CHL12, a Gene Essential for the Fidelity of Chromosome Transmission in the Yeast *Saccharomyces cerevisiae*

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ABSTRACT

We have analyzed the *CHL12* gene, earlier identified in a screen for yeast mutants with increased rates of mitotic loss of chromosome *III* and circular centromeric plasmids. A genomic clone of *CHL12* was isolated and used to map its physical position on the right arm of chromosome *XIII* near the *ADH3* locus. Nucleotide sequence analysis of *CHL12* revealed a 2.2-kb open reading frame with a 84-kD predicted protein sequence. Analysis of the sequence upstream of the *CHL12* open reading frame revealed the presence of two imperfect copies of *Mlu*I motif, ACGCGT, a sequence associated with many DNA metabolism genes in yeast. Analysis of the amino acid sequence revealed that the protein contains a NTP-binding domain and shares a low degree of homology with subunits of replication factor C (RF-C). A strain containing a null allele of *CHL12* was viable under standard growth conditions, and as well as original mutants exhibited an increase in the level of spontaneous mitotic recombination, slow growth and cold-sensitive phenotypes. Most of cells carrying the null *chl12* mutation arrested as large budded cells with the nucleus in the neck at nonpermissive temperature that typical for cell division cycle (*cdc*) mutants that arrest in the cell cycle at a point either immediately preceding M phase or during S phase. Cell cycle arrest of the *chl12* mutant requires the *RAD9* gene. We conclude that the *CHL12* gene product has critical role in DNA metabolism.

THE isolation of DNA replication mutants is an essential step in studying the mechanism of DNA replication in eukaryotic cells. The earliest attempts at obtaining such mutants in yeast involved the isolation of cell-division-cycle (*cdc*) mutants which arrest in the cell cycle at points immediately preceding or during S phase (for review see PRINGLE and HARTWELL 1981). Several such *cdc* mutants have been obtained that exhibit a variety of effects on DNA replication. The analysis of conditional lethal mutants of *Saccharomyces cerevisiae* that are defective in S phase or the entry into S phase has led to the identification of genes with established biochemical roles in DNA replication. Among them are the *CDC9* gene encoding DNA ligase (JOHNSTON and NASMYTH 1978), the *CDC17* gene encoding DNA polymerase I (LUCCHINI *et al.* 1985; BUDD and CAMPBELL 1987), the *CDC2* gene encoding the large subunit of DNA polymerase III (CONRAD and NEWLON 1983; BOULET *et al.* 1989; SITNEY *et al.* 1989) and the *CDC8* and *CDC21* genes encoding thymidylate kinase and thymidylate synthetase, respectively (BISSON and THORNER 1977; SCLAFANI and FANGMAN 1984). The functions of many other genes identified using cell-cycle arrest criteria [*e.g.*, *CDC7* (YOON and CAMPBELL 1990), *CDC13* (HARTWELL and SMITH 1985], *CDC45*, *CDC47* and *CDC54* (HENNESSY *et al.* 1991), *DBF1-DBF4* (JOHNSTON *et al.* 1990), *DNA43* and *DNA52* (SOLOMON *et al.* 1992)] in chromosome replication has not been clarified.

To date the total number of genes known to control yeast chromosome replication (including those which were identified by reverse genetics approach) is about 30 (for review see CAMPBELL and NEWLON 1991). Although we do not know the entire repertoire of genes involved in chromosome replication, it is obvious that many genes are yet to be discovered.

New collections of mutants with a high frequency of chromosome loss in mitosis can serve as an excellent source for identifying genes involved in DNA replication. The isolation of such mutants has been described by several laboratories. A search for mutants that destabilize linear, but not circular, chromosomes *lcs* (for *l*inear *c*hromosome *s*tability) was undertaken by RUNGE and ZAKIAN (1993). Although analysis of these mutants has not yet been carried out, among them might be mutants with impaired replication of telomeric ends. Two laboratories have recently described collections of mutants with impaired mitotic chromosome transmission, *ctf*s and *chl*s (SPENCER *et al.* 1988; KOUPRINA *et al.* 1988). *ctf* mutants (for *c*hromosome *t*ransmission *f*idelity) were selected using a visual color assay to monitor the inheritance of an artificially generated nonessential marker chromosome. *chl* mutants (for *ch*romosome *l*oss) were identified using the criteria of chromosome *III* and circular centromeric plasmids instability. The criteria used for selecting *ctf* and *chl* mutants were expected to identify previously unknown genes controlling chromosome segregation and replication in yeast. It has been shown recently that most *chl* mutations complement *ctf* mutations

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(KOUPRINA *et al.* 1993), so these two collections appear to increase the spectrum of potentially newly identified genes.

As shown earlier, chromosome replication appears to be impaired in mutants of the *CHL2, CHL5, CHL12* and *CHL15* genes. Analysis of the segregation properties of circular minichromosomes indicated that sister chromatid loss (1:0 segregation) is the predominant mode of chromosome destabilization in these mutants (KOUPRINA *et al.* 1988, 1993). Although the mode of chromosome destabilization might be caused by different reasons (*e.g.*, physical chromosome loss during nuclear division), a defect in chromosome replication is a plausible explanation of chromosome instability in these *chl* mutants. This point of view was supported by analysis of one of the mutants from this group, *chl15*. Yeast genomic DNA that complements *chl15* mutations has been cloned. It was shown that *CHL15* (*CTF4/POB1*) codes for a 105-kD helix-loop-helix (HLH) protein that binds to yeast DNA polymerase I (KOUPRINA *et al.* 1992; MILES and FORMOSA 1992). Thus, a previously unknown gene encoding a yeast DNA replication accessory protein has been identified among *chl* mutants exhibiting sister chromatid loss in mitosis.

In this report we present a detailed study of the second gene, *CHL12*. The data below show that the product of *CHL12* is essential for chromosome transmission fidelity. Recently *CHL12* was cloned independently by F. SPENCER as *CTF18* (F. SPENCER and P. HIETER, personal communication). These authors have also demonstrated that mutation of the *CHL12/CTF18* gene affects the rate of a test chromosome fragment loss. Both studies indicate a critical role for *CHL12* in DNA metabolism.

MATERIALS AND METHODS

Strains and media: Media recipes from SHERMAN *et al.* (1979) were used. Yeast transformation was performed by the LiCl procedure of ITO *et al.* (1983). The *Escherichia coli* strain DH5α (Bethesda Research Laboratories) was used for routine cloning and library construction. Genetic analysis was performed using standard protocols (SHERMAN *et al.* 1979) for mating, diploid selection, sporulation and tetrad dissection.

