CSE4 Genetically Interacts With the *Saccharomyces cerevisiae* Centromere DNA Elements CDE I and CDE II but Not CDE III: Implications for the Path of the Centromere DNA Around a Cse4p Variant Nucleosome

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ABSTRACT

Each *Saccharomyces cerevisiae* chromosome contains a single centromere composed of three conserved DNA elements, CDE I, II, and III. The histone H3 variant, Cse4p, is an essential component of the *S. cerevisiae* centromere and is thought to replace H3 in specialized nucleosomes at the yeast centromere. To investigate the genetic interactions between Cse4p and centromere DNA, we measured the chromosome loss rates exhibited by *cse4 cen3* double-mutant cells that express mutant Cse4 proteins and carry chromosomes containing mutant centromere DNA (*cen3*). When compared to loss rates for cells carrying the same *cen3* DNA mutants but expressing wild-type Cse4p, we found that mutations throughout the Cse4p histone-fold domain caused surprisingly large increases in the loss of chromosomes carrying CDE I or CDE II mutant centromeres, but had no effect on chromosomes with CDE III mutant centromeres. Our genetic evidence is consistent with direct interactions between Cse4p and the CDE I-CDE II region of the centromere DNA. On the basis of these and other results from genetic, biochemical, and structural studies, we propose a model that best describes the path of the centromere DNA around a specialized Cse4p-nucleosome.

CSE4P and CENP-A are variant histone H3 proteins involved in centromere structure and function in *Saccharomyces cerevisiae* and mammals, respectively. Cse4p and CENP-A have C-terminal histone-fold domains that are >60% identical to the histone-fold domain of H3 (Sullivan *et al.* 1994; Stoler *et al.* 1995). Amino acids throughout the histone-fold domains of Cse4p and CENP-A participate in a cooperative manner to specify centromere structure and function, indicating that the two proteins mediate formation of centromeric chromatin by a common mechanism despite the differences in centromere DNA (Shelby *et al.* 1997; Keith *et al.* 1999). The highly divergent N termini of Cse4p, CENP-A, and H3 are localized outside the nucleosome and interact with transcription factors and chromatin remodeling factors in the case of H3 and kinetochore proteins in the case of Cse4p.

The histone-fold domain is an evolutionarily conserved protein motif shared by the four core histones and a variety of proteins involved in DNA metabolism (Baxevanis *et al.* 1995). The X-ray crystal structure of the core histones shows that the histone-fold domain consists of three α-helical structures (helix I, II, and III) separated by β-loop strands (loop I and II; Luger *et al.* 1997). The histone-fold domain mediates H3-H4 and H2A-H2B dimer interactions in which the two long central helices (helix II) cross each other in an antiparallel fashion, juxtapositioning loop I of one protein with loop II of the other protein (Luger *et al.* 1997). The dimers have three major sites, two loop I-loop II sites and a helix I-helix I site, that contact the minor groove of the DNA approximately every 10 bp. The histone-fold domain is involved in H4-H3/H3-H4 tetramer formation that is mediated through a four helix bundle interaction involving the C terminus of helix II, loop II, and helix III of each H3 molecule. The $(H3\text{-}H4)_2$ tetramer mediates initial contact with the DNA at the dyad axis. Two H2A-H2B dimers bind separately to the $(H3\text{-}H4)_2$ tetramer through four helix bundle interactions involving H2B and H4. The resulting core octamer binds ~150 bp of DNA.

CENP-A is a mammalian centromere-specific H3 variant that is always associated with active centromeres and is a key component of centromeric chromatin. CENP-A copurifies with core histones (Palmer *et al.* 1987) and forms homodimers *in vivo*, suggesting that CENP-A replaces both H3 molecules in specialized nucleosomes (Shelby *et al.* 1997). The histone-fold domain of CENP-A functions to localize the protein to the mammalian centromere where it binds predominantly to α-satellite DNA in phased arrays of nucleosomes (Vafa and Sullivan 1997).

The yeast H3 centromere variant, Cse4p, is essential

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for cell viability and proper mitotic chromosome transmission (Stoler *et al.* 1995). Mutations in *CSE4* can cause cell cycle arrest at G2/M and increased chromosome missegregation. The high amino acid homology among the histone-fold domains of Cse4p, H3, and CENP-A indicates that Cse4p most likely replaces H3 in a subset of yeast nucleosomes. In addition to similarities with H3 and CENP-A, several lines of evidence show that Cse4p functions as a nucleosome core histone in yeast. Biochemically, Cse4p is an integral chromatin protein with physical and chemical properties resembling histone H3 (Stoler *et al.* 1995). Mutations in the histone-fold domain of Cse4p disrupt chromatin structure at the centromere (Meluh *et al.* 1998). Furthermore, evidence also supports interactions between Cse4p and other histones. Overexpression of *CSE4* suppresses *hhf1-20*, a mitosis-specific H4 allele that causes mitotic arrest at the nonpermissive temperature and increased chromosome missegregation (Smith *et al.* 1996). High dosage of *CSE4* also rescues the defective centromeric chromatin structure observed in *hhf1-20* cells, further supporting a direct interaction between Cse4p and H4 at the centromere (Meluh *et al.* 1998).

