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The *Schizosaccharomyces pombe* MBF Complex Requires Heterodimerization for Entry into S Phase

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In *Schizosaccharomyces pombe*, MBF is a DNA-binding complex suspected to activate the transcription of genes necessary for entry into S phase. The MBF complex contains both p85^{cdc10} and p72$^{res1/sct1}$. To obtain a better understanding of how the MBF complex regulates gene expression at the G_1/S transition, we have performed a genetic analysis of p72^{res1}. We determined that p72^{res1} can bind specifically to the *cdc22* promoter, when analyzed by gel mobility shift assay, and that the N-terminal 157 amino acids of p72^{res1} are sufficient for this specific binding. When overexpressed in vivo, a fragment of p72^{res1} containing this DNA-binding domain could rescue a strain carrying a temperature-sensitive *cdc10* allele at the restrictive temperature as well as a strain with a *cdc10* null allele. We also determined that the C-terminal region of p72^{res1} is necessary and sufficient for binding to p85^{cdc10}. Overexpression of the cdc10-binding domain of p72^{res1} leads to a G_1 arrest with a *cdc* phenotype and a decrease on MBF activity. Overexpression of full-length p72^{res1} also leads to a growth arrest that can be rescued by overexpression of p85^{cdc10}. These results imply that the MBF activity in vivo is dependent on the interaction of p85^{cdc10} with p72^{res1}.

Genetic and biochemical analysis of the fission yeast *Schizosaccharomyces pombe* has led to the identification of several genes that regulate entry into S phase of the cell cycle. Depending on environmental conditions, *S. pombe* can enter either premitotic or premeiotic DNA synthesis or remain in stationary phase. This decision point is called Start. The cell cycle-dependent kinase cdc2 is required for the passage through Start (34–36). Another essential gene, *cdc10*, is also required for the completion of Start in *S. pombe* (2, 3, 35). Yeast cells carrying a temperature-sensitive *cdc10* allele become arrested at Start when grown at the restrictive temperature (2, 35, 50).

The *cdc10* gene product, p85^{cdc10}, has been demonstrated to be a component of the specific DNA-binding complex MBF (*Mlu*I cell cycle box-binding factor), which is also known as DSC1 (DNA synthesis control complex) (25, 40). The MBF complex binds specifically to its target DNA sequence in a cell cycle-dependent manner, with its DNA-binding activity peaking during the G_1/S transition and diminishing during G_2/M (2a, 25, 40). Furthermore, by using a synthetic promoter containing three tandem *Mlu*I sites, it has been shown that the MBF complex can activate the transcription of a reporter gene after Start (25). MBF DNA-binding activity participates in the cell cycle-specific expression of at least three genes necessary for entry into S phase: *cdc22*, which encodes the regulatory subunit of ribonucleotide reductase (11, 25); *cdc18* (17); and *cdt1* (15).

Recently, p72$^{res1/sct1}$ has been identified as an extragenic suppressor of *cdc10* and a component of the MBF complex (8, 52). While *cdc10* is an essential gene, causing a *cdc* phenotype and a G_1/S arrest when functionally inactivated, *res1* can be deleted and viability can be maintained under certain conditions (52). Two recent reports detail the cloning of a new partner of p85^{cdc10}, the *res2*/*pct1* gene product (30, 56). The *res2*/*pct1* gene product, p73$^{res2/pct1}$, can also form a heterocomplex with p85^{cdc10} (56). The expression of *res2*/*pct1* is induced during nitrogen starvation, premeiotic DNA synthesis, and conjugation (30). In addition, p73$^{res2/pct1}$ apparently also recognizes the MCB elements (*Mlu*I cell cycle box, ACGCGT), found in the promoters of *cdc18* and *cdc22*, and may provide a role similar to that of p72^{res1} in the transcriptional activation of genes required for premeiotic DNA synthesis (30).

In *Saccharomyces cerevisiae*, at least two DNA-binding activities, MBF and SBF, are involved in the cell cycle-dependent transcription of genes necessary for entry into S phase (1, 5, 10, 12, 33, 37, 54). The *S. cerevisiae* MBF complex contains Mbp1 and Swi6 and, similarly to MBF in *S. pombe*, recognizes the MCB elements found upstream of genes involved in DNA synthesis (19, 54; see reference 24 for a review). At least 30 genes have been found in *S. cerevisiae* to be regulated in a cell cycle-dependent manner by the MBF complex. Some of these genes encode intrinsic enzymatic components necessary for DNA synthesis (POL1, POL2, etc.), and others encode regulators of the initiation of S phase, including CLB5, CLB6, and CDC6 (41, 47). The SBF complex contains Swi4 and Swi6 and binds to the SCB elements (Swi4/Swi6 cell cycle box, CAC GAAA) (1, 5, 10, 12, 23, 31, 33, 37). The SCB element is found upstream of several genes expressed in late G_1, including the genes encoding HO endonuclease and the G_1 cyclins CLN1, CLN2, and HCS26 (6, 33, 37). Expression of these genes is dependent on the activity of Swi4 and Swi6.

p85^{cdc10}, p72^{res1}, and p73^{res2} show extensive sequence homologies to Swi6, Swi4, and Mbp1 in three regions (1, 2, 8, 19, 30, 49, 52, 56). There is a high degree of homology among the N-terminal regions of p85^{cdc10}, p72^{res1}, p73^{res2}, Swi4, and Mbp1. This domain of Swi4 has been demonstrated to be able to bind directly to the SCB element (1, 39, 49). Furthermore, UV cross-linking experiments have also demonstrated that Mbp1 also can bind directly to DNA (19). The high degree of similarity among these genes suggests that they define a new class of DNA-binding proteins. However, the ability of either

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TABLE 1. *S. pombe* strains used in this study

Strain	Genotype
SP223	*h⁺ leu1-32 ura4-D18 ade6-M210*
JL1	*h⁻ cdc10-129 leu1-32 ura4-D18 ade6-M210*
SP1070	*h⁺/h⁹⁰ cdc10::ura4⁺/cdc10⁺ leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M210/ade6-M210*
K123-14D	*h⁻ res1::ura4⁺ leu1-32 ura4-D18 ade6-M210*

p72^{res1} or p85^{cdc10} to bind to directly to DNA has not yet been demonstrated. A second region of homology exists between the C-terminal regions of p85^{cdc10} and Swi6. This domain of Swi6 has been demonstrated to be necessary and sufficient for binding to Swi4 (1). Furthermore, each of these proteins contains two copies of the 33-amino-acid domain known as the cdc10/Swi6 motif or ankyrin repeat (7). This motif has been identified in several other DNA-binding proteins, including the GA-binding protein (53), dorsal (18), and NF-κB (4) as well as the cell cycle-regulatory protein p16^{INK4} (48; see reference 9 for a review). This motif has been implicated in protein-protein interactions, although its role in cdc10-res1-res2 or Swi6-Swi4-Mbp1 interactions has not been well defined.

We have performed a genetic and biochemical analysis of p72^{res1} in order to understand how the MBF complex participates in passage through Start. Here we report that the N-terminal region of p72^{res1} can specifically bind to its target DNA site and that overexpression of this region alone is necessary and sufficient for complementation of the lethal phenotype of a *cdc10* null strain. In addition, the C-terminal domain of p72^{res1} is necessary and sufficient for specific binding to p85^{cdc10}. Furthermore, we found that overexpression of either full-length p72^{res1} or its C-terminal domain leads to a growth arrest in G_1 with a *cdc* phenotype. These results suggest that under physiological conditions, p85^{cdc10} is required for the correct activation of the DNA-binding activity of p72^{res1} in the MBF complex.

MATERIALS AND METHODS

Strains and media. All of the *S. pombe* strains used (Table 1) are isogenic to the wild-type strain 972h⁻ and were transformed by the lithium acetate method (32). Media were prepared as previously described (32).

DNA manipulation. Res1 mutants were prepared by PCR amplification of a *res1* cDNA with *Pfu* DNA polymerase (Stratagene), using the following oligonucleotides: full-length p72^{res1}, 551 (5′-GGGGGATCCATGTATAACGACCAAATACATAA-3′) and 550 (5′-CCCGAATTCTTAAGATCCACTTTGATCTGT-3′); Δ398, 707 (5′-GGGGGATCCTCTTCGAAAGACATGTCTTTTCC-3′) and 550; 398-590, 707 and 776 (5′-CCCGAATTCCAGCTGTAAAAAGATCAACTTCG-3′); 398-544, 707 and 777 (5′-CCCGAATTCAATTGATGAAGCTGAACTGTTTC-3′); Δ448, 842 (5′-GGGGGATCCACTCACCAAACCCTTCCCAATGT-3′) and 550; 448-590, 842 and 776; 448-544, 842 and 777; Δ498, 708 (5′-GGGGGATCCTGGAATGAACGTACCAATAATGAC-3′) and 550; and 398-498, 707 and 752 (5′-CCCGAATTCCCATAATCTTTGGCAAGTTTCAGO-3′).

