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The Cdc2 Protein Kinase Controls Cdc10/Sct1 Complex Formation

Timothy Connolly,*† Maureen Caligiuri,*‡ and David Beach§

Howard Hughes Medical Institute, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

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In the fission yeast *Schizosaccharomyces pombe*, the execution of Start requires the activity of the Cdc2 protein kinase and the Cdc10/Sct1 transcription complex. The loss of any of these genes leads to G_1 arrest and activation of the mating pathway under appropriate conditions. We have undertaken a genetic and biochemical analysis of these genes and their protein products to elucidate the molecular mechanism that governs the regulation of Start. We demonstrate that serine-196 of Cdc10 is phosphorylated in vivo and provide evidence that suggests that phosphorylation of this residue is required for Cdc10 function. Substitution of serine-196 of Cdc10 with alanine (Cdc10 S196A) leads to inactivation of Cdc10. We show that Cdc10 S196A is incapable of associating with Sct1 to form a heteromeric complex, whereas substitution of this serine with aspartic acid (S196D) restores DNA-binding activity by allowing Cdc10 to associate with Sct1. Furthermore, we demonstrate that Cdc2 activity is required for the formation of the heteromeric Sct1/Cdc10 transcription complex and that the Cdc10 S196D mutation alleviates this requirement. We thus provide biochemical evidence to demonstrate one mechanism by which the Cdc2 protein kinase may regulate Start in the fission yeast cell cycle.

INTRODUCTION

In eukaryotes, the passage of cells through the cell cycle is regulated in part by the association of multiple cyclin-dependent kinases (cdk) with different cyclin regulatory subunits. In yeast, a single 34-kDa cdk (Cdc2/CDC28) sequentially associates with G_1, S-phase, or mitotic cyclins to form active holoenzyme complexes and thereby direct distinct cell cycle transitions. The developmental fate of the cell is determined by a series of regulatory events in the G_1 termed Start. At this control point, a cell monitors both internal cues, such as cell size, and external cues, such as nutrient availability, and subsequently decides to proceed into a new round of mitotic cell division or to exit the cell cycle by activating functions leading to cellular differentiation.

In *Saccharomyces cerevisiae*, passage through Start requires the association of the CDC28 protein kinase with positive regulatory subunits known as G_1 cyclins, CLNs. Three CLN type cyclins form a redundant multigene family that associates with the CDC28 protein kinase to control passage through G_1 (Richardson *et al.*, 1989). Whereas the activity of any CLN gene product is sufficient to promote cell cycle progression, the loss of all three *CLN* genes results in cell cycle arrest at Start. In turn, overexpression of any CLN results in a shortened G_1 and accelerates passage through Start. Thus, the decision to maintain mitotic cell cycle progression or to enter an alternative developmental fate is controlled in part by the association of CDC28 with CLN-type cyclins in G_1.

Although the concept of cell cycle commitment at Start has been useful in describing yeast cell physiology, the underlying molecular basis of cell cycle commitment remains unclear. It is likely that one role of the Cdc2/CDC28 kinase in G_1 is to directly activate transcription factors involved in regulating genes required for the activation of DNA replication. In budding yeast, the SWI4 and SWI6 proteins have been

* These two authors have contributed equally to this work.
† Present address: Osiris Therapeutics, Inc. 2001 Aliceanna Street, Baltimore, MD 21231.
‡ Present address: Mitotix, Inc., 1 Kendall Square, Cambridge, MA 02139.
§ Corresponding author.

identified as components of the SBF transcription complex that is required for the CDC28 (Start) dependent expression of the *HO*, *CLN1*, *CLN2*, *HCS26*, and *PCL2* (ORFD) genes at the G_1/S-phase boundary (Breeden and Nasmyth, 1987a; Andrews and Herskowitz, 1989a; Nasmyth and Dirick, 1991; Ogas *et al.*, 1991; Measday *et al.*, 1994). The SWI4 and SWI6 proteins have been implicated in the transcriptional control of the *CLN1* and *CLN2* genes through a positive feedback loop (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991; Ogas *et al.*, 1991;). The CLN3/CDC28 kinase complex is present throughout the cell cycle, and consequently, it has been proposed that this complex induces CLN1 and CLN2, which in turn leads to an abrupt rise in CDC28 kinase activity that is associated with the commitment of cells to enter the S-phase (Cross and Tinkelenberg, 1991). Recent experiments, however, have shown that CLN3 is normally responsible for SBF-dependent transcription when cells reach a critical size at Start (Dirick *et al.*, 1995; Stuart and Wittenberg, 1995). Activation of the SWI4/SWI6 complex at Start is thus thought to require phosphorylation by one or more members of the CLN/CDC28 family of kinases (Cross and Tinkelenberg, 1991; Tyers *et al.*, 1993; Koch *et al.*, 1996).

In the fission yeast *S. pombe*, three genes are known to be required for the execution of Start. These encode the Cdc10 and Sct1/Res1 transcription factors and the Cdc2 protein kinase, which is also required in G_2 (Nurse and Bisset, 1981, Tanaka *et al.*, 1992, Caligiuri and Beach, 1993). Haploid strains carrying inactivating mutations in any of these genes arrest in G_1 and mate to form diploid cells under appropriate nutritional conditions. Cdc10 and Sct1 share amino acid sequence similarity with the SWI4, SWI6, and MBP1 transcription factors from budding yeast (Aves *et al.*, 1985; Breeden and Nasmyth, 1987b; Andrews and Herskowitz, 1989b; Tanaka *et al.*, 1992; Caligiuri and Beach, 1993; Koch *et al.*, 1993). Cdc10 and Sct1 function together as a heteromeric transcription complex to activate the expression of the *cdc22*, *cdt1*, and *cdc18* genes at the G_1-S-phase transition (Lowndes *et al.*, 1992a; Caligiuri and Beach, 1993; Hofmann and Beach, 1994; Kelley *et al.*, 1993). Sct1 and Cdc10 are thought to be directed to the promoters of these periodically expressed genes by the presence of Mlu 1-like elements (MCB) in their upstream regions, which have been shown to be sufficient to confer cell cycle periodicity on heterologous promoters (Lowndes *et al.*, 1992a,b). Sct1 and Cdc10 are both expressed constitutively throughout the cell cycle; however, the DNA-binding activity of the transcription complex has been shown to oscillate (M.C., unpublished observation; Aves *et al.*, 1985; Reymond *et al.*, 1993). This activity is dependent on Cdc2 function, thus phosphorylation of Sct1 or Cdc10 may contribute to the regulation of the complex (Reymond *et al.*, 1993).

