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Functional Domains of Rep2, a Transcriptional Activator Subunit for Res2–Cdc10, Controlling the Cell Cycle "Start"

Sayaka Tahara,*† Koichi Tanaka,† Yasuhito Yuasa,* and Hiroto Okayama†‡

*Department of Hygiene and Oncology, Tokyo Medical and Dental University, School of Medicine, Bunkyo-ku, Tokyo 113, Japan; and †Department of Biochemistry and Molecular Biology, The University of Tokyo Graduate School of Medicine, Bunkyo-ku, Tokyo 113, Japan

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In the fission yeast *Schizosaccharomyces pombe*, passage from G_1 to S-phase requires the execution of the transcriptional factor complex that consists of the Cdc10 and Res1/2 molecules. This complex activates the *Mlu*I cell cycle box *cis*-element contained in genes essential for S-phase onset and progression. The *rep2*$^+$ gene, isolated as a multicopy suppressor of a temperature-sensitive *cdc10* mutant, has been postulated to encode a putative transcriptional activator subunit for the Res2–Cdc10 complex. To identify the *rep2*$^+$ function and molecularly define its domain organization, we reconstituted the Res2–Cdc10 complex-dependent transcriptional activation in *Saccharomyces cerevisiae*. Reconstitution experiments, deletion analyses using one and two hybrid systems, and in vivo Res2 coimmunoprecipitation assays show that the Res2–Cdc10 complex itself can recognize but cannot activate *Mlu*I cell cycle box without Rep2, and that consistent with its postulated function, Rep2 contains 45-amino acid Res2 binding and 22-amino acid transcriptional activation domains in the middle and C terminus of the molecule, respectively. The functional essentiality of these domains is also demonstrated by their requirement for rescue of the cold-sensitive *rep2* deletion mutant of fission yeast.

INTRODUCTION

The periodic expression of genes required for cell cycle progression is a common feature of cell cycle regulation in eukaryotes. A number of genes required for the onset of DNA synthesis are expressed during the G_1–S transition. In yeast a few such genes contain a *cis*-regulatory element called *Mlu*I cell cycle box (MCB) (ACGCGTNA) in their promoter (Lowndes *et al*., 1991; McIntosh *et al.*, 1991; for review, see Johnston *et al*., 1991; Andrews, 1992). A factor complex that specifically binds to the MCB *cis*-element was initially identified in budding yeast by gel shift assay and called DSC (Lowndes *et al*., 1991). Subsequently, a DSC-like factor was also detected from the fission yeast *Schizosaccharomyces pombe* (Lowndes *et al*., 1992b). DSC consists of at least two distinct molecules, which in *S. pombe* are Cdc10, a structural homologue of *Saccharomyces cerevisiae* Swi6, and Res2 or Res1, homologues of budding yeast Swi4 or Mbp1. Cdc10 forms a complex with Res2 or Res1. The Res and Mbp1 subunits possess an MCB binding domain in their N-terminal region (Lowndes *et al*., 1992b; Tanaka *et al*., 1992; Caligiuri and Beach, 1993; Koch *et al*., 1993; Reymond *et al.*, 1993; Miyamoto *et al*., 1994; Zhu *et al*., 1994; Ayte *et al*., 1995; Zhu *et al*., 1997; for review, see Moll *et al*., 1993). Although Cdc10 and Swi6 are essential for the DSC activity (Dirick *et al.*, 1992; Lowndes *et al*., 1992a,b; Verma *et al*., 1992; Reymond *et al.*, 1993), their role remains unknown. DSC was initially thought to be a transcriptional factor complex that activates MCB, but recent analysis indicates that it is rather a transcriptionally inactive complex responsible for transcriptional repression in late S–G_2 for fission yeast (McInerny *et al*., 1995; Baum *et al.*, 1997). Although the active transcriptional complex requires the same components (Tanaka *et al*., 1992; Caligiuri *et al*., 1993; Reymond *et al.*, 1993; Miyamoto *et al*., 1994; Zhu

‡ Corresponding author.

et al., 1994, 1997), its biochemical nature is little understood.

Recently we identified a new component for the active Res2–Cdc10 complex that functions to start the mitotic cell cycle. It is a zinc-finger protein encoded by *rep2*$^+$, which was isolated as a multicopy suppressor of a *cdc10* mutant (Nakashima *et al.*, 1995). *rep2*$^+$ suppresses growth defects of cells lacking *res1*$^+$. The Rep2 molecule binds Res2 in vitro and forms a complex with Res2–Cdc10 in vivo (Nakashima *et al.*, 1995). In the cells deleted for *rep2*$^+$, MCB is only partially activated, as evident from reduced induction of *cdc18*$^+$, a key target gene for Res–Cdc10, yet additional deletion of *res2*$^+$ restores the activation of MCB (Baum *et al.*, 1997). Consequently, these genetic and biochemical data strongly suggested that Rep2 is a transcriptional activator subunit for the Res2–Cdc10 complex that functions as an MCB binding complex.

To obtain definitive evidence and identify the functional domains of Rep2, we reconstituted Res2–Cdc10-dependent transcriptional regulation in the budding yeast *S. cerevisiae* and carried out deletion analysis of the Rep2 molecule. In this article we provide solid evidence that Rep2 is a transcriptional activator subunit for Res2–Cdc10 and show that the Rep2 molecule contains a Res2 binding and a transcriptional activation domain in the C-terminal half, both of which are essential for Rep2 function.

MATERIALS AND METHODS

Strain and Media

The strains of *S. cerevisiae* and *S. pombe* used in this study are listed in Table 1. Media were prepared as described (Guthrie and Fink, 1991; Sturm and Okayama, 1996).

Construction of Assay System in S. cerevisiae

A *LacZ* transcriptional unit driven by the triple *Mlu*I sequence containing the core promoter of the *S. cerevisiae* cytochrome *c* gene (*CYC1* $-1 \sim -178$) was excised from pSPΔ*178.3 M* (Lowndes *et al.*, 1991) and subcloned into the single-copy plasmid pRS313 (Sikorski and Hieter, 1989) with the *ADH* transcriptional terminator. This plasmid was used as a *LacZ* reporter for monitoring the activation of MCB by Cdc10–Res2. The *S. cerevisiae* wild-type strain YPH499 (Sikorski and Hieter, 1989) was disrupted for the *SWI6* gene by a one-step gene replacement (YPH-ls). The *res2*$^+$-coding region fused with the *GAL1* promoter (Tanaka *et al.*, 1990) was subcloned into the YIP vector containing the *ADE2* gene as a selective marker and integrated at the *ade2* locus in the cells disrupted for the *SWI6* gene (YPH-lsr2).

The *HIS3* reporter gene was constructed as follows. The coding region of the *HIS3* gene and the 166 bp promoter of the *S. cerevisiae* thymidine synthase gene (*TMP1* -1 to -166 bp) containing two MCB elements (McIntosh *et al.*, 1991) were ligated, subcloned into a *GAPDH* terminator-containing YIP vector with the *LYS2* gene as a selective marker, and integrated into the *lys2* locus of YPH499 followed by disruption of the *SWI6* gene (YPH-ts). The *res2*$^+$ gene driven by the *GAL1* promoter was integrated at the *ade2* locus in YPH-ts to obtain a final host strain YPH-tsr2. Disruption and integration were confirmed by genomic Southern blotting. The *vp16*-fused *cdc10*$^+$ gene was constructed by ligating the *VP16* activation domain (78 amino acids from 413 to 490 aa) (Sadowski *et al.*, 1988) to the initiation codon of *cdc10*$^+$, joined to the *GAL1* promoter, and inserted into a *LEU2* marker-containing single-copy plasmid (Sikorski and Hieter, 1989). The *rep2*$^+$ coding region was cloned into the multicopy plasmid pKT10-GAPDH driven by the *GAPDH* promoter (Tanaka *et al.*, 1990).

