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Ran1 Functions to Control the Cdc10/Sct1 Complex through Puc1

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We have undertaken a biochemical analysis of the regulation of the G_1/S-phase transition and commitment to the cell cycle in the fission yeast *Schizosaccharomyces pombe*. The execution of Start requires the activity of the Cdc2 protein kinase and the Sct1/Cdc10 transcription complex. Progression through G_1 also requires the Ran1 protein kinase whose inactivation leads to activation of the meiotic pathway under conditions normally inhibitory to this process. We have found that in addition to Cdc2, Sct1/Cdc10 complex formation requires Ran1. We demonstrate that the Puc1 cyclin associates with Ran1 and Cdc10 in vivo and that the Ran1 protein kinase functions to control the association between Puc1 and Cdc10. In addition, we present evidence that the phosphorylation state of Cdc10 is altered upon inactivation of Ran1. These results provide biochemical evidence that demonstrate one mechanism by which the Ran1 protein kinase serves to control cell fate through Cdc10 and Puc1.

INTRODUCTION

The mechanism by which yeast cells monitor environmental conditions and respond by either arresting in G_1 to initiate mating and subsequent meiotic functions or by proceeding through the mitotic cell cycle is not well understood. It is clear that passage through Start and commitment to the mitotic cell cycle requires the function of the Cdc2/CDC28 protein kinase (Hartwell *et al.*, 1974; for review, see Reed, 1992). Genetic screens in the budding yeast *Saccharomyces cerevisiae* have identified three *CLN* genes, *CLN1*, *-2*, and *-3*, that function to activate the CDC28 kinase at Start (Cross and Blake, 1988; Nash *et al.*, 1988; Richardson *et al.*, 1989). The three *CLN* genes exhibit some functional redundancy because inactivation of any CLN pair results in a viable cell whereas inactivation of all three genes results in cell cycle arrest at Start. A single CLN-type cyclin, Puc1, that shares considerable amino acid similarity with CLN3 has been identified in fission yeast (Forsburg and Nurse, 1991).

It is likely that one role of the CLN/CDC28 protein kinase at Start is to directly activate transcription factors involved in regulating genes required for DNA replication. The SWI4, SWI6, and MBP1 proteins were identified in *S. cerevisiae* as factors required for the expression of the HO endonuclease and DNA replication genes, respectively (Breeden and Nasmyth, 1985; Andrews and Herskowitz, 1989a; Koch *et al.*, 1993). SWI6 associates with MBP1 and binds to the Mlu cell cycle box to activate many genes involved in DNA synthesis (for review, see Andrews, 1992; Dirick *et al.*, 1992; Lowndes *et al.*, 1992a). In addition, SWI6 associates with SWI4 and binds to the *cis*-acting regulatory motif known as the Swi-dependent cell cycle box to activate the expression of the HO, CLN1, CLN2, HCS26, and PCL2 (ORFD) genes at the G_1/S-phase transition (Andrews and Herskowitz, 1989a; Nasmyth and Dirick, 1991; Ogas *et al.*, 1991; Measday *et al.*, 1994). The transcriptional regulation of CLN1 and CLN2 has been implicated in coupling cell size to growth rate through cAMP regulation (Baroni *et al.* 1994; Tokiwa *et al.*, 1994).

In the fission yeast *Schizosaccharomyces pombe*, the Cdc2 protein kinase is required for the execution of

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Start as well as for entry into mitosis. Cells that arrest in G_1 due to inactivation of *cdc2* are capable of conjugation if challenged by a cell of the opposite mating type under appropriate nutritional conditions (Nurse and Bisset, 1981). To date, three B-type cyclins, *cdc13*, *cig1*, and *cig2* and one Cln-type cyclin, *puc1*, have been identified in fission yeast (Booher and Beach, 1988; Hagan *et al.* 1988; Bueno *et al.*, 1991; Forsburg and Nurse, 1991; Bueno and Russell, 1993; Connolly and Beach, 1994). Of the three B-type cyclins, only Cdc13 is essential for growth (Booher and Beach, 1988). The inactivation of *cig1* or *cig2* does not lead to loss of cell viability (Bueno *et al.*, 1991; Bueno and Russell, 1993; Connolly and Beach, 1994). Similarly, cells that carry a *puc1* null allele are viable and display no abnormal cell cycle phenotype, suggesting that additional, as yet unidentified, CLNs might exist in fission yeast (Forsburg and Nurse, 1994; T.C., unpublished results).

In addition to the Cdc2 protein kinase, the execution of Start in fission yeast requires the *cdc10* and *sct1* (*res1*) genes (Nurse and Bisset, 1981; Tanaka *et al.*, 1992; Caligiuri and Beach, 1993). Haploid cells carrying mutations in either *cdc10* or *sct1* arrest in G_1 and mate to form diploid cells under appropriate nutritional conditions. The Sct1 and Cdc10 proteins share amino acid sequence similarity to the *S. pombe pct1* gene product and the SWI4, SWI6, and MBP1 family of transcriptional regulators in *S. cerevisiae* (Aves *et al.*, 1985; Breeden and Nasmyth, 1987b; Andrews and Herskowitz, 1989b; Tanaka *et al.*, 1992; Caligiuri and Beach, 1993; Koch *et al.*, 1993; Miyamoto *et al.*, 1994; Zhu *et al.*, 1994). The results of both genetic and biochemical studies suggest that Cdc10 and Sct1 function together as a heteromeric transcription complex to activate several periodically expressed genes at the G_1/S-phase transition, which include *cdc22*, *cdc18*, and *cdt1* (Kelley *et al.*, 1993; Hofmann and Beach, 1994). The Cdc10/Sct1 complex is thought to bind to Mlu cell cycle box-like elements, which have been shown to be sufficient to confer cell cycle periodicity to a reporter gene (Lowndes *et al.* 1992b).

Cells that lack the *sct1* gene, unlike those that lack *cdc2* or *cdc10*, will conjugate even when grown on rich medium and thus bypass the normal requirement of nutritional deprivation for the activation of the mating pathway (Caligiuri and Beach, 1993). This phenotype is reminiscent of that which results from the loss of another cell cycle regulator, *ran1* (*pat1*; Iino and Yamamoto, 1985; Nurse, 1985). The *ran1* gene encodes a protein kinase that functions to prevent activation of the meiotic pathway under conditions that normally promote vegetative growth (McLeod and Beach, 1986). Cells that carry inactivating mutations in *ran1* also bypass the requirement of nutritional starvation for conjugation. In addition, *ran1* mutants undergo meiosis and sporulation from the haploid state and are thus alleviated of the requirement for heterozygosity at the mating type locus for the activation of these functions. Temperature-sensitive mutations in *ran1* thus cause deregulation of the molecular events at Start and allow the meiotic and cell division pathways to become insensitive to the normal environmental cues that govern cell fate.

