

Detection and Functional Characterization of p180, a Novel Cell Cycle Regulated Yeast Transcription Factor That Binds Retinoblastoma Control Elements*

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In recent years it has become apparent that the cellular machinery governing cell cycle progression and transcription control are often homologous in yeast and mammalian cells. We and others have previously shown that the SP family of mammalian transcription factors regulates the transcription of a number of genes whose activities are governed by the product of the retinoblastoma (Rb) susceptibility gene, including *c-FOS*, *c-MYC*, *TGF β -1*, *IGF-II*, and *c-JUN*. To determine whether a similar pathway of transcriptional regulation may function in yeast, we explored the possibility that transcription factors with nucleotide-binding specificities akin to those of the SP family are expressed in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Here we report the detection of novel yeast proteins (*S. cerevisiae*, p180; *S. pombe*, p200) that specifically bind Rb-regulated promoter elements *in vitro* dependent on nucleotides that are also required for binding and *trans*-activation by SP family members *in vivo*. Our results indicate that the *S. cerevisiae* retinoblastoma control element-binding activity 1) requires zinc for association with DNA; 2) does not bind to SCB, MCB, or E2F sites *in vitro*; 3) is cell cycle-regulated in a *SWI6*-independent fashion; and 4) maximally stimulates retinoblastoma control element-mediated transcription in early- to mid-S phase. Taken together, these data suggest that p180 may regulate the transcription of a subset of yeast genes whose expression is coincident with the onset and/or progression of DNA replication.

DNA in a sequence-nonspecific fashion, Rb regulates transcription indirectly via its physical or functional interaction with *trans*-acting factors that specifically bind to DNA (3). To date, nearly a dozen sequence-specific DNA-binding proteins have been shown to be targets of Rb function *in vivo*. Interestingly, the functional consequence of Rb's interaction with these transcription factors is dependent on the factors themselves and the cell types in which their interaction is analyzed.

The transcriptional response of a subset of Rb-regulated genes, including *c-FOS*, *c-MYC*, *TGF β -1*, *IGF-II*, and *c-JUN*, is dependent on GC-rich promoter elements termed retinoblastoma control elements (RCEs, Refs. 3-8). At least three ubiquitously expressed nuclear proteins (retinoblastoma control proteins, RCPs), including SP1 and SP3, bind to RCEs *in vitro*, and the interaction of one or more of these proteins with RCEs *in vivo* is required for RCE-mediated transcription (7, 9-11). Co-expression of Rb and SP1 or SP3 in transient transfection assays leads to a marked stimulation of RCE transcription, a phenomenon we have termed "superactivation" (10, 11). Regions of Rb that are targets of mutation in human tumors are required for Rb-mediated superactivation, suggesting that the functional interaction of Rb with SP1/SP3 plays a significant role in the regulation of cell cycle progression (11). The mechanism(s) by which Rb stimulates SP-mediated transcription has yet to be clearly defined. Physical interactions between Rb and members of the SP family of transcription factors have not as yet been detected *in vitro* or *in vivo* perhaps suggesting that Rb interacts with these transcription factors in the context of a large macromolecular complex. Consistent with this supposi-

Functional inactivation of the retinoblastoma (Rb) protein is associated with the genesis of a number of human tumors, including retinoblastoma, osteosarcoma, and breast, bladder, and small cell lung carcinomas (for reviews see Refs. 1, 2). The Rb protein is believed to control cell proliferation at least in part via the transcriptional regulation of a wide variety of growth-related genes. Although capable of associating with

tion, Rb has been proposed to modulate the transcriptional activity of SP family members via their cooperative regulators or by indirectly "bridging" their *trans*-activation domains to components of the basal transcription machinery (12, 13). *Trans*-activation mediated by transcription factors such as ATF-2, NF-IL6, MYOD, and myogenin is stimulated and/or facilitated by Rb *in vivo* (14-17). Unlike SP family members, Rb forms physical complexes with these transcription factors although the mechanism by which Rb augments transcriptional activity has not as yet been determined. In contrast to these functional effects, interactions between Rb and transcription factors such as E2F, ELF-1, and UBF1 suppress transcriptional activity (18-20). Rb forms cell cycle-regulated complexes with factors such as E2F and UBF1 *in vivo*, sequestering their *trans*-activation domain components of the basal transcription complex (18, 19). Rb is believed to control the transcription of at least a subset of cell cycle-regulated genes via periodic interactions with sequence-specific DNA-binding proteins.

The Rb protein is phosphorylated in concert with progression of the mammalian cell cycle. Quiescent and non-dividing mitotic cells, and cells in early G₁ carry un-

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¹ The abbreviations used are: Rb, retinoblastoma; RCP, retinoblastoma control protein; RCE, retinoblastoma control element; kb, kilobase pair(s); BUdR, bromodeoxyuridine.

TABLE I
Yeast strain list and source

Strain	Relevant genotype
<i>S. cerevisiae</i>	
YRC1	<i>MATa ura3 his3 leu2 TRP1 ade8 ras1::HIS3 bar1::LEU2 RAS2 CAN cyh^r</i> (this study)
BY600	<i>MATa swi6 ΔTRP1-197 ade2 holacZ ura3 leu2-3,-112 trp1-1 can1-100 met⁻</i> (Linda Breeden, University of Washington)
BY602	<i>MATa SWI⁺</i> (Linda Breeden)
BY606	<i>MATa swi4 Δleu2-194</i> (Linda Breeden)
H6C1A1	<i>MATa ural his7 cdc6</i> (Daniel Burke, University of Virginia)
H13C1A1	<i>MATa ural his7 cdc13</i> (Daniel Burke)
H28C1A5	<i>MATa his7 hom3 can1 cdc28</i> (Daniel Burke)
H16C1A2	<i>MATa ural his7 cdc16-2</i> (Daniel Burke)
H23C1A1	<i>MATa ural his7 cdc23-1</i> (Daniel Burke)
H17C1A1	<i>MATa ural his7 cdc17-1</i> (Daniel Burke)
H2C2A2	<i>MATa ural his7 cdc2-2</i> (Daniel Burke)
MSY272	<i>MATa bar1 his6 cdc7-1 leu2,3,112 ura3-52 trp1-287</i> (Daniel Burke)
7546-22	<i>MATa ura3 cdc14 his7 can1 cyh2</i> (Daniel Burke)
753F16	<i>MATa ura3 cdc15 his7 can1 cyh2</i> (Daniel Burke)
DLY204	<i>MATa cln2::LEU2 ura3 trp1 his2 ade1 bar1</i> (Daniel Lew, Duke University)
<i>S. pombe</i>	
972h ⁻	(Thomas Chappell, I.C.R.F., UK)