Assay for Transcriptional Activity of Res2–Cdc10–Rep2 in the Reconstitution System

The assay host strains transfected with the indicated expression constructs and the *LacZ* reporter plasmid were inoculated in synthetic minimal medium (SD) containing 3% galactose and 0.2% sucrose at 30°C and grown to log phase. The cells were harvested and ruptured by freeze and thaw, and then β-galactosidase activity was measured as described (Clontech, Cambridge, United Kingdom).

The *HIS3* selection host cells were transfected with the indicated constructs and selected on SD plate containing 2% glucose at 30°C for 3 d. The transfectants were spotted on 3% galactose/0.2% sucrose SD plate containing 4 mM 3-aminotriazole (3AT) and incubated at 30°C for 10 d. 3AT was used to inhibit the basal activity of the *HIS3* gene product in this strain.

Yeast One- and Two-Hybrid Systems

The yeast one- and two-hybrid assay systems were performed using the commercial Matchmaker two-hybrid system (Clontech). To construct the full-length and deletion mutants of *rep2*$^+$, fragments from the indicated amino acid to the end of ORF were excised from pcL-*rep2*$^+$ by PCR. To destroy the zinc-finger motif in Rep2, 177 Cys (tgc) was changed to Gly (ggt) and 180 Cys (tgc) to Ser (tcc). These constructs were inserted into pGBT9. The *res2*$^+$ gene fused with the *GAL4* transactivation domain (pGAD424) was constructed as described previously (Nakashima *et al.*, 1995)

The *S. cerevisiae* reporter strain SFY526 was transfected with pGAD424-X and pGBT9-*rep2*$^+$ for the one-hybrid system and with pGAD424-*res2*$^+$ and pGBT9-*rep2*$^+$ for the two-hybrid system. Transformants were cultured to log phase in SD medium at 30°C and then harvested and ruptured by freeze and thaw, and β-galactosidase activity was measured as described (Clontech).

Antibodies

The anti-Res1/2 antibody was described previously (Nakashima *et al.*, 1995). The anti-FLAG M2 affinity gel was purchased from IBI (New Haven CT), and the anti-FLAG D8 polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Protein Extraction and Immunoprecipitation

The *rep2* (N3–141S), *res1* (K156-D1), and *res2* (M222) disruptants were transfected with FLAG-tagged deletion mutants of *rep2*$^+$, which were induced by culturing in thiamine-free pombe minimal medium (PM) at 30°C for 13–17 h depending on the constructs to produce similar levels of protein. The crude extract was prepared as described (Nakashima *et al.*, 1995), and protein concentration was determined by the Bradford method. Crude cell extracts (4.5 mg/ml) were incubated at 4°C for 1 h with 20 μl of anti-FLAG M2 affinity gel containing 0.15 M NaCl followed by centrifugal sedimentation of immunoaffinity gel-bound proteins. Affinity gel-bound proteins were washed five times with 500 μl of buffer H (Booher *et al.*, 1989) containing 0.15 M NaCl, 1 mM PMSF, 20 μg/ml pepstatin A and leupeptin, and 10 μg/ml aprotinin, separated by SDS-PAGE (7.5% gel for anti- Res1/2 and 15% for anti-FLAG), and analyzed by Western blotting using anti-Res1/2 and anti-FLAG D8 antibodies as described previously (Jinno *et al.*, 1994) and in the IBI protocol.

Suppression of rep2 Disruptant Cells by Deletion Mutants of the rep2$^+$ Gene

For assay in *S. pombe*, all of the constructs were expressed from the SV40 promoter (Okazaki *et al.*, 1990). The deletion mutants of *rep2*$^+$ were constructed by PCR amplification followed by insertion into the pcL vector.

The *rep2* disruptant was transformed with the indicated constructs as described (Okazaki *et al.*, 1990). One-half of the transfectant was selected for *Leu*$^+$ at 30°C, and the other half was selected first at 30°C for 16 h and then at 18°C for 2 wk. The ratios of colonies formed at the nonpermissive temperature to those formed at the permissive temperature were expressed as percentage suppression.

Cell Cycle Distribution and Expression of cdc18$^+$ mRNA

The *rep2* disruptant (N3–141S) was transfected with the indicated plasmids and selected for *Leu*$^+$ at 30°C. Transformants were cultured at 30°C to midlog phase in PM medium and shifted to 18°C. Flow cytometry and Northern blotting of *cdc18*$^+$ mRNA were performed for the cells before and after a 53 h incubation at 18°C. The probe was the ^{32}P-labeled *Bam*HI fragment of *cdc18*$^+$. Flow cytometry was performed as described previously (Nakashima *et al.*, 1995) using the FACScan system and CellFIT cell cycle analysis program and the software LYSISII (Becton Dickinson, San Jose, CA).

RESULTS

Reconstruction of Transcriptional Activation by Res2–Cdc10 in Budding Yeast

One clear demonstration of the requirement for Rep2 in the Res2–Cdc10 activity is the reconstitution of this transcriptional system in an evolutionary distant organism. *S. cerevisiae* is a suitable organism for such an experiment because Swi6 cannot functionally be substituted with its fission yeast homologue Cdc10 (Lowndes *et al.*, 1992a). Figure 1 schematically represents the reconstitution system we constructed. In this system, two reporter genes were used to confirm the dependence of Res–Cdc10-activated transcription on MCB and its independence from the core promoter used. One is the *LacZ* coding sequence ligated to a core sequence ($-1 \sim -178$) of the *CYC* (cytochrome *c*) gene promoter fused with artificially synthesized three repeats of the *Mlu*I sequence as a Res–Cdc10-responsive MCB *cis*-element. The other is the His3 coding sequence ligated to the *TMP* promoter ($-1 \sim -166$) containing the two endogenous MCB motifs. The resulting reporter genes were expressed in appropriate host cells from a single-copy plasmid or integrant. Accordingly, promoter activation was assayed by determining induced β-galactosidase activity or the cell's ability to grow without histidine.

As already noted, the budding yeast contains Cdc10–Res homologues that can activate the *Mlu*I sequence used as MCB. To reduce background levels caused by this homologous system, the *SWI6* gene was deleted from the host cells. In addition, the *res2*$^+$ gene under the control of the *GAL1* promoter was integrated into the *ade2* locus of host cells. The proper function of the enhancer and the inducibility of the artificial promoter were confirmed by β-galactosidase activity strongly induced by coexpression of Res2 and vp16 (transactivator activity)-fused Cdc10. This induction was absolutely dependent on the MCB *cis*-element. When the *MluI* sequence was mutated to ACtaGT, induction of β-galactosidase was completely failed (Figure 2A). In this control experiment, expression of Res2 alone slightly induced β-galactosidase activity.

However, coexpression of Cdc10 did not elevate β-galactosidase activity but rather repressed its level. Coexpression of vp16-fused Cdc10 induced β-galactosidase activity >10-fold. This induction depended on the coexpression of Res2. Similar results were obtained with the His3 selection host (Figure 2C). These results show that Res2–Cdc10 alone can recognize, but cannot activate, MCB.