S. cerevisiae accomplishes very high fidelity chromosome segregation using just 125 bp of centromere DNA present on each chromosome. All 16 *S. cerevisiae* centromeres contain three conserved centromere DNA elements (CDE), CDE I, CDE II, and CDE III (Fitzgerald-Hayes *et al.* 1982; Fleig *et al.* 1995). CDE I is bound by a homodimer of CP1 (also called Cbf1p, Cpf1p; Bram and Kornberg 1987; Baker *et al.* 1989; Cai and Davis 1989), but neither CDE I nor the bound CP1 protein is essential for centromere function and abolishing either one increases chromosome missegregation 10- to 30-fold (Baker and Masison 1990). Apparently, the CDE I/CP1 complex functions to enhance centromere function and promote high fidelity mitotic chromosome transmission, probably by maintaining favorable chromatin structures at the centromere. CDE III is a 26-bp DNA element containing a highly conserved central region that is absolutely essential, since a single base-pair mutation in CDE III can completely abolish centromere function (McGrew *et al.* 1986). CDE III is bound by the CBF3 complex, which consists of three structural proteins, p110, p64, and p58, and a regulatory protein, Skp1p. The CBF3 complex, along with at least three other proteins, Ctf19p, Mcm21p, and Okp1p, are thought to mediate a connection between the centromere DNA and microtubule (Ortiz *et al.* 1999). Recently, we have shown that a unique domain in the N terminus of Cse4p interacts directly with the Ctf19p-Mcm21p-Okp1p outer kinetochore complex (Chen *et al.* 2000) The CDE II DNA elements from all 16 yeast chromosomes have similar characteristics, including conserved length (78–87 bp), intrinsic bends, high A + T base composition (>90%), and short repeated tracts of A or T residues. All four features are important determinants that contribute to the function of CDE II in chromosome segregation (Cumberledge and Carbon 1987; Gaudet and Fitzgerald-Hayes 1987; Murphy *et al.* 1991).

Yeast centromere DNA is organized into a unique chromatin structure, where 160–220 bp of DNA, including CDE I, II, and III, are protected from nuclease digestion and are flanked by arrays of phased nucleosomes (Schulman and Bloom 1991). Histone proteins are directly involved in this unique chromatin structure, since depletion of either histone H4 or H2B renders the centromere DNA sensitive to nuclease digestion (Saunders *et al.* 1990).

Current models of the yeast kinetochore propose that the yeast centromere DNA is wrapped around a Cse4p variant nucleosome. Here we present evidence that the histone-fold domain of Cse4p interacts specifically with CDE I and CDE II centromere DNA. We made a series of mutations distributed throughout the histone-fold domain of Cse4p that alter amino acids that, by analogy with H3 in the nucleosome crystal structure, contact or are adjacent to regions of the protein that directly interact with the DNA (Luger *et al.* 1997). The *cse4* histone-fold domain mutations were analyzed for synthetic chromosome loss phenotypes when combined in the same cell with chromosomes carrying mutant *cen3* centromere DNA. Our results show genetic interactions between Cse4p and CDE I and CDE II, but no detectable interactions between Cse4p and CDE III DNA. We propose a model supported by current genetic and biochemical evidence that predicts the most likely position of the centromere DNA around a specialized Cse4p variant nucleosome. The implications of this novel chromatin structure on centromere function and chromosome segregation in yeast and humans are discussed.

MATERIALS AND METHODS

Yeast strains and plasmids: To integrate mutant centromeres into chromosome III, *cen3* mutants cloned in pJUP [3B14, BCT1 (McGrew *et al.* 1986); CAT1, P130-3, X78, X69 (Gaudet and Fitzgerald-Hayes 1987); and GA/TG] or pJII (MOI, MO4, and MO4B, Murphy *et al.* 1991) were linearized with *Eco*RI and used to transform KC99 or KC140 cells (Table 1). Proper integrants, verified by Southern blot analysis (data not shown), were mated with KC148 or KC151 to generate the 10 strains used in the chromosome loss assays. Mutant alleles of *CSE4* were made by site-directed mutagenesis using the Clontech transformer site-directed mutagenesis kit (CLONTECH, Palo Alto, CA) following the vendor's instructions and using pCSE4HA as template DNA (Stoler *et al.* 1995). Plasmids were transformed into the chromosome loss strains and transformants were cured of pHCC4 or pC4/RS318 (YEp351 and pRS318, respectively, containing the *Cla*I-*Dra*I fragment of *CSE4*) by isolating LEU− auxotrophs. Plasmid pCSE4HA contains the HA epitope-tagged allele of wild-type *CSE4* cloned into pRS314 (*CEN4/ARS/TRP1*; Stoler *et al.* 1995).

Mitotic chromosome loss assays: The rate of mitotic loss of marked copies of chromosome III was quantified using

TABLE 1

Yeast strains used in this study

Strain	Relevant genotype	Source
KC99	*MAT*α *ade2-101 his3-11,15 leu2-3 lys2-801 trp1Δ901 ura3-52 cyh2 cse4::HIS3* plus pC4/RS318 (*CSE4* in pRS318)	This study
KC140	*MAT*α *ade2-101 his3-11,15 leu2-3 lys2-801 trp1Δ901 ura3-52 cse4::HIS3* plus pHCC4 (*CSE4* in YEp351)	This study
KC148	*MAT***a** *ade2-101 his3-11,15 leu2-3 trp1Δ901 ura3-52 cse4::HIS3 cyh2* plus pC4/RS318 (*CSE4* in pRS318)	This study
KC151	*MAT***a** *ade2-101 his3-11,15 leu2-3 trp1Δ901 ura3-52 cse4::HIS3* plus pHCC4 (*CSE4* in YEp351)	This study

