

Centromere Function on Minichromosomes Isolated from Budding Yeast

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Centromeres are a complex of centromere DNA (CEN DNA) and specific factors that help mediate microtubule-dependent movement of chromosomes during mitosis. Minichromosomes can be isolated from budding yeast in a way that their centromeres retain the ability to bind microtubules *in vitro*. Here, we use the binding of these minichromosomes to microtubules to gain insight into the properties of centromeres assembled *in vivo*. Our results suggest that neither chromosomal DNA topology nor proximity of telomeres influence the cell's ability to assemble centromeres with microtubule-binding activity. The microtubule-binding activity of the minichromosome's centromere is stable in the presence of competitor CEN DNA, suggesting that the complex between the minichromosome CEN DNA and proteins directly bound to it is very stable. The efficiency of centromere binding to microtubules is dependent upon the concentration of microtubule polymer and is inhibited by ATP. These properties are similar to those exhibited by mechanochemical motors. The binding of minichromosomes to microtubules can be inactivated by the presence of 0.2 M NaCl and then reactivated by restoring NaCl to 0.1 M. In 0.2 M NaCl, some centromere factor(s) bind to microtubules, whereas other(s) apparently remain bound to the minichromosome's CEN DNA. Therefore, the yeast centromere appears to consist of two domains: the first consists of a stable core containing CEN DNA and CEN DNA-binding proteins; the second contains a microtubule-binding component(s). The molecular functions of this second domain are discussed.

INTRODUCTION

Chromosome movement during mitosis depends upon the microtubules of the mitotic spindle and upon the centromere, a specific chromosomal organelle. The centromere is structurally complex; it contains DNA, proteins, and perhaps other unidentified factors. The centromere is also functionally complex; it not only binds microtubules but also is capable of generating the forces that move chromosomes along microtubules both toward and away from the spindle poles. Researchers now are trying to understand the assembly of the centromere, to elucidate the molecular mechanisms of centromere activities like microtubule binding or force production, and to unravel the complex regulation of the centromere necessary for highly ordered chromosome movement.

In the budding yeast *Saccharomyces cerevisiae*, progress has been made in identifying some of the structural components of centromeres. Centromere DNA (CEN DNA) from different yeast chromosomes has been isolated (Clarke and Carbon, 1980; Fitzgerald-Hayes *et al.*,

1982a; Hieter *et al.*, 1985) and sequenced (Fitzgerald-Hayes *et al.*, 1982b; Hieter *et al.*, 1985). All CEN DNA sequences share 125 base pairs (bp) of homology that can be divided into three distinct centromere DNA elements (CDEI, II, and III). The importance of these DNA elements for mitotic centromere function has been assessed by generating mutations in cloned CEN DNA, substituting the mutant CEN DNA into an endogenous chromosome and then monitoring the chromosome's segregation during cell division (McGrew *et al.*, 1986; Cumberland and Carbon, 1987; Gaudet and Fitzgerald-Hayes, 1987; Hegemann *et al.*, 1988). From these studies, CDEII and CDEIII have been shown to be essential for mitotic centromere function. Recently, several putative centromere proteins have also been identified. Affinity chromatography with wild-type CEN DNA has been used to purify a fraction of yeast proteins (CBF3) whose binding to CEN DNA is dependent upon the presence of wild-type CDEIII (Lechner and Carbon, 1991). This fraction has a complex protein profile but contains three prominent proteins of 58 kDa, 64 kDa,

and 110 kDa. Mutants with conditional defects in the 110- and 58-kDa proteins have been isolated (Doheny *et al.*, 1993; Goh and Kilmartin, 1993). The phenotype of these mutants demonstrates that the 58- and 110-kDa proteins are required for proper chromosome segregation, consistent with their identification as centromere components.

The study of the molecular function of centromeres from budding yeast has also progressed recently. Small circular minichromosomes can be generated by adding CEN DNA and an ARS (autonomously replicating sequence) to recombinant DNA vectors (Clarke and Carbon, 1980). When introduced into yeast, these minichromosomes are replicated and segregated with a fidelity as high as 99% per cell division. Minichromosomes can be recovered in cleared lysates of yeast, and these soluble minichromosomes bind to microtubules assembled from pure bovine tubulin (Kingsbury and Koshland, 1991). Using CEN DNA mutations, this binding has been shown to be centromere dependent. Moreover, the centromere's ability to mediate microtubule binding *in vitro* correlates with its ability to ensure minichromosome segregation *in vivo*. The efficiency of binding is greater in lysates made from mitotic cells than G1 cells. This result suggests that centromere binding to microtubules may be cell cycle regulated. As an alternative approach to study the molecular function of yeast centromeres, complexes assembled with CEN DNA and the CBF3 fraction of proteins have been analyzed for microtubule-dependent activities (Hyman *et al.*, 1992). These complexes have been shown to contain a minus-end-directed microtubule motor. The association of this motor with the centromere complex requires the presence of wild-type CEN DNA. Although these results suggest that the microtubule motor is a component of the centromere, they do not exclude the fortuitous association *in vitro* between a "noncentromere" motor protein and a CEN DNA binding protein. In addition, if yeast centromeres exhibit any of the complexity of other eukaryotic centromeres, then not all yeast centromere activities are likely to be reconstituted by the simple addition of the CBF3 fraction to CEN DNA. Therefore, it is valuable to compare the properties of these putative reconstituted centromeres with those assembled *in vivo*.

In this study the binding of microtubules to the centromeres of isolated yeast minichromosomes is used to gain insight into the properties of *in vivo* assembled centromeres. We address whether centromere assembly or function is affected by chromosomal DNA topology or proximity to telomeres. We also address whether efficiency of centromere binding to microtubules is dependent upon the polymer or end concentration of microtubules, and whether it is modulated by ATP and other nucleoside triphosphates (NTPs). Finally, we begin to examine the stability of the interaction of centromere factors to each other, to CEN DNA, and to microtubules.

MATERIALS AND METHODS

Reagents

Bovine serum albumin (BSA) fraction V, sorbitol, nocodazole, and alpha factor were purchased from Sigma (St. Louis, MO). Proteinase K, NTPs, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN), and gluculase was purchased from Dupont Biomedical Products Department (Wilmington, DE). Ethylene glyco-bis-succinimidylsuccinate (EGS) was purchased from Pierce (Rockford, IL). Restriction enzymes and poly-linkers were purchased from New England Biolabs (Beverly, MA). Taxol was provided by the Drug Synthesis and Chemistry Branch of National Cancer Institute. Tubulin was kindly provided by Andrew Murray (UCSF).

Strains and Plasmids

The strain 4513-216 (*MATa his3 leu2-3,112 ade2 ade3 ura3-52 can1 sap3*) was constructed in this laboratory. All minichromosomes were introduced into 4513-216 by transformation (Ito *et al.*, 1983). The circular minichromosomes pDK370 and YCp41 have been described previously (Clarke and Carbon, 1980; Kingsbury and Koshland, 1991). The linear minichromosome pDK416L was constructed in 4513-216 as follows. A Kpn linker was introduced into the Nru1 site of Ylp5 generating the plasmid Ylp5-L3. A 0.8-kilobase (kb) *EcoRI-HindIII* fragment containing ARS1 and a 0.3-kb *BamHI* fragment containing CEN3 were ligated sequentially into the *EcoRI-HindIII* and *BamHI* sites respectively of Ylp5-L3 generating the plasmid pDK416. pDK416 was linearized by digestion with *Kpn I*, and the ends were phosphatase-treated. A 0.7-kb *BamHI-Kpn I* restriction fragment containing *Tetrahymena* rDNA repeats was isolated from pVG72 such that the 5' phosphate at the *BamHI* but not the *Kpn I* site had been removed. The *Tetrahymena* ends were then ligated to linearized pDK416, and the ligation mix was transformed directly into 4513-216. Transformants were analyzed by the method of Southern to verify that they contained a linear molecule of expected size of 8–10 kb (6.3 kb from pDK416, 0.7 kb of *Tetrahymena* rDNA at each end and ~0.3 kb of yeast telomeric sequences).

