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Cell Cycle–regulated Phosphorylation of Swi6 Controls its Nuclear Localization

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The Swi6 transcription factor, required for G1/S-specific gene expression in *Saccharomyces cerevisiae*, is highly phosphorylated in vivo. Within the limits of resolution of the peptide analysis, the synchrony, and the time intervals tested, serine 160 appears to be the only site of phosphorylation in Swi6 that varies during the cell cycle. Serine 160 resides within a Cdc28 consensus phosphorylation site and its phosphorylation occurs at about the time of maximal transcription of Swi6- and Cdc28-dependent genes containing SCB or MCB elements. However, phosphorylation at this site is not Cdc28-dependent, nor does it control G1/S-specific transcription. The role of the cell cycle–regulated phosphorylation is to control the subcellular localization of Swi6. Phosphorylation of serine 160 persists from late G1 until late M phase, and Swi6 is predominantly cytoplasmic during this time. Aspartate substitution for serine 160 inhibits nuclear localization throughout the cycle. Swi6 enters the nucleus late in M phase and throughout G1, when serine 160 is hypophosphorylated. Alanine substitution at position 160 allows nuclear entry of Swi6 throughout the cell cycle. GFP fusions with the N-terminal one-third of Swi6 display the same cell cycle–regulated localization as Swi6.

INTRODUCTION

The Swi6 protein of *Saccharomyces cerevisiae* is involved in the transcription of dozens of genes specifically at the G1/S transition of the cell cycle. These include the genes encoding the *HO* endonuclease, the periodically expressed G1 cyclins (Nasmyth and Dirick, 1991; Ogas *et al.*, 1991; Dirick *et al.*, 1992; Measday *et al.*, 1994), and many of the genes involved in DNA replication (Toyn *et al.*, 1995). Swi6 associates with at least two DNA-binding proteins: Swi4 and Mbp1, which confer the ability to bind to SCB (Swi4 and Swi6 cell cycle binding) elements, and MCB (*Mlu*I cell cycle binding) elements. Although Swi4 and Mbp1 contain the DNA binding domains, Swi6 is also required for DNA binding and activation in wild-type cells. Thus, Swi6 may be modulating the activity of both of these transcription complexes (Andrews and Moore, 1992; Primig *et al.*, 1992; Koch *et al.*, 1993; Sidorova and Breeden, 1993).

It is still largely unknown how activation of transcription by Swi4/Swi6 and Mbp1/Swi6 complexes is achieved in late G1. Swi4 is itself transcribed in a cell cycle–specific manner and its restricted expression contributes to the G1/S-specific activation of its target genes, but this is not the sole source of regulation (Breeden and Mikesell, 1991). In fact, Swi4/Swi6 and Mbp1/Swi6 complexes can be formed on DNA in vitro from whole cell extracts prepared from cells at all stages of the cell cycle (Taba *et al.*, 1991; Dirick *et al.*, 1992), so it is not the production of these proteins or the ability of their complexes to bind DNA that is responsible for their cell cycle–specific activity.

Transcriptional activation of SCB elements in the *HO* promoter and in isolation also requires Cdc28 activity (Breeden and Nasmyth, 1985, 1987b; Nasmyth, 1985). MCB-regulated promoters and the G1 cyclin promoters, which contain both SCB and MCB elements, are also maximally expressed after Cdc28 activation in G1 (Johnston and Thomas, 1982; Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991). In addition, renaturation of Cdc28 activity is sufficient to induce a burst of MCB- and SCB-regulated transcription in the absence of any further protein synthesis (Marini and Reed, 1992). These observations suggest that Cdc28 may directly activate these transcription

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complexes at the beginning of the cell cycle. To explore this possibility, we have used phosphopeptide analysis to monitor Swi6 phosphorylation through the cell cycle.

Swi6 is phosphorylated on multiple serine and threonine residues, but most of these sites are phosphorylated throughout the cell cycle. Our data indicate that there is one prominent phosphorylation, which occurs on a Cdc28 phosphorylation consensus site and is differentially phosphorylated during the cell cycle. This serine, at position 160, is hypophosphorylated during most of G1. It is phosphorylated just before S phase, around the time of maximal SCB and MCB-mediated transcription, and it remains phosphorylated until late in mitosis. Despite the coincidence between phosphorylation of serine 160 (S-160) and transcription, phosphorylation is not a prerequisite for activation or repression of Swi6-regulated genes. Instead, this regulated phosphorylation of S-160 controls the subcellular localization of Swi6. S-160 is adjacent to a critical nuclear localization signal (NLS) in Swi6 and when S-160 is in its hypophosphorylated form, Swi6 is detectable only in the nucleus, whereas when it is phosphorylated or when the NLS is mutated, Swi6 accumulates in the cytoplasm. Substitution of S-160 for alanine results in constitutive nuclear localization, and insertion of a negatively charged aspartate at the same site significantly impairs nuclear localization. This data suggests that phosphorylation disrupts the NLS activity of adjacent residues and prevents nuclear localization of Swi6.

MATERIALS AND METHODS

Strains and Plasmids

The yeast strains used in this study are listed in Table 1. The plasmid Bd176 is a 2μ-based yeast shuttle vector and was described previously under the name of YEpSWI6 (Sidorova and Breeden, 1993). The Bd1378 plasmid is a centromere vector pRS316 (Sikorski and Hieter, 1989) containing the *Hin*dIII–*Sma*I fragment from Bd176 that contains the *SWI6* promoter and open reading frame (Breeden and Nasmyth, 1987a).

Swi6 Mutant Construction

The A-160, D-160, and SA4 mutants of Swi6 were constructed by site-directed mutagenesis (Amersham kit, version 2.1; Arlington Heights, IL) using *SWI6* DNA cloned into M13 mp19. The 2.4-kb *Xho*I–*Sph*I fragments containing mutated *SWI6* open reading frames were then used to replace the wild-type *SWI6* open reading frame in Bd176, giving rise to Bd1651 (A-160), Bd1436 (D-160), and Bd1756 (SA4). To generate Bd1646, Bd1437, and Bd1435, 2.1-kb *Hin*dIII–*Eco*RI fragments of plasmids Bd1651, Bd1436, and Bd1756, respectively, were subcloned into Bd1378 to substitute for the wild-type *SWI6* open reading frame. The NLS mutation of Swi6 was introduced by oligonucleotide-directed mutagenesis (Kunkel, 1985) of Bd1378. A *Hin*dIII–*Sph*I fragment of this mutagenized plasmid was subcloned into Bd176 to substitute for the wild-type *SWI6* gene and that plasmid is called Bd1734.

Growth Conditions

All rich (YEPD) and minimal (YC) media and growth conditions were as previously described (Breeden and Mikesell, 1991). Cultures used for labeling with $^{32}PO_4$ were allowed to double at least once in low phosphate YEPD media (Rubin, 1975) before the experiment. Typically, a culture at OD660 = 0.2–0.3 was arrested by addition of α factor or by shifting to high temperature. Arrest with α factor was carried out with 5 μg/ml of the pheromone in low phosphate or YEPD media typically for 90–120 min. Cells were released from the arrest by filtration. *cdc* strains were arrested at 37° for 3–4 h unless otherwise indicated. Arrest with hydroxyurea was carried out for 5 h at a concentration of 200 mM.

RNA Isolation and S1 Protection

These procedures were performed exactly as described previously (Breeden and Mikesell, 1991). The probes used for detection of *CLN1* and *SWI4* transcripts are described by Foster *et al.* (1993) and Breeden and Mikesell (1994).

$^{32}PO_4$ Labeling Conditions and Extract Preparation

Cells were labeled with 1 mCi of $^{32}PO_4$ (ICN Biomedicals, Costa Mesa, CA) per 1 ml of culture. Five to ten milliliters of culture were used to generate a phosphopeptide map. Cells were harvested, resuspended in cold 0.1 M sodium phosphate, pH 7.0, pelleted, and resuspended in breaking buffer (Sidorova and Breeden, 1993) supplemented with 10 mM sodium fluoride and 2 mM sodium orthovanadate. Glass beads (45 μm) were added to the cells in breaking buffer and cells were vortexed for 10 min at 4°. SDS was added to the mixture to 1% and it was incubated at 95° for 5 min. Cellular debris was then cleared by a 10-min centrifugation in a microfuge.

Table 1. Strains used in this study

Strain	Genotype	Source
BY600	*MAT***a** *swi6::TRP1 ade2 ho::lacZ ura3 his3 leu2-3, 112 trp1-1 can1-100 met1*	(Sidorova and Breeden, 1993)
BY602	*MAT***a** *SWI ade2 ho::lacZ ura3 his3 leu2-3, 112 trp1-1 can1-100 met2*	(Sidorova and Breeden, 1993)
BY662	*MAT*α *cdc28-4 met2 tyr1 ura1*	L. Hartwell
BY665	*MAT*α *cdc4-1 ade1 ade2 leu2 lys2 ura1*	L. Hartwell
BY707	*MAT***a** *cdc34 ade1 ade2 lys2 ura1 his7 tyr1 gal1*	L. Hartwell
BY1230	*MAT***a** *swi6::LEU2 ade2 his3 leu2-3, 112 trp1-1 ura3*	(Sidorova and Breeden, 1993)
BY1231	*MAT***a** *SWI ade2 his3 leu2-3, 112 trp1-1 ura3*	(Sidorova and Breeden, 1993)
BY1365	*MAT***a** *cdc28-13 ade2 ade3 leu2 trp1-1 ura3*	J. Roberts
BY1601	*MAT*α *cdc28-13 ade2 leu2 ura3 trp1-1 his3*	BY1365 × BY1230
BY1782	*MAT***a** *cdc15-2 tyr1 leu2 ura3 his7 gal1 can1*	L. Hartwell
BY1991	*MAT***a** *cdc28-4 ade2 his3 leu2 ura3 tyr1*	BY1231 × BY662

Immunoprecipitation, Phosphopeptide Mapping, and Phosphoamino Acid Analysis

Yeast extracts prepared as described above, were diluted with AB buffer [20 mM Tris HCl, pH 7.5, 50 mM NaCl, 0.5% NP40, 0.3% SDS, 0.5% deoxycholic acid (DOC)] such that the final concentration of SDS was lowered to 0.5%, and anti-Swi6 serum and protein A-agarose beads (Life Technologies, Gaithersburg, MD) were added. Reaction mixes were incubated on a roller at 4° for at least 1.5 h. Immunoprecipitates were washed with several changes of RIPA buffer (10 mM Tris HCl, pH 7.5, 150 mM NaCl, 1% NP40, 1% DOC, 0.1% SDS) and then with two to four changes of high salt buffer (10 mM Tris HCl, pH 7.4, 2 M NaCl, 1% NP40, 0.5% DOC), resuspended in 30 μl of SDS sample buffer, boiled, and loaded onto a 12% SDS-polyacrylamide gel. After electrophoresis, the gel was exposed and bands corresponding to Swi6 were cut out. Trypsin cleavage and phosphopeptide separation were carried out essentially as described by Boyle *et al.* (1991). Polyacrylamide gel pieces containing Swi6 were soaked in 50 mM $(NH_4)_2CO_3$, 1% β-mercaptoethanol, 1% SDS for at least 8 h. The eluate was separated from polyacrylamide by centrifugation and then the eluted Swi6 was precipitated with trichloroacetic acid. Swi6 precipitates were resuspended in 50 mM $(NH_4)_2CO_3$, treated with performic acid, and lyophilized. Swi6 was then resuspended in 50 mM $(NH_4)_2CO_3$ and digested with TPCK-trypsin (Sigma, St. Louis, MO) overnight. Tryptic peptides were lyophilized several times and loaded onto thin layer cellulose plates (Merck, Darmstadt, Germany). Phosphopeptides were resolved first by electrophoresis in a pH 1.9 buffer, and then by ascending chromatography in an isobutyrate buffer (Boyle *et al.*, 1991). Plates were dried and exposed to film and/or to a PhosphorImage screen (Molecular Dynamics, Sunnyvale, CA). Phosphoamino acid analysis was performed exactly as described by Boyle *et al.* (1991) and showed that P1 and P2 contain only phosphoserine.

