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) (Hietter 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-
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 binds to CDEI and altered sensitivities to benzimidazole compounds, which are absolutely essential for centromere function; mutations would not. 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 to CDEII (Lechner and Carbon 1997; Gaudet and Fitzgerald-Hayes 1987; Hengemann 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 to CDEII (Lechner and Carbon 1997). The Cbf3 complex consists of four subunits p110 (NDC10/CBF2/CTF14), p46 (CBF3b/CBF3), p38 (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. For example, Davis et al. (1988) have identified a novel gene which they named CDP1 (Cbf1-dependent). Both cdp1-1 and cdp1Δ 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 (For eman and Davis 1996). MEL and Kos hland (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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
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<tr>
<td>381G</td>
<td>MATα cry1 ade2-1 trp1 his4-580 tyr1 lys2 SUP4-3</td>
</tr>
<tr>
<td>D37-1A</td>
<td>[381G] MATα ura3 leu2 ade8 cep1::ura3-10</td>
</tr>
<tr>
<td>D102-4A</td>
<td>[381G] MATα ura3 leu2 TRP1 ade8 cep1::ura3-10 cyh2</td>
</tr>
<tr>
<td>R95-1-1</td>
<td>[381G] MATα ura3 leu2 TRP1 ade8 cep1α cyh2</td>
</tr>
<tr>
<td>D102-5D</td>
<td>[381G] MATα ura3 leu2 TRP1 ade8 cep1::ura3-10 cyh2 can1</td>
</tr>
<tr>
<td>R85-1D</td>
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</tr>
<tr>
<td>R67-RA</td>
<td>[381G] MATα ura3 leu2 TRP1 ade8 cep1::ura3-10 cyh2 can1</td>
</tr>
<tr>
<td>R108</td>
<td>[381G] MATα MATα ura3 ura3 leu2 leu2 trp1/TPR1 [CFIII(D8B.DJ147-1-2.URA3-SUP11-3CDEI)]</td>
</tr>
<tr>
<td>R109</td>
<td>[381G] MATα MATα ura3 ura3 leu2 leu2 trp1/TPR1 [CFIII(D8B.DJ147-1-2.URA3-SUP11-3CDEI)]</td>
</tr>
<tr>
<td>R98</td>
<td>[R108] CEP1/cep1::ura3-10</td>
</tr>
<tr>
<td>R99</td>
<td>[R109] CEP1/cep1::ura3-10</td>
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<tr>
<td>R100</td>
<td>[R108] cep1::ura3-10 cep1::ura3-10</td>
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</tr>
<tr>
<td>R116</td>
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<td>R218</td>
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</tr>
<tr>
<td>R226</td>
<td>[R229] ura3/URA3::pRB267</td>
</tr>
<tr>
<td>R231</td>
<td>[R229] CSL4/cs4Δ::ura3::LEU2::pRB289</td>
</tr>
<tr>
<td>R230</td>
<td>[R229] CSL4/cs4Δ::ura3::LEU2::pRB289</td>
</tr>
<tr>
<td>R203-8A</td>
<td>[381G] MATα ura3 leu2</td>
</tr>
<tr>
<td>R57-7B</td>
<td>[381G] MATα ura3 leu2 TRP1 ade8 cep1::ura3-10 cyh2 cs1-1 [pAP2]</td>
</tr>
<tr>
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</tr>
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</tr>
<tr>
<td>BM 3-40a</td>
<td>[381G] MATα ura3 leu2 TRP1 ade8 cep1::ura3-10 cyh2 cs4-1 [pAP2]</td>
</tr>
<tr>
<td>BM 3-64a</td>
<td>[381G] MATα ura3 leu2 TRP1 ade8 cep1::ura3-10 cyh2 cs5-1 [pAP2]</td>
</tr>
</tbody>
</table>

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

Complete 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°C.