Table 1. List of *S. pombe* strains

Strain	Genotype
SP 191	*h⁻ˢ ran1-114 leu1-32 ura4-d18*
SP 201	*h⁺ⁿ leu1-32 ade6-210*
SP 315	*h⁺ⁿ ran1-114 leu1-32 ade6-216*
SP 357	*h⁻ˢ cdc2-33 leu1-32 ura4-D12 ade6-210*
SP 871	*h⁹⁰ ran1::adh leu1-32 ura4-D12 ade6-210*
SP 963	*h⁻ˢ cdc22-M45 leu1-32*
SP 965	*h⁻ˢ cdc10-129 leu1-32 ura4-D12 ade6-210*
SP 1296	*h⁻ˢ cdc2-M72 leu1-32 ura4-D12 ade6-210*
SP 1297	*h⁻ˢ cdc10::ura4 sct1-1 ura4-D18 leu1-32*
JZ 420	*h⁻ˢ ran1::ura mei2::ura ura4-D18 leu1-32 ade6-M216*

It is clear that both Cdc2 and Ran1 are essential for maintenance of the mitotic cell cycle and progression through Start, though the substrates of both of these protein kinases have yet to be identified. We have thus undertaken a biochemical analysis of these proteins to obtain a better understanding of their role in the regulation of Start. We demonstrate that the Puc1 cyclin is associated with both the Cdc2 and Ran1 protein kinases in vivo. In addition, we show that Puc1 is complexed with Cdc10 in vivo and that Ran1 is required for this association. Finally, we show that Ran1 is required for formation of the heteromeric Sct1/Cdc10 transcription complex and that inactivation of this protein kinase leads to an alteration in the phosphorylation state of Cdc10. In the accompanying article, we present data that demonstrates that Cdc2 also functions to stabilize the Sct1/Cdc10, thus promoting progression through the G_1/S-phase transition. Thus, these results provide biochemical evidence that suggests that the Ran1 protein kinase affects cell fate by promoting Sct1/Cdc10 complex formation through Puc1 thereby influencing the expression of genes under the control of this transcription complex and ultimately the onset of DNA replication.

EXPERIMENTAL PROCEDURES

Strains and Media

All fission yeast strains were derived from wild-type strains originally described by Leupold (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). Standard genetic procedures previously described for *S. pombe* were followed (Gutz *et al.*, 1974).

Epitope Addition

Puc1 and Sct1 were tagged at the carboxyl terminus with a triple tandem HA1 epitope (Caligiuri and Beach, 1993; this study). The plasmid pGETP1 contains this epitope bounded by *Not*I restriction sites (Field *et al.*, 1988; Tyers *et al.*, 1992). Site-directed mutagenesis was employed to place *Not*I restriction sites at the carboxyl terminus of both *puc1* and *sct1* reading frames and the triple tandem hemagglutinin (HA) epitope *Not*I cassette was ligated into the *puc1* and *sct1* cDNA constructs. The addition of the epitope in the correct reading frame was confirmed by DNA sequence analysis with a semiautomatic DNA sequencer (Applied Biosystems, Foster City, CA). The monoclonal (12CA5) and polyclonal (HA1.1) anti-HA antibodies recognizing the HA epitope were purchased from Boehringer Mannheim (Indianapolis, IN) and Babco Berkeley Laboratory (Richmond, CA), respectively.

Immunoprecipitation and Western Blotting

S. pombe cell-free lysates were prepared essentially as described (Booher *et al.*, 1989). Cells were quickly disrupted by vortex mixing in the presence of glass beads in buffer H [25 mM Tris(hydroxymethyl)aminomethane (Tris), pH 7.5, 1 mM dithiothreitol (DTT), 60 mM β-glycerol phosphate, 15 mM *p*-nitrophenyl phosphate, 15 mM EGTA, 15 mM $MgCl_2$, 0.1 mM NaF, 0.1 mM sodium orthovanadate, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 10 μg/ml L-1-tosylamide-2-phenylethyl chloromethyl ketone, and 7-amino-1-chloro-3-tosylamido-2-heptanone]. After lysis, an equal volume of ice-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). Cig2 does not quantitatively associate with Cdc2 in extracts prepared from asynchronously growing cultures even when expressed constitutively from a heterologous promoter. Therefore, the *cig2-HA* gene was expressed in a temperature-sensitive *cdc22* strain (SP 963) arrested for 3 h in S-phase and released at the permissive temperature for the preparation of protein extracts from a synchronized culture.

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, 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% Nonidet P-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 1:1000 dilution of antibody in TBST containing 5% milk for 2 h at room temperature. The blots were washed for three 20-min periods in TBST then incubated with a 1:50,000 dilution of an anti-mouse or anti-rabbit Fc antibody as appropriate (Jackson Immuno Research Laboratories, West Grove, PA) in TBST containing 5% milk for 1 h at room temperature. The proteins were visualized with an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL) and conventional autoradiography.

Metabolic Labeling and Two-Dimensional Tryptic Peptide Mapping

S. pombe cells were grown for approximately 16 h in phosphate-free minimal medium 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, in 5–10 ml containing 1×10^7 cells per ml, washed with H_2O, and resuspended in phosphate-free minimal medium supplemented with 50 μM NaH_2PO_4. The volume of the *cdc2-M72* culture was increased to 35 ml to allow the recovery of enough Cdc10 protein for two-dimensional peptide analysis. The cultures were incubated for 2 h prior to the addition of [^{32}P]orthophosphate (1 mCi/5 ml of 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-phenylethyl chloromethyl ketone-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 buffer at pH 1.9 and the second dimension was chromatography in isobutyric acid buffer (van der Geer *et al.*, 1993).

Protein Kinase Assays

Histone H1 kinase assays were performed as described (Booher *et al.*, 1989). The immunoprecipitates used in kinase reactions were washed once with 1.0 ml of kinase reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 1 mM DTT) and resuspended in 20 μl of kinase reaction buffer containing 0.125 mg/ml histone H1. The immunoprecipitates were preincubated for 15 min at the indicated temperature, 5 μl of [γ-^{32}P]ATP solution (0.5 μM ATP, 0.5 μCi/μl) was added, and the incubation was continued for an additional 10 min. The reactions were terminated with the addition of Lamelli buffer.

Gel Retardation Assays

Cells were grown to a density of 5×10^6 to 1×10^7 cells/ml in minimal selective medium, 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-glycerophosphate, 5 mM EDTA, 0.1 mM sodium vanadate, 1 mM DTT, 0.2% Nonidet P-40, 1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 2 μg/ml 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. All manipulations were performed at 4°C. The supernatant was collected and the protein concentration was determined by a colorimetric assay using Bradford reagent (Bradford, 1976).

The restriction fragments used for probes were recovered from low-melting-temperature agarose in Tris-acetate buffer and end labeled with the Klenow fragment of DNA polymerase I 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 mM Tris (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 5% glycerol, 2 mM DTT, 1 mM ATP, and 0.1 mg/ml poly(dIdC)·poly(dIdC) (Pharmacia). After the addition of the protein extracts (20 μg), 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. Where noted, a 50-fold molar excess of unlabeled promoter fragment was added at the same time as the probe. The anti-cdc10 antibody and preimmune sera (from the same rabbit) were diluted 1:10 in binding buffer and 1 μl was added after the binding the reaction. The HA1 monoclonal antibody 12CA1 (Boehringer) was purified on protein A-Sepharose as described by the manufacturer. The antibody was diluted 1:10 in binding buffer and 1 μl 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 3 MM paper and subjected to autoradiography.

