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Determination of the binding constants of the centromere protein Cbf1 to all 16 centromere DNAs of *Saccharomyces cerevisiae*

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ABSTRACT

Cbf1p is a *Saccharomyces cerevisiae* chromatin protein belonging to the basic region helix–loop–helix leucine zipper (bHLHzip) family of DNA binding proteins. Cbf1p binds to a conserved element in the 5′-flanking region of methionine biosynthetic genes and to centromere DNA element I (CDEI) of *S.cerevisiae* centromeric DNA. We have determined the apparent equilibrium dissociation constants of Cbf1p binding to all 16 CDEI DNAs in gel retardation assays. Binding constants of full-length Cbf1p vary between 1.7 and 3.8 nM. However, the dissociation constants of a Cbf1p deletion variant that has been shown to be fully sufficient for Cbf1p function *in vivo* vary in a range between 3.2 and 12 nM. In addition, native polyacrylamide gel electrophoresis revealed distinct changes in the 3D structure of the Cbf1p/*CEN* complexes. We also show that the previously reported DNA binding stimulation activity of the centromere protein p64 functions on both the Cbf1 full-length protein and a deletion variant containing only the bHLHzip domain of Cbf1p. Our results suggest that centromeric DNA outside the consensus CDEI sequence and interaction of Cbf1p with adjacent centromere proteins contribute to the complex formation between Cbf1p and *CEN* DNA.

INTRODUCTION

Chromosome segregation in mitosis and meiosis depends on a particular chromosomal structure, the centromere. It provides a chromosomal attachment site, the kinetochore, for the spindle microtubules. Once attached, kinetochore proteins resembling molecular motors actively move chromosomes along microtubules. The centromere of *Saccharomyces cerevisiae* is ideal for a molecular dissection and *in vitro* reconstitution because its structure is much less complex than that of higher eukaryotes. The DNA involved in building the *S.cerevisiae* kinetochore, the centromere DNA, is short (125 bp) whereas it is very long and repetitive in higher eukaryotes (for reviews see 1–5).

The centromere DNA (*CEN* DNA) of *S.cerevisiae* is organized into three elements, the centromere DNA element I (CDEI), CDEII and CDEIII (3,6). CDEI (8 bp) and CDEIII (26 bp) represent consensus sequences, whereas CDEII (78–86 bp) is an AT-rich sequence that separates CDEI and CDEIII. The CDEI element contains the DNA binding site for the Cbf1 protein. So far, no protein has been reported that specifically binds to the CDEII element although Cse4, a histone H3 homolog (7), has been genetically linked to CDEII (8). CDEII likely contributes to the structure of the centromere with no protein specifically bound to it (9). Deletions or insertions altering the length of the AT-rich CDEII domain decrease mitotic chromosome stability (reviewed in 10). High AT content and specific length rather than nucleotide sequence seem to be critical for CDEII (11). CDEIII is essential for centromere function and specifies the binding site for the multi-subunit protein complex CBF3 (reviewed in 5;12).

Cbf1p is not only involved in kinetochore complex formation at the CDEI site but is also part of a transcription activator complex regulating the biosynthesis of the amino acid L-methionine (13) as well as the transcription activator complexes CYT1 (14), GAL2 and TRP1 (15). Inactivation of Cbf1p leads to a partial loss of centromere function and methionine auxotrophy in *S.cerevisiae* (13,16). Furthermore, Cbf1p deletion mutants show a 10-fold reduction in chromosome segregation efficiency (15–17). It has been suggested that the major cellular role of Cbf1p is to safeguard the biochemical integrity of the kinetochore (18). Cbf1p consists of 351 amino acids and binds to the CDEI concensus sequence 5′-dRTCACRTG-3′ (R = purine) (19,20) as a homodimer via a basic region helix–loop–helix zipper (bHLHzip) domain at its C-terminal end (15–17). Protein function is mediated by the bHLHzip domain since deletion of the N-terminal 209 amino acids has no effect on chromosome segregation (15). Two α-helical basic regions of bHLH dimers bind into the major grooves of the DNA (for comparison see 21). Cbf1p was shown to bend CDEI upon binding by ~70° towards the major groove of the CDEI binding site (22).

