# A Colony Color Assay for *Saccharomyces cerevisiae* Mutants Defective in Kinetochore Structure and Function

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Manuscript received August 20, 1991
Accepted for publication May 12, 1992

ABSTRACT

We have designed a colony color assay for monitoring centromere DNA-protein interactions in yeast (*Saccharomyces cerevisiae*). The assay is based on the ability of centromere DNA sequences to block (in *cis*) transcription initiated from a hybrid *CEN-GAL1* promoter. Using a *lacZ* reporter gene under control of the *CEN-GAL1* promoter, we screened colonies derived from mutagenized cells for a blue color phenotype indicative of derepression of the hybrid construct. A limited screen in which a 61-bp *CEN11* DNA fragment containing an intact CDEIII subregion plus flanking sequences was used as the "pseudo-operator" led to the identification of mutations (*blu*) in three complementation groups. The *blu1* mutants exhibited a decrease in activity of the major *CEN* DNA-binding proteins *in vitro*. The *BLU1* gene was shown to be identical to the previously isolated *SPT3* gene, known to be involved in the transcriptional regulation of a subset of yeast genes. Our results indicate that the *BLU1/SPT3* gene product may also be required to maintain optimal levels of functional centromere DNA-binding proteins.

THE centromere/kinetochore of higher eukaryotic chromosomes is a highly complex structure involving many proteins and large amounts of DNA (RIS and WITT 1981; BRINKLEY *et al.* 1989; PLUTA, COOKE and EARNSHAW 1990). In lower eukaryotes, functional centromere DNA (*CEN* DNA) has been isolated from both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (reviewed in CARBON and CLARKE 1990; CLARKE 1990). The *CEN* DNA from *S. cerevisiae* appears to be extremely simple both in terms of its size (about 125 bp) and organization (HIETER *et al.* 1985; FITZGERALD-HAYES 1987; COTTAREL *et al.* 1989). The consensus *CEN* sequence contains three functional domains, termed CDEs (*c*entromere *D*NA *e*lements). The CDEI subregion appears to be important but not essential for centromere function (CUMBERLEDGE and CARBON 1987; PANZERI *et al.* 1984); the CDEII subregion acts as a high (A + T) spacer between CDEI and CDEIII (CUMBERLEDGE and CARBON 1987; GAUDET and FITZGERALD-HAYES 1987; HEGEMANN *et al.* 1988); and the CDEIII subregion (Figure 1B) is critical for centromere function. Deletions or single point mutations within the central portion of CDEIII completely inactivate the centromere (MCGREW, DIEHL and FITZGERALD-HAYES 1986; NG and CARBON 1987; HEGEMANN *et al.* 1988), suggesting that proteins interacting directly with the CDEIII sequence must play an important role in centromere function.

Two different experimental strategies have been used in our laboratory to identify these CDEIII binding factors. Purification of proteins specifically binding to a DNA fragment containing a wild-type *CEN3* sequence led to the identification of a multisubunit complex (CBF3) made up of three different polypeptides (CBF3A, CBF3B, CBF3C) (NG and CARBON 1987; LECHNER and CARBON 1991). In this paper, we describe a genetic screen for mutations leading to a decrease in the levels of CDEIII binding factors. Our assay is based on the ability of specific centromeric DNA fragments to act as "pseudo-operators" in *S. cerevisiae* when inserted within the *GAL1* promoter of a *GAL1-lacZ* fusion (NG, NESS and CARBON 1986). Cells harboring a hybrid plasmid with a 61-bp *CEN11* fragment containing an intact CDEIII region as an insert in the *GAL1* promoter form pale blue colonies when grown on galactose medium in the presence of X-gal, whereas cells containing a *GAL1-lacZ* fusion lacking the CDEIII insert form dark blue colonies. This phenomenon is presumably due to the binding of specific CDEIII-binding proteins to their recognition sequence within the hybrid promoter and the subsequent inhibition of *GAL1*-specific transcription. We reasoned that mutant cells containing decreased levels of the CDEIII binding activity would exhibit at least a partial release of the CDEIII-mediated repression of the *GAL1-lacZ* fusion, leading to increased intensity of the blue color of the resulting colonies grown on galactose indicator medium. The mutants obtained by this method could then be used to isolate and characterize the genes involved. In principle, the use of this genetic screen should make possible the identification of not only the structural genes for the CBF3 proteins, but also genes specifying factors in-

TABLE 1

*S. cerevisiae* strains used in this study

| Strain[a] | Genotype | Source |
|---|---|---|
| DBY745 | *MATα ura3-52 ade1-100 leu2-3 leu2-112* | D. BOTSTEIN |
| FPY1 | *MATa ura3-52 ade1-100 trp1* | This laboratory |
| EMS-30.3.1 | *MATa ura3-52 ade1-100 trp1 blu2-1* | This laboratory |
| EMS-30.3.2 | *MATα ura3-52 ade1-100 leu2-3 leu2-112 blu2-1* | This laboratory |
| EMS-39.1.2 | *MATa ura3-52 ade1-100 trp1 blu2-2* | This laboratory |
| EMS-39.9.5 | *MATα ura3-52 ade1-100 leu2-3 leu2-112 blu2-2* | This laboratory |
| EMS-53.23.82 | *MATa ura3-52 ade1-100 trp1 blu1-1* | This laboratory |
| EMS-53.23.22 | *MATα ura3-52 ade1-100 leu2-3 leu2-112 blu1-1* | This laboratory |
| EMS-56.4.3 | *MATa ura3-52 ade1-100 trp1 blu3-1* | This laboratory |
| EMS-56.4.14 | *MATα ura3-52 ade1-100 leu2-3 leu2-112 blu3-1* | This laboratory |
| EMS-79.4.8 | *MATa ura3-52 ade1-100 trp1 blu1-2* | This laboratory |
| EMS-79.4.2 | *MATα ura3-52 ade1-100 leu2-3 leu2-112 blu1-2* | This laboratory |
| blu1-52 | *MATa ura3-52 ade1-100 leu2-3 leu2-112 trp1 blu1-1* | This laboratory |
| blu1-116 | *MATα ura3-52 ade1-100 leu2-3 leu2-112 trp1 blu1-1* | This laboratory |
| blu1-191 | *MATa ura3-52 ade1-100 blu1-1* | This laboratory |
| blu2-22 | *MATa ura3-52 ade1-100 leu2-3 leu2-112 trp1 blu2-1* | This laboratory |
| wt161 | *MATα ura3-52 ade1-100 leu2-3 leu2-112 trp1* | This laboratory |
| wt163 | *MATa ura3-52 ade1-100 leu2-3 leu2-112 trp1* | This laboratory |
| FY41 | *MATa leu2Δ1 trp1Δ63 ura3-52 his4-917d* | F. WINSTON |
| FY51 | *MATa leu2Δ1 trp1Δ63 ura3-52 his4-917d spt3Δ202::TRP1* | F. WINSTON |

[a] Diploid strains are referred to in the text, in the tables, and in the figure legends by the cross from which they were derived; *e.g.*, DBY745 × FPY1 is a diploid strain derived from a cross between haploid strains DBY745 and FPY1.

volved in the regulation of their synthesis and DNA binding activity.

## MATERIALS AND METHODS

**Strains and media:** All strains used in this study and their corresponding genotypes are listed in Table 1. Strains constructed in this laboratory were obtained by standard genetic methods involving crossing, sporulation, and tetrad dissections as described in the literature (MORTIMER and HAWTHORNE 1969; SHERMAN, FINK and HICKS 1986). All media were prepared as described by SHERMAN, FINK and HICKS (1986).