RESULTS

Puc1 Associates with the Cdc10 Transcription Factor In Vivo

To investigate the role of the various cyclins in the fission yeast cell cycle, a triple tandem copy of the influenza HA epitope (HA1) was placed in-frame at the carboxyl terminus of the *puc1*, *cig1*, and *cig2* cDNAs for the immunological detection of these proteins (Field *et al.*, 1988; Tyers *et al.*, 1992). Cell-free lysates were prepared from strains expressing the *puc1-HA*, *cig1-HA*, and *cig2-HA* genes under the control of the thiamine-repressible *nmt1* promoter (Maundrel, 1993). Immunoblots of Puc1-HA, Cig1-HA, and Cig2-HA, immunoprecipitated with the monoclonal anti-HA antibody (12CA5) and probed with a rabbit anti-HA polyclonal antibody (HA1.1), revealed bands of 43, 50, and 55 kDa, corresponding to the predicted molecular weights of these proteins, respectively (Figure 1A, lanes 2–4). The specificity of the immunoprecipitations is shown by the lack of these bands when normal mouse serum was substituted for the anti-HA antibody (Figure 1A, lane 1). In addition, these band were absent in lysates prepared from wild-type strains (our unpublished observation).

The cyclins were assayed for association with Cdc2 by blotting anti-HA immunoprecipitates (12CA5) prepared from strains producing the HA-tagged cyclins, with a polyclonal antibody (G8) directed against fission yeast Cdc2. Cdc2 was found to coimmunoprecipitate with Puc1-HA, Cig1-HA, and Cig2-HA (Figure 1B, lanes 2–4). Histone H1 kinase activity was detected in Puc1-HA and Cig1-HA (a representative B-type cyclin) immunoprecipitates upon addition of histone H1 and [γ-^{32}P]ATP (Figure 1B, lanes 6 and 7 and lanes 10 and 11, respectively). The kinase activity was temperature-sensitive when Puc1-HA or Cig1-HA were immunoprecipitated from strains harboring the *cdc2-M72* mutation (SP 1296; Figure 1B, lanes 9 and 13, respectively). Histone H1 kinase activity was not detected in immunoprecipitates with normal mouse serum, indicating that this activity was specific to the Cdc2/cyclin complex (Figure 1B, lane 5). Identical results were obtained with Cig2-HA and Cdc13 immunoprecipitates (our unpublished observation). These results demonstrate that these cyclins associate with and activate the Cdc2 protein kinase.

It has been demonstrated previously that Cdc10 is a phosphoprotein whose activity is required for the execution of Start (Nurse and Bisset, 1981; Simanis and Nurse, 1989). The identity of the protein kinase required for this phosphorylation has, however, remained elusive. We therefore set out to determine whether Cdc10 was associated with any of the Cdc2/cyclin complexes identified thus far in fission yeast. Cyclin-HA immunoprecipitates (Puc1-HA, Cig1-HA, and Cig2-HA) were prepared from the appropriate strains, resolved by SDS-PAGE, and blotted to nitrocellulose membrane. The Western blot was probed with a polyclonal antisera directed against fission yeast Cdc10, and a band corresponding to Cdc10 was, in fact, detected in the Puc1-HA immunoprecipitate (Figure 1C, lane 2). This association, however, was not detected in either the Cig1-HA or Cig2-HA immuno-

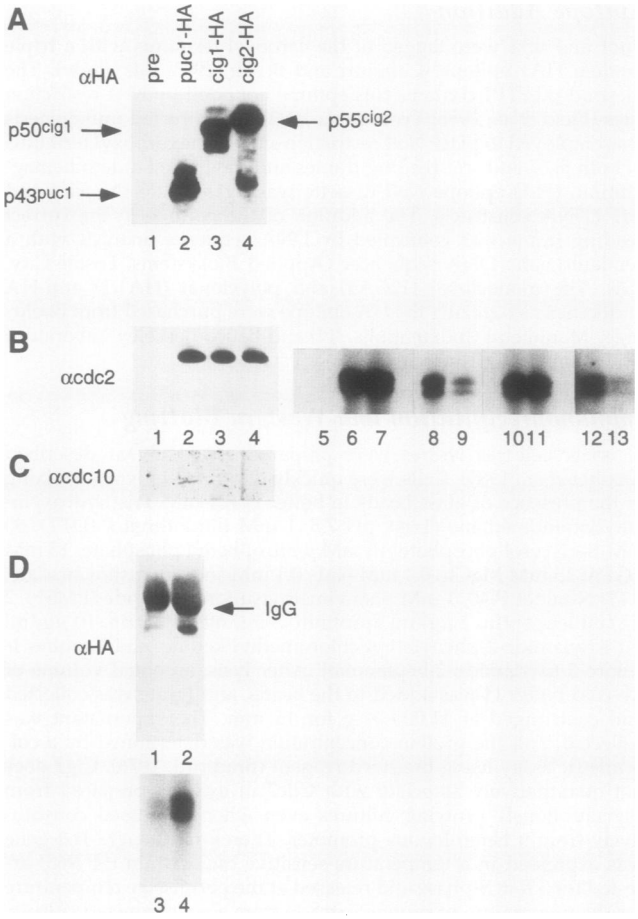

Figure 1. Puc1-HA associates with the Cdc10 transcription factor in vivo. (A) Anti-HA immunoblot of immunoprecipitates with normal mouse serum (pre, lane 1), Puc1-HA (lane 2), Cig1-HA (lane 3), and Cig2-HA (lane 4) anti-HA antibodies. (B) Anti-Cdc2 immunoblot of immunoprecipitates with normal mouse serum (lane 1), Puc1-HA (lane 2), Cig1-HA (lane 3), or Cig2-HA (lane 4) anti-HA antibodies. Histone H1 kinase assays were performed with either Puc1-HA (lanes 6–9) or Cig1-HA (lanes 10–13) immunoprecipitates from wild-type cells expressing the HA-tagged genes (lanes 6, 7, 10, and 11) or a *cdc2-M72* mutant expressing the HA-tagged genes (lanes 8, 9, 12, and 13). The kinase assays were performed at either 25°C (lanes 6, 8, 10, and 12) or 37°C (lanes 7, 9, 11, and 13). (C) Anti-cdc10 immunoblot of immunoprecipitates with normal mouse serum (lane 1), Puc1-HA (lane 2), Cig1-HA (lane 3), and Cig2-HA (lane 4) anti-HA antibodies. (D) Anti-HA immunoblot of immunoprecipitates with normal mouse serum (lane 1) and anti-Cdc10 antibodies (lane 2). Histone H1 kinase assays of the normal mouse serum (lane 3) and anti-Cdc10 immunoprecipitates (lane 4) from the Puc1-HA extract.

