Functional characterization of the fission yeast Start-specific transcription factor Res2

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In the fission yeast *Schizosaccharomyces pombe*, transcriptional activation at Start is mediated by complexes that bind the MCB. Two such complexes have been identified; both contain the Cdc10 protein in partnership with either the Res1 or Res2 protein. Characterization of null mutants suggests that the Res1–Cdc10 complex predominantly functions in mitotic cells whereas the Res2–Cdc10 complex is required for meiosis and spore formation. Here we have characterized the functional domains of the Res2 protein. The N-terminus is both necessary and sufficient for DNA binding, whereas the C-terminus is the region involved in the interaction with the Cdc10 protein. The centrally located ankyrin repeats are dispensable for both functions. Res2 binds to DNA as a dimer. In addition, complexes containing both Res1 and Res2 can form and bind to DNA *in vitro*. Furthermore, the major MCB-specific complex detected in extracts from wild-type cells contains Res1 and Res2; the complex is lost when either gene is deleted and can be recognized by antibodies specific to both proteins. In order to understand the basis for the specific function of Res2 in meiosis, hybrids between Res1 and Res2 were constructed and their functions analysed. The results indicate an absolute requirement for the Res2 C-terminus for normal meiosis to occur whereas the origin of the DNA-binding region is irrelevant. The implications of these results for the regulation of the MCB-binding complexes will be discussed.

Keywords: cell cycle/meiosis/Res1/Res2/Start

Introduction

A central point of regulation of the eukaryotic cell cycle occurs in the late G₁ phase where mitogenic and inhibitory signals are integrated. Passage through this point results in the initiation of S phase and commitment to completion of the cell cycle, irrespective of whether growth-stimulatory signals continue to prevail. In mammalian cells, this regulatory point has been termed the restriction point (Pardee, 1989). In almost all tumour cells, alterations are present that result in loss of the normal regulation that operates at the restriction point, emphasizing its importance in the control of cell proliferation (Strauss *et al.*, 1995).

An equivalent point has been described in yeast and termed Start (for review, see Nasmyth, 1993). Once cells have progressed through Start they are no longer capable of entering the sexual differentiation pathway that leads to meiosis and spore formation until they have completed the cell division cycle and re-entered the G₁ phase. The molecular mechanisms that underlie the regulation that occurs at the restriction point or Start are therefore of considerable interest. Two components of this regulation have been clearly elucidated. The first is that progression through this regulatory point requires the activity of one or more specific cyclin-dependent kinases (cdks). The second is that progression requires the transcriptional activation of specific genes encoding products required for S phase. If this activation is prevented, then cells arrest at the G₁–S transition.

In the fission yeast, *Schizosaccharomyces pombe*, transcriptional activation at Start is mediated by the products of the *cdc10*⁺ (Lowndes *et al.*, 1992), *res1*⁺/*sct1*⁺ (Tanaka *et al.*, 1992; Caligiuri and Beach, 1993) and *res2*⁺/*pct1*⁺ (Miyamoto *et al.*, 1994; Zhu *et al.*, 1994) genes. The Res1 and Res2 proteins interact with the Cdc10 protein to form complexes that can bind specifically to an element known as the MCB box (Caligiuri and Beach, 1993; Zhu *et al.*, 1994); the Res component of the complexes provides the DNA-binding function. The MCB element regulates the Start-specific transcription of several genes required for the process of DNA replication. One key target is the *cdc18*⁺ gene which, when ectopically expressed, can rescue the G₁ arrest induced in *cdc10*^{ts} mutants at the restrictive temperature (Kelly *et al.*, 1993). Furthermore, overexpression of *cdc18*⁺ stimulates repeated rounds of DNA synthesis in the absence of mitosis, suggesting that it links events at Start with the initiation of DNA replication (Nishitani and Nurse, 1995). In mitotic cells, the most important complex that regulates Start-specific transcription is the Res1–Cdc10 complex. When *res1* is deleted, although cells are viable at 30°C, they are severely cold and heat sensitive, resulting from arrest at Start (Tanaka *et al.*, 1992). In contrast, deletion of *res2* has little effect on the viability of mitotically growing cells (Miyamoto *et al.*, 1994; Zhu *et al.*, 1994). However, they are defective in meiosis and pre-meiotic DNA replication, indicating the requirement for the Res2–Cdc10 complex at one or more steps in the sexual differentiation pathway. The growth defect of Δ *res1* cells can be suppressed by the high level expression of *res2*, indicating that both the Res1–Cdc10 and Res2–Cdc10 complexes can recognize and activate target genes critical for cell cycle progression (Miyamoto *et al.*, 1994). However, this is not the case in meiosis since overexpression of *res1* will not suppress the meiotic defect of Δ *res2* cells (Miyamoto *et al.*, 1994).

The Res1 and Res2 proteins are structurally homologous, showing considerable similarity in their N-termini

Fig. 1. The Res2 N-terminus is necessary and sufficient for binding to DNA. **(A)** Schematic of the Res2 mutants used in this study. Shown are the DNA-binding domain (grey box) and the ankyrin repeats (black boxes); the amino acid positions of the truncations and internal deletions are indicated. **(B)** Mobility retardation assays using rabbit reticulocyte lysates containing full-length p73^{res2} protein or mutant derivatives. The radioactive probe used derives from the *cdc18*⁺ promoter and has been described previously (Zhu *et al.*, 1994). The left-hand side panel indicates binding in the absence of p85^{cdc10} whereas the right-hand side panel represents binding in the presence of lysates containing p85^{cdc10}. The arrows indicate the major, specific complexes that are generated without p85^{cdc10} and the dots indicate major complexes that were only seen in the presence of p85^{cdc10}.

and less but still significant similarity in their C-termini. In addition, they contain two good and two weak consensus ankyrin repeat domains that are located centrally. Similar repeat sequences are found in the central portion of the Cdc10 protein (Aves *et al.*, 1985; Breeden and Nasmyth, 1987). These structural features are shared by factors in the budding yeast *Saccharomyces cerevisiae*, that also regulate expression of numerous genes at Start (Andrews and Herskowitz, 1989; Koch *et al.*, 1993). Two different complexes are again evident, with each containing a common subunit, Swi6, together with one of two interacting DNA-binding subunits, Swi4 or Mbp1. The two resulting complexes have slightly different DNA binding specificities and consequently regulate the expression of different but overlapping sets of target genes (for review, see Breeden, 1996).