**β-Galactosidase assays:** Assays on liquid cultures were done essentially as described by GUARENTE (1983) with some modifications. Cells were typically grown in minimal medium containing 2% galactose (SG) for two days; 0.75 ml of cell suspension was then spun down in an Eppendorf microcentrifuge in a 1.5 ml centrifuge tube, and resuspended in 0.75 ml of Z buffer (per liter, 16.1 g $Na_2HPO_4 \cdot 7\ H_2O$, 5.5 g $NaH_2PO_4 \cdot H_2O$, 0.75 g KCl, 0.25 g $MgSO_4 \cdot 7\ H_2O$, and 2.7 ml of 2-mercaptoethanol; pH adjusted to 7.0). Three drops of $CHCl_3$ and 2 drops of 0.1% sodium dodecyl sulfate (SDS) were added, and the cells were vortexed at high speed for 10 sec. *o*-Nitrophenyl-β-D-galactoside (ONPG) (Sigma) hydrolysis was measured by adding ONPG (4 mg/ml in $H_2O$) to the above samples preincubated at 28°. The reaction was stopped by adding 0.4 ml of 1 M $Na_2CO_3$, the cell debris spun out, and the absorbance measured at 420 nm. Assays were normalized to the $OD_{600}$ of the culture measured after vigorous vortexing to disrupt cell clumps. Results are expressed in terms of β-galactosidase units calculated from the following formula:

$$\{OD_{420}/[OD_{600} \times \text{time of incubation (min)} \times \text{volume assayed (ml)}]\} \times 1{,}000.$$

Preparation of indicator plates was as follows: 4 g of yeast nitrogen base without amino acids (Difco) were dissolved in 235 ml $H_2O$ with the required supplements according to the requirements of the strain to be tested. This mixture was autoclaved, cooled to 57°, and 100 ml of sterile 12% galactose in $H_2O$, 70 ml of 1 M potassium phosphate buffer (pH 7.0) and 1.2 ml of 20 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Boehringer) were added in that order. An autoclaved molten mixture of 12 g of agar (Difco) in 200 ml $H_2O$ was added to the above; the resulting mixture was thoroughly mixed and poured into approximately 20 Petri dishes. These plates are referred to in the text as X-gal plates or indicator plates.

**Plasmid descriptions and constructions:** The pFP1 plasmid (Figure 1, panel A) was constructed by blunt end ligation of the 61-bp *Dra*I/*Mae*III fragment of pYe (*CEN11*)5 (FITZGERALD-HAYES *et al.* 1982) into the *Xho*I site of vector pLR1Δ20B (WEST, YOCUM and PTASHNE 1984). This 61-bp *CEN11* fragment contains the 26-bp CDEIII region flanked on one side by 23-bp of CDEII sequence and on the other by an additional 12-bp of *CEN11* DNA (Figure 1, panel B). Orientation of the insert within pFP1 was determined by restriction analysis, taking advantage of the fact that the original *Xho*I site of pLR1Δ20B is reconstituted on one side of the insertion. In pFP1, the 61-bp *CEN11* fragment is located between the *GAL1–10* upstream activating sequence (UAS) and the TATA box of *GAL1* (Figure 1, panel B), with the 23-bp CDEII flanking sequence proximal to the UAS. Plasmid pFP1 maintenance in yeast is provided by the 2 micron sequence, whereas selection for the plasmid in a *ura3* strain on minimal media lacking uracil is accomplished by the presence of a *URA3* allele on this vector. Replication in *Escherichia coli* is assured by the presence of a pBR322-derived Ori locus, with ampicillin-resistance as a selectable marker. A restriction map of vector pFP1 is shown in Figure 1 as well as a more detailed representation of the pFP1 region containing the *CEN11* fragment.

**Measurements of plasmid copy number:** Genomic DNA was isolated from cells containing either pFP1 or the parent vector pLR1Δ20B growing on minimal galactose medium. The DNA was digested with *EcoRI* to completion, and

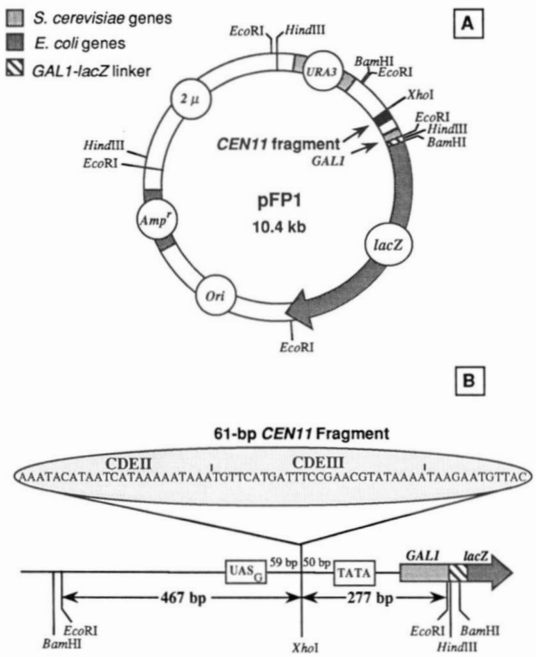


FIGURE 1.—Restriction map of pFP1 and detailed view of the hybrid *GAL1-CEN11* promoter of pFP1. (A) Schematic representation of vector pFP1 indicating major restriction sites as well as the relevant *E. coli* and *S. cerevisiae* genes. Approximately to scale. (B) Detailed representation of the hybrid *GAL1* promoter region of pFP1 showing the sequence and site of insertion of the 61-bp *CEN11* fragment. $UAS_G$ and TATA refer to the upstream activating sequence and the TATA box of the *GAL1* promoter. Direction of transcription of the *GAL1-lacZ* fusion gene is indicated by the arrow. Approximately to scale.

approximately 10 μg of DNA for each transformant were subjected to electrophoretic separation on a 0.7% agarose gel, transferred to a Nytran (Schleicher and Schuell) membrane, and hybridized to a $^{32}$P-labeled 0.5-kb *XhoI/BamHI* fragment of pLR1Δ20B containing part of the *GAL1–10* promoter (Figure 1). This probe hybridizes to a 0.74-kb *EcoRI* fragment of pLR1Δ20B, a 0.81-kb *EcoRI* fragment of pFP1 (see Figure 1), and a 2.0-kb *EcoRI* genomic fragment of the endogenous *GAL1* gene (ST. JOHN and DAVIS 1981). Autoradiography was performed, and the intensity of the signals was determined by laser densitometry scanning. The apparent copy number of each plasmid was then calculated by dividing the signal intensities corresponding to the plasmid-derived fragment and the genomic single copy sequence. To measure the fraction of cells containing the plasmid in the population, an aliquot of the cells used for the DNA preparation was diluted appropriately, and plated onto YPD plates. After a 3-day incubation at 32°, colonies were counted and replica-plated to minimal media lacking uracil. The frequency of each plasmid in the population is the ratio of the number of Ura$^+$ colonies to the total number of colonies. The plasmid copy number per cell was calculated as the ratio of the apparent copy number to the plasmid frequency in the population.

**Fragment mobility shift assays:** Fragment mobility shift assays were performed as described previously (NG and CARBON 1987; LECHNER and CARBON 1991) with modifications as follows. The binding reactions were performed in a total volume of 25 μl and contained 10 mM HEPES, pH 8.0, 6 mM $MgCl_2$, 2 mM NaF, 100 mM KCl, 0.5 mM dithiothreitol, 10% glycerol, 1–6 μg denatured salmon sperm DNA (Sigma) and 4–12 fmol of *CEN3* or *CEN11* DNA probe ($^{32}$P-end-labeled by using the Klenow fragment of DNA polymerase to fill in the restriction ends with labeled deoxynucleotides). The *CEN3* DNA probe (containing CDEI, CDEII, and CDEIII DNA sequences) was a 350-bp *XbaI/EcoRI* fragment of pRN505 (NG and CARBON 1987); the *CEN11* probe (containing CDEIII and 25-bp of flanking *CEN11* DNA sequences) was an 85-bp *EcoRI/BamHI* fragment of pFP2 (PÉRIER 1991). After addition of approximately 20 μg of protein extract, the reaction mix was incubated at the indicated temperature for 20 min, loaded on a 4% nondenaturing polyacrylamide gel and electrophoresed at 15 V/cm for 1.5–2.5 hr in 45 mM Tris, 290 mM glycine, pH 8.3. Gels were dried and autoradiographed at −70° with an intensifier screen. When films were to be scanned for quantification purposes, no intensifier screen was used.

