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Two genes required for the binding of an essential *Saccharomyces cerevisiae* kinetochore complex to DNA

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ABSTRACT **Kinetochores are DNA–protein structures that assemble on centromeric DNA and attach chromosomes to spindle microtubules. Because of their simplicity, the 125-bp centromeres of *Saccharomyces cerevisiae* are particularly amenable to molecular analysis. Budding yeast centromeres contain three sequence elements of which centromere DNA sequence element III (CDEIII) appears to be particularly important. cis-acting mutations in CDEIII and trans-acting mutations in genes encoding subunits of the CDEIII-binding complex (CBF3) prevent correct chromosome transmission. Using temperature-sensitive mutations in CBF3 subunits, we show a strong correlation between DNA-binding activity measured *in vitro* and kinetochore activity *in vivo*. We extend previous findings by Goh and Kilmartin [Goh, P.-Y. & Kilmartin, J. V. (1993) *J. Cell Biol.* 121, 503–512] to argue that DNA-bound CBF3 may be involved in the operation of a mitotic checkpoint but that functional CBF3 is not required for the assembly of a bipolar spindle.**

CBF3 binds to the highly conserved centromere DNA sequence element III (CDEIII) region of the *Saccharomyces cerevisiae* centromere and is the only essential centromere-binding complex thus far identified in budding yeast. When purified by DNA affinity chromatography, CBF3 contains major proteins of 58 kDa, 64 kDa, and 110 kDa (referred to as CBF3-p58, etc.), as well as a number of minor species (1). Recently, clones were isolated for CBF3-p58 (encoded by the *CTF13* gene; ref. 2), CBF3-p64 (encoded by the *CBF3b* gene; ref. 3), and CBF3-p110 (encoded by the *NDC10/CBF2/CTF14* gene; refs. 4 and 5). Each of these three genes is essential for cell growth, and a mutation in any one of them increases the rate of chromosome loss.

In this paper, we use a bandshift assay in whole-cell extracts (2) together with temperature-sensitive mutants in CBF3 subunits to analyze the role of cis- and trans-acting factors in the binding of CBF3 to DNA. We show that CBF3 contacts an extended DNA sequence that includes the highly conserved CDEIII core and flanking DNA. Both CBF3-p58 and CBF3-p110 are required for this DNA-binding activity.

MATERIALS AND METHODS

Strains. Extracts in Figs. 1, 3, and 4 were prepared from a *leu2, trp1, ura3-52, prb1-1122, pep4-3, prc1-407, GAL, Mat***a**/α strain. *ndc10-2* was isolated by growing a collection of 700 temperature-sensitive mutants at 30°C, shifting the cells to 14°C or 37°C, and then visualizing microtubules and DNA using fluorescence microscopy (6). In one mutant, cells with anaphase spindles had DNA associated with only one of the poles. Linkage analysis demonstrated that the mutant was an allele of *NDC10* (5). All other strains have been described previously (see text).

Plasmids, Probes, and Oligonucleotides. A 350-bp fragment containing 289 bp of *CEN3* DNA and derived from pRN505 was used to generate all *CEN3* constructs (7). *CEN6* sequences were derived from plasmids kindly provided by J. Hegemann (8). Variant *CEN3* sequences were constructed using PCR and oligonucleotide-directed mutagenesis. Centromere-derived sequences in CDEIII-inverted DNA are identical to those in the previously described MOIII construct (9).

To generate various probe DNAs, 20 independent PCR reactions were pooled, and fragments were purified on agarose gels (10). The sequences of the probes and primers are shown in Fig. 2. For the 88-bp *CEN3* probe, the left-hand primer lies in the pRN505 polylinker, and the fragment was liberated from a larger 241-bp fragment by digestion with *Ssp* I. Purified DNA was labeled with ^{32}P using polynucleotide kinase.

Extract Preparation and DNA-Binding Assay. For whole-cell extracts, cells at 2–5 × 10^7 cells/ml were washed by pelleting in breakage buffer [30 mM sodium phosphate, pH 7.0/60 mM β-glycerophosphate/1 M KCl/6 mM EGTA/6 mM EDTA/6 mM NaF/10% (vol/vol) glycerol], resuspended in a minimal volume of breakage buffer with protease inhibitors (1 mM phenylmethylsulfonyl fluoride and pepstatin, leupeptin, and chymostatin each at 10 μg/ml), frozen in liquid nitrogen, and fragmented in a mortar and pestle (2). Cell debris was removed by centrifugation at 15,000 × *g* for 15 min. Total protein concentration was 25–50 mg/ml. Nuclear extracts were prepared as described (1, 7).