regulated Rb protein (22, 25). In cycling cells, Ph-hormon-MRE and their interaction with G₁/S boundary elements, respectively, phosphorylated on serine and threonine residues beginning in late G₁ (the "restriction point") and extending through G₂ and then is abruptly dephosphorylated in anaphase (26). Rb is a substrate of a number of cyclin-dependent kinases (cdks), including cyclin D/CDK4 and cyclin E/CDK2 (27). In addition, a novel cell cycle-regulated Rb and histone H1 kinase has recently been described that associates with the Rb amino terminus in G₂/M phases (28, 29). Given that the initiation of DNA synthesis occurs subsequent to Rb phosphorylation, it is widely suspected that phosphorylation of Rb is a necessary step for normal cells to transit through the G₁/S boundary. This view is consistent with the observations that 1) transcription factors that control gene expression at the G₁/S boundary, such as E2F, are bound exclusively by un- or underphosphorylated Rb; and 2) phosphorylation of Rb by cdks *in vitro* inactivates Rb as an inhibitor of E2F-mediated transcription (18, 21, 30).

The recent identification of homologues of E2F and Rb in *Drosophila* and suggestions of an Rb-like protein in plants serves to support the contention that a conserved pathway of transcriptional regulation may operate in many, if not all, eukaryotic cells (31-35). To date, a structural homologue of Rb has not been identified in yeast, but proteins that are similar to mammalian targets of Rb function have been noted. For example, a 47-kDa factor in *Saccharomyces cerevisiae* that specifically binds E2F sites in a cell cycle-dependent fashion has been reported, and a similar factor has been detected in *Schizosaccharomyces pombe* (36, 37). In concert with these findings, when expressed in *S. cerevisiae* Rb is phosphorylated by yeast cdks at sites that are targets of phosphorylation in mammalian cells (38). Moreover, Rb phosphorylation in yeast appears to be temporally controlled in a manner that is similar to that which occurs in mammalian cells; Rb is phosphorylated prior to the initiation of DNA synthesis in yeast coincident with the cell cycle checkpoint termed "Start" (27). Given these observations, it is tempting to speculate that yeast may harbor proteins functionally analogous to Rb that integrate progression of the cell cycle with transcriptional regulation. Yet, exogenous expression of human Rb in yeast does not appreciably alter cell cycle progression, suggesting that should yeast carry Rb-like proteins their targets of function may not be closely related to their mammalian counterparts (37).

In *S. cerevisiae*, cell cycle-regulated transcription of a number of critical genes has been shown to be at least partly dependent on two heterodimeric transcription factors, SBF and

SCB promoter elements (5'-CACGAAA-3') govern the periodic transcription of genes such as the *HO* gene (39, 40). MCB (*Mitotic Cell Cycle*) promoter elements, including many required at the G₁/S boundary for entry into phase, such as thymidylate synthase (*TMPI*) and B-type cyclins (*CLB5* and *CLB6*). SBF is composed of the SWI4 and SWI6 proteins, whereas active MBF complexes result from the heterodimerization of MBP1 and SWI6 proteins. Transcription factors that are structurally and functionally similar to SWI4, SWI6, and MBP1 have also been isolated from *S. pombe* (41). Given that RCEs share limited sequence homology (5'-GCCACC-3') with yeast SCB and MCB elements, we hypothesize that RCEs might represent a related family of cell cycle-regulated yeast promoter elements. Furthermore, we speculate that yeast RCE-binding proteins might be functionally, and perhaps structurally, homologous with mammalian RCPs. In this report we characterize the biochemical and functional properties of a novel cell cycle-regulated RCE-binding protein p180, that is synthesized in *S. cerevisiae* and whose DNA binding domain is functionally homologous to that of SP1, SBF, and perhaps other members of the SP family of mammalian transcription factors.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—Table I lists the yeast strains used in this study. Cells were grown in YEPD (1% yeast extract, 2% peptone, 2% dextrose) at 30 °C or in selective SDmin media for strains transformed with *URA3*-based plasmids. For cell cycle studies, temperature-sensitive *cdc* mutants were grown in YEPD at 23 °C to an A_{600 nm} of 0.4, pelleted, resuspended in prewarmed YEPD, and incubated at 37 °C for 5 h. Arrest was confirmed by documenting terminal arrest phenotype under light microscopy. *Cdc17* cells arrest heterogeneously at S phase (38 °C) or G₂ (34 °C) and were treated at 38 °C in this study (41).

Plasmid Constructions—A high copy, *URA3*-based plasmid (pJLB; 42) containing a UAS-less *cytochrome c* (*CYC1*) promoter upstream of the *LACZ* gene was a kind gift from Stephen Johnston (University of Texas-Southwestern, Dallas, TX). A synthetic oligonucleotide and its complement carrying three tandem copies of an octameric p180-binding site (pNUT; 5'-TCGAGCGCACCGCGCCACCGCGCCACC-3') or a mutated derivative and its complement lacking a p180-binding site (pWEE; 5'-TCGAGCTTCACCGCTTCACCGCTTCACC-3') were cloned upstream of the *CYC1* promoter at a unique *XhoI* site. Plasmids containing one or two copies of pNUT or one to three copies of pWEE upstream of *LACZ* were identified by double-stranded DNA sequencing (43) and named pNUT1, pNUT2, pWEE1, pWEE2, and pWEE3, respectively. To further differentiate between independent clones that were

TABLE II
Wildtype and mutated oligonucleotides

Nucleotides required for binding of relevant transcription factors are underlined. Mutated nucleotides are denoted by italics.