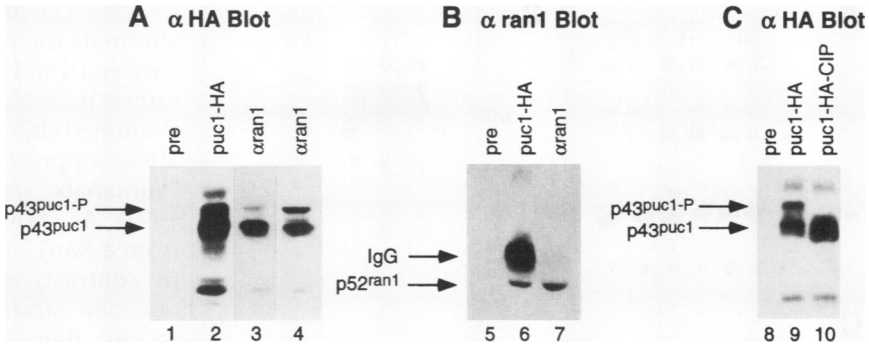

Figure 2. Puc1 and the Ran1 kinase associate in vivo. (A) Anti-HA immunoblot of immunoprecipitates with normal mouse serum or the anti-HA antibody from a lysate prepared from a wild-type strain producing Puc1-HA protein (lanes 1 and 2, respectively) and anti-Ran1 immunoprecipitates from lysates prepared from a wild-type or a Ran1 overproducing strain, both producing Puc1-HA (SP 871) (lanes 3 and 4, respectively). (B) Anti-Ran1 immunoblot of immunoprecipitates from the Puc1-HA lysate using a normal mouse serum, the anti-HA antibody of the anti-Ran1 antibody (lanes 5–7, respectively). (C) Anti-HA immunoblot of immunoprecipitates from the Puc1-HA lysate using either normal mouse serum (lane 8) or the anti-HA antibody and subjected to treatment with either buffer only (lane 9) or 1 U of calf intestinal alkaline phosphatase (lane 10) for 15 min at 37°C.

precipitates (Figure 1C, lanes 3 and 4). In the reciprocal experiment, Puc1-HA was detected in an anti-Cdc10 immunoprecipitate (Figure 1D, lane 2). The presence of Cdc2 in the Cdc10 immunoprecipitate was suggested by the presence of histone H1 kinase activity (Figure 1D, lane 4). The specificity of the interaction between Puc1 and Cdc10 is shown by the lack of a band when normal mouse serum or rabbit preimmune serum were used in the immunoprecipitation (Figure 1, A, lane 1, and D, lane 3, respectively). We conclude that Puc1, and presumably Cdc2, is physically associated with the Cdc10 transcription factor in vivo.

Puc1 Is Associated with Ran1 In Vivo

Overexpression of *puc1* partially rescues strains carrying the *ran1–114* temperature-sensitive mutation (Forsburg and Nurse, 1994). We therefore investigated the possibility that Puc1 and Ran1 may be physically associated in a multiprotein complex in vivo. Cell-free lysates were prepared from strains that produce Puc1-HA and immunoprecipitations were performed with either anti-HA or anti-Ran1 monoclonal antibodies. The immunoprecipitates were resolved by SDS-PAGE, blotted to nitrocellulose, and probed with either anti-HA or anti-Ran1 polyclonal antibodies. Anti-Ran1 immunoprecipitates probed with the anti-HA antibody revealed the presence of the full-length form of Puc1-HA (Figure 2A, lane 3). Similarly, anti-HA immunoprecipitates probed with an anti-Ran1 polyclonal antibody revealed the presence of the 52-kDa Ran1 protein (Figure 2B, lane 6). These results demonstrate that Ran1 and Puc1 are associated in vivo. Ran1 was not detected in anti-HA immunoprecipitates from lysates prepared from strains that produced Cig1-HA or Cig2-HA, indicating that the interaction between Ran1 and cyclin is specific to Puc1, the CLN-type cyclin (our unpublished observation).

Anti-Puc1-HA immunoprecipitates from concentrated lysates probed with the anti-HA antibody revealed three specific bands (Figure 2A, lane 2). To investigate whether the slower-migrating form was phosphorylated Puc1, a Puc1-HA immunoprecipitate was treated with calf intestinal alkaline phosphatase. This slower migrating from of Puc1-HA did indeed collapse into the intermediate form after phosphatase treatment (Figure 2C, lanes 9 and 10). Thus, in all probability, the slower-migrating band represents the phosphorylated form of Puc1-HA and the intermediate band represents the nonphosphorylated form. The fastest migrating band is presumably a degradation product. Immunoprecipitation of Puc1-HA, from extracts prepared from a strain that overexpresses the Ran1 kinase, reveals an substantial increase in the phosphorylated form of Puc1-HA (Figure 2A, lane 4). These results suggest that Puc1 is a phosphoprotein in vivo and that Puc1 may be a substrate of the Ran1 kinase. We cannot draw any conclusions regarding the relative stoichiometries of these proteins, because to resolve the phosphorylated and nonphosphorylated forms of Puc1, it was necessary to perform the anti-HA immunoprecipitate from less protein than the anti-Ran1 immunoprecipitate (10 and 50 mg, respectively) and to obtain a shorter exposure of the autoradiograph of the Puc1-HA immunoprecipitates than the Ran1 immunoprecipitates.

The Association between Puc1 and Cdc10 Requires Ran1

We next addressed whether the association between Puc1-HA and Cdc10 was dependent on either Ran1 or Cdc2 protein kinase activity. A wild-type strain and strains carrying either the *cdc2-M72* (SP 1296) or *ran1–114* (SP 315) mutations expressing *puc1-HA* were grown to mid-logarithmic phase at the permissive temperature and then half of the culture was shifted to the restrictive temperature (36°C) for 2 h. Protein extracts were prepared and immunoprecipitations were performed with the anti-HA antibody. The immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with either the anti-Cdc10 or anti-HA antibody. Cdc10 was clearly

