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Dicentric Chromosome Stretching during Anaphase Reveals Roles of Sir2/Ku in Chromatin Compaction in Budding Yeast[V]

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We have used mitotic spindle forces to examine the role of Sir2 and Ku in chromatin compaction. *Escherichia coli* lac operator DNA was placed between two centromeres on a conditional dicentric chromosome in budding yeast cells and made visible by expression of a lac repressor–green fluorescent fusion protein. Centromeres on the same chromatid of a dicentric chromosome attach to opposite poles ~50% of the time, resulting in chromosome bridges during anaphase. In cells deleted for *yKU70*, *yKU80*, or *SIR2*, a 10-kb region of the dicentric chromosome stretched along the spindle axis to a length of 6 μm during anaphase. On spindle disassembly, stretched chromatin recoiled to the bud neck and was partitioned to mother and daughter cells after cytokinesis and cell separation. Chromatin immunoprecipitation revealed that Sir2 localizes to the lacO region in response to activation of the dicentric chromosome. These findings indicate that Ku and Sir proteins are required for proper chromatin compaction within regions of a chromosome experiencing tension or DNA damage. The association of Sir2 with the affected region suggests a direct role in this process, which may include the formation of heterochromatic DNA.

INTRODUCTION

Eukaryotic chromosomes consist of euchromatic and heterochromatic regions. Euchromatic DNA contains a relative abundance of transcriptionally active genes, whereas heterochromatic regions of the genome exhibit transcriptional repression. The silent nature of heterochromatin results from the concerted roles of *cis*-acting DNA sequences and *trans*-acting proteins. Heterochromatic domains in *Saccharomyes cerevisiae* include the mating type loci, telomeres along with subtelomeric sequences, and rDNA. Genes implicated in the establishment and maintenance of yeast heterochromatin include members of the silent information regulator (SIR) family (Moretti *et al.*, 1994). *SIR1, SIR2, SIR3,* and *SIR4* are required for silencing within the heterothallic mating loci *HML* and *HMR. SIR2, SIR3,* and *SIR4* contribute to telomere-associated transcriptional repression. *SIR2* acts independently of the other *SIR* genes to promote rDNA silencing and also suppresses recombination between exogenous DNA repeats inserted within the rDNA locus. It is not known whether the two processes are linked mechanistically.

[V] Online version of this article contains video material for some figures. Online version is available at www.molbiolcell.org.
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There is strong evidence that the Sir proteins promote heterochromatic silencing by direct interaction with chromatin of the affected region. This was first suggested by mutational studies and in vitro binding assays that revealed the binding of Sir3 and Sir4 to the N-terminal domains of histones H3 and H4 (Hecht *et al.*, 1995). Further support for this model has been provided by the in vivo localization of Sir proteins to heterochromatic regions as demonstrated by chromatin immunoprecipitation (Hecht *et al.*, 1996; Gotta *et al.*, 1997). Heterochromatin can be distinguished from euchromatin on a molecular level by the presence of hypoacetyled histones within the transcriptionally silenced regions. Hypoacetylation of *HML* is abrogated by deletion of *SIR2, SIR3,* or *SIR4* and that overexpression of *SIR2* promotes a general reduction in histone acetylation throughout the genome (Braunstein *et al.*, 1993). It has been shown recently that Sir2 has histone deacetylase activity (Imai *et al.*, 2000, Landry *et al.*, 2000; Smith *et al.*, 2000), providing a functional link between Sir2 and the establishment of heterochromatin.

The association between Sir2, Sir3, and Sir4 with DNA at telomeres requires additional proteins, including the yKu70/yKu80 heterodimer. Ku, like the Sir2, -3, -4 complex localizes at telomeres (Gotta *et al.*, 1997), and Sir4 interacts with yKu70 in vivo (Tsukamoto *et al.*, 1997). The Ku heterodimer is best known for its role in nonhomologous end joining (NHEJ). Additional functions of yKu include telomere length maintenance, telomere clustering, and the formation of telomeric heterochromatin (reviewed by Feather-

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Table 1. Yeast strains used in this study

Strain	Genotype	Source
J178#24	*MATa, ade1, met14, ura3-52, his4::URA3, GALCEN3*	Brock and Bloom, 1994
J178124	*MATa, ade1, met14, ura3-52, his4::URA3, GALCEN3, yku70*::KAN^r	This study
AFS173	*MATa, ade2-1, can1-100, his3-11,15::GFP-LacI-HIS3, leu2-3,112::lacO-LEU2, trp1-1, ura3-1*	Straight *et al.*, 1996
KBY3024	*MATa, ade2-1, can1-100, his3-11,15::GFP-LacI-HIS3, his4::URA3, GALCEN3 leu2-3,112::lacO-LEU2, trp1-1, ura3-1*	This study
KBY3124	*MATa, ade2-1, can1-100, his3-11,15::GFP-LacI-HIS3, his4::URA3, GALCEN3 leu2-3,112::lacO-LEU2, trp1-1, ura3-1 yku70*::KAN^r	This study
KBY3134	*MATa, ade2-1, can1-100, his3-11,15::GFP-LacI-HIS3, leu2-3,112::lacO-LEU2, trp1-1, ura3-1 yku70*::KAN^r	This study
KBY3224	*MATa, ade2-1, can1-100, his3-11,15::GFP-LacI-HIS3, his4::URA3, GALCEN3 leu2-3,112::lacO-LEU2, trp1-1, ura3-1 yku80*::KAN^r	This study
KBY3324	*MATa, ade2-1, can1-100, his3-11,15::GFP-LacI-HIS3, his4::URA3, GALCEN3 leu2-3,112::lacO-LEU2, trp1-1, ura3-1, yku70*::KAN^r *rad9::TRP1*	This study
KBY3424	*MATa, ade2-1, can1-100, his3-11,15::GFP-LacI-HIS3, his4::URA3, GALCEN3 leu2-3,112::lacO-LEU2, trp1-1, ura3-1 sir2*::KAN^r	This study
KBY3524	*MATa, ade2-1, can1-100, his3-11,15::GFP-LacI-HIS3, his4::URA3, GALCEN3 leu2-3,112::lacO-LEU2, trp1-1, ura3-1, sir2*::KAN^r *rad9::TRP1*	This study
KBY3624	*MATa, ade2-1, can1-100, his3-11,15::GFP-LacI-HIS3, his4::URA3, GALCEN3 leu2-3,112::lacO-LEU2, trp1-1, ura3-1 rad50*::KAN^r	This study
KBY3724	*MATa, ade2-1, can1-100, his3-11,15::GFP-LacI-HIS3, his4::URA3, GALCEN3 leu2-3,112::lacO-LEU2, trp1-1, ura3-1 rad52*::TRP1	This study
KBY3824	*MATa, ade2-1, can1-100, his3-11,15::GFP-LacI-HIS3, his4::URA3, GALCEN3 leu2-3,112::lacO-LEU2, trp1-1, ura3-1 mre11*::KAN^r	This study
KBY3924	*MATa, ade2-1, can1-100, his3-11,15::GFP-LacI-HIS3, his4::URA3, GALCEN3 leu2-3,112::lacO-LEU2, trp1-1, ura3-1, yku70*::KAN^r *rad52::TRP1*	This study
KBY4024	*MATa, ade2-1, can1-100, his3-11,15::GFP-LacI-HIS3, his4::URA3, GALCEN3 leu2-3,112::lacO-LEU2, trp1-1, ura3-1 yku70*::HPH^r, *SPC29-CFP*::KAN^r	This study

stone and Jackson, 1999; Haber, 1999). yKu70 is also involved in the process of adaptation and escape from a cell cycle arrest caused by a DNA double-strand break (DSB) (Lee *et al.*, 1998).

A number of studies have revealed that the Sir proteins are necessary for the same types of DNA repair that require Ku. Yeast strains mutant for *SIR2, SIR3,* or *SIR4* are deficient in the circularization of linearized plasmids, dicentric plasmid rearrangement, and the repair of DSBs associated with continuous expression of endonucleases (Tsukamoto *et al.*, 1997; Lewis *et al.*, 1998); however, the mechanistic roles of Ku and Sir proteins in DNA repair are not well understood. Recent studies have shown that Ku and Sir proteins dissociate from telomeres in response to a DSB and relocalize to DNA surrounding the breakage site (Martin *et al.*, 1999; Mills *et al.*, 1999). Given the role of Sir and Ku proteins in the establishment of heterochromatin, it has been suggested that the involvement of these proteins in DNA repair might reflect the formation of heterochromatic DNA at locations of DSBs (Critchlow and Jackson, 1998).

