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Phosphorylation of the *Saccharomyces cerevisiae* La protein does not appear to be required for its functions in tRNA maturation and nascent RNA stabilization

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ABSTRACT

An abundant nuclear phosphoprotein, the La autoantigen, is the first protein to bind all newly synthesized RNA polymerase III transcripts. Binding by the La protein to the 3′ ends of these RNAs stabilizes the nascent transcripts from exonucleolytic degradation. In the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, the La protein is required for the normal pathway of tRNA maturation. Experiments in which the human protein was expressed in *S. pombe* have suggested that phosphorylation of the La protein regulates tRNA maturation. To dissect the role of phosphorylation in La protein function, we used mass spectrometry to identify three sites of serine phosphorylation in the *S. cerevisiae* La protein Lhp1p. Mutant versions of Lhp1p, in which each of the serines was mutated to alanine, were expressed in yeast cells lacking Lhp1p. Using two-dimensional gel electrophoresis, we determined that we had identified and mutated all major sites of phosphorylation in Lhp1p. Lhp1p lacking all three phosphorylation sites was functional in several yeast strains that require Lhp1p for growth. Northern blotting revealed no effects of Lhp1p phosphorylation status on either pre-tRNA maturation or stabilization of nascent RNAs. Both wild-type and mutant Lhp1 proteins localized to both nucleoplasm and nucleoli, demonstrating that phosphorylation does not affect subcellular location. Thus, although La proteins from yeast to humans are phosphoproteins, phosphorylation does not appear to be required for any of the identified functions of the *S. cerevisiae* protein.

Keywords: Lhp1p; mass spectrometry; nucleolus; RNA-binding proteins; RNA–protein interactions

INTRODUCTION

The first protein that binds newly synthesized RNA polymerase III transcripts is an abundant phosphoprotein known as the La protein. First identified as an autoantigen in patients suffering from the rheumatic diseases Sjogren's syndrome and systemic lupus erythematosus, the La protein is a component of all eukaryotic nuclei. The La protein binds nascent RNA polymerase III transcripts through specific recognition of their 3′-UUU_{OH} terminal sequences (Stefano, 1984). RNAs bound by the La protein include precursors to tRNAs, 5S rRNA, U6 snRNA, SRP RNA, and the cytoplasmic Y RNAs (Hendrick et al., 1981; Rinke & Steitz, 1982, 1985; Chambers et al., 1983; Stefano, 1984). In addition to these RNA polymerase III transcripts, the La protein also binds some transcripts made by other RNA polymerases. In yeast, precursors to certain RNA polymerase II-transcribed small nuclear and nucleolar RNAs that terminate in UUU_{OH} are bound by the La protein (Kufel et al., 2000; Xue et al., 2000).

Genetic analyses in the yeast *Saccharomyces cerevisiae* have revealed that binding by the La protein Lhp1p to precursor tRNAs is required for the normal pathway of tRNA maturation (Yoo & Wolin, 1997). Experiments in *Schizosaccharomyces pombe* cells and in vertebrate cell extracts have revealed that this is a conserved function of La proteins (Van Horn et al., 1997; Fan et al., 1998; Lin-Marq & Clarkson, 1998). Although the La protein is dispensable in wild-type yeast

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(Yoo & Wolin, 1994), genetic screens have identified a number of mutations in other genes that cause cells to require the La protein. These investigations revealed that Lhp1p stabilizes U6 snRNA and several other polymerase III transcripts from degradation (Pannone et al., 1998; Calvo et al., 1999) and also functions in the assembly of RNA polymerase II-transcribed U snRNAs into snRNPs (Xue et al., 2000). The human La protein has also been proposed to function in termination and reinitiation by RNA polymerase III (Gottlieb & Steitz, 1989; Maraia et al., 1994), however others have failed to detect a requirement for the human protein in transcription (Weser et al., 2000; Chong et al., 2001).

La proteins from humans to yeast are phosphoproteins (Pizer et al., 1983; Francoeur et al., 1985; Van Horn et al., 1997). Phosphorylation has been proposed to regulate several aspects of La protein function. In humans, the major phosphorylation site was mapped to S366 in the carboxy-terminus (Fan et al., 1997). Phosphorylation of S366 was proposed to regulate the recycling of RNA polymerase III transcription complexes, as only the unphosphorylated form of the La protein was active in stimulating transcription in vitro (Fan et al., 1997). More recently, the human La protein was expressed in La-deficient *S. pombe* cells and examined for its ability to promote maturation of a mutant *S. pombe* pre-tRNA. Although the human protein stabilized the 3′ ends of the pre-tRNA, removal of the pre-tRNA 5′ leader sequence was inhibited (Intine et al., 2000). Because a human La protein that could undergo phosphorylation of S366 was less inhibitory to pre-tRNA maturation than mutants lacking S366, phosphorylation on S366 was proposed to also regulate tRNA maturation (Intine et al., 2000). A more recent analysis of the phosphorylation of the human protein using nanoelectrospray mass spectrometry identified three additional C-terminal phosphorylation sites at Thr302, Ser325, and Thr 362 (Broekhuis et al., 2000). No functions have yet been assigned to these sites.

In this study, mass spectrometry was used to identify phosphorylation sites in the *S. cerevisiae* La protein. Three sites of phosphorylation were determined, including two serines in the N-terminus (Ser14 and Ser18) and one in the C-terminus (Ser234) of the protein. Versions of Lhp1p, in which each of the serines was mutated to alanines, were expressed in yeast cells lacking Lhp1p. Two-dimensional electrophoresis of cell extracts, followed by western blotting, revealed that all major sites of phosphorylation in Lhp1p had been identified and mutated. Northern blotting revealed no effects of Lhp1p phosphorylation status on either pre-tRNA maturation or mature tRNA accumulation. Consistent with the work of Broekhuis et al. (2000) on human La, phosphorylation of Lhp1p did not affect subcellular localization. Because yeast strains containing mutations in an essential tRNA gene or certain snRNA-binding proteins require Lhp1p for viability (Yoo & Wolin, 1997; Pannone et al., 1998; Xue et al., 2000), we examined whether phosphorylation was required for Lhp1p function in these strains. A mutant Lhp1p lacking all three major phosphorylation sites functioned similarly to wild-type Lhp1p in all tested strains, revealing that phosphorylation at these sites is not required for the roles of Lhp1p in tRNA maturation or nascent RNA stabilization.

RESULTS

Multiple isoforms of Lhp1p are due to phosphorylation

To examine Lhp1p phosphorylation, we subjected yeast cell extracts to two-dimensional nonequilibrium gradient electrophoresis (NEPHGE)/SDS-polyacrylamide gel electrophoresis (O'Farrell et al., 1977), followed by western blotting to detect Lhp1p. Because addition of a phosphate group affects the overall net charge of a protein, phosphorylations can appear as multiple isoforms. Three distinct Lhp1p isoforms were apparent in wild-type extracts (Fig. 1A, spots a, b, and c). All three species were undetectable when extracts were prepared from strains lacking *LHP1* (Fig. 1B). In addition to Lhp1p, the polyclonal antiserum recognizes several unrelated proteins (Yoo & Wolin, 1994). Although the identity of these proteins are unknown, they were useful for aligning the blots.

To determine whether the multiple isoforms were due to phosphorylation, we examined whether addition of phosphatase to the extract would reduce the number of species. In our initial experiments, calf intestinal alkaline phosphatase was added to the cell extract and the mixture incubated at 37 °C. Although the three isoforms collapsed into the most basic species upon incubation (data not shown), an experiment in which the phosphatase was omitted revealed that the 37 °C incubation alone reduced these isoforms to a single species (Fig. 1D). However, if either of two phosphatase inhibitors, β-glycerophosphate (Fig. 1E) and sodium fluoride (Fig. 1F), was added to the extract, all three isoforms were unaffected by heating. These results suggested that endogenous phosphatases were activated at 37 °C and that the two acidic isoelectric species were due to phosphorylation of Lhp1p.

To determine the role of phosphorylation in Lhp1p function, we purified the protein from a yeast strain in which Lhp1p was expressed under control of the *GAL1* promoter (Yoo & Wolin, 1997). Subjection of the purified protein to two dimensional gel analysis again revealed three isoforms (Fig. 1G). Upon treatment of the protein with λ phosphatase, the two most acidic isoforms collapsed to the most basic species (Fig. 1H). The collapse to a single species was not observed when β-glycerophosphate was included in the reaction (data not shown). We conclude that the two most acidic spots represent phosphorylated forms of Lhp1p.