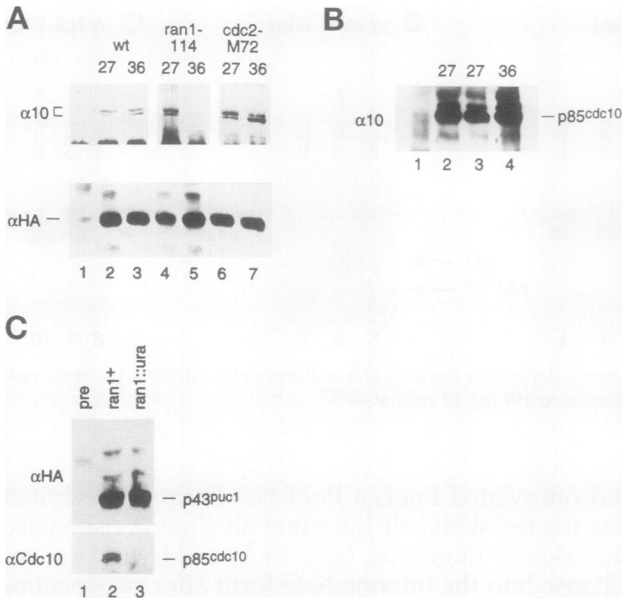

Figure 3. Association between Puc1 and Cdc10 requires Ran1. Cultures of a wild-type strain or strains carrying either the *cdc2-M72* or *ran1–114* temperature-sensitive alleles that produce Puc1-HA were grown at 27°C, and then one-half of the culture was shifted to 36°C for 3 h. Cell-free extracts were prepared for immunoprecipitation. (A) Anti-Cdc10 (Top) or anti-HA (Bottom) immunoblots of immunoprecipitations from extracts prepared from the indicated strains with either normal mouse serum (lane 1) or the anti-HA antibody (lanes 2–7). (B) Anti-Cdc10 immunoblot of immunoprecipitations using either preimmune serum (lane 1) or the anti-Cdc10 antibody from lysates prepared from wild-type cells grown at 27°C (lane 2) or *ran1–114* cells grown at 27°C (lane 3) or 36°C (lane 4). (C) Anti-HA (top) or anti-Cdc10 (bottom) immunoblots of immunoprecipitations with normal mouse serum from the *ran1+* extract (pre, lane 1) or the anti-HA antibody from the *ran1+* or *ran1::ura* extracts (lanes 2 and 3, respectively).

present in Puc1-HA immunoprecipitates from lysates prepared from wild-type cells cultured at both permissive (27°C) and restrictive (36°C) temperatures (Figure 3A Top, lanes 2 and 3). Similarly, Cdc10 was present in Puc1-HA immunoprecipitates from lysates prepared from the *cdc2-M72* strain cultured at both temperatures (Figure 3A, top, lanes 6 and 7). The lack of a requirement for Cdc2 is not specific to this allele, Cdc10 and Puc1-HA were also found to be associated upon inactivation of the *cdc2–33* allele (our unpublished observation). The association between Puc1-HA and Cdc10 was, however, abolished upon inactivation of the thermolabile Ran1 kinase (Figure 3A, top, lane 5). The lack of Cdc10 in the Puc1-HA immunoprecipitate from the *ran1–114* lysate is not due to a loss of Puc1-HA, as approximately equal amounts of the protein are present in the wild-type, *ran1–114*, and *cdc2-M72* extracts (Figure 3A, bottom, lanes 2–7). Similarly, the level of Cdc10 is not affected by inactivation of Ran1 (Figure 3B, lanes 2–4, respectively). Growth of *ran1–114* strains at elevated temperature leads to cell cycle arrest and activation of meiotic functions. To eliminate the possibility that the loss of the association between Puc1-HA and Cdc10 was due to this effect rather than the specific loss of the Ran1 kinase, we examined this interaction in cells that carry a *ran1* null allele suppressed by deletion of the *mei2* gene (JZ 420; Wantanabe *et al.*, 1988; Wantanabe and Yamamoto, 1994). As shown above, anti-HA immunoprecipitates from a Ran1$^+$ strain contain Cdc10 (Figure 3C, lane 2). In contrast, anti-HA immunoprecipitates from the *ran1::ura* strain do not (Figure 3C, lanes 3). These results demonstrate that the association between Puc1-HA and Cdc10 requires Ran1, but not Cdc2, protein kinase activity.

The Association between Cdc10 and Sct1 Requires Ran1

As discussed above, the association between Puc1 and Cdc10 requires a functionally active Ran1 kinase. It was therefore of interest to determine whether the loss of *ran1* would have an effect on Cdc10/Sct1 complex formation since, as described in the accompanying article, Cdc2 activity is required for the formation of this transcription complex. Cultures of a wild-type strain and a *ran1–114* temperature-sensitive strain both expressing the *sct1-HA* gene were grown to mid-logarithmic phase at the semipermissive temperature for the *ran1–114* mutation (27°C), divided in half, and then grown at either 27°C or 36°C. In this experiment, 27°C was chosen because we found this to be the optimal temperature for the growth of other strains used in these studies. Cell-free lysates were prepared, and Cdc10 and Sct1-HA were immunoprecipitated. The immunoprecipitates were resolved by SDS-PAGE, blotted to nitrocellulose, and probed with either anti-Cdc10 or anti-HA antibodies. The Sct1/Cdc10 complex was clearly detected at both temperatures when Sct1-HA was immunoprecipitated from wild-type extracts and probed with the anti-Cdc10 antibody (Figure 4A, lanes 6 and 7). Interestingly, Cdc10 was not present in anti-Sct1-HA immunoprecipitates from the *ran1–114* extracts prepared from cells cultured at either the permissive and restrictive temperatures (Figure 4A, lanes 8 and 9, respectively). Anti-Cdc10 immunoprecipitates from each extract were blotted with the anti-Cdc10 antibody to assess whether the loss of the interaction was due to a loss of Cdc10. We found that approximately equal amounts of Cdc10 were present in each extract (Figure 4A, lanes 2–5). Similarly, we found that Sct1-HA was clearly present in all of the extracts (Figure 4B, lanes 2–5). The level of Sct1-HA was, however, reduced upon the complete inactivation of Ran1 (Figure 4B, lane 5). Thus, under conditions where Ran1 is inactivated, Sct1 and Cdc10 are both present yet their interaction is abolished.

These results suggest that Ran1 function is required for the association of Sct1 with Cdc10.

The Cdc10 S196D Mutant Bypasses the Requirement of Ran1 for Sct1/Cdc10 Complex Formation

It has been shown previously that Cdc2 function is required for the DNA-binding activity of the Sct1/Cdc10 complex (Reymond *et al.*, 1993). A Cdc2 consensus site within Cdc10 that contains Ser-196 has been identified. Results from the accompanying article suggest that phosphorylation of Cdc10 at Ser-196 is necessary for its stable association with Sct1. The results presented above suggest that Ran1 might influence Cdc10/Sct1 complex formation by controlling the ability of the Puc1/Cdc2 protein kinase to associate with and thus presumably phosphorylate Cdc10. If this is true, then one prediction that follows is that the loss of Ran1 kinase activity would prevent the phosphorylation of Cdc10 at Ser-196. To address this issue, we performed a metabolic labeling experiment. Plasmids carrying the *cdc10+* gene were transformed into a *ran1*$^+$ strain (SP 1297) and a *ran1*$^-$ strain (SP 191) that carries the temperature-sensitive *ran1–114* mutation. Cultures of these transformants were grown at the restrictive temperature for the *ran1–114* mutation (36°C) and labeled with [^{32}P]orthophosphate. After 3 h of growth at the restrictive temperature, cell-free lysates were prepared and Cdc10 was immunoprecipitated with the anti-Cdc10 antibody. The Cdc10 immunoprecipitates were resolved by SDS-PAGE and subjected to autoradiography. The ^{32}P-labeled Cdc10 was eluted from the gel and digested with trypsin. The tryptic peptides were resolved by two-dimensional chromatography, and the maps were visualized by phosphorimage analysis (van der Geer *et al.*, 1993; Fuji, Stamford, CT). The tryptic digestion of Cdc10$^+$ from the extract prepared from the Ran1$^+$ strain generated two major spots (Figure 5, spots 1 and 2) and a minor spot (spot 3). The tryptic digestion of Cdc10$^+$ immunoprecipitated from the Ran1$^-$ extract also generated two major spots; however, spot 2 is missing and an additional spot (spot 4) appears (Figure 5). The results from the accompanying article demonstrate that spot 2 results from phosphorylation of Ser-196. Identification of the residue whose phosphorylation is responsible for spot 4 awaits further analysis. Thus, inactivation of the Ran1 kinase results in the loss of phosphorylation at Ser-196 and the consequent phosphorylation of an additional, as yet unidentified, site within Cdc10.

