migrated just slightly faster than the major Coomassie Blue-stained histone H4 band and therefore appears to be from a contaminating protein or possibly a modified form of H4. In order to determine whether the methylation of histone H2A was specific for Hsl7, we looked at the methylation of histone H1, H2A, H2B, H3 and H4 by a GST-fusion of *Rmt1* purified from *E. coli*. GST-Rmt1 did favour histone H2A as a substrate (Figure 5B). However, GST-Rmt1 was also able to methylate contaminants that co-purified with the histones. The elution position of the histones is shown in the Coomassie Blue-stained gel in Figure 5(C).

Since calf thymus histone H2A is a very good *in vitro* substrate for yeast Hsl7 and *Rmt1*, we next looked to see whether yeast histone H2A is methylated at arginine residues *in vivo*. Wild-type cells were labelled *in vivo* with [³H]AdoMet and labelled histones were purified from these cells as described in the Experimental section. Purified histones were fractionated by SDS/15% PAGE and Coomassie Blue-stained bands corresponding to each histone were excised from the gel. Gel slices were then acid-hydrolysed, and the free amino acids were separated on a high-resolution cation-exchange column. We were able to detect the presence of ADMA in bands corresponding to histone H2A, H2B, H3 and H4 (Figure 6). When this was repeated in cells in which *RMT1* was deleted, no arginine methylation was detected in the bands corresponding to histone H2A, H2B, H3 and H4 (Figure 7). Therefore *Rmt1* is the major PRMT that modifies yeast histones *in vivo*. There is no evidence that Hsl7 methylates histone H2A

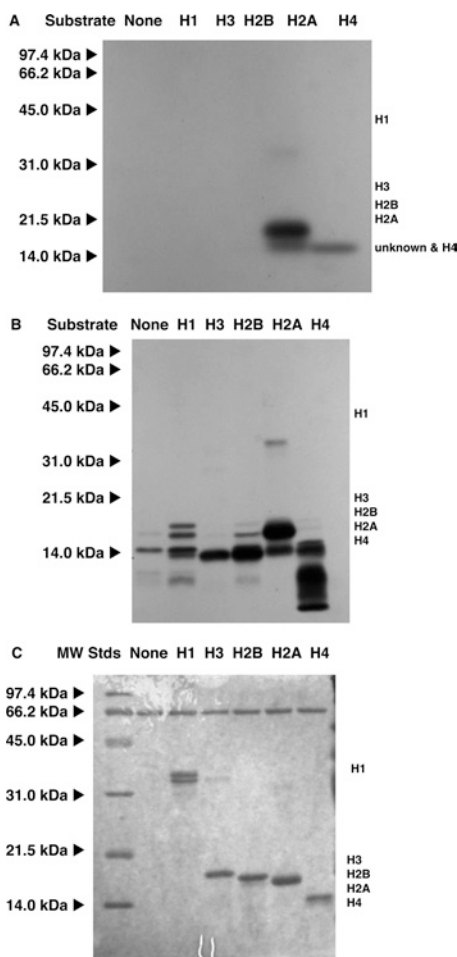


Figure 5 Specificity of FLAG-Hsl7 for methylation of histone H2A

Methylation reactions with either FLAG-Hsl7 (A) or GST-Rmt1 (B) were performed with purified calf thymus histone H1, H2A, H2B, H3 and H4 as described in the Experimental section. Polypeptides were fractionated by SDS/PAGE as described in the Experimental section, and radiolabelled species were visualized after gels were exposed to film for 1 week. The Coomassie Blue-stained gel for the GST-Rmt1 experiment is shown in (C). The position of marker proteins (Bio-Rad low-molecular-mass standards; MW Stds) electrophoresed in a parallel lane are shown by the lines on the left (rabbit muscle phosphorylase, 97.4 kDa; BSA, 66.2 kDa; hen's-egg ovalbumin, 45.0 kDa; bovine carbonic anhydrase, 31 kDa; soya bean trypsin inhibitor, 21.5 kDa; and hen's-egg lysozyme, 14.0 kDa).

in *S. cerevisiae* *in vivo* under the conditions of our incubation, although we may not be able to detect the modification of a small fraction of this protein. This result is consistent with the cytoplasmic localization of Hsl7 shown previously [18].

