Saccharomyces cerevisiae Cdc6 Stimulates Abf1 DNA Binding Activity*

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In budding yeast Saccharomyces cerevisiae, an ARS binding factor 1 (Abf1) binds to the sequence-specific DNA element involved in DNA replication and transcription. We describe in this study how yeast Cdc6 protein stimulates Abf1 protein DNA binding activities. The Abf1 binding activity was reduced approximately 20-fold in a cdc6-1 mutant than in the wild-type strain. Introducing a copy of the wild-type CDC6 gene into the cdc6-1 mutant strain restored the Abf1 DNA binding activity. We demonstrated that recombinant Abf1 binds to ARS1 in vitro, and its DNA binding activity can be highly stimulated by the addition of a fusion glutathione S-transferase (GST)-Cdc6 protein. Deletion analysis revealed that the stimulating region is located at the amino terminus of the Cdc6 protein. However, we could not find the direct physical interaction between Cdc6 and Abf1. Instead, we found that the GST-Cdc6 can compete with distamycin A for binding to the DNA molecule. As distamycin A is a specific reagent that binds noncovalently to DNA at (A + T)-rich tracks, the stimulation of Abf1 DNA binding activity may be mediated by the Cdc6/Abf1 DNA interaction. Our results favor a hypothesis that Cdc6 may function as an architectural factor in the assembly of a functional initiation replication complex.

In Saccharomyces cerevisiae, Cdc6 is essential for the initiation of DNA replication. The cdc6-1 mutation fails to control the initiation of DNA replication (1) and has a high rate of plasmid loss. The loss of plasmid can be suppressed by increasing tandem copies of short cis-acting ARS sequences (ARS, autonomously replicating sequences) (2), suggesting its direct role at replication origins. The nucleotide sequence of the CDC6 gene predicts a 58-kDa protein with a nucleotide binding domain and multiple potential cyclin-dependent kinase (Cdk) phosphorylation sites (3, 4). The CDC6 gene product is unstable, and its synthesis must take place during the G1 phase for the onset of S phase. The CDC6 mRNA content fluctuates periodically during the cell cycle (5, 6), and the nuclear entry of Cdc6 protein is also cell cycle-dependent (7). Yeast cells that exit from mitosis without de novo Cdc6 protein synthesis fail to initiate DNA replication (6).

The B3 element at ARS1 sequence functions as an enhancer for replication origins and contains binding sites for an ARS-binding factor 1 (Abf1) (18). Evidence suggests that Abf1 is required for diverse functions in regulation of DNA replication and gene expression (19, 20). The binding of Abf1 to the B3 site at ARS1 may be required for the full origin function (18). It is still not clear how the activity of the Abf1 protein is regulated for binding origins of replication. In this study, we report that Cdc6 protein stimulates the binding of Abf1 to ARS1. Protein extracts prepared from the cdc6-1 mutant have a low Abf1 DNA binding activity based on DNA gel mobility shift assays. The Abf1 binding activity could be restored by integrating a copy of normal CDC6 gene back into the cdc6-1 mutant cell. We demonstrate that Escherichia coli expressed Abf1 can bind to ARS1 DNA fragment and that the addition of GST-Cdc6 can further enhance the Abf1 DNA binding activity. The GST-Cdc6 appears to interact with DNA and increases the affinity of Abf1 for DNA because it competes with distamycin A (a specific reagent that binds (A + T)-rich tracks noncovalently) for Abf1 DNA binding activity. The interrelationship among Cdc6, Abf1, and Abf1.

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1 The abbreviations used are: ORC, origin recognition complex; RC, replication complex; GST, glutathione S-transferase; PCR, polymerase chain reaction; bp, base pair(s).
and ARS1 and the possible physiological significance are discussed in this report.

MATERIALS AND METHODS

Yeast Strains and Culture—B32168 (a prc1-407 prb1-1122 pep4-3 leu2 trpl ura3-52) was used as CDC6 wild type strain. Strain 611 (a cdc6-1, leu2, his3), kindly provided by Dr. Ray Deshire at California Institute of Technology, was derived from the temperature-sensitive (ts) mutant cdc6-1. Strain 5D611 was constructed by transformation of 611 cells with an integration plasmid pLEU-5D6E which carried the LEU2 marker gene and the wild type CDC6 gene. Southern blot hybridization was performed to verify the specific CDC6 gene integration by using yeast genomic DNA prepared from the 5D611, which is indicated as WT/CDC6 in the legend to Fig. 1.

