

Yeast Centromere DNA Is in a Unique and Highly Ordered Structure in Chromosomes and Small Circular Minichromosomes

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Summary

We have examined the chromatin structure of the centromere regions of chromosomes III and XI in yeast by using cloned functional centromere DNAs (*CEN3* and *CEN11*) as labeled probes. When chromatin from isolated nuclei is digested with micrococcal nuclease and the resulting DNA fragments separated electrophoretically and blotted to nitrocellulose filters, the centromeric nucleosomal subunits are resolved into significantly more distinct ladders than are those from the bulk of the chromatin. A discrete protected region of 220–250 bp of *CEN* sequence flanked by highly nuclease-sensitive sites was revealed by mapping the exact nuclease cleavage sites within the centromeric chromatin. On both sides of this protected region, highly phased and specific nuclease cutting sites exist at nucleosomal intervals (160 bp) for a total length of 12–15 nucleosomal subunits. The central protected region in the chromatin of both centromeres spans the 130 bp segment that exhibits the highest degree of sequence homology (71%) between functional *CEN3* and *CEN11* DNAs. This unique chromatin structure is maintained on *CEN* sequences introduced into yeast on autonomously replicating plasmids, but is not propagated through foreign DNA sequences flanking the inserted yeast DNA.

Introduction

The centromeres of eucaryotic chromosomes are specific regions along the chromatin fiber that play a fundamental role in chromosome movement during cell division. The apparatus that regulates the distribution of chromosomes to daughter cells is composed of protein subunits assembled into spindle tubules. The function of the centromere, at least in part, is to serve as the chromatin attachment site for the spindle tubules and to direct the chromosomes through mitosis and meiosis. An understanding of the molecular mechanisms by which centromere regions become associated with microtubules will require a knowledge of the organization of centromere DNA, centromere proteins and other chromatin components specifically associated with the centromere.

Functional centromere DNA sequences have been isolated from chromosomes III and XI of the yeast, *Saccharomyces cerevisiae* (Clarke and Carbon, 1980; Fitzgerald-Hayes et al., 1982a). When DNA segments containing either *CEN3* or *CEN11* are introduced into yeast on autonomously replicating plas-

mids, genetic markers on these plasmids are stably maintained through mitosis in the absence of selective pressure and segregate through meiosis in a manner similar to that expected for centromere-linked genes (Clarke and Carbon, 1980; Fitzgerald-Hayes et al., 1982a). Autonomously replicating plasmids containing a functional replicator (*ars*) but no *CEN* sequence are extremely unstable during mitosis and quickly lost under nonselective growth conditions (Stinchcomb et al., 1979). The *CEN* sequences therefore stabilize autonomously replicating plasmids in yeast, and enable plasmids to undergo Mendelian segregation as minichromosomes.

The DNA fragments containing functional *CEN3* and *CEN11* activity differ in overall nucleotide sequence but share several features of sequence organization (Figure 1; Fitzgerald-Hayes et al., 1982b). Both contain a 90 bp region (element II), more than 90% of which is A+T base pairs. There are three regions of complete homology between the two centromeres, element I (14 bp), element III (11 bp) and element IV (10 bp), which surround the A+T-rich core segment and are positioned in an almost identical spatial arrangement within the two centromere sequences, as shown in Figure 1. In both centromeres the most highly conserved region (71%) is a 130 bp segment from element I to about 20 bp beyond element III. These unique aspects of sequence organization, common to both centromeres, indicate that this may be the functional unit of the centromere DNA.

The DNA in yeast cells is organized into nucleosomal subunits typical of the DNA in other eucaryotic cells (Lohr and van Holde, 1975; Thomas and Furber, 1976; Lohr et al., 1977; Lohr, 1981). The immediate consequence of this folding of the DNA around a histone core is that distant regions of a chromosome are brought into proximity in the cell nucleus. The highly conserved sequence and spatial arrangement of elements I–IV in the two centromeres (Fitzgerald-Hayes et al., 1982b) indicate that this region may be a recognition site for sequence-specific DNA-binding proteins (Weintraub, 1980). To determine specifically how the centromere DNA sequence is folded in the yeast chromosome and how this region of DNA gives rise to a functional centromeric unit, we have analyzed the structural organization of centromeric chromatin. Our results show that a specific region of 220–250 bp in the centromere DNA sequence is in a unique chromatin conformation, and the surrounding chromosomal DNA directs the nucleosomal subunits into a very ordered and specifically aligned array. The centromere DNA sequences are in this same conformation in the genome and on autonomously replicating plasmids in yeast.

Results

Restriction maps of the centromere regions of yeast



Figure 1. Spatial Arrangement of Sequence Elements Common to *CEN3* and *CEN11*

The regions of perfect sequence homology between *CEN3* and *CEN11*, elements I (14 bp), III (11 bp) and IV (10 bp), are positioned in an almost identical spatial arrangement within the two centromere sequences. The A+T-rich core segment (element II) lies between elements I and III in both DNA sequences. An overall homology of 71% is seen in the element I-II-III regions of *CEN3* and *CEN11*. The total length encompassed by sequence elements I-IV is 370 bp in *CEN3* and 374 bp in *CEN11*.

chromosomes III and XI, including the positions of the restriction fragments containing functional centromere activity (*CEN3*, 627 bp, and *CEN11*, 858 bp) and the relevant fragments cloned into various plasmids, are shown in Figure 2. The DNA fragments used as hybridization probes are indicated below the restriction maps. More detailed restriction maps are shown in Clarke and Carbon (1980) and Fitzgerald-Hayes et al. (1982a and 1982b).

Distinct Nucleosomal Ladders Are Characteristic of Centromere Sequences

To determine whether the centromere DNA is organized into nucleosomal subunits typical of the bulk of yeast DNA, we examined the chromatin structure along chromosomes III and XI in yeast by using DNA fragments containing functional centromeres (*CEN3* and *CEN11*) as labeled probes. Chromatin from isolated yeast nuclei was partially digested with micrococcal nuclease, and after deproteinization, the resulting DNA fragments were separated electrophoretically and blotted as single strands onto nitrocellulose filter paper. Upon visualization of the centromeric DNA by hybridization with the labeled 627 bp DNA fragment containing *CEN3* or the labeled 858 bp DNA fragment containing *CEN11* (Figures 2A and 2B), the centromeric nucleosomal subunits were seen to be resolved into significantly more distinct ladders than the bulk of the chromatin, or the chromatin from the *LEU2* region, 20 kb downstream from the centromere (Figure 3). To demonstrate that this pattern is dependent on the structure of the DNA in chromatin, a sample of the chromatin DNA was completely deproteinized before nuclease digestion. As shown in Figure 4A (naked DNA, unrestricted lanes), no repeating pattern is evident in the centromeric DNA fragments produced upon partial micrococcal nuclease cleavage of naked DNA. The highly repetitive pattern of centromeric chromatin is therefore dependent upon the folding of these DNA sequences in chromatin. The largest centromere fragments observed in Figure 3 correspond to 15-16 nucleosomal subunits, which indicate that

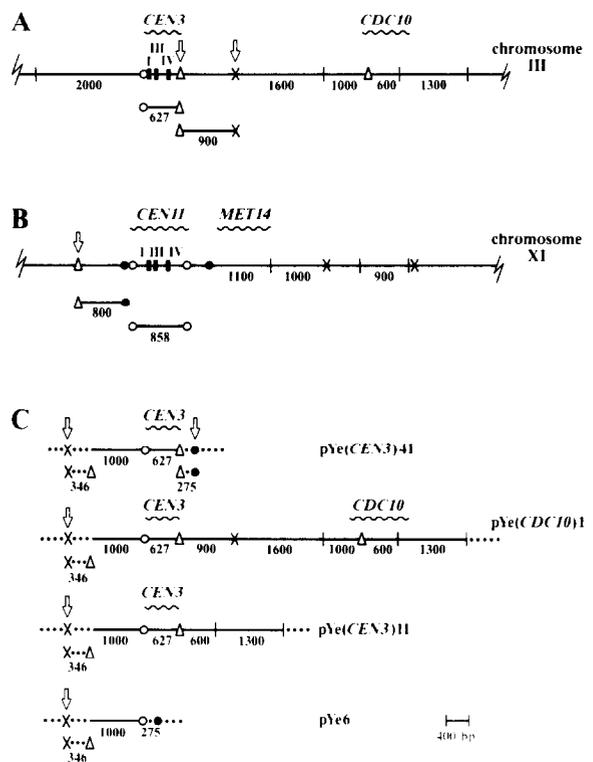


Figure 2. Comparative Restriction Maps of the Centromere Regions of Yeast Chromosomes III and XI and the Relevant Subcloned DNA Fragments

