Formation of functional centromeric chromatin is specified epigenetically in *Candida albicans*

Mary Baum, Kaustuv Sanyal[†], Prashant K. Mishra, Nathaniel Thaler, and John Carbon[‡]

Department of Molecular, Cellular, and Developmental Biology, University of California, Santa Barbara, CA 93106-9610

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In the pathogenic yeast *Candida albicans*, the 3-kb centromeric DNA regions (*CEN*) of each of the eight chromosomes have different and unique DNA sequences. The centromeric histone CaCse4p (CENP-A homolog) occurs only within these 3-kb *CEN* regions to form specialized centromeric chromatin. Centromere activity was maintained on small chromosome fragments derived *in vivo* by homologous recombination of a native chromosome with linear DNA fragments containing a telomere and a selectable marker. An *in vivo* derived 85-kb truncated chromosome containing the 3-kb *CEN7* locus on 69 kb of chromosome 7 DNA was stably and autonomously maintained in mitosis, indicating that preexisting active *CEN* chromatin remains functional through many generations. This same 85-kb chromosome fragment, isolated as naked DNA (devoid of chromatin proteins) from *C. albicans* and reintroduced back into *C. albicans* cells by standard DNA transformation techniques, was unable to reform functional *CEN* chromatin and was mitotically unstable. Comparison of active and inactive *CEN* chromatin digested with micrococcal nuclease revealed that periodic nucleosome arrays are disrupted at active centromeres. Chromatin immunoprecipitation with antibodies against CaCse4p confirmed that *CEN7* introduced into *C. albicans* cells as naked DNA did not recruit CaCse4p or induce its spread to a duplicate region only 7 kb away from active *CEN7* chromatin. These results indicate that CaCse4p recruitment and centromere activation are epigenetically specified and maintained in *C. albicans.*

CENP-A | centromere | Cse4p | kinetochore

The centromere (*CEN*) is the *cis*-acting DNA site of kinetochore assembly and spindle attachment during chromosome segregation in mitosis and meiosis. The identity of these sites is determined by some combination of organism-specific DNA sequence motifs, the deposition of a *CEN*-specific histone protein, and often, the presence of adjacent heterochromatic (silenced) DNA (reviewed in ref. 1). *CEN* DNA in most eukaryotes is relatively large (40–4,000 kb) and contains species-specific arrays of satellite, microsatellite, or retrotransposon-like repeated DNAs. However, in most budding yeasts, including *Saccharomyces cerevisiae*, functional *CEN* DNA is quite small (<0.4 kb) and contains conserved protein binding motifs that are found on each chromosome. Despite this DNA sequence heterogeneity, centromeres in all eukaryotes studied to date are assembled into specialized chromatin containing a *CEN*-specific histone H3 variant in the CENP-A/Cse4p family (1, 2). It has been proposed that CENP-A/Cse4p serves as the epigenetic marker of *CEN* identity, but the mechanism by which this marking event takes place remains unknown (2, 3). The nucleosomal packaging of CENP-A/Cse4p-containing chromatin is also different from bulk chromatin in the yeasts. In *S. cerevisiae*, *CEN* chromatin is packaged into a single, Cse4p-containing, nuclease-resistant particle that is flanked by hypersensitive sites and phased nucleosomes (4–6). In the fission yeast *Schizosaccharomyces pombe*, the ≈10-kb central domain of the centromere, containing a 4- to 7-kb central core and the innermost portion of flanking inverted repeat DNA, is rich in CENP-A (Cnp1p), but appears nearly devoid of nucleosomes (7–10). Moreover, centromeric heterochromatin, formed usually on repeated DNA sequences at the *CEN* regions of most eukaryotes, including fission yeast, appears to be important for establishment of centromeres (1, 11). In flies, neocentromerization was reported on an otherwise euchromatic DNA region when this DNA was placed close to centric heterochromatin (12). Therefore, *CEN* identity is determined by many genetic and epigenetic factors, some of which might be species-specific.

In this study, we address how *CEN* identity is specified in an important pathogenic yeast, *Candida albicans* (13), in the apparent absence of conserved DNA sequences or repeated DNA arrays. We previously found that the eight *C. albicans* chromosomes contain different, unique CaCse4p-associated DNA sequences (≈3 kb), referred to here as the *CEN* regions, that lack conserved motifs and large proximal arrays of repeated DNAs (14). Instead, these 3-kb sites occur within 4- to 18-kb gene-free regions that appear to be flanked by euchromatin. Only one *CEN* region is found per chromosome, and deletion of the *CEN* severely destabilizes the chromosome from which it is removed. CaMif2p, a conserved *C. albicans* centromere protein homologous to mammalian CENP-C, colocalizes with CaCse4p, indicating that each 3-kb *CEN* region is indeed the site of kinetochore assembly (14).

An artificial chromosome containing species-specific *CEN* DNA, a selective marker gene, and autonomous DNA replication signal (*ARS*), introduced by standard DNA transformation methods into fission yeast or most budding yeasts, assembles *CEN* chromatin and a kinetochore *de novo* and exhibits *CEN* activity (9, 10, 15–19). However, *in C. albicans*, exogenously introduced *CEN* DNA does not load *de novo* with CaCse4p nor does the *CEN* activate, indicating that DNA sequence cues are insufficient alone to drive CaCse4p recruitment to the centromere. Therefore, *in vivo* recombination was used to truncate chromosome 7 to form a small chromosome fragment (CF) with an active *CEN*. This CF was isolated as naked DNA and shuttled back into *C. albicans* to examine factors that determine *CEN* identity (Fig. 1*A*). In *C. albicans*, the centromere appears to be entirely dependent on the preexisting chromatin state for its propagation as functional *CEN* chromatin.