To insert a point mutation in amino acid 56 of p72^{res1} (lysine 56 for glutamic acid [E56→K]), the cDNA for *res1* was amplified with *Pfu* DNA polymerase and primers 551 and 679 (5′-GAGGACAAGGATTCTGGAAAAATTCGC-3′) or primers 550 and 680 (5′-GTCCCTTTTGAGCGAATTTTTCCAGAATC-3′). After 25 cycles of amplification, aliquots of the two reaction mixtures were mixed and reamplified by using primers 551 and 550. All of the amplified DNAs were subcloned into Bluescript II SK⁻ as *Bam*HI-*Eco*RI fragments, sequenced by the dideoxynucleotide method (45), and subcloned into pGEX-2TK (16).

The deletions 1→192, 1→192K, 1→157, 1→157K, 1→79, and 1→45 were made by digesting full-length *res1* or the point mutant (E56→K) with *Hin*dIII, *Xba*I, *Kpn*I, or *Sac*I and subcloning into pGEX-2TK. ΔB→S, ΔS→K, and ΔB→H, which represent deletions of the first 45 residues, from residues, 45 to 79, and of the first 192 residues, respectively, were prepared by digesting pGEX-2TK/*res1* with *Bam*HI and *Sac*I, *Sac*I and *Kpn*I, or *Bam*HI and *Hin*dIII, filling in with Klenow fragment, and ligating.

The expression of glutathione *S*-transferase (GST) fusion proteins in *Escherichia coli* and purification on glutathione-Sepharose beads was carried out as described previously (51). The concentration of the purified fusion proteins was determined by the Bradford method (Bio-Rad protein assay kit), and the purity was assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining or Western blotting (immunoblotting) with anti-GST antibody DG122 (Upstate Biotechnology Inc.).

To generate the hemagglutinin (HA)-tagged proteins, we cloned upstream of the different cDNAs (into the *Bam*HI site) an oligonucleotide encoding the HA epitope (844 [5′-GATCCATGGCCTACCCCTACGACGTGCCCGACTACGCCTCCCTCC-3′] or 843 [5′-GATCGGAGGGAGGCGTAGTCGGGCACGTCGTAGGGGTAGGCCATG-3′]). The resulting plasmids were confirmed by sequencing, digested with *Bam*HI and *Hin*cII, and subcloned in the expression vector pREP1 (26, 27).

pART1/cdc10 was constructed by insertion of the *cdc10* cDNA into pART1 (28) as a *Bam*HI-*Sma*I fragment. pARTU/cdc10 was constructed as a derivative of pART1/cdc10, replacing the *LEU2* gene with *URA4*, as *Hin*dIII fragments.

Antibodies. Monoclonal antibodies to p72^{res1} and p85^{cdc10} were generated by injecting mice with either GST-res1 or GST-cdc10 according to standard procedures (14). Hybridoma supernatants were screened for the ability to immunoprecipitate ^{35}S-labeled p72^{res1} or p85^{cdc10} as well as the ability to supershift the MBF complex when analyzed by electrophoretic mobility shift assays (EMSA) (see below).

Protein extraction and immunoprecipitations. Exponentially growing cells were harvested by centrifugation, washed once with distilled water, resuspended in lysis buffer (50 mM Tris [pH 7.5], 120 mM KCl, 5 mM EDTA, 0.1% Nonidet P-40, 10% glycerol, 10 mM NaF, 1 mM β-glycerophosphate, 0.1 mM sodium vanadate, 1 mM dithiothreitol, 0.5 mM phenylmethysulfonyl fluoride, 5 μg each of leupeptin, chymostatin, pepstatin, and aprotinin per ml) and disrupted by vortexing with glass beads for 50 s. Lysates were cleared by centrifugation at 14,000 × *g* for 15 min at 4°C. Protein concentration was determined by the Bradford method.

For the in vitro binding experiments, p85^{cdc10} was synthesized by coupled transcription-translation (TNT system; Promega) in the presence of [^{35}S]methionine. Radiolabeled p85^{cdc10} was mixed with 500 ng of GST fusion proteins bound to glutathione-Sepharose in NET-N (20 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg each of leupeptin and aprotinin per ml) and incubated at 4°C for 30 min. After five washes with 500 μl of NET-N, the proteins were separated in an SDS–7.5% polyacrylamide gel (20) and detected by fluorography (14).

For the in vivo binding experiments, extracts were incubated for 1 h at 4°C with anti-HA monoclonal antibody 12CA5 (Boehringer Mannheim). Immunoprecipitates were isolated with protein A-coupled Sepharose and washed four times in NET-N. Proteins present in the immunoprecipitates were resolved in an SDS–6% polyacrylamide gel, transferred to polyvinylidene difluoride membranes, and incubated with a mix of anti-Cdc10 monoclonal antibody tissue culture supernatants (YS140 and YS144). Immunoreactive bands were detected with alkaline phosphatase-labeled secondary antibody (14).

EMSA. The *cdc22* promoter fragment was amplified by PCR from genomic DNA prepared from strain 972h⁻ using *Pfu* DNA polymerase and the primers 174 (5′-CGGGATCCCTGTTTACGACTGAATGTA-3′) and 47 (5′-CGGAATTCAATCTCATAGAGCAGGTTG-3′), digested with *Eco*RI and *Bam*HI, and subcloned in Bluescript II SK⁻. This fragment contains one *Mlu*I site (ACGCGT) and three *Mlu*I-like sites (NCGCGN). Binding reactions were performed as described by Lowndes et al. (25), using 0.5 ng of ^{32}P-labeled 132-bp *cdc22* promoter probe and 10 μg of native protein extract or 30 to 200 ng of GST fusion proteins. Binding reactions (20-μl mixtures) were allowed to proceed for 20 min on ice. When noted, 1 μl of anti-HA (12CA5), 2 μl of anti-p85^{cdc10} (YS140 or YS144), or 4 μl of anti-p72^{res1} (RY115) monoclonal antibody was added to the binding reaction mixture. When indicated, a 50-fold molar excess of unlabeled competitor (3×MluI [3×ACGCGT] or 3×Mut [3×ACATGT]) was added to the binding reaction mixture (25). The DNA-protein complexes were analyzed by electrophoresis through a 4% polyacrylamide (40:1 cross-linking) gel in 0.5 × Tris-borate-EDTA at 4°C and 200 V of constant voltage.

RNA analysis. RNA was prepared by glass bead lysis in the presence of guanidinium thiocyanate, using the RNAzol B kit (Tel-Test, Inc.). Equal amounts of RNA, as measured by optical density at 260 nm, were loaded in formaldehyde agarose gels (44) and transferred to GeneScreen Plus membranes (Dupont). Hybridization and washes were performed as recommended by the manufacturer. *cdc18* cDNA was amplified by reverse transcription-mediated PCR using primers 883 (5′-GGGGGATCCATGTGTGAAACTCCAATAGGTTG-3′) and 884 (5′-CCCGAATTCGGTTATATAATTCATTGGCGGAC-3′). Probes were prepared with [α-^{32}P]dCTP by random priming (Boehringer Mannheim).

Flow cytometry analysis and DAPI staining. Flow cytometry was performed on ethanol-fixed cells as previously described (46), using the FACScan system and the LYSYS II cell cycle analysis program (Becton Dickinson); 10,000 cells were analyzed at each time point. For the photomicrographs, cells were fixed in methanol and stained with 4′,6-diamidino-2-phenylindole (DAPI) as described previously (32).

