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Identification and purification of a factor that binds to the *Mlu* I cell cycle box of yeast DNA replication genes

(*Saccharomyces cerevisiae*/G_1 phase/S phase/periodic transcription/DNA polymerase α)

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ABSTRACT **In *Saccharomyces cerevisiae*, the genes encoding at least 10 enzymes involved in DNA replication are periodically expressed in the late G_1 and S phases of the cell cycle. All of these genes have one copy or more of the sequence ACGCGT, which conforms to the recognition site for the *Mlu* I restriction endonuclease. For the *CDC21*, *CDC9*, and *POL1* genes, the *Mlu* I site has been shown to be absolutely required for periodic transcription. Using nuclear extracts fractionated by conventional and oligonucleotide affinity chromatography, we have purified a 17-kDa protein that recognizes the *Mlu* I motif. Synthetic oligonucleotides containing mutated *Mlu* I sites do not bind the protein. In contrast, synthetic oligonucleotides derived from the *CDC2*, *CDC6*, and *CDC21* genes, which are expressed with the same timing as *POL1*, bind purified protein efficiently.**

In *Saccharomyces cerevisiae*, cells make the decision to enter a round of DNA synthesis and duplication at a point in G_1 called Start (1). Although we know little about the events that occur between Start and S phase, one landmark is an induction of the RNAs encoding at least 10 genes involved in DNA replication. Thus, genes encoding proteins at the replication fork such as *POL1* (DNA polymerase α) (2, 3), *PRI1* and *PRI2* (subunits of primase) (4, 5), *CDC2* (DNA polymerase δ), and *POL30* (proliferating cell nuclear antigen) (6) are all maximally transcribed at the G_1/S boundary. In addition, the genes encoding some of the enzymes involved in the production of dNTPs are also expressed periodically with the same peak of timing. Examples include *CDC8*, encoding thymidylate kinase (7), *CDC21*, encoding thymidylate synthase (8), and ribonucleotide reductase (9, 10). Another example is *CDC9*, encoding DNA ligase, required for completion of DNA synthesis (11, 12). The expression of these genes depends, either directly or indirectly, on Start (7, 13), and studies that elucidate the molecular mechanism of this Start dependence should complement the use of *CDC* mutants in defining events set in motion by Start.

The sequence 5′-ACGCGT-3′, the recognition sequence for *Mlu* I restriction endonuclease, is the only sequence conserved among the promoters of the replication genes (14) and is necessary for periodic transcription for the *CDC21* (15, 16), *CDC9* (17), and *POL1* genes (18). A region of 54 nucleotides in the *POL1* promoter, having two *Mlu* I sites spaced 29 base pairs (bp) apart, contains all the information necessary for transcriptional expression and periodic activation. Oligonucleotides containing either one of the *Mlu* I sites can confer UAS activity on a heterologous gene. Since the flanking sequences differ at each site, the *Mlu* I sequence alone appears to be sufficient for cell cycle regulation. This conclusion is supported by the demonstration that an oligonucleotide carrying a single *Mlu* I site flanked by sequences that are a mixture of the sequences adjacent to both sites in the natural promoter confers periodicity (18). The *Mlu* I site is necessary for the periodic expression of the *CDC21* and *CDC9* promoters as well (16, 17), suggesting coordinate regulation of the entire set of periodic replication genes through *Mlu* I sites. We have designated the UAS that is involved the *Mlu* I cell cycle box (MCB).

The MCB is a potential target for a trans-acting factor that responds either directly or indirectly to Start. Identification of such a factor in an organism such as yeast should facilitate a combined biochemical and genetic analysis of this distinctive type of regulated gene expression. In this study, we report the purification of such a specific binding factor. We have designated the protein MCBF for MCB binding factor. The purified protein binds in a *Mlu* I-specific fashion to the *POL1* promoter as well as to oligonucleotides derived from three additional periodic genes containing *Mlu* I sites in their promoters.

MATERIALS AND METHODS

Preparation of Labeled Probes for DNA Binding Assays. Complementary strands of the oligonucleotides listed in Table 1 were annealed and labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. The 60-bp oligonucleotide (PU1) has previously been shown to function as a UAS that confers periodicity on the heterologous *CYC1* promoter (18). For footprinting analysis, PU1 was cloned into the *Bam*HI site of the Bluescript vector pBS (Stratagene). Cleavage at the *Not* I site in the pBS polylinker, followed by phosphatase end-labeling with T4 polynucleotide kinase, and digestion with *Xho* I generated the 130-bp noncoding strand. The coding strand was prepared by cutting with *Xho* I, labeling with T4 kinase, and then cleaving with *Sac* I to yield a 150-bp fragment.