Isolation of Minichromosomes from Yeast Cells and Assay for Binding of Minichromosomes to Bovine Microtubules

Isolation of minichromosomes from yeast cells and assaying their binding to microtubules has been described in detail previously (Kingsbury and Koshland, 1991). An abbreviated version of these methods is provided below. Yeast cultures were grown to 1×10^7 cells/ml. Cells from these asynchronous cultures were converted to spheroplasts and then osmotically lysed in EBB buffer (10 mM tris(hydroxymethyl)aminomethane, DTT pH 7.5, 10 mM MgCl₂, 0.5 mM PMSF, 0.1 mM dithiothreitol). Minichromosomes were eluted from nuclei by increasing the salt concentration to 0.3 M for 5 min. This salt shock step increased yield about fivefold but was not essential to recovering minichromosomes with microtubule-binding activity. The extracts were diluted threefold with EBB buffer containing BSA to bring the NaCl to 0.1 M and the BSA to 1 mg/ml. The lysate was subjected to two high speed spins to remove debris and most large organelles. We refer to these lysates as asynchronous lysates. Alternatively, after reaching a density of 1×10^7 cells/ml, exponentially dividing cultures were grown in the presence of nocodazole for an additional 3 h to arrest them in mitosis. Minichromosomes were isolated from these mitotic cells as described for asynchronous cells except nocodazole was added to spheroplasting buffer to maintain cells in mitosis while they were being converted to spheroplasts. These lysates are referred to as mitotic lysates.

To assay the ability of minichromosomes in cleared extracts to bind to microtubules, microtubule seeds (1–5 μ m in length) were assembled *in vitro* from bovine tubulin and stabilized with taxol (Kingsbury and

Koshland, 1991). These microtubule seeds were added to the 500 μ l aliquots of asynchronous or mitotic extracts (10 μ M taxol final) to initiate binding. Fifteen minutes after addition of microtubules, the binding reactions were spun in a microfuge (15 000 rpm, 8 min) to sediment microtubules. The pellet and supernatant fractions were separated. Minichromosome DNA from each fraction was isolated, subjected to agarose electrophoresis, and visualized by the method of Southern using probes homologous to the minichromosomes. The intensities of the bands corresponding to the minichromosomes in the supernatant and pellet fractions were determined using a computing densitometer. The percentage of minichromosome in each fraction was calculated by dividing the intensity of the band in the supernatant or pellet fraction by the sum of the intensities of the supernatant and pellet fractions.

Mitotic Stability of pDK416L, a Short Linear Chromosome, In Vivo and Microtubule-binding Activity of Its Centromeres In Vitro

The mitotic stability of pDK416L and pDK370 was calculated by measuring the fraction of 4513-216 cells that retain these plasmids after growth in selective medium and after growth for 7–9 generations in nonselective medium (Kingsbury and Koshland, 1991). To measure the microtubule-binding activity of the centromeres on pDK416L, two exponentially growing cultures, 4513-216 cells with YCp41 and 4513-216 cells with DK416L1, were prepared (Kingsbury and Koshland, 1991). Equal volumes of the two asynchronous cultures were mixed. Alternatively, nocodazole (15 μ g/ml final) was added to the mixed cultures. After 3 h, between 80–90% of the cells were synchronized in mitosis. From these mixed cultures, asynchronous and mitotic lysates were prepared that contained both minichromosomes (see above). The ability of minichromosomes to bind to microtubules was assayed over a range of microtubule concentrations using our standard microtubule-binding assay (see above). LEU2 and URA3 probes (Kingsbury and Koshland, 1991) were used to follow YCp41 and pDK416L respectively in the microtubule-bound (pellet) and unbound (supernatant) fractions.

Binding of Minichromosomes to Microtubules in the Presence of Exogenously Added CEN DNA

A 1-kb *Bam*HI-*Sal*I restriction fragment that contained the wild-type CEN6 was purified by standard methods from plasmid pDK381 (Kingsbury and Koshland, 1991). Increasing amounts (30, 10, 3, 1, 0.3, or 0 ng) of this restriction fragment were added to 500 μ l aliquots of a mitotic extract. After 10 min taxol and then taxol-stabilized microtubules (1.3 \times 10⁹ microtubules/ml final) were added to each aliquot. The amount of minichromosome pDK370 bound to microtubules after a 15 min incubation was determined for each aliquot as described above. As part of this procedure, DNA is extracted from the supernatant and pellet fractions and then subjected to agarose electrophoresis (see above). Staining of the agarose gel with ethidium bromide revealed bands for the carrier ϕ X174 DNA and the competitor DNA. The position and intensity of the competitor DNA band were used to measure its stability in the extract. The number of competitor DNA molecules in each binding reaction was calculated from the initial amount added and fragment molecular weight. The number of minichromosomes in each binding reaction was determined from the number of cell equivalents and the calculated yield of minichromosomes from nocodazole arrested cells (Kingsbury and Koshland, 1991). The molar ratio of competitor DNA molecules to minichromosomes was then determined. To determine whether exogenous DNA was being sequestered by specific or nonspecific DNA binding proteins in the extract, exogenous pDK370 DNA (30 ng) was added to mitotic lysates containing the pDK370 minichromosome. After 10 min, the restriction enzyme DRA1 was added; sites for this enzyme existed in pDK370 DNA both within the centromere DNA and within vector sequences. The digestion of the exogenous pDK370 DNA was analyzed as was the digestion of the pDK370 DNA in the minichromosome isolated from yeast.

Centromere Binding to Microtubules as a Function of the Concentration of Microtubule End and Polymer

Long microtubules were grown by mixing 2 μ l of microtubule seeds to a 100 μ l of solution containing tubulin (4 mg/ml), 3.3 mM MgCl₂, and 1.3 mM GTP. After 45 min at 37°C, 15 μ l of 1 mM taxol was added. A 75- μ l aliquot of the microtubules was passed through a 25-gauge needle to shear the microtubules to a short length. Pipetmen were used for all subsequent manipulation of microtubules; the disposable tips were clipped near their ends to ensure that the long microtubules were not sheared during pipeting. The length and number of microtubules in the sheared and unsheared microtubule populations were determined as described previously (Koshland *et al.*, 1988). Mitotic extracts were prepared from 4513-216/pDK370 cells. Aliquots of the long or short microtubules were added to 500- μ l aliquots of the extracts. The percentage of pDK370 minichromosomes bound to microtubules in each binding reaction was determined by our standard microtubule-binding assay.

Effect of NTPs on Minichromosome Binding to Microtubules

Appropriate amounts of stock NTPs (100 mM stock in EBB, pH 7.4) were added to the lysates. After 15 min at 23°C, microtubules (1.5 \times 10⁹ Mt/ml final) were added to a mitotic lysates. Binding of minichromosomes to microtubules was monitored as described above. An aliquot of the lysate was removed just after the addition of the NTP and after separation of the supernatant and microtubule pellet fractions. These aliquots were immediately put into a mixture of phenol and water to kill all protein activity. The NTP concentration in the aqueous phase was determined using luciferase (Koshland *et al.*, 1988).

Centromere Binding to Microtubules with Increasing NaCl Concentration

NaCl concentration of an asynchronous extract was changed by adding increasing volumes of 5 M NaCl stock to 500 μ l aliquots of extract. Five minutes after the addition of the salt, microtubules were added to a final concentration of 2 \times 10⁹ Mt/ml. The binding reactions were incubated at 23°C for an additional 15 min. The percentage of minichromosomes bound to microtubules was determined as described above. To determine the stability of microtubules in lysates containing different salt concentrations, aliquots of the binding reactions containing 0.1, 0.2, and 0.3 M NaCl were diluted into the fixative EGS (100 mM in BRB80); microtubule length and number were quantitated as described previously (Koshland *et al.*, 1988).