fluctuation analysis as described previously (Hegemann *et al.* 1988; Keith *et al.* 1999). A pink colony was picked from nonselective color indicator plates [0.6% yeast nitrogen base without amino acids (Difco, Detroit), 0.5% casamino acids, 2% glucose, and 30 mg of uracil and 4.5 mg of adenine per liter] and grown 4–6 hr at 30° in media that selects for the *cse4* mutant plasmid (*TRP1*) and the marked chromosome (*URA3*). Cultures were diluted and ~50 cells were plated onto color indicator plates and incubated at 30° for 18–20 hr. Six to eight equally sized test colonies for each mutant tested were picked with a Pasteur pipette on an agar plug, resuspended in water, vortexed, and half the volume was spread onto color indicator plates (150 × 15 mm). Plates were incubated at 30° for 5 days and at 4° for 4–6 days to allow colony colors to fully develop. The total number of cells plated onto the large indicator plates represents the number of cell divisions that occurred during test colony growth and the number of red colonies represents the number of cells that lost the marked chromosome prior to plating. The total number of colonies and the total number of red colonies were counted and the values doubled to reflect the total number of cells originally picked. From the total number of colonies and the number of red colonies the median number of cells without the marked chromosome was calculated and used to determine the mean number of chromosome loss events during growth of the test colony (Lea and Coulson 1949). The mean number of chromosome loss events divided by the total number of cell divisions that occurred during test colony growth is the rate of chromosome loss per cell division.

RESULTS

Analysis of *cse4-cen3* double mutants: To investigate the functional relationship between Cse4p and centromere DNA, we employed a genetic approach in which we tested the effect of *cse4* mutations on the segregation of chromosomes carrying mutations in either CDE I, CDE II, or CDE III DNA. It has been previously shown that mutant centromere proteins that have little or no effect on the function of wild-type centromeres can significantly increase the missegregation events involving chromosomes carrying mutant centromeres (McGrew *et al.* 1989; Meluh and Koshland 1995; Strunnikov *et al.* 1995; Baker *et al.* 1998). Such synergistic effects can result when the mutations alter regions usually involved in protein-centromere DNA interactions.

The mitotic loss rates of marked (*URA3*, *SUP11*) copies of chromosome III containing either wild-type *CEN3* or mutant *cen3* DNA were determined by fluctuation assays performed in *cse4* null diploid cells with the lethal phenotype covered by wild-type Cse4p expressed from a plasmid (materials and methods). Fluctuation assays are highly sensitive, allowing recognition of loss rates as low as two- to threefold (Hegemann *et al.* 1988). The loss rate for chromosomes with wild-type *CEN3* was 0.17×10^{-3} (approximately two loss events per 10,000 cell divisions; Figure 1). The *cen3* centromere DNA mutations studied cause a range of increases in chromosome loss. For instance, the X78 centromere, which has a small deletion mutation in CDE II, causes only a slight increase in chromosome loss (0.60×10^{-3}), while BCT1, which has a single base-pair change in CDE III, causes dramatic chromosome loss (47.5×10^{-3}).

***cse4* mutants have little or no effect on the segregation of chromosomes with wild-type centromeres:** We made several *cse4* alleles containing mutations that alter regions containing potential Cse4p-centromere DNA contact sites (Figure 2; Luger *et al.* 1997). To preserve the nucleosome structure and limit disruption of histone-histone interactions, we replaced the Cse4p residues with the specific amino acids found at the analogous position in yeast H3. The two *cse4* alleles with H3 residue substitutions in the N-helix, *cse4-52* and *cse4-53*, overlap, as do the N-loop and helix I substitution alleles, *cse4-49* and *cse4-50*. The *cse4* loop I alleles contain a point mutation or a single amino acid deletion in *cse4W-F* and *cse4ΔD*, respectively. Three residues in loop II/helix III are changed in *cse4-56* and a single helix III amino acid is substituted in *cse4-101*. Immunoblot analysis shows that the *cse4* mutant and wild-type proteins have identical steady-state expression levels in yeast cells containing wild-type or mutant centromeres (data not shown; Keith *et al.* 1999).

We tested whether the mutant *cse4* alleles affect the segregation of chromosomes carrying wild-type *CEN3* DNA (Table 2). Diploid yeast shuttle strains were constructed containing a marked chromosome III copy carrying either a wild-type *CEN3* or mutant *cen3* centromeres and with both *CSE4* genes disrupted (*cse4::HIS3/*