Results

Mitotically Stable Truncated Chromosomes Could Be Generated *in Vivo* from *C. albicans*. Chromosomes 6 and 7 were truncated *in vivo* proximal to *CEN6* and *CEN7*, respectively, using a targeting vector containing a homologous region for recombination, a selectable marker (*ARG4* or *URA3*), and a telomere (20, 21). A

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Abbreviations: *CEN*, centromere; CF, chromosome fragment.

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[†]Present address: Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore 560064, India.

[‡]To whom correspondence should be addressed. E-mail: carbon@lifesci.ucsb.edu.

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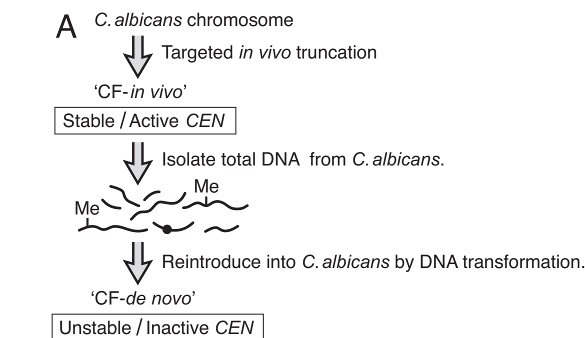

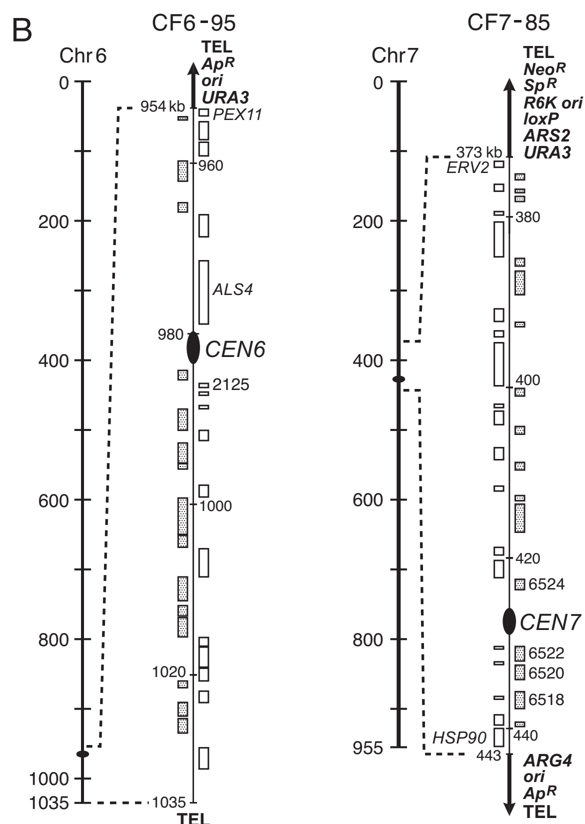

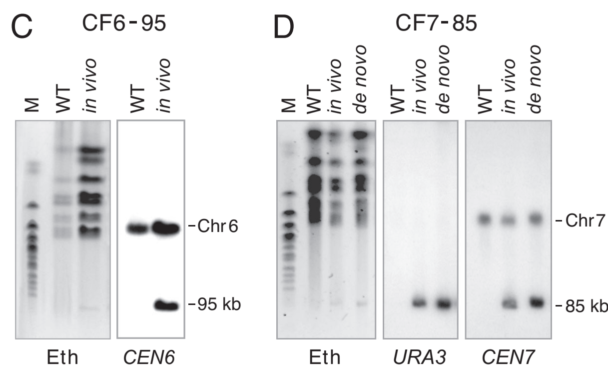

Fig. 1. Targeted *in vivo* truncation of chromosomes 6 and 7. (*A*) Strategy to evaluate the contribution of *CEN* DNA, *CEN*-proximal DNA, and epigenetic DNA modifications to *de novo* kinetochore assembly in *C. albicans*. The resulting phenotypes (boxed), CF *CEN* DNA (marked with black dot) and possible methylated bases (Me) are shown. (*B*) Derivation of CF6-95 and CF7-85. The regions are drawn to scale and numbered in kilobases. *CEN* DNA (filled oval), targeting vector sequences (thick lines), and ORFs (boxes) are indicated for chromosomes (Chr) 6 and 7. Pertinent ORFs are labeled with their assembly 19 designations or their putative homologous genes. Open and shaded boxes are transcribed from top to bottom and bottom to top, respectively. Chromosome maps are based on the following resources: Chibana *et al.* (22), http://candida.bri.nrc.ca, and www.candidagenome.org. (*C* and *D*) Electrophoretic karyotypes of *C. albicans* strains containing CF6-95 or CF7-85. Undigested chromosomal DNA prepared in agarose plugs was separated by clamped homogeneous electrical field electrophoresis to resolve CFs from native chromosomes. A reverse image of each ethidium-stained gel is compared with a Southern blot hybridized with a ^{32}P-labeled probe as indicated. Lane M, *S. cerevisiae* chromosome size markers; WT, strain BWP17; *in vivo*, CF strain derived by *in vivo* truncation; *de novo*, strain CF7-85 *de novo*, containing the CF introduced as naked DNA prepared from the *in vivo* truncation strain.