Plasmids and DNA—5’ primer, 5-AAAATAGCAAATTTCGTCAAA-AATGC-3, and 3’ primer, 5-CAAATC AATCGAAAAGCCAAATG-3, were used in polymerase chain reaction (PCR) to amplify the 173-bp ARS1 DNA fragment (745–917) (18) from yeast genomic DNA. Deletion probes were also constructed: probe ARS1-B3 contained a 45-bp fragment of B3 subunit of the ARS1 sequence (nucleotides 745–789), while probe ARS1-AB contained a 106-bp fragment of domain A and B1 element.

DNA Electrophoresis Mobility Shift Assay—Whole-cell protein extracts which contain both cytoplasmic and nuclear proteins were prepared from yeast cells cultured at 25 °C according to Kimmery et al. (21). The 173-bp ARS1 was labeled with [α-32P]ATP (3000 C/mmol, NEN Life Science Products) using a fill-in reaction with Klenow enzyme. DNA and protein interactions were performed in a 20-μl mixture containing 25 mM HEPES, pH 7.6, 5 mM MgCl2, 50 μg/ml bovine serum albumin, 0.5 mM ATP, 1 μg/ml poly(dA-dG)-poly(dC-dT) (Pharmacia), 2 μg of yeast whole cell protein extracts, 2.5 fmol of the probe (15 × 10^9 cpm). DNA binding reactions were conducted for 10 min at 25 °C and were then loaded immediately on a 6% non-denaturing polyacrylamide gel (acylamide:bisacrylamide, 80:1) that had been pre-run for 1 h at 25 °C in 0.5 × TBE. The gel was run for 4 h at 120 V and 25 °C, dried, and then autoradiographed.

In Vitro Expression and Protein Purification—5’-End primer 5-CTGGATCCATGGACAAATTAGTCGTG-3 and 3’-end primer 5-AAGCCTTGGACCTCTTAATTCGTC-3 were designed for PCR amplification of the yeast Abf1 gene (20). The PCR products were subcloned into pBlueScript and sequenced. DNA fragment containing the yeast Abf1 coding region was then cloned into the plasmid vector pET28a (Novagen) for expression in histidine-tagged protein. Abf1 protein produced in E. coli was purified according to the manufacturer’s description. pGEX (Pharmacia Biotech Inc.) was used to construct glutathione S-transferase-Cdc6 (GST-Cdc6) expression construct and its truncated GST fusion proteins as described (Fig. 2B) (22). SDS-polyacrylamide gel electrophoresis, gel staining, and Western blot analysis were performed by using a PhastSystem (Pharmacia).

RESULTS

Abf1 Binding Activity Is Reduced in the cdc6-1 Mutant Compared with the Wild-type Strain—We have studied the ARS1 binding activity in yeast cell extracts to determine the role of Cdc6 in controlling the initiation complex formation. ARS1 was selected as the probe because it is by far the most well characterized eukaryotic replication origin. By using the whole cell protein extracts in a DNA gel retardation assay, we observed one predominant protein-DNA complex (Fig. 1A, lane 1). This complex appeared to be a result of binding of Abf1 to the B3 element of ARS1 since it was diminished upon the addition of a 100- to 500-fold molar excess of the 45-bp fragment containing the B3 element of ARS1 (ARS1-B3) (Fig. 1A, lanes 7–10 and lanes 15–17). In contrast, the addition of a 500-fold molar excess of the 106-bp fragment containing the entire domain A and the B1 element (ARS1-AB) was unable to reduce the binding activity (Fig. 1A, lanes 3–6 and lanes 12–14). The specificity of this binding activity was confirmed by the formation of supershifts when an anti-Abf1 rabbit serum (20) was used in the gel retardation assay (data not shown).