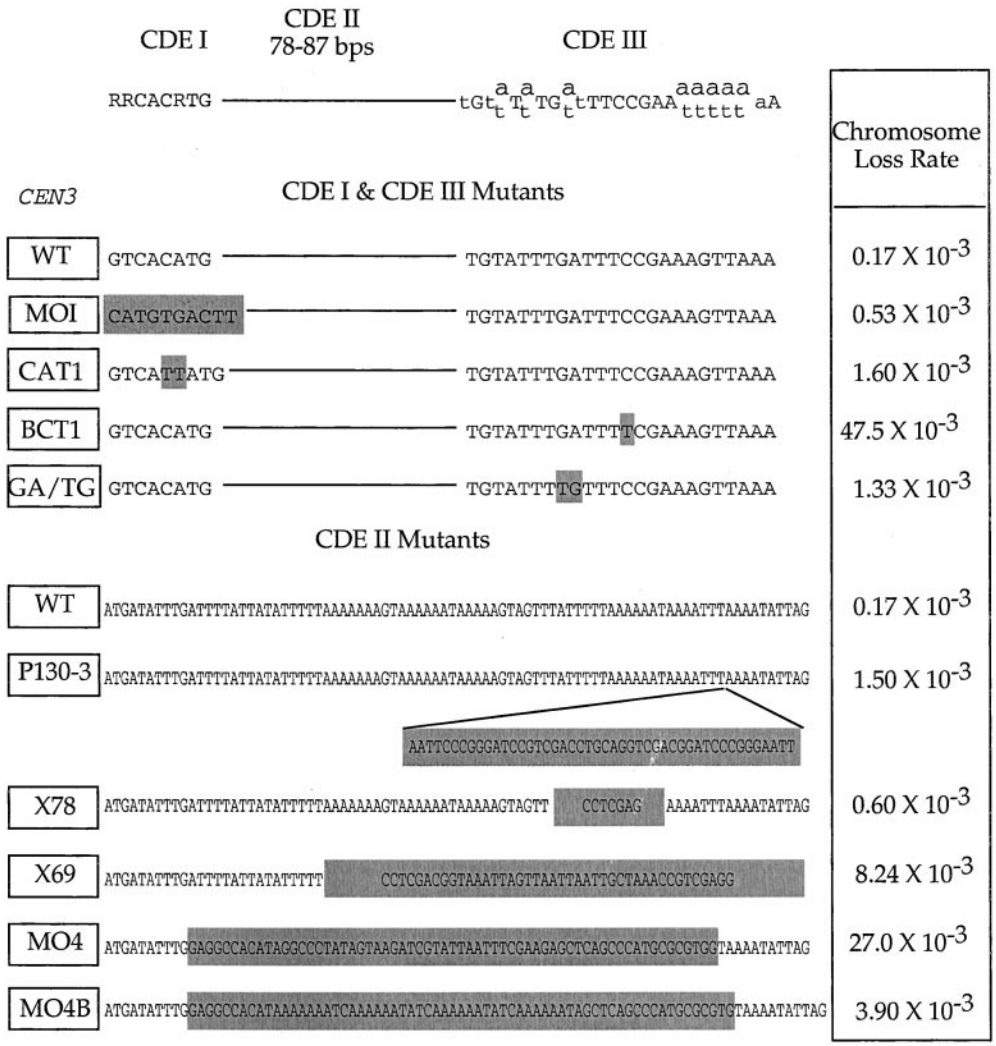


FIGURE 1.—*Cis*-acting mutations in *CEN3* DNA. The sequences of the nine different *cen3* mutations tested in the mitotic chromosome loss assays are shown compared to the wild-type *CEN3* sequence and the centromere consensus sequence derived from all 16 budding yeast centromeres. The shaded regions indicate the base pairs altered by each mutation. The chromosome loss rates determined using fluctuation analysis are shown at the right (chromosome loss events per cell division). The loss rates of chromosome III copies containing mutant *cen3* centromeres were measured in wild-type Cse4HAp cells.

cse4::HIS3) and the lethal phenotype covered with a plasmid containing *CSE4*. These strains were transformed with low-copy plasmids carrying a mutant *cse4* allele, after which the wild-type *CSE4* plasmid was removed so that the function of the mutant Cse4 protein could be studied in the absence of wild-type Cse4 protein. The chromosome loss rates per cell division for the mutant and wild-type *CEN3* chromosomes were measured in cells that express either a wild-type *CSE4* or mutant *cse4* allele. The fold increases in chromosome loss rates caused by the mutant *cse4* alleles were obtained by comparison to congenic cells expressing wild-type *CSE4* (Figure 3). Notably, none of the *cse4* alleles caused a significant increase in the loss of chromosomes with wild-type *CEN3* DNA (1.1- to 1.9-fold; Table 2).

Mutations in both *CSE4* and CDE I or CDE II cause synergistic increases in chromosome loss: The CAT1 *cen3* mutation changes a highly conserved C in CDE I to TT, which severely decreases CP1 binding (BAKER *et al.* 1989) and results in a chromosome loss rate of 1.6×10^{-3} in wild-type *CSE4* cells (Figure 1). Some CAT1 CDE I, *cse4* double mutants exhibited synthetically high chromosome loss rates compared to the rate for the identical *cen3* mutant chromosome in wild-type *CSE4* cells. Three *cse4* alleles, *cse4ΔD*, *cse4W-F*, and *cse4-101*, cause large increases in mitotic loss of the CAT1 chromosome (12- to 29-fold), while *cse4-49* causes a moderate increase (6.8-fold) and *cse4-53* had a small effect (2.9-fold; Table 2 and Figure 3). The MOI *cen3* mutation contains an inversion of CDE I relative to CDE II and CDE III and causes a chromosome loss rate of 0.53×10^{-3} (Figure 1; MURPHY *et al.* 1991). Moderate increases in loss rates of chromosomes with MOI mutant centromeres were observed for cells with *cse4-49* (4.0-fold), *cse4W-F* (4.1-fold), and *cse4-101* (2.8-fold) alleles, while the other *cse4* alleles had small or no detectable effects (Table 2 and Figure 3).

Five CDE II *cen3* mutations were tested that either alter the length or reduce the A + T base composition of CDE II, or both (Figure 1). The P130-3 *cen3* mutation increases the length of CDE II from 84 to 130 bp, resulting in a chromosome loss rate of 1.5×10^{-3} (Figure 1; GAUDET and FITZGERALD-HAYES 1987). The *cse4W-F* and *cse4-101* alleles caused high increases in P130-3 chromosome loss (16.6- and 14.2-fold, respectively), while *cse4-49* and *cse4-53* caused a moderate increase (4.6-fold;

