Chromatin Conformation of Yeast Centromeres

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ABSTRACT The centromere region of *Saccharomyces cerevisiae* chromosome III has been replaced by various DNA fragments from the centromere regions of yeast chromosomes III and XI. A 289-base pair centromere (*CEN3*) sequence can stabilize yeast chromosome III through mitosis and meiosis. The orientation of the centromeric fragments within chromosome III has no effect on the normal mitotic or meiotic behavior of the chromosome. The structural integrity of the centromere region in these genomic substitution strains was examined by mapping nucleolytic cleavage sites within the chromatin DNA. A nuclease-protected centromere core of 220–250 base pairs was evident in all of the genomic substitution strains. The position of the protected region is determined strictly by the centromere DNA sequence. These results indicate that the functional centromere core is contained within 220–250 base pairs of the chromatin DNA that is structurally distinct from the flanking nucleosomal chromatin.

A mechanism to transmit genetic material to daughter cells during cell division is required by both procaryotic and eucaryotic organisms. Although the mitotic apparatus may range in complexity from a simple DNA–membrane attachment in procaryotes to the highly elaborate processes of the eucaryotic spindle, one common feature is the establishment of a specific DNA locus that interacts with the segregation mechanism.

In procaryotes, plasmid systems have proved the most tractable in identifying DNA sequences involved in segregation phenomena. The par locus is a *cis*-acting DNA element needed to promote the equipartition of replicating plasmid DNA molecules in *Escherichia coli* (1, 2). The par sequences are functional in either orientation in the plasmid molecule and par loci from different plasmids are interchangeable (2, 3).

The experimental strategy for isolation of DNA elements required for the proper segregation of eucaryotic chromosomes also depends upon the relative simplicity of plasmid systems. The yeast *Saccharomyces cerevisiae* can be transformed with plasmid molecules containing a selectable genetic marker and DNA sequences providing autonomous replication. These molecules can be followed through many successive generations of mitotic cell division, and upon sporulation of the diploid yeast host, plasmid segregation can be followed through meiosis. Plasmids containing a selectable genetic marker plus an autonomous replication sequence are not efficiently distributed from mother to daughter cells during mitosis (4) and are rapidly lost from the population. Fragments of chromosomal DNA isolated by virtue of their ability to confer mitotic stability on these plasmids map to the centromere region of yeast chromosomes (5). The centromere DNAs (*CEN*) isolated from five chromosomes in yeast, *CEN3*(5), *CEN4*(6), *CEN5*(7), *CEN6*(8), and *CEN11*(9), have the common ability to stabilize autonomously replicating plasmids through mitosis, and direct the segregation of plasmid molecules in a predominantly Mendelian fashion through meiosis.

The nucleotide sequences of DNA fragments carrying *CEN3*(10), *CEN4* (Mann, C., and R. Davis, Stanford University, personal communication), *CEN6*(8), and *CEN11*(10) have been determined. Sequence comparison reveals that short regions of DNA are conserved in their nucleotide sequence and spatial arrangement in the centromere regions from the different chromosomes (Fig. 1). Sequence element III (11 base pairs [bp][1] is completely homologous in *CEN3* and *CEN11* and exhibits strong homology (10/11 bp) to similar elements in *CEN4* and *CEN6*. An extremely (A+T)-rich region (>93% A+T, element II) spanning 82–89 bp occurs immediately adjacent to element III in all the centromeres. The element II region is flanked on the other side by sequence element I (14 bp), which is completely homologous in *CEN3* and *CEN11*, and exhibits partial homology in *CEN4*

[1] *Abbreviations used in this paper:* bp, base pair; kb, kilobase pair; SPCM, standard digestion buffer as described in Materials and Methods.

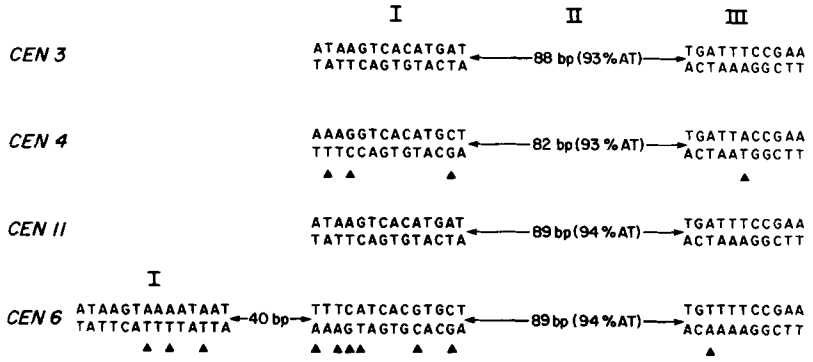

FIGURE 1 Regions of sequence homology between *CEN3*(10), *CEN4* (C. Mann and R. Davis, Stanford University, personal communication), *CEN6*(8), and *CEN11*(10) in the yeast, *Saccharomyces cerevisiae*. The sequence elements I–III are arranged in an almost identical spatial arrangement in the centromeres of four different chromosomes. The regions of nonhomology are indicated by the arrowheads below the nucleotide pair

(11/14 bp) and *CEN6* (11/14 and 8/14 bp). Deletion of the sequence element I–III region completely inactivates the centromere (11), indicating that these sequences comprise all or an important part of the functional yeast centromere.

A more detailed description of the eucaryotic centromere requires an assay that is not encumbered by problems that may be unique to a plasmid molecule. For example, the centromeric plasmids do not segregate with the fidelity of the parental chromosomes; the frequency of centromeric plasmid loss is about once in every 100 cell divisions (5), whereas mitotic chromosomes are lost about once in every 50,000 cell divisions (12). A recent development in the yeast system allows specific DNA sequences, isolated and manipulated in vitro, to be directed into the yeast cell to substitute for sequences normally occurring within the host genome (13). By using this technique, a series of genomic substitution strains were constructed by replacing the host centromere region in chromosome III with altered DNA fragments (14). When the 624-bp *CEN3* fragment is deleted from chromosome III, an acentric chromosome results and is rapidly lost from the population. Thus the *CEN3* fragment is required to stabilize the entire yeast chromosome. Chromosome stability is recovered when the centromere DNAs from chromosomes III (624-bp fragment) or XI (858-bp fragment), are substituted in either orientation for the *CEN3* region in chromosome III. The resulting chromosome segregates normally through mitosis and meiosis in a manner identical to that of the normal chromosome III. These results indicate the yeast centromeres are fully functional in either orientation and are not necessarily chromosome specific. Thus the *CEN* sequences in eucaryotes, as well as the par loci in procaryotes, function as autonomous units stabilizing the plasmid or chromosome in which they reside.

To begin to understand how the centromere DNA interacts with the segregation machinery, we have initiated studies on the conformation of the centromere DNA sequences in the yeast chromosomes. The DNA in eucaryotic cells is wrapped around histone core particles to create a periodic array of 146-bp nucleosomal subunits and 20–50-bp linker sequences. A consequence of the histone-core linker organization of DNA in chromatin is that the linker DNA is more accessible to nucleolytic cleavage than is the DNA in the nucleosomal core particles. Mild digestion of chromatin with the enzyme, micrococcal nuclease, generates a series of nucleoprotein particles containing DNA fragments whose molecular weights are multiples of the basic nucleosomal subunit. Digestion of the chromatin DNA from yeast with micrococcal nuclease reveals this typical nucleosomal subunit structure. Analysis of centromeric chromatin, in contrast, indicates that the centromere DNA sequences are in a nuclease-protected structure that encompasses 220–250 base pairs of DNA (15). This protected region is distinct from the nucleosomal core particle and maps to the sequence element I–III region in both chromosomes III and XI. This region of structural differentiation is associated with the centromere sequences whether they are present within the yeast genome or on autonomously replicating plasmids. We now demonstrate that the protected centromere core seen in the parental chromosomes and on centromeric plasmid molecules is determined strictly by the element I–III DNA sequences, and includes these sequences regardless of where they are positioned in the genome. This protected 220–250 bp of centromeric chromatin may therefore be the fundamental structural unit of the yeast centromere.