Polyacrylamide gel electrophoresis (PAGE) revealed different degrees of retarded migration behavior of all 16 *CEN*

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DNAs indicating intrinsic curvature (9) and providing further evidence for a model of budding yeast centromeres in which *CEN* DNA structure is important for the assembly, activity and/or regulation of the centromere protein–DNA complex.

There is increasing genetic and biochemical evidence for a regulated network of protein–protein and protein–DNA interactions that contributes to the structure of the *S.cerevisiae* kinetochore complex (23). The CBF3 multiprotein complex consists of the four essential subunits: p110, p64, p58 and p23, which form the core element of the centromere (reviewed in 5). In analyses of genetic interactions between centromere protein genes, synthetic lethality was observed between the *CEP3* gene encoding p64 and *Cbf1*. These analyses suggested that Cbf1p may physically interact with p64 *in vivo* (18,24). This idea was supported by the finding that CBF3 subunits can directly interact with Cbf1p *in vitro* and that p64 and p23 stimulate the binding of Cbf1p to *CEN* DNA (25).

In this paper, the apparent affinity constants for the interaction of Cbf1p to all 16 *CEN* DNAs were determined in saturation binding experiments followed by gel retardation analyses. The data reveal that the affinities of a truncated Cbf1p version carrying only the DNA binding domain (Cbf1ΔN209p) vary in a broad range when compared with full-length Cbf1p. The variations appear to depend on CDEI flanking centromere DNA as well as on the N-terminal part of Cbf1p. The CBF3 subunit p64 stimulates the *CEN* DNA binding activity of both, Cbf1ΔN209p and the full-length Cbf1p protein indicating that differences in the binding affinities of the two proteins may be overruled through interaction of Cbf1p/CDEI with p64.

MATERIALS AND METHODS

Centromere DNAs

DNA fragments (300 bp) carrying the centromere DNA from each of the 16 yeast chromosomes were cloned recently (9) in pBlueScript II SK+ vectors (Amersham, Freiburg, Germany) yielding the plasmids p*CEN1* to p*CEN16*. The cloned 300 bp fragments comprising CDEI, CDEII and CDEIII are flanked by the centromere-specific 90 or 91 bp of genomic budding yeast DNA (9). The consensus binding site of Cbf1p, 5′-dRTCACRTG-3′, within CDEI is present only once per centromere fragment. The 16 different *CEN* DNA fragments were amplified by PCR using the primers PH9 (5′-dCGCTCTAGAACTAGTGGATC-3′, 20 bases) and PH10 (5′-TCGAGGTCGACGGTATC-3′, 17 bases) which are complementary to the *CEN* DNA flanking vector sequences. Thus, the amplified fragments are 337 bp in length. PCR products were purified (QIAquick kit, Qiagen, Hilden, Germany) and analyzed on 4% polyacrylamide gels. DNA purity and concentration were determined in a UV spectrophotometer (Carl Zeiss, Specord M 500, Jena) between 200 and 300 nm wavelength. All fragments were diluted to a concentration of 5 nM and stored at –20°C.

Recombinant proteins

Full-length Cbf1p protein and Cbf1ΔN209p containing only the DNA binding domain of Cbf1p were expressed as histidine-tagged fusion proteins in *Escherichia coli* BL21(DE3) from plasmids pET-Cbf1p and pET-Cbf1ΔN209, respectively (kindly provided by D.Thomas), and purified according to a protocol detailed in Kuras *et al.* (26). His-tagged p64 protein was expressed and purified as described previously (27,28). Protein concentrations were determined using the micro BSA protein assay kit (Pierce, USA).

Bandshift assay

The binding constants were determined by bandshift assays in polyacrylamide gels (29). In the reaction mixtures, the amount and concentration of DNA was kept constant while the protein concentration was varied. The binding reactions contained protein at the indicated concentrations, 0.5 nM *CEN* DNA, 25 mM HEPES buffer pH 7.6, 50% glycerine and 10 mg/ml BSA. Reaction mixtures were kept on ice for 20 min. The reaction mixture was loaded onto 4% native polyacrylamide gels pre-electrophoresed for 1 h. DNA binding stimulation assays were performed as described previously (25). Gels were run in 0.5× TBE (1× TBE contains 100 mM Tris, 83 mM borate, 0.1 mM EDTA pH 8.0) at 20 mA at room temperature and stained in SYBR Gold nucleic acid stain solution (Molecular Probes, Eugene, USA) for 10 min in the dark. The fluorescence of the gel bands was visualized by UV light. Digital pictures were taken with a high resolution CCD camera and stored as tag image files (TIFF). The digital files were analyzed using the gel scan program MacCAM (Cybertec, Berlin, Germany). The intensity of free DNA bands and complex bands in each lane were determined. For calculating the binding constants, the intensities of those bands representing DNA complexes with one or more Cbf1 proteins (multimers) were added. For some *CEN* DNAs the binding constant of Cbf1p was determined in four completely independent experiments. Deviations of a few percent were observed at concentrations at which most of the DNA is complexed. The deviations of the binding constants from the mean value (for *CEN6*, mean = 3.69 nM) were <1%.