Reactivation of Centromeres Inactivated by 0.2 M NaCl

Taxol-stabilized microtubules (1.5 \times 10⁹ Mt/ml final) were added to 500 μ l aliquots of a mitotic lysate. After 15 min binding reactions were spun to sediment microtubules. One binding reaction was processed as described above to measure the percentage of minichromosomes in the original lysate bound to microtubules. For the remaining samples all but 10 μ l of the supernatant was removed and discarded. Five hundred microliters of EBB with either 0.1 or 0.2 M NaCl was added to the pellet and remaining supernatant. The pellets were resuspended by pipeting the pellet and buffer up and down with a 1-ml pipetman. After 5 min the sample containing 0.2 M salt was separated into two 250- μ l aliquots. One aliquot received an equal volume of EBB with 0.2 M NaCl (keeping the NaCl concentration at 0.2 M); whereas the other got an equal volume of just EBB (reducing the NaCl concentration to a final of 0.1 M). Fresh taxol-stabilized bovine microtubules (3 \times 10⁹ Mt/ml final) were added to all aliquots. The binding reactions were incubated for 15 min, and then the percentage of minichromosome bound to microtubules was determined as described above.

Inactivation of Microtubule-binding Activity of Centromeres by Sedimentation of Microtubules Through Extracts in 0.2 M NaCl

Tubes containing 500- μ l aliquots of a mitotic lysate were brought to 0.2 M NaCl. Microtubules (1.5×10^9 Mt/ml final) were added to a subset of tubes. After 15 min at 23°C, all tubes were spun (15 000 rpm, 8 min). One-half of the supernatant was transferred from the original tubes to new tubes. The microtubule pellet and the remaining supernatant in the original tubes were pipeted up and down with a 1-ml pipetman multiple times to resuspend the pellets. EBB with or without NaCl was added to each tube to either reduce the NaCl concentration to 0.1 M or to maintain it at 0.2 M. Microtubules (1.5×10^9 Mt/ml final) were added to the appropriate subset of tubes. Fifteen minutes later, all tubes were spun (15 000 rpm, 8 min) for a second time. The percentage of minichromosome in the pellet and supernatant fractions was determined (see above).

RESULTS

Chromosome Topology and Centromere Function: Microtubule-binding Activity of Centromeres on Linear and Circular Minichromosomes

A yeast strain was constructed that contained the linear minichromosome pDK416L1 (Figure 1). The loss rate of pDK416L1 was 10 times the loss rate of circular minichromosomes with a wild-type centromeres (Figure 1). Similar results have been obtained with other linear and circular minichromosomes (Murray and Szostak, 1983). However, circular minichromosomes with certain CEN DNA mutations can exhibit loss rates almost as high as pDK416L1. These circular minichromosomes are significantly impaired in their binding to microtubules in our in vitro assay (Kingsbury and Koshland, 1991). Therefore, if the high loss of pDK416L1 was caused by a defect in its centromere's ability to bind microtubules, we should be able to detect this defect with our in vitro assay. With this in mind, mitotic and asynchronous lysates were prepared from cells harboring pDK416L1 (see MATERIALS AND METHODS). Increasing amounts of microtubules were added to these lysates, and the binding of the pDK416L1 minichromosomes to microtubules was assayed. The results were qualitatively similar to the binding observed previously for circular minichromosomes (Kingsbury and Koshland, 1991).

To obtain a more quantitative comparison of the binding of linear and circular minichromosomes, asynchronous and mitotic lysates were prepared from a mixture of cells containing the linear minichromosome pDK416L1 and the circular minichromosome YCp41 (Figure 1). In this way we ensured that the reaction conditions for measuring the binding of the two minichromosomes to microtubules were identical. In the mitotic lysate the percentages of YCp41 and pDK416L1 that bound to microtubules were indistinguishable (Figure 2A). Moreover, the percentage of pDK416L1 bound at saturating microtubule concentrations (~ 50 – 60%) was also similar to what we had previously reported for other URA3-based circular minichromosomes

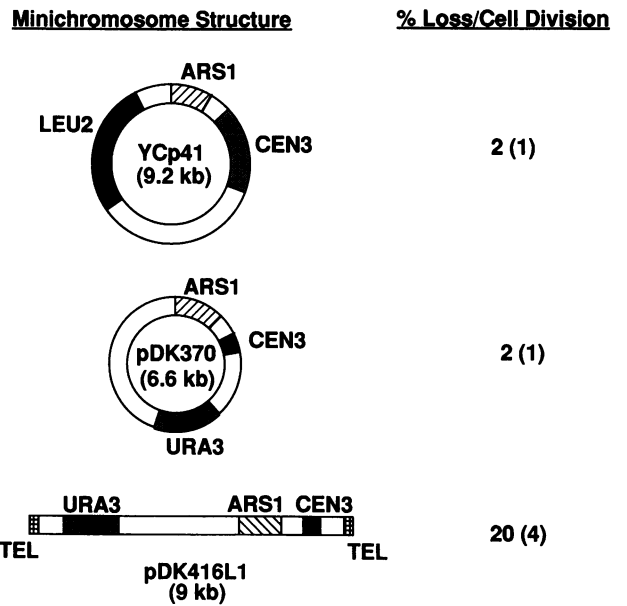


Figure 1. Minichromosomes used to assay microtubule-binding activity of yeast centromeres. Minichromosomes contain an origin of replication, ARS1, and a selectable marker, either LEU2 or URA3. The plasmid YCp41 (Clarke and Carbon, 1980) has a 1.6-kb fragment from chromosome III that contains the 117 bp of CEN3. The plasmid pDK370 (Kingsbury and Koshland, 1991) has a 580-bp fragment from chromosome III that contains the 117 bp of CEN3. The plasmid pDK416L1 is a linear derivative of pDK370 that includes *Tetrahymena* rDNA and yeast telomeres (TEL) (see MATERIALS AND METHODS). The mean mitotic loss rates for each minichromosome in the strain 4513-216 were determined (see MATERIALS AND METHODS). The mitotic loss rates are expressed as % Loss/Cell Division. Standard deviations are presented in the parentheses.

that were structurally identical to pDK416L1 except they lacked telomeres (Kingsbury and Koshland, 1991). These results suggested that the efficient assembly and function of the yeast centromere as measured by microtubule binding was independent of the topology of chromosomal DNA and the proximity of the centromere to telomeres. A caveat to this conclusion was that the centromeres of the linear chromosomes might have acquired the ability to bind microtubules in vitro. However, when linear or circular DNA molecules harboring CEN DNA were added to our extracts, they failed to bind microtubules (see below, Kingsbury and Koshland, 1991). These results suggested that centromeres with microtubule-binding activity could not assemble in our extracts; therefore, the microtubule-binding activity of the YCp41 and pDK416L1 minichromosomes presumably resulted from the assembly of their centromeres in vivo.

The percentage of both the linear and circular minichromosomes bound to microtubules was three- to fivefold greater in the mitotic lysate than in the asynchronous lysate (Figure 2, A and B). This result suggested that the microtubule-binding activity for centromeres

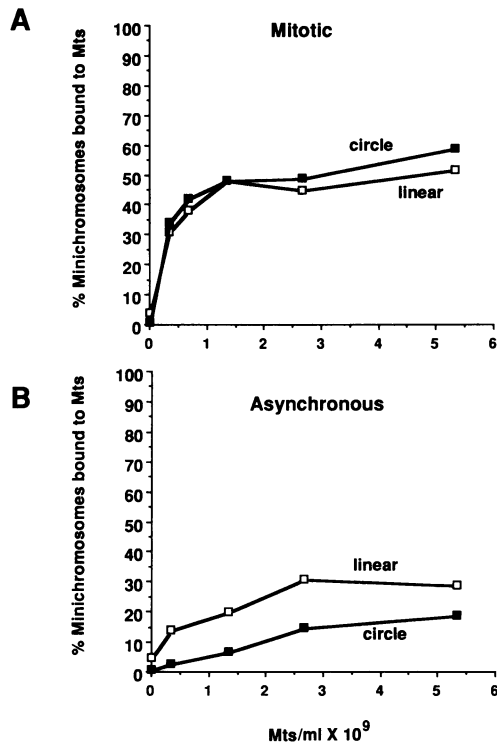


Figure 2. Binding of linear and circular minichromosome to microtubules. Cleared lysates were prepared from a mixture of cells containing the linear and circular minichromosomes pDK416L1 and YCp41 respectively (see MATERIALS AND METHODS). Increasing amounts of taxol stabilized bovine microtubules were added to a mitotic lysate (top) or an asynchronous lysate (bottom), and the percentage of minichromosomes bound to microtubules was determined from the fraction of minichromosomes that co-sedimented with microtubules (see MATERIALS AND METHODS).

on linear chromosomes was induced in mitosis as we had suggested previously for circular minichromosomes (Kingsbury and Koshland, 1991). However, in the asynchronous lysate, a greater percentage of linear minichromosomes was capable of binding microtubules than circular minichromosomes (Figure 2B). This difference may have reflected that dividing cells with linear minichromosomes are more likely to be in mitosis and hence have activated centromeres (see DISCUSSION).

