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, TGFβ-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.

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 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, TGFβ-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 supposition, Rb has been proposed to modulate the transcriptional activity of SP family members via their liberation from negative regulators or by indirectly “bridging” their trans-activation domains to components of the basal transcription machinery (8, 12, 13). Trans-activation mediated by transcription factors such as ATF-2, NF-IL6, MYOD, and myogenin is also stimulated and/or facilitated by Rb in vivo (14–17). Unlike SP family members, Rb forms physical complexes with these latter factors although the mechanism by which Rb augments their transcriptional activity has not as yet been determined. In contrast to these functional effects, interactions of Rb with transcription factors such as E2F, ELF-1, and UBF lead to the suppression of transcriptional activity (18–20). Rb forms cell cycle-regulated complexes with factors such as E2F and ELF-1 in vivo, sequestering their trans-activation domains from components of the basal transcription complex (18, 19, 21). Hence, Rb is believed to control the transcription of at least some cell cycle-regulated genes via periodic interactions with sequence-specific DNA-binding proteins.

The Rb protein is phosphorylated in concert with the progression of the mammalian cell cycle. Quiescent cells, postmitotic cells, and cells in early G1 carry un- or underphospho-
rlylated Rb protein (22–25). In cycling cells, Rb becomes increasingly phosphorylated on serine and threonine residues beginning in late G1 (the “restriction point”) and extending through G2 and then is abruptly de-phosphorylated 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 transcription factor 1 in Saccharomyces cerevisiae has not been identified in yeast, but proteins that are structurally and functionally similar to SWI4, SWI6, and MBP1 have also been isolated from S. pombe (36, 37). In concert with these findings, 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, exogenously expressed 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 MBF, and their interaction with SCB (SWI4-SWI6 cell cycle box) and MCB (Mlu1 cell cycle box) promoter elements, respectively (39, 40). SBF promoter elements (5′-CACGAAA-3′) govern the periodic transcription of genes such as the HO endonuclease and cyclins (CLN1 and CLN2). MCB elements (5′-GGCGCACC-3′) direct the transcription of a variety of genes including many required at the G1/S boundary for entry into S 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 (40). Given that RCEs share limited sequence homology (5′-GGCGCACC-3′) with yeast SBF and MCB elements, we hypothesized that RCEs might represent a related family of cell cycle-regulated yeast promoter elements. Furthermore, we speculated 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, SP3, 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 A600 of 0.4, pelleted, resuspended in prewarmed YEPD, and incubated at 37°C for 5 h. Arrest was confirmed by documenting terminal arrest phenotypes (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 (CYCI) 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′-TCGAGCCACCGCGCCACCGCGGAGG-3′) or a mutated derivative and its complement lacking a p180-binding site (pWEE; 5′-TCGAGCTCCCCCTCACCGCGCCCGGGC-3′) were cloned upstream of the CYCI promoter at a unique Xhol 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
This study

moter, a 1.5-kb DNA fragment of pUC19CLN2x/s,2 and plasmid pWEE3aCln2x/s, carry the defective 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′-GGCCACCCCTCT-3′ (RCE7, Table II) was cleaved with EcoRI and XbaI liberating a DNA fragment that was used as a template for primer extension from the EcoRI end of the probe. Primer extension was carried out using a synthetic primer (5′-GAGGTGCCTGCC-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 10^8 cpm/ng was employed in a protein-DNA binding assay with 35 μg 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 a Factor—Yeast cultures were grown in YEPD to approximately A_{600 nm}=0.5 at 30 °C, and a 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 μM NaCl to establish a budding index by microscopic inspection. 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 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 pUC19CLN2x/s, 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, pNUT2bCLN2x/s and pWEE3aCLN2x/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, producing plasmids P5Cln202 and W1Cln202, respectively.

### Table II

| Name  | Sequence                                      | Reference |
|-------|-----------------------------------------------|-----------|
| pNUT  | 5′-TGAGGCACCCCGCCACCGCCACCGCCACCGACCT-3′      | This study|
| pWEE  | 5′-TGAGCCTCGCCTCGCCTCGCCTCGCCTCGCCTCGACCT-3′ | This study|
| HIP   | 5′-ATTTCGCAATTCGCAATTCGCAATTCGCAATTCGACCTG-3′ | 49        |
| E/J   | 5′-ATTTCGCAATTCGCAATTCGCAATTCGCAATTCGACCTG-3′ | 49        |
| MCB   | 5′-GACGGTTCGACCGCGTCGACCGCGTCGACCGCGTCGAC-3′ | 58        |
| SCB   | 5′-GACATGCGCTGACGACGACGACGACGACGACGACGAC-3′  | 59        |
| AP-1  | 5′-GATCTAAATTATGATGACGATGACGATGACGATGAC-3′    | 60        |
| GCN4  | 5′-CTAGACGGCCGCGATGATGATGATGATGATGATGATGATG-3′ | 46        |
| HIS3  | 5′-TGAGCGGAGATGATGATGATGATGATGATGATGATGATGATG-3′ | 61        |