The formation of heterochromatin is associated with structural changes within the affected region. The establishment of heterochromatic silencing on a plasmid containing *HML* or *HMR* sequences is accompanied by alterations in plasmid supercoiling (Bi and Broach, 1997). In metazoans, heterochromatic DNA seems more tightly compacted than euchromatic DNA (Heitz, 1928). Chromosome compaction, and heterochromatin in particular, has been difficult to visualize in yeast. Previously, Trf4, topoisomerase1, Pds5, and members of the condensin complex have been shown to have roles in maintaining chromosome compaction in yeast (Castano *et al.*, 1996; Freeman *et al.*, 2000; Hartman *et al.*, 2000; Lavoie *et al.*, 2000). The function of these proteins in chromatin compaction was revealed in fixed cells by means of fluorescence in situ hybridization of probes specific for rDNA. With the advent of the lac repressor–green fluorescent protein construct (lacI-GFP) marker to visualize discrete lacO loci along the chromosome (Straight *et al.* 1996), it has become possible to study changes in yeast chromosome substructure in living cells. With the use of a lacO array positioned between two centromeres on the same chromosome, we have shown that the compact structure of the lacO marker was lost on exposure to anaphase spindle forces in cells lacking yKu70, yKu80, or Sir2. The dicentric chromosome decondensed from its normal compact (spot) distribution to form an extended filament spanning the distance from spindle pole to spindle pole. Additionally, we found that Sir2 associates with the lacO region after activation of the dicentric chromosome. These findings are indicative of a role of Ku and Sir in chromatin compaction and may reflect the formation of heterochromatic DNA in response to chromatin distortion or DNA double-strand breaks.

MATERIALS AND METHODS

Strains, Media, and Determination of Viability

Genotypes of strains used in this study are given in Table 1. A dicentric *yku70Δ* strain J178124 (*yku70::KAN^r*) was derived from J178#24 (Brock and Bloom, 1994) by fragment-mediated transforma-

tion. KBY3134 (*yku70::KAN^r*) was derived from AFS173. A PCR-derived fragment kindly provided by J. Haber (Brandeis University, Waltham, MA) was used to delete the entire coding region of the *YKU70* gene. A *Pvu*I/*Pvu*II fragment from pJB2#4 containing *GAL-CEN3* (Brock and Bloom, 1994) was inserted into the *HIS4* locus of AFS173 (Straight *et al.*, 1996), a gift from A. Straight (Harvard University, Boston, MA), to generate KBY3024. KBY3124 (*yku70::KAN^r*) and KBY3324 (*rad9::TRP1*) were derived from KBY3024 and KBY3124, respectively.

The *rad9::TRP1* disruption fragment was generated with the oligonucleotides: 5′-aagagcatagtgagaaaatcttcaacatcagggctatgtcaggccagttagattgtactgagagtgcacc-3′ and 5′-ccactcctggcgtgtgggaggatgttcttagacttaattaagaatctctatgcggtatttcacaccg-3′, with the use of pRS304 as template. KBY4042 was made by replacing the G418 (KAN^r) resistance marker in KBY3124 with a gene coding for hygromycin B resistance (HPH^r) (Goldstein and McCusker, 1999). The *rad52::TRP1* fragment was generated with a *Bam*HI digestion fragment from pSM21 (Schild *et al.*, 1983). *KAN^r* transformation fragments were synthesized by PCR with the use of the oligonucleotide sets and the pkanMX2 template (Wach *et al.*, 1994) as follows: *yku80::KAN^r*: 5′-taacgagagtgcaggatatgcacaaataatatatctcacaccataatcagctgaagcttcgtacgc-3′and 5′-ttctctttaactgtggtgacgaaaacataaactcaaaggatgttagacctttaggccactagtcgatctg-3′; *sir2::KAN^r*: 5′-gaagagatgtaaagcccattctcacgtatttcaagaaattaggcatcgctcagctgaagcttcgtacgc-3′ and 5′-agtgagatgggcggtacatgtaatatttcacccggtacaatgaaaatagctaggccactagtcgatctg-3′; *mre11::KAN^r*: 5′-actctttacgcttgtaaggaagacaatgtggaaacaacattaagagaatcgctgaagcttcgtacgc-3′ and 5′-acctctaattaatcaatcaaagtctatcccttcgtagatattatggggtctaggccactagtcgatctg-3′; *rad50::KAN^r* : 5′-tactctttacgttgtaaggaagacaatgtggaaacaacattaagagaatcagctgaagcttcgtacgc-3′ and 5′-caagcccttggttataaataggatataatataatatagggatcaagtacataggccactagtcgatctg-3′. The spindle pole body protein and cyan fluorescent protein (SPC29-CFP)-*KAN^r* transforming PCR fragment was constructed with the use of an oligonucleotide with homology to the C-terminus of the *SPC29* coding region and the N-terminus of the CFP-coding region of pDH3 (pDH3 was obtained from the University of Washington Yeast Resource Center): 5′-gaaaatgaaagtacggaggatatactaaaaatattgtcttcgtcttttcacaatatgagtaaaggagaagaacttttc-3′ and an oligonucleotide with homology to the C-terminus of the *KAN^r* coding region and genomic sequences downstream of the *SPC29* coding region: 5′-ggagacggatatattggttattctggtaactacaactggggcggtgacacctattggatggcggcgttagtatcggcgttagtat-3′. Transformation results were confirmed by PCR and/or microscopic analysis for GFP and CFP constructs. Yeast cultures were maintained at 32°C in rich medium (2% peptone and 1% yeast extract) containing 2% galactose (YPG) for strains with a conditional dicentric chromosome. Viability of strains on glucose was determined by plating equal numbers of cells on YPG or rich media with 2% glucose (YPD) (Brock and Bloom, 1994).

Southern Blot Analysis

Total genomic DNA was isolated from strains KBY3024 and KBY3124 and digested with *Sal*I and *Kpn*I. A Southern blot of these samples was probed with a digoxigenin-labeled PCR fragment made from an eight-repeat lacO template (Robinett *et al.*, 1996). Hybridization and detection were accomplished with the DIG Easy Hyb system (Roche, Indianapolis, IN). Images of Southern blots were collected with a Storm Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Microscopy and Image Quantitation

Cells were induced for expression of GFP-lacI and activation of the dicentric chromosome by growth for 1–2 h in glucose-based synthetic dropout media lacking histidine, followed by the addition of 3-aminotriazole (Sigma, St. Louis, MO) at a final concentration of 10 mM for 30 min. An aliquot of washed cells was added to the surface of a 25% gelatin slab containing 2% glucose, 0.5% casamino acids, 50 μg/ml tryptophan, 50 μg/ml adenine either alone or with 16.5 μg/ml uracil. Coverslips were applied and sealed with Valap (1:1:1 vaseline:lanolin:paraffin), and cells were observed at room temperature (22°C). To demonstrate the requirement for microtubules in lacO stretching, KBY3124 cells were cultured for 1 h in YPG containing 20 μg/ml nocodazole and 0.5% DMSO (Sigma), transferred to glucose-based synthetic dropout media, induced with 3-AT, and examined microscopically on gelatin slabs containing 20 μg/ml nocodazole. The microscopy system and GFP fluorescence imaging are described in Shaw *et al.* (1997), except that a 100×/1.4 NA Plan Apo objective was used for live cell imaging and a 40×/1.3 NA Plan Fluor objective was used to obtain the images in Figure 8. Metamorph software (Universal Imaging, Downingtown, PA) was used to control a Ludl filter wheel (Ludl Electronics, Hawthorne, NY). CFP fluorescence images were obtained with an exposure setting of 0.2 s and a 436/470 nm (excitation/emission) filter cube (Chroma, Battleboro, VT). Images were collected at intervals ranging from 10 s to 5 min. Five *z*-axis fluorescence images were acquired at 0.75-μm intervals through the cell and projected into a single three-dimensional (3-D) reconstruction image with the use of Metamorph software. A single bright-field or differential interference contrast (DIC) image was collected at the medial *z*-step by rotating the analyzer into the polarized light path and taking a 0.25-s exposure. Image analysis was accomplished with Metamorph software. Lengths of stretched lacO arrays were measured from single plane images with the use of appropriate calibrations from a stage micrometer. Distances between spindle poles were measured from 3-D reconstruction images and corrected for specimen tilt angle through a Microsoft Excel algorithm containing the formula c^2 = square root ($a^2 + b^2$), where a is the measured distance taken from the 3-D image, and b is the distance between the uppermost and lowest focal planes in which the poles are visible.

