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Purification of a Yeast Centromere-Binding ProteinThat Is Able To Distinguish Single Base-Pair Mutations in Its Recognition Site

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A centromere-specific DNA-binding protein has been purified to homogeneity by a combination of conventional and sequence-affinity chromatography from the yeast *Saccharomyces cerevisiae*. This protein (designated CBP-I) has an apparent molecular weight of 16,000. It binds specifically to the CDEI (centromere DNA element I) region of yeast centromere DNA, as shown by the electrophoretic mobility retardation assay and DNase I protection analysis, but does not bind specifically to other regions of yeast centromere DNA such as CDEII and CDEIII. The relative binding affinity of purified CBP-I to five different point mutations of CDEI correlates directly with the previously determined ability of each point mutation to convey centromere function in a mitotic chromosome segregation assay (J. H. Hegemann, J. H. Shero, G. Cottarel, P. Philippsen, and P. Hieter, Mol. Cell. Biol. 8:2523–2535, 1988). This supports the authenticity of CBP-I as a functional component of the yeast kinetochore.

Eucaryotic cells possess a highly reliable mechanism for chromosome segregation to ensure the accurate transmission of genetic information during cell division. A vital element involved in chromosome segregation is the unique chromosomal locus called the centromere. In prometaphase, a complex structure known as the kinetochore develops at the centromere region. Microtubule fibers attach to the kinetochore, and chromosomes are thereby able to align and migrate to spindle poles in later stages of division (for a review, see reference 16). Although the events of chromosome segregation have been well recorded cytologically, the molecular mechanism of this process is as yet unknown.

In recent years, our knowledge of the role of the centromere in chromosome segregation has been expanded by advances in two areas of research. The first area is the identification of a number of centromere-associated proteins. This work is facilitated by the discovery that human autoantibodies from scleroderma CREST patients specifically recognize centromeres of metaphase chromosomes, as detected by indirect immunofluorescence (25). By using these anticentromere antibodies, a set of centromere-specific proteins from human and other mammalian cells has been identified (2, 5). Although there are certain discrepancies among the different reports regarding the number and size of centromere proteins, at least one such protein appears to be consistently encountered (2, 5, 11, 19, 23, 24, 27, 31). The protein, named CENP-A, has an apparent molecular mass of 17 to 19 kilodaltons (kDa). Two additional centromere-specific antigens have also been found in human cells: CENP-B (80 kDa) and CENP-C (140 kDa) (5, 6).

The second area of research involves the isolation and genetic characterization of centromeric DNA from the yeast *Saccharomyces cerevisiae*. Centromeric DNAs from 12 different yeast chromosomes have been isolated and characterized (7, 15, 20, 29). Deletion studies have defined the functional centromeric DNA to approximately 120 base pairs (13, 28). Sequence comparison of these CEN DNAs has revealed three conserved sequence elements: centromere DNA element I (CDEI), CDEII, and CDEIII (Fig. 1). Extensive mutagenesis studies have been carried out for each of the three elements to determine the significance of the conserved structural features. These studies have shown that the ability of centromeres to confer mitotic and meiotic stability on artificial or native chromosomes can be impaired by mutations in each of the three CDEs (3, 10, 13, 14, 22, 26, 28). The most profound effect results from changes in the center of the partial dyad symmetry in CDEIII (14, 22, 26).

The current studies on centromere function in both of the systems described above have their respective limitations. Although the immunological techniques in the mammalian system have established the authenticity of centromere-specific proteins, there is no functional assay available in this system for the centromere and its associated proteins. Studies of the yeast system, on the other hand, have focused largely on the genetic analysis of centromeric DNA, while information on the nature and function of the proteins associated with the centromere is lacking. Although there have been a few reports on the detection of yeast centromere-binding proteins in crude extracts (1, 26), these proteins have not been purified to homogeneity to allow accurate characterization.

In this report, the purification and characterization of a yeast centromere-binding protein is presented. The protein, designated CBP-I (centromere-binding protein I), is the first centromere-binding protein to have been purified to homogeneity. This protein binds CDEI, and the sequence specificity of its binding in vitro correlates with the known sequence requirements determined for CDEI function in vivo.

MATERIALS AND METHODS

Centromere-containing plasmids and yeast strains. A 150-base-pair *Eco*RI fragment of the plasmid pCT116 (gift of C.

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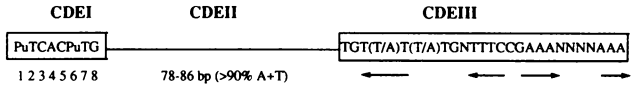

FIG. 1. The consensus structure of the centromere DNA of *S. cerevisiae*. CDEI contains an 8-base-pair (bp) sequence (Pu, purine) which is 100% conserved in all known yeast centromeres. Numbers below CDEI designate the position of each base pair referred to in the text. CDEII (78 to 86 base pairs) has an unusually high A+T content (>90%) but lacks recognizable conservation of primary sequence. CDEIII (25 base pairs) contains an axis of partial dyad symmetry indicated by arrows beneath it.