Preparation of Oligonucleotide Affinity Column. The 60-bp PU1 oligonucleotide was phosphorylated with T4 kinase, annealed, ligated, and coupled to Sepharose CL2B essentially according to the method of Kadonaga and Tjian (19). A column consisting of 1.5 mg of PU1 oligonucleotide coupled to 10 ml (settled volume) of Sepharose was used.

DNA Binding Reactions. DNA binding activity was monitored by altered electrophoretic mobility of the protein–DNA complex compared to free DNA (20, 21) as described (22).

For DNase I footprinting, samples were digested with DNase I at room temperature for 1 min using different dilutions of a stock solution of enzyme (5 mg/ml).

Preparation of Nuclear Extract: *S. cerevisiae*. PEP4D (*MATa*/α,*his1*/+,*trp1*/+,*pep4-3*/*pep4-3*,*prc1-126*/*prc1-126*,*prb1-1122*/*prb1-1122*,*can1*/*can1*) was grown in a 350-liter

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Abbreviations: UAS, upstream activation sequence; MCB, *Mlu* I cell cycle box; MCBF, MCB binding factor.

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Table 1. Oligonucleotides used

Oligonucleotide	Length, bp	Sequence
PU1	60	GATCACGCGACGCGTTTTACTTATAGGCTAACAAAAAAATTTTAACGCGTTTGAATAGAG TGCGCTGCGCAAAATGAATATCCGATTGTTTTTTTAAAATTGCGCAAACTTATCTCCTAG
PU4	48	GATCTCGCGTTTACTTATAGGCTAACAAAAAAATTTTATTGAATAGAG AGCGCAAATGAATATCCGATTGTTTTTTTAAAATAACTTATCTCAGCT
C21	60	AATTTTGGTGACGCGTTAAATAGAAAAAATGAAAAAGACCTTAATTGACGCGTTTCCTGA AACCACTGCGCAATTTATCTTTTTTACTTTTTCTGGAATTAACTGCGCAAAGGACTTTAA
C6	60	GATCCCAAAGAAGAAAGCGACGCGAGGCCTCACGCGTCGGAGTTTCCAGATCAGG GGTTTCTTCTTTCGCTGCGCTCGGGAGTGCGCAGCCTCAAAGGTCTAGTCCCTAG
C2	60	GATCCCTTAACGTAATAGGCAATTTTTCGTATTACGCGTAACTTTTTATTCTATAAAATG GGAATTGCATTATCCGTTAAAAAGCATAATGCGCATTGAAAAATAAGATATTTTACCTAG
PU2	25	GATCATAAGTAAAACGCGTCGCGTG TATTCATTTTGCGCAGCGCACCTAG
PU3	25	GATCTCTATTCAAACGCGTTAAAAT AGATAAGTTTGCGCAATTTTACTAG
PU8	25	GATCTCTATTCAAACtCGTTAAAAT AGATAAGTTTGaGCAATTTTACTAG
PU9	25	GATCTCTATTCAAACGCaTTAAAAT AGATAAGTTTGCGtAATTTTACTAG
PU10	25	GATCTCGCGACtCcTTTGAATAGAG AGCGCTGaGgAAACTTATCTCCTAG
PU11	25	GATCATAAGTAAAACtCcTtGCGTG TATTCATTTTGaGgAaCTCACCTAG
PU12	25	GATCTCTATTCAAACtCcTTgAAAT AGATAAGTTTGaGgAACTTTACTAG
ABF1	33	GAATTCATTTCTTAGCATTTTTGACGAAATTTG TAAAGAATCGTAAAAACTGCTTTAAACCTTAAG
Bluescript polylinker	24	CTAGTGGATCCCCCGGGCTGCAGG ACCTAGGGGGCCCGACGTCCTTAA

The top strand is listed 5′ to 3′. Base-pair changes in the mutant oligonucleotides are shown in lowercase letters.