Plasmid pAP2 was derived from YEp352 (Hill et al. 1993) by inserting DNA fragments containing ade2-2p (Sall-Smal) and CEP1 (BamHI-BglII). Plasmid pKM35 was derived from pRS328 (Sikorski and Boeke 1991) by inserting the wild-type CEP1 gene (BamHI-BglII) at the unique BamHI site. Plasmid pRB267 was obtained by inserting the 1.3-kbp EcoRI-HindIII 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 EcoRI fragment spanning positions −468 to −113 relative to the CSL4 initiator ATG and inserting this fragment into the EcoRI site of pRB267. The resulting plasmid, pRB289, has the EcoRI fragment in the native orientation. Plasmid pRB288 is identical to pRB289 except that the CDEI site located within the EcoRI fragment was mutated to GTCATTATG using bidirectional recombinant PCR. This mutation (CAT) overlapping the CDEI site and containing the CAT mutation was used to amplify both halves of the EcoRI 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 EcoRI and inserted into the EcoRI 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 Sphi-MluI 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 EcoRI and HindIII 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 254::URA3 deletion allele. To allow subsequent transformation with URA3 plasmids, the csd4-linked URA3 marker in R211 was changed to LEU2 using the marker change plasmid pDM2.
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 XbaI and XhoI 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 XbaI and XhoI and inserted between the Sp6 and XhoI 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 (Cyc+). The first protocol, adapted from Bender and Pringle (1991), employed parent strains (D37-1A, D102-4A, or R95-1) of genotype MATα leu2 ura3 ade2 ade3 cep1 URA3 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 pheno-typically Cep1+ (Met+) Lee− 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 recovers the cep1+ synthetic lethality. Cells were mutatedized 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 streaking, the candidate csl mutant strains 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 csl4-1 mutation and carried a CEN plasmid (pKM35) bearing the wild-type CYH2 allele along with CEP1. Plasmid dependence (Sect−) was scored by replicating mutated colonies onto cycloheximide plates. The csl mutants cannot give rise to Cyc+ papillae, which would normally arise from mitotic loss of pKM35. Except for scoring the Sect− phenotype, other aspects of the Cyc+ 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, csl segregants were obtained in both mating types and in CEP1 and ADE3 genetic backgrounds.

| TABLE 2 |
| Results of Csl screens |
| Sectoring | Trial 1 | Trial 2 | Cyh+ |
| Parent strain | D37-1A | R95-1-1 | D102-4A |
| Mutagenized survivors | 14,000 | 7500 | 15,000 |
| 1st candidates | 520 | 75 | 100 |
| Positive on retest | 15 | 12 | 22 |
| Passed 2nd shuffle test | 6 | 6 | 10 |
| 2:2 segregation | 2 | 2 | 1 |
| Complementation groups | csl1, csl2, csl4, csl5, csl3 |

The remaining mutants were cloned by complementation. In some 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, SacI (−4); CSL2, NdeI (−2); CSL3, BglII (−4); and CSL5, BglII (−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 csl1-1 (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 BglII-ClaI, 7:0:0; CSL2, 2.7-kbp EcoRI-BglII, 13:0:0; CSL3, 2.7-kbp HindIII-Smal (located 5 kbp downstream of CSL3), 9:1:0; CSL4, 1.3-kbp EcoRI-HindIII, 11:0:0; and CSL5, 2-kbp XbaI, 10:0:0.

Analysis of human CS44 (hCS4): A BLAST search (Altschul et al. 1990) of the predicted yeast CS4 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 × 10^-19) was to human cDNA clone z84h09.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 z84h09.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 (Heggemann et al. 1988). The CFs were derived from chromosome III and were generated in strain D147-1-2 by transformation with derivatives of plasmid pJS2 linearized with EcoRI (Sher o et al. 1991). The pJS2 derivatives used for the fragmentation had the CEN6 segment replaced with either wild-type CEN3 or CEN3 lacking the CDE1 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 Imageer (Bio-Rad Laboratories, Hercules, CA). CSL Xcm plasmids were 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 PstI-Dral; CSL2, 640-bp NsiI-Sphl; CSL3, 1050-bp Smal-NsiI; CSL4, 1150-bp Sphl-HindIII; CSL5, 1340-bp EcoRI; and ACT1, 300-bp BglII. The strains used for the analyses were as follows: wild-type, R203-8A; csl1-1, R95-1-1; csl2-1, 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 NsiI site at the initiator ATG.)

RESULTS

csl1 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 csl1 allele but harbored a plasmid containing wild-type CEP1. The csl mutants are plasmid-dependent, because plasmid loss uncovers the chromosomal csl1 mutation, exposing the csl1 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 csl1 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 csl1 synthetic lethal phenotype was accomplished by tetrad analysis. Diploids of genotype CEP1/csl1 cslx/ cslx yielded tetrads with two viable and two inviable spores (Figure 1). Inviability was genetically linked to cep1; CEP1 spores (Met+) were viable, csl1 spores (Met-) were not. The csl5-1 homozygous diploids germinated very poorly (<1%); therefore, csl5-1 csl1 synthetic lethality was tested using cep1 csl1-1 double heterozygotes. In this case, 25% of spores are expected to cosegregate csl1 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 cep1-1 spore, one-sixth (parental ditypes) will contain two csl5-1 cep1-1 spores, and one-sixth (nonparental ditypes) will contain no csl5-1 cep1-1 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 “inviiable” 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 csl-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 mitotuble inhibitor benomyl; however, csl5-1 strains were found to be temperature sensitive (Ts) for growth at 38°C.