MATERIALS AND METHODS

Yeast Strains: *S. cerevisiae* genomic substitution strains were constructed as previously described (14) by transformation of strain SB9882-4 (*a trp1-289 ura3-52 leu2-3 leu2-112 his4-519/α trp1-289 ura3-52 can1*) with the appropriate centromere-substitution fragments diagrammed in Fig. 3 (g.v.). The haploid strains used for the chromatin-mapping experiments were derived by sporulation of the resulting stable *URA3*$^+$ diploid transformants. The haploid genotypes are: SB303-6A(3C), *α URA3*$^+$(III) *ura3*(V) *trp1 leu2 his4*; SB303-4A(2C), *α URA3*$^+$(III) *ura3*(V) *trp1 leu2 his4*; SB311-9B(1A), *α URA3*$^+$*(III) ura3*(V) *trp1 leu2 his4*; SB311-11A(10D), *α URA3*$^+$(III) *ura3*(V) *trp1 LEU2*$^+$ *HIS4*$^+$ *can*; SB303-14(29D), *α URA3*$^+$(III) *ura3*(V) *trp1 leu2 his4*; SB303-12(2B), *α URA3*$^+$(III) *ura3*(V) *trp1 leu2 his4*. Yeast strain X2180a was obtained from the Yeast Genetic Stock Center, University of California, Berkeley. Yeast stain J17 (*α his2 ade1 trp1 met14 ura3*) has been described previously (9). A proteinase deficient strain 20B-12α, carrying the *pep4-3* mutation (16), was used for preparing the protein extracts in DNA-binding experiments.

Isolation and Digestion of Yeast Nuclei: Yeast cells were grown in rich media containing 1% yeast extract, 2% bacto-peptone, and 2% glucose. Cells were harvested in mid-logarithmic growth phase, washed, and converted to spheroplasts by treatment with 1% Glusulase (DuPont Pharmaceuticals, Wilmington, DE), as described by Forte and Fangman (17). Nuclei were isolated from spheroplasts as described by Nelson and Fangman (18). The nuclei were resuspended in standard digestion buffer (SPCM) that contains 1 M sorbitol, 20 mM 1,4-piperazone-diethanesulfonic acid (pH 6.3), 0.1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 1 mM phenylmethylsulfonyl fluoride. The suspension was prewarmed to 32°C for 3 min, and DNAase I (5 μg/ml) was added for the times (in minutes) indicated in the text. After incubation, samples were adjusted to 1% SDS, 1 M NaCl, and 20 mM EDTA to stop the digestion.

Preparation and Analysis of DNA: DNA was extensively deproteinized and treated with RNase A as described by Bloom and Carbon (15). DNA samples were digested with restriction enzymes according to the specifications provided by the suppliers. DNA fragments were analyzed on 1.4% agarose slab gels containing 0.09 M Tris-borate (pH 8.3) and 2.5 mM EDTA. To visualize unique DNA sequences, DNA fragments were transferred to nitrocellulose filters (19) and hybridized to radiolabeled DNA probes as described previously (15). Autoradiography was performed for 24–72 h at −80°C with Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY) and a Du Pont Cronex Lightning-Plus intensifying screen (DuPont Instruments, Wilmington, DE).

Isolation and Analysis of DNA-binding Proteins: Extracts used for isolation of centromere-binding proteins were prepared from nuclei isolated from 200 ml of mid-logarithmic growth-phase cultures of yeast strain

20B-12α as described above. Nuclei were resuspended in 2 ml of SPCM and sonicated using three 15-s bursts with intermittent cooling to an average double-stranded DNA length of 1,000 base pairs, as determined by agarose gel electrophoresis. The samples were diluted to 5 ml with 10 mM sodium phosphate (pH 7.0) and centrifuged at 20,000 *g* for 15 min. The solubilized chromatin supernatant was applied to a hydroxyapatite column equilibrated with 10 mM sodium phosphate (pH 7.0). The proteins were selectively dissociated by eluting the column with solutions containing increasing concentrations of NaCl as described by Bloom and Anderson (20). Centromere-binding proteins were eluted with 2 M NaCl and dialyzed against 10 mM Tris-HCl (pH 7.0), 50 mM NaCl, 5 mM $MgCl_2$, and 0.1 mM EDTA. Proteins were reconstituted with restriction digested yeast genomic DNA for 45 min at 32°C. After incubation the samples were diluted to 500 μl in dialysis buffer and passed through a nitrocellulose filter. The filters were sequentially washed with 0.4 M NaCl and 1.0 M NaCl followed by 1.0 M NaCl in the presence of 1% SDS as described by Bloom et al. (11). The eluants were precipitated with ethanol, and the pelleted DNAs were redissolved and electrophoresed on a 1.4% agarose gel. Specific DNA fragments were visualized after Southern blot transfer and hybridization as described above.

RESULTS

Chromatin Structure of Yeast Centromeres

We have previously assayed the structure of centromeric regions in chromatin from yeast chromosomes III and XI by examining the susceptibility of centromeric DNA to nucleolytic cleavage (15). Here, we have utilized the enzyme, DNAase I, to map specific nuclease cutting sites in the centromeric region of chromosome IV in yeast (Fig. 2). To determine if the enzyme is actually recognizing structural parameters of the chromatin template, rather than specific DNA sequences, we have also examined the cutting sites in the centromeric region of protein-free chromosomal DNA. Chromatin DNA from yeast nuclei was partially digested with DNAase I, before (Fig. 2, chromatin) or after (Fig. 2, naked DNA) extraction of chromosomal proteins. The purified DNA samples were digested to completion with a restriction enzyme, *Xho*I, which cleaves at a fixed site close to the sequence element I–III region of *CEN4* (6) (Fig. 2, *left*). The lengths of the sequences that hybridize to a radiolabeled probe extending from the restriction site towards the centromere therefore provide a direct map of the points of nucleolytic cleavage within the chromatin or DNA fiber relative to the restriction site (21).

The most striking feature of the fragment pattern shown in Fig. 2 is the 220–250-bp nuclease-resistant region of DNA, occurring between 720 and 950 bp in a centromere-proximal direction from the *Xho*I site (open arrow in Fig. 2). The molecular weight standards on the gel (Fig. 2, lane *MW*) confirm the restriction map of the chromosome, and allow the nuclease cleavage sites to be mapped relative to the DNA sequence. Strong nuclease cleavage sites occur on both sides of the region of sequence elements *I–III*, leaving it in a protected region of ~220–250 bp. These results indicate the regions of highest sequence homology, elements *I–III*, are protected in a centromere core particle. In the control lanes (Fig. 2, naked DNA), there were no specific nuclease cutting sites visualized. The protected region therefore reflects chromatin components associated with the centromere DNA in the yeast cell nucleus. A similar protected region was also found around the centromere region in chromosomes III and XI, and was determined to include sequence elements I–III (15). In fact, no matter where we map along the chromatin fiber in the centromere regions of chromosomes III, IV, and XI, we find a protected region of 220–250 bp which includes sequence elements I and III.