In the present study, we have shown that Hsl7 is an arginine methyltransferase that catalyses the formation of MMA *in vitro*. Hsl7 appears to be very specific in the substrates and sequences that it methylates. In our study, the only significant methyl-accepting substrate *in vitro* was found to be purified calf thymus histone H2A. We also find that GST-Rmt1 can methylate histone H2A along with other contaminating proteins in purified calf thymus histones. The fact that purified histones contain contaminants which are substrates for arginine methyltransferases makes it difficult to analyse histones as possible substrates for these arginine methyltransferases. In *in vivo*-labelled histones, we detected RMT1-dependent arginine methylation in bands containing purified histones. However, since other possible Rmt1 substrates may

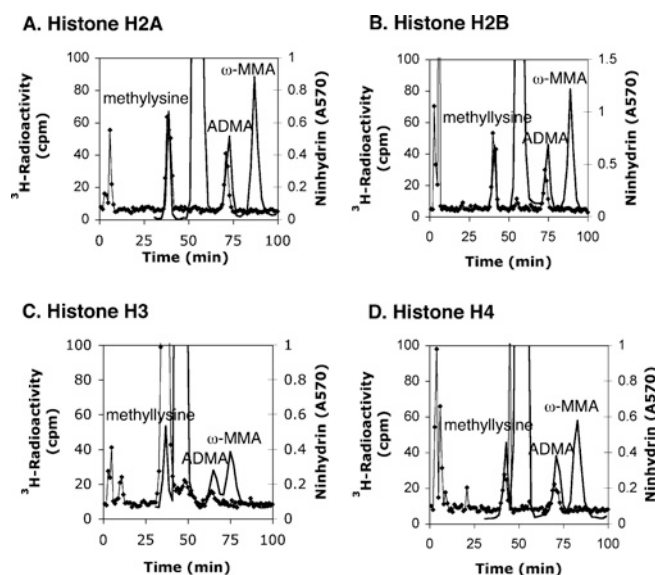


Figure 6 Detection of arginine methylation in intact yeast cell histones

Yeast CH9100 α cells (RMT1⁺, HSL7⁺) were *in vivo*-labelled, and endogenous histones H2A (A), H2B (B), H3 (C) and H4 (D) were isolated and acid-hydrolysed as described in the Experimental section. Analysis for methylated amino acids was carried out as described in the legend to Figure 2. Radioactivity (\blacklozenge) and the ninhydrin colour (solid line) are indicated. The methyl-lysine peak in (C) is approx. 850 c.p.m.

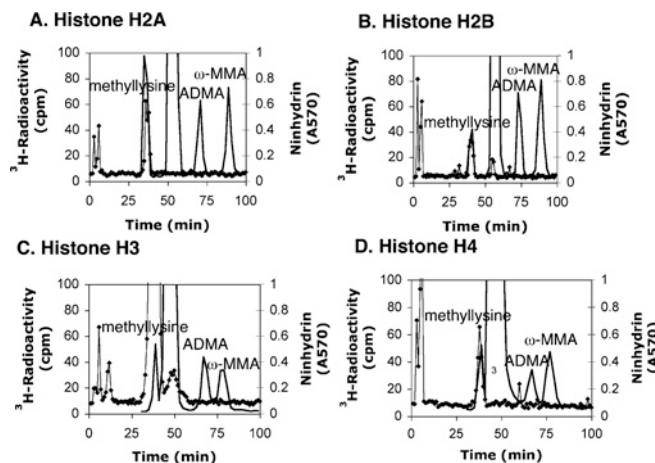


Figure 7 Loss of arginine methylation of purified histones from *rmt1* cells

JDG9100 α (RMT1⁻, HSL7⁺) cells were *in vivo*-labelled with [³H]AdoMet and histones H2A (A), H2B (B), H3 (C) and H4 (D) were isolated, acid-hydrolysed and analysed for methylated amino acids as described in the legend to Figure 6. Radioactivity (\blacklozenge) and the ninhydrin colour (solid line) are indicated. The methyl-lysine peak in (C) is approx. 2000 c.p.m.