Traver) containing functionally intact CEN4 DNA was used as a probe for electrophoretic mobility retardation assays throughout purification. The following plasmids, containing the wild type or one of the five point mutations in positions 7 and 8 of CDEI sequence of CEN6, were provided by J. Shero and P. Hieter: pJS31 (wild type), pJS41 (CDEI-7A), pJS42 (CDEI-7C), pJS43 (CDEI-7G), pJS51 (CDEI-8T), and pJS52 (CDEI-8A). Plasmids containing CDEI or CDEIII consensus sequences were gifts of S. Elledge. A wild-type diploid yeast strain, SK1 (18), was used as a source for protein purification.

Nuclear extracts. The method for isolation of yeast nuclei was derived from that of Mann and Mecke (21). One liter of rich medium (1% yeast extract, 2% Bacto-Peptone, 2% glucose) was inoculated with SK1 and allowed to grow until the optical density at 600 nm reached 3 at 30°C. Cells were collected, washed once with 20 ml of 1.3 M sorbitol, and suspended in 20 ml of 1.3 M sorbitol–50 mM phosphate buffer (pH 6.5)–1 μl of β-mercaptoethanol per ml. Zymolyase 100T (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) was added to a final concentration of 0.4 mg/ml. After 1 h of digestion at 30°C, cells were washed three times with 20 ml of 1.3 M sorbitol and then suspended in 10 ml of 20 mM phosphate buffer (pH 6.5)–1 mM $MgCl_2$–1 mM phenylmethylsulfonyl fluoride–20% Ficoll (Pharmacia Fine Chemicals, Piscataway, N.J.). The suspension was homogenized with a glass Dounce tissue grinder and centrifuged at 4,000 × *g* for 15 min. The supernatant was transferred to a clean tube and centrifuged again at 25,000 × *g* for 30 min. The pellet was suspended with 3 ml of 20 mM phosphate buffer (pH 6.5)–0.5 mM $MgCl_2$–1 mM phenylmethylsulfonyl fluoride–20% Ficoll and overlaid on 2 ml of the same buffer containing 50% Ficoll, and the nuclei were pelleted at 100,000 × *g* for 90 min in a rotor (model SW50.1; Beckman Instruments, Inc., Fullerton, Calif.). The soluble nuclear extract was made from the pellet by the procedure of Dignam et al. (4).

Electrophoretic mobility retardation assay for DNA-protein interaction. Centromere DNA was end labeled by the Klenow fragment of DNA polymerase I. The typical binding reactions contained 10 mM Tris hydrochloride (pH 7.4), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 1 to 5 μg of poly(dI · dC) (Sigma Chemical Co., St. Louis, Mo.), and 10 fmol of ^{32}P-labeled CEN4 probe in a volume of 50 μl. Protein fractions were added, and the reactions were incubated at room temperature for 30 min. A 20-μl portion of each reaction mixture was loaded onto a 5% nondenaturing polyacrylamide gel. Electrophoresis was allowed to proceed at 20 V/cm of gel for 45 min to 1 h in 6.7 mM Tris hydrochloride (pH 8.0)–3.3 mM sodium acetate–1 mM EDTA. Gels were wrapped with Saran Wrap and autoradiographed at −80°C with an intensifying screen.

DNase I protection analysis. The DNase I footprinting method of Galas and Schmitz (8) was modified as follows. The binding reaction was same as in the electrophoretic mobility retardation assays, except that probes were labeled at one end only. After a 30-min incubation, 5 μl of 10× DNase I buffer (1× DNase I buffer is 10 mM $MgCl_2$ plus 5 mM $CaCl_2$) and 3 ng of DNase I (Worthington Diagnostics, Freehold, N.J.) were added. After 1 min at room temperature, an equal volume of stop buffer (200 mM NaCl, 20 mM EDTA, 1% sodium dodecyl sulfate [SDS], 2 μg of yeast tRNA per ml) was added. The mixture was phenol extracted, ethanol precipitated, and electrophoresed on an 8% denaturing polyacrylamide gel.

Renaturation of proteins from SDS-polyacrylamide gels. The method of Hager and Burgess (12) was used with the following modifications. (i) Slab gels instead of cylindrical gels were used. (ii) Gels were usually not stained before slicing. When they were, Coomassie blue R-250 instead of KCl was used. (iii) Acetone precipitation was performed at −80°C overnight instead of for 30 min in dry ice.