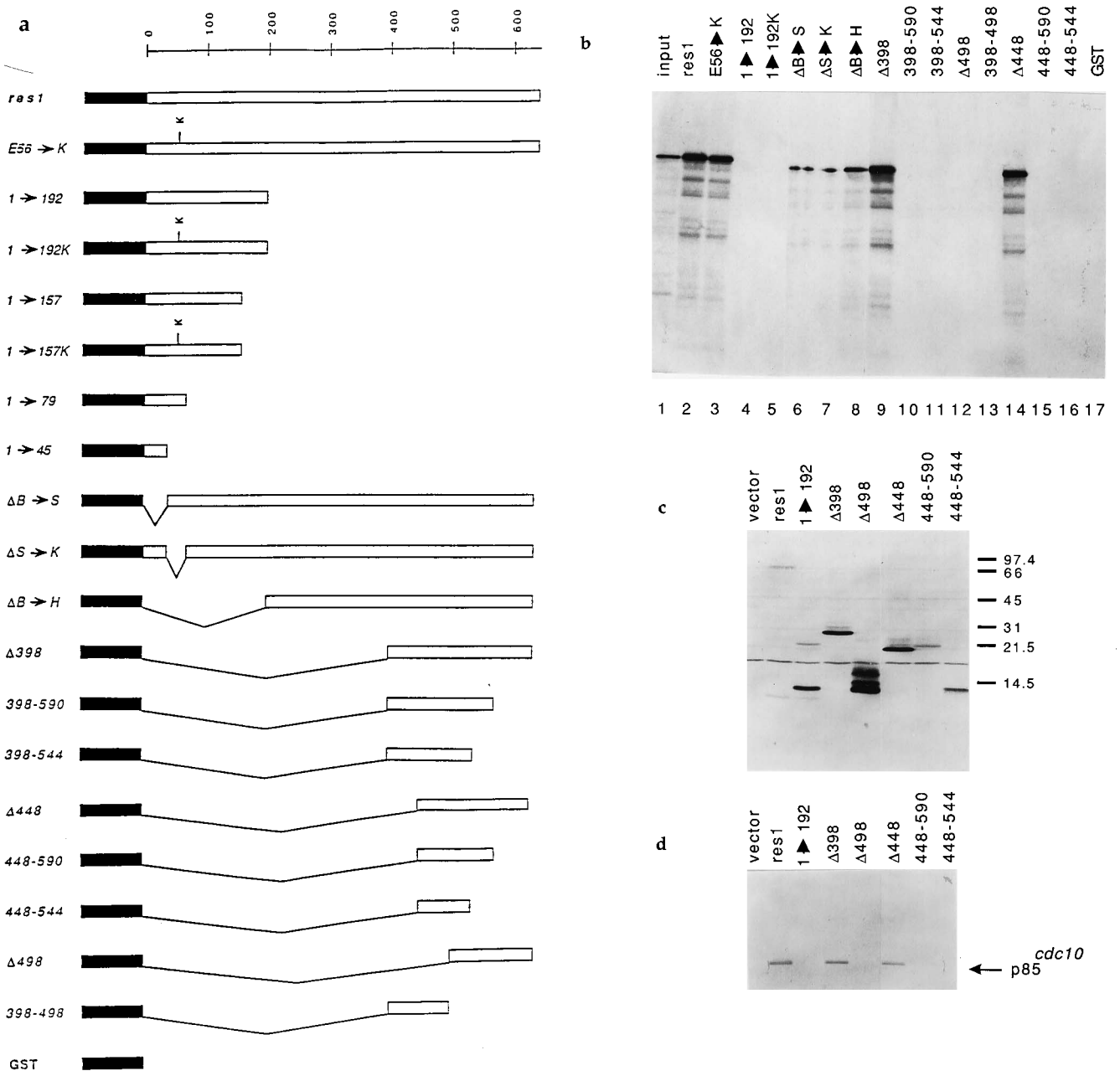


FIG. 1. p72^{res1} binds to p85^{cdc10} through its C-terminal region. (a) Schematic of the GST-res1 constructs. A filled box represents the GST portion, and an open box represents the res1 peptide of each fusion protein expressed in *E. coli*. E56→K, 1→192K, and 1→157K represent constructs in which glutamine 56 is replaced by lysine. ΔB→S, ΔS→K, and ΔB→H represent deletions of the first 45 residues, from residues 45 to 79, and of the first 192 residues of p72^{res1}, respectively. The scale at the top represents amino acid residues. (b) ^{35}S-radiolabeled p85^{cdc10} (lane 1) was incubated with derivatives of bacterially produced GST-res1, precipitated with glutathione-Sepharose beads, separated in SDS–7.5% polyacrylamide gels, and autoradiographed. (c) Wild-type *S. pombe* cells were transformed with plasmids that directed the expression of full-length p72^{res1} or deleted derivatives, under the control of the inducible *nmt* promoter. Cells were grown in medium without thiamine (promoter on) for 24 h until early log phase (2×10^6 to 6×10^6 cells per ml). Extracts (100 μg) were prepared and separated in an SDS–15% polyacrylamide gel and analyzed by Western blotting with monoclonal antibody 12CA5 to detect expression of the HA-tagged proteins. The positions of molecular mass markers are indicated on the right in kilodaltons. (d) Aliquots (400 μg) of the extracts used for panel c were immunoprecipitated with the anti-HA antibody. Immunoprecipitates were separated in an SDS–6% polyacrylamide gel and analyzed by Western blotting with anti-Cdc10 monoclonal antibodies YS140 and YS144 as probes.

RESULTS

p72^{res1} binds p85^{cdc10} through its carboxy-terminal region. To determine whether p85^{cdc10} and p72^{res1} bind directly to each other, ^{35}S-radiolabeled *cdc10* gene product was produced in a cell-free transcription-translation system (Fig. 1b, lane 1) and incubated with bacterially produced p72^{res1} fused to GST. As shown in Fig. 1b, p85^{cdc10} could be coprecipitated by GST-res1 but not by GST alone (compare lanes 2 and 17). Deletion analysis demonstrated that Δ448, containing the C-terminal 188 amino acids of p72^{res1}, was sufficient for binding to p85^{cdc10} (lane 14). Any further deletion of this region within p72^{res1}, from either the N or C terminus, abolished any binding to

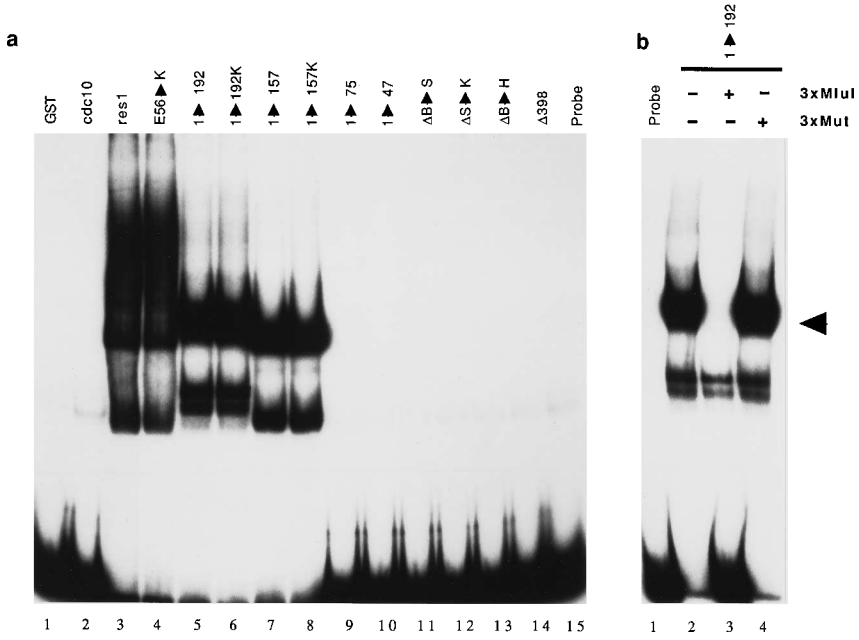


FIG. 2. The N-terminal domain of p72^{res1} binds to the *cdc22* promoter. (a) GST-res1 fusion proteins were expressed in *E. coli*, purified on glutathione-Sepharose beads, and eluted (16). An aliquot of each (33 ng in lanes 3 to 8 and 200 ng in lanes 1, 2, and 9 to 14) was analyzed by EMSA for binding to the *cdc22* promoter as described in Materials and Methods. (b) Unlabeled DNA was added in a 50-fold molar excess to the binding reaction mixtures prior to the addition of 1→192. The arrowhead points to the specific DNA-binding complex.

p85^{cdc10} (lanes 12, 15, and 16). Expression of each GST construct was confirmed by Coomassie blue staining. Under these assay conditions, the ankyrin domains did not appear to contribute to the ability of p72^{res1} to bind to p85^{cdc10} (lanes 8 and 9).