Data analysis

Free protein Pf binds to free DNA Df to form the complex DP according to:

$$Df + Pf \leftrightarrow DP$$

For the total concentrations of DNA [Dt] and protein [Pt] holds:

$$[Dt] = [Df] + [DP] \quad \mathbf{1}$$

$$[Pt] = [Pf] + [DP] \quad \mathbf{2}$$

The dissociation constant K_d is given by:

$$K_d = [Df][Pf]/[DP] \quad \mathbf{3}$$

K_d is related to the equilibrium constant K_E by:

$$K_E = 1/K_d \quad \mathbf{4}$$

For K_d we can write:

$$K_d = ([Dt] - [DP])([Pt] - [DP])/[DP] \quad \mathbf{5}$$

For equal gel band intensities of free DNA and DNA complexed with protein we have:

$$[Df] = [DP] \quad \mathbf{6}$$

Thus:

$$[DP] = 0.5[Dt] \quad \mathbf{7}$$

For this protein concentration [Pt,50] we thus have:

$$K_d = [Pt,50] - 0.5[Dt] \quad \mathbf{8}$$

Table 1. Binding sequence and dissociation constants of Cbf1p and Cbf1ΔN209p binding to all 16 *CEN* DNAs of *S.cerevisiae*

CEN	CDEI	K_d ± error Cbf1 [nM]	K_d ± error Cbf1ΔN209 [nM]	K_d (Cbf1ΔN209)/K_d (Cbf1)
1	GTCACATGAC	1.94 ± 0.16	5.45 ± 0.44	2.8
2	ATCATGTGAC	3.11 ± 0.25	5.02 ± 0.40	1.6
3	GTCACATGAT	2.52 ± 0.20	3.21 ± 0.26	1.3
4	GTCACATGCT	2.43 ± 0.19	10.81 ± 0.86	4.4
5	ATCACGTGCT	3.35 ± 0.27	4.11 ± 0.33	1.2
6	ATCACGTGCT	3.69 ± 0.30	6.12 ± 0.49	1.7
7	ATCACGTGTT	3.27 ± 0.26	7.12 ± 0.57	2.2
8	ATCACATGAC	3.77 ± 0.30	11.48 ± 0.92	3.0
9	TTCACGTGAA	2.80 ± 0.22	12.11 ± 0.97	4.3
10	ATCACGTGTT	1.76 ± 0.14	3.78 ± 0.30	2.1
11	GTCACATGAT	1.68 ± 0.13	5.11 ± 0.41	3.0
12	ATCACGTGTA	1.93 ± 0.15	3.44 ± 0.28	1.8
13	ATCACATGAC	2.43 ± 0.19	5.11 ± 0.41	2.1
14	GTCACGTGCA	2.85 ± 0.23	6.45 ± 0.52	2.3
15	ATCACGTGAA	2.43 ± 0.19	6.12 ± 0.52	2.5
16	ATCACATGAT	2.77 ± 0.22	8.80 ± 0.70	3.2
Mean value	RTCACRTG	2.67	6.52	2.5

In addition, the consensus sequence and the mean binding constants of the two proteins to the 16 *CEN* DNAs are shown. For every *CEN* DNA the ratio of the two K_ds is given.

For known [Dt] we take [Pt,50] at 50% binding from the binding curve (Fig. 3) and calculate the dissociation constant K_d according to equation **8**.