Table 1. List of *S. pombe* strains

Strain	Genotype
SP 201	*h^{+n} leu1-32 ade6-210*
SP 357	*h^{-s} cdc2-33 leu1-32 ura4-D18 ade6-210*
SP 812	*h^{-s} leu1-32 ura4-d18 ade6-210*
SP 965	*h^{-s} cdc10-129 leu1-32 ura4-D18 ade6-210*
SP 1296	*h^{-s} cdc2-M72 leu1-32 ura4-D18 ade6-210*
SP 1297	*h^{-s} cdc10::ura4 sct1-1 ura4-D18 leu1-32*

We have undertaken a biochemical and genetic analysis of the Sct1/Cdc10 transcription complex in *S. pombe*. We demonstrate that Cdc2 kinase activity is necessary for the formation of the Cdc10/Sct1 transcription complex. An analysis of the primary structure of Cdc10 revealed a single canonical Cdc2 phosphorylation site. We show that this site, which includes serine-196 (Ser-196), is phosphorylated in vivo. Substitution of the serine residue within this site with an alanine (S196A), thus preventing phosphorylation, led to the dissociation of the Cdc10/Sct1 complex. As a consequence, the *Cdc10 S196A* gene is incapable of functioning in vivo. Substitution of this serine with the negatively charged aspartic acid, thus potentially mimicking phosphorylation, restored the ability of Cdc10 to associate with Sct1 and consequently to function in vivo. Furthermore, we demonstrate that Cdc2 kinase activity is essential for Cdc10/Sct1 complex formation, and that the Cdc10 S196D mutant bypasses this requirement. Thus, we provide biochemical and genetic evidence to suggest one mechanism by which the Cdc2 protein kinase may serve to regulate Start in the fission yeast cell cycle.

MATERIALS AND METHODS

Strains and Media

All *S. pombe* strains were derived from a wild-type strain originally described by Leupold (1970). The strains used in this study are listed in Table 1. *S. pombe* was grown in standard YEA, YE, PM, and PMA media containing additional amino acids when indicated at 75–150 μg/ml (Beach *et al.*, 1985). The standard genetic procedures previously described for *S. pombe* were followed (Gutz *et al.*, 1974).

Immunoprecipitation and Western Blotting

S. pombe cell-free lysates were prepared essentially as described (Booher *et al.*, 1989). Cells were quickly disrupted by vortexing in the presence of glass beads in buffer H [25 mM Tris, pH 7.5, 1 mM dithiothreitol (DTT), 60 mM β-glycerol phosphate, 15 mM p-nitrophenylphosphate, 15 mM EGTA, 15 mM $MgCl_2$, 0.1 mM NaF, 0.1 mM sodium orthovanadate and 0.1% Nonidet P-40 (NP-40), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin, aprotinin, and pepstatin, 10 μg/ml L-1-tosylamide-2-phenylethylchloromethyl, and 7-amino-1-chloro-3-tosylamido-2-heptanone]. After lysis, an equal volume of cold buffer H was added to the beads, and lysate was collected and centrifuged at 11,000 × *g* for 15 min. The supernatant was collected, and the protein concentration was determined by a colorimetric assay using Bradford reagent (Bradford, 1976).

Immunoprecipitations were performed with 10–50 mg of soluble protein extract and 1.0 μl of antibody as specified and were rotated at 4°C. After 2 h, 20 μl of a 1:1 slurry of protein-A Sepharose (Pharmacia Biotech, Piscataway, NJ) was added, and the incubation was continued for an additional 30 min at 4°C. The immune complexes were recovered by centrifugation and washed three times in 1.0 ml of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM DTT, and 1 mM PMSF. The samples were resuspended in Laemmli buffer, and the proteins were resolved by conventional SDS-PAGE.

For immunoblotting, the proteins were transferred to nitrocellulose for 30 to 60 min at 400 mA with a semidry transfer apparatus (Millipore, Bedford, MA). After the transfer, the immunoblots were blocked in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat dry milk for 30 min at 37°C. Blots were incubated with a 1:1,000 dilution of antibody in TBST 5% milk for 2 h at room temperature. The blots were washed three times (20 min each) in TBST and then incubated with a 1:50,000 dilution of an anti-mouse or anti-rabbit Fc antibody as appropriate (Jackson ImmunoResearch Laboratories, West Grove, PA) in TBST 5% milk for 1 h at room temperature. The proteins were visualized using an enhanced chemiluminescence kit (Amersham Life Science, Arlington Heights, IL) and conventional autoradiography.

Cdc25 Block Release

A strain (SP 530) carrying *cdc25–22* was grown to mid-exponential phase in rich medium (YEA) at 25°C, and the cells were collected by centrifugation and inoculated into fresh media at 36°C for 3 h. The culture was then shifted back to 25°C, and 50-ml aliquots of cells were harvested at 30-min intervals. Cell extracts were prepared as described above.

Metabolic Labeling and Two-Dimensional Tryptic Peptide Mapping

S. pombe cells were grown for approximately 16 h in phosphate-free minimal media in which the NaH_2PO_4 was reduced to 1 mM and the potassium phthalate was replaced with sodium acetate (Moreno *et al.*, 1991). The cells were harvested, 5–10 ml at 1×10^7 cells per ml, washed with H_2O, and resuspended in phosphate-free minimal media supplemented with 50 μM NaH_2PO_4. The cultures were incubated for 2 h before the addition of [^{32}P]orthophosphate (1 mCi/5 ml culture) and labeled for 3 to 4 h. Extracts were prepared for immunoprecipitation as described above.

The proteins subjected to tryptic mapping were eluted from gel slices in 50 mM ammonium bicarbonate overnight in the presence of 20 μg of RNase. The proteins were precipitated with trichloroacetic acid and digested with 10 μg of L-1-tosylamide-2-phenylethylchloromethyl-trypsin as described (van der Geer *et al.*, 1993). Peptide maps were produced by two-dimensional separation of the phosphopeptides on TLC plates. The first dimension was electrophoresis in pH 1.9 buffer, and the second dimension was chromatography in isobutyric acid buffer (van der Geer *et al.*, 1993).

Gel Retardation Assays

Cells were grown into a density of 5×10^6 to 1×10^7 cells/ml in minimal selective media, harvested by centrifugation, and washed once in lysis buffer (50 mM Tris, pH 7.5, 0.3 M KCl, 10% glycerol, 10 mM $MgCl_2$, 20 mM β-glycero-phosphate, 5 mM EDTA, 0.1 mM sodium vanadate, 1 mM DTT, 0.2% NP40, 1 mM PMSF, 2 μg/ml leupeptin, aprotinin, and pepstatin). The cells were disrupted by vortexing with glass beads. Cellular debris was cleared from the lysate by centrifugation for 20 min in a microcentrifuge. The supernatant was collected, and the protein concentration was determined by a colorimetric assay using Bradford reagent (Bradford, 1976).

