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Chromatin structure of altered yeast centromeres

(nuclease chromatin mapping/*Dra* I sites/sequence-specific structures)

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ABSTRACT **We have investigated the chromatin structure of wild-type and mutationally altered centromere sequences in the yeast *Saccharomyces cerevisiae* by using an indirect end-labeling mapping strategy. Wild-type centromere DNA from chromosome III (*CEN3*) exhibits a nuclease-resistant chromatin structure 220–250 base pairs long, centered around the conserved centromere DNA element (CDE) III. A point mutation in CDE III that changes a central cytidine to a thymidine and completely disrupts centromere function has lost the chromatin conformation typically associated with the wild-type centromere. A second conserved DNA element, CDE I, is spatially separated from CDE III by 78–86 A+T-rich base pairs, which is termed CDE II. The sequence and spatial requirements for CDE II are less stringent; alterations in CDE II length and sequence can be tolerated to a limited extent. Nuclease-resistant cores are altered in dimension in two CDE II *CEN3* mutations. Two CDE I deletion mutations that retain partial centromere function also show nuclease-resistant regions of reduced size and intensity. The results from a number of such altered centromeres indicate a correlation between the presence of a protected core and centromere function.**

The centromere is a specific domain on eukaryotic chromosomes that plays an essential role in the faithful segregation of the chromosomes during mitotic and meiotic cell divisions. Cytologically, the centromere has been defined as the region of the chromosome that interacts with the spindle fibers during cell division. It has been proposed that centromere-specific DNA-binding proteins mediate the interaction between centromeres and spindle fibers. Following development of a functional assay for centromeres of the yeast *Saccharomyces cerevisiae*, the centromeres of 11 of the 16 chromosomes have been isolated (1–6). These centromere (*CEN*) sequences confer stable inheritance on autonomously replicating plasmids during mitosis and meiosis (1). Analysis and comparison of the DNA sequences of the 11 centromeres isolated has allowed description of several conserved sequence elements and their spatial organization (3, 6, 7). Each centromere has a highly conserved 8-base-pair (bp) region known as centromere DNA element (CDE) I and a 25-bp region of partial dyad symmetry known as CDE III. These elements are separated from one another by a region of DNA that is 78–86 bp long and that is >90% A+T-rich, termed CDE II. These conserved structural features of yeast centromeres are suggestive of protein-binding domains.

The centromeric DNA is complexed with chromatin components in the cell nucleus such that a 220- to 250-bp core centered around CDE III is resistant to nuclease digestion of the chromatin DNA (8–10). It has been demonstrated that high-salt washes (0.75–1.25 M NaCl) of the chromatin prior to nuclease treatment destroy the nuclease-resistant core. The nuclease-resistant core associated with yeast centromeres is, therefore, believed to result from interactions between *CEN* DNA, *CEN*-specific DNA-binding proteins, and histone proteins.

The relationship between *CEN* DNA sequence and centromere function has been investigated by constructing centromere mutations *in vitro* and then assaying their segregation capacity *in vivo* (11–15). These experiments have indicated that alterations of certain conserved bases, such as the central cytidine residue in CDE III, result in centromere dysfunction. Similarly, deletion of CDE III yields a nonfunctional *CEN*. However, when CDE I is deleted, the centromere retains partial mitotic function (13, 14). Alteration of the normal length of CDE II decreases *CEN* function in a gradual fashion.

In this report we have investigated the chromatin structure of several centromere mutations. We demonstrate that the nuclease-resistant chromatin core associated with the wild-type centromere is correlated with function. Specifically, point mutations in CDE III that destroy centromere function also destroy the nuclease-resistant region. The use of restriction enzymes as probes for chromatin structure confirms the change in structure associated with loss of centromere function. Centromere mutations that alter the distance between CDE I and CDE III alter the overall dimensions of the nuclease-resistant core. *CEN* mutations deleted for CDE I retain a residual nuclease-resistant structure that is reduced in intensity proportional to their loss of function.