Intensity Measurement and Calculation of Segregation Coefficient

Pixel intensity values (PIVs) of GFP spots were obtained by integration of pixel intensities within a 10 × 10 pixel region surrounding each of the spots. Background PIVs were obtained by integrating the pixel values for two regions of identical dimensions adjacent to each GFP spot. Background PIVs for each image were averaged and subtracted from PIVs of adjacent spots. The segregation coefficient (SC) (the difference between the background-corrected PIVs for a pair of daughter cells) was calculated from normalized PIVs to account for cell-to-cell differences in GFP-lacI expression with the use of the formula: segregation coefficient = absolute value [PIV-(daughter1) − PIV(daughter2)/PIV(daughter1) + PIV(daughter2)]. Background-corrected PIVs were also used in quantifying changes in spot intensity in Figure 6.

Chromatin Immunoprecipitation

Cells were grown in YPG to an OD_{660} of 1.0, washed and transferred to YPD or YPG containing 20 μg/ml nocodazole, and reincubated for 2.5 h until >90% of cells arrested in G2/M. The cells were washed and resuspended in nocodozole-free media and reincubated until >80% of cells were in anaphase (large budded cells with bilobed nuclei as revealed by DAPI staining). The cells were formaldehyde-fixed and processed for chromatin immunoprecipitation (ChIP) analysis as described in Hecht and Grunstein (1999). PCR of nonimmunopreciptated (input) DNA and immunoprecipitated DNA was performed with primers to the lacO 8mer sequence: 5′-aggcgccgaattccaca-3′ and 5′-agaattcgtcgaggaattc-3′, which produced a 314-bp fragment when genomic DNA containing the 256-repeat lacO marker was used as template; primers to a 351-bp fragment of *MATA1*: 5′-gctaattctggagcgattg-3′and 5′-ctcactactaccattactg-3′; and primers to a 395-bp fragment of *ACT1*: 5′-tgttctagcgcttgcacc-3′ and 5′-tagatgggaagacagcacg-3′. PCR products were separated on 1.5% agarose gels and visualized with ethidium bromide staining.

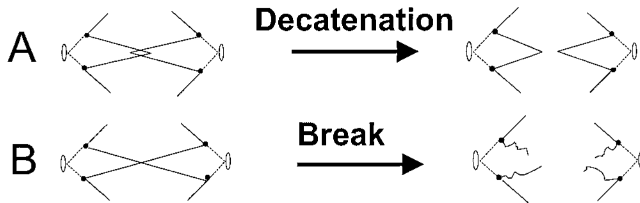


Figure 1. Spindle attachments and dicentric chromosome breakage. (A) Attachment of both centromeres of a given sister chromatid to the same spindle pole. Decatenation of DNA strands allows sister chromatids to separate and segregate normally. (B) Attachment of the centromeres of a given sister chromatid to opposite spindle poles. Resolution occurs through chromosome breakage. Filled circles, Centromeres; thin lines, endogenous DNA; dashed lines, kinetochore microtubules; open circles, spindle pole bodies.

Table 2. Viability of dicentric strains on glucose media

Strain	Percent viability (mean ± 1 SD)
J178#24 (dicentric)	62.2 ± 14.8
J178124 (dicentric, yku70)	23.1 ± 7.8
KBY3024 (dicentric, lacO)	64.2 ± 15.7
KBY3124 (dicentric, lacO, yku70)	27.8 ± 10.6
KBY3224 (dicentric, lacO, yku80)	28.8 ± 6.3
KBY3424 (dicentric, lacO, sir2)	25.2 ± 4.5
KBY3324 (dicentric, lacO, yku70, rad9)	59.3 ± 10.0
KBY3524 (dicentric, lacO, sir2, rad9)	56.4 ± 7.8

Viability was determined by comparing the number of colonies that formed on glucose plates (dicentric) with the number on galactose plates (monocentric). n = 4 determinations for each strain.

RESULTS

Deletion of YKU70 *Results in Dicentric Chromosome Stretching during Anaphase*

A conditional dicentric chromosome has been constructed in *S. cerevisiae* by means of site-directed integration of a second copy of centromere 3 (*CEN3)* at the *HIS4* locus (Hill and Bloom, 1989). This extra copy of *CEN3* is regulated by the *GAL1* promoter (*GALCEN)*, allowing cells to be propagated in the presence or absence of a functional dicentric chromosome. The dicentric chromosome is functionally monocentric when cells are grown on galactose and functionally dicentric in the presence of glucose. If both centromeres on the dicentric chromosome attach to the same spindle pole, chromosome segregation can occur without chromosome breakage (Figure 1A). If centromeres from the same sister chromatid attach to opposite poles, the chromosome breaks (Figure 1B).

The viability of cells containing a dicentric chromosome is reduced 100- to 200-fold after deletion of *RAD52,* indicating that repair of broken dicentric chromosomes occurs primarily through homologous recombination (Brock and Bloom, 1994). We found that deletion of *yKU70* is also associated with reduced survival of a dicentric yeast strain, with viability decreased two-to threefold relative to dicentric cells containing a wild-type copy of *yKU70* (Table 2). This finding was unexpected given that several previous studies have concluded that *yKU70* is not required for homologous repair of damaged DNA in yeast (Boulton and Jackson, 1996; Siede *et al.*, 1996). Furthermore, dicentric chromosome rearrangements characteristic of NHEJ have been detected only in populations lacking *RAD52* (Kramer *et al.*, 1994). This suggested that a function of Ku other than its role in NHEJ might be responsible for the reduction in viability associated with deletion of *yKU70* in a dicentric strain.

We deleted *YKU70* in cells containing a lacO-marked dicentric chromosome to determine whether the Ku proteins play a role in the dynamic behavior of a dicentric chromosome. The 10-kb lac operator repeat was integrated at *LEU2*, placing it nearly equidistant from both the endogenous *CEN3* and the *GALCEN3* (Figure 2A). The marked chromosome was made visible by expression of lacI-GFP. A Southern blot confirmed that the lacO repeat sequence was maintained at a stable length in both dicentric *yKU70* and dicentric *yku70* mutant strains (Figure 2B). Activation of a marked dicentric chromosome in *yku70* mutant cells (KBY3124) resulted in a viability loss similar to that observed with the unmarked strain J178#124 (Table 2). The lacI-GFP/lacO marker system has been used previously to track individual chromosomes in living yeast cells and changes in chromosome condensation within domains of mammalian chromosomes (reviewed in Belmont and Straight, 1998).

Cells containing either a lacO-marked monocentric chromosome or a lacO-marked dicentric chromosome were imaged by time-lapse digital fluorescence microscopy. A lacO array located on a monocentric chromosome appeared as one spot that subsequently divided into two spots as the cells entered anaphase (Figure 3, A–E). A lacO-marked dicentric chromosome behaved similarly, although in some cells the spot pairs underwent repeated cycles of overlap and separation (Figure 3, F–I). In contrast, we observed the formation of a linear filament after activation of a lacO-marked dicentric chromosome in *yku70* mutant cells (Figure 3, O and P). Quantification of filament formation revealed that 18% of large budded cells in a dicentric *yku70* strain exhibited extended lacO arrays (Table 3). Stretched lacO arrays were not observed in unbudded or small budded cells, indicating that their formation was restricted to G2/M, when dicentric chromosome breakage occurs (Brock and Bloom, 1994). The dependency of lacO stretching on the dicentric chromosome was further supported by the low frequency of lacO DNA stretching events when dicentric *yku70* cells were maintained on galactose (Table 3).

