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Inner Kinetochore of the Pathogenic Yeast *Candida glabrata*†

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The human pathogenic yeast *Candida glabrata* is the second most common *Candida* pathogen after *Candida albicans*, causing both bloodstream and mucosal infections. The centromere (*CEN*) DNA of *C. glabrata* (*CgCEN*), although structurally very similar to that of *Saccharomyces cerevisiae*, is not functional in *S. cerevisiae*. To further examine the structure of the *C. glabrata* inner kinetochore, we isolated several *C. glabrata* homologs of *S. cerevisiae* inner kinetochore protein genes, namely, genes for components of the CBF3 complex (Ndc10p, Cep3p, and Ctf13p) and genes for the proteins Mif2p and Cse4p. The amino acid sequence identities of these proteins were 32 to 49% relative to *S. cerevisiae*. *CgNDC10*, *CgCEP3*, and *CgCTF13* are required for growth in *C. glabrata* and are specifically found at *CgCEN*, as demonstrated by chromatin immunoprecipitation experiments. Cross-complementation experiments revealed that the isolated genes, with the exception of *CgCSE4*, are species specific and cannot functionally substitute for the corresponding genes in *S. cerevisiae* deletion strains. Likewise, the *S. cerevisiae* CBF3 genes *NDC10*, *CEP3*, and *CTF13* cannot functionally replace their homologs in *C. glabrata* CBF3 deletion strains. Two-hybrid analysis revealed several interactions between these proteins, all of which were previously reported for the inner kinetochore proteins of *S. cerevisiae*. Our findings indicate that although many of the inner kinetochore components have evolved considerably between the two closely related species, the organization of the *C. glabrata* inner kinetochore is similar to that in *S. cerevisiae*.

Candida glabrata is a major fungal pathogen which causes severe, often life-threatening infections in humans (20). Currently, *C. glabrata* is the second most common *Candida* pathogen after *Candida albicans*, which is isolated from the human bloodstream as well as from mucosal sites (13, 50). *C. glabrata* infections are difficult to treat because of innate and acquired resistance of clinical strains to antifungal agents, specifically, the azoles and amphotericin B (50). *C. glabrata* is phylogenetically closely related to the well-studied model organism *Saccharomyces cerevisiae*, and many of their structural genes, such as *URA3*, *TRP1*, and *HIS3*, are functionally exchangeable (33, 37, 67). However, *C. glabrata* is a haploid organism, and a sexual cycle has not been found, although three mating type loci have been identified (57).

C. glabrata, like *S. cerevisiae*, contains "point" centromeres, short stretches of DNA that are required for chromosome segregation during mitosis and that provide the attachment sites for spindle microtubules along which the chromosomes are moved (6, 34, 42). In contrast to the small point centromeres of budding yeasts, centromeres of most other eukaryotes, including humans, contain very large stretches of DNA spanning several megabases and are therefore referred to as "regional" centromeres (14). Only one *C. glabrata CEN* sequence (out of 14) has been identified and characterized so far (34, 35). It is highly homologous to *CEN16* from *S. cerevisiae* and contains three centromere DNA elements (CDEs), CDEI (8 bp) and CDEIII (26 bp), separated by a 79-bp nonconserved AT-rich (>90%) sequence, CDEII. In spite of their high homology, the *CEN* DNAs of these two species are not functionally exchangeable. *C. glabrata CEN* does not function in *S. cerevisiae*, and *S. cerevisiae CEN* has only partial activity in *C. glabrata*. The species specificity of the *S. cerevisiae* and *C. glabrata* centromeres is determined by both CDEII and CDEIII (35).

The centromeres of *S. cerevisiae* have been well studied over the last 15 years (42). The *S. cerevisiae* kinetochore (*CEN* DNA and binding proteins) is a highly complex structure consisting of >60 proteins, which are organized in multiple subcomplexes that form several layers (10, 15, 47). The inner kinetochore consists of the proteins Cbf1p, Mif2p, and Cse4p and the CBF3 protein complex. The CBF3 protein complex, consisting of the four subunits Ndc10p, Cep3p, Ctf13p, and Skp1p, binds directly to the CDEIII region (39). The CBF3 components have been shown to interact directly with the Cbf1p homodimer binding to CDEI (9, 24). CBF3 is essential for kinetochore assembly, and all four subunits are necessary for DNA binding and cell viability (6, 31, 51). The histone H3-like protein Cse4p is a homolog of the human kinetochore protein CENP-A, and evidence suggests that, together with histone H4, it forms a specialized nucleosome around which *CEN* DNA is wrapped (21, 32, 65). Mif2p is homologous to the essential mammalian kinetochore protein CENP-C and specifically copurifies with histones H2A, H2B, and H4 and with Cse4p (8, 65). It serves as a linker molecule for the central and outer kinetochore complexes, which consist of at least five key protein complexes, including multiple microtubule binding proteins and kinesin-related motor proteins (10, 15, 47). Together, these protein complexes connect the DNA to the microtubules and to the mitotic spindle. All known kinetochore proteins require CBF3 activity for in vivo association with *CEN* DNA. Whereas several outer kinetochore proteins have human homologs, no species-specific DNA binding proteins in complex kineto-

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† Supplemental material for this article may be found at http://ec.asm.org/.

chores that are analogous to budding yeast Cbf1p and CBF3 have been identified.

In previous work, two of the inner kinetochore proteins of *C. glabrata*, Skp1p and Cbf1p (CgSkp1p and CgCbf1p, respectively), were cloned and characterized (60, 61). CgSkp1p is 76% identical to and functionally exchangeable with the *S. cerevisiae* homolog. CgCbf1p is 34% identical to the *S. cerevisiae* homolog and functionally complements the methionine biosynthesis defect of an *S. cerevisiae cbf1* (*Sccbf1*) deletion mutant. In contrast to the situation in *S. cerevisiae*, *C. glabrata CBF1* (*CgCBF1*) gene disruption is lethal in *C. glabrata*, and the phenotype of Cbf1p-depleted cells indicates a defect in chromosome segregation (61). Furthermore, the CDEI region and sequences upstream of *CEN* DNA are required for *CEN* function in *C. glabrata*, whereas in *S. cerevisiae*, deletion of the CDEI region and sequences upstream results only in a partial loss of *CEN* function (61). These findings indicate that despite their high homology, the centromeres of *S. cerevisiae* and *C. glabrata* are functionally quite different.

To further examine the *C. glabrata* inner kinetochore, we searched the *C. glabrata* genome database (B. Dujon, Institut Pasteur, unpublished data) for genes with homology to known *S. cerevisiae CEN* binding protein genes: *NDC10*, *CEP3*, *CTF13*, *MIF2*, and *CSE4*. Several *C. glabrata* homologs were identified, and the corresponding genes subsequently were cloned from a *C. glabrata* genomic library. These genes were tested for their functionality in *S. cerevisiae* deletion strains, and their interactions were examined in vivo by two-hybrid analysis. We show here that despite their considerable homology to *S. cerevisiae* proteins, the CBF3 subunits Ndc10p, Cep3p, and Ctf13p and Mif2p are not functionally exchangeable between *C. glabrata* and *S. cerevisiae*. The interactions between the CBF3 subunits and Mif2p that we found by two-hybrid analysis, however, have all been reported for *S. cerevisiae*, indicating that the overall structure of the inner kinetochore complex of *C. glabrata* is similar to that in *S. cerevisiae*. Our findings indicate that an unusually rapid evolutionary drift has occurred in the inner kinetochores of these otherwise closely related species.