fermenter to midlogarithmic phase in medium containing 1% yeast extract, 2% peptone, and 2% cerelose at 30°C. Cells were harvested in a Sharples centrifuge and the cell pellets were frozen in liquid nitrogen and stored at −70°C. Nuclear extract was prepared as described by Yoon and Campbell (23). Nuclei were resuspended in 50 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$/3 mM dithiothreitol containing a protease inhibitor mixture of 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and leupeptin, pepstatin, and soybean trypsin inhibitor at 10 μg/ml. They were lysed by addition of ammonium sulfate to a final concentration of 0.4 M. The lysate was centrifuged at 35,000 rpm for 20 min in a Beckman Ti50 rotor. Ammonium sulfate (0.56 g/ml) was added and the precipitate was collected by centrifugation at 35,000 rpm for 30 min at 4°C. The pellet was dissolved in a buffer containing 20 mM Hepes (pH 8), 10% (vol/vol) glycerol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine (buffer A), and 100 mM KCl and dialyzed against the same buffer.

RESULTS

Identification of a Specific MCB Binding Activity. Standard DNA binding studies were conducted with oligonucleotide probes derived from the *POL1* promoter (Table 1). PU1 contains 54 bp from the *POL1* promoter with two *Mlu* I sites spaced 29 bp apart and is sufficient for cell cycle-regulated transcription of a heterologous promoter (18). At least five protein–DNA complexes were observed when nuclear extract was incubated with PU1 as a probe. Although some of them were blocked by competition with PU1 and not by the heat shock element, they were also blocked by competition with PU4. PU4 does not confer periodicity to a heterologous promoter *in vivo* (18). To enrich for *Mlu* I-specific proteins, nuclear extracts prepared on an analytical scale were fractionated on a PU1 oligonucleotide affinity column as described below. Several proteins that recognized the cloned PU1 probe were observed eluting at different salt concentrations. A protein eluting at 0.7 M salt that gave rise to a complex with relatively high mobility was judged to be specific for the MCB, as demonstrated by the data in Fig. 1. Lane 1 shows complex formation in the presence of 1 μg of poly[d(IC)] but in the absence of added test competitor. Formation of this complex was not blocked by competition with nonspecific DNA, such as the recognition site for *ARS* binding factor 1 (22) or an oligonucleotide carrying sequences from the Bluescript polylinker. Similarly, PU4, which contains no *Mlu* I sites but is otherwise identical to PU1, does not compete. Binding was blocked by competition with PU1 itself. A single *Mlu* I site is sufficient to confer periodic transcription *in vivo*, and, consistent with this, a single *Mlu* I site is sufficient for formation of this complex, as evidenced by complete competition by the PU2 and PU3 oligonucleotides. These 25-bp oligonucleotides contain the proximal and distal *Mlu* I sites, respectively, which have different flanking DNA sequences. [The *Mlu* I site nearest the ATG translation start site (−173 to −168) is referred to as the proximal site, and the *Mlu* I site at −208 to −203 is referred to as the distal

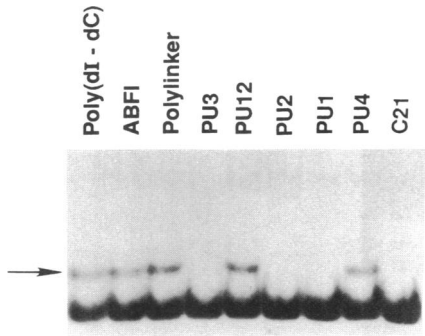

FIG. 1. Identification of a specific MCB binding activity. Nuclear extract was fractionated on a PU1 oligonucleotide affinity column and assayed with labeled PU1 cloned into Bluescript. Binding activity eluting at 0.7 M salt was assayed in the presence of 1 μg of poly(dI·dC) (lane 1) or 850 ng of poly(dI·dC) and 150 ng of competitor DNA (30-fold excess over probe DNA; all other lanes). Competitors used are listed above the lanes and their sequences are presented in Table 1. The protein–DNA complex judged to be specific is marked by an arrow.