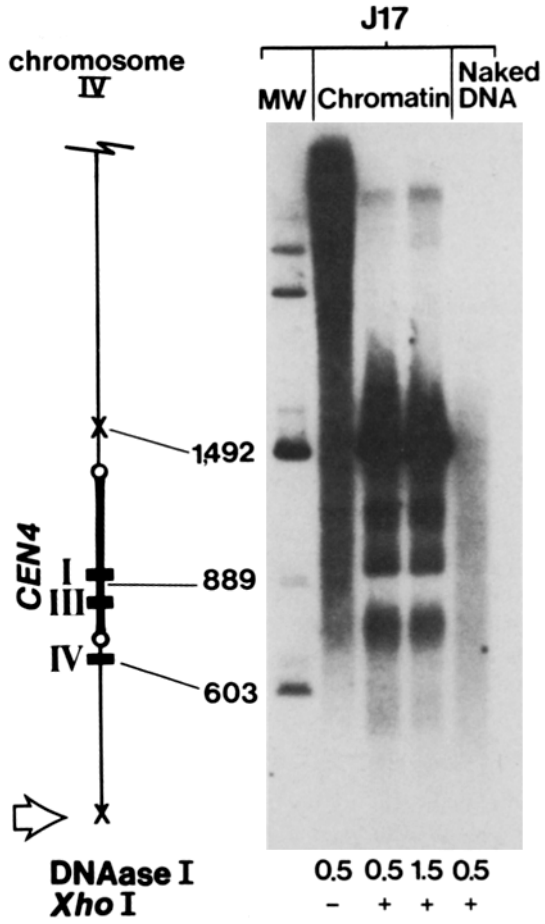

FIGURE 2 Mapping nuclease-sensitive sites on centromeric chromatin from yeast chromosome IV. Nuclei were prepared from yeast strain J17 and digested with DNAase I (5 μg/ml) for the times (in minutes) indicated, as described in Materials and Methods. For the experiments with naked DNA, nuclei were prepared as described for the chromatin digests, but immediately before nuclease cleavage, the DNA was extensively deproteinized. The naked DNA samples were resuspended in SPCM and digested with DNAase I (50 ng/ml) for the times (in minutes) indicated. After partial DNAase I cleavage, DNA samples were incubated in the presence (+), or absence (–) of *Xho*I, and electrophoresed on a 1.4% agarose gel. The DNAs were transferred to nitrocellulose filters and hybridized to the radiolabeled 603-bp probe from chromosome IV that extends from the *Xho*I site (large arrow) toward the *CEN4* region (elements I–III). At left is a partial restriction-site map of the centromere region of yeast chromosome IV (6). The elements of sequence homology (I–III) between the different centromeres (Fig. 1) are indicated by darkened boxes. Sequence element IV is homologous to similar elements in the other centromeres, but is expendable with respect to active *CEN* function. Molecular weight markers (*MW*) indicate yeast nuclear DNA fragments cut with *Xho*I, and *Xho*I-*Hpa*I. These fragments contain regions complementary to the radiolabeled probe (6). Restriction enzyme sites are *Xho*I (x).

Genomic Substitution of Yeast Centromeres

To define the functional boundaries of the centromere in the chromosome, we have utilized a unique property of the yeast system that allows replacement of DNA sequences in the host chromosome (13). DNA fragments constructed in vitro were introduced into yeast by transformation (14). The transforming DNA fragments were directed into the centromeric region of chromosome III by virtue of the DNA sequence homology between their free ends and regions flanking

the centromere in chromosome III (Fig. 3, regions *A* and *B*). The internal portion of each transforming fragment contains a genetically selectable marker, *URA3*$^+$, and an inverted, deleted, or foreign centromeric DNA sequence. A number of these transforming fragments containing different internal sequences are diagrammed in the inset to Fig. 3. These include the 624-bp *CEN3* or 858-bp *CEN11* fragments, either properly oriented or inverted, or a 289-bp fragment containing *CEN3*, including sequence elements I–III, in either orientation.

When diploid yeast cells are transformed with any one of these fragments, a recombination event occurs between regions *A* and *B* from the transforming fragment and the corresponding regions in the host chromosome. Using a genetic selection for the *URA3*$^+$ gene, the transformants with a genomic substitution in one copy of chromosome III are identified. When a fragment lacking the 624-bp *CEN3* sequence replaces *CEN3* in one of the host chromosomes no. III, an acentric chromosome results and thus, missing a spindle attachment site, is rapidly lost from the population, presumably through mitotic nondisjunction (Table I and reference 14). Thus the centromere sequence is required to stabilize the entire yeast chromosome. If the 624-bp *CEN3* or 858-bp *CEN11* fragments, in either orientation, replaces *CEN3* in the host chromosome, the substituted chromosome behaves completely normally in both mitosis and meiosis (Table I and reference 14).