**Note:**

a D. Stuart, unpublished data.

b P. Hieter, personal communication.
RESULTS

Proteins That Specifically Bind Retinoblastoma Control Elements (RCEs) Are Expressed in S. cerevisiae and S. pombe—To determine whether S. cerevisiae and/or S. pombe express RCE-binding proteins (RCE-BPs), a 31-base pair oligonucleotide corresponding to the human c-FOS RCE was radiolabeled and employed with yeast extracts in protein-DNA binding (“gel-shift”) assays. As shown in Fig. 1, several protein-DNA complexes resulted when this oligonucleotide was incubated with either yeast extract. To determine if one or more of these protein-DNA complexes results from the specific association of yeast proteins with the c-FOS RCE oligonucleotide, excess unlabeled homologous or heterologous oligonucleotides were added as competitor DNAs. For both S. cerevisiae and S. pombe extracts, a single slowly migrating protein-DNA complex is eliminated by excess unlabeled homologous oligonucleotides (Fig. 1, +Fos) but not by an irrelevant oligonucleotide carrying binding sites for the transcription factor AP-1 (Fig. 1, +AP-1). The abundance of additional faster migrating protein-DNA complexes was not appreciably affected by the inclusion of either competitor oligonucleotide, and we have found that their abundance in yeast extracts is quite variable, often being undetectable (for example see Fig. 2). To determine if yeast RCE-BPs require nucleotides for complex formation that are shared with mammalian RCPs, we employed two mutated RCE oligonucleotides that have previously been shown to strongly interact (Fig. 1, 5’Fos-4) or not interact (Fig. 1, 5’Fos-5) with S1 and SP3 (9). As shown in Table II, oligonucleotides 5’Fos-4 and 5’Fos-5 include 18 nucleotides derived from the 5’-half of the c-FOS RCE and carry dinucleotide substitutions that respectively increase or abolish mammalian RCP-binding activity relative to wild-type sequences. Akin to our previous result with mammalian RCPs, the abundance of the slowest migrating yeast protein-DNA complexes was abolished by inclusion of an excess of 5’Fos-4 and not appreciably diminished by 5’Fos-5 (Fig. 1).

Wild-type oligonucleotides corresponding to RCEs within the TGFβ-1 and c-MYC promoters also eliminated the slowest migrating protein-DNA complex when employed as competitors in parallel protein-DNA binding assays (data not shown). Thus, S. cerevisiae and S. pombe express at least one protein that specifically binds RCEs in vitro. As is more clearly illustrated in the rightmost panel of Fig. 1, we note that the specific protein-DNA complex detected in S. cerevisiae extracts migrates slightly faster than its counterpart in S. pombe extracts.

Yeast and Mammalian RCE-binding Proteins Require Identical Nucleotides and Zinc for DNA Binding—To extend our comparison of mammalian and yeast RCE-binding proteins and map nucleotides that are important for protein-DNA complex formation, protein-DNA binding assays were performed in the presence of a large panel of synthetic oligonucleotides whose RCP-binding activities have been previously established with mammalian cell extracts (9). As shown in Table II, these additional oligonucleotides carry hexanucleotide or dinucleotide substitutions within discrete portions of the c-FOS RCE. Consistent with our previous results for SP1, the slowly migrating protein-DNA complex detected in S. cerevisiae extracts was abolished in competition experiments with the 5’ (5’Fos-WT) and the 3’ (3’Fos-WT) octameric repeats of the c-FOS RCE (Fig. 2A; Ref. 10). These results indicate that as for SP1, both octamer sites within the c-FOS RCE are bound by yeast RCE-BPs. This conclusion is supported by evidence that mutational disruption of both octamer sites (dbl RCP-) generates an oligonucleotide with little or no protein-binding activity, whereas mutations within either the 5’ (5’RCP-) or 3’ (3’RCP-) octameric repeat result in oligonucleotides that function as competitors (Fig. 2A). To further map the nucleotides required for yeast RCE-binding activity, synthetic oligonucleotides containing dinucleotide mutations within the 5’ 18 bases of the c-FOS RCE were employed as competitor DNAs. An excess of mutants 5’Fos-1, -2, -3, and -5, which contain dinucleotide mutations within the octameric repeat, were unable to compete for the formation of the single yeast protein-DNA complex. In contrast, inclusion of synthetic oligonucleotides carrying dinucleotide substitutions outside the octamer site (mutants 5’Fos-4, -6, -7, and -8) did not diminish RCE-binding activity. Taken together, these data indicate that the minimum RCE nucleotides required for binding of yeast RCE-BPs is the octameric 5’-GCGCCACC-3’ sequence. A similar series of experiments performed with S. pombe extracts gave identical results (data not shown). To confirm that mutated oligonucleotides carrying dinucleotide substitutions retained or lost their ability to bind yeast RCE-BPs, a panel of wild-type and mutated c-FOS oligonucleotides were examined directly for yeast RCE-BP-binding activity. Each oligonucleotide was radio labeled and incubated with yeast extracts, and protein-DNA complexes were resolved by electrophoresis. Consistent with earlier protein-DNA binding assays, those oligonucleotides
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. 2B). Additionally, mutated oligonucleotides that function as competitors had wild-type protein-binding activity (Fig. 2B, mutants 5'Fos-6, -7, and -8). Interestingly and consistent with our previous results using mammalian cell extracts, mutant 5'Fos-4 has greater protein-binding activity than wild-type RCE oligonucleotides (Fig. 2B).