The positions of the functional 627 bp *CEN3* DNA fragment on yeast chromosome III (A) and the 858 bp *CEN11* DNA fragment on yeast chromosome XI (B) are indicated. The regions of complete homology between the two centromeres, elements I, III and IV, are indicated on *CEN3* (A) and *CEN11* (B). The very A+T-rich region (element II) lies between elements I and III in both centromeres. The relevant subcloned fragments are shown in (C). The solid lines indicate the yeast DNA inserts, and the dotted lines denote vector (pBR322) sequences. The complete structure of pYe(*CEN3*)41, pYe(*CDC10*)1 and pYe(*CEN3*)11 are given in Clarke and Carbon (1980). Plasmid pYe6 is described in Fitzgerald-Hayes et al. (1982b). Only selected restriction sites are shown. The DNA fragments shown below each restriction map were used as hybridization probes; lengths are given in base pairs. The large arrows denote the restriction sites chosen for the nuclease mapping studies. Restriction enzyme cleavage sites are Bam HI (Δ), Sau 3A (\circ), Sal I (\bullet), Hind III (\times) and Eco RI (\square).

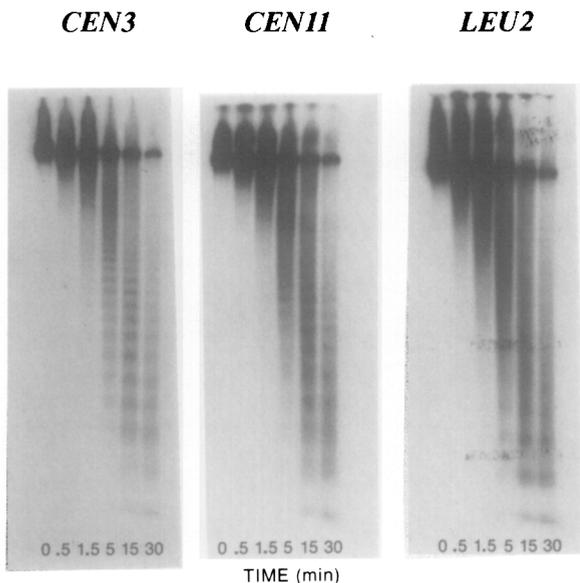


Figure 3. Micrococcal Nuclease Digestion Patterns of Centromeric and *LEU2* Chromatin

Nuclei from mid-logarithmic-phase cultures of yeast strain X2180a were digested with micrococcal nuclease (50 units/ml) for the times indicated. Purified DNA from each time point was subjected to electrophoresis on a 1.45% agarose gel. The DNAs were transferred to nitrocellulose filters (Southern, 1975) and hybridized to a ^{32}P -labeled nick-translated probe and autoradiographed. In the part marked *CEN3*, the probe was the 627 bp *CEN3* fragment from yeast chromosome III (Fitzgerald-Hayes et al., 1982b) (Figure 2A); the *CEN11* probe was the 858 bp *CEN11* fragment from yeast chromosome XI (Fitzgerald-Hayes et al., 1982a) (Figure 2B); the *LEU2* probe was the 2.2 kb *Pst* I DNA fragment that complements *leu2* mutations in yeast (Tschumper and Carbon, 1980). The *LEU2* region occurs 20 kb downstream from the *CEN3* sequence on yeast chromosome III (Clarke and Carbon, 1980).

at least 2.0–2.5 kb of DNA surrounding the small *CEN* fragments (627 and 858 bp) are in a highly ordered array. The simplest interpretation is that nucleosomal subunits are uniformly spaced in the centromere region. The internucleosomal spacer, which tends to be somewhat variable in the bulk of the chromatin DNA, may be very constant in this region of the chromosome, and would thus lead to a dramatic increase in the resolution of these centromeric nucleosomal subunits.

Mapping Nuclease-Sensitive Sites in Centromeric Chromatin

The nucleosome mapping technique of Wu (1980) was used to determine precisely the position of the nucleosomal subunits on the chromosome fiber. In this procedure, chromatin DNA in isolated nuclei is partially digested with micrococcal nuclease or DNAase I. Following nuclease cleavage, the DNA is completely deproteinized, and the purified DNA is digested to completion with a restriction endonuclease that cleaves DNA at a site adjacent to the region to be mapped. The DNA fragments are separated

electrophoretically, blotted to nitrocellulose and hybridized to a labeled DNA probe homologous to the region immediately proximal to the chosen restriction enzyme site (Figure 2). Only those fragments that contain the DNA in the same direction from the restriction enzyme site as the radiolabeled probe will be visualized following hybridization. The lengths of the hybridizing sequences that are cleaved by the restriction enzyme provide a direct map of the points of micrococcal nuclease or DNAase I cleavage within the chromatin relative to that restriction site.

We used micrococcal nuclease and DNAase I to probe the structure of centromeric chromatin. These enzymes differ markedly in their modes of nucleolytic cleavage (reviewed in McGhee and Felsenfeld, 1980). Micrococcal nuclease cleaves primarily internucleosomal chromatin DNA (Noll, 1974a; Axel, 1975), whereas DNAase I cleaves the chromatin DNA at nucleosomal and internucleosomal regions (Noll, 1974b; 1977). If the same chromatin structure is deduced when either of these enzymes is used, then this structure is probably characteristic of the chromatin region and not a result of the action of the enzymes on naked, deproteinized DNA.

A time course of micrococcal nuclease and DNAase I cleavage within the centromere III region of chromatin DNA and naked, deproteinized DNA is shown in Figure 4. After partial nucleolytic digestion of chromatin from isolated yeast nuclei, the DNA fragments were extracted and subsequently digested to completion with the restriction endonuclease *Bam* HI, which cleaves the DNA immediately adjacent to *CEN3* (Figure 2A). The DNA fragments were separated electrophoretically, blotted to nitrocellulose and probed with the 627 bp *CEN3* fragment shown in Figure 2A. The higher order nucleosomal subunit pattern of centromeric chromatin visualized after micrococcal nuclease digestion is again evident (Figure 4A, chromatin, unrestricted lanes). The higher order pattern is also generated after DNAase I cleavage, but is not clearly visualized until secondary restriction enzyme digestion is carried out (Figure 4B, chromatin, restricted lanes). If these nucleosomal subunits were randomly placed with respect to DNA sequence, then in different cells there would be different sequences protected by the nucleosomes, and upon secondary restriction enzyme cleavage and hybridization, a relatively uniform smear of hybridizing labeled probe would be seen. However, upon secondary restriction enzyme cleavage a distinct and ordered pattern is evident (Figure 4). The appearance of specific DNA fragments indicates that, in all cells, the same micrococcal nuclease cutting sites (Figure 4A) and DNAase I cutting sites (Figure 4B) occur at regular intervals in the centromere.

