Selective Excision of the Centromere Chromatin Complex from *Saccharomyces cerevisiae*

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Abstract. We have taken advantage of the known structural parameters associated with centromere DNA in vivo to construct a *CEN* fragment that can be selectively excised from the chromatin DNA with restriction endonucleases. *CEN3* DNA is organized in chromatin such that a 220–250-bp region encompassing the elements of centromere homology is resistant to nuclease digestion. Restriction enzyme linkers encoding the Bam HI–recognition site were ligated to a 289 base pair DNA segment that spans the 220–250-bp protected core (Bloom et al., 1984). Replacement of this *CEN3*–Bam HI linker cassette into a chromosome or plasmid results in formation of a complete structural and functional centromeric unit. A centromere core complex that retains its protected chromatin conformation can be selectively excised from intact nuclei by restriction with the enzyme Bam HI. The centromeric protein–DNA complex is therefore not dependent upon the intact torsional constraints on linear chromosomes for its structural integrity. Isolation of this complex provides a novel approach to characterizing authentic centromeric proteins bound to DNA in their native state.

THE centromere region functions as a *cis*-acting element for chromosomal segregation in both mitotic and meiotic cell divisions in the yeast *Saccharomyces cerevisiae*. Specific centromeric DNA sequences (*CEN*) are required in *cis* for proper chromosomal transmission during cell growth. The sequence requirements have been investigated by altering DNA in vitro and determining *CEN* function in an in vivo assay for chromosomal stability (Carbon and Clarke, 1984; Panzeri et al., 1985; Hegemann et al., 1986; McGrew et al., 1986; Gaudet and Fitzgerald-Hayes, 1987). A 25-bp region of dyad symmetry (centromere DNA element III, CDE III) is conserved in all centromere DNA's thus far examined (Fitzgerald-Hayes et al., 1982; Heiter et al., 1985). Single point mutations in this element can completely impair the segregation function (McGrew et al., 1986; Panzeri et al., 1985; Saunders et al., 1988). Another centromere DNA element (CDE I), defines an 8-bp element that is separated from CDE III by 76-86 bp of predominantly A+T residues (CDE II). The base requirements for these elements are less stringent than CDE III.

The chromatin organization of the centromere is distinct from the nucleosomal organization of the remaining chromatin DNA. A discrete region of the 220–250 base pairs encompassing the centromere is resistant to DNAase I or micrococcal nuclease digestion (Bloom and Carbon, 1982). This complex is centered around the conserved CDE III, and is flanked by DNAase I hypersensitive nuclease cleavage sites. The complex can be dissociated by treatment of the nuclei with high concentrations of NaCl before nuclease digestion (Bloom and Carbon, 1982; Bloom et al., 1984).

Alternatively, the complex is disrupted in selected point mutants that inactivate *CEN* function (Saunders et al., 1988). These data reveal a direct correlation between the structural organization and the ability of *CEN* to impart chromosomal stability. The chromatin components involved in the structural integrity of this chromosomal domain are therefore requisite for function. Isolation and characterization of the *CEN* DNA-binding proteins will be essential in a description of the molecular mechanisms responsible for chromosomal segregation and cell division.

The power of the yeast system has been in the development of in vivo assays for gene function. In the case of centromere function an accompanying in vitro assay has not been developed. Specific DNA binding is just one expectation for centromere proteins in vitro. Other functional expectations can only be speculated upon with our current state of knowledge. We have pioneered an in vivo approach for isolating *CEN* proteins by direct isolation of the *CEN* DNA-protein complex from budding yeast cells. A 289-bp *CEN3* DNA fragment that includes the 250-bp protected region was isolated in vitro, and restriction enzyme oligonucleotide linkers were ligated in tandem to both ends of the fragment. The fragment was introduced into chromosome IV by fragment mediated transformation, substituting for the wild-type *CEN4* sequences. Digestion of the nuclei isolated from cells harboring the heterocentric chromosome IV with the appropriate enzyme selectively and quantitatively excises the *CEN* complex. This approach provides a physiological assessment of proteins bound to centromere DNA in vivo.

Materials and Methods

Yeast Strains

S. cerevisiae strains J17 (*Mata, his2, adel, trpl, metl4, ura3*) and ABDE1 (*Mata, arg4-8, cupl::URA3+, thrl-1, leu2-3.112, ade2, ade5, trpl-289, ura 3-52, his4, his7-2*) were used for nuclei isolations and transformations. Substitution strain yC3BX289 is J17 transformed with the chromosome substitution vector described in Fig. 1.

