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Mutations Synthetically Lethal with *cep1* Target *S. cerevisiae* Kinetochore Components

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ABSTRACT

CP1 (encoded by *CEP1*) is a *Saccharomyces cerevisiae* chromatin protein that binds a DNA element conserved in centromeres and in the 5′-flanking DNA of methionine biosynthetic (*MET*) genes. Strains lacking CP1 are defective in chromosome segregation and *MET* gene transcription, leading to the hypothesis that CP1 plays a general role in assembling higher order chromatin structures at genomic sites where it is bound. A screen for mutations synthetically lethal with a *cep1* null allele yielded five recessive *csl* (*cep1* synthetic lethal) mutations, each defining a unique complementation group. Four of the five mutations synergistically increased the loss rate of marker chromosomes carrying a centromere lacking the CP1 binding site, suggesting that the *cep1* synthetic lethality was due to chromosome segregation defects. Three of these four *CSL* genes were subsequently found to be known or imputed kinetochore genes: *CEP3*, *NDC10*, and *CSE4*. The fourth, *CSL4*, corresponded to ORF YNL232w on chromosome *XIV*, and was found to be essential. A human cDNA was identified that encoded a protein homologous to Csl4 and that complemented the *csl4-1* mutation. The results are consistent with the view that the major cellular role of CP1 is to safeguard the biochemical integrity of the kinetochore.

CP1 (also known as Cbf1 or Cpf1), encoded by the gene *CEP1*, is an abundant, nonessential, sequence-specific DNA-binding protein of *Saccharomyces cerevisiae* (yeast) that recognizes the degenerate octanucleotide RTCACRTG (R = purine) (Baker *et al.* 1989; Bram and Kornberg 1987; Cai and Davis 1989). CP1 binding sites are scattered more or less randomly throughout the yeast genome (Baker *et al.* 1989) and are functionally important in two contexts. First, the site is present at the same position and orientation in all yeast centromeres where it is known as centromere DNA element I (CDEI) (Hieter *et al.* 1985). Neither CP1 nor the CDEI site are essential for centromere function; however, *cep1* null mutants exhibit defects in chromosome segregation (Baker and Masison 1990; Cai and Davis 1990; Mellor *et al.* 1990). Mitotic chromosome nondisjunction is increased 10- to 30-fold, and high rates of premature sister chromatid separation are observed at meiosis (Masison and Baker 1992). The quantitative effects of *cis*-acting CDEI mutations and *trans*-acting *cep1* mutations are equal and nonadditive, indicating that the role of CP1 in chromosome segregation is executed at CDEI (Baker and Masison 1990). Second, CP1 binding sites are found near the promoters of genes of the methionine biosynthetic pathway (O'Connell and Baker 1992; Thomas *et al.* 1989). The best characterized example is *MET16* where it has been shown that CP1 modulates two different activation pathways, and both CP1 and an intact CDEI site are required for full *MET16* expression (O'Connell *et al.* 1995). (We refer to the RTCACRTG element as CDEI, regardless of context.) The *cep1* null mutant is a methionine auxotroph (Baker and Masison 1990; Cai and Davis 1990; Mellor *et al.* 1990), although the *MET16* defect alone does not account for this phenotype (O'Connell *et al.* 1995). CP1, along with Met28 is required for the binding of Met4 to the *MET16* UAS *in vitro* (Kuras *et al.* 1997). As Met4 is the positive transactivator for most if not all *MET* genes (Thomas *et al.* 1992), *cep1* methionine auxotrophy probably results from cumulative reductions in enzyme levels throughout the sulfate utilization pathway.

In addition to chromosome segregation and transcription defects, *cep1* null mutants double more slowly than isogenic wild-type strains. The physiological basis for the 35% increase in generation time is not known, but measured rates of chromosome missegregation are insufficient to account for a growth defect of this magnitude (Baker and Masison 1990). The pleiotropic *cep1* phenotype as well as the widespread distribution of CDEI sites and its cellular abundance have led to the notion that CP1 is a "general regulatory factor" whose function is to promote the assembly of higher order structures in yeast chromatin (Buchman *et al.* 1988): at *MET* promoters, CP1 enhances assembly of the RNA polymerase II initiation complex, while at centromeres, CP1 facilitates kinetochore assembly.

The kinetochore is the centromere-associated organ-

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elle at which spindle microtubules attach to chromosomes during mitosis and meiosis. Besides providing the physical connection between spindle and chromosome, the kinetochore participates in checkpoint regulation of the cell cycle and receives the signal for sister chromatid disjunction upon anaphase onset (reviewed by Pluta *et al.* 1995). The *S. cerevisiae* kinetochore is a multiprotein complex that assembles over the conserved 120-bp centromere (CEN) DNA sequence. In addition to CDEI described above, the yeast centromere contains two other defined sequence elements: CDEIII, a 25-bp partially palindromic sequence located at the right-hand end of the centromere; and CDEII, a 78- to 86-bp segment of highly A+T-rich (>87%) DNA (Hieter *et al.* 1985). CDEII acts as a spacer between CDEIII and CDEI, located at the left-hand end of the centromere. CDEIII is absolutely essential for centromere function; mutations of CDEI and CDEII are tolerated but can significantly compromise function (Cumberledge and Carbon 1987; Gaudet and Fitzgerald-Hayes 1987; Hegemann *et al.* 1988; McGrew *et al.* 1986).

Yeast kinetochore proteins have been characterized by both biochemical and genetic means. CP1, the first centromere protein purified, was identified by virtue of its specific binding to CDEI (Baker *et al.* 1989; Cai and Davis 1989). Cbf3 is a multiprotein complex that binds CDEIII (Lechner and Carbon 1991). The Cbf3 complex consists of four subunits, p110 (*NDC10*/*CBF2*/*CTF14*), p64 (*CBF3b*,*CEP3*), p58 (*CTF13*), and p23 (*SKP1*) (Connelly and Hieter 1996; Doheny *et al.* 1993; Goh and Kilmartin 1993; Jiang *et al.* 1993; Lechner 1994; Strunnikov *et al.* 1995). Mif2p, an essential protein required for chromosome segregation, is presumed to be part of the kinetochore by virtue of observed genetic interactions with *cep3*, *ndc10*, and *cep1* (Meluh and Koshland 1995). Because it contains an "AT hook," Mif2p is presumed to interact with CDEII (Brown 1995). Lastly, Cse4, a variant of histone H3 and a homolog of CENP-A, appears to play some role in kinetochore structure or assembly (Stoler *et al.* 1995).

To gain additional perspective on the biochemical function of CP1 at kinetochores and promoters and possibly to identify other pathways in which CP1 might play a critical role, we carried out a genetic screen for mutations that were synthetically lethal with *cep1*, *i.e.*, mutations lethal in a *cep1* genetic background but not in a wild-type background. Synthetic lethal gene interactions often define similarity or functional relatedness between gene products (Botstein 1988). Mutations synthetically lethal with *cep1* (*cep1* synthetic lethal, or *csl*) might identify gene products having functions related to, redundant with, or overlapping those of CP1. At least two different classes of *csl* mutations were anticipated. The first class were mutations in kinetochore components or kinetochore assembly factors. If a major role of CP1 is to facilitate kinetochore assembly, nonlethal mutations in other essential kinetochore components could become lethal in a *cep1* background. Synthetic lethality would result from inefficient or failed kinetochore assembly and consequent catastrophic increases in chromosome loss. The second class of mutations would be related to the transcription function of CP1. If CP1 were to participate along with other transcription factors in the activation of one or more essential genes, mutations in the other factors could render transcription CP1-dependent. Synthetic lethality would result from the failure to express the essential gene(s). For both classes of mutation, we assumed that the Csl target would interact directly or indirectly with DNA-bound CP1 and, therefore, that the mutations could be distinguished by their sites of action, *i.e.*, kinetochore-related *csl* mutations might exhibit genetic interactions with *cis*-acting centromere CDEI mutations, while transcriptional (or other) *csl* mutations would not.

Mutations giving rise to a Csl phenotype have already been described. Foreman and Davis (1996) screened for such mutations and identified a novel gene which they named *CDP1* (Cbf1-dependent). Both *cdp1-1* and *cdp1*Δ mutants exhibit defects in nuclear division and chromosome segregation, although the *CDP1* gene product is probably not directly involved in kinetochore assembly. Additional *cdp1* phenotypes include spindle abnormalities and altered sensitivities to benzimidazole compounds, suggesting that Cdp1 might interact directly with microtubules or tubulin (Foreman and Davis 1996). Meluh and Koshland (1995), in an analysis of genetic interactions between kinetochore genes, detected synthetic lethality between *cep1* and *mif2*, *ndc10*, and *cep3*. Their findings confirm the prediction that kinetochore components will be targets of *csl* mutations.

