Molecular Biology of the Cell
Vol. 10, 3705–3715, November 1999

Cell Cycle–regulated Transcription in Fission Yeast: Cdc10–Res Protein Interactions during the Cell Cycle and Domains Required for Regulated Transcription

Simon Whitehall,* Peter Stacey,† Keren Dawson,†‡ and Nic Jones†‡§

†Laboratory of Gene Regulation, Imperial Cancer Research Fund, London WC2A 3PX, United Kingdom; and *School of Biochemistry and Genetics, The Medical School, The University of Newcastle, Newcastle-upon-Tyne NE2 4HH, United Kingdom

Submitted April 2, 1999; Accepted August 17, 1999
Monitoring Editor: Mitsuhiro Yanagida

In *Schizosaccharomyces pombe* the MBF (DSC1) complex mediates transcriptional activation at Start and is composed of a common subunit called Cdc10 in combination with two alternative DNA-binding partners, Res1 and Res2. It has been suggested that a high-activity MBF complex (at G1/S) is switched to a low-activity complex (in G2) by the incorporation of the negative regulatory subunit Res2. We have analyzed MBF protein–protein interactions and find that both Res proteins are associated with Cdc10 throughout the cell cycle, arguing against this model. Furthermore we demonstrate that Res2 is capable of interacting with a mutant form of Cdc10 that has high transcriptional activity. It has been shown previously that both Res proteins are required for periodic cell cycle–regulated transcription. Therefore a series of Res1–Res2 hybrid molecules was used to determine the domains that are specifically required to regulate periodic transcription. In Res2 the nature of the C-terminal region is critical, and in both Res1 and Res2, a domain overlapping the N-terminal ankyrin repeat and a recently identified activation domain is important for mediating cell cycle–regulated transcription.

INTRODUCTION

In eukaryotic cells a key regulatory step of the cell cycle occurs at late G1, which in yeast cells has been termed "Start" (for review, see Nasmyth, 1993). At this regulatory point the cell determines whether it will commit to a new round of proliferation or choose alternative pathways leading to arrest and sexual differentiation. Progression through Start requires the activity of one or more cyclin-dependent kinases (cdks) and also the transcriptional activation of specific genes encoding products for S phase.

In the fission yeast *Schizosaccharomyces pombe,* transcriptional activation at Start is mediated by the multisubunit factor MBF (DSC1). The major components of this factor are encoded by the *cdc10*$^+$ (Lowndes *et al.*, 1992), *res1*$^+$/sct1$^+$ (Tanaka *et al.*, 1992; Caligiuri and Beach, 1993), *res2*$^+$/*pct1* $^+$ (Miyamoto *et al.*, 1994; Zhu *et al.*, 1994), and *rep2*$^+$ (Nakashima *et al.*, 1995) genes. The proteins encoded by these genes form a complex, which binds to the *MluI* cell cycle box (MCB) enhancer elements that are found in the promoters of genes, which are transiently activated at the G1/S phase of the cell cycle. One of the critical target genes of MBF is *cdc18*$^+$ that, when ectopically expressed, can overcome the G1 arrest that *cdc10*^{ts} cells undergo at the restrictive temperature (Kelly *et al.*, 1993). Cdc10 is an integral part of the MBF complex, but it does not bind to DNA directly; rather the DNA-binding function is provided by one of two Res subunits. Res1 and Res2 are structurally homologous proteins and contain highly similar N-terminal DNA-binding domains. In addition they have centrally located ankyrin repeats and interact with Cdc10 via their C termini (Ayte *et al.*, 1995; Zhu *et al.*, 1997). Moreover, these structural features are shared by their budding yeast counterparts Swi6, Swi4, and Mbp1 (for review, see Breeden, 1996). Despite being highly related, Res1 and Res2 are functionally nonidentical. Cells deleted for *res1*$^+$ have deficiencies in the mitotic cycle and have a cold- and heat-sensitive phenotype resulting in a G1 arrest (Tanaka *et al.*, 1992). In contrast Δ*res2* cells have no obvious defects in the mitotic cell cycle but are severely impaired in their ability to enter into premeiotic DNA synthesis and meiosis, indicating that Res2 has roles in the sexual differentiation process (Miyamoto *et al.*, 1994; Zhu *et al.*, 1994). The defects of Δ*res1* cells can be suppressed by overexpression of *res2*$^+$, but the reverse is not true, because the meiotic phenotypes of Δ*res2* cells cannot be rescued by *res1*$^+$ overexpression (Miyamoto *et al.*, 1994; Zhu *et al.*, 1997). The genetic phenotypes lead to a model whereby two dif-

‡ Present address: The Paterson Institute for Cancer Research, Christie Hospital NHS Trust, Wilmslow Road, Manchester M20 4BX, UK.
§ Corresponding author. E-mail address: njones@picr.man.ac.uk.

ferent but overlapping MBF complexes operate, with Res1–Cdc10 playing the major role at mitotic Start and Res2–Cdc10 controlling entry into premeiotic S phase. However, recent evidence has necessitated a reevaluation of this model. Res1 and Res2 can heterodimerize in a Cdc10-dependent manner in vitro (Zhu *et al*., 1997), and the MBF complex, detectable in crude extracts by bandshift experiments, contains Res1, Res2, and Cdc10 (Ayte *et al*., 1995; Zhu *et al*., 1997). Furthermore, both Res subunits are required for periodically regulated transcription because *cdc18*$^+$ levels are constitutively low throughout the cell cycle in Δ*res1* cells and constitutively high in Δ*res2* cells (Baum *et al*., 1997). Thus, Res2 and by implication Res subunit heterodimerization is required for the downregulation of transcription in G2. Indeed, it has been suggested that Res2 functions as a repressive subunit of MBF in mitotically growing cells (Baum *et al*., 1997). The results have led to the suggestion that regulation of MBF activity may be achieved by Res subunit "switching" with alternative complexes having different activation potentials (Baum *et al*., 1997; Zhu *et al*., 1997). We have tested this model directly and assessed the influence of cell cycle progression upon the ability of the Res subunits to interact with Cdc10. Contrary to previous predictions, no evidence of subunit switching was found, and our evidence suggests that the complex remains a Res1–Res2–Cdc10 heteromeric complex throughout the cell cycle. This implies that all three components are required in the complex for it to be a regulatory target. We also present evidence that argues against Res2 being only a negative regulatory subunit because it is able to interact with a mutant form of Cdc10 that mediates constitutively high levels of target gene expression. We have used a series of hybrid molecules between Res1 and Res2 to determine the domains in the Res proteins that are specifically required to regulate transcription periodically. In Res2 the nature of the C terminus is critical, and in both Res1 and Res2 a domain overlapping the N-terminal portion of the ankyrin repeats is crucial for mediating cell cycle–regulated transcription. The implication of these findings for the mechanism of transcription regulation at Start is discussed.

MATERIALS AND METHODS

Plasmids

The plasmid pRep41-FLAG Cdc10 was constructed by digesting pT7Cdc10 (Zhu *et al*., 1997) with *Xba*I and infilling with Klenow. After further digestion with *Nco*I, the 2.4-kb *cdc10* fragment was cloned in the *Sma*I and *Nco*I sites of pRHA41. The plasmid pRHA41 is a derivative of pRep41 and contains three copies of the hemagglutinin (HA) epitope upstream of the *Nde*I site. Digestion with *Nco*I and *Sma*I results in the complete excision of the HA epitope sequence. N-terminal tagging of Cdc10 with the FLAG epitope was achieved by annealing oligonucleotides 1 (5′-CATGGATTACAAGGACGATGACGACAAGGG-3′) and 2 (5′-CATGCCCTTGTCGTCATCGTCCTTGTAATC-3′) and inserting the resulting double-stranded oligonucleotide into the unique *Nco*I site of pRep41-Cdc10. The pRep41-FLAG Cdc10 C4 plasmid (in which the C-terminal 61 codons of *cdc10* are deleted) was created by PCR mutagenesis. Oligonucleotides 5′-CCTGTACAGATCCAGCTGGACGGACGG-3′ and 5′-GCACCTGTCTCTAGATTATTGCCAAAGTTTGTTCGCTAA-3′ were used to amplify a fragment from *cdc10*$^+$ that was cleaved with *Pst*I and *Xba*I. This fragment was subcloned into pT7Cdc10 cleaved with *Xba*I and *Pst*I. The *cdc10-C4* gene was subcloned into pRHA41 and tagged as described above. The plasmids used for the "one-hybrid" analysis of *res1*$^+$ are to be described in detail elsewhere (Stacey, Whitehall, and Jones, unpublished data). Briefly, pPapX expresses the N-terminal DNA-binding domain of *pap1*$^+$ from the attenuated *nmt41* promoter, and pPapRes1 expresses a fusion of the N terminus of *pap1*$^+$ and amino acids 46–637 of *res1*$^+$. Other plasmids used in this study have been described previously (Zhu *et al*., 1997).

