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

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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+/pc11+ (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 cdc10Δ 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 res1Δ 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 Δres2 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 Δres1 cells can be suppressed by overexpression of res2Δ, but the reverse is not true, because the meiotic phenotypes of Δres2 cells cannot be rescued by res1Δ overexpression (Miyamoto et al., 1994; Zhu et al., 1997). The genetic phenotypes lead to a model whereby two dif-
different 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 ∆res1 cells and constitutively high in ∆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 XbaI and infilling with Klenow. After further digestion with NcoI, the 2.4-kb cdc10 fragment was cloned in the Smal and NcoI sites of PRHA41. The plasmid pRHA41 is a derivative of pRep41 and contains three copies of the hemagglutinin (HA) epitope upstream of the NdeI site. Digestion with NcoI and Smal 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'-CATGGATACAAG-GACGATACGACGACAGG-3') and 2 (5'-CATGCGCTTGGTG-CATGGCTCTGGTAAATC-3') and inserting the resulting double-stranded oligonucleotide into the unique NcoI 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'-GCCATGTTCTACATTGCGGACCGACGCGG-3' and 5'-GCCCTGCTCATTAGATTTCTGCGAAGTCCGCTAAA-3' were used to amplify a fragment from cdc10+ that was cleaved with PstI and XbaI. This fragment was subcloned into pT7Cdc10 cleaved with XbaI and PstI. 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 mtf41 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-D18, sc1-1 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 pap1::ura4+, h− 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 NcoI site of pRep41-FLAG Cdc10. A 1.4-kb fragment containing the promoter and the cdc10 sequence to the PstI 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 Nuclon, Boston, MA). cdc18+ and his+ probes for RNA-DNA hybridization have been described by Baum et al. (1997). The cdc11+ probe was produced by PCR amplification from genomic DNA using the following primers: 5'-GTCGTTAATTCATCGATCTCAGTCAGGTC-3' and 5'-GGATGGAATGATGTGTTCC-3'. The psf1+ probe was a 1.2-kb HindIII-EcoRI fragment derived from a CDNA clone in the two-hybrid library vector pGADGH. The psf1+ 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-32P]dCTP by use of a DNA megaprime labeling kit (Amersham, Arlington Heights, IL).

Denatured Cell Extracts

Approximately 2.5 × 108 cells were harvested by centrifugation and washed once in dH2O. 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.
beads (425–600 μm; Sigma, St. Louis, MO) and vortexed twice for 1 min. The beads were washed with 100 ml 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 mg 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, 1 mM benzamidine, and 5 μg/ml each of aprotinin, leupeptin, and pepstatin. Anti-FLAG immunoprecipitations were performed by adding 20 ml 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. 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.

**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.
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. (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 $cyc18^{+}$ mRNA levels. The addition of HU caused an increase in $cyc18^{+}$ levels indicating that activation of the Res–Cdc10 complex had taken place. The levels of $cyc18^{+}$ 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 $cyc10^{+}$-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 $\Delta res2^{+}$ 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 $\Delta cyc10^{+}$ sc1-1 cells. The sc1-1 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 $cyc18^{+}$ expression (Baum et al., 1997). However in the $\Delta cyc10$ sc1-1 background, $cyc18^{+}$ levels are low, and no cell cycle periodicity of transcription is observed (Baum et al., 1997) (see Figure 4A). Expressing full-length Cdc10 increased $cyc18^{+}$ 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 $cyc18^{+}$ transcription both in the presence and absence of HU. We prepared whole-cell extracts from exponentially growing cells and cells blocked by...
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 Δrep2 cells (Nakashima et al., 1995; Baum et al., 1997). Moreover, low cdc18**\(^+\)** mRNA levels are dependent on Res2, because transcription is constitutively high in Δrep2 Δres2 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 Δrep2 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 Δres1 cells and constitutively high in Δres2 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 Δres1 cells cdc18**\(^+\)** mRNA levels are low both before and after HU addition. Furthermore, cdc18**\(^+\)** expression levels are high in exponentially growing cultures of Δres2 cells, and addition of HU has no effect. Also, synchronization of cdc10-C4 cells has shown MBF-dependent
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 \(\Delta\)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 \(\Delta\)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 \(\Delta\)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 \(\Delta\)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 \(\Delta\)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 Δres1 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 Δres2 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 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
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 Δres1 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 (McInerney et al., 1995). Also, recent work with Swi6 has revealed...
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<sup>rep2</sup>, 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 Δrep2 cells in which cdc18<sup>trans</sup> 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 <i>S. cerevisiae</i> 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 <i>Muil</i> cell cycle box elements (ACCGTNTA) 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 down-regulation 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<sup>trans</sup> in the regulation of the <i>S. pombe</i> 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 Δres2 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 Δrep2 and Δres1 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; Connelly et al., 1997). However, experiments that separated the effects of inactivating pat1<sup>trans</sup> upon entry into meiosis and transcription have shown that pat1<sup>trans</sup> 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<sup>trans</sup> 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.).

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