To deduce the identity of the cell cycle–regulated phosphopeptides, we calculated the theoretical migration values for each tryptic peptide (Boyle *et al.*, 1991). Few peptides within Swi6 have the high hydrophobicity and positive charge required to migrate with P1 and P2. Of these, only the peptide sequence around serine 228 and 229 and S-160 contain cleavage sites for V8 protease (glutamic and aspartic acid), proline-specific endopeptidase, and thermolysin (mainly leucine, isoleucine, and valine), which we showed could all cleave P1 and P2. In each case, these secondary digests of P1 and P2 produced one phosphopeptide of similar mobility, suggesting that P1 and P2 contained the same phosphorylation site at C-terminal end. Substitution of serines 228 and 229 to aspartate (D) did not change the phosphopeptide map, however, substitution of S-160 to D eliminated both the P1 and P2 phosphopeptides. Furthermore, elimination of the potential trypsin cleavage sites adjacent to S-160 led to the disappearance of both P1 and P2, and the appearance of new spots in the tryptic peptide map.

Immunofluorescence

For immunofluorescence, mutant and wild-type *SWI6* gene products were expressed from a 2μ plasmid, and Swi6 levels in the mutant strains were comparable with wild type as judged by Western blotting of total yeast extracts with anti-Swi6 antibodies (our unpublished observations). Immunofluorescence was performed as described by Kilmartin and Adams (1984). Cells were fixed in 4% paraformaldehyde at room temperature for 2 h, washed three times in 0.1 M K phosphate, pH 6.5, and spheroplasts were produced by digesting with 1/10 vol of glusulase (NEN, Boston, MA) and 0.5 mg/ml of zymolyase-100T (ICN) for 90 min at 30° in sorbitol buffer (1.2 M sorbitol, 0.12 M K_2HPO_4, 33 mM citric acid, pH 5.8). Suspensions of these spheroplasts were applied to polylysine-coated slides. Slides were fixed in methanol and acetone at −20° and then incubated with phosphate-buffered saline (PBS) plus 1% ovalbumin for 20 min. They were then incubated overnight at 4° with a mixture of an affinity-purified Swi6 antibody (Sidorova and Breeden, 1993) and theYOL1/34 tubulin antibody. After several washes in PBS, rodamine-conjugated anti-rabbit and fluorescein-conjugated anti-rat (Boehringer Mannheim, Indianapolis, IN) antibodies were applied to the slides and incubated for 1–2 h. Slides were washed and mounted in 90% glycerol, 10% PBS, 2 ng/ml 4,6-diamidino-2-phenylindole (DAPI). Images of immunofluorescence were obtained either on EPH135–36 (Kodak, Rochester, NY) slide film, or via the Wide Field Deconvolution DeltaVision microscope system (Applied Precision)

Construction of $Swi6_N$:GFP Fusion and Visualization of the Protein

The 0.7-kb *Bam*HI–*Xho*I fragment containing the *GFP10* open reading frame (Prasher *et al.*, 1992) was cloned into the pYES2 plasmid (Invitrogen, San Diego, CA) to make Bd1814. A 1.2-kb fragment of the *SWI6* gene containing the promoter and encoding amino acids 1 to 252 of the open reading frame was amplified by polymerase chain reaction and cloned into the TA vector (Invitrogen). To generate the $Swi6_N$:GFP fusion, the 1.2-kb fragment of *SWI6* was cut out of the TA vector with *Bam*HI and recloned into Bd1814. The resulting construct is called Bd1815 and the fusion protein produced was of the predicted size, as confirmed by Western blotting. Fluorescence of the fusion protein was visualized in live cells with a fluorescein 5-isothiocyanate filter. Cells were examined either immediately or after freezing at −20° in 15% glycerol. The freezing step did not alter the distribution of the fusion protein.

RESULTS

Phosphorylation of Individual Tryptic Peptides of Swi6 Varies during the Cell Cycle

To study the phosphorylation state of Swi6 in vivo, we labeled yeast cells with $^{32}PO_4$, immunoprecipitated the protein, and resolved it by SDS-PAGE. A single band, migrating at 100 kDa, was precipitated from wild-type cell extracts by Swi6 antibodies. This band was cut out of the gel and subjected to phosphoamino acid analysis (Boyle *et al.*, 1991), which showed that the protein was phosphorylated only on serine and threonine residues (our unpublished observations). We also immunoprecipitated Swi6 from a synchronized culture obtained by α factor arrest and then release into $^{32}PO_4$-containing medium. A roughly constant level of phosphate is incorporated into Swi6 over the course of nearly two cell cycles as judged by one-dimensional PAGE analysis (our unpublished observations).

If Swi6 is phosphorylated on several sites, then a cell cycle-dependent phosphorylation of a subset of these sites could be masked by the constitutive phosphorylation of the others. We therefore applied phosphopeptide mapping to study the phosphorylation of individual sites in Swi6 during the cell cycle. Wild-type cells were arrested with α factor and then either labeled immediately with $^{32}PO_4$, or released from the arrest into a $^{32}PO_4$-containing medium and allowed to progress through the cell cycle synchronously. Swi6 was immunoprecipitated from the α factor–arrested cells and from cycling cells harvested at 30, 50, and 90 min after release. These samples were resolved on

SDS-PAGE, eluted from the gel, and digested with trypsin. Tryptic peptides were separated in two dimensions by electrophoresis and chromatography as described in MATERIALS AND METHODS. As shown in Figure 1A, these tryptic digests indicate that Swi6 is phosphorylated on multiple sites and that the majority of these phosphopeptides are present constitutively, yet there are two phosphopeptides (marked P1 and P2 on Figure 1) that are hypophosphorylated in Swi6 isolated from α factor–arrested cells. After the cells are released from the arrest, but before the onset of budding, $^{32}PO_4$ incorporation into P1 and P2 increases and remains high in the 50-min and 90-min time points.

To find out if Swi6 hypophosphorylation is specific to the α factor arrest, or if it normally occurs during G1, we used a *cdc15–2* strain to arrest cells in mitosis, and then monitored a synchronous cell cycle after release from the arrest. We found that P1 and P2 are phosphorylated during the *cdc15–2* arrest (Figure 1B). However, 40 min after release from the arrest, when the culture is comprised mostly of unbudded G1 cells, phosphorylation of P1 and P2 is very low. At later time points, after cells undergo budding, the phos-

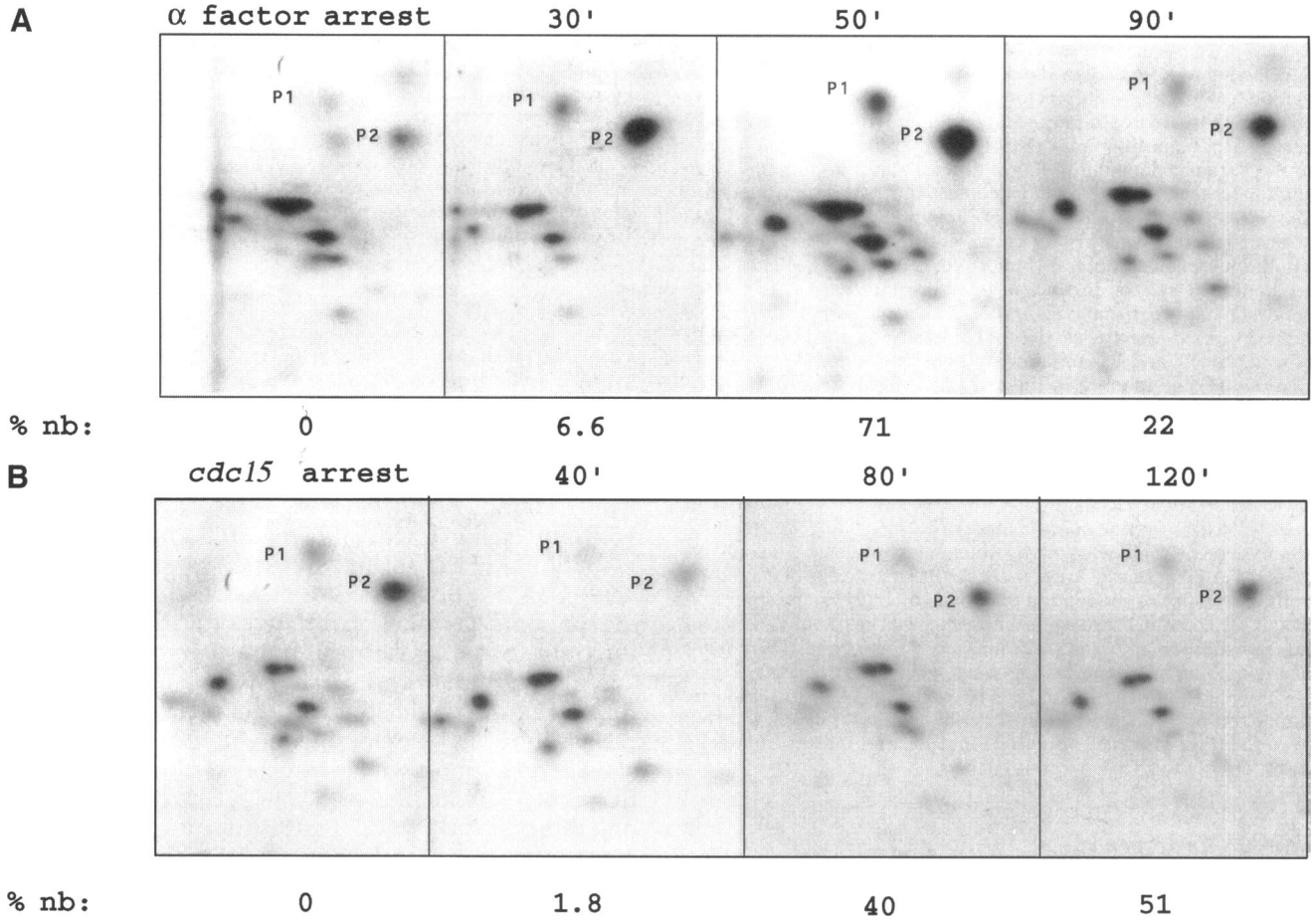

Figure 1. Swi6 is hypophosphorylated on two peptides (P1 and P2) in the G1 phase of the cell cycle. (A and B) Four tryptic phosphopeptide maps of Swi6 isolated from synchronized cell cultures. Cells were labeled with $^{32}PO_4$ during the arrest and/or at several time points after release. Swi6 was immunoprecipitated from these samples, digested with trypsin, and the resulting peptides were resolved in two dimensions on a thin layer chromatography plate as described in MATERIALS AND METHODS. Cells from respective time points were examined microscopically to verify their progression through the cell cycle and the percent of cells with newly formed buds (% nb) are shown beneath the phosphopeptide maps. A newly budded cell was defined as one in which the daughter bud is smaller than its mother. The formation of new buds can be unambiguously scored after release from *cdc15* arrest and this number directly reflects progression through the next cell cycle. (A) Wild-type BY602 strain was synchronized with α factor and labeled with $^{32}PO_4$ during the arrest. The rest of the culture was released from the α factor arrest into $^{32}PO_4$-containing media and allowed to progress through the cell cycle synchronously. Swi6 was isolated from labeled α factor–arrested cells and from cells taken at 30, 50, and 90 min after the release. (B) BY1782 *cdc15–2* was arrested at 37° for 3.5 h and released by returning to 25°. $^{32}PO_4$ was added to 1 aliquot of culture during the last 30 min of the arrest. To the rest of the culture, $^{32}PO_4$ was added immediately upon the release, and samples were taken at 40, 80, and 120 min after the release and Swi6 was isolated for phosphopeptide mapping.

phorylation is restored. We conclude from Figure 1, A and B, that hypophosphorylation of P1 and P2 occurs during G1 phase of the cell cycle.