The most striking feature of the centromeric chromatin pattern visualized by the experiment shown in Figure 4 is that a 250 bp region of DNA occurring

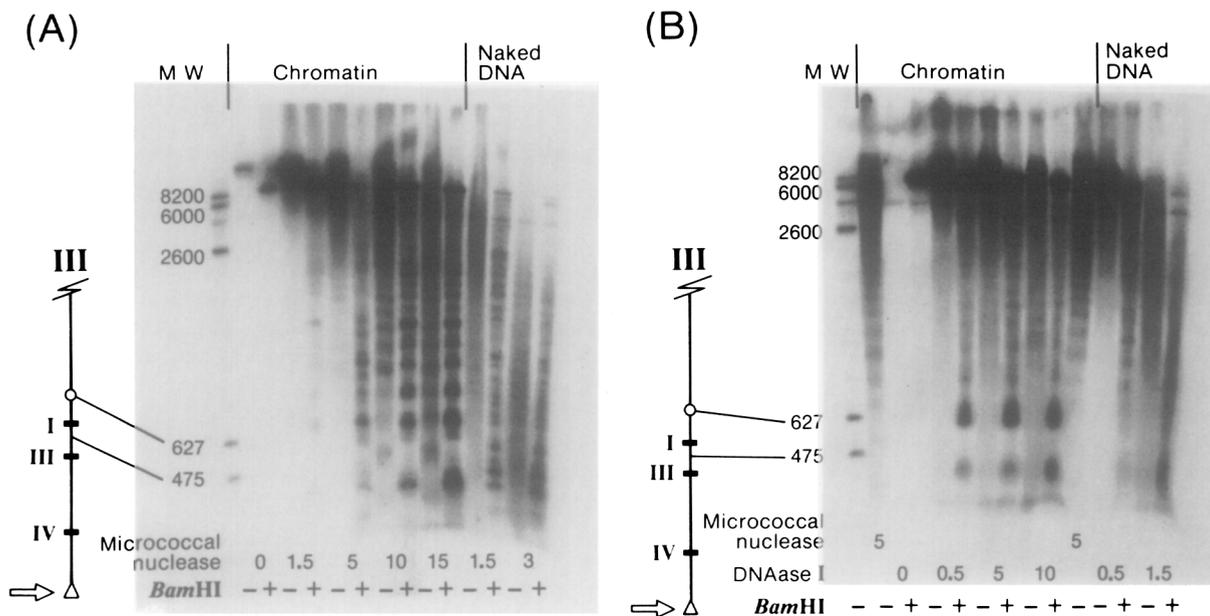


Figure 4. Mapping Nuclease-Sensitive Sites on the Centromeric Chromatin from Yeast Chromosome III

Nuclei were prepared from mid-logarithmic-phase cells of yeast strain X2180a and were digested with micrococcal nuclease (50 units/ml) (A) or DNAase I (5 μ g/ml) (B), for the times (min) indicated, as described in Experimental Procedures. For the experiments with naked DNA, nuclei were prepared exactly as described for the chromatin digests, but immediately before nuclease cleavage, DNA was extensively deproteinized as outlined in Experimental Procedures. The deproteinized DNA was subsequently digested under the same conditions as described for the chromatin digests with micrococcal nuclease (0.5 units/ml) (A) or with DNAase I (50 ng/ml) (B), for the times (min) indicated. After partial micrococcal nuclease or DNAase I cleavage, DNA samples were deproteinized, incubated in the presence (+) or absence (-) of Bam HI and subjected to electrophoresis on 1.4% agarose gels. The DNAs were transferred to nitrocellulose filters and hybridized to the radiolabeled 627 bp *CEN3* fragment shown in Figure 2A. The identical pattern was obtained following hybridization to a radiolabeled 150 bp fragment extending toward the centromere from the Bam HI site (data not shown). Molecular weight markers (MW) indicate yeast nuclear DNA fragments cut with Bam HI, Hind III, Bam HI-Eco RI, Sau 3A and Sau 3A-Hinf I. These fragments contain regions complementary to the radiolabeled probe (Clarke and Carbon, 1980). To the left is shown a partial restriction site map of the centromere region of yeast chromosome III. Restriction enzyme sites are Bam HI (Δ) and Sau 3A (\circ). The large arrows indicate the Bam HI site on yeast chromosome III that is immediately proximal to the 627 bp labeled DNA probe.

between 350 and 600 bp in a centromere-proximal direction from the Bam HI site (see Figures 2A and 4) is completely protected from nucleolytic cleavage, and is bounded on both sides by micrococcal nuclease and hypersensitive DNAase I cleavage sites. A specific micrococcal-nuclease-sensitive site occurring in naked, deproteinized DNA occurs 500 bp from the Bam HI site and is totally masked in the chromatin DNA (Figure 4A, naked DNA lanes). Thus the 250 bp protected region of DNA results mostly from the conformation of the DNA in chromatin, rather than from intrinsically nuclease-insensitive sites in naked DNA. The sites that are hypersensitive to DNAase I cleavage also result from the chromatin conformation, for they are not preferentially cleaved by DNAase I in unprotected, deproteinized DNA (Figure 4B, naked DNA lanes). In the region extending 600 to 2500 bp in a centromere-proximal direction from the Bam HI site (Figure 2A), micrococcal nuclease and DNAase I cutting sites are evident at regular intervals (Figure 4). Calibration with markers of known molecular weight shows that these cleavage sites occur at intervals of about 160 bp.

Specific micrococcal nuclease cleavage sites that

are visualized after digestion of naked, deproteinized DNA (Figure 4A, naked DNA lanes) confirm the DNA sequence specificity of this enzyme seen by other investigators (Dingwall et al., 1981; Horz and Altenburger, 1981; Keene and Elgin, 1981). The cleavage pattern given by unprotected DNA is similar to that given by whole chromatin, however specific sites in naked DNA are masked from nuclease action in the chromatin. The sensitivity of individual cleavage sites in the chromatin also differs from that in naked DNA, as demonstrated by the different intensities of various bands seen after nuclease digestion (Figure 4A, compare chromatin and naked DNA lanes). We therefore believe that the fragment patterns obtained from digestion of the chromatin from the centromere region (Figure 4) are for the most part dependent on the conformation of the DNA in the cell nucleus.

A similar structural organization emerges from mapping the centromeric chromatin from chromosome XI. As described above, the DNA fragments resulting from partial nucleolytic cleavage of chromatin from isolated yeast nuclei were extracted and digested to completion with Bam HI. The DNA fragments were separated electrophoretically, blotted to nitrocellulose and

probed with the labeled 800 bp fragment extending toward the centromere from the Bam HI site (Figure 2B). A very ordered pattern is revealed upon secondary restriction endonuclease digestion (Figure 5). A 250 bp region of chromatin occurring 1200 to 1450 bp from the Bam HI site in a centromere-proximal direction (Figure 2B) is resistant to enzymatic digestion and is bounded by micrococcal nuclease and DNAase I cleavage sites (Figure 5). This appears to be a result of the conformation of the DNA in chromatin, for a different pattern is evident after micrococcal nuclease digestion of naked, deproteinized DNA (Figure 5A, naked DNA lanes). A micrococcal-nuclease-sensitive site occurring about 1350 bp from the Bam HI site in naked DNA is totally masked in the same region of the chromatin (Figure 5A, naked DNA lanes). In the 2 kb region extending from the Bam HI site toward the centromere, specific micrococcal nuclease and DNAase I cleavage sites are evident at regular intervals and correspond to the nucleosomal array. The fragmentation pattern becomes less distinct about 400 bp on the other side of *CEN11*, approximately where the *MET14* gene is thought to occur on chromosome XI.

The Protected Region in Centromeric Chromatin Is Surrounded by Highly Ordered Nucleosomal Subunits

As the first step in delimiting the yeast centromere on the host chromosome we have mapped the chromatin structure in the centromere-flanking regions of chromosome III. Chromatin DNA from isolated yeast nuclei was partially digested with micrococcal nuclease or DNAase I, deproteinized and cleaved with restriction endonuclease Hind III. This restriction site is located 900 bp in a centromere-distal direction from the Bam HI site used in the mapping studies shown in Figure 4 and is approximately 1250 bp from the 250 bp protected region (Figure 2A). The resulting DNA fragments were subjected to electrophoresis, blotted as

described in Figure 4 and hybridized to the 900 bp DNA fragment from chromosome III shown in Figure 2A. The most striking feature of the centromeric chromatin pattern visualized in Figures 6A and 6B is the 250 bp nuclease-resistant region occurring in the interval between 1250 bp and 1500 bp from the Hind III site in a centromere-proximal direction. This nuclease-resistant region is bounded by micrococcal nuclease and DNAase I cutting sites, and occurs at the identical position on the chromosome III DNA sequence previously determined by mapping from the Bam HI site. Extending from the Hind III site to *CEN3*, specific micrococcal nuclease and DNAase I cutting sites are apparent, spaced at approximately nucleosomal intervals (Figures 6A and 6B). To determine how far the ordered nucleosomal subunits extend from the Bam HI site in a direction away from *CEN3* (see Figure 2A), the DNA fragments prepared by DNAase I and Bam HI digestion as described in Figure 4B were hybridized to the same labeled 900 bp fragment. Preferential DNAase I cutting sites are evident at nucleosomal intervals extending away from the centromere for a total distance of about 1500 bp from the Bam HI site (Figure 6C). No hypersensitive DNAase I cutting sites similar to those flanking the 250 bp resistant region of *CEN3* (Figures 4B and 6B) are evident in this region of the chromosome. Thus the 220–250 bp protected region of *CEN3* DNA is flanked by micrococcal nuclease and hypersensitive DNAase I cleavage sites (Figures 4 and 6), and 3.5 kb of the surrounding chromatin is organized into a highly ordered and specifically aligned nucleosomal array.