MATERIALS AND METHODS

Strains. All strains used and generated in this study are listed in Table 1.

Plasmids. All plasmids used and generated in this study are listed in Table S1 in the supplemental material.

Primers. All primers used for cloning and sequencing in this study are listed in Table S2 in the supplemental material.

Media. Rich medium (yeast-peptone-dextrose medium), synthetic minimal dextrose (SD) medium (0.7% yeast nitrogen base, 2% glucose, 2% agar), and 5-fluoroorotic acid (5-FOA)-containing medium were prepared as described previously (30). Complete medium (YPDA) and selective medium (SC-dropout medium, lacking various amino acids) for the two-hybrid analysis were made according to the manufacturer's instructions (Clontech). *Escherichia coli* strain DH5α was used for the propagation of plasmids. Bacterial media were prepared as described previously (52).

Transformation. Yeast transformation with linear or supercoiled plasmid DNA was performed by the lithium acetate method (3).

PCRs. For PCRs, genomic DNA of strain CgHTU2001 was digested with EcoRI, XhoI, or BamHI, and 300 ng of the genomic DNA was used for each reaction with the degenerate PCR primers listed in Table S2 in the supplemental material. Predigestion of the genomic DNA enhanced the performance of the PCRs. PCRs were performed with *Taq* polymerase (Fisher) by using a Peltier thermal cycler; the cycles were as follows: initial denaturation for 3 min at 94°C and then 40 cycles of 1 min at 94°C, 1 min at 40 or 45°C (annealing), 2 min at 72°C, and 5 min at 72°C. The PCR products were purified with a gel extraction kit (Qiagen, Valencia, Calif.), cloned into TOPO cloning vectors (Invitrogen, Carlsbad, Calif.), and sequenced. For screening of genomic libraries, the PCR fragments were radioactively labeled with a Prime-It-II random prime labeling kit (Stratagene, La Jolla, Calif.).

Cloning of centromere protein homolog genes from a *C. glabrata* genomic library. All manipulations of DNA and Southern blot analysis were performed by using standard protocols (3, 52). A *C. glabrata* genomic library (53) was transformed into *E. coli* DH5α, and transformants were transferred to colony/plaque screen hybridization transfer membrane filter disks (Perkin-Elmer). The membranes were treated according to the supplier's instructions and probed with the radioactively labeled PCR fragments. Positive colonies were reprobed, and plasmid DNA was prepared with a plasmid Midi kit (Qiagen). Plasmids were digested with several restriction enzymes, and the resulting restriction fragments were analyzed by Southern blotting with the radioactively labeled PCR fragments as probes. Restriction fragments containing the gene of interest were subcloned into pUC19 and sequenced. DNA sequencing was performed by sequencing in two directions with an ABI 377 automated sequencing apparatus and fluorescent Big Dye terminator chemistry (PE-ABI Inc., Foster City, Calif.).

Construction of deletion strains. In order to prevent the possibly lethal effects of a gene knockout in haploid *C. glabrata*, wild-type copies of the genes to be deleted were introduced into strain CgHTU2001 on *ScURA3*-bearing plasmids. After selection of the transformants on plates of SD medium lacking Ura, gene deletion was performed by a standard gene transplacement procedure with DNA cassettes that contained a *CgTRP1* gene as a selectable marker flanked by gene-specific sequences created by PCR. Briefly, DNA cassettes were released from pTS53 (*CgNDC10*), pTS55 (*CgCEP3*), and pTS54 (*CgCTF13*) by digestion with restriction enzymes KpnI and XbaI. The purified DNA fragments were transformed into Ura^+ strain CgHTU2001 carrying pTS46 (*CgNDC10*), pTS48 (*CgCEP3*), or pTS50 (*CgCTF13*), with selection for Trp^+ transformants on SD medium plates. The resulting colonies were streak purified, and the integration of the fragment at the correct genomic locus was confirmed by genomic Southern blotting with gene-specific fragments as probes. This procedure resulted in strains CgTS10, CgTS11, and CgTS12 (Table 1).

The effects of the gene deletions were tested by growing the deletion strains on plates of SD medium containing Ura and 1 mg of 5-FOA/liter. Strains that require *ScURA3*-bearing plasmids with the wild-type copies of the examined genes are expected not to grow on medium containing 5-FOA. Growth was monitored after the plates were incubated for 48 h at 30°C.

Epitope tagging of CgNDC10, CgCEP3, and CgCTF13. *C. glabrata* strains expressing epitope-tagged CgNdc10p, CgCep3p, and CgCtf13p were generated by introducing *HIS3*-bearing plasmids carrying the genes fused to the hemagglutinin (HA) epitope into CgTS10/pTS46, CgTS12/pTS48, and CgTS11/pTS50. Plasmid shuffling was carried out by growing the strains on medium containing 5-FOA to delete the *URA3*-bearing plasmids expressing the wild-type copies of the genes. The resulting strains were CgTS10/pTS131 (*CgNDC10*-HA), CgTS12/pTS132 (*CgCEP3*-HA), and CgTS11/pTS124 (*CgCTF13*-HA).

In vivo cross-linking and ChIP analysis. Chromatin immunoprecipitation (ChIP) experiments were carried out as described previously (2, 5). *C. glabrata* strains were exponentially grown to an optical density at 600 nm of 1 ($\sim 4 \times 10^7$ cells/ml) and fixed with 1% formaldehyde for 15 min at room temperature. Formaldehyde-induced cross-linking was quenched for 5 min at room temperature by the addition of glycine to a final concentration of 120 mM. The cells were washed and spheroplasted with Zymolyase 100T (1 mg/ml), and the washed spheroplasts were lysed with extraction buffer (50 mM HEPES-KOH [pH 7.5], 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium desoxycholate, protease inhibitor mixture). The chromatin was sheared by sonication to an average length of 0.8 kb. Purified monoclonal antibody HA.11 16B12 (Berkeley Antibody Co.) was added to the sheared chromatin to a final concentration of 5 μg/ml, and immunocomplexes were captured with protein G-Sepharose beads for 4 h at 4°C. For DNA analysis, 2 μl of a 1:40 dilution of total chromatin or 2 μl of a 1:10 dilution of immunoprecipitated chromatin was subjected to PCR (25 cycles of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C) with primer pairs CgCEN1-CgCEN2, CgACT1-CgACT4, and CgLEU1-CgLEU2. The PCR products were electrophoresed on 2% NuSieve 3:1 agarose gels, stained with ethidium bromide, and digitally photographed with an AlphaImager 2000 system (Alpha Innotech, San Leandro, Calif.).