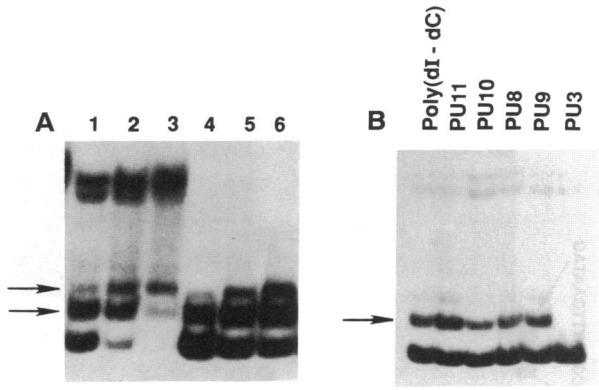

FIG. 2. Characterization of the purified protein. (*A*) Gel mobility-shift analysis. Increasing amounts of oligonucleotide affinity (lanes 1–3; 3, 6, and 10 μl, respectively) and Mono S purified MCBF (lanes 4–6; 3, 6, and 10 μl, respectively) were incubated with a fixed amount of probe DNA. The specific complexes are marked by arrows. (*B*) Competition analysis of affinity peak. Purified protein was assayed in the presence of poly(dI·dC) (1.0 μg) alone (lane 1) or poly(dI·dC) (0.7 μg) and competitor DNA (0.3 μg) (all other lanes). Lanes: 2, triple mutant, PU11; 3, double mutant, PU10; 4 and 5, single mutants PU8 and PU9, respectively; 6, wild-type competitor, PU2.

site in this work.] Finally, an oligonucleotide derived from PU3 but containing three point mutations (PU12) did not compete for binding.

An oligonucleotide derived from the *CDC21* promoter, C21, contains two *Mlu* I motifs, which have been shown to be necessary for periodic UAS function (15, 16). There is no other sequence similarity between the two promoters. As shown in lane 9, C21 also competed for binding.

Purification of MCBF. To further characterize the DNA binding protein, which we refer to as MCBF, we used formation of this complex as an assay to purify the protein. The dialyzed nuclear extract, containing 3 g of protein, was loaded onto a heparin-Sepharose column. The peak of binding activity eluted at 0.4 M KCl and contained 300 mg of protein. The activity was loaded onto the oligonucleotide affinity column in three samples of 100 mg each. Specific binding activity eluted at 0.5–0.6 M salt. The peaks of activity from the three different runs were pooled and contained 2.2 mg of protein. The fraction was dialyzed and loaded onto a Mono S FPLC column. Specific binding activity eluted at 0.7 M salt. Approximately 150 μg of protein was recovered from this step, representing a 10,200-fold purification from the nuclear extract, based on protein recovery.

A dosage binding experiment was carried out with Mono S purified fractions (Fig. 2 *A* and *B*). The Mono S fraction is composed only of the specific DNA binding species. As the protein concentration is increased, multimeric forms of the complex are observed, all of which can be blocked by competition with specific oligonucleotides (data not shown). The multimeric forms could be a consequence of the factor binding on the two *Mlu* I sites of PU1 or multimeric interactions at a single *Mlu* I site.

Specificity of binding of the purified protein was confirmed by using the mutant oligonucleotides listed in Table 1 in competition binding experiments. The mutants to be tested were designed based on conservation of the nucleotides at specific positions within and surrounding the *Mlu* I sites in the promoters of 10 similarly regulated genes. Although the entire ACGCGT sequence is conserved in at least 8 replication genes (24), a few of them such as *PRI1* and *TS26* that are periodically expressed have 5/6-bp matches (4, 25). If these are functional, the consensus sequence is ACGNG. Oligonucleotides carrying single, double, and triple point mutations were synthesized. The single mutations tested were made in the two internal G residues, as these are absolutely conserved nucleotides. PU8 and PU9, containing a single G to T transversion or a G to A transition, respectively, do not compete for binding (Fig. 2). In addition, PU11 mutant oligonucleotide, when labeled with ^{32}P and used as a probe, does not form a complex (data not shown).

Footprinting Analysis. Standard DNase I footprinting reactions shown in Fig. 3*A* (noncoding strand) and *B* (coding strand), demonstrate that binding of MCBF is at the *Mlu* I sites. Both the proximal as well as the distal *Mlu* I sites were well resolved in the noncoding strand (Fig. 3*A*), and protection was observed within each *Mlu* I site extending to a few bases beyond the site. Similarly, the distal *Mlu* I site is clearly protected in the coding strand (Fig. 3*B*). The proximal *Mlu* I site is not very well resolved in the coding strand (Fig. 3*B*), but an effect on the site is still apparent. Additional evidence that protection from DNase I digestion occurs at specific sites is demonstrated by the competition experiment in Fig. 3*C*. Using the well resolved noncoding strand, protection is observed in the absence of added oligonucleotide competitor. This protection is abolished by inclusion of cold PU1, containing both *Mlu* I sites, as well as by PU2, containing the single proximal *Mlu* I site. In contrast, the protection is not blocked by competition with mutant *Mlu* I oligonucleotides PU11 or PU12. Thus, footprinting confirms the *Mlu* I specificity of this protein.