**Population average DNA content:** DNA content was determined by the diaminobenzoic acid (DABA) procedure described by HOPPER and HALL (1975). Cells ($1.0 \times 10^7$) were hydrolyzed in 1 N sodium hydroxide for 24 hr at 30° with shaking. The samples were sequentially precipitated for 15 min on ice with the following solutions: 0.6 M trichloroacetic acid (TCA); 0.3 M TCA; 0.1 M potassium acetate in 95% ethanol; 100% ethanol. The final pellet was then vacuum dried, resuspended in 0.1 ml of a 0.4 g/ml solution of DABA (Sigma) in $H_2O$, and allowed to react with the dye for 30 min in a 60° water bath. One milliliter of 0.6 N $HClO_4$ was then added to the DABA-DNA reaction mix, and 1 ml of this mixture was added to 2.0 ml of 0.6 N $HClO_4$ for measurement of fluorescence. Fluorescence was measured (excitation at 400 nm, emission at 490 nm) with a Perkin-Elmer fluorescence spectrophotometer and the fluorescence intensity of a control DABA solution lacking DNA was used as a blank.

**Acriflavine staining of *S. cerevisiae* for flow cytometry:** Cells were sonicated at 200 watts for 10 sec to disrupt clumps, counted using a hemocytometer, and $10^6$ cells were fixed in 1 ml 70% ethanol for 1–2 hr in a 1.5-ml Eppendorf centrifuge tube at room temperature. After fixing, cells were spun down, washed once with double-distilled water, resuspended in 1 ml 4 N HCl and incubated at room temperature for 20 min. After a wash step with 1 ml $H_2O$, cells were resuspended in 1 ml acriflavine solution prepared as follows: 20 mg neutral acriflavine (Sigma) and 500 mg potassium metabisulfite were dissolved in 100 ml $H_2O$ and 10 ml 0.5 N HCl were added; the solution was then filtered through a 0.22-mm filter and stored at 4°. Cells were then spun out of the staining solution, washed once with 1 ml of acid-alcohol (1 ml concentrated HCl in 99 ml 70% ethanol), once with 1 ml of $H_2O$ and resuspended in 1 ml $H_2O$. Stained cells were kept at 4° for up to 2 weeks without alteration in flow cytometric properties. All centrifugations were performed for 15 sec in 1.5-ml Eppendorf centrifuge tubes at 4°. This staining protocol was kindly provided by DANE WITTRUP as a personal communication. Aliquots of stained cells were sonicated to disrupt clumps, examined under light and fluorescence microscopy to confirm the disruption of aggregates and nuclear staining, respectively, and analyzed with a Coulter EPICS 541 flow cytometer. Excitation wavelength was set at 488 nm, and emission larger than 530 nm was monitored by using the appropriate filters. Calibration of the apparatus was performed with 2-μm yellow/green beads obtained from PolySciences.

## RESULTS

**Isolation of mutants; experimental strategy:** Various DNA fragments containing all or portions of the

TABLE 2

**β-Galactosidase activity derived from various indicator plasmids**

| Plasmid[a] | DNA segments in *Xho*I site[b] | β-Galactosidase activity[c] | Color on X-gal medium[d] |
|---|---|---|---|
| pLR1Δ20B | No insert | 12,636 | Blue |
| pRN20B-12D | 20-bp synthetic CDEIII | 12,734 | Blue |
| pRN20B-7G | 36-bp *Ssp*I/*Bgl*II (*CEN3*) | 796 | Light blue |
| pFP1 | 61-bp *Dra*I/*Mae*III (*CEN11*) | 86 | White to light blue |
| pRN20B-8D | 139-bp *Bam*HI/*Bgl*II (*CEN3*) | 6 | White |

[a] Plasmids contained in the various DBY745 transformants tested for β-galactosidase activity in selective galactose medium.

[b] The DNA fragments inserted into the *Xho*I site of the pLR1Δ20B parent vector are as follows: 20-bp synthetic CDEIII: 5'-TGATTTCCGAAAGTTAAAA-3';36-bp *Ssp*I/*Bgl*II (*CEN3*) fragment of pRN-mp7-30-1 containing CDEIII of *CEN3*: 5'-ATTAGTGT-ATTTGATTTCCGAAAGTTAAAAAAGATC-3' (NG, CUMBERLEDGE and CARBON 1986); 61-bp *Dra*I/*Mae*III (*CEN11*), see Figure 1; 139-bp *Bam*HI/*Bgl*II (*CEN3*): fragment of pRN-mp7-30-1 containing CDEI, CDEII and CDEIII of *CEN3* (NG, CUMBERLEDGE and CARBON 1986).

[c] β-Galactosidase levels measured in the corresponding transformants growing on galactose at room temperature. β-galactosidase units = $\{OD_{420}/[OD_{600} \times \text{volume used (ml)} \times \text{time (minutes)}]\} \times 1{,}000$.

[d] Color of colonies from the corresponding transformants growing on X-gal indicator plates.

consensus *CEN* sequence were inserted at the *XhoI* site located upstream of the *GAL1-lacZ* gene fusion in plasmid pLR1Δ20B (Figure 1, see also NG, NESS and CARBON 1986). The induced levels of β-galactosidase expression in yeast strains containing these plasmids are shown in Table 2. A 139-bp DNA fragment containing the intact *CEN3* region gives essentially complete repression of expression of the indicator gene, and the colonies of this strain growing on X-gal plates are white. On the other hand, insertion of the 20-bp CDEIII sequence alone does not repress. However, addition of flanking DNA sequences surrounding the central CDEIII core leads to increased ability to repress expression of the indicator gene (NG, NESS and CARBON 1986). A 36-bp sequence containing CDEIII gives partial repression, while the 61-bp CEN11 fragment in pFP1, containing CDEIII plus 23 bp of flanking sequence to the left and 12 bp to the right (see Figure 1), gives almost complete repression; *in vivo* β-galactosidase levels are reduced to less than 1% of the control pLR1Δ20B strain. These results correlate well with the binding of CBF3 *in vitro* to DNA segments of various lengths containing the CDEIII consensus sequence (R. NG, F. PÉRIER and J. CARBON, unpublished observations). Colonies of cells containing plasmid pFP1 are white to very light blue on indicator plates. The very low level of β-galactosidase activity observed in pFP1 cells is not due to a dramatic decrease in plasmid copy number (see Table 5, below), and thus is most likely due to binding of CBF3 to the CDEIII sequence.

Yeast cells containing the indicator plasmid pFP1 (Figure 1, panel A) were mutagenized, plated on medium selective for the presence of the plasmid, allowed to grow at a permissive temperature, and replica-plated to two X-gal indicator plates. After appropriate growth of the replicate colonies at the permissive temperature, one of the two replicas was left at the permissive temperature, while the other was incubated at 37°. Colonies exhibiting a blue color on the X-gal plates indicative of expression of the *GAL1-lacZ* gene at either of the incubation temperatures were picked and submitted to further tests to assess their phenotype in terms of chromosome stability and centromere-associated functions.