RESULTS

The centromere binding protein Cbf1p and its deletion variant Cbf1ΔN209p containing only the bHLHzip DNA binding motif (Fig. 1A) were expressed in *E.coli* and purified. The purity of the two proteins was analysed in denaturing SDS–PAGE indicating a protein purity >95% (Fig. 1B). The binding constants of the full-length protein $(His)_6$-Cbf1p and the deletion mutant $(His)_6$-Cbf1ΔN209 to all 16 *CEN* DNAs of *S.cerevisiae* were determined in gel retardation experiments. In binding reactions, 0.5 nM *CEN* DNA was incubated with increasing amounts of recombinant proteins: 0.625–25 nM $(His)_6$-Cbf1p and 2.82–112.5 nM $(His)_6$-Cbf1ΔN209 (Fig. 2). Binding mixtures were incubated on ice for 20 min and loaded on native polyacrylamide gels, which separate unbound DNA from the protein–DNA complexes. Typical results are displayed for *CEN15* DNA in Figure 2. With increasing amounts of protein in the binding mixture, the intensities of the bands representing free DNA decrease and those of the protein–DNA complex bands increase. At high protein concentrations, high molecular weight complexes appear which most likely represent multimere aggregates. The quantitative analyses of the gels in Figure 2 are shown in Figure 3.

The binding constants of the recombinant proteins were deduced from the protein concentration at which 50% DNA was bound, and represent mean values from three to four independent binding curves. Table 1 lists binding constants, DNA binding sequences, relative values of the binding constants for the two proteins and the mean values for the binding constants. The analysis of gel band intensities has particularly small errors for that protein concentration [Pt,50] for which we measure equal distribution of the DNA in the free [Df] and the complex gel band (including multimer complexes, [DP]). For smaller protein concentrations, the band representing the complex becomes faint while at higher protein concentrations we observed multimer complex bands and only a faint band for free DNA. At the protein concentration [Pt,50] we have [Df] = [DP].

The binding constants of Cbf1p binding to the 16 *CEN* DNAs vary between 1.8 nM found for *CEN10* and 3.8 nM found for *CEN8* (Table 1) with a mean value of 2.7 nM. The binding of the deletion variant Cbf1ΔN209p, however, varies between 3.2 nM found for *CEN3* and 12.1 nM found for *CEN9* with a mean value of 6.5 nM. This indicates that the N-terminus of Cbf1p contributes to optimal binding to the 16 CDEI elements. While the binding constant of both proteins to *CEN3* or *CEN5* is similar (K_d difference 20–30%), the binding constant of the two proteins to *CEN4* or *CEN9* differs by a factor of 4 (Table 1). This result strongly suggests that CDEI flanking *CEN* DNA contributes to the complex formation between Cbf1p and CDEI.

A previous analysis had shown that the 16 *CEN* DNAs display different degrees of reduced mobility in native polyacrylamide gels, indicating intrinsic curvature of *S.cerevisiae* centromeric DNA (9). Since the Cbf1p protein has been shown to bend the CDEI sequence of at least three of the 16 *CEN* DNAs (22), we tested whether Cbf1p would show any additional

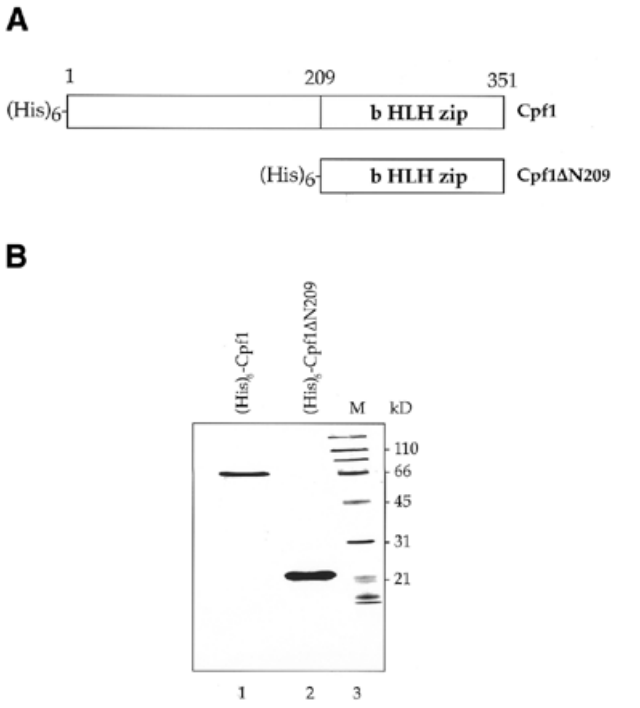

Figure 1. Expression of Cbf1 proteins. (**A**) Schematic drawing of the two proteins $(His)_6$-Cbf1p and $(His)_6$-Cbf1ΔN209p. The full-length protein is 351 amino acids in length while the deletion variant only contains the C-terminal amino acids 209–351 carrying the bHLHzip DNA binding motif. (**B**) Denaturing SDS–polyacrylamide gel of $(His)_6$-Cbf1p (lane 1) and $(His)_6$-Cbf1ΔN209p (lane 2). M, marker proteins with molecular weight indicated (kDa).