Strains and Genetic Methods

General genetic methods for *S. pombe* were followed according to the procedures of Gutz *et al.* (1974) and Moreno *et al.* (1991). The following strains were used in this study: *h*$^-$ *cdc25-22 leu1-32, h*$^-$ *leu1-32 ura4-D18, h*$^-$ *ade6-M210 leu1-32 ura4-D18 res2::ura4*$^+$, *h*$^-$ *ura4-D18 res1::ura4*$^+$, *h*$^-$ *leu1-32 ura4-D-18, sct1-1 cdc10::ura4*$^+$, *h*$^-$ *leu1-32 cdc10-C4, h*$^-$ *ura4-D18 leu1-32 rep2::ura4*$^+$, *h*$^-$ *ura4-D18 rep2::ura4*$^+$ *cdc10-C4, h*$^-$ *leu1-32 ura4-D18 pap::ura4*$^+$, *h*$^-$ *leu1-32 ura4-D18 pap1::ura4*$^+$ *cdc2-33, h*$^-$ *leu1-32,* and *h*$^-$ *ura4-D18 res1::ura4*$^+$. A strain in which the *cdc10-129* locus was replaced with *FLAGcdc10* was created by inserting 149 bp of *cdc10*$^+$ promoter sequence into the *Nco*I site of pRep41-FLAG Cdc10. A 1.4-kb fragment containing the promoter and the *cdc10* sequence to the *Pst*I site was used to transform an *h*$^+$ *cdc10-129 leu1-32* temperature-sensitive strain. Potential integrants were isolated as colonies able to grow at 36°C on EMM supplemented with leucine. PCR analysis was used to confirm that the *cdc10-129* locus had been replaced with *FLAGcdc10*. The ability of isolated clones to produce epitope-tagged Cdc10 was confirmed by Western blotting.

Cell cycle blocks with strains carrying the *cdc25-22* allele were performed by shifting early log-phase cells from 25 to 36°C for 4 h. The culture was then chilled rapidly to 25°C on ice water, and incubation continued at this temperature. Microscopic examination was used to determine the proportion of cells with septa and thus to measure the synchronicity of the culture.

Hydroxyurea (HU) cell cycle blocks were performed by adding HU to early log-phase cultures to a final concentration of 11 mM. Incubation was continued for 3–4.5 h, and microscopic examination showed the cells to be slightly elongated, indicating that a cell cycle block had taken place. Release from the HU block was performed by washing the cells twice in an equal volume of EMM and resuspending the cells in fresh EMM media.

RNA Analysis

RNA was prepared by vortexing cell pellets with glass beads as described by Zhu *et al.* (1997). RNA analysis was as described previously (White *et al*., 1986). Briefly a 10- to 15-μg sample of total RNA was denatured with glyoxal, separated on a 1.2% agarose gel prepared in 15 mM sodium phosphate (pH 6.5), and transferred to a GeneScreen hybridization membrane (DuPont New England Nuclear, Boston, MA). *cdc18*$^+$ and *his3*$^+$ probes for RNA–DNA hybridization have been described by Baum *et al.* (1997). The *cdt1*$^+$ probe was produced by PCR amplification from genomic DNA using the following primers: 5′-GTCCGTAAACTCGATCCTCA-3′ and 5′-GGATCGCAAGTATGGTTTCCC-3′. The *pps1*$^+$ probe was a 1.2-kb *Hin*dIII-*Eco*RI fragment derived from a cDNA clone in the two-hybrid library vector pGADGH. The *pps1*$^+$ gene encodes a putative 26 S proteasome subunit, and its 1.5-kb transcript has been shown not to vary during the mitotic and meiotic cell cycles or upon the addition of HU or other DNA-damaging agents (our unpublished data). All probes were labeled with [α-^{32}P]dCTP by use of a DNA megaprime labeling kit (Amersham, Arlington Heights, IL).

Denatured Cell Extracts

Approximately 2.5 × 10^8 cells were harvested by centrifugation and washed once in dH_2O. The cell pellets were resuspended in 50 μl of 1× SDS loading buffer (with no bromophenol blue) and incubated for 3 min at 90°C. The reactions were transferred to 1.5-ml Eppendorf tubes (Scotlab, Strathclyde, Scotland) containing 1 ml of glass

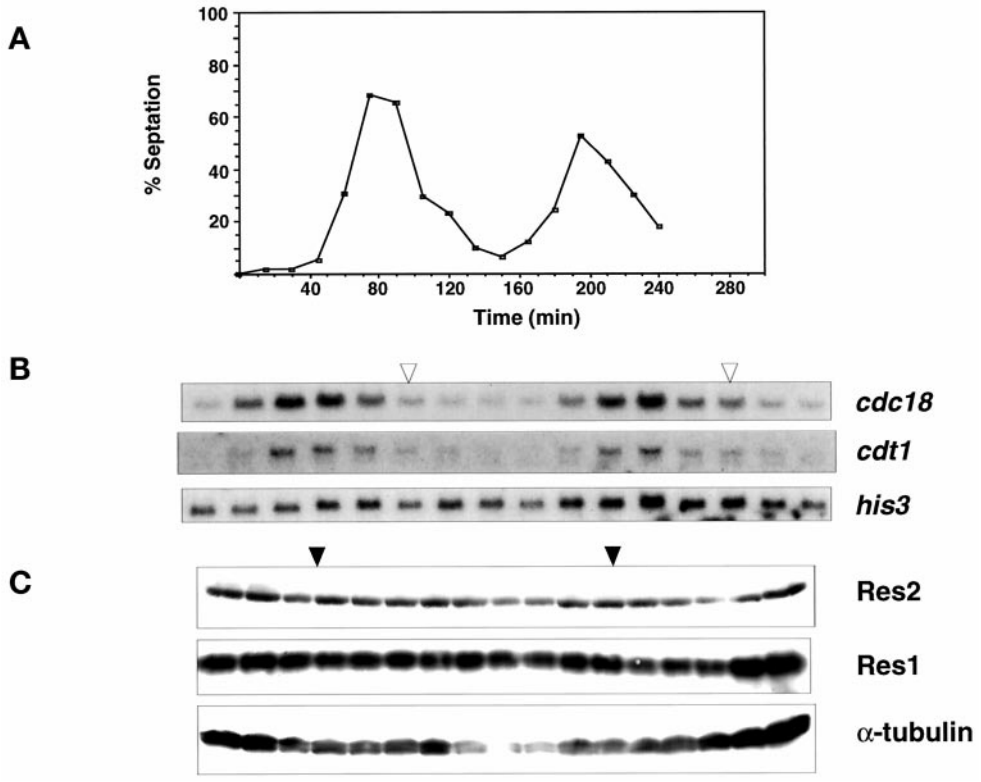

Figure 1. Res2 and Res1 protein levels remain constant throughout the cell cycle. *cdc25-22* cells were synchronized as described in MATERIALS AND METHODS. (A) The level of synchrony for the culture is indicated by the septation index (percentage of septated cells). (B) Samples of the synchronous culture were analyzed by Northern blotting for *cdc18*$^+$ and *cdt1*$^+$ mRNA levels, with *his3*$^+$ mRNA serving as a loading control. Open arrowheads indicate the peaks of septation. (C) Western blot analysis of denatured protein extracts (40 μg) prepared from synchronized cells is shown. Extracts were probed with anti-Res2 antibody, anti-α-tubulin antibody, and monoclonal anti-Res1 antibody. Closed arrowheads indicate the peaks of *cdc18*$^+$ mRNA. Note that the Res1 panel represents a different blot of the same samples used for the Res2 and α-tubulin analysis.

beads (425–600 μm; Sigma, St. Louis, MO) and vortexed twice for 1 min. The beads were washed with 100 μl of 1x SDS loading buffer, and the supernatant was removed from the glass beads. The lysates were cleared by spinning in a microcentrifuge for 10 min. Approximately 40 μg of total protein was analyzed by SDS-PAGE followed by Western blotting as described below.

Immunoprecipitations

Native whole-cell extracts were prepared as described (Zhu *et al.*, 1997) except that the lysis buffer used was as follows: 50 mM Tris-HCl (pH 7.5), 120 mM KCl, 5 mM EDTA, 0.1% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 5 μg/ml each of aprotinin, leupeptin, and pepstatin. Anti-FLAG immunoprecipitations were performed by adding 20 μl of a 50% slurry of anti-FLAG M2 affinity gel (Kodak, Rochester, NY) to 1–1.5 mg of protein extract, and the reactions were gently agitated for 2 h at 4°C. Reactions were washed four times in lysis buffer and analyzed by SDS-PAGE. For Western blotting, proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA) and probed with the appropriate antibody. Anti-Res1 immunoprecipitations were performed as described by Ayte *et al.* (1995). The antibodies used in this study were as follows: polyclonal anti-Res2 (Zhu *et al.*, 1997), polyclonal anti-Res1 (Nakashima *et al.*, 1995), monoclonal anti-Res1 (Ayte *et al.*, 1995), polyclonal anti-Cdc10 (Baum *et al.*, 1997), and monoclonal anti-α-tubulin (Sigma).