Interaction of CEN DNA and Centromere DNA Binding Factors

Yeast centromere DNA is unable to bind microtubules directly (Kingsbury and Koshland, 1991; Hyman *et al.*, 1992). Moreover, the ability of minichromosomes in our lysates to bind microtubules can be inactivated by trypsin. A purified fraction of yeast proteins can bind to CEN DNA (Lechner and Carbon, 1991), and the complex is capable of binding to and moving along microtubules (Hyman *et al.*, 1992). These observations suggest

proteins assemble on centromere DNA, and this protein-DNA complex binds microtubules.

We assessed the stability of the protein-DNA interactions of the centromere by an approach originally developed to study the stability of transcription complexes (Bogenhagen *et al.*, 1982). Lysates were preincubated with increasing amounts of a 1.0-kb restriction fragment harboring CEN6 DNA; this CEN6 DNA was competent to bind centromere factors *in vivo* and *in vitro* as evidenced by its ability to promote normal minichromosome segregation *in vivo* and normal microtubule binding *in vitro* (Kingsbury and Koshland, 1991). If the factors bound to the CEN DNA of the minichromosome were undergoing cycles of association and disassociation, then the CEN DNA on the restriction fragment should have competed with the minichromosome CEN DNA for the binding of these factors. This competition should have consequently disrupted binding of the minichromosome to microtubules. In contrast, if the proteins bound to the minichromosome CEN DNA formed a stable complex, then the addition of CEN6 DNA to the extract should have had no effect on minichromosome binding to microtubules. In fact, the presence of CEN6 DNA did not alter the binding of minichromosomes to microtubules even when it was in 2000 molar excess to minichromosomes (Figure 3A). The exposure of the minichromosomes to CEN6 DNA lasted ≥ 25 minutes (a 10-min preincubation period followed by 15 min during the binding reaction). These results suggested that the complex between the minichromosome's CEN DNA and centromere factors was stable for ≥ 25 min.

The validity of our conclusions from these competition experiments requires that the number of exogenous CEN DNA molecules vastly exceeded the number of free centromere proteins in the lysate. To determine whether this was the case, we analyzed the stability of the exogenous CEN DNA and pool size of the CEN DNA binding proteins in the lysate. As no significant degradation of the exogenous CEN DNA was observed after its addition to the lysate (Figure 3B), this DNA was stable in the lysate. It had been shown previously that naked CEN3 DNA was digested readily with *Dra I*, whereas the CEN3 DNA of centromeres assembled *in vivo* was very resistant to digestion by this enzyme (Saunders *et al.*, 1988). Similarly, in our lysate $>95\%$ of the pDK370 DNA associated with the minichromosomes was resistant to *Dra I* digestion, whereas 95% of the exogenous pDK370 DNA was digested. This result suggested that centromere factors remained bound to the CEN DNA on the minichromosome and that the pool size of free centromere factors was insufficient to bind and protect significant amounts of exogenous CEN DNA added to the lysate. In support of this conclusion, the exogenous CEN DNA did not acquire centromere function as evidenced by the fact that $<5\%$ of the re-

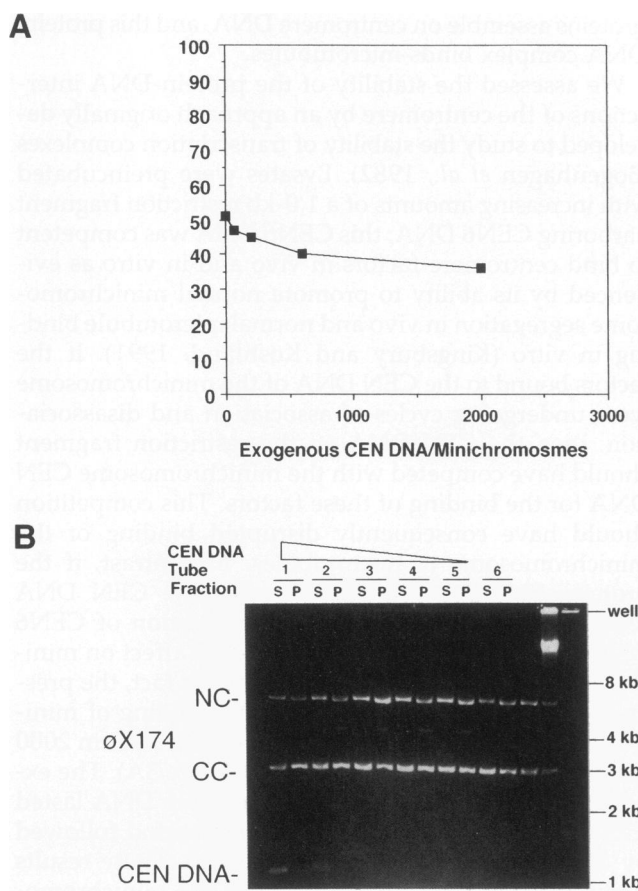


Figure 3. Binding of minichromosomes to microtubules in the presence of exogenously added CEN DNA. Mitotic lysates were prepared from the strain 4513-216 containing the minichromosome pDK370. Increasing amounts of a 1-kb restriction fragment that contained the centromere DNA from chromosome VI (CEN6) was added to aliquots of the lysate. After 15 min a fixed number of microtubules was added to each aliquot and then the percentage of pDK370 minichromosome bound to microtubules was assayed (see MATERIALS AND METHODS). (A) The percentage of pDK370 minichromosome bound to microtubules in the presence of increasing molar amounts of exogenous CEN6 DNA (see MATERIALS AND METHODS). (B) Six microtubule-binding reactions received 30, 10, 3, 1, 0.3, or 0 ng of CEN6 DNA (tubes 1–6 respectively). DNA was extracted from supernatant (S) and microtubule pellet (P) fractions of each binding assay. DNA was subjected to agarose electrophoresis and then visualized with EtBr. The positions of migration of exogenous CEN6 DNA and the different forms of ϕ X174 are indicated. Note the ϕ X174 DNA is added to the supernatant and pellet fractions before isolation of DNA as a means to assess how quantitative is the recovery of DNA by the extraction procedure. The faint band at around 4 kb is killer RNA. The majority of killer RNA was recovered in the supernatant but is degraded during RNase treatment of that fraction (see MATERIALS AND METHODS).

striction fragment molecules sedimented with microtubules (Figure 3B).

Microtubule Polymer Determines the Efficiency of Centromere Binding to Microtubules

We asked whether the efficiency of centromeres binding to microtubules was dependent upon the concentration

of microtubule polymer or ends. To address this question we made two populations of microtubules, sheared and unsheared, in which polymer concentration was fixed but microtubule end concentration varied from 4- to 20-fold (see MATERIALS AND METHODS). We then added equivalent volumes of these microtubules to mitotic lysates and measured the percentage of minichromosomes that bound to them. The results from two different experiments are presented in Figure 4. In each case the percentage of minichromosomes bound to microtubules was the same for sheared and unsheared microtubules when the binding was plotted as a function of polymer concentration. These results suggested that efficiency of minichromosome binding to microtubules depended upon the polymer concentration and not the end concentration.