The restriction fragments to be used for probes were recovered from low-melting-temperature agarose in Tris-acetate buffer and end labeled with Klenow polymerase in the presence of [α-^{32}P]dATP (Sambrook *et al.*, 1989). The binding reactions were performed in a volume of 20 μl and contained 20 μg of lysate in 20 mM Tris (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 5% glycerol, 2 mM DTT, 1 mM ATP, 0.1 mg/ml poly(dIdC)·poly(dIdC) (Pharmacia, Piscataway, NJ). After the addition of the protein extracts, the reaction mixtures were incubated at room temperature for 10 min. Approximately 1 ng of probe at 10^7 cpm/μg was added to the binding reaction, and the incubation was continued for an additional 10 min. When noted, unlabeled promoter fragment, at an approximately 50-fold M excess to the probe, was added at the same time as the probe. The anti-Cdc10 antibody and preimmune sera were diluted 10-fold in binding buffer, and 1 μl was added after the binding reaction. The HA1 monoclonal antibody 12CA1 (Boehringer Mannheim, Indianapolis, IN) was purified on protein A-Sepharose as described by the manufacturer. The antibody was diluted 1:10 in binding buffer, and 1 ml was added to the reaction as above. The binding reaction was incubated for an additional 10 min after the addition of antibodies. The DNA protein complexes were resolved on a 4% polyacrylamide gel (30:0.8 cross-linking) in 0.5× Tris-borate EDTA. The gels were dried on Whatman 3MM paper and subjected to autoradiography.

RESULTS

Cdc10/Sct1 Function Requires the Cdc2 Consensus Site Within Cdc10

The Cdc10 transcription factor is a phosphoprotein in vivo (Simanis and Nurse, 1989), and analysis of its primary structure revealed a single Cdc2 consensus site (SPLR) at Ser-196. We therefore explored the consequences of amino acid substitutions at Ser-196 by mutating this residue to the nonphosphorylatable alanine (S196A) and to the negatively charged aspartic acid (S196D) to potentially mimic phosphorylation. These mutant *cdc10* genes were then analyzed for their ability to complement the *cdc10–129* temperature-sensitive mutation. The *cdc10+*, *cdc10 S196A*, and *cdc10S196D* genes expressed on plasmids under the control of the *cdc10* promoter were transformed into a *cdc10–129* strain (SP 965) under permissive conditions for this mutation (25°C). Individual transformants were then assayed for growth under permissive or restrictive (36°C) conditions. Transformants harboring an empty vector or the S196A mutant failed to grow at elevated temperature, indicating that Ser-196 is essential for Cdc10 function in vivo (Figure 1A). The S196D mutant was, however, capable of fully complementing the temperature-sensitive *cdc10–129* mutation, suggesting that phosphorylation of this residue is critical for Cdc10 function. The *cdc10 S196D* mutant displays a mild cell elongation phenotype indicative of cell cycle delay, which is similar to that observed upon overexpression of *cdc10+/sct1+* (Ayte *et al.*, 1995).

The lack of *cdc10–129* complementing activity by *cdc10 S196A* could be explained if this mutant gene was not expressed or if its protein product was unstable. We therefore investigated the expression of