RESULTS

Both Res1 and Res2 are required for the periodic transcription of MBF-dependent target genes such as *cdc18*$^+$ (Baum *et al.*, 1997; Zhu *et al.*, 1997). It has therefore been proposed that the ability of the MBF complex to mediate cell cycle–regulated transcription could be caused by phase-dependent Res subunit switching (Baum *et al.*, 1997; Zhu *et al.*, 1997). If different Cdc10-Res complexes had different activation potentials, then periodic transcription would ensue. It is also known that Res1 and Res2 heterodimerize in the presence of Cdc10, because the MBF complex detectable in crude extracts contains both Res subunits (Ayte *et al.*, 1997; Zhu *et al.*, 1997). An alternative model suggests that the composition of the MBF complex remains constant and that to be a target for regulatory mechanisms, all three components must be present. We therefore decided to investigate the influence of cell cycle progression on the composition of the MBF complex.

Res2 Protein Levels Are Constitutive throughout the Cell Cycle

We initially determined whether the level of the Res protein changes in a cell cycle–dependent manner, particularly because Northern analysis has shown that *res2*$^+$ mRNA has slight cell cycle periodicity (Obara-Ishihara and Okayama, 1994). Cells were synchronized by growing a *cdc25-22* temperature-sensitive strain at the nonpermissive temperature for 4 h so that the cells accumulated at the G2–M boundary. The cells were then shifted to the permissive temperature at which they proceeded through highly synchronous cell cycles as determined by measuring the septation index (Figure 1A). The level of *cdc18*$^+$ and *cdt1*$^+$ mRNA was monitored as a measure of MBF activity and was found to be periodic

(Figure 1B). However, Western blotting demonstrated that Res2 protein levels remained constant throughout the cell cycle as did the levels of Res1 protein. The level of Cdc10 protein has also been shown to be constant throughout the cell cycle (Simanis and Nurse, 1989), and so the periodic activation–deactivation of the MBF complex is not achieved by regulating the protein levels of the major subunits.

Cell Cycle Progression and the Composition of the MBF Complex

We then investigated the subunit composition of the MBF complex at different stages of the cell cycle. To facilitate this analysis we expressed Cdc10 tagged with the FLAG epitope in *cdc25-22* cells. Whole-cell extracts were prepared from nonsynchronous, exponentially growing cells, and Cdc10 was immunoprecipitated with FLAG monoclonal antibody. The immunoprecipitates were probed by Western blotting with anti-Res1, anti-Res2, and anti-Cdc10 antibodies. Cdc10 was efficiently immunoprecipitated from cells expressing tagged Cdc10, and furthermore both Res1 and Res2 were coimmunoprecipitated (see Figure 2A). The presence of Cdc10, Res1, and Res2 was specific, as demonstrated by their absence from control immunoprecipitations in extracts derived from cells containing empty vector (Figure 2A). Because asynchronous, exponentially growing *S. pombe* cells are mainly in the G2 phase of the cell cycle, we analyzed Cdc10–Res interactions after release from a *cdc25-22* cell cycle block. Cells expressing either empty vector or tagged Cdc10 were synchronized by a block-and-release protocol as described above. Expression of *cdc18*$^+$ and *cdt1*$^+$ mRNA increased dramatically 20 and 40 min after shifting the cells to the permissive temperature and then decreased at longer time points, indicating that the MBF complex had been activated and then downregulated. Almost identical results were obtained with cells expressing FLAG-tagged Cdc10 and with cells carrying empty vector (see Figure 2B). Thus moderate overexpression of tagged Cdc10 did not disrupt cell cycle progression or the transcriptional pattern of Cdc10-dependent genes. We therefore used this strain to investigate the interaction of Cdc10 and Res subunits during the cell cycle. Whole-cell extracts were prepared from cells during the *cdc25-22* block-and-release experiment and analyzed using FLAG Cdc10 immunoprecipitation followed by Western blotting. Res1 coimmunoprecipitated with Cdc10 at each point in the experiment, and importantly, Res2 was also found to be constitutively associated with Cdc10. This is significant because Res2 has been termed a negative regulatory subunit of MBF (Baum *et al*., 1997), and these experiments indicate that Res2 was bound to Cdc10, not only when the activation potential of the complex was low, but also during periods of high MBF-dependent gene expression.

It was important to exclude the possibility that the ability of Res2 to interact with Cdc10 during a *cdc25-22* block-and-release experiment was an artifact of increased levels of Cdc10. We therefore integrated a FLAG-tagged version of Cdc10 into its natural chromosomal locus, resulting in physiological levels of the epitope-tagged protein. We then examined the ability of the Res subunits to interact with Cdc10 during an HU block-and-release experiment. Exponentially growing cells were blocked at early S phase by the addition of hydroxyurea for 4 h, and the cells were then released from this block by washing away the HU. The ability of the MBF

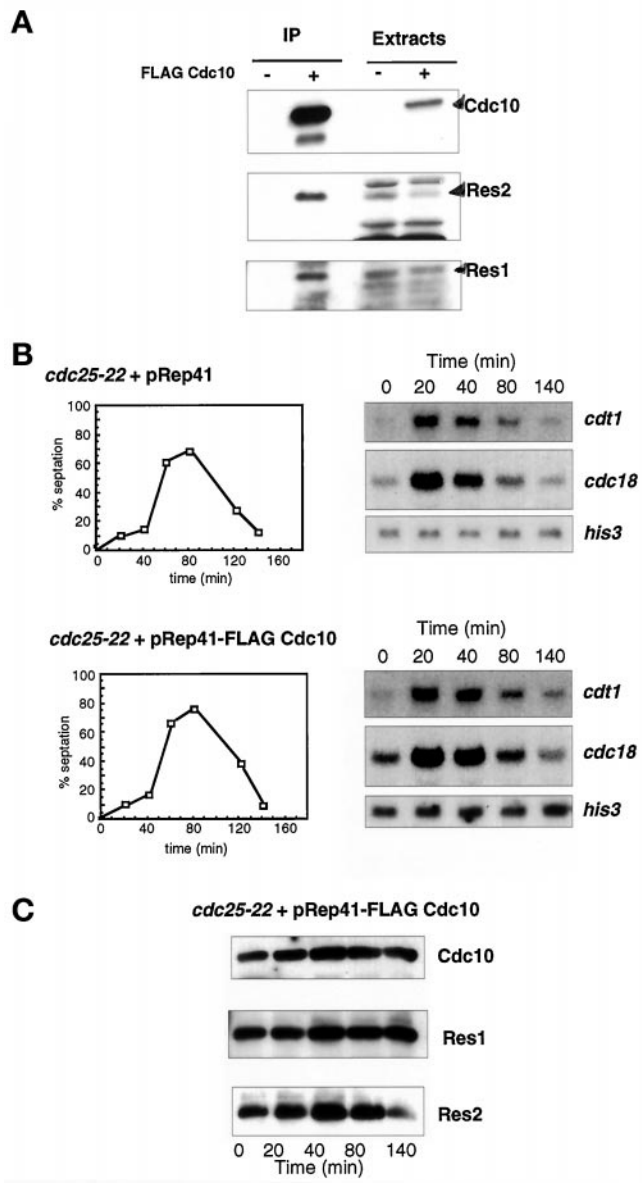

Figure 2. Both Res1 and Res2 remain associated with Cdc10 during a *cdc25-22* block and release. (A) Whole-cell native extracts were prepared from exponential asynchronous *cdc25-22* cells transformed with pRep41 or pRep41-FLAG Cdc10 and were immunoprecipitated with anti-FLAG antibody. Whole-cell extracts and immunoprecipitates (IPs) were analyzed by Western blotting with anti-Cdc10, anti-Res1, and anti-Res2 polyclonal antibodies. (B) Moderate overexpression of Cdc10 does not disrupt cell cycle progression or alter the timing of transcription of Cdc10-dependent genes. *cdc25-22* cells carrying pRep41 or pRep41-FLAG Cdc10 were synchronized as described in MATERIALS AND METHODS; the synchrony of the cultures is indicated by the septation index (left). Northern blotting was used to probe *cdc18*$^+$ and *cdt1*$^+$ mRNA levels (right). The time after release from the temperature block is indicated above the lanes. (C) Whole-cell extracts from the *cdc25-22* + pRep41-FLAG Cdc10 cells described in b were immunoprecipitated with anti-FLAG antibody. Immunoprecipitates were analyzed by Western blotting with anti-Cdc10, anti-Res1, and anti-Res2 polyclonal antibodies.

complex to activate transcription was followed by Northern blot analysis of *cdc18*$^+$ mRNA levels. The addition of HU caused an increase in *cdc18*$^+$ levels indicating that activation of the Res–Cdc10 complex had taken place. The levels of *cdc18*$^+$ mRNA decreased 0.5 and 1 h after removal of the HU, indicating a switch to a low-activity complex (Figure 3A). Whole-cell extracts were prepared from each of the experimental time points, and FLAG-Cdc10 was immunoprecipitated. The composition of the Res subunits was assayed by Western blotting with an anti-Res1 antibody that cross-reacts with Res2 (Nakashima *et al.*,1995). By the use of this antibody it was possible to demonstrate that not only did both Res1 and Res2 remain associated with Cdc10 during the entire HU block-and-release experiment but also that the relative levels of both proteins also remain constant (Figure 3B).