Salt Elution of the Chromatin Complex Results in Disorganization of the Centromeric Chromatin Structure

Since the primary nucleotide sequences of the *CEN3* and *CEN11* regions are mostly unique (Fitzgerald-Hayes et al., 1982b), the similar fragmentation patterns obtained from mapping the 2–3 kb of centromere

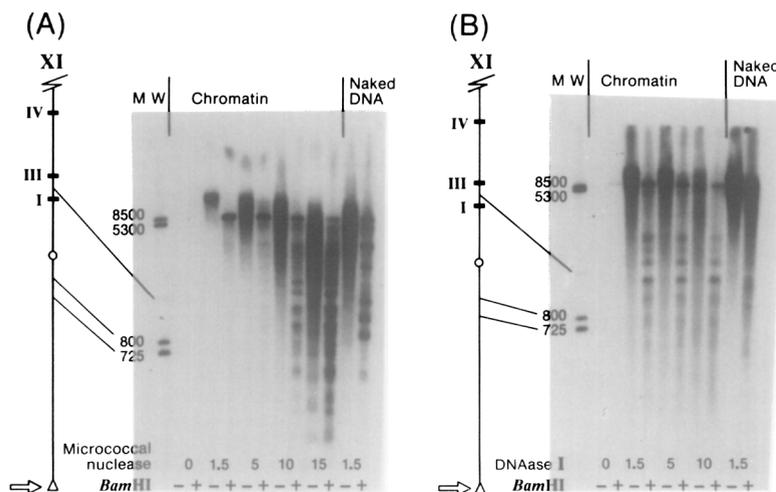


Figure 5. Mapping Nuclease-Sensitive Sites on Centromeric Chromatin from Yeast Chromosome XI

Conditions for digestion of chromatin or deproteinized DNA, cleavage with Bam HI, gel electrophoresis and Southern transfer were as described in Figure 4 and Experimental Procedures. After transfer to nitrocellulose, filters were hybridized to the radiolabeled 800 bp fragment from chromosome XI (Figure 2B). Molecular weight markers indicate yeast DNA fragments containing regions complementary to the probe, cut with Bam HI, Eco RI, Bam HI-Sal I, Hind III-Sal I and Hind III (Fitzgerald-Hayes et al., 1982a). To the left is a partial restriction site map of the centromere region of yeast chromosome XI. Restriction enzyme sites are Bam HI (Δ) and Sau 3A (○). Large arrows indicate the Bam HI site on yeast chromosome XI immediately proximal to the radiolabeled 800 bp DNA probe.

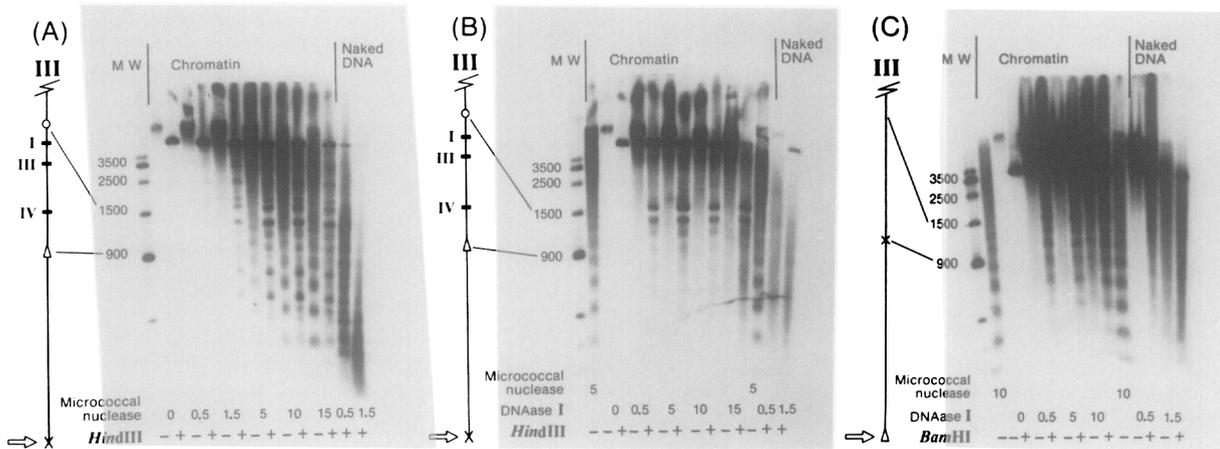


Figure 6. Nuclease Cleavage Sites in the Chromatin Surrounding the Centromere on Yeast Chromosome III

Nuclei from mid-logarithmic-phase X2180a cells were digested with micrococcal nuclease (50 units/ml) (A) or with DNAase I (5 μ g/ml) (B), for the times (min) indicated. Naked DNA was prepared as described in the legend to Figure 4. Deproteinized DNA was incubated with (+) or without (-) Hind III and subjected to electrophoresis on a 1.4% agarose gel. In (C), selected samples from the experiment shown in Figure 4B were subjected to electrophoresis on a 1.4% agarose gel. Following transfer to nitrocellulose paper, DNA was hybridized to the 900 bp fragment from chromosome III, shown in Figure 2A. Molecular weight markers (MW) are derived from the *CEN3* region as described in the legend to Figure 4. Yeast nuclear DNA fragments were cut with Hind III-Eco RI, Bam HI-Eco RI and Bam HI-Hind III. To the left is a partial restriction site map from this region of yeast chromosome III (Clarke and Carbon, 1980). Restriction enzyme sites are Bam HI (Δ), Sau 3A (O) and Hind III (X). The large arrows in (A) and (B) indicate the Hind III site on yeast chromosome III immediately proximal to the labeled 900 bp DNA probe. The large arrow in (C) indicates the Bam HI site on yeast chromosome III immediately proximal to the same probe.

meric chromatin are certainly the result of the conformation of these sequences in chromatin. This unique structural organization associated with centromeric chromatin must result from specific protein-DNA interactions or RNA-DNA interactions or both. The experiment shown in Figure 7 was performed to demonstrate that nucleoproteins are responsible for the unique structural order of the centromere. Chromosomal proteins can be removed from DNA by treatment of chromatin with NaCl (Spelsberg and Hnilica, 1971; Bloom and Anderson, 1978), and the structure of the protein-depleted chromatin complex can be determined (Adolph et al., 1977; Paulson and Laemmli, 1977). Chromatin from isolated yeast nuclei was washed extensively with standard digestion buffer (NaCl-free) or with buffer containing 0.4, 1.0 or 2.0 M NaCl. Following the salt washes, chromatin was re-equilibrated with the standard digestion buffer and partially cleaved with micrococcal nuclease. DNA was prepared from these samples, deproteinized and restricted with Bam HI. The DNA fragments were separated electrophoretically, blotted to nitrocellulose and probed with the 627 bp *CEN3* fragment as described in Figure 4A. The pattern visualized after exhaustive washing in standard digestion buffer (Figure 7, no NaCl) was virtually identical to the pattern observed in Figure 4A. This indicates that extensive washing of the chromatin complex with low ionic strength buffer does not affect the chromatin structure as determined by these mapping techniques. The pattern visualized after treatment of the chromatin with 0.4 M NaCl was also very similar to the pattern shown in Figure 4A. Thus the chromatin components that confer this unique structure to the centromeric chromatin remain

bound following dissociation of loosely bound chromosomal proteins. More tightly bound proteins, including the core histone proteins, are not dissociated from the chromatin complex until higher concentrations of salt (1-2 M NaCl) are used (Spelsberg and Hnilica, 1971; Bloom and Anderson, 1978). Upon dissociation of these more tightly bound chromosomal proteins, the protected region of chromatin in the 350 to 600 bp interval from the Bam HI site becomes exposed to nucleolytic cleavage (Figure 7, 1 M and 2 M NaCl). The sites that are cleaved by micrococcal nuclease in unprotected DNA are now cleaved in this region of chromatin (compare Figure 4A, naked DNA lanes, and Figure 7, 1 M and 2 M NaCl). Furthermore, the very ordered fragmentation pattern in chromatin in the region extending 600 to 2500 bp from the Bam HI site (Figures 4A and 7) becomes less distinct following salt extraction, and begins to resemble the fragmentation pattern of naked, deproteinized DNA. These observations provide compelling evidence that upon dissociation of proteins from the chromatin complex, the centromeric chromatin DNA becomes more exposed to nucleolytic digestion. The unique structural organization of centromeric chromatin is therefore dependent on the association of chromatin components in the cell nucleus, including nuclear proteins and DNA.