Yeast strains used in this study are listed in Table 1. The *chl12-Δ1::URA3* insertion mutation constructed *in vitro* was used to replace the wild-type locus by a one step replacement technique (ROTHSTEIN 1983). A 2.5-kb fragment of genomic DNA complementing the *chl12–1* and *chl12–2* mutations was cloned as a *Eco*RI-*Not*I fragment into the *Eco*RI-*Eag*I cleaved plasmid pBR325. A 1.1-kb *Hin*dIII fragment containing the *URA3* gene was then inserted into the unique *Hin*dIII site interrupting *CHL12* at the codon 583 making plasmid p12D1U. Digestion of this plasmid with *Eco*RI and *Eag*I gives a fragment containing the *chl12-Δ1::URA3* allele. For interruption of the *CHL12* locus the fragment containing the mutant allele was used to replace the wild-type *CHL12* by transformation of the *ura3* diploid strain YPH49. Several Ura$^+$ transformants were checked by Southern analysis. All diploid transformants appeared to have the expected size of insert (data not shown). The obtained diploid strain YE78 heterozygous for insertion into the *CHL12* gene was further analyzed. To obtain the null deletion mutant *chl12-Δ2::LEU2*, the plasmid p12D2L was constructed as follows. Plasmid p12D1U was digested by *Mlu*I and *Hin*dIII and the largest fragment containing sequences from the beginning of the insert to a *Mlu*I site, from a *Hin*dIII site to the end of the insert and sequences of pBR325 plasmid was gel-purified, mixed with a 2.2-kb *Sal*I-*Xho*I fragment containing the *LEU2* gene (ends made blunt) and ligated (see Fig. 2). Then, plasmid p12D2L was cut by *Sma*I and *Spe*I and transformed into the diploid YPH501. Integration of the *Sma*I-*Spe*I fragment at the *CHL12* gene in the yeast genome creates a genomic *chl12-Δ2* from positions +38 to +2266. In this allele the first 546 codons of the 742 were replaced by *LEU2*. To confirm gene disruption, genomic DNA from *CHL12* and *chl12-Δ2* diploid strains was digested with *Eco*RI (a *Eco*RI site presents in the *LEU2* gene that replaced a coding reading frame of *CHL12* in p12D2L) and blot-hybridized with the *CHL12* probe (a 2.5-kb *Eco*RI-*Not*I fragment from the plasmid p12). Southern hybridization analysis of genomic DNA has shown the presence of two additional bands (5.8 and 2.3 kb) in *chl12-Δ2 vs.* a 8.0-kb band in *CHL12* strains. The *chl12-Δ2/CHL12* diploid was designated YE102.

rad52 disruption alleles of YE104 and YE105, respectively, were made by one step gene replacement (ROTHSTEIN 1983) using a *Sal*I digest of the *RAD52* disruption plasmid p52BLAST (kindly provided by A. PERKINS) producing the isogenic strains YNK84 (*rad52-Δ1*) and YNK85 (*chl12-Δ2 rad52-Δ1*). Ura$^+$ transformants were confirmed as *rad52* disruption by sensitivity to γ-ray irradiation and by Southern analysis.

Isogenic *rad9* strains were made as follows. The *rad9-Δ1::URA3* deletion was introduced into the *chl12* mutant (YE105) and wild-type (YE104) strains by a one step gene replacement technique (ROTHSTEIN 1983) using a *Sal*I-*Eco*RI digest of the *RAD9* disruption plasmid pRR300 obtained from R. SCHIESTL (SCHIESTL *et al.* 1989) resulting in strains YNK88 and YNK89. Ura$^+$ transformants were confirmed as the *rad9-Δ1* disruption by increased sensitivity to UV irradiation and by Southern analysis (data not shown). Integration of the *Sal*I-*Eco*RI fragment at the *RAD9* site of the yeast genome creates a genomic *rad9-Δ1* from positions +1166 to + 3546 (SCHIESTL *et al.* 1989).

Assays of recombination, spontaneous mutation and chromosome loss rates and frequencies: The frequency of chromosome *III* loss in disomic cells was determined by measuring concurrent hemizygotization of three markers of chromosome *III: MAT, his4* and *thr4*. Loss of chromosome *III* in diploid strains homozygous for *chl12* was also recorded in this way (LARIONOV *et al.* 1987).

The frequency of spontaneous mitotic recombination in the *chl12* mutants was determined by characterizing the missegregation of chromosome *III* markers (*his4 thr4/HIS4 THR4*) (LARIONOV *et al.* 1987).

Spontaneous mitotic intragenic recombination was measured in disomic and diploid cells carrying *leu2* heteroalleles (*leu2–1* and *leu2–27*). To select for Leu$^+$ recombinants, 10 parallel cultures for mutants and 10 for wild-type strains were grown on synthetic complete medium and the cells were then plated onto medium lacking leucine. Because recombinants do not have a growth advantage, the number of Leu$^+$ prototrophs are a measure of the recombination frequency.

Spontaneous mutation frequencies were measured as reversion of Ade$^-$ auxotrophy to Ade$^+$ prototrophy and by selection for canavanine-resistant colonies originating by forward mutation of the *CAN1^s* wild-type allele to *can1^r*.

Fluctuation analysis was used to determine recombination, mutation and chromosome *III* loss rates. To determine the