To examine further the nature of the MBF complex, we prepared extracts from an HU block-and-release experiment and subjected them to immunoprecipitation with monoclonal anti-Res1 antibody. The levels of Res1, Res2, and Cdc10 in these immunoprecipitates was investigated by Western blotting (Figure 3C). This experiment is important because it provides a measure of Res1–Res2 interactions and thus the level of the heteromeric complex. Thus, any changes in MBF stoichiometry should be revealed by this experiment. However, we found that the levels of Res2 and Cdc10 in the immunoprecipitates were very similar throughout the HU block-and-release experiment, implying that the level of Res1–Res2–Cdc10 is not altered. Taken together these results argue against a "subunit switch"–based model for MBF regulation and for a model in which all three components are required in the complex for its activity to be regulated.

Both Res1 and Res2 Interact with the Cdc10-C4 Mutant

We next investigated the ability of the Res subunits to interact with the mutant Cdc10-C4. The Cdc10-C4 mutant lacks 61 C-terminal amino acids and confers temperature sensitivity to cells (Reymond and Simanis, 1993). Furthermore in the *cdc10-C4* background, transcription of target genes has no periodicity and, at the permissive temperature, is high throughout the cell cycle (McInerny *et al.*, 1995). This phenotype is somewhat similar to that seen in Δ*res2* cells in which the Res–Cdc10 complex contains only Res1. A possible explanation for this phenotype is that Cdc10-C4 is unable to interact with Res2. To test this possibility directly, we expressed FLAG-tagged full-length and C4 versions of Cdc10 in Δ*cdc10 sct1-1* cells. The *sct1-1* mutation is a single amino acid change in *res1* (E56K) that allows cells to be viable in the absence of Cdc10 (Marks *et al.*, 1992; Caligiuri and Beach, 1993). It should be noted that this mutation by itself does not appear to influence the periodic nature of *cdc18*$^+$ expression (Baum *et al.*, 1997). However in the Δ*cdc10 sct1-1* background, *cdc18*$^+$ levels are low, and no cell cycle periodicity of transcription is observed (Baum *et al.*, 1997) (see Figure 4A). Expressing full-length Cdc10 increased *cdc18*$^+$ transcription in an HU-dependent manner. As would be expected from previous observations the expression of the C4 mutant from a multicopy plasmid in this same background resulted in high *cdc18*$^+$ transcription both in the presence and absence of HU. We prepared whole-cell extracts from exponentially growing cells and cells blocked by

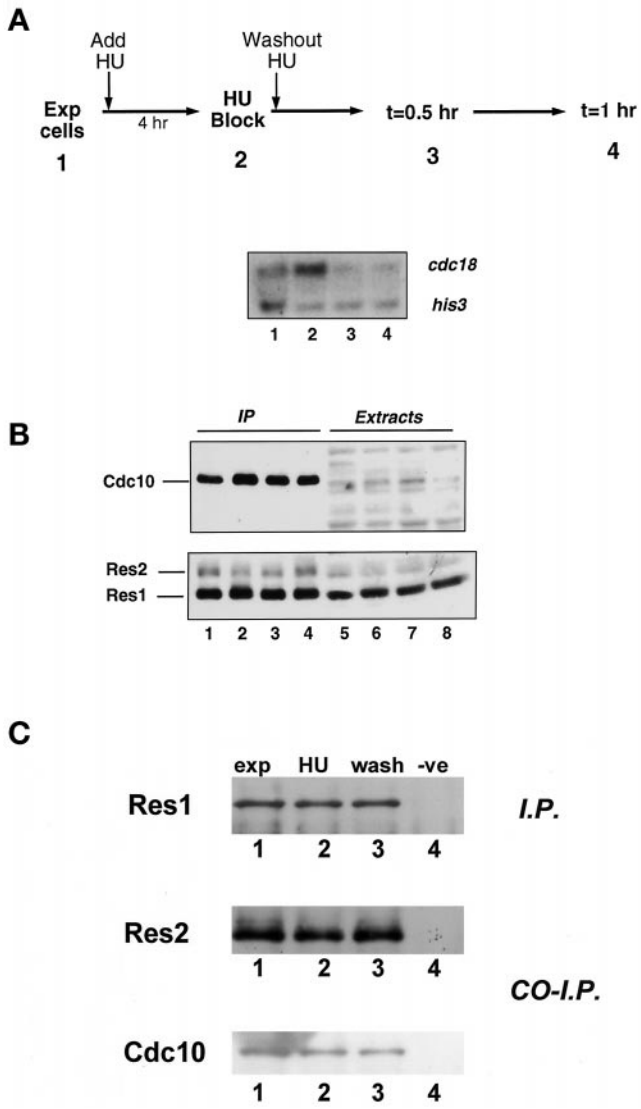

Figure 3. Both Res1 and Res2 remain associated with Cdc10 during a hydroxyurea block and release. (A and B) Exponentially growing (Exp) *FLAGcdc10* cells (lanes 1 and 5) were treated with hydroxyurea (11 mM) for 4 h (lanes 2 and 6). Cells were released from the hydroxyurea block by washing out the hydroxyurea, and samples were taken after 0.5 h (lanes 3 and 7) and 1 h (lanes 4 and 8). (A) Samples were analyzed by Northern blotting for *cdc18*$^+$ and *his3*$^+$. (B) Whole-cell extracts prepared from cells treated as described above were subjected to immunoprecipitation with anti-FLAG antibody. Extracts (lanes 5–8) and immunoprecipitates (lanes 1–4) were subjected to Western analysis with anti-Cdc10 and anti-Res1 antibodies. (C) The level of Res2 and Cdc10 associated with Res1 does not change during an HU block and release. Exponentially growing (exp) wild-type cells (lane 1) were blocked with hydroxyurea (11 mM) for 4 h (lane 2) and then released from the block for 1 h (lane 3). Whole-cell extracts from these cells and from exponentially growing Δ*res1* cells (lane 4) were immunoprecipitated using monoclonal anti-Res1 antibody (Ayte *et al.*, 1995) and probed by Western blotting with polyclonal anti-Res1, anti-Res2, and anti-Cdc10 antibodies. CO-I.P., coimmunoprecipitate; I.P., immunoprecipitate.

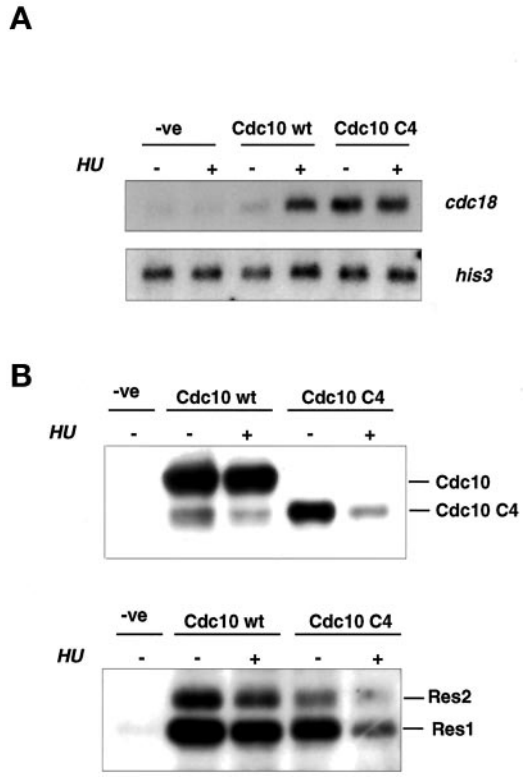

Figure 4. Res2 interacts with Cdc10-C4. Δ*cdc10 sct1-1* cells were transformed with pRep41, pRep41-FLAG Cdc10, or pRep41-FLAG Cdc10-C4. Samples were taken from exponentially growing cells (HU−) or cells treated with hydroxyurea (11 mM) for 4 h (HU+). (A) RNA prepared from these samples was analyzed by Northern blotting with the indicated probes. (B) Whole-cell extracts prepared from these samples were subjected to anti-FLAG immunoprecipitation followed by Western blotting with anti-Cdc10 antibody (top) and anti-Res1 antibody (bottom). wt, wild type.