MATERIALS AND METHODS

Strains. *S. cerevisiae* strain J17 (*MATα, his2, ade1, trp1, met14, ura3-52*) was used as the host for the plasmid transformations and for the *CEN3* chromosomal substitutions. Substitution strain 193-8b containing mutant *CEN3* BS154 substituted in chromosome III is *MATα,URA3* (III), *CEN3* BS154 (III), *ura3-52*(V), *ade1, met14* (12). The other *CEN3* substitution strains detailed in Fig. 1*B* were constructed by transforming the plasmids 303-7, 303-31, 303-42, and 303-6 (kindly supplied by J. Carbon, University of California, Santa Barbara, CA) in J17 as described (13, 16).

Medium. Cells were grown in synthetic medium [0.67% yeast nitrogen base, 2% (wt/vol) glucose, and 0.5% Casamino acids]. Adenine, tryptophan, or uracil (50 μg/ml) was added when appropriate.

Plasmids. Plasmids pd1314 and pBCT2 were obtained from McGrew *et al.* (11). Plasmid pYe(*CEN3*)30 has been described (3), and plasmids pYe(*CEN3*)30-C→A and pYe(*CEN3*)30-C→G are the equivalent plasmids containing CDE III point mutations described in Fig. 1*A*. The orientation of the *CEN3* fragment is reversed with respect to that in pYe(*CEN3*)30. Plasmid 303-6 AR was constructed by inserting *ARS2* into the unique *Xho* I site of 303-6 (16).

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Abbreviations: *CEN*, centromere; CDE, centromere DNA element.

Preparation and Digestion of Yeast Nuclei. This procedure was as described (8). Yeast cells were always grown in selective medium. For the restriction analysis of centromeric chromatin structure, nuclei were resuspended in TC buffer (10 mM Tris·HCl, pH 7.6/0.1 mM $CaCl_2$/20 mM NaCl/10 mM $MgCl_2$) and incubated 30 min with *Dra* I at 150 units/ml.

Preparation and Analysis of DNA. Chromatin DNA was extensively deproteinized as described (8). A portion of the total DNA was cleaved with DNase I (50 ng/ml) following deproteinization (see Figs. 2 and 4). All DNA samples were then digested to completion with *Hin*dIII. Molecular weight markers were constructed from yeast DNA by appropriate restriction endonuclease digestions. DNA fragments were electrophoresed in 1.5% or 1.9% agarose slab gels, transferred to nitrocellulose sheets, and hybridized to a radiolabeled 346-bp *Hin*dIII–*Bam*HI fragment of pBR322 as described (8). The 346-bp fragment of pBR322 is adjacent to all the centromere DNAs mapped.

RESULTS

Chromatin Mapping of *CEN3* CDE III Point Mutations. The central cytidine residue in the highly conserved, partially symmetric 25-bp CDE III is conserved in all 11 *CEN* sequences analyzed. This degree of conservation suggests an essential function for this nucleotide. *In vitro* mutagenesis has been used to alter this residue in a 624-bp *CEN3* fragment creating a series of point mutations with an adenosine or guanosine replacing the wild-type cytidine (R. Ogden and K.B., unpublished results). A C→T mutation at this position in a 211-bp *CEN3* derivative has been characterized (pBCT2, ref. 11). Both the wild-type 624-bp and the 211-bp *CEN3* DNAs contain CDE I, II, and III and have been shown to promote accurate chromosome segregation during mitosis and meiosis. When the mutated centromeres were tested for their ability to stabilize autonomously replicating plasmids in yeast, the thymidine mutation (pBCT2, ref. 11) was found to be essentially nonfunctional, and the adenosine and guanosine mutations [pYE(CEN3)30-C→A and pYE(CEN3)30-C→G] were severely reduced in function (Fig. 1*A*). The plasmids pBCT2 and pYe(*CEN3*)30-C→A and -C→G were only present in a small percent of the population due to their unequal partitioning at each cell division. Their copy number in the plasmid-bearing cells was elevated to 20 copies per cell (unpublished observations). Thus a single base-pair change in the *CEN3* sequence could alter the segregation capabilities