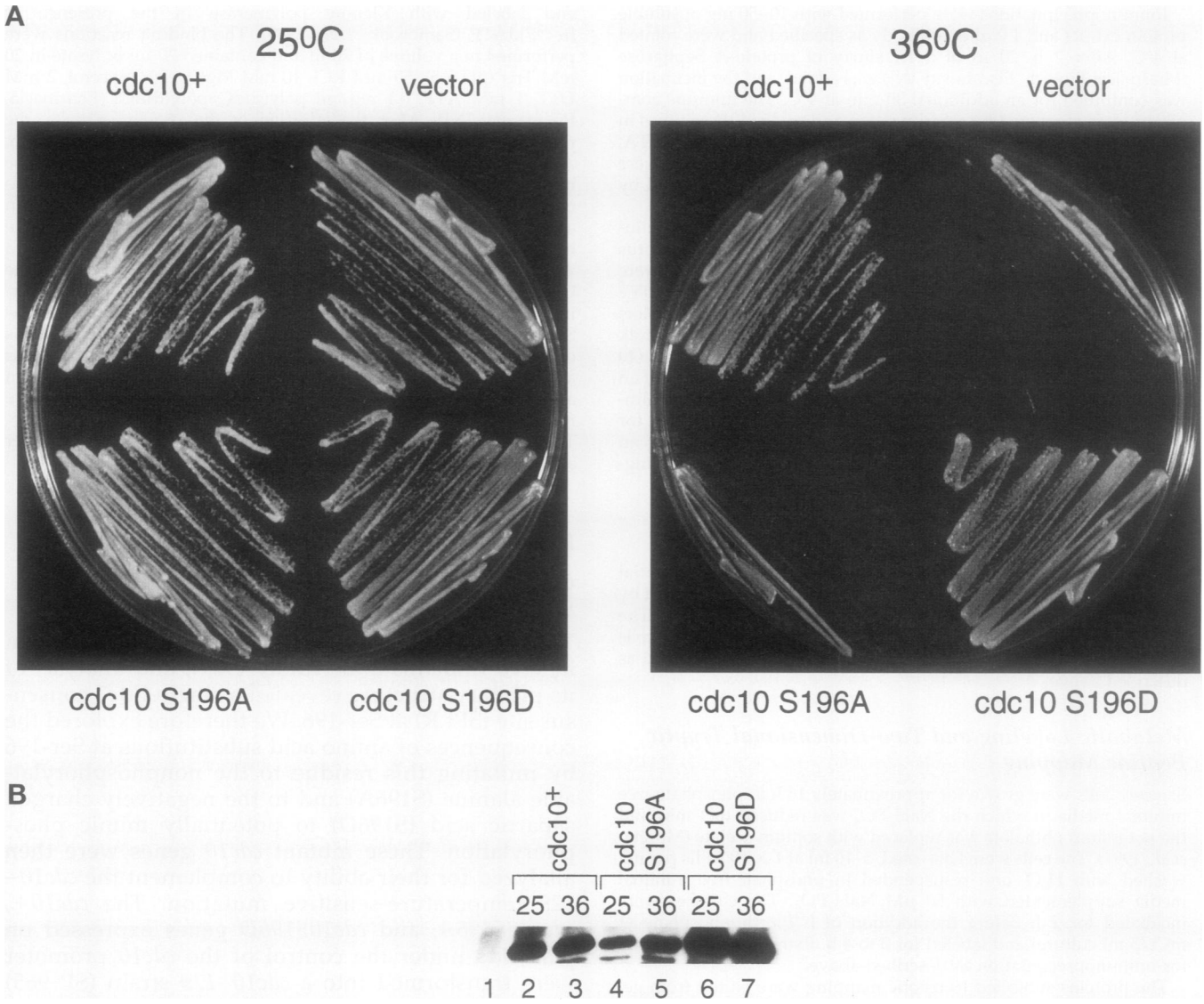

Figure 1. Ser-196 is essential for Cdc10 function in vivo. (A) The *cdc10–129* mutant strain was transformed with either *cdc10+*, *cdc10 S196A*, or *cdc10 S196D* on a multicopy plasmid and grown on selective media at either 25°C (permissive) or 36°C (restrictive) as indicated. (B) Anti-Cdc10 immunoblot of immunoprecipitates using either preimmune serum (lane 1) or the anti-Cdc10 antibody (lanes 2–7) from extracts prepared from cells producing Cdc10+ (lanes 2 and 3), Cdc10 S196A (lanes 4 and 5), or Cdc10 S196D (lanes 6 and 7) grown at either 25°C or 36°C as indicated.

cdc10+, *cdc10 S196A*, and *cdc10 S196D* in the *cdc10–129* transformants at both the permissive and nonpermissive temperatures for this mutation (Figure 1B). Individual transformants, expressing either *cdc10+*, *cdc10 S196A*, or *cdc10S196D* were cultured at either 25 or 36°C, and cell-free lysates were prepared. Cdc10 was immunoprecipitated with a polyclonal antibody raised against full-length Cdc10. The Cdc10 immunoprecipitates were resolved by SDS-PAGE and then transferred to nitrocellulose for Western blot analysis. We found that the level of Cdc10 S196A was approximately equal to that of $Cdc10^{+}$ in cells grown at 36°C. Thus, a reduction in the level of Cdc10S196A does not account for its inability to complement the *cdc10–129* mutation.

Cdc10 Is Phosphorylated at Ser-196 In Vivo

We have demonstrated that Ser-196 is essential for Cdc10 function in vivo. It was therefore of interest to determine if this residue was phosphorylated in vivo. Plasmids carrying the *cdc10+* or *cdc10 S196A* genes were transformed into a *cdc10::ura sct1–1* strain, SP 1297, which contains no endogenous immunologically

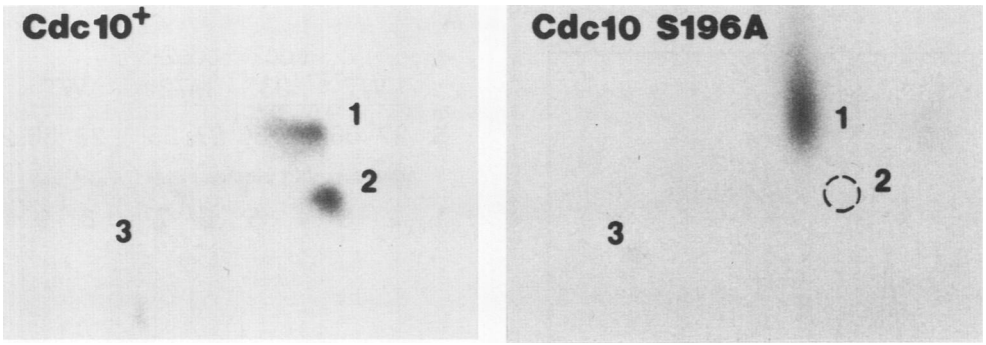

Figure 2. Ser-196 in Cdc10 is phosphorylated in vivo. ^{32}P-labeled Cdc10 immunoprecipitates were resolved by SDS-PAGE, recovered from gel slices, and subjected to proteolytic digestion with trypsin. The ^{32}P-labeled tryptic peptides were resolved by two-dimensional chromatography and visualized by phosphoimage analysis.

reactive Cdc10 (Marks *et al.*, 1992). Cultures of these transformants were labeled with [^{32}P]orthophosphate for the preparation of cell-free lysates, and Cdc10 was immunoprecipitated with a polyclonal antibody raised against full-length Cdc10. The Cdc10 immunoprecipitates were resolved by SDS-PAGE and subjected to autoradiography. The ^{32}P-labeled Cdc10 protein was eluted from the gel and digested with trypsin. The tryptic peptides were resolved by two-dimensional chromatography, and the maps were visualized by phosphoimage analysis (van der Geer *et al.*, 1993; Fuji Medical Systems USA, Stamford, CT). The tryptic digestion of wild-type Cdc10 generated two major spots (Figure 2, spots 1 and 2) and a minor spot (spot 3), whereas the digestion of Cdc10 S196A generated only 1 major spot and a minor spot (spot 3); spot 2 is clearly missing. This provides strong evidence that Ser-196 in Cdc10 is phosphorylated in vivo.

The Association of Cdc10 with Sct1 Is Not Cell Cycle Dependent

Formation of the heteromeric Cdc10/Sct1 complex is required for the activity of this transcription complex (this work; Ayte *et al.*, 1995). We investigated whether the cell cycle periodicity of the DNA binding activity of this complex was due to an oscillation in Cdc10/Sct1 association across the cell cycle (Reymond *et al.*, 1993). *S. pombe* cells carrying a temperature-sensitive allele of *cdc25* (*cdc25–22*) display a tight cell cycle arrest in G_2 when shifted to the nonpermissive temperature of 36°C and undergo synchronous cell division upon reintroduction into media at the permissive temperature (25°C) (Russell and Nurse, 1986). A culture of a strain (SP 530) carrying the *cdc25–22* allele harboring a plasmid expressing the *sct1* gene tagged at the 3′ end with a triple tandem copy of the hemagglutinin (HA) epitope (Sct1-HA) was synchronized by restricting growth at 36°C for 3 h (Caligiuri and Beach, 1993). The cells were then released into fresh media at the permissive temperature of 25°C, and aliquots were removed at 30-min intervals over a period of 6 h, corresponding to two cell cycles. Cell-free extracts were prepared, and the cell cycle profiles were monitored by measuring septation index (Figure 3A). Anti-HA immunoprecipitates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with the anti-Cdc10 polyclonal antibody (Figure 3B). The results demonstrate that there are no major and long-lasting fluctuations in the association of Cdc10 with Sct1, but do not rule out the possibility that a transient dissociation may occur in G_1.