In order to understand the function and regulation of the Res–Cdc10 complexes, we have characterized the functionally important domains of the Res2 factor and addressed the basis for the different functions of the Res1–Cdc10 and Res2–Cdc10 complexes in meiotic cells. We show that the N-terminus of Res2 is both necessary and sufficient to bind to DNA, whereas the C-terminal region interacts with Cdc10. Neither of these functions of Res2 requires the ankyrin repeats. We also show that *in vitro* complexes generated by Res2 and Cdc10 are homodimeric for the Res2 subunit. However, the major complex detected in extracts from wild-type cells contains both Res1 and Res2. Finally, we show that the C-terminus of Res2 is a critical determinant in specifying its unique role in meiotic cells. The implications of these findings for the regulation of transcription at Start are discussed.

Results

The N-terminus of Res2 is necessary and sufficient to bind to DNA

In order to map functional domains of the Res2 protein, a series of N- and C-terminal mutants and internal deletion mutants were constructed as depicted in Figure 1A. The mutants were designed particularly to address the functions of the conserved regions in the N- and C-termini and the centrally located ankyrin repeats.

The ability of the mutant proteins to bind to DNA was assayed by electrophoretic mobility shift assay (EMSA) following their translation *in vitro*. Each of the mutant constructs was translated efficiently to give one major product of the expected size (data not shown). The labelled probe used in the assay was a 162 bp fragment of the *cdc18*⁺ promoter, and contains six MCB-like sites (Zhu *et al.*, 1994). Incubation of a lysate containing full-length p73^{res2} with the *cdc18* probe gave rise to three complexes (Figure 1B); the fastest migrating complex is non-specific and was observed with all the primed lysates. The other two complexes were specific; they were competed by an unlabelled oligonucleotide containing three MCB sites (data not shown). The middle complex, which was the major complex of the two, resulted from the occupation of a single MCB site on the probe whereas the minor, upper complex possibly resulted from the occupation of two sites. The presence of this minor complex was dependent upon the total level of binding activity and was not observed when a probe containing a single MCB element was used in the reaction (data not shown). The C-terminal deletions (ct1–ct5) also gave rise to a major

Fig. 2. The *res2* mutants *iap1* and *iap2* are functionally inactive. *cdc10-129* cells (NT8) were transformed with the pRep41 vector alone or containing *res2*, *iap1* or *iap2*. The growth of the transformants on EMM plates lacking thiamine was compared at 25 and 36°C.

complex whose migration correlated with the size of the translated product (Figure 1B). The smallest product (ct5) contains the N-terminal 152 amino acids only and showed very strong binding compared with the full-length protein or the other mutants. Clearly, therefore, this N-terminal region is sufficient for DNA binding. This was supported by the fact that both of the N-terminal deletion mutants (*nt1* and *nt2*) failed to form a complex. Surprisingly, very poor binding was also observed with the two internally deleted mutants (*iap1* and *iap2*) even though the N-terminal 150 amino acids were intact. This might suggest that fusion of the C-terminus to the N-terminal domain affected its conformation sufficiently to negate DNA-binding activity.

We had shown previously that binding of $p73^{\text{res2}}$ to DNA was significantly enhanced in the presence of the $p85^{\text{cdc10}}$ protein (Zhu *et al.*, 1994). Accordingly, binding of each of the mutant proteins was assayed in the presence of *in vitro* translated $p85^{\text{cdc10}}$ (Figure 1B). Co-expression of $p85^{\text{cdc10}}$ and $p73^{\text{res2}}$ resulted in a slower migrating and stronger complex than that seen with $p73^{\text{res2}}$ alone. However, there was no effect on the binding of any of the C-terminal deletion mutants, suggesting that they were unable to interact with the $p85^{\text{cdc10}}$ protein. Interestingly, the two internal deletion mutants also produced slow migrating complexes, indicating that they could interact with $p85^{\text{cdc10}}$ and, as a result, their binding to DNA was enhanced. Three conclusions can be made from these results: the N-terminal 150 amino acids are necessary and sufficient for $p73^{\text{res2}}$ to bind to DNA; the C-terminal region including some or all of the terminal 53 amino acid residues is required for interaction with $p85^{\text{cdc10}}$; and the central region spanning from amino acid 152 to 435, which includes the ankyrin repeats, is not required for either binding to DNA or to $p85^{\text{cdc10}}$.

The central region was, however, found to be necessary for function. As shown in Figure 2, overexpression of *res2* can suppress the cell cycle arrest phenotype of *cdc10^{ts}* mutants at the restrictive temperature; *cdc10-129* cells containing *res2* in the pRep41 vector were able to grow at 36°C whereas cells containing the empty vector failed to do so. Cells containing pRep *iap1* or pRep *iap2* were also unable to grow at the restrictive temperature,

suggesting that this central region of Res2 encodes an essential function.

Interaction of Res2 with $p85^{\text{cdc10}}$ is mediated by the C-terminal domain

The results of the binding assays suggested that the C-terminal region of $p73^{\text{res2}}$ was required for complex formation with $p85^{\text{cdc10}}$. To confirm these results and to determine whether the C-terminal region was sufficient, we assayed interaction with $p85^{\text{cdc10}}$ in two ways. The first took advantage of the interaction assay in budding yeast, that was used by us to clone *res2⁺/pct1⁺* (Zhu *et al.*, 1994). Full-length $p73^{\text{res2}}$ or mutant derivatives were expressed together with a VP16-Cdc10 fusion gene in a ΔSWI6 mutant of *S.cerevisiae* containing an integrated LacZ reporter controlled by MCB elements. Thus $p73^{\text{res2}}$ could bind to the MCB elements of the reporter together with the VP16-Cdc10 fusion protein resulting in activated transcription. Failure to bind to DNA or to the fusion protein would result in loss of reporter expression. Expression of the fusion gene was controlled by the inducible *gal* promoter and so growth on glucose or galactose medium allowed reporter expression to be determined in the absence or presence of the fusion. The results are shown in Figure 3A. As described previously (Zhu *et al.*, 1994), co-expression of $p73^{\text{res2}}$ and the VP16-Cdc10 fusion resulted in high levels of LacZ expression. This was also found with the internal deletions *iap1* and *iap2*, although in the latter case expression was decreased. However, none of the C-terminal deletions could support expression of the reporter, confirming the importance of this region for interaction with $p85^{\text{cdc10}}$.

