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The Fission Yeast Protein p73^{res2} Is an Essential Component of the Mitotic MBF Complex and a Master Regulator of Meiosis

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Depending on environmental conditions, *Schizosaccharomyces pombe* can remain in the stationary phase or enter into either premitotic or premeiotic DNA synthesis. This decision point is known as Start. In the mitotic cell cycle, regulation of G_1/S-specific gene expression is dependent upon the MBF (Mlu1 binding factor) complex, known to contain p85^{cdc10} and p72^{res1}. Here we demonstrate that p73^{res2} controls cell cycle progression via its participation in the MBF complex, interacting directly with both p85^{cdc10} and p72^{res1}. In contrast, when cells enter into meiosis, the MBF complex is disrupted, and p73^{res2} shifts its regulatory function towards the transactivation of genes required for meiotic progression. These observations suggest that p73^{res2} plays a pivotal role at Start and constitutes an example of a transcription factor involved in the control of both mitotic and meiotic progression.

Under optimal growth conditions, fission yeast cells proliferate in a haploid state. When the nitrogen source becomes limited, haploid cells arrest in the G_1 phase of the cell cycle (34). At this point, known as Start, cells enter into meiosis and undergo sporulation if cells of the opposite mating type are present or reenter the mitotic cell cycle if nutrients are restored (11, 31).

Genetic screenings have shown that exit from Start into the mitotic cell cycle is dependent upon several genes. One of these genes, *cdc2*, encodes the prototypic cyclin-dependent protein kinase (references 32 to 34 and see reference 38 for a review). *cdc10* and *res1*/*sct1* constitute two additional genes that participate in Start and are required for progression into the cell cycle (2, 3, 7, 37, 39). p85^{cdc10} and p72^{res1} have been shown to be components of the DNA-binding complex MBF (Mlu1 cell cycle box binding factor), also known as DSC1 (DNA synthesis control complex) (3, 22, 35). This complex binds specifically to its target DNA sequence, the MCB (Mlu1 cell cycle box; ACGCGT) element, in a cell cycle-dependent manner, with its DNA-binding activity peaking during the G_1/S transition and diminishing during G_2/M (3a, 22, 35). Furthermore, with a synthetic promoter containing three tandem MCB elements, it has been shown that the MBF complex can activate the transcription of a reporter gene after Start (22). The MBF complex is responsible for the cell cycle-specific expression of at least three genes required for entry into the S phase, namely, *cdc22*, which encodes the regulatory subunit of ribonucleotide reductase (12, 22), *cdc18* (18), and *cdt1* (16). The latter two are required for DNA replication as well as in the checkpoint control that prevents the onset of mitosis until the DNA is fully replicated.

An additional Start factor, p73$^{res2/pct1}$, was isolated as a multicopy suppressor of *res1* null cells as well as an MCB-binding partner of p85^{cdc10} (29, 42). p85^{cdc10}, p72^{res1}, and p73^{res2} have extensive sequence homologies to each other and to the *Saccharomyces cerevisiae* proteins Swi6, Swi4, and Mbp1. Each of these proteins contains two copies of the 33-amino-acid domain known as the cdc10/Swi6 motif or ankyrin repeat (6). This motif has been implicated in protein-protein interactions, although its role in the regulation of the MBF complex has not been well defined. We have previously shown that the ankyrin repeat domains in p72^{res1} were not necessary for DNA-binding activity or for association to p85^{cdc10} (3). Recently, Zhu et al. have shown similar results for p73^{res2} (43). In addition, there is a high degree of homology among the N-terminal regions of p72^{res1}, p73^{res2}, Swi4, and Mbp1. This domain of p72^{res1}, p73^{res2}, and Mbp1 has been demonstrated to be able to bind directly to the MCB element (3, 7, 19, 42). The high degree of similarity among these proteins suggests that they define a new class of DNA-binding proteins.

In *S. cerevisae*, there are two independent DNA-binding complexes, MBF, which contains Mbp1 and Swi6, and SBF, which contains Swi4 and Swi6. These complexes are responsible for the transcription of two different sets of genes required for cell cycle progression. In *Schizosaccharomyces pombe*, it has been assumed that there are also two functionally overlapping systems, p72^{res1}-p85^{cdc10} and p73^{res2}-p85^{cdc10} complexes, whose activity is required for exit from Start (29, 42). Genetic experiments suggest that the former is essential during mitotic growth, while the latter has its major role during meiosis. *S. pombe* cells with a disrupted *res1* gene show both heat and cold sensitivity, arresting at Start at nonpermissive temperatures (39). Fission yeast cells deficient in *res2* also show impaired vegetative growth (a longer doubling time than that of wild-type cells), but their main phenotype is their inability to complete meiosis (29, 42). However, it is not clear whether p72^{res1} cooperates with p73^{res2} during vegetative growth or what the molecular mechanisms are by which p73^{res2} exerts its influence on both mitosis and meiosis. Here we show that in vegetatively growing cells, the MBF complex contains both p72^{res1} and p73^{res2}. Upon entry into meiosis, p72^{res1} levels decrease and the MBF complex is disrupted. Conversely, p73^{res2} levels increase and *res2* activity is involved in the control of the expression of genes required during the meiotic process.

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MATERIALS AND METHODS

Strains and media. All *S. pombe* strains used are isogenic to the wild type 972h$^-$ and are summarized in Table 1. Media was prepared as described previously (30). *S. pombe* cells were transformed by the lithium acetate method (30)

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

Strain	Genotype
SP223	h^+ *leu1-32 ura4-D18 ade6-M210*
FYC23	h^{90} *leu1-32 ura4-D18 ade6-M210*
SP1070	h^+/h^{90} *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*
M106	h^{90} *res2::ura4*$^+$ *leu1-32 ura4-D18*
TE397	h^+/h^- *leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M210/ade6-M210*
TE330	*pat1-114 leu1-32 ura4-D18*

with plasmids directing the expression of full-length p72^{res1}, full-length p73^{res2}, or the first 154 amino acids of p73^{res2} (DBD-res2) under the control of the inducible *nmt* (no message in thiamine) promoter (24).

To obtain a synchronous culture of cells entering into meiosis, a culture of the *pat1-114* strain was grown at 25°C until the mid-log phase (5×10^6 cells/ml). For half of the culture, the temperature was shifted to 35°C for 8 h (1.1×10^7 cells/ml). To monitor the haploid meiosis, cells were examined by microscopic observation following DAPI (4′,6-diamidino-2-phenylindole) staining (3). When indicated, hydroxyurea (HU) was added to the culture at a final concentration of 12 mM.