To measure DNA-binding activities, extracts were incubated at room temperature for 30–45 min with 40–60 fmol of radiolabeled DNA in 30 μl of 100 mM Hepes (pH 8.0), 6 mM $MgCl_2$, 1 mM NaF, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride containing 10 μg of sheared salmon sperm DNA, 1 μg of poly(dI-dC), 10 μg of bovine serum albumin, and KCl to a final concentration of 125 mM and then electrophoresed at 15 V/cm on prerun 4% polyacrylamide gels as described (7). Competition experiments were performed by premixing CDEIII probe with an excess of unlabeled DNA. The intensity of the CBF3-dependent band in each lane was determined using a PhosphorImager (Molecular Dynamics).

RESULTS

An Assay for CDEIII-Binding Activity. When whole-cell or nuclear extracts were incubated with a 350-bp *CEN3* probe as described (7) and DNA–protein complexes were resolved on nondenaturing gels, a complicated pattern of bands was observed. Because the 350-bp probe contained binding sites for several different proteins, we generated an 88-bp subfragment that includes only sequences within the DNase I footprint of CBF3 (1). A single strong band (bracketed) was observed with this shorter probe (Fig. 1). The band's intensity was reduced by

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Abbreviations: CDE, centromere DNA sequence element; CBF3, CDEIII-binding complex; wt, wild type.

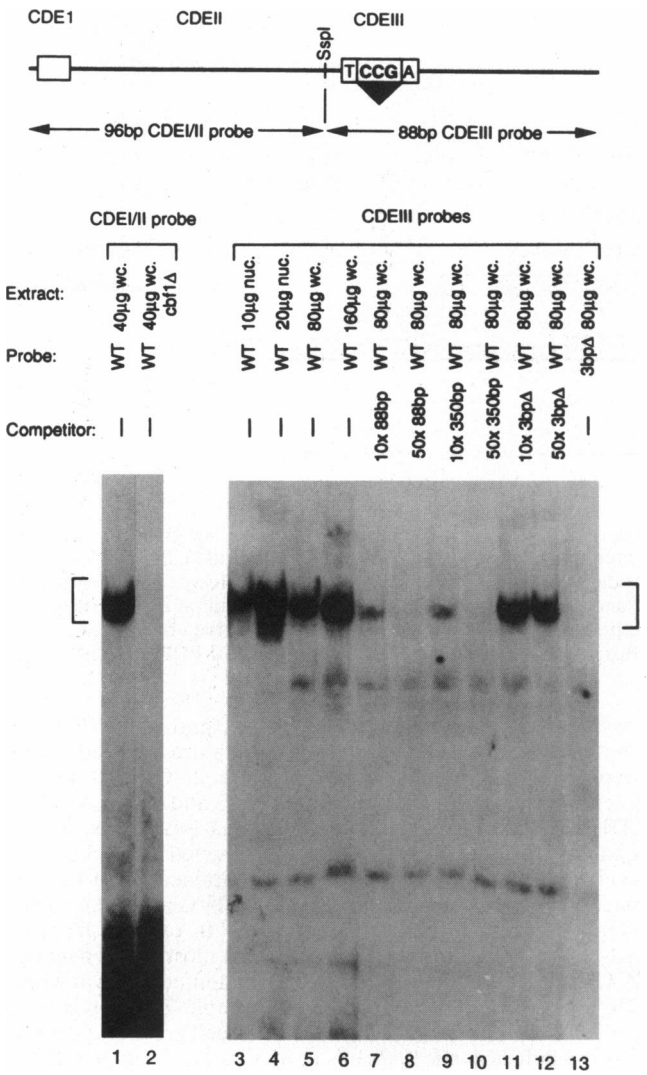

FIG. 1. Analysis on nondenaturing gels of DNA–protein complexes formed with CDEI and CDEIII DNAs. Lanes 1 and 2, complexes formed with a 153-bp probe containing 96 bp of CDEI/II DNA and 57 bp of vector, in extracts from congenic wt cells and cells carrying the *cbf1Δ1* deletion (11). Lanes 3–12, gel of complexes formed with an 88-bp CDEIII probe in nuclear extracts (nuc.) and whole-cell extracts (wc.). The identities of nonradioactive competitor DNAs and their molar excess over radiolabeled probe are indicated: 88bp and 3bpΔ denote wt and CDEIII-3bpΔ DNA, respectively; 350bp denotes a fragment that encompasses an extended region of *CEN3* (7).