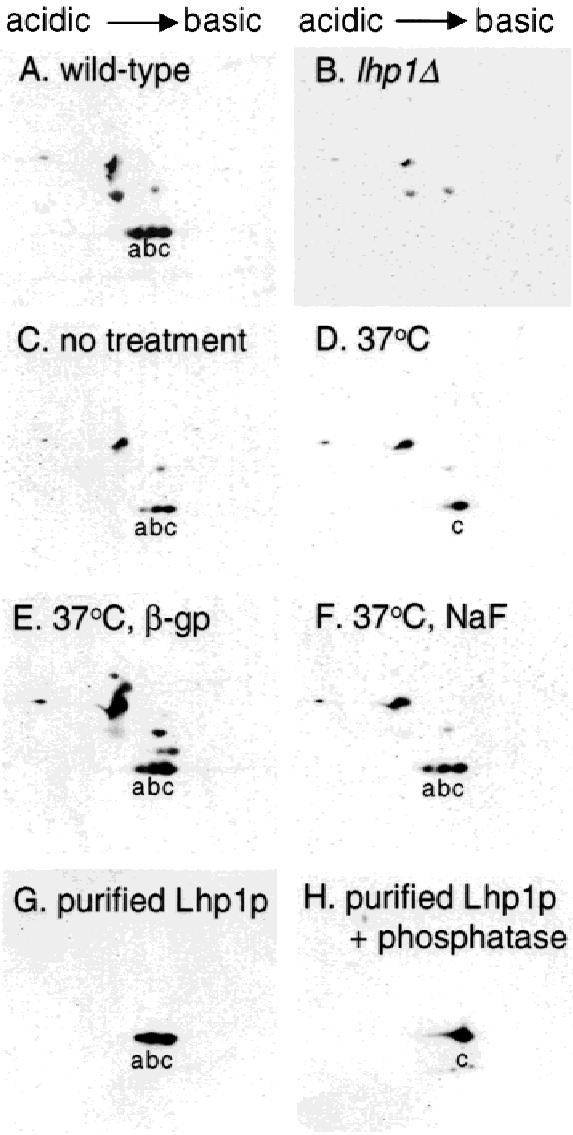


FIGURE 1. Multiple isoforms of Lhp1p are due to phosphorylation. **A,B:** Extracts from wild-type cells (**A**) and cells lacking Lhp1p (**B**) were subjected to two-dimensional gel electrophoresis and western blotting using anti-Lhp1p antibodies. Three major isoforms of Lhp1p are designated a, b, and c. **C–F:** Extracts from wild-type cells were either not heated (**C**) or heated to 37 °C for 45 min, followed by two-dimensional gel electrophoresis and Western blotting to detect Lhp1p (**D**, **E**, **F**). To determine if the change in the pattern of isoforms on heating was due to endogenous phosphatases, extracts were heated with 50 mM β-glycerophosphate (**E**) or 100 mM sodium fluoride (**F**). **G,H:** Lhp1p purified from yeast cells was subjected to two-dimensional gel electrophoresis followed by western blotting. In **H**, Lhp1p was incubated with 400 U λ pyrophosphatase (30 min at 30 °C) prior to electrophoresis.

Phosphorylation of Lhp1p does not affect RNA binding affinity

To compare the RNA-binding properties of the phosphorylated and unphosphorylated forms of Lhp1p, we performed electrophoretic mobility shift assays. Two forms of Lhp1p were examined, the partially phosphorylated protein purified from yeast (Fig. 1G) and the recombinant protein produced in *Escherichia coli*. For the RNA substrate, pre-tRNA$^{Ser}_{CGA}$ was transcribed with T7 RNA polymerase in the presence of [α-^{32}P]rUTP. Increasing amounts of protein were added to a constant amount of labeled RNA. The protein-bound and free forms of the RNA were separated in native gels. Upon addition of phosphorylated or recombinant Lhp1p, complex formation was observed (Fig. 2A,B, lanes 6–10). Quantitation of the gels using a phosphorimager revealed that the amount of Lhp1p required to shift 50% of the labeled pre-tRNA into complexes was similar for the two sources of protein, and occurred at a protein concentration of ~10 nM. Moreover, upon addition of increasing amounts of either the phosphorylated or recombinant protein, a higher order complex migrating with slower mobility was observed (Fig. 2A,B, asterisk).

The specificity of the complexes formed in the mobility shift assays was investigated with competition experiments. The competitors consisted of pre-tRNA$^{Ser}_{CGA}$ containing different 3′ ends. One competitor was the same RNA used to form the complexes, which terminates in UUU$_{OH}$. The other was the identical RNA that had been modified at the 3′ end to produce a 3′-UU$_p$ terminus. The former was expected to be an excellent competitor whereas the latter was expected to compete more poorly for binding to Lhp1p (Stefano, 1984; Terns et al., 1992; Yoo & Wolin, 1994).

For these experiments, we chose an Lhp1p concentration that resulted in 50–80% of the radiolabeled RNA forming RNP complexes. Labeled RNA was mixed with increasing amounts of unlabeled competitor RNA and then incubated with Lhp1p (Fig. 2C–F) For both forms of Lhp1p, the 3′-UUU$_{OH}$ competitor competed for binding more efficiently than the 3′-UU$_p$ competitor. In addition, the higher order complexes were preferentially dissociated by both competitors (Fig. 2C–F, asterisks), revealing that binding by Lhp1p in these higher order complexes is of lower specificity. The amount of competitor needed to dissociate 50% of the labeled RNA–protein complex, $K_d^{1/2}$, was estimated for each experiment. For the 3′-UUU$_{OH}$ competitors, the $K_d^{1/2}$ value was approximately 22.5 nM for both the partially phosphorylated yeast protein and the recombinant protein, indicating that for both proteins, a 15-fold excess of competitor was required to dissociate the complex. For the 3′-UU$_p$ competitors, the $K_d^{1/2}$ values were approximately 900 nM for the yeast protein and 600 nM for the recombinant protein. Thus for both preparations of Lhp1p, significantly more 3′-UU$_p$ is necessary to compete away 50% of the labeled complex (Fig. 2G).

Relative binding affinities were obtained by taking ratios of the $K_d^{1/2}$ values, providing a measure of how well each form of Lhp1p discriminates between the two RNAs. This revealed that for the partially phosphorylated yeast protein, the relative binding affinity (i.e., $K_{rel} = K_d^{1/2}$ UU$_p$/$K_d^{1/2}$ UUU$_{OH}$) was 40, compared to a value