The cis-trans test: As loss of CEP1 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
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- integration and tetrad analysis (see materials and methods) the loss rate of the wild-type chromosome (R110), and the original 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 csl3ΔCDEI chromosome. Expressed relative to the wild-type rate (3.8 \times 10^{-4}), the loss rate of the csl3ΔCDEI 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 cen3ΔCDEI mutations individually. The csl3-1 mutation behaved like cep1; the loss rate of the cen3ΔCDEI chromosome (R111) was the same as that of the wild-type chromosome (R110). On the basis of these results, we concluded that the csl1, csl2-1, csl4-1, and csl5-1 synthetic lethality was derived 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 (Stolarski 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 (Stolarski 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

![csl1 CEP1 csl1 cep1Δ](image)

![csl2 CEP1 csl2 cep1Δ](image)

![csl3 CEP1 csl3 cep1Δ](image)

![csl4 CEP1 csl4 cep1Δ](image)

![csl5 CEP1 csl5 cep1Δ](image)

Figure 1.—Dissected tetrads of cslx diploids. Viable segregants were scored for Met prototrophy to determine cep1 genotype: csl1, 1/4 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).

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 cen3ΔCDEI 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 csl3ΔCDEI chromosome. Expressed relative to the wild-type rate (3.8 \times 10^{-4}), the loss rate of the csl3ΔCDEI 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 cen3ΔCDEI mutations individually. The csl3-1 mutation behaved like cep1; the loss rate of the cen3ΔCDEI chromosome (R111) was the same as that of the wild-type chromosome (R110). On the basis of these results, we concluded that the csl1, csl2-1, csl4-1, and csl5-1 synthetic lethality was derived from kinetochore defects, while the synthetic lethality of csl3-1 appeared to be unrelated to centromeres.

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from the doubly heterozygous diploid yielded four via-
the available genetic evidence supports the hypothesis
cep1 (the S. cerevisiae gene was deleted in a diploid strain heterozygous for
CSL3 1997). To determine if the al.
MET16. dent gene
csl3-1 of perturbations in RNA polymerase II transcription, two genes were predicted to have centromere-speci®c
csl1-1 met16-47 and
are unlinked). The same result was obtained with
met16-47 methionine bradytrophy of
csl3-1 spores and two Met
met16-47 csl3-1 between
double heterozygote yielded two Met
tromeric site of CP1 action. The
met16-47 MET16
steady-state
centromere. The
met16-47
methionine (Met
1 nine (Met
the promoter. As a result, the dissociation 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 CDEII-binding complex Cbf3. CSL1 is identical to CEP3 (Cbf3b) which encodes the 64-kD subunit (Lechner 1994; Strun
nikov et al. 1995), while CSL5 is identical to NDC10 (CBF2, CEP2) encoding the 110-kD subunit (Goh and Kil
martin 1993; jiang et al. 1993). Significantly, these two genes were predicted to have centromere-specific functionson 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 3

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Loss rate</th>
<th>Events\cell/\division × 10⁴</th>
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<tr>
<td>R108/ R109</td>
<td>+/+ +</td>
<td>CF[CEP3]</td>
<td>3.8 (1.0) 22 (5.8) 5.8</td>
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<tr>
<td>R98/ R99</td>
<td>+/cep1/ +/ +</td>
<td>CF[cep1]</td>
<td>3.4 (0.9) 27 (7.1) 7.9</td>
</tr>
<tr>
<td>R100/ R101</td>
<td>cep1/cep1/ +/+</td>
<td>CF[cep1]</td>
<td>26 (6.8) 27 (7.1) 1.0</td>
</tr>
<tr>
<td>R116/ R119</td>
<td>+/ + csl1-1/csl1-1</td>
<td>CF[cen3ΔCDEI]</td>
<td>21 (5.5) 340 (89) 16.2</td>
</tr>
<tr>
<td>R129/ R127</td>
<td>+/ + csl2-1/csl2-1</td>
<td></td>
<td>9.0 (2.4) 630 (166) 70</td>
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<tr>
<td>R110/ R111</td>
<td>+/ + csl3-1/csl3-1</td>
<td></td>
<td>32 (8.4) 36 (9.5) 1.1</td>
</tr>
<tr>
<td>R224/ R220</td>
<td>+/ + csl4-1/csl4-1</td>
<td></td>
<td>6.9 (1.7) 175 (46) 25</td>
</tr>
<tr>
<td>R225/ R228</td>
<td>+/ + csl5-1/csl5-1</td>
<td></td>
<td>30 (7.9) 960 (253) 32</td>
</tr>
<tr>
<td>R221/ R222</td>
<td>+/ + Δcsl3Δcsl3</td>
<td></td>
<td>4.2 (1.1) 26 (6.9) 6.2</td>
</tr>
</tbody>
</table>