This article describes the results of our independent Csl screen. Five *csl* complementation groups were identified and the corresponding genes cloned. Consistent with expectations, the *csl* mutations targeted both kinetochore and transcription components, but kinetochore mutations predominated. One of the Csl gene products, Csl4, is an uncharacterized yeast protein for which a human homolog exists, and genetic evidence is presented implicating Csl4 in centromere function. Overall, the results imply that CP1 is important for stabilizing kinetochore structure and are discussed in light of the model for the yeast kinetochore proposed by Meluh and Koshland (1995). Specifically, our findings confirm interaction between CP1 and Cbf3 and provide the first evidence suggesting interaction between CP1 and the presumed centromeric histone Cse4.

MATERIALS AND METHODS

Strains, media, and plasmids: The yeast strains used in this study are listed in Table 1. Strain construction and genetic analysis were carried out using standard methods (Mortimer and Hawthorne 1969). All media were as described (Baker and Masison 1990). Color indicator plates were synthetic com-

TABLE 1

Strains

Strain	Genotype
381G	*MAT***a** *cry1 ade2-1 trp1 his4-580 tyr1 lys2 SUP4-3*
D37-1A	[381G] *MAT***a** *ura3 leu2 ade3 cep1::ura3-10*
D102-4A	[381G] *MAT***a** *ura3 leu2 TRP1 ade3 cep1::ura3-10 cyh2*
R95-1-1	[381G] *MAT***a** *ura3 leu2 TRP1 ade3 cep1Δ cyh2*
D102-5D	[381G] *MATα ura3 leu2 TRP1 ade3 cep1::ura3-10 cyh2 can1*
R85-1D	[381G] *MAT***a** *ura3 leu2 TYR1* [CFIII(D8B.DJ147-1-2.*URA3-SUP11-CEN3*)]
R67-R2	[381G] *MAT***a** *ura3 leu2 TYR1* [CFIII(D8B.DJ147-1-2.*URA3-SUP11-cen3ΔCDEI*)]
R108	[381G] *MAT***a**/*MATα ura3/ura3 leu2/leu2 trp1/TRP1* [CFIII(D8B.DJ147-1-2.*URA3-SUP11-CEN3*)]
R109	[381G] *MAT***a**/*MATα ura3/ura3 leu2/leu2 trp1/TRP1* [CFIII(D8B.DJ147-1-2.*URA3-SUP11-cen3ΔCDEI*)]
R98	[R108] *CEP1/cep1::ura3-10*
R99	[R109] *CEP1/cep1::ura3-10*
R100	[R108] *cep1::ura3-10/cep1::ura3-10*
R101	[R109] *cep1::ura3-10/cep1::ura3-10*
R116	[R108] *csl1-1/csl1-1*
R119	[R109] *csl1-1/csl1-1*
R129	[R108] *csl2-1/csl2-1*
R127	[R109] *csl2-1/csl2-1*
R110	[R108] *csl3-1/csl3-1*
R111	[R109] *csl3-1/csl3-1*
R221	[R108] *csl3::URA3/csl3::URA3*
R222	[R109] *csl3::URA3/csl3::URA3*
R224	[R108] *csl4-1/csl4-1*
R220	[R109] *csl4-1/csl4-1*
R225	[R108] *csl5-1/csl5-1*
R218	[R109] *csl5-1/csl5-1*
R117	[381G] *MAT***a**/*MATα ura3/ura3 leu2/leu2 trp1/TRP1 ade3/ade3 CEP1/cep1Δ*
R211	[381G] *MAT***a**/*MATα ura3/ura3 leu2/leu2 trp1/TRP1 ade3/ade3 CEP1/cep1Δ CSL4/csl4Δ::URA3*
R229	[381G] *MAT***a**/*MATα ura3/ura3 leu2/leu2 trp1/TRP1 ade3/ade3 CEP1/cep1Δ CSL4/csl4Δ::ura3::LEU2*
R226	[R229] *ura3/URA3::pRB267*
R231	[R229] *CSL4/csl4Δ::ura3::LEU2::pRB289*
R230	[R229] *CSL4/csl4Δ::ura3::LEU2::pRB288*
R203-8A	[381G] *MAT***a** *ura3 leu2*
R57-7B	[381G] *MATα ura3 leu2 TRP1 ade3 cep1::ura3-10 cyh2 can1 csl1-1* [pAP2]
R56-7B	[381G] *MAT***a** *ura3 leu2 TRP1 ade3 cep1::ura3-10 cyh2 csl2-1* [pAP2]
R82-10D	[381G] *MAT***a** *ura3 leu2 TRP1 ade3 cep1::ura3-10 cyh2 csl3-1* [pKM35]
BM3-40a	[381G] *MAT***a** *ura3 leu2 TRP1 ade3 cep1Δ cyh2 csl4-1* [pAP2]
BM3-64a	[381G] *MAT***a** *ura3 leu2 TRP1 ade3 cep1Δ cyh2 csl5-1* [pAP2]

All strains are congenic to 381G (Hartwell 1980), and the genotypes given are in addition to the 381G markers.

plete medium containing 6 μg/ml adenine (1/3 normal concentration). Cycloheximide plates contained 5 μg/ml cycloheximide. Unless noted otherwise, all strain growth was at 30°.

Plasmid pAP2 was derived from YEp352 (Hill *et al.* 1993) by inserting DNA fragments containing *ade3-2p* (*Sal*I-*Sma*I) and *CEP1* (*Bam*HI-*Bgl*II). Plasmid pKM35 was derived from pRS328 (Sikorski and Boeke 1991) by inserting the wild-type *CEP1* gene (*Bam*HI-*Bgl*II) at the unique *Bam*HI site. Plasmid pRB267 was obtained by inserting the 1.3-kbp *Eco*RI-*Hin*dIII fragment carrying *CSL4* into pRS306 (Sikorski and Hieter 1989). Additional 5′-flanking sequences were restored to the *CSL4* gene in pRB267 using PCR to amplify DNA containing the 356-bp *Eco*RI fragment spanning positions −468 to −113 relative to the *CSL4* initiator ATG and inserting this fragment into the *Eco*RI site of pRB267. The resulting plasmid, pRB289, has the *Eco*RI fragment in the native orientation. Plasmid pRB288 is identical to pRB289 except that the CDEI site located within the *Eco*RI fragment was mutated to GTCATTATG using bidirectional recombinant PCR. This mutation (CAT) was characterized previously (Baker *et al.* 1989). PCR primers overlapping the CDEI site and containing the CAT mutation were used to amplify both halves of the *Eco*RI fragment in separate reactions with flanking primers. The two amplification products were gel-purified, mixed, and used as template in a third PCR reaction with only the flanking primers. The resulting product was cleaved with *Eco*RI and inserted into the *Eco*RI site of pRB267. The Expand High Fidelity PCR System (Boehringer Mannheim, Indianapolis) was used for the recombinant PCR reaction; otherwise, Taq polymerase (Boehringer Mannheim) was used.

To disrupt *CSL4*, the *Sph*I-*Mlu*I fragment containing the *CSL4* ORF (starting at codon 2 and including 30 bp of 3′-flanking DNA) was removed from pRB267 and replaced with the *URA3* gene. The resulting plasmid, pRB274, was cleaved with *Eco*RI and *Hin*dIII and used to transform diploid strain R117. One of the Ura^+ transformants (strain R211) was shown by Southern blot analysis to have one copy of *CSL4* replaced by the *csl4::URA3* deletion allele. To allow subsequent transformation with *URA3* plasmids, the *csl4*-linked *URA3* marker in R211 was changed to *LEU2* using the marker change plasmid pDM2

(Masison and Baker 1992). The resulting strain (R229) retains *ura3* sequences flanking *LEU2* (*csl4::ura3::LEU2*).