Name	Sequence	Reference
Fos	5'- <u>CCC</u> GC <u>CGCC</u> ACCCTCTGGCCACCCTG-3'	9
5'RCP-3'RCP	5'- <u>CCC</u> GC <u>AAAA</u> ACCCTCTGGCCACCCTG-3'	This study
dbl RCP-dbl E2F-3'Fos-WT	5'- <u>CCC</u> GC <u>CGCC</u> ACCCTCTG <u>AAAA</u> ACCCTG-3'	This study
5'Fos-WT	5'- <u>CCC</u> GC <u>CGCC</u> ACCCTCT-3'	9
5'Fos-8	5'- <u>CTT</u> GC <u>CGCC</u> ACCCTCT-3'	9
5'Fos-4	5'- <u>CCC</u> T <u>TG</u> CCACCCTCT-3'	9
5'Fos-3	5'- <u>CCC</u> GC <u>TG</u> CCACCCTCT-3'	9
5'Fos-2	5'- <u>CCC</u> GC <u>TT</u> CACCCTCT-3'	9
5'Fos-1	5'- <u>CCC</u> GC <u>CGC</u> TTCCCTCT-3'	9
5'Fos-5	5'- <u>CCC</u> GC <u>CGC</u> CAATCTCT-3'	9
5'Fos-6	5'- <u>CCC</u> GC <u>CGC</u> CACTTCT-3'	9
5'Fos-7	5'- <u>CCC</u> GC <u>CGC</u> CAAT-3'	9
RCE7	(5'- <u>GCC</u> ACCCTCT-3') × 6	This study
pNUT	5'-TCGAG <u>CGCC</u> ACC <u>CGCC</u> ACC <u>CGCC</u> ACC-3'	This study
pWEE	5'-TCGAG <u>C</u> TT <u>C</u> ACC <u>G</u> TT <u>C</u> ACC <u>G</u> TT <u>C</u> ACC-3'	This study
HIP	5'AATTCTGCGATTTCGCGCAA <u>AA</u> CTTGACG-3'	49
E/J	5'AATTCTGCGATTTCGCGCAA <u>AA</u> CTTGACG-3'	49
MCB	5'-GACGCGTCTCGAGACGCGTC-3'	58
SCB	5'-GACATGTGCGT <u>CACG</u> AAAAAAGAAATCAATC-3'	59
AP-1	5'-GATCTAAAA <u>TGAG</u> TCAAGTGG-3'	60
GCN4	5'-CTAGACGGGCGATGACTCATCGCCCGT-3'	46
HIS3	5'-TCGAGCGGATGACTCTTTTTTTTTC-3'	61

examined for β -galactosidase activity, a lowercase letter was appended to each plasmid name (e.g. pNUT2b is a second clone with two copies of a wild-type trimer). Cell transformations and β -galactosidase assays were performed using previously described protocols (44). To quantify p180-mediated transcription as a function of cell cycle progression, a mutated derivative of the *CLN2* gene (*ClN2x/s*; a kind gift of David Stuart, Scripps Research Institute, La Jolla, CA) encoding a nonfunctional protein was cloned downstream of a p180-dependent promoter by linking together a 1.5-kb DNA fragment containing the pNUT2b promoter, a 1.5-kb DNA fragment of pUC19*CLN2x/s*,² and plasmid pRS306, a single copy *URA3*-containing vector (45). A plasmid dependent on a promoter lacking a p180-binding site, pWEE3a, was prepared in a similar manner. The resulting plasmids, pNUT2b*CLN2x/s* and pWEE3a*ClN2x/s*, carry the defective *CLN2* gene in the same transcriptional orientation as *URA3*. High copy plasmids carrying these *CLN2* reporter genes were prepared by transferring pNUT- or pWEE-dependent genes to plasmid pRS202, a *URA3*-containing vector,³ producing plasmids P5*ClN202* and W1*ClN202*, respectively.

Protein-DNA Binding Assays—Yeast extracts were prepared by bead-beating 1×10^9 cells for 30 min at 4 °C following their suspension in 1.0 M Tris-HCl (pH 7.5), 0.2 M NaCl, 5 mM EDTA, 20% glycerol, 90 mM β -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 20 μ g/ml pepstatin A and leupeptin (Sigma). This suspension was clarified by centrifugation at $16,000 \times g$ at 4 °C. Supernatants were transferred to tubes containing an equal volume of 0.5 M HEPES (pH 7.4), 30 mM KCl, 0.4 mM EDTA, 20% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20 μ g/ml pepstatin A, and 20 μ g/ml leupeptin; protein concentrations were determined by a colorimetric assay (Bio-Rad), and aliquots were stored at -80 °C. Radiolabeled probes were prepared and employed in protein-DNA binding assays as described previously (9, 46). Oligonucleotides used as radiolabeled probes or competitor DNAs are listed in Table II. Radiolabeled probes had a specific activity of 10^6 cpm/ng DNA, and 1–2 $\times 10^5$ cpm of probe was typically combined with 5 μ g of yeast proteins. Competition experiments with unlabeled oligonucleotides typically employed a 50–200-fold molar excess of DNA relative to radiolabeled probes. Following resolution on polyacrylamide

gels and transfer to paper, protein-DNA binding assays were exposed to film (Kodak XAR-5) for 2 days at -80 °C or directly analyzed in a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

Photoaffinity Labeling—A plasmid carrying six copies of the sequence 5'-GCCACCCTCT-3' (RCE7, Table II) was cleaved with *Eco*RI and *Xba*I liberating a DNA fragment that was used as a template for primer extension from the *Eco*RI end of the probe. Primer extension was carried out using a synthetic primer (5'-CGAGCTCGCC-3'), bromodeoxyuridine (BUdR, Sigma), dTTP, dATP, radiolabeled dGTP and dCTP (3000 Ci/mmol; ICN Biomedicals, Inc., Irvine, CA), and Klenow enzyme (New England BioLabs, Beverly, MA) as described elsewhere (9). A BUdR-substituted probe (12×10^6 cpm) with a specific activity of 10^9 cpm/ng was employed in a protein-DNA binding assay with 35 μ g of yeast proteins. Following resolution on a polyacrylamide gel, protein-DNA complexes were irradiated by UV light *in situ* and visualized by exposure of the gel to film, and excised complexes were applied to an SDS-polyacrylamide gel. Following electrophoresis, dried gels were exposed to film for 2 days at -80 °C. Apparent molecular weights of resulting protein-DNA complexes were determined by comparison with molecular weight markers resolved in parallel.

Synchronization of Yeast Cells with α Factor—Yeast cultures were grown in YEPD to approximately $A_{600\text{ nm}} = 0.5$ at 30 °C, and α factor pheromone (Sigma) was added to a final concentration of 10 ng/ml. After 2.5 h of incubation, cell cycle arrest was confirmed by microscopic examination. Cells were then pelleted, resuspended in 3 ml of sterile deionized water, sonicated, and added to fresh, prewarmed YEPD media. Subsequently, 50 ml of cells were collected by centrifugation at 15-min intervals for the preparation of protein extracts or RNA. Small aliquots of cells were also fixed in 4 volumes of 3.7% formaldehyde, 0.15 M NaCl to establish a budding index by microscopic inspection. Fresh, prewarmed YEPD was added every 45 min during synchronous growth to maintain a stable concentration of cells. Cultures of cells containing high copy reporter plasmids were grown in YEPD because synchrony was not optimal in selective minimal media. No more than 7% of cells (as determined by replica plating onto YEPD and selective minimal media plates) lost their respective plasmids during synchronous growth in YEPD.

Northern Blot Analysis—RNA extracts were obtained using a modification of a previously described procedure (47). Briefly, cells were

² D. Stuart, unpublished data.³ P. Hieter, personal communication.