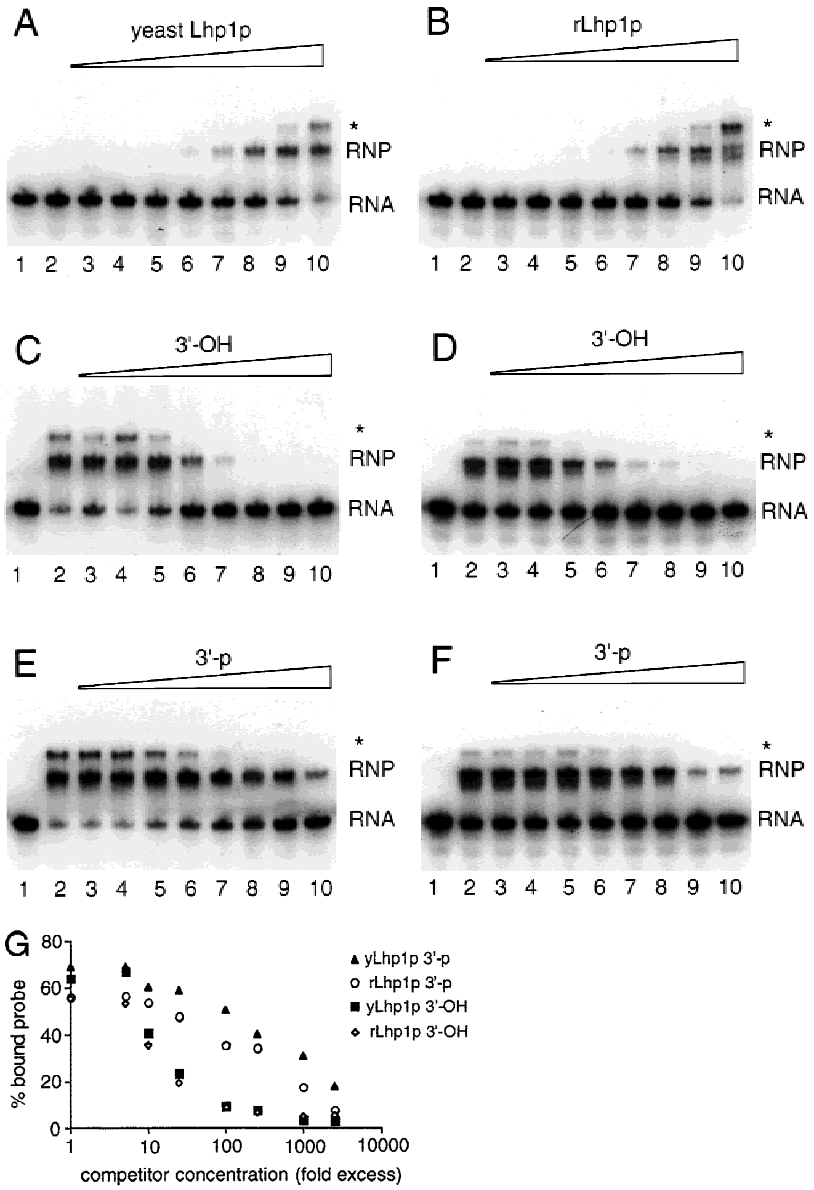


FIGURE 2. Partially phosphorylated and recombinant Lhp1p are similar in RNA binding affinity. **A,B:** 1.5 fmol of ^{32}P-labeled pre-tRNA$^{Ser}_{CGA}$ were incubated either without protein (lanes 1 and 2) or with 1.5 (lane 3), 3 (lane 4), 6 (lane 5), 12 (lane 6), 24 (lane 7), 48 (lane 8), 96 (lane 9), or 192 fmol (lane 10) purified Lhp1p. Naked RNAs and RNPs were separated by native gel electrophoresis. In **A**, partially phosphorylated Lhp1p was purified from yeast, and in **B**, recombinant Lhp1p was expressed and purified from *E. coli*. The asterisk denotes a higher order complex that forms in the presence of excess Lhp1p. The slightly smaller bands that appear below the major shifted product in **B** (as well as **D**, and **F**) are likely due to a small amount of an Lhp1p breakdown product that is present in the preparation of recombinant protein. **C,D:** 96 fmol of partially phosphorylated Lhp1p (**C**) or recombinant Lhp1p (**D**) were mixed with 1.5 fmol of ^{32}P-labeled pre-tRNA$^{Ser}_{CGA}$ in the presence of 0 (lane 2), 1.5 (lane 3), 3.75 (lane 4), 15 (lane 5), 37.5 (lane 6), 150 (lane 7), 375 (lane 8), 1,500 (lane 9), or 3,750 fmol unlabeled competitor pre-tRNA$^{Ser}_{CGA}$ terminating in UUU$_{OH}$. Naked RNAs and RNPs were separated by native gel electrophoresis. Lane 1: no added protein. **E,F:** 96 fmol of partially phosphorylated Lhp1p (**E**) or recombinant Lhp1p (**F**) were mixed with 1.5 fmol of ^{32}P-labeled pre-tRNA$^{Ser}_{CGA}$ in the presence of 0 (lane 2), 1.5 (lane 3), 3.75 (lane 4), 15 (lane 5), 37.5 (lane 6), 150 (lane 7), 375 (lane 8), 1,500 (lane 9), or 3,750 fmol unlabeled competitor pre-tRNA$^{Ser}_{CGA}$ in which the 3′ terminus was converted to UU$_p$. Naked RNAs and RNPs were separated by native gel electrophoresis. Lane 1: no added protein. **G:** Competition titrations for Lhp1p purified from yeast (yLhp1p) and recombinant Lhp1p (rLhp1p). The data are plotted as the fraction of radiolabeled probe bound at each concentration of unlabeled competitor. The data points represent averages from two experiments.

of 27 for the recombinant protein. Thus, Lhp1p discriminates between RNAs containing 3′-UUU$_{OH}$ and 3′-UU$_p$ termini, which are otherwise identical, with a specificity ratio of about 30. Our results are consistent with those obtained for the human protein (Stefano, 1984) in that a significant portion of the RNA-binding specificity comes from relatively minor changes in the RNA 3′ end. Our results also suggest that the partially phosphorylated protein is marginally better at discriminating between the two RNAs. Although the difference between the two proteins is small, similar relative binding affinities were observed with at least three different preparations of the yeast protein. Thus, the partially phosphorylated protein purified from yeast may have slightly higher specificity than the recombinant protein in discriminating between RNAs with 3′-UUU$_{OH}$ and 3′-UU$_p$ termini.

Mapping of Lhp1p phosphorylation sites

To identify sites of phosphorylation, the purified phosphoprotein was digested with trypsin and analyzed using a multidimensional electrospray mass spectrometry (MS)-based phosphopeptide mapping strategy (Annan et al., 2001). The digest was fractionated by reverse phase HPLC coupled on-line to a mass spectrometer operating in a manner that detects phosphopeptides selectively (Huddleston et al., 1993). The phosphopeptide-containing fractions were analyzed by nanoelectrospray MS using a precursor ion scan to selectively record the molecular weight of the phosphopeptides (Carr et al., 1996). If the amount of phosphopeptide was sufficient, the peptide was sequenced using tandem mass spectrometry.

Using this strategy, we identified four peptides that contained two unique phosphorylation sites, Ser14 (fraction 11, Fig. 3A,B and Table 1) and Ser18 (fraction 27, Fig. 3A,C, and Table 1). Using this digest we quantitated the extent of phosphorylation by positive ion liquid chromatography–mass spectrometry (LC-MS), measuring the ion abundance of the phosphorylated and nonphosphorylated peptide for each sequence. The stoichiometry of phosphorylation for Ser14 and Ser18 was 8% and 63%, respectively.

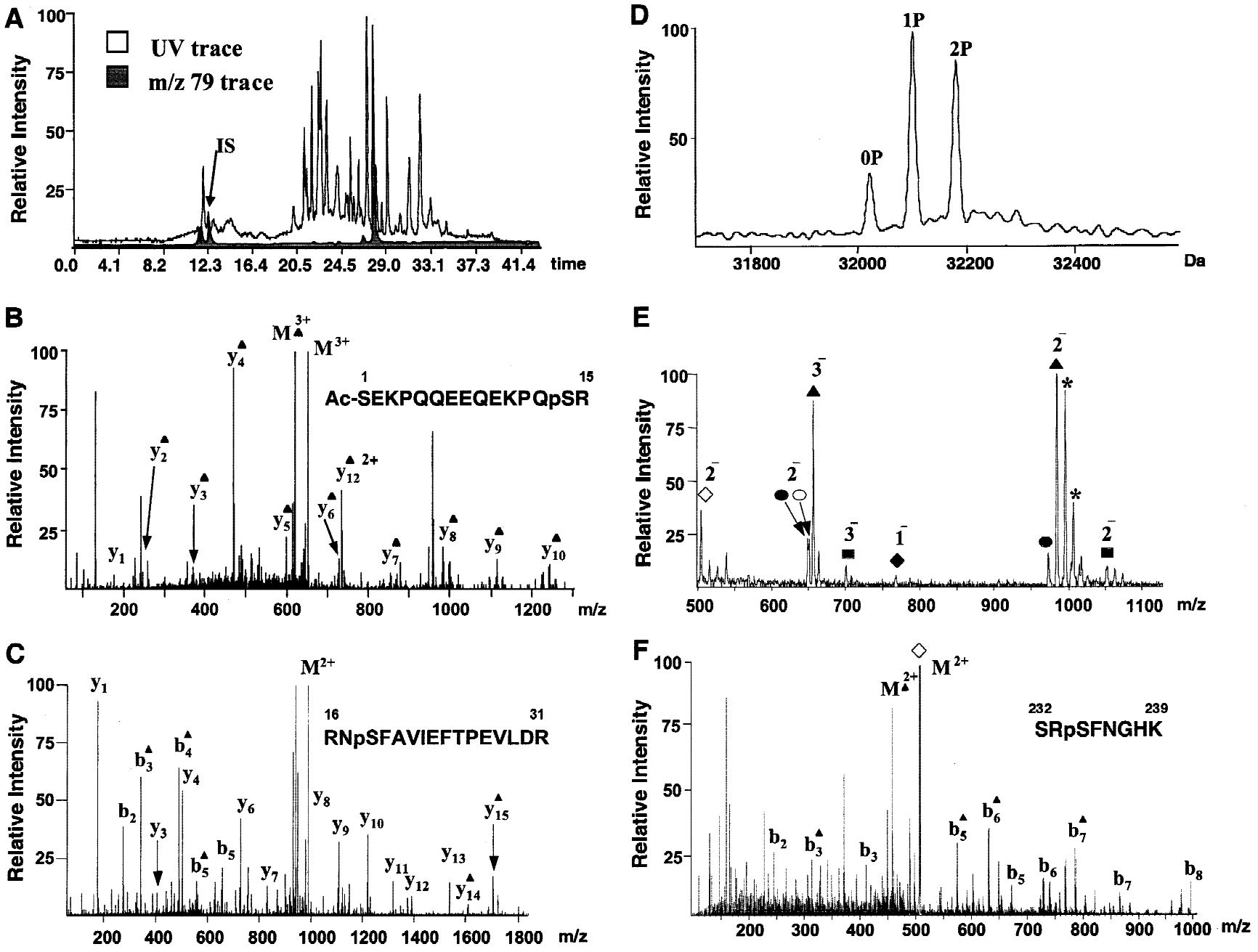
Analysis of the data revealed that we recovered relatively few peptides from the C-terminus of Lhp1p. Most peptides produced from the C-terminus by trypsin digestion are small due to the large number of lysines and arginines, and may not be retained by the reverse phase HPLC column. As the C-terminus contains many serines and human La is phosphorylated in this region (Fan et al., 1997; Broekhuis et al., 2000), we repeated the analysis using chymotrypsin. The phosphopeptide-selective LC-MS trace of the chymotryptic digest showed a large number of phosphopeptides (data not shown), however phosphopeptide-selective precursor ion analysis revealed that most were overlapping peptides containing the two previously identified phosphoserines. In fractions 17 and 20, we identified several potentially new phosphorylation sites. Fraction 17 contained two overlapping peptides that we assigned to residues 229–235 and 232–235 (Table 1). Fraction 20 contained two peptides, containing 1 and 2 mol of phosphate, which we assigned to residues 242–272 (Table 1). Using the nanoES spectrum of fraction 20, the approximate stoichiometry for the monophosphorylated form of the 242–272 peptide was 3%. As not all the nonphosphorylated peptide may have been recovered in the fraction, 3% represents the maximum possible level for the phosphorylated peptide. The diphosphorylated form was too low to be estimated accurately. We also detected two peptides, containing 1 and 2 mol of phosphate, which we assigned to residues 231–241 (Table 1). Although these peptides were derived from cleavage after glycine, which is not a preferred chymotryptic cleavage site, chymotrypsin is prone to random cleavages, especially at higher enzyme to substrate ratios (M.J. Huddleston & R.S. Annan, unpubl. data). However, we were unable to verify any of the assignments by sequencing, due to overlapping peptides or low recovery in the fractions.