Figure 2 shows another *cdc15–2* arrest-release experiment carried out to compare the timing of P1 and P2 phosphorylation with that of G1/S-specific transcription. Two parallel cultures were arrested at the *cdc15–2* block and $^{32}PO_4$ was added to one of them (see legend to Figure 2A for details). We then took parallel samples from the labeled and nonlabeled cultures and monitored budding (% nb, Figure 2B) to ensure that both cultures were growing with similar timing and synchrony throughout the experiment. Phosphopeptide maps of Swi6 were made with the labeled samples and the nonlabeled samples were used to measure the levels of *HO* and *CLN1* mRNA over the same time course. This allowed us to compare the kinetics of phosphorylation directly with the interval of maximal Swi4/Swi6-dependent transcription (Figure 2B). We found that P1 and P2 phosphorylation is low 30 min after the release from the *cdc15–2* block and is restored no later than 45 min after release (Figure 2A). This increase occurs before cells start to bud and during the interval when transcription of both *HO* and *CLN1* is highest. Therefore, the phosphorylation of P1 and P2 occurs either before or shortly after the interval of maximal Swi4/Swi6 transcriptional activity.

Another means to determine the position in the cell cycle when Swi6 phosphorylation occurs is to use temperature-sensitive *cdc4* and *cdc34* strains. At the nonpermissive temperature these strains arrest at a point when G1/S-specific transcription is occurring but DNA synthesis has not yet been initiated (Pringle and Hartwell, 1981; Nasmyth, 1983). As seen in Figure 3, the phosphorylation of P1 and P2 is detectable both in the culture growing at 25° and the one stalled at the *cdc4* arrest point. A similar result was obtained with *cdc34*-arrested cells (our unpublished observations). The level of phosphorylation is somewhat reduced in both arrests, but it is clear from these results and those depicted in Figure 2 that phosphorylation of P1 and P2 occurs before or during the interval of maximal transcription activated by the Swi4/Swi6 complex.

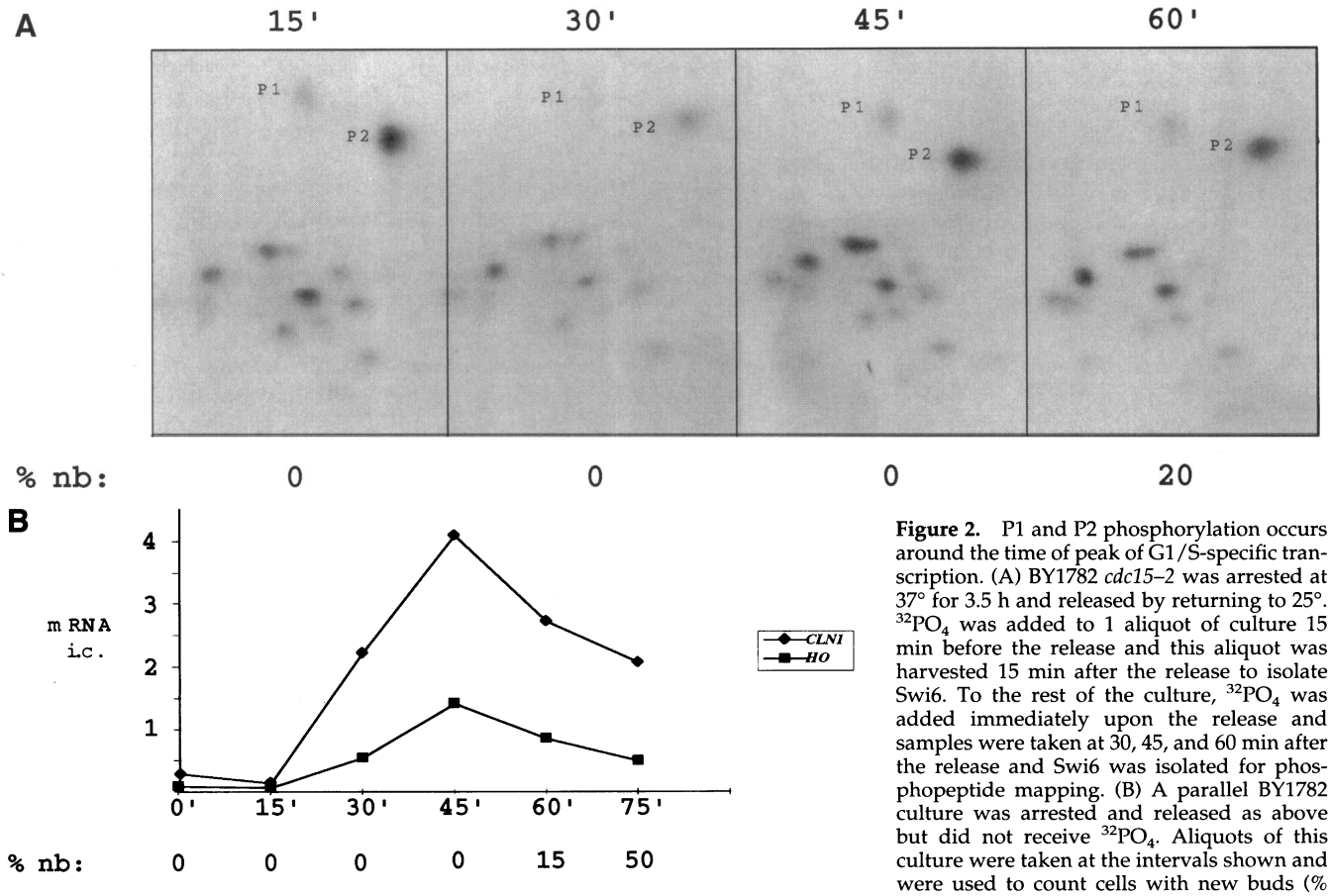

Figure 2. P1 and P2 phosphorylation occurs around the time of peak of G1/S-specific transcription. (A) BY1782 *cdc15–2* was arrested at 37° for 3.5 h and released by returning to 25°. $^{32}PO_4$ was added to 1 aliquot of culture 15 min before the release and this aliquot was harvested 15 min after the release to isolate Swi6. To the rest of the culture, $^{32}PO_4$ was added immediately upon the release and samples were taken at 30, 45, and 60 min after the release and Swi6 was isolated for phosphopeptide mapping. (B) A parallel BY1782 culture was arrested and released as above but did not receive $^{32}PO_4$. Aliquots of this culture were taken at the intervals shown and were used to count cells with new buds (% nb) and to isolate RNA. *HO* and *CLN1* transcript levels in these samples were compared with an internal control (*MATa1*) by S1 protection. The graph represents quantitation of these data performed by PhosphorImage analysis. Relative levels of RNAs were obtained by normalizing *HO* and *CLN1* transcript levels to the internal control (i.c.). These data are plotted as a function of time after release.

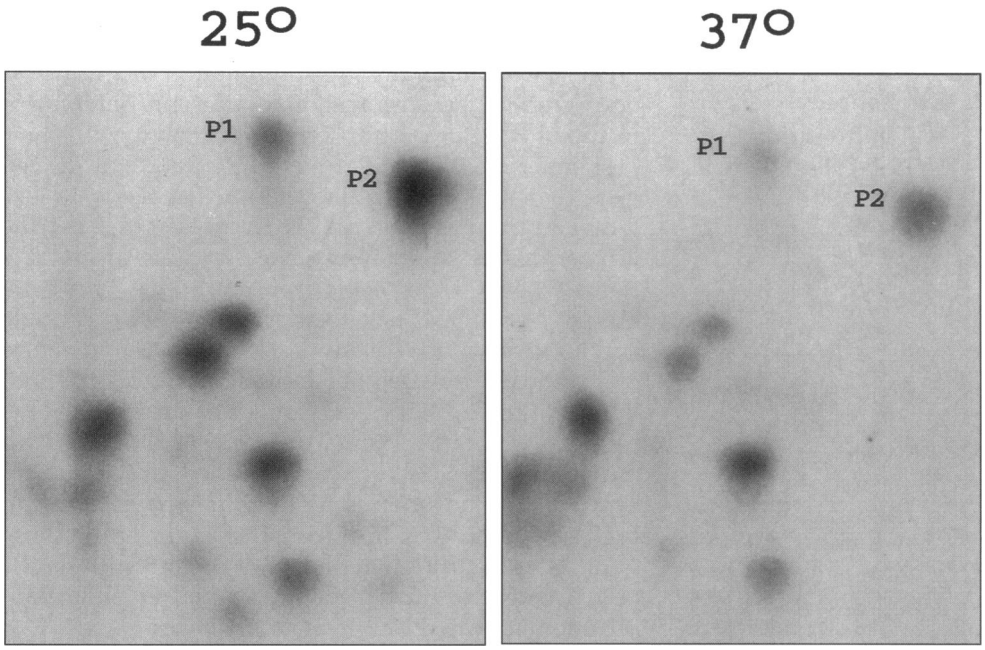

Figure 3. Phosphorylation of Swi6 P1 and P2 occurs before the Cdc4-dependent step in the cell cycle. BY665 *cdc4–1* culture was grown at 25°, and then split, incubated for 3 h at 25° or 37°C, and labeled for 40 min with $^{32}PO_4$. Phosphopeptide maps of Swi6 from these 25° and 37° cultures were produced as in Figure 1.