HU addition and immunoprecipitated the Cdc10 protein with FLAG antibody. Both Res1 and Res2 coimmunoprecipitated with wild-type Cdc10 in both exponentially and HU-treated extracts, consistent with the previous results (see Figure 4). Importantly, both Res1 and Res2 also coimmunoprecipitated with the C4 version of Cdc10. This indicated that the high constitutive transactivation potential of an MBF complex containing Cdc10 C4 does not result from the exclusion of the Res2 subunit. Indeed, these results support a conclusion that Res2 is a component of the high-activity Cdc10-C4 MBF complex.

High Levels of cdc18$^+$ Transcription in *cdc10-C4* Cells Require the Res2-specific Coactivator Rep2

Evidence indicates that the zinc finger protein Rep2 is a Res2-specific coactivator; Rep2 interacts directly with Res2, and *cdc18*$^+$ mRNA levels are low in Δ*rep2* cells (Nakashima *et al.*, 1995; Baum *et al.*, 1997). Moreover low *cdc18*$^+$ mRNA levels are dependent on Res2, because transcription is constitutively high in Δ*rep2* Δ*res2* cells (Baum *et al.*, 1997). To investigate further the role of Res2 in the high level of transcriptional activity associated with the Cdc10-C4 mutant, we examined the requirement for Rep2. Deletion of *rep2*$^+$ in the *cdc10-C4* background caused *cdc18*$^+$ levels to become low, similar to those observed in Δ*rep2* cells (Figure 5). This indicates that the high levels of target gene expression in *cdc10-C4* cells require the Res2 specific coactivator and strongly supports the conclusion that Res2 interacts with Cdc10-C4. In combination these results imply that Res2 is not simply a negative regulatory subunit but has a positive role in the transcription of MBF target genes during the mitotic cell cycle.

Domains Determining Functional Specificity of Res1 and Res2 for Regulation of Cell Cycle–dependent Transcription

As described previously, properly regulated periodic transcription of genes such as *cdc18*$^+$ requires both Res subunits. Examination of synchronous cultures showed *cdc18*$^+$ mRNA levels to be constitutively low in Δ*res1* cells and constitutively high in Δ*res2* cells (Baum *et al.*, 1997). This pattern of expression is also detected using an HU cell cycle block (Baum *et al.*, 1997; Zhu *et al.*, 1997). In wild-type cells the addition of HU causes increases in *cdc18*$^+$ mRNA levels (Figure 6), whereas in Δ*res1* cells *cdc18*$^+$mRNA levels are low both before and after HU addition. Furthermore *cdc18*$^+$ expression levels are high in exponentially growing cultures of Δ*res2* cells, and addition of HU has no effect. Also synchronization of *cdc10-C4* cells has shown MBF-dependent

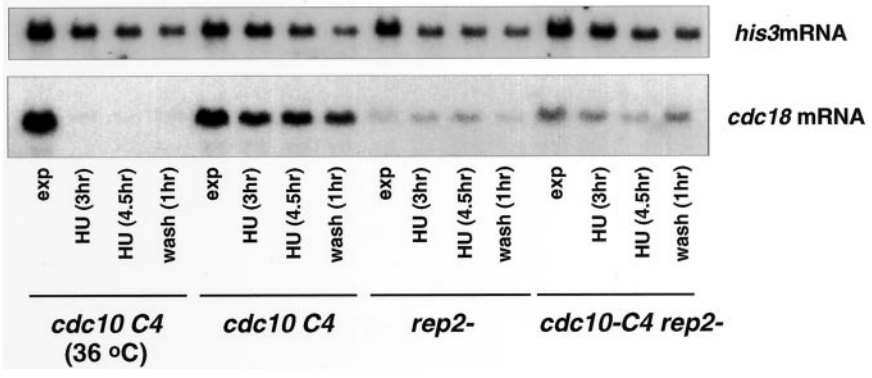

Figure 5. The high transcriptional activity of Cdc10-C4 is Rep2 dependent. Strains *cdc10-C4*, Δ*rep2*, and *cdc10-C4* Δ*rep2* were grown at 25°C and arrested by incubation with hydroxyurea (11 mM) for 3 and 4.5 h. Hydroxyurea was washed out, and incubation was continued for 1 h in fresh media. Note that in samples labeled cdc10 C4 (36°C), cells were shifted from 25 to 36°C upon addition of hydroxyurea. Samples were taken for Northern analysis and probed for *cdc18*$^+$ and *his3*$^+$ mRNA.

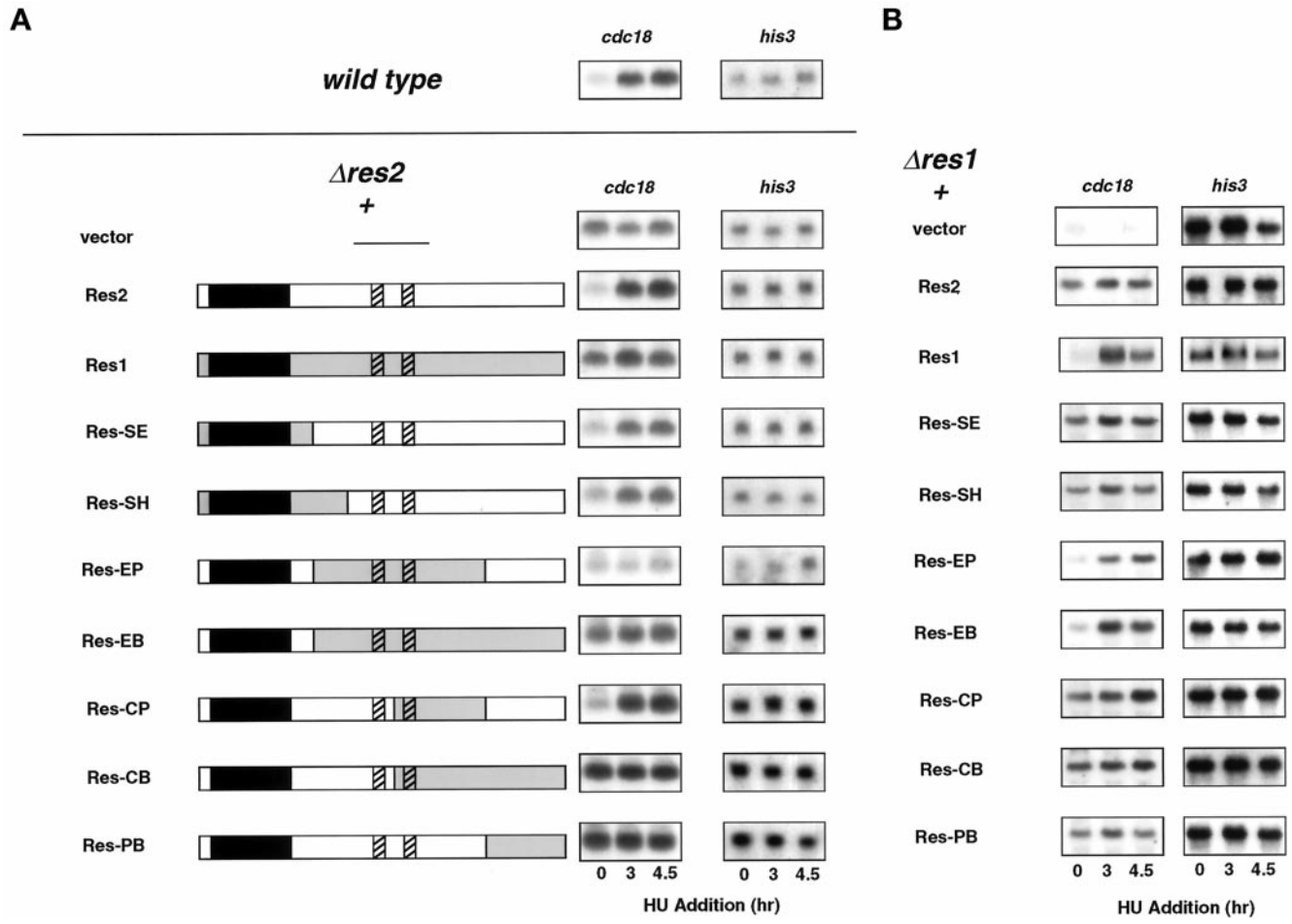

Figure 6. Domains of Res1 and Res2 required for periodic regulation of *cdc18*$^+$ transcription. Schematics describing the Res1–Res2 hybrids used in this study are shown in A. The small striped boxes represent the ankyrin repeats, and the solid box indicates the DNA-binding domain. Res2 sequences are represented by open boxes; Res1 sequences are shaded gray. Δ*res2* cells (A) or Δ*res1* cells (B) were transformed with the appropriate plasmid. Exponentially growing cells were treated with hydroxyurea (11 mM), and incubation was continued for 3 and 4.5 h. RNA was prepared from samples and subjected to Northern analysis with *cdc18*$^+$ and *his3*$^+$. A comparison with wild-type cells is shown above in a.