The fragment-mediated transformation system has recently

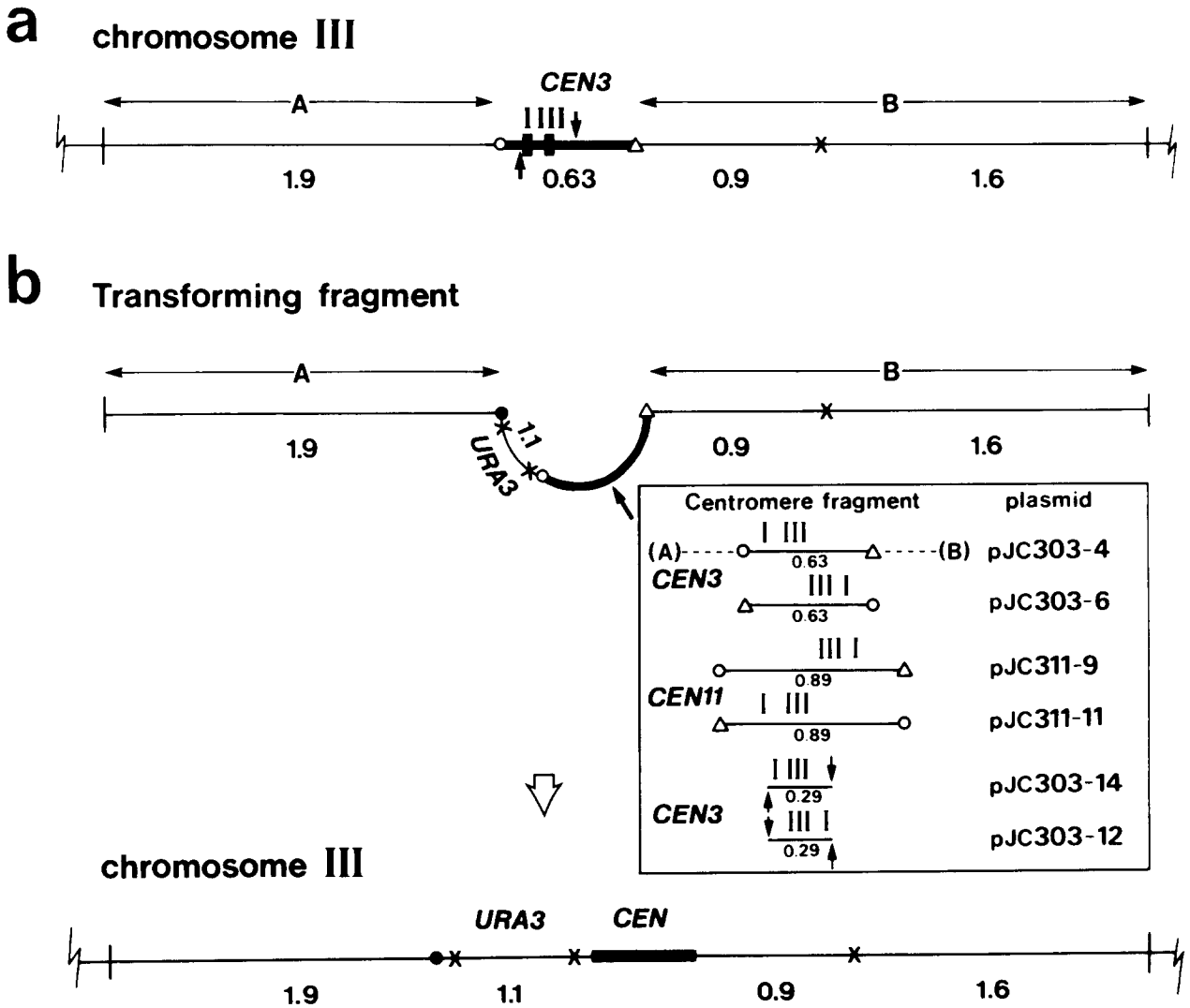

FIGURE 3 Schematic representation of the centromere region of yeast chromosome III and conversion of this region in the genomic substitution strains. (a) The physical map gives the location of the 624-bp *CEN3* fragment (darkened line) along the wild-type yeast chromosome III. Restriction sites are *Eco*RI (|), *Bam*HI (△), *Hin*dIII (x), and selected *Alu*I (↓), *Rsa*I (↑), and *Sau*3A (○) sites. Numbers denote kilobase pairs (kb). The flanking regions *A* (1.9 kb) and *B* (2.5 kb) are as indicated (5). Panel *b* illustrates the genomic substitution fragments that are utilized in construction of the *CEN3* substitution strains (14). The transforming fragment contains a selectable genetic marker, *URA3*, regions *A* and *B* flanking the centromere in chromosome III, and any one of the centromere fragments in the inset at right. These insertions include the 624-bp *CEN3* fragment in either orientation (JC303-4, JC303-6), the 858-bp *CEN11* fragment in either orientation (JC311-9, JC311-11), and the 289-bp *Rsa*I-*Alu*I *CEN3* fragment in either orientation (JC303-12, JC303-14). Roman numerals above the centromere fragments give the orientation of centromere sequence elements I and III. Transformation of diploid yeast strains with *Eco*RI-restricted pJC303-4, pJC303-6, pJC311-9, pJC311-11, pJC303-12, and pJC303-14 DNAs yield conversion constructions 303-4, 303-6, 311-9, 311-11, 303-12, and 303-14, which include at the *CEN3* region *URA3* plus the various *CEN* fragments in the orientations as shown.

been used to more accurately define the structural features necessary for proper functioning of *CEN3*. Various restriction fragments contained within the 624-bp *CEN3 Bam*HI-*Sau*3A fragment were used individually or in various combinations as substitutes for the 624-bp *CEN3* region in the genome (22). For example, cleavage of the 624-bp fragment with both *Rsa*I and *Alu*I yields a 289-bp segment that extends from a point 4 bp immediately to the left of element I through elements I–III, and ends 172 bp to the right of element III (see Fig. 3*b*). Substitution of the 624-bp *CEN3* sequence in the genome with this 289-bp fragment effectively deletes sequences occurring on both sides of the key element I–III region. Plasmid pJC303-14 contains the 289-bp sequence in the same orientation as occurs in the yeast genome, whereas the orientation is reversed in pJC303-12. Plasmid pJC303-12 and pJC303-14 DNAs were individually cleaved with *Eco*RI, and the resulting DNA fragments were used to transform diploid yeast strain SB9882-4CR (see the legend to Table II for genotype) to *URA3*$^+$. The transformant colonies (~1,000 per microgram of transforming DNA) were normal in appearance and growth rate, and the Ura$^+$ phenotype was mitotically stable. After growth of individual transformants in nonselective media for several generations, no Ura$^-$ cells could be detected among the 1,000 cells that were examined. The rate of loss of chromosome III in these transformants was determined by scoring for the number of mating-competent cells in a growing population. The loss of one copy of chromosome III in an a/α diploid (sterile) results in a competent mater, because the mating type locus (*MATa* or *MAT*α) is located on that chromosome (see reference 14 for details). As shown in Table I, the chromosome III loss rate in these transformants was no greater than 10^{-5} per cell division, a value not significantly different from that obtained with the untransformed parent diploid strain.

TABLE I

Chromosome III Nondisjunction in Strains with Altered Centromeres

Sequences at centromeres III of diploid	Structural alteration of *CEN3* (orientation)	Frequency of appearance of competent *a* or α cells (per cell division)
		$\times 10^5$
CEN3/*CEN3*(SB9882-4)	Wild-type	0.7
CEN3/*CEN3*(SB9882-4CR)	Wlld-type	0.1
303-4/*CEN3*	624 bp *CEN3* (correct)	0.9
303-6/*CEN3*	624 bp *CEN3* (reverse)	0.3
303-9/*CEN3*	858 bp *CEN11* (reverse)	1.1
303-11/*CEN3*	858 bp *CEN11* (correct)	0.6
303-12/*CEN3*	289 bp *CEN3* (reverse)	0.2
303-14/*CEN3*	289 bp *CEN3* (correct)	0.5

The observed frequencies of appearance of mating-competent cells represent the sum of mitotic gene conversion and recombination at the *MAT* locus, plus nondisjunction of chromosome III to form 2n-1 cells. Some of these data are taken from reference 14. See that reference for a more detailed description of experimental methods.

The meiotic behavior of the altered chromosomes no. III in the transformants of the 303-12 and 303-14 classes was examined by the classical methods of yeast genetic analysis. The diploid transformants were induced to sporulate, the individual spores in the tetrads were separated by microdissection, and the resulting haploids were scored for the distribution of appropriate genetic markers. In addition, haploid progeny containing the altered *CEN3* region (*Ura3*$^+$) were back-crossed to examine the effect of having two altered *CEN3* regions in opposition. The results of these meiotic analyses, summarized in Table II, indicate normal behavior of the chromosome III containing the 289-bp *CEN3* fragment in place of the normal 624-bp sequence. Spore viability was uniformly high (>90% in most cases), and the *URA3* marker on the altered chromosome III segregated as a centromere-linked gene, tightly linked to *LEU2* on chromosome III. Recombination frequencies between markers on chromosome III, and the number of gene conversions observed also fell within normal ranges. Finally, the predicted structure of the DNA in the *CEN3* region in the *URA3* haploids was verified by using a standard genomic Southern blot hybridization analysis (data not shown, see reference 14 for details).