Human Csl4 (hCsl4) was expressed in yeast using the cDNA expression vectors p416GPD and p426GPD (Mumberg *et al.* 1995), which are single- and multicopy plasmids, respectively, containing the yeast glyceraldehyde 3-phosphate dehydrogenase promoter and terminator flanking a polylinker cloning site. The hCsl4 coding region and 16 bp of 3′ untranslated sequences were obtained by PCR using I.M.A.G.E Consortium cDNA clone 511361 (Research Genetics, Inc., Birmingham, AL) as template and primers incorporating *Xba*I and *Xho*I restriction sites. The upstream primer also contained the sequence ATTATA immediately preceding the initiator ATG to serve as a ribosome binding site. This is the sequence found in yeast *CSL4*. The PCR product was cleaved with *Xba*I and *Xho*I and inserted between the *Spe*I and *Xho*I sites of p416GPD and p426GPD, generating plasmids pRB305 and pRB308, respectively. As controls, analogous expression plasmids containing the yeast *CSL4* coding DNA were constructed in an identical fashion. The yeast *CSL4* plasmids, pRB306 and pRB307, respectively, contained *CSL4* nucleotides −6 to 1170 relative to the initiator ATG. The authors will gladly supply the sequences of all PCR primers upon request.

Csl screens: Two screens were used to isolate *csl* mutants, one a visual screen based on colony color sectoring, the other based on cycloheximide sensitivity (Cyh^s). The first protocol, adapted from Bender and Pringle (1991), employed parental strains (D37-1A, D102-4A, or R95-1-1) of genotype *MAT***a** *leu2 ura3 ade2 ade3 cep1*Δ carrying an episomal plasmid (pAP2) bearing the wild-type *CEP1* gene. The plasmid also contained the yeast *ADE3* and *LEU2* genes. The parent strains are phenotypically Cep1$^+$ (Met$^+$) Leu$^+$ Ade3$^+$ Ade2$^-$ and form red colonies with multiple white sectors. The red color is the result of a block in adenine biosynthesis due to the *ade2* mutation; the white sectors arise through mitotic loss of pAP2, uncovering the chromosomal *ade3* mutation, which blocks formation of the red pigment. The *csl* mutants are unable to form sectored colonies, because plasmid loss also uncovers the *cep1*Δ allele and exposes the *cep1* synthetic lethality. Cells were mutagenized with ethylmethane sulfonate to 30–50% survival and spread directly on indicator plates. Survivors were visually screened for a red, nonsectoring colony color phenotype (Sect$^-$). After verifying the Sect$^-$ phenotype by restreaking, the candidate *csl* mutants were transformed with pKO56, an episomal plasmid carrying *CEP1* and *URA3* (but not *ADE3*). Mutants that regained the ability to sector (showing dependence on *CEP1* but not *LEU2* or *ADE3*) were backcrossed to strain D102-5D to test for dominance and for 2:2 segregation of the Sect$^-$ phenotype.

As an alternative to the sectoring screen, a *csl* screen based on cycloheximide sensitivity was also used. In this case, the parent (D102-4A) contained the chromosomal *cyh2^r* mutation and carried a CEN plasmid (pKM35) bearing the wild-type *CYH2* allele along with *CEP1*. Plasmid dependence (Sect$^-$) was scored by replicating mutagenized colonies onto cycloheximide plates. The *csl* mutants cannot give rise to Cyh^r papillae, which would normally arise from mitotic loss of pKM35. Except for scoring the Sect$^-$ phenotype, other aspects of the Cyh^s *csl* screen were the same as for the color sectoring screen.

Results of the screens are summarized in Table 2. Each of the *csl* mutants was backcrossed at least three times to congenic parents. During this process, *cslx* segregants were obtained in both mating types and in *CEP1* and *ADE3* genetic backgrounds.

Identification of *CSL* genes: Wild-type alleles of the *csl* genes were cloned by complementation. *CSL3* was obtained by transforming a *csl3-1* strain with the YEp24-based *S. cerevisiae* genomic library of Carlson and Botstein (1982) and screening by replica plating for reversion of the Cyh^s phenotype. *CSL2* was obtained by transforming a *csl2-1* mutant with the YCp50-based *S. cerevisiae* library of Rose (1987) and plating the transformants directly on indicator medium to score for reversion of Sect$^-$. It was found that the Cyh^s system was more efficient; therefore, the remaining *CSL* genes were cloned from the YCp50 library using the Cyh^s selection system after first introducing pKM35 into each mutant. In addition to *csl*-complementing DNAs, *CEP1* clones were obtained in all cases. These were recognized by their Met$^+$ phenotype. The *csl*-complementing plasmids were rescued into *Escherichia coli* for subsequent manipulation and analysis.

TABLE 2

Results of Csl screens

	Sectoring		
	Trial 1	Trial 2	Cyh^s
Parent strain	D37-1A	R95-1-1	D102-4A
Mutagenized survivors	14,000	7500	15,000
1° candidates	520	75	100
Positive on retest	15	12	22
Passed 2° shuffle test	6	6	10
2:2 segregation	2	2	1
Complementation groups	*csl1*, *csl2*	*csl4*, *csl5*	*csl3*

CSL1, *CSL2*, and *CSL3* were obtained prior to completion of the *S. cerevisiae* genome sequencing project. The complementing DNAs were first localized within the genomic insert by subcloning and then partially sequenced. Matches to *CSL1* and *CSL2* were found in GenBank, accession numbers X81396 (CBF3b) and Z28049 (ORF YKL049c; Cse4), respectively. *CSL3* was physically mapped by hybridizing the complementing DNA to ordered phage blots (ATCC 76269) and found to lie very near the *RTF1* locus on chromosome *VII*. The sequence of *RTF1* (GenBank accession no. U86702) was provided by Karen Arndt (University of Pittsburgh, PA) and found to be identical to *CSL3*. *CSL4* and *CSL5* were obtained after the yeast genome sequence was made public. Genomic inserts from the original *CSL4*- and *CSL5*-complementing library plasmids were partially sequenced and the data used to search the *S. cerevisiae* genome database. After identifying the chromosomal locations of the cloned DNAs, potential ORFs in the region were tested singly and in combination to determine which one complemented the *csl* mutation. The GenBank accession numbers for *CSL4* (ORF YNL232w) and *CSL5* (*NDC10*) are Z71508 and X69300, respectively.

The identity of the *cslx*-complementing ORFs was confirmed by introducing frameshift mutations and retesting complementation. The frameshift mutations were generated by cleaving the respective ORF-containing plasmids at unique restriction sites within the identified ORF and blunting the DNA ends with Klenow polymerase. After religation, the plasmids were transformed into the *cslx* strains to test for complementation. In all cases (*csl1-1*, *csl2-1*, *csl3-1*, *csl5-1*), the frameshift plasmids failed to complement. The restriction sites used to generate the mutations were as follows: *CSL1*, *Sac*I (−4); *CSL2*, *Nde*I (+2); *CSL3*, *Bgl* II (+4); and *CSL5*, *Bgl* II (+4). (Numbers in parentheses indicate the number of base pairs added or deleted.) Assignment of *CSL4* was confirmed by expressing YNL232w from a heterologous promoter and observing complementation of *csl4-1* (plasmid pRB306, Figure 3).

Restriction fragments from the cloned DNAs were inserted into the integrating vector pRS306 (Sikorski and Hieter 1989) and used to target *URA3* to the corresponding chromosomal loci by homologous recombination. After confirming

the integrations by Southern blotting, meiotic analysis was performed to test linkage between the integrated *URA3* and the respective *csl* mutations. In all cases, direct linkage was observed, demonstrating that the cloned DNAs carried the respective *CSL* genes and not phenotypic suppressors. The restriction fragments used to target integration and the PD:T:NPD ratios for *cslx-URA3* linkage were as follows: *CSL1*, 2-kbp *Bgl*II-*Cla*I, 7:0:0; *CSL2*, 2.7-kbp *Eco*RI-*Bgl*II, 13:0:0; *CSL3*, 2.7-kbp *Hin*dIII-*Sma*I (located 5 kbp downstream of *CSL3*), 9:1:0; *CSL4*, 1.3-kbp *Eco*RI-*Hin*dIII, 11:0:0; and *CSL5*, 2-kbp *Xba*I, 10:0:0.

Analysis of human Csl4 (hCsl4): A BLAST search (Altschul *et al.* 1990) of the predicted yeast Csl4 amino acid sequence against the GenBank expressed sequence log (EST) database translated in all six reading frames (tblastn) was performed using the NCBI default parameters. The highest scoring homology ($P = 1.1 \times 10^{-15}$) was to human cDNA clone zl84h09.r1 (accession no. AA088342). Several other EST homologies of lesser significance were also returned, all apparently the same or related cDNAs present in different libraries including colon, pregnant uterus, fetal heart, pineal gland, placenta, and parathyroid tumor. Fifteen EST sequences homologous to zl84h09.r1 were collected from the database and assembled by computer to generate a cDNA consensus sequence.