TABLE 1

Genotypes of the strains used in this study

Strain	Genotype	Source
Z4221-3c1	*MATa HIS4 leu2-27 THR4* / *MATα his4 leu2-1 thr4* *ade2 met2 ura3-52*	J. ROTH
YE63	*MATa HIS4 leu2-27 THR4* / *MATα his4 leu2-1 thr4* *ade2 met2 ura3-52 chl12-Δ1::URA3*	This study
CL12-1	*MATa HIS4 leu2-27 THR4* / *MATα his4 leu2-1 thr4* *ade2 met2 ura3-52 chl12-1*	KOUPRINA *et al.* (1993)
CL12-2	*MATa HIS4 leu2-27 THR4* / *MATα his4 leu2-1 thr4* *ade2 met2 ura3-52 chl12-2*	KOUPRINA *et al.* (1993)
YPH49	*MATa ura3-52 ade2-101 trp1-Δ1 lys2-801* / *MATα ura3-52 ade2-101 trp1-Δ1 lys2-801*	GERRING *et al.* (1991)
YE78	*MATa ura3-52 ade2-101 trp1-Δ1 lys2-801 CHL12* / *MATα ura3-52 ade2-101 trp1-Δ1 lys2-801 chl12-Δ1::URA3*	This study
YPH102	*MATα ura3-52 lys2-801 ade2-101 his3-Δ200 leu2-Δ1*	GERRING *et al.* (1991)
YPH501	*MATa ura3-52 ade2-101 trp1-Δ63 lys2-801 his3-Δ200 leu2-Δ1* / *MATα ura3-52 ade2-101 trp1-Δ63 lys2-801 his3-Δ200 leu2-Δ1*	P. HIETER
YE102	*MATa ura3-52 ade2-101 trp1-Δ63 lys2-801 his3-Δ200 leu2-Δ1 CHL12* / *MATα ura3-52 ade2-101 trp1-Δ63 lys2-801 his3-Δ200 leu2-Δ1 chl12-Δ2::LEU2*	This study
YNK40[a]	*MATα leu2-1 his4 thr4 ade2 met2 CAN1[s] ura3-52*	This study
YNK41[a]	*MATa leu2-27 ade2 met2 ura3-52*	This study
YNK42	*MATα leu2-1 his4 thr4 ade2 met2 ura3-52* / *MATa leu2-27 HIS4 THR4 ade2 met2 ura3-52*	YNK40 × YNK41
YNK54[a]	*MATα leu2-1 his4 thr4 ade2 met2 ura3-52 CAN1[s] chl12-1*	This study
YNK55[a]	*MATa leu2-27 ade2 met2 ura3-52 chl12-1*	This study
YNK56[a]	*MATα leu2-1 his4 thr4 ade2 met2 ura3-52 CAN1[s] chl12-2*	This study
YNK57[a]	*MATa leu2-27 ade2 met2 ura3-52 chl12-2*	This study
YNK58	*MATα leu2-1 his4 thr4 ade2 met2 ura3-52 chl12-1* / *MATa leu2-27 HIS4 THR4 ade2 met2 ura3-52 chl12-1*	YNK54 × YNK55
YNK59	*MATα leu2-1 his4 thr4 ade2 met2 ura3-52 chl12-2* / *MATa leu2-27 HIS4 THR4 ade2 met2 ura3-52 chl12-2*	YNK56 × YNK57
YNK63	*MATα leu2-1 his4 thr4 ade2 met2 ura3-52 chl12-Δ1:URA3*	This study
YNK64	*MATa leu2-27 ade2 met2 ura3-52 chl12-Δ1::URA3*	This study
YNK65	*MATα leu2-1 his4 thr4 ade2 met2 ura3-52 chl12-Δ1::URA3* / *MATa leu2-27 HIS4 THR4 ade2 met2 ura3-52 chl12-Δ1::URA3*	YNK63 × YNK64
YNK71	*MATα ura3-52 lys2-801 ade2-101 his3-Δ200 leu2-Δ1 MET2 CHL12* / *MATa ura3-52 LYS2 ade2-101 HIS3 leu2-27 met2 chl12-1*	YNK55 × YPH102
YNK98	*MATα leu2-1 his4 thr4 ade2 met2 ura3-52 chl12-Δ2::LEU2*	This study
YNK99	*MATa leu2-27 ade2 met2 ura3-52 chl12-Δ2::LEU2*	This study
YNK76	*MATα leu2-1 his4 thr4 ade2 met2 ura3-52 chl12-Δ2::LEU2* / *MATa leu2-27 HIS4 THR4 ade2 met2 ura3-52 chl12-Δ1::LEU2*	YNK98 × YNK99
YNK80	*MATα leu2-1 his4 thr4 ade2 met2 ura3-52 CHL12* / *MATa leu2-27 HIS4 THR4 ade2 met2 ura3-52 chl12-1*	YNK40 × YNK55
YNK81	*MATα leu2-1 his4 thr4 ade2 met2 ura3-52 CHL12* / *MATa leu2-27 HIS4 THR4 ade2 met2 ura3-52 chl12-2*	YNK40 × YNK57
YE104[b]	*MATα ade2-101 ura3-52 lys2-801 leu2-Δ1 trp1-Δ63 his3-Δ200 CAN1[s]*	This study
YE105[b]	*MATα ade2-101 ura3-52 lys2-801 leu2-Δ1 trp1-Δ63 his3-Δ200 CAN1[s] chl12-Δ2::LEU2*	This study
YNK84	*MATα ade2-101 ura3-52 lys2-801 leu2-Δ1 trp1-Δ63 his3-Δ200 rad52-Δ1::URA3*	This study
YNK85	*MATα ade2-101 ura3-52 lys2-801 leu2-Δ1 trp1-Δ63 his3-Δ200 rad52-Δ1::URA3 chl12-Δ2::LEU2*	This study
YNK86[c]	*MATa HIS4 leu2-27 THR4* / *MATα his4 leu2-1 thr4* *ade2 met2 ura3-52 chl12-1 rad52-Δ1::URA3*	This study
YNK87[c]	*MATa HIS4 leu2-27 THR4* / *MATα his4 leu2-1 thr4* *ade2 met2 ura3-52 rad52-Δ1::URA3*	This study
YNK88	*MATα ade2-101 ura3-52 lys2-801 leu2-Δ1 trp1-Δ63 his3-Δ200 CAN1[s] rad9-Δ1*	This study
YNK89	*MATα ade2-101 ura3-52 lys2-801 leu2-Δ1 trp1-Δ63 his3-Δ200 CAN1[s] chl12-Δ2::LEU2 rad9-Δ1*	This study

[a] YNK54, YNK55 and YNK56, YNK57 are haploid mitotic segregants of the CL12-1 and CL12-2 strains disomic for chromosome *III*. Strains YNK40 and YNK41 are haploid mitotic segregants of Z4221-3c1 disomic for chromosome *III*.

[b] YE104 and YE105 are meiotic product of YE102.