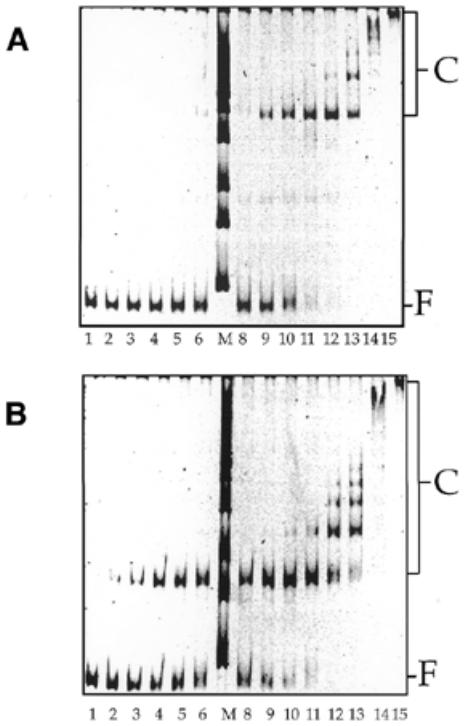

Figure 2. Binding of Cbf1p and Cbf1ΔN209p to *CEN* DNA. (**A**) Cbf1p binding. Lanes 1–15, increasing Cbf1p concentrations: lane 1, 0.63 nM; lane 2, 0.89 nM; lane 3, 1.04 nM; lane 4, 1.25 nM; lane 5, 1.39 nM; lane 6, 1.56 nM; lane 8, 1.79 nM; lane 9, 2.08 nM; lane 10, 2.50 nM; lane 11, 3.13 nM; lane 12, 4.17 nM; lane 13, 6.25 nM; lane 14, 12.5 nM; lane 15, 25 nM. (**B**) Cbf1ΔN209p binding. Lanes 1–15, increasing Cbf1ΔN209p concentrations: lane 1, 1.41 nM; lane 2, 2.01 nM; lane 3, 2.35 nM; lane 4, 2.82 nM; lane 5, 3.13 nM; lane 6, 3.52 nM; lane 8, 4.02 nM; lane 9, 4.69 nM; lane 10, 5.63 nM; lane 11, 7.05 nM; lane 12, 9.37 nM; lane 13, 14.07 nM; lane 14, 28.13 nM; lane 15, 56.25 nM. M, marker DNA in steps of 1 kb; F, free *CEN* DNA; C, complexed *CEN* DNA.

effects on the *CEN* DNA migration behavior (Fig. 4). Bandshift experiments were performed that detected all 16 *CEN* DNAs and the corresponding Cbf1ΔN209–*CEN* complex in the same gel (Fig. 4A). The pattern of migration of free *CEN* DNAs was identical to the one observed previously (9). The Cbf1p–*CEN* complexes displayed different mobilities. However, the pattern of migration differences of the Cbf1p–*CEN* complexes was clearly distinct from the pattern of the free *CEN* DNAs. Pairwise comparison revealed that *CEN* DNAs that migrated at different positions in their free form were detected at the same position when in complex with Cbf1p (compare *CEN4* with *CEN5*, or *CEN14* with *CEN15*, Fig. 4A), or, vice versa, *CEN*-DNAs which migrated at similar positions in their free form, were retarded to distinct positions with Cbf1p bound to it (compare *CEN8* with *CEN9*, Fig. 4A). Figure 4B represents an evaluation of the band shift results for each of the *CEN* DNAs as the ratio between the migration length of the free *CEN* and the respective Cbf1p–*CEN* complex. The largest difference (14%) of the complex:*CEN* migration ratio was observed between *CEN5* and *CEN12* (Fig. 4B). We obtained similar migration patterns using the full-length Cbf1p derivative indicating that the N-terminal part of Cbf1p does not influence the Cbf1p–*CEN* structure (data not shown). These results indicate that the altered migration behavior of Cbf1p-complexed *CEN* may be the result of both Cbf1p-induced bending of CDEI (22) and the intrinsic curvature of the *CEN* DNA (9).