Chromosome loss assays: Mitotic loss rates of nonessential chromosome fragments (CFs) were determined by fluctuation test as described (Hegemann *et al.* 1988). The CFs were derived from chromosome *III* and were generated in strain DJ147-1-2 by transformation with derivatives of plasmid pJS2 linearized with *Eco*RI (Shero *et al.* 1991). The pJS2 derivatives used for the fragmentation had the CEN6 segment replaced with either wild-type CEN3 or CEN3 lacking the CDEI site (Baker *et al.* 1989). The resulting CFs carry *URA3* and *SUP11* on their short arms and chromosome *III* sequences distal to D8B on their long arms. It was observed that *ade2-1* haploid strains containing either CF (white) grew slightly faster than strains lacking the CF (red). This is probably related to the red pigment formation, because the opposite effect was observed in *ade2-1 ade3* strains when the CF carried *ADE3*. In any event, the growth differential was not observed in diploid strains carrying only one copy of the CF (2N+1); therefore, 2N+1 diploids were used for all quantitative CF loss assays.

Determination of *CSL* mRNA levels: Steady-state RNA levels were determined by northern blot analysis as described (O'Connell *et al.* 1995). Hybridization signals were quantitated using a Betascope Blot Analyzer or GS-525 Molecular Imager (Bio-Rad Laboratories, Hercules, CA). *CSLX* cpm are reported relative to the level of *ACT1* RNA which was used as an internal standard to correct for differences in RNA recovery and gel loading. The following DNA fragments were labeled by random primed DNA synthesis and used as hybridization probes: *CSL1*, 690-bp *Pst*I-*Dra*I; *CSL2*, 640-bp *Nsi*I-*Sph*I; *CSL3*, 1050-bp *Sma*I-*Nsi*I; *CSL4*, 1150-bp *Sph*I-*Hin*dIII; *CSL5*, 1340-bp *Eco*RI; and *ACT1*, 300-bp *Bgl*II. The strains used for the analyses were as follows: wild-type, R203-8A; *cep1*, R95-1-1; *csl1-1*, R57-7B; *csl2-1*, R56-7B; *csl3-1*, R82-10D; *csl4-1*, BM3-40a; and *csl5-1*, BM3-64a. (The *CSL2* probe was isolated from plasmid pRB190 in which the *CSL2* gene had been modified to contain an *Nsi*I site at the initiator ATG.)

RESULTS

***cep1* synthetic lethal (*csl*) mutants:** Two screening systems were developed to isolate *csl* mutants; both utilized *CEP1* plasmid dependence as the primary screening phenotype (see materials and methods). Parental strains carried a disrupted or deleted chromosomal *cep1* allele but harbored a plasmid containing wild-type *CEP1*. The *csl* mutants are plasmid-dependent, because plasmid loss uncovers the chromosomal *cep1* mutation, exposing the *cep1* synthetic lethality. A "plasmid shuffle" test was used to eliminate plasmid dependence due to sequences on the screening plasmid other than *CEP1*, *i.e.*, candidate mutants were transformed with an unrelated *CEP1* plasmid, and only mutants that regained the ability to segregate the original plasmid were characterized further. In three separate trials, 36,500 colonies were screened to obtain 22 candidate *csl* mutants that passed the shuffle test. Of those, five segregated the plasmid-dependent phenotype 2:2 when backcrossed to a *cep1* parent, and these were designated *csl1-csl5* (Table 2). All five *csl* mutations were recessive and each defined a unique complementation group.

The *csl* screens were based on *CEP1* plasmid dependence, an indirect phenotype. Direct demonstration of the *cep1* synthetic lethal phenotype was accomplished by tetrad analysis. Diploids of genotype *CEP1*/*cep1 cslx*/*cslx* yielded tetrads with two viable and two inviable spores (Figure 1). Inviability was genetically linked to *cep1*; *CEP1* spores (Met^+) were viable, *cep1* spores (Met^-) were not. The *csl5-1* homozygous diploids germinated very poorly (<1%); therefore, *csl5-1 cep1* synthetic lethality was tested using *cep1 csl5-1* double heterozygotes. In this case, 25% of spores are expected to cosegregate *cep1* and *csl5-1* and be inviable. This was the observed result. Assuming no linkage, two-thirds of the tetrads (tetratypes) will contain one *csl5-1 cep1* spore, one-sixth (parental ditypes) will contain two *csl5-1 cep1* spores, and one-sixth (nonparental ditypes) will contain no *csl5-1 cep1* spores. The *csl5-1* mutation causes a Ts phenotype, allowing it to be scored in the *CEP1* background. No viable Ts^- Met^- (*csl5-1 cep1*) spores were recovered.

Microscopic examination revealed that most of the "inviable" spores were able to germinate and complete several cell divisions. In the case of *csl1-1* and *csl4-1*, microcolonies became visible after 5–7 days, while *csl2-1* and *csl5-1* produced microcolonies of only 4–50 cells (not visible to the naked eye). The phenotype of *csl3-1* was the most leaky, with microcolonies obvious after 3–4 days. No clear cell division cycle arrest phenotype was observed for any of the slow-growing *cep1 cslx* segregants. After backcrossing at least three times, strains containing each *csl* mutation in the $CEP1^+$ background were tested for additional phenotypes. None of the mutations caused methionine auxotrophy or altered sensitivity to the microtubule inhibitor benomyl; however, *csl5-1* strains were found to be temperature sensitive (Ts) for growth at 38°.

The *cis-trans* test: As loss of CP1 leads to defects in both centromere function and gene transcription, the synthetic lethality of *csl* mutations could be due to defects in either of these essential genetic processes. To

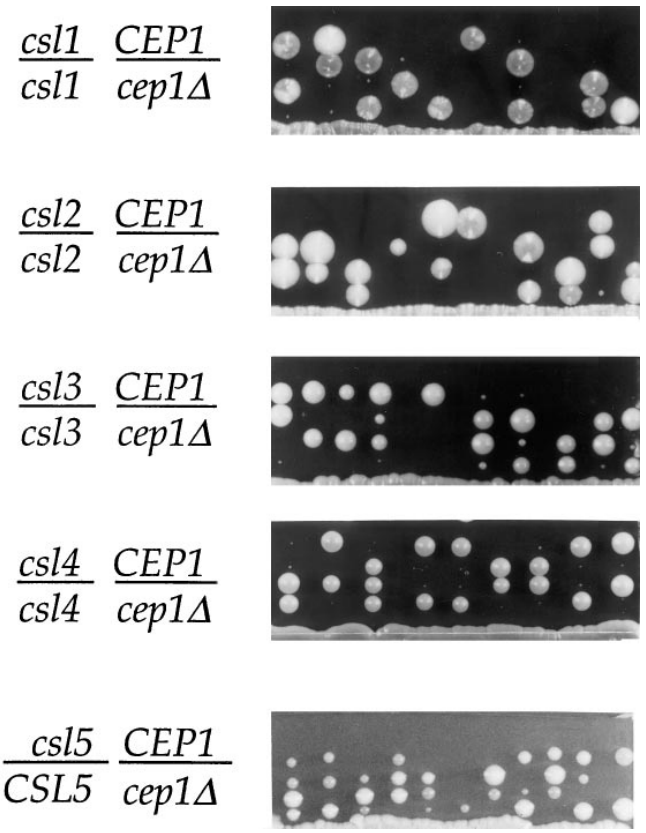

Figure 1.—Dissected tetrads of *cslx* diploids. Viable segregants were scored for Met prototrophy to determine *cep1* genotype: *csl1*, 14/14 Met^+; *csl2*, 18/18 Met^+; *csl3*, 20/20 Met^+ (tetrad #4 from left segregated Met 3^+:1^-); *csl4*, 19/19 Met^+ (tetrad #3 from left segregated Met 3^+:1^-). To avoid the sporulation defect of *csl5-1* homozygotes, *csl5-1* synthetic lethality was analyzed using a *csl5-1 cep1* double heterozygote, and *csl5-1* was scored by its Ts phenotype. No Met^- Ts^- segregants were recovered (see text).

distinguish the subset of *csl* gene products that interact directly or indirectly with CP1 at centromeres, we tested the effect of a *cis*-acting CDEI mutation on chromosome stability in the *csl* mutants. In carrying out its CEN-related function, CP1 acts through the CDEI site (Baker and Masison 1990). If the synthetic lethality of a given *csl* were due to a gross defect in centromere function, *e.g.*, the inability of the mutant to assemble a functional kinetochore in the absence of CP1, the same defect should exist for centromeres lacking the CDEI site even when CP1 is present. That is, with respect to an individual chromosome, the phenotypes of *cslx cep1* (*cep1* acting in *trans*) and *cslx* Δ*CDEI* (Δ*CDEI* acting in *cis*) should be equivalent, and centromere-related *csl* mutations should selectively and synergistically destabilize a Δ*CDEI*-mutated chromosome, leading to a "synthetic acentric" phenotype (Strunnikov *et al.* 1995).