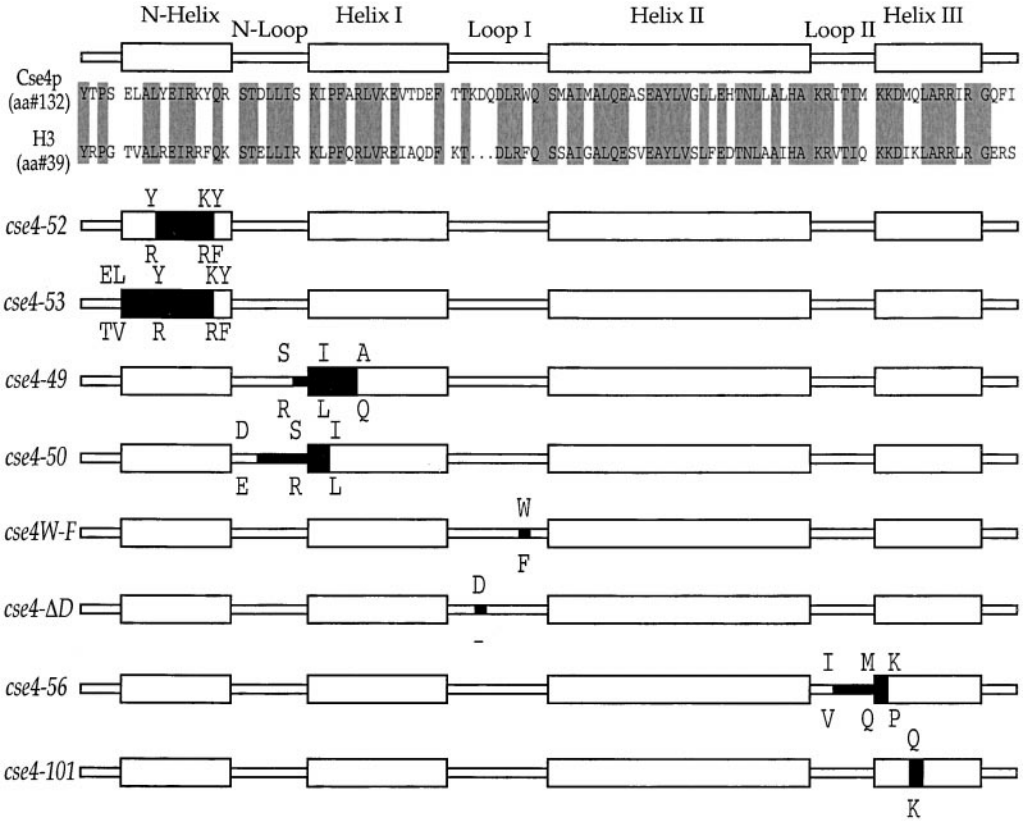


FIGURE 2.—Cse4p histone-fold domain mutants. Residues in the histone-fold domain of Cse4p were substituted with amino acids from analogous positions in histone H3. Sequence alignment of the histone-fold domains of *S. cerevisiae* H3 and Cse4p proteins is shown at the top of the figure; amino acids that are identical between the two proteins are shaded. The positions of the α-helices and β-loops determined from the nucleosome X-ray crystal structure are noted above the Cse4p and H3 amino acid sequences.

Table 2 and Figure 3). Centromere mutants X78 and X69 have shortened CDE II regions that cause chromosome loss rates of 0.60×10^{-3} and 8.24×10^{-3}, respectively (Figure 1; GAUDET and FITZGERALD-HAYES 1987). The *cse4-49* and *cse4W-F* allele had a moderate effect on X78 *cen3* chromosome loss (3.9- and 4.4-fold, respectively), while *cse4-52*, *cse4-53*, *cse4-49*, *cse4-50*, *cse4ΔD*, *cse4W-F*, and *cse4-101* all caused moderate increases in the loss rate of X69 *cen3* chromosomes (4.3- to 7.1-fold; Table 2 and Figure 3). The MO4 and MO4B *cen3* mutations maintain the length of CDE II while changing the A + T composition or the intrinsic bend of CDE II (MURPHY *et al.* 1991). The MO4 mutation reduces the A + T composition from 93 to 53% and results in a chromosome loss rate of 27×10^{-3} (Figure 1). Alleles *cse4-52*, *cse4-53*, *cse4-49*, and *cse4ΔD* caused moderate increases in the chromosome loss rates of MO4 *cen3* chromosomes (4.2- to 5.0-fold; Table 2 and Figure 3). The MO4 *cen3* chromosomes exhibit high loss rates even in wild-type *CSE4* cells and were too unstable for fluctuation assays when combined with *cse4* mutations that cause increases >7- to 8-fold, such as *cse4W-F* and *cse4-101*. MO4B enhances the intrinsic bend in CDE II, resulting in a chromosome loss rate of 3.9×10^{-3} (Figure 1). Alleles *cse4-49*, *cse4ΔD*, and *cse4W-F* caused moderate increases and *cse4-101* caused a high increase (12.4-fold) in the loss rate of MO4B *cen3* chromosomes (Table 2 and Figure 3).

Mutations in *CSE4* do not increase missegregation of CDE III mutant chromosomes: Two CDE III mutations were tested, BCT1 and GA/TG *cen3*. The integrity of CDE III is essential for centromere function because specific CDE III sequences are required for the recognition and assembly of the CBF3 kinetochore complex. Mutations that change bases in the central region of CDE III have drastic effects on centromere function even in *CSE4* wild-type cells. For instance, the BCT1 mutation, which changes the essential central CCG triplet to TCG in CDE III (MCGREW *et al.* 1986), causes a chromosome loss rate of 47.5×10^{-3} in *CSE4* cells (Figure 1). Mutations that change bases flanking the central region, such as the GA/TG CDE III mutation, have relatively low chromosome loss rates (1.33×10^{-3}). In contrast to CDE I and CDE II, none of the *cse4* mutant alleles significantly affected the loss rates of chromosomes carrying either the BCT1 or GA/TG CDE III centromere mutations (Table 2 and Figure 3). In fact, all of the CDE I and CDE II mutant centromeres we tested showed increased chromosome loss rates of up to 28-fold when combined with the various *cse4* mutant alleles, while the largest increase observed for the CDE III mutants was 1.4-fold.