Table 1. Mitotic stability of *C. albicans CEN* plasmids

CEN construct in *C. albicans*	Size, kb/form*	Ura$^-$ or His$^-$ colonies/total	Loss frequency†
CF6-95 *in vivo*	95/L	4/962	4.2×10^{-3}
CF7-85 *in vivo*	85/L	10/1,623	6.2×10^{-3}
CF7-85 *de novo*	85/L	159/279	5.7×10^{-1}‡
CF7-440 *in vivo*	440/L	2/2,319	8.6×10^{-4}
CF7-590 *in vivo*	590/L	0/2,882	$<3.4 \times 10^{-4}$
CAKS4/pAB1CEN7	7.6/C	680/850	8.0×10^{-1}
CAI8/pMB4CEN6	16/C	539/554	9.7×10^{-1}
CAI8/pMB4CEN1	17/C	1,160/1,165	9.9×10^{-1}
BWP17/pCaCEN7-L	18/L	243/1,127	2.2×10^{-1}
BWP17/pAB1	4.7/C	1,041/1,161	9.0×10^{-1}
CAI8/pMB4	10.9/C	911/922	9.9×10^{-1}

*L, linear; C, circle.

†Loss frequency equals the number of colonies plated on rich media minus the number of Ura$^+$ or His$^+$ colonies divided by the number of colonies on rich media.

‡This value is an underestimate because the strain grows as a mixture of budding and pseudohyphal cells.

95-kb CF (CF6-95) containing 90 kb of genomic DNA from chromosome 6 and an 85-kb CF (CF7-85) containing 69 kb of genomic DNA from chromosome 7 were generated (Fig. 1*B*). CF6-95 *in vivo* is ≈10-kb larger than predicted from the *C. albicans* chromosome web site (http://candida.bri.nrc.ca), which we attribute to the presence of subtelomeric DNA that has not been reported in the database or a possible sequence insertion relative to the database reference strain SC5314. CF7-85 *in vivo* closely matches its predicted size. The electrophoretic karyotype of each strain reveals a new, autonomously replicating, linear CF that is not seen in a strain with a WT electrophoretic karyotype (Fig. 1 *C* and *D*; compare WT and *in vivo* lanes). Both CF6-95 *in vivo* and CF7-85 *in vivo* are mitotically stable; they are lost approximately once in 200 cell divisions (Table 1). This level of mitotic stability is comparable to that reported (≈10^{-3}) for *S. cerevisiae* linear artificial chromosomes of a similar size (23) containing the well characterized 125-bp point centromere. However, the small CFs are 5- to 12-fold less stable than large CFs containing *CEN7* and either centromere-distal half of *C. albicans* chromosome 7 (Table 1; compare to CF7-440 containing chromosome 7 from map positions 0–437 kb and CF7-590 containing chromosome 7 from map positions 373–955 kb; CF7-440 and CF7-590 share the region 373–437, which contains the *CEN*). Thus, each small CF contains an active *CEN* region that maintains a functional kinetochore.

An 85-kb CF That Exhibits *CEN* Function *in Vivo* Is Not Sufficient for *de Novo CEN* Activation If Shuttled As Naked DNA from *C. albicans* Back Into *C. albicans*. From strain CF7-85 *in vivo*, we isolated naked genomic DNA devoid of chromatin proteins, but retaining any DNA modifications, including DNA methylation, which might mark the *CEN* region for deposition of specialized chromatin (Fig. 1*A*). With 69 kb of collinear genomic DNA, this CF would

also contain *CEN*-proximal DNA that *in cis* could promote *de novo* assembly of specialized chromatin and kinetochore assembly, as previously demonstrated for fission yeast (10, 18, 24). CF7-85, representing ≈1/200 of the total genomic DNA of strain CF7-85 *in vivo*, transformed *C. albicans* strain BWP17 to Arg^+ Ura^+ prototrophy with low efficiency (three transformants per 30 μg of genomic DNA). Presence of the CF, designated CF7-85 *de novo*, was confirmed by genomic Southern blot analysis in two of the three isolates (Fig. 1*D*, a representative clone is shown). Naked *CEN* DNAs carrying native patterns of DNA modifications, however, did not assemble active kinetochores *de novo*. The "*de novo*" CF is 100-fold less stable mitotically than the identical "*in vivo*" CF (Table 1). The instability of CF7-85 *de novo* demonstrates that *CEN7* pericentric DNA does not promote assembly of *CEN* chromatin. In addition, DNA methylation, observed at a level of 0.1 and 0.05 mole percent 5-methyldeoxycytidine, respectively, for the budding and filamentous cell forms of *C. albicans* (25), is an insufficient signal for assembly of specialized *CEN* chromatin. Moreover, we found no evidence for methylation of *CEN1* DNA based on digestion with methylation-sensitive restriction enzymes and bisulfite sequencing (Fig. 5, which is published as supporting information on the PNAS web site).