Rep2 Activates the Res2–Cdc10 Complex

We next examined whether Rep2 could activate Res2–Cdc10 in the reconstitution system. In this experiment, the *rep2*$^+$ coding sequence was inserted into a *GAPDH* promoter-driven multicopy expression vector and expressed in the host cells with or without expression of Res2 and Cdc10. As shown in Figure 2B, in the presence of Res2 and Cdc10, Rep2 coexpression activated the MCB-containing *CYC* promoter six- to sevenfold as measured by β-galactosidase activity. Interestingly, coexpression of only Res2 and Rep2 induced β-galac-

Table 1. Strains used in this study

Strain	Genotype
YPH499	*MAT***a** *ura3-52 ade2-101 leu2-1 trp1-Δ63 his3-Δ200 lys2-Δ1*
YPH-ls	*MAT***a** *ura3-52 ade2-101 leu2-Δ1 trp1-Δ63 his3-Δ200 lys2-Δ1 swi6::TRP1*
YPH-lsr2	*MAT***a** *ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 lys2-Δ1 swi6::TRP1 ade2-101- GAL1 res2*$^+$ *-ADE2*
YPH-ts	*MAT***a** *ura3-52 ade2-101 leu2-Δ1 trp1-Δ63 his3-Δ20 swi6::TRP1 lys2-Δ1-TMP-HIS3-LYS2*
YPH-tsr2	*MAT***a** *ura3-52 ade2-101 leu2-Δ1 trp1-Δ63 swi6::TRP1 ade2-101- GAL1 res2*$^+$*-ADE2 lys2-Δ1-TMP-HIS3-LYS2*
N3-141S	*h*$^-$ *leu1-32 ura4-D18 rep2::ura4*$^+$
K156-D1	*h*$^-$ *leu1-32 ura4-D18 res1::ura4*$^+$
M222	*h*$^-$ *leu1-32 ura4-D18 res2::ura4*$^+$

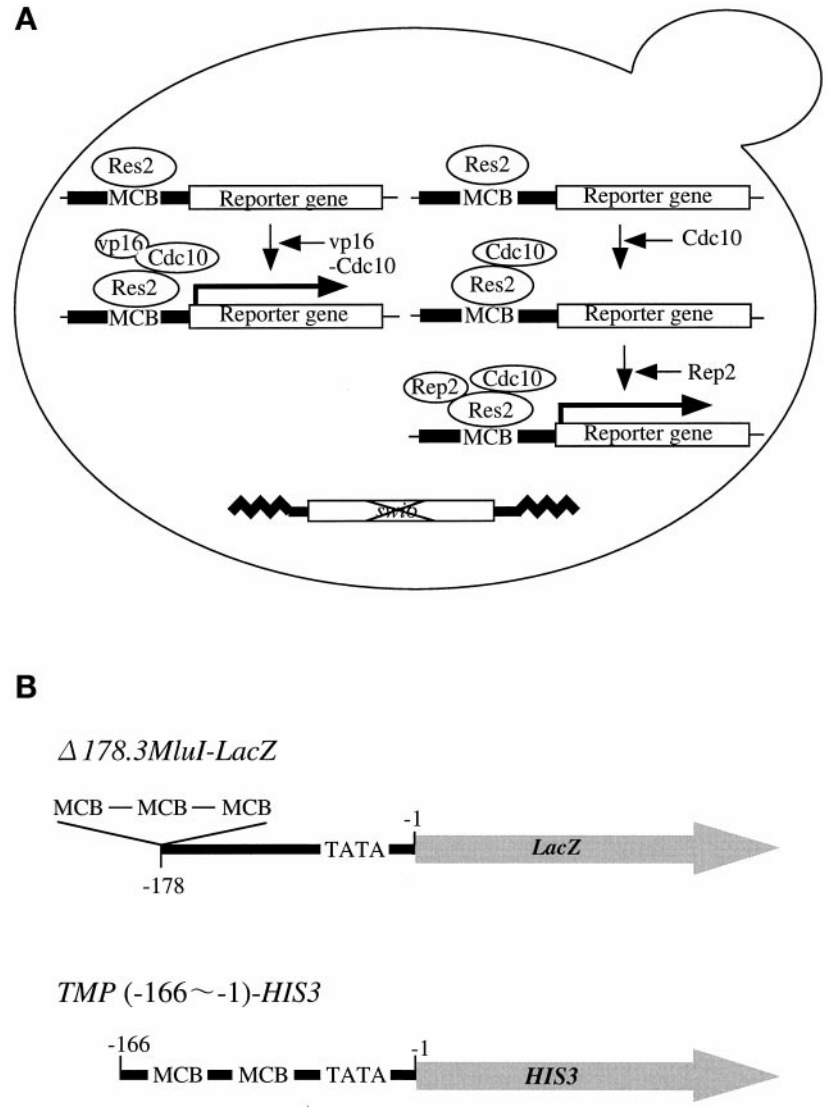

Figure 1. Schematic representation of the assay systems for *in vivo* transcriptional activation by Res2–Cdc10–Rep2. (A) Assays for transcription activation by Res2–Cdc10–Rep2 were carried out with a Δ*swi6* strain of *S. cerevisiae* and a single copy of a reporter gene whose promoter contains two or three MCB elements. Res2 by itself would not activate reporter gene, whereas the Res2–vp16–Cdc10 complex would. Res2–Cdc10 would have no or very low transcriptional activity, but coexpression of Rep2 with Res2–Cdc10 is expected to activate the reporter gene. (B) Structure of reporter genes in Δ*178.3 MluI-LacZ*, the promoter of *S. cerevisiae CYC1* (−1 ～ −178) containing exogenously inserted triple *Mlu*I sequences (MCB element) is fused with the *LacZ* coding sequence. In *TMP-HIS3*, the *S. cerevisiae TMP* promoter (−1 ～ −166) containing two endogenous MCB motifs at −159 and −122 is ligated to the *S. cerevisiae* His3 coding sequence.

tosidase activity, but to a slightly low level. Because the basal level of β-galactosidase activity induced by Res2 alone was a bit higher, the induction by Rep2 coexpression was only twofold. In fission yeast, overexpression of Res1 completely and overexpression of Res2 at least largely dispense Cdc10 (Tanaka *et al.*, 1992; Caligiuri and Beach, 1993; Miyamoto *et al.*, 1994; Zhu *et al.*, 1994). This situation thus appears to be reproduced with the reconstitution system in a heterogeneous organism. As expected, the activation of the promoter by Rep2 absolutely depended on Res2. These results were confirmed with the reconstitution system using the *HIS3* selection host. Virtually identical or even clearer results were obtained by using this system (Figure 2C). These results support our previous assignment of Rep2 to a transcriptional activator subunit for the Res2–Cdc10 complex.

Transactivation Domain of Rep2 Is Located at the C Terminus

If Rep2 is indeed a transcriptional activator subunit for Res2–Cdc10, it must contain a domain that is responsible for transcriptional activation. We examined this question by using a budding yeast one-hybrid system. A series of N- and C-terminal and internal deletion mutants of $rep2^+$ were constructed, fused with the Gal4 DNA binding domain at their N terminus, and expressed in *S. cerevisiae* containing a *LacZ* reporter gene with a Gal4 binding element in the promoter (Figure 3A). The transcriptional activation ability of the deletion mutants was then assayed by measuring induced β-galactosidase activity (Figure 3B, black bar).