Two-hybrid analysis. Two haploid *S. cerevisiae* strains, AH109 and Y187 (Table 1), were used for the Gal4-based two-hybrid analysis. The open reading frames (ORFs) for centromere binding protein homologs from *C. glabrata* were generated by PCR and sequenced (see Tables S1 and S2 in the supplemental material for cloning of all plasmids and for primers). The ORFs were cloned into plasmid pGAD-C1, encoding the Gal4 activation domain (GAD) (amino acids

TABLE 1. Yeast strains used in this study

Strain	Genotype[a]	Reference or source
Candida glabrata		
CgHTU2001 (wild type)	*his3 trp1 ura3*	34
CgTS1/p112Cp1 (*Cgcbf1*Δ)	*his3 trp1 ura3 CgCBF1*::*TRP1* + p112Cp1 (*CgCBF1 CEN ARS URA3)*	This study
CgTS10/pTS46 (*Cgndc10*Δ)	*his3 trp1 ura3 CgNDC10*::*TRP1* + pTS46 (*CgNDC10 CEN ARS HIS3*)	This study
CgTS11/pTS48 (*Cgcep3*Δ)	*his3 trp1 ura3 CgCEP3*::*TRP1* + pTS48 (*CgCEP3 CEN ARS HIS3*)	This study
CgTS12/pTS50 (*Cgctf13*Δ)	*his3 trp1 ura3 CgCTF13*::*TRP1* + pTS50 (*CgCTF13 CEN ARS HIS3*)	This study
CgTS10/pTS131 (*Cgndc10*Δ)	*his3 trp1 ura3 CgNDC10*::*TRP1* + pTS131 (*CgNDC10*-HA *CEN ARS HIS3*)	This study
CgTS11/pTS132 (*Cgcep3*Δ)	*his3 trp1 ura3 CgCEP3*::*TRP1* + pTS132 (*CgCEP3*-HA *CEN ARS HIS3*)	This study
CgTS12/pTS124 (*Cgctf13*Δ)	*his3 trp1 ura3 CgCTF13*::*TRP1* + pTS124 (*CgCTF13*-HA *CEN ARS HIS3*)	This study
Saccharomyces cerevisiae		
ScCC718-1A (*Sccbf1*Δ)	*MAT***a** *ade2-101^{ochre} trp1-*Δ*63 leu2-*Δ*1 ura3-52 his3-*Δ*200 ScCBF1*::*TRP1*	36
ScYJL49/pWJ110B (*Scndc10*Δ)	*MAT***a** *ade2-101^{ochre} trp1-*Δ*63 leu2-*Δ*1 ura3-52 his3-*Δ*200 lys2-801^{amber} cyh2^{R} ScNDC10*::*TRP1* + pWJ110B (*ScNDC10 CEN ARS URA3*)	J. Lechner (personal communication)
ScYJO28/pJO53 (*Sccep3*Δ)	*MAT*α *ade2-101^{ochre} trp1-*Δ*63 leu2-*Δ*1 ura3-52 his3-*Δ*200 lys2-801^{amber} cyh2^{R} ScCEP3*::*TRP1* + pJO53 (*ScCEP3 CEN ARS URA3*)	Lechner (personal communication)
ScYJL33/pJL114 (*Scctf13*Δ)	*MAT*α *ade2-101^{ochre} trp1-*Δ*63 leu2-*Δ*1 ura3-52 his3*Δ*200 lys2-801^{amber} cyh2^{R} ScCTF13*::*TRP1* + pJL114(*ScCTF13 CEN ARS URA3*)	Lechner (personal communication)
ScOS71/pOS221a (*Scskp1*Δ)	*MAT*α *ade2-101^{ochre} trp1-*Δ*63 leu2-*Δ*1 ura3-52 his3*Δ*200 lys2-801^{amber} cyh2^{R} ScSKP1*::*HIS3* + pOS221a (*ScSKP1 CEN ARS URA3*)	58
6764-181 (*Scmif2*Δ)	*MAT*α *his3 ura3 trp1 leu2 met2 can1 sap3 MIF2*::*HIS3* [pMB030]	7
HY13 (*Scndc10^{ts}*)	*MAT***a** *ade2 leu2-3,112 his3 lys2 trp1 ura3-52 ndc10-1*	66
MW169 (*Scndc10^{ts}*)	*MAT*α *leu2-3,112 lys2 trp1-*Δ*1 ura3-52 ndc10-169*	E. A. Siewert and M. Winey (unpublished data)
MW209 (*Scndc10^{ts}*)	*MAT***a** *leu2-3,112 his3-*Δ*206 ura3-52 ndc10-209*	Siewert and Winey (unpublished)
ctf14-42 (*Scndc10^{ts}*)	*MAT*α *ura3-52 lys2-801 ade2-101 his3-*Δ*200 leu2-*Δ*1 ctf14-42 CFIII* (*CEN3.L.YPH278*) *URA3 SUP11*	56
Sc3dAS282 (*Sccep3^{ts}*)	*cep3-2 ade2 his3 leu2 lys2 trp1 ura3*	D. Koshland (personal communication)
ctf13-30 (*Scctf13^{ts}*)	*MAT*α *ura3-52 lys2-801 ade2-101 his3*Δ*200 leu2-*Δ*1 ctf13-30* CFIII (*CEN3.L.YPH278*) *URA3 SUP11*	16
SB404 (*Sccse4^{ts}*)	*MAT*α *ade2-101 his3-11,15 leu2-3 trp1*Δ*901 ura3-52 cse4-1*	59
ScD102-1D (*ScCBF1^{+}*/CF)	*MAT***a** *CBF1^{+} cyh2 leu2 ura3 lys2 ade2 ade3*/CFIII (*URA3 ADE3*)	R. Baker (unpublished data)
ScD102-7A (*Sccbf1*Δ/CF)	*MAT***a** *cbf1*Δ::URA*3 cyh2 leu2 ura3 lys2 ade2 ade3*/CFIII (*URA3 ADE3*)	Baker (unpublished)
AH109	*MAT***a** *trp1-901 leu2-3,112 ura3-52 his3-200 gal4*Δ *gal80*Δ *LYS2*::*GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*HIS3 GAL2*$_{UAS}$-*GAL2*$_{TATA}$-*ADE2 URA3*::*MEL1*$_{UAS}$-*MEL1*$_{TATA}$-LacZ	Clontech
Y187	*MAT*α *ura3-52 his3-200 ade2-101 trp1-901 leu2-3,112 gal4*Δ *met-gal80*Δ *URA3*::*GAL1*$_{UAS}$-*GAL1*$_{TATA}$-LacZ	Clontech

[a] CFIII, chromosome fragment III.

[aa] 768 to 881), and plasmid pGBD-C1, encoding the Gal4 binding domain (GBD) (aa 1 to 147) (27), and junctions were confirmed by sequencing. The pGAD plasmids were transformed into AH109, and transformants were selected on supplemented minimal medium lacking leucine. The pGBD plasmids were transformed into Y187, and transformants were selected on supplemented minimal medium lacking tryptophan. After colony purification of the transformants, the strains were mated by inoculating a single colony of an AH109 transformant with a single colony of a Y187 transformant and growing them overnight in 0.5 ml of yeast-peptone-dextrose medium. The mating products were streaked on supplemented minimal medium lacking leucine and tryptophan in the appropriate dilutions (1:10 to 1:100). The resulting colonies were tested on various indicator media.