The interaction of $p85^{\text{cdc10}}$ with $p73^{\text{res2}}$ was also examined using a GST fusion protein chromatography assay. The various *res2* mutants were expressed as GST fusions in bacteria, purified by binding to GST beads and incubated with *in vitro* translated [³⁵S]methionine-labelled $p85^{\text{cdc10}}$. Following extensive washing, proteins bound to the beads were analysed by SDS-PAGE. As shown in Figure 3B, the GST-Res2 full-length fusion retained a significant proportion of the input $p85^{\text{cdc10}}$ protein. Deletion of N-terminal residues had very little effect (*Gnt1* and *Gnt2*). In addition, deletion of the central ankyrin repeat-containing

A **B**

Fig. 3. The C-terminus of Res2 is sufficient for interaction with Cdc10. **(A)** Measurement of interaction by the two-hybrid assay. The interaction of Res2 and mutant derivatives with VP16-Cdc10 was assayed essentially as described previously (Zhu *et al.*, 1994). The assay was carried out in a *SWI6*-disrupted strain of *S.cerevisiae* containing a *TMP1* promoter-*lacZ* hybrid gene integrated at the *URA3* locus. The promoter contains two copies of the MCB element. Two plasmids were introduced into this strain: one contained a *VP16-cdc10* hybrid gene under the transcriptional control of the galactose-inducible promoter and the other contained *res2* or mutant derivatives under the control of the constitutive ADH promoter. The resulting transformants were assayed for LacZ activity following growth on glucose (open bar) or galactose (black bar) medium. **(B)** GST pull-down assay with Res2 or mutant derivatives. Full-length or mutant Res2 proteins were fused with GST at their N-terminus. The fusion proteins were purified on glutathione beads and incubated with *in vitro* translated, radioactively labelled p85^{cdc10}. The bound proteins were separated by SDS-PAGE and visualized by autoradiography. GST protein alone was used as a negative control and the total p85^{cdc10} added to each binding reaction shown as input.

region (Giap1 and Giap2) also failed to negate binding, although interaction with Giap2 was again lower. However, interaction was abolished by deletion of C-terminal residues (Gct1–4). Thus, in three different assays, it was clear that the C-terminal region of Res2 was essential for interaction. To show that this region was sufficient, a further fusion was synthesized (Gntnco1) containing Res2

residues from 415 to 657. Efficient interaction of p85^{cdc10} with this fusion was observed.

p73^{res2} binds to DNA as a dimer

Many transcription factors bind to DNA as dimers rather than monomers. The EMSA assay was used to test whether this was also the case with the p73^{res2}-containing complex.

Fig. 4. $p73^{\text{res}2}$ binds to DNA as a homodimer. (A) Equivalent amounts of *in vitro* translated $p73^{\text{res}2}$ protein, or the mutant iap2 or a mixture of both were incubated with labelled *cdc18* probe in the presence of *in vitro* translated $p85^{\text{cdc}10}$. Complexes binding to the probe were revealed by EMSA. The proposed composition of these complexes with respect to the Res2 proteins is indicated. (B) *In vitro* translated and labelled $p73^{\text{res}2}$ or mutant derivatives were incubated with either GST or the GST-Res2 or GST-ct5 fusion proteins. The amount of bound protein is shown together with the input protein added to each binding reaction (input).

Full-length $p73^{\text{res}2}$ and the mutant iap2 were used; both can bind to DNA together with $p85^{\text{cdc}10}$, giving rise to complexes of distinct mobility (Figures 4A and 1C). However, co-expression of both Res2 proteins together with $p85^{\text{cdc}10}$ gave rise to a new complex of intermediate mobility indicative of dimerization between Res2 and iap2 (Figure 4A). The results clearly indicate that $p73^{\text{res}2}$ binds to DNA as a dimer. The stoichiometry of $p85^{\text{cdc}10}$ in the complex was not studied; however, it is also likely to be dimeric, resulting in a final DNA-binding complex that is tetrameric. $p85^{\text{cdc}10}$ does not participate directly in DNA binding although it does enhance the DNA-binding efficiency of the Res subunits. To avoid confusion, therefore, in the remainder of the discussion homo- or heterodimerization will refer specifically to the interaction of the Res subunits.

The results also indicated that Res2 dimerization did not require the centrally located ankyrin repeat-containing region which is absent in the mutant iap2. In other proteins, such repeats have been shown to mediate protein-protein interactions. In order to identify which region was necessary for dimerization, the GST fusion assay was used. ^{35}S -Labelled $p73^{\text{res}2}$ protein and mutant derivatives were incubated with the GST-Res2 protein. The full-length Res2 protein, the C-terminal deletions (ct2, ct4 and ct5) and the two internal deletions (iap1 and iap2) were all retained by the fusion protein (Figure 4B). In contrast, there was no retention of the N-terminal deletions (nt1 and nt2). These results indicate that the dimerization of Res2 requires the N-terminus. We showed above that the N-terminal 150 amino acids were sufficient for DNA binding; since it is unlikely that binding could occur in the absence of dimerization, this suggests that this N-terminal region must also contain the dimerization domain. To confirm that this was the case, the GST-ct5 fusion protein, containing the N-terminal 150 amino acids, was incubated with *in vitro* synthesized ct5 protein; efficient retention was observed (Figure 4B).

Heterodimer formation between $p72^{\text{res}1}$ and $p73^{\text{res}2}$

The demonstration that $p73^{\text{res}2}$ binds to DNA as a homodimer opened up the possibility that $p72^{\text{res}1}$ and $p73^{\text{res}2}$ could form DNA-binding heterodimers. This possibility was tested initially using a similar *in vitro* EMSA assay to that described above for demonstrating $p73^{\text{res}2}$ homodimerization. In this assay, the binding of similar amounts of *in vitro* translated full-length $p72^{\text{res}1}$ and the Res2 mutant iap2 were tested together with $p85^{\text{cdc}10}$. $p72^{\text{res}1}$, together with $p85^{\text{cdc}10}$, produced a weak DNA-binding complex (Figure 5A) and, in this respect, it was far less efficient than $p73^{\text{res}2}$. It would appear, therefore, that the majority of $p72^{\text{res}1}$ did not participate in a complex that bound to DNA. The reason for this is unclear but could result from either inefficient dimer formation, inefficient interaction with the $p85^{\text{cdc}10}$ or low affinity of the complex for DNA. The mutant iap2 produced a faster migrating complex, as described earlier. When both Res proteins were present together with $p85^{\text{cdc}10}$, a new complex of intermediate mobility was detected, indicative of heterodimerization. Increasing the concentration of iap2 resulted in an increase in this intermediate complex.