In this hybrid construct, full-length Rep2 showed a relatively low LacZ induction. Progressive N-terminal

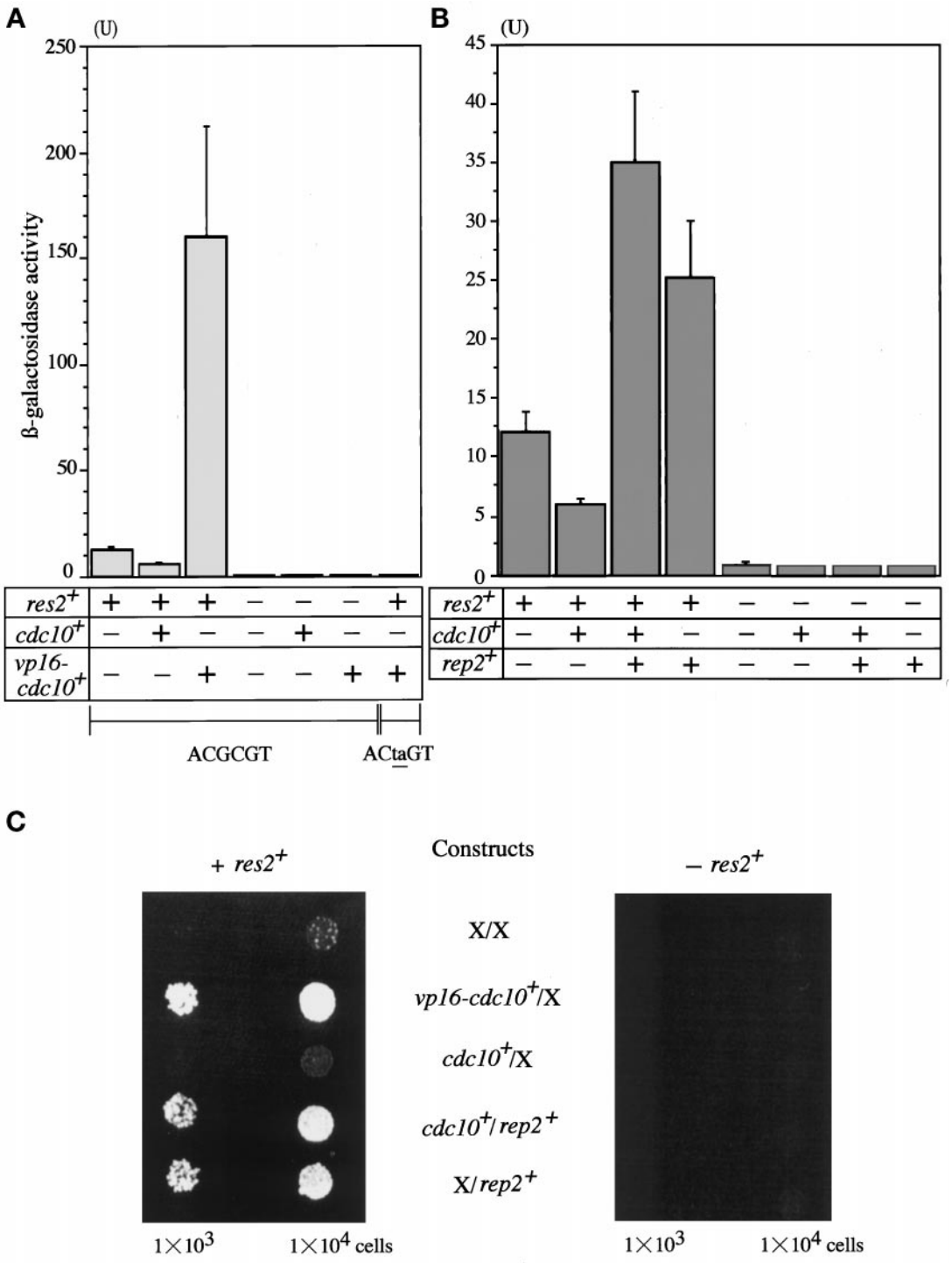

Figure 2. (A and B) Reconstruction of transcriptional activation by Res2–Cdc10 in budding yeast. Rep2–Res2–Cdc10 complex activates MCB (triple *Mlu*I sequences). The *LacZ* reporter host cells were transfected with the indicated plasmids. The transformants were then induced for the expression of *res2*$^+$ and *cdc10*$^+$ by incubating in SD medium containing 3% galactose and 0.2% sucrose at 30°C for 16 h. Cells were harvested and ruptured by freeze and thaw followed by colorimetric assay of β-galactosidase. For the experiment shown in the far right column of A, the *LacZ* reporter gene driven by the *CYC* minimal promoter containing triple repeats of the mutated *Mlu*I sequences (ACtaGT) was used. Experiments were repeated three times, and values are expressed as means ± SD. (C) Rep2–Res2–Cdc10 complex activates the *TMP* promoter. The *HIS3* reporter host cells with or without expressing *res2*$^+$ were transfected with the indicated constructs. The transfectants were spotted on 3% galactose/0.2% sucrose SD plate containing 4 mM 3-AT and incubated at 30°C for 10 d.