[c] YNK86 and YNK87 are derivatives of disomic strains CL12-1 and Z421-3c1, respectively.

rates of recombination and chromosome loss, 5–10 independent single colonies were removed from YPD and suspended in 1 ml of sterile water. Appropriate dilutions were plated on YPD (approximately 200–300 colonies per plate) and then replica plated onto selective medium lacking histidine or threonine. Plates were incubated at 30° for 2–3 days before counting. To determine intragenic recombination and spontaneous mutations, 5–10 independent test colonies were grown in the appropriate medium to an approximate density of 10^7 cells per ml at 30°. Afterward, cells were collected by centrifugation and were washed once with distilled water. Cells were counted and their appropriate dilutions were spread onto synthetic complete medium for determining plating efficiency and onto selective medium for selecting recombinants or mutants. Plates were incubated at 30° for 3–5 days. The rates were obtained from the median number of cells after loss, recombination or mutation using the mathematical expression derived by LEA and COULSON (1949).

Mitotic stability of the centromere-containing plasmids was determined as the ratio of plasmid-carrying to plasmidless cells in individual colonies of transformants grown on a medium selective for the plasmid marker (minimal without leucine) as described earlier (KOUPRINA *et al.* 1988).

Growth kinetics: To determine doubling time, individual colonies of the *chl12* mutant and wild-type strains were picked from YPD plates and transferred into liquid YPD medium. The cultures were grown to an A_{600} of 0.1. Cells were harvested at 1–2-hr intervals at 30°. The increase in the optical density (A_{600}) observed for 5–10 hr after inoculation was used to compute doubling times. We have observed that twofold increase in the optical density corresponded to about twofold increase in the number of viable cells for each culture analyzed (determined by plating on YPD). Three independent colonies were assessed for each strain. The difference in doubling times between clones was never more than 15%.

Cell viability assays: Cell viability was assessed in two ways. In the first procedure, cultures of different strains were grown to logarithmic phase, sonicated for 5 sec, diluted and plated on YPD plates to determine the number of viable cells in the growing cultures. The same diluted cells were used to estimate the total number of cells in each culture by a Coulter counter. Cell viability was determined as a percentage of viable cells to total number of log-phase growing cells for each strain. In the second procedure, several independent clones were grown in YPD liquid medium to logarithmic phase, sonicated for 5 sec, then diluted and plated on to solid agar YPD plates. Colony formation was determined by light microscopy after approximately 1 day of growth (16–24 hr) at 25° and 11° (24 and 48 hr). Cells were counted as inviable if they contained typically <10 cells bodies (buds). At least three determinations were made for each strain, and 200–300 colonies were observed in each case. The mean values of cell viability determined from the second approach are included in Table 5.

Screens for conditional lethality: Assays for secondary conditional lethal phenotypes were carried out in patch tests with serial dilutions containing from 10^5 to 10^1 test cells per patch. Temperature conditional growth (at 37° or 11°) was determined by incubating test YPD plates at the desired temperature (for 3 or 21 days, respectively) and comparing growth on the test plates to growth on control plates cultured at 25° for 5 days. Temperature conditional lethality was scored visibly as no growth in the most concentrated patch. A screen for benomyl sensitivity was done by replica plating on to YPD plus 15 μg/ml benomyl. Media containing benomyl were made by addition of the stock solution (15 mg/ml in DMSO) to hot YPD medium. Benomyl treatment was generally carried out at 25°. UV light sensitivity were assayed by exposing the cells to various fluences of UV radiation. After irradiation, cells were grown at 30° in the dark to avoid photoreactivation. For γ-rays irradiation, cells were suspended in sterile distilled water at various concentrations and irradiated with ^{137}Cs source at a dose rate of 3.25 krad/min.

Morphological studies: For microscopy yeast cell cultures were grown to logarithmic phase (at A_{600} of 0.2–0.6) and then divided for incubation at both permissive and restrictive temperatures. Cells were then fixed in 70% EtOH, 0.2 M Tris HCl, pH 7.5 for 60 min, treated with RNase (1 mg/ml) for 30 min at 37° and stained with 4,6-diamidino-2-phenylindole essentially as described (HUTTER and EIPEL 1978). The fixed cell samples were observed under visible light for observing cell morphology or UV illumination for scoring nuclear morphology using a rhodamine filter set. Morphology of the cells was monitored at 1, 24 and 48 hr of incubation at the restrictive temperature. The positively scored class was defined as large budded cells where the diameter of the smallest spheroid >0.5 the diameter of the largest one.

Cloning of the *CHL12* gene: *CHL12* was cloned from a library of 10–12-kb fragments of yeast genomic DNA inserted into pBR322-based *LEU2/CEN4/ARS1* shuttle vector pSB32 (the library was kindly provided by F. SPENCER, unpublished). Putative *CHL12*-containing clones were identified by screening Leu$^+$ transformants of YNK58 diploid for complementation of instability of centromeric plasmids and chromosome *III* loss. This occurred at a frequency of 1 in approximately 600 transformants. Plasmids were rescued by *E. coli* transformation of yeast genomic DNA preparation. To subclone the gene within the cloned DNA segment, one of five genomic clones obtained, p194, was partially digested with *Sau*3A, and 1–2-kb, 2–3-kb and 3–5-kb fractions were agarose gel purified and cloned into the *Bam*HI site of a pBluescript-based *CEN6/LEU2/ARSH4* pRS315 shuttle vector (SIKORSKI and HIETER 1989). *CHL12* complementing subclones were identified by their ability to complement the chromosome *III* loss and minichromosome instability phenotypes in the *chl12 leu2* × *chl12 leu2* diploids (YNK58 and YNK59 strains). Plasmid p12 carried the smallest insertion of approximately 2.5 kb that complemented both phenotypes.

Proof that the cloned DNA corresponded to the *CHL12* locus was obtained as follows. A 3.6-kb *Hin*dIII-fragment from plasmid p194 was inserted into the *Hin*dIII site of plasmid YCF3 (VOLLRATH *et al.* 1988) to generate plasmid YCF-194. The *CHL12* gene was marked with *URA3* by integrative transformation of the diploid YNK71 (YNK71 was constructed by crossing the mutant YNK55 and the wild-type YPH102 strains) with plasmid YCF-194 linearized at a unique *Cla*I site within the inserted *Hin*dIII fragment. The diploid was then sporulated and tetrads were dissected. Haploid spore segregants from four-spore tetrads were examined for mitotic stability of a centromeric plasmid and of chromosome *III* (each segregant was crossed with the original *chl12* mutant of either *MAT***a** or *MATα* mating types and then the obtained diploids were tested for ability to mate with *MAT***a** and *MATα* haploid tester strains). In 15 four-spored tetrads 30 spores were Ura$^+$ Chl$^-$ and 30 others were Ura$^-$ Chl$^+$ (Chl$^+$ and Chl$^-$ indicate either mitotic stability or instability of a minichromosome and chromosome *III*). Data obtained indicate that the genomic fragment complementing the *chl12-1* and *chl12-2* mutations is identical to the *CHL12* gene.