the addition of excess unlabeled 88-bp DNA but not by a control fragment (CDEIII-3bpΔ) lacking three bases in CDEIII required for centromere function *in vivo* (8). The intensity of the CBF3–DNA band varied in a linear fashion in the range of 20–120 μg of whole-cell protein per 30-μl binding reaction. This allowed us to determine that the specific activity of nuclear extracts (7.5×10^{-18} mol of probe bound per μg of total protein) was ≈5-fold greater than that of whole-cell extracts (1.5×10^{-18} mol per μg of protein). However, CDEIII-binding activity was more uniform in a series of independently generated whole-cell extracts that nuclear extracts, making the former preferable for the comparison of wild-type (wt) and mutant stains.

Delineating the CBF3-Binding Site. Next we investigated the minimum region of CDEIII required for optimal CBF3 binding. Competition experiments by others had suggested that CBF3 could bind to a short 34-bp fragment spanning the 25 bases in CDEIII that are highly conserved among different centromeres (Fig. 2). In these earlier competition experiments, however, the molar ratio of competitor DNA to probe DNA was in excess of 3000 (7). We found that a 39-mer covering the core competes poorly for CBF3 binding (Fig. 2). In contrast, the 88-bp CDEIII fragment described above is as good a competitor as a 350-bp fragment covering all of the chromosomal *CEN3* locus (Fig. 1 and data not shown). This shows that efficient binding by CBF3 requires sequences to the right of the CDEIII core that are contained within the 88-bp fragment. Because centromere-distal sequences can be altered *in vivo* without impairing centromere function (7), CBF3 appears to make nonspecific contact with them.

An extensive series of point mutations has been generated in the CDEIII core region of *CEN6*, and the effects of these mutations have been determined by measuring the increase in mitotic loss rates they cause when present on artificial chromosomes (8, 12). The data are summarized in the shaded bar in Fig. 2. Dark boxes denote bases whose mutation increases chromosome loss the most, and light boxes denote bases whose mutation increases loss the least. To analyze these mutations *in vitro*, a set of wt and mutated CDEIII fragments was generated from *CEN6*, and competition analysis was performed using the 88-bp fragment as a probe. In general, there was a close correspondence between the degree of functional impairment of a mutant *in vivo* and the reduction in its binding to CBF3 *in vitro* (Fig. 3). Asymmetry in the important bases on the left and right sides of the central CCG is observed *in vivo* and *in vitro*. For example, 12A and 16T are symmetrical mutations with respect to the dyad axis of CDEIII, but they have different effects on centromere function (Fig. 3). Bases 1–4 appear to be more important for centromere function than the analogous bases on the right side of the CDEIII core (12), and the use of the *CEN3Δ2* derivative as a competitor (Fig. 2) shows that CBF3 makes important contacts with these bases. These data support the simple conclusion that point mutations in the left and center of the CDEIII core sequence increase chromosome loss rates as a consequence of their reduced ability to bind CBF3.

Next we examined the binding of CBF3 to centromeres in which mutations have been made outside the CDEIII core. These derivatives have been critical to the delineation of centromere structure but have not previously been examined for their ability to bind to CBF3. One *CEN3* truncation, used to demonstrate a requirement for CDEII *in vivo* (X-35; refs. 13 and 14), removes all centromere sequences on the CDEII-side of the conserved CDEIII core, but it also deletes 6 bp of CDEII that lie inside the CBF3 footprint. To test whether this truncation might interfere with CBF3 binding, a 95-bp mutant centromere fragment was generated in which CDEIII was truncated at the same position as in X-35 (*CEN3Δ1*). Competition analysis revealed that *CEN3Δ1* bound to CBF3 essentially as well as wt *CEN3* (Fig. 3). A second *CEN* derivative inverts the CDEIII core sequence (and 9 bp of flanking DNA) with respect to CDEI and CDEII. This CDEIII-inverted centromere contains all of the sequences present in wt *CEN3* but is inactive *in vivo* (9). Competition analysis demonstrated that this centromere derivative bound to CBF3 about as well as 12A *CEN6*, which exhibits only a 3-fold increase in the rate of chromosome loss (Fig. 3). Thus centromeres with nearly normal binding to CBF3 can be strongly defective for centromere activity *in vivo*. In particular, the failure of the CDEIII-only (X-35) and CDEIII-inverted *CEN* sequences to function *in vivo* cannot be a consequence of a disruption of the CBF3-binding site. Instead, we speculate that proteins bound to CDEII and CDEIII interact in an orientation-dependent manner.