Antibody preparation. Monoclonal antibodies to p73^{res2} were generated by injecting mice with a maltose-binding protein (MBP)-p73^{res2} chimera by standard procedures (15). Hybridoma supernatants were screened for their ability to immunoprecipitate ^{35}S-labeled p73^{res2} as well as their ability to supershift the MBF complex when analyzed by electrophoretic mobility shift assay (EMSA; see below). Monoclonal antibodies to p72^{res1}, p85^{cdc10}, and hemagglutinin (HA) epitope were described previously (3, 41).

Protein extraction and immunoprecipitations. Extracts were prepared as described previously (3). Immunoprecipitations were performed with 1 mg of whole-cell lysate and 50 μl of the indicated tissue culture supernatant monoclonal antibody. Immunoprecipitates were washed three times in NET-N buffer (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 of leupeptin per ml, and 5 μg of aprotinin per ml) resolved in a sodium dodecyl sulfate–6% polyacrylamide gel electrophoresis (SDS–6% PAGE) gel (20), transferred to polyvinylidene difluoride membranes, and incubated with the indicated monoclonal antibody tissue culture supernatants. Immunoreactive bands were detected with alkaline phosphatase-labeled secondary antibody (15).

For the in vitro binding experiments, p72^{res1} or p73^{res2} was synthesized by coupled transcription-translation (TNT; Promega) in the presence of [^{35}S]methionine. Radiolabeled products were mixed with 100 ng of glutathione *S*-transferase (GST) fusion proteins bound to glutathione-Sepharose in NET-N buffer and incubated at 4°C for 30 min. After washing (five times, with 1 ml of NET-N buffer), the proteins were separated in a SDS–6% PAGE gel (20), transferred to polyvinylidene difluoride membranes, and exposed to autoradiographic film.

Chimeric proteins between the dimerization domain of ZEBRA and p72^{res1} were prepared by PCR amplification of cDNAs with either a wild-type or a mutant ZEBRA defective in dimerization (described as Z214R or Z218R in reference 13) with *Pfu* polymerase (Stratagene), by use of the following oligonucleotides: JA1185 (5′-AAAAGTACTGATCAAGCTTTTAAGCAACTGCT GCAGCAC-3′) and JA1186 (5′-CCGGAATTCTTAGAAATTTAAGAGATC CTCGTG-3′).

EMSA. A 123-bp fragment of the *cdc22* promoter was labeled and used as a probe (3, 22). Binding reactions were performed with 10 μg of whole-cell lysate. The DNA-protein complexes were analyzed as described previously (3). Where indicated, 4 μl of anti-p85^{cdc10} (YS140), 6 μl of anti-p72^{res1} (RY115), or 4 μl of anti-p73^{res2} (AG14) tissue culture supernatants was added to the binding reaction mixture prior to the addition of the probe.

Northern blot analysis. RNA was prepared by glass bead lysis in the presence of guanidinium thiocyanate (9). The RNA concentration was measured by the optical density at 260 nm, and equal amounts were loaded into formaldehyde-agarose gels (36) and transferred to GeneScreen Plus membranes (Dupont). Hybridization and washes were performed as recommended by the manufacturer. *cdc2*, *cdc18*, *res1*, *mei2*, and *mei3* probes contained the open reading frame of these genes. The following specific probes for the *mat1* genes were obtained by PCR amplification of genomic DNA: *mat1-Mm* probe, from nucleotides 145 to 454 of the reported sequence (17); *mat1-Mc* probe, from nucleotides 455 to 1175; *mat1-Pc* and *mat1-Pm* probe, from nucleotides 150 to 1171. *mei3* cDNA was amplified from genomic DNA with the primers JAmei3s (5′-CGCGGATC CGCATTGTGGAATGTAGCAATCTC-3′) and JAmei3a (5′-CCGGAATTCT TAGCGAGAGGTGTTGTTTACAC-3′). Probes were labeled with [α-^{32}P] dCTP by random priming (Boehringer Mannheim).

DAPI staining. DAPI staining was performed on methanol-fixed cells as described previously (3).

RESULTS

In vitro dimerization of p72^{res1} enhances its DNA-binding activity. In vitro-translated p73^{res2} binds to its target sequence in the *cdc22* promoter in an EMSA (42, 43). In contrast, in vitro-translated full-length p72^{res1} binds very weakly to DNA, in either the presence or absence of p85^{cdc10} (data not shown and reference 43). Since most transcription factors bind to DNA as dimers, the differences observed between p72^{res1} and p73^{res2} may reflect their ability to form homodimers in vitro. To test this possibility, we examined the ability of p72^{res1} and p73^{res2} to form homo- and heterodimers with an in vitro binding assay. ^{35}S-radiolabeled *res1* gene product was produced in a cell-free transcription-translation system (Fig. 1A, lane 1) and incubated with bacterially produced p85^{cdc10}, p72^{res1}, or p73^{res2} fused to GST. As shown in Fig. 1A, in vitro-translated p72^{res1} could bind to GST-p85^{cdc10} or GST-p73^{res2} but was unable to form homodimers with GST-p72^{res1}. On the other hand, p73^{res2} was able to form heterodimers with GST-p85^{cdc10} or GST-p72^{res1} as well as homodimers with GST-p73^{res2} (Fig. 1B).

To test the possibility that forced dimerization of p72^{res1} would improve its DNA-binding activity in vitro, we fused p72^{res1} to either the 51-amino-acid dimerization domain of the Epstein-Barr virus transactivator ZEBRA or to a mutant defective in dimerization (res1-ZEBRA and res1-ZEBRARR, respectively) (13). The ability of these two chimeric proteins to

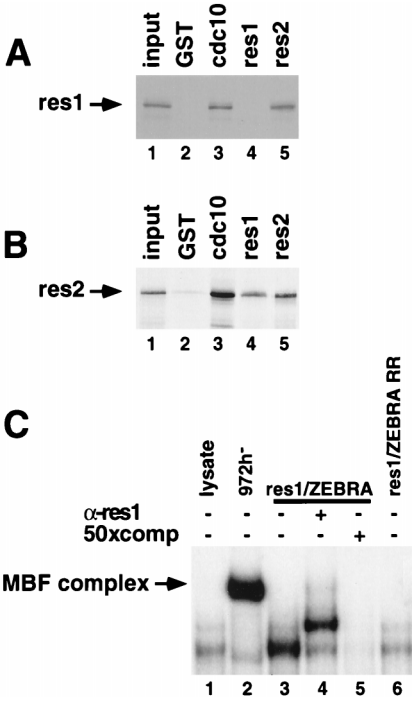

FIG. 1. Forced dimerization of p72^{res1} enhances its DNA-binding activity. (A) ^{35}S-radiolabeled p72^{res1} (lane 1) was incubated with bacterially produced GST (lane 2), GST-p85^{cdc10} (lane 3), GST-p72^{res1} (lane 4), or GST-p73^{res2} (lane 5), precipitated with glutathione-Sepharose beads, separated in a SDS–6% PAGE gel, and autoradiographed. Lane 1 contains 1/10 of the amount of in vitro translate used in the binding reaction mixtures. (B) ^{35}S-radiolabeled p73^{res2} (lane 1) was analyzed for binding to the same proteins as those described for panel A. (C) Gel shift analysis of different products of in vitro translation (4 μl), with the *cdc22* promoter as a probe. Where indicated, antibodies against p72^{res1} (α-res1) were used in the binding reaction mixture. In lane 1, 4 μl of unprogrammed lysate was used. In lane 5, a 50-fold molar excess of unlabeled probe was added to the binding reaction mixture (50xcomp). 972h$^-$ corresponds to whole-cell lysates (10 μg) prepared from wild-type cells.