The results described above suggest the functional centromere to be completely contained within the 289-bp *Rsa*I-*Alu*I

TABLE II

Meiotic Behavior of Chromosome III Containing a 289-bp CEN3 Substitution

a/α diploid strain	Tetrads scored	Spore viability	Apparent map distance			Gene conversions		
			HIS4-LEU2	*LEU2-URA3* *LEU2-CEN3*	*URA3-MAT*	*HIS4*	*LEU2*	*MAT*
	n	%		*centimorgans*			%	
303-14/*CEN3*	33	95	21	9	33	10	6	0
303-12/*CEN3*	41	98	20	10	45	18	6	0
Back-crosses								
303-14(29D) × BF305-18A	22	91	5	0	32	9	0	0
303-14(38A) × 303-14(18B)	26	95	21	9	31*	8	8	0
303-12(2A) × 303-14(18B)	13	77	29	8	21* (*LEU2-MAT*)	0	8	0
Literature values			9.9–19.9	3.4–11.6 (*LEU-2-CEN3*)	20–23.1 (*CEN3-MAT*)	8.4	2.5	0.6

The *URA3*/*ura3* heterozygous diploids, 303-14/*CEN3* and 303-12/*CEN3*, were obtained by transformation of yeast strain SB9882-4CR (*a ura3-52 trip1-289 leu2-3 leu2-112 his4-519 cry1*/α *ura3-52 trp1-289 can1*) with the centromere-substitution *Eco*RI fragments as described in the text. The 303-14 construction contains the *URA3* gene plus the 289 bp *Rsal-Alul CEN3* fragment in the correct orientation, whereas the 303-12 construction contains the*CEN3* sequence inserted in the orientation opposite from that seen in normal chromosome III. The back-cross diploids were constructed by mating selected Ura+ haploid progeny from the first two groups of tetrads. The genotypes are: 303-14 (29D), α *URA3*(III) *ura3*(V) *trp1 leu2 his4*; BF305-18A, *a arg5,6 ura3 met14 ade1*; 303-14(38A), α *URA3*(III) *ura3*(V) *trp1*; 303-14(18B), *a URA3*(III) *ura3*(V) *trp1 leu2 his 4 met14 ade1*; 303-12(2A), α *URA*(III) *ura3*(V) *trp1*. As expected, *URA3*+ segregated 2+:2− always in sister spores in all tetrads from the first three diploids (*URA3* thus maps 0 cM from *CEN3*). In the homozygous *URA3* back-cross diploids, *URA3* segregated 4+:0− in all tetrads. For experimental details, see reference 14.

fragment. Sequences within flanking regions *A* and *B* (Fig. 3) may be required, but need not be contiguous with the centromere core.

Chromatin Structure of the Altered CEN3 Regions

We have examined the chromatin conformation associated with the altered centromeres in the genomic substitution strains described above. Chromatin was isolated from the haploid genomic substitution strains with *CEN3*, *CEN11*, or the 289-bp *CEN3* fragments in either orientation in chromosome III (Fig. 3 *b*), and was partially digested with the nucleolytic enzyme, DNAase I, as described above. The DNA was purified and cut to completion with the restriction enzyme, *Hin*dIII. The DNA fragments were separated on agarose gels, transferred to nitrocellulose, and probed with a radiolabeled DNA sequence originating from the *Hin*dIII site in region *B* (Fig. 3 *a*) and extending 900 bp toward the centromere region. This region remains intact in all of the genomic substitutions shown in Fig. 3, thereby enabling the same mapping strategy to be employed for each strain.

The chromatin mapping results from the genomic substitution strains are shown in Fig. 4. The fragment patterns obtained by using chromatin from strains containing the 624-bp *CEN3* fragment in the proper orientation (strain 303-4, *left*) and an inverted orientation (strain 303-6, *right*) are shown in Fig. 4 *a*. The orientation of the *CEN* fragments can be visualized by the change in position of selected restriction sites. In strain 303-4, the *Bam*HI site to the element III side of the 624-bp *CEN3* fragment occurs 900 bp from the *Hin*dIII restriction site, whereas when the 624-bp *CEN3* fragment is inverted (strain 303-6), the *Bam*HI site is 1,500 bp from the