To test the ability of p72^{res1} to bind to p85^{cdc10} in vivo, we cloned some of these *res1* constructs into the high-copy-number yeast vector pREP1 (27), which directs expression by the inducible *nmt* (no message in thiamine) promoter (26). An influenza virus HA epitope was also added at the N-terminal end of each construct to distinguish it from the endogenously expressed p72^{res1} (55). Whole-cell lysates from wild-type yeast strain SP223, induced to overexpress either full-length or truncated forms of p72^{res1}, were separated in an SDS-polyacrylamide gel and transferred to a polyvinylidene membrane. The membrane was probed with the anti-HA antibody 12CA5 (55). As shown in Fig. 1c, each of the HA-res1 constructs directed the expression of a protein of the expected size. Truncated forms of various sizes were also observed for some of these constructs. To determine the region of p72^{res1} that could bind to endogenously expressed p85^{cdc10}, immunoprecipitations were performed with the anti-HA monoclonal antibody, separated by SDS-PAGE, transferred to a polyvinylidene membrane, and probed with an anti-p85^{cdc10} monoclonal antibody (Fig. 1d). Consistent with the results obtained from the in vitro analysis, only full-length res1, Δ398, and Δ448, containing the last 188 residues, could be found associated with p85^{cdc10} in vivo. Although 1→192, Δ498, 448-590, and 448-541 were expressed, as shown in Fig. 1c, they were unable to coprecipitate p85^{cdc10}.

p72^{res1} binds to the *cdc22* promoter through its amino-terminal domain. To determine the DNA-binding domain within the MBF complex, bacterially produced full-length p72^{res1} and p85^{cdc10}, fused to GST, were prepared. Expression of every fusion protein was confirmed by Western blotting with an anti-GST monoclonal antibody (data not shown). The ability of purified fusion proteins to bind to a 132-bp fragment of the *cdc22* promoter, previously demonstrated to bind the MBF complex (25), was analyzed by EMSA. This fragment of the *cdc22* promoter contains one *Mlu*I site (ACGCGT) and three *Mlu*I-like sites (NCGCGN) (25). As shown in Fig. 2a, GST-res1, but neither GST-cdc10 nor GST alone, could bind to the *cdc22* promoter (lanes 1 to 3).

Deletion mutants of p72^{res1} were prepared and assayed by EMSA to map the specific DNA-binding domain. Only those proteins that contained at least the first 157 residues of p72^{res1} demonstrated DNA-binding activity (Fig. 2a, lanes 3 to 8). Several deletions within this region completely abolished the DNA-binding activity of the fusion protein (lanes 9 to 14). This finding suggested that the DNA-binding activity of p72^{res1} was contained entirely within the first 157 residues. We also tested the effect on the DNA-binding activity of the E56→K point mutation. This construct corresponds to the *sct1-b1* allele (1→192K; Fig. 1a), which was originally isolated as an extragenic suppressor of a *cdc10* temperature-sensitive strain (8). We could not detect any differences in the DNA-binding activities of the wild-type and mutated proteins, expressed either as full length or as C-terminal truncations, when assayed by EMSA (compare lanes 4, 6, and 8 with lanes 3, 5, and 7). The gel mobility shift for the wild-type full-length p72^{res1} and the point mutant E56→K (lanes 3 and 4) smeared over a broad area compared with the truncated N-terminal constructs (lanes 5 to 8). This effect may be due to the presence of proteolytic fragments of the full-length protein that retain DNA-binding activity.

The specificity of the DNA-binding activity of the fusion proteins for the *Mlu*I sites contained within the *cdc22* promoter was determined by competition analysis. As shown in Fig. 2b, the specific DNA-binding activity of the 1→192 construct for the *cdc22* promoter is demonstrated by competition with an unlabeled fragment of DNA containing three *Mlu*I sites (3×MluI) but not by a DNA fragment with mutated *Mlu*I

sites (3×Mut). The large arrowhead in Fig. 2b points to the specific DNA-binding complex that is competed for by 3×MluI but not by 3×Mut. This result demonstrates that bacterially produced 1→192 can bind specifically to the *Mlu*I sites within the *cdc22* promoter.

The DNA-binding domain of p72^{res1} is able to override *cdc10* mutations. Tanaka and colleagues found that overexpression of wild-type p72^{res1}, driven by the moderately strong simian virus 40 promoter, could rescue a *cdc10* null strain (52). Similarly, we found that overexpression of p72^{res1}, driven by the constitutive *adh* promoter, could also rescue a *cdc10* null strain (data not shown). We tested whether expression of the DNA-binding domain of p72^{res1} alone was sufficient to rescue strains carrying *cdc10* mutations. As shown in Fig. 3a, expression of high levels of 1→192, under the control of the inducible *nmt* promoter (pREP1-1→192), rescued both a temperature-sensitive *cdc10* strain (*cdc10*$^{-}$129), when grown at the restrictive temperature (35.5°C), and a null strain (*cdc10*::*ura4*). The temperature-sensitive strain was also rescued by the constitutive expression of p85^{cdc10} (pART1-cdc10) (Fig. 3a). Similarly, the *cdc10* null strain (*cdc10*::*ura4*) could also be rescued by pART1-cdc10 (data not shown) (Fig. 3a). This *cdc10* null strain, carrying pREP1-1→192, grew well in liquid medium without thiamine. However, when thiamine was added to the medium to repress the expression of 1→192 in the *cdc10* null strain, nearly all of the cells developed a *cdc* phenotype within 6 h and appeared to be nonviable by 18 h. Flow cytometry analysis demonstrated a G_1 block, with most cells shifting from 2N to 1N DNA content within 6 h after addition of thiamine (Fig. 3b). Although aliquots were also taken at 14 and 18 h after the addition of thiamine, the flow cytometry analysis data were difficult to interpret because of the elevated number of dead cells. Therefore, in the absence of functional p85^{cdc10}, the expression of 1→192 was necessary for growth. To determine the effect on MBF-dependent gene expression, Northern (RNA) blots were prepared with total RNA isolated from this strain at several intervals after the addition of thiamine. As demonstrated in Fig. 3c and d, the levels of *cdc22* and *cdc18* mRNAs decreased soon after thiamine addition. Notably, the level of *cdc18* mRNA was reduced by 50% within 2 h, while the *cdc22* mRNA level tapered gradually to a level of 40% by 18 h.

The DNA binding and cdc10 binding of p72^{res1} are required for MBF complex formation. To determine if the overexpression of p72^{res1} and some of the various truncated forms listed above could affect MBF DNA-binding activity in vivo, extracts were prepared from wild-type cells (SP223) induced to express high levels of these proteins and assayed by EMSA using the *cdc22* promoter as a probe. Extracts prepared from cells carrying the backbone vector, pREP1 (Fig. 4, lanes 1 to 6), demonstrated a specific MBF complex (large arrowhead). Monoclonal antibodies against p85^{cdc10} or p72^{res1} were able to supershift the MBF complex, while the anti-HA antibody had no effect on the native endogenous complex (lanes 2 to 4). The DNA-binding specificity of this complex was demonstrated by competition with unlabeled 3×MluI DNA and not by 3×Mut (lanes 5 and 6). When HA-tagged full-length p72^{res1} was expressed, there was a slight increase in the amount of MBF complex (compare lanes 1 and 7). The MBF complex could be supershifted by the anti-HA antibody as well as by the anti-res1 antibody. An additional DNA-protein complex was detected in the presence of the anti-HA antibody that was not evident in the vector-only control (noted by an asterisk). This DNA-binding activity could be competed for with the 3×MluI competitor (lane 10).

The DNA-binding activity of the endogenous MBF complex was not affected in cells that overexpressed the DNA-binding domain of p72^{res1}, 1→192 (lane 11). The anti-HA antibody did not affect the MBF complex but did increase the amount of the faster-migrating complex also observed when the full-length HA-tagged protein was expressed (compare lanes 12 and 9). This faster-migrating complex may have been derived from proteolytic fragments resulting from the overexpression of both the full-length and 1→192 constructs (see also Fig. 1c, lanes 2 and 3). Notably, the addition of anti-Cdc10 antibodies in addition to the anti-HA antibody did not affect the faster-migrating band (noted by the asterisk) but did supershift the MBF complex (data not shown).