Time-lapse images of dicentric *yku70* cells revealed that lacO filaments formed through an ordered series of events (Figure 3, K–O). Filament formation was preceded by the formation of a row of approximately eight spots (Figure 3, L–N). The chain of spots subsequently formed a filament with one or two compact "terminal spots" at each end (Figure 3, O and P). The extension of the lacO array occurred at an average rate of 0.47 ± 0.33 μm/min (mean ± 1 SD; n = 10 cells). The mean length of a fully extended filament was 6.4 ± 2.4 μm (n = 38 cells). Stretched lacO filaments persisted for an average of 32.0 ± 9.0 min (n = 38 cells). In 89% of cells with stretched filaments, extension of the lacO region was followed by a symmetrical contraction of the filament into the bud neck before septum formation (Figure 3, P–T). The average rate of this collapse was 2.03 ± 0.40 μm/min

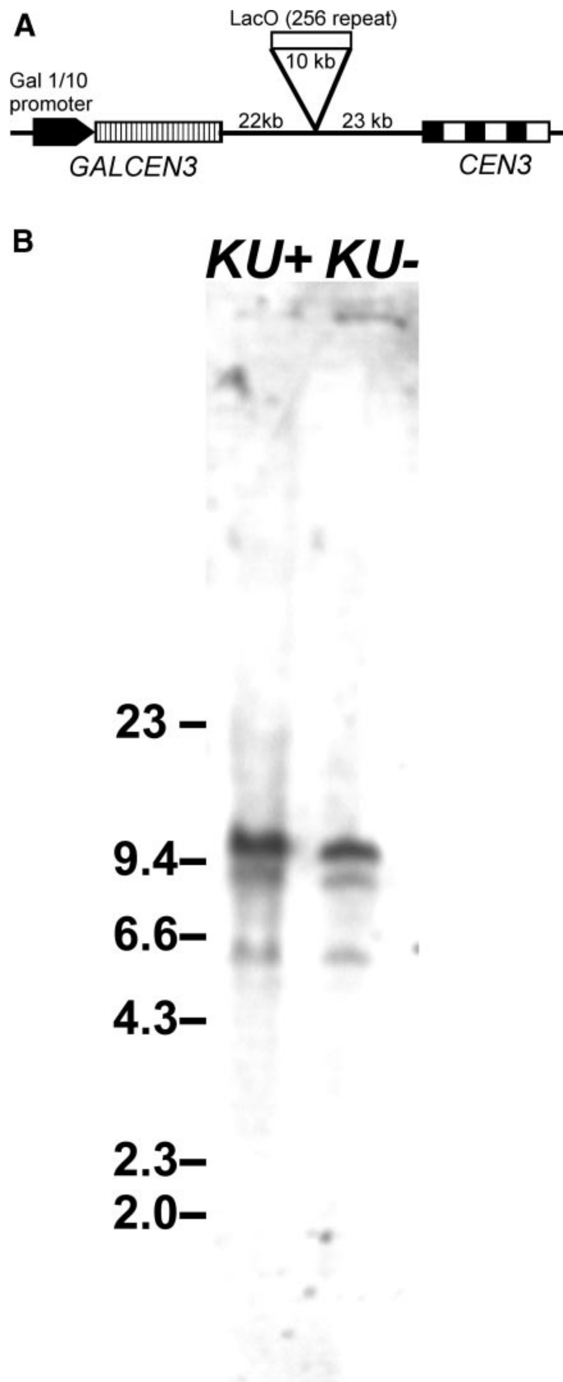


Figure 2. Construction and characterization of the lac Operator (lacO)-marked conditional dicentric chromosome III. (A) Schematic showing components of the lacO-marked dicentric chromosome. (B) The lacO repeat sequence is maintained at a stable length of 10 kb in dicentric cells and dicentric cells lacking *yku70*. Southern blot of *Sal*I/*Kpn*I-digested genomic DNA from the lacO-marked dicentric strain KBY3024 and the *yku70* mutant dicentric strain KBY3124 (both strains were cultured for >20 generations in galactose media). The blot was probed with a labeled DNA fragment containing eight tandem lacO repeats. Sizes of molecular weight markers (kilobase) are indicated at left margin of blot.

(n = 10 cells). The collapse of the filament coincided with the appearance of two to four spots at the bud neck. Spots persisted at the bud neck for an average of 13 ± 8 min (n = 10 cells) before migrating into the daughter cells (Figure 3, S and T). In the remaining 11% of cells the septum formed, while the dicentric chromosome remained stretched across the bud neck (our unpublished results). This is analogous to the *cut* phenotype of the fission yeast *Schizosaccharomyces pombe* (reviewed in Yanagida, 1998).

The length and kinetics of stretched lacO arrays were similar to previous real time observations of anaphase mitotic spindles (Yeh *et al.*, 1995; Straight *et al.*, 1996), suggesting that spindle forces were involved in their formation. We tested this hypothesis by incubating dicentric *yku70* cells in media containing 20 μg/ml nocodazole. No stretched lacO arrays were observed in dicentric *yku70* cells after the nocodazole treatment. Additionally, we constructed a strain expressing a fusion product of the *SPC29* spindle pole body protein and cyan fluorescent protein. Simultaneous measurements of the distance between spindle poles and the length of the lacO filament confirmed that expansion of this region paralleled the elongation and collapse of the spindle during anaphase (Figure 3U).

Most of the stretched lacO filaments in dicentric *yku70* deletion cells appear as single elements (Figure 3, O and P). In y*KU70* wild-type cells, this marker separates into two distinct spots during anaphase (Figure 3, A–J). LacO DNA also separated normally during anaphase in *yku70* mutant cells where the lacO marker was located on a monocentric chromosome (our unpublished observations). Careful examination of sequential time-lapse images of 38 cells containing stretched lacO filaments revealed two examples in which a filament transiently separated into two axially wound elements. A cell with paired filaments is shown in Figure 3V. The fact that lacO spots did eventually separate once spindle tension was released suggests that sister chromatid separation is inhibited while the lacO region remains in a stretched configuration.

Dicentric Chromosome Stretching Occurs in Cells Lacking yKU70, yKU80, or SIR2

Several genes involved in heterochromatic silencing and DNA repair were deleted to determine whether the lacO array on a dicentric chromosome would undergo a similar decondensation event. We found that deletion of either *YKU80* or *SIR2* resulted in stretched lacO filaments with a similar frequency and appearance identical to those observed with the *yku70* mutant (Figure 3, W and X, and Table 3). Furthermore, the viability of these strains was reduced to a similar degree in y*ku70*, *yku80*, and *sir2* mutant cells (Table 2). In contrast, deletion of the DNA repair genes *RAD50*, *MRE11*, or *RAD52* in dicentric cells was not accompanied by formation of lacO filaments (Table 3). Stretching of lacO DNA was not altered in either frequency or kinetics by the double deletion of *YKU70* and *RAD52* (Table 3), demonstrating that this behavior is not a consequence of *RAD52*-dependent DNA repair events.