Differential Phosphorylation of Swi6 Occurs at S-160

Peptides 1 and 2 of Swi6 were subjected to phosphoamino acid analysis and found to both contain phosphoserine (our unpublished observations). Secondary digestions with specific proteases were then performed on these peptides to obtain information about their amino acid composition. This analysis indicated that P1 and P2 are related and that S-160 was the most likely target of phosphorylation in both peptides (see MATERIALS AND METHODS). We then substituted aspartic acid for S-160 and performed phosphopeptide analysis on this mutant as before. Neither P1 nor P2 is phosphorylated when S-160 is changed to aspartic acid (Figure 4A). The simplest interpretation of this is that P1 and P2 both contain a single phosphoserine corresponding to position 160 and that these two peptides are different digestion products of the same region of Swi6. Further corroboration that S-160 is the site of phosphorylation came from the phenotype of the D-160 mutant. The aspartate at position 160 causes Swi6 to localize predominantly to the cytoplasm. Thus, the negatively charged aspartate at 160 mimics the behavior of Swi6 when P1 and P2 are phosphorylated.

S-160 Can Be Phosphorylated in the Strains with Defective Cdc28 Kinase

S-160 is followed by proline, leucine, and lysine (SPLK) and as such they form a Cdc28 phosphorylation consensus sequence (Moreno and Nurse, 1990). This sequence is located in the N-terminal part of Swi6 within a cluster of three other similar sites (Figure 4B). To examine the role of Cdc28 in phosphorylation of S-160 in vivo, we carried out a series of experiments. Phosphopeptide maps of Swi6 isolated from *cdc28–13* and *cdc28–6* strains that were either growing at 25° or arrested at 37° indicated that there is much less S-160 phosphorylation in *cdc28*-arrested cells as compared with growing cells (Figure 5B; our unpublished observation). Although a similar result has been used to suggest that Swi5 is a substrate for Cdc28 (Moll *et al.*, 1991), this experiment is inconclusive. S-160 of Swi6 and the relevant sites in Swi5 are not normally phosphorylated during G1, so the accumulation of cells in G1 by any means would decrease the amount of phosphorylation of these residues. Because *cdc28–13* or *cdc28–6* strains arrest in G1 (Hartwell *et al.*, 1973; Reed and Wittenberg, 1990) it is not possible to conclude that the reduction in S-160 phosphorylation that is observed in these arrests is a direct effect of loss of Cdc28 kinase activity. To overcome this difficulty, we have used conditions that cause *cdc28*-deficient cells to arrest in G2, the phase when S-160 should normally be phosphorylated.

A *cdc28–4* strain can be arrested in G2 by first arresting the cells in S phase with hydroxyurea (HU), raising the temperature to denature Cdc28, and then withdrawing the HU (Reed and Wittenberg, 1990). If cells are shifted to the permissive temperature after removal of HU, they resume their cell cycle and after 4 h they are in M phase, as judged by tubulin staining (Figure 5A). However, cells that are maintained at the nonpermissive tem-

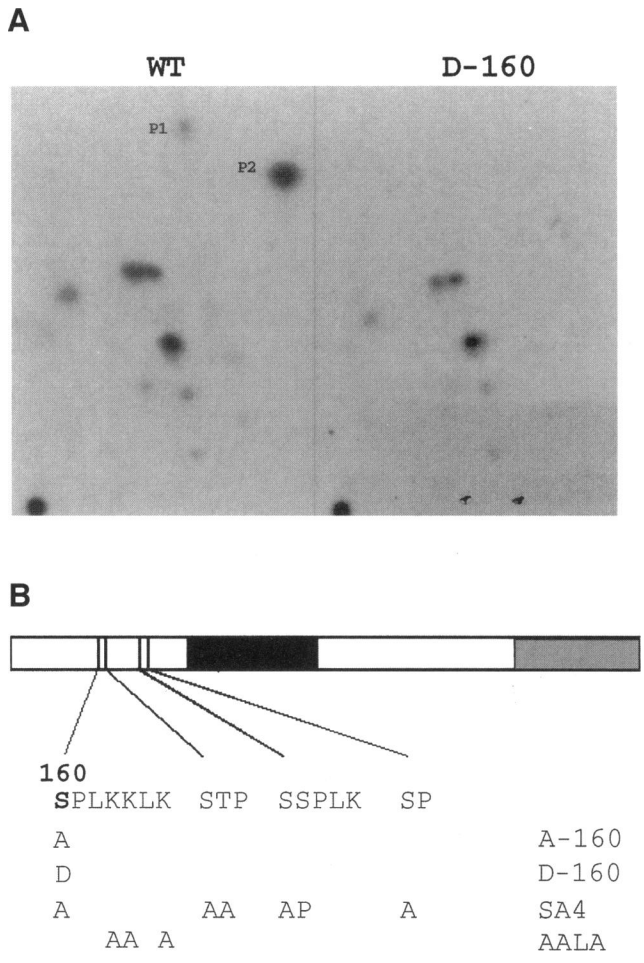

Figure 4. S-160 is hypophosphorylated during G1. (A) Swi6 P1 and P2 are not phosphorylated in a mutant Swi6 with S-160 changed to aspartate. Asynchronous cultures of BY600 Swi6Δ transformed with plasmids expressing the wild-type Swi6 or the mutant with S-160 changed to aspartate (D-160) were labeled with $^{32}PO_4$ for 45 min. Phosphopeptide maps of Swi6 from these cells were generated, and positions of P1 and P2 are marked. (B) Positions of the Swi6 mutations used in this study. The black box shows the region containing four potential Swi6/cdc10 repeats (Bork, 1993; Ewaskow, Sidorova, and Breeden, unpublished data). The grey box shows the region required for association with Swi4. The region near S-160 is shown in greater detail below. The positions of the four potential sites of Cdc28 phosphorylation located in the N-terminal half of the protein are shown. S-160 is shown in bold. Below the sequence, there is a summary of amino acid substitutions in Swi6 used in this study and their names.

perature after HU release retain the phenotype characteristic of a G2 arrest. They display short spindles and elongated buds, and their nuclei are not separated (Reed and Wittenberg, 1990; Figure 5A; our unpublished observations). This phenotype is plainly evident 2 h after the shift to 37°. To determine the phosphorylation state of Swi6 under these conditions, cells were labeled during the HU block at 25°, and also 2 h after removing the HU and shifting to 25° or 37° medium. As shown in Figure 5A, S-160 can be phosphorylated during the HU arrest in S phase and after release into 25° media. S-160 can also be phosphorylated after the HU-arrested cells are transferred to 37° media and arrested in G2 due to inactivation of Cdc28. It is unlikely that the phosphorylation that is observed under these conditions is due to the residual activity of the *cdc28–4* gene product, because no kinase activity can be detected in vitro at the nonpermissive temperature with this mutant (Reed *et al.*, 1985; Ghiara *et al.*, 1991; Moll *et al.*, 1991; Donovan *et al.*, 1994). Despite this highly defective Cdc28 kinase, S-160 of Swi6 is still being phosphorylated during G2 to nearly the same extent as in the growing 25° cells.

The timing of S-160 phosphorylation suggests that this site may be a substrate for a late G1-specific Cdc28 kinase activity, so we asked whether S-160 phosphorylation can occur upon reactivation of a renaturable allele of *CDC28* during G1. A *cdc28–13* strain was arrested in G1 at 37° and an aliquot of the culture was $^{32}PO_4$-labeled as before. The rest of the cells were released from the arrest by filtration and resuspended in $^{32}PO_4$-containing medium at 25°, with or without cycloheximide (CHX), and incubated for 60 min. Marini and Reed (1992) observed that renaturation of Cdc28–13 in this way was sufficient to induce G1/S-specific transcription even in the presence of CHX. We have monitored S-160 phosphorylation under the same conditions, and found that although we can observe the induction of G1/S-specific transcription in parallel cultures (our unpublished observations), there is no indication that S-160 phosphorylation is increased in the presence of cycloheximide (+CHX), whereas cells released and allowed to grow (−CHX) can phosphorylate S-160 over the same time course (Figure 5B). This shows that the renaturation of Cdc28 kinase that occurs in the absence of protein synthesis is not sufficient to allow phosphorylation of Swi6 on S-160. S-160 phosphorylation cannot be a prerequisite for the activation of the G1/S-specific transcription that is observed under these conditions, nor is it likely that the G1 form of Cdc28, renatured in this way, is responsible for S-160 phosphorylation.

Regulation of HO, CLN1, and SWI4 Transcripts Does Not Depend upon S-160 Phosphorylation

To further characterize the relationship between G1/S-specific transcription and Swi6 phosphorylation we substituted either alanine or aspartic acid for S-160, with the idea that the negatively charged aspartic acid (D-160) might partially phenocopy the constitutive presence of a phosphate on that site, and the alanine substitution (A-160) would pheno-