Western immunoblot analysis. The yeast strains for two-hybrid analysis were tested for protein expression by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Western blotting. Protein extracts were made according to the manufacturer's instructions (Clontech). Briefly, cells were grown in selective medium overnight at 30°C, and cell pellets were resuspended in cracking buffer, containing urea-SDS and a protease inhibitor solution. Cells then were vortexed with glass beads to remove cell walls and to release proteins. SDS-polyacrylamide gel electrophoresis was performed, and gels were blotted onto nitrocellulose membranes for Western blot analysis. Western blotting was carried out for 1 h at room temperature at 1 mA/cm^2. For immunoblot analysis, a 1:1,000 dilution of a mouse primary antibody against GAD was used (Clontech). The primary antibody was detected by incubation with an anti-mouse secondary antibody (1:2,000) conjugated to horseradish peroxidase, and the protein was visualized after incubation with enhanced chemiluminescence solution (Amersham) by exposure to autoradiographic film.

Amino acid sequence analysis and multiple-sequence alignments. Multiple-sequence alignments of protein sequences were performed with the CLUSTALW program (63) at the Baylor College of Medicine website (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html). Printouts of the aligned amino acid sequences were created with the Boxshade program (see Fig. S1 in the supplemental material). Amino acid sequence comparisons (Table 2) were performed with the BLASTP program (BLOSUM62 matrix) (1) by using the National Center for Biotechnology Information (NCBI) server (http://www.nlm.nih.gov/BLAST/bl2seq/bl2.html).

Nucleotide sequence accession numbers. The nucleotide sequences reported in this article have been submitted to the GenBank database under accession numbers AY230407 (*CgNDC10*), AY230408 (*CgCEP3*), AY230409 (*CgCTF13*), AY230410 (*CgMIF2*), and AY230411 (*CgCSE4*).

RESULTS

Identification of inner kinetochore protein genes in *C. glabrata*. Preliminary attempts to detect homologs of the *S. cerevisiae* inner kinetochore protein genes in *C. glabrata* by low-

TABLE 2. Amino acid sequence comparisons of *C. glabrata* and *S. cerevisiae* proteins[a]

Protein	%		No. of aa compared	Cross-complementation[b]	Source or reference
	Identity	Similarity			
Centromere binding					
Ndc10p	32	49	898	−	This study
Cep3p	42	63	610	−	This study
Ctf13p	35	56	441	−	This study
Skp1p	76	79	171	+	60
Mif2p	40	57	539	−	This study
Cse4p	49	61	207	+	This study
Cbf1p	25	34	441	+	61
Other					
His3p	72	82	210	+	33
Leu2p	80	86	364	+	Kitoda et al.
Trp1p	56	72	217	+	33
Ura3p	82	90	265	+	67
Act1p	100	100	375	+	64
Amt1p	33	51	225	+	68
Cdr1p	72	83	1,500	+	53
Gas1p	60	73	559	ND	64
Gas2p	60	71	559	ND	64
Hlpp	69	79	508	ND	38
Kex2p	52	68	803	ND	4
Knh1p	51	69	265	+	46
Kre9p	57	73	276	+	46

[a] Amino acid sequence alignments were obtained with the BLASTP program (BLOSUM62 matrix) (1) by using the NCBI server (http://www.nlm.nih.gov/blast/bl2seq/bl2.html.

[b] +, cross-complementation with *C. glabrata* gene in *S. cerevisiae*; −, no cross-complementation with *C. glabrata* gene in *S. cerevisiae*; ND, not determined.

stringency Southern blotting with the *S. cerevisiae* genes *NDC10*, *CEP3*, and *CTF13* as probes were unsuccessful, indicating that the genes are not highly conserved (data not shown). We also failed to detect cross-reactions with antibodies against the corresponding *S. cerevisiae* proteins on Western blots with *C. glabrata* crude cell extracts (data not shown). However, a BLAST search of the unassembled *C. glabrata* genome database with amino acid sequences of the inner kinetochore proteins of *S. cerevisiae* identified several stretches of amino acid sequences homologous to the proteins Ndc10p, Cep3p, Ctf13p, Mif2p and Cse4p. Based on the sequence homologies, we designed sets of degenerate primers and amplified portions of the genes from genomic DNA by PCR. The PCR fragments then were used to isolate the complete genes from a *C. glabrata* genomic library (see Materials and Methods).

The results of amino acid sequence comparisons of *S. cerevisiae* and *C. glabrata* inner kinetochore proteins are summarized in Table 2. The multiple-sequence alignments of these sequences are shown in Fig. S1a to e in the supplemental material. With the exception of that of Skp1p, the amino acid sequences of the inner kinetochore proteins of *C. glabrata* are moderately conserved; they are only 32 to 49% identical to the corresponding *S. cerevisiae* homologs, and their average amino acid sequence identity is 39%. In contrast, other proteins of *C. glabrata* are much more highly homologous to *S. cerevisiae* (Table 2), and their average amino acid sequence identity is 65%

CgNdc10p has the lowest identity with its *S. cerevisiae* homolog, and the alignment revealed clusters of homology that were distributed quite evenly throughout the whole sequence (Fig. S1a). A putative GTP binding consensus sequence in *S. cerevisiae* Ndc10p (ScNdc10p) that had been reported previously (aa 235 to 305) (29) is not conserved in CgNdc10p (aa 187 to 253 in the alignment). The translational start site of CgNdc10p overlaps the third methionine of ScNdc10p. It was reported that an expression construct containing ScNdc10p was more active in DNA binding when fused to a His_6 tag at Met-12 than when fused at Met-1 (51), and it is possible that the third methionine in ScNdc10p is the correct translational start site of the protein.

Of the CBF3 components, CgCep3p shows the highest homology to the corresponding *S. cerevisiae* protein (42% identity and 63% similarity) (Fig. S1b). Like the *S. cerevisiae* protein, it contains a Zn_2Cys_6 zinc cluster at its N terminus that is nearly identical to the zinc cluster in ScCep3p (aa 14 to 42) and an acidic domain at its C terminus (aa 577 to 607; negative net charge of −10) (40). A region between aa 298 and 428 is more highly conserved than the remainder of ScCep3p and is 57% identical between the proteins.

CgCtf13p is only 35% identical to the *S. cerevisiae* protein (Fig. S1c) but is fairly well conserved at its N terminus (aa 1 to 59; 43% identity and 63% similarity), which contains the F box (51). Most F-box residues that have been found to be essential in ScCtf13p are identical in CgCtf13p, with the exception of L-12, which is Q-12 in CgCtf13p (51). The putative PEST domain (aa 211 to 230) of ScCtf13p and a region required for binding to Skp1p and showing homology to the CCL1 domain (aa 360 to 420) (51) are not conserved in CgCtf13p.

The identity of CgMif2p to the *S. cerevisiae* homolog is 40%. The N terminus of CgMif2p and other parts of the protein are highly conserved (82% identity between aa 1 and 52 in *C. glabrata* and aa 1 and 55 in *S. cerevisiae*), including the CENP-C box (56% identity between aa 478 and 529 in *C.*

glabrata and aa 476 and 527 in *S. cerevisiae*) (Fig. S1d). The latter motif is conserved in eukaryotic species ranging from plants to humans (8).

CgCse4p is 49% identical to the *S. cerevisiae* homolog. The identity is primarily in the C-terminal histone H3-like half of the protein, which is known to be evolutionarily conserved among different organisms (54). The N-terminal half of the protein is only moderately conserved (aa 145 to 240; 24% identity and 41% similarity) (Fig. 1Ae). A stretch of 33 aa (END domain; aa 31 to 63) that has been identified in *S. cerevisiae* as being essential for function (11) is also moderately conserved in *C. glabrata*.