CDEI sequence affinity column. The following oligonucleotide sequences were synthesized by Operon Technologies, Inc. (San Pablo, Calif.): 5′-AATTCAAAAGGTCACATGCT TATAAG-3′ and 5′-AATTCTTATAAGCATGTGACCTTT TG-3′. The sequences were allowed to anneal and polymerize in the presence of DNA ligase. The ligation products ranged from trimers to high-molecular-weight molecules that could not enter 4% polyacrylamide gel (data not shown). The procedure of Kadonaga and Tjian (17) was used to couple the ligation mixture to Sepharose CL-2B. The coupling efficiency was estimated to be about 10%.

Purification of CBP-I. All steps were performed at 4°C. Frozen cells (SK1) (300 to 400 g) were allowed to thaw overnight at 4°C, washed once with 0.7 M NaCl in TEG buffer (50 mM Tris hydrochloride (pH 7.4), 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 μl of β-mercaptoethanol per ml), and suspended in same buffer to give a final volume of 800 ml. Cell suspension (200 ml) was mixed with glass beads (100 ml) and disrupted with the Beadbeater (Biospec Products, Bartlesville, Okla.) (four 2-min beatings with a 5-min cooling interval). Glass beads, unbroken cells, and some cell debris were pelleted by low-speed centrifugation (4,000 × *g* for 10 min). The supernatant was spun again at 15,000 × *g* for 1 h, and the second supernatant was passed through a DE52 column (300-ml bed volume) equilibrated with TEG plus 0.7 M NaCl. The column was washed with 1 volume of the same buffer, and the flowthrough fractions were combined (crude cell extracts).

Crude cell extracts, diluted with 3 volumes of TEG without NaCl, were loaded onto a Biorex 70 column (300-ml bed volume) equilibrated with TEG plus 0.1 M NaCl. The column was washed with the same buffer until no protein was detected (A_{280}) in the flowthrough. Bound proteins were eluted with a linear salt gradient (3,000 ml, 0.1 to 1.0 M NaCl). Fractions containing centromere-binding activity were pooled, dialyzed overnight against 10 volumes of TEG plus 0.1 M NaCl with two changes, and the desalted fractions were loaded on a DNA-cellulose column (Pharmacia; 60-ml bed volume) equilibrated with TEG plus 0.1 M NaCl. The column was washed with 500 ml of TEG plus 0.1 M NaCl and eluted with a linear salt gradient (800 ml, 0.1 to 1.0 M NaCl). Active fractions were pooled and diluted with TEG to a final salt concentration of 0.15 M and immediately loaded on a CDEI sequence affinity column (10-ml bed volume). The column was washed with 300 ml of TEG plus 0.15 M NaCl and eluted with a linear salt gradient (200 ml, 0.15 M to 1.0 M NaCl). Centromere-binding fractions were

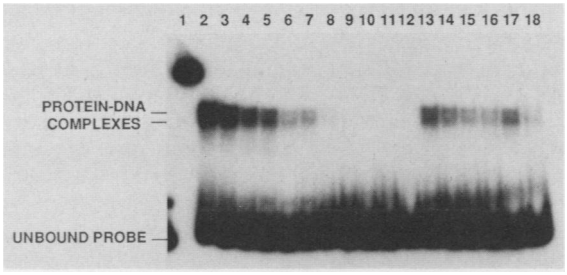

FIG. 2. Centromere-specific DNA-binding proteins in yeast nuclear extracts detected by electrophoretic mobility retardation assay. The binding reactions were performed as described in Materials and Methods. Each reaction had 0.5 ng (5 fmol) of ^{32}P-CEN4 DNA probe and 1.5 μl (1.8 mg of protein per ml) of nuclear extracts. Poly(dI · dC), which was used as a nonspecific competitor, was present at 0 (lane 1), 0.5 (lane 2), 1 (lane 3), 2 (lane 4), 4 (lane 5), 10 (lane 6), and 2 (lanes 7 through 18) μg. The specific competitor for binding, unlabeled wild-type CEN6 DNA, was present at 10 (lane 7), 30 (lane 8), 50 (lane 9), 100 (lane 10), 200 (lane 11), and 500 (lane 12) fmol. Unlabeled centromere DNA with a point mutation in the CDEI region, CDEI-8A, was present at 10 (lane 13), 30 (lane 14), 50 (lane 15), 100 (lane 16), 200 (lane 17), and 500 (lane 18) fmol.

pooled, and the fractions were divided in small volumes and stored at −80°C.