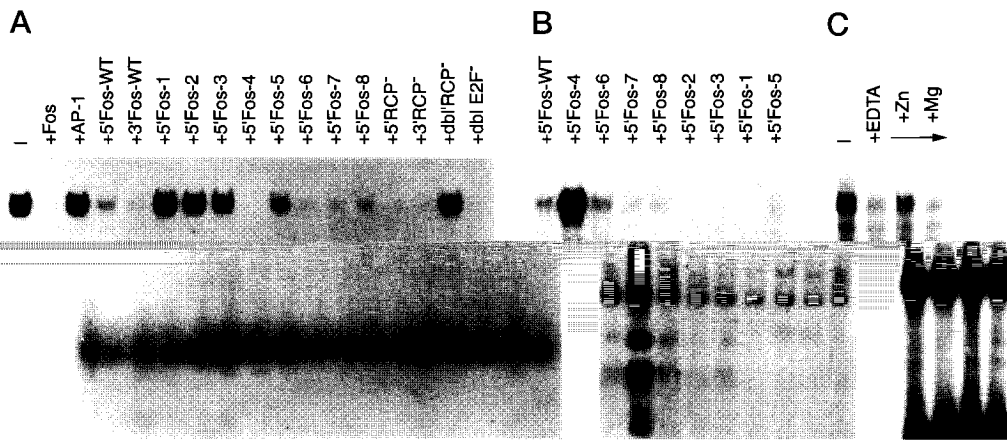


FIG. 2. Protein-DNA binding assays with *S. cerevisiae* cell extracts and radiolabeled wild-type and mutated *c-FOS* RCE oligonucleotides. Protein-DNA binding assays were performed as in Fig. 1. *A*, radiolabeled wild-type *c-FOS* oligonucleotides were incubated with yeast cell extracts alone (-) or in conjunction with a 200-fold molar excess of competitor oligonucleotides that carry (+*Fos*, +5'*Fos*-WT, +3'*Fos*-WT, +5'*Fos*-4, +5'*Fos*-6, +5'*Fos*-7, +5'*Fos*-8, +5'*RCP*-, +3'*RCP*-, +*dbl E2F*-) or lack (+*AP*-1, +5'*Fos*-1, +5'*Fos*-2, +5'*Fos*-3, +5'*Fos*-5, +*dbl RCP*-) mammalian RCP-binding activity. *B*, wild-type (WT) and mutated *c-FOS* RCE oligonucleotides were radiolabeled and incubated with cell extracts as above. *C*, effect of zinc chelation of the formation of yeast protein-RCE complexes. Yeast extracts were incubated with (+*EDTA*) or without (-) *EDTA* for 10 min at room temperature prior to addition of radiolabeled wild-type *c-FOS* RCE oligonucleotides supplemented with excess $ZnCl_2$ (+*Zn*) or $MgCl_2$ (+*Mg*). Final concentrations of $ZnCl_2$ and $MgCl_2$ were 1 mM and *EDTA* was 0.25 mM.

that do not function as competitors (mutants 5'*Fos*-1, -2, -3, and -5) also did not form protein-DNA complexes when examined as radiolabeled probes (Fig. 2*B*). Additionally, mutated oligonucleotides that function as competitors had wild-type protein-binding activity (Fig. 2*B*, mutants 5'*Fos*-6, -7, and -8).

Interestingly and consistent with our previous results using yeast cell extracts, mammalian RCE-BPs bound to SCB and MCB elements in *S. cerevisiae* cell extracts. To determine if yeast RCE-BPs bound to SCB and MCB elements, wild-type and mutated oligonucleotides carrying consensus elements were examined in protein-DNA binding assays as shown in Fig. 3*A*. A 100-fold molar excess of these radiolabeled oligonucleotides did not diminish the recovery of RCE protein-DNA complexes. These results indicate that SCB and MCB elements are functional in yeast. In addition, SCB and MCB elements may be necessary for yeast RCE-binding activity, extracts were prepared from yeast strains carrying disruptions of *SWI4* or *SWI6*, these extracts were employed in protein-DNA binding assays. As shown in Fig. 3*B*, disruption of these genes had no discernible effect on the abundance or mobility of protein-DNA complexes. Thus, we conclude that 1) under the conditions we employed, yeast RCE-BPs do not bind to E2F, SCB, and MCB elements, and 2) yeast RCE-BPs bind to SCB and MCB

cleotide (*E2*-WT) derived from the *E2* promoter diminished protein-RCE complexes; however, a mutated derivative (*E2*-Mut) in which the *E2F* sites were destroyed could also effectively compete for DNA-binding activity (52). Taken together, these data indicate that the DNA-binding domains of yeast

RCE-binding proteins are not likely to be functionally analogous to that of mammalian RCE-BPs. In mammalian cell extracts, mutant 5'*Fos*-4 has greater protein-binding activity than wild-type RCE oligonucleotides (Fig. 2*B*). In summary, we conclude from these results that extracts prepared from *S. cerevisiae* and *S. pombe* carry at least one protein that forms specific protein-DNA complexes with the *c-FOS* RCE. Moreover, mutated oligonucleotides derived from any of these complexes are identical to those required for the binding of mammalian RCPs, such as SP1 and SP3. These data suggest that the DNA-binding domains of yeast and mammalian RCE-binding proteins are functionally, and perhaps structurally, conserved. Consistent with the notion that yeast RCE-BPs may be structurally related to SP family members, addition of potent zinc chelating agents, such as *EDTA* or 1,10-phenanthroline, to yeast extracts inhibits the formation of protein-RCE complexes in a zinc-dependent manner (Fig. 2*C*).