Sequence of *CHL12*: DNA sequencing was performed by the chain-termination method (SANGER *et al.* 1977). For sequencing the *CHL12* gene, DNA fragments of plasmid p12 were subcloned into M13mp18 and M13mp19 and sets of deletions were generated with exonuclease III and S1. Single-stranded M13 DNA was sequenced with the Sequenase kit

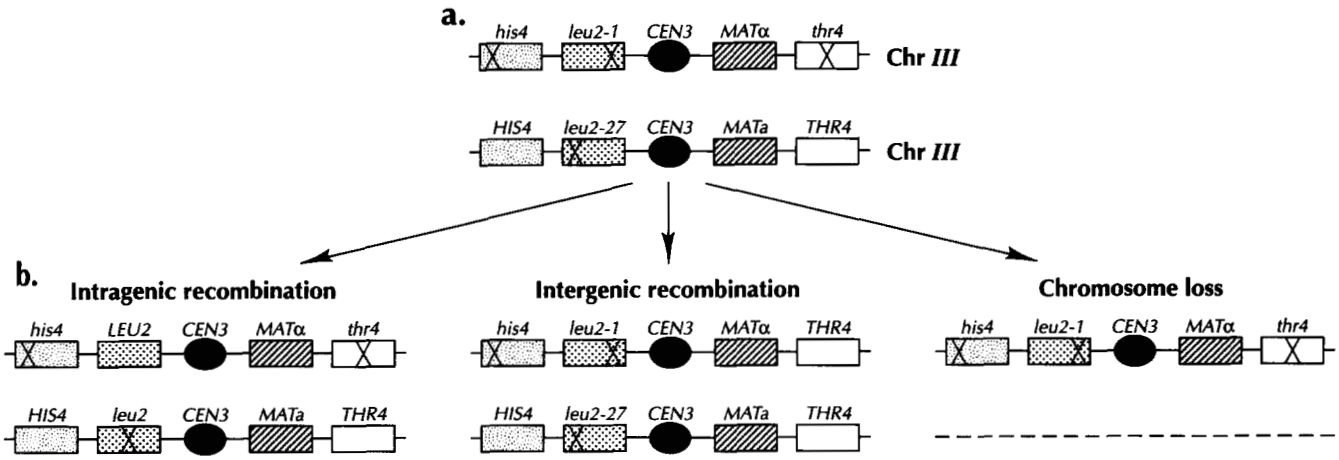

FIGURE 1.—Chromosome system to study mitotic recombination. Genetic consequences of chromosome *III* loss and interchromosomal recombination. (a) The basic genetic features of the strains used for this study. (b) Missegregation of chromosome *III* bearing *MAT***a** *HIS4 THR4* will allow mating with a *MAT***a** tester and lead to His$^-$ Thr$^-$ phenotype. Homozygotization by mitotic recombination will allow mating with *MATα or MAT***a** mating testers or will result in His$^-$ Thr$^+$ or His$^+$ Thr$^-$ phenotypes.

(Pharmacia Biotech Inc.) under conditions recommended by the manufacturer.

GenBank/EMBL accession number: The GenBank/EMBL accession number for the nucleotide sequence of *CHL12* is L24514.

RESULTS

Genetic characterizations of the *chl12* alleles: Previously we described the identification of 15 *CHL* genes essential for maintaining the fidelity of chromosome transmission in mitosis (KOUPRINA *et al.* 1988, 1993). There were two mutagenesis-induced alleles of *CHL12*, *chl12-1* and *chl12-2*, recovered in the collection of *chl* mutants. Both *chl12* mutations were characterized by a high frequency of chromosome *III* loss and an inability to maintain centromeric plasmids (KOUPRINA *et al.* 1993).

To determine whether defects in the *CHL12* gene would also affect mitotic recombination, we used a genetic system capable of measuring both recombination between homologs and interallelic recombination as well as chromosome loss. Briefly, the system is based on haploid strains disomic for chromosome *III* carrying heteroalleles *leu2-1/leu2-27*, *HIS4/his4* and *THR4/thr4* of chromosome *III* markers (Figure 1). The relative contributions of mitotic recombination and chromosome loss were determined by characterizing the missegregation of the chromosome *III* markers in *MATα/MAT***a** disomic strains. The culture frequencies of cells resulting from recombination (His$^-$ Thr$^+$ and His$^+$ Thr$^-$) and of cells resulting from chromosome loss (His$^-$ Thr$^-$ that mate as *MATα*) were elevated in the *chl12* mutants about 10- and 70-fold, respectively (Table 2). Chromosome *III* instability and increased mitotic recombination observed in the *chl12* mutants is not a peculiarity of disomic strains. Diploid strains YNK58 and YNK59, which were obtained by crossing *MAT***a**- and *MATα*-mitotic segregants of CL12-1 and CL12-2 disomic strains, exhibited the same elevated frequencies of chromosome loss and interchromosomal recombination (data not shown). A high frequency of chromosome *III* loss in the *chl12* mutants correlated with unstable maintenance of centromeric plasmids: the mitotic stability of plasmid YCp41 (CLARKE and CARBON 1980) was approximately 50% in the *chl12-1* and *chl12-2* mutants, whereas in the isogenic wild-type strains it was 95%.

The relative rates of spontaneous mitotic intragenic recombination were also determined for *chl12* mutant and wild-type strains. The *chl12* strains YNK58, YNK59, CL12-1 and CL12-2 strains and the wild-type strains Z4221-3c1, YNK80 and YNK81, all of which carry *leu2* heteroalleles (*leu2-1* and *leu2-27*), were assayed for recombination events leading to restoration of a Leu$^+$ phenotype (interchromosomal exchange). The rate of heteroallelic recombination at the *LEU2* locus was observed to increase about 10-fold in the *chl12* mutants

TABLE 2

Chromosome *III* missegregation and recombination in the *chl12* mutants

Strain	Relevant genotype	Rate[a] ($\times 10^{-5}$) of Missegregation (*MAT*αHis$^-$Thr$^-$)	Rate[a] ($\times 10^{-5}$) of Recombination[b] (His$^+$Thr$^-$ and His$^-$Thr$^+$)
Z4221-3c1	*CHL12*	3.7	5.7
CL12-1	*chl12-1*	250 (×68)[c]	56 (×10)
CL12-2	*chl12-2*	264 (×72)	48 (×8)
YNK42	*CHL12/CHL12*	4.1	7.3
YNK76	*chl12-Δ2/chl12-Δ2*	180 (×44)	65 (×9)
YNK65	*chl12-Δ1/chl12-Δ1*	215 (×52)	84 (×12)

[a] Determined from five independent clones.
[b] Recombination was measured as a frequency of homozygotozation of the *TRH4* or *HIS4* markers.
[c] Numbers in parentheses indicate increase over the wild type rate of chromosome loss and recombination.