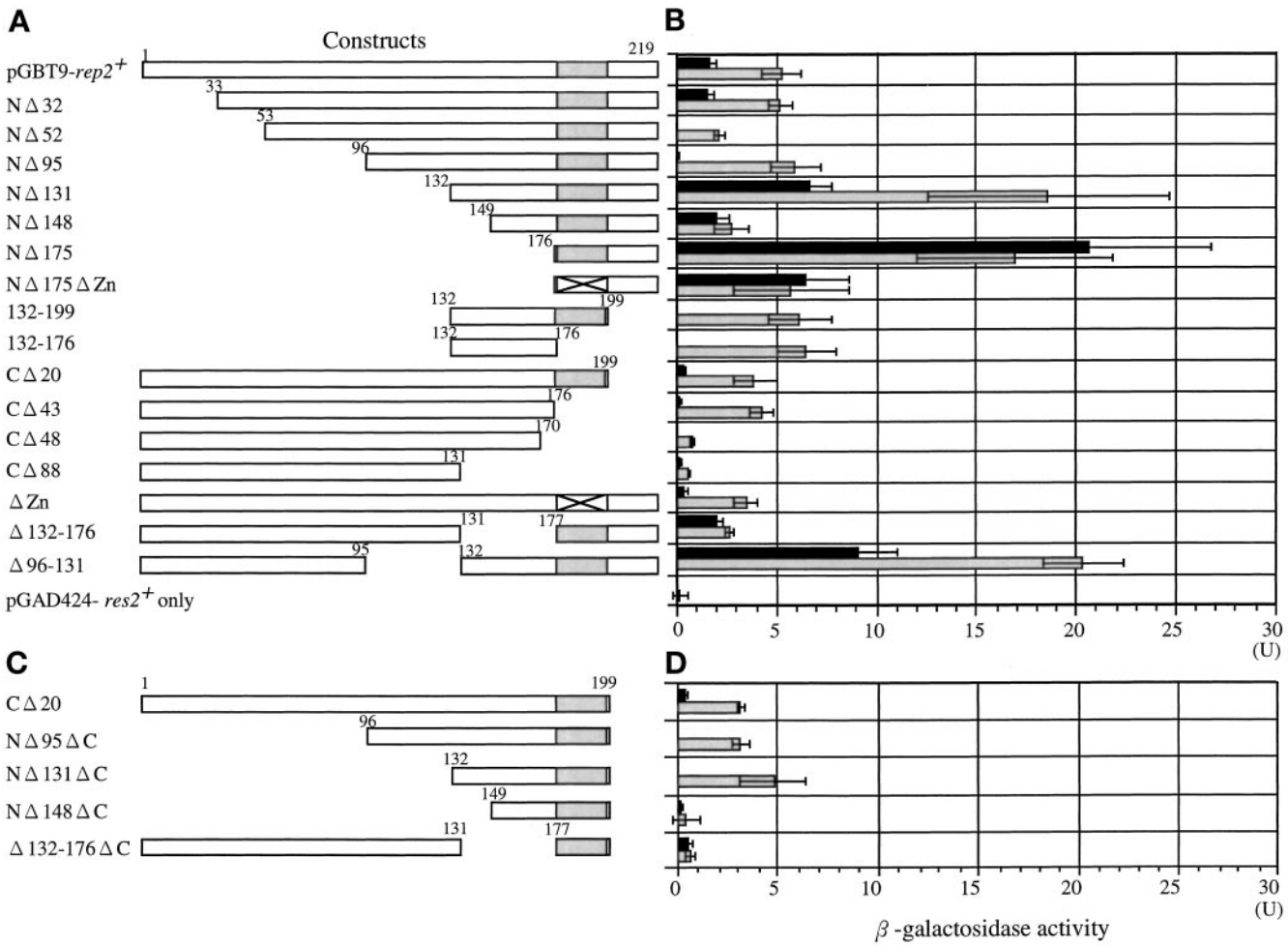

Figure 3. (A and C) Identification of transactivation and Res2 binding domains in Rep2. Schematic representation of deletion mutants analyzed in this study. The intact *rep2*$^+$ gene is shown at the top. The zinc-finger motif is shaded. The amino acid numbers are shown at each protein coding region. Full-length and deletion mutants of *rep2*$^+$ were fused to a DNA binding domain of Gal4. (D) One-hybrid and two-hybrid analysis for transcriptional activator and Res2 binding domains in Rep2. The DNA binding domain hybrid was transfected into the yeast reporter strain SFY526 together with (gray bar) or without (black bar) Res2-fused Gal4 activation domain. After growth on selective medium, each transformant was assayed for β-galactosidase activity. Black bars indicate transactivation activity, and the difference between gray and black bars reflects Res2 binding activity. Experiments were repeated four times in B and three times in D; data are presented as means ± SD.

deletion of Rep2 initially abolished, but further deletion restored, transcriptional activator activities, which reached the maximum (an ~10-fold greater induction than intact Rep2) when all the molecules but the C-terminal 44 amino acids were deleted. This result suggests that the transcriptional activator activity is localized within this region. Consistently, deletion of the C-terminal 20 amino acids from NΔ131 abolished the transcriptional activator activity. Analysis with progressive C-terminal or internal deletion mutants yielded results consistent with the N-terminal deletion analysis data.

The 44-amino acid region contains a zinc-finger motif (Nakashima *et al.*, 1995). The next question we examined is whether this finger is essential for activity. Two cysteine residues forming the zinc-finger motif were substituted with glycine and serine, respectively. The resulting zinc-fingerless Rep2 molecule showed a reduced ability to activate the reporter gene, suggesting that this motif is important for transcriptional activator function. However, the importance of this motif for Rep2 activity appears to occur only in the budding yeast one-hybrid system. We failed to find any significant requirement for this motif in fission yeast and in the budding yeast reconstitution system (see below). In addition, the apparent requirement for the N-terminal 33–95 amino acid region in Rep2 transcriptional activator function was seen only

in budding yeast. In fission yeast, this region was dispensable (see below).

Identification of Res2 Binding Domain

The second key property required for Rep2 function as a transcriptional activator subunit is the ability to physically interact with Res2, because Rep2 is required for the activity of Res2–Cdc10 but not Res1–Cdc10 (Nakashima *et al.*, 1995; Baum *et al.*, 1997). Rep2 forms a complex with Res2–Cdc10 in vivo as well as in vitro (Nakashima *et al.*, 1995). To confirm this and identify the region essential for Res2 binding, we used a two-hybrid binding analysis, in which the same Rep2 deletion constructs fused with the Gal4 DNA binding domain (GD-Rep2 deletion mutant) and Res2 fused with the Gal4 transcriptional activator domain (GT-Res2) were coexpressed. Because at least some GD-Rep2 deletion mutants contain transcriptional activator function, their Res2 binding abilities were initially measured by assaying increases in β-galactosidase activity from the level obtained by expression of GD-Rep2 mutants alone (black bars) to that obtained by coexpression of GD-Rep2 and GT-Res2 (gray bars). Such indirect assays tended to yield quantitatively less accurate results, which would be improved by use of transcriptional activation domainless Rep2; however, we did not initially use such a construct because the Res2 binding domain might overlap with, or be proximal to, the transcriptional activator domain.

Deletion of 131 amino acids from the N terminus was judged to have no marked effect on binding to Res2, but deletion of 148 amino acids or more abolished binding activity as indicated by NΔ148 and NΔ175. On the other hand, deletion of the 43-amino acid transcriptional activator domain (CΔ43) did not affect binding to Res2, but deletion of an additional 5 amino acids (CΔ48) almost completely abolished binding ability. These results suggest that the region of amino acid 132 to 176 is required for Res2 binding. Consistently, internal deletion of this region (Δ132–176) completely abolished binding ability. Because the tentatively assigned Res2 binding region did not overlap with the tentatively assigned transactivator domain, we performed the same assay with transactivator domainless Rep2 constructs shown in Figure 3C, to confirm the results with the original constructs. As shown in Figure 3D, the same results were obtained with the transactivator domainless constructs. These results indicate that the 45-amino acid sequence from amino acid 132 to 176 contains a Res2 binding ability.

Res2 Binding Domain Is Required for Res2 Binding In Vivo

To confirm the assignment of the Res2 binding domain, we examined the ability of Rep2 deletion mutants to associate with Res2 in an in vivo situation. FLAG-tagged, Res2 binding domainless *rep2*$^+$ was placed under the control of the thiamine-inducible promoter, expressed in the *rep2* (Figure 4A) or *res1* (Figure 4B) disruptant, immunoprecipitated with anti-FLAG antibody, and assayed for coprecipitation of Res2 by Western blotting with anti-Res1/2 antibody. The anti-Res1/2 antibody detects Res2 (top band) and Res1 (bottom band) (Nakashima *et al.*, 1995) (Figure 4A, lanes 6 and 7). As we showed previously, not only Res2 but also Res1 coprecipitates with Rep2 (Figure 4A) (Nakashima *et al.*, 1995), but the biological significance of the association of Rep2 with Res1 is still unclear. It could be an artifact of an overexpressed situation because we failed to obtain any results suggesting their functional interaction (Nakashima *et al.*, 1995; Sturm and Okayama, 1996).

In good agreement with the two-hybrid assay results, the same or higher amount of Res2 protein coprecipitated with any of the tested mutant Rep2 proteins that lack the regions N- or C-terminal to the border of the Res2 binding domain but hold the intact Res2 binding domain. Furthermore, no Res2 protein coprecipitated with the Rep2 lacking the putative Res2 binding domain (Δ132–176), despite the presence of the same amount of Res2 in this cell extract as in others.

Recent analysis indicated that Res2 forms a heteroduplex complex with Res1 in *S. pombe* (Ayte *et al.*, 1997; Baum *et al.*, 1997; Zhu *et al.*, 1997). To further confirm the results in the absence of such a complication, we performed the same assay as above, but with the *res1* disruptant as a host, and obtained identical results. Again, Res2 protein coprecipitated with all the Rep2 constructs containing the putative Res2 binding domain but not with the one lacking the binding domain (Δ132–176). In this experiment, even a faint amount of Res2 failed to coprecipitate with the binding domainless Rep2. These results show that the 45-amino acid sequence from 132 to 176 is essential and sufficient for Res2 binding in vivo, leading us to conclude that the Res2 binding domain resides in this region.