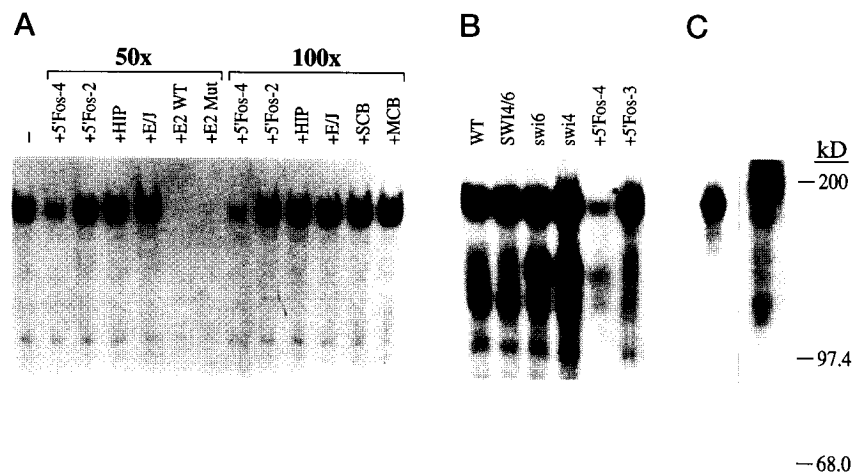


FIG. 3. Protein-DNA binding assays with *S. cerevisiae* cell extracts and radiolabeled wild-type c-FOS RCE oligonucleotides and photoaffinity labeling of yeast RCE-binding proteins. Protein-DNA binding assays were performed as in Fig. 1. A, radiolabeled 5'Fos-4 oligonucleotides were incubated with yeast cell extracts alone (–) or in conjunction with a 50- or 100-fold molar excess of competitor oligonucleotides that carry (+5'Fos-4) or lack (+5'Fos-2) mammalian RCP-binding activity, oligonucleotides that carry wild-type (+HIP, +E2 WT) or mutated (+E/J, +E2 Mut) E2F sites, or oligonucleotides that carry wild-type SCB (+SCB) or MCB (+MCB) yeast promoter elements. B, radiolabeled 5'Fos-4 oligonucleotides were incubated with extracts prepared from wild-type (YRC1, WT; BY602, *swi4/6*) yeast cells or cells carrying gene disruptions (BY600, *swi6*; BY606, *swi4*). Extracts of BY606 were also examined for 5'Fos-4 binding activity in the presence of unlabeled competitor oligonucleotides (+5'Fos-4, +5'Fos-3). C, photoaffinity labeling of *S. cerevisiae* (left) and *S. pombe* (right) RCE-binding proteins covalently linked to radiolabeled, BUdR-substituted RCE7 oligonucleotides. Molecular mass markers are indicated on the right.

p180) was recovered from *S. cerevisiae* extracts. Consistent with the lessened relative mobility of protein-DNA complexes produced by extracts prepared from *S. pombe*, similar cross-linking assays performed with *S. pombe* extracts resulted in the detection of a single protein-DNA complex of approximately 200 kDa (Fig. 3C). The simplest interpretation of these data is that *S. cerevisiae* and *S. pombe* express a single large RCE-binding protein that may be cross-linked to DNA. However, we are mindful of the possibility that additional yeast proteins may participate in RCE protein-DNA complexes that were not detected by UV cross-linking. The remaining studies in this report were performed with *S. cerevisiae* cells and extracts.

RCE-mediated Transcription Is Dependent on an Intact p180-binding Site—To determine if an intact p180-binding site is required for RCE-mediated transcription, a multimer carrying three tandem copies of the octameric site shown to be a target for p180 binding (pNUT; Fig. 4A and Table II) or a multimer carrying three copies of a mutated octamer that lacks a binding site for p180 (pWEE; Fig. 4A and Table II) were cloned upstream of a *LACZ* reporter gene whose expression is directed by a basal *CYC1* promoter (42). In control experiments, p180 bound pNUT but not pWEE when examined in protein-DNA binding assays both as probes and unlabeled competitors (data not shown). Asynchronously growing populations of yeast transformed with either construction or the parent plasmid were permeabilized with SDS and chloroform and assayed for β -galactosidase activity. As shown in Fig. 4B, plasmids carrying one or two copies of the pNUT trimer increased β -galactosidase activity 31- and 370-fold, respectively, relative to the parent plasmid lacking a p180-binding site. Similar levels of pNUT *trans*-activation were also obtained in cells lacking *SWI6* function (BY600; data not shown). pNUT-mediated *trans*-activation was also independent of the orientation of the octameric repeat cloned upstream of β -galactosidase (Fig. 4B). In contrast to results with pNUT, one to three copies of the pWEE trimer resulted in little or no increase in β -galactosidase activity (Fig. 4B). We conclude from these results that an intact p180-binding site is required for RCE-mediated yeast transcription. Given that a dinucleotide substitution that ablates p180-binding activity *in vitro* also inactivates transcriptional activity *in vivo*, these data also strongly suggest that p180 can function as

a stimulatory transcription factor.

p180 Abundance and/or DNA-binding Activity and Resulting Transcription Is Cell Cycle Regulated—Should RCEs function as transcriptional targets of a Rb-like pathway in yeast, we speculated that p180 DNA-binding and/or transcriptional activity might vary in concert with cell cycle progression. This speculation was buoyed by the observation that extracts prepared from cells arrested with nocodazole showed significantly lower p180 DNA-binding activity relative to extracts prepared from asynchronously growing cultures (data not shown). To further examine the abundance of p180 DNA-binding activity during the cell cycle, extracts were prepared from a panel of *S. cerevisiae* temperature-sensitive *cdc* mutants grown at the permissive and nonpermissive temperatures. All *cdc* mutant strains grown asynchronously at the permissive temperature (30 °C) contained significant amounts of p180 DNA-binding activity (Fig. 5, top row of left panel). At the nonpermissive temperature (38 °C), mutants whose functions are required during G₁ or M phases showed little or no p180 DNA-binding activity (Fig. 5, center row of left panel). Yet, these cell extracts are clearly functional since they retained GCN4 DNA-binding activity (Fig. 5, bottom row of left panel). In contrast, mutants whose functions are required in early to mid-S phase (*cdc6*, *cdc7*, and *cdc17*) contained wild-type levels of p180 DNA-binding activity. Interestingly, a distinct S phase mutant, *cdc2*, did not have detectable p180 DNA-binding activity at the nonpermissive temperature for function (Fig. 5, center row of left panel). Importantly, the abundance of p180-DNA complexes were not appreciably altered in extracts prepared from a wild-type strain grown at either temperature. Although these results suggested that p180 abundance and/or DNA-binding activity is maximal during early to mid-S phase, we were concerned that these results might be compromised by artifacts induced by cell cycle arrest and wished to perform similar analyses with synchronously growing cell populations. Thus, cells were synchronized by incubation with α factor, extensively washed and incubated in growth medium, and protein extracts were prepared in 15-min intervals. Equivalent amounts of total cell proteins were subsequently examined in protein-DNA binding assays (Fig. 6A). Microscopic inspection of α factor-treated cells showed that greater than 95% of cells