$p72^{\text{res}1}$ on its own binds very weakly to DNA. This binding is enhanced by complex formation with $p85^{\text{cdc}10}$, although how this enhancement takes place is not clear at present. However, as shown in Figure 5B, it can be mimicked by the addition of anti-Res1 antibodies. In the presence of such an antibody, a strong Res1 complex was seen; such enhancement was not seen with control antibodies. The level of complex formation was equivalent to that seen in the presence of $p85^{\text{cdc}10}$.

The *in vitro* results showed that heterodimers between $p72^{\text{res}1}$ and iap2 could be generated. Of major importance, therefore, was the question of whether Res1 and Res2 formed such heterodimers *in vivo*. Examining MCB-binding complexes in $\Delta\text{res}1$ and $\Delta\text{res}2$ cell extracts indicated that this was likely to be the case. In wild-type extracts, a major slow mobility complex that bound

Fig. 5. p72^{res1} and p73^{res2} can heterodimerize in the presence of p85^{cdc10}. (A) Radioactive probe derived from the *cdc18* promoter was incubated with rabbit reticulocyte lysates containing iap2, p72^{res1} and p85^{cdc10}. The amount of lysate is indicated above the lanes. The arrow indicates the positions of the various homo- and heteromeric complexes. All indicated complexes are dependent upon the presence of p85^{cdc10}. (B) Res1 binding to DNA can be stimulated by interaction with specific antibody. *In vitro* translated proteins were incubated with a radioactive probe derived from the *cdc22* promoter (Zhu *et al.*, 1994). The lysate used and the antibodies added are indicated above the lanes. 'Control' indicates unprogrammed reticulocyte lysate.

to the *cdc18* probe could be readily detected and shown to be specific by competition with an excess of unlabelled oligonucleotide containing MCB-binding sites (Figure 6A). A number of faster migrating complexes were also detected but they were judged to be unspecific since they were not competed by the MCB-containing oligonucleotide. Surprisingly, the major, specific complex was lost in Δ *res1*- and Δ *res2*-derived extracts (Figure 6B). Furthermore, overexpression of Res2 in Δ *res1* cells failed to regenerate the complex although it was regenerated upon Res1 expression (Figure 6B). Similarly, overexpression of Res1 in Δ *res2* cells failed to generate a detectable complex; the complex was restored upon ectopic expression of Res2 (data not shown). These results strongly suggested that the complex required both Res1 and Res2 and was, therefore, most likely heterodimeric in nature. This conclusion was supported by testing the sensitivity of the complex to Cdc10- and Res-specific antibodies (Figure 6C). As expected, the complex was supershifted by an antibody to p85^{cdc10}, but was also supershifted by antibodies to p72^{res1} or p73^{res2}. The anti-Res1 supershifted complex did not have a discrete mobility and was rather broad; nevertheless it was clear that the complex was shifted. Furthermore, in the presence of both anti-Res1 and anti-Res2 antibodies, a supershifted complex that migrated at an even slower position than that generated by either antibody alone was seen. Taken together, these results indicate very strongly that the major complex detected by EMSA is a heterodimer between the Res1 and Res2 proteins. Additional confirmation came from studying complexes derived from extracts of Δ *res2* cells ectopically expressing iap2. In these extracts, a complex was detected that migrated slightly faster than that seen in wild-type extracts, indicative of the presence of the smaller iap2 protein rather than full-length p73^{res2}

(Figure 6D). The complex was supershifted by the anti-Res1 antibody showing that p72^{res1} was also present.

The loss of the major specific complex in Δ *res1* and Δ *res2* extracts was very surprising since *in vitro*, in the presence of p85^{cdc10}, both p73^{res2} and p72^{res1} (albeit poorly) could bind to DNA. Furthermore, there was poor correlation between the presence of the complex and mitotic growth. Both Δ *res2* cells and Δ *res1* cells overexpressing Res2 grow normally and yet, in both cases, complexes were not detected. Even more surprising was a lack of good correlation between the presence of a detectable complex and expression of *cdc18* (Figure 6E). We examined the expression of *cdc18* in Δ *res1* and Δ *res2* cells before and after the addition of hydroxyurea (HU) to block DNA synthesis. Prior to HU treatment, expression levels were found to be low but equivalent in wild-type and Δ *res1* cells, whereas expression was significantly elevated in Δ *res2* cells. Hence, even under conditions where we could not detect a DNA-binding complex by EMSA, expression of *cdc18* could be detected. A direct comparison of expression levels using asynchronous cultures is complicated by the fact that a large proportion of Δ *res1* cells are in G₁ (Tanaka *et al.*, 1992) whereas the vast majority of wild-type and Δ *res2* cells are in G₂. In order to circumvent this problem, we also examined the *cdc18* expression levels in cells treated with HU which causes arrest in S phase. In the wild-type case, HU treatment resulted in elevated expression levels due to the fact that cells had proceeded through Start and activation of the MBF complex had taken place but that the down-regulation that occurs in late S–G₂ was prevented. This activation was significantly affected in the mutant cells, being lower in Δ *res1* cells and absent in Δ *res2* cells (Figure 6E). Moreover, after HU treatment, the level of expression was approximately equivalent in wild-type and

Fig. 6. The MCB-binding complex contains both p72^{res1} and p73^{res2}. (A) EMSA analysis of extracts prepared from wild-type cells (NT110) with radioactive probe derived from the *cdc18* promoter (Zhu *et al.*, 1994) either in the absence or presence of the indicated molar excess of unlabelled oligonucleotide containing three tandem MCB elements. (B) EMSA analysis of extracts prepared from wild-type (NT110) cells, $\Delta res1$ (NT70) cells, $\Delta res2$ (NT38) cells or $\Delta res1$ (NT70) cells overexpressing *res2* or *res1* from the pRep41 vector. (C) Extracts prepared from wild-type (NT110) cells were incubated with radioactive probe derived from the *cdc18* promoter. The composition of the MCB-binding complex was probed by antibody supershift. The antibody additions are indicated above the lane. (D) EMSA analysis of extracts prepared from wild-type cells (NT110) (lanes 1–3) and $\Delta res2$ cells (NT38) overexpressing *iap2* from the pRep41 vector (lanes 4–6). Antibody additions are indicated above the lane. (E) The influence of *res1* and *res2* deletion on the level of target gene expression. Northern analysis of *cdc18* mRNAs from wild-type (NT110) cells, $\Delta res2$ (NT38) cells and $\Delta res1$ (NT70) cells before, 3 and 4.5 h after the addition of hydroxyurea. The level of *his3* mRNA was used as a loading control.