SDS/Polyacrylamide Gel Analysis of MCBF. The purity of MCBF was assessed by SDS/polyacrylamide gel analysis (Fig. 4). The Mono S fraction is enriched in a species of ≈17 kDa. A lower abundance 15-kDa species is also seen, which may or may not represent degradation of the 17-kDa protein. For Southwestern analysis (26), the Mono S fraction was run on a separate SDS gel, and the proteins were transferred to nitrocellulose. After protein renaturation, the filter was incubated with ^{32}P-labeled probe. A major DNA binding species of 17 kDa was detected, together with a faint lower band of 15 kDa. Thus, the 17-kDa species is most likely MCBF.

Binding of MCBF to Promoters of Other Periodic Genes Containing *Mlu* I Motifs. A major question raised by the occurrence of the *Mlu* I site in several promoters regulated with similar timing is the extent to which coordinate control occurs. We have investigated the *CDC21*, *CDC2* (DNA polymerase δ), and *CDC6* (gene product required for DNA synthesis initiation) genes. Sixty-base-pair oligonucleotides from the regions containing the *Mlu* I sites were synthesized. The C21 oligonucleotide derived from *CDC21* has two *Mlu* I

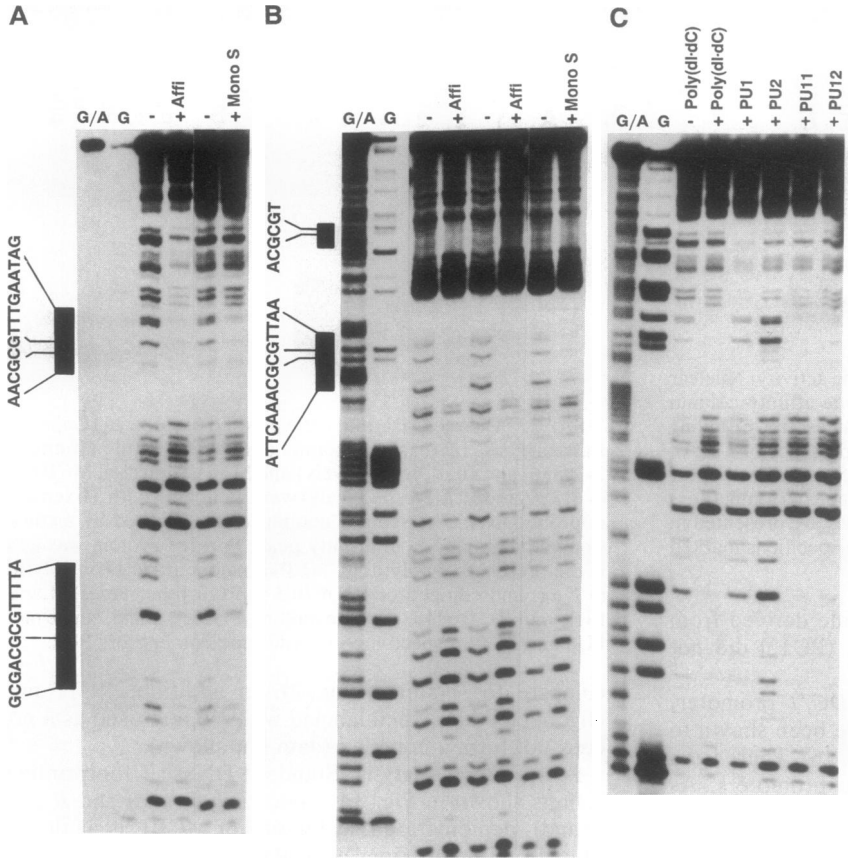

FIG. 3. Specificity of DNase I protection by MCBF. (*A*) Noncoding strand. The extent of protection observed using both affinity and Mono S purified protein is delineated by solid boxes. Lanes −, no protein added; lanes +, protein added. Three times more protein as well as poly(dI·dC) was used for the affinity-purified MCBF reactions. The sequence protected is shown on the left and tick marks indicate the two invariant G residues in the consensus ACGCGT. G and G/A represent Maxam–Gilbert sequencing ladders. (*B*) Coding strand probe. (*C*) Competition binding. Footprinting reactions used the noncoding strand and affinity-purified MCBF. Competitors tested were wild-type PU1 or PU2 and mutants PU11 or PU12 as marked.