Components of the essential CBF3 complex can directly interact with Cbf1ΔN209p and increase the affinity of

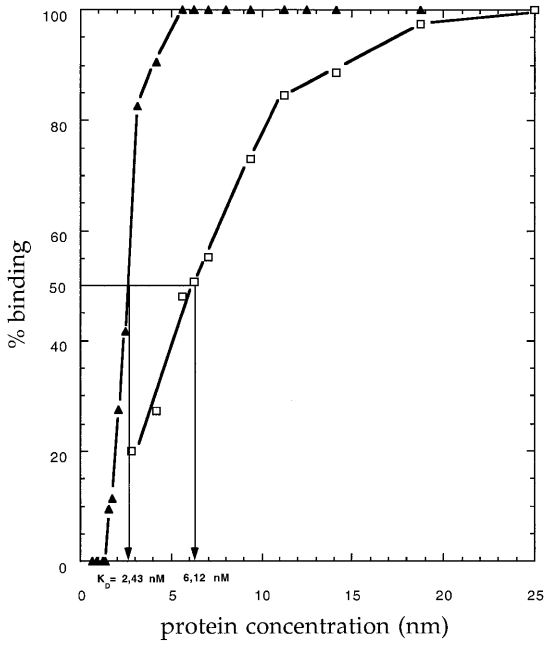

Figure 3. Binding analysis of Cbf1p and Cbf1ΔN209p to *CEN15* DNA. The plot displays the data of gels shown in Figure 2. Triangles, binding values for Cbf1p; squares, binding values for Cbf1ΔN209p.

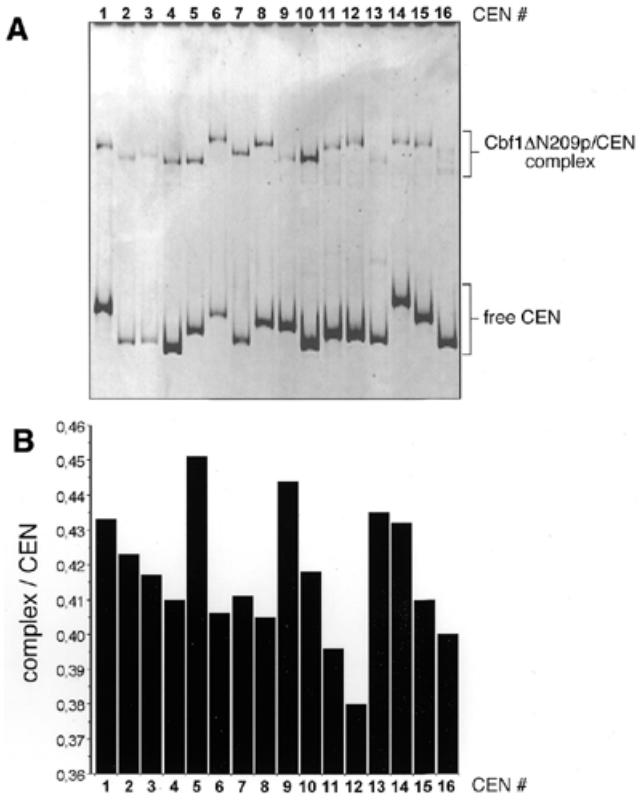

Figure 4. PAGE migration behavior of Cbf1p–*CEN* complexes. (**A**) All 16 *CEN* DNAs were incubated with Cbf1ΔN209p and analysed by native PAGE. (**B**) The migration length of the free *CEN* DNA was divided by the migration length of the respective Cbf1ΔN209p–*CEN* complex and the values are plotted for each *CEN* DNA. The data were derived from three independent experiments and represent mean values. Standard deviation was <5%.