Mitotic lysates from *Xenopus* contain a microtubule-severing activity (Vale, 1992). Therefore, in our experiments the number of ends for the sheared and unsheared microtubules might have changed after addition to the mitotic lysate. To address this caveat, long microtubules were assembled in buffer, and an aliquot of these microtubules was added to a mitotic lysate. Subsequently, the microtubules were fixed and visualized by indirect immunofluorescence. The average lengths of the microtubules were 31 μ m (\pm 11) in buffer and 38 μ m (\pm 15) in the lysate. The average numbers of microtubules/ml were 2.5×10^9 ml in buffer and 2.8×10^9

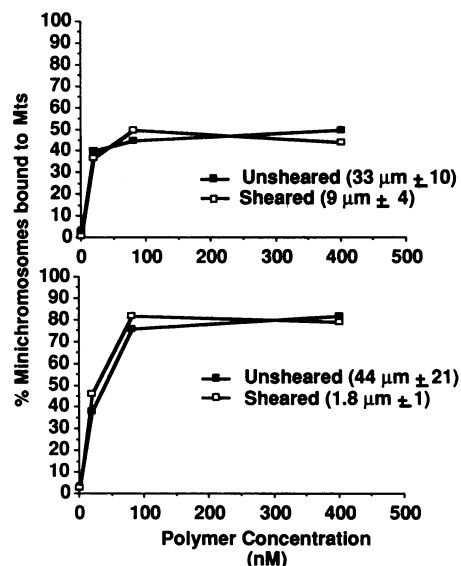


Figure 4. Centromere binding to Mts varying the concentration of Mt polymer and ends. Equal volumes of the sheared and unsheared microtubules were added to a lysate and the percentage of pDK370 minichromosomes that bound to microtubules was determined (see MATERIALS AND METHODS). Results from two different experiments are presented. The mean lengths and the standard errors in the length measurements of the sheared and unsheared microtubules are indicated.

in the lysate. Therefore, the length and number of microtubules were not affected significantly by the mitotic lysate.

Inhibition of the Microtubule-binding Activity of Centromeres by NTPs

In the presence of 1 mM ATP, the mechanochemical-microtubule motor in the CBF3 fraction briefly translocates along microtubules and then disassociates from them (Hyman *et al.*, 1992). However, we showed that the addition of 1 mM ATP to lysates had no effect on the binding of minichromosomes to microtubules, suggesting that *in vivo*-assembled centromeres bound to microtubules in an ATP independent fashion (Kingsbury and Koshland, 1991). To investigate further this apparent discrepancy we reinvestigated the effect of NTPs on the ability of *in vivo*-assembled centromeres to bind microtubules (see MATERIALS AND METHODS). In fact higher concentrations of ATP did inhibit binding (Figure 5A). At a concentration of 20 mM, CTP and ATP gamma-S inhibited binding to 1–5% like ATP. However UTP was a significantly poorer inhibitor indicating that the amount of inhibition was nucleotide specific (Figure 5B). The high ATP concentration required for inhibition may have been an artifact of its degradation or sequestration in the lysate. However, free ATP concentration was measured at the time of addition and at the end of the binding reaction (see MATERIALS AND METHODS) and found to vary <10%. Therefore, ATP and other NTPs could inhibit binding of microtubules to *in vivo*-assembled centromeres in the extract, but the apparent ATP concentration required for this inhibition was greater than the ATP concentration required to disassociate reconstituted centromeres from microtubules (Hyman *et al.*, 1992).

Interaction Between Centromere Factors: Inactivation and Reactivation of Minichromosome Binding to Microtubules by Varying NaCl Concentration

To determine whether we could disassemble centromeres *in vitro*, we altered the reaction conditions for the binding of minichromosomes to microtubules. Neither the addition of Ca^{2+} (1 mM final) nor ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid EGTA (3 mM final) to the lysate affected binding. We also varied the concentration of NaCl. As all previous binding experiments had been done with lysates that contained 0.1 M NaCl (Kingsbury and Koshland, 1991), we tried increasing the NaCl concentration of the lysate (see MATERIALS AND METHODS). When NaCl concentration was increased to 0.2 M or greater (Figure 6A), the amount of minichromosomes bound to microtubules was reduced 5- to 10-fold compared to the amount bound in 0.1 M NaCl. In contrast, the stability of microtubules in the lysates was unaffected by in-

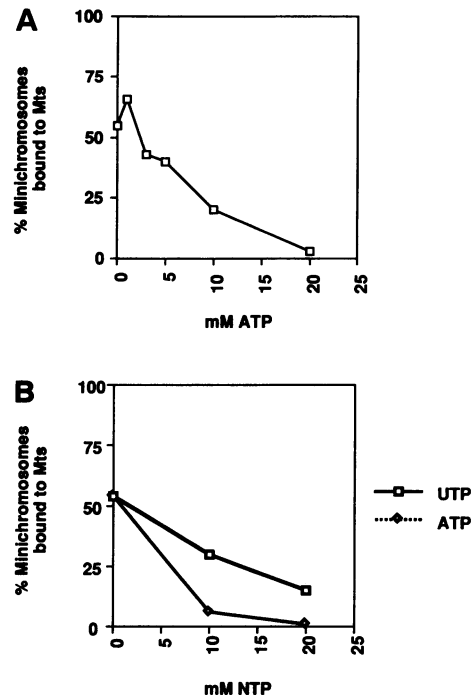


Figure 5. Centromere binding to Mts in the presence of NTPs. A mitotic lysate was prepared from cells harboring the plasmid pDK370. Increasing amounts of ATP (A and B) or UTP were added to aliquots of the lysate. After 5 min a constant amount of microtubules was added to each aliquot and binding of the minichromosome to microtubules was measured (see MATERIALS AND METHODS).

creasing NaCl (Figure 6A). Thus the inhibition of minichromosome binding to microtubules in 0.2 M NaCl presumably reflected the disruption of the centromere structure.

We tested whether centromeres inactivated by 0.2 M NaCl could be reactivated by lowering the NaCl concentration (see MATERIALS AND METHODS). Minichromosomes in a standard 0.1 M lysate were bound to microtubules, and the complexes were sedimented. The complexes were resuspended in buffer containing either 0.1 or 0.2 M NaCl, and the microtubules were resedimented. In 0.1 M NaCl, the percentage of minichromosomes that resedimented with microtubules (87%) (Figure 6B) was even greater than the percentage that sedimented with microtubules from the original lysate (53%). This result is consistent with the enrichment for minichromosomes in the lysate with active centromeres during the first sedimentation of microtubules. When the complexes were resuspended in 0.2 M NaCl, only 3–5% of the minichromosomes resedimented with microtubules (Figure 6B). This result corroborated our previous observation that centromere binding to microtubules was inhibited by 0.2 M NaCl. However, when the salt concentration was shifted from 0.2 M back to 0.1 M, now ~40% of the minichromosomes resedimented with microtubules (Figure 6B). This

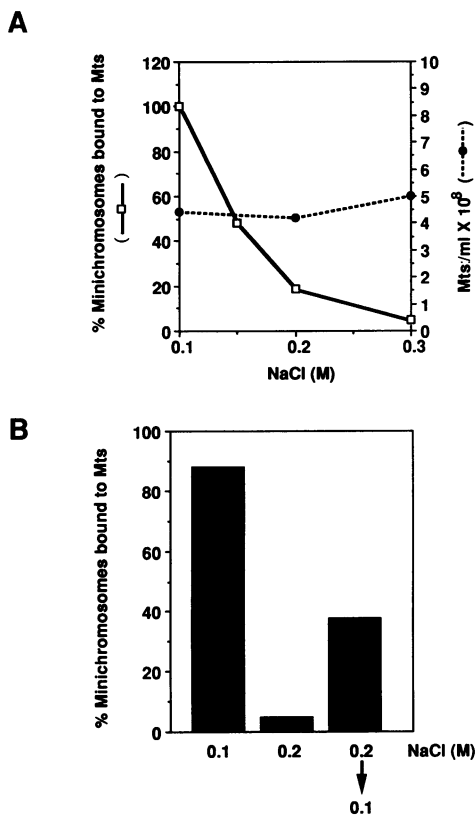


Figure 6. Inactivation and reactivation of the binding of minichromosomes to microtubules by changing the NaCl concentration. (A) Asynchronous lysates were prepared from cells harboring pDK370 (see MATERIALS AND METHODS). Initial buffer contained 0.1 M NaCl. Increasing amounts of NaCl were added to aliquots of the lysate to increase the NaCl concentration. After 10 min a constant amount of microtubules were added to each aliquot and the binding of minichromosomes to microtubules was measured. The amount of binding in each aliquot was normalized to the binding observed under standard binding conditions (0.1 M NaCl). (B) Mitotic lysates were prepared from cells with pDK370. Pellets containing microtubules and bound minichromosomes were resuspended in either 0.1 or 0.2 M NaCl. After 5 min the sample containing 0.2 M NaCl was split in two and each half was diluted twofold with buffer containing 0.2 M NaCl (leaving the NaCl concentration unchanged) or no NaCl (bringing the final NaCl concentration to 0.1 M). The microtubules were then resedimented and the percentage of the minichromosomes that bound to Mt was measured.

amount of binding represented a 13-fold increase relative to complexes that remained in 0.2 M NaCl. This amount of binding was also only 2-fold less than the amount observed for complexes that never experienced 0.2 M NaCl. These results suggest that ~50% of the centromeres that were inactivated for microtubule-binding by 0.2 M NaCl could be reactivated by returning the salt concentration to 0.1 M NaCl.