Dicentric Chromosome Stretching May Occur in Endogenous DNA

To determine whether chromatin stretching was restricted to lacO DNA, we examined the behavior of lacO DNA spots in

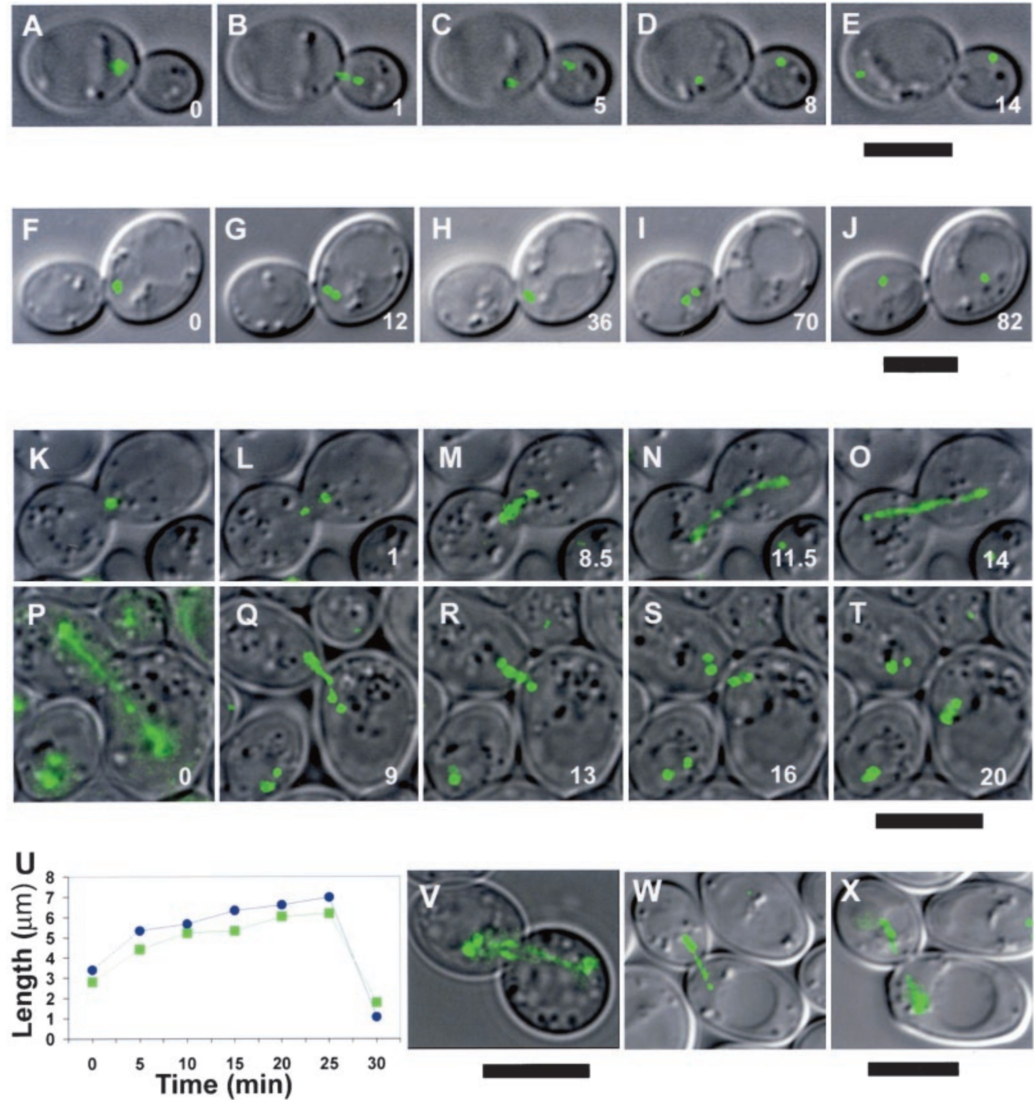


Figure 3. The lacO DNA sequence located between the centromeres of a dicentric chromosome becomes stretched in cells deleted for *YKU70, YKU80,* and *SIR2.* Pseudocolored images of GFP-lacI (green), marking a 10-kb region of chromosome III, or SPC29-CFP (blue), marking spindle poles, were overlaid onto transmitted light images (bright field for K–T and DIC for the others). Elapsed time appears in the bottom right corner of each frame. Bar, 5 μm (bar below X applies to W and X). (A–E) Anaphase separation of sister chromatids of a lacO-marked monocentric chromosome III in *YKU70* wild-type strain AFS173. (F–J) Anaphase separation of sister chromatids of a lacO-marked dicentric chromosome III in *YKU70* wild-type strain KBY3024. (K–O) Sequential images of a *yku70* mutant cell (strain KBY3124) containing a lacO-marked dicentric chromosome III showing critical stages in the formation of a stretched lacO array. (P–T) Sequential images of a KBY3124 cell showing steps in the resolution of a stretched lacO array. (U) LacO DNA stretching of a dicentric chromosome occurs in parallel with changes in spindle length during anaphase. Graph shows spindle length (blue circles) and length of a stretched lacO filament (green squares) in a dicentric *yku70* cell containing the SPC29-CFP spindle pole marker. (V) Partially separated lacO filaments in KBY3124 cells. Separation between filaments is most obvious where the filaments cross the bud neck. (W) A stretched lacO array in dicentric *yku80* strain KBY3224. (X) A stretched lacO array in dicentric *sir2* strain KBY3424. The gap in the GFP filament was due to loss of the fluorescence signal where the stretched chromosome crossed the bud neck.

dicentric *yku70* cells that did not form lacO filaments. We reasoned that any significant unfolding of chromatin adjacent to the lacO region might displace the GFP marker toward one spindle pole. We recorded spot movements in cells that exhibited a cell cycle arrest at midanaphase (Yang *et al.*, 1997), because previous characterizations revealed that the midanaphase pause preceded lacO stretching (our unpublished results). Of the 25 cells examined, 14 formed lacO filaments. In three cells that did not form filaments, the lacO marker separated into two closely spaced spots, both of which moved to a location near one pole as the spindle elongated (Figure 4, A and B). The spots persisted near one pole throughout late anaphase (spindle length >5 μm) (Figure 4, B and C). After spindle collapse, one spot remained associated with the proximal spindle pole, whereas the other spot moved to the opposite pole (Figure 4D). This behavior, which was not observed in *yKU* and *SIR* wild-type cells containing a dicentric chromosome, is consistent with displacement of the spots toward one pole by the stretching of adjacent endogenous DNA.

Chromatin Stretching and Dicentric Chromosome Breakage

We observed that *yku70* dicentric cells exhibit a high incidence of unequal partitioning of GFP-lacI–marked DNA to daughter cells (Figure 5A). In some instances the marked DNA was partitioned into a single daughter cell (Figure 5B). Quantification of integrated fluorescence pixel intensities confirmed these observations. The pixel intensity difference

Table 3. Frequency of stretched lacO arrays in large budded cells

Strain	Frequency (percentage of large budded cells)
KBY3124 (dicentric, lacO, yku70)	18.2
KBY3124 (galactose media)	1.2
KBY3024 (dicentric, lacO)	0.4
KBY3134 (monocentric, lacO)	<0.1
KBY3224 (dicentric, lacO, yku80)	17.3
KBY3424 (dicentric, lacO, sir2)	20.3
KBY3624 (dicentric, lacO, rad50)	0.4
KBY3724 (dicentric, lacO, rad52)	0.1
KBY3824 (dicentric, lacO, mre11)	0.5
KBY3124 (dicentric, lacO, yku70, rad52)	16.7
KBY3324 (dicentric, lacO, yku70, rad9)	3.8
KBY3524 (dicentric, lacO, sir2, rad9)	5.0

lacO/lacI-GFP distributions were categorized in 1000 large-budded cells for each strain. All values were determined after a 2 h incubation of cells on glucose, except as indicated above.

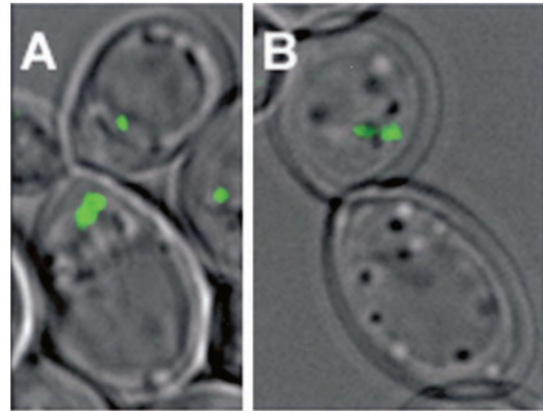


Figure 5. Unequal distribution of lacO DNA is observed in KBY3124 cells after the resolution of DNA stretching events. (A) Fluorescence/bright-field overlay images show examples of unequal distribution of lacO DNA in a pair of daughter cells. (B) Complete partitioning of lacO DNA into a single cell. Bar, 5 μm.

between the two spots was reported as a segregation coefficient, defined by the ratio of the difference in pixel intensity of a pair of daughter cells to the total pixel intensity of the two cells (see MATERIALS AND METHODS). Equal segregation of marked DNA would theoretically yield an SC value of 0, whereas the segregation of all of the lacO DNA into one cell would give an SC value of 1. The average SC value for cells that exhibited lacO stretching was 0.534 ± 0.322 (n = 20), consistent with unequal separation of the marked DNA. We interpret these results as evidence that stretched regions of dicentric chromosomes frequently develop breaks that are not repaired before segregation of the broken sister chromatids into daughter cells.