Centromeric DNA on Minichromosomes Is Organized in a Chromatin Structure Indistinguishable from Chromosomal Centromeric DNA

The unique structure of centromeric chromatin is highly conserved between the two centromeres from

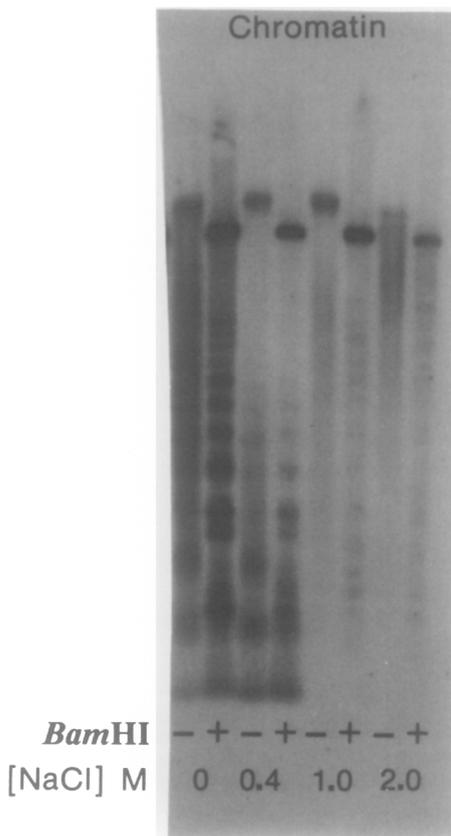


Figure 7. Mapping Nuclease-Sensitive Sites on Centromeric Chromatin from Protein-Depleted Yeast Chromosomes

Nuclei were prepared from mid-logarithmic-phase X2180a yeast cells, as described in Experimental Procedures. Prior to nuclease digestion, samples were resuspended in SPC or in SPC plus 0.4 M NaCl, 1.0 M NaCl or 2 M NaCl. Each sample was immediately sedimented at $10,000 \times g$ for 10 min and washed twice in the same solution of SPC plus salt. The resulting salt-washed nuclear pellets were resuspended in SPC and sedimented at $10,000 \times g$ for 10 min. The final nuclear pellets were resuspended in SPC and digested with micrococcal nuclease (50 units/ml) for 5 min at 32°C . Purified DNA was subjected to electrophoresis on 1.4% agarose gels after pre-digestion in the presence (+) or absence (-) of Bam HI. The DNA was transferred to nitrocellulose filters and hybridized to the radiolabeled 627 bp *CEN3* fragment shown in Figure 2A.

chromosome III and XI in yeast. If this structure is an essential property of the functional centromere, then a similar organization would be expected to be associated with any yeast centromere sequence that serves as a stabilizing and directing element for chromosomes or minichromosomes through mitosis and meiosis. To determine whether the unique structural organization characteristic of chromosomal *CEN* sequences is maintained on these fragments when they are cloned into autonomously replicating plasmids in yeast, we prepared nuclei from yeast cells transformed with plasmids carrying *CEN3* sequences. The plasmid used for this study, pYe(*CEN3*)41, contains a 1600 bp DNA segment from chromosome III including the functional *CEN3* element (Clarke and Carbon,

1980; see Figure 2C). A time course of micrococcal nuclease and DNAase I cleavage of chromatin DNA in nuclei from the transformed yeast cells is shown in Figure 8. To map only the centromere region cloned on the plasmid, we cleaved the isolated DNA fragments with Sal I, which cuts the plasmid once in the pBR322 vector DNA, and used a 275 bp pBR322 DNA fragment as hybridization probe (Figure 2C). Only the centromere sequences contiguous to the pBR322 vector DNA should be visualized after hybridization and autoradiography.

A 250 bp nuclease-resistant region occurring between 600 and 850 bp from the pBR322 Sal I site shown in Figure 2C is bounded by micrococcal nuclease and hypersensitive DNAase I cleavage sites (Figures 8A and 8B). This region is the same 220–250 bp sequence of *CEN3* DNA that is resistant to nuclease digestion in the chromosome. In the adjacent 1000 bp of yeast DNA cloned into pYe(*CEN3*)41, extending 900 bp to 1900 bp from the Sal I site, specific micrococcal nuclease and DNAase I cutting sites are present at nucleosomal intervals, in accordance with previous results obtained by mapping nuclease cleavage sites on these sequences in yeast chromosome III (Figure 4A). Similarly, the nuclease cleavage sites on the *CEN11* sequence cloned into autonomously replicating plasmids map to the same positions relative to the DNA sequence as those sites on the chromosomal *CEN11* sequence (data not shown).

Sequences Flanking *CEN* DNA Are Requisite for the Highly Ordered Nucleosomal Array Characteristic of This Region of the Chromosome

The highly ordered nucleosomal array occurring in the 3.5 kb region surrounding *CEN3* could depend upon the existence of specific DNA sequences or base distributions in the flanking regions. An alternative possibility would be that the nucleosomes are naturally brought into phase for a limited distance by the discontinuity occurring at the centromere boundary, and thus the phasing could be independent of the nature of the flanking DNA sequences. To distinguish between these possibilities, we mapped the nuclease cleavage sites on plasmid pYe(*CEN3*)41, containing 1.6 kb, and on plasmid pYe(*CDC10*)1, containing 7 kb of yeast DNA from the centromere region of chromosome III (Figure 2C). The plasmid pYe(*CDC10*)1 carries the 1.6 kb of yeast DNA cloned into pYe(*CEN3*)41 plus an additional 5.4 kb of yeast DNA contiguous with the *CEN3* sequence on the other side (Figure 2C). A time course of micrococcal nuclease digestion of chromatin DNA in isolated nuclei from yeast cells transformed with pYe(*CDC10*)1 or pYe(*CEN3*)41 is shown in Figures 9A and 9B. To map only the centromere sequences on the plasmid DNA, we cleaved the isolated DNA fragments with Hind III and hybridized them to the 346 bp pBR322 vector DNA fragment shown in Figure 2C. Only the centro-