DISCUSSION

Cse4p has two distinct domains, an essential, unique N terminus necessary for interactions with the Ctf13p/Mcm21p/Okp1p/CBF3 complex at CDE III and the highly conserved C-terminal histone-fold domain that is thought to be involved in a unique centric nucleosome

TABLE 2

Chromosome loss rates per mitotic division of *cen3* mutants ($\times 10^3$)

CSE4 Allele	*CEN3* Wild type	CDE I Mutant *cen3*		CDE II Mutant *cen3*					CDE III Mutant *cen3*	
		CAT1	MOI	P130-3	X78	X69	MO4	MO4B	GA/TG	BCT1
CSE4	0.17	1.6	0.53	1.50	0.60	8.24	27	3.9	1.33	47.5
cse4-52	0.37	1.9	1.03	1.52	1.13	26.7	93	4.3	1.31	51.5
cse4-53	0.23	4.7	0.76	7.0	0.80	28.8	133	5.3	1.40	50.4
cse4-49	0.30	10.9	2.13	7.0	2.31	59.9	118	23.2	1.56	21.2
cse4-50	0.27	1.7	0.87	2.7	0.80	28.4	55	4.2	0.86	44.5
cse4W-F	0.32	23.5	2.19	24.9	2.64	47.9	Acentric	20.3	1.46	50.1
cse4ΔD	0.32	26.7	0.50	2.1	1.34	35.7	114	16.3	1.01	50.9
cse4-56	0.19	1.9	0.70	2.7	0.87	8.4	52	5.2	0.72	34.0
cse4-101	0.21	45.9	1.50	21.4	1.15	55.7	Acentric	47.9	1.26	66.4

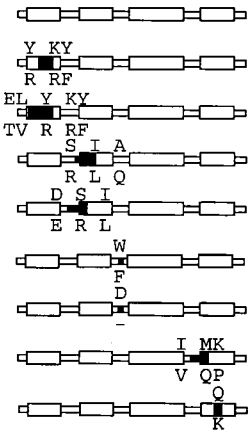
Chromosome loss rates were determined by fluctuation analysis using the method of the median (Lea and Coulson 1949; materials and methods).

(Stoler *et al.* 1995 and Chen *et al.* 2000). Our genetic results reported here indicate that the histone-fold domain of Cse4p engages in critical interactions with the CDE I and CDE II regions of the centromere DNA, but does not interact with CDE III DNA. Synthetic lethal phenotypes can occur when two proteins that usually interact are mutated and coexpressed in the same cell (Huffaker *et al.* 1987). The single mutants alone are partially functional and viable, but the double mutant exhibits a strongly enhanced phenotype. In a similar manner, synthetic phenotypes can be elicited by combining mutations that affect interactions involving proteins and DNA.

Increases in chromosome loss phenotypes are exhibited by cells carrying certain combinations of mutant proteins and chromosomes with mutant centromere DNA (McGrew *et al.* 1989; Meluh and Koshland 1995; Strunnikov *et al.* 1995; Baker *et al.* 1998). If a DNA-binding protein is mutated so that it can no longer bind to a specific site in the DNA, then a subsequent mutation in the DNA is unlikely to cause a further loss of function. However, if the first mutation confers partial function, then a second mutation altering an appropriate site in the DNA might significantly exacerbate the chromosome loss phenotype. The synthetic phenotypes between *cse4* mutants and mutations in CDE I and CDE II, but not CDE III, support a model where the histone-fold domain of Cse4p associates with CDE I and CDE II DNA and wraps the centromere DNA elements into a nucleosome-like structure, with CDE III positioned in the linker DNA.

In standard nucleosomes, the H3/H4 tetramer interacts with 60 bp of DNA, centered at the dyad axis, and makes a series of interactions that act cooperatively to stabilize the overall structure. We found a similar pattern of potential centromere DNA contact sites distributed across the histone-fold domain of Cse4p as revealed by mutagenesis studies (Keith *et al.* 1999) and by the synthetic interactions between *cse4* histone-fold domain mutants and *cen3* CDE II mutants. If the histone-fold domain of Cse4p interacts at multiple sites with the DNA helix, then the synthetic effects we observed most likely result from changes in the DNA-protein interactions. It is possible that specific protein-DNA contacts between Cse4p in a nucleosome-like structure and the centromere DNA have been disrupted, causing synthetic phenotypes. Alternatively, the two mutations might affect separate sites that usually interact cooperatively, resulting in a general weakening of the multicomponent structure. By analogy with H3, these results suggest that the CDE II region of the centromere DNA might lie across the dyad axis where Cse4p would replace both copies of H3 in a centric nucleosome. Furthermore, the nonspecific DNA-binding properties of H3 indicate that Cse4p probably interacts nonspecifically with CDE II DNA. Analysis of CDE II DNA from 16 different *S. cerevisiae* budding yeast centromeres shows no primary consensus sequence, but reveals three common features: conserved length, high A + T base composition, and an intrinsic bend, all of which are important features for centromere function *in vivo* (Murphy *et al.* 1991). The CDE II mutants studied here affect these characteristics, suggesting that structural properties conferred by DNA composition and not the primary DNA sequence *per se* is important to Cse4p-CDE II recognition and interactions.