The CBF3 components are required for growth in *C. glabrata* and localize to the centromere. In *S. cerevisiae*, the CBF3 components Ndc10p, Cep3p, and Ctf13p as well as Mif2p and Cse4p are essentially required for growth. Depletion of any one of these gene products leads to cell death due to a chromosome segregation defect during mitosis (22, 40, 16, 7, 59). Haploid *S. cerevisiae* strains in which one of these genes is deleted in the genome can grow only if a plasmid bearing a wild-type copy of the gene is present in the cells. These strains, however, cannot grow on medium containing 5-FOA if the plasmid carries a *URA3* marker, because 5-FOA is toxic to cells that produce the *URA3* gene product. We constructed *C. glabrata* strains with deletions of the genomic copies of *CgNDC10*, *CgCEP3*, and *CgCTF13* (see Materials and Methods) (Table 1). As expected, these CBF3 deletion mutants require a wild-type copy of the deleted gene on a plasmid (Ura^+) for growth and do not grow on medium containing 5-FOA (data not shown), indicating that in *C. glabrata*, as in *S. cerevisiae*, these genes are essential.

To verify that these proteins are associated in vivo with *CEN* DNA, we carried out a standard ChIP analysis. To this end, *CgNDC10*, *CgCEP3*, and *CgCTF13* were fused at their 3′ ends to a PCR fragment expressing an HA epitope, and the fusion constructs on *HIS3*-bearing plasmids were introduced into *C. glabrata* cells. Plasmid shuffling then was performed by growing the transformants on 5-FOA-containing medium and selecting for cells that had lost *URA*-bearing plasmids containing the wild-type gene (see Materials and Methods; see also Table S1 in the supplemental material for plasmid constructs). The HA tag did not impair the functions of CgNdc10p and CgCep3p, but CgTS11/pTS124 (CgCtf13-HA) showed slow growth at 37°C, indicating that the HA tag at the C-terminal end of the protein might affect the function of the protein at the centromere (data not shown).

To determine whether the proteins are localized at the centromere in vivo, exponentially grown cells were treated with formaldehyde to cross-link cellular structures and then were lysed and sonicated to shear the chromatin. An HA-specific antibody was used to immunoprecipitate chromatin containing tagged CgNdc10p, CgCep3p, and CgCtf13p. After the cross-links were reversed, coimmunoprecipitated *CEN* DNA was amplified by PCR with *CEN*-specific primers. As a control, PCR was performed with primer pairs specific for *CgACT1* and *CgLEU2*. *CEN* DNA was found to be present only in the anti-HA antibody immunoprecipitate and not in the mock-treated control (no antibody) (Fig. 1). PCR products obtained with primer pairs specific for the *ACT1* and *LEU2* genes were detected only when the starting material (total) was used as the template. These results indicate that CgNdc10p, CgCep3p, and CgCtf13p are bound in vivo to the *C. glabrata CEN* region.

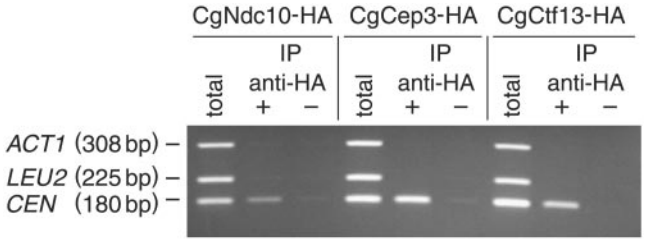

FIG. 1. Localization of CgNdc10-HA, CgCep3-HA, and CgCtf13-HA to *C. glabrata CEN* DNA by ChIP. *C. glabrata* strains CgTS10 (*Cgndc10*), CgTS12 (*Cgcep3*), and CgTS11 (*Cgctf13*) bearing plasmids expressing HA-tagged versions of *CgNDC10*, *CgCEP3*, and *CgCTF13* (pTS130, pTS131, and pTS124; see Table S1 in the supplemental material for plasmid constructs) were grown to late logarithmic stage. After in vivo cross-linking, extracts of these strains were immunoprecipitated (IP) with anti-HA antibody. Aliquots of the extracts (the load was 0.05 μl of chromatin solution, with the exception of CgCtf13-HA, for which the load was 0.025 μl) and the immunoprecipitates (2 μl of chromatin solution, except for CgCtf13-HA, for which 0.2 μl was used) were analyzed by PCR. PCR mixtures (see Table S2 in the supplemental material for primers) were designed to amplify the *CEN* DNA fragment in addition to two noncentromeric control fragments (*ACT1* and *LEU2*).

The CBF3 proteins are species specific. Although the *CEN* DNA sequences are highly homologous, the only *C. glabrata CEN* sequence (out of 14) that has been isolated so far is not functional in *S. cerevisiae*, and *S. cerevisiae CEN16*, which has the CDEIII sequence most similar to the *C. glabrata* centromere, functions only partially in *C. glabrata*. The species specificity is determined by both CDEII and CDEIII (35). Thus, it was of interest to determine whether the inner kinetochore protein homologs are species specific. Based on the ability of an *Scskp1* deletion strain containing *CgSKP1* on a *LEU2*-bearing plasmid to grow on 5-FOA (which means that the *ScSKP1* gene on a *URA3*-bearing plasmid can be lost), CgSkp1p was previously shown to complement an *Scskp1*Δ mutant (60). To determine whether the other *C. glabrata* inner kinetochore proteins could complement deletions of the corresponding genes in *S. cerevisiae*, we inserted the *C. glabrata* genes under the control of their own promoters into plasmids that contained either an *ScLEU2* or an *ScHIS3* marker and tested them in the corresponding *S. cerevisiae* deletion strains (plasmids pRSCg1, pTS32, pTS39, pTS37, pRSCg3D, and pTS34) (Table 1; see also Table S1 in the supplemental material for plasmid constructs). These strains contained the corresponding wild-type gene on *URA3*-bearing plasmids before the plasmids were shuffled in and were unable to grow on 5-FOA. We then grew these strains carrying two plasmids in the presence of 5-FOA. None of the transformant deletion strains with a mutation in *ScNDC10*, *ScCEP3*, *ScCTF13*, or *ScMIF2* was able to grow on 5-FOA-containing medium, indicating that the *C. glabrata* genes were not functional in *S. cerevisiae* (Tables 2 and 3). As a control, plasmids containing *CgNDC10*, *CgCEP3*, and *CgCTF13* were tested in the corresponding *C. glabrata* deletion strains (see below) and were found to be functional.