The marker chromosome used for the *cis-trans* test was derived from chromosome *III* and carried either a wild-type or CDEI-deleted version of CEN3. Mitotic loss rates were determined for wild-type, *cep1*, and *cslx* strains (Table 3). The assays were carried out using diploids (2N+1) to avoid the slight growth advantage conferred by the marker chromosome in haploids (see materials and methods). Loss rates were determined by fluctuation test. The phenotypic equivalence of *cep1* and *cen3*Δ-*CDEI* mutations was confirmed for this marker chromosome by the results reported on the first three lines of Table 3. Deleting CDEI from the centromere caused a 5.8-fold increase in loss rate (R109 *vs.* R108), while deleting *cep1* caused a not significantly different 6.8-fold increase (R100 *vs.* R108). Combining the *cis* and *trans* mutations led to no significant additional increase (R101 *vs.* R100). The *csl* mutations caused only moderate increases in the mitotic loss rate of the wild-type marker chromosome; the 1.7- to 8.4-fold increases were quantitatively similar to or less than the 6.8-fold effect of *cep1*. However, unlike *cep1*, all of the *csl* mutations except *csl3-1* synergistically increased the loss rate of the *cen3*Δ*CDEI* chromosome. Expressed relative to the wild-type rate (3.8×10^{-4}), the loss rate of the *cen3*Δ*CDEI* chromosome in the *csl1-1*, *csl2-1*, *csl4-1*, and *csl5-1* mutants was increased 89-, 166-, 46-, and 253-fold, respectively. In each case, the increase was significantly greater than the additive effect of the *cslx* and *cen3*Δ*CDEI* mutations individually. The *csl3-1* mutation behaved like *cep1*; the loss rate of the *cen3*Δ*CDEI* chromosome (R111) was the same as that of the wild-type chromosome (R110). On the basis of these results, we concluded that the *csl1-1*, *csl2-1*, *csl4-1*, and *csl5-1* synthetic lethalities probably resulted from kinetochore defects, while the synthetic lethality of *csl3-1* appeared to be unrelated to centromeres.

Identification of *csl* genes: The wild-type alleles corresponding to each of the *csl* mutations were cloned by complementation of the plasmid-dependent phenotype. In all cases, complementing activity was shown to be conferred by a single open reading frame (ORF), and genetic linkage between the complementing ORF and the original *csl* mutation was verified by targeted integration and tetrad analysis (see materials and methods). The results are summarized in Table 4.

CSL3 is identical to *RTF1*, a recently characterized gene whose product appears to regulate transcription initiation by RNA polymerase II (Stolinski *et al.* 1997). Missense and loss of function *rtf1* alleles suppress mutations in TATA-binding protein and affect TATA element utilization. Rtf1 appears to act directly or indirectly at many promoters in the genome and may exert its effect by modulating chromatin structure (Stolinski *et al.* 1997). The possible involvement of Rtf1/Csl3 in promoter recognition by RNA polymerase II is consistent with the conclusion that Csl3 does not act at centromeres and suggests that the Csl phenotype of *csl3-1* is related to the transcription function of CP1.

The *csl3-1* mutant is a methionine prototroph, indicating that *MET* gene transcription is not grossly defective. To test for a more subtle phenotype, genetic interaction

TABLE 3

Mitotic loss rates of marker chromosomes containing either a wild-type (*CEN3*) or CDEI-deleted (*cen3ΔCDEI*) centromere

	Genotype		Loss rate (Events/cell/division × 10^4)		
Strains	*CEP1*	*CSL*	CF[*CEN3*]	CF[*cen3ΔCDEI*]	Relative[a]
R108/R109	+/+	+/+	3.8 (1.0)	22 (5.8)	5.8
R98/R99	+/*cep1*	+/+	3.4 (0.9)	27 (7.1)	7.9
R100/R101	*cep1/cep1*	+/+	26 (6.8)	27 (7.1)	1.0
R116/R119	+/+	*csl1-1/csl1-1*	21 (5.5)	340 (89)	16.2
R129/R127	+/+	*csl2-1/csl2-1*	9.0 (2.4)	630 (166)	70
R110/R111	+/+	*csl3-1/csl3-1*	32 (8.4)	36 (9.5)	1.1
R224/R220	+/+	*csl4-1/csl4-1*	6.9 (1.7)	175 (46)	25
R225/R218	+/+	*csl5-1/csl5-1*	30 (7.9)	960 (253)	32
R221/R222	+/+	Δ*csl3*/Δ*csl3*	4.2 (1.1)	26 (6.9)	6.2

[a] CF[*cen3ΔCDEI*]/CF[*CEN3*]

between *csl3-1* and a *cis*-acting *MET16* promoter mutant was analyzed, *i.e.*, the *cis-trans* test applied to an extracentromeric site of CP1 action. The *met16-47* mutation inserts a TA dinucleotide at the center of the CP1 binding site in the *MET16* promoter. As a result, the dissociation constant for CP1 binding is increased by 170-fold, steady-state *MET16* RNA levels are reduced by 90%, and *met16-47* strains grow slowly on media lacking methionine ($Met^{+/-}$) (O'Connell *et al.* 1995). Tetrads of a *met16-47 csl3-1* double heterozygote yielded two Met^+ spores and two $Met^{+/-}$ spores (data not shown), indicating that *csl3-1* neither enhanced nor suppressed the methionine bradytrophy of *met16-47* (*csl3-1* and *met16-47* are unlinked). The same result was obtained with *csl1-1 met16-47* and *csl2-1 met16-47* double heterozygotes. If the synthetic lethal phenotype of *csl3-1* is the result of perturbations in RNA polymerase II transcription, the defect apparently does not extend to the CP1-dependent gene *MET16*.

RTF1 (*CSL3*) is not an essential gene (Stolinski *et al.* 1997). To determine if the *csl3-1* synthetic lethal phenotype was due to loss of Rtf1 function, the *CSL3* gene was deleted in a diploid strain heterozygous for *cep1* (the *csl3* deletion was marked with *URA3*). Tetrads from the doubly heterozygous diploid yielded four viable spores, and Ura^+ and Met^+ phenotypes segregated 2:2 in all tetrads. Therefore, total loss of Rtf1 led to neither *cep1* synthetic lethality nor methionine auxotrophy. Also, the chromosome loss phenotype of the *csl3-1* mutant is not due to loss of Rtf1 function, as the mitotic loss rates of neither wild-type nor *cen3ΔCDEI* marker chromosomes are significantly changed in the *csl3* null mutant (Table 3). Thus, it appears that even though *csl3-1* is recessive, the observed phenotypes result from altered Rtf1 function rather than loss of function.