RESULTS

CDEI-binding activities in yeast nuclear extract. The electrophoretic mobility retardation assay (9) was used to identify centromere-specific binding proteins in nuclear extracts. Two bands with retarded mobilities presumably corresponding to DNA-protein complexes emerged when the nonspecific competitor poly(dI · dC) was added to the binding reactions (Fig. 2). Both bands persisted when the ratio (by weight) of poly(dI · dC) to CEN4 probe was increased to 20,000:1 (Fig. 2, lane 6). Their appearance, however, was sensitive to competition by wild-type CEN6 DNA, and both bands vanished completely when the molar ratio of unlabeled CEN6 to CEN4 probe reached 20:1 (Fig. 2, lane 10). In contrast, unlabeled CEN6 DNA that contained a single base-pair mutation in CDEI competed poorly and failed to eliminate the retardation of CEN4 probe even when present in 100-fold molar excess (Fig. 2, lane 18). The mutation in CDEI used here, CDEI-8A, is a substitution of A for G at position 8 of CDEI and has been shown previously to impair centromere function in an in vivo assay (14). This result demonstrates that the protein-DNA complexes seen on the nondenaturing gel were formed between CEN DNA and CDEI-specific binding proteins. It also suggests that the effect of CDEI point mutations on chromosome stability as revealed by genetic studies (14) was due to their weakened interaction with specific binding proteins (see below). Similar results were obtained with labeled oligonucleotides containing only the CDEI sequence. This CDEI probe gave rise to two bands with retarded mobilities which resemble the pattern in Fig. 2. These bands were also sensitive to competition by excess unlabeled wild-type CEN6 DNA (data not shown). Proteins which bind specifically to other regions of CEN DNA were not detected in these nuclear extracts, although in the later stage of this work, a weak CDEIII-binding activity was observed in a partially purified fraction (see Discussion).

Purification of CBP-I to homogeneity. Sequence-specific DNA-binding proteins can be purified efficiently by sequence affinity columns (17). Because of the small capacity of such columns, it is often necessary to first obtain a significant enrichment by conventional chromatographic methods. Whole-cell extracts were prepared as described in Materials and Methods and loaded onto a DEAE-cellulose column to deplete nucleic acids. The crude cell extracts were diluted fourfold to decrease the salt concentration and fractionated on a Biorex 70 ion exchange column. One centromere-binding activity was detected (Fig. 3a). The activity was found in fractions ranging from 0.4 to 0.55 M NaCl. These fractions were pooled, dialyzed against low-salt buffer, and fractionated on a DNA-cellulose column. Centromere-binding activity was detected in 0.4 to 0.55 M NaCl fractions (Fig. 3b). Active fractions were combined and adjusted to 0.15 M salt concentration by dilution, loaded on a sequence affinity column, and eluted with a linear salt gradient (0.1 to 1.0 M NaCl). Fractions were analyzed for protein composition (Fig. 4a) and CEN-binding activity (Fig. 4b). It is evident that the majority of DNA-binding proteins (present in the DNA-cellulose-purified fractions [Fig. 4a, lane F]) were unable to bind to the sequence affinity column at 0.15 M NaCl, and those which did bind to the column could be eluted before the CEN-binding activity, which coincided with the elution of a 16-kDa protein (Fig. 4a, lane J; Fig. 4b, fraction 6). Lanes A and B of Fig. 4a represent 5- and 10-fold, respectively, the amount of fraction 6 loaded in lane J. The 16-kDa protein in this fraction was apparently homogeneous.

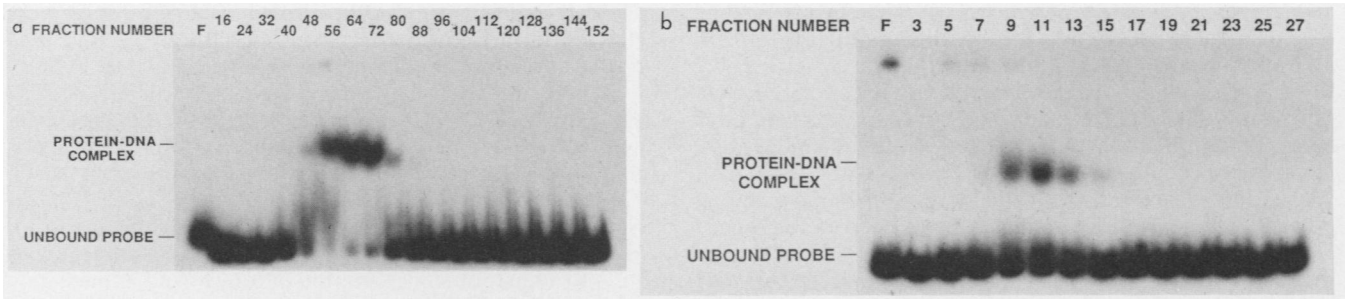

FIG. 3. Electrophoretic mobility retardation assays of fractions from Biorex 70 (a) and DNA cellulose (b) column chromatographies. ^{32}P-labeled CEN4 DNA was used as a probe. Poly(dI · dC) (2 μg) was present in each binding reaction, regardless of protein concentration of the fractions. A 3-μl portion of each fraction was taken into assay. After 30 min of binding reaction, the mixtures were loaded onto a 5% nondenaturing polyacrylamide gel and electrophoresed for 1 h. One centromere-binding activity was detected.