On the basis of nuclease protection experiments, Bloom and Carbon (1982) proposed that CDE I, CDE II, and CDE III are assembled into a nucleosome *in vivo*. Further mapping studies demonstrated that several different yeast centromeres have similar nuclease-resistant chromatin structures, which are characterized by hypersensitive sites flanking a core region of ~220 bp of centromere DNA beginning upstream of CDE I and extending past CDE III (reviewed by Schulman and Bloom 1991). The CDE II DNA is the length (~80 bp)



	WT	CDE I		CDE II					CDE III	
	CEN3	CAT1	MOI	p130-3	X78	X69	MO4	MO4B	BCT1	GA/TG
CSE4HA	1	1	1	1	1	1	1	1	1	1
cse4-52	1.9	1.2	2.0	1.0	1.8	3.2	3.4	1.1	1.0	1.1
cse4-53	1.4	2.9	1.4	4.6	1.3	3.5	5.0	1.4	1.1	1.1
cse4-49	1.8	6.8	4.0	4.6	3.9	7.1	4.4	6.0	1.2	0.5
cse4-50	1.5	1.1	1.6	1.8	1.3	3.4	2.0	1.1	0.6	0.9
cse4W-F	1.9	14.7	4.1	16.6	4.4	5.8	Acen	5.2	1.1	1.1
cse4ΔD	1.9	16.7	1.0	1.4	2.2	4.3	4.2	4.2	0.8	1.1
cse4-56	1.1	1.2	1.3	1.8	1.5	1.0	1.9	1.4	0.5	0.7
cse4-101	1.2	28.7	2.8	14.2	2.0	6.8	Acen	12.4	0.9	1.4

FIGURE 3.—The fold increases in loss rates of chromosomes with mutant centromeres in different *cse4* mutant cells. The fold increases were determined by fluctuation tests (MATERIALS AND METHODS). Values for each *cse4* allele are normalized in each column by comparison to the loss rate of wild-type chromosomes carrying the same centromere in *CSE4HA* cells.

required to wrap once around a histone octamer, which would position the CDE I and CDE III DNA elements on the same side of the nucleosome. This early model is further supported by evidence that the CP1/CDE I and CBF3/CDE III protein-DNA complexes interact *in vivo* (BAKER *et al.* 1998; ORTIZ *et al.* 1999) and that core histones are important for centromere structure (SAUNDERS *et al.* 1990). The similarities between Cse4p and H3 and the genetic interactions between Cse4p and histone H4 provide strong support for a specialized Cse4p-nucleosome(s) at the centromere. MELUH *et al.* (1998) modified this model on the basis of chromatin immunoprecipitation experiments that placed Cse4p at the centromere and genetic interactions between Cse4p and H4. They proposed that CDE I DNA is located outside the Cse4p nucleosome structure while CDE III is placed across the dyad axis where Cse4p replaces histone H3. The synthetic interactions between *cse4* and *cen3* reported here are not easily accommodated by the DNA path around the specialized Cse4p nucleosome shown in MELUH *et al.* (1998). Furthermore, recent studies show that two CBF3 subunits bind to at least three distinct sites on opposite sides of the CDE III DNA helix (RUSSELL *et al.* 1999). If the CDE III DNA is in continuous physical contact with the core histones, access to regions of the CDE III helix by proteins should be significantly limited.

Our genetic results are consistent with the model shown in Figure 4, where the CDE III DNA is located in the linker region, permitting the CBF3 proteins to have access to the CDE III DNA helix (RUSSELL *et al.* 1999). CDE I and CDE II DNA are located within the 60 bp where Cse4p would interact if it replaces H3, with CDE II across the dyad axis where it could interact with Cse4p proteins that replace H3. In this configuration, interactions between the CBF3 proteins and the Cse4p histone-fold domain and other histones in the nucleosome are minimized. One potential discrepancy stems from the location of the CDE I DNA element relative to an upstream nuclease hypersensitive site in the centromeric chromatin. One study reports a shorter nuclease-resistant core of 150–160 bp for *CEN14*, beginning 11 bp upstream of CDE I (FUNK *et al.* 1989), compared to the 220 bp reported for other centromeres (SCHULMAN and BLOOM 1991). Our model is most consistent with a nuclease-resistant core region containing ~220 bp of centromere DNA, including CDE I. Nuclease accessibility upstream might be affected during the cell cycle or by the bend in the CDE I DNA helix caused by CP1 binding. Alternatively, CDE may be within the first 20 bp that enter the centric nucleosome. In this configuration CDE II would remain across the dyad axis of the nucleosome in contact with Cse4p, and the part of CDE II at the dyad axis would be shifted ~30–40 bp toward CDE III. This would place CDE III within the nucleosome, where CDE III would interact mainly with histones H2A and H2B, not Cse4p. This alternative model does accommodate our results, however; as we argued above, it is unlikely that the CBF3 subunits and the nucleosomal histones could interact at the same sites simultaneously.

The CP1 protein binds to CDE I elements in centromeres and also mediates the regulation of some biosynthetic genes by inducing bends and changing the chromatin structures associated with promoter DNA (KENT *et al.* 1994). Interestingly, the *cse4* histone-fold domain mutant, *cse4*^{csl2}, is synthetically lethal in *cep1* null cells, indicating a functional connection between the histone-fold domain of Cse4p and CP1 (BAKER *et al.* 1998). In this study we observed synthetic chromosome loss phenotypes when the mutant *cen3* CDE I CAT1 centromere was combined in cells with a *cse4* allele. The CAT1 centromere DNA mutation alone dramatically lowers the binding affinity of the CP1 protein to the altered CDE I DNA and causes an increase in loss events involving the chromosome with the mutant centromere