bind to DNA was assayed by an EMSA, with the *cdc22* promoter as a probe. This probe contains 1 *Mlu1* site (ACGCGT) and 3 *Mlu1*-like sites (NCGCGN) (3). When a lysate prepared from wild-type cells was incubated with this probe, a specific MBF complex could be detected (Fig. 1C, lane 2). Likewise, when the in vitro-translated chimera of p72^{res1} and the dimerization domain of ZEBRA (p72^{res1}-ZEBRA) was incubated with the same probe, a specific complex was detected (lane 3). This complex was smaller than the MBF complex, as indicated by its increased mobility on the gel. The specificity of this DNA-binding complex was demonstrated since the complex could be supershifted with anti-p72^{res1} antibodies and could be competed with unlabeled probe (lanes 4 and 5, respectively). However, when p72^{res1} was fused to a defective dimerization domain (p72^{res1}-ZEBRARR), no specific DNA-binding complex was detected (lane 6). This result suggested that the lack of DNA-binding activity in vitro of p72^{res1} alone (reference 43 and data not shown) may have been due to the inability of p72^{res1} to form homodimers (Fig. 1A). Forced homodimerization of p72^{res1} through a heterologous dimerization domain provided binding to the *cdc22* promoter.

The MBF complex contains both p72^{res1} and p73^{res2}. Since forced dimerization of p72^{res1} could enhance binding to DNA in vitro, it raised the possibility that, in vivo, the active MBF complex contained homodimers of p72^{res1} or p73^{res2} or even heterodimers of p72^{res1} and p73^{res2}. To test this hypothesis, we first sought to determine if p73^{res2} was a component of the MBF complex. When extracts prepared from vegetatively growing wild-type *S. pombe* cells were analyzed in a gel mobility shift assay using the *cdc22* promoter as a probe, a specific MBF complex was detected (Fig. 2A). The MBF complex contains p85^{cdc10} and p72^{res1} (lanes 2 and 3) as well as p73^{res2} (lane 5), as shown by mobility shift when specific monoclonal antibodies were added to the binding reaction mixture. When the extracts were incubated with both anti-p72^{res1} and anti-p73^{res2} antibodies, the entire complex could be supersupershifted, indicating that the MBF complex contained both p72^{res1} and p73^{res2} in addition to p85^{cdc10} (lanes 4 and 6). To establish the specific contributions of p72^{res1} and p73^{res2} to the DNA-binding activity of the MBF complex, extracts were prepared from cells deleted for each of these genes. With conditions identical to those used for wild-type cells, we were unable to detect any MBF DNA-binding activity in extracts prepared from strains deleted for either of these two genes (lanes 9 to 12). During the preparation of this manuscript, similar results were reported by Zhu et al. (43). Consistent with the loss of MBF DNA-binding activity, the expression of two known MBF-dependent genes, *cdc22* and *cdc18*, was altered (Fig. 2B). *cdc22* mRNA levels were decreased in *res1* or *res2* null strains compared to the levels in the wild-type strain. Unexpectedly, *cdc18* mRNA levels were decreased in *res1* null cells and increased in *res2* null cells.

The observation that both p72^{res1} and p73^{res2} contributed to the DNA-binding activity of a single MBF complex raised the possibility that p72^{res1} and p73^{res2} could interact with each other directly. To test this hypothesis, extracts prepared from wild-type cells were immunoprecipitated with specific monoclonal antibodies against p85^{cdc10}, p72^{res1}, or p73^{res2} and then subjected to Western blotting (immunoblotting). As shown in Fig. 2C, p85^{cdc10} coprecipitated with p72^{res1} and p73^{res2} (lanes 2 and 3), while p72^{res1} and p73^{res2} coprecipitated with each other in extracts prepared from wild-type cells (lanes 6 and 12). 4No antibody cross-reactivity between p72^{res1} and p73^{res2} was detected, as shown by immunoprecipitation followed by Western blotting of extracts prepared from either *res1* or *res2* null cells: antibodies to p72^{res1} were able to detect p72^{res1} only in extracts prepared from *res1*$^{+}$ cells (lane 9), while antibodies to