Both Transactivation and Res2 Binding Domains Are Required for Rep2 Function in the Reconstitution System and in Fission Yeast

To corroborate the results obtained by the one- and two-hybrid systems and the in vivo coimmunoprecipitation assay, the functional importance of the identified domains was examined in the reconstitution system (Figure 5). In the reconstitution system, deletion of the C-terminal 20 amino acids (CΔ20), which were essential for transcriptional activator function in the one-hybrid system, completely abrogated the ability of Rep2 to induce β-galactosidase. Similarly, deletion of the Res2 binding domain (Δ132–176) completely

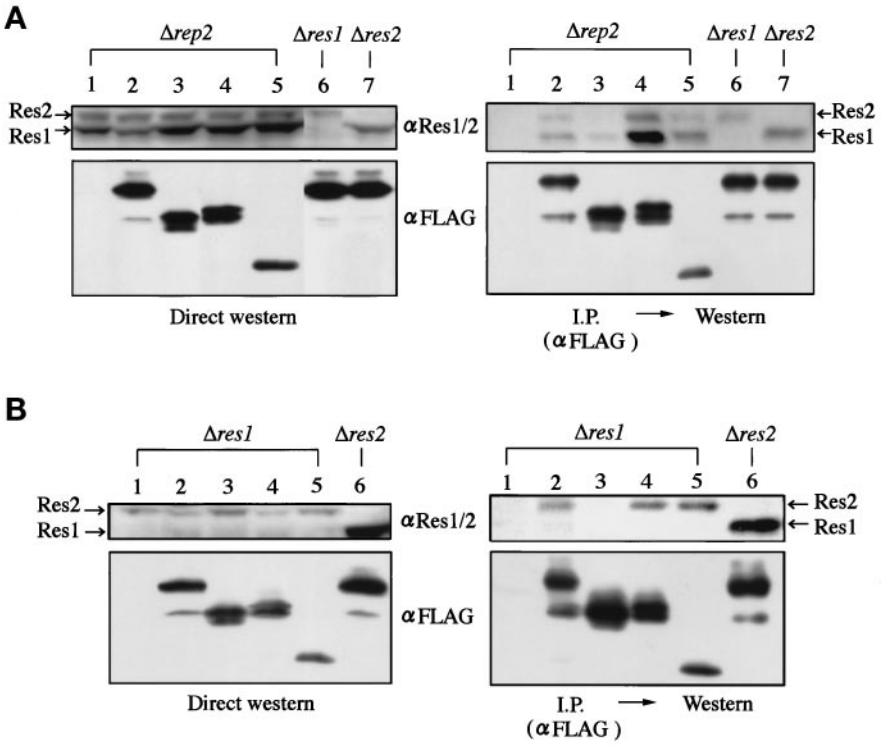

Figure 4. The putative Res2 binding domain in Rep2 is required for its Res2 binding in vivo. Full-length and deletion mutants of *rep2*$^+$ tagged with FLAG epitope were inserted into the pREP1 vector and transfected into the *rep2* (N3–141s), *res1* (K156-D1), and *res2* disruptants (M222). The structures of *rep2*$^+$ deletion mutants are shown in Figure 3A except for the Δ22–131 aa mutant, which was constructed by fusing the sequence of amino acids 1–21 with NΔ131. Cell extracts (4.5 mg protein) prepared from the transformants grown in thiamine-free medium at 30°C to express the FLAG-tagged Rep2 were immnoprecipitated with the anti-FLAG antibody. Cell extracts (200 μg protein) (left panels) and immunoprecipitates (right panels) were separated in SDS-polyacrylamide gels and analyzed by Western blotting with anti-Res1/2 or anti-FLAG antibody. Lane 1, empty vector; lanes 2, 6, 7, full-length *rep2*$^+$; lane 3, Δ132–176 aa; lane 4, CΔ43; lane 5, Δ22–131 aa.

abolished Rep2 function. Interestingly, deletion of the zinc-finger motif (ΔZn) only slightly decreased Rep2 activity.

To further corroborate the experimental data, the activity of the *rep2*$^+$ deletion mutant genes was assayed in fission yeast. The fission yeast host used was a *rep2* deletion mutant cell. This mutant cell can grow at regular growth temperatures but is slow in traversing the G_1–S transition. Consequently, a significant G_1 population is noticeable. In addition, this cell cannot grow at 18°C or lower temperatures (Nakashima *et al.*, 1995). Accordingly, the activity of the *rep2*$^+$ gene mutants was assayed by two methods. One was rescue of the cold-sensitive growth arrest of the mutant. The other was acceleration of the slow cell cycle start of the mutant and simultaneous induction of *cdc18*$^+$ mRNA at 30°C. To carry out these assays, various deletion mutants of *rep2*$^+$ were inserted in the SV40 early promoter-based pcL vector, transfected into the *rep2* disruptant, and selected at 18°C. In this assay, the activity of the *rep2*$^+$ gene deletion mutants was measured as percentage colony formation at 18°C against at 30°C. As shown in Figure 6A, the *rep2* genes lacking the putative Res2 binding domain or the C-terminal 20 amino acids completely or nearly completely failed to rescue the mutant, confirming the results obtained with the one- or two-hybrid and reconstitution systems. Similar results were also obtained with the second assay for G_1 acceleration as well as elevation of *cdc18*$^+$ mRNA (Figure 6, B and C). The *rep2* gene lacking the putative Res2 binding domain showed no detectable activity in this assay either. On the other hand, the C-terminal 20-amino acid-deleted *rep2* gene had only a marginal activity with a slight decrease in G_1 population but no apparent changes in *cdc18*$^+$ mRNA upon its expression.

The one-hybrid assay showed that the zinc-finger motif was important for transcriptional activator function, although its role was obscure when assayed in the reconstitution system. In fission yeast, however, this motif was totally dispensable under our assay conditions. Moreover, deletion of the 21-amino acid region containing the zinc-finger motif had no apparent effect on the Rep2 activity in this assay. We therefore tentatively conclude that not only the zinc finger but also the entire 21 amino acids containing this motif