Inactivation of Cdc2 Leads to the Dissociation of Cdc10 and Sct1

We have demonstrated that Cdc10 is inactivated when Ser-196, which lies within a Cdc2 consensus site, is mutated to alanine and that activity is restored when it is replaced with aspartic acid. One possibility we considered was that phosphorylation of Cdc10 on Ser-196 was required for its association with Sct1. It was therefore of interest to determine if the Cdc10/Sct1 complex was dissociated upon loss of *cdc2* in vivo. For this purpose, protein extracts were prepared from a wild-type strain and strains carrying the *cdc2–33* and *cdc2-M72* mutations (SP 201, SP 357, and SP 1296, respectively) harboring a plasmid expressing the *sct1-HA* gene (Caligiuri and Beach, 1993). Cells that carry the *cdc2–33* and *cdc2-M72* alleles are temperature sensitive for growth. Both of these mutants arrest in G_1 and G_2, although the *cdc2-M72* mutant appears to have a more profound G_1 defect that the *cdc2–33* mutant. These extracts were immunoprecipitated with either a polyclonal antibody raised against Cdc10 or with a monoclonal antibody raised against the HA epitope (12CA5). These immunoprecipitates were resolved by SDS-PAGE, and the proteins were transferred to nitrocellulose to visualize Cdc10 and Sct1 by Western blotting. The association between Cdc10 and

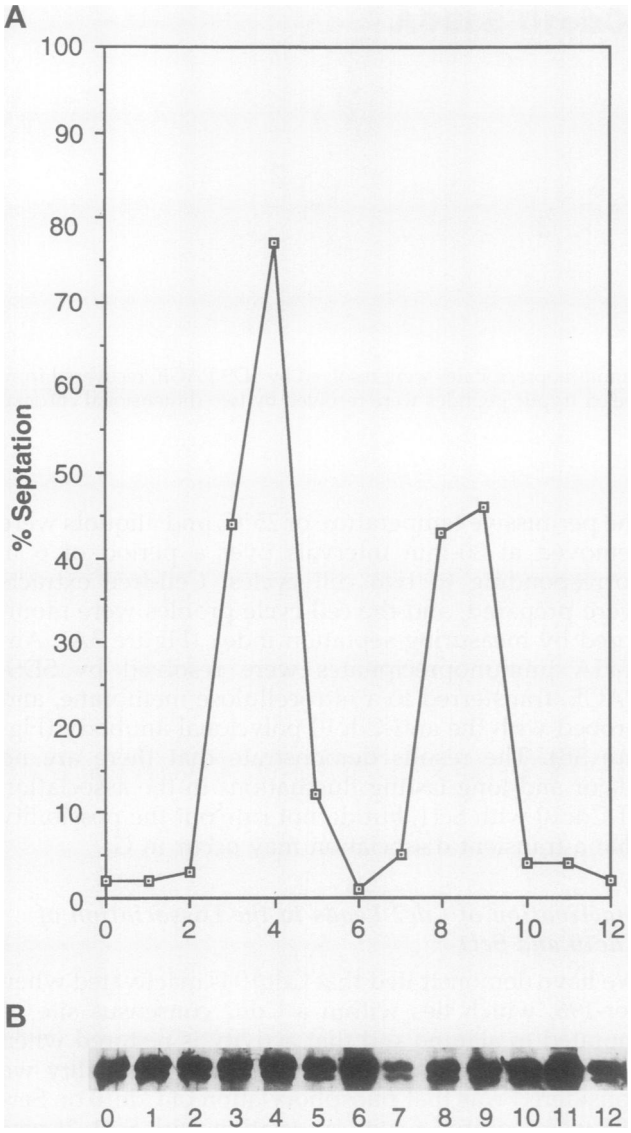

Figure 3. Cdc 10 and Sct 1 are associated throughout the cell cycle. A strain (SP 530) carrying a temperature-sensitive allele of *cdc25* (*cdc25–22*) harboring a plasmid expressing the *sct1-HA* gene was synchronized by arresting cell growth at the restrictive temperature (36°C) for 3 h followed by release at the permissive temperature (25°C). Aliquots of cells were removed at 30-min intervals after release to permissive conditions for the preparation of cell-free extracts. (A) The cell cycle profile was monitored by measuring the percentage of septated cells at each time point. (B) Anti-HA immunoprecipitates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with an anti-Cdc10 antibody. The lanes numbered 1–12 correspond to each 30-min interval with the first lane corresponding to an an aliquot taken before release to the permissive temperature.

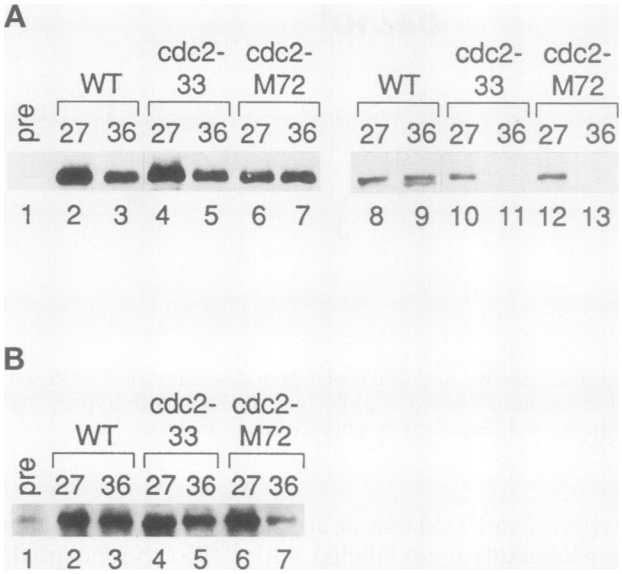

Figure 4. The association between Cdc10 and Sct1 is dependent upon Cdc2 function in vivo. Cell-free extracts were prepared from wild-type, *cdc2–33*, and *cdc2-M72* strains producing Sct1-HA grown at 27°C or 36°C as indicated. Immunoprecipitations were performed using either the Cdc10 preimmune sera or normal mouse serum (A and B, lane 1, respectively), the anti-Cdc10 antibody (A, lanes 2–7) or the anti-HA antibody (A, lanes 8–13; B, lanes 2–7). These immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies raised against Cdc10 (A, lanes 1–13) or Sct1-HA (B, lanes 1–7) to visualize the Cdc10 and Sct1-HA proteins, respectively.

Sct1 seen in immunoprecipitates from extracts prepared from wild-type cells (Figure 4A, lanes 8 and 9) is abolished upon inactivation of Cdc2 by growth of either the *cdc2–33* or *cdc2-M72* cells under restrictive conditions (36°C) and is thus not specific to a single allele of *cdc2* (Figure 4A, lanes 11 and 13). Growth of the wild-type or *cdc2* mutant strains at elevated temperature had no effect on the level of Cdc10 (Figure 4A, lanes 3, 5, and 7). Similarly, the level of Sct1 was not affected in the wild-type or *cdc2–33* strains grown at elevated temperature, but was, however, reduced in the *cdc2-M72* strain (Figure 4B, lanes 3, 5, and 7). These results suggest, therefore, that Cdc2 function, which has been shown previously to be required for the DNA binding activity of the complex (Reymond *et al.*, 1993), is required for the stabilization of the interaction between Sct1 and Cdc10.