Media

Cells were grown in either rich medium ([YPD] 1% yeast extract, 2% bacto-peptone, 2% glucose) or synthetic medium (0.67% yeast nitrogen base, 2% glucose, 0.5% casamino acids). Tryptophan (50 μg/ml) or adenine (50 μg/ml) were added as necessary. For selection of CUP1 containing plasmids, YCp5T and YCp5ΔA, copper sulfate (100 μM) was added.

Plasmids

Plasmids YCp5T and YCp5ΔA were used for analysis of *CEN3* chromatin structure in selected experiments described in the text. YCp5T and YCp5ΔA were derived from pGALCEN3 described by Hill and Bloom (1987). A *CUP1* gene (Karin et al., 1984) was inserted into pGALCEN3 and the 627 bp *CEN3* fragment was replaced with the wild-type (YCp5T) or mutant (YCp5ΔA) 289 bp *CEN3*-Bam HI linker cassette. The plasmids have the following arrangement of markers: YCp5T; *GAL1* promoter − *CEN3*/Bam HI linker cassette − *CUP1* − *ARS1* − *TRP1* and for YCp5ΔA; *GAL1* promoter − *CEN3* C→ A/Bam HI linker cassette − *CUP1* − *ARS1*. The cytidine to adenine mutation occurs in the central cytidine base of the conserved DNA element III (CDE III) as described by Saunders et al. (1988).

Construction of Restriction Enzyme Linker-CEN3 Fragment

The 289 base pair Rsa I-Alu I fragment containing *CEN3* DNA was isolated as described by Bloom et al. (1984). The fragment was incubated with synthetic oligonucleotide linkers encoding the Bam HI restriction recognition site in the presence of T4 DNA ligase. Enzymes were purchased from New England Biolabs (Beverly, MA) and used according to the manufacturers specifications. Oligonucleotide linkers were from P-L Biochemicals (Milwaukee, WI). The reaction was terminated and unincorporated linkers were removed by ethanol precipitation. The Bam HI linker-*CEN3* DNA complex was subsequently incubated with Xho I oligonucleotide linkers in the presence of T4 DNA ligase. The reaction was terminated and restricted with Xho I. The *CEN3*-Bam HI linker cassette could be shuttled into substitution vectors at unique Xho I or Sal I restriction sites without disrupting the tandem Bam HI sites. This fragment was introduced into a chromosome IV substitution vector as described in Fig. 1. Recombinant clones containing *CEN3* DNA were identified by colony hybridization. The *CEN3* positive clones were subsequently screened by hybridization with [32P]-labeled Bam HI linkers. A number of clones were identified as candidates with multiple Bam HI sites. *Escherichia coli* cells containing these clones were grown up and plasmid DNA isolated as described by Maniatis et al. 1982. *CEN3* fragments were released from the substitution vectors by Xho I or Bam HI digestion. An estimate of the number of linkers was obtained by comparing the migration of the *CEN3* DNA after Bam HI or Xho I digestion, respectively, in 6% polyacrylamide gels. The clones used in this text contained ∼2-3 Bam HI linkers per end.

Analysis of Fragment-mediated Transformation

The Bam HI linker *CEN3* chromosome substitution vector was used to transform yeast strain J17 to Ura3+ by the method of fragment mediated transformation as described by Rothstein (1983). Randomly chosen transformants were grown overnight in selective media and harvested by centrifugation. Cells were washed once with water, suspended in buffer (0.1 M Tris-HCl [pH 8.0], 50 mM EDTA, 1% SDS) and lysed with glass beads by shaking for 20 min. The supernatants were extracted with phenol and chloroform and DNA precipitated with 2 vol ethanol. Cellular DNA was resuspended in 50 μl of 10 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (STE), followed by RNase digestion (50 μg/ml for 60 min at 37°C) and an additional chloroform extraction and ethanol precipitation. The DNA obtained from these minipreps was digested with Bam HI, and analyzed by gel electrophoresis and Southern hybridizations to a [32P]-labeled *CEN3* probe.

Positive transformants for the Bam HI linker-*CEN3* DNA construction were confirmed by physical analysis in this manner.

Isolation and Digestion of Yeast Nuclei

1-liter cultures of yeast cells were grown in rich medium (YPD). Cells were harvested in mid-logarithmic growth phase, washed, and converted to spheroplasts by treatment with 1% Glusulase (Forte and Fangman, 1976). Nuclei were isolated from spheroplasts (Nelson and Fangman, 1979) and resuspended in PC buffer (20 mM Pipes, 0.1 mM CaCl₂, 75 mM NaCl, and 6 mM MgCl₂). Aliquots were incubated with Bam HI (150 U/ml) for 30 min at 37°C.