$\Delta res2$ cells but significantly lower in $\Delta res1$ cells. The significance of these results will be discussed later.

Characterization of Res1–Res2 hybrids

The phenotypes of $\Delta res1$ and $\Delta res2$ mutants indicated that the p72^{res1}–p85^{cdc10} complex is required predominantly

during the mitotic cell cycle whereas the p73^{res2}–p85^{cdc10} complex is required for meiosis (Tanaka *et al.*, 1992; Caligiuri and Beach, 1993; Miyamoto *et al.*, 1994; Zhu *et al.*, 1994). There is some redundancy in function since overexpression of *res2* can suppress a $\Delta res1$ phenotype (Miyamoto *et al.*, 1994). However, the converse is not the

case; *res1*⁺ overexpression does not suppress the meiotic defect of Δ *res2* cells (Miyamoto *et al.*, 1994). In order to investigate the nature of their functional difference, we constructed several hybrid genes between *res1* and *res2*. A schematic of the different hybrids analysed is shown in Figure 7A.

Overexpression of either *res1* or *res2* can suppress the cell cycle arrest phenotype of *cdc10*^{ts} mutants at the restrictive temperature. Accordingly, it would be expected that Res1-Res2 hybrids would also show suppression, assuming the hybrid proteins were stable and active. We therefore used this assay to assess the activity of the different hybrids. The hybrids were expressed condition-

ally in *cdc10-129* cells using the pRep41 vector. Growth at 36°C in the absence of thiamine was tested and the results are shown in Figure 7B. Under these conditions, *cdc10-129* cells containing the empty pRep41 vector were completely unable to grow. However, as expected from previous reports, expression of either *res1* or *res2* rescued this phenotype (Figure 2; Tanaka *et al.*, 1992; Caligiuri and Beach, 1993; Miyamoto *et al.*, 1994; Zhu *et al.*, 1994). This was also the case for all the hybrid genes, with the exception of pRep41 Hyb-CP which rescued very poorly. These results demonstrated that the majority of the hybrid genes, when expressed, gave rise to functionally active proteins. This conclusion was confirmed by expres-

sion of the hybrids in a *res1* deletion strain which grows, albeit poorly, at 30°C but shows severe heat and cold sensitivities (Tanaka *et al.*, 1992). Thus as shown in Figure 7C, $\Delta res1$ cells containing the pRep41 empty vector showed no growth at 20°C. Again, this phenotype was rescued by expression of *res1* or *res2* or any of the different *res1-res2* hybrids. In this case some rescue with pRep41 Hyb-CP was detected but it was poor and the resulting colonies were very small. Rescue by the different hybrids of the G₁ arrest phenotype, which $\Delta res1$ cells demonstrate at 20°C, was confirmed by FACS analysis (data not shown).

Given that the different hybrid proteins were shown to be functional, we assessed their ability to rescue the meiotic defect shown by $\Delta res2$ cells. As described previously and in Figure 7D, such mutant cells failed to undergo normal meiosis, with the majority of resulting asci containing no spores as compared with the normal four-spore asci of wild-type cells (Miyamoto *et al.*, 1994; Zhu *et al.*, 1994). Whereas significant rescue of this defect was observed in cells containing the pRep41 *Res2* vector as judged by the number of asci containing four spores, there was no significant rescue with pRep41 *Res1* (Figure 7D). This is similar to previous findings (Miyamoto *et al.*, 1994). Efficient rescue was obtained with pRep41Hyb-SE and pRep41Hyb-SH; in both these cases the N-terminal DNA-binding domain of *Res2* had been replaced with the corresponding region of *Res1*. Therefore, the different activities of *Res1* and *Res2* during meiosis cannot be attributed to different DNA-binding specificities; the origin of the binding domain appeared to be irrelevant. These results focused attention on other regions of *Res2* and in particular the C-terminal domain and the centrally located ankyrin repeats. Both regions appeared to contribute to

the functional specificity of *Res2*. The importance of the C-terminal region was highlighted by pRep41Hyb-PB which, when compared with cells containing the empty vector, was completely ineffective in rescuing the $\Delta res2$ meiotic defect. In this construct, the C-terminus of *Res2* is replaced with the corresponding region of *Res1*, a switch which does not affect function during the mitotic cycle. Similarly, pRep41Hyb-CB and pRep41Hyb-EB, which contain more extensive C-terminal substitutions, were also ineffective in rescue, although in the latter case there was some increase in two spore asci. Nevertheless, it is clear that even in this case meiosis is severely defective. In contrast, pRep41Hyb-CP, which contains an internal substitution and therefore has the *Res2* C-terminal domain, rescued fairly well although not as efficiently as pRep41 *Res2*. However, the C-terminal domain is not the only region that contributes to specificity. The hybrid pRep41Hyb-EP showed no rescue; in this construct the central region of *Res2*, including the ankyrin repeats, was substituted with the analogous region of *Res1*.