Consistent with the result for CF7-85 *de novo*, centromeres on various circular and linear *CEN* plasmids constructed in *E. coli* and introduced as naked DNAs into *C. albicans* failed to activate and the plasmids were mitotically unstable. The mitotic stabilities of small circular plasmids pAB1CEN7 (2.9-kb *CEN7* insert, Fig. 6*A*, which is published as supporting information on the PNAS web site), pMB4CEN6 (5.4-kb *CEN6* insert), and pMB4CEN1 (6.1-kb *CEN1* insert) were equivalent to that of their respective vectors, pAB1 and pMB4; they were lost in at least eight of every 10 cell divisions (Table 1). The small linear plasmid pCaCEN7-L (7.2-kb *CEN7* insert, Fig. 6*B*) was also mitotically unstable; its loss frequency ranged from 96% to 22% in five *C. albicans* isolates (96%, 89%, 26%, 26%, and 22% loss; Table 1; not all data shown). We attribute this variation in stabilities to plasmid copy number differences among the five transformants. Likewise, the centromere on a large *CEN7* insert (145 kb corresponding to chromosome 7 map positions 373–520 kb) cloned in a linear BAC (26–28) modified with *C. albicans* telomeres (21) and *ARS2 URA3* for shuttling from *E. coli* into *C. albicans* did not activate (pBACRetroCEN7-L; plasmid construction is described in *Supporting Text*, which is published as supporting information on the PNAS web site). Because of the high rate of recombination in *C. albicans*, large circular BACs with 87- to 180-kb *CEN1*, *CEN6*, or *CEN7* inserts integrated into the genome and large linear BACs caused *in vivo* truncation of native chromosomes (data not shown). Analysis of the electrophoretic karyotype of five Ura^+ isolates transformed with plasmid pBACRetroCEN7-L indicated that none contained the 160-kb linear artificial chromosome, but four contained a new mitotically stable 590-kb CF that hybridized with a *CEN7* probe. CF7-590 contained BAC vector sequences at only one end, indicating that the linear BAC underwent a single recombination event with chromosome 7. This 590-kb CF is mitotically stable, indicating it received the native copy of *CEN7*. The reciprocal 530-kb recombination product, containing the other half of chromosome 7 and the exogenous *CEN7* region from the BAC clone, was not detected by Southern hybridization, suggesting that it was functionally acentric. Thus, for every *CEN* construct introduced as naked DNA, the *CEN* region failed to activate to form a functional centromere. Apparently, DNA sequence cues alone are insufficient to initiate functional kinetochore assembly at the *C. albicans CEN* loci. Overall, these observations strongly suggest that functional centromeric chromatin is epigenetically inherited in *C. albicans*.

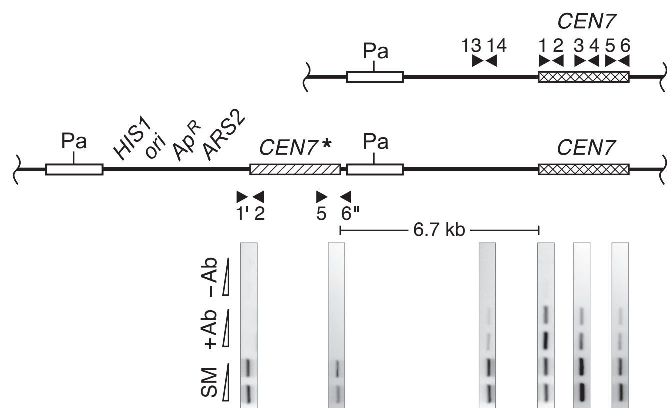

Fig. 2. CaCse4p is present only at native *CEN* loci. Formaldehyde cross-linked chromatin from *C. albicans* strain CAKS5, carrying an extra 3-kb *CEN7* sequence (*CEN7**) integrated 6.7 kb away from its native location, was fragmented and immunoprecipitated with antibodies against CaCse4p. Enrichment of CaCse4p bound to endogenous *CEN7* and exogenous *CEN7** was assayed by PCR using primers corresponding to the numbered black arrowheads (Table 3). Cross-hatched box, native *CEN7* region; hatched box, *CEN7** region introduced by recombination as naked DNA; open box, target sequence for integration; targeting site Pa, PacI; SM, starting material; +Ab, ChIP with anti-CaCse4p; −Ab, mock ChIP. Primers 1′ and 6″ anneal to vector sequences adjacent to *CEN7**. Primers 1–6 anneal to both *CEN7* and *CEN7**. Primers 13 and 14 anneal within the non-ORF region between *CEN7* and Orf19.6522.

Naked *CEN* DNA Cannot Activate and Is Unable to Recruit the *CEN* Histone CaCse4p. In flies, the homolog of CaCse4p, Cid, has been observed to spread from its native site to neighboring pericentric regions (12). We investigated whether a second *CEN7* region (*CEN7**), integrated 6.7 kb from the native locus on *C. albicans* chromosome 7, would induce recruitment of CaCse4p (Fig. 2). Circular plasmid pAB1IntCEN was linearized with PacI to target insertion of the entire plasmid into chromosome 7 by site-directed integration between ORFs 6518 and 6520 (Fig. 1*B*). Formaldehyde cross-linked chromatin from this strain (CAKS5) was immunoprecipitated with antibody against aa1–18 of CaCse4p (29) and deproteinized, and the DNA was examined with PCR primers that distinguish between loading of CaCse4p at native and nonnative loci. PCR with *CEN7**-specific primer pairs 1′–2 and 5–6″ showed *CEN7** to be free of CaCse4p, indicating that CaCse4p is present only on the preestablished *CEN7* locus (Fig. 2, primer pairs 1–2 and 5–6). Thus, the mechanism that directs CaCse4p to the *C. albicans CEN* is so precise that a duplicate sequence introduced as naked DNA 6.7 kb from its native cognate site is incapable of recruiting CaCse4p or inducing its spread. Consistent with this result, ChIP-PCR revealed that 2.9- and 7.2-kb *CEN7* regions introduced by transformation into *C. albicans* on circular pAB1CEN7 or linear pCaCEN7-L plasmids, respectively, do not load CaCse4p (Fig. 6). Thus, when introduced into *C. albicans* cells as naked DNA by transformation, neither circular nor linear plasmids containing the *CEN7* region loaded the centromeric histone CaCse4p into *CEN* chromatin, and the centromere did not activate.