To improve recovery of the C-terminal phosphopeptides, we performed a limited tryptic digest and conducted the precursor ion scan analysis on the unfractionated mixture, eliminating the reverse phase HPLC separation of the peptides. Prior to analysis, intact Lhp1p was desalted using the online LC-MS. This allowed us to record the molecular weight of the intact protein, revealing a distribution of Lhp1p containing 0, 1, and 2 mol of phosphate (Fig. 3D). The precursor ion scan analysis of the unfractionated limited tryptic digest showed, in addition to peptides containing the two N-terminal phosphoserines, two peptides that we assigned as residues 232–239 and 234–239 (◇ and ◆, respectively, in Fig. 3E and Table 1). We were able to sequence the larger peptide (◇) and show that it was phosphorylated on Ser234 (Fig. 3F). Comparing the ion abundance in the positive ion nanoES spectrum of the phosphorylated and nonphosphorylated peptides revealed between 45 and 60% phosphorylation at Ser234. Stoichiometry at Ser14 and Ser18 was similar to previous measurements, being 8% and 60%, respectively. As noted above, stoichiometry calculated from analyses of HPLC fractions should be considered a maximum estimate.

TABLE 1. Phosphopeptides of Lhp1p determined by mass spectrometry.

Residues[a]	Enzyme digest	Molecular size measured/calculated	Peptide	Mole %[b]	Modified residue[c]
1–15 + P	Trypsin	1948.8/1948.8	Ac-SEKPQQEEQEKPQ**pS**R	8%	Ser14
1–16 + P	Trypsin	2105.2/2104.9	Ac-SEKPQQEEQEKPQ**pS**RR		Ser14
16–31 + P	Trypsin	1972.8/1973.1	RN**pS**FAVIEFTPEVLDR	63%	Ser18
16–31 + P	Trypsin	1955.1/1955.1	RN**pS**FAVIE*TPEVLDR[d]		Ser18
229–235 + P	Chymo	875.2/875.4	SGRSRSF		
232–235 + P	Chymo	575.4/575.2	SRSF		
231–241 + P	Chymo	1422.8/1423.7	RSRSFNGHKKK		
231–241 + 2P	Chymo	1502.8/1503.7	RSRSFNGHKKK		
242–274 + P	Chymo	3865.8/3866.1	NLPKFPKNKKKNGKEESKEDSSAIADDDEEHKE	3%	
242–274 + 2P	Chymo	3945.0/3946.1	NLPKFPKNKKKNGKEESKEDSSAIADDDEEHKE		
232–239 + P	Trypsin[e]	1012.0/1011.4	SR**pS**FNGHK	45%	Ser234
234–239 + P	Trypsin[e]	769.0/768.3	SFNGHK	60%	

[a]Initiator methionine has been removed. Sequence begins with acetylserine at residue one.
[b]Mole percent is calculated by dividing the ion intensity of the phosphorylated peptide by the sum of the ion intensities for the phosphorylated and nonphosphorylated peptides.
[c]Specific site of phosphorylation determined by MS/MS.
[d]The residue at position 24 is modified such that the mass difference is 18 Da less than Phe at this position. The nature of the modification is unknown.
[e]Limited tryptic digest.


A
Relative Intensity
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IS
time
B
Ac-SEKPQQEEQEKPQpSR
m/z
C
RNpSFAVIEFTPEVLDR
m/z
D
1P
2P
0P
Da
E
m/z
F
SRpSFNGHK
m/z

Nonetheless, the measured stoichiometry at Ser14, Ser18, and Ser234 predicts a distribution of 17, 47, 33, and 3% for 0, 1, 2, and 3 mol of phosphate on the intact protein, which agrees with the measured distribution of 12, 50, 37, and 0% shown in Figure 3D. This suggests that phosphorylation on other sites, particularly the C-terminal 242–274 peptide identified in the chymotryptic digest, must be of very low stoichiometry.

Phosphorylation does not appear required for the roles of Lhp1p in tRNA and snRNP biogenesis

To determine the role of phosphorylation in Lhp1p function, each of the three major sites of serine phosphorylation was mutated to the nonphosphorylatable residue alanine. We examined the function of each mutant Lhp1p by determining whether the mutant protein could replace wild-type Lhp1p in yeast strains that require Lhp1p for viability. Strains containing mutations in *sup61*$^+$, which encodes $tRNA^{Ser}_{CGA}$, require Lhp1p for maturation of the mutant pre-tRNA (Yoo & Wolin, 1997). In addition, strains carrying mutations in either *LSM8*, which encodes a core protein of the U6 snRNP, or *SMD1*, which encodes a core protein of the RNA polymerase II-transcribed spliceosomal U snRNPs, require *LHP1* (Pannone et al., 1998; Xue et al., 2000). For these experiments, each strain contained a chromosomal disruption of *LHP1* (*lhp1::LEU2*), with the only copy of *LHP1* supplied on a *URA3*-containing plasmid. Following transformation with low copy plasmids containing serine to alanine Lhp1p mutants, colonies were plated onto media containing 5-fluoro-orotic acid (5-FOA) and screened for the ability to lose the *LHP1/URA3*-containing plasmid. Because 5-FOA selects against *URA3*, only cells able to lose the plasmid will grow under these conditions. For all mutant Lhp1p proteins, including a version of Lhp1p in which all three major phosphorylation sites were mutated to alanines, the extent of growth on 5-FOA was comparable to that seen when a wild-type *LHP1* gene was introduced into the strains (Fig. 4A; also data not shown).

To confirm that the mutations altered phosphorylation of Lhp1p, we isolated *sup61* strains in which the only copy of Lhp1p was the mutated form on the low copy plasmid. Protein extracts were separated on two-dimensional gels and Lhp1p detected by western blotting. For the single mutant S14A, only the tail of the most acidic spot was reduced (Fig. 5B, spot a), consistent with the low abundance of this phosphorylation in the purified protein. However, the single mutant S18A (Fig. 5C) and the double mutant S14A S18A (Fig. 5F) resulted in a more distinct change in the pattern of isoelectric species, as the most acidic spot was significantly reduced. Although mutation of S234 alone resulted in minor changes to the gel pattern (Fig. 5D), only the most basic isoform was detected in the triple mutant S14A S18A S234A (Fig. 5H), suggesting that we had mutated all major sites of phosphorylation in Lhp1p. This was confirmed by mutating each of the remaining serines in the two C-terminal phosphopeptides (S232, S258, S262, and S263) to alanines. Each mutant protein functioned in the *sup61* strain (data not shown), and none of the mutations changed the pattern of isoforms detected in the two-dimensional gels (shown for S258A, Fig. 5E). We conclude that S14, S18, and S234 are the major sites of phosphorylation in Lhp1p. Furthermore, in the presence of the triple mutation (Fig. 5H), phosphorylation of minor sites does not increase significantly.