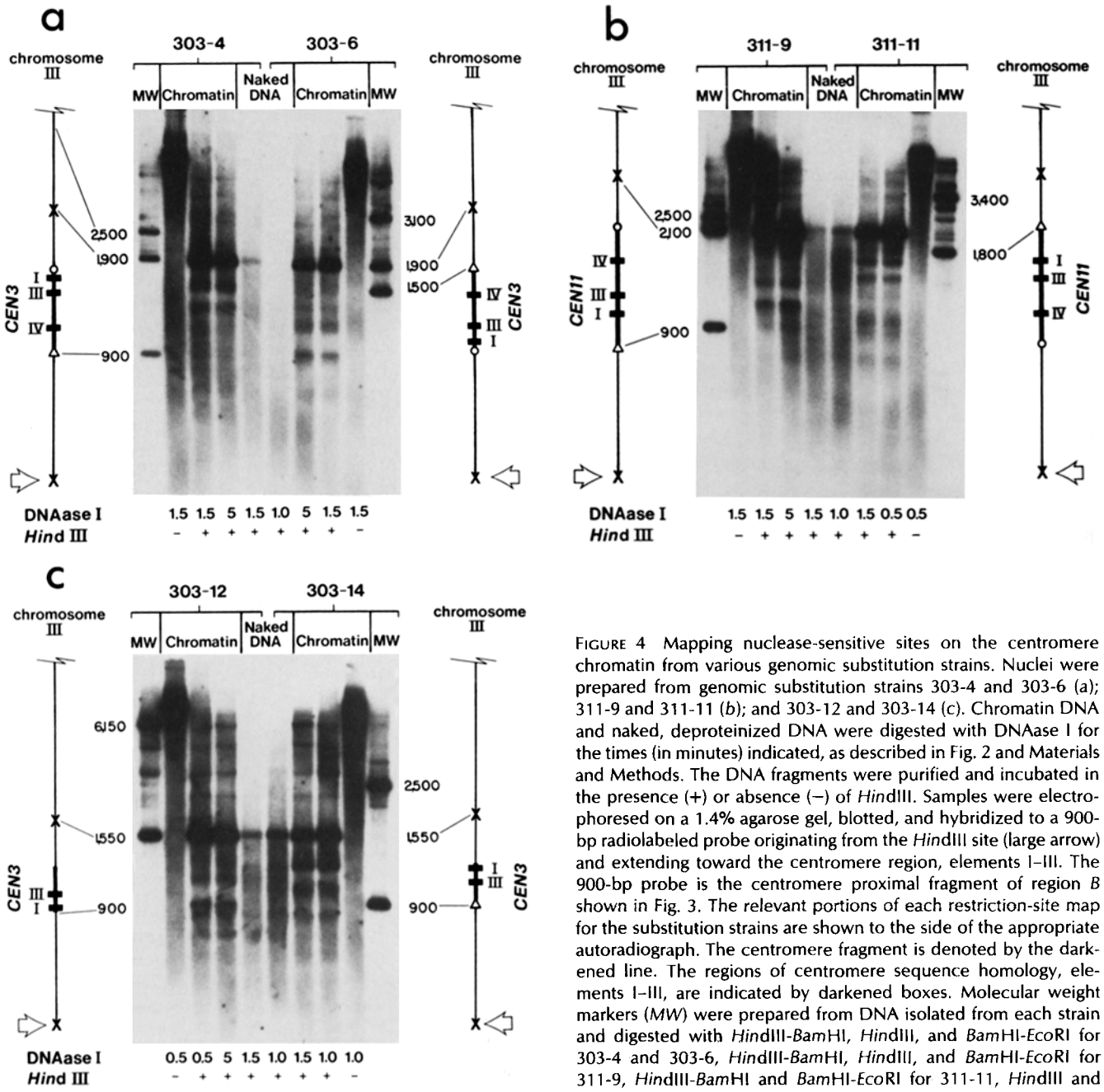

FIGURE 4 Mapping nuclease-sensitive sites on the centromere chromatin from various genomic substitution strains. Nuclei were prepared from genomic substitution strains 303-4 and 303-6 (a); 311-9 and 311-11 (*b*); and 303-12 and 303-14 (*c*). Chromatin DNA and naked, deproteinized DNA were digested with DNAase I for the times (in minutes) indicated, as described in Fig. 2 and Materials and Methods. The DNA fragments were purified and incubated in the presence (+) or absence (−) of *Hin*dIII. Samples were electrophoresed on a 1.4% agarose gel, blotted, and hybridized to a 900-bp radiolabeled probe originating from the *Hin*dIII site (large arrow) and extending toward the centromere region, elements I–III. The 900-bp probe is the centromere proximal fragment of region *B* shown in Fig. 3. The relevant portions of each restriction-site map for the substitution strains are shown to the side of the appropriate autoradiograph. The centromere fragment is denoted by the darkened line. The regions of centromere sequence homology, elements I–III, are indicated by darkened boxes. Molecular weight markers (*MW*) were prepared from DNA isolated from each strain and digested with *Hin*dIII-*Bam*HI, *Hin*dIII, and *Bam*HI-*Eco*RI for 303-4 and 303-6, *Hin*dIII-*Bam*HI, *Hin*dIII, and *Bam*HI-*Eco*RI for 311-9, *Hin*dIII-*Bam*HI and *Bam*HI-*Eco*RI for 311-11, *Hin*dIII and *Eco*RI for 303-12, and *Hin*dIII-*Bam*HI and *Bam*HI-*Eco*RI for 303-14. The size of these DNA fragments serves to confirm the actual restriction map of the genomic substitution chromosomes in the yeast cell. Restriction sites are *Bam*HI (Δ), *Hin*dIII (x), and selected *Sau*3A (O) sites.

same *Hin*dIII site (Fig. 4*a*). The chromatin structure in the *CEN3* regions of strains 303-4 and 303-6 was revealed after secondary restriction endonuclease digestion (Fig. 4*a*, chromatin). The nucleolytic cleavage pattern within the centromeric region reveals two prominent cutting sites that delineate a nuclease resistant region. With molecular weight standards on the gel, these cutting sites could be mapped relative to the DNA sequence. In the wild-type orientation (303-4), the nuclease cutting sites occur ~1250 and 1500 bp from the *Hin*dIII site. The sequence element I–III region occurs between 1350 and 1450 bp from the *Hin*dIII site; thus, the cleavage sites in chromatin flank a 220–250-bp protected centromere core that encompasses sequence elements I–III. This cleavage pattern is clearly absent in naked DNA, and therefore reflects the chromatin conformation at this chromosomal locus. In the inverted orientation (303-6), the protected region of centromere chromatin is altered in its position in the gel (Fig. 4*a*, *right*), corresponding to the altered position of the inverted element I–III region.

A protected chromatin structure is also maintained on the element I–III region from chromosome XI when these sequences are used to replace *CEN3* in chromosome III (Fig. 4*b*). The orientation of the 858-bp *CEN11* fragment can be visualized by the position of the *Bam*HI site 900 bp from the *Hin*dIII site in strain 311-9, and 1,800 bp from the *Hin*dIII site in strain 311-11. The chromatin mapping lanes (Fig. 4*b*, chromatin), again reveal two prominent cutting sites that delineate a nuclease resistant core, and map to either side of the sequence element I–III region. Again, the protein-free DNA does not contain these specific nuclease-sensitive sites (Fig. 4*b*, naked DNA).

When the 289-bp *CEN3* fragment was substituted into chromosome III (Fig. 4*c*), the same protected region encompassing elements I–III is seen as occurs in the wild-type chromosome. The truncated 289-bp *CEN3* fragment contains only the DNA sequences from element I to ~150 bp past element III (Fig. 3). The DNA sequences normally present at the nuclease-sensitive site flanking element I are deleted in the 289-bp *CEN3* fragment, and foreign DNA sequences originating either from the bacterial vector or flanking yeast chromosomal DNA juxtapose element I in strains 303-14 and 303-12. Nevertheless, the nuclease cleavage pattern of the 289-bp centromeric chromatin (Fig. 4*c*) exhibits striking similarities to the patterns shown in Fig. 4, *a* and *b*. Two prominent nuclease cleavage sites flank the element I–III sequences regardless of the orientation of this region in the chromosome. The substitution of foreign DNA sequences for the DNA normally located adjacent to sequence element I apparently does not effect the structural integrity of the centromere core in the chromosome.

Protein Binding to the Centromere Core In Vivo

Because the yeast centromeres do not appear to be chromosome specific, and the chromatin structure surrounding the element I–III region in the various chromosomes is very similar or identical, it seems likely that the same centromeric proteins and/or RNA molecules recognize and bind to the different centromeres. We have measured the strength of the protein–DNA interaction at the centromere core in both the wild-type and structurally altered *CEN3* regions by dissociating chromosomal proteins with NaCl and by subsequently determining the structure of the protein-depleted chromatin complex. Chromatin from isolated yeast nuclei was washed extensively with SPCM or with buffer containing 0.4, 0.75, or 1.25 M NaCl. After the salt washes, chromatin was re-equilibrated with the standard digestion buffer and partially cleaved with DNAase I. The DNA was isolated from these samples, deproteinized and restricted with *Hin*dIII. The DNA fragments were separated electrophoretically, blotted to nitrocellulose, and probed with the 900-bp *Hin*dIII-*Bam*HI fragment from chromosome III (Fig. 3), as described in Fig. 4. The dissociation pattern of nucleoproteins from the centromeric chromatin of the wild-type strain, X2180a, is shown in Fig. 5. The pattern visualized after exhaustive washing in standard digestion buffer (Fig. 5, X2180a, no NaCl) revealed two prominent cutting sites flanking elements I–III, with the characteristic 220–250-bp spacing, indistinguishable from the pattern previously obtained with the conventional nuclei preparations (see Fig. 6*B* in reference 15). The pattern visualized after treatment of the chromatin complex with 0.4 M NaCl was comparable to the "no salt" lanes. Thus, the protein or RNA components that confer this unique structure to the centromeric chromatin remain bound after dissociation of loosely bound chromosomal proteins. More tightly bound chromosomal proteins, including the core histone proteins, are not dissociated until higher salt concentrations (1–2 M NaCl) are employed (20, 23). Upon dissociation of these more tightly bound chromosomal proteins, the protected region of chromatin becomes accessible to nucleolytic digestion (Fig. 5, X2180a, 0.75 and 1.25 M NaCl lanes), and the specific cleavage pattern in chromatin begins to resemble the cleavage pattern of naked DNA. Similar results were obtained by using chromatin from the genomic substitution stains. An example using the 289-bp *CEN3* substitution in chromosome III (303-12) is shown in Fig. 5. The protected centromere core was intact after exhaustive washing in SPCM (Fig. 5, 303-12, no NaCl lanes) and was very similar to the pattern visualized in Fig. 4*c*. After treatment with 0.4 M NaCl, again dissociating loosely bound chromosomal proteins, centromeric chromatin in this altered strain was unaffected. Washes with higher salt concentrations disrupted the protected centromere core (Fig. 5, 303-12, 0.75 and 1.25 M NaCl lanes). These results indicate that upon dissociation of tightly bound chromosomal proteins, the centromeric chromatin DNA becomes more accessible to nucleolytic digestion. The unique structure of the centromere is therefore dependent on the association of chromatin components in the cell nucleus. Furthermore, the chromatin components protecting the centromere DNA from cleavage bind with equal affinity to the various structurally altered *CEN3* regions studied in this work.