To address how Cdc6 might affect the DNA binding activity of Abf1, we analyzed the binding of Abf1 to the 173-bp ARS1 probe by using equal amounts of protein derived from the whole cell extracts prepared from the wild-type CDC6 strain BJ2168 and from the cdc6-1 mutant strain. Both extracts showed specific Abf1-DNA complexes; however, the level of the Abf1 activity in the cdc6 mutant was at least 20 times lower than that in the wild-type strain (Fig. 1A, compare lane 2 with lane 11). The Abf1 binding activity was compared in the competition assay. As shown in Fig. 1A, 50- to 500-fold molar excess of the ARS1-AB did not compete for binding (lanes 3–6 and lanes 12–14), yet the addition of a 50-fold molar excess of ARS1-B3 in the cdc6 extracts (lane 7) caused a ~90% reduction of Abf1 DNA binding activity. The addition of 100- to 500-fold molar excess of the competitor resulted in less than 1% of the Abf1 activity left in cdc6 extracts (lanes 8–10). However, approximately 10–20% of the Abf1 activity could still be observed in the wild-type strain even when 100- to 500-fold molar excess of the same ARS1-B3 DNA was used in the competition (lanes 15–17). Thus, our results show that the DNA binding activity of Abf1 is significantly lower in the cdc6 mutant extracts compared with the wild-type ones.

Functional Cdc6 Protein Is Required for High Abf1 Binding
Activity—The CDC6 gene is essential for DNA replication, and the cdc6 mutant causes G₁/S phase defects in the cell cycle. To address the question of whether lower Abf1 activity is due to the defective cdc6, we constructed an isogenic strain 5D611 by introducing a normal CDC6 gene into the mutant cdc6 cells. Upon yeast transformation, the wild-type CDC6 gene was integrated into the chromosomal cdc6 DNA region. The integrated wild-type CDC6 gene rescued the temperature-sensitive cdc6 cells at the nonpermissive temperature (37 °C) (data not shown). The Abf1 DNA binding activity was determined by electrophoretic mobility shift assay using whole cell protein extracts prepared from the 5D611 and from the cdc6. Compared with the Abf1 activity shown in the cdc6 extracts (Fig. 1B, lanes 2 and 3), the elevated Abf1 DNA binding activity was detected in the extracts prepared from the integrated strain 5D611 (Fig. 1B, lanes 6 and 7). The protein-DNA complexes can be competed out by the addition of cold probe ARS1-B3 (lanes 4 and 8), but not by cold probe ARS1-AB (lanes 5 and 9). The results indicate that the normal Abf1 DNA binding may require functional Cdc6 at ARS1. To further determine the effect of Cdc6 on the stimulation of Abf1 activity, the DNA binding assay was performed by using whole cell protein extracts prepared from the cdc6 mutant and other cell cycle mutants such as cdc7, cdc34, cdc28, and cdc4, as well as the wild type strain BJ2168. Significantly low Abf1 activity could only be observed in the extracts prepared from the cdc6 mutant (Fig. 1B, lanes 2–5); in contrast, the DNA binding activity was rather constant in extracts prepared from the other cdc mutant and wild-type strains (Fig. 1B, lanes 10–19). We have used Western blots to examine the Abf1 and Cdc6 levels in these tested strains. Both protein concentrations are rather constant (data not shown), suggesting that the change of Abf1 binding activities is not due to alteration at their protein levels. Therefore, the low Abf1 DNA binding activity is specifically related to the defective cdc6 in the cdc6-1 mutant cells.

