Factors Required for the Binding of Reassembled Yeast Kinetochores to Microtubules In Vitro

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Abstract. Kinetochores are structures that assemble on centromeric DNA and mediate the attachment of chromosomes to the microtubules of the mitotic spindle. The protein components of kinetochores are poorly understood, but the simplicity of the *S. cerevisiae* kinetochore makes it an attractive candidate for molecular dissection. Mutations in genes encoding CBF1 and CBF3, proteins that bind to yeast centromeres, interfere with chromosome segregation in vivo. To determine the roles played by these factors and by various regions of centromeric DNA in kinetochore function, we have developed a method to partially reassemble kinetochores on exogenous centromeric templates in vitro and to visualize the attachment of these reassembled kinetochore complexes to microtubules. In this assay, single reassembled complexes appear to mediate microtubule binding. We find that CBF3 is absolutely essential for this attachment but, contrary to previous reports (Hyman, A. A., K. Middleton, M. Centola, T. J. Mitchison, and J. Carbon. 1992. Microtubule-motor activity of a yeast centromere-binding protein complex. *Nature (Lond.).* 359:533–536) is not sufficient. Additional cellular factors interact with CBF3 to form active microtubule-binding complexes. This is mediated primarily by the CDEIII region of centromeric DNA but CDEII plays an essential modulatory role. Thus, the attachment of kinetochores to microtubules appears to involve a hierarchy of interactions by factors that assemble on a core complex consisting of DNA-bound CBF3.

Mitosis is the process by which replicated chromosomes are evenly segregated to the two daughters of a dividing cell. During mitosis, kinetochores attach chromosomes to spindle microtubules and direct a complex series of movements (see Mitchison, 1988; McIntosh and Pfarr, 1991; Earnshaw and Tomkiel, 1992). Kinetochores are composed of centromeric DNA and a set of associated proteins. One of the most important functions of these proteins is to bind DNA to microtubules, but the identities and properties of kinetochore proteins are largely unknown. In animal cells, centromeres span several megabases and have a complex organization. In contrast, fungal centromeres are much simpler, and those of *S. cerevisiae* are only 125 bp long. The structure of microtubules is very highly conserved among eukaryotes (Shatz et al., 1986) and both simple and complex kinetochores mediate chromosome segregation on a bipolar microtubule array. Thus, kinetochores in higher and lower eukaryotes are likely to share many important properties despite their morphological differences. The relative simplicity of the *S. cerevisiae* kinetochore (Bloom and Carbon, 1982) makes it a particularly good candidate for the molecular analysis of proteins involved in microtubule binding.

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The dynamic attachment of animal cell kinetochores to microtubules has been studied extensively in vivo with video microscopy (e.g., Skibbens et al., 1993 and references therein) and in vitro with isolated chromatin (Mitchison, 1988). Animal cell kinetochores contain both motor and nonmotor microtubule-binding proteins (Bernat et al., 1990) and the relative activities of plus and minus-end directed motors appear to be regulated by phosphorylation (Hyman and Mitchison, 1991). Unfortunately, the poor cytology and small size of budding yeast has prevented similarly detailed studies of chromosome movement in living cells. One unique feature of the *S. cerevisiae* kinetochore is that it binds to a single microtubule (Peterson and Ris, 1976) whereas complex kinetochores bind several microtubules. Budding yeast chromosomes may be substantially less motile than those in higher cells and their attachment to microtubules may be dominated by nonmotor proteins, as evidenced by the inability of ATP to dissociate yeast minichromosomes from microtubules in vitro (Kingsbury and Koshland, 1991).

S. cerevisiae centromeres are comprised of three sequence elements: CDEI, CDEII, and CDEIII (Fitzgerald-Hayes et al., 1982). CDEI is a conserved palindromic sequence and mutations in CDEI increase the frequency of chromosome loss 10–20-fold (Niedenthal et al., 1991 and references therein). CDEII is a 75–85 bp A/T rich element that shows little conservation in sequence among different chromo-

somes. However, CDEII is essential for centromere function in vivo (Gaudet and Fitzgerald-Hayes, 1987). CDEIII is a highly conserved imperfect palindrome. Single point mutations in CDEIII abolish centromere activity in vivo (e.g., Hegemann et al., 1988; McGrew et al., 1986) and the binding of minichromosomes to microtubules in vitro (Kingsbury and Koshland, 1991). For this reason, CDEIII is often called the "core" region of the centromere.

Two protein complexes have been identified that bind to *S. cerevisiae* centromeres: CBF1 and CBF3. CBF1 is a member of the helix-loop-helix family of DNA-binding proteins that interacts with CDEI. Its deletion increases the frequency of chromosome loss 10-fold (Cai and Davis, 1990). CBF1 is also thought to act as a transcription factor at several promoters (but there is some controversy about this; see Mellor et al., 1990). Although CBF1 is not required for cell growth, CDEI sequences are found in all centromeres, implying that they play an important role in kinetochore function.

CBF3 is a protein complex that binds to the core CDEIII element. When purified by DNA affinity chromatography, CBF3 contains major proteins of 58, 64, and 110 kD, as well as a number of minor species (Lechner and Carbon, 1991; Jiang et al., 1993*b*). The CBF3 complex has an apparent native molecular mass of 240 kD, consistent with the possibility that it is a trimer containing one of each of the 58-, 64-, and 110-kD subunits. Recent experiments have shown that two genes required for chromosome transmission, *CTF13* and *CBF2/NDC10/CTF14* encode the 58-kD and 110-kD components of CBF3 (Doheny et al., 1993; Goh and Kilmartin, 1993; Jiang et al., 1993*a*). Both *CTF13* and *CBF2/NDC10/CTF14* are essential genes whose activity is required for the binding of CBF3 to DNA (Sorger, P. K., and A. A. Hyman, manuscript in preparation). Cbf2p contains a sequence with the hallmarks of a GTP-binding motif but Ctf13p lacks homology to other known proteins. CBF3 preparations contain a microtubule based motor activity with the pharmacological properties of kinesin (Hyman et al., 1992), but the isolation and sequencing of the major proteins in CBF3 has failed thus far to identify those that are involved in the motor activity. The functions of individual CBF3 subunits therefore remain unknown.

In vitro experiments have suggested that the CBF3 complex is itself responsible for the binding of centromeres to microtubules (Hyman et al., 1992). However, CDEIII is not sufficient for centromere function in cells (Clarke, 1990) presumably because factors in addition to CBF3 are required to assemble active kinetochores. It has been difficult to demonstrate directly the existence of these factors due to the lack of a method to generate kinetochore complexes containing different sets of components and to measure the activities of the complexes. We now report the development of such a method. It allows genetic and biochemical approaches to studying kinetochore function to be combined. Beads carrying centromeric DNA are incubated with crude or fractionated extracts to initiate the assembly of kinetochore complexes. The microtubule-binding activity of the reassembled complexes is then determined by fluorescence microscopy. Using this approach, we show that CBF3 is absolutely essential for the binding of kinetochores to microtubules, but that it is not sufficient. Additional cellular factors that interact with both CDEII and CDEIII DNA are required for microtubule attachment.

Materials and Methods

Yeast Strains

The *ctf13-30* and *ctf14-42* strains (Spencer et al., 1990) were provided by Dr. P. Hieter; *ndc10-1* by Dr. J. Kilmartin (Goh and Kilmartin, 1993); *ndc10-2* by Dr. T. Huffaker (Sorger, P. K., and A. A. Hyman, manuscript in preparation), and *cbf1Δ1* by Dr. P. Foreman (Cai and Davis, 1990). The cell cycle regulation of microtubule-binding activity (Table III) was analyzed using the strain PS612: A364A, *leu2*, *ura3-52*, *trp1-1*, *LEU2::bar1*, *Mat a*. CBF3 was purified from the strain PS886: *leu2*, *trp1*, *ura3-52*, *prb1-1122*, *pep4-3*, *prc1-407*, *GAL*, *Mat a/α*. To analyze the contribution of Kar3p to microtubule-binding activity, extracts from the strain MS1617, *ura3-52*, *leu2-3,112*, *his3Δ200*, *ade2-101*, and *trp1Δ1 KAR3::LEU2*, were compared to extracts from a strain with the same background but carrying the YCp50-*KAR3* plasmid pMR820 (Meluh and Rose, 1990). To assess the contributions of Kip1p and Cin8p to microtubule binding, the wild-type strain MAY589, *his3-Δ200*, *leu2-3,112*, *ura3-52*, *ade2-101 Mata* was compared to congenic strains MAY2169 *cin8-3*, *kip1::HIS3* and MAY2733 *kip1::HIS3*, *kar3::LEU2*, *lys2-801*, *can1^R* (Hoyt et al., 1992; Saunders and Hoyt, 1992). *S. pombe* extracts were prepared from the strain SP812, *h^{-s}*, *ade6-210*, *leu1-32*, *ura4*.

Preparation of Beads Carrying Centromeric DNA

Wild-type *CEN3* sequences were derived from pRN505 (Ng and Carbon, 1987). The CDEIII-3bpΔ, CDEIII-only and CDEIII-inverted centromere derivatives were constructed using oligonucleotide-directed mutagenesis and are described in detail elsewhere (note that the CDEIII-only *CEN* is identical to *CEN3Δ1* of Sorger, P. K., and A. A. Hyman, manuscript in preparation.) A 184-bp fragment of wild-type *CEN3* to be coupled to beads was amplified by PCR using a biotinylated "right-hand" primer (biotin-CCACCAGTAAACGTTTC) that anneals 68 bp to the right of the central "C" of CDEIII (bp 14 in Hegemann et al., 1988) and a "left-hand" primer (GTACAAATAAGTCACATGATGATATTTG) that anneals 10 bp to the left of CDEI. In the case of the CDEIII-3bpΔ construct, these primers generate a 181-bp fragment. With the CDEIII-only *CEN* construct, the left-hand primer was CCGGCTCGTATGTTGTG and with CDEIII-inverted *CEN* it was AGTCATTATGATGATATTTGATTTTATT. For each DNA fragment, 20–40 independent PCR reactions were pooled and gel purified.