In contrast, extracts prepared from yeast cells that overexpressed either Δ398 or Δ448 contained reduced levels of MBF DNA-binding activity (Fig. 4; compare lanes 14 and 17 with lane 1). These N-terminal truncations, unable to bind to DNA, may have bound to the endogenous p85^{cdc10} and prevented it from binding to the endogenous p72^{res1}, thereby decreasing the overall MBF DNA-binding activity. Therefore, both the DNA-binding domain and the cdc10-binding domain of p72^{res1} are required for the DNA-binding activity of the MBF complex.

Overexpression of full-length p72^{res1} or C-terminal truncations induces growth arrest in wild-type *S. pombe*. When testing the ability of p72^{res1} to bind to p85^{cdc10} and form the MBF complex in vivo (Fig. 1d and 4), we observed that overexpression of some of these constructs led to a decrease in the growth rate. To investigate this further, wild-type *S. pombe* cells (SP223), transformed with plasmids that direct the expression of full-length p72^{res1}, 1→192, or the N-terminal truncations Δ398 and Δ448, under the control of the inducible *nmt* promoter, were grown on minimal medium plates in the presence or absence of thiamine (Fig. 5a). Cells induced to express Δ398 or Δ448 were unable to form colonies (Fig. 5a). Overexpression of full-length p72^{res1} led to the formation of small colonies. However, these small colonies were unable to grow upon restreaking on plates without thiamine. Conversely, the expression of the DNA-binding domain, 1→192, or vector alone did not visibly affect viability.

When colonies were grown in liquid culture and observed microscopically, overexpression of the DNA-binding domain (1→192) did not affect the morphology compared with vector alone (Fig. 5b; compare panels 1 and 4). However, overexpression of Δ398 or Δ448 led to a characteristic *cdc* phenotype within 24 h after the removal of thiamine (Fig. 5b, panels 5 and 6). DAPI staining demonstrated a *cdc* phenotype with a single, centrally located nucleus. Notably, full-length p72^{res1} also produced a *cdc* phenotype, but it was delayed until 30 h after induction of its expression. DAPI staining of these cells 24 h after the induction of the expression revealed a punctuated DNA-staining pattern without an increase in cell length (Fig. 5b, panel 2). However, when the induction of full-length p72^{res1} was allowed to continue for several hours more, the cells increased in length, until a *cdc* morphology was observed (panel 3). DAPI staining revealed that most cells retained a single, centrally located nucleus.

To determine if the growth arrest resulted from a block at a specific point in the cell cycle, we analyzed cells for DNA content by flow cytometry before and at several time points after the depletion of thiamine from the medium (Fig. 5c). Consistent with the observations noted above, overexpression of the DNA-binding domain alone, 1→192, had only a minor effect on the cell cycle distribution compared with the effect of vector alone. However, when thiamine was removed from the medium and Δ448 and Δ398 were expressed, the DNA content of these cells shifted from 2N to 1N. The increase in the number of cells in G_1 phase began by 22 h from the removal of thiamine, and by 30 h the shift was nearly complete.

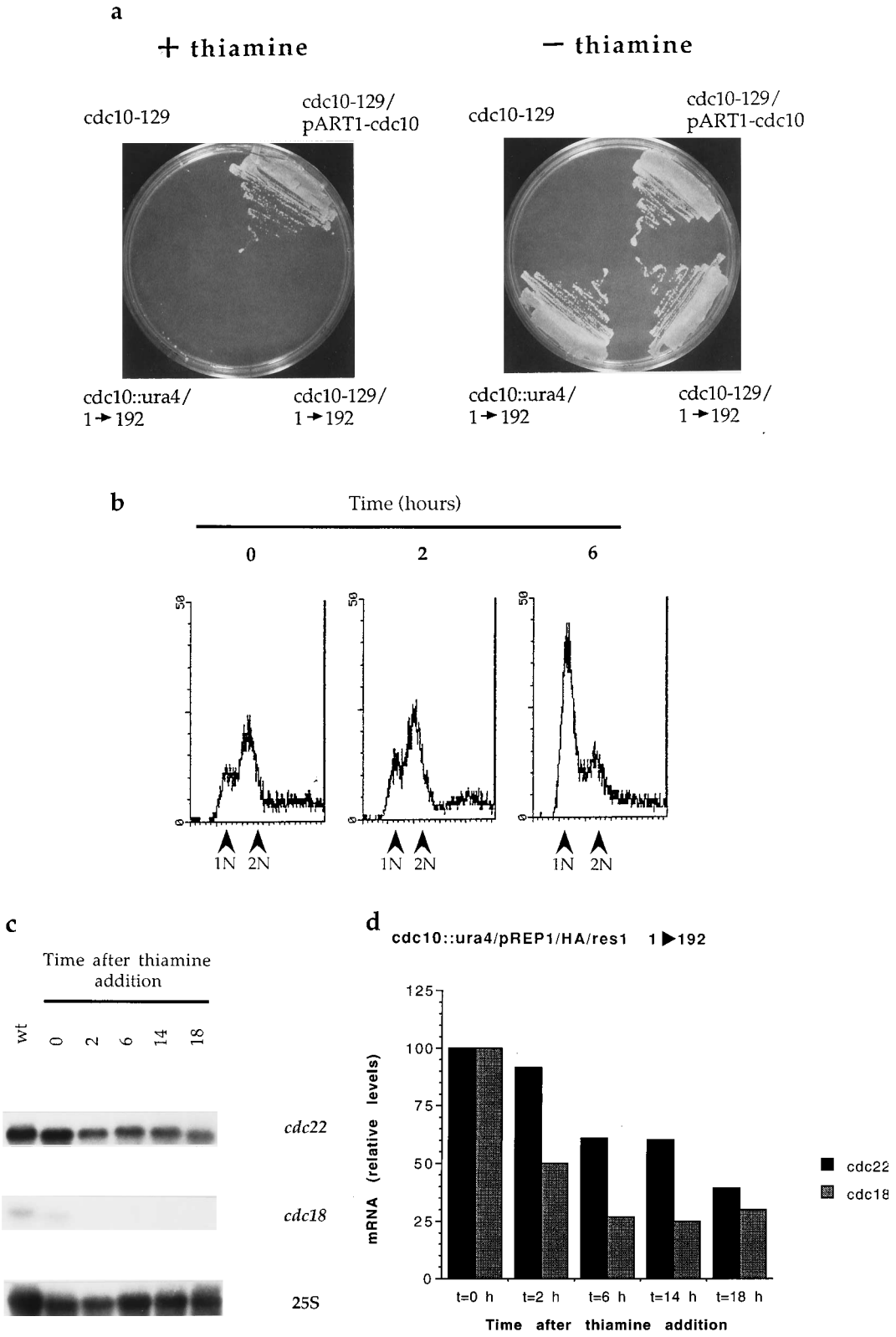


FIG. 3. res1 1→192 can rescue strains with *cdc10* mutations. (a) *cdc10-129* or *cdc10*::*ura4*$^+$ cells transformed with the indicated plasmids were streaked on plates, in the presence (promoter off) or absence (promoter on) of thiamine (2 μM), at 35.5°C for 4 days. (b) Flow cytometry analysis of *cdc10*::*ura4*$^+$ cells overexpressing 1→192, grown to early exponential phase at 30°C in the absence of thiamine. Thiamine was added to the culture, and at the time points indicated at the top (in hours), cells were fixed and prepared for flow cytometry analysis of DNA content. (c) Total RNA was prepared from the same experiment as in panel b. Total RNA (7.5 μg) was analyzed by Northern blotting and hybridized with a *cdc18*, *cdc22*, or 25S rRNA probe. wt indicates total RNA prepared from wild-type *S. pombe* cells (972h$^-$) in the logarithmic phase of growth. (d) The membranes shown in panel c were scanned with a Betascope 603, and the relative values, normalized with the 25S probe, were plotted.