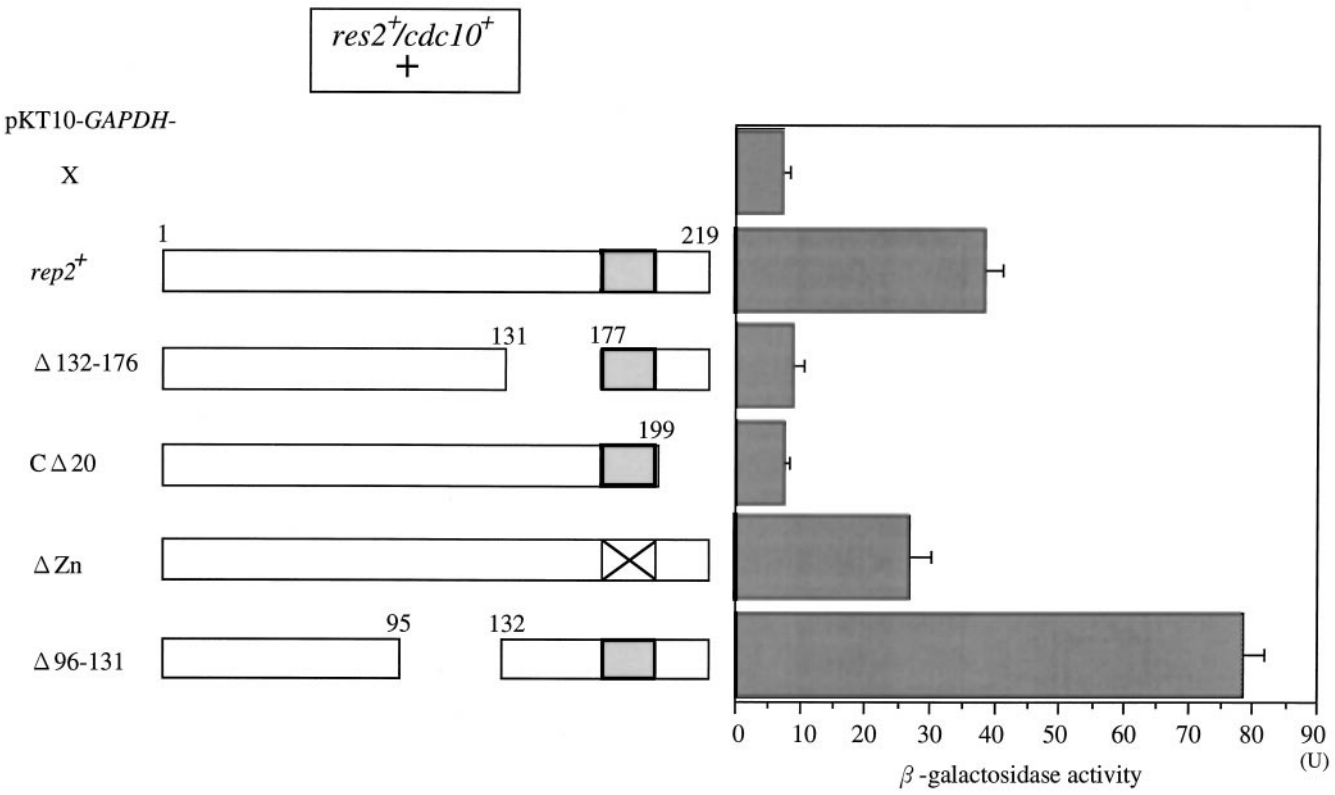

Figure 5. Transactivation and Res2 binding domains are required for Rep2 function in the reconstitution system. Full-length and deletion mutants of *rep2*$^+$ were inserted into the pKT10-*GAPDH* vector and transfected into the *LacZ* reporter host cells harboring *res2*$^+$ and *cdc10*$^+$, which were then induced for *res2*$^+$ and *cdc10*$^+$ by incubating at 30°C for 16 h in SD medium containing 3% galactose and 0.2% sucrose followed by β-galactosidase assay. Experiments were repeated three times, and data are presented as means ± SD.

are dispensable for Rep2 function as a transcriptional activator subunit for the Res2–Cdc10 complex under the assay conditions used.

As shown already, in the one- and two-hybrid and reconstitution systems, the region (36 amino acids) just upstream of the Res2 binding domain was totally dispensable or rather inhibitory to Rep2 function (Figures 3 and 5). This region, however, was found to be essential for Rep2 function in this rescue assay. Deletion of this region nearly completely eliminated the ability of Rep2 to rescue the low-temperature growth arrest of the *rep2* disruptant. The reason for this apparent contradiction became clear in the second assay.

The *rep2* disruptant displays a large G_1 peak during exponential growth at 30°C, which is due to slow cell cycle start caused by partial sequestering of MCB by the inactive Res2–Cdc10 complex. Expression of wild-type *rep2*$^+$ suppressed this G_1 peak and concurrently increased the level of *cdc18*$^+$ mRNA (Figure 6, B and C). In this assay, *rep2*$^+$ lacking the 36-amino acid region was as active as wild-type gene; however, when this assay was carried out at 18°C, the same temperature as for the *rep2* rescue assay, the *rep2*$^+$ mutant gene failed to show any significant activity. These results suggest that this 36-amino acid region is essential for Rep2 at low temperatures but dispensable at the regular growth temperatures.

On the basis of all these results taken together, we conclude that the Rep2 molecule contains a Res2 binding domain within amino acids 132–176 and a main transcriptional activator domain within the C-terminal 22 amino acids.

DISCUSSION

The Res–Cdc10-led transcriptional regulation of genes required for S-phase onset, particularly of *cdc18*$^+$ that encodes a component of the prereplicative complex, is a key step in regulating the start of the cell cycle. One critical question concerning this transcriptional system is how it is regulated. We recently showed that at least the Res2–Cdc10 complex itself is inactive as transcriptional activator and requires Rep2, a nitrogen starvation-repressible zinc-finger protein, for activity in the mitotic cell cycle (Nakashima *et al.*, 1995). Because Rep2 has a transcriptional activation ability in the one-hybrid system, binds to Res2 in vivo as well as in vitro, and is essential for MCB activation but not for