TABLE 3

Heteroallelic recombination in *leu2* in the *chl12* mutants during mitosis

Strain	Relevant genotype	Rate[a] of Leu$^+$ recombinants ($\times 10^{-7}$)
Z4221-3c1	*CHL12*	1.2
CL12-1	*chl12-1*	12.2 ($\times$10)[b]
CL12-2	*chl12-2*	14.1 ($\times$12)
YNK80	*CHL12/chl12-1*	1.3
YNK81	*CHL12/chl12-2*	1.1
YNK58	*chl12-1/chl12-1*	13.3 ($\times$11)
YNK59	*chl12-2/chl12-2*	15.4 ($\times$13)
YNK65	*chl12-Δ1/chl12-Δ1*	12.5 ($\times$10)
YNK86	*chl12-1 rad52-Δ1*	5.8
YNK87	*CHL12 rad52-Δ1*	4.9

[a] Determined from 10 independent clones.
[b] Number in parentheses indicate factor of increase over the wild-type rate of recombination.

TABLE 4

Rates of spontaneous mutations in the *chl12* mutants

Strain	Relevant genotype	Spontaneous mutation rate[a] in *ade2* ($\times 10^{-8}$)	Spontaneous mutation rate[a] in *CAN1*^{s} ($\times 10^{-7}$)
YNK40	*CHL12*	0.65	0.56
YNK54	*chl12-1*	1.10	0.48
YNK56	*chl12-2*	0.91	0.56
YE104	*CHL12*	0.24	1.15
YE105	*chl12-Δ2*	0.53	2.1

[a] Spontaneous mutation was measured as reversion of Ade$^-$ auxotrophs to adenine prototrophy or, in the case of *CAN1*, as forward mutation to canavanine resistance.

TABLE 5

Doubling time and viability of cells in log-phase cultures of chl12 mutants

Strain	Relevant genotype	Percent dead cells	Doubling time (min)
YNK40	*CHL12*	2[a]	92
YNK54	*chl12-1*	54	150
YNK56	*chl12-2*	24	116
YNK84	*rad52-Δ1*	32	120
YNK85	*chl12-Δ2 rad52-Δ1*	62	211
YE104	*CHL12*	1 (8)[b]	103
YE105	*chl12-Δ2*	26 (58)	126
YNK109	*chl12-Δ2 rad9-Δ1*	30 (43)	ND

[a] Viability was determined from three cultures for each strain at 25°. Cells were counted as inviable if they contained typically <10 cells bodies (buds). The total number of observed cells for each strain was at least 300.
[b] In parentheses there is viability determined after 48 hr at nonpermissive temperature (11°). ND, not determined.

compared with that in the wild-type strain. The increase of intragenic recombination in the *chl12* mutant is *RAD52*-dependent (Table 3).

To summarize, both interchomosomal recombination and chromosome loss are increased in the *chl12* mutants.

The observed phenotype of *chl12* mutations raised the possibility that the *chl12* mutants might exhibit defects in DNA replication or DNA repair that would result in decreased fidelity of these processes. To determine whether *CHL12* plays any role in mutagenic processes, we examined spontaneous mutation rates in the *chl12* mutant and isogenic wild-type strains. Two mutant haploid strains (YNK54 and YNK56) were compared with the isogenic wild-type strain (YNK40). The rates of spontaneous mutation at the *CAN1*^{s} locus were approximately the same in the *chl12* mutant and wild-type strains (Table 4). Spontaneous mutation rates measured at the *ADE2* locus in the *chl12-1* and *chl12-2* strains were also not different from that in wild type. Two mutant alleles of the *CHL12* gene were assayed for sensitivity to both ultraviolet and γ-ray irradiation as well as for sensitivity to benomyl. Both alleles were indistinguishable from wild type under all conditions tested (data not shown). These results indicate that the fidelity with which chromosomal DNA is replicated in the *chl12* mutants is equivalent to that in the wild type.

Both mutations identified caused a severe growth defect. The doubling time for *chl12* cells was significantly higher than that of the isogenic wild-type strain. The growth defect caused by *chl12* mutations is due to poor cell viability (Table 5). A strain containing a *chl12-1* allele was unable to grow at a low temperature (11°). A genomic clone of the *CHL12* (plasmid p12) complemented the cold sensitivity as well as chromosome *III* instability and slow growth phenotypes of *chl12-1*. Meiotic analysis of the chromosome *III* loss, centromeric plasmid instability, growth defect and cold sensitivity phenotypes showed that cosegregation was observed in 5 of 5 scorable spores. Thus, all characteristics result from mutation in a single gene.

Cloning of the *CHL12* gene: Originally the *chl12* mutants were selected for instability of chromosome *III* (measured as ability of a disomic strain heterozygous for *MAT* to mate with *MAT***a**- and *MATα*-tester strains with a high frequency) and for centromeric plasmid instability. The mitotic stability of CEN/ARS plasmids was about 45% in *chl12* strains, compared to 95% in wild-type strains. This value corresponds to a 4–5-fold increase in the rate of plasmid loss per generation in *chl* mutants compared with wild type (KOUPRINA *et al.* 1988).