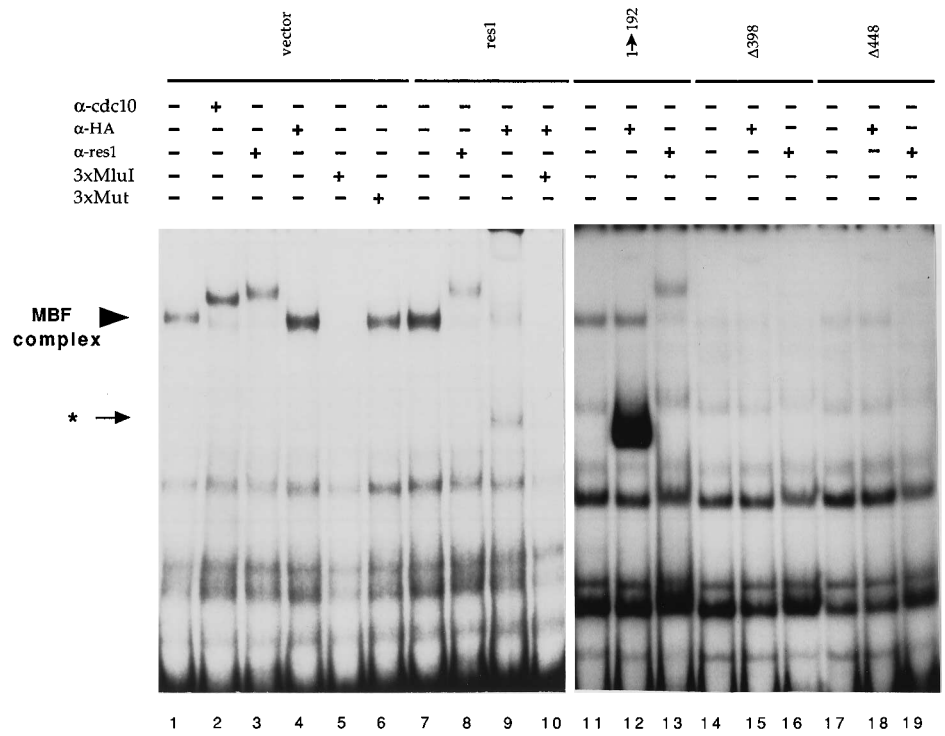


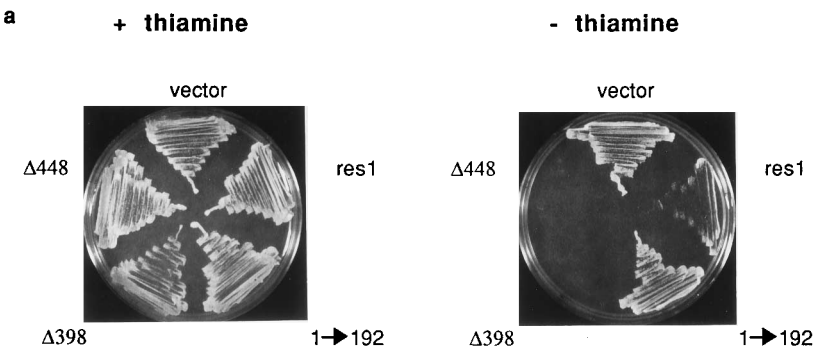
FIG. 4. The DNA-binding and cdc10-binding domains of p72^{res1} are required for the MBF complex formation. EMSA, with end-labeled *cdc22* promoter as a probe, were performed with extracts prepared from wild-type strain SP223 induced to express full-length p72^{res1} and several deletions under the control of the *nmt* promoter. Cells were grown to exponential phase in the absence of thiamine for 24 h. Additions of antibody (anti-cdc10 [α-cdc10], anti-res1, and anti-HA) or unlabeled competitor (50-fold molar excess) are indicated by + signs. The large arrowhead points to the MBF complex. The nature of the complex indicated by the asterisk is discussed in the text.

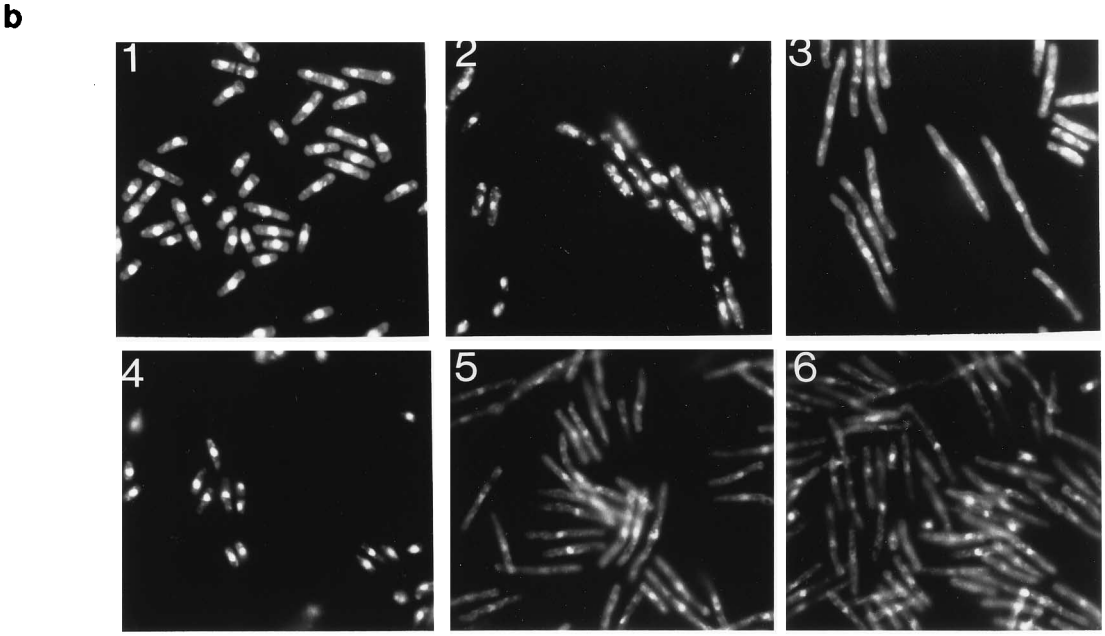
Overexpression of full-length p72^{res1} also led to growth delay in wild-type *S. pombe*. However, 22 h after thiamine was removed, cells increased their DNA content to greater than 2N (Fig. 5c; see also Fig. 7c). Several hours later, most of the cells accumulated with a 1N DNA content. Notably, this growth arrest by full-length p72^{res1} was observed only when its expression was driven by the *nmt* promoter. When overexpression of full-length p72^{res1} was driven by the weaker but constitutive *adh* promoter, the growth rate or the DNA content analyzed by flow cytometry analysis was not affected (data not shown).

Since high-level expression of wild-type p72^{res1} and the N-terminal truncations led to an apparent G_1 arrest in wild-type cells, we wanted to determine if the same effect could also be observed in a haploid strain deleted for *res1* (K123-14D) (52). We confirmed the *res1* deletion by PCR analysis of genomic DNA and by Western blotting of whole-cell lysates with several anti-res1 monoclonal antibodies (data not shown). Similarly to the effect seen in wild-type cells, the expression of full-length p72^{res1} and of Δ448 induced a growth arrest and accumulation of cells in the G_1 phase (data not shown). However, just as in wild-type *S. pombe* cells, full-length p72^{res1} initially induced an increase in DNA content, which was followed by an arrest in G_1.

To test the effect of p72^{res1} overexpression on the levels of the MBF-dependent induction of *cdc18* and *cdc22* transcription, total RNA was isolated from wild-type yeast cells carrying the pREP-derived plasmids described above, 24 h after the depletion of thiamine. Northern blots were prepared and hybridized with a probe for *cdc22* or *cdc18*. The mRNA signal was quantitated and normalized to the levels detected with an 25S rRNA probe (21). As shown in Fig. 6, overexpression of the p72^{res1} DNA-binding domain, 1→192, led to a modest 1.5-fold increase in the level of both *cdc18* and *cdc22* mRNAs. On the other hand, overexpression of full-length p72^{res1} yielded a 12-fold increase in the mRNA levels for both *cdc18* and *cdc22*. Conversely, expression of either Δ398 or Δ448 led to a 40 to 60% decrease in the mRNA levels of both *cdc22* and *cdc18* (Fig. 6). Hence, while overexpression of either full-length p72^{res1} or the C-terminal domain of p72^{res1} could induce a growth arrest, these proteins had opposite effects on the level of MBF-dependent gene expression.

p85^{cdc10} rescues the growth arrest produced by overexpression of p72^{res1}. Since moderate levels of p72^{res1} overexpression did not lead to a growth arrest, we wondered if the growth arrest by the *nmt*-driven overexpression of p72^{res1} could be rescued by overexpression of p85^{cdc10}. To test this, the strain carrying the pREP1-res1 construct was retransformed either with a plasmid expressing p85^{cdc10} under the control of the constitutive *adh* promoter (pARTU-cdc10) or with the backbone plasmid (pARTU) (28). Extracts were prepared at different times after the removal of thiamine and analyzed by Western blotting. As shown in Fig. 7a, p72^{res1} overexpression was detectable 12 h after the removal of thiamine. This same Western blot was also probed with anti-cdc10 antibodies and demonstrated increased expression of p85^{cdc10} with pARTU-cdc10. When both proteins were overexpressed, this strain grew as well as the wild-type yeast strain in terms of growth rate and morphology (data not shown). Notably, coexpression of both proteins did not lead to significant changes in the DNA contents when they were measured by flow cytometry analysis (Fig. 7b). We also did not observe any noticeable changes when cells were stained by DAPI. However, overexpression of