Discussion

Functional domains of *Res2*

Analysis of the properties of N- and C-terminal truncations of *Res2* has shown that the N-terminus is important and sufficient for specific binding to MCB elements whereas the C-terminus is sufficient for complex formation with the Cdc10 protein. Importantly, neither of these functions requires the central region of *Res2* which contains the well-conserved ankyrin repeats. These results are similar to those described for the other known Cdc10 partner *Res1* (Ayte *et al.*, 1995) and are consistent with the conservation that exists between these two proteins in

D

	No of spores				
	0	1	2	3	4
Rep41	76.1±0.7	5.4±1.6	9.7±2.1	3.9±0.8	4.7±0.7
Rep41 <i>Res2</i>	29.3±2.2	3.6±1.2	9.4±1.8	3.3±1.0	54.3±2.4
Rep41 <i>Res1</i>	68.3±1.6	5.9±0.6	10.7±0.8	5.1±0.8	8.8±1.1
Rep41 Hyb-SE	30.0±5.1	3.2±0.4	4.3±1.0	2.7±0.2	59.7±4.5
Rep41 Hyb-SH	33.5±5.3	3.5±1.6	6.5±1.9	2.1±0.5	54.4±1.6
Rep41 Hyb-EP	81.3±1.7	6.1±0.3	5.9±0.4	4.0±2.0	3.8±1.7
Rep41 Hyb-EB	66.2±3.1	8.6±0.8	22.9±3.1	5.4±1.0	2.9±0.7
Rep41 Hyb-CP	54.8±2.5	3.6±0.9	8.2±4.3	3.0±0.8	31.0±0.6
Rep41 Hyb-CB	72.5±2.7	5.5±0.3	13.9±0.8	3.9±1.5	4.1±0.4
Rep41 Hyb-PB	78.4±3.4	4.9±1.9	10.5±1.7	3.5±0.9	2.6±0.5

Fig. 7. (A) Schematic of the *Res1-Res2* hybrids analysed in this study. The solid and striped large boxes represent the DNA-binding domains of *Res2* and *Res1* respectively. The small striped and open boxes represent the ankyrin repeats of *Res2* and *Res1* respectively. (B) Suppression of *cdc10-129* by *Res1-Res2* hybrids. *cdc10-129* cells (NT8) were transformed with each of the *Res1-Res2* hybrids in the pRep41 vector as indicated and grown at the permissive temperature of 25°C. Colonies were streaked onto EMM plates lacking thiamine and incubated at 36°C for 4 days. (C) Suppression of the cold sensitivity of $\Delta res1$ cells by the *Res1-Res2* hybrids. $\Delta res1$ cells (NT70) transformed with each of the indicated hybrids were streaked onto EMM plates lacking thiamine and incubated at 20°C for 7 days. (D) Suppression of the meiotic defect of $\Delta res2$ cells by the *Res1-Res2* hybrids. A diploid strain deleted for *res2* (NT40) was transformed with each of the indicated hybrids. They were grown in EMM medium and then transferred to EMM-N medium to induce sporulation at 30°C. After incubation for 24 h, spore formation was analysed by phase-contrast microscopy. The percentage of the population of asci with the indicated number of spores is given. The results represent the analysis of three independent cultures for each of the transformants.

their respective N- and C-termini. It is also clear that similar regions of the Swi4 and Mbp1 proteins of *S.cerevisiae* perform analogous functions, namely binding to SCB or MCB elements and interacting with Swi6 protein (Andrews and Moore, 1992; Primig *et al.*, 1992; Koch *et al.*, 1993; Sidorova and Breeden, 1993; Siegmund and Nasmyth, 1996).

The interaction of p73^{res2} with p85^{cdc10} is clearly important for its ability to bind to DNA. In the absence of p85^{cdc10}, full-length Res2 binds to DNA *in vitro* very weakly, although significantly better binding is observed with the N-terminal domain alone. This may suggest that the C-terminus of the protein inhibits its ability to bind to DNA, perhaps by affecting the conformation of the DNA-binding region or by an intramolecular interaction. Therefore, enhancement of DNA binding by interaction of the C-terminus with p85^{cdc10} might arise from removal of this inhibition. Such a model has been suggested to explain the positive effect of Swi6 interaction on the DNA-binding properties of Swi4 and Mbp1 (Sidorova and Breeden, 1993; Siegmund and Nasmyth, 1996). Alternatively, interaction with p85^{cdc10} may promote or stabilize dimerization. Whatever the mechanism, it appears that the interaction of Res1 with a specific anti-Res1 antibody can mimic the effect since efficient binding of Res1 is seen in the absence of p85^{cdc10}. It has also been shown that efficient and MCB-specific DNA binding can be observed *in vitro* with a GST-Res1 fusion protein (Ayte *et al.*, 1995) which contrasts dramatically with what we observe with the unfused protein. Since it is known that the GST moiety has oligomerization potential, a likely explanation for these apparently conflicting results is that the dimerization of p72^{res1} is stabilized by GST.

The observation that the central region of p73^{res2} containing the ankyrin repeats is not required for interaction with DNA or with p85^{cdc10} but is required for function poses the question of what role this central region plays. Preliminary results suggest that it is involved in transcriptional activation. We have mapped transcriptional activation domains in the Res1 and Res2 proteins and in both cases these domains reside within the central region although the ankyrin repeats do not appear to be necessary (P.Stacey and N.Jones, unpublished observations).

Heterodimerization between p72^{res1} and p73^{res2}

We present evidence in this report that p73^{res2} is homodimeric in the complex that binds to DNA and that the dimerization domain is located within the N-terminal 152 amino acids. This location is perhaps not too surprising since this same region contains the requirements for DNA binding, and invariably DNA-binding and dimerization domains are situated adjacently or overlap. The stoichiometry of the complete p73^{res2}-p85^{cdc10} complex is unknown but we suggest that it is likely to be a tetramer containing two p73^{res2} and two p85^{cdc10} subunits. Some supporting evidence for this was reported by Reymond *et al.* (1993) who showed that truncated forms of p85^{cdc10} could give rise to a complex in *cdc10^{ts}* but not *cdc10* deletion strains. Furthermore, the resulting complexes were smaller than the wild-type complex. They suggested, therefore, that the regenerated complex contained both the truncated and full-length Cdc10 proteins.

The demonstration of p73^{res2} dimerization suggested

the possibility of heterodimerization between the p72^{res1} and p73^{res2} proteins. If this was found to be the case it would increase the possibilities for regulation of the complex and necessitate a re-evaluation of the role of Res1 and Res2 as deduced from the phenotypes of mutants where each gene has been deleted. We present a number of lines of evidence that together strongly suggest that such heterodimers do indeed occur: (i) using full-length and mutant forms of each protein, DNA-binding heterodimers of p72^{res1} and p73^{res2} were shown to form *in vitro*; (ii) the major *in vivo* complex detected by EMSA was lost when either *res1* or *res2* was deleted; (iii) the *in vivo* complex was supershifted by antibodies to both Res1 and Res2; (iv) in cells deleted for wild-type Res2 but expressing a shorter version, the major complex migrated more rapidly and was supershifted by antibodies to Res1.