Plasmids containing the *CHL12* gene were identified by complementation of the mitotic instability of a centromeric plasmid and of chromosome *III* in a *chl12 leu2* × *chl12 leu2* diploid strain (YNK58). Strain YNK58 was transformed with a yeast genomic library of 10–12-kb fragments inserted into an *ARS1/CEN4/LEU2*-based vector. About 3,000 Leu$^+$ transformants were analyzed for stability of the library centromeric plasmids. The mitotic stability of centromeric plasmids was approximately 95% in five independent transformants. These five potential candidates were then examined for rescue of chromosome *III* loss by mating five diploids carrying the

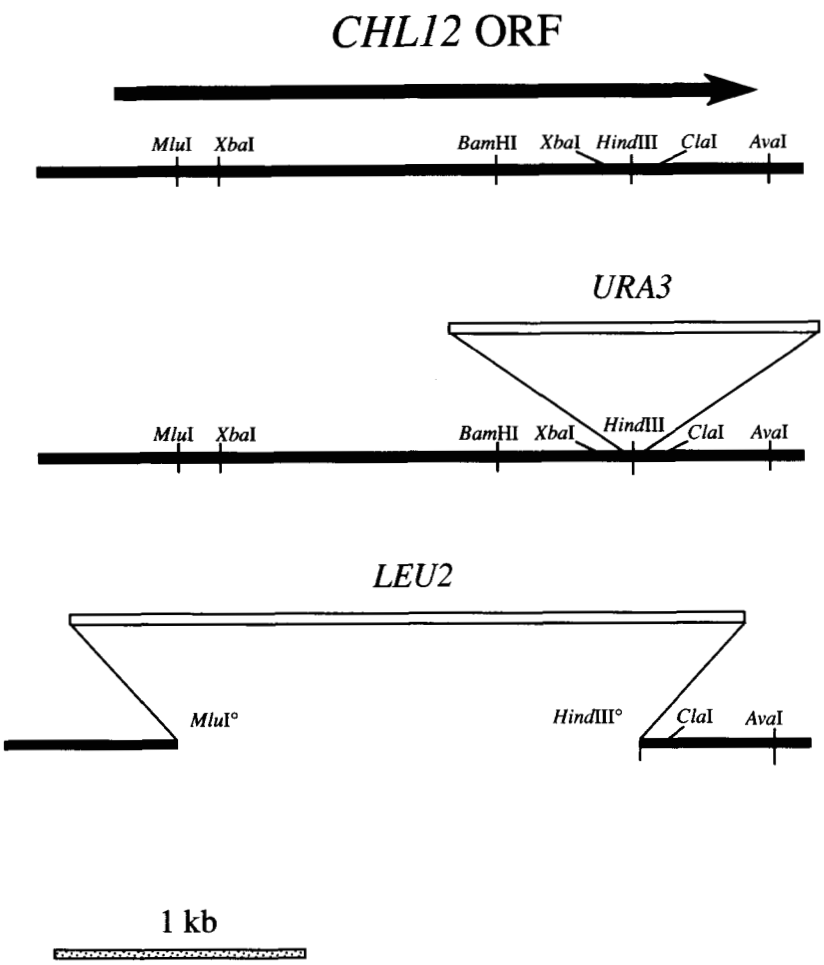

FIGURE 2.—Structure of the *chl12-Δ1::URA3* and *chl12-Δ2::LEU2* alleles. The plasmids p12D2L (see MATERIALS AND METHODS) were used for insertion and disruption at the *CHL12* locus by homologous recombination. The 2.5-kb fragment was the smallest complementing clone defined. The thick solid bar represents the *CHL12* sequence. The open bar represents the *URA3* and *LEU2* selectable markers. The arrow indicates the extent of coding sequence.

plasmids with complementing inserts to *MAT***a**- and *MATα*-mating tester strains. The transformants did not exhibit chromosome *III* loss. After loss of the library plasmids, all five of them showed a clear coreversion to *chl12 leu2*. Plasmid DNA from five independent transformants of YNK58 was recovered in *E. coli*. Restriction fragment analysis indicated that two plasmids contained identical inserts and three others had overlapping inserts. All five recovered plasmids rescued the *chl12* phenotype of minichromosome instability and chromosome *III* loss upon retransformation and, therefore, contained a *chl12* complementing genomic segment. The identity of the cloned genomic fragment as the *CHL12* gene was confirmed by homologous integration of the *URA3*-marked fragment into the yeast chromosome and subsequent linkage analysis (see MATERIALS AND METHODS). One of the plasmids, p194, was used for subsequent analysis. Fragments of p194 were subcloned into the multipurpose vector pRS315 (*CEN6/ARSH4/LEU2*). The minimum size fragment complementing *chl12* mutations in the plasmid p12 was 2.5 kb (Figure 2). This fragment was used for sequencing the *CHL12* gene.

Sequence analysis of *CHL12*: A 2.5-kb fragment of plasmid p12 that complemented the *chl12-1* and *chl12-2* mutations was subcloned into M13-based plasmids and sequenced using the SANGER *et al.* (1977) dideoxy method. Computer analysis of the sequence revealed one open reading frame of 2223 bp. Figure 3 shows the nucleotide sequence of the *CHL12* gene. The open reading frame of *CHL12* encodes a predicted protein of 741 amino acid residues with a molecular mass of 84 kD. The primary sequence predicted for the *CHL12* gene product was compared with both GenBank and EMBL data bases using the FASTP algorithm (LIPMAN and PEARSON 1985). A search of protein data base (release of June 1994) detected limited homology between the CHL12 protein and two highly related subunits (37 and 40 kD) of human activator 1 or RF-C (CHEN 1992a,b). Whereas overall degree of homology is low (25% identical and 50% homologous between CHL12 and two subunits of RF-C), most of it distributed unevenly throughout the sequence of all three proteins, clustering in three domains. Notably, these regions of homology are positioned in the same relative order in CHL12, 37- and 40-kD subunits of RF-C. These domains have been also identified in recently cloned 128-kD subunit of human RF-C and two subunits of yeast RF-C (Figure 4).