Purified DNA was then linked to fluorescent latex microspheres of uniform size (coefficient of variation in diameter 3%; Molecular Probes, Eugene, OR). These *CEN*-bearing beads were prepared as follows. Biotinylated BSA was made by reaction of BSA at 25 mg/ml with NHS-XX-biotin (Molecular Probes) in 50 mM Pipes, pH 7.0 and free biotin removed by fractionation on a P10 column (BioRad Labs., Hercules, CA). Typically, the BSA was derivatized with 6 mol of biotin per mol of BSA. 0.2 μm diam carboxylate-modified rhodamine-labeled latex beads were coupled to the biotinylated BSA with 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide and *N*-hydroxysulfosuccinamide in 50 mM Hepes, pH 7.0. Beads were washed with 50 mM Hepes, pH 7.0, incubated with streptavidin (Molecular Probes), washed again in 0.5 M NaCl, and then incubated overnight with biotinylated *CEN3* DNA, a small amount of which was radiolabeled as a tracer. Unbound DNA was removed by washing beads in 50 mM Hepes, pH 8.0, 0.5 M NaCl. To make beads with different numbers of active centromeric DNA molecules, the total amount of DNA per bead was kept constant at 800–1,000 molecules per bead by mixing active wild-type, CDEIII-only or CDEIII-inverted *CEN* DNA and inactive CDEIII-3bpΔ DNA. The average number of DNA molecules per bead, which varied from 0.2 to 1,000, was then calculated by determining the amount of *CEN* DNA bound and the number of beads per unit volume. In this calculation, the most difficult value to determine accurately is the concentration of *CEN* DNA. To measure it, we compared band intensities of serially diluted *CEN* DNA samples to a HaeIII-digested φX174 molecular mass marker DNA of known concentration (GIBCO-BRL) after electrophoresis on ethidium bromide–containing agarose gels. In a typical measurement performed three times on different gels, the concentration of wild-type (WT)[1] *CEN* DNA was determined by comparing three or four dilutions of DNA to two different batches of φX174 DNA standard. The concentration estimates for the three gels were 1.0 μM, 0.75 μM, and 0.84 μM. We therefore estimate that the determination of DNA concentration is subject to an error of approximately ±20%. The standard deviation in counting the number of beads in a reaction is typically ±10%, suggesting that the overall error in determining the number of DNA molecules per bead is about ±30%.

1. *Abbreviation used in this paper*: WT, wild-type.

Preparation of Extracts and Purification of CBF3

We wanted to prepare extracts from yeast cells carrying temperature sensitive mutations in chromosome transmission frequency mutants (Spencer et al., 1990) and harvested under well-controlled permissive and nonpermissive conditions; we have found that the mechanical disruption of cells in liquid nitrogen is the most satisfactory way to make such extracts (see Sorger, P. K., and A. A. Hyman, manuscript in preparation for further details). In their purification of CBF3, Lechner and Carbon (1992) used 1 M KCl to extract centromere-binding proteins from cells. However, the dilution or dialysis of these high-salt extracts into low salt assay buffers results in extensive and uncontrolled precipitation. We have found that whole-cell extracts made in 150 mM KCl contain 50–80% as much microtubule-binding activity as extracts made in 1 M KCl. No solubility problems are encountered when these low-salt extracts are incubated in assay buffer. We postulate that buffers containing 150 mM KCl efficiently remove kinetochore components from chromatin because EDTA present in the buffer at 6 mM chelates the magnesium required for the binding of CBF3 to DNA (Ng and Carbon, 1987).

Whole-cell extracts were prepared from cells grown at 25–30°C in rich medium to a density of 2–5 × 10^7 cells ml^{-1}. Cells were washed by pelleting once in water and once in breakage buffer (30 mM sodium phosphate, pH 7.0, 60 mM β-glycerophosphate, 150 M KCl, 6 mM EGTA, 6 mM EDTA, 6 mM NaF, 10% glycerol), resuspended in a minimal volume of breakage buffer with protease inhibitors (final concentrations: 1 mM PMSF, 10 μg ml^{-1} each of pepstatin, leupeptin, and chymostatin) frozen in liquid nitrogen and fragmented in a mortar and pestle cooled in liquid nitrogen. Cell debris was removed by centrifugation at 15,000 *g* for 30 min. Extracts from START-arrested and mitotic *bar1* cells were prepared by treating asynchronous cultures of PS612 with 2 μg ml^{-1} α-factor or 15 μg ml^{-1} of nocodazole for 3 h at room temperature before harvest.

CDEIII-binding activity was measured on non-denaturing (bandshift) gels using an 88-bp CDEIII-containing *CEN3* fragment as described (Sorger, P. K., and A. A. Hyman, manuscript in preparation). Gels were quantitated on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

To isolate CBF3, the strain PS886 was grown to OD_{600} = 0.8 in a fermenter, harvested by centrifugation and frozen in liquid nitrogen as a thin "vermicelli" (Sorger et al., 1988). 100 g wet weight of frozen cells was broken by blending in a Waring Blender, and then warmed to 4° by addition of 2× breakage buffer (see above). CBF3 purification was monitored using band-shift gels. The extract was spun at 100,000 *g* for 1 h and ammonium sulfate was added to the supernatant to 60% saturation and left for 1 h with continuous stirring. The precipitate was recovered by centrifugation and resuspended in column buffer (100 mM β-glycerophosphate, 50 mM Tris Bis Propane, 100 mM KCl, 5 mM EGTA, 5 mM EDTA, 10% glycerol, 1 mM PMSF, 0.1% 2-mercaptoethanol, 10 μg/ml each of leupeptin and chymostatin, pH 7.0), and then dialyzed into the same buffer. The dialysate was clarified by centrifugation and 200 mg was applied to a 10-ml Mono Q column (Pharmacia LKB Biotechnology, Piscataway, NJ) equilibrated in column buffer. The column was eluted with a 0.1–1 M KCl gradient in column buffer. CBF3 elution was monitored on bandshift gels. CBF3 eluted from the MonoQ column between 0.15 and 0.25 M KCl.

Microtubule-binding Assays

To measure centromere-dependent microtubule-binding activity, beads were incubated with 15–200 μg whole-cell extract from WT or mutant cells grown at 25–30°C. Typically, extract was added to a 30-μl reaction containing 3 × 10^7 beads and 10 μg of sonicated salmon sperm DNA in bead binding buffer (10 mM Hepes pH 8.0, 6 mM $MgCl_2$, 10% glycerol and adjusted to a final KCl concentration of 150 mM). With semipurified CBF3, the amount of salmon sperm DNA was reduced to 1 μg. Casein or BSA was occasionally included at 0.3 mg ml^{-1}. After 30–40 min of incubation, samples were removed from the binding reaction and diluted 1:1 into antifade mix which had been incubated at 37°C for 10 min before use. Antifade contained 40 mM potassium Pipes, pH 6.8, 3.5 mM $MgCl_2$, 0.1% 2-mercaptoethanol, 1.5 mg ml^{-1} casein, 0.1 mg ml^{-1} glucose oxidase, 0.1 mg ml^{-1} catalase, 10 mM glucose and 10 μM taxol. Polarity marked microtubules, constructed as described (Howard and Hyman, 1993) and diluted into 80 mM potassium Pipes pH 6.8, 1 mM EGTA, 10 mM $MgCl_2$, 10 mM taxol, were perfused into a 5-μl chamber (Hyman et al., 1992) and allowed to adsorb for 5 min. 3 mg ml^{-1} casein in 50 mM Hepes pH 8.0 was then perfused into the chamber followed immediately by bead-binding reaction diluted into antifade (see above). Bead binding was observed after 5–40 min of incubation in the chamber. Chambers were observed with a Zeiss Axioskop microscope and a 63× PlanApo 1.4 lens using a field of known area. Microtubule-bound beads were counted manually and values from 10–30 fields were averaged. The errors reported in this study are the standard deviation for observation of multiple fields. To prevent interference from the occasional bead that sticks directly to the glass surface of the perfusion chamber, beads were counted as bound only if they were linked to microtubules tethered to the perfusion chamber by one end (usually this occurs by binding of the heavily rhodamine-derivatized microtubule seeds to glass). Beads bound to tethered microtubules are easily distinguished from free beads because they undergo slow brownian movement. The field size was typically 10–25 × 10^3 μm^2 but values in this paper (unless otherwise noted) are normalized to a 25 × 10^3 μm^2 field and 10^6 beads μl^{-1} of binding reaction.

To demonstrate that the batch of CDEIII-only DNA used in Fig. 6 was fully active and thus, had similar CBF3-binding activity to WT *CEN* DNA under the conditions of the bead assay, reactions were set up with 800-WT beads whose residual biotin-binding sites had been blocked with free biotin. Varying amounts of CDEIII-only or WT *CEN* DNA were included in these reactions as soluble competitor DNAs. Both WT and CDEIII-only DNA were observed to half maximally inhibit microtubule binding at the same concentration. This shows that the difference in the microtubule-binding activities of WT and CDEIII-only DNA is not a consequence of non-specific defects in the CDEIII-only *CEN* DNA preparation.

Isolation and Washing of Beads Carrying Kinetochore Complexes

To determine the microtubule-binding activities of kinetochore complexes free of soluble factors and to determine the effect of washing kinetochore complexes with varying concentrations of salt, complexes were assembled on beads, and then isolated by low speed centrifugation. A 20-fold reaction (600 μl) was prepared in bead binding buffer with 2.5 mg WT extract, 200 μg salmon sperm DNA and 10^9 800-WT beads. After a 40-min incubation, the reaction mix was divided into four aliquots, each of which was spun in an Eppendorf centrifuge at 4°C for 10′ to pellet the beads. The beads were then resuspended in 50 μl wash buffer (10 mM Hepes, 6 mM $MgCl_2$, 1 mg ml^{-1} BSA, 10% glycerol, pH 7.0) with 100 mM KCl, spun a second time, resuspended in wash buffer with 100 mM to 600 mM KCl to elute various centromere components, spun a third time, and then finally resuspended in 30 μl wash buffer with 150 mM KCl and supplemented with 4 μg salmon sperm DNA. Washed beads were then assayed for microtubule-binding directly, or 10-μl samples were incubated with 30 μg of whole cell extract from *ctf13-30* or *ctf14-42* cells for 30 min at 25°C (or 37°C for reactions with *ctf13-30* extract), and then assayed. Because up to 50% of the beads can be lost in this procedure, microtubule-binding activities were normalized to the number of beads present in each binding reaction.