The finding that p72^{res1} and p73^{res2} form heterodimers poses a number of important questions. One question concerns the lack of detectable DNA-binding complexes in Δ *res1* and Δ *res2* cells. Such complexes can be detected *in vitro*, albeit poorly in the case of Res1-Cdc10. This might suggest that the nature of the complex is different *in vivo* versus *in vitro*, for example via modification of one or more components of the complex. It is important to stress that the detection of the complex does not correlate with the expression of MBF-dependent genes such as *cdc18*; in particular, *cdc18* expression is constitutively high in Δ *res2* cells. Since expression is Cdc10-dependent, the results suggest that although a Cdc10-containing complex cannot be detected, it must nevertheless exist and be active. A similar situation has been reported for the mutant *cdc10-C4*, which is a temperature-sensitive mutant of *cdc10* that derives from the truncation of the C-terminal 61 amino acids. At the permissive temperature, the mutation causes elevated expression of Cdc10 target genes as well as a loss in their periodicity (McInery *et al.*, 1995). However, no DNA-binding complexes could be detected in extracts from these cells (Reymond *et al.*, 1993; McInery *et al.*, 1995).

A second question concerns the function of the heterodimeric complex. The periodic activity of Cdc10-containing complexes seen through the cell cycle could arise from the regulation of the heterodimeric complex through post-translational modification or through its interaction with accessory proteins such as a co-activator. Alternatively, however, it is possible that the composition of the DNA-binding subunits of the complex could change in a cell cycle-dependent manner, switching from heterodimers to homodimers. If the different complexes had different transcriptional activation potentials, then periodic expression of target genes would ensue. What does seem to be the case is that the ability to form heterodimers is important for cell cycle regulation of transcription since in either Δ *res1* or Δ *res2* cells such periodicity is lost (this report and B.Baum and P.Nurse, personal communication).

The specificity of Res2 function in meiosis

Res1 and Res2 can both function in mitotic cells to allow passage through Start, although they do differ quite considerably in their efficiency in so doing. Res1 is significantly better and this may reflect the fact that in mitotic cells it is a much stronger activator of transcription (P.Stacey and N.Jones, unpublished observations). The situation in meiotic cells, however, is different; Res2 is

specifically required and its role cannot be subsumed by Res1 (Miyomoto *et al.*, 1994; Zhu *et al.*, 1994). Furthermore, consistent with its exclusive role, the *res2* but not the *res1* gene is activated upon induction of conjugation (Miyomoto *et al.*, 1994). We have addressed the basis for this functional specificity by characterizing a number of hybrid molecules containing different regions of the Res1 and Res2 proteins. What has emerged is quite surprising and unexpected. A critical determinant of this specificity is the C-terminal region of Res2, since a hybrid where the C-terminal 174 amino acids have been replaced with the corresponding region of Res1 can no longer function in meiosis. This is the region of Res1 and Res2 that is involved in binding to Cdc10. The results must imply, therefore, that either some other function is encoded in this region or that there is regulation of Cdc10 interaction that is dictated by the Res C-terminal region. Whatever the explanation, it is only manifested in meiotic cells since the hybrid functions efficiently in mitotic cells. Two different models can be envisaged. In one, it is assumed that this region is required for transcriptional activation in meiotic but not mitotic cells by, for example, interaction with a meiotic-specific co-activator. A Res2-specific co-activator has been described (Nakashima *et al.*, 1995), although it appears to function specifically in mitotic cells. However, based on this precedence, a similar co-activator may exist that functions together with Res2 in meiosis. An alternative model could involve regulated interaction with the Cdc10 protein. It is possible that modification of either of the Res proteins or of Cdc10 could restrict the interaction of Cdc10 in meiosis to Res2; this modification-induced specificity might be dependent upon the Res2 C-terminus. Thus any molecule that does not have this C-terminal region would be unable to interact with Cdc10 and thus be unable to bind to DNA and function efficiently. Which of these models might be operating, if either, currently is being tested.

It is also clear from the hybrids that no functional specificity can be assigned to the respective DNA-binding domains. This suggests that they have similar DNA-binding specificities and thus are capable of targeting similar promoters. However, an additional specificity determinant resides in the central portion of the Res2 protein since replacement of amino acids 152–483 with the corresponding region of Res1 resulted in a hybrid that could function in mitotic but not meiotic cells. As described earlier, this region does contain activation domains and so the source of the activation domain may be important for function in meiotic cells. Further characterization of the hybrids should prove useful in understanding the regulation and function of the Res proteins.

Materials and methods

Strains and media and genetic methods

The *S.pombe* strains used in this study are summarised in Table I. Complete medium YES and minimal media SD (Sherman *et al.*, 1986) or EMM (Mitchison, 1970) were used for routine culture of *S.pombe* strains. SPA medium (Gutz *et al.*, 1974) was used to induce mating and sporulation. Minimal medium EMM and its nitrogen-free derivative EMM-N (Beach *et al.*, 1985) were used for nitrogen starvation experiments. General genetic methods for *S.pombe* were followed according to Gutz *et al.* (1974) and Moreno *et al.* (1991). The *S.cerevisiae* strain YZ100 has been described previously (Zhu *et al.*, 1994).

Table I. *Schizosaccharomyces pombe* strains used in this study

Strain	Genotype
NT8	<i>h⁻ cdc10-129 leu1-32</i>
NT38	<i>h⁻ ade6-M210 leu1-32 ura4-D18 res2::ura4⁺</i>
NT40	<i>h⁺/h⁻ ade6-M216/ade6-M210 leu1-32/leu1-32 ura4-D18/ura4-D18 res2::ura4⁺/res2::ura4⁺</i>
NT70	<i>h⁻ ade6-M216 leu1-32 ura4-D18 res1::ura4⁺</i>
NT110	<i>h⁻ leu1-32 ura4-D18</i>