We have provided strong evidence that phosphorylation of Cdc10 at Ser-196 and thus its association with Sct1 is controlled, in part, by the Ran1 kinase. It follows, therefore, that substitution of Ser-196 with Asp, thus potentially mimicking phosphorylation at this site, would bypass the requirement of Ran1 for Sct1/Cdc10 complex formation, as described in the accompanying article for Cdc2. Cultures of strains carrying the *ran1–114* mutation (SP 191) expressing Sct1-HA and either Cdc10$^+$ or mutants in which Ser-196 was replaced with Asp (S196D) or Ala (S196A) were grown at 27°C and then split and grown at either 27°C or 36°C for 3 h. Cell-free extracts were prepared and anti-Cdc10, and anti-HA immunoprecipitates were blotted with either the anti-Cdc10 (Figure 6A) or the anti-HA (Figure 6B) antibodies. As expected from our results described above, Cdc10$^+$ was found to be associated with Sct1-HA when the strain was grown at the permissive temperature (Figure 6A, lane 8) but not, however, upon inactivation of Ran1 by incubation at the restrictive temperature (Figure 6A, lane 9). The Cdc10 S196A mutant was unable to complex with Sct1 at either temperature, suggesting that phosphorylation of Ser-196 is essential for association with Sct1 (Figure 6A, lanes 10 and 11). Interestingly, Cdc10 S196D was found complexed with Sct1 at both the permissive and restrictive temperatures for this mutation (Figure 6A, lanes 12 and 13). The overall level of Cdc10$^+$, S196A, S196D (Figure 6A, lanes 1 and 2, lanes 3 and 4, and lanes 5 and 6, respectively), or Sct1 (Figure 6B, lanes 2–7) did not change significantly upon incubation at the restrictive temperature. These results demonstrate that the Cdc10 S196D mutant bypasses the requirement for Ran1 activity for Sct1/

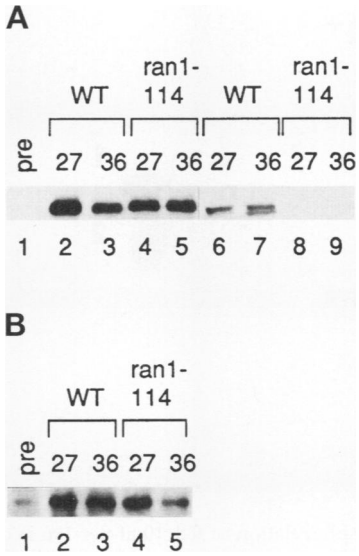

Figure 4. Association between Cdc10 and Sct1 requires Ran 1. Cultures of wild-type or *ran1–114* cells producing the Sct1-HA protein were grown at 27°C to mid-logarithmic phase and then half of the culture was shifted to 36°C for 3 h. Cell-free extracts were prepared for immunoprecipitation. (A) Anti-Cdc10 immunoblot of immunoprecipitations using a preimmune serum (lane 1), the anti-Cdc10 antibody (lanes 2–5), or the anti-HA antibody (lanes 6–9). (B) Anti-HA immunoblot of immunoprecipitations using normal mouse serum (lane 1) or the anti-HA antibody (lanes 2–5).

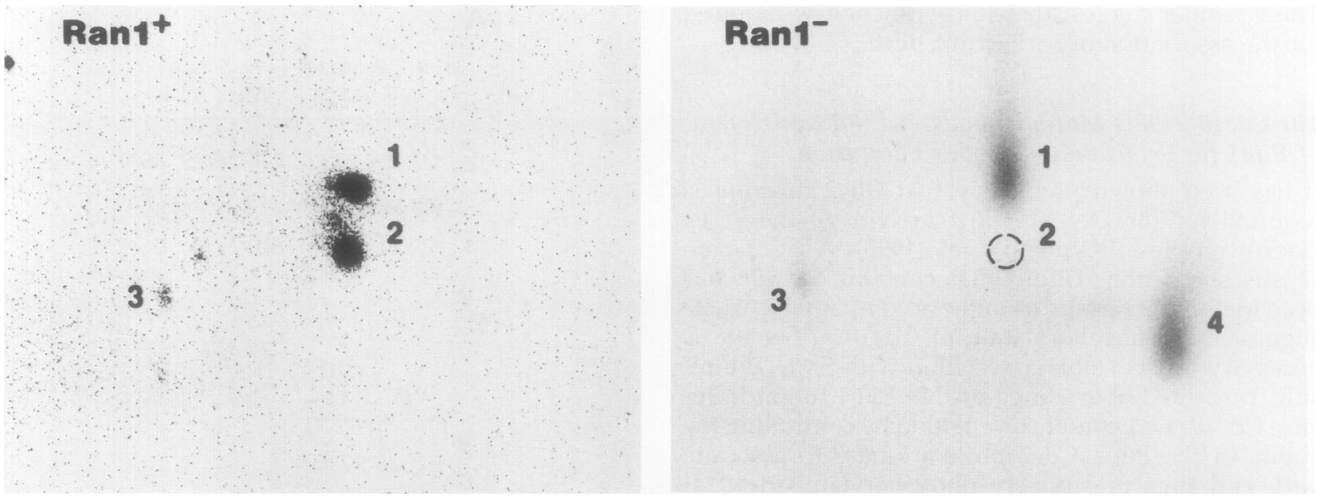

Figure 5. Phosphorylation of Cdc10 at Ser-196 is controlled by Ran1. ^{32}P-labeled Cdc10 immunoprecipitates from extracts prepared from Ran1$^+$ and Ran1$^-$ cells were resolved by SDS-PAGE, recovered from gel slices, and subjected to proteolytic digestion with trypsin. The ^{32}P-labeled tryptic peptides were resolved by two-dimensional chromatography and visualized by phosphorimage analysis.

Cdc10 complex formation. In addition, the results suggest that Ran1 functions in cooperation with Cdc2 to control the phosphorylation of Ser-196 within Cdc10. As described above, Ran1 appears to control the ability of Puc1, and presumably Cdc2, to associate with Cdc10. The phosphorylation of Cdc10 at Ser-196 may thus be regulated by Ran1 through Puc1. In this manner, Ran1 may function to control the association between Cdc10 and Sct1.