Miscellaneous Methods

ATP concentrations were determined using luciferase luciferin (Sigma Chem. Co., St. Louis, MO) and a Bertold Luminometer. To deplete ATP in binding reactions, 11 U of apyrase were added to 30-μl reactions and incubated at room temperature for 10 min. The endogenous ATP level in extracts was typically 3 μM (for an extract made at 50 mg/ml total protein) and was reduced to <0.2 μM by apyrase treatment. To confirm that beads bound to microtubules were resistant to dissociation by ATP, chambers were perfused with 2 mM ATP or a buffer control (assays with luciferase confirmed that ATP concentrations remained above 1 mM during the course of the binding reaction). No significant difference was observed in the number of beads bound. We have found however, that the perfusion of chambers containing microtubule-bound beads with buffer and 150 mM KCl, 15 mM ATP dissociated 95% of the beads. But, buffer containing 150 mM KCl, 15 mM ATP that had been hydrolyzed with apyrase to 0.25 μM was equally potent.

The values in Table IV were derived as follows: (Line 1) From Hyman et al. (1992), Table I. The reaction contains sufficient CBF3 (~0.003 μg) to bind 5 fmol of 350 bp *CEN* DNA. This CBF3 is derived from 2.5 × 10^8 cells assuming a yield of 12 mol CBF3 per mole of cells (as calculated by Lechner and Carbon, 1991). The number of CBF3 molecules per bead is calculated to be 300; (Line 2) Data from this work, Table II. The reaction contains sufficient CBF3 to bind 0.15 fmol of CDEIII DNA representing 50 μg of whole-cell extract (note that this reflects a 10-μl binding reaction) derived from 10^7 cells. The number of CBF3 molecules per bead is estimated to be 10; (Line 3) Data from this work, Table II. The reaction contains the same amount of CDEIII-binding activity as the reaction in line 2; for simplicity, both are assumed to derive from the same number of cells (i.e., no correction has been made for the loss of CBF3 activity during

purification, ~40%). The value "Beads bound per field normalized to CDEIII-binding activity" is expressed in beads bound to microtubules per field per fmol of *CEN* DNA bound. All reactions contain 10^6 beads μl^{-1}.

Results

Reassembly of Yeast Kinetochore Complexes in Whole-Cell Extracts

To assay the activity that binds centromeric (*CEN*) DNA to microtubules, we attached biotinylated *CEN* DNA to fluorescently labeled streptavidin-coated beads and incubated the beads in whole-cell extracts. These whole-cell extracts were made from cells maintained in well-defined conditions and the extracts are therefore suitable for the analysis of temperature sensitive mutants and of cultures arrested at discrete points in the cell division cycle; the preparation of these extracts is described in detail elsewhere (Sorger, P. K., and A. A. Hyman, manuscript in preparation). After the incubation of beads with extracts, taxol-stabilized microtubules were adsorbed to the glass surface of a thin perfusion chamber and beads and extract were then introduced into the chamber. The binding of beads to microtubules was observed by fluorescence microscopy. This method is conceptually similar to one previously used by Hyman et al. (1992) to analyze the microtubule-binding properties of purified CBF3. To apply the method to the analysis of cell extracts, however, changes were required in the compositions of the beads and the reaction buffers.

When beads carrying WT *CEN* DNA were incubated with extract prepared from an asynchronous cell culture, and then assayed for microtubule binding, a large fraction of the beads was observed to bind along the lengths of microtubules (Fig. 1 *a*). To demonstrate that this binding required functional centromeric DNA, a mutant sequence was constructed by deleting 3 bp of CDEIII required for centromere function in vivo (Sorger, P. K., and A. A. Hyman, manuscript in preparation). Beads carrying this CDEIII-3bpΔ mutant were at least 50-fold less active in binding to microtubules than beads carrying WT centromeres (Fig. 1 *b*). Similar results were obtained with whole-cell and nuclear extracts (prepared as described by Lechner and Carbon, 1991) except that the nuclear extracts had higher specific activity (data not shown; as discussed in Materials and Methods, we have chosen to use whole-cell extracts in preference to nuclear extracts). These data show that factors that mediate microtubule attachment appear to interact specifically with functional *CEN* DNA when *CEN*-bearing beads are incubated with cell extracts. This assembly is quite efficient: in an optimized reaction containing 150 μg of whole-cell extract derived from ca. 3×10^7 cells, $3–5 \times 10^7$ beads are active in microtubule binding. Because each *S. cerevisiae* cell contains 16 chromosomes, this suggests that ~10% of the kinetochores present in vivo can reassemble in the in vitro system.

For simplicity, we will refer to the complexes reassembled on centromeric DNA in our in vitro system as "kinetochore

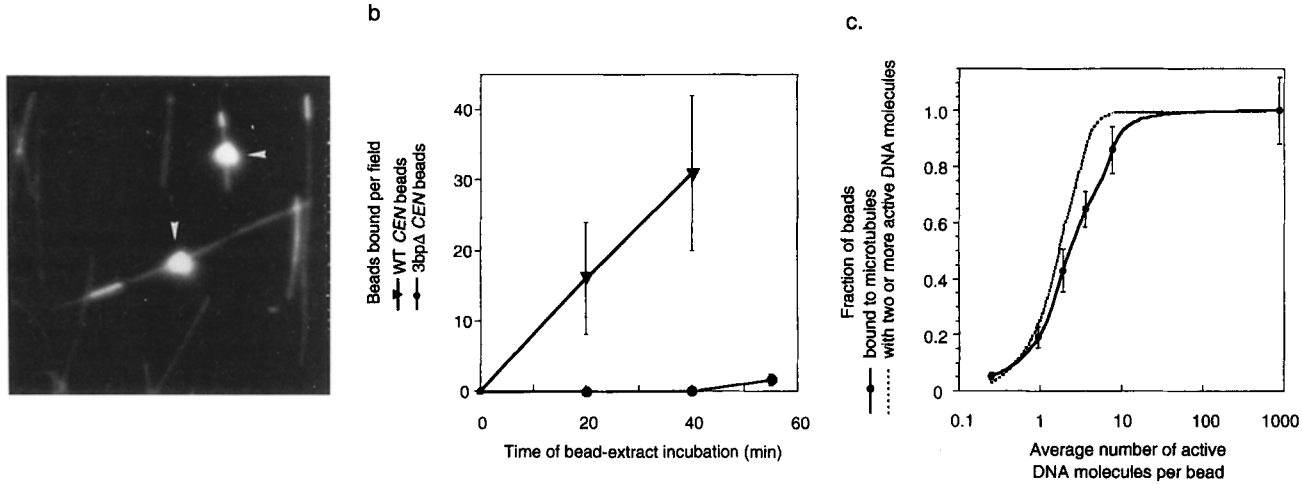

Figure 1. Binding of beads carrying centromeric DNA to microtubules. (*a*) Photograph of rhodamine-latex beads that carry *CEN* DNA bound to rhodamine-labeled microtubules in extracts from WT cells. (*b*) Time course of binding of beads carrying WT *CEN* DNA (*triangles*) or CDEIII-3bpΔ mutant *CEN* DNA (*circles*) to microtubules. Binding reactions contained 120 μg of extract from WT cells. Extracts were prepared in breakage buffer containing 1 M KCl. In all subsequent experiments we switched to extracts made in breakage buffer containing 150 mM KCl. Error bars show a high variance in the measurement of beads bound per field. This is a consequence of the relatively short reaction times and the need to count beads quickly. In subsequent experiments, we settled on a standard time for bead-extract incubation of 40 min. (*c*) Microtubule-binding activities of beads carrying varying numbers of WT *CEN* DNA molecules per bead. Active and inactive *CEN* DNAs were linked to beads in varying ratios so that the total amount of DNA on each bead was constant. The dotted line shows the fraction of beads with two or more active DNA molecules (given by the equation $1-e^{-x}-xe^{-x}$, where x is the average number of active DNA molecules). The solid line shows the dependence of the fraction of beads bound to microtubules on the average number of active *CEN* DNA molecules present per bead. By measuring the volume of the chamber, we estimate that 80–100% of all 800-WT beads present in the reaction are actually bound to microtubules. However, in this plot, the number of beads carrying 800 molecules of active DNA and bound to microtubules is arbitrarily defined as 100% and the number bound of beads carrying 800 molecules of inactive CDEIII-3bpΔ DNA and bound to microtubules is defined as 0% binding. Each point represents a single reaction containing 200 μg whole-cell extract and incubated with microtubules for 40 min. Microtubule binding was still rising at 40 min but because the antifade had started to lose its effectiveness, data could not be gathered reliably from longer incubation times. Thus, the extent of microtubule binding shown here may be an underestimate of the maximum binding achievable. Results from a typical experiment are shown.

complexes." In the discussion to this paper, we review the evidence that these reassembled complexes have many properties of kinetochores found in living yeast cells. It is likely however, that there are important aspects of kinetochore function that we have not yet reconstituted in vitro. In particular, reassembled complexes do not appear to translocate along microtubules (see below).

Beads Carrying as Few as Two Reassembled Kinetochore Complexes Binding to a Microtubule

In cells, single kinetochores (and pairs of sister kinetochores) mediate the binding of chromosomes to microtubules, but in our assay we were using beads with about 800 molecules of *CEN* DNA per bead (called 800-WT beads). We therefore wanted to determine how many kinetochore complexes were required in vitro to attach a bead to a microtubule. A set of beads was constructed in which the number of active DNA molecules was progressively reduced and inactive mutant centromeric DNA substituted to keep the total amount of DNA per bead constant. This set of "low active density" beads was then examined for microtubule binding. Half-maximal binding was observed with an average of 2 ± 0.6 active molecules per bead (Fig. 1 *c*, *solid line*). For a population of beads with a known average number of DNA molecules, the fraction of beads with a particular number of DNA molecules can be determined from a Poisson distribution. The best fit to the observed dependence of microtubule binding on DNA number at low numbers of DNA molecules per bead was the distribution "two or more" (Fig. 1 *c*, *dashed line*). This indicates that beads with as few as two active DNA molecules (2-WT beads) bind tightly to microtubules. However, simple geometric considerations indicate that in only a subset of beads with two active DNA molecules (ca. 20%) are both molecules found on the same face of a bead and therefore positioned to bind simultaneously to a microtubule. We therefore believe that most of the binding events observed with 2-WT beads probably involve the interaction of single kinetochore complexes with microtubules. For simplicity, we will refer to this as "single kinetochore" binding, but we cannot exclude the possibility that two or perhaps three kinetochore complexes are in fact required for efficient microtubule attachment in vitro. These data with low active density beads suggest, however, that we have reproduced an interaction between kinetochore complexes and microtubules that is similar in an important respect to the interaction found in living cells.