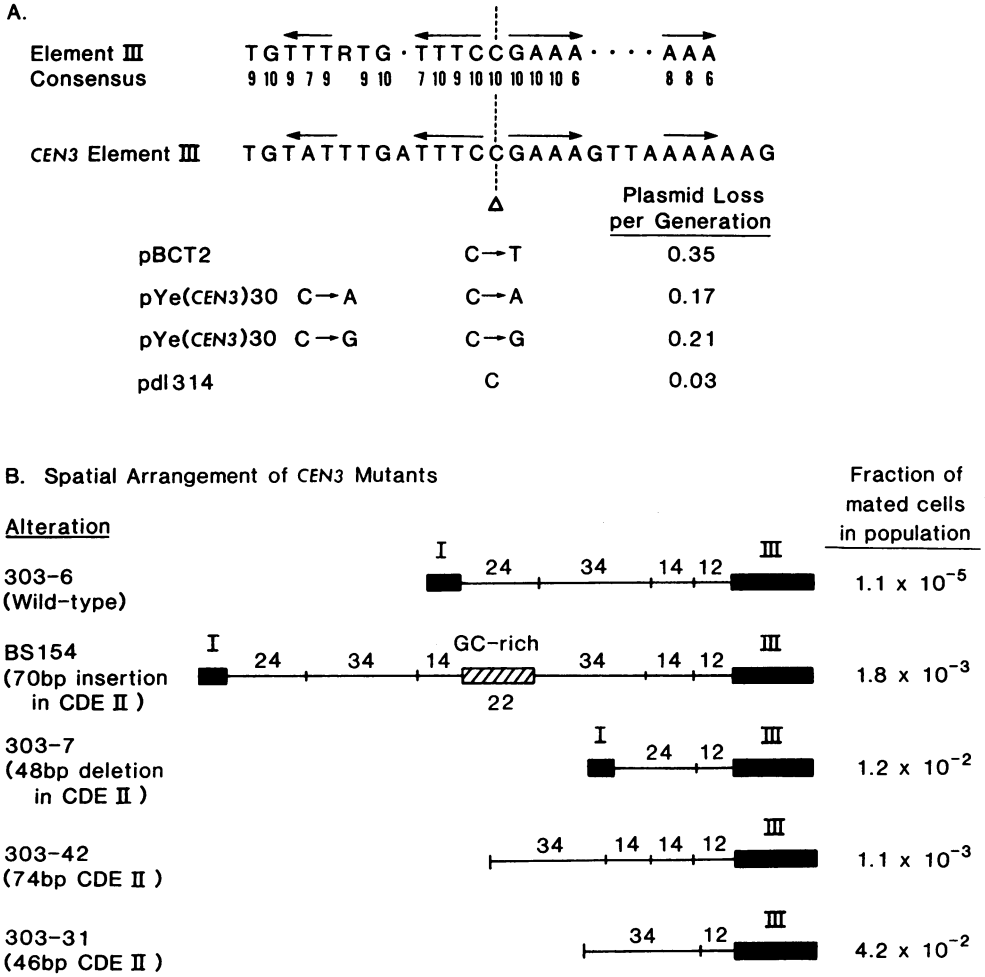

FIG. 1. Summary of the centromere mutations used in these chromatin mapping studies. (*A*) The consensus sequence of CDE III and its actual sequence in *CEN3* are shown. The numerical subscripts indicate the proportion of 10 sequenced centromeres that have the consensus nucleotide at each position. The vertical line marks the 100% conserved cytidine nucleotide that is the center of partial dyad symmetry. This is the nucleotide that is altered in *CEN3* to produce the series of CDE III point mutations. Mitotic stability assays were performed as described (3). (*B*) Outline of the centromere mutations with CDE II and CDE I deletions. CDE I and CDE III are indicated for each centromere by black boxes I and III. 303-6 is wild-type *CEN3*. CDE I has been deleted in 303-42 and 303-31. The physical separation of CDE I and CDE III in each case represents the dimensions of CDE II. *Dra* I sites (tick marks) and the fragment sizes in base pairs are indicated in the wild-type and mutated CDE II (13). The frequency of chromosome III loss in cells containing an altered *CEN3* was estimated by a quantitative mating assay (13, 16). Values are taken from refs. 12 and 13. The structure and function of the mutations are described in refs. 11–13 and 16.

from active partitioning at each cell division to the highly biased patterns characteristic of autonomously replicating plasmids (17).