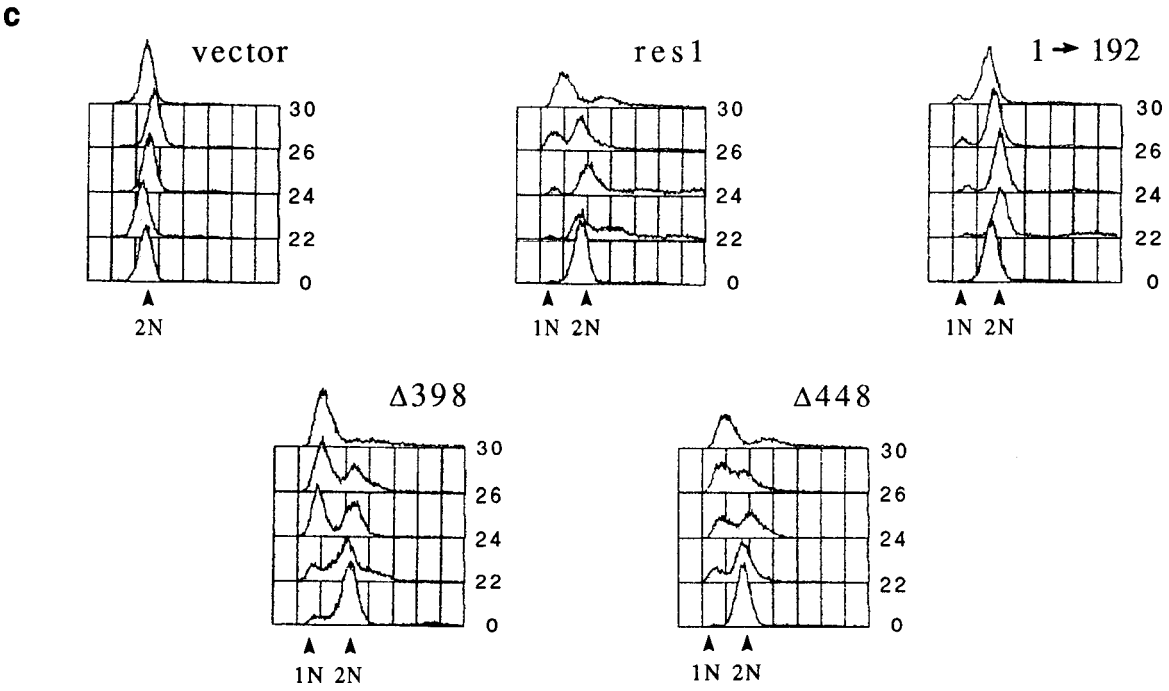


FIG. 5. Overexpression of N-terminally deleted derivatives of p72^{res1}, as well as full-length p72^{res1}, induces a growth arrest. (a) Wild-type *S. pombe* cells (SP223), transformed with the indicated plasmids, were streaked on plates, in the presence (promoter off) or absence (promoter on) of thiamine, at 30°C for 4 days. (b) Photomicrograph of DAPI-stained cells (magnification, ×1,000) grown in liquid medium at 34°C to exponential phase in the absence of thiamine. 1, wild-type cells carrying vector alone (pREP1); 2 and 3, cells overexpressing full-length p72^{res1} for 24 and 30 h, respectively; 4, cells overexpressing 1→192 for 24 h; 5, cells overexpressing Δ398 for 24 h; 6, cells overexpressing Δ448 for 24 h. (c) DNA flow cytometry analysis of cells carrying the indicated plasmids and grown to exponential phase at 34°C, in the absence of thiamine, for the times (hours) indicated on the right. Cells were ethanol fixed at the indicated times and stained with propidium iodide.

p72^{res1} alone induced changes in the DNA content and a growth arrest, as previously described. As shown in Fig. 7c, extracts prepared from cells overexpressing both p85^{cdc10} and p72^{res1} have a large increase in MBF DNA-binding activity compared with cells that overexpress p72^{res1} alone. Overexpression of both proteins also led to a 10- to 12-fold increase in the levels of the *cdc22* mRNA. As shown in Fig. 7d, overexpression of p72^{res1} led to a similar increase in the *cdc22* mRNA level, independently of overexpression of p85^{cdc10}. Therefore, high levels of p85^{cdc10} could rescue the growth-inhibitory effects of overexpression of p72^{res1} without decreasing the mRNA levels of at least one of the MBF-dependent genes.

DISCUSSION

The MBF complex is thought to play a key role in the passage through Start in *S. pombe* by activating the expression of certain genes required for entry into S phase. However, the mechanism for activation of MBF at Start remains unknown. One approach for understanding its regulation has been to search for extragenic suppressors of *cdc10* mutant alleles. This approach has led to the cloning of *res1* as well as of *cdc18* (8, 15). For example, it has been shown that overexpression of *cdc18* can rescue strains with defective *cdc10* function (15). An alternative approach to understanding MBF regulation is to perform a mutational analysis of p72^{res1} and p85^{cdc10} and determine the effects of overexpression of these mutations on MBF activity.

The C terminus of p72^{res1} can bind directly to p85^{cdc10}. We have found that this binding activity resides in the C-terminal 188 residues of p72^{res1}. Overexpression of this domain (Δ448 or

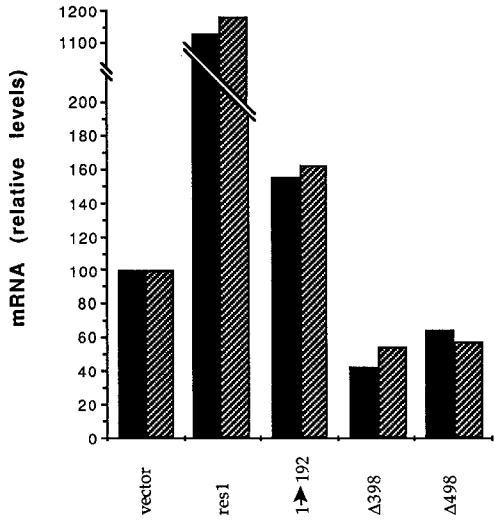


FIG. 6. Effect of overexpression of p72^{res1} on the RNA levels of MBF-dependent genes. Total RNA was obtained from exponential-phase cultures after 24 h of thiamine deprivation. RNA (10 μg) was analyzed by Northern blotting and hybridized with a *cdc18*, *cdc22*, or 25S rRNA probe. Membranes were scanned with a Betascope 603, and the relative values, normalized with the 25S probe, of *cdc18* (black bars) and *cdc22* (striped bars) mRNAs were plotted.