As phosphorylation of human La was reported to be required for its role in tRNA maturation (Intine et al., 2000), we examined the pattern of pre-tRNA processing intermediates in cells containing the triple phosphorylation mutant. For these experiments, we created *sup61* strains in which the only copy of *LHP1* was the S14A S18A S234A mutant on a low copy plas-

FIGURE 3. Identification of phosphorylation sites on Lhp1p using mass spectrometry. **A:** Purified Lhp1p was digested with trypsin and injected onto a 0.5-mm i.d. reversed phase HPLC column. The MS was set to detect only the phosphopeptide-specific marker ions m/z 63 and 79. The m/z 79 trace (gray) is compared with the LC-UV trace (white). Four fractions were taken for further analysis by nanoES using precursor scans for m/z 79. Fractions correspond to the whole number integer of the elution time, such that any peak eluting between 12.0 and 12.9 will be in fraction 12. I.S. refers to the 50-pmol phosphopeptide internal standard, KRPpSQRHGSKY, which is added to the sample as a control. **B:** NanoES (+) ion MS/MS spectrum of m/z 650.5, the triply charged ion for the Lhp1p phosphopeptide 1–15 + P found in fraction 11. The y_n series assigns the phosphate to Ser14. **C:** NanoES (+) ion MS/MS spectrum of m/z 987.5, the doubly charged ion for the Lhp1p phosphopeptide 16–31 + P found in fraction 27. The b_n and y_n series locate the phosphate on Ser18. Peptide fragment ion nomenclature is that of Biemann (1990), except that b_n- or y_n- refer to $(b_n\text{-}H_3PO_4)^+$ and $(y_n\text{-}H_3PO_4)^+$ and M^{2+} and $M-^{2+}$ refer to $[M+2H]^{2+}$ and $[(M+2H)\text{-}(H_3PO_4)]^{2+}$ respectively. **D:** LC-MS (+) ion spectrum showing the distribution and relative stoichiometry of phosphate incorporation in purified Lhp1p. Raw MS data was smoothed and transformed into the molecular weight domain. Measured M_r for zero phosphates was 32,178 Da (calculated 32,175 Da). **E:** NanoES (−) ion m/z 79 precursor scan of the unfractionated limited tryptic digest of Lhp1p. Six phosphopeptides representing three unique phosphorylation sites were detected, including two peptides from the C-terminus of Lhp1p (Table 1). The peptide marked with the diamond was sequenced by MS/MS. **F:** NanoES (+) ion MS/MS spectrum of m/z 506.5, the doubly charged ion for the phosphopeptide 232–239 + P. The b_n series assigns the phosphate to Ser234.

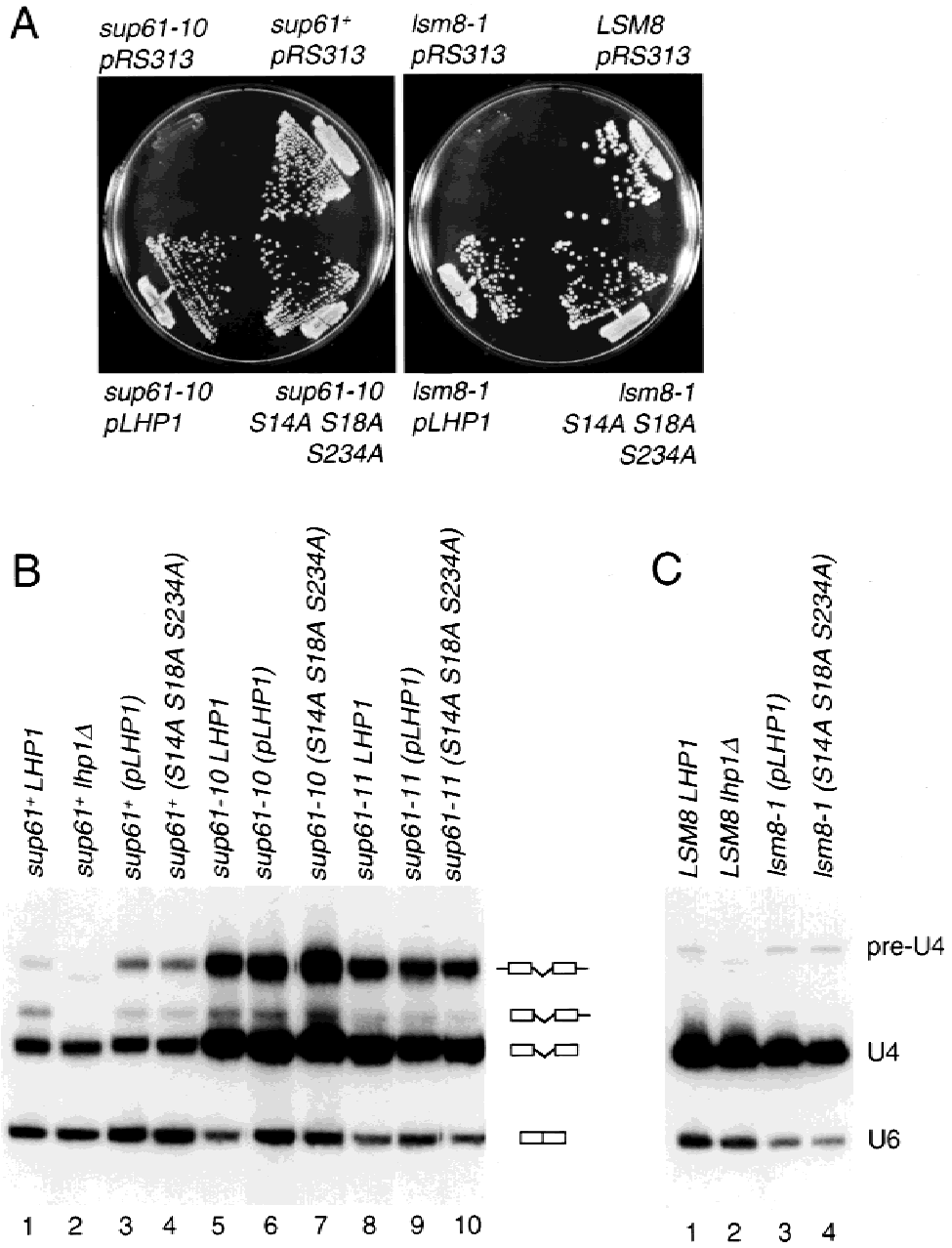

FIGURE 4. Phosphorylation of Lhp1p does not appear to be required for its functions in tRNA and U snRNA biogenesis. **A:** Either wild-type *LHP1* or *LHP1* carrying the S14A S18A S234A mutations was cloned into the *HIS3/CEN* plasmid pRS313 and introduced into *lhp1::LEU2* strains carrying either the *sup61-10* (left panel) or the *lsm8-1* (right panel) mutation. All strains also carried pSLL28 (*LHP1 URA3 LYS3*; Yoo & Wolin, 1997). Strains were streaked on media containing 5-FOA and grown at 25 °C. For comparison, the growth of a *lhp1::LEU2* strain carrying pSLL28, an *lhp1::LEU2 sup61-10* pSLL28 strain carrying pRS313, and an *lhp1::LEU2 lsm8-1* pSLL28 strain carrying pRS313 are shown. **B:** RNA was extracted from *sup61*$^+$ (lanes 1–4) *sup61-10* (lanes 5–7), and *sup61-11* (lanes 8–10) strains carrying either chromosomal *LHP1* (lanes 1, 5, 8), no *LHP1* (lane 2), pRS313-LHP1 (lanes 3, 6, 9), or pRS313-LHP1 carrying the S14A S18A S234A triple mutation (lanes 4, 7, 10) and subjected to northern analyses to detect $tRNA^{Ser}_{CGA}$. Note that the *sup61-10* mutation reduces the level of mature $tRNA^{Ser}_{CGA}$ (lane 5), and that *LHP1* on the low copy plasmid increases the levels of mature tRNA in the mutant strain (lanes 6–7). The *sup61*$^+$ strains in lanes 3 and 4 also contain the *lsm8-1* mutation. **C:** RNA was extracted from wild-type (lanes 1 and 2) and *lsm8-1* (lanes 3 and 4) strains carrying either chromosomal LHP1 (lane 1), no LHP1 (lane 2), pRS313-LHP1 (lane 3), or pRS313-LHP1 carrying the S14A S18A S234A triple mutation (lane 4) and subjected to Northern analyses to detect U4 and U6 snRNAs.

mid. These strains carried either the original *sup61-10* mutant allele (Yoo & Wolin, 1997) or a second allele, *sup61-11*, which contains a G-to-A change in the D-loop of $tRNA^{Ser}_{CGA}$ (D. Rubinson & S. Wolin, unpubl. data). In both *sup61* strains, Lhp1p is required for pre-tRNA maturation (Yoo & Wolin, 1997; N. Carmi &