In the majority of cells with stretched lacO DNA, the timing of chromosome breakage could not be determined precisely. In 7 of 67 cells, however, a visible interruption appeared in the stretched filament. After breakage, the filament retracted toward its terminal spots. An example of a cell experiencing filament breakage and retraction is shown in Figure 6, A–H. Measurements of the length of the lower filament segment and the intensity of the spot attached at its lower end revealed an inverse relationship between filament length and spot intensity (Figure 6I). These observations demonstrate that a dicentric chromosome can break while stretched. The increase in terminal spot intensity that accompanied retraction of the broken lacO filament suggests that release of tension allows recompaction of a stretched chromosome.

Sir2 Associates with LacO DNA on a Dicentric Chromosome

Ku and Sir proteins might act directly or indirectly by regulating the expression of other proteins to prevent dicentric chromosome decompaction. We used ChIP, previously used

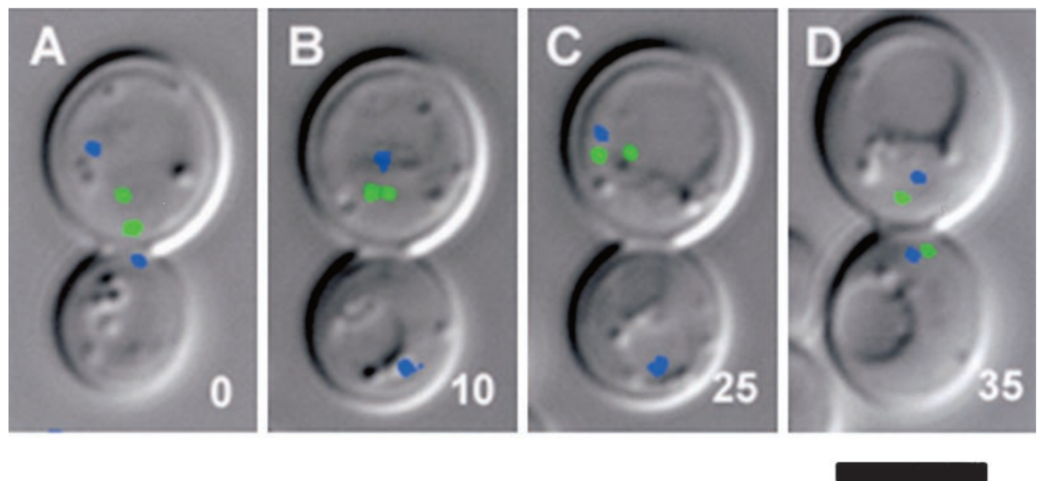
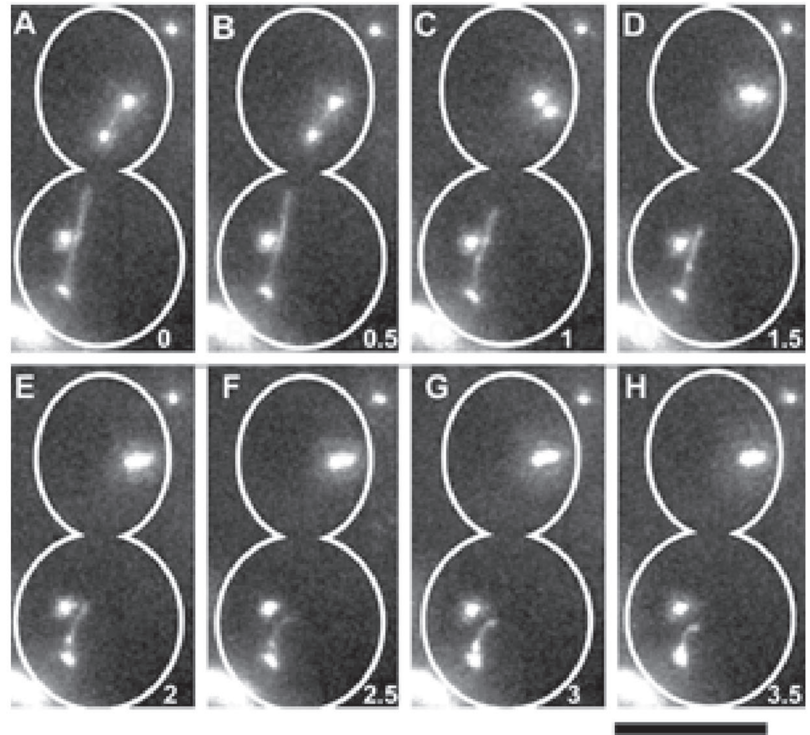


Figure 4. DNA stretching on a dicentric chromosome in *yku70* strain KBY4024, containing both the lacO chromosome marker (green) and SPC29-CFP spindle pole body marker (blue). A–D show a cell during successive time points after anaphase onset. Elapsed time is indicated in minutes in bottom right corner of each panel. Evidence for stretching of endogenous yeast DNA is revealed by displacement of the lacO marker (green spots) toward one spindle pole (blue spot). Images are composed from GFP and CFP signals overlaid on DIC images. Bar, 5 μm.



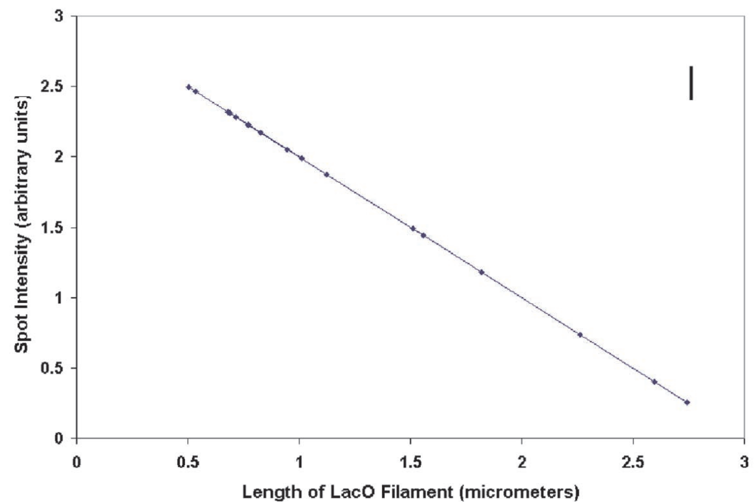


Figure 6. (A–H) Breakage and condensation of a stretched lacO DNA filament. Breakage of a filament occurred just before the first panel (note gap at bud neck between filament segment in top cell and segment in bottom cell in A). Spots in the top cell fuse together as the connecting filament disappears (A–D), and the filament segment in the lower cell retracts into the bottom-most spot (A–H). The yeast cell outline was copied from phase images and overlaid onto fluorescence images. Elapsed time is in minutes. Bar, 5 μm. (I) The intensity of a terminal spot increases as the length of an attached broken filament decreases. Fluorescence intensity measurements of the lower-most spot in the bottom cell in A–H were plotted against the length of the attached filament.

to demonstrate the association of Ku and Sir proteins with silenced regions and DNA DSBs (Hecht *et al.*, 1995, 1996; Martin *et al.*, 1999; Mills *et al.*, 1999), to address whether a direct mode of action might be the case for Sir2. Sheared chromatin from dicentric cells was immunoprecipitated with antibodies directed against Sir2 and probed with PCR primers complementary to the lacO 8mer sequence from which the 10-kb lacO repeat was constructed (Belmont *et al.*, 1999). We found that Sir2 was localized to the lacO region of an active dicentric chromosome during anaphase (Figure 7). This suggests that Sir2 has a direct role in suppressing chromosome stretching. Significantly, Sir2 was not associated with this region in cells maintained in galactose media so as to prevent activation of the dicentric chromosome (Figure 7). Thus the affinity of Sir2 for the lacO marker on a dicentric chromosome does not simply reflect the association of this protein with the repeated lacO array. Tension generated by either the spindle or dicentric chromosome breakage may promote the association of Sir2 with this region.