Functional CBF3 Is Required for the Binding of Kinetochore Complexes to Microtubules

Functional CBF3 is required for chromosome segregation in vivo (Doheny et al., 1993; Goh and Kilmartin, 1993). To determine if this reflects a requirement for CBF3 in the binding of kinetochores to microtubules, we prepared extracts from cells carrying temperature sensitive mutations in the *CTF13* or *CBF2/NDC10/CTF14* genes. The *CTF13* gene encodes the 58-kD subunit of CBF3 and *CBF2/NDC10/CTF14* encodes the 110-kD subunit (Doheny et al., 1993; Goh and Kilmartin, 1993; Jiang et al., 1993*a*). We have recently shown that the 58-kD and 110-kD subunits are required for the DNA-binding activity of CBF3, and thus, that there is an almost complete absence of functional CBF3 in *ctf13* and *ctf14/ndc10* cells (Sorger, P. K., and A. A. Hyman, manuscript in preparation). To measure microtubule-binding activity in these strains, extracts were prepared from cells grown at permissive temperatures, and then incubated with beads at 37°C. Only background levels of bead binding were observed in extracts from *ctf13-40*, *ctf14-42*, or *ndc10-1* cells (Table I, rows *1–4*). Extracts from *ndc10-2* cells prepared at 25°C exhibited microtubule-binding activity that was strongly temperature sensitive in vitro (lines 6–7). From this we conclude that extracts lacking CBF3 cannot assemble a kinetochore complex active in microtubule binding.

In many cases, the loss of a single subunit in a multiprotein complex leads to inactivation or degradation of the other subunits (Blanton et al., 1992; Brown and Beggs, 1992). To determine if the low microtubule-binding activity of beads incubated in *ctf14/ndc10* or *ctf13* extracts reflects the loss of a single protein, or whether multiple kinetochore components are lacking, we asked whether extracts carrying different mutations could complement each other biochemically. When *ctf13* and *ctf14* extracts were mixed at 37°C, efficient reconstitution of microtubule-binding activity was observed. This indicates that kinetochore subunits other than Ctf13p and Cbf2p are present in the extracts in an active state (Table I, line 5) and argues against indirect explanations for the low activity of the mutant extracts. We conclude from this that the products of both *CTF13* and *CBF2/NDC10/CTF14* are required for the microtubule-binding activity of budding yeast kinetochores.

CBF3 Is Not Sufficient for the Microtubule-binding Activity of Kinetochore Complexes

Next, we asked whether CBF3 is sufficient for the microtubule-binding activity of kinetochores. Our use of low-salt extraction buffers allowed the development of a simplified scheme for the partial purification of CBF3. During the course of this purification, CDEIII and microtubule-binding activities were compared (Table II). In the first purification step, whole cell extracts were precipitated with 60% ammo-

Table I. Microtubule-binding Activities of Extracts from WT Cells or Cells Carrying Mutations in CBF3 Subunits

Number	Sample	Reaction temperature (°C)	Microtubule-binding activity (beads per field)
1	Wild-type extract	25	81 ± 10
2	Wild-type extract	37	42 ± 5
3	*ctf13-30* extract	37	5 ± 3
4	*ctf14-42* extract	37	3 ± 1
5	*ctf13-30* extract + *ctf14-42* extract	37	41 ± 8
6	*ndc10-2* extract	25	90 ± 8
7	*ndc10-2* extract	37	2 ± 1

All reactions contain 100 μg of whole-cell extract except that the reaction in line 5 contained 100 μg of each extract. 25°C is the permissive and 37°C the restrictive temperature for the *ctf13-30*, *ctf14-42*, and *ndc10-2* strains. Although not measured in parallel with these samples, the background level of binding observed with beads carrying non-functional centromeric DNA is typically 3 per field. We believe that the twofold reduction in binding activity observed with wild-type extract incubated at 37°C is a consequence of proteolysis or nonspecific inactivation. Microtubule-binding activities are normalized to a standard field size of 25,000 μm^2 and a standard bead concentration of 10^6 μl^{-1}.

nium sulfate, and then dialyzed into low-salt buffer. This was then further fractionated on a MonoQ resin. CBF3 eluted from MonoQ was purified up to 60-fold and the yield of CDEIII-binding activity was ~60% (as estimated on "band-shift" gels; Fig. 2). Lechner and Carbon (1992) have previously shown that CBF3 purified to near-homogeneity by DNA affinity chromatography will not bind efficiently to DNA unless assembly factors are added. This does not appear to be true of our semi-pure preparations and CBF3 isolated by chromatography on MonoQ had high CDEIII-binding activity (Fig. 2, lanes *4–5*). This may reflect the lower purity of our preparation relative to that of Lechner and Carbon (1992).

To measure microtubule-binding activity during the purification, samples of whole-cell extract, dialyzed ammonium sulfate precipitate or MonoQ eluate containing equal CDEIII DNA-binding activities (Fig. 2 *b*) were incubated with 800-WT and 2-WT beads. Strikingly, the microtubule-binding activity of the MonoQ-purified CBF3 was at least 200-fold lower than that of whole-cell extract (Table II, lines 1–4). By the criterion of "single kinetochore binding," semi-pure CBF3 was completely inactive in promoting attachment to microtubules (Table II, 2-WT column).

We reasoned that the low microtubule-binding activity of complexes assembled with the semipurified CBF3 might be a consequence of the absence of specific factors required for microtubule attachment. To test this, we added whole-cell extract from *ctf14/ndc10* or *ctf13* cells to the semi-pure CBF3. As described above, *ctf14/ndc10* and *ctf13* extracts lack functional CBF3 but appear to contain other kinetochore components in an active form. When semi-pure CBF3 and *ctf14* extract were mixed, the microtubule activity of kinetochore complexes was reconstituted (Table II, lines 6–7) without a significant change in CDEIII-binding activity (Fig. 2; lanes *10–12*). Similar reconstitution was obtained with *ndc10-1* and *ctf13* extracts (Table II, lines 8–10 and data not shown). The reconstituting activity was abolished if the extract was denatured (data not shown).

The amount of the reconstituting activity in extracts appears to be similar to that of CBF3 because efficient reconstitution required the addition of *ctf14* extract and CBF3 in amounts that derive from equal numbers of cells (in line 6, this is ca. 3×10^7 cells). This suggests that the activity is required in stoichiometric amounts and does not merely modify CBF3. However, to test directly whether factors present in *ctf14* extract might mediate changes in the phosphorylation state of kinetochore components during the reassembly reaction, we asked whether ATP or protein phosphatases were required for reconstitution. We observed that neither the depletion of ATP to <0.2 μM with apyrase nor the inhibition of protein phosphatases with 15 μM microcystin affected the efficiency of reconstitution (data not shown). The biochemical properties of the reconstituting activity are not yet known but it appears to be cell-type specific since it is not present in the fission yeast *S. pombe* (lines 11–12). We interpret these results to mean that *ctf14/ndc10* and *ctf13* extracts provide to the assembly reaction factors other than CBF3 that are required for the formation of active kinetochore complexes. It seems likely that these factors include components of the yeast kinetochore that directly contact microtubules.

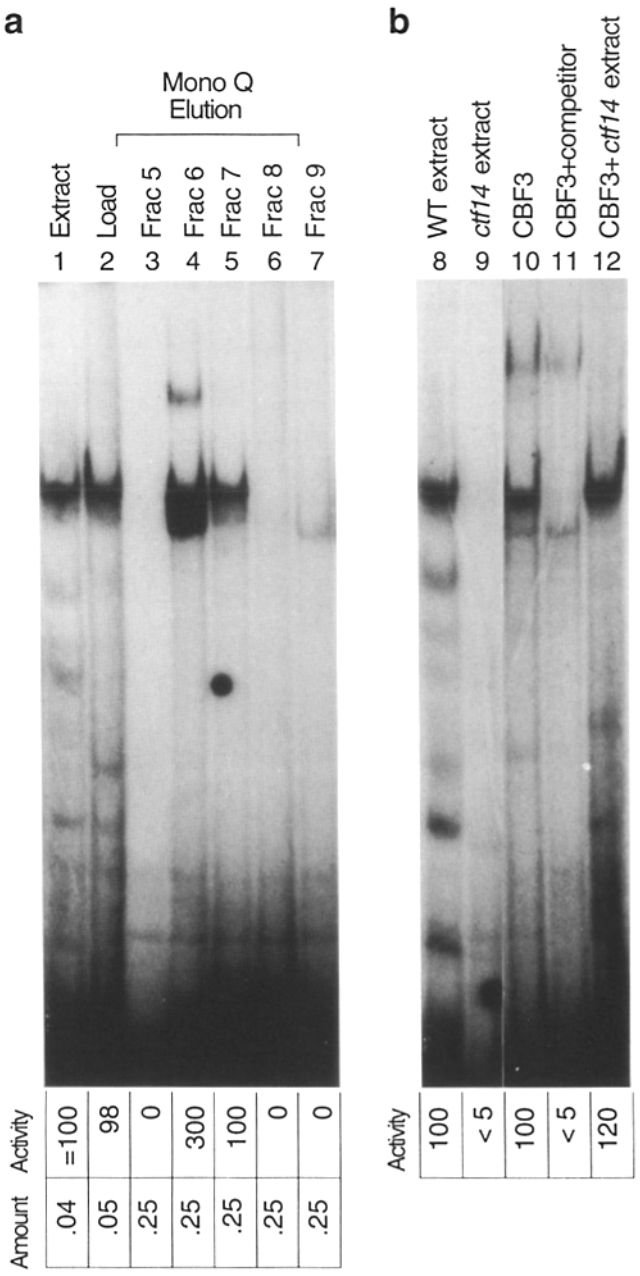

Figure 2. Analysis of CDEIII-binding activity in extracts and in partially-purified CBF3 preparations. The amounts of sample analyzed on these nondenaturing gels are identical to the amounts used in the microtubule-binding assays of Table II. (*a*) Successive samples during the course of CBF3 purification; *extract*: starting whole-cell extract in 150 mM KCl; *load*: dialyzed ammonium sulfate precipitate as loaded on the MonoQ column; *Frac 5 to Frac 9*: successive fractions eluted from the MonoQ column between 0.17 M KCl and 0.30 M KCl. Activity indicates the relative intensity of the band corresponding to the CBF3-CDEIII complex; this band is observed only with functional CDEIII DNA (as demonstrated by cross-competition analysis with a series of point mutations in CDEIII; Sorger, P. K., and A. A. Hyman, manuscript in preparation). Amount indicates percent of each fraction from the purification that was used in the binding reaction (e.g., for the MonoQ fractions this is 5 μl out of 2 ml or 0.25%). (*b*) Analysis of CDEIII-binding in WT and *ctf14* extracts, in semi-purified CBF3 preparations (5 μl of MonoQ fraction 7) alone or with 2 pM of non-radiolabeled competitor DNA, and in a mixture of CBF3 and *ctf14* extract.