sites spaced 31 bp apart, almost the same spacing as *POL1*. There is no sequence similarity in the region between the two promoters except for an A+T-rich base composition. The C6 oligonucleotide derived from *CDC6* contains one exact *Mlu* I site and a 5/6 match spaced 6 bp apart (27). The C2 oligonucleotide derived from the *CDC2* promoter contains a single *Mlu* I site.

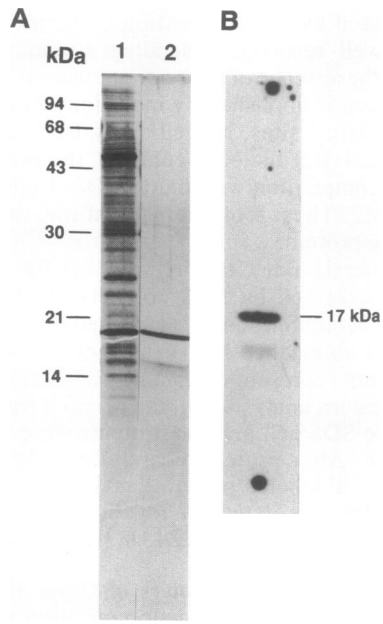

FIG. 4. SDS/polyacrylamide gel analysis of MCBF. (*A*) Oligonucleotide affinity (lane 1) and Mono S (lane 2) purified MCBF preparations were resolved on a SDS/15% polyacrylamide gel and the proteins present were visualized by silver staining. (*B*) Mono S FPLC purified MCBF was resolved on a SDS/13% polyacrylamide gel, transferred to nitrocellulose after renaturation, and probed with [^{32}P]DNA.

C21, C2, C6, as well as PU1 were labeled and used as binding probes. The C21 oligonucleotide formed a complex with MCBF of similar mobility to that with PU1 (Fig. 5 *A* and *B*). Complex formation was efficiently blocked by competition with cold C21 as well as PU1 and PU2. In contrast, the mutant PU11 did not compete. Since the sequences between the two *Mlu* I sites are A+T-rich, while the *Mlu* I site has the CGCG motif, poly(dT) and poly(dC·dG) were also tested as competitors. As shown in lanes 7, poly(dT) did not compete and actually enhanced complex formation. Poly(dC·dG) does compete, although its efficiency as a competitor on a molar basis is less than that of PU1. Almost identical results were obtained with C2 oligonucleotide (Fig. 5*C*) and with C6 oligonucleotide (Fig. 5*D*). Thus, MCBF has the DNA binding properties expected of a protein involved in coordinate regulation of these genes in the cell cycle.

DISCUSSION

In this report, we describe the purification of MCBF, a protein that binds to the MCB element that we have previously shown is part of a UAS that is necessary and sufficient for periodic expression of the *POL1* gene in the yeast cell cycle (18). The protein has an apparent molecular mass of 17 kDa. Southwestern analysis of the fraction shows that *Mlu* I binding activity resides in the 17-kDa polypeptide (Fig. 4). The report of a highly purified yeast protein that recognizes a DNA sequence involved in cell cycle regulation is of interest because it allows a reverse genetic analysis of how this protein senses position in the cell cycle.

Specificity of binding was established by footprinting and competition analysis and by the demonstration that MCBF binds to three other *Mlu* I-containing promoters. The critical determinant for binding seems to be the *Mlu* I site *per se* and not the flanking sequences since (*i*) footprinting reactions