GST-Cdc6 Stimulates Purified Abf1 DNA Binding Activity—To understand how Cdc6 regulates Abf1 DNA binding activity, purified E. coli produced Abf1 was employed in the gel mobility shift assays (Fig. 2A). GST-Cdc6 was also prepared to test whether it could stimulate Abf1 binding to 173-bp ARS1 probe. When 50 ng of purified protein was used, the protein-DNA complexes of the recombinant Abf1 and the labeled ARS1 were barely detectable in our assay condition (Fig. 2A, lane 2). At lower concentrations of Abf1 (from 20 to 5 ng per reaction), the ARS1 binding activity of Abf1 was essentially undetectable (lanes 3–5). Increased Abf1 activity, however, could be achieved upon the addition of the GST-Cdc6 fusion protein into the reactions (lanes 6–8). We estimate the difference in Abf1 DNA binding between the presence and absence of GST-Cdc6 to be at least 50-fold (Fig. 2A, compare lane 8 with lanes 2–5). Under the condition used, no DNA binding was found for the GST-Cdc6 fusion protein itself (0.5–2 ng per reaction). GST protein only, however, had no effect on the stimulation (Fig. 2A, lanes 9–11). When 45-bp B3 DNA element which contains Abf1 consensus sequence is used as the probe, a similar stimulation can be observed (Fig. 2A, lanes 12–17), i.e. when the B3 probe, 10 ng of Abf1, or 2 ng of GST-Cdc6 was used individually, no shifted band can be observed (Fig. 2A, lanes 12, 13, and 14). However, the Abf1-DNA complex can be seen in the presence of 10 ng of Abf1 and 1 ng (lane 15) or 2 ng (lane 16) of GST-Cdc6. GST alone has no effect (lane 17). This effect was specific to the Cdc6 protein, since proteins such as bovine serum albumin, ovalbumin, yeast histidine-tagged nucleoside diphosphate kinase, could not stimulate DNA binding (data not shown). Thus, the stimulation of Abf1 DNA binding is specific to Cdc6 protein.

Deletion Analysis to Define the Stimulation Domain of the

![Fig. 2. GST-Cdc6 stimulates recombinant Abf1 DNA binding activity. A, from 50 down to 5 ng of the purified Abf1 protein were used in the gel retardation assay (lanes 2–5). Lane 1, 173-bp ARS1 probe only. 0.5 to 2 ng of GST-Cdc6 (lanes 6–8) or 10 to 100 ng of GST protein (lanes 9–11) were added in the reactions containing 10 ng of Abf1 and 2.5 fmol of the 173-bp probe. A similar experiment was carried out, except using the 45-bp ARS-B3 probe. The B3 probe, 10 ng of Abf1, or 2 ng of GST-Cdc6 was used individually as controls (lanes 12, 13, and 14). 1 ng or 2 ng of GST-Cdc6 was used to stimulate 10 ng of Abf1 DNA binding activities (lanes 15 or 16). 100 ng of GST was used in lane 17. The shifted bands from the exposed films are quantitated by the Molecular Imager® (Hercules) System (Bio-Rad, model GS-250). B, deletion analysis of Cdc6 domains that stimulate Abf1 binding. Schematic illustration of the GST-Cdc6 and the truncated fusion proteins. GST is indicated as solid boxes, whereas Cdc6 and its truncated proteins are indicated as open boxes. C, 10 ng of the Abf1 protein (lanes 3–17) were incubated with 0.5, 1, or 2 ng of the different GST fusion proteins, except that only 0.5 or 1 ng of GST-NP6 or 5, 10, or 20 ng of GST protein was used in the gel retardation assay. Lane 1, 173-bp probe only.
Cdc6—Truncated GST-Cdc6 fusion proteins were generated to identify a specific region responsible for the stimulation of Abf1 binding (Fig. 2B). The following proteins were produced and analyzed: GST-NB6 (46 kDa) is that GST fused to residues 1–191 of the Cdc6 protein; GST-NP6 (35 kDa), GST fused to residues 1–47; and GST-BE6 (63 kDa), GST fused to residues 191–513. As a control, 2 ng of the GST-Cdc6 itself showed no detectable DNA binding activity (Fig. 2C, lane 2), but could stimulate the Abf1 binding activity (lane 3). The stimulation was dependent on the amount of GST-Cdc6 added (lanes 4–6). The stimulation could also be observed in GST fusion proteins GST-NB6, containing 191 amino acid residues of the amino-terminal Cdc6 (lanes 10–12), and GST-NP6, containing only the first 47 amino acid residues of the amino-terminal Cdc6 (lanes 16 and 17). However, a GST-fusion protein containing the 322-amino acid carboxyl-terminal peptide of the Cdc6 (GST-BE6) did not stimulate DNA binding (Fig. 2C, lanes 13–15). GST-NP6 exhibited a more significant stimulation. It could be because, under the same amount of protein used, the molar ratio of GST-NP6 is higher because of its smallest size. Currently, we cannot exclude the possibility that the removal of the carboxyl terminus deletes a negative regulatory element resulting in a stronger stimulation. In any case, the results suggest that the first 47-amino acid amino terminus of the Cdc6 (GST-NP6) is the major region that contains the necessary sequence for the stimulation of Abf1 DNA binding.