Gel Electrophoresis

Nuclear samples containing DNA were resuspended in 1% SDS, 1 M NaCl, and 20 mM EDTA (pH 8.0) and extracted with phenol and chloroform, treated with RNase A, and precipitated with ethanol as described above. After precipitation and centrifugation, DNA pellets were resuspended in small volumes of STE and subjected to electrophoresis on 1.4% agarose gels in 1× TBE (Maniatis et al., 1982). Nucleic acids were transfered to nitrocellulose filters as described by Southern (1975), and hybridized to [32P]-radio-labeled *CEN3* DNA for direct visualization of the *CEN* complex or *CEN* DNA.

Results

Construction of CEN3-Bam HI Linker Cassette

We have taken advantage of the known structural parameters associated with centromere DNA in vivo to construct a *CEN*
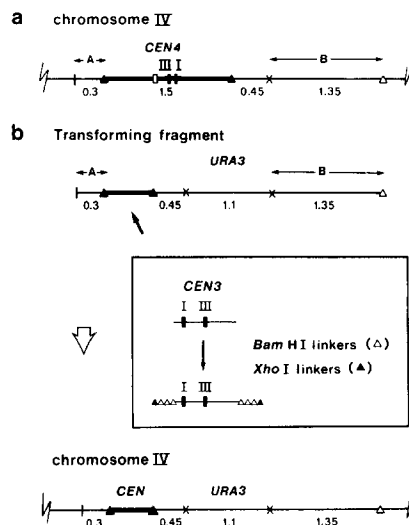

Figure 1. Substitution of *CEN3* DNA flanked by Bam HI linkers into chromosome IV. A 289 bp *CEN3* fragment was isolated in vitro and ligated to Bam HI oligonucleotide linkers. Xho I linkers were attached subsequently (*inset*) and the *CEN3* construction was introduced into the appropriate restriction site (Xho I) of a *CEN4* substitution plasmid vector. In *a*, regions *A* (0.3 kb) and *B* (1.35 kb) flanking the *CEN4* sequence in chromosome IV are indicated. The location of *CEN4* is denoted (*darkened line*) along the wild-type chromosome IV. *b* Illustrates the genomic substitution fragment that was used in construction of the *CEN3*/Bam HI linker cassette. The transforming fragment contains a selectable genetic marker, *URA3+*, regions A and B flanking the centromere in chromosome IV, and the *CEN3*/Bam HI linker cassette shown (*inset*). Selected Ura+ transformants (yC3BX289) contain *CEN3* flanked by Bam HI linkers in chromosome IV. Restriction sites are Bam HI (△), Hind III (X), Eco RI (I), and Xho I (▲).
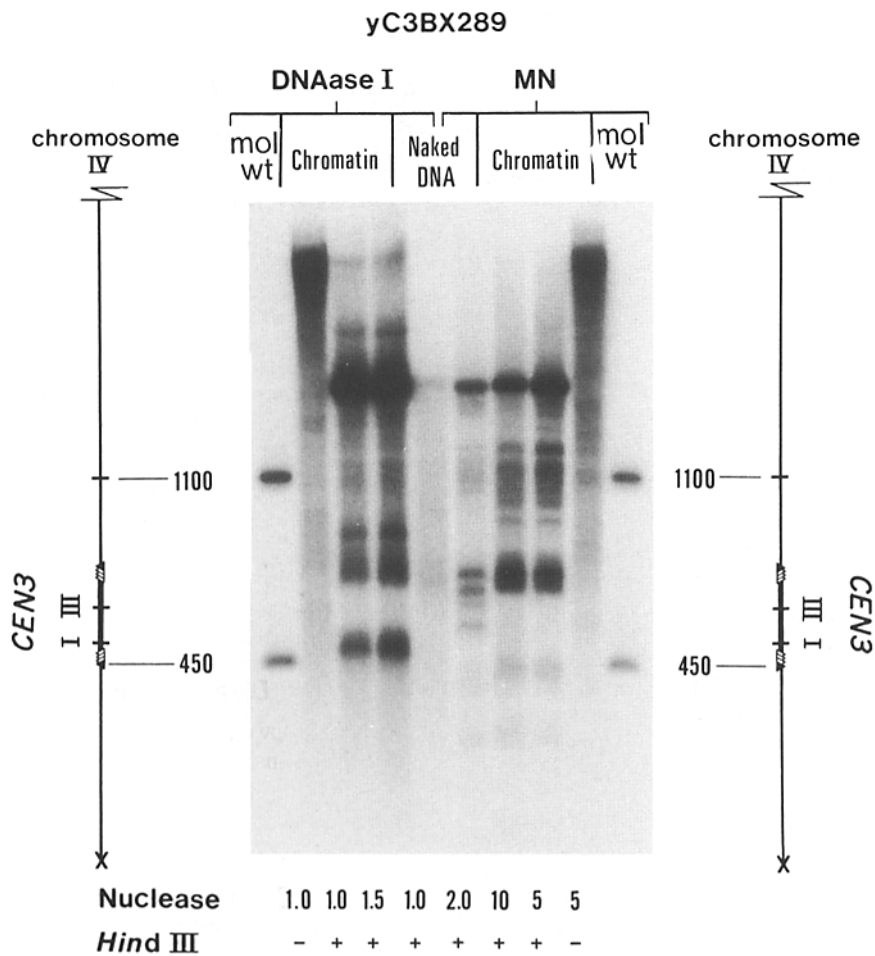
Figure 2. Mapping *CEN3* chromatin structure in yC3BX289. Nuclei were prepared from yeast strain yC3BX289. Chromatin DNA (*chromatin* lanes) and naked, deproteinized DNA (*Naked DNA* lanes) were digested in SPC buffer (1M Sorbitol, 20 mM Pipes (pH 6.3), 0.1 mM CaCl$_2$) with either DNAase I or micrococcal nuclease for the times (in minutes) indicated as described previously (Bloom and Carbon, 1982). The DNA fragments were purified and incubated with (+) or without (−) Hind III. Samples were subjected to electrophoresis on a 1.4% agarose gel, transferred to nitrocellulose and hybridized to a radiolabeled probe originating from the Hind III site and extending toward the centromere region. The probe is the 450 bp Hind III–Xho I fragment adjacent to the *CEN3* region substituted in chromosome IV as shown in Fig. 1. Elements I and III of centromere homology lie within the nuclease protected structure, while the Bam HI linkers are situated in nuclease hypersensitive sites (visualized as the intense bands at 450 and 700 bp, respectively). Molecular weight markers (*mol wt*) were prepared by digestion with Hind III and Hind III/Xho I. The sizes of these fragments confirm the actual restriction map of the genomic substitution strain. Restriction sites are the same as in Fig. 1.