To rule out a promoter problem, *CgNDC10*, *CgCEP3*, and *CgCTF13* were brought under the control of an inducible *ScGAL10* promoter, tested again, and found to be not func-

TABLE 3. Species specificity of inner kinetochore proteins of *C. glabrata* and *S. cerevisiae*

Yeast strain[a]	Plasmids[b]	Species specificity[c] of:						
		Cbf1p	Ndc10p	Cep3p	Ctf13p	Skp1p	Mif2p	Cse4p
ScΔ/Sc-ts	ScP-ScG	+++	+++	+++	+++	+++	+++	ND
	CgP-CgG	++[d]	−	−	−	++[e]	−	+
	CgP-ScG	++	++	+++	+++	ND	ND	ND
	GaIP-CgG	ND	−	−	−	ND	ND	ND
	GalP-ScG	ND	+++	+++	+++	ND	ND	ND
CgΔ	CgP-CgG	+++	+++	+++	+++	ND	ND	ND
	CgP-ScG	++	−	−	−	ND	ND	ND

[a] Yeast strains carrying deletions in the genes that were tested for species specificity (ScΔ or CgΔ) or *ts* mutant strains (Sc-ts) are described in Table 1. The host strains for complementation carry mutations in the same genes as those that were tested.
[b] Abbreviations: Sc, *S. cerevisiae*; Cg, *C. glabrata*; P, promoter; G, gene. All plasmids are described in Table S1 in the supplemental material.
[c] +++, fast growth; ++, intermediate growth; +, slow growth; −, no growth; ND, not determined.
[d] See reference 61.
[e] See reference 60.

tional in the corresponding *S. cerevisiae* deletion strains (plasmids pTS78, pTS76, and pTS109) (Table 3). As a control, we tested the corresponding genes from *S. cerevisiae*, *ScNDC10*, *ScCEP3*, and *ScCTF13*, under the control of the *ScGAL10* promoter, in these strains and found that they were functional (plasmids pTS100, pTS96, and pTS98) (Table 3). Plasmid constructs carrying *C. glabrata* genes under the control of their own promoters were also transformed into several different *S. cerevisiae* temperature-sensitive (ts) mutant strains (*Scndc10*^{ts} [four different strains], *Sccep3*^{ts}, *Scctf13*^{ts}, and *Sccse4*^{ts} strains) (Table 1). These mutants grew normally at 25°C but stopped growing when shifted to the nonpermissive temperature (38°C) due to a chromosome segregation defect. Again, none of the *S. cerevisiae* ts mutants bearing mutations in CBF3 could be complemented by the *C. glabrata* genes, with the exception of the *Sccse4*^{ts} strain containing *CgCSE4*, which grew very slowly at the nonpermissive temperature (Table 3). We thus conclude that most of the inner kinetochore proteins, including the CBF3 subunits (with the exception of Skp1p) and Mif2p, are species specific for *C. glabrata*. The centromeric histone H3 variant CgCse4p, however, is somewhat functional in *S. cerevisiae*.

We also sought to determine whether the *S. cerevisiae* inner kinetochore proteins could function in *C. glabrata*. To this end, we introduced plasmids (His$^+$) carrying the corresponding *S. cerevisiae* genes under the control of a *C. glabrata* promoter (*CgNDC10*, *CgCEP3*, and *CgCTF13*; pTS128, pTS72, and pTS73) into *C. glabrata* deletion strains CgTS10/pTS46, CgTS11/pTS48, and CgTS12/pTS50. We found that none of the resulting *C. glabrata* strains could grow in the presence of 5-FOA. We thus conclude that the *S. cerevisiae* CBF3 genes are also species specific. As a control, we tested these constructs in the corresponding *S. cerevisiae* deletion strains and found them to be functional. These results indicate that the *C. glabrata* promoters for *NDC10*, *CEP3*, and *CTF13* are generally able to drive the expression of the corresponding genes in *S. cerevisiae*. The results of these experiments are summarized in Table 3.

TABLE 4. Effect of *CgCBF1* on the mitotic stability of a chromosome fragment in an *Sccbf1*Δ strain

Strain	Mitotic loss frequency[a]
ScD102-7A *CBF1*$^+$ (wild type)	1.4×10^{-3} (6,211)
ScD102-7A *cbf1*Δ[b]	2.3×10^{-2} (2,606)
ScD102-7A *cbf1*Δ/pADNS-*CgCBF1*	7.2×10^{-3} (8,212)
ScD102-7A *cbf1*Δ/pADNS-*SpCBF1*[b]	6.5×10^{-3} (7,087)

[a] Half-sector colonies/(total colonies − red colonies). Numbers in parentheses indicate the total number of colonies counted.
[b] See reference 45.

Determination of the species specificity of Cbf1p. In *S. cerevisiae*, Cbf1p is required both for methionine biosynthesis and for efficient centromere function (9, 43). In a previous study, Stoyan et al. showed that *CgCBF1* functionally complements the methionine biosynthesis defect of an *Sccbf1*Δ strain (61). To determine whether the effect of *CgCBF1* complements the chromosome segregation defect of an *Sccbf1*Δ strain, we introduced the *CgCBF1* gene under the control of an *ADH* promoter on a plasmid into an *Sccbf1*Δ strain carrying a nonessential *CEN*-bearing chromosome fragment (*Sccbf1*Δ/CF strain). The mitotic loss frequency of the chromosome fragment in a *CBF1* wild-type strain is 1.4×10^{-3} per generation; whereas in a *cbf1*Δ strain, the chromosome fragment is lost at a frequency of 2.3×10^{-2} per generation. The introduction of *CgCBF1* into the *Sccbf1*Δ/CF strain only partially complemented this chromosome segregation defect (Table 4). Similar partial complementation was observed when Cbf1p from the phylogenetically distant fission yeast *Schizosaccharomyces pombe* was introduced into the *Sccbf1*Δ/CF strain (45). The results are summarized in Table 4.

Although deletion of *ScCBF1* is not lethal in *S. cerevisiae*, *Cgcbf1* deletion has been shown to be lethal in *C. glabrata* due to a pronounced defect in chromosome segregation (61). We tested whether an *ScCBF1* gene under the control of a *C. glabrata* promoter could complement the *Cgcbf1* deletion. A *Cgcbf1*Δ strain carrying wild-type *CgCBF1* on a *URA3*-bearing plasmid was able to grow on 5-FOA when *ScCBF1* on a *HIS3*-bearing plasmid was present in the cells (Table 3). Taken together, these results show that *CBF1* can, to a certain extent, be functionally exchanged between these two yeasts.

Two-hybrid analysis reveals interactions between the *C. glabrata* inner kinetochore proteins. To gain insight into the organization of the inner kinetochore proteins of *C. glabrata*, we carried out an analysis of protein-protein interactions by using an *S. cerevisiae* two-hybrid system. The ORFs for the inner kinetochore genes were placed behind either a GAD or a GBD

TABLE 5. Yeast two-hybrid analysis of protein-protein interactions between *C. glabrata* and *S. cerevisiae* inner kinetochore proteins

Organism	GAD × GBD[a]	Growth of colonies on[b]:	
		AHB	HB
C. glabrata	CgCep3p × CgCtf13p	+++	+++
	CgNdc10p × CgCtf13p	+	+++
	CgCep3p × CgMif2p	+	+++
	CgSkp1p × CgCtf13p	−	+++
	CgCtf13p × CgCep3p	−	++
	CgNdc10p × CgNdc10p	−	++
	CgCep3p × CgCep3p	−	++
	CgNdc10p × CgCep3p	−	−
	GAD × CgMif2p	−	++
S. cerevisiae	ScCep3p × ScCtf13p	+++	+++
	ScNdc10p × ScCtf13p	+++	+++
	ScNdc10p × ScCep3p	−	−
Both (cross-species interactions)	ScCep3p × CgCtf13p	++	+++
	ScNdc10p × CgCtf13p	−	−

[a] Mating products expressing GAD and GBD fusion proteins; proteins on the left are GAD fusion proteins, and proteins on the right are GBD fusion proteins (see Table S1 in the supplemental material for vector constructions).
[b] AHB, growth on SD medium lacking Ade, lacking His, and containing α-galactoside, blue colony color; HB, growth on SD medium containing Ade, lacking His, and containing α-galactoside, blue colony color. +++, ++, +, and −, fast, intermediate, slow, and no growth, respectively.

in *S. cerevisiae* plasmid vectors and were expressed under the control of the constitutive *ADH* promoter in two yeast strains of opposite mating types (see Table S1 in the supplemental material for plasmid constructs). The expression of the GAD fusion proteins could be confirmed by Western blot analysis with an anti-GAD antibody, however an anti-GBD antibody failed to detect any of the proteins (data not shown). We used a two-hybrid system based on the three reporter genes, *ADE2*, *HIS3*, and *MEL1*, that are activated upon interaction of a GAD fusion protein with a GBD fusion protein (Matchmaker System 3; Clontech) (see Materials and Methods) (Table 1). The results of this study are summarized in Table 5.