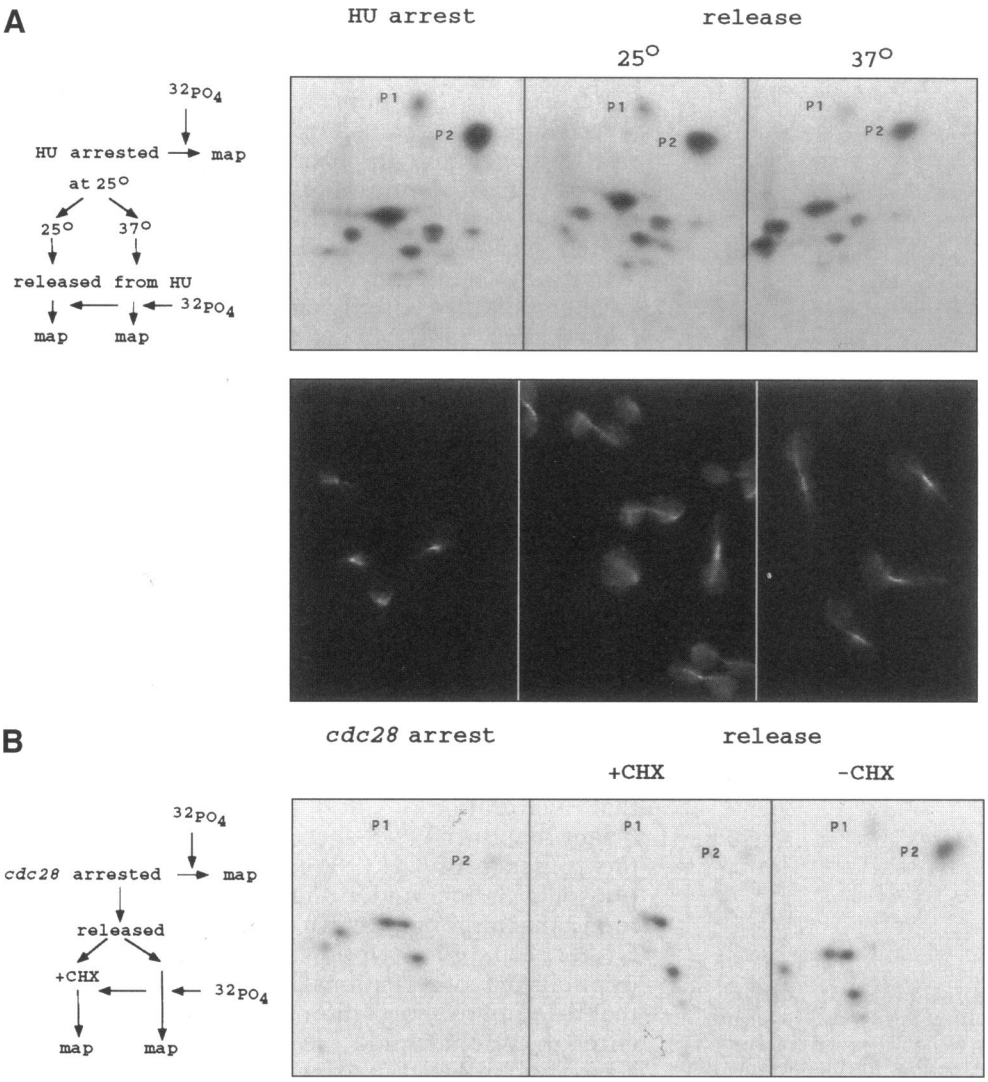

Figure 5. (A) S-160 can be phosphorylated in G2 in strains with defective Cdc28. BY1991 *cdc28–4* cells transformed with the wild-type Swi6 plasmid were arrested in S phase with hydroxyurea (HU) for 5 h and an aliquot was labeled with $^{32}PO_4$ for 45 min. The remaining culture was split and aliquots were incubated at 25° or 37° for 1 h. Then both aliquots were released from the HU block by filtration and transferred to new 25° or 37° media and grown for 2 h. $^{32}PO_4$ was added and cells were incubated for another 45 min and then harvested. Swi6 was immunoprecipitated from these three samples and subjected to phosphopeptide analysis (upper row). In the second row of images, the same strain was subjected to the identical arrest-release conditions, only the cells were fixed and stained with YOL1/34 anti-tubulin antibodies. The cells were harvested after 6 h of HU treatment, and after 2.5 h following release into 25°C and 37°C media respectively. (B) S-160 is not phosphorylated in G1 upon reactivation of the renaturable *cdc28–13* gene product. BY1601 *cdc28–13* was arrested in G1 by incubation at 37° for 3 h 45 min, an aliquot was labeled with $^{32}PO_4$ for 30 min, and a phosphopeptide map of Swi6 was generated (left panel). The rest of the culture was split into two aliquots and $^{32}PO_4$ was added to both. One of these aliquots received cycloheximide (CHX) at a concentration of 10 mg/l (+CHX, middle panel). Both cultures were released from the *cdc28* arrest by shifting back to 25°, incubated for 60 min, and then harvested to generate Swi6 phosphopeptide maps.

copy the absence of phosphorylation. Both mutants and the wild-type Swi6 gene were cloned into a CEN-based plasmid and introduced into a *swi6*Δ strain. The resulting strains produce comparable levels of the mutant and wild-type Swi6 proteins (our unpublished observations). We then monitored the effect of these Swi6 mutations on the profiles of three different Swi6-dependent transcripts: *HO*, *CLN1*, and *SWI4*. Figure 6 shows the data for *HO* transcription, because *HO* is the most Swi6-dependent promoter known (Breeden and Nasmyth, 1987b; Ogas *et al.*, 1991; Nasmyth and Dirick, 1991), however, the results were similar with *CLN1* and *SWI4*. Neither the A-160 or D-160 mutation had any appreciable effect on the timing or periodicity of *HO* transcription. The D-160 mutant produced considerably less *HO* mRNA than the strain carrying wild-type Swi6, but peak transcript levels were attained at the same time (Figure 6B).

In addition to the SPLK site at position 160, Swi6 contains three other S/TP sites (see Figure 4B). One more potential phosphorylation sites (TPLH) is in the first Swi6/cdc10 (or ankyrin) repeat of Swi6. However, this threonine has been mutated to alanine in the swi6-A mutant (Sidorova and Breeden, 1993) and this substitution does not change the phosphopeptide map of Swi6 (our unpublished observations). Thus, the threonine in the TPLH sequence is not phosphorylated in vivo in an exponentially growing culture. We then mutated the other three potential phosphorylation sites along with S-160 and found that the peptide map of the

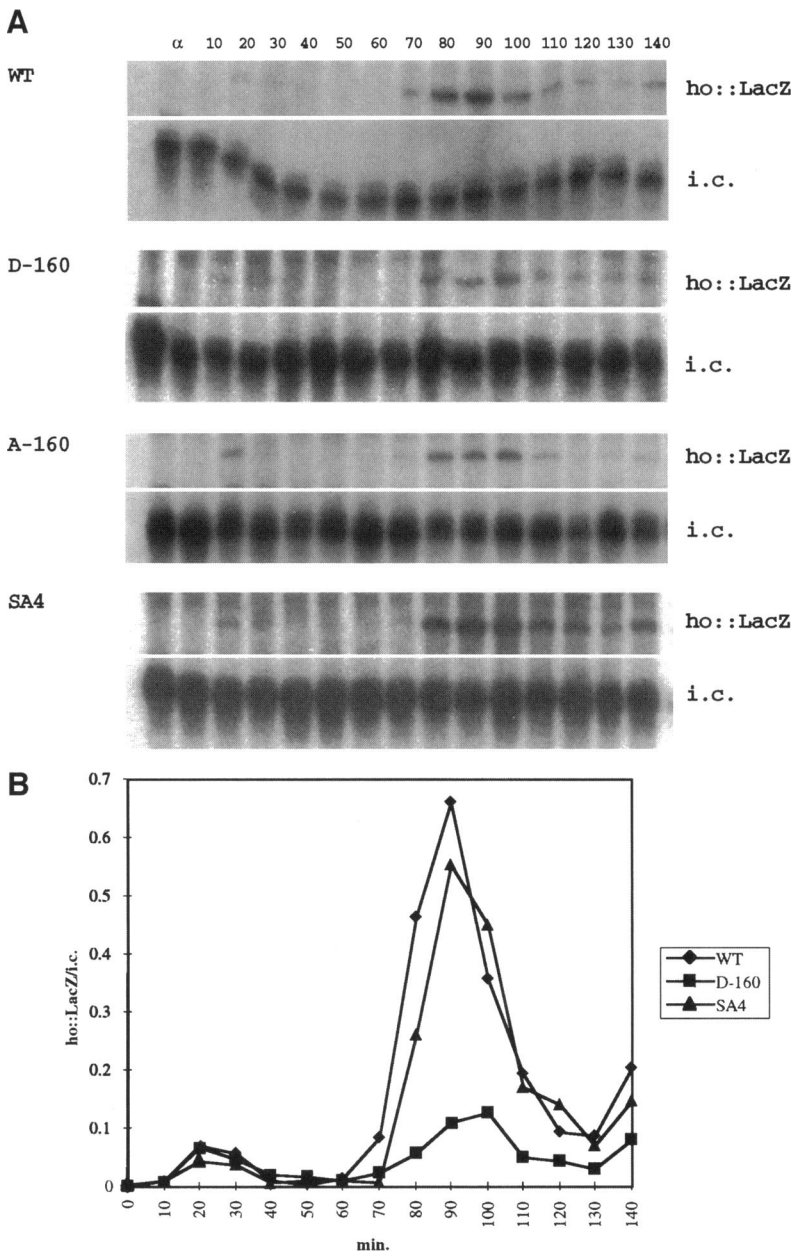

Figure 6. S-160 phosphorylation is not required for cell cycle–specific accumulation of SCB- and MCB-driven transcripts. (A) BY600 *swi6::TRP1* was transformed with plasmids expressing the wild-type Swi6 (Bd1378), D-160 Swi6 (Bd1437), A-160 Swi6 (Bd1646), and SA4 Swi6 (Bd1435). Figure 4B shows the positions of these mutations in Swi6. These strains were arrested with α factor, released, and allowed to undergo two synchronous cell cycles. Samples for S1 protection analysis of RNA levels were taken every 10 min. Positions of the *HO* and *MATa1* (internal control, i.c.) are marked on the right. (B) Quantitation of the S1 analysis data on the wild-type, D-160, and SA4 mutants of Swi6. These three sets of RNAs were analyzed at the same time, with the same radiolabelled probes so they can be directly compared. The levels of *HO* RNA were normalized to the internal control message and plotted. As previously seen, the level of *HO* mRNA in the first cycle after release from α factor arrest is quite low (Breeden and Nasmyth, 1987b).

resulting quadruple mutant, SA4, lacks one other phosphopeptide in addition to P1 and P2 (our unpublished observations). This indicates that at least one of these other S/TP sites is phosphorylated in vivo. However, the quadruple mutant is still able to activate periodic transcription of *HO*, *CLN1*, and *SWI4* mRNAs and thus behaves like the single mutant (Figure 6; our unpublished observations). We conclude that phosphorylation is not required upon S-160 or any of the other S/TP sites for the cell cycle–specific activation or repression of transcription of these Swi6-regulated promoters.

Phosphorylation of S-160 Controls Subcellular Localization of Swi6

Swi6 is concentrated in the nucleus during an α factor or *cdc28* arrest and is mainly cytoplasmic in nocodazole-treated cells (Taba *et al.*, 1991). To monitor Swi6 localization during a normal cell cycle, we synchronized cells with α factor and followed localization of Swi6 throughout the cell cycle using Swi6-specific antibodies. The position of each cell within the cell cycle was determined by its budding, DNA, and spindle morphology (see MATERIALS AND METHODS). As

shown in Figure 7, Swi6 is nuclear during α factor arrest and 30 min after release from the arrest. A short time later, before the cells start to bud, Swi6 gradually redistributes until it is predominantly cytoplasmic, and then it remains in the cytoplasm until late M phase. Nuclear localization of Swi6 is restored at the end of mitosis, when the cells have highly elongated spindles, and Swi6 remains in the nucleus until the beginning of the next S phase.

The interval during which Swi6 is nuclear localized correlates with the interval during which S-160 is not phosphorylated. To test whether S-160 phosphorylation is regulating Swi6 localization, we studied the subcellular distribution of the A-160 and D-160 mutants of Swi6. Alanine substitution at position 160 leads to constitutive nuclear localization of Swi6. Conversely, the D-160 mutation with the negatively charged aspartic acid causes Swi6 to remain predominantly in the cytoplasm throughout the cell cycle. This is best illustrated by comparing the localization of wild-type Swi6 to that of the D-160 and A-160 mutants at the relevant time points throughout a synchronous cell cycle. In an α factor arrest and 30 min after release, wild-type Swi6 is nuclear (Figure 8) but the D-160 substitution severely reduces the extent of its nuclear localization (Figure 8). Later, after 50 or 70 min, when cells are in S or G2, the wild-type Swi6 is largely cytoplasmic but the A-160 mutant remains in the nucleus (Figure 9). These localization defects caused by the A-160 and D-160 mutations demonstrate that the phosphorylation state of S-160 determines the subcellular localization of Swi6. We conclude that phosphorylation of S-160 at the end of G1 prevents nuclear localization of Swi6. At some time late in mitosis when the relevant phosphatase is activated or the kinase is inactivated, S-160 phosphorylation declines and this leads to the accumulation of Swi6 in the nucleus.