fragment that can be selectively excised from the chromatin DNA with restriction endonucleases. *CEN3* DNA is organized in chromatin such that a 220–250-bp region encompassing the elements of centromere homology is resistant to nuclease digestion. We have localized the functional centromere core to a 289-bp DNA segment that spans the 220–250-bp protected core (Bloom et al., 1984). This fragment was isolated in vitro and restriction enzyme linkers encoding the Bam HI recognition site were ligated as described in Materials and Methods and Fig. 1. Oligonucleotide linkers for Bam HI were chosen due to the infrequent number of Bam HI cutting sites in the *Saccharomyces cerevisiae* genome. We estimate on the average that Bam HI cuts yeast DNA every 8–10 kilobase pairs.

The *CEN3*–Bam HI linker cassette was inserted into autonomously replicating plasmids, or the chromosome IV substitution vector shown in Fig. 1. Yeast transformants were picked by conversion of cells to uracil prototrophy and confirmed by physical analysis of isolated DNA. Plasmids containing this *CEN3*–Bam HI linker cassette are as stable as their wild-type *CEN3* counterparts in a mitotic segregation assay (data not shown). The resulting chromosome substitution strain (yC3BX289), containing the wild-type *CEN3* in chromosome III and the *CEN3*–Bam HI linker in chromosome IV, grows with wild-type doubling times. Thus insertion of Bam HI linkers in the centromere adjacent regions

does not affect the ability of these sequences to impart mitotic segregation function.

The structural integrity of the *CEN3*–Bam HI linker in the heterocentric chromosome is demonstrated in Fig. 2. Cells containing this construction were grown to mid-logarithmic growth phase and nuclei were isolated as previously described (Bloom and Carbon, 1982). Chromatin DNA was digested with micrococcal nuclease (MN) or DNAase I for the times indicated in Fig. 2. DNA was isolated and cut to completion with Hind III. This enzyme cuts the DNA 450 bp from the *CEN3* region in chromosome IV. Hybridization with a chromosome IV-specific probe that originates from the Hind III site and extends in a centromere proximal direction provides a unique mapping strategy for the *CEN3* region in chromosome IV. As shown in Fig. 2, the protected core of 220–250 bp is associated with this *CEN3* region. A protected core is visualized upon digestion of the chromatin DNA with either enzyme. The DNAase I lanes clearly reveal the nuclease hypersensitive cleavage sites flanking the protected *CEN* core. Thus, the *CEN3*–Bam HI linker does not compromise the ability of the sequence to stabilize a chromosome or plasmid, nor is the structural integrity perturbed by the flanking restriction sites.