Functional CBF3-p58 and CBF3-p110 Are Required for the DNA-Binding Activity of CBF3. To investigate the roles of various subunits in the binding of CBF3 to DNA, we measured CDEIII-binding activity in extracts grown from strains carrying mutations in CBF3-p58 (*ctf13-30*) and CBF3-p110

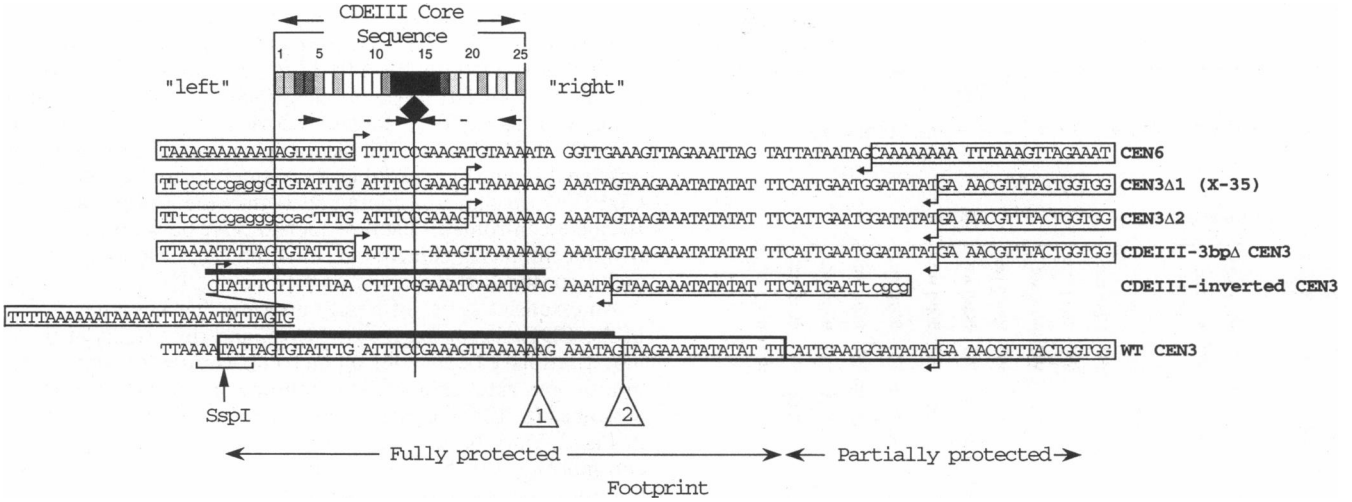

FIG. 2. Structure of CDEIII sequences used in this study, the conserved core of CDEIII is denoted by a horizontal bar graph in which the darkness of boxes under each residue number shows the severity *in vivo* of a mutation in that position (12). In *CEN3Δ1* and *CEN3Δ2*, lowercase letters indicate mutations introduced by PCR primers. In CDEIII-3bpΔ, dashes indicate bases deleted by site-directed mutagenesis. In CDEIII-inverted *CEN3*, the heavy bar indicates the region that was inverted and a similar bar in wt *CEN3* shows its position in wt centromeric DNA. Triangle 1 denotes the endpoint of a truncation that has wt centromere function *in vivo* (7), and triangle 2 denotes the endpoint of a 39-bp DNA fragment used to determine the optimal extent of the CDEIII probe. Boxes with small arrows show the positions of PCR primers used to generate various centromeric fragments.