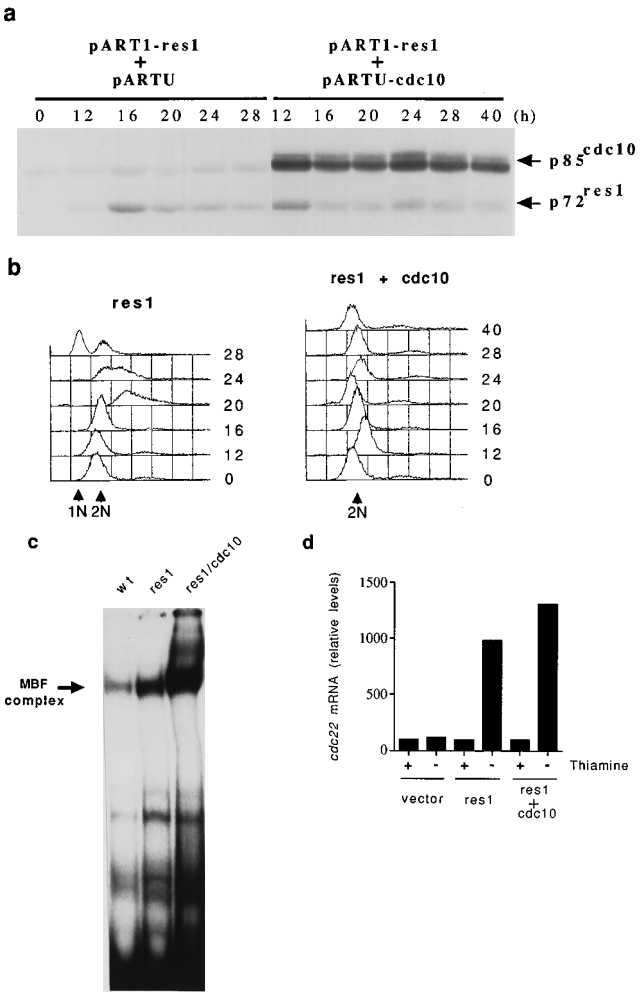


FIG. 7. p85^{cdc10} rescues the G_1 block caused by overexpression of p72^{res1}. (a) Western blot of cells overexpressing either full-length p72^{res1} alone (pART1-res1 + pARTU) or p72^{res1} and p85^{cdc10} (pART1-res1 + pARTU-cdc10). Cells were grown to exponential phase at 34°C in medium with thiamine. Extracts (50 μg) were prepared at different time points after the removal of thiamine, separated in an SDS–6% polyacrylamide gel, and analyzed by Western blotting with the specific monoclonal antibodies YS140 (anti-cdc10) and RY115 (anti-res1). The positions of p85^{cdc10} and p72^{res1} are indicated on the right. (b) DNA flow cytometry analysis of cells overexpressing either full-length p72^{res1} alone (res1) or p72^{res1} and p85^{cdc10} (res1 + cdc10) and grown to exponential phase at 34°C in the absence of thiamine for the time (hours) indicated on the right. Cells were ethanol fixed and stained with propidium iodide. (c) EMSA using end-labeled *cdc22* promoter as a probe, incubated with extracts (10 μg) prepared from the wild-type (wt) strain (SP223) or strains induced to express either full-length p72^{res1} alone (res1) or p72^{res1} and p85^{cdc10} (res1/cdc10). Cells were grown to exponential phase in the absence of thiamine for 24 h before the extracts were prepared. (d) Total RNA was prepared from exponential-phase cultures grown in the presence or absence of thiamine for 24 h. RNA (10 μg) was analyzed by Northern blotting and hybridized with a *cdc22* or 25S rRNA probe. Vector, wild-type cells (SP223) transformed with pREP1; res1, wild-type cells overexpressing p72^{res1} under the control of the *nmt* promoter; res1 + cdc10, cells that were also overexpressing p85^{cdc10} under the control of the constitutive *adh* promoter.

Δ398) led to a growth arrest in the G_1 phase of the cell cycle and a *cdc* phenotype. Expression of other truncated forms of p72^{res1} that were unable to bind to p85^{cdc10} did not induce a growth arrest. In addition, overexpression of this C-terminal domain led to a decrease in the detectable levels of MBF DNA-binding activity as well as a decrease in the levels of *cdc18* and *cdc22* mRNA. These results suggest that the overexpressed C terminus of p72^{res1} was able to bind to the endogenous p85^{cdc10} and prevent it from entering into the MBF complex. Therefore, overexpression of the cdc10-binding domain of p72^{res1} resulted in a transdominant negative effect on *cdc10* function.

In *S. cerevisiae*, the C-terminal 259 amino acids of Swi4 are necessary and sufficient for binding to Swi6 (1, 49). While there is a high level of homology between the C termini of Swi6 and p85^{cdc10}, there is little homology between the C termini of p72^{res1}, Swi4, and p85^{cdc10}. However, all members of this family are predicted to contain three putative α-helical regions in the C termini when analyzed for secondary structure by the profile-fed neural network system (42, 43).

We have demonstrated that p72^{res1} itself can bind directly and specifically to the MCB elements within the *cdc22* promoter. As shown in Fig. 2, the first 157 amino acids of p72^{res1} were sufficient for binding in vitro to the *cdc22* promoter. We were not able to detect any specific MCB-binding activity by p85^{cdc10} alone, despite sequence homology to the N-terminal, DNA-binding domain of Swi4 (39). It has been reported that the expression of the first 192 residues of p72^{res1}, containing an internal point mutation (*sct1-b1*, E→K56), could rescue a *cdc10* null strain (8). In our hands, overexpression of the wild-type DNA-binding domain of p72^{res1}, 1→192, could rescue the *cdc10* temperature-sensitive strain as well as a *cdc10* null strain. The higher level of expression achieved under the control of the *nmt* promoter may have been responsible for this complementation not previously observed. Indeed, when the *nmt* promoter was repressed by the addition of thiamine to the culture, the mRNA level for the MBF-dependent genes *cdc18* and *cdc22* decreased and the cells became arrested in G_1 with a *cdc* phenotype. Therefore, the expression of the mRNAs for *cdc18* and *cdc22* in the *cdc10* null strain was absolutely dependent on res1 1→192 expression. Interestingly, the fact that 1→192 can rescue a *cdc10* null strain by itself suggests that this small region of p72^{res1} may also contain a transactivation domain.

The demonstration that the N-terminal region of p72^{res1} can bind directly to DNA confirms that it is also a member of the Swi4 family of DNA-binding proteins. Other factors that have been identified by sequence homology that may also have DNA-binding activity include StuA of *Aspergillus nidulans* as well as PHD1 of *S. cerevisiae* (13, 29). In higher eukaryotes, including *Drosophila melanogaster*, *Xenopus laevis*, and mammals, the expression of genes required for DNA synthesis is dependent, at least in part, on the E2F family of transcription factors (16, 22, 38). However, E2F demonstrates very little sequence homology to p72^{res1} or p85^{cdc10}. Thus, structural homologs of these yeast proteins have not yet been identified in higher organisms, nor has an E2F-like protein been identified in yeast cells.

Overexpression of full-length p72^{res1} led to a growth arrest that could be rescued by constitutive overexpression of p85^{cdc10}. When we compared the effects of p72^{res1} overexpression on MBF activity, we observed several key differences when p85^{cdc10} was also overexpressed. When p72^{res1} alone was overexpressed in wild-type cells, there was a slight increase in the amount of the MBF complex, as measured by EMSA. However, when p85^{cdc10} was also overexpressed, a severalfold increase in this specific DNA-binding activity was observed. This result is consistent with the observation that both p85^{cdc10} and p72^{res1} contribute to the formation of the MBF complex. Flow cytometry analysis of DNA content after induction of full-length p72^{res1} overexpression demonstrated an unusual effect that was not observed when p85^{cdc10} was also overexpressed. High levels of p72^{res1} led to an initial increase in DNA content, greater than 2N. DAPI staining also suggested that there was an overall increase in DNA content in each cell. It is possible that the high levels of p72^{res1} expression in the absence of simultaneous p85^{cdc10} overexpression may have led to an early or aberrant initiation of DNA synthesis, perhaps even before the cells had completed the previous cell cycle. Continued overexpression of p72^{res1} led to a growth arrest, with a *cdc* phenotype, and flow cytometry analysis suggested that the cells had 1N DNA content.

Overexpression of p72^{res1} induced high levels of *cdc22* mRNA. It is unlikely that the growth arrest induced by p72^{res1} resulted from overexpression of MBF-dependent genes for several reasons. For example, when p72^{res1} overexpression was induced in a strain that also overexpressed p85^{cdc10}, a similar increase in the expression of *cdc22* was also observed. However, this strain was now viable and could apparently tolerate high levels of MBF-dependent gene expression. Similarly, it has also been reported that *nmt*-driven expression of *cdc18* or *cdt1*, two known MBF-dependent genes, did not lead to a growth arrest (15, 17). Overexpression of p72^{res1}, in the absence of compensating amounts of p85^{cdc10}, may result in p72^{res1} becoming aberrantly active, perhaps transactivating genes whose transcription is not normally dependent on p72^{res1}. Alternatively, p72^{res1} may interact with another protein in addition to p85^{cdc10}, and increasing production of p72^{res1} may lead to a dominant negative effect on this factor. There are several other possible explanations for the growth arrest induced by p72^{res1}, but none of them can be confirmed with the data at hand.

Deletions of the ankyrin repeats within p72^{res1} did not appear to affect the results of any of the experiments performed here. We have not found a role for the ankyrin repeats in DNA-binding activity, expression of *cdc18* or *cdc22* mRNA, p85^{cdc10} binding, cell cycle distribution, or viability. Possible roles of this domain in protein folding or interaction with proteins different from p85^{cdc10} are not discarded. While this domain is conserved among all members of this family of transcription factors, there is no adequate explanation yet for its role in passage through Start.

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