Enzymatic Release of CEN3 Chromatin

The placement of Bam HI linkers in a chromatin hypersensi-
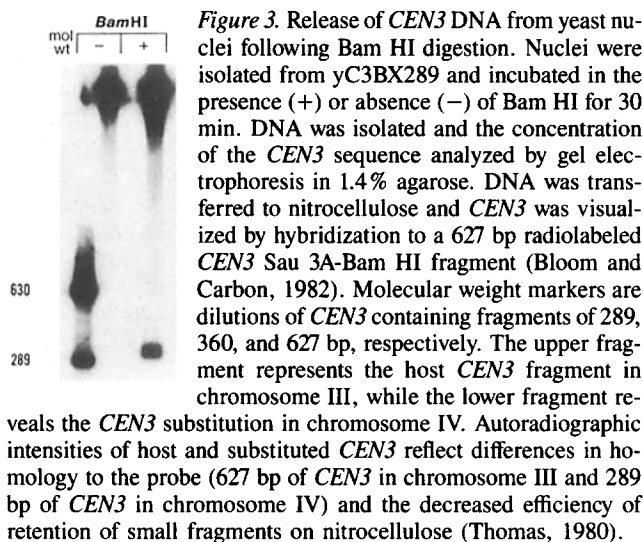
BamHI

mol
wt | − | + |

630

289

Figure 3. Release of *CEN3* DNA from yeast nuclei following Bam HI digestion. Nuclei were isolated from yC3BX289 and incubated in the presence (+) or absence (−) of Bam HI for 30 min. DNA was isolated and the concentration of the *CEN3* sequence analyzed by gel electrophoresis in 1.4% agarose. DNA was transferred to nitrocellulose and *CEN3* was visualized by hybridization to a 627 bp radiolabeled *CEN3* Sau 3A-Bam HI fragment (Bloom and Carbon, 1982). Molecular weight markers are dilutions of *CEN3* containing fragments of 289, 360, and 627 bp, respectively. The upper fragment represents the host *CEN3* fragment in chromosome III, while the lower fragment reveals the *CEN3* substitution in chromosome IV. Autoradiographic intensities of host and substituted *CEN3* reflect differences in homology to the probe (627 bp of *CEN3* in chromosome III and 289 bp of *CEN3* in chromosome IV) and the decreased efficiency of retention of small fragments on nitrocellulose (Thomas, 1980).

tive region should allow for the excision of this region from the chromatin DNA with Bam HI. To examine the efficacy of Bam HI cleavage of the *CEN* region, yeast cells (yC3BX289) were grown to mid-logarithmic phase and nuclei were isolated as described above. Nuclei were incubated with Bam HI for 30 min in a low salt digestion buffer (PC buffer). The selective release of centromere DNA from yeast nuclei after Bam HI digestion is shown in Fig. 3. Excision of the 289 *CEN3* fragment is evident only after Bam HI treatment. The upper signal present in the autoradiograph represents the contribution of host *CEN3* sequences in chromosome III. Similar results are obtained when deproteinized DNA from the same cells is digested to completion with Bam HI (data not shown). Direct comparisons between the digestion of deproteinized DNA and chromatin samples reveal that the *CEN3* fragment is quantitatively released from chromosome IV after in situ restriction digestion. The intensities on the autoradiographs reflect the decreased retention of the small fragments on nitrocellulose filters (Thomas, 1980) and their reduced hybridization efficiency to the radiolabeled probe.

Structural Integrity of the Centromere Complex

It is likely that restriction digestion could disrupt the integrity of the protein-DNA complex. Releasing any potential tension on the DNA by effectively linearizing the fragment in situ could result in dissociation of a portion of the proteins at the centromere. In fact, Harland et al., (1983) have demonstrated that linearization of supercoiled DNA in *Xenopus* oocytes compromised the ability of plasmid sequences to serve as templates for RNA synthesis. The alteration in DNA structure itself, or the subsequent release of specific *trans*-acting factors could have been the causative factor. It was important to demonstrate that at least a portion of centromere proteins remain associated after restriction digestion for any subsequent attempts in isolation of proteins from this complex.

A modified indirect end-labeling experiment was performed by digesting yeast nuclei with restriction enzyme in situ, followed by micrococcal nuclease. The results of such an experiment using with wild-type J17 cells are shown in
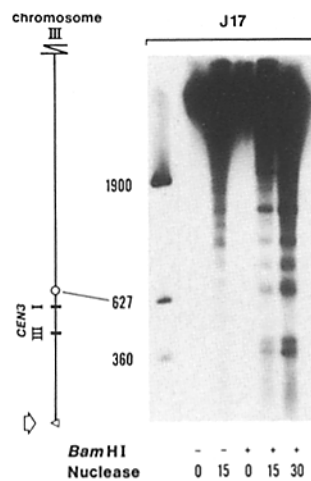