(*ndc10-1, ndc10-2*, and *ctf14-42*). These mutant strains grow normally at 25°C but die at 37°C. In extracts from *ctf13-30* cells grown at 25°C and assayed at 37°C, CDEIII DNA-binding activity was dramatically reduced relative to wt extracts (Fig. 4, lanes 7 and 8). Residual activity (≈6% of wt) was present in *ctf13-30* extracts, even when they were assayed at fully restrictive temperatures. In extracts from *ndc10-1* or *ctf14-42* cells grown and assayed at 37°C, all activity was absent (Fig. 4*B* and data not shown). When *ctf13* and *ctf14* mutant extracts were mixed, they complemented each other, showing that the defect in CDEIII binding is specific (Fig. 4*C*). We conclude that CBF3-p58 and CBF3-p110 are required for the DNA-binding activity of CBF3.

Correlation Between Defects *in Vivo* and *in Vitro*. To determine whether defects in the DNA-binding activity of CBF3 observed *in vitro* are representative of what occurs *in vivo*, we compared cell growth and CDEIII-binding activity in *ndc10-2* cells at various temperatures. *ndc10-2* cells grew well at 30°C, very slowly at 33°C, and not at all at 36°C and above (Fig. 4*f*), whereas wt cells grew well at all temperatures. Extracts were prepared from *ndc10-2* cells grown at 25°C, and binding reactions were performed between 25°C and 39°C. At 25°C, CDEIII-binding activity was present at nearly wt levels, at 33°C it was 10-fold lower, and at 36°C it dropped to background levels (Fig. 4*D*). Similar results were obtained when binding reactions were incubated for 30 min at 25°C and then subsequently shifted to higher temperatures. In extracts from wt cells, CDEIII-binding activity dropped at most 2-fold between 25°C and 36°C. These data show that the temperature at which DNA–protein complexes become unstable *in vitro* is very similar to the restrictive temperature for cell growth and argue that the defect in *ndc10-2* cells at 37°C is an absence of DNA binding by CBF3. The data also suggest that after temperature upshift, all of the CBF3 complexes in *ndc10-2* cells are disrupted and functional kinetochores are absent.

To investigate whether spindles could assemble *de novo* in cells lacking functional kinetochores, congenic wt and *ndc10-2*

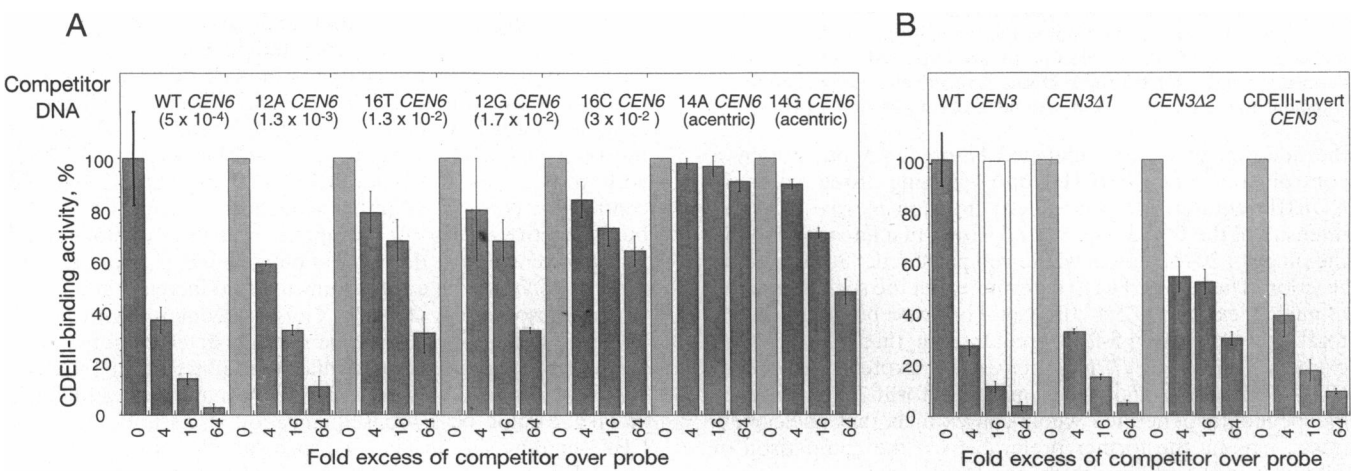

FIG. 3. Binding of *CEN* derivatives to CBF3. (*A*) Reactions containing 60 fmol of 95-bp radiolabeled *CEN6* DNA and 60 μg of whole-cell extract. The identities of *CEN6* point mutations are indicated, as are chromosome loss rates in events per generation (12). The amount of CDEIII-binding activity is expressed as a percentage of the amount with no competitor present; vertical lines denote the range of each measurement. The lightly shaded bars are repeats of the first bar included for clarity, and open bars show the amount of CDEIII bound when CDEI/II DNA was used as a negative control competitor. (*B*) Reactions containing 40 fmol of radiolabeled 88-bp *CEN3* probe DNA and various amounts of *CEN3* competitor DNAs.