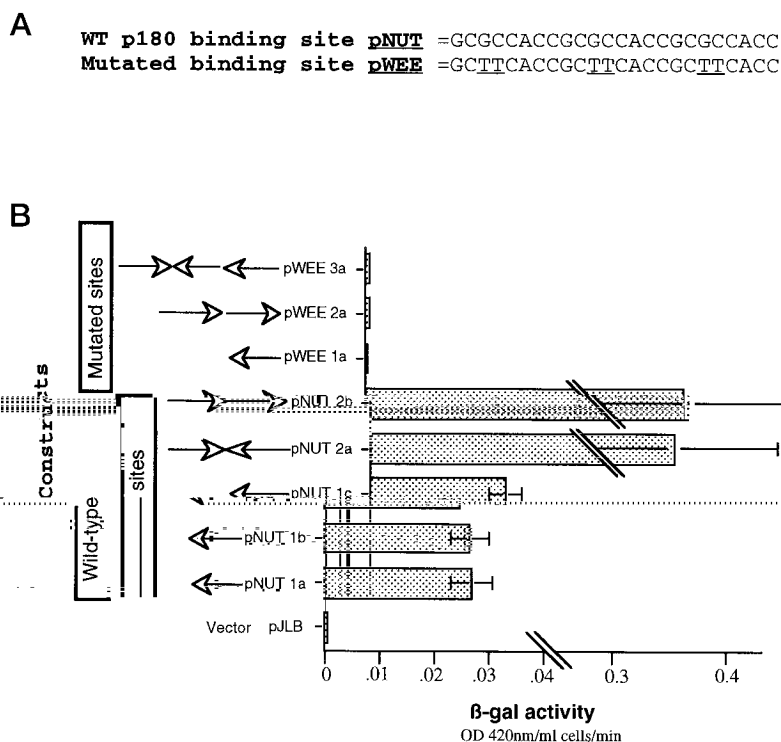


FIG. 4. Intact p180-binding sites are required for RCE-mediated transcription *in vivo*. A, sequence of oligonucleotides cloned in single copies or as multimers upstream of the *CYC1* promoter and *LACZ* that carry (pNUT) or lack (pWEE) p180-binding sites. Mutated nucleotides that abrogate p180-binding activity are underlined in pWEE. B, β -galactosidase activities of cells transformed with plasmids carrying single (e.g. pNUT1a) or multiple copies (e.g. pNUT2a) of oligonucleotides that do (pNUT) or do not (pWEE) bind p180 *in vitro*. Measurements presented represent the average β -galactosidase activities of two or three independent cell clones carrying the indicated constructions. Arrowheads indicate the orientation of individual oligonucleotides relative to the site of transcriptional initiation.

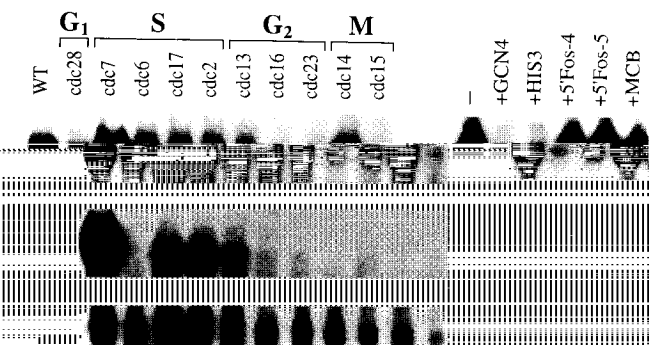


FIG. 5. Protein-DNA binding assays using extracts prepared from wild-type and *cdc* strains grown at the permissive (30 °C) or nonpermissive (38 °C) temperatures for function. Radiolabeled 5'Fos-4 or GCN4 oligonucleotides were incubated with cell extracts, and protein-DNA complexes were detected as indicated in Fig. 1. *Cdc* strains used for these studies as well as the approximate cell cycle position within which their functions are required are indicated at the top left panel of the figure. Protein-DNA complexes resulting from cell extracts from a wild-type strain (YRC1) grown at 30 and 38 °C are shown in the leftmost lane of the left panels for comparison. Left panel, extracts prepared from strains grown at the permissive temperature (top row) and the nonpermissive temperature (center row) for function were examined for p180 binding activity. As a control for extract integrity, extracts prepared from strains grown at the nonpermissive temperature were also examined for GCN4 DNA-binding activity (bottom row). Right panel, extracts prepared from wild-type cells were incubated with radiolabeled GCN4 oligonucleotides alone (-) or in the presence of a 200-fold molar excess of unlabeled homologous (+GCN4, +HIS3) or heterologous (+5Fos-4, 5Fos-5, +MCB) competitor oligonucleotides.

entered S phase synchronously during the course of two population doublings (Fig. 6B). Consistent with evidence from growth-arrested *cdc* mutants, maximal p180 DNA-binding activity was detected at time points that immediately precede the peak of accumulated budded cells (Fig. 6). The abundance of p180 DNA-binding activity throughout the cell cycle was directly quantified by PhosphorImaging and determined to vary at least 2-fold during the course of two cell cycles.

during cell cycle progression, becoming maximal during S phase, we wished to determine whether p180-mediated transcription was similarly regulated. The promoter regions from pNUT2b, containing six copies of a wild-type p180-binding site upstream of a basal *CYC1* promoter, or from pWEE3a, contain-

ing nine copies of a mutated p180-binding site, were cloned upstream of a disrupted *CLN2* gene (*clnX's*) and inserted into a high copy *URA3*-containing vector (pRS202). The resulting constructions (P5Cln202 and W1Cln202, respectively) were subsequently used to transform a yeast strain (DLY204) carrying a disruption of *CLN2*, and Northern blots carrying total RNAs

from cells containing either construction were examined for exogenous *CLN2* mRNA with a *CLN2*-specific probe. DLY204 has previously been shown to express an unstable *CLN2* mRNA of 0.6 kb.⁴ Each transformed yeast strain expressed a minor transcript of 2.0 kb (Fig. 7A, top panel; asterisk) whose synthesis was independent of the integrity of plasmid-borne p180-binding sites. In contrast, a single prominent transcript of 3.0 kb was expressed exclusively in cells carrying plasmids with intact p180-binding sites (Fig. 7A, top panel; *CLN*). To determine whether the abundance of the 3.0-kb pair mRNA was cell cycle-dependent, cells containing P5Cln202 were synchronized with α factor and then incubated in growth medium. Total RNA was isolated from synchronized cells at 15-min intervals; *CLN2* expression was examined with a *CLN2*-specific probe, and the abundance of the 3.0-kb *CLN2* mRNA was directly quantified in a PhosphorImager and normalized to the abundance of actin mRNA (Fig. 7A, middle panel). In successive experiments, the abundance of p180-dependent *CLN2* mRNA varied by an average of 3-4-fold during the cell cycle with peaks of transcription occurring coincident with maximal p180 DNA-binding activity (compare Figs. 6B and 7B). In contrast to these results, the abundance of the 2.0-kb *CLN2*-related mRNA was largely unchanged during the course of these experiments (Fig. 7A, top panel). As an additional meas-

⁴ D. Low personal communication.