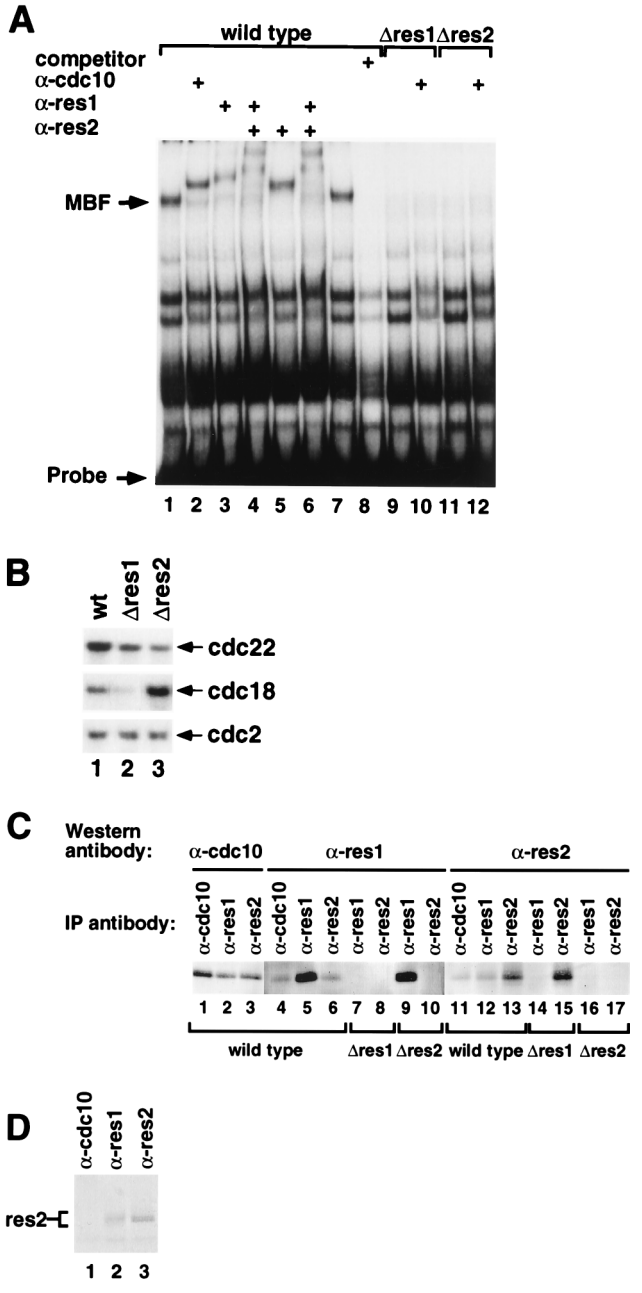

FIG. 2. p85^{cdc10}, p72^{res1}, and p73^{res2} are present in the MBF complex. (A) Gel shift analysis of whole-cell lysates prepared from wild-type cells (lanes 1 to 8), *res1* null cells (Δres1; lanes 9 and 10), or *res2* null cells (Δres2; lanes 11 and 12). In lane 8, a 50-fold molar excess of unlabeled probe was added to the binding reaction mixture. Where indicated (+), tissue culture supernatants of monoclonal hybridomas were added to the binding reaction mixture. In lanes 4 and 6, anti-p73^{res2} and anti-p72^{res1} monoclonal antibodies, respectively, were added after the binding reaction was performed in the presence of anti-p72^{res1} or anti-p73^{res2} antibodies. (B) Total RNA (10 μg) prepared from asynchronous cultures of wild-type (lane 1), *res1* null (lane 2), or *res2* null (lane 3) cells was analyzed by Northern blotting and hybridized with the probes indicated to the right of the gel. The *cdc2* probe was used as a load control. (C) Whole-cell lysates (1 mg) from wild-type, Δres1, or Δres2 cells were immunoprecipitated (IP antibody) and analyzed by Western blotting (Western antibody) with the antibodies indicated. (D) Whole-cell lysates from *cdc10* null cells (3) were immunoprecipitated with anti-HA (41) (lane 1), anti-p72^{res1} (lane 2), or anti-p73^{res2} (lane 3) monoclonal antibodies. The immunoprecipitates were analyzed by Western blotting for the presence of p73^{res2} with anti-p73^{res2} monoclonal antibody.

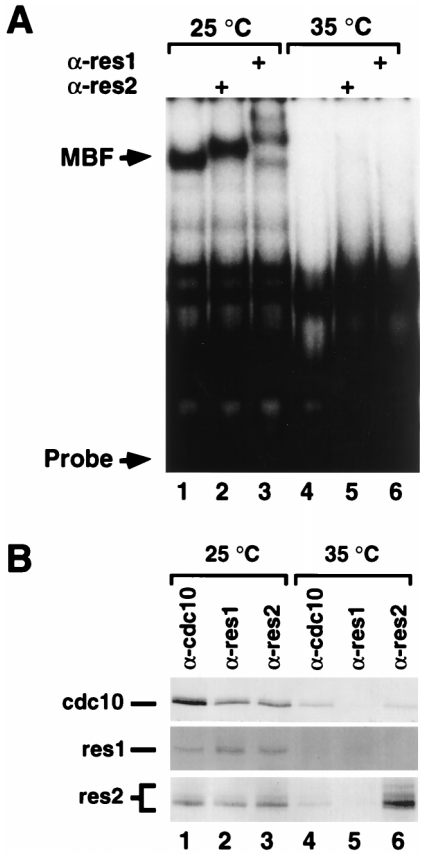

FIG. 3. The MBF complex is disrupted during meiosis. (A) Gel shift analysis of whole-cell lysates (10 μg) from *pat1-114* cells grown at the permissive (25°C) or restrictive (35°C) temperature. Where indicated (+), tissue culture supernatants of monoclonal hybridomas were added to the binding reaction mixture. (B) Whole-cell lysates from *pat1-114* cells grown at the permissive (25°C) or restrictive (35°C) temperature were immunoprecipitated with the antibodies indicated at the top of the gel. The immunoprecipitates were analyzed by Western blotting, probed with anti-p85^{cdc10}, anti-p72^{res1}, or anti-p73^{res2} monoclonal antibodies. The positions of p85^{cdc10}, p73^{res2}, and p72^{res1} are indicated to the left of the gel.

p73^{res2} detected p73^{res2} in extracts prepared from *res2*$^{+}$ cells (lane 15). To determine whether the interaction of p72^{res1} with p73^{res2} required p85^{cdc10}, extracts were prepared from a strain in which the *cdc10* gene was deleted. The lethality of the *cdc10* null phenotype was suppressed by overexpression of the HA-tagged amino-terminal 192 residues of p72^{res1}, which includes its DNA-binding domain (3). As shown in Fig. 2D, p72^{res1} and p73^{res2} were coprecipitated by each other, indicating that the interaction between these two proteins was independent of p85^{cdc10} (lanes 2 and 3). Notably, the N-terminal 192 residues of p72^{res1} were not sufficient to coprecipitate p73^{res2} (lane 1).

The MBF complex is disrupted during meiosis. As noted above, *res2* null cells are unable to complete meiosis (29, 42). Conversely, the temperature-sensitive *pat1-114* strain undergoes a lethal haploid meiosis at the nonpermissive temperature (5, 26) that can be rescued by overexpression of p72^{res1} (29, 39). These observations led us to examine the fate of the MBF complex during meiosis. In extracts prepared from the *pat1-114* strain (TE330) grown at the permissive temperature, i.e., while cells remained in the vegetative cell cycle, an intact MBF complex was observed (Fig. 3A, lane 1), which could be supershifted with either anti-p73^{res2} or anti-p72^{res1} antibodies (lanes 2 and 3). After 8 hours at the nonpermissive temperature, most of the cells had three or more nuclei, indicating that they had undergone haploid meiosis (data not shown). Extracts prepared from these cells showed that the MBF complex was no longer detectable (lanes 4 to 6). In lysates prepared from these same cultures, p73^{res2} levels were increased and p73^{res2} could coprecipitate p85^{cdc10} (Fig. 3B, lanes 4 to 6). Strikingly, p72^{res1} protein levels decreased below the detection limit of Western blot analysis, and we were unable to detect coprecipitation of p72^{res1} with either p73^{res2} or p85^{cdc10}.

To distinguish the effect of *pat1* inactivation from entry into meiosis, a culture of the *pat1-114* strain was divided in two and incubated at the nonpermissive temperature, in the presence or absence of HU. At hourly intervals, a sample of each culture was taken, and entry into meiosis was monitored by DAPI staining (Table 2). After 4 hours, the cells still contained one nucleus, but after 6 h, most cells contained two nuclei, indicating that the bulk of the culture had finished meiosis I. Finally, after 8 h at the nonpermissive temperature, the majority of the cells had three or more nuclei, indicating that at that time point they had completed meiosis II. However, when the culture was set at the nonpermissive temperature in the presence of HU, the cells were arrested in the S phase and were unable to enter into meiosis.