Table II. Microtubule and CDEIII-binding Activities of Whole-Cell Extract and CBF3

Number	Sample	Amount %	DNA binding activity %	Microtubule-binding activity (beads per field) 800-WT beads	2-WT beads
1	Wild-type extract	150	= 100 ± 15	95 ± 12	33 ± 7
2	Dialyzed ammonium sulfate precipitate	75	98	23 ± 4	2 ± 2
3	CBF3 (fraction 7)	10	102	0.4	0.9
4	CBF3 (fraction 6)	10	600	3 ± 2	nd
5	*ctf14* extract	100	bknd	4 ± 2	nd
6	CBF3 (fraction 7)	10	120	84 ± 10	39 ± 7
	+ *ctf14* extract	+100			
7	CBF3 (fraction 7)	10	nd	23 ± 4	nd
	+ *ctf14* extract	+20			

Number	Sample	Amount	Conditions	800-WT beads
8	CBF3 (fraction 7)	10	at 37°C	0.4
9	*ctf13* extract	100	at 37°C	9 ± 4
10	CBF3 (fraction 7)	10	at 37°C	39 ± 5
	+ *ctf13* extract	+100		
11	CBF3 (fraction 7)	10		2 ± 1
	+ *S. pombe* extract	+100		
12	CBF3 (fraction 7)	10		73 ± 14
	+ *ctf14* extract	+100		
	+ *S. pombe* extract	+100		

Radiolabeled CDEIII DNA was used to measure DNA binding activity on bandshift gels; the values reported here are derived from the gel shown in Fig. 2. The error in these measurements is estimated to be ±15%. Microtubule binding is normalized to 10^6 800-WT beads μl^{-1} or 2×10^6 2-WT beads μl^{-1} and a field size of 25,000 μm^2.

Additional Factors Associate with CBF3 and CEN DNA to Form Microtubule-binding Complexes

To learn more about the microtubule-binding components of kinetochores, we asked whether the components were associated with centromeric DNA. Beads were incubated in whole-cell extract, recovered from the binding reaction by low-speed centrifugation, and then washed two times with buffer containing 100 mM KCl. These washed beads retained 60–80% of the microtubule-binding activity of the original reaction, demonstrating that the primary microtubule-binding proteins are tightly associated with centromeric DNA (Fig. 3 and data not shown). Since CBF3 is necessary for kinetochore activity, we reasoned that these components might bind to DNA via association with CBF3 and that it would be possible to differentially elute microtubule-binding components from the CBF3-DNA complex by washing beads with increasingly high concentrations of salt. We found that a 100-mM KCl wash left binding activity intact and removed virtually no CDEIII-binding activity. After a 250 mM KCl wash, the microtubule-binding activity of beads declined 15-fold but only 20% of the bead-bound CDEIII-binding activity was eluted (Fig. 3 *a*). This suggested that we had efficiently removed the microtubule-binding proteins but left the great majority of the CBF3 still bound. To test this interpretation, we asked whether microtubule-binding activity could be restored to the 250 mM KCl-washed beads by the addition of factors present in *ctf14* extract. We found that the addition of *ctf14* extract stimulated microtubule binding about 20-fold up to wild-type levels (Fig. 3 *b*; similar results were obtained with *ctf13* extracts—data not shown). This stimulation suggests that the *ctf14* extract had donated microtubule-binding factors to the bead-bound CBF3 and thereby reconstituted functional kinetochore complexes. To demonstrate that bead-bound CBF3 was in fact necessary for this reconstitution, beads were washed with 600 mM KCl. This resulted in the elution of all (>95%) of the CBF3. As expected, the addition of *ctf14* extract to these beads did not cause any to bind to microtubules.

We conclude from these observations that the essential microtubule-binding components of kinetochore complexes interact with centromeric DNA but that they can be selectively eluted under conditions that leave CBF3 still bound. A similar model has been proposed to explain the microtubule-binding properties of salt-washed minichromosomes (Kingsbury and Koshland, 1993). The only *S. cerevisiae* kinetochore protein other than CBF3 to have been identified thus far is CBF1. We therefore investigated the possibility that CBF1 plays a role in assembling active kinetochore complexes. Minichromosomes with a mutation in CDEI have been reported to bind to microtubules as efficiently as minichromosomes with wild-type centromeres (Kingsbury and Koshland, 1991) but it is now clear that the mutant used in that study is only partially defective in CDEI function (Niedenthal et al., 1991). To investigate directly the role of CBF1, we used a strain in which the *CBF1* gene had been deleted. Extracts were prepared from congenic WT and *cbf1Δl* strains and the binding of 800-WT beads to microtubules was assayed over a range of extract concentrations. Alternatively, the binding of a set of limited active DNA beads was assayed with high concentrations of WT and *cbf1Δl* ex-

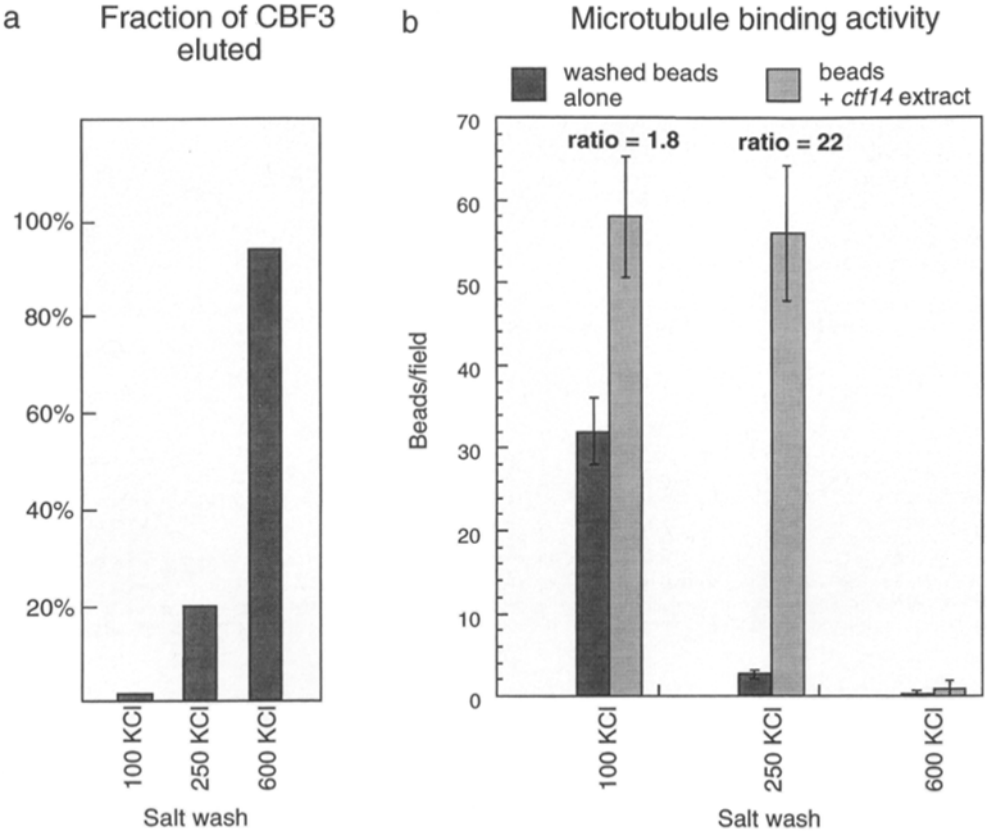

Figure 3. Analysis of salt-washed kinetochore complexes. 800-WT beads were incubated with whole-cell extract in buffer containing 150 mM KCl for 40 min, washed once with buffer containing 100 mM KCl, and then washed with either 100 mM, 250 mM, or 600 mM KCl. Washed beads were then resuspended in buffer containing 100 mM KCl and either assayed for microtubule-binding activity directly or incubated with 100 μg *ctf14* extract for 30 min, and then assayed. (*a*) Fraction of input CDEIII-binding activity eluted by various salt washes as measured on "bandshift" gels. (*b*) Microtubule-binding activity of washed beads alone (*dark bars*) and following the addition of *ctf14* extract (*light bars*). Bold numbers indicate the ratio of microtubule-binding activities of beads with and without the addition of *ctf14* extract. The addition of *ctf14* extract to the original binding reaction containing unwashed beads resulted in a 1.5-fold stimulation of microtubule-binding activity (data not shown).

tracts (Fig. 4). In neither case was a difference in microtubule-binding activity observed. This indicates that CBF1 does not play a role in the in vitro assembly of kinetochore complexes active in binding to microtubules.

Microtubule Binding Is Mediated Primarily by CDEIII but CDEII Plays an Essential Modulatory Role

Next we asked whether microtubule-binding factors interact with the CDEII or CDEIII of the centromere. Genetic analysis has revealed that both CDEII and CDEIII are required for chromosome transmission and an examination of the binding of mini-chromosomes to microtubules has demonstrated that both are essential for the assembly of active kinetochores in vivo (Kingsbury and Koshland, 1991). The comparison of CDEII sequences from different chromosomes shows that while all are A/T rich, there is no consensus CDEII sequence. CDEII and CDEIII sequences must lie in the correct orientation with respect to each other in vivo and in this sense, they appear to interact (Murphy et al., 1991). However, no CDEII-binding proteins have been identified and it has not been possible by genetic means to determine the specific functions of CDEII and CDEIII in kinetochore assembly and microtubule binding.