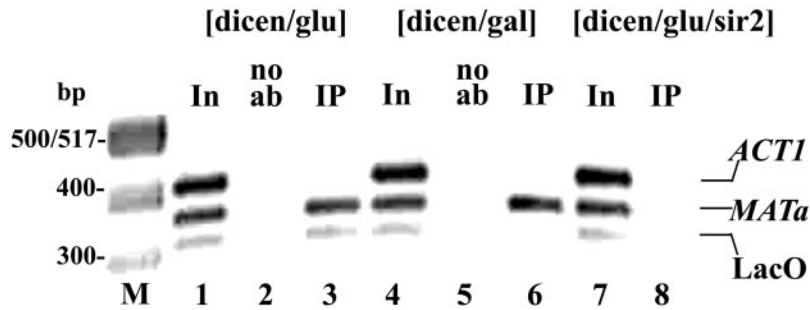


Figure 7. Sir2 localizes to the lacO array on an activated dicentric chromosome. Dicentric KBY3024 cells were blocked in mitosis with nocodozole, then released from the nocodozole block and allowed to enter anaphase. The cells were then subjected to ChIP analysis (see MATERIALS AND METHODS). Chromatin was cross-linked with formalin and immunoprecipitated with anti-Sir2 sera. DNA was then purified from the immunoprecipitate and analyzed by PCR with the use of primers recognizing the 314-bp sequence of the 8mer subunit of the lacO array, and primers that hybridized to 351- and 395-bp sequences of *MATa1* and *ACT1*, respectively, as positive and negative controls for the specificity of the immunoprecipitation reaction. Additional controls included omission of the antibody and substitution of a *sir2* deletion strain (KBY3424). The lacO region was immunoprecipitated from cells containing an active dicentric chromosome (shifted to glucose) but not from cells containing an inactive dicentric chromosome (maintained on galactose). In, Input DNA (cell lysate); no ab, mock immunoprecipitation (no antisera); IP, immunoprecipitated DNA. Lanes 1–3 are samples from dicentric cells grown on glucose; lanes 4–6 are from the same strain maintained in galactose. Lanes 7 and 8 were from a dicentric *sir2* deletion strain.

RAD9 *Facilitates lacO DNA Stretching*

The *RAD9* gene is involved in the establishment of delays at multiple points in the cell cycle in response to DNA damage (Elledge, 1996), including the midanaphase pause that follows activation of a conditional dicentric chromosome (Yang *et al.*, 1997). Dicentric cells with a double deletion of *YKU70* and *RAD9* or deleted for both *SIR2* and *RAD9* had a similar viability when compared with dicentric cells containing wild-type copies of *YKU70* and *SIR2* (Table 2). Unexpectedly, we found that the frequency of lacO stretching events in dicentric *yku70 rad9* cells was nearly fivefold lower than that observed with dicentric *yku70* cells (Figure 8 and Table 3). Similar results were observed with *sir2 rad9* cells containing a dicentric chromosome. The requirement for *RAD9* in the stretching of a dicentric chromosome may result from its function in promoting the midanaphase pause (Yang *et al.*, 1997). The midanaphase pause prolongs the period during which the dicentric chromosome is exposed to forces generated by the mitotic spindle. This may make the dicentric chromosome more likely to unfold when the stabilizing activities of Ku and Sir proteins are absent. Alternatively, *RAD9* might have a role in the regulation of chromatin structure.

DISCUSSION

Components of the DNA repair and heterochromatic silencing machinery *yKu70*, *yKu80*, and *Sir2* are necessary for maintaining a condensed chromatin structure between the centromeres of a dicentric chromosome. In their absence, a GFP-labeled region of a dicentric chromosome bridge forms a highly extended filament in response to forces generated by the mitotic spindle (Figure 3). Spindle disassembly results in chromatin compaction and reveals evidence of DNA double-strand breaks. Sir2 may have a direct role in chromatin compaction because it localizes to the lacO region in response to activation of the dicentric chromosome. Additionally, chromatin decompaction induced by dicentric chromosome stretching is dependent on the DNA damage checkpoint gene *RAD9*.

Sir- and Ku-dependent alterations in chromosome structure have been postulated to accompany the formation of

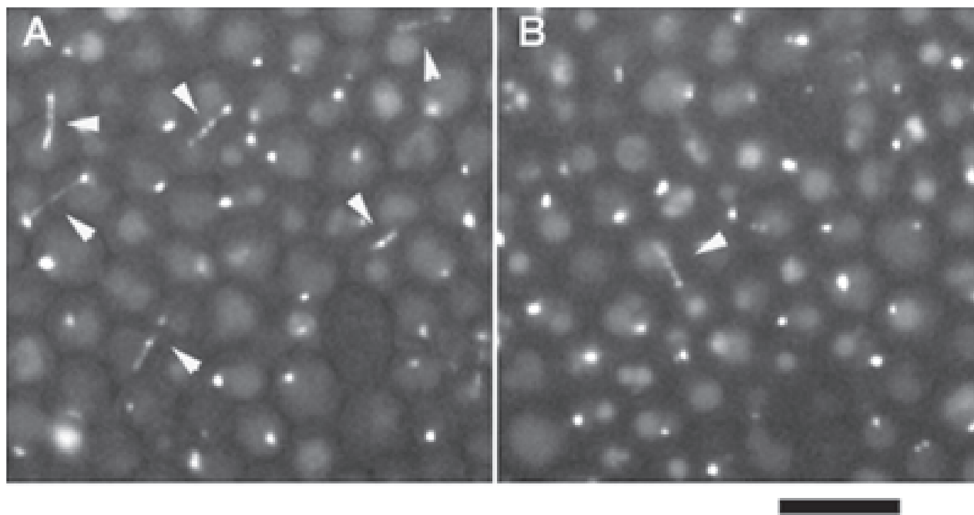


Figure 8. The incidence of stretched lacO arrays in dicentric *yku70* cells is *RAD9* dependent. GFP fluorescence images of representative fields of view of (A) lacO/lacI-marked dicentric *yku70* cells (KBY3124) and (B) lacO/lacI-marked dicentric *yku70 rad9* cells (KBY3324). Stretched lacO arrays are indicated by arrowheads. Bar, 10 μm.

heterochromatin, but the difficulty in imaging individual yeast chromosomes has hindered detection of such changes. The lacO/lacI-GFP marker system and a conditional dicentric chromosome have made it possible to observe changes in chromosome behavior that may reflect the loss of heterochromatic DNA within a defined region of the chromosome. A study using the lacI-GFP/lacO reporter system to study chromatin structure in mammalian cells during interphase revealed the formation of an unfolded chromosomal fiber and the recruitment of histone acetyl transferases and hyperacetylation of histones within lacO DNA (Tumbar *et al.*, 1999). Chromatin decompaction on a yeast dicentric chromosome in *yku70/80* or *sir2* mutants may similarly reflect a change in chromatin structure facilitated by changes in histone acetylation. Sir2 is a histone deacetylase and could play a direct role in generating a region of silenced chromatin (Imai *et al.*, 2000).

Yeast chromatin is compacted on the order of 100- to 200-fold during mitosis (Guacci *et al.*, 1994). The average maximum length of stretched lacO DNA filaments (6.4 μm) is nearly twice the predicted length of a 10-kb segment of B DNA (3.4 μm), as calculated from measurements of linearized, deproteinated DNA (Pietrasanta *et al.*, 1999). Our findings are consistent with studies that tested the extensibility of DNA in vitro. The application of forces in the range of 70 pN to one end of a tethered DNA molecule resulted in a 1.7- to 1.8-fold increase in the length of the DNA fragment relative to its length when not under tension (Thundat *et al.*, 1994; Cluzel *et al.*, 1996; Smith *et al.*, 1996; Strick *et al.*, 1998). Measurements of the force required to stop the movement of a chromosome in grasshopper spermatocytes revealed that a single microtubule could exert a force of $\sim 10^{-6}$ dynes or 47 pN during anaphase (Nicklas, 1983). Electron microscopic examination of the total number of microtubules in yeast spindles indicates that there is a single microtubule attached to each sister chromatid (Winey *et al.*, 1995). In the case of a dicentric chromosome, this would amount to a maximum force ranging from 94 to 198 pN, depending on whether the force is transmitted through two or four microtubules, i.e., whether sister chromatids are completely separate or remain joined within the stretched region (see below). These calculations suggest that the force required to produce the degree of dicentric chromosome stretching that we observed is within the range of forces generated by the anaphase spindle.