CSL1 and *CSL5* encode subunits of the CDEIII-binding complex Cbf3. *CSL1* is identical to *CEP3* (*CBF3b*) which encodes the 64-kD subunit (Lechner 1994; Strunnikov *et al.* 1995), while *CSL5* is identical to *NDC10* (*CBF2*, *CEP2*) encoding the 110-kD subunit (Goh and Kilmartin 1993; Jiang *et al.* 1993). Significantly, these two genes were predicted to have centromere-specific functions on the basis of the *cis-trans* test. *CSL2* is identical to *CSE4*, another essential gene associated with the centromere. *CSE4* was first identified in a screen for mutations that enhanced the phenotypic effect of a *cis*-acting mutation in CDEII. Cse4 appears to be the *S. cerevisiae* homolog of the mammalian centromere protein CENP-A, and while biochemical evidence is lacking, the available genetic evidence supports the hypothesis

TABLE 4

Identification of *CSL* alleles

Mutation	Complementing ORF	Chromosome	Gene	Essential?
csl1-1	YMR168C	*XIII*	*CEP3/CBF3B*	Yes
csl2-1	YKL049C	*XI*	*CSE4*	Yes
csl3-1	YGL244W	*VII*	*RTF1*	No
csl4-1	YNL232W	*XIV*	*CSL4*	Yes
csl5-1	YGR140W	*VII*	*NDC10/CBF3A/CEP2*	Yes

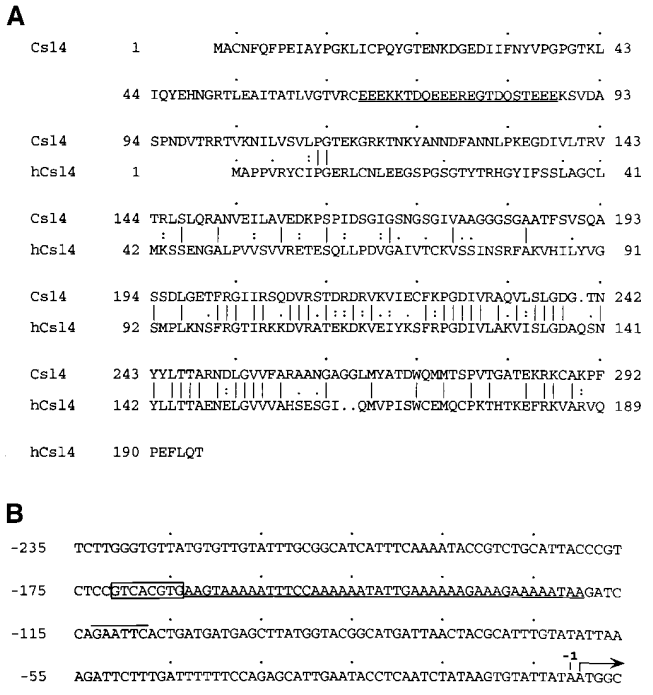

Figure 2.—Sequences of the *CSL4* gene product and 5′ flanking DNA. (A) The translated sequence of the *CSL4* ORF is displayed with the glutamate-rich motif underlined. Also shown is an alignment between Csl4 and its human homolog (hCsl4) made using the Genetics Computer Group (Madison, WI) GAP program. (B) The *CSL4* 5′-flanking DNA sequence is shown with the CDEI site boxed. The CDEII-like sequence is underlined and the proximal *Eco*RI site is overscored.

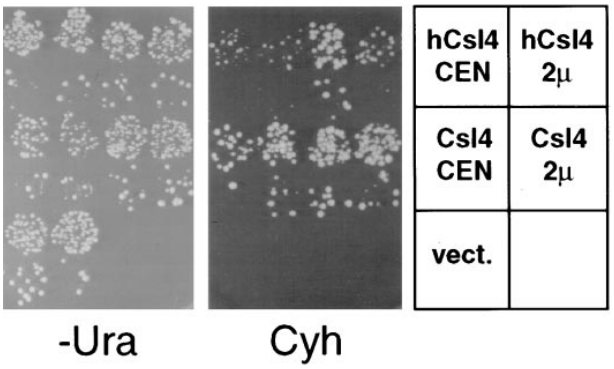

Figure 3.—hCsl4 complements *csl4-1*. A *cep1 csl4-1 cyh2*^{r} strain maintained by pKM35 (*CEP1-CYH2*) was transformed with hCsl4 (pRB305, pRB308) or *CSL4* (pRB306, pRB307) expression vectors (single- and multicopy, respectively). Transformant colonies (Ura^{+}) were picked, resuspended, and plated to test for cycloheximide resistance. Plasmids complementing *csl4-1* allow the mitotic loss of pKM35 which is detected by the appearance of cycloheximide-resistant cells. Three independent transformants were tested for each plasmid, and "spots" of approximately 10^3 and 10^2 cells were plated in each case. Plasmid pRS306, which carries the selectable marker *URA3* but no *CSL4* sequences, was included as a control.

that Cse4 is a centromere-specific histone (Smith *et al.* 1996; Stoler *et al.* 1995). Thus, three of four Csl proteins predicted by the *cis-trans* test to have centromere-related functions are known or imputed kinetochore components. This supported the validity of the *cis-trans* test for distinguishing centromere-related *csl* targets and suggested that the fourth member of this class, *CSL4*, also encoded a gene product that acts at centromeres. The *csl4-1* mutation had relatively little effect (less than 2-fold) on the loss rate of the wild-type marker chromosome; however, *csl4-1* dramatically increased the loss rate of the *cen3ΔCDEI* chromosome. In relative terms, the synergism between *csl4-1* and *cen3ΔCDEI* was similar to that observed for *csl1-1* and *csl5-1* (25-fold *vs.* 16-fold and 32-fold, respectively; Table 3).

***CSL4* is an essential gene with a human homolog:** *CSL4* corresponds to the uncharacterized *S. cerevisiae* ORF YNL232w located on the left arm of chromosome *XIV*. It potentially encodes a 292-amino-acid protein of molecular weight 32,000 (Figure 2A). A BLAST search against the GenBank protein databases produced numerous hits of low significance ($0.1 < P < 0.9$), with apparent homologies occurring mainly in a region of the hypothetical Csl4 that is rich in glutamic acid residues. When these regions were filtered (Altschul *et al.* 1994), no significant similarity between Csl4 and other proteins in the database was found. Nonetheless, the limited homology to other proteins with glutamic acid-rich regions may suggest that Csl4 is a chromatin protein, like many of the proteins in which such Glu-rich regions are found, *e.g.*, AT motif binding factor (Ido *et al.* 1996), *X*-linked nuclear protein (Gecz *et al.* 1994), HMG protein (Pentecost and Dixon 1984).

A BLAST search of the Csl4 ORF against the human EST database yielded several significant hits, all apparently to the same or closely related cDNAs. The most extensive homology was observed with EST zl84h09.r1 (GenBank AA088342) from the Stratagene (La Jolla, CA) human colon cDNA library (#937204). The homology to yeast ORF YNL232w (*CSL4*) had been noted when the EST was entered into the database. By collecting additional ESTs homologous to zl84h09.r1, a cDNA consensus sequence was assembled. The assembled sequence contained a good Kozak initiator ATG consensus sequence (Kozak 1992) at the 5′ end (AATCATGG) and encoded an open reading frame of 195 amino acids (M_r = 21,400) which was designated human Csl4 (hCsl4). The putative human protein is smaller than Csl4, but the two proteins share significant homology over their C-terminal regions (Figure 2A). In this 107-residue region, the proteins are 57% similar and 48% identical. The possible functional significance of this homology was assessed by testing whether hCsl4 could complement *csl4-1*. As shown in Figure 3, when expressed from either a single- or multicopy vector, hCsl4 rescued the synthetic lethal phenotype of *csl4-1*. The high copy hCsl4 vector was also able to partially complement the *csl4* deletion mutation (data not shown).

The possibility that Csl4 might be a kinetochore component led us to determine if *CSL4* was an essential gene. One copy of *CSL4* was disrupted in a *cep1* heterozy-

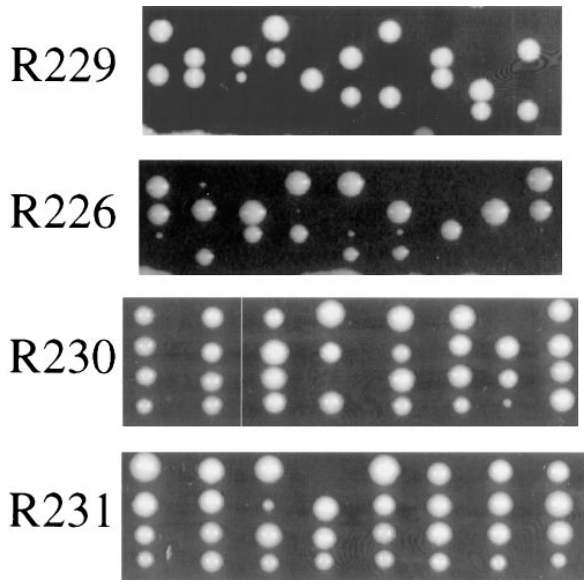

Figure 4.—Effect of *CSL4* gene disruption and dispensability of the upstream CDEI site. Shown are dissected tetrads of diploid strain R229 and its derivatives. R229 is heterozygous for *csl4*::*LEU2*. Strain R226 was obtained from R229 by integrating plasmid pRB267 (carrying the 1.3-kbp *Eco*RI-*Hin*dIII fragment of *CSL4*) at the *ura3* locus on chromosome *V*. In strains R231 and R230, the integrated *CSL4* alleles contained additional 5′ sequences, either with (pRB289) or without (pRB288) the intact CDEI site, respectively. In the case of R230 and R231, the *CSL4* alleles integrated at *ura3* sequences flanking the *LEU2* gene at *csl4::LEU2* (see materials and methods); therefore, only $4^+:0^-$ segregation is observed. The *CSL4* locus from two independent Ura^+ Leu^+ segregants of R230 was amplified by PCR and subjected directly to DNA sequencing. The results confirmed that the CAT mutation was present.