The chromatin structure of the centromere regions of plasmids bearing these *CEN* mutations was investigated using an indirect end-labeling technique (18) and compared to that of the wild-type centromere. Each plasmid was introduced into yeast strain J17 by transformation. Isolated nuclei were prepared, and the chromatin was partially digested with DNase I. Proteins were subsequently extracted from the DNA, which was then digested to completion with *Hin*dIII. The DNA fragments were separated electrophoretically, transferred to nitrocellulose, and probed with a radiolabeled 346-bp *Hin*dIII–*Bam*HI fragment from pBR322 that extends toward the centromeres from the *Hin*dIII site in the respective plasmids. The lengths of the hybridizing fragments provided a map of the points of nuclease cleavage within the chromatin DNA relative to the *Hin*dIII site. The chromatin map of the wild-type centromeres on plasmids pd1314 (Fig. 2*A*, lanes wt), and 303-6AR (Fig. 2*B*, lanes wt) showed the typical nuclease-resistant core associated with the centromere region that was centered around CDE III. The nuclease-resistant region was not visualized when deproteinized DNA was treated with DNase I (Fig. 2, lanes labeled Naked). In the adenosine, guanosine, and thymidine CDE III mutations, no nuclease-resistant region mapping to the mutated centromeres was apparent in either chromatin or deproteinized DNA samples (Fig. 2 *A*, lanes T, and *B*, lanes A and G). Since these plasmids were elevated in copy number relative to their wild-type counterparts, it was possible that a limited supply of *CEN* components would result in loss of the nuclease-resistant core. The adenosine and guanosine point-mutated centromeres were, therefore, introduced into a plasmid containing a functional *CEN4*. These molecules were stably maintained at low copy number through successive cell divisions, and did not exhibit physical rearrangements characteristic of dicentric plasmids containing two functional centromeres (19–21). The chromatin of the mutated centromeres on these stable, low-copy plasmids was mapped as described above, and again no DNase I-resistant region was present (data not shown). These results indicate that the typical nuclease-resistant chromatin structure associated with *CEN* DNA is correlated with centromere function.

Restriction Enzymes as Probes of Centromeric Chromatin. We also have utilized the specificity of restriction endonucleases to map the internal structure of the centromere core. The restriction enzyme *Dra* I recognizes a base sequence that occurs three times in the CDE II element (see Fig. 3 *Lower*). Yeast cells transformed with wild-type centromere plasmid (pd1314) or plasmid carrying the thymidine point-mutated *CEN3*(pBCT2) were used for nuclei preparation as described above. Nuclei were incubated with *Dra* I as described for DNase I digestion. DNA was isolated from digested chromatin samples and cleaved to completion with a secondary enzyme, *Eco*RI. *Dra* I cleavage at individual recognition sites was then visualized by a modified end-labeling technique (Fig. 3). Complete digestion of the deproteinized DNA by *Eco*RI following *Dra* I treatment of chromatin allowed visualization of the three centromeric *Eco*RI–*Dra* I fragments (of 475, 509, and 523 bp) as well as the extracentromeric *Eco*RI–*Dra* I fragment (XCEN of 418 bp) located on opposite sides of the *TRP1-ARS1* sequence. The extracentromeric *Dra* I site in both plasmids provided an internal control for *Dra* I digestion of the chromatin DNA.