To investigate further the reactivation of salt-inactivated centromeres, we carried out a series of experiments outlined in Figure 7. A lysate was made in which ~60% of the minichromosomes were capable of bind-

ing microtubules. The lysates were brought to 0.2 M NaCl to inactivate microtubule binding of these minichromosomes. When the lysate containing 0.2 M NaCl was centrifuged in the absence of microtubules, all of the minichromosomes remained in the supernatant. Thirtytwo percent of the minichromosomes in the supernatant could be sedimented during a second centrifugation (Figure 7, 1) as long as the NaCl concentration was restored to 0.1 M and microtubules were added (Figure 7, 2, and 3); thus ~50% of the minichromosomes' centromeres that were initially active could be reactivated. The similarity of these results with our previous results suggested that the centrifugation of the lysate while in 0.2 M salt had no effect on subsequent reactivation of centromere function. When the lysate in 0.2 M NaCl was spun in the presence of microtubules, the majority of the minichromosomes remained in the supernatant. However, after the salt concentration of the supernatant fraction was returned to 0.1 M, only 7% of the minichromosomes in the supernatant fraction could be reactivated to bind newly-added microtubules (Figure 7, 4). This result suggested that centrifugation of a lysate containing both 0.2 M salt and microtubules inhibited subsequent reactivation of centromere function four- to fivefold.

To explain the latter observation, we proposed the following hypothesis. While in high salt, the active form of a centromere factor(s) sedimented with the microtubules rather than remaining in the supernatant with the minichromosomes. Because most minichromosomes in the supernatant lacked this factor, they could not bind newly added microtubules. If this model were correct, minichromosomes in the supernatant might be competent to bind the "conditioned" microtubules (Mts*, Figure 7) that had passed through the lysate while in high salt. In fact, minichromosomes in the supernatant fraction sedimented with the conditioned microtubules four times better than to newly added microtubules (Figure 7, 4 and 5). Furthermore, the absolute amount of reactivation with these conditioned microtubules (Figure 7, 5) was comparable to what we had observed when no microtubules were added to lysates in 0.2 M NaCl (Figure 7, 1). These results supported the hypothesis that during the exposure of lysates to 0.2 M NaCl and microtubules, a factor necessary for the minichromosome's centromere to bind microtubules was bound to microtubules.

Disruption of centromere function by 0.2 M NaCl might have occurred by reducing the affinity of the putative centromere protein complex for CEN DNA; centromere reactivation would occur by the reassembly of this complex onto the CEN DNA. If so, the addition of CEN6 DNA to the lysate in 0.2 M NaCl might have inhibited subsequent reactivation of the minichromosome's centromere in 0.1 M NaCl by competing for the binding of the centromere factors. In fact salt-inactivated minichromosomes reactivated to bind microtubules with

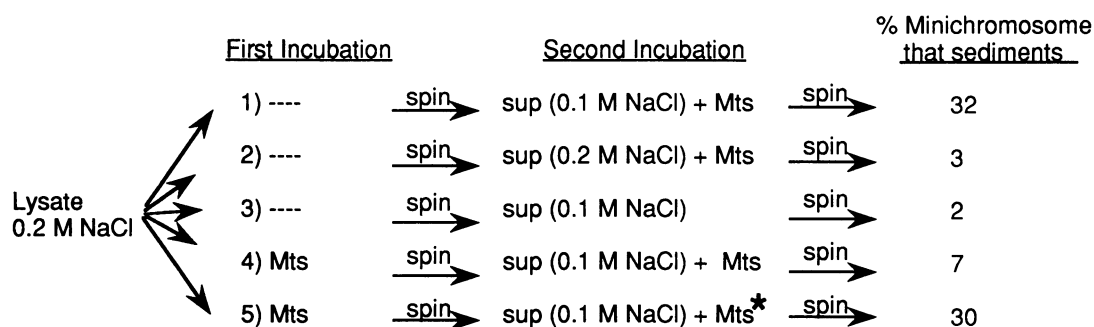


Figure 7. Reactivation of centromeres after inactivation in the presence or absence of microtubules. Mitotic lysates were prepared from cells with pDK370. The lysate was brought to 0.2 M NaCl. Aliquots (1, 2, and 3) were centrifuged and the supernatant (sup) fractions were brought to 0.1 M NaCl (1 and 3) or maintained at 0.2 M NaCl. Microtubules were (1 and 2) or were not (3) added to the aliquots and then the aliquots were centrifuged for a second time. The percentage of minichromosomes that sedimented was determined (see MATERIALS AND METHODS). Alternatively, aliquots of the lysate in 0.2 M NaCl were centrifuged in the presence of microtubules (4 and 5). The supernatant fractions were then restored to 0.1 M NaCl. Fresh microtubules, Mts (4), or the microtubules that passed through the lysate whereas at 0.2 M NaCl, Mts* (5), were added. The aliquots were spun for a second time and the percentage of minichromosomes that sedimented was determined.

the same efficiency in the presence or absence of CEN6 DNA. These results were consistent with the idea that the centromere proteins bound directly to minichromosome's CEN DNA did not disassociate in 0.2 M NaCl. Taken together these data suggested that 0.2 M salt inhibited minichromosome binding to microtubules in a way that left some centromere factor(s) bound to the CEN DNA and other(s) bound to microtubules.

DISCUSSION

Centromere Function on Linear Minichromosomes

The high mitotic loss of linear minichromosomes compared to circular minichromosomes or large linear chromosomes indicates that chromosome structure plays an important role in chromosome transmission. One could imagine that chromosome structure plays a role in centromere function. For example, efficient assembly of the centromere may require that the CEN DNA be part of a superhelical domain in which only large linear or circular chromosomes can form. However, in this study, we showed that linear and circular minichromosomes isolated from mitotic cells were equally proficient to bind microtubules *in vitro*. This result suggests that *in vivo* a specific topology of chromosomal DNA is not required for the efficient assembly of centromeres with microtubule-binding function. In support of this conclusion, rearrangements of dicentric linear minichromosomes are detected *in vivo*, suggesting that centromeres on linear minichromosomes must at least occasionally bind to the mitotic apparatus (Murray *et al.*, 1986). Chromosome topology may be important for other potential centromere functions that we can not currently assay (like sister chromatid cohesion or bipolar attachment to the spindle), and the high mitotic loss of linear minichro-

somes may reflect impairment of one of these functions.

Transcription of sequences 850 bp from chromosomal telomeres is repressed by as much as 30% (Gottschling *et al.*, 1990). In this study, the centromere on the linear minichromosome is <550 bp away from the telomere, yet its microtubule-binding activity is indistinguishable from the centromeres on circular minichromosomes. Therefore, the assembly and function of the microtubule-binding component of the centromere may be refractive to the mechanism by which telomeres repress transcription. This difference may reflect a hierarchy of assembly of centromeres, telomeres, and transcription complexes during DNA replication. It will be interesting to pursue this possibility by a quantitative comparison of the activities of centromeres and promoters under conditions where their spacing from the telomere and chromosomal context are carefully controlled.