gous diploid strain by deleting the presumptive *CSL4* ORF and marking the deletion with either the *URA3* or *LEU2* gene. Tetrads obtained from these heterozygous diploids yielded two viable and two inviable spores (*e.g.*, strain R229, Figure 4). The inviability was genetically linked to *csl4*Δ (no Ura^+ or Leu^+ prototrophs were recovered among 65 viable spores) and independent of *cep1*. Thus, *CSL4* is an essential gene. To show that the observed lethality was complemented by *CSL4*, a *URA3* plasmid carrying the 1.3-kbp genomic *CSL4 Eco*RI-*Hin*dIII fragment was integrated at the endogenous *ura3* locus in strain R229. This *CSL4* subclone complemented *csl4-1* (not shown); therefore, it was somewhat surprising that the same fragment was unable to completely rescue *csl4::LEU2*. The *csl4::LEU2* spores cosegregating the integrated plasmid (Leu^+ Ura^+) grew very slowly compared to the wild-type spores (Figure 4, strain R226), and the slow growth phenotype was independent of *cep1* (3/8 Leu^+ Ura^+ spores were Met^-). The most likely explanation for the poor complementation was that the 1.3-kbp *Eco*RI-*Hin*dIII fragment lacked 5′-flanking sequences required for full *CSL4* expression.

Examination of the *CSL4* 5′-flanking DNA upstream of the proximal *Eco*RI site revealed sequences resembling an *S. cerevisiae* centromere. A perfect CDEI site is present at position −171 (relative to the initiator ATG) adjacent to a stretch of AT-rich DNA (Figure 2B). Although shorter in length than CDEII elements found at centromeres, the nucleotide composition of this 44-bp segment (86% AT) is similar. Since poly (dA:dT) is known to be a ubiquitous transcriptional activation sequence in yeast (Iyer and Struhl 1995), it seemed plausible that the CDEII-like element provides this function for *CSL4*, explaining the failure of the 1.3-kbp *Eco*RI-*Hin*dIII fragment to provide wild-type *CSL4* function. To test this hypothesis, the AT-rich element, either with or without an intact CDEI site, was restored to the 1.3-kbp *CSL4 Eco*RI/*Hin*dIII fragment and the resulting constructs (pRB289 and pRB288, respectively) were reintegrated at the *csl4* locus to test their ability to provide Csl4 function. In pRB288, the CDEI site was disabled by changing the CA dinucleotide at position 5/6 to TTA. This mutation (CAT) increases the dissociation constant for CP1 binding by 1400-fold and inactivates CDEI with respect to centromere function (Baker *et al.* 1989). Both constructs complemented *csl4::LEU2* (Figure 4; strains R230, R231), indicating that full expression of *CSL4* requires sequences upstream of −113, but not a functional CDEI element.

***CSL* mRNA levels are independent of CP1:** A trivial explanation for the synthetic lethality of *csl1-1*, *csl2-1*, *csl4-1*, and *csl5-1* would be that these mutations render transcription of the respective essential genes CP1-dependent. (This model does not explain *csl3-1*, because *CSL3* is not an essential gene.) Such a model is inconsistent with the results of the *cis-trans* test, which argue that the Csl gene products interact with CP1 at the level of CEN DNA; however, CP1-dependent expression could contribute to the phenotypes observed. For example, if full expression of an essential kinetochore gene (*CSLX*) required CP1, a partial loss of function mutation (*cslx*) might be synthetically lethal in a *cep1* genetic background due to reduced expression of the mutant Cslx gene product. In the $CEP1^+$ background, the hypomorph would be viable but especially sensitive to *cis*-acting CEN mutations. To determine if *CSL* gene transcription was dependent on CP1, steady-state *CSL* RNA levels were measured in wild-type and *cep1* strains. The results (Figure 5) showed no significant differences. In fact, except for *CSL1*, RNA levels were actually higher in *cep1* cells. Also, the *csl* mutations themselves did not dramatically alter *CSL* RNA levels; increases or decreases of more than 50% were not observed. In sum, these results offer no evidence that *CSL* gene expression is defective in *cep1* strains or altered in *csl* mutants.

DISCUSSION

The Csl screen is an efficient genetic method for identifying genes involved in centromere function. Of the five *csl* genes obtained so far, two encode Cbf3 subunits, while a third encodes Cse4, presumed to be an essential centromere histone. As only a single allele was

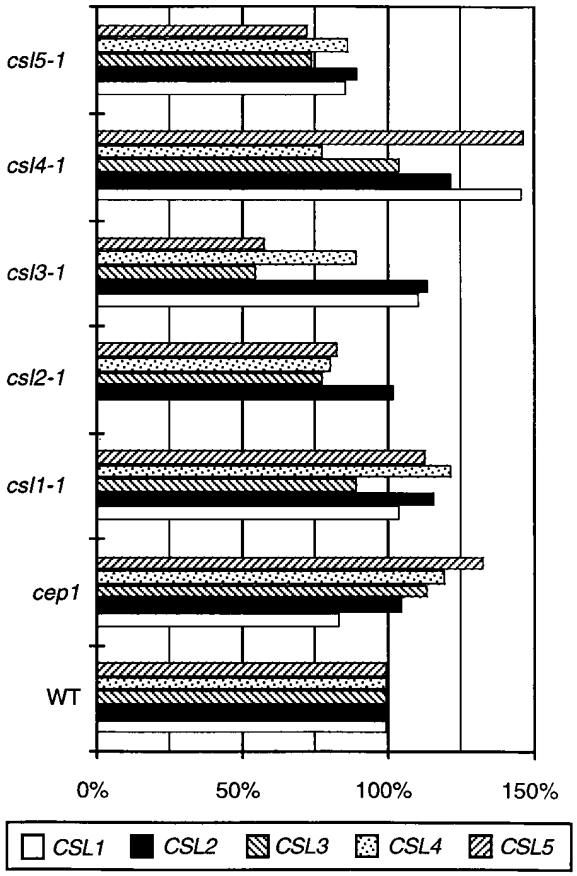

Figure 5.—Determination of *CSLX* RNA levels. Steady state levels of *CSL* RNAs were determined as described in materials and methods and are expressed relative to levels found in the wild-type strain.

obtained for each of the five *csl* complementation groups, the screen is not saturated. Also, no alleles of *mif2* or *cdp1* were obtained, and both of these genes are known to yield mutations conferring a Csl phenotype (Foreman and Davis 1996; Meluh and Koshland 1995). Continued screening should yield additional Csl complementation groups, at least some of which may identify new kinetochore-related gene products. In this regard, the *cis-trans* test proved to be an effective means of classifying the *csl* mutants. All three of the *csl* mutations targeting known or imputed kinetochore components (*csl1-1*, *csl2-1*, *csl5-1*) showed synergism in the *cis-trans* test. On this basis, we propose that a fourth *csl* gene product, Csl4, also has a kinetochore-related function. Like Csl1/Cep3, Csl2/Cse4, and Csl5/Ndc10, Csl4 is essential for cell viability.

Obtaining an allele of *rtf1* (*csl3-1*) in the Csl screen is further evidence that the function of CP1 is not restricted to centromeres. Rtf1 apparently acts at gene promoters to influence transcription site selection by RNA polymerase II (Stolinski *et al.* 1997). While the *csl3-1* mutation does increase chromosome loss, the lack of interaction between *csl3-1* and *cen3ΔCDEI* implies that the *cep1* synthetic lethality of *csl3-1* is not the direct result of kinetochore defects. The *csl3* null mutant has no phenotype that we detect, again arguing that Rtf1 plays no positive role in centromere function. Our favored explanation for synthetic lethality of *csl3-1* is that pol II transcription is lethally misregulated by the mutant Rtf1 protein in the absence of CP1. The number and identity of the promoters involved is unknown. The chromosome loss phenotype of *csl3-1* may be the cumulative result of multiple subtle defects in chromosome structure introduced by the mutant Rtf1 protein (possibly at all pol II promoters) and, in effect, be independent of kinetochore function. In this case, the presence or absence of CDEI at the centromere would be expected to have at most an additive effect.