chromosome III   J17

1900

627

360

BamHI   − − • • •
Nuclease   0 15 0 15 30

Figure 4. Structural analysis of *CEN3* chromatin after in situ restriction digestion. Nuclei were isolated from J17 yeast cells as described above. Samples were digested with Bam HI (300 U/ml) (+ lanes) for 30 min at 37°C. Nuclei were pelleted by centrifugation at 13,000 g for 10 min at 4°C, to clear samples of Bam HI and resuspended in SPC buffer for subsequent micrococcal nuclease digestions. Nuclease was added (50 U/ml) and digestions proceeded for times indicated in minutes. DNA was extracted and directly prepared for electrophoresis in a 1.4% agarose gel. Fragments were transferred to nitrocellulose and hybridized to a 627 bp *CEN3* probe originating from the *Bam* HI site in chromosome III and extending in a centromere proximal direction. This strategy reveals the protected nuclease core as well as the flanking nuclesome arrays (Bloom and Carbon, 1982). Molecular weight markers are dilutions of *CEN3* containing fragments of 360, 627 and 1900 bp, respectively. A partial restriction map of chromosome III is shown to the left. Restriction sites are Bam HI (△) and Sau 3A (○).

Fig. 4. A naturally occurring Bam HI site lies ~350 bp from the protected structure surrounding *CEN3* in chromosome III. The next Bam HI site is 8 kp downstream. Saunders and Bloom (unpublished results) have demonstrated that restriction sites outside the protected core are accessible to nucleolytic cleavage. The centromere proximal site that lies in the linker region between nucleosomes (Bloom and Carbon, 1982) is also accessible to in situ digestion. Nuclei were isolated and incubated with Bam HI for 30 min. Soluble enzyme was cleared from the sample by centrifugal fractionation, with the bulk of the nuclei pelleting (Bloom and Anderson, 1978). Nuclear suspensions containing the centromere complex were resuspended in SPC and subsequently digested with micrococcal nuclease. DNA was extracted and directly subjected to electrophoresis on 1.4% agarose gels. To visualize the centromere, DNA was hybridized to a radiolabeled probe originating from the Bam HI site and extending in a centromere proximal direction. As shown in Fig. 4, after primary Bam HI treatment in situ, micrococcal nuclease cutting sites delineate a 220–250–bp protected structure that surrounds the elements of centromere homology. Furthermore, cutting sites are apparent at 160-bp intervals in the flanking region for 1–2 kp. These results are typical of the chromatin structure previously determined after cleavage of micrococcal nuclease treated chromatin with the restriction enzyme Bam HI (Bloom and Carbon, 1982). If Bam HI is not included in the pretreatment, or if the digestion is not complete, this structural organization is not visualized (Fig. 4, *left*). Therefore excision of the chromosome adjacent to the centromere does not perturb the structural organization at this level of resolution.

We have also made use of restriction enzymes to directly assay the integrity of centromere structure after release of the 289 bp *CEN* chromatin complex. Nuclei were isolated
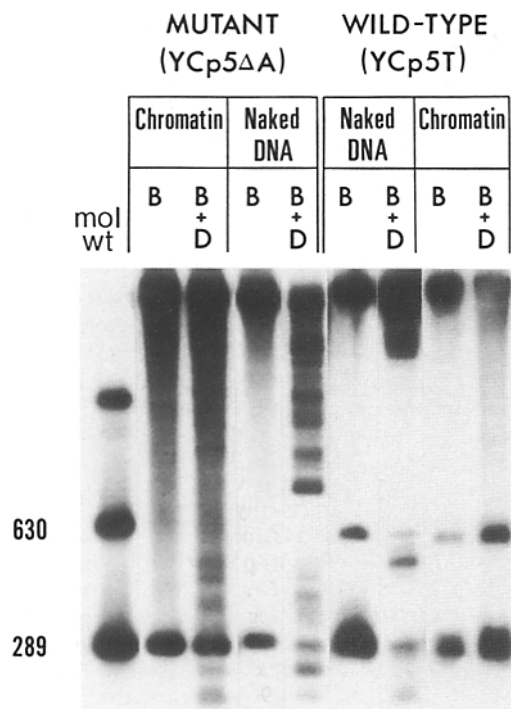
MUTANT
(YCp5ΔA)

WILD-TYPE
(YCp5T)

	Chromatin	Naked DNA		Naked DNA		Chromatin	
mol wt	B	B + D	B	B + D	B	B + D	B	B + D