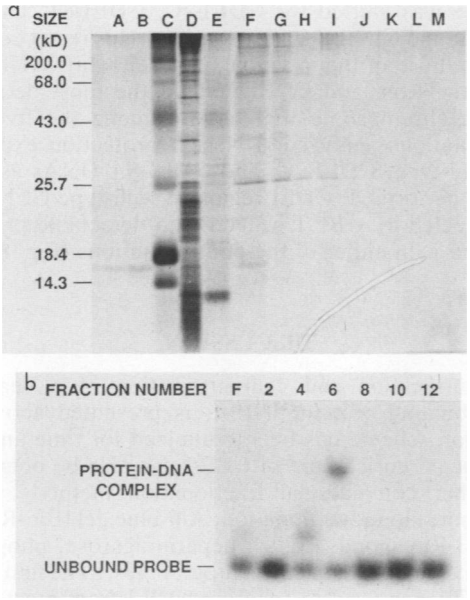

FIG. 4. Final purification of a centromere-binding protein by sequence affinity column chromatography. (a) Silver staining of the SDS-polyacrylamide gel run with proteins from different fractions. Gel concentration was 12.5%. Lanes: A, 25 μl of fraction 6 which had been concentrated 10-fold by Centricon microconcentrators (Amicon Corp., Lexington, Mass.); B, 50 μl of the material as in lane A; C, molecular weight standards; D, crude cell extracts (7 μg of proteins); E, Biorex 70-purified binding activity (4.0 μg of proteins); F, DNA-cellulose-purified binding activity (3.5 μg of proteins); G through M, fractions from CDEI affinity column. They were flowthrough fraction (lane G), linear salt gradient fraction 2 (lane H), fraction 4 (lane I), fraction 6 (lane J), fraction 8 (lane K), fraction 10 (lane L), and fraction 12 (lane M). A 50-μl portion of each fraction was loaded on gel. (b) Electrophoresis mobility retardation assay for CDEI affinity column fractions. ^{32}P-labeled CEN4 was used as a probe. A 5-μl portion of each fraction was taken into assay. Fraction numbers are indicated at the top.

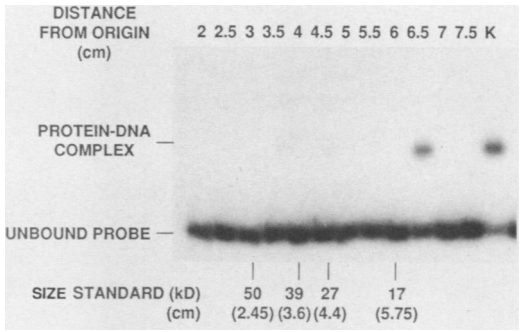

FIG. 5. Electrophoretic mobility retardation assay of centromere-binding activity for fractions renatured from SDS-polyacrylamide gel slices. Proteins were electrophoresed in a 12.5% SDS-polyacrylamide gel. The gel was then cut into 0.5-cm slices without prior staining. Numbers at the top represent the distance (in centimeters) of each gel slice from the origin of the separation gel. Lane K, Assay of renatured 16-kDa protein sliced out directly from a Coomassie blue-stained gel. Molecular weight standards are shown at the bottom.

To confirm that the CEN-binding activity in fraction 6 of the sequence affinity column chromatography was due to the 16-kDa protein rather than to another protein(s) undetected on the SDS gel, a protein renaturation experiment was performed. Fraction 6 (20 μl, ten-fold concentrated) was subjected to SDS-polyacrylamide gel electrophoresis, and the gel was cut into 0.5-cm slices. Protein was extracted and allowed to renature by the procedure of Hager and Burgess (12). Figure 5 shows the results of the electrophoretic mobility retardation assay of the renatured fractions. The CEN-binding activity was present at the position where the 16-kDa protein migrated (Fig. 5, 6.5-cm fraction). This protein was also able to renature after Coomassie blue staining (Fig. 5, lane K), and recovery of the activity in both cases was over 80% (data not shown). Thus, the 16-kDa protein alone was responsible for the centromere-binding activity.