gene expression to be high across the cell cycle (McInerny *et al.*, 1995), and this pattern of transcription is reflected in an HU block experiment (see Figure 5). Thus in every case the pattern observed in an HU block experiment correlates extremely well with the more extensive examination of transcript periodicity using synchronized cultures. Therefore, we used this method to address which regions of the Res proteins are critical for mediating periodic activity. Expression of Res2 from a plasmid under the control of the attenuated *nmt* promoter restored a wild-type pattern of *cdc18*$^+$ expression to Δ*res2* cells; low *cdc18*$^+$ levels were observed in exponentially growing cells, and an increase was detected 3 and 4.5 h after the addition of HU (Figure 6A). In contrast, ectopically expressing Res1 in Δ*res2* cells did not restore periodic transcription, indicating that the inability to regulate transcription properly was not attributable to limiting levels of Res1. Res1 and Res2 are highly related proteins with the following similar domain structures: N-terminal DNA-binding domains, centrally located ankyrin repeats, and C-terminal Cdc10 interaction domains (Ayte *et al.*, 1997; Zhu *et al.*, 1997). However, they clearly have functional differences in terms of mediating regulated transcription. We therefore used a series of Res1–Res2 hybrid molecules to investigate the nature of this functional specificity. It should be noted that all of these hybrids, with the exception of Res-CP, are able to suppress the temperature-sensitive phenotype of *cdc10-129* cells and the cold sensitivity of Δ*res1* cells (Zhu *et al.*, 1997), indicating that they are functional and expressed. The structure of the various hybrids and their influence upon *cdc18*$^+$ expression when they are expressed in Δ*res2* cells are shown in Figure 6A. Hybrid subunits Res-SE and Res-SH have Res1-derived N-terminal DNA-binding regions (1–141 and 1–191 amino acids, respectively), whereas the remainder of these molecules is from Res2. These subunits were clearly able to mediate a wild-type pattern of *cdc18*$^+$ expression in Δ*res2* cells. Conversely, the Res-CB hybrid that has a Res2 DNA-binding domain and a Res1-derived C-terminal domain was unable to do this.

Thus the origin of the DNA-binding domain appears to be unimportant, whereas the C-terminal region is critical. This is most clearly demonstrated by the Res-PB hybrid in which only the last 103 amino acids in Res2 are replaced with Res1 sequence, yet this hybrid is unable to bring about regulated transcription. The C-terminal sequence however is not the only domain contributing to Res2 functional specificity. A comparison of the hybrids Res-EP (which did not restore regulated transcription) and Res-CP (which did restore regulated transcription) showed that sequences between amino acid residues 203 and 350 were also important. This also demonstrates that the Res-CP hybrid is functional. Furthermore, it is interesting that the regions in Res2 required for regulating cell cycle–dependent expression are also involved in other aspects of Res2 functional specificity. The C-terminal region of Res2 is essential for its meiotic function (Sturm and Okayama, 1996; Zhu *et al.*, 1997), and the extreme Res2 C terminus has been shown to confer a requirement for the coactivator Rep2 (Sturm and Okayama, 1996). Also, the region just N-terminal to the ankyrin repeats overlaps the domain identified as the Rep2-binding site (Sturm and Okayama, 1996).

We extended our analysis to Δ*res1* cells in which MBF-dependent genes are expressed at constitutively low levels (Baum *et al.*, 1997; Zhu *et al.*, 1997) (see also Figure 6B) and examined the influence of expressing the various hybrid subunits on transcription in this genetic background. Expression of all of the Res subunits brought about increased levels of *cdc18*$^+$ transcription. However, we were particularly interested in the pattern of gene expression, that is, the level of transcription in exponentially growing cells compared with that in HU-blocked cells. The expression of Res2 in this background resulted in *cdc18*$^+$ expression that was unaffected by HU addition. In contrast Res1 restored a wild-type pattern of transcription because HU addition led to an increase in *cdc18*$^+$ expression. Again the origin of the DNA-binding domain had no influence because the hybrid Res-EB that only differs from Res1 in its N-terminal region restored regulated transcription. Surprisingly, the nature of the extreme C terminus was not important in this background; Res-EP expression gave regulated transcription despite having Res2 C-terminal sequences. However, a comparison between Res-EP and Res-CP that differ only in the N-terminal half of the ankyrin repeat region shows that these sequences are as important as they are in the Δ*res2* background.

These experiments clearly demonstrate that the different activities of Res1 and Res2 in regulating transcription in a cell cycle–dependent manner are not caused by subtly different DNA-binding activities. They also indicate that for Res2 the C-terminal Cdc10 interaction domain is required for downregulation of transcription. These results also imply an important role for a region immediately N-terminal of the ankyrin repeats. This region of Res1 is not known to be required for the binding of any factor nor is it required for DNA binding or Cdc10 interaction. However recent one-hybrid analysis has shown this region to contain the major activation domain of Res1 (Stacey, Whitehall, and Jones, unpublished data). In this analysis, Res1 was fused to the DNA-binding domain of the transcription factor Pap1. In a *pap1*$^-$ background, the Pap–Res1 fusion is able to drive expression of the *apt1*$^+$ (*p25*) reporter gene that has Pap1-binding sites in its promoter (see Figure 7). This activation is

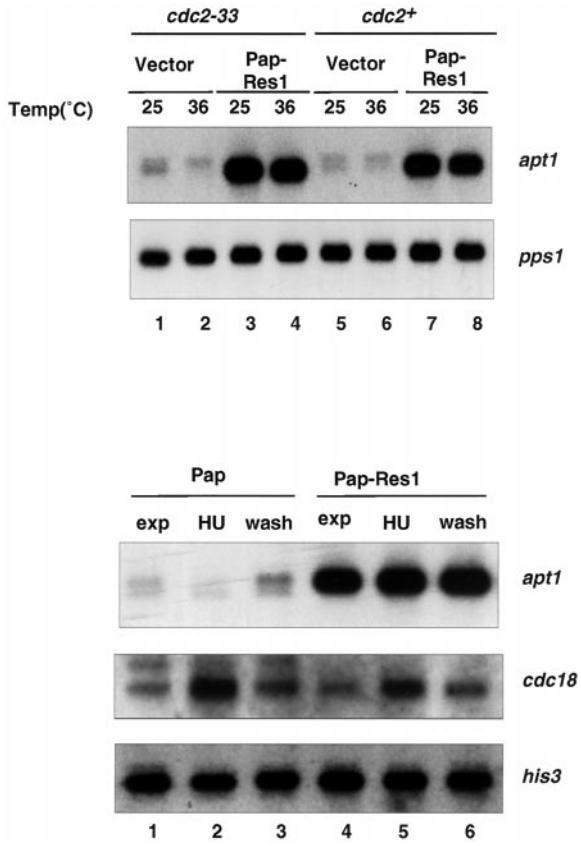

Figure 7. Top, activation of transcription by a Pap–Res1 fusion is not Cdc2 dependent. Δ*pap1 cdc2*$^+$ cells (lanes 5–8) and Δ*pap1 cdc2-33* cells (lanes 1–4) expressing a Pap1 DNA-binding domain (lanes 1, 2, 5, and 6) or a Pap1–Res1 fusion (lanes 3, 4, 7, and 8) were grown at the permissive temperature of 25°C. Cultures were split in two, and incubation was continued at 25 or 36°C for 5 h. Samples were taken and analyzed by Northern blotting with the indicated probes. Bottom, transcription activation by the Pap–Res1 fusion is unaffected by a hydroxyurea block. Δ*pap1* cells were transformed with plasmids expressing the DNA-binding domain of Pap1 or a Pap1–Res1 fusion. Exponentially growing cells (lanes 1 and 4) were arrested by incubation with hydroxyurea for 4 h (lanes 2 and 5). Hydroxyurea was washed out, and incubation was continued in fresh media for 1 h (lanes 3 and 6). RNA prepared from the samples was analyzed by Northern blotting with the indicated probe.

dependent on a short stretch of sequence N-terminal to the ankyrin repeats (Stacey, Whitehall, and Jones, unpublished data). Considering the results described above that show an importance of this region in regulated transcription, we investigated whether the Res1 activation domain itself was regulated in a cell cycle–dependent manner. Initially we asked whether the Cdc2 cyclin–dependent kinase activity was required for its activity. The Pap–Res1 hybrid was expressed in a *cdc2-33 pap1*$^-$ background, and expression of *apt1*$^+$ was compared at the permissive and nonpermissive temperatures (see Figure 7, top). No difference in expression was detected, indicating that the activation potential of Res1 was independent of Cdc2 kinase activity. This supports the findings of Baum *et al.* (1997), which indicated that *cdc2*$^+$ is

not required for the maintenance of MBF activity. Furthermore the transcription activation of Pap–Res1 did not vary in an HU block-and-release experiment (see Figure 7, bottom), further supporting the findings that all components of the MBF complex are required to bring about cell cycle–dependent transcription.