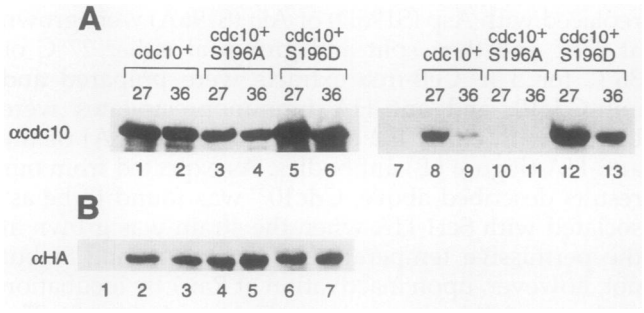

Figure 6. Cdc10 S196D mutant bypasses the requirement of Ran1 for Sct1/Cdc10 complex formation. Cultures of *ran1–114* strains that coexpressed *sct1-HA* and *cdc10$^+$*, *cdc10 S196A*, or *cdc10 S196D* were grown to mid-logarithmic phase at 27°C and half of the culture was then shifted to 36°C for 3 h as indicated. Cell-free extracts were prepared for immunoprecipitation. (A) Anti-Cdc10 immunoblot of anti-Cdc10 immunoprecipitates from the Cdc10$^+$ extract (lanes 1 and 2), the Cdc10 S196A extract (lanes 3 and 4), the Cdc10 S196D extract (lanes 5 and 6), normal mouse serum immunoprecipitate from the Cdc10$^+$ extract (lane 7), anti-HA immunoprecipitates from the Cdc10$^+$ extract (lanes 8 and 9), the Cdc10 S196A extract (lanes 10 and 11), or the Cdc10 S196D extract (lanes 12 and 13). (B) Anti-HA immunoblot of a normal mouse serum immunoprecipitate from the Cdc10$^+$ extract (lane 1) and anti-HA immunoprecipitates from the Cdc10$^+$ extract (lanes 2 and 3), the Cdc10 S196A extract (lanes 4 and 5), and the Cdc10 S196D extract (lanes 6 and 7).

The Cdc10/Sct1 DNA-binding Activity Requires the Ran1 Kinase

As discussed above, the association between Puc1 and Cdc10 is dependent on a functionally active Ran1 kinase. It was therefore of interest to determine whether the loss of Ran1 had a deleterious effect on Cdc10/Sct1-dependent DNA-binding activity, which has been shown previously to require Cdc2 (Reymond *et al.*, 1993). Cultures of a wild-type strain (SP 201) and one carrying the *ran1–114* mutation (SP 315) were shifted from the permissive (25°C) to the restrictive (36°C) temperature for the *ran1–114* mutation for 4 h. Aliquots of cells were removed at hourly intervals and used for the preparation of cell-free extracts. The DNA-binding activity of Cdc10/Sct1 in these extracts was assessed with a gel mobility shift assay using a probe from the *cdc22* promoter (Lowndes *et al.*, 1992b; Caligiuri and Beach, 1993). We found that the DNA-binding activity was completely abolished after 3 h of growth at elevated temperature in extracts prepared from the *ran1–114* strain (Figure 7, lanes 11 and 12). The kinetics of this loss of activity parallels the loss of viability of the *ran1–114* strain upon incubation at the restrictive temperature (our unpublished observation). The temperature shift had no effect on the DNA-binding activity when the extracts were prepared from the wild-type strain (Figure 7, lanes 2–6). This indicates that the Ran1 kinase is required for the DNA-binding activity of the Cdc10/Sct1 complex as assayed in vitro. It is likely that this requirement is due to the loss of the Puc1/Cdc10 interaction, and potentially Cdc10 phosphorylation, upon inactivation of Ran1.

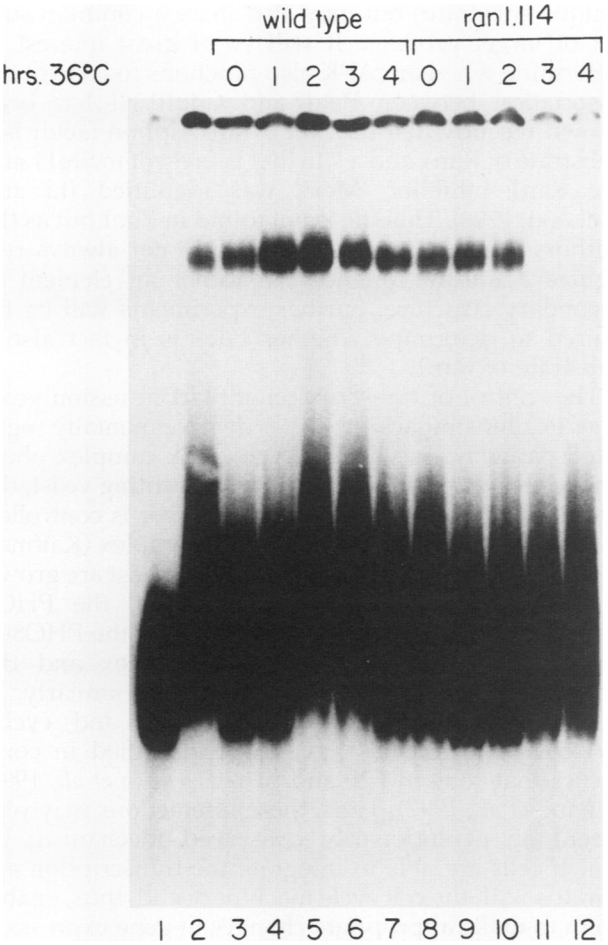

Figure 7. Cdc10/Sct1 function requires the Ran1 kinase. Cultures of a wild-type and *ran1–114* strains were shifted from 25 to 36°C for 4 h. Aliquots of cells were removed at hourly intervals and used for the preparation of cell-free extracts. The DNA-binding activity of Cdc10/Sct1 was assessed with a gel mobility shift assay using a probe from the *cdc22* promoter. No extract (lane 1), wild-type extract (lanes 2–7), and *ran1–114* extract (lanes 8–12) are shown.

DISCUSSION

The results presented in this article support a molecular model for the regulation of Start in which the Ran1 kinase cooperates with Cdc2 to control Cdc10/Sct1-dependent transcription during the fission yeast cell cycle (Figure 8). Our data demonstrate that the association of Sct1 with Cdc10 to form an active transcription complex requires the activity of the Ran1 protein kinase in vivo. Inactivation of *ran1* under restrictive conditions leads to the dissociation of Cdc10 and Sct1 and concomitant loss of DNA-binding activity. This leads to G_1 arrest and the activation of the mating pathway regardless of nutritional conditions.