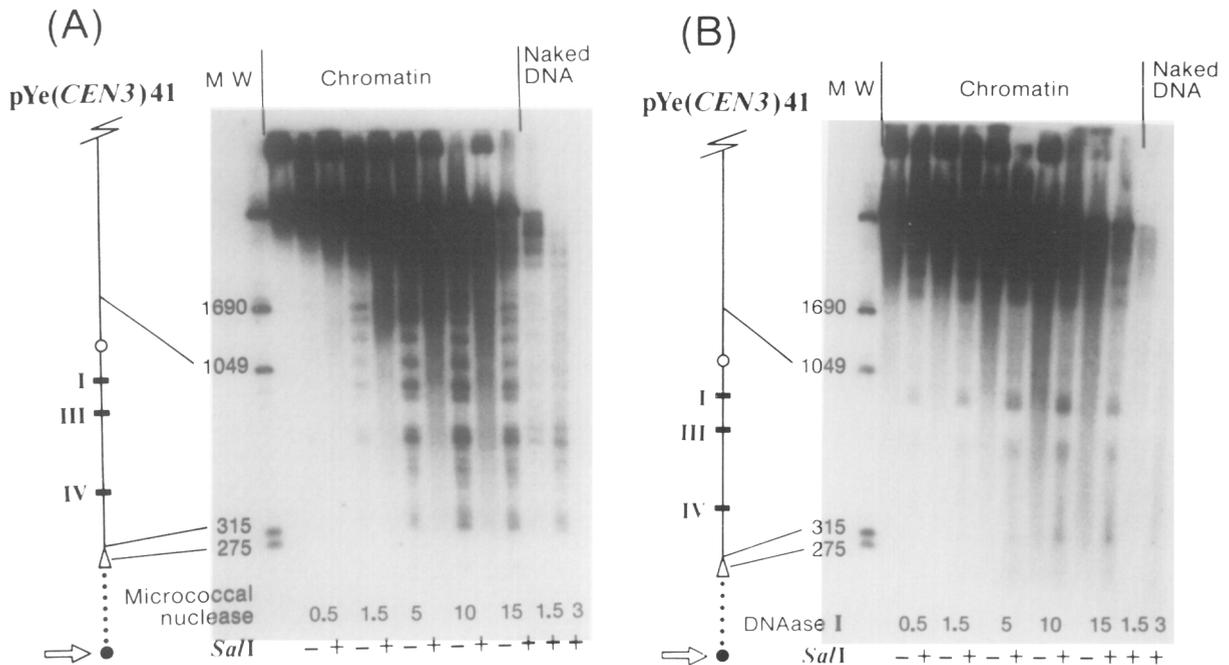


Figure 8. Mapping Nuclease Cleavage Sites on Plasmid pYe(CEN3)41 Chromatin from Transformed Yeast Cells

Yeast cells (DC04) were transformed as described by Hsiao and Carbon (1979) with plasmid pYe(CEN3)41 DNA. The plasmid pYe(CEN3)41 (Clarke and Carbon, 1980) contains the 627 bp *CEN3* fragment and 1000 bp of flanking yeast DNA (Figure 2C). The relevant portion of the restriction site map is shown to the left. Restriction enzyme sites are Sal I (●), Bam HI (Δ) and Sau 3A (○). Nuclei were prepared from these cultures as described in Experimental Procedures. Micrococcal nuclease digestion (A) and DNAase I digestion (B) were performed as described in Figure 4 for the times (min) indicated. The resulting DNA fragments were purified and incubated in the presence (+) or absence (-) of Sal I. The samples were subjected to electrophoresis on a 1.4% agarose gel, blotted and hybridized with the 275 bp pBR322 fragment shown in Figure 2C. Molecular weight markers (MW) were prepared from DNA isolated from the same cultures and restricted with Sal I-Bam HI, Sal I-Rsa, Bam HI-Ava and Bam HI-Pvu II. The size of these DNA fragments confirms the restriction map of the plasmid chromatin as it occurs in the yeast cell. The labeled 275 bp pBR322 probe hybridizes to fragments extending from the Sal I site (large arrow) toward the centromere.

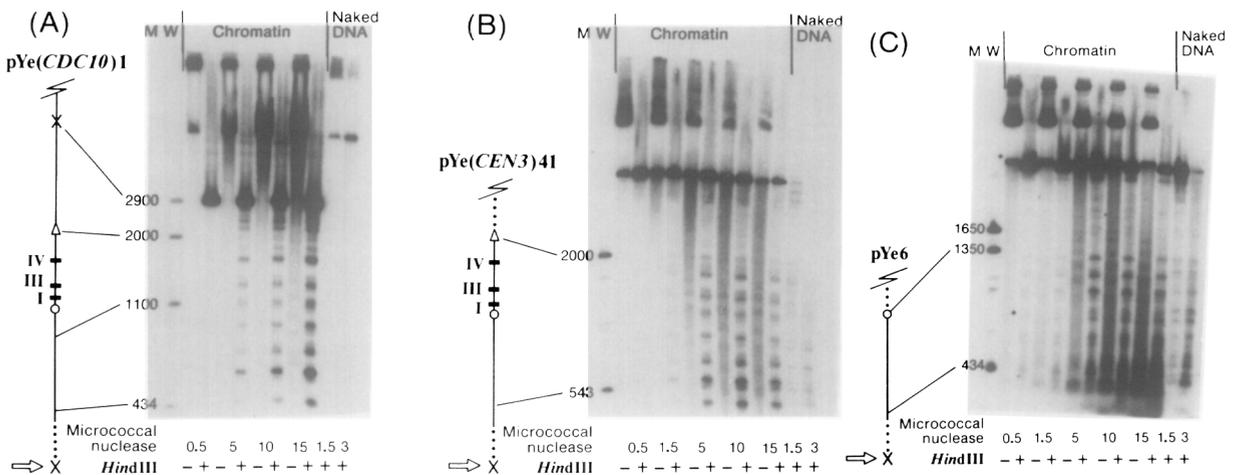


Figure 9. Mapping Nuclease Cleavage Sites on Plasmid pYe(CEN3)41, pYe(CDC10)1 and pYe6 Chromatin from Transformed Yeast Cells

Yeast cells (J17) were transformed with the appropriate plasmid DNA as described by Hsiao and Carbon (1979). Restriction maps of the plasmid DNAs are shown in Figure 2C. The relevant portion of each restriction enzyme map is shown to the left. Restriction enzyme sites are Hind III (×), Bam HI (Δ) and Sau 3A (○). Nuclei were prepared and digested for the times (min) indicated with micrococcal nuclease as described in the legend to Figure 4. The resulting DNA fragments were purified, digested with (+) or without (-) Hind III and fractionated by electrophoresis. Samples were blotted and hybridized to the 346 bp pBR322 fragment shown in Figure 2C. Molecular weight markers (MW) were prepared from DNA isolated from the appropriate yeast strain; for pYe(CDC10)1 (A), DNA was restricted with Hind III, Hind III-Bam HI, Hinf I and Sau 3A; for pYe(CEN3)41 (B), DNA was restricted with Bam HI-Hind III and Sau 3A, and for pYe6 (C), DNA was restricted with Hind III-Bam HI, Hinf I and Sau 3A. The size of these DNA fragments confirms the restriction map of the plasmid chromatin as it occurs in the transformed yeast cell. The large arrows indicate the Hind III site on the respective plasmid DNAs that is immediately proximal to the labeled 346 bp probe.

mere sequences contiguous with the 346 bp pBR322 fragment should be visualized after hybridization and autoradiography. The results (Figures 9A and 9B) demonstrate the specific micrococcal nuclease cleavage sites at nucleosomal intervals on the 1000 bp region of yeast DNA (400 to 1400 bp from the vector Hind III site) that is contiguous with the *CEN3* sequence in both plasmids (to the left of *CEN3* in Figure 2C). The *CEN3* region, 1400–2000 bp from the vector Hind III site, occurs in a structure analogous to the chromosomal *CEN3* region, and contains the expected 220–250 bp region of protected chromatin DNA (revealed by the occurrence of 1400 bp and 1600 bp DNA fragments). On pYe(*CEN3*)41 (Figure 9B), vector pBR322 DNA sequences are adjacent to the Bam HI site in the yeast DNA segment shown in Figure 2C, whereas pYe(*CDC10*)1 contains an additional 5.4 kb of yeast DNA before pBR322 sequences are encountered. The yeast DNA contiguous with the Bam HI site on pYe(*CDC10*)1 is cut into very ordered DNA fragments (Figure 9A), differing by approximately 160 bp in length, similar to the ordered pattern visualized on these sequences in chromosomal DNA (Figures 6A and 6C). The ordered pattern is evident up to the Hind III site on the plasmid DNA, the point at which the DNA was cleaved in the experiment shown in Figure 9A. However, when we use Eco RI for the mapping studies, the ordered pattern extends about 200–400 bp past the Hind III site (data not shown), consistent with the extent to which the ordered pattern was seen in the analogous chromosomal sequences (Figure 6C). In marked contrast, the pBR322 DNA sequences immediately adjacent to the yeast DNA in the analogous region of pYe(*CEN3*)41 (Figure 2C) are not cleaved in the chromatin at specific sites by micrococcal nuclease (Figure 9B, 2000–4000 bp region). A uniform smear is visualized in the 2000–4000 bp region following micrococcal nuclease cleavage of either intact chromatin or deproteinized pYe(*CEN3*)41 DNA. The pBR322 DNA sequences on pYe(*CEN3*)41 chromatin are therefore devoid of proteins or, more probably, the nucleohistones are asynchronously aligned on this DNA sequence.