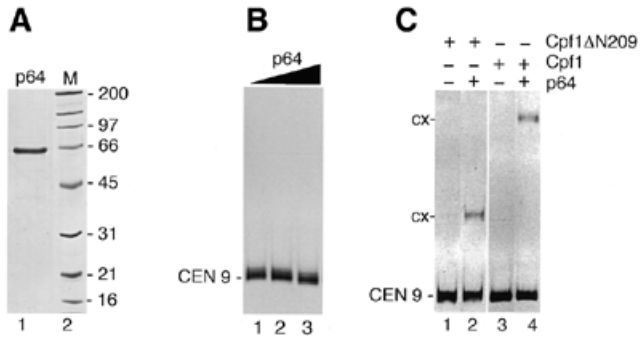

Figure 5. p64-induced stimulation of Cbf1p DNA binding. (**A**) Coomassie stained SDS–gel of purified, recombinant CBF3 subunit p64 (lane 1). M, molecular weight standard proteins. (**B**) *CEN* 9 DNA was incubated with 10, 20 and 40 nM p64, and reaction mixtures analysed by gel shift (lanes 1, 2 and 3, respectively). No complex is observed. (**C**) *CEN* 9 DNA was incubated with 0.2 nM of Cbf1p (lanes 1 and 2) or Cbf1ΔN209p (lanes 3 and 4) in the absence (lanes 1 and 3) or presence (lane 2 and 4) of 20 nM p64.

Cbf1ΔN209p for *CEN* DNA (25). We therefore examined how the addition of p64 would effect the binding of the full-length Cbf1p to *CEN* DNA. Recombinant p64 was purified from bacteria (Fig. 5A) and showed no intrinsic *CEN* DNA binding activity (Fig. 5B). We then added p64 to reaction mixtures that contained very low amounts of Cbf1 proteins (Fig. 5C). Although without p64 there was no detectable Cbf1p–*CEN9* complex formation (Fig. 5C, lanes 1 and 3), we observed formation of such complexes in the presence of p64 (Fig. 5C, lanes 2 and 4). The p64-induced increase in complex formation between Cbf1ΔN209p and *CEN* DNA is p64 concentration-dependent (~40-fold at 20 nm concentration; 25). Figure 5C shows that p64 exhibited a similar DNA binding stimulation activity on Cbf1p (lane 4) indicating that p64 functions on both the Cbf1ΔN209–*CEN* complex and the Cbf1p–*CEN* complex with similar activity.

DISCUSSION

We analyzed the binding of the Cbf1p protein and its deletion mutant containing only the DNA binding domain to 16 different centromeric DNA fragments representing a degenerate set of specific binding sites. Each of the 337 bp DNA centromere fragments used in this study contains a single CDEI binding site of the consensus sequence 5′-RTCACRTG-3′ specific for *CEN1* to *CEN16*, respectively. Cbf1p binds to these sites as a homodimer. In addition, the DNA fragments carry the CDEIII region, which represents the binding site of the hetero-oligomeric protein complex Cbf3 (5). The binding constant of Cbf1p to DNA sequences other than the consensus site was determined to be 2×10^{-4} M in competition experiments (26). Assuming 329 independent non-specific binding sites per centromere fragment, one can estimate their contribution to the binding constant as two orders of magnitude off the constants for specific binding (Table 1). Thus, non-specific binding to the fragments can be neglected.

The binding constants of full-length Cbf1p agree with literature values obtained for related proteins. Studies by Baker *et al.* (30) revealed that the Cbf1p binding equilibrium constant K_E to *CEN3* DNA is 3×10^8 M^{-1} (±40%) which corresponds (equation **4**) to a K_d of 3.3 nM. Baker *et al.* (30) had purified the Cbf1p protein from yeast, thus the endogenous protein from yeast exhibits a very similar binding constant when compared to the binding constant of 2.5 nM obtained for the recombinant Cbf1 protein used in this work. The binding constant of the human upstream stimulatory factor USF, also containing a bHLH DNA binding motif, to its binding sequence 5′-dCACGTG-3′ was determined to 1.3 nM (31).

The 16 *CEN* DNAs offer a variation of binding sequences. It could be speculated that the binding constants are related to the particular CDEI DNA sequence suggesting similar binding constants for those *CEN* fragments with 5′-dRTCACATG-3′ or with 5′-dRTCACGTG-3′. However, this is not observed. For example, within the CDEI sequence group 5′-dRTCACATG-3′ we find the strongest (*CEN11*) and the weakest (*CEN8*) binding by full-length Cbf1p. Very weak (*CEN8*) and very strong (*CEN3*) binding within this DNA sequence group is also observed for Cbf1pΔN209p. Thus, DNA sequences next to the consensus binding site seem to influence the DNA binding of Cbf1p and Cbf1ΔN209p. Similar findings have been reported previously by Wilmen *et al.* (20) who showed that Cbf1p binding *in vitro* as well as full *in vivo* centromere function requires a 10 bp recognition sequence including the 8 bp CDEI. Moreover, these studies indicated that Cbf1p interaction with CDEI is different *in vitro* and *in vivo* suggesting that the

CDEI–Cbf1p complex interacts with other centromere components in the *CEN* complex (20).