Recent studies have shown that sister centromeres in yeast separate before anaphase onset and pericentric chromatin is more readily extendable than chromatin located in other portions of the chromosome (Goshima and Yanagida, 2000; He *et al.*, 2000; Tanaka *et al.*, 2000; Pearson *et al.*, 2001). These preanaphase pericentric stretching events are transient in nature (between 2 and 10 min in duration) and are observed only within a 12-kb region flanking the centromere. In contrast, the dicentric chromosome-dependent stretching events persist for an average of 32 min after anaphase onset and occur in regions between two centromeres (at least 20 kb from either centromere). Importantly, chromatin expansion between the two centromeres is dependent on the loss of *KU70, KU80,* or *SIR2*. Pericentric stretching events similar in appearance to those generated after dicentric chromosome activation have been observed during anaphase (He *et al.*, 2000, their Figure 6); however, as with preanaphase centromere stretching, the events were independent of either *SIR* or *KU*. It is likely that separate mechanisms are responsible for stretching in the pericentric regions versus compaction of chromosome arms, because the latter do not exhibit preanaphase stretching.

Visualization of DNA stretching of these lacO or tetO sequences have prompted the question of whether stretching within repeated sequences of operator DNA accurately reflects the behavior of endogenous yeast DNA. The use of a spindle pole body marker (SPC29-CFP) made it possible for us to observe stretching of endogenous DNA in the intercentromeric region surrounding the lacO repeat region. In a minority of cells that did not exhibit lacO DNA stretching, both spots are displaced toward one pole and remain at that location until spindle disassembly at the end of anaphase (Figure 4). Such an arrangement would be expected if adjacent DNA underwent stretching. Stretching of lacO DNA seems to occur more frequently (14/25 cells) than stretching of non-lacO DNA (3/25 cells), suggesting that chromosome regions containing repeated DNA sequences have a heightened susceptibility to chromatin decondensation. Interestingly, it has been shown that the most pronounced defect in yeast cells containing mutations in condensin proteins is a failure to properly compact the rDNA locus, the largest naturally occurring direct repeat sequence in the yeast genome (Freeman *et al.*, 2000). It is possible that Sir2 also contributes to the compaction of rDNA, given its localization to sites within the rDNA locus and the requirement of Sir2 for heterochromatic silencing within this region (Gotta *et al.*, 1997; Freeman *et al.*, 2000).

The discovery that condensin mutants are defective in sister chromatid separation during anaphase may help to explain why only one lacO filament was observed in most cells that exhibited dicentric chromosome stretching. If chromatin compaction promotes sister chromatid separation as was suggested by Freeman *et al.* (2000), then decompaction of the lacO sequence in the dicentric chromosome system would likely prevent separation of sister chromatids. Consistent with this idea, recompaction of a stretched filament after spindle breakdown was accompanied by separation of the lacO marker into at least two spots. It was reported recently that the *SMC4* condensin component in *Drosophila* is required for separation of sister chromatids (Steffensen *et al.*, 2001), indicating that chromatin compaction and sister chromatid resolution are also functionally linked in metazoans.

An unequal distribution of lacO DNA between mother and daughter cell pairs was observed after the collapse of stretched lacO arrays (Figure 5A). This distribution is indicative of the formation of DNA double-strand breaks within the lacO array. The presence of more than one GFP spot per cell in most mother/daughter cell pairs indicates that chromosome breakage has occurred (Figure 3, S and T), as does visualization of DNA breakage within a stretched lacO filament (Figure 6, A–H). DNA breakage events do not occur exclusively within lacO DNA. Some dicentric *yku70* cells distribute the entire complement of lacO DNA to a single cell (Figure 5B). Breakage events can also occur during cell separation, with septum formation occurring while lacO DNA remains stretched across the bud neck.

The *RAD9*-dependent DNA damage checkpoint system promotes delays at critical points in the cell cycle to allow time for repair after DNA damage (Weinert and Hartwell,

1988). Results of recent studies indicate that Rad9 is involved in other functions in addition to its role as a checkpoint protein, including regulation of a transcriptional response that facilitates DNA repair (Aboussekhra *et al.*, 1996) and the release of Ku and Sir proteins from telomeres in response to DNA damage (Martin *et al.*, 1999; Mills *et al.*, 1999). We found that *RAD9* is required for efficient formation of stretched lacO arrays (Figure 8 and Table 3), and deletion of *RAD9* improves the viability of dicentric cells that carry deletions of *yKU70* or *SIR2* (Table 2). The role of *RAD9* in the lacO DNA stretching process could be a consequence of its action in promoting the midanaphase pause (Yang *et al.*, 1997). The midanaphase pause prolongs the exposure of a dicentric chromosome to pulling forces of the mitotic spindle, increasing the probability of disrupting protein interactions that stabilize chromatin structure. Consistent with this explanation, experiments in which repeated stretching forces were applied in vitro to chromatin fibers under physiological conditions resulted in the irreversible lengthening of the fibers (Cui and Bustamante, 2000). Permanent chromatin distortions may occur on a dicentric chromosome that could hinder repair and contribute to the loss in viability observed for dicentric strains deficient in Ku or Sir proteins. Alternatively, *RAD9* might sense alterations in chromatin structure resulting from the absence of Ku and Sir proteins and activate chromatin-remodeling factors with actions that could make the dicentric chromosome more susceptible to chromatin decompaction.

The genes with mutations that lead to stretching of chromatin on a dicentric chromosome, *YKU70, YKU80,* and *SIR2*, function both in DNA repair by the NHEJ pathway and in the silencing of constitutive heterochromatin (Tsukamoto *et al.*, 1997). In contrast, mutations in proteins with known functions that are limited to DNA repair, *RAD50, XRS2,* and *RAD52,* do not promote chromatin stretching. This suggests that the silencing activities of Ku and Sir proteins rather than their DNA repair functions may inhibit lacO stretching. To explore the link between Sir proteins, heterochromatin formation, and chromosome stretching, we used chromatin immunoprecipitation to determine whether Sir2 is targeted to the lacO region during activation of the dicentric chromosome. We found that Sir2 associates with lacO DNA in functional dicentric chromosomes but not lacO arrays in monocentric chromosomes. Thus Sir2 is most likely recruited to lacO after attachment of the non-sister centromeres to opposite spindle poles, rather than the result of any inherent affinity between Sir2 and repeated DNA. The structural aberrations that are responsible for recruiting Sir2 during non-sister centromere attachment are not known. Chromatin distortions induced by spindle forces rather than actual breaks (see above) may induce the association of Sir2 with the lacO sequence, or DNA double-strand breaks may directly recruit Sir2. In either case these results are consistent with a model in which Sir2 acts directly on the dicentric chromosome to prevent chromosome decompaction. We propose that stretching of a dicentric chromosome is symptomatic of a loss of chromatin structure that would otherwise be maintained in a heterochromatin-like state as part of the cellular response to DNA damage. These data extend the recent discovery that yeast Ku and Sir proteins dissociate from subtelomeric regions and relocalize to an extended region surrounding a DNA break site (Martin *et al.*, 1999; Mills *et al.*, 1999).

Deletion of *yKU70* also results in an increased rate of 5′–3′ excision of DNA initiating at the site of the break (Lee *et al.*, 1998). According to current models, the excision of nonhomologous DNA located between homologous repeats is an early step in the process of homologous repair (Paques and Haber 1999**;** Aguilera *et al.*, 2000). The rate of 5′–3′ single-strand excision may well be regulated by the heterochromatic state established via Ku binding to sites of DNA damage. Our observation that the loss of Ku alters the behavior of an extended region of DNA leads to the prediction that effects of a *KU70* deletion would be observed at a significant distance from the original break site. The altered rate of excision in *yku70* mutant strains in fact does extend over a region several kilobase pairs from the original break site (Lee *et al.*, 1998). In the absence of Ku and Sir, the actions of repair proteins and chromatin remodeling complexes recruited to sites of DNA damage may generate a region of loosely organized chromatin that is susceptible to unfolding in response to spindle force.

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