S-160 Is Located Adjacent to a Nuclear Localization Signal

In yeast, as well as in higher eukaryotes, protein targeting to the nucleus is determined by NLS. These are short stretches of positively charged amino acids, and it is the charge rather than primary sequence that is crucial for the NLS activity (Osborne and Silver, 1993). One of the longest stretches of basic amino acids in Swi6 occurs immediately after S-160 in the sequence SPLKKLK. To determine whether this sequence is required for nuclear localization, we changed these three lysines to alanines (Swi6-AALA). As seen in Figure 10, these substitutions abolish nuclear localization. Unlike the wild-type protein, which exhibits cell cycle–specific nuclear localization, the swi6-AALA mutant appears uniformly cytoplasmic at all stages of the cell cycle. Even during prolonged G1 arrest induced by α factor, we see no accumulation of swi6-AALA in the nucleus (Figure 10). This result is consistent with the view that the KKLK sequence is critical for nuclear localization of Swi6. It is also likely that phosphorylation of the adjacent S-160 prevents nuclear localization by interfering with the activity of this NLS.

This putative NLS lies within the N-terminal region of Swi6 (see Figure 4). To see whether this region is sufficient to promote cell cycle–regulated nuclear localization, we fused the N-terminal one-third of Swi6 to the green fluorescent protein (GFP) from *Aequorea victoria* (Prasher *et al.*, 1992). GFP fluoresces in living yeast, and this allows one to directly monitor its distribution in vivo. GFP, expressed in yeast from the *GAL1–10* promoter, is distributed uniformly within the cell (our unpublished observations). However, the $Swi6_N$-GFP fusion behaves just like native Swi6 as detected by immunofluorescence (Figure 11). $Swi6_N$-GFP is concentrated in the nucleus in cells arrested in G1 by α factor. In the G2 arrest induced by nocodazole, $Swi6_N$-GFP is present in the cytoplasm. This indicates that cell cycle–regulated nuclear localization is conferred by the N-terminal one-third of Swi6. It also demonstrates that these changes in subcellular localization are not artifacts induced by the permeabilization step required for visualization by immunofluorescence. This is important because in permeabilized cells, variation in retention of a transcription factor in the nucleus could easily be a secondary consequence of its DNA binding properties. The behavior of the $Swi6_N$-GFP fusion in vivo clearly demonstrates that the N terminal one-third of Swi6 contains a cell cycle–regulated nuclear localization activity.

DISCUSSION

Swi6 is crucial for G1/S-specific transcription of SCB- and MCB-containing promoters. Swi6 is targeted to these promoters by binding to Swi4 or Mbp1 but it is unclear what regulates the activity of these complexes such that their target genes are only expressed during late G1/early S phase. The prominent role of Cdc28 in the G1/S transition, and the requirement for Cdc28 activity for SCB and MCB-regulated transcription led us to search for cell cycle–specific phosphorylation of Swi6. Our experiments indicate that S-160 in Swi6, which resides within a Cdc28 consensus sequence (SPLK), is variably phosphorylated during the cell cycle in a manner that is summarized in Figure 12. S-160 phosphorylation occurs in late G1, around the time of maximal G1/S-specific transcription. Phosphorylation occurs after the Cdc28 execution point in G1, but before the Cdc34 and Cdc4 execution points in G1, and before budding and S phase occur in the unperturbed cycle. S-160 phosphorylation is reduced after the Cdc15 execution point, very late in M phase

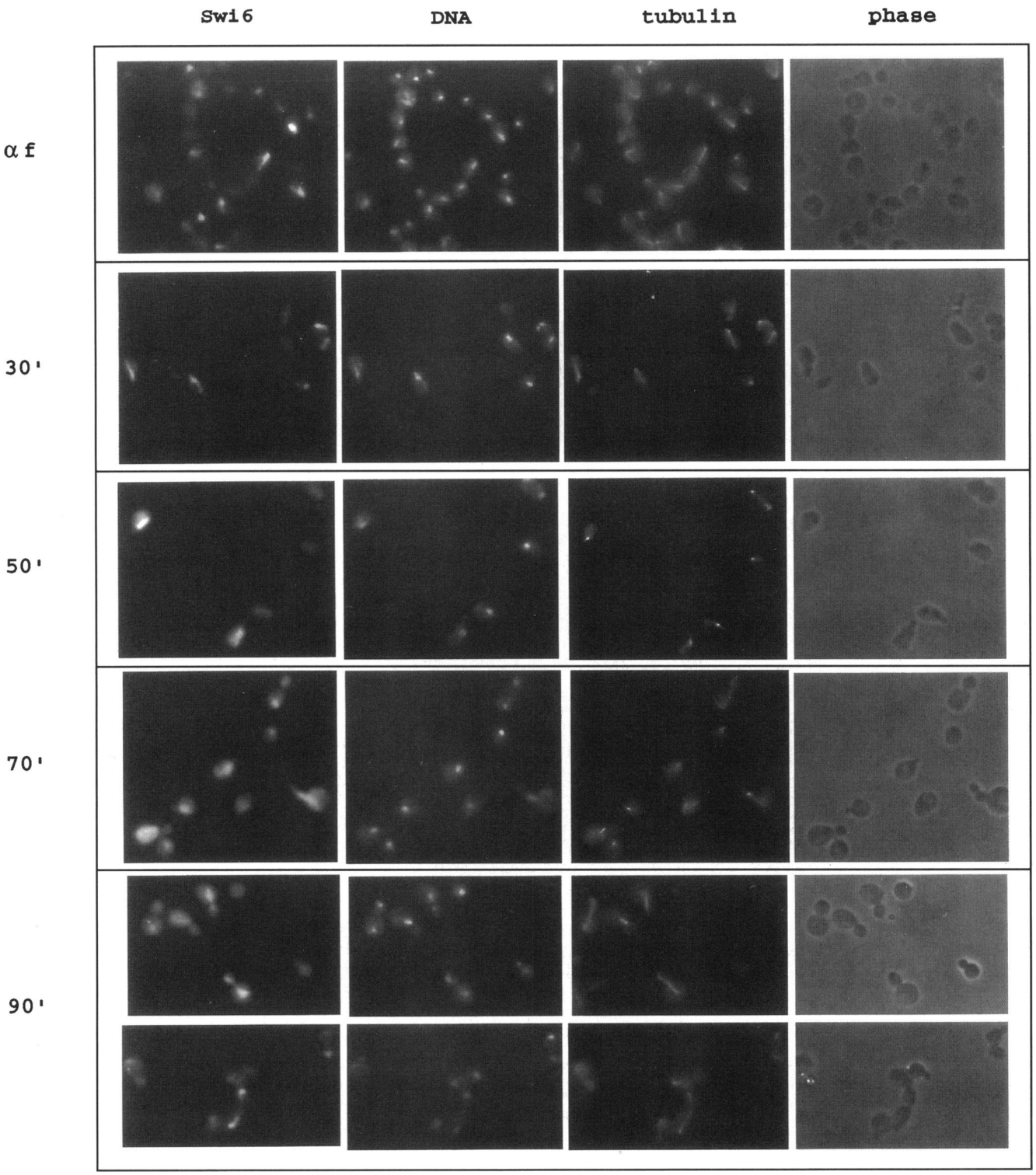

Figure 7. The subcellular localization of Swi6 changes during the cell cycle. BY600 *swi6::TRP1* transformed with the wild-type Swi6 plasmid Bd176 was arrested with α factor and released. Samples were taken during the arrest and at 30, 50, and 90 min after release, cells were fixed and stained with Swi6 antibodies (Swi6), DAPI (DNA), and tubulin antibodies (tubulin) as described in MATERIALS AND METHODS. The rightmost column shows phase contrast photomicrographs of the same field of cells made with the EPH135–36 slide film. The specificity of the Swi6 antibodies has been demonstrated in immunoblots and immunoprecipitation (Sidorova and Breeden, 1993). Immunofluorescence of *swi6* deletion strains with these antibodies are also negative (our unpublished observations).

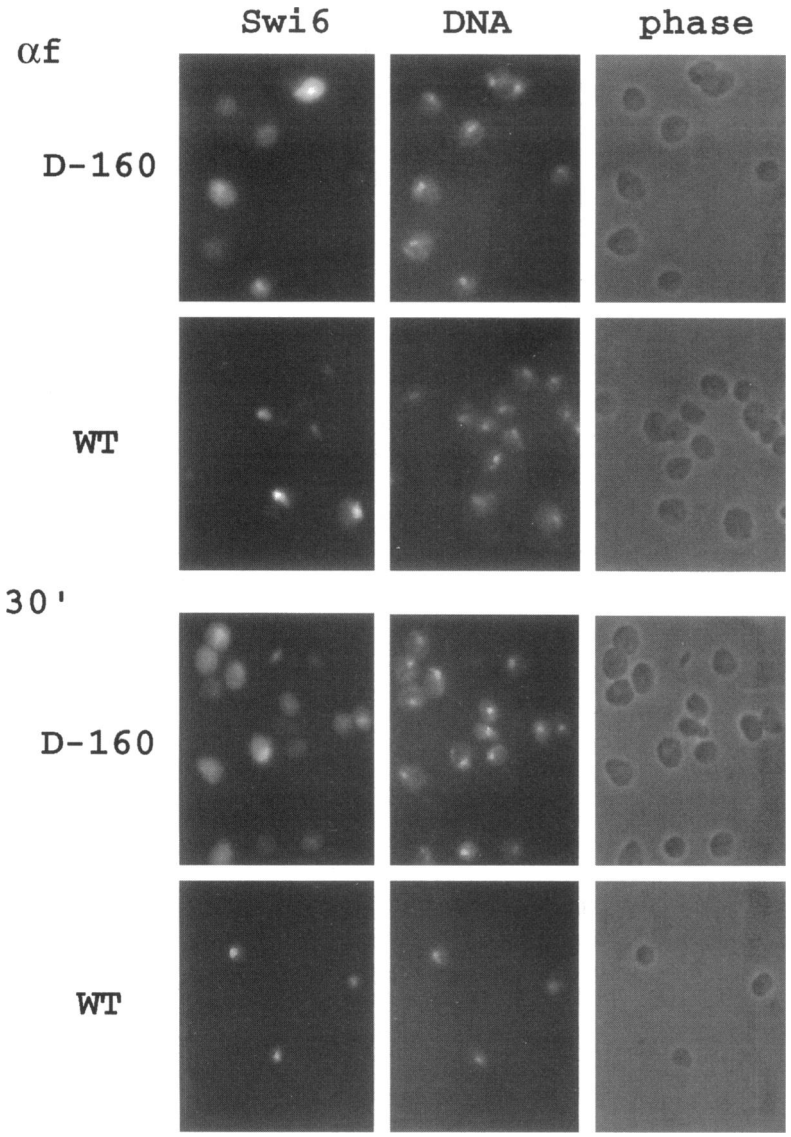

Figure 8. Aspartic acid substitution for S-160 changes the subcellular localization of Swi6. BY600 *swi6::TRP1* transformed with either the D-160 *SWI6* plasmid (Bd1436) or the wild-type *SWI6* plasmid (Bd176) were synchronized with α factor and samples were collected during α factor arrest and 30 min after release. These samples were stained with Swi6 antibodies and DAPI, or pictured using phase contrast micrography.

or at the onset of G1 phase. Then, phosphorylation remains low until shortly before the next S phase.