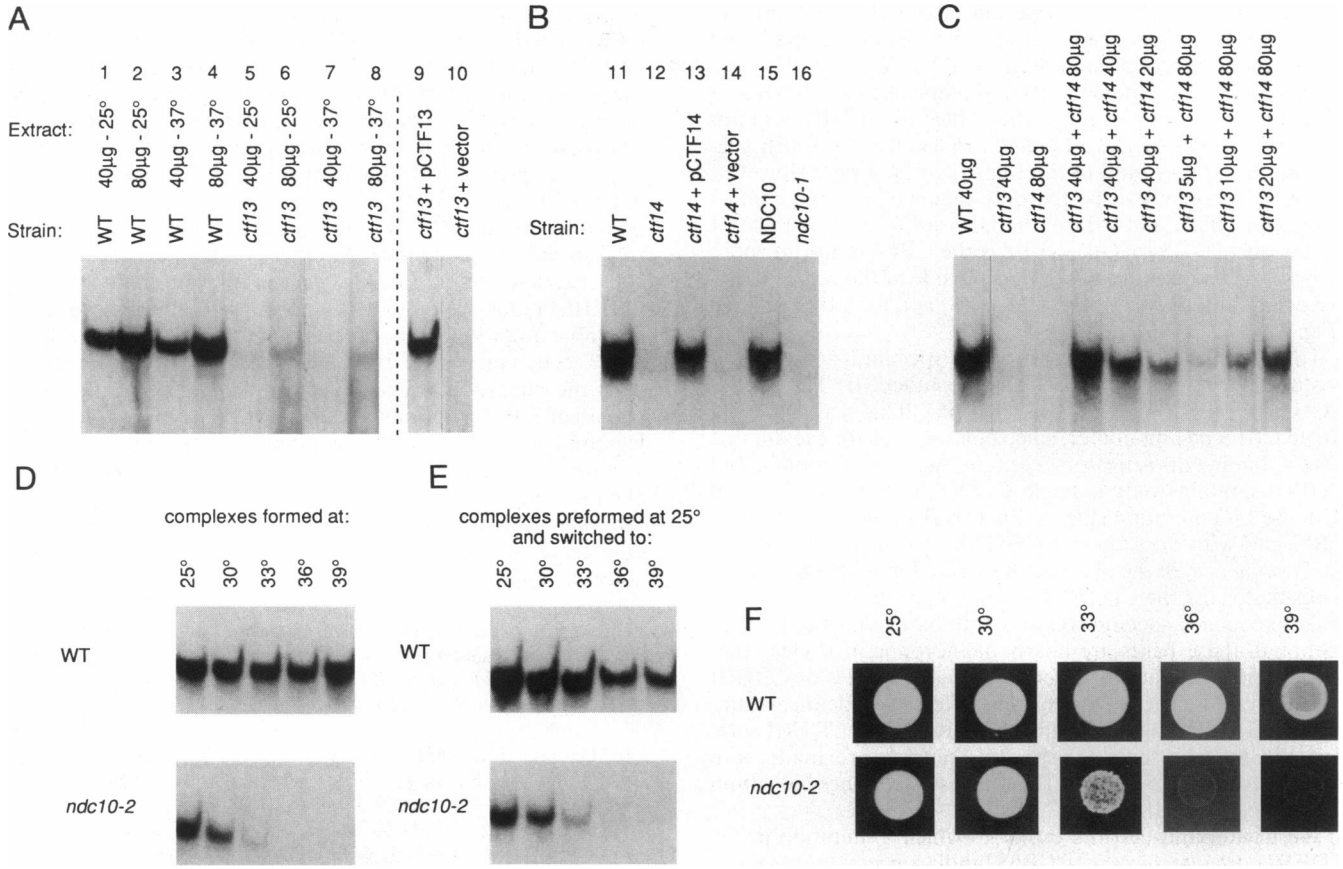

FIG. 4. Nondenaturing gels showing CDEIII-binding activity in extracts from *ctf13-30* and *ndc10/ctf14* cells. (*A*) Extracts were prepared from congenic wt or *ctf13-30* cells grown continuously at 25°C or shifted to 37°C for 3 hr as indicated or from *ctf13-30* cells transformed with a *CTF13*-encoding plasmid (lane 9) or a control vector (lane 10) and grown at 25°C. Binding reactions and electrophoresis were performed at 37°C. (*B*) Extracts were prepared from congenic wt (lane 11) and *ctf14-42* (lane 12) cells, or from *ctf14-42* cells transformed with a *CTF14*-encoding plasmid (lane 13) or a control vector (lane 14), or from congenic *NDC10:URA3* wt (lane 15) and *ndc10-1* (lane 16) cells. Binding reactions and electrophoresis were performed at 27°C. (*C*) Extracts from *ctf13-30* and *ctf14-42* cells were mixed in different amounts and incubated at 37°C. (*D*) Extracts from congenic wt and *ndc10-2* cells were grown at 25°C and incubated with probe DNA at the indicated temperatures; electrophoresis was performed at 25°C. (*E*) Extracts from wt and *ndc10-2* cells grown at 25°C were preincubated with probe at 25°C for 30 min and then shifted to the indicated temperatures for a further 30 min; electrophoresis was performed at 25°C. (*F*) Growth on solid medium of wt and *ndc10-2* cells at various temperatures.

cells were synchronized at START with α factor and then released into fresh medium at 37°C. Two hours after release from α factor, 85% of the *ndc10-2* cells possessed large buds. Ninety-seven percent of these had failed to segregate their DNA as determined by 4′,6-diamidino-2-phenylindole staining, but 82% of the wt large-budded cells had completed DNA segregation (Fig. 5). Thus, *ndc10-2* cells exhibited first-cycle defects in DNA segregation consistent with the hypothesis that both new and existing CBF3 complexes have been disrupted.