The purification of the 16-kDa CEN-binding protein, CBP-I, is summarized in Table 1. By three chromatographic steps, the protein was enriched 7,690-fold, with an overall recovery of 10% of the binding activity present in crude extracts. It should be noted, however, that the specific activity of the crude cell extracts might be underestimated because of the possible presence of binding inhibitors.

Throughout purification, the intact CEN4 sequence was used as a probe to monitor CEN-binding activity. Although CBP-I was eventually purified by its ability to bind to a CDEI sequence affinity column, more experiments were needed to define its recognition site on CEN DNA. As analyzed in an electrophoretic mobility retardation assay, purified CBP-I binds both intact CEN4 and CDEI fragments but not CDEIII fragments, and its binding to CEN4 probe was sensitive to the competition of unlabeled CDEI oligonucleotides (data not shown). The CBP-I recognition site on CEN DNA was further localized by DNase I protection analysis (Fig. 6). Clearly, the CDEI sequence is the only region of the centromere protected from DNase I digestion by CBP-I.

Interaction of purified CBP-I with various CDEI point mutations of CEN6 DNA. Hegemann et al. (14) generated a series of 26 mutations in CEN6 DNA, 6 of which resided in CDEI. All of the six CDEI point mutations give rise to impaired centromere function, albeit to different degrees (14). One of these point mutations, CDEI-8A, which had a significant effect on centromere function, failed to compete with CEN4 probe for CDEI-specific binding proteins in the electrophoretic mobility retardation assay performed with

TABLE 1. Purification of CBP-I

Activity fraction	Volume (ml)	Total protein (mg)	Activity[a]		% Yield		Purification (fold)	
			Total	Specific	Per step	Overall	Per step	Overall
Crude extract	1,440	18,720	748,800	0.04				
Biorex 70	675	270	945,000	3.5	126	126	87.5	87.5
DNA-cellulose	141	12.21	223,443	18.3	23.6	29.7	5.2	455
CDEI affinity	60	0.24	74,400	310	33.3	9.9	16.9	7,690

[a] Femtomoles of ^{32}P-CEN DNA retarded per microgram of protein.

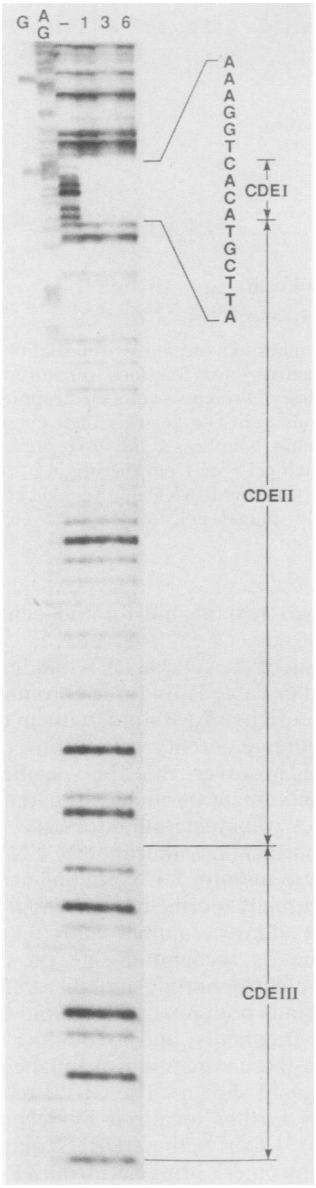

FIG. 6. DNase I protection experiment of CEN4 probe with purified CBP-I. Shown are G-specific (lane G) and AG-specific (lane AG) sequencing reactions and DNaseI digestions in the absence of CBP-I (lane −) and in the presence of 1 (lane 1), 3 (lane 3), and 6 (lane 6) μl of CBP-I. Each reaction contained 1 ng of probe. CBP-I was present at a concentration of 4 ng/μl. CDEI, CDEII, and CDEIII regions are indicated. The protected sequence is also shown.

yeast nuclear extracts (Fig. 2). If purified CBP-I is the protein that interacts with CDEI and contributes to centromere functions in vivo, mutations in CDEI known to diminish centromere function will likely lead to a decrease of binding affinity of CBP-I. To test this hypothesis, five of the CEN6 CDEI mutations were tested for their affinity to purified CBP-I protein. Figure 7 shows a quantitative measurement of CBP-I-binding activity of five point mutations with wild-type CEN6 as a control. All mutants exhibited a significant decrease in binding activity compared with the wild type (Fig. 7b). Under conditions in which 50% of the wild-type probe was retarded, only 15, 10, 4, 2, and 0% of the probe was shifted for CDEI-7C, CDEI-8T, CDEI-7G, CDEI-7A, and CDEI-8A, respectively. This result correlates well with those of the in vivo experiments, in which CDEI-7C had the least and CDEI-8A had the most detrimental effect on chromosome segregation among the five CDEI point mutations analyzed. DNase I protection experiment using wild-type, CDEI-7C, and CDEI-8A DNAs as probes was also performed. CDEI region of wild-type CEN6 DNA was protected by CBP-I, whereas no detectable protection was found with either of the point mutations (Fig. 8).