Phosphorylation of S-160 is temporally coincident with the G1/S-specific pulse of SCB- and MCB-driven transcription. However, this phosphorylation is not required for the activation or repression of Swi6-dependent transcription. Alanine or aspartate substitutions at position 160 of Swi6 prevent its phosphorylation but have no effect on the periodicity of G1/S-specific transcription. Rather than having a critical role in regulating transcription, the phosphorylation state of S-160 determines the subcellular localization of Swi6. In a normal cell cycle, Swi6 is localized exclusively to the nucleus from the end of M phase to the beginning of S, when S-160 is not phosphorylated. Throughout S and G2, when S-160 is phosphorylated, Swi6 accumulates in the cytoplasm. Amino acid substitutions at position 160 disrupt the normal pattern of localization in predicted ways. The Swi6 mutant with alanine substituted for serine at position 160 constitutively localizes to the nucleus, while the mutant with a negatively charged aspartate at this position is cytoplasmic throughout the cell cycle. Therefore, the phosphorylation status of S-160 seems to determine the localization of Swi6. Phosphorylation-regulated localization has also been documented in the case of Swi5, another *S. cerevisiae* transcription factor (Moll *et al.*, 1991), and of *Xenopus* nuclear factor 7 (Li *et al.*, 1994). One simplistic explanation of the mechanism of this regulation is that phosphorylation neutralizes the necessary positive charge of an adjacent nuclear localization signal. Indeed, this may be the case in Swi6 be-

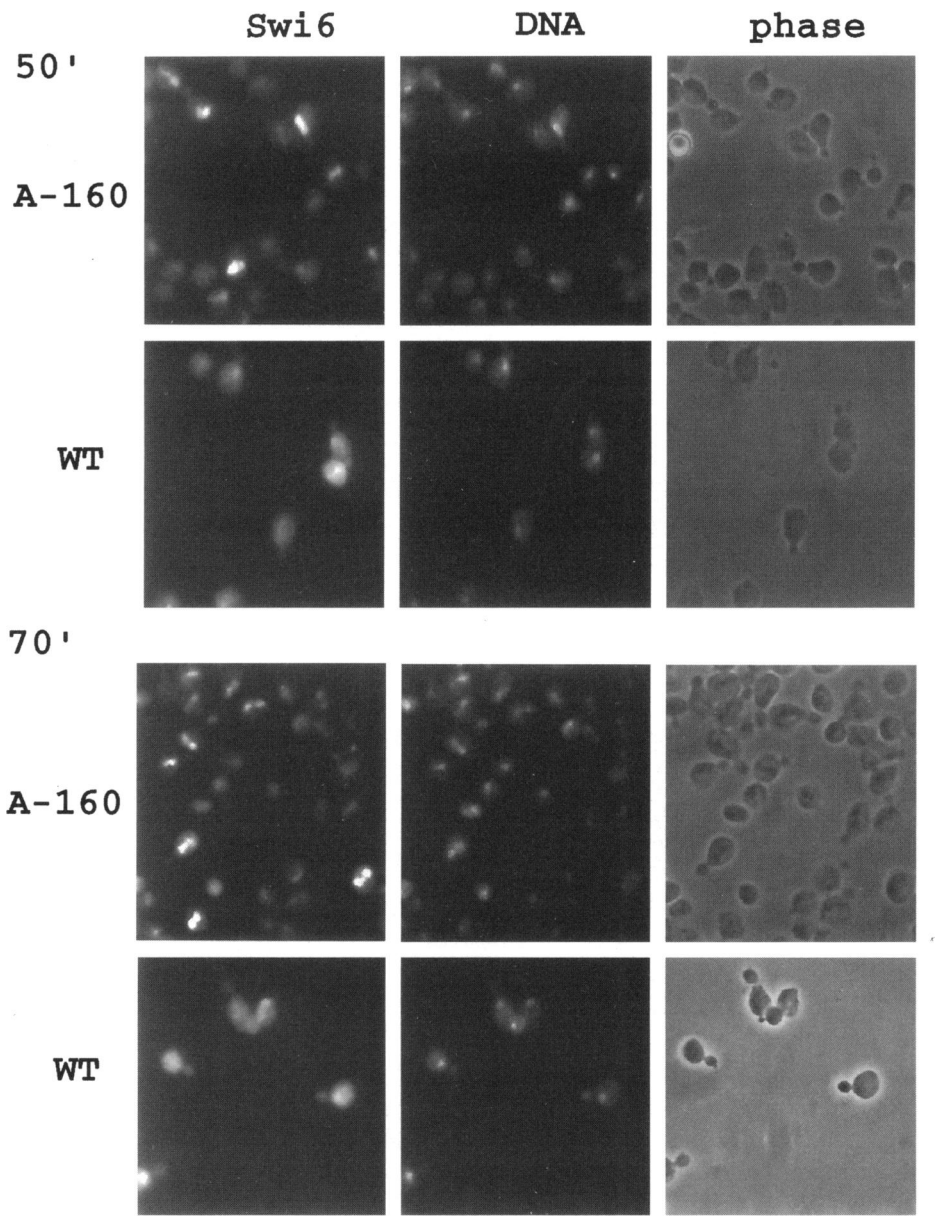

Figure 9. Alanine substitution for S-160 changes the subcellular localization of Swi6. BY600 *swi6::TRP1* transformed with either the A-160 *SWI6* plasmid (Bd1651) or wild-type *SWI6* plasmid (Bd176) was synchronized with α factor and samples were collected. Cells taken at 50 and 70 min after release were stained with Swi6 antibodies and DAPI and photographed as above.

cause a single negative charge at position 160 can disrupt nuclear localization, and an NLS sequence is located adjacent to S-160 of Swi6.

The fact that we have not observed any profound loss of periodicity of *HO*, *CLN1*, or *SWI4* transcription driven by the S-160 and NLS mutants of Swi6 indicates that regulated localization of Swi6 is not a required component of the cell cycle–specific regulation of these promoters. However, regulated nuclear entry of Swi6 may still contribute to the regulation of Swi6-dependent genes. G1/S-specific transcription of *CLN1*, *CLN2*, *HO*, and other SCB and MCB-driven genes are regulated by multiple activities and Swi6 is only one of them (Breeden and Mikesell, 1994; Cross *et al.*, 1994; Stuart and Wittenberg, 1994). Thus, it is not surprising that constitutive nuclear localization of the A-160 Swi6 mutant is not sufficient to deregulate SCB- and MCB-mediated transcription. Constitutive cytoplasmic localization of Swi6 has a negative impact on Swi6 function as indicated by the low level of *HO* transcription produced in the D-160 strain, but the *HO* mRNA that is produced is still regulated. In this context it is worth noting that close examination of cells carrying the D-160 and NLS mutants of Swi6 indicated that neither mutant is completely excluded from the nucleus, so some Swi6 is still available in the nucleus to activate transcription. This is probably explained by the fact that aspartate carries only one negative charge and

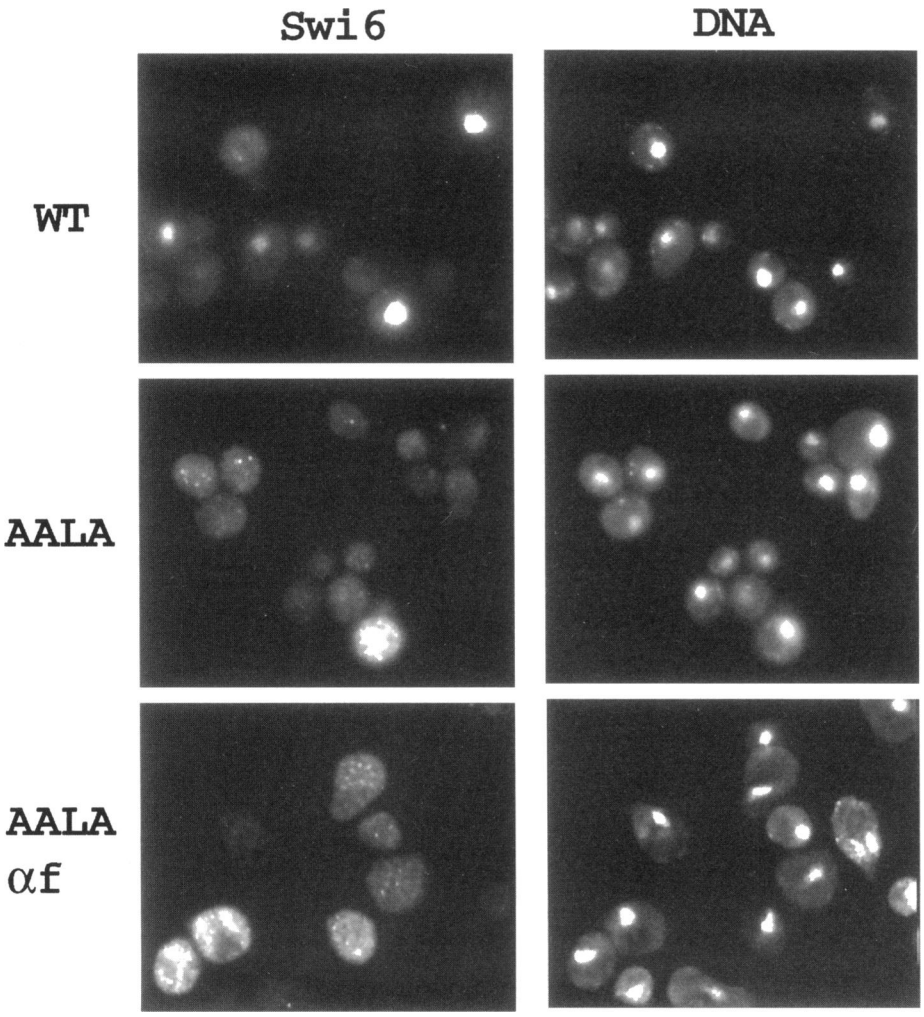

Figure 10. Mutation of the basic amino acids adjacent to S-160 abolish nuclear localization of Swi6. BY600 *swi6::TRP1* was transformed with the plasmid expressing the AALA mutant of Swi6 (Bd1734), which has substitutions of alanines for three lysines within the S(160)PLKKLK sequence. Asynchronous cultures of this strain were compared with the same strain carrying the wild-type *SWI6* plasmid (as noted in the upper and middle rows). Cells were fixed and stained with Swi6 antibodies and DAPI. In the lower row BY600 with Swi6-AALA was arrested in G1 with α factor and cells were collected and stained with Swi6 antibodies and DAPI. The localization pattern of this Swi6 mutant can be compared with the α factor arrested wild-type Swi6 shown in Figures 7 and 8. Note that in Figure 10 images were generated by direct scanning of the slides by the DeltaVision microscope system.

phosphate carries two. Also it is possible that there are other NLS sequences in Swi6 or that Swi6 can also be transported to the nucleus via NLS sequences within its known binding partners.