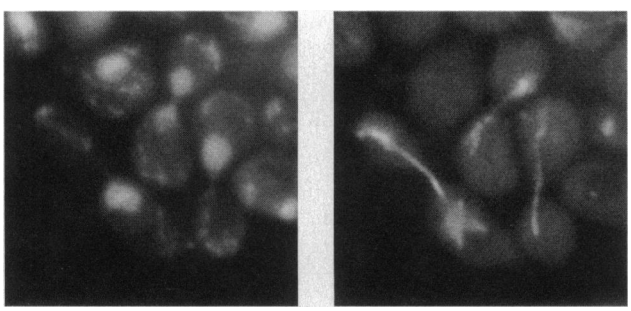

FIG. 5. Spindle assembly is observed in *ndc10-2* cells. Cells arrested for 3 hr in alpha-factor (5 µg/ml) were resuspended in prewarmed yeast extract/peptone/glucose at 37°C for 2 hr and then fixed and processed for immunofluorescence. (*Left*) 4′,6-Diamidino-2-phenylindole staining. (*Right*) Anti-tubulin staining.

Anti-tubulin and anti-spindle pole antibodies revealed the formation of bipolar anaphase spindles of normal length in the *ndc10-2* cells (although the spindles appeared to contain fewer microtubules than normal spindles, as noted for *ndc10-1*; ref. 5). At later time points after release from α factor, the percentage of large-budded *ndc10-2* cells diminished, and unbudded cells appeared. Many of the unbudded cells lacked chromosomal DNA, indicating that cytokinesis had occurred in the absence of DNA segregation. Thus an apparent failure of chromosome–microtubule attachment in *ndc10-2* cells does not prevent formation of a bipolar spindle and does not cause an obvious delay in the onset of anaphase.

DISCUSSION

The binding of CBF3 to DNA appears to involve both specific and nonspecific contacts with bases spread out >50 bp of DNA (1). We postulate that this reflects the interaction of more than one subunit of CBF3 with DNA. Consistent with this, we have found that DNA binding by CBF3 is impaired by conditional mutations in CBF3-p58 and CBF3-p110, neither of which is similar in sequence to known DNA-binding proteins. Our data do not demonstrate that these proteins make direct contact with DNA, but they do suggest that the quaternary structure of the CBF3 complex is necessary for tight DNA binding.