This experimental strategy is dependent on several assumptions. We assumed that the repression characteristics of *CEN* DNA fragments when inserted in a *GAL1* promoter are indeed due to specific binding of centromere DNA-binding proteins to the *CEN* sequence, thus acting as pseudorepressors. This is very likely since DNA fragments containing nonspecific sequences do not affect transcription when inserted within a *GAL1* promoter, and fragments containing specific binding sites display pseudo-operator characteristics only in the presence of their specific binding proteins (MILLER, MCKAY and NASMYTH 1985; SILICIANO and TATCHELL 1986; BRENT and PTASHNE 1984). We also assumed that a decrease in the levels of centromere-proteins in the cells would cause derepression of the hybrid promoter, even though this type of mutation might prevent cell division. To meet this possibility, we screened for conditional mutations and pre-grew the test colonies at the permissive temperature. In addition, our screen would be most effective if repression of the hybrid promoter is dependent upon a unique species of centromere DNA-binding protein encoded by a single gene; if several proteins contributed to this repression effect in a noncooperative fashion, inactivation of a single component might not result in derepression. Thus, we used a plasmid containing the CDEIII protein binding site flanked by short adjacent DNA sequences, since the intact CEN sequence contains at least two protein binding sites (CDEI and CDEIII). Finally, we hoped that an alteration in levels of centromere DNA-binding proteins would have no detrimental effect on the level of general cell transcription and translation, since

the whole assay is based on expression of the *GAL1-lacZ* gene fusion.

**Identification of mutants:** Approximately 5,000 individual colonies derived from EMS-treated DBY745 cells (59% killing) and 6,000 colonies derived from UV-irradiated DBY745 cells (50% killing) were screened for a blue color phenotype either at room temperature, or after incubation at 37° for 1–2 days. (Incubation at 37° for periods longer than 2 days resulted in all colonies turning blue when insertion vector pFP1 was used.) Eighty colonies with a reproducible blue color phenotype on X-gal at either room temperature or 37° were picked for further screening. Of these primary isolates, 14 exhibited a 2–11-fold increase in intracellular levels of β-galactosidase activity in the presence of an intact pFP1 plasmid (after replacement of the original pFP1 plasmid present during the mutagenesis) as compared to wild-type β-galactosidase levels; these were subcloned, and five individual subclones were tested for blue color on X-gal plates under the conditions indicated above.

Five candidates (derived from the EMS-mutagenized cells) giving a reproducible dark blue color on galactose plates in the presence of X-gal and a 4–11-fold increase in β-galactosidase activity in the presence of indicator plasmid pFP1 were identified. These strains gave blue colonies at both the permissive and nonpermissive temperatures. They all displayed a 2+:2− segregation pattern of the blue colony color trait after tetrad analysis of the progeny of heterozygotes, indicating that a mutation affecting a single gene was causing the phenotype of these candidates. Mutations causing derepression of the hybrid promoter will be called *blu* mutations (for *blue* color of colonies on indicator plates) in this study.

**Dominance/recessiveness of *blu* mutations:** To determine whether the five *blu* mutations that were identified were recessive or dominant, *MAT***a** *ura3 trp1* or *MATα ura3 leu2* strains containing each mutation were mated to strains DBY745 and FPY1, respectively, the latter two containing indicator plasmid pFP1. In addition, homozygous diploids for each *blu* mutation containing pFP1 were prepared. Diploids containing pFP1 were picked from selective plates and two clones of each were assayed for β-galactosidase activity after growth in liquid SG medium. A DBY745 × FPY1 diploid was used as a control in these experiments.

The results of this analysis (Table 3) indicate that all five *blu* mutations are recessive. We observed that, under our experimental conditions, wild-type diploids consistently expressed significantly lower levels of β-galactosidase in the presence of the pFP1 plasmid as compared to the corresponding wild-type haploids (see Tables 2 and 3). However, levels of β-galactosidase were reduced in heterozygotes to values close to the basal level obtained with wild-type diploids. We therefore concluded that the five *blu* mutations are recessive to wild-type.

**TABLE 3**

**Dominance/recessiveness of *blu* mutations**

| Diploid[a] | β-Galactosidase units[b] | Normalized β-galactosidase units[c] |
|---|---|---|
| FPY1 × DBY745 | 16 | 1 |
| EMS-30.3.1 × DBY745 | 34 | 2.1 |
| EMS-30.3.2 × FPY1 | 41 | 2.6 |
| EMS-30.3.1 × EMS-30.3.2 | 253 | 15.8 |
| EMS-39.1.2 × DBY745 | 59 | 3.7 |
| EMS-39.9.5 × FPY1 | 50 | 3.1 |
| EMS-39.1.2 × EMS-39.9.5 | 720 | 45 |
| EMS-53.23.82 × DBY745 | 20 | 1.3 |
| EMS-53.23.22 × FPY1 | 36 | 2.3 |
| EMS-53.23.82 × EMS-53.23.22 | 453 | 28 |
| EMS-56.4.14 × FPY1 | 53 | 3.3 |
| EMS-56.4.3 × EMS-56.4.14 | 342 | 21.4 |
| EMS-79.4.2 × FPY1 | 30 | 1.9 |
| EMS-79.4.8 × EMS-79.4.2 | 397 | 24.8 |

[a] DBY745 and FPY1 are wild-type *BLU* controls (Table 1). Haploid *blu* mutants crossed with DBY745 have a *MAT***a** *ade1 ura3 trp1 blu* genotype, those crossed to FPY1 have a *MATα ade1 ura3 leu2 blu* genotype.

[b] Results of β-galactosidase assays are expressed as β-galactosidase units as described in MATERIALS AND METHODS. Values are the average of two duplicate measurements.

[c] Normalized β-galactosidase units were obtained by dividing the number of units obtained for each diploid by those obtained with the wild-type diploid DBY745 × FPY1.

**Complementation analysis of *blu* mutations:** The above five recessive *blu* mutants were placed into complementation groups by testing *blu/blu* heterozygotes for β-galactosidase activity. These heterozygotes were constructed by crossing a *MATαleu2 TRP1 blu* mutant strain with a *MAT***a***LEU2 trp1* strain containing a different *blu* mutation. Diploids containing the pFP1 vector were selected, grown in liquid SG medium, and β-galactosidase levels were determined as described in MATERIALS AND METHODS. Controls for these experiments were provided by *blu*/wild-type heterozygotes and a wild-type diploid strain. The EMS-30 and EMS-39 mutants did not complement one another in terms of their increased β-galactosidase levels, indicating that these mutants belonged to the same complementation group (Figure 2). Similarly, crosses between EMS-53 and EMS-79 produced diploids that still exhibited significantly higher β-galactosidase levels than either the wild-type diploid or EMS-30/wild-type and EMS-39/wild-type heterozygotes. These two mutations were therefore assigned to another complementation group. EMS-56 mutants, on the other hand, seemed to complement both the other groups, indicating that this mutation involved a third and different genetic locus. Complementation group *blu1* is defined by mutants EMS-53 (*blu1-1*) and EMS-79 (*blu1-2*);

| *MATa LEU2 trp1* Mater | DBY745 | EMS-30.3.2 | EMS-39.9.5 | EMS-53.23.22 | EMS-56.4.14 | EMS-79.4.2 |
|---|---|---|---|---|---|---|
| *MATα leu2 TRP1* Mater | | | | | | |
| FPY1 | 1.0 | 2.6 | 3.1 | 2.3 | 3.3 | 1.9 |
| EMS-30.3.1 | 2.1 | **15.8** | **39.7** | 3.5 | 5.2 | 2.6 |
| EMS-39.1.2 | 3.7 | **25.4** | **45.0** | 4.7 | 10.3 | 3.6 |
| EMS-53-23-82 | 1.3 | N.D. | N.D. | **28.3** | 7.3 | **26.1** |
| EMS-56.4.3 | N.D. | 9.3 | 7.8 | 7.3 | **21.4** | 7.4 |
| EMS-79.4.8 | N.D. | N.D. | 1.8 | **35.2** | 4.7 | **24.8** |

FIGURE 2.—Complementation matrix for *blu* mutants. Each number within the matrix corresponds to normalized β-galactosidase units obtained for the corresponding diploid. Units are normalized to the β-galactosidase levels obtained for a homozygous wild-type strain, DBY745 × FPY1, whose β-galactosidase levels were assigned the arbitrary value of 1 unit. Bold characters identify probable noncomplementing crosses. N.D., not determined.

complementation group *blu2* is defined by mutants EMS-30 (*blu2–1*) and EMS-39 (*blu2–2*), and mutant EMS-56 was designated *blu3–1*, belonging to a third complementation group, designated *blu3*.