The *Dra* I sites in the chromatin DNA of wild-type centromeres were resistant to digestion as shown in Fig. 3. The release of a 418-bp fragment from the chromatin digestion indicates the efficacy of hydrolysis of extracentromeric (XCEN) *Dra* I sites in this experiment. Furthermore, the centromeric *Dra* I sites were readily cleaved in deproteinized

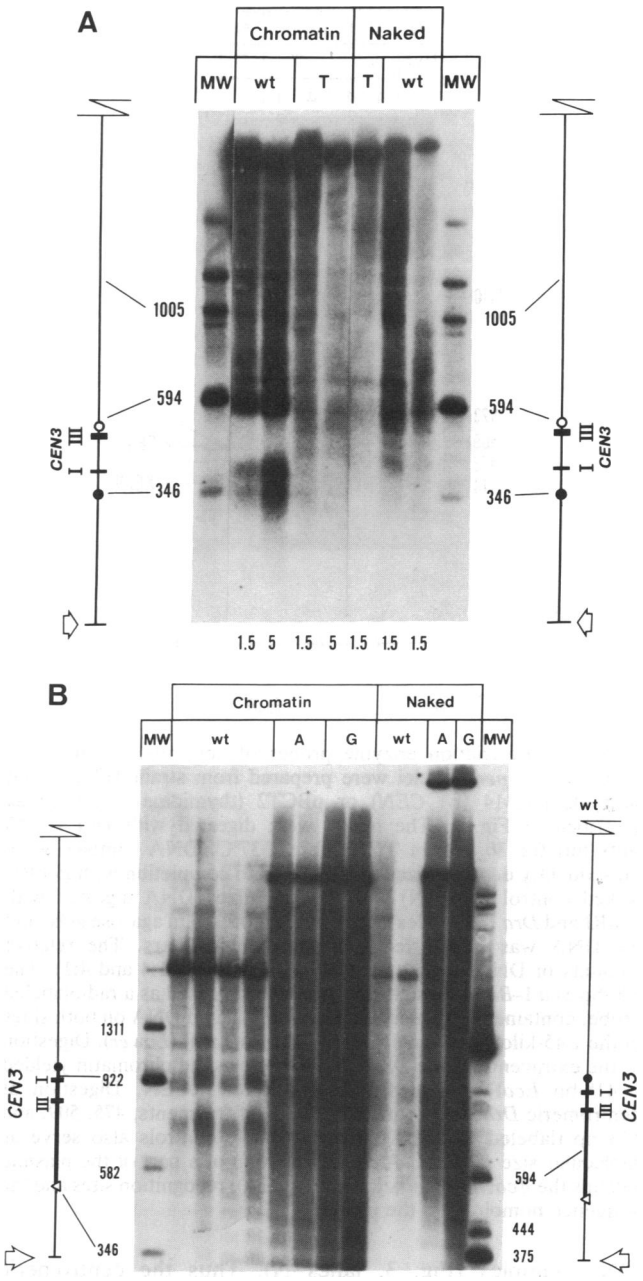

FIG. 2. Mapping the centromere chromatin structure of the CDE III point mutations. Nuclei were prepared from yeast cells (J17) containing the following plasmids. (*A*) pd1314 (wild-type *CEN3*, lanes wt) and pBCT2 (C→T point mutation, lanes T). (*B*) 303-6 AR (wild-type *CEN3*, lanes wt), pYe(*CEN3*)30-C→A, and pYe(*CEN3*)-30-C→G (lanes A and G, respectively). Chromatin lanes represent nuclei that were directly treated with DNase I (5 μg/ml) for the times indicated in min. All samples were digested for 1.5 min in *B*. Lanes labeled Naked represent nuclei that were deproteinized prior to the nuclease treatment. Subsequent DNase I digestion of these samples provides a measure of the sequence specificity of this enzyme. Deproteinized DNA samples were digested with DNase I (50 ng/ml) as indicated. After partial DNase I digestion, DNA samples were digested to completion with *Hin*dIII and then electrophoresed on a 1.9% agarose gel (*A*) or a 1.5% agarose gel (*B*). The DNA was then transferred to nitrocellulose filters and hybridized to the radiolabeled 346-bp probe from pBR322 that is homologous to the DNA extending from the *Hin*dIII site (large arrow) toward the *CEN3* region. Beside each panel is a partial restriction map of the centromere region of the various plasmids. CDE I and CDE III are indicated by solid boxes. Molecular size markers (lanes labeled MW in bases) indicate plasmid sequences hydrolyzed with appropriate restriction endonucleases that contain regions complementary to the radiolabeled probe. ○, *Sal* I; △, *Bam*HI; ●, *Sau*3AI; −, *Hin*dIII.