Understanding the mechanism through which subcellular localization of Swi6 is regulated requires identification of the kinases and/or phosphatases that modulate S-160 phosphorylation and determining their location. One difference we observe between the kinetics of phosphorylation and localization is relevant in this context. During the G1/S transition, S-160 achieves a high steady state level of phosphorylation over a short interval of time, but the Swi6 protein is only slowly redistributed until it is predominantly cytoplasmic. In contrast, at the end of M phase, both the loss of phosphorylation and nuclear localization occur rapidly and coordinately (see Figure 12). This behavior is consistent with the idea that loss of phosphorylation leads to rapid transport of Swi6 into the nucleus, whereas phosphorylation prevents nuclear entry and only gradually, as the nuclear Swi6 is degraded, is the protein redistributed and localized to the cytoplasm. Passive proteolysis could account for the redistribution of Swi6 to the cytoplasm during the G1/S transition. If this is the case, this proteolytic pathway is probably not nucleus specific, because the A-160 mutant of Swi6, although always nuclear, is maintained at the same levels as the wild-type protein. Furthermore, the kinase(s) that phosphorylate S-160 are probably present in both the nuclear and cytoplasmic compartments. This is based on the fact that at the G1/S transition S-160 phosphorylation occurs when Swi6 is only detectable in the nucleus, and our labeling experiments show that later in the cycle, phosphorylation readily occurs on Swi6 that is predominantly cytoplasmic.

In a search for the kinase/phosphatase system responsible for the cell cycle–specific phosphorylation of Swi6, we first turned to the Cdc28 kinase because the temporal pattern of Swi6 phosphorylation correlates

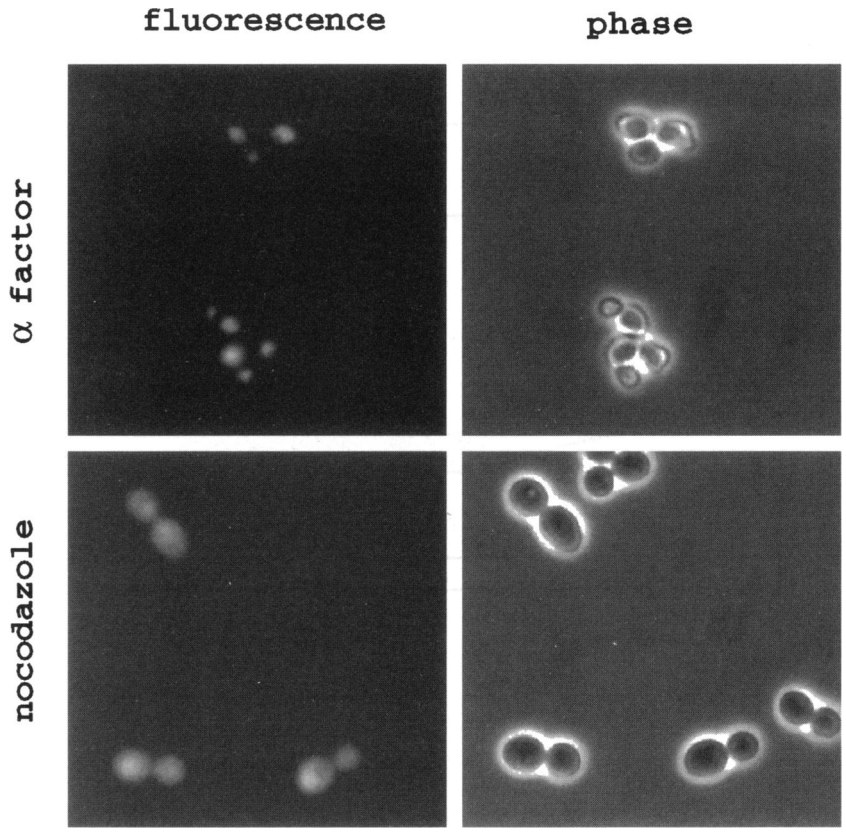

Figure 11. The pattern of localization of the $Swi6_N$-GFP fusion protein is similar to the native Swi6. BY602 wild-type transformed with Bd1815 was grown in raffinose then galactose was added along with either 5 μg/ml of α factor (upper row) or 15 μg/ml of nocodazole (lower row) for 2–3 h. Cells were mounted on slides and examined under the microscope. Representative fields were photographed in fluorescent light (right column) or in phase contrast (left column).

with the activation pattern of the Cdc28 kinase (assuming that all cyclin/Cdc28 complexes are capable of phosphorylating Swi6), and S-160 lies within a sequence that matches the Cdc28 consensus phosphorylation site. Furthermore it is known that activation of transcription by Swi6-dependent SCB and MCB elements requires Cdc28 activity (Johnston and Thomas, 1982; Breeden and Nasmyth, 1987b; Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991), and SCB and MCB activation can occur upon direct reactivation of Cdc28 without additional protein synthesis (Marini and Reed, 1992). The results of our studies indicate that Cdc28 cannot be the predominant source of S-160 phosphorylation in vivo. It is possible that Cdc28 can phosphorylate S-160, but it is not required for this phosphorylation, nor is it required for any of the other phosphorylations that can be monitored with tryptic peptide maps. There must be at least one other kinase involved. Furthermore, S/TP phosphorylation does not appear to modulate G1/S specific transcription, so the question of how the Cdc28 activation in late G1 affects G1/S-specific transcription remains unanswered. It is possible that Swi4 or another unidentified protein in these complexes is regulated by Cdc28 phosphorylation. Another possibility is that it is the association with the kinase, rather than the phosphorylation of Swi6 that is important in activating G1/S-specific transcription. There is evidence that Swi4 can bind to Cdc28/Clb2 complex (Amon *et al.*, 1993). Perhaps the Cdc28 kinase binds to the Swi4/Swi6 or Mbp1/Swi6 complexes and this localizes its kinase activity to particular cell cycle–regulated promoters. Thus positioned at these promoters, the kinase could phosphorylate and activate other proteins in the transcription complex and induce transcription.

Swi6 is not the only *S. cerevisiae* protein that is differentially localized during the cell cycle. Swi5 (Nasmyth *et al.*, 1990), Ace2 (Dohrmann *et al.*, 1992), Mcm2, Mcm3, and Cdc46/Mcm5 (Hennessy *et al.*, 1990; Yan *et al.*, 1993) all shift their location during the cell cycle in the same way as Swi6 shifts. The differential localization of Swi5 (Moll *et al.*, 1991) and Swi6 (this work) is regulated by phosphorylation and Mcm3 has a region that is organized remarkably similarly to the phosphorylation site-NLS regions of Swi5 and Swi6 (Tye, 1994 and Laskey, 1988). Hence, it is plausible that the same mechanism of regulated subcellular localization is utilized by other members of the group as well. Swi5 and Ace2 are transcription factors (Stillman *et al.*, 1989; Dohrmann *et al.*, 1992), Mcm2 and Mcm3 are required for the initiation of DNA replication (Yan *et al.*, 1993), and Cdc46/Mcm5 is also crucially involved in replication (Hennessy *et al.*, 1990; Yan *et al.*, 1993). The regulated nuclear localization of the Mcm pro-

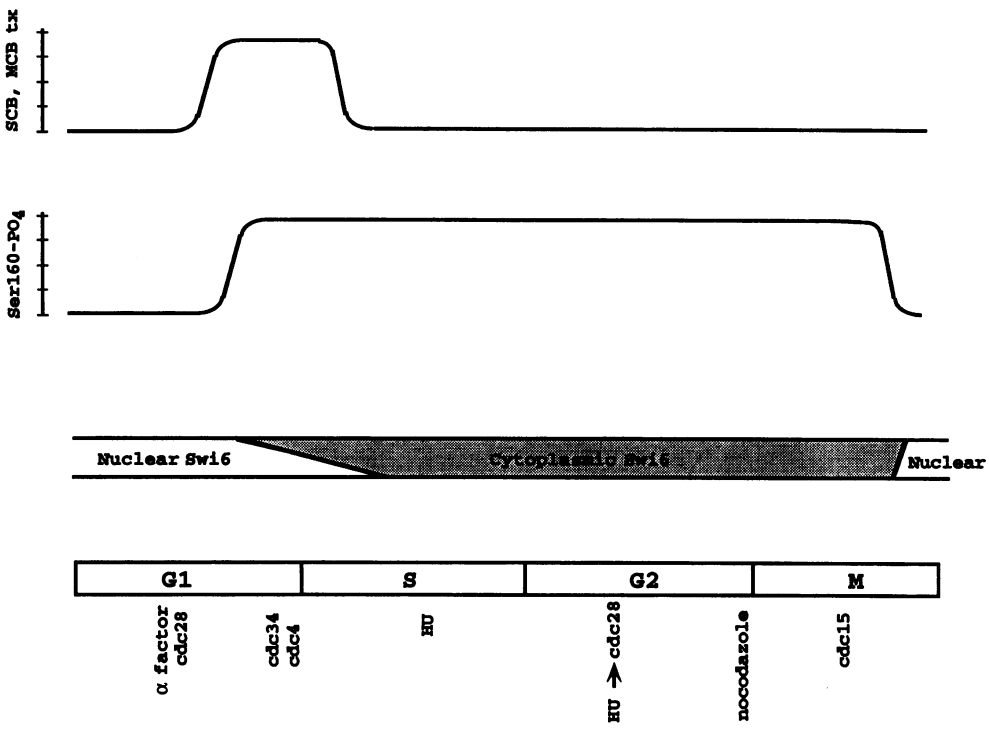

Figure 12. Changes in Swi6 phosphorylation correlate with its subcellular distribution and vary during the cell cycle. The cell cycle arrests in which the localization and/or phosphorylation state of Swi6 was studied are outlined beneath the bar representation of the cell cycle. The upper plot denotes the timing of SCB- and MCB-driven transcription. The second plot and the bar below it summarize our observations regarding the rapid transition in the phosphorylation state of S-160 in Swi6, compared with the kinetics of redistribution of Swi6 between the nucleus and cytoplasm.

teins at the beginning of S phase has led to speculation that they play the role of licensing factor(s) to ensure that only one S phase occurs per cell cycle (Blow and Laskey, 1988; Hennessy *et al.*, 1990; Tye, 1994). If this is so, then delocalization of these proteins may lead to the disruption of re-replication control. So far, there are no known mutants of *S. cerevisiae* that conditionally over-replicate their DNA by multiple discrete rounds of replication. In contrast, in *Schizosaccharomyces pombe* the mere inactivation of the G2 form of a cdc2 kinase by heat treatment of temperature-sensitive mutants of *cdc2* or *cdc13* (encoding mitotic cyclin B) leads to an extra S phase without an intervening M phase (Broek *et al.*, 1991; Hayles *et al.*, 1994). In *S. cerevisiae*, inactivation of Cdc28 in G2 by elimination of mitotic cyclins does not have this effect (Fitch *et al.*, 1992; Richardson *et al.*, 1992; Amon *et al.*, 1993). This suggests that an additional negative control is imposed over S phase in *S. cerevisiae* that is independent of Cdc28 activity. Our results show that nuclear entry of Swi6 is regulated by phosphorylation, and this phosphorylation occurs in the absence of Cdc28 activity. Thus, it is likely that nuclear localization of Swi6 is Cdc28-independent in vivo. If the mechanism that restricts nuclear entry of Swi6 also affects the nuclear entry of Mcm2, Mcm3, and Mcm5/Cdc46, this could represent the second negative control imposed over S phase in *S. cerevisiae*.

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