Plasmids

The plasmid pGal1-10-vp16cdc10 has been described previously (Zhu *et al.*, 1994). For *in vitro* translations, the cDNAs of *res1*, *res2* and *cdc10* were cloned into the T7plink vector (Dalton and Treisman, 1992). For the construction of the various Res2 mutants, fragments were removed through the use of appropriate restriction sites. For the C-terminal deletions, sequences were removed extending from the C-terminus to the *HindIII* site at nucleotide position 1799 relative to the translation initiation site (ct1), the *PstI* site at position 1511 (ct2), the *NcoI* site at position 1142 (ct3), the *Clal* site at position 1051 (ct4) and the *EcoRI* site at position 453 (ct5). For the N-terminal deletions, sequences were removed extending from the N-terminus to the *FspI* site at position 194 (nt1) and the *EcoRI* site at position 453 (nt2). For the internal deletions, sequences were removed between the *EcoRI* and *Clal* sites at positions 453 and 1052 (iap1) and the *EcoRI* and *BspEI* sites at positions 453 and 1300 (iap2). For *in vitro* translations, the mutants were cloned into T7plink, for GST fusions in the vector GST-KG (Guan and Dixon, 1991) and for budding yeast expression cloned behind the ADH promoter in a high copy expression vector (Fikes *et al.*, 1990). The *res1-res2* fusion plasmids were constructed by introducing appropriate restriction sites into the indicated positions by PCR mutagenesis; derivatives of *res1* were constructed containing *EcoRI*, *PstI* and *Clal* sites at equivalent positions to these sites in *res2* and a derivative of *res2* was constructed with a *HindIII* site at the equivalent position to the *HindIII* site in *res1*. Restriction fragments from wild-type or modified *res1* and *res2* were then combined to give the various hybrids. All hybrids were cloned into the plasmid pRep41 containing a thiamine-repressible, mutant *nmt1* promoter (Maundrell, 1993).

Cell extract preparation

Exponentially growing cells were harvested by centrifugation, washed once with distilled water, resuspended in lysis buffer [25 mM HEPES (pH 7.6), 0.1 mM EDTA, 150 mM KCl, 0.1% Triton X-100, 25% glycerol, 1 M urea, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 5 µg/ml each of aprotinin, leupeptin and pepstatin] and disrupted by vortexing with glass beads twice for 45 s. The lysates were cleared by centrifugation at 13 500 g for 10 min at 4°C. Protein concentration was determined by standard Bradford assay.

Northern analysis

Exponentially growing cells were harvested by centrifugation, washed once with distilled water and resuspended in cold RNA buffer [100 mM EDTA, 100 mM NaCl and 50 mM Tris (pH 8.0)] and 0.5% SDS. After the addition of an equal volume of phenol:chloroform, cell breakage was carried out by vortexing with glass beads three times for 1 min. After a further phenol:chloroform extraction, the RNA was precipitated with 0.6 vol. of isopropanol, washed with 70% ethanol, dried, resuspended in 1 mM EDTA/0.1% SDS and stored at -70°C. For Northern analysis, 15 µg of RNA was loaded onto a 1.2% formaldehyde agarose gel and transferred to GeneScreen membrane (DuPont). Hybridization and washes were performed as recommended by the manufacturer. Probes were prepared from 1.3 kb *cdc18⁺* and 1.0 kb *his3⁺* fragments with [α -³²P]dCTP by random priming (Amersham Megaprime kit). The hydroxyurea block was performed by adding HU (Sigma) to a final concentration of 11 mM to exponentially growing cells. After the appropriate incubation period, cells were harvested and treated as described above.

Electrophoretic mobility shift assays

The radiolabelled DNA fragments and EMSA assays employing *in vitro* translated proteins have been described in detail previously (Zhu *et al.*, 1994). Note that the *in vitro* translated proteins used in EMSA assays were produced in reactions containing [³⁵S]methionine so that their relative levels could be assessed. Autoradiography of the EMSA assays

was performed using two films: the first (inner film) filters out the contribution of the ^{35}S -labelled proteins so that the second (outer film) specifically detects the ^{32}P -labelled DNA. Binding reactions using cell extracts were performed by adding 10 μl of native extract (10–100 μg protein) in lysis buffer to 20 μl reactions containing 25 mM HEPES (pH 7.6), 34 mM KCl, 5 mM MgCl_2 and 2 μg of poly[d(I-C)]. Reactions were incubated for 10 min at room temperature before the addition of ~0.5 ng of [^{32}P]dNTP-labelled probe followed by a further 20 min incubation. At this point, samples were analysed by electrophoresis through 4% polyacrylamide gels run in 0.25 $\times$ Tris-borate-EDTA buffer. Antibody supershifts were performed by adding an appropriate amount of antibody 10 min following the addition of probe DNA. Anti-p72^{res1} (RY115) monoclonal antibody and anti-p85^{cdc10} (YS140) monoclonal antibody were the kind gift of J.DeCaprio (Harvard University) and their use has been described (Ayte *et al.*, 1995). The p73^{res2} antibody was a polyclonal antibody raised against a GST-Res2 fusion. The anti-myc antibody (9E10) was a gift of G.Evan (ICRF) and the anti-HA (12CAS) antibody was purchased from Boehringer Mannheim.

GST pull-down assays

GST fusion proteins were synthesized and bound to glutathione beads as previously described (Kaelin *et al.*, 1991). For the binding assays, ~500 ng of the GST fusion protein loaded on beads was incubated with 2–4 μl of *in vitro* translated proteins in a final volume of 200 μl in EBC buffer [140 mM NaCl, 0.5% (v/v) NP-40, 100 mM NaF, 200 mM sodium orthovanadate, 50 mM Tris-HCl, pH 8.0] at 4°C on a rotating platform. The beads were washed three times in 1 ml of pre-chilled NETN buffer, pelleted at 500 r.p.m. in an Eppendorf microfuge for 30 s and boiled in 4 $\times$ Laemmli buffer. Bound proteins were resolved by 12% SDS-PAGE.

Assay of β -galactosidase activity

Approximately 10^6 cells were harvested, permeabilized with chloroform and SDS and the β -galactosidase activity measured as described previously (Guarente, 1983).

Acknowledgements

We are grateful to Jerome Wuarin for advice on extract preparation for EMSA and to B.Baum and P.Nurse for communication of results before publication. We thank H.Okayama, P.Nurse and J.DeCaprio for yeast strains and plasmids and for anti-Res1 and anti-Cdc10 antibodies. We are grateful to Jerome Wuarin for comments on the manuscript and members of the Gene Regulation Laboratory for general advice and comment. This work was funded by ICRF and the HFSP.

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Received on August 6, 1996; revised on October 30, 1996