The percentage of circular minichromosomes bound to microtubules increased dramatically in lysates made from mitotic cells compared to those made from asynchronous or G1 cells, suggesting that centromere function is regulated during the cell cycle (Kingsbury and Koshland, 1991). In this study, we observed that the percentage of linear minichromosomes bound to microtubules was several fold greater in lysates made from mitotic cultures compared with those made from asynchronous cultures. This result suggests that the centromeres on linear minichromosomes also are activated in mitosis. In lysates made from asynchronous cultures, the fraction of minichromosomes with active centromeres was greater for linear minichromosomes than circular ones (this study). A comparison of asynchronous cultures showed that the fraction of large budded cells (potential mitotic cells) within a culture increased when the cells contained linear minichromosomes (Murray,

1991). This correlation between the percentage of mitotic cells in an asynchronous culture and in vitro centromere function supports the model of the mitotic induction of centromere function.

The Role of Microtubule Polymer and NTPs in the Binding of Centromeres to Microtubules

In this study we showed that centromere-mediated binding of minichromosomes to microtubules was equally efficient when the microtubule-polymer concentration remained constant but the microtubule-end concentration varied as much as 20-fold. These results strongly suggest that binding efficiency is determined by polymer and not end concentration. Beads coated with yeast CEN DNA and the CBF3 fraction bind to the lateral surface of microtubules (Hyman *et al.*, 1992). Therefore, our results with in vivo-assembled centromeres are in agreement with results obtained with partially reconstituted centromeres. This polymer dependence may greatly facilitate association of centromeres with the microtubules of the spindle in vivo because polymer concentration is much higher than end concentration inside any eukaryotic cell. Our results do not exclude the possibility that once centromeres bind anywhere along the length of the microtubule, they migrate to one of the microtubule's ends where they remain stably attached. This type of behavior has been observed with centromeres of larger eukaryotes (Rieder *et al.*, 1990). Although the reconstituted yeast centromeres did not show any stable association with microtubule ends (Hyman *et al.*, 1992), it is possible that these centromeres lack important factors. The analysis of the position of our minichromosomes on microtubules may help to resolve this issue.

The polymer dependence of the microtubule-binding activity of yeast centromeres is reminiscent of mechanochemical microtubule motors. Interestingly, the binding of centromeres to microtubules can be inhibited by 10–20 mM ATP, CTP, and ATP- γ -S (this study). Given that the NTP concentration must be high to see an inhibitory effect, it is possible that the inhibition is not physiological. However, the binding of some mechanochemical motors to microtubules is inhibited by a spectrum of NTPs (Shimizu *et al.*, 1991), and in at least one case the NTP concentration must be in the 10–20 mM range (Yen *et al.*, 1992). Therefore our observations with NTP inhibition are consistent with the notion that the microtubule-binding activity associated with the centromeres of isolated minichromosomes may be mediated by a mechanochemical motor.

In fact, mechanochemical motors have been implicated in mediating the binding of microtubules to in vivo-assembled centromeres in larger eukaryotic cells (Mitchison and Kirschner, 1985; Plarr *et al.*, 1990; Steuer *et al.*, 1990; Hyman and Mitchison, 1991; Yen *et al.*, 1992) and reconstituted centromeres from yeast (Hyman

et al., 1992). However, the difference in ATP concentration required to disassociate the minichromosomes' centromeres (10–20 mM) (this study) and reconstituted centromeres (1 mM) (Hyman *et al.*, 1992) suggests that the putative motor on isolated minichromosomes may have a much higher affinity for microtubules than the motor associated with reconstituted centromeres. Or, the affinity of centromeres assembled in vivo for microtubules may reflect the binding by a mechanochemical motor and other microtubule-binding components yet to be identified. We note that a high affinity of centromeres for microtubules is expected in vivo. Anaphase in yeast lasts 20 min, and centromeres bind to one or two microtubules at most. If chromosomes are to segregate properly, they must remain bound to these microtubules for most of anaphase, i.e., for ≥ 10 –20 min (Palmer *et al.*, 1989). Interestingly, one newly identified kinesin-like molecule that is associated with the mammalian centromere requires high levels of ATP to be disassociated from microtubules in vitro (Yen *et al.*, 1992).

Stability and Reactivation of Centromeres In Vitro

In this study we observed that the centromere's ability to bind microtubules could be inactivated by raising the salt concentration to 0.2 M and then partially reactivated by lowering the salt concentration to 0.1 M. When lysates were exposed transiently to microtubules and 0.2 M salt, the minichromosomes in the lysate reactivated to bind these conditioned microtubules much more efficiently than to naive microtubules. From these observations we propose that when centromeres are inactivated by 0.2 M salt in the presence of microtubules, the active form of a centromere factor(s) necessary for microtubule binding associates with microtubules. We also observed that the addition of centromere DNA to the lysates in 0.1 M salt did not inhibit the minichromosomes' centromeres from binding to microtubules. Furthermore, the addition of CEN DNA to lysates in 0.2 M salt did not prevent the reactivation of the minichromosomes' centromeres. These observations suggest that some centromere proteins bind to CEN DNA to form an extremely stable core even in 0.2 M salt.

A simple and attractive model to explain these observations is that the centromere consists of two domains whose interaction can be influenced in vitro by salt (Figure 8). The first domain is formed by a stable core found between CEN DNA and CEN DNA binding proteins (Figure 8, ■). The presence of this core is supported by other in vitro and in vivo observations. Proteins in yeast lysates that bind to CEN DNA were monitored by a band shift assay (Ng and Carbon, 1987). In these studies complexes formed between the putative CEN proteins and CEN DNA were stable to the subsequent addition of molar excess of CEN DNA. Furthermore, the analysis of yeast chromatin structure in

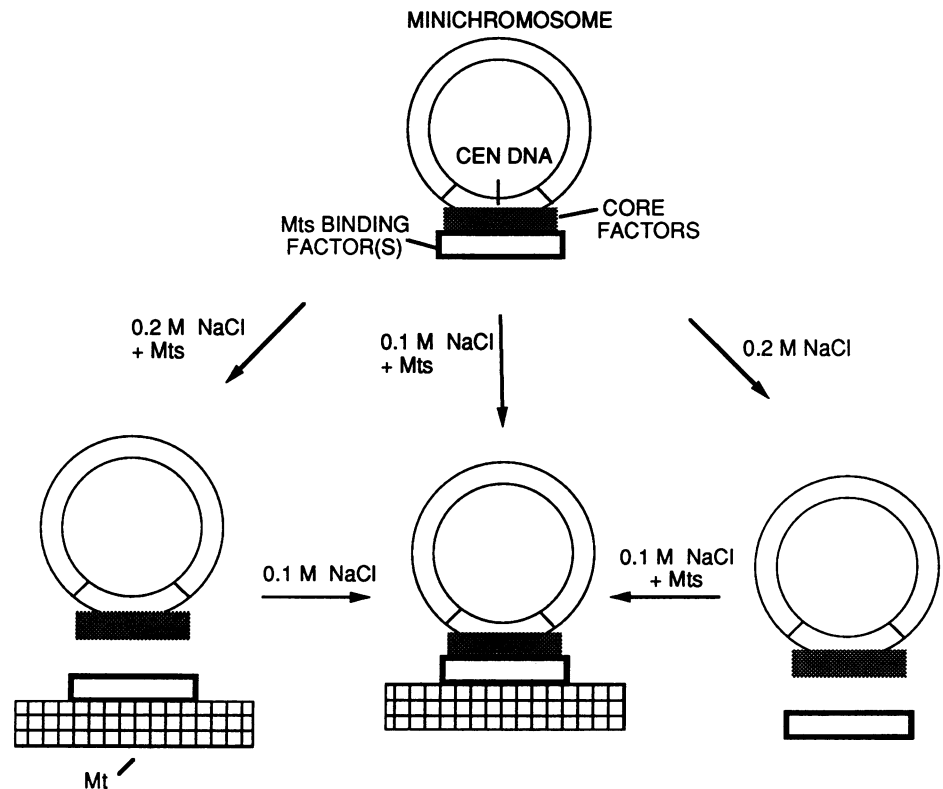


Figure 8. Working model for the structure and function of yeast centromeres in vitro. A schematic drawing summarizes our conclusions on centromere-mediated binding of minichromosomes to microtubules (see text) in the presence of 0.1 or 0.2 M NaCl.

vivo suggests that the CEN DNA is complexed with proteins throughout the cell cycle (K. Bloom, personal communication).