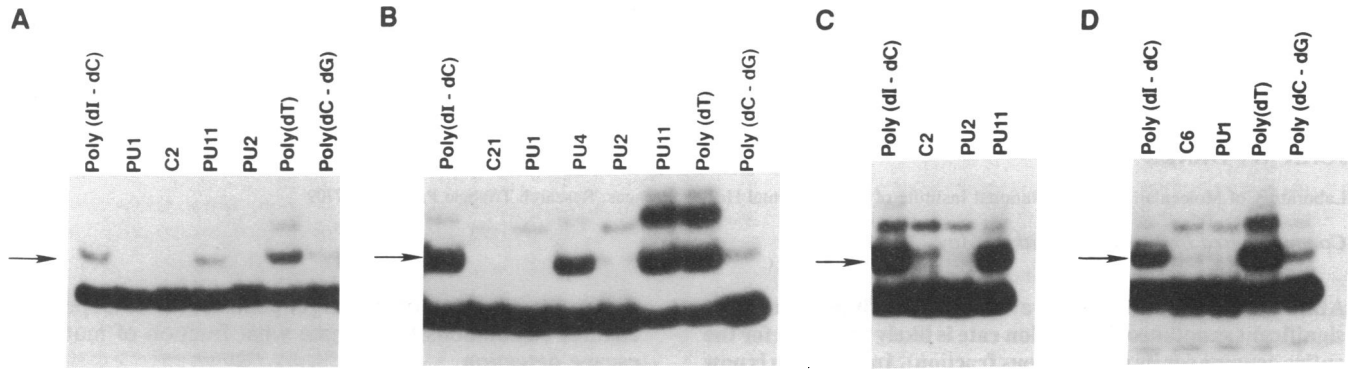

FIG. 5. Binding of MCBF to different promoters containing *Mlu* I sites. Affinity-purified MCBF was tested for binding with *POL1*-derived PU1 oligonucleotide (*A*), *CDC21*-derived C21 oligonucleotide probe (*B*), *CDC2*-derived C2 oligonucleotide probe (*C*), and *CDC6*-derived C6 oligonucleotide probe (*D*) (arrows). Total DNA concentration was kept constant at 1.2 μg for all binding reactions using poly(dI·dC) alone (lane 1) or 800 ng of poly(dI·dC) and 400 ng of competitors (80× over probe DNA; all other lanes). Competitors tested are indicated and their sequences are given in Table 1.

showed protection of both the proximal and distal *POL1 Mlu* I sites, which have dissimilar flanking sequences (Fig. 3), and (*ii*) sequences shared by the *CDC21, CDC2, CDC6*, and *POL1* promoters are limited exclusively to the *Mlu* I site. The sequences adjacent to *Mlu* I therefore seem unnecessary for recognition. Since the *Mlu* I site has been shown to be necessary and sufficient for periodic expression *in vivo*, MCBF is a good candidate for the transcription factor that mediates this timing.

Footprinting data and competitive binding allow some new conclusions about the bases important for recognition. Although the entire ACGCGT sequence is conserved in at least eight periodically expressed replication genes (24), *PRI1* and *TS26* have only 5/6 matches, while CDC6 has one exact site and a 5/6 match. Assuming these 5/6 matches contribute to periodic expression of these genes, the functional consensus sequence ACGNG can be derived. Changing the 5′ G to a T or the 3′ G to an A abolishes the competition observed with wild-type sequence *in vitro*. Changing both G residues is also deleterious (Fig. 2*B*). Thus, the two invariant G residues seem to be very important for recognition. It is interesting that McIntosh *et al.* (16) have shown by site-directed mutagenesis that mutations in either G at the distal *Mlu* I site in the *CDC21* promoter almost completely abolish transcription from that promoter *in vivo*.

The ready availability of large quantities of pure MCBF will allow us to use a concurrent genetic and biochemical investigation of the mechanism by which MCBF confers periodicity to *POL1*. Recent studies that have demonstrated *Mlu* I-specific protein–DNA complex formation in yeast extracts suggest that the *Mlu* I binding activity may fluctuate in the cell cycle with approximately the same periodicity as the mRNA of *CDC9* (17). Thus, MCBF may be periodically either synthesized or phosphorylated in G_1 by CDC28 kinase, the activity of which is essential for completion of Start (28).

Although the *HO* gene is periodically transcribed with apparently the same peak of timing as the DNA synthesis genes (29), the periodicity is due to a cis-acting element (RNYCACGAAAA) (R, purine; Y, pyrimidine) quite distinct from the *Mlu* I motif (30). Two genes, *SWI4* and *SWI6*, have been shown to be required for activation of *HO* transcription in G_1 (31). The pleiotropic phenotypes associated with *swi4* and *swi6* mutants suggest that they may participate in controlling essential processes in the cell besides regulating *HO* gene expression. The identification of MCBF allows us to test its presence and activation in *swi4* and *swi6* mutants.

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