Nucleosome Ladders Are Disrupted at Specialized *CEN* Chromatin Containing CaCse4p. In budding and fission yeasts, the nucleosome profile of Cse4p/CENP-A-assembled chromatin is distinct from that of bulk chromatin, which contains regularly spaced nucleosomes, recognizable as a ladder of DNA fragments after partial digestion with micrococcal nuclease (MNase), deproteinization, and gel electrophoresis (4, 8, 9). We found that *C. albicans CEN* chromatin also gives a distinctive nucleosomal pattern in this assay. Mononucleosomes, dinucleosomes, and a smeared pattern

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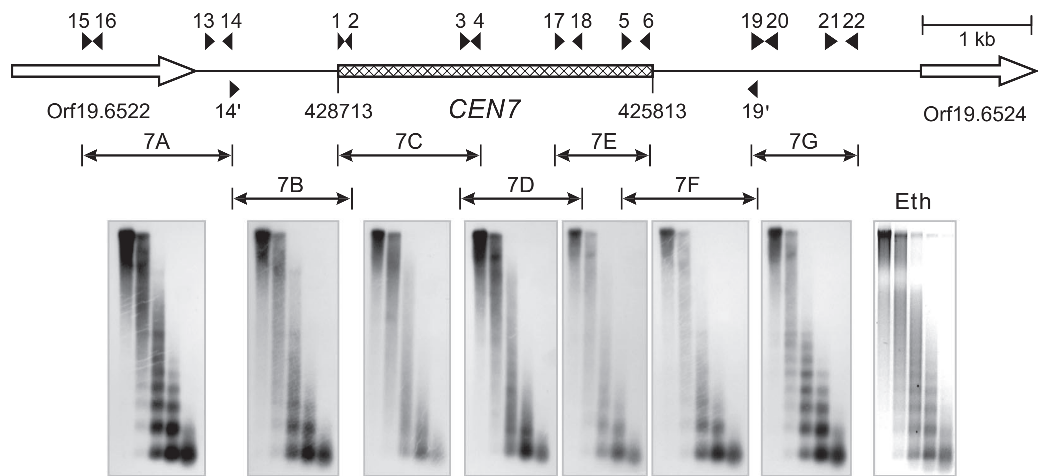

Fig. 3. Micrococcal nuclease treatment reveals an unusual chromatin structure at the *C. albicans CEN*. Chromatin from strain CAI4 was digested with MNase for 2, 5, 10, 20, and 40 min. Purified DNA was separated by gel electrophoresis, Southern transferred to membranes, and hybridized to labeled probes (see below). Bulk nucleosomes are shown in a reverse image of one ethidium bromide-stained gel for comparison to the nucleosome profiles detected by hybridization with ^{32}P-labeled probes 7A–7G. Probes are aligned with a map of the *CEN7* region drawn to scale. The boundary map positions of *CEN7* are shown in base pairs. Arrowheads, PCR primers used to amplify probes (7A, 1,434 bp; 7B, 1,141 bp; 7C, 1,346 bp; 7D, 1,130 bp;7E, 882 bp; 7F, 1,216 bp; 7G, 872 bp).

were detected at *CEN7* (Fig. 3, probes 7C–7E), but there was an absence of nucleosome ladders. Ordered arrays of nucleosomes identical to ethidium bromide-stained bulk nucleosomes begin ≈1 kb from either side of the CaCse4p-bound *CEN7* region (Fig. 3, probes 7A and 7G). A similar smeared chromatin pattern is seen for the *Schizosaccharomyces pombe* centromeric central core region, which appears nearly devoid of nucleosomes (8). In both *Schizosaccharomyces pombe* and *C. albicans* the regions with unusual chromatin patterns correspond to the regions enriched for the *CEN*-specific histones Cnp1p and CaCse4p, respectively (7, 14).

Unusual *CEN* Chromatin Packaging Is Correlated with *CEN* Function. In fission yeast, the unusual smeared chromatin seen at active *CEN*

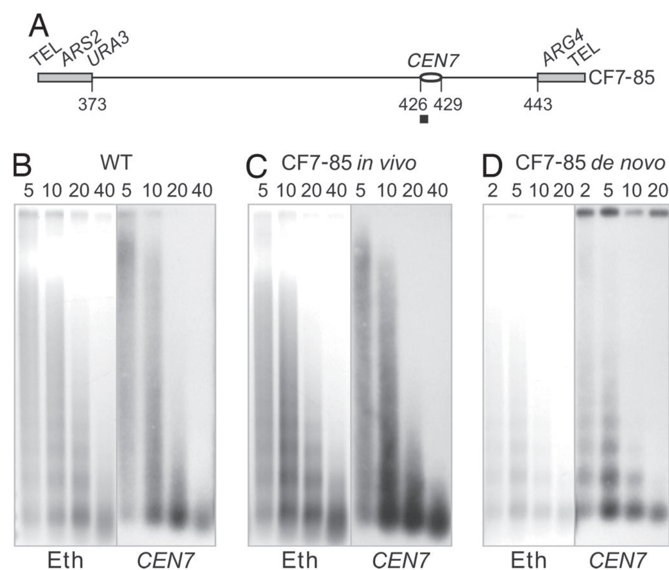

Fig. 4. Chromatin structure of *CEN7* on *CEN*-active and *CEN*-inactive CFs. (*A*) CF7-85 schematic. Black box, *CEN7* probe 7C (see Fig. 3). (*B–D*) Chromatin from the indicated strains (WT, strain BWP17) were digested with MNase for the length of time (min) shown above each lane. Purified DNA was separated by electrophoresis and a reverse image of each ethidium bromide (eth)-stained gel is shown next to its nucleosome profile detected with a ^{32}P-labeled 1.4-kb *CEN7* probe.