Based on the ability of the strains to grow on various indicator media, we classified the interactions as strong, intermediate, and weak (Table 5). As might be expected for proteins functioning in a multisubunit complex (CBF3), we found a strong interaction between CgCep3p and CgCtf13p, an intermediate interaction between CgNdc10p and CgCtf13p, and several weak interactions between the inner kinetochore proteins (Table 5). A model summarizing these kinetochore protein interactions is shown in Fig. 2. No interactions were found for CgCbf1p and CgCse4p with any of the other proteins. GAD-CgCbf1p was functional in a *Cgcbf1*Δ strain (data not shown), but its interactions with the other proteins, if any, might have been too weak to be detected in the two-hybrid system. The inability of GAD-CgCse4p and GBD-CgCse4p to interact with the other proteins might have been due to steric hindrance by the GAD or GBD domains on the N terminus of CgCse4p. It is also possible that GBD-CgCbf1p and GBD-Cse4p were not sufficiently expressed in the yeast cells. None of the proteins, with the exception of CgMif2p and CgSkp1p, interacted nonspecifically with either GAD or GBD alone (data not shown).

Like the *C. glabrata* proteins, GAD-ScCep3p and GBD-ScCtf13p and GAD-ScNdc10p and GBD-ScCtf13p strongly interacted in the two-hybrid system (Table 5). No interaction could be detected between ScNdc10p and ScCep3p. None of the *S. cerevisiae* fusion constructs interacted with either GAD or GBD alone. We then tested whether we could detect any cross-species interactions between the CBF3 subunits from both species in the two-hybrid system. An intermediate interaction between GAD-ScCep3p and GBD-CgCtf13p was detected, indicating that the interspecies interaction is not as strong as the interaction of proteins from the same species (Table 5). Apparently, either the interspecies protein-protein interaction is not strong enough for CgCtf13p to complement the *ctf13*-null mutation in *S. cerevisiae* or the complete CBF3 complex does not form with a heterologous Ctf13p subunit. ScNdc10p and CgCtf13p did not interact (Table 5).

Analysis of the CgCep3p-CgCtf13p interaction. Deletion analysis was performed to further delineate the binding site of

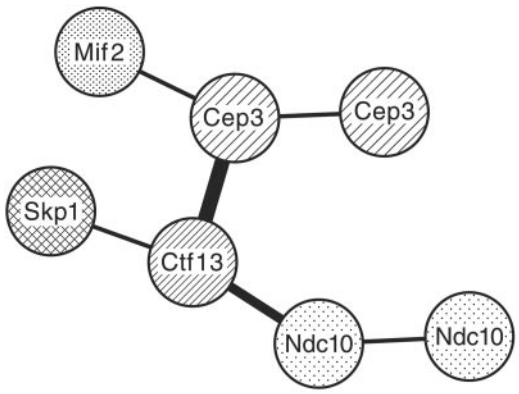

FIG. 2. Protein-protein interactions between inner kinetochore proteins of *C. glabrata*. The strength of the interaction is indicated by the line thickness: thick line, fast growth and blue colony color on SD medium lacking Ade, lacking His, and containing α-galactoside; medium line, slow growth and blue colony color on SD medium lacking Ade, lacking His, and containing α-galactoside; thin line, no growth on SD medium lacking Ade but fast to intermediate growth and blue colony color on SD medium lacking His and containing α-galactoside.

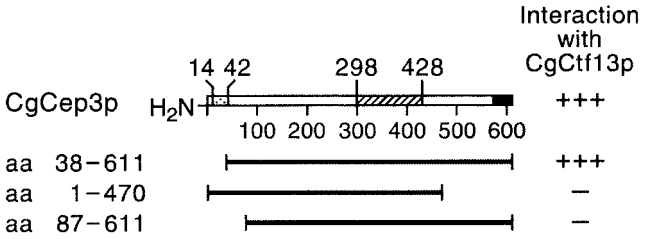

FIG. 3. Cep3p-Ctf13p interactions in a yeast two-hybrid system. Shown is the interaction of truncated GAD-CgCep3p with full-length GBD-CgCtf13p. Truncated versions of CgCep3p were generated by restriction digestion of pTS101 or by PCR (see Table S1 in the supplemental material). Symbols: +++, strong interaction, with growth on SD medium lacking Ade and lacking His and blue colony color; –, no interaction.

CgCep3p for CgCtf13p by using the two-hybrid system to assay for interactions (Fig. 3). Deletion of the Zn_2Cys_6 zinc cluster domain at the N terminus of CgCep3p (aa 1 to 38) did not affect CgCep3p binding to CgCtf13p. However, further deletion of the N terminus to aa 86 led to disruption of the interaction with CgCtf13p. Deletion of a portion of the C terminus of CgCep3p (aa 470 to 611) likewise led to abolishment of the interaction with CgCtf13p. We conclude that both the N terminus and the C terminus of CgCep3p are required for the interaction with CgCtf13p.

DISCUSSION

We have identified five inner kinetochore homologs in *C. glabrata* and have shown that the CBF3 components Ndc10p, Cep3p, and Ctf13p and Mif2p are species specific. This is the first example of a comparative study of the inner kinetochores of two closely related budding yeasts. Compared to those of other budding yeast species, the *CEN* DNAs of *S. cerevisiae* and *C. glabrata* are rather unique in their similarity. *CEN* DNAs that have been isolated from *Kluyveromyces* species, *Candida maltosa*, and *Yarrowia lipolytica* differ in one or more of the CDEI, CDEII, or CDEIII sequences (12, 25, 49). The close similarity of the *S. cerevisiae* and *C. glabrata* centromeres and the fact that they are functionally not exchangeable make them interesting objects for a comparative study of inner kinetochore protein structure and function.