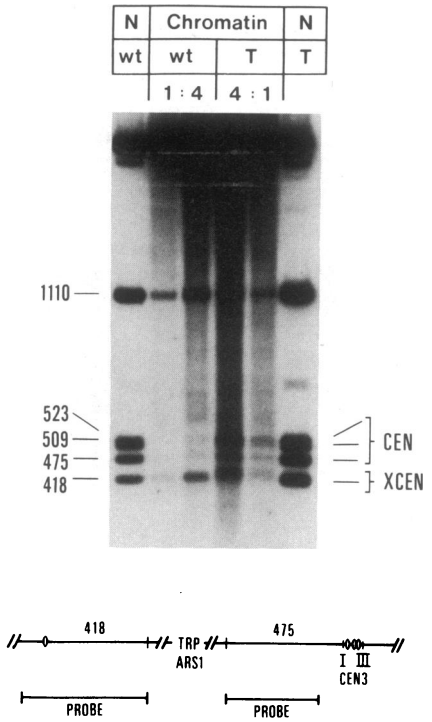

FIG. 3. Restriction enzyme probes of centromeric chromatin structure. (*Upper*) Nuclei were prepared from strain J17 carrying plasmids pd1314 (wt *CEN*) or pBCT2 (thymidine mutation) as described in Fig. 2. The nuclei were digested with *Dra* I (150 units/ml) for 30 min in TC buffer at 37°C. DNA samples were subsequently deproteinized and digested to completion with *Eco*RI. Naked control lanes (N) were deproteinized DNA digested with *Eco*RI and *Dra* I. Samples were electrophoresed in agarose gels, and the DNA was transferred to nitrocellulose filters. The relative amounts of DNA loaded per lane are indicated (1:4 and 4:1). The 890-bp *Sca* I–*Bam*HI fragment from pBR322, used as a radiolabeled probe, contained sequence homology to plasmid DNA on both sides of the 1.45-kilobase *Eco*RI *TRP1-ARS1* fragment (*Lower*). Digestion at the extracentromeric *Dra* I site in the plasmid chromatin yielded a 418-bp *Eco*RI–*Dra* I fragment denoted XCEN. Digestion at centromeric *Dra* I sites yielded a series of fragments, 475, 509, and 523 bp (labeled CEN). Deproteinized (N) controls also serve as molecular size markers. A restriction map of a part of the plasmid reveals the *Eco*RI (tick marks) and *Dra* I (◊) recognition sites and the sequence homology to the probe.

DNA samples (Fig. 3, lanes N). Thus the centromeric chromatin was protected from DNase I and restriction endonuclease attack. In contrast, the centromeric *Dra* I sites in plasmids harboring the thymidine point mutation in *CEN3* (BCT2, ref. 11) were accessible to cleavage with apparent efficiency equal to the extracentromeric recognition site (Fig. 3, lanes T). No site preferences were observed in the deproteinized DNA control lanes (lanes N). This data provides additional support for the absence of centromeric components in the CDE III point-mutated centromeres.

Chromatin Structure of Partially Functional Centromere Mutations. To further investigate the relationship between centromere function and chromatin conformation, the structure of several partially functional, altered centromeres was determined. The centromere mutations chosen for this study and their degree of function are outlined in Fig. 1*B*. These mutations represent alterations in the distance between CDE I and III (see mutations BS154 and 303-7) or complete deletions of CDE I. Each of these mutated centromeres exhibits significant segregational capacities. The chromatin structures associated with each of the centromere mutations 303-7, 303-31, 303-42 (13), and BS154 (12) were mapped in chromosome III genomic substitution strains.