**GST-Cdc6 Activates the Abf1 Binding Activity by Competing with Distamycin A, an (A + T)-Track DNA Binder**—The results of the stimulation of the Abf1 activity by GST-Cdc6 prompted us to determine how the Cdc6 promotes Abf1 DNA binding activity. The stimulation observed in the DNA gel retardation assay occurred only in the presence of the purified GST-Cdc6, Abf1, and the ARS1 probe. This suggested that Cdc6 regulates Abf1 DNA binding via direct physical interaction with the Abf1 or that the interaction of Cdc6 with DNA facilitates the Abf1-DNA binding. To test these, the CDC6 gene driven by the ADH1 promoter (pADH-CDC6) was analyzed for suppression of abf-1, abf-3, and abf-5 temperature-sensitive strains (23), individually. None of these abf1 mutants were rescued by the plasmid pADH-CDC6 at 37 °C (data not shown), indicating that Cdc6 does not interact with Abf1 genetically in these mutants. In addition, immunoprecipitation studies could not detect any association between Cdc6 and Abf1, and Abf1 could not be retained on a GST-Cdc6 protein affinity column (data not shown). Together, these studies do not favor the possibility that the Cdc6 directly interacts with Abf1 to regulate its DNA binding activity. We therefore analyzed the possibility that the Cdc6 itself may behave like an E. coli HU-like or high mobility group protein that may interact with DNA to stimulate the binding of sequence-specific DNA-binding proteins.

Yeast ARS elements have high A + T content (>75%). To understand how Cdc6 increases Abf1 binding activity, we set up Abf1 DNA binding reactions in the presence of distamycin A, a DNA-binding reagent which recognizes (A + T)-rich tracks and associates with DNA noncovalently (24, 31). In the absence of distamycin A, GST-Cdc6 stimulated Abf1 binding, but the GST protein alone could not (Fig. 3, lanes 3 and 4, respectively). Simultaneous incubation of distamycin A (increased from 5 to 25 μM) with GST-Cdc6 (0.5 ng), however, caused suppression of Abf1 DNA binding (lanes 6–8). No protein-DNA complex was formed by the incubation of distamycin A with the DNA probe only (lane 5) or the incubation of 25 μM distamycin A with 10 ng of Abf1 and DNA probe (lane 11). In another set of reactions, distamycin A was maintained constant at 25 μM, and the Abf1 DNA binding activity was restored along with the addition of increased amounts of GST-Cdc6 (lanes 8–10). The competition between GST-Cdc6 and distamycin A was specific since GST protein alone could not restore the Abf1 binding (lane 12). Thus, GST-Cdc6 may interact with DNA at the (A + T)-rich tracks by the removal of distamycin A from the DNA and increase the affinity for sequence-specific DNA-binding protein, Abf1, to bind DNA.

**DISCUSSION**

Abf1 is an abundant, multifunctional protein in budding yeast. It binds to sequence-specific DNA elements involved in DNA replication and transcription. Different Abf1 gene functions may be regulated in accordance with different physiological responses during cell cycle progression. Our combined data demonstrate that Cdc6 stimulates Abf1 DNA binding to the ARS origin sequences. The comparison of the DNA binding activities of proteins derived from the wild-type strain and the cdc6-1 mutant reveals that the functional Cdc6 contributes to a higher Abf1 binding activity (Fig. 1). In our control studies, both Abf1 and Cdc6 protein levels are rather constant in wild-type and cdc6-1 mutant cells (data not shown). We could not detect direct protein-protein interaction between Abf1 and Cdc6 either. Instead we found that Cdc6 can compete with distamycin A to restore the Abf1 DNA binding activity, suggesting an interaction between the Cdc6 protein and DNA probe. If higher concentrations of Abf1 or the excess amount of GST-Cdc6 was used alone, both can induce the protein-DNA complex formation, respectively; however, the synergistic effect of Abf1 and Cdc6 on DNA binding is at least ~20-fold more significant. In addition, the effect of the Cdc6 on Abf1 DNA binding is subtle, since the GST-Cdc6 cannot generate a distinguishable DNAse I footprint pattern using ARS1 probe, and no sign of an altered Abf1 footprint could be found in the presence or absence of the Cdc6 either. This discrepancy between footprinting and gel shifting results may be due to that the former techniques can only detect the sequence-specific binding through protein complex formation at defined region(s) but the latter technique can detect bent or altered structures of the DNA probes. Instead of binding to the specific nucleotide sequences, the Cdc6 protein may recognize certain DNA structures, such as (A + T)-rich tracks (Fig. 3). The (A + T)-rich tracks usually exist in many ARS sequences and in eukaryotic DNA containing palindromic sequences that are involved in...