Isolation of Centromeric DNA-binding Proteins

The disruption of centromeric structure with high-salt treatment indicates that at least some of the essential centromere DNA-binding proteins are concomitantly released from the chromatin fiber. We have isolated the protein fraction that dissociates from the chromatin complex at the same salt concentrations required to dissociate the centromere chromatin structure. A soluble chromatin fraction was prepared as described in Materials and Methods, and immobilized on hydroxyapatite. The chromosomal proteins were subsequently dissociated by washing the column with increasing concentrations of NaCl. This method is useful for the isolation of specific chromosomal proteins, in that the protein fractions obtained by dissociation of chromatin in higher ionic strengths are devoid of proteins dissociated at lower NaCl

levels (20). Loosely bound chromosomal proteins were dissociated and eluted from the immobilized chromatin by extensive washing with 0.4 M NaCl. From the results shown in Fig. 5, it was evident that the centromere core was somewhat less distinct in chromatin washed with 0.75 M NaCl, and completely disrupted after 1.25 M NaCl treatment. Therefore, to collect the majority of specific binding proteins, we eluted the immobilized chromatin with 2 M NaCl and collected the dissociated proteins for DNA-binding studies.

We tested the sequence specificity of the fractionated chromatin proteins using a nitrocellulose filter binding assay (11, 24). Duplex DNA passes through nitrocellulose, whereas proteins remain bound. If a protein binds a specific DNA sequence, that sequence will appear to be retained on the nitrocellulose. The affinity of the interaction can be studied by dissociating the protein–DNA complex with increasing concentrations of NaCl and determining when the bound DNA is eluted from the filter. Restriction endonuclease-digested yeast genomic DNA was complexed with increasing concentrations of the 2 M NaCl-dissociated fraction of chromosomal proteins prepared as described above. The complexes were passed through nitrocellulose filters, the unbound fraction was collected (Fig. 6, lane *1*) and the bound DNA was eluted with increasing concentrations of NaCl (Fig. 6, lanes *2* and *3*) and NaCl in the presence of SDS (Fig. 6, lanes *4*). The DNA was separated by size on agarose gels and transferred to nitrocellulose filters. To visualize specific DNA sequences, the DNA was hybridized to the radiolabeled 624-bp *CEN3* fragment (Fig. 3) and, as a control, to a radiolabeled 1.7-kilobase pair (kb) DNA fragment containing the yeast *HIS3* gene (not centromere-linked) (25). In the absence of DNA-binding proteins, both the *CEN3* and *HIS3* sequences flow through the filter (Fig. 6, extreme left, lane *1*) and no DNA is retained. With increasing protein concentrations (right lanes) the *CEN3* fragment was specifically retained on the nitrocellulose filter; *CEN3* sequences were reduced in the flow-through fractions, (Fig. 6, lane *1*) but were eluted with increasing salt concentrations (lanes *3* and *4*). In the presence of higher protein concentrations, the specificity was most marked (Fig. 6, far right, lanes 1–4). The *HIS3* gene as well as the bulk of the yeast genomic DNA were not bound, whereas the *CEN3* fragment was completely retained, and not eluted from the filters until high concentrations of NaCl (>1 M) were employed to dissociate the complex. These DNA-binding proteins therefore specifically recognize the centromere DNA sequences, even when challenged with the entire complement of yeast genomic DNA. The same protein fraction binds both centromeres that have been tested (*CEN3* and *CEN11*), consistent with genetic data that indicates these centromere DNAs are interchangeable on chromosome III (14).

DISCUSSION

A deletion of a 624-bp region containing *CEN3* from chromosome III results in dramatic instability of the entire chromosome (14). Replacement of these sequences with the truncated 289-bp *CEN3* fragment in either orientation results in a completely functional chromosome that segregates through mitosis and meiosis with the fidelity of a wild-type chromosome. The 289-bp *CEN3* fragment is also functional whether present in only one or in both copies of the chromosome in diploid cells. Previous results have shown that the presence of

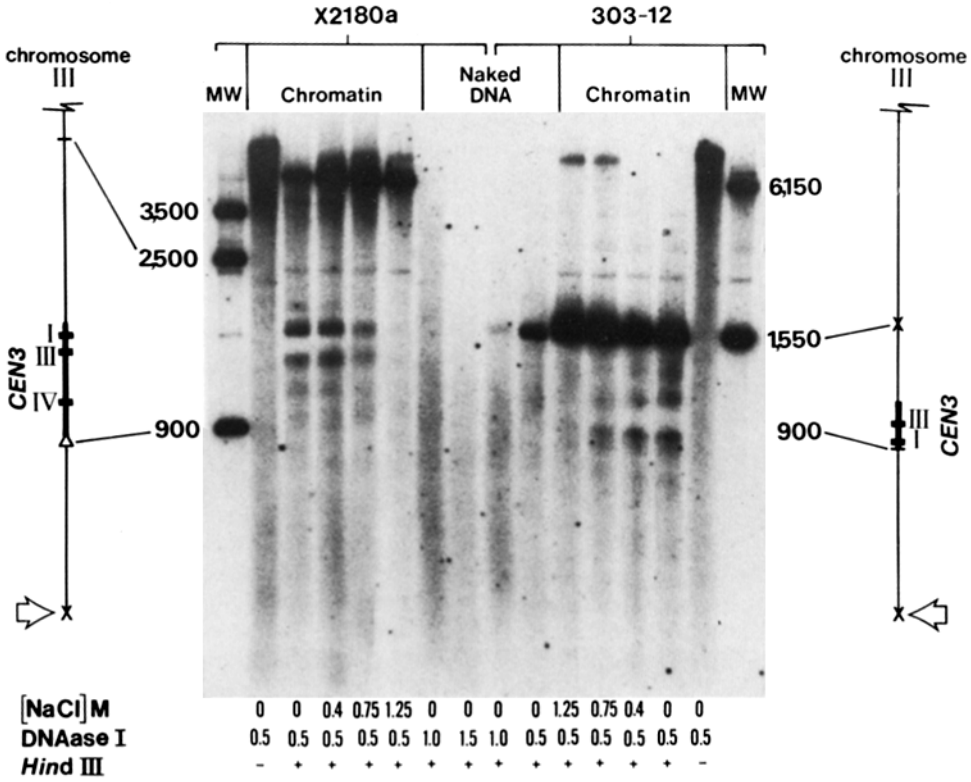

FIGURE 5 Mapping nuclease-sensitive sites on centromeric chromatin from protein-depleted yeast chromosomes. Nuclei were prepared from wild-type strain X2180a, or the genomic substitution strain 303-12. Before nuclease digestion, samples were resuspended in SPCM or in SPCM plus 0.4 M NaCl, 0.75 M NaCl, or 1.25 M NaCl. Each sample was immediately sedimented at 10,000 *g* for 10 min and washed twice in the same solution of SPCM plus salt. The final nuclear pellets were equilibrated with SPCM and digested with DNAase I (5 μg/ml) for the times (in minutes) indicated. The DNA samples were prepared and analyzed as described in Fig. 4. Molecular weight markers (*MW*) indicate yeast DNA fragments containing regions complementary to the probe, cut with *Hin*dIII-*Bam*HI, *Bam*HI-*Eco*RI, and *Hin*dIII-*Eco*RI for X2180a, and *Hin*dIII *Eco*RI for 303-12. The portion of the chromosome containing *CEN3* is shown to the side of the appropriate autoradiograph. *CEN3* is indicated by the darkened line. Restriction sites are *Eco*RI (–), *Bam*HI (Δ), and *Hin*dIII (x).