The second domain (Figure 8, □) has a microtubule-binding activity that associates reversibly with the centromere. One possibility is that the microtubule-binding component in this domain is a mechanochemical motor (this study; Hyman *et al.*, 1992). Alternatively, this domain may never be an intricate part of the centromere. Rather, it may be a soluble factor in the extract that associates with microtubules and facilitates their binding to centromeres much like enhancers are thought to bring transcription complexes to promoters. Although we cannot exclude this latter model, several observations are consistent with the second domain being a component of the centromere, perhaps a mechanochemical motor. Cytoplasmic dynein has been shown to associate with the kinetochore and spindle microtubules in larger eukaryotic cells (Schroer *et al.*, 1989; Plarr *et al.*, 1990) and can be disassociated from the kinetochore in vitro by 0.15 M salt (Wordeman *et al.*, 1991). In addition CENPE, a kinesin-like molecule has been shown to reversibly associate with kinetochores in vivo; upon disassociation from the kinetochore, it appears bound to microtubules (Compton *et al.*, 1991; Yen *et al.*, 1992). Thus, the association of these mechanochemical motors with the kinetochore and microtubules is strongly reminiscent of the microtubule-binding component observed in this study.

In conclusion, in our in vitro system we can apparently modulate the affinity of a microtubule-binding component to the centromere core by changing the salt concentration. Although this is unlikely to be physiological, it is possible that in vivo the association of the microtubule-binding component with the core is regulated by some other mechanism (e.g., phosphorylation) and that this regulated assembly is important for the control of centromere function. Currently we are purifying the microtubule-binding component in this second domain to address its molecular function and to determine whether its binding to centromeres is regulated.

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REFERENCES

Bogenhagen, D.F., Wormington, W.M., and Brown, D.D. (1982). Stable transcription complexes of *Xenopus* 5S RNA genes: a means to maintain the differentiated state. *Cell* 28, 413-421.

- Clarke, L., and Carbon, J. (1980). Isolation of a yeast centromere and construction of functional small circular chromosomes. *Nature* 287, 504–509.
- Compton, D.A., Yen, T.J., and Cleveland, D.W. (1991). Identification of novel centromere/kinetochore-associated proteins using monoclonal antibodies generated against human mitotic chromosome scaffolds. *J. Cell Biol.* 112, 1083–1097.
- Cumberledge, S., and Carbon, J. (1987). Mutational analysis of meiotic and mitotic centromere function in *Saccharomyces cerevisiae*. *Genetics* 117, 203–212.
- Doheny, K.F., Sorger, P.K., Hyman, A.A., Tugendreich, S., Spencer, F., and Hieter, P. (1993). Identification of essential components of the *Saccharomyces cerevisiae* kinetochore. *Cell* 73, 761–774.
- Fitzgerald-Hayes, M., Buhler, J.-M., Cooper, T.G., and Carbon, J. (1982a). Isolation and subcloning analysis of functional centromere DNA (CEN11) from *Saccharomyces cerevisiae* chromosome XI. *Mol. Cell Biol.* 2, 82–87.
- Fitzgerald-Hayes, M., Clarke, L., and Carbon, J. (1982b). Nucleotide sequence comparisons and functional analysis of yeast centromere DNAs. *Cell* 29, 235–244.
- Gaudet, A., and Fitzgerald-Hayes, M. (1987). Alterations in the adenine-plus-thymine-rich region of CEN3 affect centromere function in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 7, 68–75.
- Goh, P.-Y., and Kilmartin, J. (1993). *NDC10*: a gene involved in chromosome segregation in *S. cerevisiae*. *J. Cell Biol.* 121, 503–512.
- Gottschling, D.E., Aparicio, O.M., Billington, B.L., and Zakian, V.A. (1990). Position effect at *S. cerevisiae* telomeres: reversible repression of Pol II transcription. *Cell* 63, 751–762.
- Hegemann, J.H., Shero, J.H., Cottarel, G., Philippsen, P., and Hieter, P. (1988). Mutational analysis of the centromere DNA from chromosome VI of *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 8, 2523–2528.
- Hieter, P., Pridmore, D., Hegemann, J.H., Thomas, M., Davis, R.W., and Philippsen, P. (1985). Functional selection and analysis of yeast centromeric DNA. *Cell* 42, 913–921.
- Hyman, A.A., Middleton, K., Centola, M., Mitchison, T.J., and Carbon, J. (1992). Microtubule-motor activity of a yeast centromere-binding protein complex. *Nature* 359, 533–536.
- Hyman, A.A., and Mitchison, T.J. (1991). Two different microtubule-based motor activities with opposite polarities in kinetochores. *Nature* 351, 206–211.
- Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* 153, 163–168.
- Kingsbury, J., and Koshland, D. (1991). Centromere-dependent binding of yeast minichromosomes to microtubules in vitro. *Cell* 66, 483–495.
- Koshland, D.E., Mitchison, T.J., and Kirschner, M.W. (1988). Polewards chromosome movement driven by microtubule depolymerization in vitro. *Nature* 331, 499–504.
- Lechner, J., and Carbon, J. (1991). A 240 kd multisubunit protein complex, CBF3, is a major component of the budding yeast centromere. *Cell* 64, 717–725.
- McGrew, J., Diehl, B., and Fitzgerald-Hayes, M. (1986). Single base-pair mutations in centromere element III cause aberrant chromosome segregation in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 6, 530–538.
- Mitchison, T.J., and Kirschner, M.W. (1985). Properties of the kinetochore in vitro. II. Microtubule capture and ATP-dependent translocation. *J. Cell Biol.* 101, 766–776.
- Murray, A.W. (1991). Coordinating cell cycle events. *Cold Spring Harbor Symp. Quant. Biol.* 56, 399–408.
- Murray, A.W., Schultes, N.P., and Szostak, J.W. (1986). Chromosome length controls mitotic chromosome segregation in yeast. *Cell* 45, 529–536.
- Murray, A.W., and Szostak, J.W. (1983). Construction of artificial chromosomes in yeast. *Nature* 305, 189–193.
- Ng, R., and Carbon, J. (1987). Mutational and in vitro protein-binding studies on centromere DNA from *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 7, 4522–4534.
- Palmer, R.E., Koval, M., and Koshland, D. (1989). The dynamics of chromosome movement in the budding yeast *Saccharomyces cerevisiae*. *J. Cell Biol.* 109, 3355–3366.
- Plarr, C.M., Coue, M., Grisson, P.M., Hays, T.S., Porter, M.E., and McIntosh, J.R. (1990). Cytoplasmic dynein is localized to kinetochores during mitosis. *Nature* 345, 263–266.
- Rieder, C.L., Alexander, S.P., and Rupp, G. (1990). Kinetochores are transported poleward along a single astral microtubule during chromosome attachment to the spindle in newt lung cells. *J. Cell Biol.* 110, 81–95.
- Saunders, M., Fitzgerald-Hayes, M., and Bloom, K. (1988). Chromatin structure of altered yeast centromeres. *Proc. Natl. Acad. Sci. USA* 85, 175–179.
- Schroer, T.A., Steuer, E.R., and Sheetz, M.P. (1989). Cytoplasmic dynein is a minus end-directed motor for membranous organelles. *Cell* 56, 937–946.
- Shimizu, T., Furusawa, K., Ohashi, S., Toyoshima, Y.Y., Okuno, M., Malik, F., and Vale, R.D. (1991). Nucleotide specificity of the enzymatic and motile activities of dynein, kinesin, and heavy meromyosin. *J. Cell Biol.* 112, 1189–1197.
- Steuer, E.R., Wordemann, L., Schroer, T.A., and Sheetz, M.P. (1990). Localization of cytoplasmic dynein to mitotic spindles and kinetochores. *Nature* 345, 266–268.
- Vale, R.D. (1992). Microtubule motors: many new models off the assembly line. *Trends Biochem. Sci.* 17, 300–304.
- Wordeman, L., Steuer, E.R., Sheetz, M.P., and Mitchison, T. (1991). Chemical subdomains within the kinetochore domain of isolated CHO mitotic chromosomes. *J. Cell Biol.* 114, 285–294.
- Yen, T.J., Li, G., Schaar, B., Szilak, I., and Cleveland, D.W. (1992). CENP-E is a putative kinetochore motor that accumulates just before mitosis. *Nature* 359, 536–539.