DISCUSSION

We have investigated the nature of the MBF complex during the cell cycle and have found, contrary to some previous suggestions, that the periodicity of MBF activity is not generated by Res subunit switching. Res1 and Res2 do not differentially interact with Cdc10 during the mitotic cell cycle. This is in contrast to the situation during entry into meiosis in which Res1 appears to be excluded from MBF (Ayte *et al.*, 1995). Although our findings suggest that the MBF complex is predominantly heteromeric (Res1–Res2–Cdc10) throughout the cell cycle, we cannot exclude the possibility that different combinations of complexes are present upon promoter DNA. Nonetheless our results, coupled with those of others, show that all three MBF components are present in the complex and all three are required for the action of cell cycle–dependent regulatory mechanisms (Figure 8).

That Res2, like Res1, appears to be an integral part of the MBF complex throughout the cell cycle suggested that Res2 plays a more active role in bringing about transcriptional activation than was thought previously. Indeed, several lines of evidence now suggest that Res2 is more than a negative regulatory subunit. 1) One-hybrid analysis has revealed that Res2 contains a potent activation domain that is important for Res2 function (Stacey, Whitehall, and Jones, unpublished data); 2) overexpression of Res2 in a Δ*res1* background increased *cdc18*$^{+}$ mRNA levels, which is inconsistent with Res2 having only a repressive function; 3) Res2 was found to interact with Cdc10-C4, which mediates high levels of transcription irrespective of the phase of the cell cycle; and 4) the high constitutive activity of the Cdc10-C4 mutant is dependent on the Res2-specific coactivator Rep2. All of these observations imply that Res2 is an active component of the high-activity MBF complex.

The important Res protein domains required for periodic expression of *cdc18*$^{+}$ were determined using a series of hybrid Res1–Res2 molecules. A similar approach has been used to identify regions contributing to other aspects of Res2 specificity (Sturm and Okayama, 1996; Zhu *et al.*, 1997). It is clear that the DNA-binding function does not play a role in generating Res subunit specificity because the DNA-binding domains of these proteins could be swapped without any loss of transcription periodicity. However this does not exclude cell cycle–dependent regulation of DNA binding as a mechanism for generating transcript periodicity. Indeed, DNA binding by the *Saccharomyces cerevisiae* SBF (Swi6–Swi4) complex is cell cycle regulated because SCB sites are occupied specifically during G1 (Harrington and Andrews, 1996; Koch *et al.*, 1996). It will be important to determine whether the DNA-binding function of *S. pombe* MBF is periodic, and one prediction of the findings of this study and those of others (Baum *et al.*, 1997) is that the loss of Res1 or Res2 should disrupt regulated DNA-binding activity.

The results indicate that the extreme C terminus and a domain overlapping the N-terminal ankyrin repeat of Res2 are both important for cell cycle–dependent transcription. The C-terminal region is also critical for the specific function of Res2 in meiosis (Res2 has roles in meiosis that cannot be subsumed by Res1). Although the C-terminal region mediates Cdc10 binding we found no evidence of differential binding of Res proteins in the mitotic cycle. Therefore, the requirement for this domain of Res2 must reflect some other aspect of its function. It is interesting to note that the extreme C-terminal amino acids of Res2 have been shown to confer a requirement for the coactivator Rep2 (Sturm and Okayama, 1996) that itself has a potent activation domain (Tahara *et al.*, 1998). Furthermore one-hybrid analysis has indicated that the activation potential of Res2 when fused to a heterologous DNA-binding domain is inhibited to some degree by the presence of the C-terminal region (Stacey, Whitehall, and Jones, unpublished data). Additionally the phenotype of the *cdc10-C4* strain indicates that the C-terminal region of Cdc10 is also important for downregulation of transcription (McInerny *et al.*, 1995). Also, recent work with Swi6 has revealed

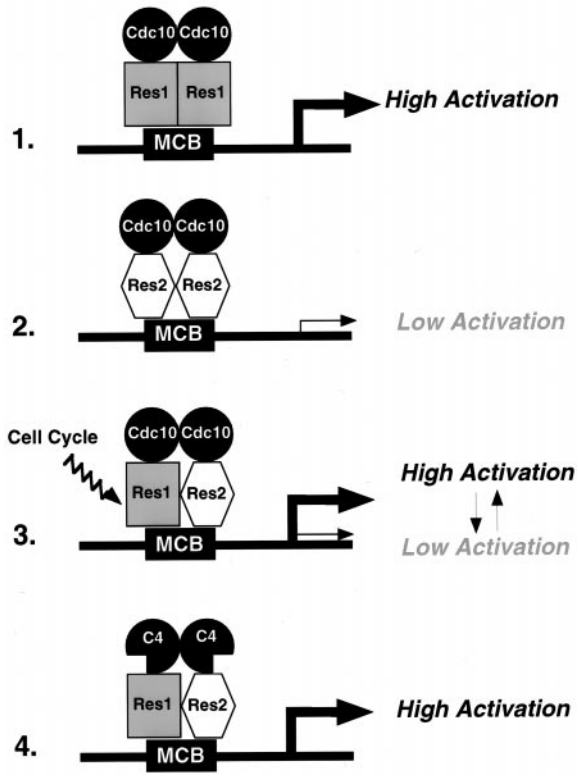

Figure 8. Model for MBF activity in the mitotic cell cycle. Loss of either Res subunit results in MBF complexes that cannot be regulated in response to cell cycle progression. (1) Cdc10–Res1 complexes (Δ*res2* cells) have high activation potentials. (2) Cdc10–Res2 complexes (Δ*res1* cells) have low activation potentials. (3) In wild-type cells the MBF complex contains both Res1 and Res2 throughout the cell cycle. The transactivation potential of this heteromeric complex can be switched from "high" in G1 and S phase to "low" in G2. The C-terminal region of Res2 and a domain overlapping the N-terminal ankyrin repeat are important for this process. (4) The C-terminal region of Cdc10 is also important for regulation because the MBF complex in *cdc10-C4* cells is unable to mediate downregulation despite containing both Res1 and Res2.

some striking parallels with the Res–Cdc10 system; Swi6 contains a potent activation domain that is located just N-terminal to its ankyrin repeats, and furthermore the activity of this domain is revealed when the C-terminal region is deleted (Sedgwick *et al*., 1998). It is possible that activation of MBF is accompanied by a conformational change, which relieves the inhibitory effects of the C termini.

In contrast to Res2 the nature of the C terminus was not important in the context of the Res1 protein. Therefore the presence of the Res2 C-terminal region is important, but it does not appear to matter whether the MBF complex contains one or two copies of it. The most important domain for functional specificity for Res1 seems to be a region overlapping the N-terminal ankyrin repeats. This domain is also important in the context of Res2, and in both proteins this region contains an activation domain (Stacey, Whitehall, and Jones, unpublished data). Furthermore the activation domain of Res1 is necessary for it to be able to suppress the phenotype of *cdc10*^{ts}, and the Res2 activation domain is required for its meiotic function (Stacey, Whitehall, and Jones, unpublished data). When isolated, these domains behave similarly; but in the context of the full-length proteins, activation by Res1 is independent of known coactivators, whereas activation by Res2 is dependent on Rep2. Moreover, the region overlapping the N-terminal ankyrin repeat in Res2 forms part of the binding site for the Rep2 coactivator (Sturm and Okayama, 1996). Thus it is tempting to suggest that Rep2 might have a function in bringing about cyclical transcription by periodically relieving the inhibitory effects of the C terminus upon the activation domain. Arguing against this possibility, however, is the phenotype of Δ*rep2* cells in which *cdc18*$^{+}$ transcription occurs at low levels but still appears to be mildly cyclical (Baum *et al*., 1997).

Although the Res1, Res2, and Cdc10 proteins are highly related to their budding yeast counterparts Swi6, Swi4, and Mbp1, it is becoming increasingly clear that these two sets of proteins are regulated in different ways. In *S. cerevisiae* two complexes are present, with Swi6 being the common component in combination with Swi4 or Mbp1. In this case DNA-binding specificity seems to be important because Swi6–Mbp1 binds to *MluI* cell cycle box elements (ACGCGTNA) whereas Swi6–Swi4 binds predominantly to a related SCB element (ACACGTTT), although some overlap in binding specificity has been described (Partridge *et al*., 1997). Nevertheless, there is no evidence of the formation of a heteromeric Mbp1–Swi4–Swi6 complex. Moreover, cdk activity is directly involved in both the activation and downregulation of Swi6-dependent transcription (Tyers *et al*., 1993; Dirick *et al*., 1995; Koch *et al*., 1996). In contrast there is conflicting evidence for the involvement of *cdc2*$^{+}$ in the regulation of the *S. pombe* MBF complex. An early report demonstrated that the detection of MBF complexes by bandshift experiments was dependent on Cdc2 kinase activity (Reymond *et al*., 1993). However more recent evidence indicates that bandshift detection does not correlate well with measurement of MBF activity as judged by expression of target genes. No MBF complex (as measured by bandshift) is observed in either Δ*res2* or *cdc10-C4* cells, and yet in both cases, target gene expression is high (Baum *et al*., 1997; Zhu *et al*., 1997) Additionally, no bandshift complex is detectable in extracts from Δ*rep2* and Δ*res1* cells in which transcription is low (Baum *et al*., 1997; Zhu *et al*., 1997). Other evidence has implied that in addition to Cdc2, the Pat1 kinase (which is a master regulator of entry into meiosis) is required for the interaction of Res1 with Cdc10 (Caligiuri *et al*., 1997; Connolly *et al*., 1997). However, experiments that separated the effects of inactivating *pat1*$^{+}$ upon entry into meiosis and transcription have shown that *pat1*$^{+}$ is not required for MBF-dependent transcription (Ayte *et al*., 1997). Importantly, recent experiments (Baum *et al*., 1997) have demonstrated that transcriptional activation of *cdc18*$^{+}$ occurs in the absence of Cdc2 kinase activity (as does the subsequent downregulation of MBF activity). We describe in this report that the transcriptional activity of Res1 when fused to a heterologous DNA-binding domain is not Cdc2 dependent. Thus the weight of experimental evidence is not consistent with a direct role of Cdc2-dependent phosphorylation in MBF regulation.