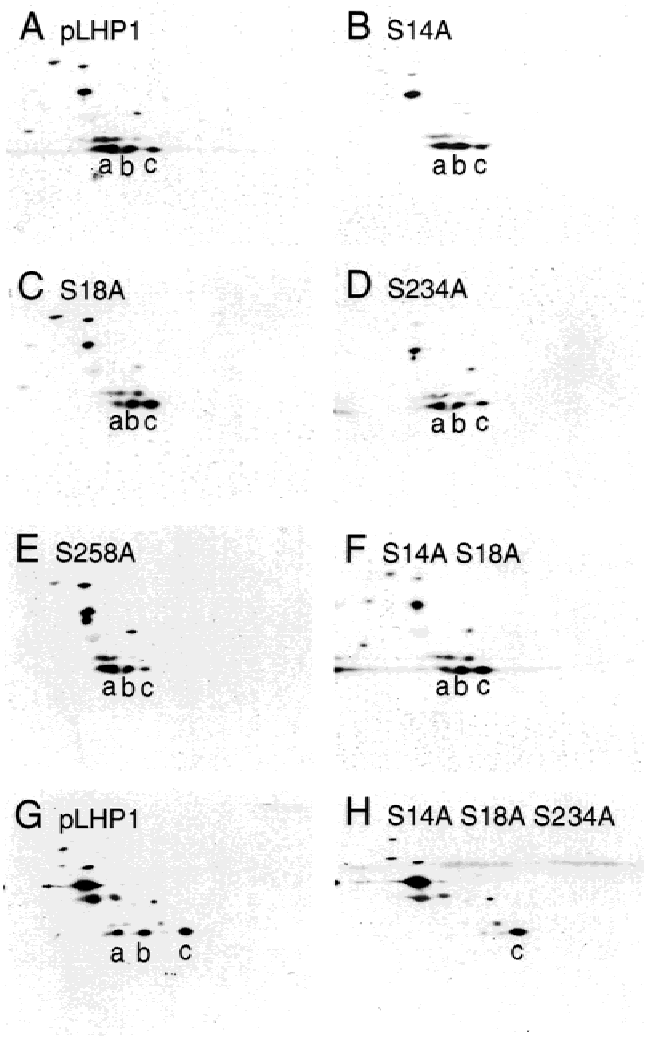

FIGURE 5. Mutations in phosphorylation sites change the isoelectric mobility of Lhp1p isoforms. Extracts were prepared from *lhp1::LEU2* yeast cells carrying wild-type (**A** and **G**) or the indicated mutant forms of *LHP1* (**B–F**, **H**) on a centromeric plasmid. Following two-dimensional gel electrophoresis, proteins were subjected to western blotting to detect Lhp1p. In **G** and **H**, the isoelectric focusing step was carried out for 2 h (vs. 1.5 h in **A–F**) to maximize separation of the different isoforms. The differences in the relative intensities of spots a, b, and c between **A** and **G** may be due to the different electrophoresis conditions. In addition, some variation was noticed in the ratio and appearance of the spots between different experiments. For this reason, a control wild-type extract was included in every experiment.

S. Wolin, unpubl. data). RNA was extracted from the strains and subjected to northern analyses. As pre-tRNA$^{Ser}_{CGA}$ contains an intron, we detected pre-tRNAs by probing with an oligonucleotide complementary to the intron. Mature tRNA was detected using an oligonucleotide complementary to the spliced anticodon loop (Yoo & Wolin, 1997). As previously described (Yoo & Wolin, 1997), yeast cells lacking Lhp1p have alterations in the pattern of pre-tRNAs processing intermediates detected on northern blots (Fig. 4B, compare lanes 1 and 2). Although both *sup61* mutations cause accumulation of pre-tRNAs, both the pattern of pre-tRNAs and the levels of mature tRNA$^{Ser}_{CGA}$ were unaffected by Lhp1p phosphorylation (Fig. 4B, lanes 5–6 and 9–10). To determine if other pre-tRNAs might be affected by Lhp1p phosphorylation, we also examined the levels of tRNA$^{Arg}_{CCG}$ in these strains. The levels of the mature tRNA$^{Arg}_{CCG}$ were identical in all strains, and precursors did not accumulate (data not shown).

Because Lhp1p is required for stable accumulation of U6 snRNA in *lsm8-1* cells, we examined whether phosphorylation altered U6 snRNA levels in the mutant strain. Northern analyses revealed that whereas U6 snRNA levels are lower in *lsm8-1* strains (Pannone et al., 1998), the phosphorylation status of Lhp1p had no effect on U6 snRNA accumulation (Fig. 4C, lanes 3–4). Probing the blot to detect the U4 snRNA revealed that pre-U4 RNA, which is stabilized by Lhp1p binding (Xue et al., 2000), was also unaffected by phosphorylation of Lhp1p (Fig. 4C, lanes 3–4).

Phosphorylation does not affect nuclear or nucleolar localization of Lhp1p

Because Lhp1p localizes to the nucleus of yeast cells (Rosenblum et al., 1997; Sobel & Wolin, 1999), we examined whether phosphorylation affected subcellular localization. Double-labeling experiments, using an antibody against the nucleolar protein Nop1p (Aris & Blobel, 1988), revealed that Lhp1p is located in both the nucleoplasm (Fig. 6A, green) and nucleolus (yellow). In cells lacking *LHP1*, only a background cytoplasmic fluorescence was detected with anti-Lhp1p antibodies (green, Fig. 6B). To determine the effect of phosphorylation on subcellular location, we examined *sup61-11* strains carrying either the wild-type *LHP1* or the triple phosphorylation mutant on a low copy plasmid. This revealed that the S14A S18A S234A mutant Lhp1p was indistinguishable in subcellular location from wild-type Lhp1p (compare Fig. 6C,D). Thus, phosphorylation at these sites is not required for targeting of Lhp1p to either the nucleoplasm or the nucleolus.

DISCUSSION

La proteins from humans to yeast are phosphoproteins. To dissect the role of phosphorylation in La protein function, we identified and mutated all major sites of phosphorylation in the *S. cerevisiae* La protein Lhp1p. Our experiments reveal that phosphorylation of Lhp1p at these sites is not essential for the roles of Lhp1p in pre-tRNA maturation, U6 snRNP biogenesis, or spliceosomal snRNP assembly. Similarly, phosphorylation does not appear required for correct subcellular localization of Lhp1p. Thus, whereas Lhp1p is phosphorylated in vivo, phosphorylation does not appear to be required for any of the identified functions of the protein.

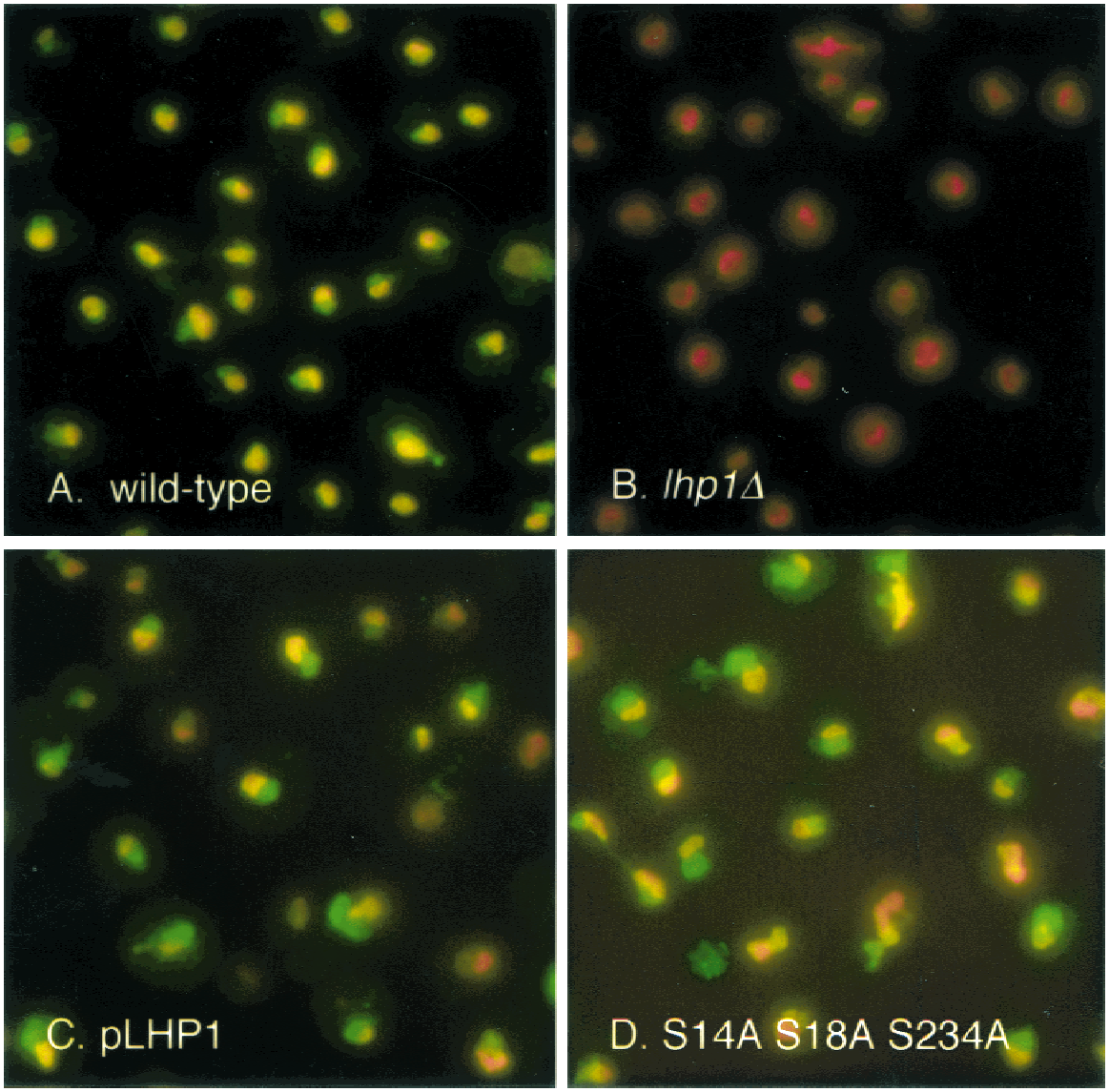

FIGURE 6. Phosphorylation does not appear to be required for nuclear or nucleolar localization of Lhp1p. Rabbit anti-Lhp1p antibodies (green) and mouse anti-Nop1 antibodies (red) were used to stain wild-type cells (**A**), *lhp1::LEU2* cells (**B**), and *lhp1::LEU2 sup61-11* cells carrying either wild-type *LHP1* (**C**) or the triple phosphorylation mutant (**D**) on a low copy plasmid. Colocalization of the Lhp1p and nucleolar Nop1p signals is indicated by yellow.