Ser-196 Within Cdc10 Is Essential for Association with Sct1

The requirement for Cdc2 for Cdc10/Sct1 complex formation suggests that the phosphorylation of either Cdc10 or Sct1 is required for their stable association. As discussed above, Cdc10 is phosphorylated on Ser-196, so we examined the possibility that the S196A mutation inactivates Cdc10 by preventing its association with Sct1. Sct1 and Cdc10 were synthesized in vitro in a coupled transcription/translation system in rabbit reticulocyte lysate (Promega, Madison, WI)

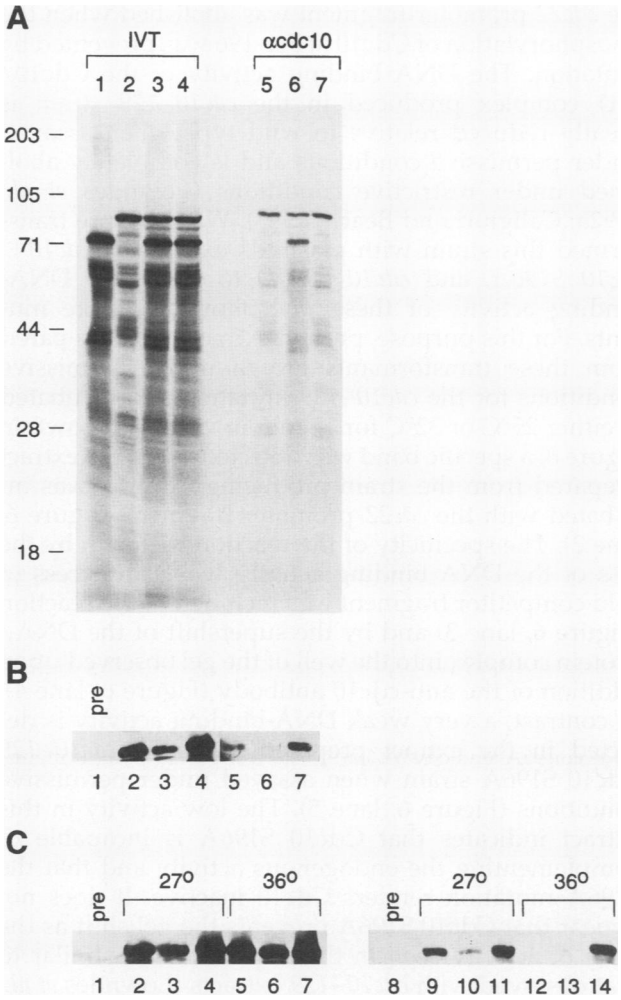

Figure 5. Ser-196 within Cdc10 is essential for complex formation with Sct1. (A) Translation products labeled with ^{35}S from rabbit reticulocytes lysates programmed with Sct1, Cdc10+, Sct1 and Cdc10+, or Sct1 and Cdc10 S196A resolved by SDS-PAGE (lanes 1–4, respectively). The Cdc10+, Cdc10+ Sct1, or Cdc10 S196A Sct1 translation products were immunoprecipitated with the anti-Cdc10 antibody and resolved by SDS-PAGE (lanes 5–7, respectively). (B) Anti-Cdc10 immunoblot of immunoprecipitates using the Cdc10 preimmune serum and Cdc10+ extract (lane 1), the anti-Cdc10 antibody and Cdc10+, Cdc10 S196A, or Cdc10 S196D extracts (lanes 2–4, respectively) and the anti-HA antibody and Cdc10+, Cdc10 S196A, or Cdc10 S196D extracts (lanes 5–7, respectively). (C) Anti-Cdc10 immunoblot of immunoprecipitates from *cdc2–33* Cdc10+, *cdc2–33* Cdc10 S196A, and *cdc2–33* Cdc10 S196D extracts prepared from strains growing at 27°C or 36°C as specified, using the Cdc10 preimmune serum or normal mouse serum and the *cdc2–33* Cdc10+ (27°C) extract (lanes 1 and 8, respectively), the anti-Cdc10 antibody and *cdc2–33* Cdc10+, *cdc2–33* Cdc10 S196A, or *cdc2–33* Cdc10 S196D extracts (lanes 2 and 5, 3 and 6, 4 and 7, respectively), and the anti-HA antibody and *cdc2–33* Cdc10+, *cdc2–33* Cdc10 S196A, or *cdc2–33* Cdc10 S196D (lanes 9 and 12, 10 and 13, 11 and 14, respectively).

(Figure 5A, lanes 1 and 2, respectively). In addition, Sct1 was cotranslated with Cdc10+ or Cdc10 S196A (Figure 5A, lanes 3 and 4, respectively). Immunoprecipitation of these ^{35}S-labeled proteins with the anti-Cdc10 antibody revealed that Sct1 was associated with Cdc10+, but not, Cdc10 S196A (Figure 5A, lanes 6 and 7, respectively). This suggests that the S196A mutation in Cdc10 does indeed prevent its association with Sct1, perhaps due to the nonphosphorylatable nature of this substitution. As described above Ser-196 is phosphorylated in vivo, we therefore infer that an activity capable of the phosphorylation of Cdc10 is present in these extracts.

We went on to examine the consequence of mutations at Ser-196 in Cdc10 on complex formation with Sct1 in vivo. For this purpose, cell-free extracts were prepared from wild-type strains (SP 812) producing either Cdc10+, Cdc10 S196A, or Cdc10 S196D and Sct1-HA, grown at 27°C. Anti-Cdc10 (Figure 5B, lanes 2–4) and anti-HA (Figure 5B, lanes 5–7) immunoprecipitates were resolved by SDS-PAGE, blotted to nitrocellulose and probed with the anti-Cdc10 antibody. Cdc10+ and Cdc10 S196D were both found to be associated with Sct1-HA (Figure 5B, lanes 5 and 7, respectively), whereas Cdc10 S196A was not (Figure 5B, lane 6). All three forms of Cdc10 were clearly detectable in these extracts (Figure 5B, lanes 2–4), indicating that the loss of the association between Cdc10 S196A and Sct1-HA was not due to a loss of Cdc10 S196A protein. This result suggests that phosphorylation of Cdc10 at Ser-196 is required for its stable association with Sct1.