To investigate the function of CDEIII, beads were linked to a centromere derivative that contains the entire CDEIII element and therefore binds CBF3 efficiently in vitro (Sorger, P. K., and A. A. Hyman, manuscript in preparation) but that has vector DNA in the place of CDEI and CDEII. This derivative is similar to the X-35 construct of Gaudet and Fitzgerald-Hayes (1987) and does not form a functional kinetochore in vivo. We observed that after incubation with extract, beads carrying 800 molecules of this CDEIII-only *CEN* DNA bound to microtubules as efficiently as WT beads (Fig. 5, constructs *A* and *B*). To exclude the possibility that vector sequences introduced in the place of CDEI and CDEII had unexpected centromere activity, beads were linked to an 88-bp *CEN* truncation that comprises only CDEIII (Fig. 5 construct *C*). Although the difference in the lengths of the CDEIII-truncated and WT *CEN* DNAs prevents a fully controlled comparison of their activities, it is clear that beads with 88 bp of CDEIII bind well to microtubules. Thus, key microtubule-binding components of kinetochores appear to interact with CDEIII DNA.

We reasoned, however, that the use of beads with large numbers of active DNA molecules on each bead might obscure subtle but important differences among the microtubule-binding activities of different centromere derivatives. We therefore constructed a set of "low active density" beads that carried progressively fewer molecules of CDEIII-only *CEN* DNA. With these beads, we observed half-maximal microtubule binding at an average of 40 molecules per bead (Fig. 6 *a*, *solid line*) as compared to 2 molecules per bead for WT centromeres (*dashed line*). Kinetochores act as single units in vivo and by the key criterion of single kineto-

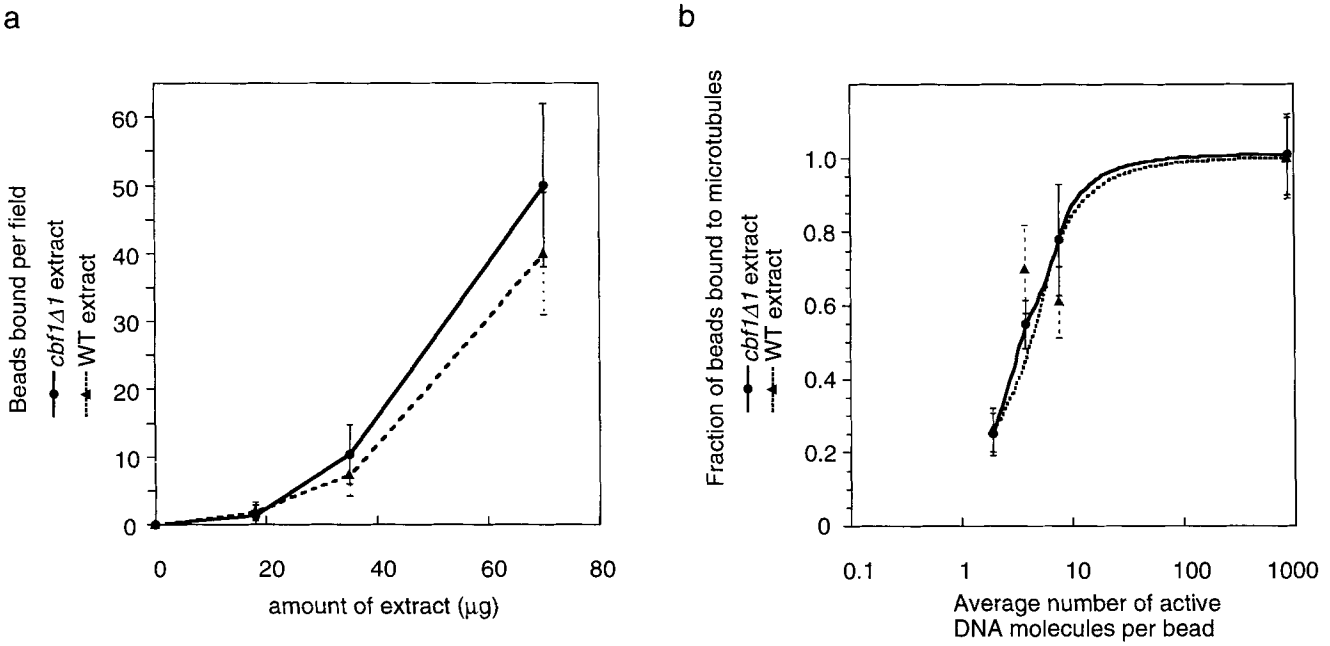

Figure 4. Analysis of the role of CBF1 in microtubule binding. (*a*) Binding of 800-WT beads to microtubules with varying amounts of extract from WT (*triangles*) or *cbf1Δ1* (*circles*) cells. (*b*) Microtubule-binding activities of beads carrying varying numbers of WT *CEN* DNA molecules per bead. Beads were incubated in 200 μg of extract from either WT (*triangles*) or *cbf1Δ1* (*circles*) cells and analyzed as described in Fig. 1.

chore binding in vitro, CDEIII-only beads exhibited no more than background levels of microtubule attachment. Thus, the efficient binding of kinetochore complexes to microtubules requires CDEII.

An important distinction between CDEII and CDEIII, is that whereas beads carrying 40 or more molecules of CDEIII-only DNA exhibit tight microtubule binding when incubated with extract, CDEII sequences alone do not mediate detectable microtubule binding even when present at 800 molecules per bead (Fig. 5, construct *E*). This suggests that

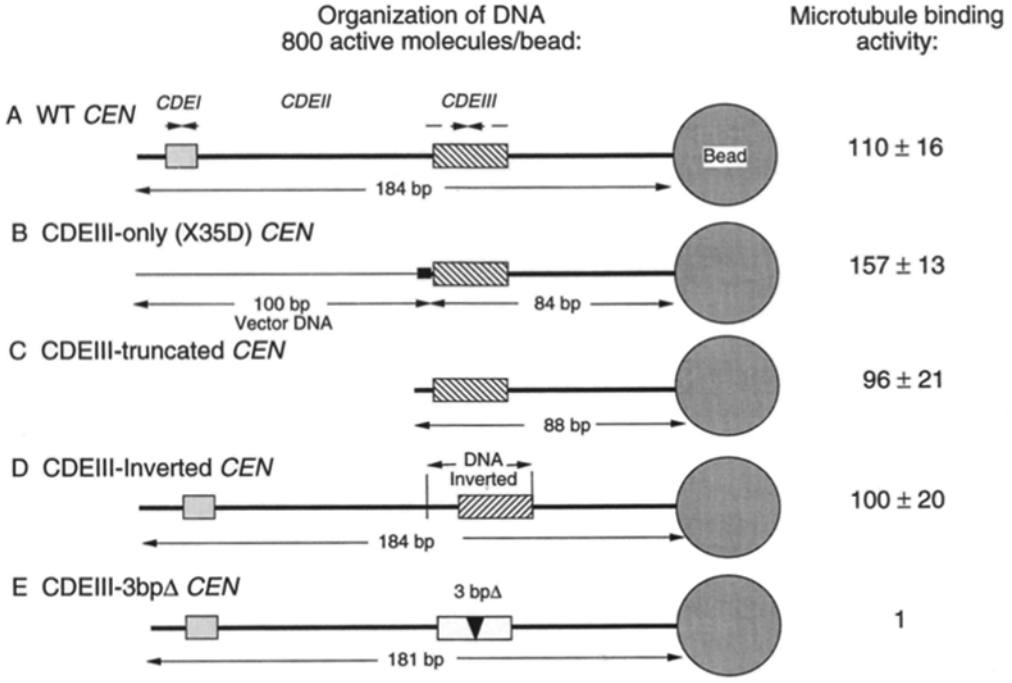

Figure 5. Structure of various centromere derivatives. CBF3 appears to bind to these derivatives with similar affinities except in the case of CDEIII-3bpΔ, which is completely inactive for CBF3 binding (Sorger, P. K., and A. A. Hyman, manuscript in preparation). The light line in the CDEIII-only *CEN* DNA denotes sequences derived from pUC19 and is 50% A-T. Binding reactions contained 200 μg of whole-cell extract and beads carried 800–1,000 DNA molecules of DNA per bead. Microtubule-binding activity is normalized as described for Table I. The difference in the first four values is not significant given the error of the measurement. This error is a combination of field-to-field variation (whose standard deviation is reported) and the error in determining the absolute number of beads present in the reaction (estimated to be ±10%; see Materials and Methods).