regions is replaced with a periodic nucleosome ladder if *CEN* function is inactivated (7, 10, 30, 31). We compared MNase-treated *CEN* chromatin from *C. albicans* strains CF7-85 *in vivo* (*CEN*-active) and CF7-85 *de novo* (*CEN*-inactive). Treatment of strain CF7-85 *in vivo*, containing active *CEN*s on both full-length chromosome 7 and CF7-85, revealed a smeared *CEN7* chromatin pattern that is indistinguishable from the WT pattern (Fig. 4 *A–C*). However, MNase digestion of chromatin from strain CF7-85 *de novo*, with an active *CEN* on full-length chromosome 7 and an inactive *CEN* on CF7-85, revealed an ordered nucleosome ladder coincident with bulk nucleosomes in the ethidium bromide-stained gel (Fig. 4*D*). The intensity of the hybridizing ladder compared with the background smear for native chromosome 7 indicates that CF7-85 is multicopy, as would be expected for cells grown on minimal medium selective for the functionally acentric CF. The correlation between the presence or absence of nucleosome ladders at inactive or active *CEN*s, respectively, suggests that kinetochore assembly precludes or masks formation of periodic nucleosome arrays. This observation presumably reflects higher order folding of specialized *CEN* chromatin at the site of kinetochore assembly and spindle attachment. The experiments described above indicate that specialized *CEN* chromatin does not form *de novo* in *C. albicans*, and suggests that *CEN* chromatin in this yeast is propagated from generation to generation via a templated mechanism.

Discussion

Mitotically stable artificial chromosomes bearing functional *CEN*s have been constructed *in vitro* in several budding yeasts, including *S. cerevisiae*, *Kluyveromyces lactis*, *Candida glabrata*, and also in the fission yeast *Schizosaccharomyces pombe* (15–19). In these organisms, when a naked DNA construct containing centromeric DNA sequences is introduced into the cell by standard DNA transformation techniques, the centromere region acquires the proper chromatin conformation *de novo* and the resulting kinetochore becomes fully functional, both in mitotic and meiotic cell divisions. Although functional kinetochores apparently form *de novo* quite rapidly on artificial chromosomes in the budding yeasts, a delay of several generations has been observed in the fission yeast system when the artificial chromosome lacks an inverted centromeric repeat or has a severely diminished inverted repeat length (24). Once activated, the mitotic stability of an artificial chromosome containing an

incomplete *CEN* is nearly indistinguishable from a full-length fission yeast *CEN*. The latter observation led Steiner and Clarke (24) to conclude that the formation of centromeric chromatin and a functional kinetochore is templated epigenetically by preexisting chromatin, especially in organisms containing regional centromeres with relatively large amounts of specialized centromeric chromatin.

The observations reported in this paper indicate that in *C. albicans* formation of functional *CEN* chromatin and kinetochores *de novo* onto naked DNA either does not occur spontaneously, or occurs so slowly that it is masked experimentally by other competing processes such as recombination. Introduced as naked DNA, neither an 85-kb truncated chromosome shown to have an active *CEN in vivo* nor artificial chromosomes containing *CEN*7 on 3–145 kb of genomic DNA plus suitable DNA replication signals, marker genes for selection (and telomeres if linear), were capable of recruiting CaCse4p and forming a functional kinetochore. It is possible that DNA sequences outside of the region examined contribute to the establishment of *CEN* chromatin, but once formed, may not be necessary for its maintenance. Such a model would preserve *CEN* function on CFs formed *in vivo*, but prevent *de novo* acquisition of centromeric chromatin on naked DNA. The locus control region at the multigene globin locus physically interacts with and affects chromatin at an actively expressed globin gene, requiring looping distances of 40–60 kb (32, 33). Interaction between remote accessory DNA sequences and the *C. albicans* CaCse4p-bound region, however, would require interaction between sequences separated by larger distances than previously observed. The most probable explanation of our results is that, in *C. albicans*, the formation of functional centromeric chromatin is templated epigenetically and is incapable of forming *de novo* on naked DNA. In many other organisms with regional centromeres, such as *Drosophila* and *Neurospora*, it also has not been possible to construct functional artificial chromosomes *in vitro*.