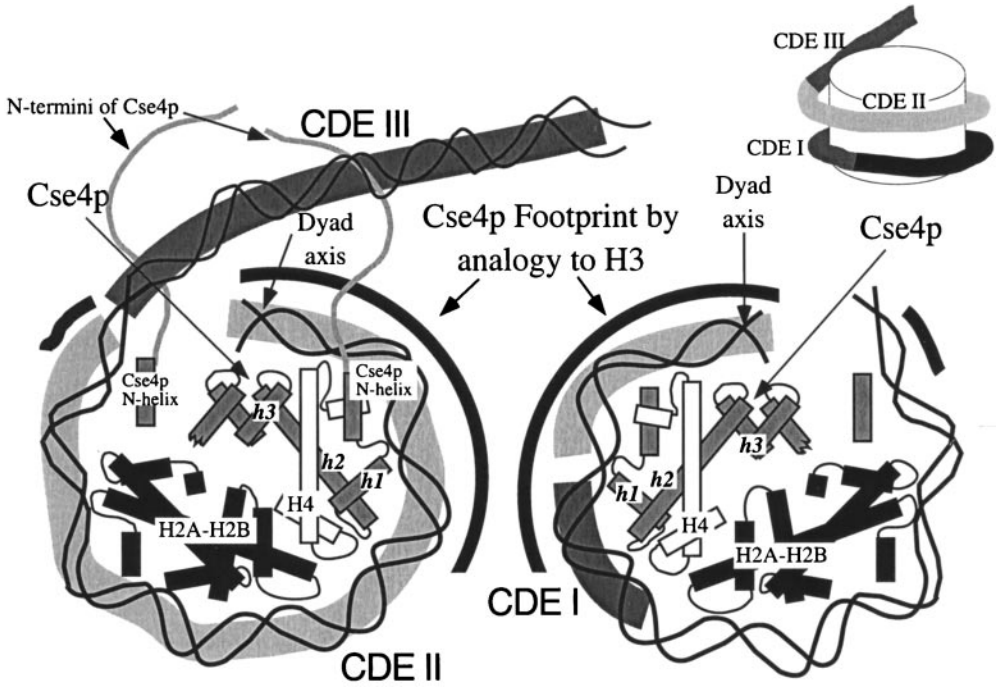


FIGURE 4.—Proposed role of Cse4p in the structure of yeast centric chromatin. Using the nucleosome structure determined by X-ray crystallography (LUGER *et al.* 1997) and the positions of known nuclease sensitive sites flanking the yeast centromere DNA (SCHULMAN and BLOOM 1991) as a template, we used our genetic results to predict the path of the CEN DNA around a novel Cse4p nucleosome. The model is drawn with the variant nucleosome split in half down the superhelical axis, with the top half on the left and the bottom half shown on the right. The dyad axis is marked with an arrow and is the point where the two halves are divided. H3 is replaced by Cse4p (gray) and the histone-fold domains of the three other core histones are shown with H4 in white and H2A and H2B shown together in black. The N-terminal region of Cse4p has been drawn only on the Cse4p molecule in the left half of the split variant nucleosome (light gray) to show that the N terminus extends out from the core nucleosome, near CDE III and the CBF3 complex. Helix I, helix II, and helix III of Cse4p are labeled in both halves of the nucleosome as *h1*, *h2*, and *h3*, respectively. The centromere CDE DNA regions are shaded. By analogy to H3 in a standard nucleosome, the region containing potential Cse4p-centromere DNA interactions is indicated with a heavy black line. The arrow marked "Cse4p" shows the position of the four helix bundle interaction predicted between the two Cse4p molecules. The second Cse4p molecule in each half of the nucleosome is truncated, showing the C-terminal half of helix II and loop II and helix III of Cse4p. The diagram at the upper right shows the centromere DNA path around an octamer represented by the cylinder.

(BAKER *et al.* 1989). CP1 may play an important role in chromatin remodeling at the centromere, similar to its function at promoters, which excludes standard H3 containing nucleosomes and promoting Cse4p specialized nucleosomes.

Positioning the CBF3/CDE III complex in the linker region does not preclude interactions between Cse4p and proteins assembled on the CDE III DNA. The crystal structure of the histone proteins in nucleosomes (LUGER *et al.* 1997) suggests that the N terminus of Cse4p should pass between the gyres of adjacent DNA helices and extend away from the nucleosome core. In this configuration, the essential region in the N terminus of the two Cse4 proteins would be on either side of the core CDE III sequence where both would be available for interactions with CBF3 subunits (ORTIZ *et al.* 1999). We have recently shown that the N terminus of Cse4p has an essential domain, which is responsible for interactions with the centromere complex containing Ctf19p, Mcm21p, and Okp1p (CHEN *et al.* 2000). In addition, *cse4* N-terminal mutants show synthetic lethality with mutant alleles of three out of four of the CBF3 components (p110, p64, and p58).

Why *S. cerevisiae* centromeres require the function of a specialized core histone protein is not yet clear, but the requirement may be universal as proteins similar to Cse4p have now been discovered in flies and worms as well as mammals and yeast (PALMER *et al.* 1987 and HENIKOFF *et al.* 2000). In budding yeast, Cse4p may play a critical role by arranging the centromere DNA sequence elements so that the kinetochore-binding site (CDE III) is accessible to microtubules in the higher-order chromatin structures in the chromosome. Indeed, CDE II DNA is clearly required for microtubule binding (KINGSBURY and KOSHLAND 1991). The tight association afforded by the integration of nucleosome structure with the kinetochore microtubule binding site may be critically important to ensure that the centromere-spindle connection has the strength necessary to withstand the physical forces of anaphase chromosome movement as well as the flexibility to change with the cell cycle.

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