***CEN*-specificity of the *blu* mutations:** If the *blu* mutants are defective in the activity of protein(s) binding specifically to the CDEIII region of the centromere, one might expect *blu*-induced derepression of the *GAL1-lacZ* gene to be specific to indicator plasmids containing a *CEN* DNA insert. However, the real possibility exists that mutations altering general levels of transcription could also lead to bypass of the *CEN*-mediated repression. The different *blu* mutants were assayed for β-galactosidase levels specified by a control plasmid in which the fusion gene is repressed by an unrelated DNA insert. A pLR1Δ20B plasmid containing a 254-bp *Alu*I fragment of the mouse immunoglobulin κ gene with nuclear matrix association region (*MAR*) activity (COCKERILL and GARRARD 1986) was obtained from BARBARA FISHEL. This insert is capable of repressing the activity of the pLR1Δ20B *GAL1* promoter in *S. cerevisiae* in a manner analogous to our *CEN* inserts and has been used in screens designed to identify mutations in genes coding for *MAR*-binding proteins in yeast (B. FISHEL and W. GARRARD, personal communication).

One *MAT***a** *ura3 leu2 blu* mutant in each complementation group was transformed with either pFP1 or the control *MAR*-plasmid named pBF20B-M; transformants were selected on SD plates supplemented with casamino acids and adenine, and three $Ura^+$ colonies from each transformation were grown in liquid SG medium supplemented with casamino acids and adenine before being assayed for β-galactosidase activity (Table 4). Mutation *blu2–1* did cause a nearly threefold increase in β-galactosidase levels expressed from the *MAR* control plasmid, indicating that *blu2* may affect the expression of the hybrid *GAL1* promoter regardless of the identity of the insert present in the promoter region. Elevated β-galactosidase levels in *blu2–1* could result from an increased plasmid copy number, alteration of a transcriptional factor, or a general defect in protein transport into the nucleus. Mutations *blu1–1* and *blu3–1*, on the other hand, do not result in an increase in β-galactosidase activity expressed from the pBF20B-M plasmid, indicating that the observed derepression of the *GAL1-CEN11* promoter may be specific for the presence of a *CEN* insert. This would suggest that mutations *blu1–1* and *blu3–1* may affect genes involved in synthesis or regulation of activity of *CEN* DNA binding proteins.

**Copy number of plasmid pFP1:** The presence of an intact functional *S. cerevisiae* centromere in a 2 micron plasmid has been shown to reduce the copy number to one to two copies per cell (TSCHUMPER and CARBON 1983). We measured pFP1 copy number in cells growing on galactose to determine if the 61-bp CDEIII insert is capable of lowering 2 micron copy number under these conditions, and to determine if the increase in β-galactosidase activity observed in *blu1* mutants containing pFP1 could be due to an increase in pFP1 copy number. The copy number of plasmid pLR1Δ20B is relatively low in wild-type cells under the conditions used (approximately eight copies per cell; Table 5). This may be due to the fact that cells were grown on galactose and the presence of the *GAL1–10* promoter at high copy number may titrate out factors such as *GAL4* required for galactose utilization by the cell, thus selecting against the subpopulation of cells containing high numbers of the 2-micron plasmid. The presence of the 61-bp *CEN11* fragment in pLR1Δ20B reduces the copy number only slightly, from eight to approximately five copies per cell. This twofold reduction in plasmid copy number cannot account for the nearly 150-fold decrease in β-galactosidase activity observed as a consequence of inserting the 61-bp CDEIII region into the *Xho*I site of plasmid pLR1Δ20B (Table 2). Therefore, these data support our premise that binding of one or more factors to the 61-bp *CEN11* fragment interferes with expression of the *GAL1-lacZ* gene on plasmid pFP1.

The apparent copy number of pFP1 in the total cell population of the *blu1–1* strains that were tested was slightly lower than in cells of the wild-type strain used as a control. This indicates that the increase in β-galactosidase levels observed in *blu1* mutants bearing pFP1 is not due to an increase in the copy number of the indicator plasmid. The plasmid copy number per cell seems to be slightly increased in the *blu1–1* mutants, however, because the frequency of occurrence of pFP1 in the *blu1–1* cell population is lower than in the wild-type control.

**TABLE 4**

***CEN*-specificity of the *blu* mutations**

| Strain[a] | Insert in *Xho*I site of pLR1Δ20B[b] | β-Galactosidase activity[c] | Normalized β-galactosidase units[d] |
|---|---|---|---|
| DBY745 (wild-type) | 61-bp *Dra*I/*Mae*III *CEN11* fragment | 138 | 1.0 |
| DBY745 (wild-type) | 254-bp *Alu*I*MAR* fragment | 266 | 1.0 |
| EMS-53.23.22 (*blu1-1*) | 61-bp *Dra*I/*Mae*III *CEN11* fragment | 989 | 7.2 |
| EMS-53.23.22 (*blu1-1*) | 254-bp *Alu*I*MAR* fragment | 248 | 0.9 |
| EMS-30.3.2 (*blu2-1*) | 61-bp *Dra*I/*Mae*III *CEN11* fragment | 891 | 6.5 |
| EMS-30.3.2 (*blu2-1*) | 254-bp *Alu*I*MAR* fragment | 719 | 2.7 |
| EMS-56.4.14 (*blu3-1*) | 61-bp *Dra*I/*Mae*III *CEN11* fragment | 702 | 5.1 |
| EMS-56.4.14 (*blu3-1*) | 254-bp *Alu*I*MAR* fragment | 269 | 1.0 |

[a] All strains were *MATα leu2 ade1 ura3*. The genotype with respect to *blu* mutations is indicated under parentheses.
[b] Plasmids contained in the corresponding strain with either a 61-bp *Dra*I/*Mae*III *CEN11* fragment (pFP1) or a 254-bp *Alu*I fragment of the mouse immunoglobulin κ gene (pBF20B-M) inserted in the *Xho*I site of pLR1Δ20B.
[c] β-Galactosidase units are defined as described in MATERIALS AND METHODS. Results are the average of triplicate experiments.
[d] Normalized β-galactosidase units were obtained by dividing the number of units observed for each strain by the number of units obtained with the wild-type strain containing the corresponding plasmid.

**TABLE 5**

**Copy number of pFP1**

| Plasmid | Strain | Apparent copy number[a] | Frequency in the population[b] | Copy number per cell[c] |
|---|---|---|---|---|
| pLR1Δ20B | wt163 | 6.8 | 0.82 | 8.3 |
| pFP1 | wt163 | 3.6 | 0.71 | 5.1 |
| pFP1 | blu1-116 | 3.1 | 0.42 | 7.4 |

[a] Apparent copy number was determined as described in MATERIALS AND METHODS and is a measure of the number of plasmids relative to the total number of cells in the population.
[b] Frequency of occurrence of the plasmid in a population of cells growing under selective conditions was determined by measuring the frequency of uracil prototrophs (*URA3*) in that population.
[c] Copy number per cell is the ratio of the absolute copy number to the frequency of the corresponding plasmid in the population.

**Pleiotropic effects of the *blu1* mutation:** Since the *blu1* and *blu3* mutations are predicted to affect genes involved in kinetochore formation and function, these mutations might be expected to result in defects in cell division and possibly even in massive chromosome nondisjunction during mitosis. Because of the potential lethality of this type of defect, we originally had attempted to identify temperature-conditional mutations. Purified strains for all five mutants were tested for growth under a range of temperature conditions (15°, 22°, 32°, 37° and 39°), and no significant change in viability with respect to wild-type controls was observed in the case of *blu2* and *blu3* mutants. However, the *blu1* strains exhibited a slow growth phenotype under all the above conditions. The generation time of *blu1* mutants was 1.5–2-fold higher than that of the wild-type cells when grown at 32° in YPD medium. This slow growth phenotype was genetically linked to the increase in β-galactosidase activity, as the 13 tetrads obtained from a *blu1–1* × wild-type diploid gave 13 parental ditypes for slow growth and blue color on X-gal.