ACKNOWLEDGMENTS

We thank Mark Toone and Paulo Pereira for advice on experiments and criticisms of the manuscript. We also thank Hiroto Okayama and Jose Ayte for providing anti-Res1 antibodies and Janet Quinn for help with immunoprecipitation experiments. This work was funded by the Imperial Cancer Research Fund and the Human Frontiers in Science Program Organization (N.J.).

REFERENCES

Ayte, J., Leis, J.F., and DeCaprio, J.A. (1997). The fission yeast Protein p73^{res2} is an essential component of the mitotic MBF complex and a master regulator of meiosis. Mol. Cell. Biol. *17*, 6246–6254.

Ayte, J., Leis, J.F., Herrera, A., Tang, E., Yang, H., and DeCaprio, J.A. (1995). The *Schizosaccharomyces pombe* MBF complex requires heterodimerisation for entry into S phase. Mol. Cell. Biol. *15*, 2589–2599.

Baum, B., Waurin, J., and Nurse, P. (1997). Control of S-phase periodic transcription in the fission yeast mitotic cycle. EMBO J. *16*, 4676–4688.

Breeden, L. (1996). Start-specific transcription in yeast. Curr. Top. Microbiol. Immunol. *208*, 95–127.

Caligiuri, M., and Beach, D. (1993). Sct1 functions in partnership with Cdc10 in a transcription complex that activates cell cycle START and inhibits differentiation. Cell *72*, 607–619.

Caligiuri, M., Connolly, T., and Beach, D. (1997). Ran1 functions to control the Cdc10/Sct1 complex through Puc1. Mol. Biol. Cell *8*, 1117–1128.

Connolly, T., Caligiuri, M., and Beach, D. (1997). The Cdc2 protein kinase controls Cdc10/Sct1 complex formation. Mol. Biol. Cell *8*, 1105–1115.

Dirick, L., Bohm, T., and Nasmyth, K. (1995). Roles and regulation of Cln-Cdc28 kinases at the start of the cell cycle of *Saccharomyces cerevisiae*. EMBO J. *14*, 4803–4813.

Gutz, H., Heslot, V., Leupold, V., and Lopriemo, N. (1974). *Schizosaccharomyces pombe.* In: Handbook of Genetics 2, ed. R.C. King, New York: Plenum Press, 395–446.

Harrington, L.A., and Andrews, B.J. (1996). Binding to the yeast Swi4,6-dependent cell cycle box CACGAA is cell cycle regulated *in vivo*. Nucleic Acids Res. *27*, 558–565.

Kelly, T.J., Martin, G.S., Forsburg, S.L., Stephen, R.J., Russo, A., and Nurse, P. (1993). The fission yeast *cdc18*$^{+}$ gene product couples S phase to START and mitosis. Cell *74*, 371–382.

Koch, C., Schleiffer, A., Ammerer, G., and Nasmyth, K. (1996). Switching transcription on and off during the cell cycle: Cln/Cdc28 kinases activate bound transcription factor SBF (Swi4/Swi6) at Start, whereas Clb/Cdc28 kinases displace it from the promoter in G2. Genes Dev. *10*, 129–141.

Lowndes, N.F., McInerny, C.J., Johnson, A.L., Fantes, P.A., and Johnston, L.H. (1992). Control of DNA synthesis genes in fission yeast by the cell cycle gene *cdc10*$^+$. Nature *355*, 449–455.

Marks, J., Frankhauser, C., Reymond, A., and Simanis, V. (1992). Cytoskeletal and DNA structure abnormalities result from bypass of requirement for the *cdc10*$^+$ start gene in the fission yeast *Schizosaccharomyces pombe.* J. Cell Sci. *101*, 517–528.

McInerny, C.J., Kersey, P.J., Creanor, J., and Fantes, P.A. (1995). Positive and negative roles for *cdc10* in cell cycle expression. Nucleic Acid Res. *23*, 4761–4768.

Miyamoto, M., Tanaka, K., and Okayama, H. (1994). *res2*$^+$, a new member of the *cdc10*$^+$*/SWI4* family, controls the "start" of mitotic and meiotic cycles in fission yeast. EMBO J. *13*, 1873–1880.

Moreno, S., Klar, A., and Nurse, P. (1991). Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe.* Methods Enzymol. *194*, 795–823.

Nakashima, N., Tanaka, K., Sturm, S., and Okayama, H. (1995). Fission yeast Rep2 is a putative transcriptional activator subunit for the cell cycle "start" function of Res2-Cdc10. EMBO J. *14*, 4794–4802.

Nasmyth, K. (1993). Control of the yeast cell cycle by the cdc28 protein kinase. Curr. Opin. Cell Biol. *5*, 166–179.

Obara-Ishihara, T., and Okayama, H. (1994). A B-type cyclin negatively regulates conjugation via interacting with cell cycle "start" genes in fission yeast. EMBO J. *13,* 1863–1872.

Partridge, J.F., Mikesell, G.E., and Breeden, L.L. (1997). Cell cycle dependent transcription of *CLN1* involves Swi4 binding to MCB like elements. J. Biol. Chem. *272*, 9071–9077.

Reymond, A., Marks, J., and Simanis, V. (1993). The activity of *S. pombe* DSC-1-like factor is cell cycle regulated and dependent on the activity of p43^{cdc2}. EMBO J. *12*, 4325–4334.

Reymond, A., and Simanis, V. (1993). Domains of p85^{cdc10} required for function of the fission yeast DSC-1 factor. Nucleic Acids Res. *21*, 3615–3621.

Sedgwick, S.G., *et al*. (1998). Structural and functional architecture of the yeast cell-cycle transcription factor Swi6. J. Mol. Biol. *281*, 763–775.

Simanis, V., and Nurse, P. (1989). Characterization of the fission yeast *cdc10* protein that is required for commitment to the cell cycle. J. Cell Sci. *92*, 51–56.

Sturm, S., and Okayama, H. (1996). Domains determining the functional distinction of the fission yeast cell cycle "start" molecules Res1 and Res2. Mol. Biol. Cell *7*, 1967–1976.

Tahara, S., Tanaka, K., Yuasa, Y., and Okayama, H. (1998). Functional domains of Rep2 a transcriptional activator subunit for Res2-Cdc10, controlling the cell cycle "Start." Mol. Biol. Cell *9*, 1577–1588.

Tanaka, K., Okazaki, K., Okazaki, N., Ueda, T., Sugiyama, A., Nojima, H., and Okayama, H. (1992). A new *cdc* gene required for S phase entry of *Schizosaccharomyces pombe* encodes a protein similar to the *cdc10*$^+$ and *SWI4* gene products. EMBO J. *11*, 4923–4932.

Tyers, M., Tokiwa, G., and Futcher, B. (1993). Comparison of the *Saccharomyces cerevisiae* G1 cyclins: Cln3 may be an upstream activator of Cln1, Cln2 and other cyclins. EMBO J. *12*, 1955–1968.

White, J.H., Barker, D.G., Nurse, P., and Johnston, L.H. (1986). Periodic transcription as a means of regulatory gene expression during the cell cycle, contrasting modes of expression of DNA ligase in budding and fission yeast. EMBO J. *5*, 1705–1709.

Zhu, Y., Takeda, T., Nasmyth, K., and Jones, N. (1994). *pct1*$^+$, which encodes a new DNA-binding partner for p85^{cdc10}, is required for meiosis in the fission yeast *Schizosaccharomyces pombe*. Genes Dev. *8*, 885–898.

Zhu, Y., Takeda, T., Whitehall, S.K., Peat, N., and Jones, N. (1997). Functional characterization of the fission yeast Start-specific transcription factor Res2. EMBO J. *16,* 1023–1034.