As described above phosphorylation of Cdc10 at Ser-196 appears to be required for Cdc10 to associate with Sct1. Because Ser-196 lies within a Cdc2 consensus site and Cdc2 is required for Cdc10/Sct1 complex formation, we examined the possibility that Cdc10 S196D might bypass this requirement because the negative charge at residue 196 may mimic constitutive phosphorylation. To test this hypothesis, cell-free extracts were prepared from strains that carry the *cdc2–33* mutation and that produce Cdc10+, Cdc10 S196A, or Cdc10 S196D and Sct1-HA grown at 27°C and then shifted to 36°C for 3 h. As expected, Cdc10+ and Cdc10 S196D were found to be associated with Sct1-HA in extracts prepared from the *cdc2–33* strain grown under permissive conditions (Figure 5C, lanes 9 and 11, respectively), whereas Cdc10 S196A was not (Figure 5C, lane 10). As shown above, inactivation of Cdc2 by growth under restrictive conditions led to the loss of the association between Cdc10+ and Sct1-HA (Figure 5C, lane 12). As predicted, Cdc10 S196D did, in fact, associate with Sct1-HA under these conditions (Figure 5C, lane 14) thus bypassing the requirement of Cdc2 for Cdc10/Sct1 complex formation. The inactivation of Cdc2 did not alter the level of Cdc10+, Cdc10 S196A, or Cdc10 S196D protein (Figure 5C, lanes 2–7). The results of this experiment suggest that the role of Cdc2 in Cdc10/Sct1 complex formation is in the phosphorylation of Cdc10 at Ser-196. The *cdc10*

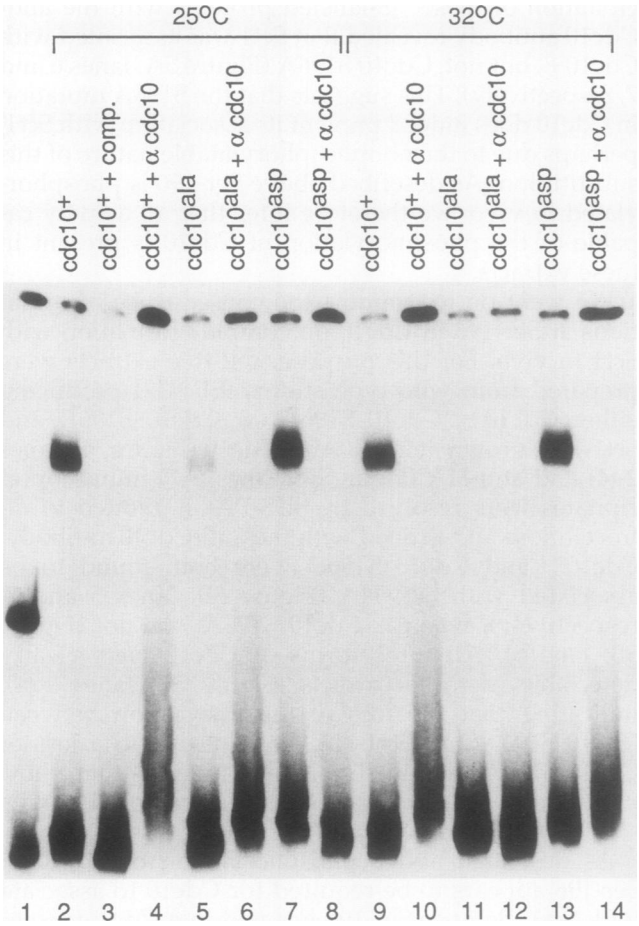

Figure 6. DNA-binding activity of the Cdc10/Sct1 complex requires the Cdc2 consensus site within Cdc10. Gel retardation assays were performed with the cdc22 promoter fragment using extracts prepared from *cdc10–129* strains producing either Cdc10+ (lanes 1–4 and 9 and 10), Cdc10 S196A (lanes 5 and 6 and 11 and 12), or Cdc10 S196D (lanes 7 and 8 and 13 and 14). The cultures were grown under permissive conditions for the *cdc10–129* mutation, and then extracts were prepared and incubated at either 25°C (lanes 1–8) or 32°C (lanes 9–14) for 15 min in vitro. Lane 1 contains the *cdc22* promoter fragment in the absence of protein extract, and lane 2 contains a binding reaction with a 50-fold M excess of unlabeled promoter fragment.

S196D gene did not, however, rescue the G_1 defect of the *cdc2* mutant, indicating that promotion of Cdc10/Sct1 complex formation is not the sole function performed by Cdc2 in the execution of Start (our unpublished observations).

Cdc10/Sct1 DNA-binding Activity Requires the Cdc2 Consensus Site Within Cdc10

Cdc2 has been shown to be required for Cdc10/Sct1 complex formation and DNA-binding activity (this study; Reymond *et al.*, 1993). It was therefore of interest to determine whether the binding of Cdc10/Sct1 to the *cdc22* promoter fragment was abolished when the phosphorylation of Cdc10 at Ser-196 was prevented by mutation. The DNA binding activity of the Cdc10/Sct1 complex produced in the *cdc10–129* strain is greatly reduced relative to wild type when assayed under permissive conditions and is completely abolished under restrictive conditions (Lowndes *et al.*, 1992a; Caligiuri and Beach, 1993). We therefore transformed this strain with plasmids expressing *cdc10+*, *cdc10 S196A*, and *cdc10 S196D* to assay the DNA-binding activity of these phosphorylation site mutants. For this purpose, protein extracts were prepared from these transformants grown under permissive conditions for the *cdc10–129* mutation and incubated at either 25°C or 32°C for 15 min in vitro. As shown in Figure 6, a specific band was detected when the extract prepared from the strain producing Cdc10+ was incubated with the *cdc22* promoter fragment (Figure 6, lane 2). The specificity of the reaction is shown by the loss of the DNA binding activity when an excess of cold competitor fragment was included in the reaction (Figure 6, lane 3) and by the supershift of the DNA/protein complex into the well of the gel observed upon addition of the anti-cdc10 antibody (Figure 6, lane 4). In contrast, a very weak DNA-binding activity is detected in the extract prepared from the *cdc10–129* Cdc10 S196A strain when assayed under permissive conditions (Figure 6, lane 5). The low activity in this extract indicates that Cdc10 S196A is incapable of complementing the endogenous activity and that the S196A mutation renders Cdc10 inactive. It does not appear that Cdc10 S196A prevents the gel shift as the level of activity seen in this experiment is similar to that observed with *cdc10–129* extracts (Lowndes *et al.*, 1992a; Caligiuri *et al.*, 1993). Cdc10 S196D was found to restore the DNA-binding activity to the level obtained with Cdc10+ (Figure 6, lane 7). This complex is supershifted into the well upon addition of the anti-Cdc10 antibody as in the wild-type case, indicating that Cdc10 S196D is, in fact, present in this DNA/protein complex (Figure 6, lane 8). When these extracts were incubated at 32°C the DNA-binding activity in the Cdc10 S196A extract was completely abolished (Figure 6, lane 11) whereas that from the Cdc10+ and Cdc10 S196D extracts was approximately the same as in the extracts incubated at 25°C (Figure 6, lanes 9 and 13, respectively). These results indicate that the *cdc10 S196A* mutation destroys the Cdc10/Sct1 DNA binding activity and suggests that phosphorylation of Cdc10 at Ser-196 is essential for this activity.