Cbf1p and Cbf1ΔN209p contain the same bHLHzip DNA binding motif. Nevertheless, both proteins bind differently to the 16 DNA sequences indicating that the N-terminal domain of the protein has an influence on binding. The relative value of the binding constants of the two proteins (Table 1) varies from 1.2 (*CEN5*) to 4.4 (*CEN4*) showing that the N-terminal part contributes to the binding in a DNA sequence specific way either by interacting with CDEI-surrounding DNA sequences or by modulating the Cbf1 protein structure and thus stabilizing the various interactions between the protein and the 16 different CDEI elements.

Cbf1p and Cbf1ΔN209p bind to the degenerate set of the 16 *CEN* DNAs with binding constants varying by a factor of 2.2 and 3.8, respectively. Since, *in vivo*, both the Cbf1p–CDEI and the Cbf1ΔN209p–CDEI complexes exert the same biological function (15), this system gives us an idea of the spectrum of binding strengths of a particular protein–DNA interaction tolerated by the cell. We are not aware of reports on other biological systems with such a DNA sequence and binding strength variation but common function within one cell. It is likely that a variety of protein–protein and protein–DNA interactions are necessary for the formation of fully assembled centromere complexes (23). Thus, the binding strength variations observed in our system represent only one contributing part of the biologically active complex. Obviously, a binding strength variation of a factor of 3.8 appears to be fully tolerated *in vivo*.

The mobility of DNA molecules in native polyacrylamide gels depends on their 3D structure. All 16 *CEN*DNAs of *S.cerevisiae* display different degrees of reduced mobility in gels (9). Here we show that all 16 *CEN* DNA–Cbf1p complexes display a migration pattern that is clearly distinct from the pattern observed for free *CEN* DNA. It is conceivable that the migration characteristics of the complexes are a result of intrinsic *CEN* DNA curvature (9) combined with bending of CDEI by Cbf1p (22). The intrinsic curvature of *CEN* DNAs may also influence the accessibilty of Cbf1 proteins to CDEI, which could also be responsible for the variety of binding constants.

Yeast mutants in which *Cbf1* is replaced by *Cbf1ΔN209* show no difference in their growth rate when compared to wild-type (32). Obviously, for centromere function the N-terminal protein domain does not seem to be essential and thus for the biological function a considerable difference in the binding constant of Cbf1p to the 16 different DNA sites, as well as differences in the Cbf1p–*CEN* DNA structure are tolerated *in vivo*. It was shown previously that binding of Cbf1p to its binding motif in the methionine promoter can be stimulated by the transcription factor Met28 (13). It is possible that similar mechanisms act at centromeres. There are more than 12 established kinetochore proteins contributing to structure and function of the centromeres in budding yeast (23). Based on biochemical and genetic data, the centromere proteins Mif2p, Cse4p, Mcm21p and the Cbf3 subunits p110 and p64 have been proposed as Cbf1p-interacting proteins (18,23,24,33). These proteins may function at the centromere to influence Cbf1p–CDEI complex formation. This has been reported recently for the p64 and p23 CBF3 subunits, which stimulate the DNA binding activity of Cbf1ΔN209p (25). p64 stimulated the *CEN* DNA binding activity of both Cbf1ΔN209p (25) and full-length Cbf1p with similar efficiencies (Fig. 5). If this interaction does also occur *in vivo*, we speculate that the observed differences in binding affinities of Cbf1ΔN209p to different *CEN*s are tolerated because p64 shifts the affinity of Cbf1ΔN209p to *CEN* DNA into a range that makes these differences negligable. Since Cbf1p does not bind to CDEI in the absence of CBF3 *in vivo* (33), the observed p64 activity may be a critical event that decides whether Cbf1p will bind to CDEI or not.

Taken together the results presented here provide further evidence that the structure of the *S.cerevisiae* centromere complex is determined by a dynamic network of specific DNA–protein and protein–protein interactions.

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