The very high mitotic loss rates of the *cen3ΔCDEI* marker chromosome in *csl1-1*, *csl2-1*, *csl4-1*, and *csl5-1* mutants lead us to conclude that the Csl phenotype of these strains is due directly to chromosome missegregation. In absolute terms, loss rates of the CP1-deficient model chromosome ranged from 1.8 to 9.6%. Assuming that these *csl* mutations affect all 16 endogenous chromosomes to similar extents, loss rates of this magnitude would extrapolate to at least one loss event in a high proportion of *cep1* mitoses (29–100%). An alternative explanation for the synthetic lethality of these mutations, that the expression of these essential genes is rendered CP1-dependent, is inconsistent with the finding that *CSL* RNA levels vary by less than twofold between wild-type, *cep1*, and *csl* backgrounds. Possible transcriptional regulation by CP1 is especially relevant to *CSL4*, which contains a CDEI site in its 5′-flanking DNA. Although this region of upstream sequence is apparently required for full expression of *CSL4*, the CDEI site is not. *CSL4* carrying an inactivating mutation in the upstream CDEI site complements the *csl4* null allele and actually produces higher than wild-type levels of *CSL4* RNA (data not shown). The juxtaposition of a CDEI site next to the AT-rich element in the *CSL4* promoter is interesting insofar as it creates a CEN-like sequence; however, at present, there is no evidence to suggest any functional relevance.

Meluh and Koshland (1995) have proposed a model for the yeast centromere-kinetochore complex invoking multiple cooperative interactions between proteins bound at CDEI and CDEIII. Our findings reinforce and extend this model. The new alleles *csl1-1* and *csl5-1* confirm the proposed interactions between CP1 and Cbf3 subunits. More significantly, the synthetic lethality detected between *csl2-1* and *cep1* extends the network of genetic interactions to include Cse4. Genetic evidence suggests that Cse4 interacts with CDEII (Stoler *et al.* 1995). That an allele of *cse4* (*csl2-1*) exhibits strong synthetic interactions with both *cep1* and *cen3ΔCDEI* can be interpreted to mean that Cse4 also interacts with CP1/CDEI. If so, a complete network of protein-protein and protein-DNA interactions would exist to intercon-

nect CDEI, CDEII, CDEIII, and the proteins associated with them. Cse4 is a variant of histone H3 and the *S. cerevisiae* homolog of the mammalian centromere protein CENP-A (Stoler *et al.* 1995). Smith *et al.* (1996) isolated *CSE4* as a high copy suppressor of *hhf1-20*, a Ts lethal mutation in histone H4 that leads to G_2-M arrest at the restrictive temperature. This finding has been interpreted to suggest that Cse4 and H4 interact and that this interaction is essential for cell cycle progression through mitosis. The observed genetic interactions between *cse4* alleles and *cis*-acting centromere mutations (*cen3-130*, *cen3*Δ*CDEI*) argue that Cse4-H4 acts at the centromere, perhaps taking the form of a novel nucleosome required for wrapping the AT-rich CDEII sequence. We note that Bloom *et al.* (1989) proposed some years ago that a modified nucleosome might serve as the structural foundation for the yeast kinetochore. CP1 might provide the targeting signal for a CEN-specific Cse4 histone complex (nucleosome?).

Histones generally impede the interaction of DNA binding proteins; however, recent findings show that many transcription factors are capable of binding to their cognate sites on the surface of a nucleosome (Owen-Hughes and Workman 1994). Therefore, it is not unreasonable to propose that CEN DNA be packaged in a nucleosome with CDEI and CDEIII accessible for binding CP1 and Cbf3. In fact, assuming that the Cse4-modified nucleosome retained the approximate molecular dimensions of a conventional nucleosome, CDEI and CDEIII, separated by an average of 82 bp, would be brought adjacent to each other on the surface of the nucleosome, allowing direct interaction between CP1 and Cbf3. Cbf3 appears to be the kinetochore component responsible for binding microtubules (Sorger *et al.* 1994); therefore, the mechanical forces transferred to the DNA fiber by kinetochore microtubules during metaphase and anaphase must be equally opposed by the sum of forces binding Cbf3 to the CEN DNA. A configuration, in which CEN DNA is wrapped around a specialized nucleosome with CDEI and CDEIII linked through CP1-Cbf3 interaction, would allow that force to be distributed over a full turn of nucleosomal DNA. In addition to the energy of sequence-specific Cbf3-CDEIII and CP1-CDEI binding, multiple histone-DNA interactions would be brought to bear in maintaining kinetochore attachment to the centromere DNA.

If a unique nucleosomal substrate containing Cse4 is indeed present at yeast centromeres, we would expect that specialized chromatin proteins are needed to position, assemble, and/or maintain the centromere-specific structure. Mif2 and Csl4 are candidates for such roles. Both proteins possess amino acid sequence characteristics of chromatin components. Mif2 has a highly acidic domain and an AT hook motif found in chromatin proteins that bind AT-rich DNA (Brown 1995), the latter attribute leading Meluh and Koshland (1995) to place Mif2 at CDEII in their kinetochore model. Protein database homology searches yield little insight to the function of Csl4. The most tangible clue is a 25-amino-acid region of charged residues, mostly glutamate (40%). Such motifs are common in transcription factors and chromatin proteins, but they are by no means restricted to this class of proteins. Csl4, like Mif2, is an essential gene product, and we interpret the interaction between *csl4-1* and *cen3*Δ*CDEI* to mean that Csl4 acts at the level of CEN DNA. It may be a *bona fide* component of the kinetochore, or Csl4 may be a kinetochore assembly factor. The analysis of conditional alleles of *csl4* is in progress and should be informative in elucidating Csl4 function. Csl4 joins Cse4 and Mif2 as likely yeast centromere proteins having structural mammalian homologs. As mentioned above, Cse4 is homologous to CENP-A, while Mif2 is homologous to the mammalian centromere protein CENP-C (Brown 1995), a component of the inner kinetochore plate (Pluta *et al.* 1995). It is tempting to speculate that hCsl4 might also be a centromere protein. As the hCsl4 cDNA complements *csl4-1*, yeast and human Csl4 share at least one function in common.

The proposal that CP1 forms a network of interactions with centromere proteins bound at CDEII and CDEIII explains how CP1 could facilitate kinetochore assembly and confer stability to the complex; however, this CP1-centric view of the kinetochore is seemingly at odds with the fact that CP1 is essential for neither kinetochore assembly nor function. What is the selective advantage for maintaining CP1 at the centromere? First, CP1 decreases the rate of mitotic chromosome loss ~10-fold (Baker and Masison 1990). As chromosome loss is lethal in haploids and aneuploidy deleterious to diploids, the improved accuracy of chromosome transmission would be strongly selected. Second, any factor that promotes the timely assembly and/or maintenance of the kinetochore complex indirectly reduces cell generation times by eliminating potential cell cycle delays. Kinetochore integrity is monitored by the spindle assembly cell cycle checkpoint (Wang and Burke 1995). A single defective centromere is sufficient to trigger the checkpoint and induce a cell cycle delay at G_2-M (Spencer and Hieter 1992; Wells and Murray 1996), implying that kinetochore assembly is or can readily become rate-limiting for cell cycle progression through mitosis. Centromeres lacking CDEI-bound CP1 to orchestrate kinetochore assembly might lag in establishing spindle attachment, trip the checkpoint, and delay mitosis. This hypothesis would explain the lengthened cell generation times of *cep1* mutants. It also predicts that the spindle integrity checkpoint is constitutively activated in *cep1* mutants and that cell cycle times would decrease in *cep1* double mutants defective in checkpoint regulation (*e.g.*, *mad*, *bub*). Of course, the cost of abrogating spindle checkpoint control in *cep1* strains would be increased segregation errors and a consequent decrease in cell viability.

The only known process for which CP1 is absolutely essential is methionine biosynthesis, where CP1 is involved in the transcriptional activation of *MET* genes. Is CP1 a transcription factor whose function was coopted to centromeres or vice versa? The results of the Csl screen focus attention to the centromere. That four of five *csl* mutations target known or imputed kinetochore proteins implies, in our opinion, that the major biological role of CP1 is to safeguard the biochemical integrity of the kinetochore. When CP1 is bound at CDEI, defects in kinetochore structure or function are tolerated that otherwise would be lethal.

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