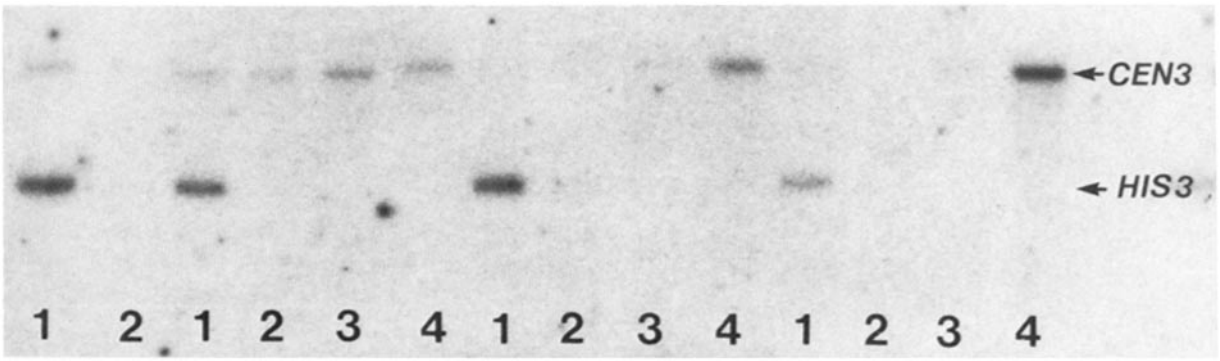

FIGURE 6 Selective affinity of centromere binding proteins to *CEN* DNAs. A protein fraction was dissociated from immobilized chromatin as described in the Materials and Methods with the salt solutions that dissociate the in vivo centromeric complex (Fig. 5). Increasing concentrations of this protein fraction (0, 0.2, 0.5, and 1.0 μg) were incubated with 1.0 μg of *Bam*HI restricted genomic DNA from yeast strain RH218, as described in Materials and Methods. The complexes were passed through nitrocellulose filters and the unbound fraction (lanes *1*), 0.4 M NaCl eluants (lanes *2*), 1.0 M NaCl eluants (lanes *3*), and 1.0 M NaCl plus 1% SDS eluants (lanes *4*) were collected. DNA samples were deproteinized and electrophoresed on a 1.0% agarose gel. The DNA fragments were transferred to nitrocellulose and hybridized with the 624-bp *CEN3* fragment shown in Fig. 3, and as a control, the 1.7-kb *HIS3* gene fragment (25). The 8.2-kb band containing the *CEN3* sequences (5) and the 1.7-kb band containing the *HIS3* sequences (25) are indicated. At left, lanes *1* and *2* show the elution pattern of DNA fragments incubated in the absence of binding protein. The three sets of lanes *1–4*, from left to right, show the elution pattern of DNA fragments incubated with 0.2, 0.5, and 1.0 μg of binding proteins, respectively.

different centromeres, or the same centromere in opposite orientations in chromosomes no. III of diploid cells, does not seem to affect the fidelity of chromosome pairing and segregation in a cell undergoing the complicated processes of meiosis (14). The centromeres may therefore be structurally autonomous units whose function is chromosome interspecific such that they are able to interact with the same components of the segregation apparatus regardless of the chromosome in which they reside.

We have examined the folding of centromere DNA in the yeast cell nucleus with the nucleolytic enzymes, micrococcal nuclease and DNAase I. A 220–250-bp region that includes the key sequence elements I–III was refractory to nucleolytic digestion (11, 15). This 220–250-bp centromere core is found to be associated with the centromere sequences on all the chromosomes that have been examined to date (Fig. 7). The maintenance of this distinctive structural differentiation in chromatin containing the 289-bp *CEN3* replacement (Fig. 4*c*) indicates that the DNA sequences within this fragment provide the necessary information for binding of centromere-specific chromatin components, independent of flanking sequence information. This region of DNA is characterized by short sequence elements whose spacing is conserved in the centromeres of different chromosomes (Figs. 1 and 7 and reference 10). If this region of chromatin is wound in a similar fashion to nucleosomal chromatin, these elements may be juxtaposed in the cell nucleus and could provide a common binding site for components of the mitotic apparatus. The components required for maintenance of the chromatin structure surrounding the centromere core are dissociated from the DNA at the same ionic strengths used to remove the histones from bulk chromatin (15, 20). Although chromatin components other than histones must be bound to the centromere at some point in the cell division cycle, this region may be wound around histone or histonelike proteins that together serve as a chromatin template for the components of the mitotic apparatus.

The ability to isolate the mitotic spindle from the yeast, *Saccharomyces cerevisiae* (27, 28), has provided a cytological view of the mitotic apparatus in a cell that is not particularly amenable to such an analysis. Although the role the spindle

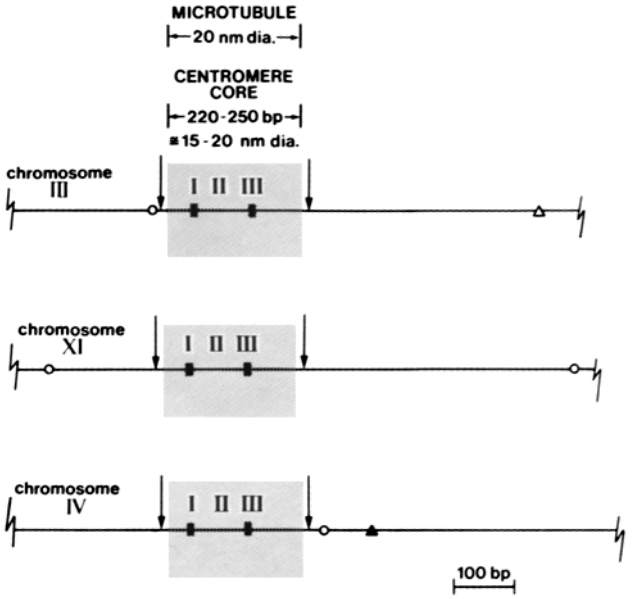

FIGURE 7 Structural organization at the centromere core in yeast chromosomes III, IV, and XI. A map of the centromere regions from chromosomes III, IV, and XI shows the region of structural differentiation encompassing the elements of sequence homology I–III in each centromere. DNAase I sites (vertical arrows) that bound the nuclease-resistant centromere core (stippled box) are indicated. Restriction enzyme sites are *Bam*HI (△), *Xho*I (▲), and *Sau*3A (○). The DNA fiber is represented in linear form to visualize the position of the 220–250-bp nuclease-resistant core and the elements I-III of centromere sequence homology. In chromatin, the bulk of the DNA is wrapped around histone proteins to form a subunit structure of 160-bp repeats. The centromere core, in contrast, is in a protected particle that contains 1.3–1.5 times more DNA than does the typical nucleosomal subunit. Although the conformation of DNA within the 220–250-bp protected core particle remains to be elucidated, a direct extrapolation from the dimensions of a nucleosome (11 × 5 nm, reference 26) would give a diameter of 15–20 nm for the centromeric core.

plays in yeast mitosis remains to be elucidated, there is a strong correlation between the number of discontinuous microtubules and the number of genetic linkage groups in yeast

(27, 29). If a single microtubule interacts with a unique point along the chromatin fiber of each chromosome, a structural discontinuity may be characteristic of that region in chromatin. The structural parameters of yeast microtubules and the nuclease-resistant centromere core (Fig. 7) are consistent with the notion that the centromere core is such a binding site. With the isolation of specific DNA-binding proteins, yeast mitotic spindles, and centromere DNA, we should eventually be able to characterize their interaction and begin to understand the molecular mechanisms that govern chromosome segregation and cell division.

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