It is possible that the nucleosomal ordering would be propagated from the centromere boundary through any yeast DNA sequence, but there could be something anomalous about the pBR322 sequences that prevents synchronous phasing. To examine this possibility, we mapped the nuclease cleavage sites in chromatin on the plasmid pYe(*CEN3*)11, in which a 3.5 kb Bam HI fragment occurring in pYe(*CDC10*)1 has been deleted (Clarke and Carbon, 1980) (Figure 2C). The yeast DNA sequences in the new position immediately adjacent to the *CEN3* sequence are not cleaved at regular 160 bp intervals after micrococcal nuclease digestion of chromatin in isolated yeast nuclei from cells transformed with pYe(*CEN3*)11 (data not shown). Thus the highly ordered nucleosomal

array is not propagated through pBR322 or yeast DNA sequences brought into new positions proximal to the *CEN3* region on plasmid DNA.

Since the sequences contiguous with the chromosomal *CEN3* DNA are required for the higher order chromatin structure observed in this region of the chromosome, these sequences might be organized into highly ordered nucleosomal arrays regardless of their proximity to *CEN3* DNA. To examine this possibility, we mapped the nuclease cleavage sites in chromatin on the plasmid pYe6, which carries 1000 bp of yeast DNA from the *CEN3* flanking region, but does not include the 627 bp *CEN3* fragment (Fitzgerald-Hayes et al., 1982b) (Figure 2C). A time course of micrococcal nuclease digestion of chromatin DNA in isolated nuclei from yeast cells transformed with pYe6 is shown in Figure 9C. The isolated DNA fragments were cleaved with Hind III and hybridized to the 346 bp pBR322 fragment as described in Figures 9A and 9B. Micrococcal nuclease cleavage sites at nucleosomal intervals on this 1000 bp region of yeast DNA (400 to 1400 bp from the vector Hind III site) are evident (Figure 9C). Thus the sequences contiguous with the *CEN3* DNA in yeast chromosome III are cleaved in the same specific manner by micrococcal nuclease when they are cloned into plasmids with or without the adjacent 627 bp *CEN3* fragment (Figure 9). These results indicate that the highly ordered nucleosomal spacing on yeast DNA adjacent to the centromere is not simply a propagation phenomenon, but rather these sequences must contain specific recognition signals for nucleosome phasing.

Discussion

We have examined the chromatin structure of the centromere region and flanking sequences on chromosomes III and XI in the yeast, *S. cerevisiae*. Mapping the exact nuclease cleavage sites within centromeric chromatin by the technique described by Wu (1980) revealed a discrete nuclease-resistant core of 220–250 bp of DNA flanked by highly nuclease-sensitive sites (Figure 10). The centromeric DNA sequences are in this same conformation whether in the genome or on autonomously replicating plasmids in yeast. This nuclease-resistant region includes the 130 bp segment that was found by nucleotide sequence comparison to exhibit the highest degree of homology (71%) between *CEN3* and *CEN11* (Figure 1; Fitzgerald-Hayes et al., 1982b). The smallest DNA fragments capable of centromere function in yeast all include this 220–250 bp segment of nuclease-resistant DNA. These results suggest that this chromosomal segment is an integral constituent of the functional centromeric unit.

Several features of the chromatin structure, as well as the sequence organization in this region, indicate this segment is a recognition site for sequence-spe-

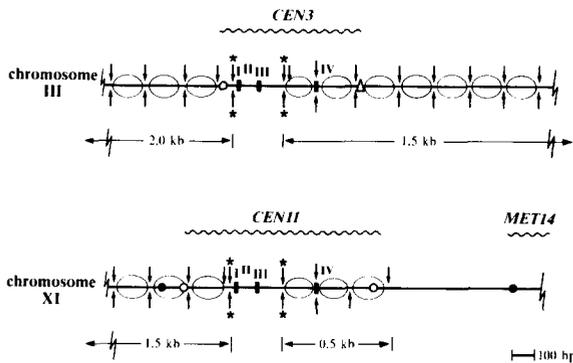


Figure 10. Schematic Map of Nuclease Cleavage Sites on Centromeric Chromatin

A map of the chromosomal region surrounding *CEN3* and *CEN11* shows the 627 bp *CEN3* fragment, the 858 bp *CEN11* fragment and the relative position of the sequence elements I, III and IV (see Figure 1) that are homologous and in identical spatial arrangements between *CEN3* and *CEN11*. The very A+T-rich region (element II) lies between elements I and III in both centromeres. The approximate position of the *MET14* gene on chromosome XI is shown (Fitzgerald-Hayes et al., 1982a, 1982b). Restriction enzyme sites are Bam HI (Δ), Sau 3A (\circ) and Sal I (\bullet). Micrococcal nuclease (\downarrow) and DNAase I (\dagger) cleavage sites on the centromeric chromatin are indicated. Asterisks indicate hypersensitive cleavage sites. The DNA fiber is presented in linear form to visualize the position of the 220–250 bp nuclease-resistant core and the nucleosomal subunits relative to the restriction maps of chromosomal DNA. In chromatin, the DNA is wrapped around the histone core to form the nucleosomal subunit (reviewed in McGhee and Felsenfeld, 1980). The conformation of DNA within the 220–250 bp nuclease-resistant core is not known. The arrows below the restriction maps indicate how far the ordered nucleosomal arrays extend from the nuclease-resistant centromere core.

cific DNA-binding proteins. The 220–250 bp nuclease-resistant region is distinct from the typical 160 bp DNA fragments protected by nucleohistones (Figure 10). Dissociation of chromosomal proteins by elution of the chromatin complex with high concentrations of salt (1 M to 2 M NaCl) (Spelsberg and Hnilica, 1971, Paulson and Laemmli, 1977; Bloom and Anderson, 1978), exposes the nuclease-resistant region to nucleolytic cleavage (Figure 7). This supports the notion that chromatin components including nuclear proteins are bound to this region of the chromosome and protect the DNA from enzymatic cleavage. The folding of the DNA double strand around the histone core or other chromatin particles brings into proximity sequences of DNA that are normally separated in deproteinized DNA. As postulated by Weintraub (1980), a recognition sequence in DNA folded into nucleosomes would be compound, 10–20 bp in length and distributed at approximately 80 bp intervals. Three short regions of perfect sequence homology, element I (14 bp), element III (11 bp) and element IV (10 bp), occur between the two centromere sequences, *CEN3* and *CEN11* (Figure 1; Fitzgerald-Hayes et al., 1982b). These homologous elements are positioned in an almost identical spatial arrangement between the two centromere DNA sequences; elements I and III are separated by 87 bp in *CEN3* and

88 bp in *CEN11*, whereas elements III and IV are separated by 248 bp in *CEN3* and 251 bp in *CEN11* (Figure 1). Thus it is possible that sequence-specific DNA-binding proteins as well as histone proteins are complexed to this region of the centromeric DNA, resulting in a 220–250 bp nuclease-resistant structure that is involved in mediating proper centromere function.

An ordered array of nucleosomes in a fixed position relative to the DNA sequence spans 2–3.5 kb of DNA surrounding the central nuclease-resistant core. The nuclease cutting sites, shown schematically in Figure 10, are at approximately 160 bp intervals in this region. A few bands are spaced 150–190 bp apart, presumably as a result of small differences in nucleosomal positioning along the chromatin fiber. Specific nuclease cleavage sites at 160 bp intervals extend only 400–600 bp to the side of *CEN11* proximal to the *MET14* gene (Figures 5 and 10). Similarly, the specific fragmentation pattern surrounding *CEN3* extends only about 1–1.5 kb to the side of *CEN3* proximal to the *CDC10* gene (Figures 2, 6 and 10). Thus the highly ordered nucleosomal array characteristic of centromere chromatin does not extend through known gene sequences, although it is possible that still unmapped genes occur in the highly organized region.