Although phosphorylation sites have only been mapped in the human and *S. cerevisiae* La proteins, a comparison of the sites suggests that the phosphorylation sites are not well conserved. Four sites of phosphorylation have been identified in the human protein. All four sites are located in the C-terminus, which is the least conserved region of La proteins (Yoo & Wolin, 1994), and none of the sites appear conserved beyond vertebrate species. Most notably, a clear counterpart to the S366 casein kinase II site of human La is not evident in either *Drosophila melanogaster* or *S. pombe*. Consistent with the lack of conservation, two of the three major phosphorylation sites in Lhp1p map to the N-terminus of the protein, and the C-terminal site does not correspond to a casein kinase II site. Although a potential casein kinase site in the C-terminus of Lhp1p (S258) was suggested to be functionally analogous to S366 (Fan et al., 1997), this residue is not appreciably phosphorylated in Lhp1p (Fig. 5E; also Table 1). Moreover, mutant versions of Lhp1p that cannot be phosphorylated on S258 function in strains that require *LHP1* for tRNA maturation.

Phosphorylation of the human La protein on S366 has been proposed to regulate several steps in the biogenesis of RNA polymerase III transcripts. Because only the unphosphorylated form of the human protein was active in stimulating transcription by RNA polymerase III, phosphorylation of La was proposed to regulate RNA polymerase III transcription (Fan et al., 1997). How-

ever, two groups recently reported that, in human cell extracts, efficient RNA polymerase III transcription occurs in the absence of detectable La protein (Weser et al., 2000; Chong et al., 2001). Consistent with these results, the La protein is not required for efficient polymerase III transcription in yeast or *Xenopus* extracts (Yoo & Wolin, 1997; Lin-Marq & Clarkson, 1998; Pannone et al., 1998). Most recently, phosphorylation of the human protein was proposed to modulate tRNA maturation. Specifically, addition of the unphosphorylated human protein to processing reactions containing *S. cerevisiae* RNase P inhibited removal of the pre-tRNA 5′ leader (Fan et al., 1998). Furthermore, experiments in which mutant human La proteins were expressed in *S. pombe* suggested that phosphorylation of the human protein on S366 was required for pre-tRNA maturation (Intine et al., 2000).

Our experiments strongly suggest that phosphorylation of the La protein is not required for tRNA maturation in *S. cerevisiae*. In both *S. cerevisiae* and *S. pombe*, RNase P-mediated removal of pre-tRNA 5′ leaders occurs in cells lacking La (Van Horn et al., 1997; Yoo & Wolin, 1997). Our finding that pre-tRNA maturation is normal in strains carrying largely unphosphorylated Lhp1p indicates that, at least in *S. cerevisiae*, RNase P-mediated removal of the 5′ leader is also not inhibited by unphosphorylated La protein. We do not know the reasons for the differences between our results and the work on the human protein. One possibility is that the human protein, which contains a longer C-terminal domain than either the *S. cerevisiae* or *S. pombe* La proteins, has evolved to carry out additional functions. It is also possible that, although the two pre-tRNAs we examined were unaffected by Lhp1p phosphorylation status of Lhp1p, the processing of other pre-tRNAs may be affected. Alternatively, differences in our experimental protocols may account for the disparate results.

The fact that La proteins from humans to yeast are phosphoproteins suggests that phosphorylation plays a role in La protein function. As the phosphorylated protein purified from yeast cells was slightly better than the recombinant protein at distinguishing between RNAs ending in UUU_{OH} and UU_p, phosphorylation may make a small contribution to RNA-binding specificity. However, experiments in which we performed immunoprecipitations from strains carrying the triple phosphorylation mutant did not reveal differences in the spectrum of RNAs bound by the mutant Lhp1p (S.L. Wolin, unpublished data). Nonetheless, there may be subtle changes in the RNAs bound by the mutant Lhp1p that we failed to detect in our experiments. It is also possible that phosphorylation of Lhp1p contributes a subtle growth advantage that we do not detect under our laboratory conditions. Lastly, we cannot rule out the possibility that minor phosphorylation sites are critical for Lhp1p function. Nonetheless, as other functions of Lhp1p are uncovered, our identification of the major phosphorylation sites will allow examination of the role of phosphorylation in these processes.

MATERIALS AND METHODS

Yeast strains and media

Yeast media and manipulations were as described by Sherman (1991). Yeast strains are listed in Table 2.

Whole-cell extracts, two-dimensional protein gels, and western blotting

For extracts, yeast cells were grown at 30 °C to OD_{600} = 0.6–0.8, pelleted, and washed once with water. Cells were lysed by vortexing with glass beads in 100 μL of NET-2 (40 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.05% Nonidet P-40) with 0.5 mM phenylmethylsulfonyl chloride (PMSF) and 125 ng each of leupeptin, chymostatin, pepstatin, and antipain. Lysates were sedimented at 1,500 $\times$ *g* for 5 min to remove debris. Extracts were focused in 4.5% acrylamide-7 M urea rod gels using nonequilibrium gradient electro-

TABLE 2. Yeast strains used in this study.

Strain	Genotype	Source or reference
CY1	*MAT*α *ura3 lys2 ade2 trp1 his3 leu2 LHP1*	Yoo & Wolin, 1997
CY4	*MAT***a** *ura3 lys2 ade2 trp1 his3 leu2 lhp1::LEU2*	Yoo & Wolin, 1997
CY9	*MAT***a** *sup61-10 ura3 lys2 ade2 trp1 his3 leu2 lhp1::LEU2* pSLL28	Yoo & Wolin, 1997
CY10	*MAT*α *sup61-10 ura3 lys2 ade2 trp1 his3 leu2 LHP1*	C. Yoo
CY21	*MAT***a** *ura3 lys2 ade2 trp1 his3 leu2 lhp1::LEU2* pLHP1GAL	Yoo & Wolin, 1997
BP3	*MAT*α *lsm8-1 ura3 lys2 ade2 trp1 his3 leu2 lhp1::LEU2* pSLL28	B. Pannone
SWY1	*MAT*α *lsm8-1 ura3 lys2 ade2 trp1 his3 leu2 lhp1::LEU2* pRS313-LHP1	This study
SWY2	*MAT*α *lsm8-1 ura3 lys2 ade2 trp1 his3 leu2 lhp1::LEU2* pRS313-LHP1 S15A S19A S235A	This study
CY113	*MAT***a** *sup61-11 ura3 lys2 ade2 trp1 his3 leu2 LHP1*	D. Rubinson & C. Yoo
DNY15	*MAT***a** *sup61-11 ura3 lys2 ade2 trp1 his3 leu2 lhp1::LEU2* pRS313-LHP1	This study
DNY16	*MAT***a** *sup61-11 ura3 lys2 ade2 trp1 his3 leu2 lhp1::LEU2* pRS313-LHP1 S15A S19A S235A	This study
DX7	*MAT***a** *smd1-1 lhp1::LEU2 ura3 lys2 ade2 trp1 his3 leu2* pSLL28	D. Xue

phoresis (NEPHGE) in the first dimension (O'Farrell et al., 1977), and 10% SDS-PAGE in the second dimension. Ampholenes with pH ranges 3.5–10 (Resolyte, BDH Chemicals) and 8–10.5 (Pharmalyte, Pharmacia) were used for NEPHGE gels in a ratio of 2:1. The rod gels were focused for 1.5 h at 250 V. Electrophoresis was performed using a Hoefer Mighty Small system (Pharmacia). After electrophoresis, proteins were transferred to nitrocellulose and visualized by immunoblotting with anti-Lhp1p antibodies (Yoo & Wolin, 1994).