When these slow growing colonies were examined by light microscopy, cells appeared to form clumps or aggregates. These cell masses could be broken up by gentle sonication indicating that cells were distinct. Approximately 59% of exponentially growing *blu1* cells were in the large bud stage, as compared to 39% large buds in an exponentially growing wild-type cell population. DAPI staining of nuclei of large budded cells did not indicate a block in mitosis since the fraction of large buds containing an elongated nucleus at the neck was identical in wild-type and *blu1* mutant cell populations, with most of the large budded *blu1* cells containing two nuclei (data not shown). Similarly, diploid *blu1–1* and *blu1–2* homozygotes and *blu1–1* × *blu1–2* heterozygotes also exhibited slow growth, and these cells formed clumps often containing one or more large cells.

The purified *blu* strains were also tested for alterations in the levels of active *CEN* DNA-binding proteins by performing *CEN3* DNA fragment mobility shift assays as described in MATERIALS AND METHODS (NG and CARBON 1987; LECHNER and CARBON 1991). This assay indicates the presence of at least three DNA-protein complexes (Figure 3). Complex A is believed to be formed by the binding of CBF1, a CDEI-binding factor, to its recognition sequence, and complexes B and C are derived from the binding of a multisubunit complex, CBF3, to the CDEIII region of the labeled fragment (LECHNER and CARBON 1991).

No differences from wild-type levels were observed under the conditions used when extracts from *blu2* and *blu3* strains were used (PÉRIER 1991). In contrast, extracts from *blu1* cells exhibited a striking reduction in the levels of *CEN3* DNA-binding activity (Figure 3, panel A). A significant reduction in ability to form complexes A, B and C is observed when *blu1* extracts

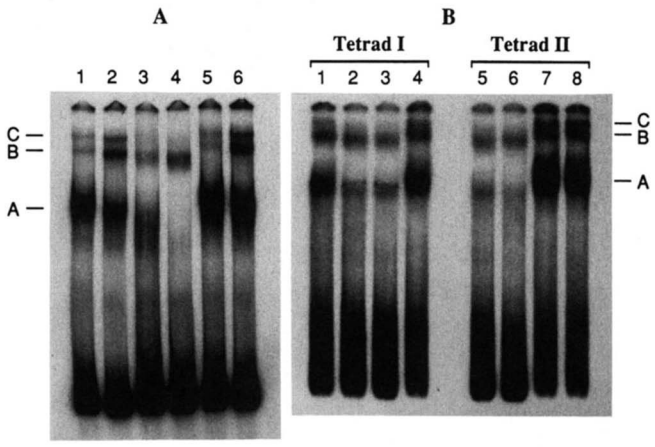


FIGURE 3.—Fragment mobility shift assays. (A) *CEN* DNA-binding activity in extracts from *blu* mutants. Approximately 20 μg of proteins from crude cellular extracts were allowed to bind to the $^{32}$P-labeled 350-bp *CEN3* fragment containing CDEI, CDEII and CDEIII at either 22° or 37° before nondenaturing polyacrylamide gel electrophoresis. FPY1 extract; binding at 22° (1); FPY1 extract; binding at 37° (2); blu1–52 extract; binding at 22° (3); blu1–52 extract; binding at 37° (4); blu2–22 extract; binding at 22° (5). blu2–22 extract; binding at 37° (6). The shift pattern displayed by *blu3* mutant extracts is not shown on this gel but is similar to that of the wild-type (FPY1). (B) *CEN* DNA-binding activity in extracts from cells derived from the spores of two tetrads from a *blu1/BLU1* heterozygous diploid strain. The meiotic products were scored for the blue color of the resulting colonies on indicator plates in the presence of plasmid pFP1; crude cellular extracts were prepared from each and 20 μg of each extract was used in CEN DNA fragment mobility shift assays (binding at 37°), using as template the $^{32}$P-labeled 350 bp CEN3 DNA fragment. **Tetrad 1:** *BLU1* (1), *blu1* (2), *blu1* (3), *BLU1* (4). **Tetrad 2:** *blu1* (5), *blu1* (6), *BLU1* (7), *BLU1* (8). A, B and C refer to the position of complexes A, B and C, respectively.

are used as compared to the levels obtained with extracts derived from wild-type cells, using the same protein concentrations. This characteristic of *blu1* extracts is not dependent upon the binding reaction temperature suggesting that the *CEN3* DNA-binding activity of *blu1* cells is not temperature-sensitive (Figure 3, panel A).

The apparent coordinate decrease in *CEN*-DNA binding activity in our extracts was genetically linked to the Blu$^-$ phenotype as shown with shift assays using extracts from cells derived from the meiotic products of a *blu1–1* × FPY1 (*BLU1–1*) diploid (Figure 3, panel B). It would appear that the *blu1* mutation has a general effect on the *in vitro* activities of all *CEN*-binding proteins, since a decrease in the levels of both CDEI and CDEIII binding activities (complexes A and B-C, respectively) is observed, suggesting that the *BLU1* gene might code for a positive regulatory factor coordinately regulating the levels of all *CEN*-binding proteins. Alternatively, the *BLU1* gene product may be involved in cooperative interactions with *CEN*-binding factors, such that alterations in the levels of the *BLU1* protein reduce the cooperative binding of the proteins to the *CEN* DNA. Other explanations are possible; for example, the centromere binding proteins may be selectively less stable in extracts derived from *blu1* cells.

**The *BLU1* gene is identical to *SPT3*:** The wild-type *BLU1* gene was cloned by screening a *CEN4/LEU2* yeast genomic library for hybrid plasmids capable of complementing the slow growth phenotype exhibited by *blu1* mutant cells. Several Blu1$^-$-complementing plasmids were obtained and these contained overlapping DNA inserts that were subsequently shown to genetically map at the *blu1* site in the genome (PÉRIER 1991). Upon further subcloning, a 1.5-kb fragment exhibiting Blu1$^-$-complementing activity was isolated from the above clones and the nucleotide sequence determined. One truncated large open reading frame of 314 codons was identified, translated into a deduced amino-acid sequence using a TRANSLATE program, and then used to look for potential homologous sequences by computer searching a GenBank library using a WORDSEARCH program (DEVEREUX, HAEBERLI and SMITHIES 1984). Perfect homology was found with the published sequence for the first 314 codons of the *S. cerevisiae SPT3* gene, originally identified as a complementation group within a class of mutations leading to a decrease in transcription involving yeast Ty elements (WINSTON *et al.* 1984; WINSTON and MINEHART 1986). Our mapping, complementation, and sequencing data indicated that the *BLU1* and *SPT3* genes were identical and that the first 314 codons of the *BLU1/SPT3* gene were sufficient for complementation of the Blu1$^-$ phenotype.

**Chromosome stability in *blu1* and *spt3* mutants:** Single chromosome loss assays and *CEN* plasmid stability assays in *blu1* mutants indicated no significant increases in non-disjunction events involving single chromosomes or *CEN*-plasmids (PÉRIER 1991). However, massive nondisjunction events involving failure to segregate entire sets of chromosomes would not be detected by these methods. If *blu1* mutants exhibit a chromosome segregation defect leading to increases in ploidy, this should result in a variable DNA content in clonal populations derived from different cells. Independent populations derived from single cells may exhibit differences in DNA content depending on the ploidy of the original cell as well as the frequency of massive nondisjunction events during clonal growth. If this were the case, population-specific DNA content measurements using various *blu1* clones should exhibit significantly higher variation than measurements performed on wild-type subpopulations.