be present in small amounts, as shown by the *in vitro* data, it is possible that some non-histone labelling occurs here. More sensitive and selective methods for determining arginine methylation at specific sites in polypeptides, such as MS, will be useful here [28].

Contradictory results have been shown previously for the methyltransferase activity of Hsl7. In an initial study, an N-terminal FLAG-tagged Hsl7 purified from wild-type yeast was shown to methylate histone H2A, MBP and histone H4 [16]. We show in the present study that the same construct when purified

from cells lacking the *RMT1* gene catalyses little or no methylation of histone H4 or MBP. In our preliminary experiments, we also detected the methylation of MBP when FLAG-Hsl7 was purified from wild-type cells. We thus conclude that the activities reported previously may have been in part or wholly due to contaminating Rmt1 activity present in the purified enzyme preparation of FLAG-Hsl7 expressed in wild-type yeast cells [16]. Additionally, we had reported previously that a GST-fusion of Hsl7 purified from *E. coli* was capable of methylating GST-GAR [17]. However, in those studies, the expression plasmid for Hsl7 may have been contaminated with a mammalian PRMT3 construct; further studies in our laboratory have not confirmed this activity (results not shown). In the studies in which Hsl7 was determined to have no methyltransferase activity *in vitro*, Hsl7-myc and GST-Hsl7 were purified from *E. coli* and *S. cerevisiae* [18]. We also found that GST-Hsl7 is an inactive enzyme (results not shown); it is possible that the bulky N-terminal tag disrupts the activity. In Hsl7-myc, the tag is placed at the C-terminus. It is possible that the C-terminus is important in Hsl7 activity, and therefore by placing a protein tag at the C-terminal end also results in an inactive enzyme.

All in all, these results do suggest that Hsl7 is an active methyltransferase. Further studies will be needed to identify its endogenous substrates in yeast. It is clear that these substrates are present in low amounts, because the loss of their methylation is not detectable in *in vivo*-labelled yeast cells lacking the *HSL7* gene. It is intriguing that calf thymus histone H2A is an effective methyl-accepting protein, since we see no evidence for the methylation of endogenous yeast histones. It is possible that Hsl7 modifies only a very small fraction of yeast histones that are appropriately modified by specific patterns of acetylation, phosphorylation or other types of methylation reactions, perhaps at nucleosomes on only one or a few genes. It is also possible that calf thymus histone H2A fortuitously matches a methylation site of a non-histone yeast protein. The identification of an endogenous substrate (or substrates) will then begin to address the physiological importance of the *HSL7* gene.

Further work will also be needed to demonstrate whether Hsl7 is indeed a type III enzyme only capable of monomethylation of the ω -nitrogen atoms of arginine residues, or whether it can catalyse additional steps of methylation leading to SDMA residues (type II activity), ADMA residues (type I activity), or perhaps even more highly modified residues on its endogenous substrates or other proteins. The similarity of the amino acid sequence of Hsl7 and the mammalian PRMT5 protein known to be a type II methyltransferase [20] does suggest the likelihood that the endogenous yeast substrate(s) of Hsl7 may be modified in a similar fashion. The identification of these substrates will be also important in resolving this issue. Finally, is it possible that there is one or more additional type II PRMTs in yeast independent of Hsl7. However, no additional homologues have been found to date and there is no clear peak attributable to SDMA in acid hydrolysates of *rmt1⁻/hsl7⁻* mutant cells labelled *in vivo* with [³H]AdoMet (Figure 2).

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