The altered centromere BS154 contains a 70-bp insert (Fig. 1*B*) in CDE II, including a partial duplication of CDE II plus insertion of a 22-bp G+C-rich palindromic DNA sequence (12). The chromatin structure of BS154 shown in Fig. 4*A* reveals nuclease cleavage sites to either side of CDE I and of CDE III and another nuclease-sensitive region that maps to the G+C-rich insert. The overall dimension of the protected region is estimated to be 300 bp long, including the 20-bp DNase I-sensitive region present in CDE II. Examination of

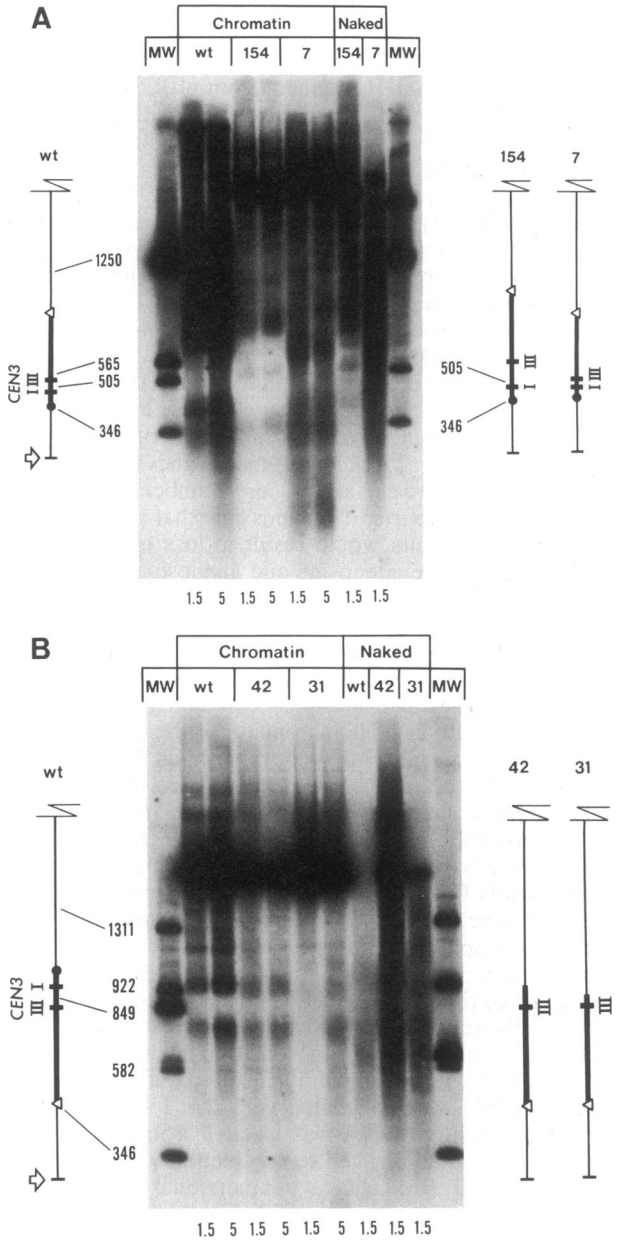

FIG. 4. Mapping nuclease-sensitive sites on the centromere chromatin from genomic substitution yeast strains containing various centromere mutations. Nuclei were prepared from genomic substitution strains carrying either a wild-type (lanes wt) or a mutated *CEN3* (*A*, 303-7 and BS154, lanes 7 and 154; *B*, 303-42 and 303-31, lanes 42 and 31). These mutated centromeres are described in Fig. 1. Chromatin and deproteinized DNA (Naked) were treated as described in Fig. 2. At the sides of each panel are partial restriction maps of chromosome III carrying wild-type or altered centromeres. CDE I and CDE III are indicated by solid boxes in the mutations, deletion of CDE I (303-42 and 303-31) is indicated by absence of the CDE I box. Molecular size markers (in bases) were prepared from DNA isolated from each strain and digested with appropriate restriction enzymes. (Restriction sites are as in Fig. 2).