The 3-kb *CEN* region in *C. albicans* appears to be analogous to the 4- to 7-kb central core region within the fission yeast centromere. Both regions bind a *CEN*-specific histone H3 variant in the CENP-A family (CaCse4p or Cnp1p) and a second conserved inner kinetochore protein in the CENP-C family (CaMif2p or SpCENP-C), indicating each region serves as the site of kinetochore assembly and spindle microtubule attachment (7, 14, 34, 35). In both yeasts, CENP-A chromatin appears to be assembled into irregularly spaced nucleosomes with arrays no longer than two nucleosomes in length. In both organisms, this unusual pattern is correlated with *CEN* function, implying that kinetochore assembly displaces some nucleosomes or masks their presence. Arrays of regularly spaced nucleosomes, as seen in bulk chromatin, resume immediately outside of CENP-A chromatin. *C. albicans* pericentric regions lack large arrays of repeated DNA in contrast to most regional centromeres, including those of fission yeast. Located within the outer repeat array of fission yeast *CEN* DNA, the *cis*-acting dg/K repeat promotes *de novo* assembly of *CEN* chromatin as assayed by MNase digestion or by mitotic stability (10, 18). The fission yeast central domain alone cannot assemble *CEN* chromatin *de novo*, analogous to the *C. albicans CEN* (10, 18). It has also been shown that heterochromatin structure is required for *de novo CEN* formation on a linear human artificial chromosome containing centromere-competent α21-I alphoid repeats (36). Thus, assembly of *CEN* chromatin may be mediated through epigenetic processes operating at heterochromatin, such as RNAi and histone modification (11). Divergent *CEN* evolution among yeasts may have resulted in the loss of pericentric heterochromatin and the inability to assemble *CEN* chromatin *de novo* in *C. albicans*.

The exact molecular cues dictating positional information for CENP-A deposition remain unclear. To account for the wide variation in centromeric DNAs among eukaryotes and the inability to activate centromeres on artificial chromosomes in many of these organisms, it is likely that centromere inheritance is propagated by a mechanism that does not rely on the underlying DNA sequence. Proposed mechanisms to account for this invoke partitioning of CENP-A nucleosomes by the advancing replication fork during DNA replication or a marking of centromeres when under spindle tension, which couples future recruitment of CENP-A to successful biorientation of kinetochores onto the mitotic spindle at metaphase (2, 11). The existence of a different *CEN* DNA sequence on each of the eight *C. albicans* chromosomes is consistent with the concept that the maintenance of functional *CEN* chromatin operates through a templated system. Unlike *Drosophila CEN* chromatin, deposition of CaCse4p appears to be tightly regulated, as duplication of its binding region close to its cognate binding site did not induce CaCse4p spreading. *C. albicans CEN* chromatin barriers may prevent spreading *in vivo*, but not contribute to *de novo* assembly of specialized *CEN* chromatin. In other eukaryotes, centromeric repeated DNA may aid *de novo* centromere formation by recruiting DNA-binding proteins and setting up a heterochromatic environment. Hence, it is possible that in the absence of heterochromatin, at least some centromere-bound CaCse4p may be constitutively required to mark the centromere domain in *C. albicans*. With relatively small centromeres, *C. albicans* promises to be a useful system to elucidate factors that control centromere-specific histone deposition and maintenance.

Materials and Methods

Strains, Media, Transformations, Primers, and Plasmids. *C. albicans* strains are listed in Table 2, which is published as supporting information on the PNAS web site, and their construction is described in *Supporting Text*. *C. albicans* strains were grown at 30°C in YPD (1% yeast extract/2% peptone/2% dextrose) supplemented with adenine and/or uridine (each 50 μg/ml) or SD (0.67% yeast nitrogen base/2% dextrose) supplemented with adenine, uridine, and amino acids (0.01%) as necessary. *C. albicans* transformations were performed by standard methods (37, 38). The PCR primers used in this study are listed in Table 3, which is published as supporting information on the PNAS web site, and detailed descriptions of the plasmids used here are described in *Supporting Text*.

Chromosome Truncation. Targeting regions for chromosome fragmentation were amplified by PCR with primer pairs CaALD1-1 and -2 (orf19.6518), CaHSP90-F and -R (orf19.6515), and CaPEX11-1 and -2 (orf19.1089). They were cloned as a 1.5-kb BamHI–KpnI *ALD1* fragment (Contig19–10248, nucleotides 121453–119973), an 0.8-kb BamHI–XhoI *HSP90* fragment (Contig19–10248, nucleotides 115561–114783), and a 1-kb BamHI–ClaI *PEX11* fragment (Contig19–10090, nucleotides 30392–29384) into vectors pCaCFU (*URA3*) or pCaCFA (*ARG4*). BamHI-digested pCaCFU-ALD1, pCaCFA-HSP90, pCaCFU-PEX11 were introduced into *C. albicans* by spheroplast transformation. Arg^+ or Ura^+ transformants that resulted from homologous recombination were identified by PCR and verified by clamped homogeneous electrical field electrophoresis.

ChIP, Antibodies, and Primers. Standard ChIP experiments used 4×10^9 cells that were fixed with formaldehyde for 15 min and disrupted by sonication with three to four 12-s bursts, resulting in chromatin sheared to an average size of 500 bp as described (3). Antibodies directed against CaCse4p (29) were used for ChIP at a final concentration of 4 μg/ml. Protein–nucleic acid cross-links were reversed overnight at 65°C, and purified DNA was recovered for use as PCR template.

Mitotic Stability Assays. A replica plating method was used to measure the mitotic stability of the plasmids described here. Serial dilutions of a single *C. albicans* colony pregrown on media

selective for the plasmid marker were plated for single colonies on rich media and grown at 30°C until colonies were ≈1 mm in diameter. These colonies were replica plated to minimal media lacking uridine or histidine and grown for 2 days at 30°C. The loss frequencies of *URA3*- or *HIS1*-marked plasmids were calculated as described in Table 1.