DISCUSSION

The purification and characterization of a yeast centromere-binding protein, CBP-I, is presented above. The purification scheme has been optimized for time and yield. (A significant enrichment of CBP-I can also be obtained by using other conventional fractionation methods such as ammonium sulfate fractionation, Affi-blue gel [Bio-Rad Laboratories, Richmond, Calif.], heparin agarose, phosphocellulose, and gel filtration chromatographies.) The first column (Biorex 70) was used to separate CBP-I from most cellular proteins (87-fold enrichment), and remaining proteins were then fractionated on the basis of their DNA-binding properties on DNA-cellulose. The contaminated DNA-binding proteins in turn were separated from CBP-I because of their weaker binding to CDEI sequence on the oligonucleotide affinity column. The same strategy has also been successfully used by others (30).

Although there are conceivably multiple protein factors interacting with CEN DNA, CBP-I was the only major centromere-binding protein detected during the purification. Other potential CEN-binding proteins could be present in considerably lower abundance than CBP-I. A CDEIII-binding protein was found in the Biorex 70 fractions overlapping CBP-I activity (data not shown). This activity was severalfold weaker than that of CBP-I and not detectable in nuclear extracts. It became observable only after Biorex 70 enrichment, and its detection required prolonged exposure of the autoradiograph (48 h with intensifying screen, compared with 6 h under the same condition for CBP-I). This CDEIII-binding activity may be the same as that described by Ng and Carbon (26). In addition, the CDEI-specific double bands detected with nuclear extracts in Fig. 2 may be evidence of additional centromere-associated proteins. When nuclear extracts used in the experiment shown in Fig. 2 were subjected to SDS-polyacrylamide gel electrophoresis and renaturation analysis, results similar to those in Fig. 5 were obtained (data not shown). The 16-kDa CBP-I was the only clearly detectable activity present in the gel slices despite the fact that the original extracts gave rise to two quantitatively comparable protein-DNA complexes. It is thus unlikely that the two complexes are the result of partial degradation of a single protein. Furthermore, these two complexes could not be generated by different CBP-I stoichiometry because purified CBP-I consistently produced only a single band, corresponding to the lower band of the doublet in crude extracts, over an at least 100-fold range of protein concentration (data not shown). These results suggest that the upper band in Fig. 2 may be a result of cooperative binding of a heterogeneous protein complex, of which CBP-I could be one component.

Our in vitro binding analysis with purified CBP-I and various CDEI point mutations correlates with the in vivo genetic studies performed by Hegemann et al. (14). Accord-