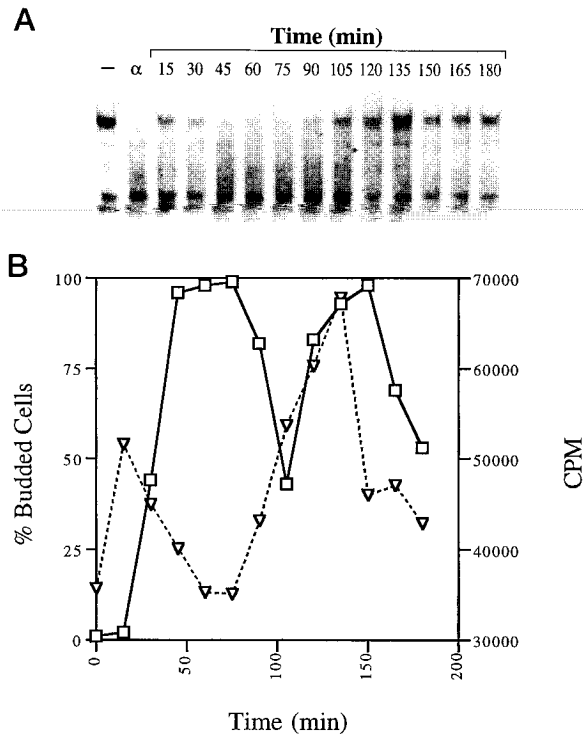


FIG. 6. Cell cycle regulation of p180-DNA complex abundance in synchronized populations of cells. A, protein-DNA binding assays. Extracts prepared from asynchronously growing cells (-), cells arrested with α factor (α), or α factor-treated cells that were washed and incubated in growth medium for varying lengths of time were examined for the abundance of p180-DNA complexes using radiolabeled 5' Fos-4 oligonucleotides. Equal quantities of yeast cell proteins were examined in each lane. B, quantification of p180-DNA complexes as a function of cell cycle progression. The abundance of p180-DNA complexes in the assay shown in A was determined directly using a PhosphorImager and plotted (indicated by triangles) against the percentage of budded cells (indicated by squares) at each time point following α factor arrest.

the abundance of histone *H4* message. With the exception of RNA harvested from α factor-arrested cells, maximal amounts of p180-dependent *CLN2* mRNA were detected coincident with the accumulation of histone *H4* mRNA (Fig. 7A, bottom panel; Ref. 53). We do not as yet understand why residual *CLN2* mRNA is apparent in α factor-arrested cells. Nonetheless, for populations of synchronously growing cells, cell cycle-regulated fluctuations in p180 DNA-binding activity *in vitro* are temporally correlated with periodicity in the abundance of RCE-mediated transcription *in vivo*. Moreover, RCE-mediated transcription is maximal during early- to mid-S phase.

DISCUSSION

The promoters of a number of Rb-responsive genes (e.g. *c-FOS*, *c-MYC*, *TGF β -1*, *IGF2*, *IL6*, *c-JUN*, and *c-NEU*) carry GC-rich sequences, termed retinoblastoma control elements (RCEs) that are necessary and sufficient for Rb-mediated transcriptional regulation (3). Previous analyses have determined that RCEs are bound by several mammalian proteins (RCPs) *in vitro*, and these proteins have been revealed to be members of the SP family of transcription factors. To determine if an RCE-like pathway of transcriptional control exists in lower eukaryotes, we sought to identify RCE-binding proteins (RCE-BPs) in yeast cells. We reasoned that if RCEs are evolutionarily conserved promoter elements then yeast RCE-BPs may be functional homologues of Rb-targeted transcription factors (e.g. SP1 or SP3), and the activities of such proteins might be regulated by a yeast Rb-like molecule. This report characterizes a novel transcription factor expressed in *S. cerevisiae*, termed

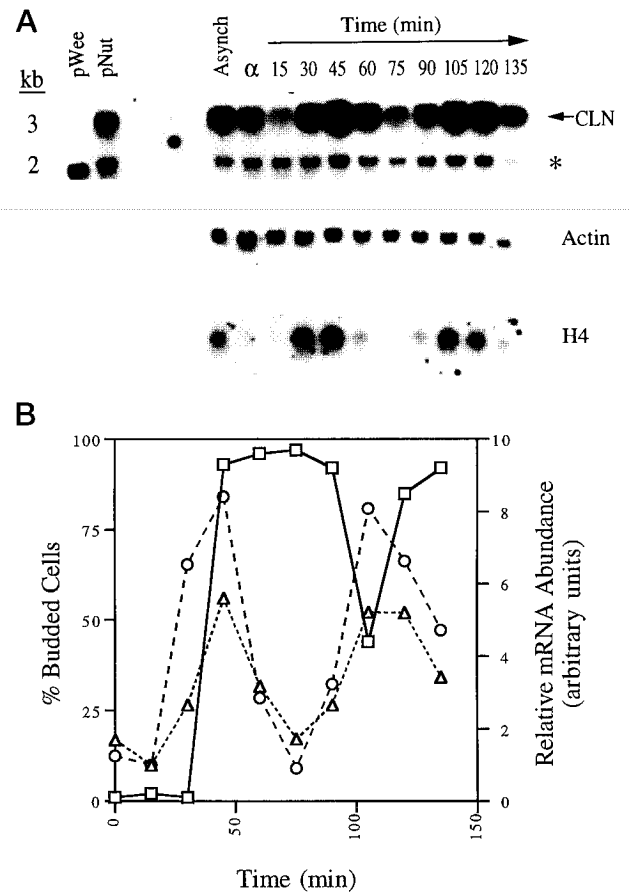


FIG. 7. Cell cycle regulation of RCE-mediated transcription in synchronized populations of cells. As in Fig. 6, cells arrested with α factor were washed and incubated in growth medium for varying lengths of time. A, Northern blots of RNAs prepared from asynchronous (*Asynch*) and synchronized cells. *CLN2* transcripts synthesized by cells carrying plasmids driven by a wild-type RCE, P5Cln202 (pNUT), or mutated RCE, W1Cln202 (pWEE), are depicted on the left. Total cell RNA was prepared from α factor-arrested cells (α) or synchronized populations of cells at the indicated time intervals and examined by Northern blotting. The Northern presented was hybridized with a *CLN2*-specific probe and then subsequently hybridized with actin and histone *H4* probes. An asterisk indicates a *CLN2*-specific transcript whose synthesis is independent of RCE integrity and cell cycle position. B, quantification of mRNA abundance in synchronized populations of cells. The abundance of each mRNA was determined directly using a PhosphorImager. Levels of RCE-mediated *CLN2* transcripts (indicated by triangles) were normalized to those of actin and plotted against the percentage of budded cells (indicated by squares) at each time point following α factor arrest. The relative abundance of *CLN2* mRNA at the 15-min time point was arbitrarily assigned a value of 1.0. The abundance of histone *H4* mRNA is also indicated (circles) as a function of cell cycle progression.

p180, that specifically binds to RCEs during early- to mid-S phase utilizing nucleotides that are required for binding and *trans*-activation by SP1/SP3. Directly correlated with this binding activity is a cell cycle-regulated stimulation of RCE-mediated transcription *in vivo*. We also report that a 200-kDa RCE-binding protein (termed p200) with similar RCE-binding activity is expressed in *S. pombe*.