Electrophoretic mobility shift assays and competition experiments

To place pre-tRNA$^{Ser}_{CGA}$ behind the T7 promoter, yptS5P 5′-CGGCGAATTCTAATACGACTCACTATAGATCGAAATGGCAC TATG-3′ and yptS3P 5′-GGCCGGATCCTTTAAATTAAAC GACACCAGC-3′ were used to amplify the gene using the plasmid pAD12SUP61$^+$ (Yoo & Wolin, 1997) as template. The product was digested with *Eco*RI and *Bam*HI and inserted into *Eco*RI/*Bam*HI sites of pSP64 (Promega). The 5′ A in the wild-type sequence was changed to a G to allow efficient transcription. After linearization with *Dra*I, transcription with T7 RNA polymerase yielded a pre-tRNA containing three Us at the 3′ end.

For gel shifts, pre-tRNA$^{Ser}_{CGA}$ was synthesized using T7 polymerase as described (Yisraeli & Melton, 1989), except that 50 μCi of [α-^{32}P]UTP (Amersham; 400 Ci/mmol) was used in place of rUTP. Competitor "unlabeled" RNAs were synthesized using 1 mM of all four rNTPs and 0.2 μCi of [α-^{32}P]rUTP in a volume of 20 μL. All RNAs were gel purified, precipitated with ethanol, resuspended in 20 μL H_2O, and quantitated by scintillation counting. The RNA was resuspended to 1.5 fmol/μL in binding buffer (20 mM Tris, pH 8.0, 3 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, 100 mM NaCl). Binding reactions contained 1.5 fmol of labeled RNA, 1.5–192 fmol of Lhp1p, 2.5 ng poly (C), 0.01% NP-40 in binding buffer. Recombinant Lhp1p was purified as described (Yoo & Wolin, 1997). Dilutions of Lhp1p were made in La storage buffer (25 mM Tris pH 8.0, 3 mM $MgCl_2$, 0.1 mM EDTA, 100 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM PMSF). Reactions were incubated at room temperature for 30 min and then at 4 °C for 30 min, before loading onto 5% acrylamide (39:1 acrylamide:bisacrylamide)/5% glycerol gels (prerun at 15 V/cm for 10 min at 4 °C). Gels were run at 4 °C for 10 min at 2 V/cm, followed by 1.75 h at 15 V/cm in 0.5 × TBE (50 mM Tris, 45 mM boric acid, 1.25 mM EDTA).

For competition experiments, 1.5-fmol-labeled RNA was mixed with competitor RNA (1.5 fmol–3.75 pmol), followed by addition of 96 fmol Lhp1p. Competitors were the identical RNA with a 3′-UUU_{OH} terminus or the RNA with a 3′-UU_P terminus. RNA with a 3′-UU_P terminus was prepared by subjecting pre-tRNA$^{Ser}_{CGA}$ to Whitfeld degradation (Stefano, 1984) by incubation in 100 μL 1 M lysine-Cl, 0.025 M $NaIO_4$, pH 8.5, for 2 h at 45 °C, followed by ethanol precipitation.

Purification of Lhp1p for mass spectrometry

Lhp1p was purified from a strain in which *LHP1* was expressed under control of the *GAL1* promoter (Yoo & Wolin, 1997). Cells were grown at 30 °C in 50 mL SC-uracil and used to inoculate 10 L of YPGalactose. Cells were grown at 30 °C and harvested at OD_{600} = ~0.6. After washing with water, cells were resuspended in 25 mM Tris, pH 8.0, 10% glycerol, 1 mM DTT, 100 mM NaCl containing protease inhibitors (complete tablets, EDTA-free; Boehringer Mannheim) and phosphatase inhibitors (20 mM NaF, 1 mM Na_3VO_4, 10 mM sodium pyrophosphate, 2 mM EDTA, and 1 μM microcystin-LR, all Sigma), and lysed by vortexing with glass beads (425–600 μM). Following sedimentation at 15,000 rpm for 30 min in an SS34 rotor, the lysate was loaded on a HiLoad Q-Sepharose column (Pharmacia) in La storage buffer and run with a 0.1 to 1.0 M NaCl gradient. All columns were run in La storage buffer unless otherwise indicated. Lhp1p eluted between 0.3 and 0.7 M NaCl. Fractions containing Lhp1p were diluted twofold in La storage buffer lacking NaCl and applied to a 5-mL Heparin Hi-Trap column (Pharmacia) run with a 0.25 to 1.0 M NaCl gradient. Lhp1p eluted between 0.6–0.9 M NaCl was dialyzed against storage buffer containing 0.1 M NaCl and loaded onto a poly(U) HiTrap column (Pharmacia). A gradient was run from 0.1 to 2 M NaCl, with Lhp1p eluting between 0.6–0.8 M NaCl. These fractions were diluted fourfold with storage buffer lacking NaCl and concentrated on a 1-mL Heparin Hi-Trap column with a 0.6 to 0.8 M NaCl gradient. Fractions containing Lhp1p (3 mL) were loaded onto an S200 gel filtration column, eluted with storage buffer, and concentrated on a Heparin Hi-Trap column run with a 0.1 to 1.0 M NaCl gradient in storage buffer lacking glycerol. Fractions containing Lhp1p were applied to a Desalting Hi-Trap column (Pharmacia) run in 1.25 mM Tris, pH 8.0, 150 μM $MgCl_2$, 5 μM EDTA, 5 mM NaCl, 50 μM DTT, 50 μM PMSF, and concentrated in a speedvac. Two-dimensional gels were run during the purification to confirm that all isoelectric species were present.

Phosphorylation site mapping by electrospray mass spectrometry

Purified phosphorylated Lhp1p was digested with either trypsin or chymotrypsin for 4 h at 37 °C. The digest was subjected to a multidimensional phosphopeptide analysis using electrospray mass spectrometry (ESMS; Annan et al., 2001). Briefly, peptides from the tryptic digest were analyzed for phosphorylation using on-line LC-ESMS. The flow from the column was split (5:1) with the majority of the eluent going to a fraction collector, taking 1-min fractions, and the balance to the mass spectrometer. A modified Sciex API III mass spectrometer (MDS Sciex, Ontario Canada) was optimized to produce and detect mass to charge ratio m/z 79 (PO_3^-) fragment ions, which are specific for phosphorylated peptides (Huddleston et al., 1993). The MS is operated in a single ion monitoring mode for enhanced sensitivity. Phosphopeptide–containing HPLC fractions were analyzed by nanoES (Wilm & Mann, 1996) in the negative-ion mode using a precursor ion scan for m/z 79. In this experiment phosphopeptides are distinguished from nonphosphopeptides and their molecular weights determined (Carr et al., 1996). Candidate phosphopeptides were sequenced by nanoES tandem MS (Annan et al., 2001).

Alternatively, the intact phosphorylated Lhp1p was injected onto the LC-MS system and the molecular weight determined by positive ion ESMS. HPLC fractions containing Lhp1p were dried, reconstituted in 25 mM NH_4CO_3, pH 8.5,

and subjected to limited trypsin digestion (enzyme to substrate ratio 1:100, 1 h at 37 °C). An aliquot of the digest was diluted 2:1 with a methanol:water solution containing 5% ammonium hydroxide and loaded into the spraying needle of the nanoES ion source. Phosphopeptides were identified using a precursor ion scan for m/z 79 (Carr et al., 1996). Phosphopeptides were sequenced from the unfractionated mixture by tandem MS using an ES quadrupole-time of flight MS (Micromass, Manchester, UK).

Phosphorylation mutants

Serine to alanine mutations were made in *LHP1* using PCR. In all cases, the primers introduced a *Spe*I site 455 nt upstream of *LHP1* and an *Xho*I site downstream of the stop codon. Products were digested with *Spe*I/*Xho*I and ligated into the *Spe*I/*Xho*I sites of pRS313 (*CEN*, *HIS3*; Sikorski & Hieter, 1989). The presence of each mutation was confirmed by sequencing. To examine the phosphorylation status of the proteins, constructs were transformed into a *sup61-11 lhp1::LEU2* strain containing the plasmid pSLL28 (*URA3 LYS2 LHP1*; Yoo & Wolin, 1997). Following removal of pSLL28 by growing the strain on media containing 5-fluorouracil, extracts were analyzed by two-dimensional gel electrophoresis and western blotting.

Northern analyses and immunofluorescence

Northern analyses were performed as described (Yoo & Wolin, 1997). Oligonucleotide probes to detect $tRNA^{Ser}_{CGA}$, U4 RNA, and U6 snRNA were described (Yoo & Wolin, 1997; Pannone et al., 1998). Immunofluorescence was performed as described (Walch-Solimena et al., 1997). The anti-Nop1p antibody was a gift of J. Aris. For immunofluorescence, the anti-Nop1p antibody was diluted 1:1,000 and anti-Lhp1p was diluted 1:10,000. To detect Lhp1p, the secondary antibodies were Alexa 488 goat anti-rabbit IgG (Molecular Probes). Nop1p was detected using Texas Red donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories). Both secondary antibodies were diluted 1:250. Images were acquired with IP Lab Scientific Imaging Software (version 3.2 from Scanalytics Inc.) driving a Quantix CCD camera (Photometrix Ltd.) attached to a Axiophot 2 microscope (Zeiss).

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