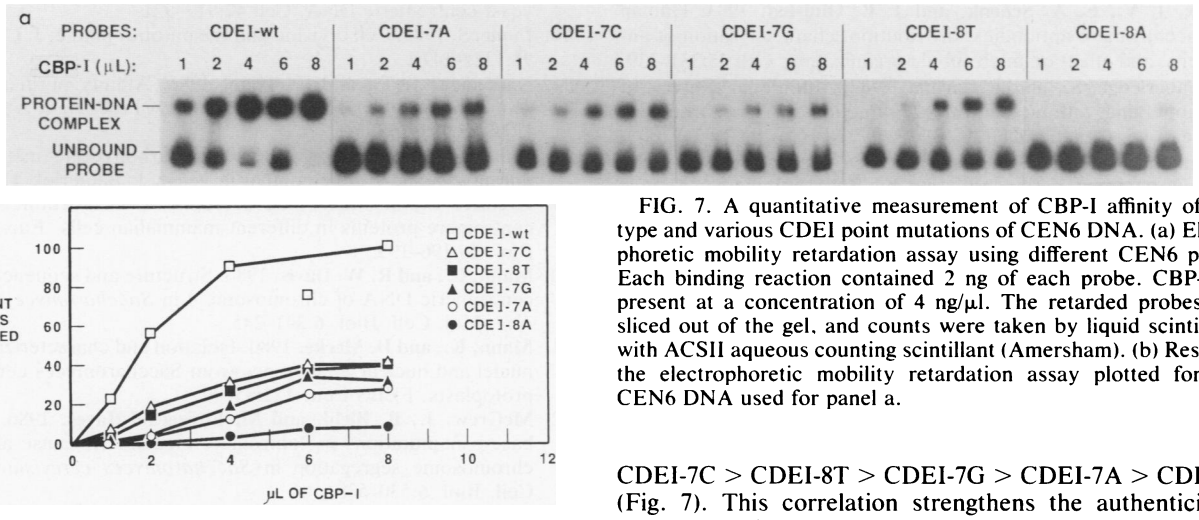

FIG. 7. A quantitative measurement of CBP-I affinity of wild-type and various CDEI point mutations of CEN6 DNA. (a) Electrophoretic mobility retardation assay using different CEN6 probes. Each binding reaction contained 2 ng of each probe. CBP-I was present at a concentration of 4 ng/μl. The retarded probes were sliced out of the gel, and counts were taken by liquid scintillation with ACSII aqueous counting scintillant (Amersham). (b) Results of the electrophoretic mobility retardation assay plotted for each CEN6 DNA used for panel a.

ing to these authors, the degree of disruption of centromere function attributable to the various point mutations of CDEI follows the order CDEI-7C (1.7) < CDEI-8T (2.3) < [CDEI-7A (2.6) CDEI-7G (2.7)] < CDEI-8A (4.4) (numbers in parentheses represent the fold decrease of mitotic chromosome stability compared with that of wild-type CEN6). This is the same order of their affinity towards purified CBP-I as assayed in our quantitative binding experiment, which is CDEI-7C > CDEI-8T > CDEI-7G > CDEI-7A > CDEI-8A (Fig. 7). This correlation strengthens the authenticity of CBP-I as a kinetochore component.

The T and G residues on positions 7 and 8 of wild-type CDEI are 100% conserved among all known yeast centromeres, implying their importance in centromere function. The in vitro binding study suggests that position 8 plays a major role in the interaction with binding protein(s), since CDEI-8A is almost totally incapable of binding CBP-I under our assay conditions.

Bram and Kornberg (1) reported identification of a CDE-I-specific binding activity (CP-1) in yeast cell lysates by filter binding assays which migrates with an apparent size of 57 to 64 kDa on an SDS-polyacrylamide gel and thus is substantially different from CBP-I in size. It is not clear whether the CP-1 is related to CBP-I, because proteins smaller than 22 kDa were not examined in their experiment (1). The 16-kDa CBP-I therefore might have been present in their partially enriched extract but escaped detection. On the other hand, we did not succeed in attempts to detect a ~60-kDa CDEI-binding protein from either crude extracts or partially enriched fractions. We cannot, however, rule out the possibility that this protein had been proteolytically degraded in the very early stage of our purification, either to CBP-I or to some other undetectable fragments. Isolation and characterization of the gene coding for CBP-I can now be directly accomplished because the protein exists in a homogeneous form. Sequence analysis of the CBP-I gene will ascertain the initial size of its protein product.

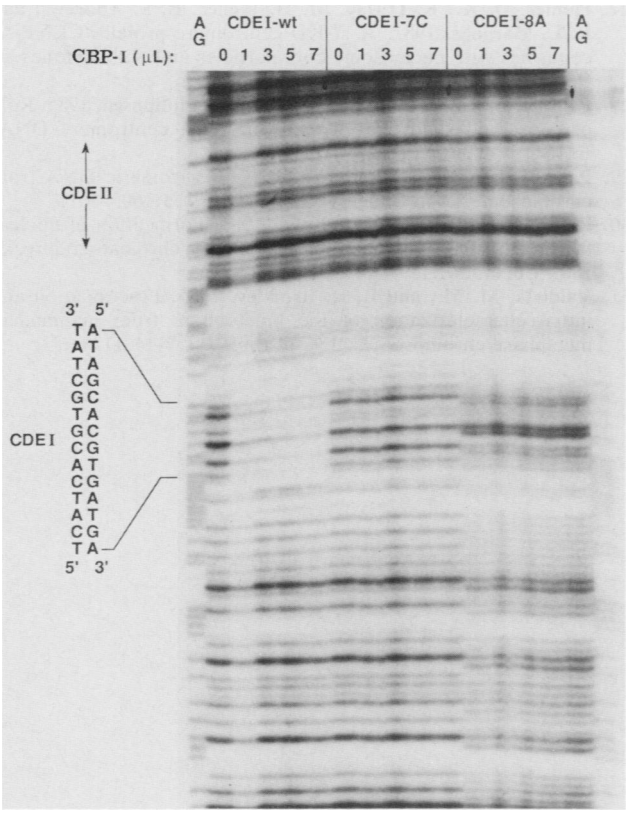

FIG. 8. DNase I protection experiment with wild-type, CDEI-7C, and CDEI-8A CEN6 DNAs. Each reaction contained 10 ng of the indicated probe. CBP-I was present at a concentration of 4 ng/μl. The CDEI sequence is indicated.

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