* CF[cen3ΔCDEI] / CF[CEP3]

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.

### TABLE 4

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Complementing ORF</th>
<th>Chromosome</th>
<th>Gene</th>
<th>Essential?</th>
</tr>
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<tbody>
<tr>
<td>csl1-1</td>
<td>YMR168C</td>
<td>XIII</td>
<td>CEP3/CBF3B</td>
<td>Yes</td>
</tr>
<tr>
<td>csl2-1</td>
<td>YKL049C</td>
<td>XI</td>
<td>CSE4</td>
<td>Yes</td>
</tr>
<tr>
<td>csl3-1</td>
<td>YGL244W</td>
<td>VII</td>
<td>RTF1</td>
<td>No</td>
</tr>
<tr>
<td>csl4-1</td>
<td>YNL232W</td>
<td>XIV</td>
<td>CSL4</td>
<td>Yes</td>
</tr>
<tr>
<td>csl5-1</td>
<td>YGR140W</td>
<td>VII</td>
<td>NDC10/CBF3A/CEP2</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Figure 3.—hCsl4 complements csl4-1. A cep1 csl4-1 cyh2 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.

Figure 2.—Sequences of the CSL4 gene product and 5' flanking DNA. (A) The translated sequence of the CSL4 ORF displayed with the glutamate-rich motif underlined. Also shown is an alignment between Csl4 and its human homolog acid-rich regions may suggest that Csl4 is a chromatin protein, like many of the proteins in which such Glu-WI) GAP program. (B) The CSL4 5'-flanking DNA sequence is shown with the CDEI site boxed. The CDEI-like sequence is underlined and the proximal EcoRI site is overscored.

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 implied 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. In relative terms, and encoded an open reading frame of 195 amino acids (Mr = 21,400) which was designated human Csl4. The putative human protein is smaller than Csl4, but the two proteins share significant homology 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 (Ito 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 AA08342) 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 EST homologous to zl84h09.r1, a cDNA consensus sequence was assembled. The assembled sequence contained a good Kozak initiator ATG consensus (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-
gous diploid strain by deleting the presumptive CSL4 ORF and marking the deletion with either the URA3 or LEU2 gene. Tetrad 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 (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 EcoRI-HindIII 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 EcoRI-HindIII fragment lacked 5'-flanking sequences required for full CSL4 expression.

Examination of the CSL4 5'-flanking DNA upstream of the proximal EcoRI 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 EcoRI-HindIII 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 EcoRI-HindIII 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 cistrons 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 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 cep1 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 cep1 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
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 cistrans test proved to be an effective means of classifying the csl mutants. All three of the csl mutations targeting known or imputed kinetochore components (csl-1, csl-2, csl-5-1) showed synergism in the cistrans 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 (csl-3-1) 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 (Stoler et al. 1997). While the csl-3-1 mutation does increase chromosome loss, the lack of interaction between csl-3-1 and cen3ΔCDEI implies that the cep1 synthetic lethality of csl-3-1 is not the direct result of kinetochore defects. The csl-3 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 csl-3-1 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 csl-3-1 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 csl-1, csl-2, csl-4, and csl-5-1 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 CDEII. Our findings reinforce and extend this model. The new alleles csl-1 and csl-5-1 confirm the proposed interactions between CP1 and Cbf3 subunits. More significantly, the synthetic lethality detected between csl-2 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 (csl-2-1) 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 (Stoller 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 G2-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 (cen-130, cen3ΔCDEI) argue that Cse4H4 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-CDEII 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 cen3ΔCDEI 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 CDEI 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 Maison 1990). As chromosome loss is lethal in haploids and aneuyploid 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 G2-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 fail 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 co-opted 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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**LITERATURE CITED**


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