Lysates were prepared at hourly intervals, and the DNA-binding activity of the MBF complex was analyzed (Fig. 4A). Within 2 h at the nonpermissive temperature (in the absence of HU), the amount of MBF complex was strongly diminished, and by 5 h, it was no longer detected. In contrast, when cells were arrested in the S phase of the cell cycle (in the presence of HU), the MBF complex was still present after 8 h at the nonpermissive temperature, indicating that the DNA-binding activity of the MBF complex was independent of *pat1*. The specificity of the complex at the nonpermissive temperature was determined with anti-p72^{res1} or anti-p73^{res2} antibodies (Fig. 4B, lanes 2 and 3).

The same lysates were analyzed for the presence of p85^{cdc10}, p72^{res1}, and p73^{res2}. As shown in Fig. 4C, p72^{res1} could not be detected after the 5-h time point at the nonpermissive temperature in the absence of HU. Interestingly, the disappearance of p72^{res1} during meiosis paralleled the loss of the MBF complex (Fig. 4A). This loss of p72^{res1} was triggered only by entry into meiosis and was independent of *pat1* activity, since the levels of p72^{res1} remained stable when the culture was shifted to the nonpermissive temperature in the presence of HU (Fig. 4C).

We also prepared RNA at the different time points and analyzed the expression of several genes (Fig. 4D). *mei2*, a marker of entry into meiosis, was, as expected, rapidly induced immediately after *pat1* inactivation in the absence of HU. *cdc18* mRNA was also induced, but after 4 h, the levels de-

TABLE 2. *pat1-114* does not enter into haploid meiosis when arrested with HU[a]

Time (h) at 35°C	% of cells with:		
	1 nucleus	2 nuclei	>2 nuclei
0	82	18	
2	86	14	
4	93	7	
6	5	82	13
8 (−HU)		8	92
8 (+HU)	97	3	

[a] A culture of the *pat1-114* strain was grown at 25°C until the mid-log phase (3×10^6 cells/ml). The culture was split, and the temperature was shifted to 35°C, in the presence or absence of HU. Cells were collected at the indicated time points and stained with DAPI. The percentage populations of cells with 1, 2, or more than 2 nuclei were calculated.

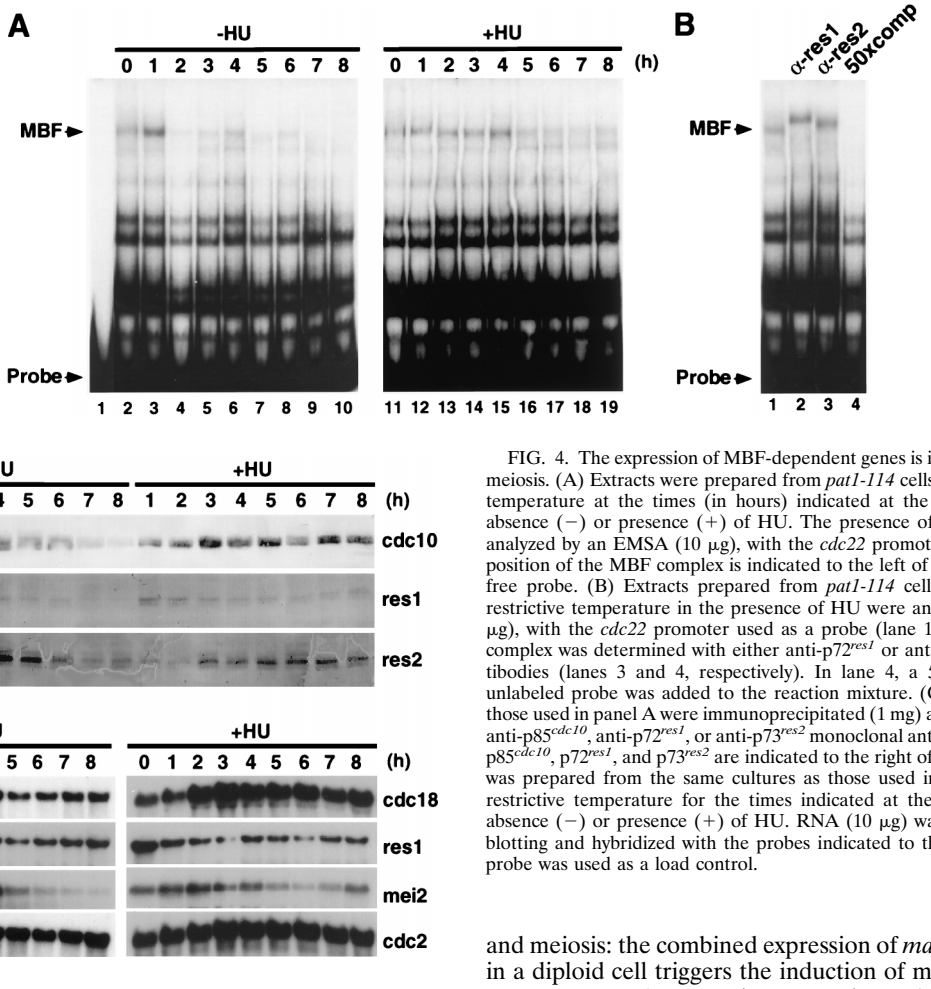

FIG. 4. The expression of MBF-dependent genes is induced at early stages of meiosis. (A) Extracts were prepared from *pat1-114* cells grown at the restrictive temperature at the times (in hours) indicated at the top of the gels, in the absence (−) or presence (+) of HU. The presence of the MBF complex was analyzed by an EMSA (10 μg), with the *cdc22* promoter used as a probe. The position of the MBF complex is indicated to the left of the gel. Lane 1 contains free probe. (B) Extracts prepared from *pat1-114* cells grown for 4 h at the restrictive temperature in the presence of HU were analyzed by an EMSA (10 μg), with the *cdc22* promoter used as a probe (lane 1). The specificity of the complex was determined with either anti-p72^{res1} or anti-p73^{res2} monoclonal antibodies (lanes 3 and 4, respectively). In lane 4, a 50-fold molar excess of unlabeled probe was added to the reaction mixture. (C) The same extracts as those used in panel A were immunoprecipitated (1 mg) and Western blotted with anti-p85^{cdc10}, anti-p72^{res1}, or anti-p73^{res2} monoclonal antibodies. The positions of p85^{cdc10}, p72^{res1}, and p73^{res2} are indicated to the right of the gel. (D) Total RNA was prepared from the same cultures as those used in panel A grown at the restrictive temperature for the times indicated at the top of the gels in the absence (−) or presence (+) of HU. RNA (10 μg) was analyzed by Northern blotting and hybridized with the probes indicated to the right of the gel. *cdc2* probe was used as a load control.

creased. However, in the presence of HU, *mei2* was not induced even though *pat1* was inactivated at the restrictive temperature. The level of *cdc18* mRNA peaked after 2 h and remained at this level during the whole process, indicating that the transcription of this MBF-dependent gene was independent of *pat1* activity. Interestingly, *res1* mRNA levels did not parallel the effect observed at the protein level. *res1* mRNA levels were slightly decreased, in both the presence and absence of HU. This result points to a specific mechanism of p72^{res1} degradation or to a decrease in the translation efficiency of its mRNA when cells enter into meiosis.