630

289

Figure 5. Analysis of centromere structure after release of the core particle. Nuclei were prepared from strain ABDE1 carrying plasmids YCp5T and YCp5ΔA. These plasmids contain the 289 bp *CEN3*–Bam HI linker fragment. They differ in a single point mutation from Cytidine (*WILD-TYPE, YCp5T*) to Adenine (*MUTANT, YCp5ΔA*) in the conserved centromere element CDE III (Saunders et al., 1988). Portions of the nuclear suspension were taken for immediate extraction and subsequent digestion with Bam HI (*B*) or Bam HI + Dra I (*B + D*) (*Naked DNA lanes*). The remaining nuclei were digested with Bam HI (150 U/ml) for 30 min at 37°C. Aliquots were taken for the Bam HI treatment alone (*B* in *Chromatin lanes*). The remaining nuclear suspension was centrifuged at 12,000 g for 10 min to clear samples of Bam HI, resuspended in TC buffer (10 mM Tris-HCl, pH 7.6; 0.1 CaCl₂; 20 mM NaCl; 10 mM MgCl₂) and incubated with Dra I (150 U/ml) for 30 min (*B + D* in *Chromatin lanes*). DNA was extracted from all samples, subjected to electrophoresis on a 1.8% agarose gel and transferred to nitrocellulose filters. *CEN3* DNA was visualized by hybridization to radiolabeled 289-bp *CEN3* probe. The upper fragments represent the contribution from *CEN3* in chromosome III. The 289-bp *CEN3* fragment from the plasmids can be visualized in all samples treated with Bam HI. The fragment visualized in the wild-type (YCp5T) lanes around 630 bp results from partial Bam HI digestion. A Bam HI site lies 275 bp downstream from the *CEN3*–Bam HI cassette in YCp5T, but not in YCp5ΔA. Fragments below 289 bp (260 and 200 bp, respectively) result from cleavage of the Dra I sites in the conserved centromere element II (CDE II). These sites in chromatin are accessible to cleavage in the mutant centromere plasmid only. Molecular weight markers are 630 and 289 bp fragments containing *CEN3* DNA.

from yeast strain ABDE1 bearing the plasmids YCp5T and YCp5Δ A. These plasmids contain the 289 bp *CEN3*–Bam HI linker cassette as described in the Materials and Methods and Fig. 1 (*box*), but differ in a single point mutation from Cytidine (*WILD-TYPE, YCp5T*) to Adenine (*MUTANT, YCp5ΔA*) in the central centromere element CDE III (Saunders et al., 1988). This single point mutation disrupts centromere function as well as structure. Saunders et al. (1988)

demonstrated that restriction enzyme recognition sites (Dra I) within the protected core are resistant to cleavage in the chromatin DNA in wild-type sequence, but were susceptible to nucleolytic digestion in cells harboring the mutant sequence. This assay provides a direct measure of structural integrity after release of the entire 289 bp fragment. Fig. 5 demonstrates that internal Dra I sites remain resistant to nucleolytic digestion after the release of the 289 bp *CEN3* particle. Nuclei were isolated from the appropriate ABDE1 transformants containing wild-type and mutant centromere plasmids. After Bam HI digestion, the nuclei were pelleted and resuspended in TC buffer and digested with Dra I for 30 min as described in the legend to Fig. 5. DNA was purified from these samples and subjected to electrophoresis on an agarose gel, transferred to nitrocellulose and probed with a ³²P-radiolabeled *CEN3* fragment. Dra I cutting sites are apparent in the non-functional mutant lanes (260 and 200 bp fragments, Fig. 5, mutant lanes), and provide direct evidence of the ability of Dra I to cut in situ. However, no cleavage of the centromere core by Dra I is apparent in cells containing the wild-type centromere sequence (wild-type lanes). Thus, whether intact nuclei (Fig. 3 in Saunders et al., 1988) or the 289 bp centromere chromatin core (Fig. 5) are exposed to Dra I, the internal Dra I recognition sites are resistant to nuclease cleavage. Therefore a portion of centromere DNA–binding proteins present in wild-type but not nonfunctional mutant sequences remain associated upon release of the centromeric complex with Bam HI.

Complex Migration of Intact CEN3 Chromatin

After excision of the *CEN3* core, the solubility properties of this complex were examined. The *CEN3* chromatin complex remains insoluble after treatment with a variety of agents including NaCl (0.25 M), Triton X-100 (0.1%), or EDTA (1 mM). Only the use of ionic or anionic detergents such as SDS and sodium deoxycholate (DOC)[1] resulted in the solubilization of bulk chromatin as well as the *CEN3* complex. Detergents such as SDS dissociate ionic interactions, and the *CEN3* DNA was released as deproteinized DNA into the supernatant. DOC is much less chaotropic, and in fact, concentrations of 0.1% (2.5 mM) are known not to disrupt histone protein–DNA interactions (Smart and Bonner, 1971). This detergent was effective in solubilizing the bulk of the chromatin without disrupting specific protein–DNA interactions that confer the nuclease protected structure (data not shown).