In order to test this hypothesis, four colonies derived from three *blu1* strains and two wild-type strains plated for single cells on YPD were picked; $10^6$ cells for each were hydrolyzed and DNA content was measured by spectrofluorometry after staining with the

TABLE 6

**Population average DNA content**

| Strain[a] | Total DNA content ($\mu$g)[b] | DNA content per cell ($\mu$g)[c] | % Standard deviation[d] |
|---|---|---|---|
| wt161 | 0.163 ± 0.015 | $(1.63 \pm 0.15) \times 10^{-8}$ | 9.2 |
| wt163 | 0.158 ± 0.015 | $(1.58 \pm 0.15) \times 10^{-8}$ | 9.5 |
| blu1-52 | 0.148 ± 0.036 | $(1.48 \pm 0.36) \times 10^{-8}$ | 24.3 |
| blu1-116 | 0.223 ± 0.022 | $(2.23 \pm 0.22) \times 10^{-8}$ | 9.9 |
| blu1-191 | 0.228 ± 0.059 | $(2.28 \pm 0.26) \times 10^{-8}$ | 25.9 |

[a] For each strain, DNA content was measured in $10^6$ cells from four independent colonies picked from a plate.

[b] Total DNA was calculated by measuring the emitted fluorescence intensity of DABA-treated DNA at 490 nm with excitation at 400 nm and deriving DNA concentration from a standard curve. Standard deviations for DNA content of 4 individual colonies are indicated.

[c] DNA content per cell is obtained by dividing total DNA content by $10^6$, the number of cells used for each measurement.

[d] Percent standard deviation obtained for cells from four subclones of each strain.

DNA-specific stain DABA (see MATERIALS AND METHODS for details). As shown in Table 6, average DNA content per cell for wild-type populations is close to the theoretical DNA content for haploid yeast cells ($1.64 \times 10^{-8}$ $\mu$g per cell), assuming a haploid genome size of $15 \times 10^6$ bp, with a standard deviation between measured DNA content of independent clones lower than 10%. In the case of the *blu1-1* mutant strains that were analyzed, standard deviations between individual clones were larger than those calculated for wild-type cells, and, in strains blu1-116 and blu1-191, the average DNA content was significantly higher than in wild-type strains, as would be expected if frequent increases in ploidy due to massive nondisjunction events were occurring in the population.

Individual cell subsets in a population were examined in terms of their individual DNA concentration by flow cytometry analysis of whole cells stained with a quantitative DNA-specific stain. Exponentially growing cultures of *blu1*, *spt3* and wild-type cells in rich medium (YPD) were fixed and stained with neutral acriflavine and analyzed by flow cytometry as described in MATERIALS AND METHODS. A plot of relative cell number *vs.* linear integrated fluorescence intensity for wild-type and *spt3* cells is shown in Figure 4 (our *blu1-1* strains gave results identical to the *spt3* strain). Typically, a wild-type sample displays two separate subpopulations of cells with distinct fluorescence levels, corresponding to $G_1$ ($1n$) and $G_2$ ($2n$) DNA content, with an approximately equal number of cells in each peak. The *spt3/blu1* cell populations, on the other hand, exhibit a markedly different pattern of fluorescence distribution, with more than 50% of the cells with $2n$ DNA content and approximately 10% of the cells exhibiting higher than $2n$ integrated fluorescence intensity. Repeated sonication of *spt3/blu1* cells did not change the fluorescence pattern exhibited by the population, indicating that clumps of cells were not likely to be responsible for the observed subpopulation with higher fluorescence. Similarly, a plot of peak fluorescence intensity *vs.* integrated fluorescence intensity did not show the presence of cell-cell doublets corresponding to this higher than expected apparent DNA content (data not shown).

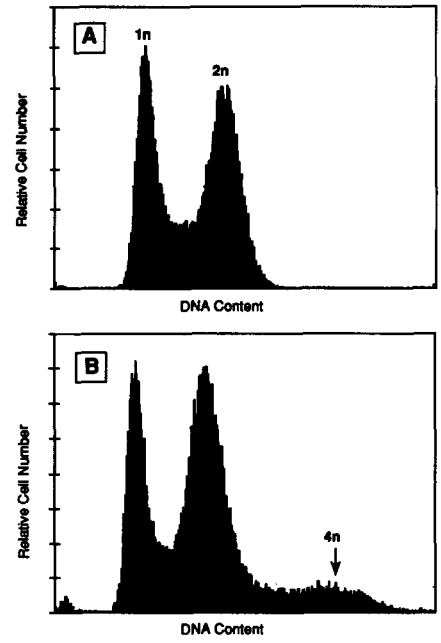


FIGURE 4.—Distributions of DNA content in cell populations of isogenic strains FY41 (*SPT3*) (A) and FY51 (*spt3Δ 202::TRP1*) (B). The two histograms shown are derived from flow cytometric measurements of individual cell fluorescence intensity in a population of acriflavine labeled, exponentially growing cells in rich medium (YPD). The *x*-axis corresponds to linear integrated fluorescence intensity and the *y*-axis indicates relative cell number. These measurements were performed on a total of approximately 40,000 cells for both wild-type and mutant populations. DNA content is indicated above the corresponding peaks. The theoretical location of cells with a $4n$ DNA content with respect to the $2n$ peak was determined by multiplying the distance between $1n$ and $2n$ peaks by a factor of 2.

Treatment of cells with the microtubule-depolymerizing drug methyl benzimidazole-2-yl carbonate (MBC) has been shown to induce chromosome loss in *S. cerevisiae* (WOOD 1982). Examination of flow cytometric data on wild-type cells treated for 6 hr with 100 mg/ml MBC (BURKE, GASDASKA and HARTWELL 1989) revealed a very similar pattern of distribution of fluorescence intensity; after the MBC treatment, the majority of cells had a $G_2$ content of DNA and a similar shoulder of cells with apparent increased DNA content could be seen. Our data therefore suggests that a subpopulation of *spt3/blu1* cells may exhibit higher than $2n$ DNA content, as predicted in the case of cells undergoing either increased levels of massive nondisjunction events or a bypass of mitosis leading to increases in ploidy. One minor difference between histograms of *spt3* cells and MBC-blocked wild-type cells is that a large number of particles with very weak

fluorescence intensity is detectable in *blu1* cell populations. These cells or debris with low DNA content may be the result of massive nondisjunction events in which one of the daughter cells lacks most or all chromosomes.

## DISCUSSION

In this study, we have used a novel approach for the isolation of mutations in genes whose products are involved in centromere structure and function. In principle, the screen we used should identify mutations in genes coding for centromere DNA-binding proteins and for factors involved in the positive regulation of the levels of functional centromere DNA-binding proteins in the cell. Several mutants exhibiting increased levels of β-galactosidase activity in the presence of an indicator plasmid containing a *CEN11* fragment acting as a pseudo-operator controlling expression of a hybrid *GAL1-lacZ* gene were isolated. These mutants were assigned to three complementation groups (*blu1, blu2* and *blu3*).

The specific derepression of a *CEN11-GAL1* hybrid promoter in *blu1* mutants observed *in vivo* was postulated to be the result of a decrease in CDEIII-binding protein activity, and this was confirmed by an *in vitro* analysis of centromere DNA-binding activity in extracts derived from these mutants. In addition, levels of *in vitro* CBF-1 activity are also decreased in *blu1* mutants.

Cloning and sequencing of the *BLU1* gene indicated that it was identical to the *SPT3* gene whose product appears to be a positive regulatory factor of transcription of Ty elements and certain haploid-specific genes involved in mating functions (Winston, Durbin and Fink 1984; Hirschhorn and Winston 1988). Our results suggest that the *BLU1* gene product may be involved in the transcription of genes coding for centromere DNA-binding proteins. Of the four centromere binding factors that have been identified so far, CBF1 (also known as CP1) is the only one for which the gene, *CEP1* (also termed *CBF1* and *CPF1*), has been cloned and sequenced (Baker and Masison 1990; Cai and Davis 1990; Mellor *et al.* 1990). Preliminary studies indicate that *spt3* mutants exhibit a significant reduction in *CEP1* mRNA levels (Périer 1991), suggesting that the *SPT3* gene product may be important in the transcriptional regulation of at least one gene coding for a centromere DNA binding protein.