The expression of the *mat1* genes is dependent on *res2*. Although the MBF complex and p72^{res1} were no longer detected during meiosis, we considered the possibility that p73^{res2} could be required for the transactivation of a set of genes directly related to the meiotic process and different from the MBF-dependent genes (i.e., *cdc22*, *cdc18*, and *cdt1*). During sexual differentiation, haploid cells of opposite mating types conjugate to form diploid cells that undergo meiotic DNA replication and, finally, sporulate (11). Two different DNA segments of the *mat1* locus specify the haploid cell mating types: *mat1-P* in the plus cells (h^+) and *mat1-M* in the minus cells (h^-) (4). Each of these loci contains two genes, *mat1-Pc* and *mat1-Pm* or *mat1-Mc* and *mat1-Mm*, respectively. It is worth noting that the *mat1-P* locus is absent in h^- cells and that in h^+ cells the *mat1-M* locus is lost. The expression of these four genes is induced during the process of conjugation and meiosis: the combined expression of *mat1-Mc* and *mat1-Pc* in a diploid cell triggers the induction of mat1-Mm and mat1-Pm. As a result, *mei3* (21, 27, 28), an inhibitor of *pat1*, is transcriptionally activated by *mat1-Mm* and *mat1-Pm* (see reference 40 and references therein).

To examine whether the expression of the *mat1* genes was dependent on *res2* function, we prepared total RNA from wild-type and *res2* null cells that had been nitrogen starved for 4 h and analyzed the expression of the *mat1* genes by Northern blotting. The levels of all *mat1* genes were severely reduced in the *res2* null strain, in either the presence or absence of nitrogen (Fig. 5A). Furthermore, *mat1-Pm* mRNA remained undetectable in *res2* null cells. However, a strong induction of the *mat1-Pm* gene occurred in wild-type cells upon nitrogen deprivation, indicating that some of those cells had already undergone conjugation and entered into meiosis. These results suggest that transcriptional control of the *mat1* locus is *res2* dependent and that *res2* function and nitrogen starvation are both required for meiosis to occur.

To determine if p73^{res2} could regulate the expression of these meiotic genes, we transformed wild-type cells of the two heterothallic mating types (h^+ and h^-) and a homothallic (h^{90}) strain with a plasmid that directs the expression of p73^{res2} under the control of the inducible nmt (no message in thiamine) promoter (23). Upon induction of p73^{res2} expression, cells (independent of their mating type) accumulated in the G_1 phase of the cell cycle and acquired a *cdc* phenotype. Concomitantly, the MBF complex was no longer detected and the mRNA levels of MBF-dependent genes (*cdc22*, *cdc18*, and *cdt1*) were strongly reduced (data not shown). RNA prepared from these cultures was analyzed by Northern blotting (Fig.

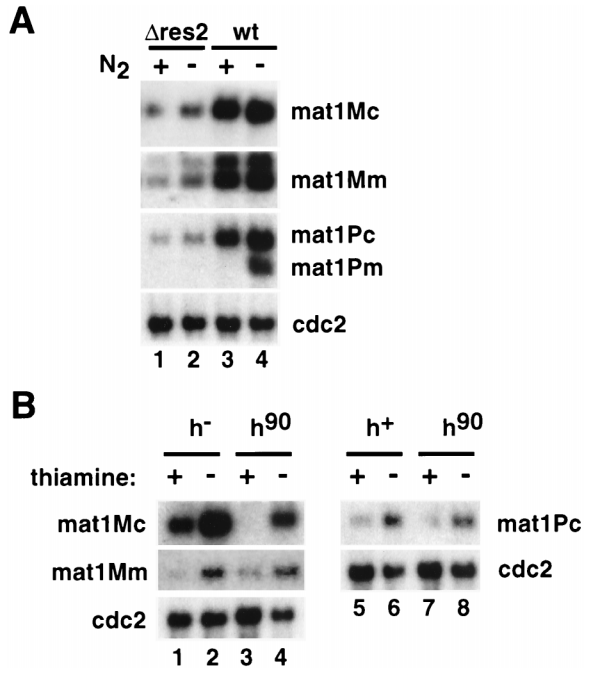

FIG. 5. The transcription of meiosis-specific genes is dependent on *res2*. (A) Total RNA was prepared from homothallic (h^{90}) wild-type (wt) or *res2* null (Δres2) cells grown in the presence (+) or absence (−) of nitrogen, analyzed (4 μg) by Northern blotting, and hybridized with the probes indicated to the right of the blot. *cdc2* probe was used as a load control. (B) Wild-type cells of the different mating types were transformed with a plasmid that directs the expression of p73^{res2} under the control of the *nmt* promoter. Total RNA was prepared from cultures in the presence (+; promoter off) or absence (−; promoter on) of thiamine, analyzed (10 μg) by Northern blotting, and hybridized with the probes indicated to the right and left of the blots.

5B). When p73^{res2} was induced, we detected an increase in the levels of three of the four mating genes, i.e., *mat1-Pc*, *mat1-Mc*, and *mat1-Mm*. Our results suggest that overexpression of p73^{res2} can overcome the nitrogen starvation requirement for the induction of some of the *mat1* genes.

p73^{res2} overexpression enhances entry into meiosis. To further study the role of p73^{res2} during meiosis, p72^{res1}, p73^{res2}, or the DNA-binding domain of p73^{res2} (res2-DBD) was overexpressed in wild-type diploid cells (TE397) and then subjected to nitrogen starvation. The main function of the *mat1* genes during meiosis is to induce the expression of the *mei3* gene, an inhibitor of Pat1 kinase and therefore a direct inducer of meiosis (28). We monitored the expression of *mei3* as a measure of entry into meiosis. As shown in Fig. 6A, expression of either p73^{res2} or its DNA-binding domain induced *mei3* mRNA levels three- and sevenfold (lanes 6 and 8), respectively, compared to that of diploid cells transformed with the backbone vector alone (lane 2). In addition, as a result of the increased levels of *mei3* mRNA, more cells had entered into meiosis (measured as a percentage of cells with four nuclei), i.e., 7 and 18% of the cells transformed with p73^{res2} or res2-DBD, respectively (compared to 3% for the backbone vector) (Fig. 4B and C). Since overexpression of full-length p73^{res2} induced a G_1 arrest, it would be possible that this arrest contributed to inducing meiosis merely because cells were appropriately positioned at Start. However, overexpression of res2-DBD did not have any effect on cell cycle progression (data not shown) but was a better inducer of meiosis. In contrast with these results, *mei3* expression was reduced when p72^{res1} was overexpressed (Fig. 6A, lane 4) and, concomitantly, meiosis was inhibited (<0.1% of cells with four nuclei) (Fig. 6B and C).