Based on photoaffinity labeling protein experiments, a single high molecular weight RCE-binding protein expressed in *S. cerevisiae* and *S. pombe* may be cross-linked to DNA *in vitro*. We note that these data do not preclude the possibility that additional yeast proteins participate in p180-DNA and p200-DNA complexes or that RCEs are bound and regulated by as yet undetected proteins *in vivo*. However, given the tight correlation

between the nucleotide binding specificity of p180 *in vitro* and the transcriptional activity of wild-type and mutated RCEs *in vivo*, this latter possibility is unlikely. Consistent with our cross-linking results, preliminary affinity chromatography experiments indicate that RCE-binding activity in *S. cerevisiae* extracts co-fractionates with a polypeptide of approximately 180 kDa as well as several smaller proteins.⁵ Determining whether one or more of these proteins account for p180 DNA-binding activity will require additional rounds of protein purification. We also note that the large apparent molecular weight of yeast RCE-binding proteins is not without precedent as a number of *S. cerevisiae* transcription factors ranging between 170 and 220 kDa have previously been noted, including MOT1, RIF1, SIN3, and SNF2 (54, 55).

p180 and p200 bind RCEs via nucleotides that are identical to those bound by SP1 and SP3. Dinucleotide substitutions introduced within a 10-base pair sequence, 5'-GCGCGCCACC-3', within the *c-FOS* RCE dramatically alter the recovery of yeast protein-RCE complexes *in vitro* and perturb RCE-mediated transcription *in vivo*. For example, several dinucleotide substitutions within the RCE (5'Fos-1, -2, -3, -5) ablate the binding of p180/p200 and SP family members to RCEs, and a single dinucleotide substitution (5'Fos-4) increases their DNA-binding activity concordantly. That identical mutations similarly affect the DNA-binding activities of yeast and mammalian RCE-binding proteins strongly suggests that their DNA-binding domains are functionally homologous. It is less certain, however, that their respective DNA-binding domains are closely related in structure. Consistent with the notion that yeast and mammalian RCE-binding proteins may be structurally related, we have shown that each requires zinc for DNA-binding activity. The SP family of transcription factors are well characterized "zinc-finger" proteins carrying three tandem zinc-binding motifs of the Cys₂-His₂ class (56). Although likely to be metalloproteins, whether p180/p200 will be "zinc-finger" proteins of the same or a similar class will require their eventual cloning and sequencing. Despite their common nucleotide specificities and zinc requirement, three additional observations suggest that the primary sequence of the SP family members are not likely to be closely related to that of p180 or other yeast transcription factors. First, using polyclonal antisera prepared against the entirety of the SP1 *trans*-activation domain, we have been unable to deplete yeast extracts of p180.⁵ Second, the expression of SP1 in *S. cerevisiae* does not result in the *trans*-activation of reporter genes regulated by several SP1-binding sites (57). Moreover, co-expression of a component of the human basal transcription complex, TATA-box binding protein, did not facilitate SP1-mediated yeast transcription. This latter result may indicate that yeast lack one or more general transcription factors necessary for SP1-mediated transcription, perhaps co-activators that bridge glutamine-rich *trans*-activation domains to the basal transcription complex. Finally, expression of human Rb in *S. cerevisiae* does not appreciably alter yeast cell growth or progression of the cell cycle (38). Thus, should p180/p200 be structurally related to SP1/SP3 we anticipate that their homology may not extend further than their respective DNA-binding domains.

In accord with the notion that p180 is a cell cycle-regulated transcription factor, we have consistently noted that maximal levels of RCE-mediated transcription are coincident with the peak of histone *H4* mRNA abundance. Although we have observed on average a 3–4-fold difference in cell cycle-regulated RCE-mediated transcription, it is possible that this may not accurately reflect the magnitude of periodic p180 activity for

the following reasons. First, our *trans*-activation assays were performed with a promoter construct carrying a wild-type *c-FOS* RCE cloned upstream of a basal promoter. It is not as yet clear that the *c-FOS* RCE is an optimal binding site for p180, and it is possible that small perturbations in sequence can greatly affect p180-mediated transcription. For example, dinucleotide substitutions at two distal positions within the RCE (5'Fos-4) result in a substantial increase in p180 DNA-binding activity and resulting *trans*-activation *in vivo*.⁵ Second, it is also likely that the promoter context of p180-binding sites and their juxtaposition to other *trans*-acting factors and the site of transcriptional initiation may play important roles in the regulation of periodic p180-mediated transcription. Thus, a more definitive cell cycle analysis of p180-mediated transcription will require the identification of genes regulated by p180 and a careful examination of the promoters of genes that p180 DNA-binding activity and RCE-mediated transcription are confined to a temporal "window" of early- to mid-S phase, it is tempting to speculate that p180 may play a role in the *trans*-activation of 1) genes associated with the biogenesis of DNA and/or 2) other S phase genes, such as histones and the B-type cyclins *CLB3* and *CLB4* (58, 59). Although sequences closely related to the *c-FOS* RCE are not contained within the promoters of these S phase genes, evaluating whether p180 plays a role in their regulation will require the cloning and functional characterization of p180. Interestingly, a computer-assisted search of the *S. cerevisiae* genome data base with the *c-FOS* RCE revealed predicted sites of p180 binding upstream of the *KIP1* and *UBC13* genes. *KIP1* is a kinesin-related protein required for spindle assembly, and its abundance is maximal in M phase (60).⁶ *UBC13* is thought to encode a ubiquitin-conjugating enzyme; however, it is not known whether its expression is cell cycle-regulated. Whether p180 plays a direct role in the synthesis of these genes remains to be determined. Since p180 does not bind SCB and MCB elements *in vitro* or require SWI4/SWI6 proteins for DNA-binding or transcriptional activity, we hypothesize that p180 may function as a regulator of a novel subset of periodically transcribed RCE-dependent yeast genes. However, since it is possible that our *in vitro* DNA binding assays may be insensitive to weak interactions of p180 with SCB/MCB elements, p180-mediated transcription may also partially account for cell viability in the absence of cell cycle regulators such as MBF (39).

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