CBF3 and Mif2p are species specific. The metabolic enzymes His3p, Leu2p, Trp1p, and Ura3p are 56 to 82% identical and functionally exchangeable between *C. glabrata* and *S. cerevisiae*, reflecting the fact that these species are closely related. Also, many other proteins that have been isolated from *C. glabrata*, including those involved in cell wall function or cell wall assembly, a regulatory endoproteinase, an ABC transport protein, and a hemolysin protein, are highly conserved between the species (51 to 72% identity), and all of those tested for cross-complementation are functionally exchangeable (Table 2). Given that their *CEN* DNA sequences are highly homologous, it is rather surprising that the inner kinetochore proteins of *C. glabrata* (with the exception of CgSkp1p) are only between 32 and 49% identical to the *S. cerevisiae* proteins and that the CBF3 subunits and CgMif2p are species specific. Reasons for this species specificity may be (i) sequence-specific binding of each protein to the *CEN* DNA, (ii) impaired DNA binding of a heterologous CBF3 complex to the *CEN* DNA, (iii) failure to correctly assemble the heterologous protein complexes, or (iv) a combination thereof. Although none of the CBF3 subunits is functionally exchangeable between the species, the interactions between them, such as those found in the two-hybrid analysis, are similar to those reported for *S. cerevisiae*. For *C. glabrata*, we found a strong interaction between CgCep3p and CgCtf13p, an intermediate interaction between CgNdc10p and CgCtf13p, and weak interactions between CgCtf13p and CgSkp1p and between CgCep3p and CgMif2p. CgNdc10p and CgCep3p interacted with each other, and no interaction was found between CgNdc10p and CgCep3p. In *S. cerevisiae*, both ScNdc10p and ScCep3p form triple complexes with the ScCtf13p-ScSkp1p heterodimer in solution. Furthermore, ScNdc10p does not bind to ScCep3p in the absence of ScCtf13p-ScSkp1p, and both ScNdc10p and ScCep3p have been shown to form homodimers (51, 28). A two-hybrid interaction between ScCep3p and ScMif2p has also been reported for *S. cerevisiae* (26, 44). The heterologous interaction between ScCtf13p and CgCep3p indicates that the binding site for ScCep3p in ScCtf13p is very similar to the corresponding binding site in CgCtf13p. Furthermore, the N-terminal zinc finger domain of CgCep3p, which is important for DNA binding, is unnecessary for CgCtf13p binding, indicating that both proteins most likely can form a complex in solution. These results suggest that the *C. glabrata* CBF3 complex is structurally quite similar to the *S. cerevisiae* CBF3 complex.

Although we were unable to detect any two-hybrid interactions between CgCse4p and the other proteins, CgCse4p has partial function in an *S. cerevisiae cse4 ts* strain, suggesting that, despite the low conservation of its N terminus, it is able to function heterologously in specialized nucleosomes. However, the *Sccse4*^{ts} mutant grew slowly when complemented by CgCse4p, indicating that its function at *S. cerevisiae CEN* is suboptimal.

CgCbf1p partially complements the chromosome segregation defect of an *Sccbf1*Δ mutant. A similar weak complementation has been observed with Cbf1p from the phylogenetically more distantly related fission yeast *S. pombe*. *S. pombe* Cbf1p does not seem to be involved in *CEN* function (45), and thus its effect on chromosome loss frequency in *S. cerevisiae* seems to be due to some general feature of the protein. A *Cgcbf1*Δ strain containing *S. pombe* Cbf1p is viable but grows somewhat more slowly than the wild type, indicating that *S. pombe* Cbf1p can, to some extent, function at the *C. glabrata* centromere. It was shown previously that Cbf1p induces bending of CDEI, which is directed toward the major groove, and bending at CDEI might contribute to centromere function (48). The DNA binding domain of CgCbf1p (helix-loop-helix leucine zipper; aa 283 to 393 in *C. glabrata*) is 66% homologous (46% identical) to that of ScCbf1p, and it is very likely that it can bend CDEI in a manner similar to that of the *C. glabrata* protein. However, the overall identity between both proteins is rather low, and other contacts between Cbf1p and CDEIII binding proteins may be impaired, explaining the weak complementation of the chromosome segregation defect. Taken together, these data indicate that although they are functionally distinct,

the inner kinetochores of *C. glabrata* and *S. cerevisiae* seem to be quite similar in their overall organization.

What determines the species specificity of *C. glabrata CEN* DNA? In *S. cerevisiae*, the proteins that have been shown to be in direct contact with DNA by UV cross-linking are the CBF3 components Ndc10p, Cep3p, and Ctf13p (17), and the three *C. glabrata* homologs have been localized in vivo to *CgCEN* DNA. It has been shown for *S. cerevisiae* that multiple subunits of Ndc10p bind to nonconserved sequences to the right of CDEIII and, together with CBF3, form an extended CBF3 complex (17). In addition, multiple subunits of Ndc10p also bind directly to CDEII, and it has been proposed that a polymeric Ndc10p complex formed on CDEII and CDEIII DNAs is the foundation for recruiting microtubule attachment proteins to kinetochores (18). Since both the CDEII region and the CDEIII core sequence along with nonconserved sequences to the right of CDEIII determine the species specificity of centromeres in both *C. glabrata* and *S. cerevisiae* (35), it can be assumed that multimeric Ndc10p binding to these elements contributes to the species specificity of *CEN* DNA. Interestingly, CgNdc10p is the least conserved of the inner kinetochore proteins examined here (32% identity to the *S. cerevisiae* homolog), and the two-hybrid analysis showed that it can interact with itself. The 26-bp CDEIII core sequence of the only *CEN* that has been identified and isolated so far from *C. glabrata* differs in only two positions from all 16 *S. cerevisiae* CDEIII core sequences, and these positions are not crucial for *CEN* function in *S. cerevisiae* (23). CgCep3p, which binds to CDEIII, carries the same zinc finger moiety at its N terminus as ScCep3p, and *CgCEN* DNA carries the same critical CCG triplet and surrounding bases in CDEIII as those required for ScCep3p binding (17). It thus seems unlikely that the DNA binding domain of Cep3p contributes to species specificity. In addition to Ndc10p, however, it is possible that Ctf13p and/or other as-yet-unidentified proteins contribute to *CEN* species specificity.

Rapid evolution of the inner kinetochore proteins. The low conservation of the inner kinetochore proteins and the fact that CgNdc10p, CgCep3p, CgCtf13p, and CgMif2p are not functional in *S. cerevisiae* suggest that the *CEN* binding proteins changed more rapidly during evolution than other proteins. Rapid evolution has been reported for genes that are involved in reproduction. For example, two genes that control mating in the unicellular green alga *Chlamydomonas reinhardtii* are very divergent from those of other *Chlamydomonas* species, and homologs could not be detected in low-stringency Southern blots (19, 62). It is assumed that the rapid evolution of reproductive proteins may be a driving force in speciation. Rapid evolution has also been found at the centromeres of higher eukaryotes, and it has been speculated that the rapid evolution of centromeres is driving speciation in *Drosophila* (41). Analysis of the genome sequence data indicates that *C. glabrata* descended from the same genome duplication event that *S. cerevisiae* underwent approximately 100 million years ago and diverged from *S. cerevisiae* after the genome duplication occurred but before the sorting process of differential gene loss was complete (55). It is tempting to speculate that the rapid changes in the inner kinetochore proteins of *S. cerevisiae* and *C. glabrata* contributed to their speciation during evolution. To be able to finally draw this conclusion, however, one would need to compare the central and outer kinetochore proteins of these two yeasts.

Finally, the CBF3 subunits are essential proteins in *C. glabrata*, and no human homologs have been identified so far. Thus, they would make excellent targets for developing antifungal drugs to be applied in prophylactic therapy against systemic *C. glabrata* infections. The identification of a strong interaction between CgCep3p and CgCtf13p will allow for the screening of small molecules that disrupt this interaction.

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