It has recently been shown that the CBF3-p64 protein contains a zinc finger similar in structure to zinc fingers found in the yeast transcriptional activators *HAP1* and *GAL4* (3). Hap1p and Gal4p bind to DNA as dimers, and each monomer contacts a CGG triplet (15). The center of CDEIII contains the complement of this sequence, CCG (bases 13–15), suggesting that it may bind to the finger of CBF3-p64. However, this is very unlikely to be the only sequence-specific contact between CDEIII and CBF3. The DNase I footprint of HAP1 covers only 20–25 bp (16), whereas the CBF3 footprint spans 56 bp (1). Furthermore, sequences outside of the central CCG are essential for centromere activity (8) and for CBF3 binding (Fig. 3).

To explain these data, we propose a speculative model that postulates three sets of CBF3–DNA contacts. (*i*) The central CCG of CDEIII is bound by CBF3-p64. This may involve a single CBF3-p64 monomer, since binding sites for the dimeric *GAL4* family of activators contain two CGG motifs, but CDEIII contains only a single CCG. The binding observed with the 14G mutation (Fig. 3*A*) may reflect the interaction of CBF3-p64 with the sequence CGG, the complement of the wt CCG sequence, in the absence of interaction with asymmetric bases that flank the CDEIII core. (*ii*) A second CBF3 subunit makes sequence-specific contacts with bases that flank CCG. Although these bases are nearly palindromic, it is clear that asymmetrical mutations in the left and right sides of CDEIII have different effects. Perhaps this reflects nonidentical binding by the second subunit to the two halves of the CDEIII core. (*iii*) Finally, non-sequence-specific contacts are made with bases outside the 25-bp CDEIII consensus sequence but within the 56-bp footprint.

We have found that the extent to which a mutation in the CDEIII core interferes with CBF3 binding *in vitro* corresponds well with the degree of impairment of centromere function *in vivo*. While this is not unexpected, it gives us a rough metric relating CBF3 binding *in vitro* to centromere function *in vivo*. This has allowed us to determine that the inactivity of a construct that retains the CDEIII core sequence but inverts it with respect to CDEII (9) is not a simple consequence of a failure to bind CBF3. It also seems that the CDEII sequences that lie within the DNase I footprint of CBF3 do not make important sequence-specific contacts with CBF3. This strengthens the argument that CDEII is required for centromere function *in vivo* because it interacts with protein factors other than CBF3 (10, 13).

The failure of chromosomes to segregate correctly in *ndc10-2* cells appears to reflect the absence of CDEIII-binding activity and the consequent failure to assemble kinetochore complexes that can attach to microtubules. Previously, Goh and Kilmartin (5) had reasoned that cells carrying the *ndc10-1* mutation lacked active kinetochores at nonpermissive temperatures but that spindles did not collapse. We have shown that in synchronized *ndc10-2* cells, normal-size anaphase spindles can form *de novo* at the nonpermissible temperature. Thus, it appears that kinetochore microtubules are dispensable for the formation and anaphase elongation of the *S. cerevisiae* spindle.

The presence of kinetochores unattached to microtubules is thought to engage a checkpoint that delays anaphase and increases the chance of microtubule capture (17–19). It is therefore surprising that a mitotic arrest is not observed in *ndc10-1* and *ndc10-2* cells (5). This can be explained if we postulate that DNA-bound CBF3 is required for operation of the mitotic checkpoint. An analogous situation appears to exist during S phase. It is thought that the assembly of replication complexes is required for the checkpoint that couples mitosis to the completion of DNA synthesis (20). Why then do cells mutant in CBF3-p58 (*ctf13-30*) accumulate in mitosis? One possibility is that CBF3-p110 and CBF3-p58 play different roles in checkpoint control. Another possibility is that *ctf13-30* cells accumulate in G_2/M because they contain residual CDEIII-binding activity (as found *in vitro*) and consequently assemble a few kinetochores competent to signal. In contrast, *ndc10* cells completely lack DNA-bound CBF3 and cannot send the checkpoint signal. This model predicts that severe alleles of CBF3 subunits will not arrest in mitosis but that hypomorphic alleles will. By examining the interaction of various *ctf13* and *ndc10* mutations with genes thought to encode components of the mitotic checkpoint pathway, such as the *mad* (21) and *bub* (22) genes, it should be possible to determine the relationship between CBF3 and checkpoint control.

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