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, nucleotides required for the formation 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, these data indicate that the DNA-binding domains of yeast RCE-binding proteins are not likely to be functionally analogous to that of E2F. To determine if yeast RCE-BP's bound SCB and MCB elements in vitro, oligonucleotides carrying consensus elements were examined in protein-DNA binding assays. As shown in Fig. 3A, a 100-fold molar excess of these unlabeled oligonucleotides did not diminish the recovery of RCE protein-DNA complexes. To establish if factors important for the binding and trans-activation of SCB and MCB elements may be necessary for yeast RCE-binding activity, extracts were prepared from yeast strains carrying disruptions of SWI4 or SWI6 for RCE-binding activity. As shown in Fig. 3B, 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 have employed, yeast RCE-BPs do not bind to E2F, SCB, and MCB elements in vitro and 2) yeast RCE-BPs do not require SWI4 or SWI6 for RCE-binding activity.

Yeast RCE-binding Activity Consists of at Least One High Molecular Weight Protein—To determine the number and apparent molecular weight(s) of the yeast RCE-binding proteins, we employed a photoaffinity labeling technique (UV cross-linking) with a multimerized RCE probe (RCE7) that was radiolabeled and substituted with bromodeoxyuridine (BUdR). This probe contains six contiguous copies of an oligonucleotide that spans the middle six bases of the octameric sequence previously shown to be critical for the binding of yeast RCE-BPs (Table II). We have previously used this multimerized probe for the analysis of mammalian RCPs, and prior to UV cross-linking experiments we confirmed that RCE7 could efficiently compete for the binding of yeast RCE-BPs (data not shown). Following radiolabeling and substitution with BUdR, RCE7 was incubated with extracts prepared from S. cerevisiae and S. pombe; protein-DNA complexes were resolved on nondenaturing gels, and bound proteins were covalently cross-linked to the probe in situ via irradiation with UV light. Protein-DNA complexes were excised and subsequently resolved on an SDS-polyacrylamide gel. As shown in Fig. 3C, a single protein-DNA complex with an apparent molecular mass of 180 kDa (denoted...
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, G2, 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
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Fig. 4. Intact p180-binding sites are required for RCE-mediated transcription in vitro. 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 (bottom 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 (+5′Fos-4, 5′Fos-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.

Since the abundance of p180 DNA-binding activity varies during cell cycle progression, becoming maximal during S phase, we wished to determine whether p180-mediated transcription was similarly regulated. The promoter regions from pNUT2h, containing six copies of a wild-type p180-binding site upstream of a basal CYC1 promoter, or from pWEE3a, containing nine copies of a mutated p180-binding site, were cloned upstream of a disrupted CLN2 gene (cln/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. 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 measure of cell cycle synchrony and to mark the position of S phase, Northern blots probed for CLN2 mRNA were re-examined for cell cycle dependencies.

4 D. Lew, personal communication.
the abundance of histone H4 message. With the exception of RNA harvested from a 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, TGFβ-1, IGF2, IL-6, 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 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 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. 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. Nucleotide substitutions introduced within a 10-base pair sequence, 5’-GGCGCCACC-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 nucleotide 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 nucleotide 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 antiserum prepared against the entirety of the SP1 trans-activation domain, we have been unable to deplete yeast extracts of p180. 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

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5 R. S. Cuevo and J. M. Horowitz, unpublished observations.

6 D. M. Roof, personal communication.
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