DISCUSSION

The biochemical and genetic results presented in this article support a molecular model for the regulation of Start in which the Cdc2 protein kinase functions to control Cdc10/Sct1 complex formation. Our data

demonstrate that the formation of the Cdc10/Sct1 transcription complex requires the activity of the Cdc2 protein kinase. We provide data demonstrating that Ser-196 in Cdc10, which lies within a Cdc2 consensus site, is phosphorylated in vivo. Phosphorylation of this serine is essential for Cdc10 function. Substitution of this serine with alanine (S196A) results in a loss of complementing activity in vivo. We show that this is due to the inability of the Cdc10 S196A mutant to associate with Sct1 in vivo. As a consequence, the Sct1/Cdc10 transcription complex is incapable of binding to its target promoters. It is formally possible that Cdc10 S196A does not localize properly to the nucleus, which could thus account for its lack of complementing activity (Reymond *et al.*, 1993). The finding, however, that Cdc10+ associates with Sct1 in vitro, whereas Cdc10 S196A does not, suggests that if this is the case complex formation precedes nuclear localization. Substitution of this serine with aspartic acid, potentially mimicking phosphorylation, restores the ability of Cdc10 to complex with Sct1 and thus its function in vivo. Interestingly, the Cdc10 S196D mutation bypasses the requirement for Cdc2 activity in Sct1/Cdc10 complex formation. We therefore propose that the Cdc2 protein kinase functions to control the affinity of Cdc10 for its cognate partner, Sct1.

The demonstration that Cdc10 S196D alleviates the requirement of Cdc2 for its association with Sct1 strongly implicates the involvement of Cdc2 in the phosphorylation of Ser-196. In the accompanying article (Caligiuri *et al.*, 1997), we demonstrate that the Ran1 kinase is also involved in Ser-196 phosphorylation. Thus, we conclude that Ran1 and Cdc2 function together to control the phosphorylation of Cdc10 at Ser-196. It is currently unclear which kinase acts directly to phosphorylate Cdc10. Ran 1 has been implicated as a mediator of the cellular response to nutritional deprivation. In this respect, Cdc10, whose phosphorylation state may influence the developmental program, leading to either cellular proliferation or differentiation, may be a substrate of both the Cdc2 and Ran 1 kinases under conditions of cellular stress.

It is clear that Cdc10 has at least two DNA binding partners, Sct1 and Pct1, giving rise to two distinct heteromeric DNA-binding complexes (Caligiuri and Beach, 1993; Miyamoto *et al.*, 1994; Zhu *et al.*, 1994). Genetic analyses of strains deleted for *sct1* or *pct1* demonstrate that their roles in the cell cycle are, in fact, different. The *pct1* gene is not essential for mitotic cell cycle progression, null mutants do not display adverse effects on cell viability or growth rate (Miyamoto *et al.*, 1994; Zhu *et al.*, 1994). Deletion of *sct1* is, however, a lethal event resulting simultaneously in G_1 arrest and derepression of mating functions (Caligiuri and Beach, 1993). Although *sct1* null mutants are derepressed for meiosis, it appears that the meiotic pathway can proceed normally (Caligiuri and Beach, 1993). In contrast, the *pct1* gene product appears to play a major role in meiosis, which is severely disrupted in the null mutant (Miyamoto *et al.*, 1994; Zhu *et al.*, 1994). These results suggest that Sct1 plays a more critical role in the mitotic cell cycle than Pct1, which appears to function predominantly in meiosis, although Pct1 can contribute to the transcriptional regulation at Start under some circumstances (Miyamoto *et al.*, 1994; Zhu *et al.*, 1994).

The *sct1* and *pct1* genes are both expressed during mitotic growth though *pct1* is greatly induced upon nitrogen starvation, thus their protein products are present simultaneously in the cell (Miyamoto *et al.*, 1994; Zhu *et al.*, 1994). The ratio of the Sct1/Cdc10 complex to the Pct1/Cdc10 therefore may be regulated by the phosphorylation state of Cdc10. In this case, Cdc10 phosphorylated on Ser-196 may display a higher affinity for Sct1, and conversely, unphosphorylated Cdc10 (at least with respect to this site) may display a higher affinity toward Pct1. It is conceivable that phosphorylation of Cdc10 at Ser-196 is not required for Pct1/Cdc10 function because unlike Cdc10, Sct1, and Cdc2, loss of Pct1 does not lead to G_1 arrest (Miyamoto *et al.*, 1994; Zhu *et al.*, 1994). Upon loss of Cdc2, Cdc10 may become under-phosphorylated at specific residues and the Pct1/Cdc10 complex would dominate. Cells that arrest in G_1 then would be competent to exit the cell cycle and activate mating functions under appropriate nutritional conditions if challenged by a cell of the opposite mating type. It follows that the *sct1* null mutant is derepressed for meiotic activation simply because Sct1 is not present to compete with Pct1 for its cognate partner, Cdc10. Alternatively, another kinase could phosphorylate Cdc10 upon meiotic activation. When cells are starved for nitrogen, no significant change in the level of Cdc10 is detected and Cdc10 remains phosphorylated (Simanis and Nurse, 1989). Subtle changes in phosphorylation sites may occur, however, and thereby account for potential differences in the affinity of Cdc10 for either Sct1 or Pct1. It will be interesting to determine whether the presence of the canonical Cdc2 phosphorylation site in Cdc10 is necessary for its association with Pct1.

A number of transcription factors contain ankyrin repeat motifs and achieve specificity in transcriptional regulation through formation of specific heteromeric complexes (Thompson *et al.*, 1991; Sawanda *et al.*, 1994). It has been suggested that ankyrin repeats play an important role by providing an interface for protein–protein interactions (Thompson *et al.*, 1991). The Cdc10/Sct1 proteins, which both contain a pair of ankyrin repeats, thus possess hallmarks of an evolutionarily conserved mechanism to regulate gene expression. The results presented here indicate that a specific phosphorylation in a non-ankyrin-like domain dramatically influences the ability of one protein to

stably associate with its heteromeric partner and form an active DNA-binding complex. It is thus possible that phosphorylation will play an important and conserved role in controlling the highly specific interactions among these proteins to regulate key developmental processes.

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