MNase Digestion, DNA Purification, and Electrophoresis. *C. albicans* nuclei were prepared from 500-ml cultures grown to logarithmic phase (≈3-g cell pellet) using the method described for fission yeast (10). In brief, cells were converted to spheroplasts in 20 mM Hepes, pH 7.4, 1.2 M sorbitol, 0.5 mM PMSF with Lysing enzymes (Sigma catalog no. L1412, St. Louis, MO) and Zymolyase 100T (MP Biomedicals, Aurora, OH). Spheroplasting was stopped by washing in 20 mM Pipes (pH 6.8), 1.2 mM sorbitol, 1 mM PMSF. Spheroplasts were lysed in 20 mM Pipes (pH 6.8), 18% Ficoll 400, 0.5 mM $MgCl_2$, 1 mM PMSF. Nuclei were released by vortexing and harvested by centrifugation through a glycerol/Ficoll cushion. Nuclei were resuspended in 20 mM Pipes (pH 6.4), 0.1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 mM PMSF. Nuclei were prewarmed for 3 min at 30°C followed by the addition of MNase to 250 units/ml (Roche, Mannheim, Germany) for the indicated times. Nuclease digestion was stopped by adjusting aliquots to 2% SDS, 20 mM EDTA. Purified DNA was electrophoresed on 1.4% agarose gels in chilled (4°C), recirculated 0.5× TAE buffer at 180 V for 140 min.

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1. Cleveland DW, Mao Y, Sullivan KF (2003) *Cell* 112:407–421.
2. Sullivan KF (2001) *Curr Opin Genet Dev* 11:182–188.
3. Henikoff S, Dalal Y (2005) *Curr Opin Genet Dev* 15:177–184.
4. Bloom KS, Carbon J (1982) *Cell* 29:305–317.
5. Schulman I, Bloom KS (1991) *Annu Rev Cell Biol* 7:311–336.
6. Meluh PB, Yang P, Glowczewski L, Koshland D, Smith MM (1998) *Cell* 94:607–613.
7. Takahashi K, Chen ES, Yanagida M (2000) *Science* 288:2215–2219.
8. Polizzi C, Clarke L (1991) *J Cell Biol* 112:191–201.
9. Takahashi K, Murakami S, Chikashige Y, Funabiki H, Niwa O, Yanagida M (1992) *Mol Biol Cell* 3:819–835.
10. Marschall LG, Clarke L (1995) *J Cell Biol* 128:445–454.
11. Pidoux AL, Allshire RC (2005) *Philos Trans R Soc B* 360:569–579.
12. Maggert KA, Karpen GH (2001) *Genetics* 158:1615–1628.
13. Odds FC (1994) *J Am Acad Dermatol* 31:S2–S5.
14. Sanyal K, Baum M, Carbon J (2004) *Proc Natl Acad Sci USA* 101:11374–11379.
15. Clarke L, Carbon J (1985) *Ann Rev Genet* 19:29–56.
16. Hahnenberger KM, Baum MP, Polizzi CM, Carbon J, Clarke L (1989) *Proc Natl Acad Sci USA* 86:577–581.
17. Heus JJ, Zonneveld BJM, Steensma HY, Van den Berg JA (1990) *Curr Genet* 18:517–522.
18. Baum M, Ngan VK, Clarke L (1994) *Mol Biol Cell* 5:747–761.
19. Kitada K, Yamaguchi E, Arisawa M (1996) *Gene* 175:105–108.
20. Vollrath D, Davis RW, Connelly C, Hieter P (1988) *Proc Natl Acad Sci USA* 85:6027–6031.
21. McEachern MJ, Hicks JB (1993) *Mol Cell Biol* 13:551–560.
22. Chibana H, Oka N, Nakayama H, Aoyama T, Magee BB, Magee PT, Mikami Y (2005) *Genetics* 170:1525–1537.
23. Newlon CS (1988) *Microbiol Rev* 52:568–601.
24. Steiner NC, Clarke L (1994) *Cell* 79:865–874.
25. Russell PJ, Welsch JA, Rachlin EM, McCloskey JA (1987) *J Bacteriol* 169:4393–4395.
26. Frengen E, Weichenhan D, Zhao B, Osoegawa K, van Geel M, de Jong PJ (1999) *Genomics* 58:250–253.
27. Magin-Lachmann C, Kotzamanis G, D'Aiuto L, Wagner E, Huxley C (2003) *BMC Biotechnol* 3:2
28. Mejia JE, Larin Z (2000) *Genomics* 70:165–170.
29. Sanyal K, Carbon J (2002) *Proc Natl Acad Sci USA* 99:12969–12974.
30. Hayashi T, Fujita Y, Iwasaki O, Adachi Y, Takahashi K, Yanagida M (2004) *Cell* 118:715–729.
31. Takahashi K, Takayama Y, Masuda F, Kobayashi Y, Saitoh S (2005) *Philos Trans R Soc B* 360:595–607.
32. Carter D, Chakalova L, Osborne CS, Dai Y, Fraser P (2002) *Nat Genet* 32:623–626.
33. Tolhuis B, Palstra R-J, Splinter E, Grosveld F, de Laat W (2002) *Mol Cell* 10:1453–1465.
34. Holland S, Ioannou D, Haines S, Brown WRA (2005) *Chromosome Res* 13:73–83.
35. Saitoh S, Ishii K, Kobayashi Y, Takahashi K (2005) *Mol Biol Cell* 16:3666–3677.
36. Nakashima H, Nakano M, Ohnishi R, Hiraoka Y, Kaneda Y, Sugino A, Masumoto H (2005) *J Cell Sci* 118:5885–5898.
37. Burgers PM, Percival KJ (1987) *Anal Biochem* 163:391–397.
38. Hull CM, Johnson AD (1999) *Science* 285:1271–1275.