The structural determinants for this type of precise nucleosomal alignment along a given region of the chromosome are not fully understood (for discussion see Kornberg, 1981; Zachau and Igo-Kemenes, 1981). Mapping the nuclease cleavage sites within plasmid chromatin permitted us to determine whether the ordered distribution of nucleosomes in the *CEN* flanking regions is determined by propagation from the centromere or directly by signals built into the flanking sequences. By deleting sequences immediately adjacent to the *CEN3* region on plasmid DNA and juxtaposing new yeast or pBR322 DNA segments and the *CEN3* region, it was found that the highly ordered nucleosomal array was not propagated through these sequences (Figure 9). This is not simply because of the torsional constraints that may be imposed on sequences occurring on supercoiled plasmids. On yeast plasmids containing *CEN3* plus 1000 bp of the yeast DNA normally present on one side of the centromere, but with only pBR322 sequences on the opposite side, the nucleosomes are ordered only on the side containing the yeast DNA (Figure 9B). Furthermore, ordered nucleosomal arrays remain evident on *CEN* flanking sequences when they are cloned into plasmids in the absence of the functional *CEN3* sequence (Figure 9C). The nucleosomal phasing in the 3.5 kb of DNA surrounding the *CEN* sequence therefore depends on the underlying DNA sequence or base composition and does not result simply from exclusion of nucleosomes from the 220–250 bp nuclease-resistant *CEN* core.

Specific sequences or relative distributions of var-

ious nucleotides could also be responsible for the specific cutting pattern observed after limited micrococcal nuclease digestion of naked, deproteinized yeast centromeric DNA. As shown in Figure 9B, the pattern of micrococcal nuclease cleavage of naked pYe(*CEN3*)41 DNA delimits the junction between yeast centromeric DNA and pBR322 DNA sequences. The pattern of regularly spaced bands generated after micrococcal nuclease cleavage of deproteinized centromeric DNA might result from recognition by micrococcal nuclease of the same type of signals responsible for nucleosomal alignment. In contrast, pBR322 DNA sequences are cleaved more randomly by micrococcal nuclease (Figure 9B, naked DNA lanes). Since pBR322 DNA is of bacterial origin, this DNA may not contain eucaryotic nucleosomal alignment signals. In related experiments, Keene and Elgin (1981) showed that micrococcal nuclease digestion of naked *Drosophila* DNA delimits the junction between coding and noncoding DNA sequences. It is possible that specific nucleosomal alignment signals are dispersed nonrandomly throughout the eucaryotic genome. However, the functional significance of the resulting highly ordered nucleosomal arrays, in particular those occurring in the centromere region of the chromosome, is not clear.

The centromere is defined in cytological terms as the primary constriction of the chromosome where the spindle tubules attach. In mammalian cells, the centromere region of metaphase chromosomes has a trilaminar architecture (Brinkley and Stubblefield, 1970; Roos, 1977). This structurally differentiated region, referred to as the kinetochore, contains proteins and nucleic acids that are essential for its structural and functional integrity (Pepper and Brinkley, 1980; Ris and Witt, 1981). The kinetochore contains tubulin (Pepper and Brinkley, 1977, 1979) and has the capacity to nucleate microtubule polymerization *in vitro* (Telzer et al., 1975; Pepper and Brinkley, 1979). In yeast, a single microtubule attaches directly to the chromatin fiber without any visible structural differentiation (Peterson and Ris, 1976). Similar direct attachments of microtubules to the chromatin fiber seem to occur in other organisms (reviewed by Kubai, 1975). It is possible that the nuclease-resistant centromere core we have observed in yeast chromatin is a structurally primitive kinetochore that serves as the microtubule attachment site. In attempts to identify the nature of the DNA-microtubule interaction, it was found that microtubule-associated proteins, but not tubulin, bound to DNA (Corces et al., 1978; Villasante et al., 1981). The association of microtubules with the chromatin fiber must therefore be mediated by chromatin components other than tubulin and DNA. The use of cloned *CEN* DNA segments as affinity probes for the isolation of specific centromere-binding proteins should eventually lead to *in vitro* reconstruction of the centromere-spindle fiber complex.

Experimental Procedures

Strains, Media and Enzymes

Yeast strain X2180a was obtained from the Yeast Genetic Stock Center, University of California, Berkeley. Yeast strain J17(*α his2 ade1 trp1 met14 ura3*) was constructed as described by Fitzgerald-Hayes et al. (1982a). Yeast strain DC04 (*a leu2-04 ade1 [cir^o]*) was from J. Broach (Broach and Hicks, 1980). Media used for yeast growth have been described previously (Hsiao and Carbon, 1979). Restriction enzymes were purchased from New England Biolabs. Micrococcal nuclease and DNAase I were purchased from Worthington Biochemicals.

Plasmids

Plasmids pYe(*CEN3*)41, pYe(*CDC10*)1 and pYe(*CEN3*)11 have been described (Clarke and Carbon, 1980). Plasmid pYe6 was described by Fitzgerald-Hayes et al. (1982b). Partial restriction maps of the various plasmids used in this study are shown in Figure 2C. Plasmid DNA was isolated as described by Tschumper and Carbon (1980).

Transformation of Yeast

A modification of the procedure of Hinnen et al. (1978) as described by Hsiao and Carbon (1979) was used.

Preparation and Digestion of Nuclei

Yeast cells of strain X2180a (unless indicated otherwise in the figure legends) in mid-logarithmic growth phase were harvested, washed and converted to spheroplasts with Glusulase as described by Forte and Fangman (1976). Nuclei were isolated from spheroplasts as described by Nelson and Fangman (1979). The nuclei were resuspended in standard digestion buffer (SPC) containing 1 M sorbitol, 20 mM 1,4-piperazine-diethanesulfonic acid (pH 6.3), 0.1 mM CaCl₂ and 1 mM phenylmethylsulfonyl fluoride. For digestions with DNAase I, 0.5 mM MgCl₂ was added. The suspension was prewarmed to 32°C for 2 min, and micrococcal nuclease (50 units/ml) or DNAase I (5 μg/ml) was added. After incubation for the times indicated in the text, samples were adjusted to 1% SDS, 1 M NaCl and 20 mM EDTA to stop the digestion.

Preparation and Restriction Digestion of DNA

DNA was deproteinized by extraction with phenol and chloroform and isoamyl alcohol (24:1). DNA was precipitated by adding 2 volumes of ethanol and was dissolved in STE (10 mM NaCl, 10 mM Tris-HCl [pH 8.0] and 1 mM EDTA). DNA samples were incubated with RNAase A (50 μg/ml) for 1 hr at 37°C, extracted with chloroform and isoamyl alcohol (24:1), ethanol-precipitated as described above and redissolved in STE. Restriction endonuclease reactions were carried out by incubating 5 μg DNA in 50 μl buffer containing 7 mM Tris-HCl (pH 7.4), 7 mM MgCl₂, 60 mM NaCl and 1–5 units enzyme overnight at 37°C. Reactions were terminated by adding 10 μl of 50 mM EDTA.

Gel Electrophoresis

DNA samples were analyzed on 1.4% agarose slab gels containing 0.09 M Tris-borate (pH 8.3) and 2.5 mM EDTA (Maniatis et al., 1975).

Southern Transfer and Hybridization to Nitrocellulose Paper

Transfer of DNA from agarose gels to nitrocellulose paper (Schleicher and Schuell, BA 85) was performed essentially as described by Southern (1975). The 2× SSC (0.3 M NaCl, 0.03 M sodium citrate) rinse after the blotting step was replaced with a 10× SSC rinse to aid in retention of small DNA fragments. After transfer of DNA, nitrocellulose sheets were heated in vacuo at 80°C for 2–3 hr.

DNA fragments for nick translation were eluted from polyacrylamide gels as described by Maxam and Gilbert (1977). The preparation of ³²P-labeled DNA probes by nick translation of the DNA fragments indicated in Figure 2 has been described (Rigby et al., 1977; Chinault and Carbon, 1979). Hybridization of the probe to single-stranded DNA fragments immobilized on nitrocellulose filters was carried out according to the method of Cameron et al. (1979).

Autoradiography was performed for 24–72 hr at -80°C with Kodak XAR-5 film with a Du Pont Cronex Lightning-Plus intensifying screen.

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