the digestion patterns in deproteinized DNA (Fig. 4*A*, lanes labeled Naked and 154) revealed that the G+C-rich region may also be preferentially cleaved in the absence of chromosomal proteins. However, DNase I sites in deproteinized DNA between the G+C-rich core and CDE I and III were protected from nucleolytic attack in the chromatin samples. Therefore, an expanded centromere, excluding perhaps the G+C-rich core, was associated with an enlarged structure in the cell nucleus. The 303-7 lanes demonstrate the pattern of nuclease sensitivity of a centromere mutation with a 48-bp deletion in CDE II. The cutting sites define a protected core that was reduced by ≈40 bp and was less distinct than that of the wild-type centromere (compare lanes labeled wt and 7 in Fig. 4*A*).

Centromere mutations, 303-42 and 303-31, in which CDE I was deleted and which contained 74 bp and 44 bp, respectively, of highly A+T-rich CDE II DNA, retained a nuclease-resistant chromatin structure that was less distinct and slightly reduced in size when compared to the wild-type centromere (Fig. 4*B*). The more stable of the two mutated centromeres, 303-42, has a protected structure of reduced clarity in comparison to wild type, but it is more distinct than 303-31.

DISCUSSION

In this report we have investigated the chromatin structures associated with altered *CEN3* DNA in the yeast *S. cerevisiae*. We have demonstrated that the distinctive nuclease-resistant chromatin structure typically associated with wild-type yeast centromeres is correlated with *CEN* function. The centromeric chromatin conformation was eradicated in a series of nonfunctional *CEN3* point mutations, in which the central conserved residue of CDE III had been altered to each of the alternative bases. The entire centromeric region of these mutations became susceptible to DNase I digestion. Additionally, when the restriction endonuclease *Dra* I was used to probe the chromatin structure, the centromeric *Dra* I recognition sites in wild-type *CEN3* chromatin was resistant to digestion, whereas those in the thymidine point mutation (BCT2) were sensitive. This data complements the DNase I chromatin mapping data above and provides additional support for the absence of centromeric components in these altered centromeres.

The central C·G base pair of CDE III is a critical base pair for *CEN* function. It was conserved in all 11 sequenced yeast centromeres and substituting it for any other possible nucleotide base pairs caused a severe reduction in centromere function. Our results from alteration of the C·G to a G·C base pair indicate that there is a component of asymmetry in CDE III (Fig. 1*A*). If CDE III was completely symmetrical, changes from C·G to G·C base pairs would be expected to retain *CEN* function. The loss of the nuclease-resistant core in each of the three CDE III point mutations suggests that alteration of this conserved base pair so dramatically alters the binding or affinity of centromeric components for CDE III that they are no longer capable of stably associating with the mutated centromere *in vivo*. If the nuclease-resistant chromatin structure of functional centromeres was brought about by a complex interaction between centromere-specific DNA-binding proteins, histones, and centromere DNA, it is possible that the highly conserved region of partial dyad symmetry, CDE III, is the primary contact site for binding or recognition of specific *CEN* proteins.

Those *CEN3* CDE I deletion mutations and CDE II insertion and deletion mutations that retain partial *CEN* function exhibited chromatin conformations of altered dimensions. The clarity of the protected structure generally reflects the degree of loss of function, as demonstrated by the altered protected regions in the two CDE I deletion mutations with various degrees of function. The mutations 303-7 and BS154, in which the length of CDE II has been reduced and increased in size, respectively, have altered protected structures that reflect the size alterations of CDE II.

Whereas the CDE III point mutations were rendered nonfunctional by loss of the protected core, presumably because *CEN*-specific binding proteins did not associate stably with the mutated *CEN* sequences, the protected region was retained in a transcriptionally inactivated conditional centromere (21). This suggests that there may be other parameters besides protein binding that are required for centromere function.

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