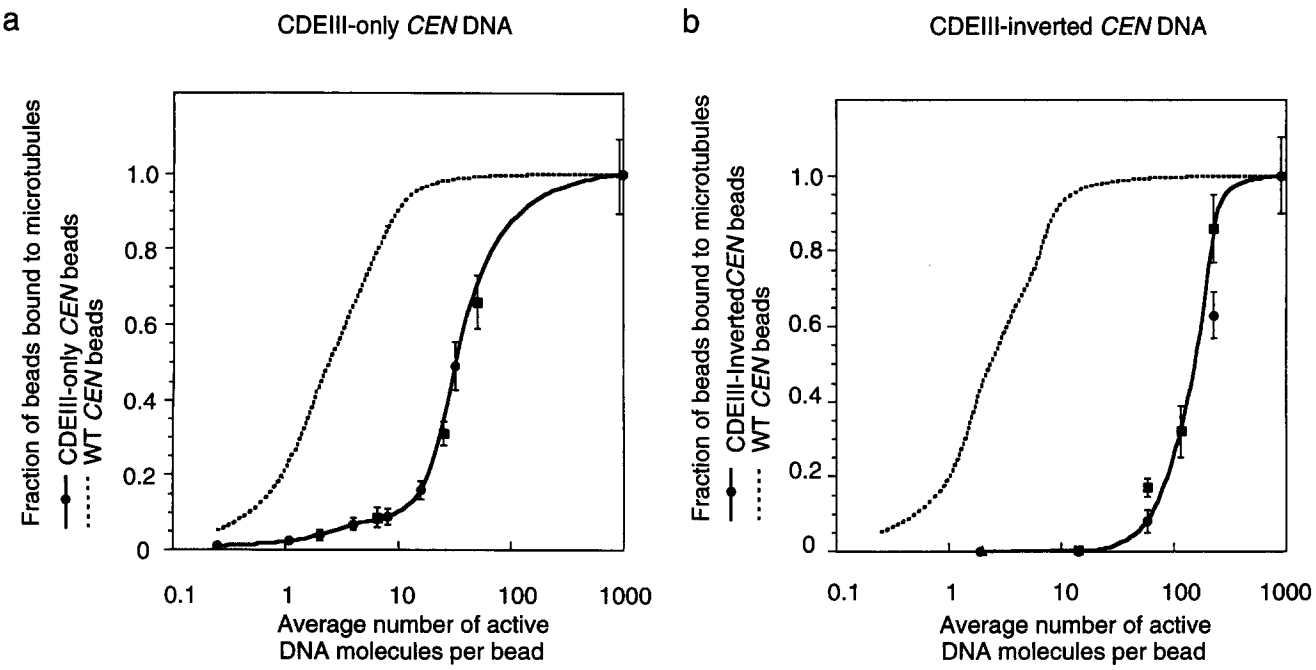

Figure 6. Microtubule-binding activities of beads carrying varying numbers of CDEIII-only and CDEIII-inverted *CEN* DNA molecules. (*a*) Microtubule-binding activity of beads carrying CDEIII-only *CEN* DNA (*solid line*) and WT *CEN* DNA as described in Fig. 1 *c* (*dotted line*). These constructs appear to bind to CBF3 with similar efficiencies (Sorger, P. K., and A. A. Hyman, manuscript in preparation). The circles and squares denote data derived from two different sets of beads that were constructed and assayed independently. The fact that these two data sets fall on the same curve is an illustration of the reproducibility of this type of assay. The CDEIII-only *CEN* has the same breakpoint as the X35 derivative analyzed in vivo by Gaudet et al. (1987). It contains all of the conserved bases in CDEIII as defined by Hegemann et al. (1988) but lacks five bases of CDEII that are within the CBF3 footprint (Lechner and Carbon, 1991). To exclude the possibility that these CDEII bases subtly alter CBF3 binding, we linked an 88-bp *CEN3* derivative containing the entire region of DNA footprinted by CBF3, to a 96-bp vector fragment to generate *CEN3Δ3*. A set of low active density DNA beads was then made with *CEN3Δ3* and microtubule binding measured. Half-maximal microtubule binding was observed to require 50 molecules of the *CEN3Δ3* derivative per bead (data not shown), confirming the results shown here for CDEIII-only *CEN* DNA. (*b*) Microtubule-binding activity of beads carrying CDEIII-inverted *CEN* DNA (*solid line*) and WT *CEN* DNA as described in Fig. 1 *c* (*dotted line*).

CDEII may operate by influencing the activity or geometry of a microtubule attachment site assembled primarily on CDEIII. One way to explore the possibility that proteins bound to CDEII and CDEIII interact, is to ask whether CDEIII must be correctly oriented with respect to CDEII for efficient microtubule binding in vitro. To do this, beads were linked to a CDEIII-inverted *CEN* in which the entire WT centromere sequence is present, but the orientation of CDEIII is reversed with respect to CDEI and CDEII. In cells, it has been observed that this CDEIII-inverted *CEN* derivative is almost completely inactive in centromere function (Murphy et al., 1991). However, it binds efficiently to CBF3 in vitro (Sorger, P. K., and A. A. Hyman, manuscript in preparation). When beads carrying CDEIII-inverted centromeres were assayed for microtubule attachment in vitro, half maximal binding was observed with 150 molecules of DNA per bead, a value 75-fold higher than that observed with WT *CEN* DNA (Fig. 6 *b*). This demonstrates that CDEII can direct the assembly of kinetochore complexes fully active in microtubule binding only when correctly oriented with respect to CDEIII. To explain these findings, we propose that CDEIII-binding factors form the primary site of attachment of kinetochores to microtubules, but that proteins bound to CDEII interact with these factors and play an essential modulatory role.

Regulation of Microtubule-binding Activity through the Cell Cycle

In organisms in which chromosome segregation can be observed directly in cells, kinetochores are assembled as the mitotic spindle is formed and disassembled as cells leave mitosis (e.g., Compton et al., 1991; Pfarr et al., 1990; Yen et al., 1991). The finding that the DNA and microtubule-binding activities of yeast kinetochores appear to be mediated by different protein complexes led us to ask which is regulated with respect to the cell division cycle (see Kingsbury and Koshland, 1991). Extracts were prepared from cycling cells, and cells arrested at START with α-factor or in mitosis with nocodazole. The DNA-binding activity of CBF3 varied by less than 30% among the extracts but microtubule-binding activity was 22-fold lower in extracts from START-arrested cells than from mitotic cells (Table III). Thus, the regulation of microtubule attachment with respect to the cell cycle involves changes in the activity of the microtubule-binding components of the kinetochore and not in the ability of CBF3 to bind to centromeric DNA.

Kinetochores from both yeast and animal cells contain kinesin-like motor proteins which may provide an important site of microtubule attachment (Hyman et al., 1992; Sawin and Scholey, 1991). The salient property of motor proteins

Table III. Cell-Cycle Regulation of the Microtubule and DNA-binding Activities of Kinetochore Complexes

Sample	Microtubule-binding activity (beads per field) 800-WT beads	800-CDEIII-3bpΔ beads	DNA-binding activity %
Cycling cells	90 ± 4	4 ± 2	= 100 ± 15
α-factor–arrested cells	4 ± 1	2 ± 1	70
Nocodazole–arrested cells	93 ± 6	5 ± 2	102

All reactions contained 100 μg of whole-cell extract and either 800-WT beads to measure microtubule-binding activity, or 800-CDEIII-3bpΔ beads to determine background levels of bead binding. DNA-binding activity was measured on non-denaturing gels using radiolabeled CDEIII DNA; an average of two determinations is shown. The error in these measurements is estimated to be ±15% (Sorger, P. K., and A. A. Hyman, manuscript in preparation). Microtubule-binding activities are normalized as described in Table I. Results of a typical experiment are shown.

is that they translocate along microtubules in the presence of ATP. To determine if yeast kinetochore complexes assembled in extracts could also slide along microtubules, beads were bound to microtubules in the presence of 1 mM caged ATP, the ATP was then uncaged with UV light, and individual beads were observed by video microscopy. Both 2-WT and 800-WT beads were filmed. In both cases, beads remained attached to microtubules in an immobile fashion. Extensive sliding of microtubules over the surface of glass was observed in the same chambers, indicating that microtubule-based motors remained active in whole-cell extracts. This shows that reassembled kinetochore complexes bind to microtubules in a static fashion and that the primary microtubule-binding component detected in vitro does not have the properties of a molecular motor. Confirming this, we observed that the deletion of the kinesin-like motor proteins *KAR3, CIN8* or *KIP1* had less than a twofold effect on microtubule-binding activity as measured by the bead assay (data not shown; see Materials and Methods for details).

Discussion

To dissect the mechanism of kinetochore action and to determine the functions of various kinetochore proteins, we have developed a method to partially reassemble kinetochores in vitro. Assembly occurs on exogenously added centromeric DNA. This DNA is linked to fluorescently labeled beads, and microtubule attachment is assayed by incubating the beads with yeast proteins, perfusing the beads into chambers containing rhodamine-labeled microtubules, and then observing binding with a fluorescence microscope. The bead assay can be used to study both the DNA and protein components of kinetochores. To study centromeric DNA, beads are constructed with progressively fewer molecules of a centromere derivative, and microtubule binding is then measured. The comparison of beads carrying different numbers of DNA molecules allows the activity of the centromere derivative to be evaluated at various levels of stringency. To study the roles of known centromere-binding factors and to identify new kinetochore components, complexes can be formed in crude extracts derived from wild-type or mutant cells. Alternatively, complexes are assembled with fractionated components and the functions of individual proteins are evaluated. We believe that the bead assay will play an important role in our long-term goal of reconstituting microtubule-binding activity in vitro from fully purified components.

The ability of the bead assay to reproduce well established genetic findings on the organization of the yeast centromere supports our conclusion that the assay measures important aspects of the attachment of kinetochores to microtubules as found in cells. In particular, we have been able to establish the following. First, kinetochores act as single units in vivo, and single or at most small numbers of reassembled kinetochore complexes appear to link centromeres to microtubules in the in vitro assay. Second, mutations in CDEIII that inactivate centromeres in vivo, abolish microtubule binding in vitro. Third, the presence of CDEII correctly oriented with respect to CDEIII is required for full centromere activity in vivo (Gaudet and Fitzgerald-Hayes, 1987) and for single kinetochore binding in vitro. Fourth, despite the importance of CDEII, CDEIII sequences are partially active in vivo on their own (Schulman and Bloom, 1993) and complexes assembled on CDEIII-only DNA exhibit some microtubule-binding activity in vitro. Fifth, *trans*-acting mutations that impair chromosome transmission in cells also inactivate kinetochore complexes in vitro. Sixth, the microtubule-binding activity detected in vitro is high in mitotic extracts and low in interphase extracts, as expected of an activity that links chromosomes to spindle microtubules during mitosis. This correspondence between in vivo and in vitro observations argues that the protein–protein and protein–DNA interactions that are responsible for microtubule binding in the bead assay are similar to the interactions that mediate the assembly of kinetochores in cells.

Under the conditions in which we observe microtubule binding by 2-WT beads, the concentration of DNA-bound CBF3 is $\sim 5 \times 10^{-12}$ molar (the total CBF3 concentration is 10–20-fold higher). Thus, only relatively high-affinity protein–protein interactions between CBF3 and other kinetochore components can be observed in the bead assay. In the cell nucleus (ca. 2 μm diam), the concentration of DNA-bound CBF3 is $\sim 2 \times 10^{-8}$ molar, assuming that a single CBF3 complex binds to each kinetochore. Thus, it seems very likely that there are protein–protein interactions mediating important aspects of kinetochore function that have not been reconstituted in vitro. For example, reassembled kinetochore complexes do not appear to translocate along microtubules (see below). We therefore believe that the current bead assay represents only a first step in the in vitro reconstitution of fully active kinetochores.