DISCUSSION

The results presented herein clarify and expand the role of p72^{res1} and p73^{res2} in regulating entry into the mitotic cell cycle and meiosis. Previously, genetic experiments suggested that p73^{res2} was required for entry into meiosis and had only a minor role in the vegetative cell cycle. For example, loss of *res2* led to a slight prolongation of the doubling time (20%) without

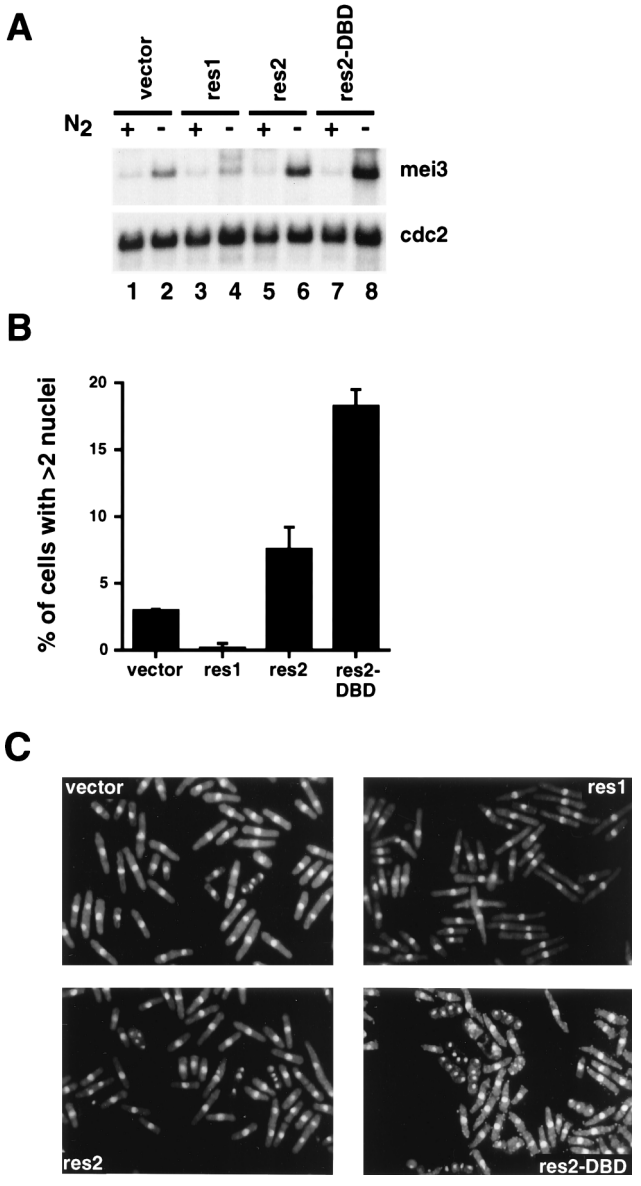

FIG. 6. Overexpression of p73^{res2} induces meiosis. (A) Total RNA was prepared from a wild-type diploid strain transformed with the indicated expression plasmids and grown in the presence (+) or absence (−) of nitrogen for 4 h. Total RNA was obtained from cultures of cells grown at 30°C (at a density of 5×10^6 cells/ml) after 16 h of the induction of the *nmt* promoter (25), analyzed (10 μg) by Northern blotting, and hybridized with probes containing the open reading frame of *mei3* or *cdc2*. (B) Aliquots of the same cultures described for panel A were fixed and stained with DAPI. The percentage of cells with more than two nuclei was calculated, and the average of four different experiments was plotted. (C) DAPI staining of the cultures described for panel B.

any obvious *cdc* phenotype (29, 42). In contrast, loss of *res1* led to a temperature-sensitive phenotype with an arrest of the cell cycle before entry into the S phase. However, *res1* null cells were able to differentiate and complete meiosis (39). Since both p72^{res1} and p73^{res2} could heterodimerize with p85^{cdc10} and bind to MCB-containing promoters (3, 7, 42), it was assumed that there were two different transcription complexes whose activity was required at Start (29, 42).

We demonstrate that both p73^{res2} and p72^{res1} are essential components of a single MBF complex and are required for the controlled expression of genes necessary for premitotic DNA synthesis. The loss of the MBF complex observed in extracts prepared from *res1* null or *res2* null cells supports the conclusion that both proteins are required for the DNA-binding activity of the MBF complex. During the preparation of this paper, similar results were reported by Zhu et al. (43). p72^{res1} and p73^{res2} have significant homology in the N-terminal DNA-binding domain, and it is presumed that both proteins make specific contact with MCB elements in the relevant promoters through their N-terminal region. In addition, p85^{cdc10} is also a component of the MBF complex and binds to both p72^{res1} and p73^{res2}. Our data support a model in which the mitotic MBF complex contains at least three proteins, p72^{res1}, p73^{res2}, and p85^{cdc10}, which are involved in the transcription of S-phase genes, with the former two interacting with each other, even in the absence of p85^{cdc10} (Fig. 2D).

Since there is a strong sequence homology between p72^{res1}, p73^{res2}, and p85^{cdc10} and the *S. cerevisae* proteins Mbp1, Swi4, and Swi6, respectively, one might imagine that a parallel system may function in budding yeast cells. However, this does not appear to be the case. *S. cerevisiae* has been shown to have two heteromeric complexes, Mbp1-Swi6 (MBF) and Swi4-Swi6 (SBF). However, both complexes appear to be involved in the transcription of genes required for S-phase progression of the cell cycle. Neither Mbp1 nor Swi4 has been shown to interact with the other or to form a homodimer with itself. In addition, neither protein has been shown to be essential for meiosis. Furthermore, the DNA-binding activity of MBF and SBF complexes in the budding yeast is contributed by only one protein, Mbp1 or Swi4, respectively. That is, deletion of either Mbp1 or Swi4 does not affect the DNA-binding activity of SBF or MBF, respectively (10, 20). In contrast, our data suggest that in fission yeast cells, p72^{res1}/p73^{res2} heterodimers are both responsible for the DNA-binding of the MBF complex. Thus, the regulation of S-phase genes in fission yeast cells is functionally analogous to that seen in higher eukaryotes in which E2F/DP1 heterodimers are responsible for transcription of genes involved in DNA synthesis (for a review, see reference 1).