Even though *spt3* and *blu1* mutants exhibit reduced levels of active CBF3 in fragment mobility shift assays, there is no evidence that the SPT3 protein directly affects the levels of transcription of genes specifying the CBF3 proteins. The CBF1 protein (binding to CDEI DNA) has been shown to be multifunctional, acting both as a kinetochore component and a transcription factor (Bram and Kornberg 1987; Baker, Fitzgerald-Hayes and O'Brien 1989). Hence, the decrease in CBF1 activity observed in *spt3* and *blu1* mutants could affect the optimal transcription of genes specifying CBF3 proteins. The *SPT3* gene product may also be involved in transcriptional activation of the gene(s) encoding putative regulators of the function of the CBF3 complex. Several potential regulatory factors involved in modulating the activity of CDEIII DNA-binding proteins have been identified. Lechner and Carbon (1991) have shown that an assembly factor is required *in vitro* for the synthesis of a competent *CEN* DNA-CBF3 complex. These investigators also demonstrated that at least one of the components of CBF3 needs to be phosphorylated to form a functional CDEIII-CBF3 complex. In addition, a protein kinase, MCK1, which appears to be involved in CDEIII function and mitotic and meiotic chromosome segregation, has been characterized (Shero and Hieter, 1991; Neigeborn and Mitchell 1991; Dailey *et al.* 1990). The reduced CDEIII DNA-binding potential that was observed in *spt3* and *blu1* mutants both by *in vivo* (levels of repression of a *CEN11-GAL1* hybrid promoter) and *in vitro* (*CEN*-DNA fragment mobility shifts) assays could be attributed to a decrease in activity of one of these factors.

In addition, either the *SPT3* gene product or CBF1 may act as assembly factors for the formation of the CBF3-CDEIII complex. We have not completely eliminated the possibility that the *SPT3* gene product may be a direct component of the kinetochore itself. However, the SPT3 protein migrates on SDS-polyacrylamide gels with an apparent molecular mass of 40 kD (Winston and Minehart 1986), significantly smaller than any of the four known *CEN* DNA binding proteins.

The slow growth of the *spt3/blu1* mutants could be attributed to an arrest at mitosis or at a checkpoint for chromosome attachment in a number of cells in the population (Zirkle 1970; Rieder and Alexander 1989). However, we did not detect a cell-cycle arrest or delay characteristic of cells blocked at mitosis in *spt3/blu1* mutant cell populations. An increase in the number of large budded cells was seen in a *spt3/blu1* population as compared to wild type, but the number of large buds with a nucleus located at the neck was identical in *spt3/blu1* and wild-type cells. A decrease in centromere DNA-binding proteins levels may lead to improper chromosome segregation events during mitosis. Even though we were unable to detect any decreases in single chromosome or *CEN*-plasmid stability in *blu1* and *spt3* mutant cells, quantitative and qualitative measurements of DNA content in a population of mutant cells indicated abnormalities which could be caused by massive nondisjunction events leading to increases in ploidy. In particular, flow cy-

tometric data indicated that a subpopulation of mutant cells exhibit a higher than expected 4*n* DNA content.

This is analogous to the situation described in the case of the *ndc1–1* mutant shifted to the nonpermissive temperature (THOMAS and BOTSTEIN 1986). This mutation results in asymmetric cell divisions in which one daughter cell doubles in ploidy while the other lacks chromosomes (aploid cell). The increase in ploidy observed in *blu1* and *spt3* mutants is consistent with a model in which the intracellular concentration of some elements of the centromere would be occasionally too low to allow synthesis of competent centromeres. Since excess centromeres on circular plasmids are toxic and result in chromosome destabilization in *S. cerevisiae,* it has been suggested that centromere DNA binding proteins may be present in limiting amounts (FUTCHER and CARBON 1986; RUNGE, WELLINGER and ZAKIAN 1991). Levels of CDEIII DNA-binding proteins appear to be extremely low in wild-type cells, possibly as little as one CBF3 molecule per chromosome (LECHNER and CARBON 1991); further reduction in the levels of these proteins would likely have major effects on centromere activity if these factors are indeed required for proper centromere function *in vivo*. Furthermore, homozygous *spt3* diploids have been shown to fail to sporulate. This defect may be directly linked to defective kinetochore function if the *SPT3* gene product is essential for the synthesis of competent kinetochores for meiosis.

Mutations in complementation groups *blu2* and *blu3* did not appear to result in loss of centromere function; no phenotypes diagnostic of defects in kinetochore activity could be detected in these mutants. The observed increase in β-galactosidase activity exhibited by *blu2* cells containing indicator plasmid pFP1 was not specific for the *CEN* DNA insert of pFP1. This lack of specificity indicates that the increase in β-galactosidase levels observed in *blu2* cells is probably caused by either an increase in indicator plasmid copy number (regardless of the type of insert) or a nonspecific increase in transcription of the *GAL1* promoter. The *blu3* mutation did appear to be specific for release of the repression caused by the *CEN* DNA insert since *blu3* did not lead to derepression of an unrelated hybrid construct. However, the lack of additional phenotypes in *blu3* mutant cells did not enable us to further characterize the *BLU3* locus.

The screen we used in this study failed to lead to the identification of genes coding for centromere DNA-binding proteins. This may be due to several reasons. We screened only 11,000 colonies and thus may have failed to identify rare mutational events. However, the fact that two of the complementation groups we identified were characterized by two independent mutations suggests that there are probably a very limited number of *BLU* genes. In addition, we did not identify conditional-lethal mutations in the screen for *blu* mutants. Since CDEIII DNA-binding proteins are believed to be essential in the formation of functional kinetochores, they are probably also essential for growth. Any mutations leading to full inactivation of one of these genes is therefore likely to be lethal. For this reason, our screen was designed to allow us to detect conditional-lethal mutations. However, we assumed that a shift of this type of temperature-sensitive mutant to nonpermissive conditions would lead not only to cell lethality but also to increased transcriptional activity of our hybrid *CEN-GAL1* promoter constructs. The requirement for active transcription in inviable cells could be difficult to fulfill, since alterations in one or many regulatory pathways in these dying cells may lead to an overall reduction in transcriptional levels. In addition, a temperature upshift may activate the heat-shock response, leading to a general decrease in transcription in the cells. We experienced difficulties in reproducing β-galactosidase activity measurements in cells growing at 37°; enzyme levels appeared to decrease as a function of the time that the cells were held at 37°, suggesting that our transcription-based system of detection could be subject to heat-shock effects. This problem possibly could be solved by using an indicator plasmid containing a *CEN* DNA fragment capable of repressing a heat-shock promoter driving a *lacZ* gene fusion or a selectable marker such as antibiotic resistance. Finally, genes encoding CDEIII-binding proteins may be members of a gene family; inactivation of one of the genes coding for homologous products may not be sufficient to suppress the function of these proteins.

We thank RAY NG for his advice and for providing plasmids pRN20B-12D, pRN20B-7G, and pRN20B-8D; LOUISE CLARKE for many helpful discussions; BARBARA FISHEL for providing plasmid pBF20B-M; ANN SPERRY for the *CEN4/LEU2* library; FRED WINSTON for his advice and providing strains FY41 and FY51 and plasmid pFR2; MARK BRZEZINSKI for help with the flow cytometry studies; and ARLECIA SALTER for technical assistance. This work was supported by National Institutes of Health research grant CA-11034 and the University of California Biotechnology Research and Training Program. J.C. holds an American Cancer Society Research Professorship in Biochemistry.

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Communicating editor: D. BOTSTEIN