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2 L. Feng, B. Wang, and A. Jong, unpublished data.
DNA replication, DNA repair, recombination, or regulation of transcription. The interaction between Cdc6 and DNA may lead to an increase of Abf1 binding to the B3 element at ARS1 and other ARS DNA elements. There are many examples where nonspecific DNA-binding proteins stimulate sequence-specific binding proteins to facilitate a higher order of DNA-protein complex formation (25, 26). The DNA binding properties of Cdc6, if true, deserves further investigation. We are currently examining Cdc6 putative DNA interacting domain(s) by mutagenesis studies and structural analysis.

Abf1 has different affinities for different ARS sequences. It binds tightly to ARS121, ARS120, and ARS131C, and weakly to ARS1, HMRE ARS, and HMLE ARS (27). It is tempting to speculate that, for some ARS sequences such as ARS1, the Abf1 binding to the B3 element can be transformed from low affinity to high affinity as functional Cdc6 increases during cell cycle. Recent data indicate that Abf1 functions as a physical barrier to restrict the nucleosome occupancy from invasion of domain A at the ARS1 replication origin (28). A nucleosome-free region is requisite for DNA replication and transcription activation. By increasing the Abf1 activity, the Cdc6 protein may determine the temporal initiation of different sets of replicative origins. Interestingly, some ARS elements such as ARS307 do not contain B3 domain. It is not yet known what the role of Cdc6 is in ARS307 or in the group of ARS elements without the Abf1 DNA binding site. One simple explanation is that Cdc6 may stimulate other sequence-specific DNA-binding protein(s) in these ARS elements. In fact, we have found that Cdc6 can stimulate Rap1 binding to the HMR DNA element in a similar manner (data not shown). This is consistent with the idea that the Cdc6 can bend or wrap certain DNA structures and may behave as an architectural factor inducing specific multiprotein/DNA formation.

Another interesting question is whether the Cdc6 protein affects the function of Abf1 in transcription. One should keep in mind that Abf1 is an abundant protein but Cdc6 is a low copy, very unstable protein (17). Besides, the nuclear entry of the Cdc6 protein is cell cycle-dependent (7). It is unlikely that the Cdc6 regulates Abf1 throughout a complete cell cycle. However, recent studies showed that ORC, Abf1, and Rap1 are involved in both transcriptional silencing and DNA replication in the HMR and HML regions. It is still possible that the Cdc6 may be involved in Abf1 transcription regulation, but it would be in a narrow window of the cell cycle or in some particular chromosomal DNA regions.

Regardless of above questions, our findings provide new important information about the Cdc6 gene function at the molecular level. Our current model is that the newly synthesized Cdc6 will be transported into nucleus in a cell cycle-dependent manner (7). The nuclear Cdc6 could be recruited by ORC components to locate its function at specific replicative origins. One of the functions of Cdc6 could be to increase Abf1 DNA binding activity to B3 domain to facilitate the formation of some high order structural organization. This would serve to preserve specific chromatin conformation or to establish and maintain the pre-RC. The Cdc6 protein itself, together with Abf1 and/or components, are among the essential components in this high-order chromatin structure. Xenopus Cdc6 has also been shown to associate with chromatin at the very early step in DNA replication (29). Recent data suggested that the loading of Mcm protein onto pre-replicative chromatin is Cdc6-dependent (30, 32). In the high order chromatin structure, the replication origins are triggered for DNA synthesis without interference from the nuclear scaffold or nucleosomes. Further study of interaction between Cdc6 and replication origins on chromatin should shed light on the initiation of eukaryotic DNA replication.

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