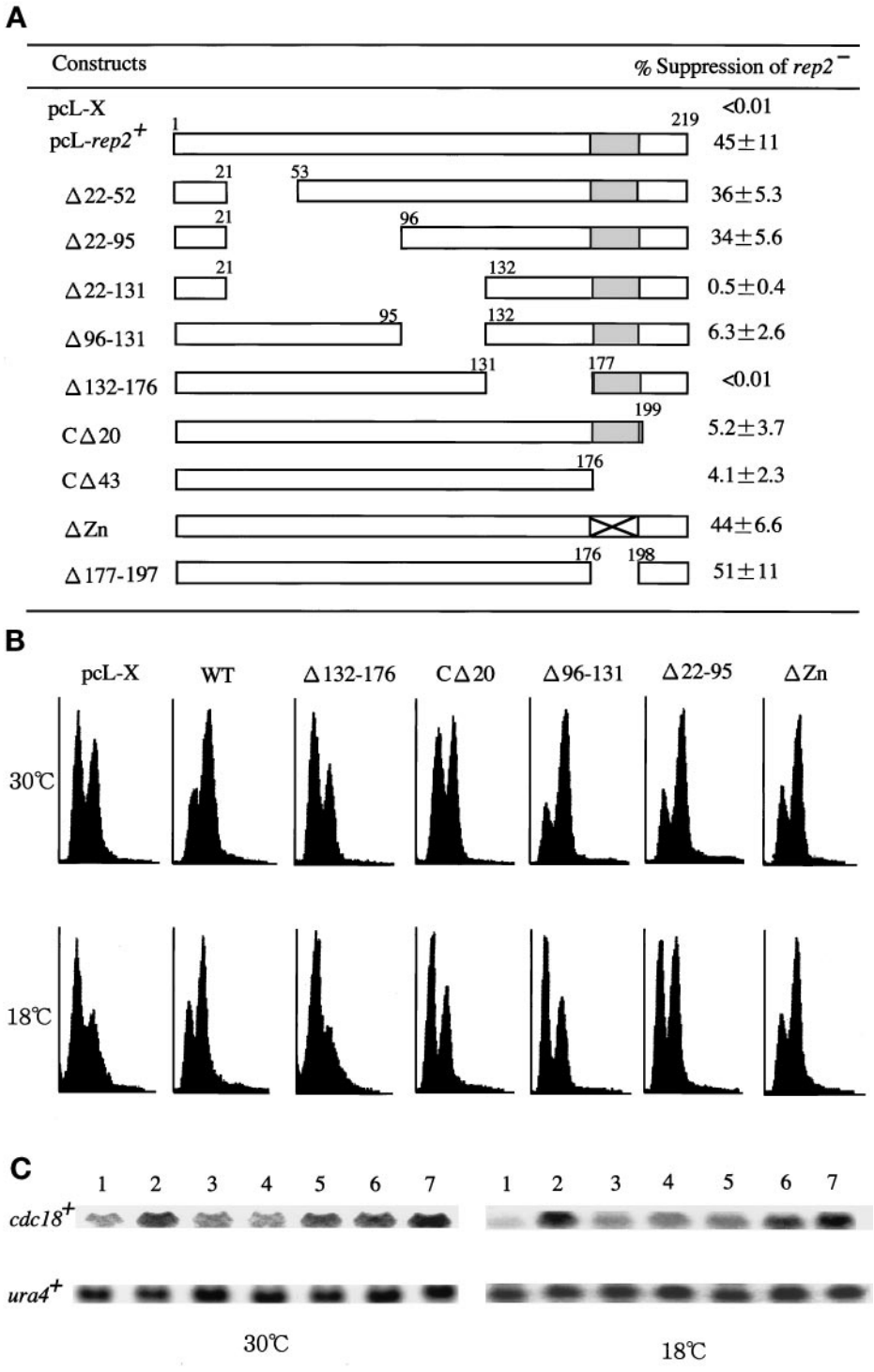

Figure 6. Both transactivation and Res2 binding domains are required for Rep2 function in fission yeast. (A) Rescue of low-temperature growth inability of *rep2*$^{-}$ cells by expression of *rep2*$^{+}$ deletion mutants. Full-length and various deletion mutants of *rep2*$^{+}$ were inserted into the pcL vector and transfected into the *rep2* disruptant (N3–141S) followed by selection at 18°C. The ratio of colonies formed at 18°C to those at 30°C is expressed as percentage suppression in the right column. pcL-X is the pcL vector with no insert and is used as a negative control. Experiments were repeated four times, and data are presented as means ± SD. (B) Flow cytometry of *rep2* disruptants expressing each *rep2*$^{+}$ deletion mutant. The *rep2* disruptant was transfected with the indicated constructs and selected for *Leu*$^{+}$ transformants. The transformants were grown to log phase at 30°C in PM medium and then incubated at 18°C for 53 h. Cells were sampled both at 30°C and after a 53 h incubation at 18°C, and analyzed by flow cytometry. (C) The level of *cdc18*$^{+}$ mRNA expressed in the *rep2* disruptants rescued by *rep2*$^{+}$ deletion mutant genes. Total RNA was prepared from the *rep2* disruptants at the same time points as described in B. The level of the *cdc18*$^{+}$ transcript was determined by Northern blot hybridization. *ura4*$^{+}$ was probed as a loading control. Lane 1, empty vector; lane 2, full-length *rep2*$^{+}$; lane 3, Δ132–176 aa; lane 4, CΔ20; lane 5, Δ96–131 aa; lane 6, Δ22–95 aa; lane 7, ΔZn finger.

the MCB binding ability of the Res2–Cdc10 complex (Zhu *et al.*, 1994; Nakashima *et al.*, 1995; Sturm and Okayama, 1996), we tentatively assigned Rep2 to a transcriptional activator subunit for Res2–Cdc10 in the mitotic cycle. In this article we show that Rep2 fulfills all the criteria required for a transcriptional activator subunit. First, Rep2 forms a complex with Res2–Cdc10 in vivo. Rep2 has a defined region required for Res2 binding in vitro as well as in vivo and an ability to activate Res2–Cdc10 in the budding yeast two-hybrid

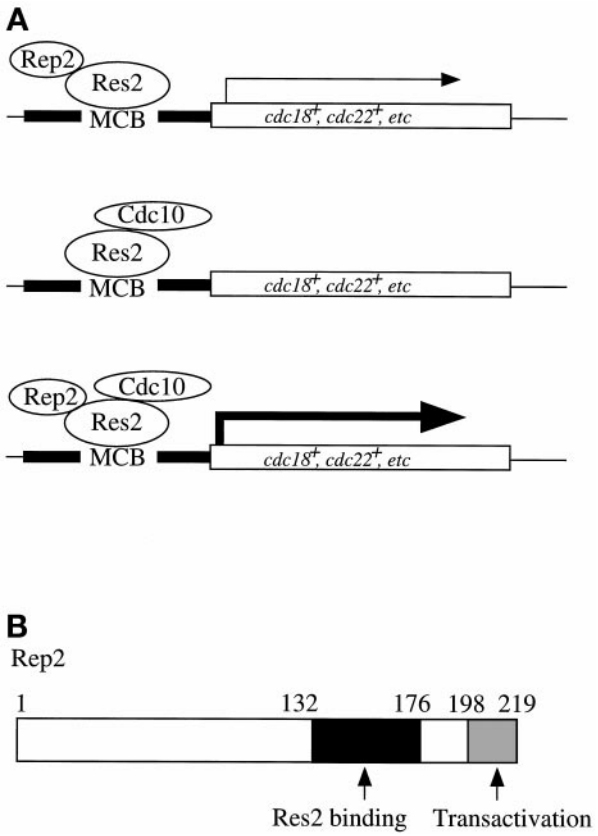

Figure 7. (A) Activity of the Res2–Cdc10–Rep2 complex suggested by analysis. When overproduced, the Res2 molecule binds and weakly activates MCB in the presence of Rep2. In the absence of Rep2, the Res2–Cdc10 complex can bind but cannot activate MCB. The Res2–Cdc10–Rep2 ternary complex strongly activates MCB. (B) Functional domains in Rep2. The Rep2 molecule contains a Res2 binding domain (black box) at amino acids 132–176 and a transactivator domain (gray box) at amino acids 198–219.

and reconstitution systems. Second, Rep2 has a defined sequence capable of transcriptional activation when fused with a DNA binding domain, and this region is also essential for Rep2's ability to activate Res2–Cdc10. Third, unless provided with Rep2, the Res2–Cdc10 complex itself has no significant ability to activate MCB in fission yeast as well as in the budding yeast reconstitution system. We therefore conclude that Rep2 is a transcriptional activator subunit for Res2–Cdc10 (Figure 7A).

Deletion analysis of *rep2*$^+$ gene led to identification of a Res2 binding and a transcriptional activator domain locating at amino acids 132–176 and 198–219, respectively (Figure 7B). All of the genetic and biochemical data are consistent with the functional assignment of these two domains. One unexpected outcome is the failure to assign function to the zinc-finger motif that is located between these two regions and that we speculated was important for either Res2 binding or transcriptional activation. In the one-hybrid analysis, this motif was required for the transcriptional activation ability of Rep2, although to a lesser extent in the reconstitution system; however, in fission yeast, not only this motif but also the entire 21-amino acid region containing this motif was dispensable for Rep2 activity under the assay conditions used. Our data do not exclude the possibility that this motif might be involved in some other functions, such as facilitation of Res2 binding and stabilization of the Rep2 protein molecule, which might be detectable only in a low level expression.

One region with an unexpected function locates at amino acids 96–131, just upstream of the Res2 binding domain. This region is either dispensable or inhibitory to Rep2 activity at the regular growth temperature, but absolutely essential at a low temperature of 18°C. The reason for this is unclear at present, but one possibility is that this region might be required for proper protein folding of Rep2 at low temperatures. No matter what the reason, the presence of such a domain in Rep2, however, seems to be reasonable because Res2 appears to preferentially function at low temperatures in the mitotic cycle. Cells deleted for *res2*$^+$ show cold sensitivity for growth (Zhu *et al.*, 1994).

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