We have shown that Puc1, and presumably Cdc2, associates with Cdc10 in vivo and that Ran1 is required for this interaction. In the accompanying article, we demonstrate that Cdc2 is required for Cdc10/Sct1 complex formation and, therefore, envisaged that since Ran1 is required for Puc1 to bind Cdc10, it too might be required for the formation of the heteromeric transcription complex. This prediction was borne out when we observed the dissociation of the Cdc10/Sct1 complex upon inactivation of Ran1. In addition, we found that as expected, inactivation of Ran1 leads to a loss of Sct1/Cdc10 DNA-binding activity. In the accompanying article (Connolly *et al.*, 1997), we show that Ser-196 of Cdc10, which lies within a canonical Cdc2 recognition site, is phosphorylated in vivo. Furthermore, we show that substitution of this serine with alanine, S196A, results in a loss of Cdc10 function in vivo due to the inability of Cdc10 S196A to associate with Sct1. Interestingly, substitution of this serine with aspartic acid, S196D, thus, potentially mimicking phosphorylation, restores Cdc10 function and bypasses the requirement for both Ran1 and Cdc2 kinase activity for Sct1/Cdc10 complex formation. We demonstrate herein that inactivation of Ran1 results in the loss of phosphorylation of Cdc10 at Ser-196 and in the appearance of a new site of phosphorylation. This suggests that the phosphorylation state of Cdc10 may constitute the signal that influences the developmental fate of the cell. Since we have demonstrated that Cdc10 is associated with Puc1 and presumably Cdc2 but have not detected an association between Ran1 and Cdc10, we propose that Ran1 functions in this capacity to control the Cdc2 execution point in G_1 by affecting the ability of Cdc2/Puc1 to associate with and phosphorylate Cdc10. It is likely that the requirement for Ran1 in the formation of an active Cdc10/Sct1 transcription complex is due to its role in promoting the Puc1/Cdc10 association.

Clearly, however, our knowledge of the role of Puc1/Cdc2 at Start is incomplete in part because no fission yeast cyclin has been definitively shown to function in G_1. Puc1 is at present the best candidate of

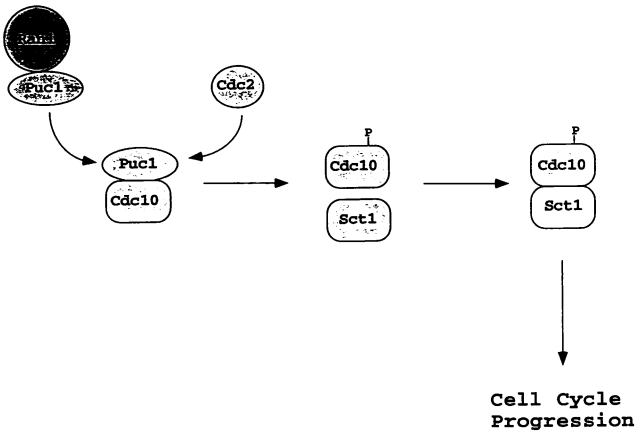

Figure 8. Model for the role of Ran1 in Cdc10/Sct1 activity.

known cyclins to function in G_1 because it shares a high degree of amino acid sequence similarity to CLN3 from *S. cerevisiae* (Forsburg and Nurse, 1991). Cells that carry the *puc1* null allele, like the budding yeast mutants deleted for any single CLN, display no defect in G_1/S-phase progression (Forsburg and Nurse, 1994). Furthermore, cells that carry the *puc1* null allele in combination with inactivating mutations in *cig1*, *cig2*, *cdc13*, or *ran1* display no cell cycle defects (T.C., unpublished observations). Thus, it appears extremely likely that an as yet unidentified cyclin must exist that functions in G_1 in the absence of Puc1 since Cdc2 is essential for G_1 progression and cell cycle commitment. Nonetheless, the observation that overexpression of *puc1* partially rescues the *ran1–114* temperature-sensitive mutation when considered with the biochemical results presented herein strongly suggests a role for the Puc1 cyclin in maintaining mitotic cell cycle progression.

As in the budding yeast, conjugation in fission yeast is controlled in part by the reciprocal action of diffusible pheromones that induce specific changes in cells of the opposite mating type (for review, see Nielsen, 1993). In budding yeast, the addition of mating pheromone to vegetatively growing cells of the opposite mating type induces a G_1 arrest through the direct inhibition of the CLN/CDC28 kinase complex by FAR1 (McKinney *et al.*, 1993; Peter *et al.*, 1993). In fission yeast, however, it has not been possible to determine whether mating pheromones are capable of inducing G_1 arrest directly because derepression of the genes necessary for activation of the pheromone response pathway requires nitrogen limitation, which itself induces G_1 arrest. Interestingly, cells that carry inactivating mutations in *ran1* undergo G_1 arrest upon addition of mating pheromone to nitrogen-rich medium (Davey and Nielsen, 1994).

The Ran1 protein kinase functions to negatively regulate sexual differentiation under conditions that promote vegetative growth. The loss of *ran1* can be suppressed by overexpression of *sct1* or *puc1*, by the loss of *ste11* or *mei2*, or by conditions that cause unregulated cAMP-dependent kinase (cAPK) activity (Sipiczki, 1988; Sugimoto *et al.*, 1991; Tanaka *et al.*, 1992; for review, see Nielsen *et al*, 1993; Forsburg and Nurse, 1994). Cells that carry a deletion of the *sct1* gene or inactivating mutations in *ran1*, adenylate cyclase (*cyr1*), or cAPK (*pka1*) undergo conjugation and subsequent meiosis regardless of nutritional conditions thereby bypassing the normal requirement for nitrogen limitation for activation of these pathways (Iino and Yamamoto, 1985; Nurse, 1985; Maeda *et al.*, 1990; Caligiuri and Beach, 1993; Maeda *et al.*, 1994). The cAMP signaling pathway functions to prevent conjugation and meiosis, whereas Ran1 appears to regulate entry into meiosis exclusively. It has therefore been suggested that Ran1 and cAPK may phosphorylate unique substrates but may also share a common subset of target proteins. It will be of great interest to determine whether cAPK also functions to control the association between Puc1 and Cdc10. It has been shown recently that the Ste11 transcription factor is a substrate of Ran1 and a site that is present in Ste11 and the Ran1 inhibitor, Mei3, was identified (Li and McLeod, 1996). This site is not found in Puc1 but as the authors mention, protein kinases do not always recognize a linear sequence but rather an element of secondary structure. Further experiments will be required to determine whether Puc1 is in fact also a substrate of Ran1.

The control of transcription at Start in fission yeast may exhibit similarity to other developmentally regulated pathways in which a cyclin/cdk complex phosphorylates a transcription factor. In budding yeast, the activity of the PHO4 transcription factor is controlled by the PHO80-PHO85 (cyclin/cdk) complex (Kaffman *et al.*, 1994). When cultures of budding yeast are grown in high-phosphate–containing medium, the PHO4 transcription factor is phosphorylated by the PHO80/PHO85 cyclin-dependent kinase complex and the transcription of the PHO5 is repressed. Similarly, in mammalian cells the cyclin A/Cdk2 and cyclin E/Cdk2 holoenzymes have been identified in complexes that contain E2F and p107 (Devoto *et al.*, 1992; Pagano *et al.*, 1992). Thus, these interactions may represent an evolutionarily conserved mechanism by which cells are able to integrate the transcription apparatus with the cell cycle machinery and, thus, enable them to make appropriate changes in gene expression in response to external stimuli.

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