Role of CBF3 in Kinetochore Function

An important unresolved question that we have addressed directly is the role played by CBF3 in attaching kinetochores to microtubules. We have shown that functional CBF3 is absolutely essential for all detectable microtubule-binding activity in vitro. However, isolated CBF3 will not mediate efficient microtubule attachment on its own. Attachment requires the presence of additional cellular factors. This finding is in marked contrast to an earlier report that CBF3 purified by DNA affinity chromatography (Lechner and Carbon, 1991) can bind centromeres to microtubules in vitro (Hyman et al., 1992). However, a quantitative comparison of the results obtained by Hyman et al. (1992) using purified

Table IV. Comparison of Microtubule-binding Activities of CBF3 and Whole-Cell Extract

Sample	Beads bound per field (not normalized)	Beads bound per field normalized to CDEIII-binding activity	Beads bound per cell-equivalent of protein
1. Purified CBF3 Hyman et al. (1992)	3	0.6	2×10^{-3}
2. Whole-cell extract	95	650	1.5
3. Semi-pure CBF3 (this work)	0.4	2.5	6×10^{-3}

A comparison of the microtubule-binding activities of 10 μl reactions containing affinity-purified CBF3 (Hyman et al., 1992), whole-cell extract or semi-pure CBF3 (this work). In a typical reaction with whole-cell extract, 30-fold more beads are bound than with purified CBF3. However, this underestimates the difference in activity between the two reactions because 35-fold more CBF3 is present in the first reaction than in the second. Viewed in another way, the CBF3 in the first reaction is derived from 25-fold more cells than the whole-cell extract. We have therefore calculated normalized values that express the microtubule-binding activities of the reactions relative to the CDEIII-binding activities they contain (third column; expressed as a number of beads bound per fmol *CEN* DNA bound) or relative to the number of cell-equivalents of protein present in the reaction (fourth column; expressed as number of beads bound per cell). Each *S. cerevisiae* cell contains 16 chromosomes and kinetochore complexes therefore reassemble in whole cell extracts with an efficiency of about 10%. The normalized values shown here are necessarily approximate and may be in error by severalfold. They nevertheless reveal that purified CBF3 is about 1,000-fold less active in binding beads to microtubules than whole-cell extracts. The calculations used to generate this table are described in Materials and Methods; all binding reactions contained 10^6 beads μl^{-1}.

CBF3 and those obtained in this study shows that the apparent contradiction is a consequence of our newly developed and more biologically meaningful definition of efficient microtubule binding. The microtubule-binding activity detected by Hyman et al. (1992) with purified CBF3 is 1,000–2,000-fold weaker than the binding that can now be detected with whole-cell extract (Table IV). By the important criterion of single kinetochore binding, CBF3 appears to be completely inactive in linking centromeres to microtubules. However, when saturating levels of CBF3 are added to the beads carrying 300 molecules of DNA used by Hyman et al. (1992), some microtubule attachment can be detected. We believe that this weak attachment is meaningful, and reflects binding by microtubule-based motor proteins present in the CBF3 preparation as minor components. Microtubule attachment in whole-cell extracts is fundamentally different however, and has the efficiency expected of true kinetochores.

Why is microtubule binding so much more efficient in unfractionated extracts? The main reason appears to be that factors required for microtubule binding do not copurify with CBF3. The analysis of salt-washed kinetochore complexes demonstrates that microtubule-binding components elute only at KCl concentrations of 250 mM and above. In our purification of CBF3, the KCl concentration never exceeds 200 mM but factors required for microtubule attachment are almost completely separated away from CBF3. Thus, CBF3 must associate with microtubule-binding factors only as part of a ternary complex containing *CEN* DNA.

What are the identities and functions of these microtubule-binding factors? They appear to be relatively low in abundance and are not found in fission yeast, an organism whose centromeres have a very different structure from those in budding yeast. Preliminary fractionation of *S. cerevisiae* extracts suggests that there are several separable activities involved in the formation of fully active kinetochore complexes (unpublished observations). Their biochemical properties are not yet known but a simple model explaining our findings is that CBF3 forms a DNA-binding scaffold onto which additional kinetochore components assemble, including proteins that directly contact microtubules. An alternative possibility is that CBF3 subunits do in fact contact microtubules but that proteins other than CBF3 are required to achieve an active microtubule-binding conformation. The purification of factors required for the assembly of active kinetochore complexes will permit these possibilities to be distinguished.

Microtubule Binding Involves a Hierarchy of Interactions

The microtubule-binding components of reassembled kinetochore complexes appear to associate tightly with centromeric DNA, and we have not observed any essential role for soluble factors in microtubule attachment. The assembly of the microtubule attachment site appears to involve a hierarchy of interactions with different regions of the centromere. With beads carrying a large number of DNA molecules, CDEIII appears to function as a site of attachment on its own. However, CDEII DNA is required for the binding of single kinetochore complexes to microtubules. In our in vitro system, CDEII is unlikely to act by influencing chromatin assembly or by altering the topology of centromeric DNA. Instead, we postulate that CDEII binds to specific proteins that are integral components of the kinetochore. These CDEII-binding proteins may contact microtubules directly. The geometry of a bead bound to a microtubule, similar at 200,000 × to a golf ball stuck to a pencil, suggests that at most 10–20% of the DNA on the surface of the bead is accessible to a microtubule. Thus, microtubule attachment by beads with 40 molecules of *CEN* DNA that lack CDEII probably reflects simultaneous attachment by 4–8 kinetochore complexes. If this is correct, CDEII may make a substantial contribution to the energy of microtubule binding. However CDEII-associated factors appear to function only as part of a large kinetochore structure. Moreover, the requirement that CDEII and CDEIII be oriented correctly with respect to each other argues that proteins bound to CDEII and CDEIII interact directly. We therefore propose that the primary site of microtubule attachment forms on CDEIII DNA and that CDEII-associated proteins modify the activity or geometry of this site.

Why is the attachment of reassembled kinetochore complexes to microtubules static? Kinetochores in animal cells form a highly dynamic attachment to microtubules via microtubule based motors (Mitchison and Kirschner, 1985; Skibbens et al., 1993). However, the small size and poor cytology of budding yeast cells limits our knowledge of chromosome movement during yeast mitosis. Perhaps yeast kinetochores contain both motor and nonmotor microtubule-binding proteins and because kinetochores in the in vitro system are only partially reassembled they contain an excess of nonmotor proteins. Tight microtubule attachment by these proteins would prevent motility. In this case, the

removal of the tight binding proteins by fractionation would reveal the presence of motor activities, as observed with affinity purified CBF3 (Hyman et al., 1992). A similar distinction between proteins required for binding to microtubules and those required for movement along them has been suggested by antibody injection experiments in human cells (Bernat et al., 1990). An alternative possibility is suggested by the finding that each *S. cerevisiae* chromosome is attached to the mitotic spindle by a single microtubule (Peterson and Ris, 1976). Because the loss of chromosomes is lethal to a cell, binding to this single microtubule must be very tight, especially during anaphase B. Thus, we may have reconstituted a tight anaphase B-like attachment peculiar to yeast but that resembles the "parked" or "neutral" states of animal cell kinetochores (Skibbens et al., 1993).

A Model for Organization of the Yeast Kinetochore

The experiments described here allow us to construct a preliminary model for the organization of the yeast kinetochore (Fig. 7). We propose that the binding of CBF3 to DNA forms a core complex that initiates all subsequent kinetochore assembly. At least two, and possibly all three CBF3 subunits are required for binding to DNA (Sorger, P. K., and A. A. Hyman, manuscript in preparation). This binding is asymmetric and relative to the center of CDEIII, and extends further away from CDEII than toward it (Lechner and Carbon, 1991). Asymmetric binding of CBF3 to DNA is essential for kinetochore assembly and is the reason for the orientation-dependent interaction of CDEII and CDEIII (Murphy et al., 1991). The CBF3 scaffold interacts with additional CDEIII-associated proteins to form the primary site of microtubule attachment. The scaffold also promotes association of another set of cellular factors with CDEII. These CDEII-associated proteins stabilize or modify the microtubule attachment site. We imagine that the microtubule-binding components of yeast kinetochores include both motor and nonmotor proteins and that modification of the activities of these two types of proteins creates dynamic attachment during metaphase and high-affinity attachment during late anaphase. The mechanism of this proposed modification is not known, but in preliminary in vitro experiments we have observed that microtubule attachment is regulated (independently of CDEIII-binding activity) by the opposing actions of a kinase and a phosphatase.

We have found that the microtubule-binding activity of kinetochore complexes is high in mitotic extracts and low in interphase extracts but that the DNA-binding activity of CBF3 is high in both. This suggests that CBF3 binds to chromosomes throughout the cell cycle. Thus, microtubule attachment may be controlled by regulating the interaction of microtubule-binding subunits with a constitutively bound CBF3 scaffold. This sort of regulation would be consistent with data on the regulation of kinetochores in animal cells. Immunofluorescence studies of animal cell chromosomes have shown that a core protein complex localizes to the kinetochore throughout the cell cycle, but that as cells enter mitosis, a large number of additional proteins become associated with this core complex and "activate" it (e.g., Compton et al., 1991; Pfarr et al., 1990; Yen et al., 1991). As cells exit mitosis, active kinetochore complexes disassemble leaving the core interphase kinetochore.

The data presented in this paper provide a biochemical explanation for many genetic observations on the organization of the yeast centromere. They also demonstrate the existence of an as yet unidentified activity that is required for the attachment of kinetochore complexes to microtubules in vitro. The conservation of microtubule structure among all eukaryotes suggests that the microtubule-binding components of the budding yeast kinetochore will be similar to their counterparts in higher cells. The methods described here for assembling yeast kinetochore complexes in vitro should allow these microtubule-binding proteins to be isolated and the mechanism of microtubule attachment to be analyzed in molecular detail.

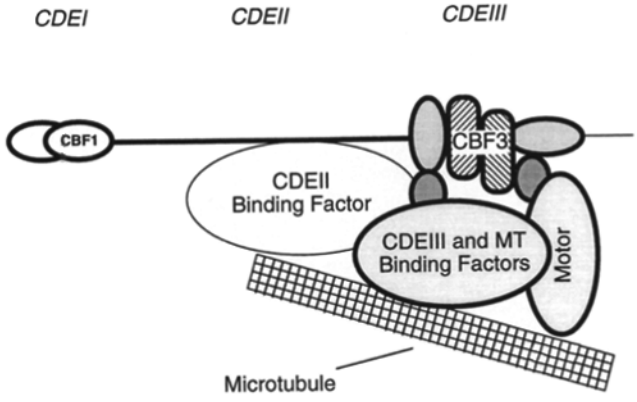

Figure 7. Speculative model for the organization of yeast kinetochore complexes that emphasizes the role of the CBF3-DNA core complex and of associated CDEII and CDEIII-binding factors.

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