The electrophoretic properties of the released centromere protein–DNA complex was examined on low ionic strength nondenaturing agarose gels as described in the Materials and Methods (Fig. 6). A soluble fraction of chromatin containing the intact centromere complex was prepared from yC3BX289 cells and incubated in the absence or presence of proteinase K. The samples were subjected to electrophoresis and protein–DNA complexes were dissociated after electrophoretic separation. The DNA fragments were transferred to nitrocellulose and *CEN3* DNA was visualized by probing with a ³²P-radiolabeled *CEN3* fragment. The autoradiograph presented in Fig. 6 demonstrates a slower migration of the *CEN3*
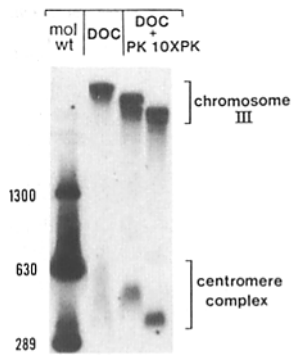
Figure 6. Electrophoretic analysis of the centromere core complex. Nuclei were isolated from yeast yC3BX289, treated with Bam HI and incubated with 0.1% DOC for 12–16 h at 4°C. Samples were centrifuged 12,000 g for 15 min. The soluble fraction was incubated in the absence (*DOC only lane*), or the presence of proteinase K (30 µg/ml, once; and 300 µg/ml, 10 times). The chromatin samples were loaded directly onto a low ionic strength, non-denaturing agarose gel system (0.045 M Tris-borate, [pH 8.3] and 1.25 mM EDTA). The gel was treated with 0.5 M NaOH, 1.5 M NaCl to dissociate proteins and denature DNA for subsequent transfer of single-strands to nitrocellulose. The nitrocellulose was probed with a 627-bp radiolabeled *CEN3* fragment as described in Fig. 3. The migration of *CEN3* in chromosome III and the centromere complex from chromosome IV is indicated to the right. Molecular weight markers are 289, 630, and 1,300-bp fragments containing *CEN3*.

complex in the absence of proteinase K. Upon increasing proteinase K digestion, the mobility of the complex increases and with further addition of protease, the migration of *CEN3* DNA approaches that of naked, deproteinized DNA. Analysis of the proteins in the DOC extract by SDS–PAGE, reveals a very complex pattern including histones and ribonuclear proteins (data not shown). An assessment of the heterogeneity of the intact complex will be revealed upon further characterization of the binding proteins that specifically retard the migration of *CEN3* DNA.

Discussion

The ability to selectively excise an intact chromatin complex after restriction digestion of nuclei has been demonstrated with centromeric chromatin from the yeast *Saccharomyces cerevisiae*. The structural integrity of this complex was assayed before and after excision from the chromosome. A modified indirect-end labeling strategy (Fig. 4) as well as protection of internal restriction sites (Fig. 5) revealed that the structural organization of centromeric chromatin remains intact after digestion with Bam HI. These results provide a direct demonstration that at least the set of proteins conferring such structures are not dependent on the torsional state of the chromosome. In contrast, DNAase I and S_1 nuclease sensitive sites associated with the 5' region of a number of transcribed genes do exhibit a dependence on the superhelical state of the flanking DNA (Larson and Weintraub, 1982; Weintraub, 1983). Introduction of such 5' hypersensitive sites onto plasmids reveals nuclease sensitivity on supercoiled, but not linear templates. Furthermore, when inverted repeats are introduced into plasmid molecules, they adopt S_1 sensitive structures on supercoiled plasmids, but not on the linearized counterparts (Lilley, 1980). The dependence of 5' nuclease cleavage sites on DNA topology would seem to indicate a difference in the nature of the binding proteins at the 5' regions of transcribed genes, or the 5' recognition sites themselves, in comparison to the

centromere structures which are retained on linearized templates.

The *trans*-acting factors bound to the 5' region of globin genes in chicken erythrocytes have been examined by a similar restriction endonuclease excision process (McGhee et al., 1981). This chromatin particle exhibited electrophoretic properties of deproteinized DNA in non-denaturing polyacrylamide gels. These investigators utilized naturally occurring Msp I sites in the DNA to release a 115-bp chromatin fragment from nuclei. The complex was solubilized and apparently exists as naked DNA in the nucleus, or alternatively protein binding in this 5' control region is dependent on the torsional state of the binding site as described above and protein release accompanied restriction digestion.

The ability to excise an intact complex provides an important approach in determining the physiological properties of DNA-binding proteins. The biochemical expectation for centromere proteins to date is exclusively DNA-binding, which requires a reliable in vitro reconstitution assay. These experiments allow proteins to be fractionated according to the properties of an intact chromatin complex, and provide a means for subsequent isolation of authentic centromere DNA-binding proteins.

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