The altered expression of MBF-dependent genes in either the *res1* null or *res2* null cells is consistent with the participation of p72^{res1} and p73^{res2} in the regulation of the expression of MBF-dependent genes. However, since cells deleted for *res1* have a more marked phenotype than those deleted for *res2*, this might indicate that, somehow, p72^{res1} can partially substitute for the loss of p73^{res2}, while p73^{res2} cannot fully compensate for p72^{res1} functions during vegetative growth. It is worth noting that in *res1* null cells, both *cdc22* and *cdc18* mRNA levels are reduced. In contrast, *cdc18* mRNA levels were increased in *res2* null cells and reduced in *res1* null cells. The disparity between these two known MBF-dependent genes may reflect additional factors that control their expression. For example, CDC6, the budding yeast homolog of *cdc18*, has recently been shown by McInerny and colleagues to be also under the control of Mcm1, which is responsible for the transcription of the gene early in the cell cycle (25). Thus, it is possible that the high levels of expression of *cdc18* observed in the *res2* null cells are a reflection of other transcriptional activity, which may be dependent on an Mcm1 homolog in *S. pombe*. Nevertheless, the reduced levels of *cdc22* in *res1* null cells and *res2* null cells support the hypothesis that both p72^{res1} and p73^{res2} contribute to the MBF complex and its transcriptional activity.

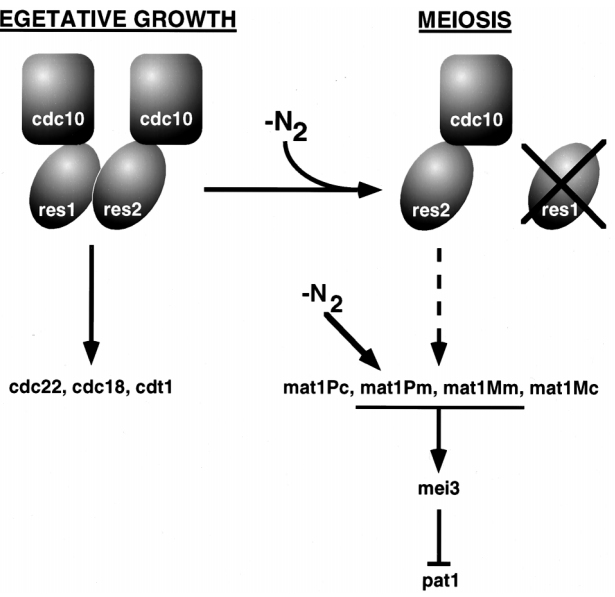

FIG. 7. Model for role of p73^{res2} in vegetative growth and meiosis. During vegetative growth, the MBF complex is composed of p72^{res1} and p73^{res2}, each bound to p85^{cdc10}. The MBF complex is responsible for the cell cycle-dependent expression of *cdc22*, *cdc18*, and *cdt1*. Upon nitrogen starvation, p72^{res1} and the MBF complex are no longer detected. Conversely, to progress into meiosis, p73^{res2} is responsible for the expression of the *mat1* genes, which in turn leads to the transcription of the Pat1 kinase inhibitor *mei3*.

Upon entry into meiosis, the scenario depicted thus far undergoes a dramatic change. p72^{res1} levels decrease until they are no longer detectable, while p73^{res2} levels increase. Consequently, there is a disruption of the MBF complex with a concomitant decrease in the expression of the MBF-dependent genes (i.e., *cdc22*, *cdc18*, and *cdt1*). During meiosis, p73^{res2} switches from being a component of the tripartite MBF complex to a new complex that lacks p72^{res1} but contains at least p85^{cdc10}. Interestingly, p72^{res1} may undergo a targeted degradation during meiosis, in a *pat1*-independent pathway. This degradation was not observed either when the *pat1-114* strain was kept at the nonpermissive temperature in the presence of HU to prevent entry into meiosis (Fig. 4C) or at any phase of the mitotic cell cycle (3b). Thus, in addition to its contribution to the formation of the mitotic MBF complex, p72^{res1} may also serve to inhibit the p73^{res2} meiosis-promoting activity. In support of this hypothesis is the fact that overexpression of p72^{res1} in a nitrogen-starved diploid cell (Fig. 6) or in the *pat1-114* strain held at the restrictive temperature (39) inhibits entry into meiosis. One possible explanation for this observation could be that p72^{res1} sequesters p73^{res2} from its meiotic function, thereby diverting the cells to the mitotic cell cycle. Conversely, p72^{res1} may compete with p73^{res2} for binding to promoters of genes necessary for meiosis. An observation that supports the former hypothesis is that overexpression of the N-terminal DNA-binding domain of p72^{res1}, which does not interact with p73^{res2} (Fig. 2D), did not inhibit entry into meiosis (3).

It is difficult to reconcile our results in the *pat1-114* strain

with data recently published by Caligiuri et al. (8). It was suggested that the interaction between p85^{cdc10} and p72^{res1} was dependent on *pat1*. However, the addition of HU allowed us to separate the effects of Pat1 kinase inactivation from entry into meiosis. It appears, therefore, that both MBF DNA-binding activity and MBF-dependent transcription (Fig. 4A and B; in the presence of HU) are independent of *pat1*.

In *S. pombe*, inactivation of Pat1 with the kinase inhibitor Mei3 is required for entry into meiosis (21, 27, 28). It is known that transcription of *mei3* is induced in a diploid cell upon nitrogen starvation (28). Here we show that *mei3* transcription is also dependent on *res2*. Our results suggest a model in which p73^{res2} has two alternative functions: it is selectively able to either trigger the transcription of S-phase genes upon heterodimerization with p72^{res1} or to promote entry into meiosis when it is not associated with p72^{res1} (Fig. 7). A possible partner of p73^{res2} in both meiosis and mitosis could be p85^{cdc10}. The fact that strains carrying a *cdc10* temperature-sensitive allele are sterile at the nonpermissive temperature is consistent with the hypothesis that a p73^{res2}-p85^{cdc10} complex is active during meiosis (14). Whether p73^{res2} interacts also with itself to form homodimers during meiosis or interacts with another as-yet-uncharacterized protein is unknown. In vitro, p73^{res2} can form homodimers, constituting a major difference with p72^{res1}, which is unable to homodimerize (Fig. 1A and B).

It is not clear whether p73^{res2} transactivates the *mat1* genes directly by binding to their promoters or acts at a point upstream of these genes in the meiotic pathway. The *mat1* genes do not appear to contain an MCB element, and attempts to detect direct binding of p73^{res2} to the promoter of the *mat1* genes have been unsuccessful to date (data not shown). Nevertheless, we cannot rule out direct binding of p73^{res2} to the promoter of the *mat1* genes. Current experiments in our laboratory are aimed at determining the direct targets of p73^{res2} during meiosis.

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