As with the human RF-C 37- and 40-kD subunits the CHL12 protein contains a match to the A consensus sequence, (A/G)X_4GK(S/T) found in NTP-binding pro-

```
caa cgt tga aaa aaa aaa tgt gga aaa aac gaa cta gcc agT CGC GTt gta cgg aac atc -231
taA CAC GTa aat aaa cat gat ttc ata tat ttt att cct aat gtg tac act att tga ccc -171
aaa agg tgg atg taa ggt cag gga tca acg ttg aaa aaa aaa atg tgg aaa aaa cga act -111
agc cag TCG CGT tgt acg gaa cat cta aca cgt aaa taa aca tga ttt cat ata ttt tat -51
tcc taa tgt gta cac tat ttg acc caa aag gtg gat gta agg tca ggg atc ATG GTT GAT 9
                                                                  M   V   D   3
ACC GCA CCA TAC ATT GGT TCA CTA GGG AGA AGT TCC CTT TTT GAT ACT GGT GAT ATA GAA 69
T   A   P   Y   I   G   S   L   G   R   S   S   L   F   D   T   G   D   I   E   23
CAA GCC CCT GGT AAT AAT GCA ATT GGT ATT AAT GAG GAA GAT ATT CAC GCG TTT GTA TCA 129
Q   A   P   G   N   N   A   I   G   I   N   E   E   D   I   H   A   F   V   S   43
AGT ACT GGC GAA ACG GTT CAA TTA AAG AAG AAG CCT GCG AAG TTA GCA ACC GGG AAT ATT 189
S   T   G   E   T   V   Q   L   K   K   K   P   A   K   L   A   T   G   N   I   63
AGT CTA TAC ACT AAC CCA GAT ACT GTT TGG AGA TCA GAC GAC ACG TAC GGC ATC AAT ATA 249
S   L   Y   T   N   P   D   T   V   W   R   S   D   D   T   Y   G   I   N   I   83
AAC TAT TTG TTA GAC AAA ATT GAG GCA TCT GGC GAT GAC CGC ACT AAC GCA CAA AAG ACT 309
N   Y   L   L   D   K   I   E   A   S   G   D   D   R   T   N   A   Q   K   T   103
TCA CCT ATA ACT GGC AAG ATA GGT AGC GAC ACA CTC TGG GTA GAG AAA TGG CGT CCT AAA 369
S   P   I   T   G   K   I   G   S   D   T   L   W   V   E   K   W   R   P   K   123
AAA TTT CTA GAT TTG GTT GGT AAT GAA AAG ACA AAC AGG AGA ATG TTA GGT TGG TTG AGA 429
K   F   L   D   L   V   G   N   E   K   T   N   R   R   M   L   G   W   L   R   143
CAA TGG ACG CCG GCT GTG TTT AAA GAG CAA TTA CCC AAA TTG CCA ACC GAA AAA GAG GTC 489
Q   W   T   P   A   V   F   K   E   Q   L   P   K   L   P   T   E   K   E   V   163
AGT GAT ATG GAA CTC GAT CCA TTG AAA AGG CCA CCA AAG AAA ATT TTA CTA CTG CAC GGG 549
S   D   M   E   L   D   P   L   K   R   P   P   K   K   I   L   L   L   H   G   183
CCA CCA GGA ATA GGC AAA ACC TCA GTA GCT CAC GTT ATT GCC AAA CAA TCA GGG TTT TCT 609
P   P   G   I   G   K   T   S   V   A   H   V   I   A   K   Q   S   G   F   S   203
GTC TCA GAA ATC AAT GCA AGT GAT GAA AGG GCT GGA CCT ATG GTA AAA GAG AAA ATA TAT 669
V   S   E   I   N   A   S   D   E   R   A   G   P   M   V   K   E   K   I   Y   223
AAT CTT TTA TTC AAT CAT ACT TTC GAT ACA AAT CCT GTG TGC TTA GTG GCG GAT GAG ATT 729
N   L   L   F   N   H   T   F   D   T   N   P   V   C   L   V   A   D   E   I   243
GAT GGA AGT ATT GAG AGT GGA TTT ATT AGA ATT TTA GTT GAC ATT ATG CAA AGC GAT ATT 789
D   G   S   I   E   S   G   F   I   R   I   L   V   D   I   M   Q   S   D   I   263
AAA GCC ACT AAT AAA CTA TTA TAC GGT CAA CCA GAT AAA AAG GAC AAA AAG CGC AAA AAG 849
K   A   T   N   K   L   L   Y   G   Q   P   D   K   K   D   K   K   R   K   K   283
AAA AGG TCT AAA TTG CTT ACG CGA CCT ATT ATT TGT ATT TGC AAT AAT CTA TAT GCT CCT 909
K   R   S   K   L   L   T   R   P   I   I   C   I   C   N   N   L   Y   A   P   303
TCT TTG GAA AAG CTG AAA CCA TTC TGT GAA ATT ATT GCT GTG AAA AGA CCT TCC GAT ACT 969
S   L   E   K   L   K   P   F   C   E   I   I   A   V   K   R   P   S   D   T   323
ACC CTA CTA GAG CGA TTG AAC CTT ATC TGC CAT AAA GAA AAC ATG AAT ATT CCT ATA AAA 1029
T   L   L   E   R   L   N   L   I   C   H   K   E   N   M   N   I   P   I   K   343
GCA ATC AAT GAC TTA ATT GAT TTG GCT CAA GGT GAC GTA AGG AAT TGT ATA AAC AAT TTA 1089
A   I   N   D   L   I   D   L   A   Q   G   D   V   R   N   C   I   N   N   L   363
CAA TTC TTG GCC TCG AAT GTT GAT TCA AGA GAT TCC TCT GCA TCA GAT AAA CCT GCT TGT 1149
Q   F   L   A   S   N   V   D   S   R   D   S   S   A   S   D   K   P   A   C   383
GCA AAA AAT ACG TGG GCG TCA TCC AAC AAG GAC TCC CCC ATA TCA TGG TTT AAA ATC GTG 1209
A   K   N   T   W   A   S   S   N   K   D   S   P   I   S   W   F   K   I   V   403
AAC CAA TTG TTT AGA AAG GAT CCA CAT CGT GAT ATA AAG GAG CAG TTT TAT GAA TTG CTA 1269
N   Q   L   F   R   K   D   P   H   R   D   I   K   E   Q   F   Y   E   L   L   423
AAC CAA GTA GAG CTT AAT GGT AAC TCT GAC AGG ATA TTG CAA GGC TGT TTT AAT ATA TTT 1329
N   Q   V   E   L   N   G   N   S   D   R   I   L   Q   G   C   F   N   I   F   443
CCC TAC GTA AAA TAT TCC GAC AAT GGT ATA AGA AAG CCA GCA AAC ATT TCA GAT TGG CTA 1389
P   Y   V   K   Y   S   D   N   G   I   R   K   P   A   N   I   S   D   W   L   463
TTT TTC CAT GAT TTA ATG TAC CAA TCA ATG TAT GCG CAT AAT GGC GAA TTG TTA CGT TAC 1449
F   F   H   D   L   M   Y   Q   S   M   Y   A   H   N   G   E   L   L   R   Y   483
TCC GCC CTT GTG CCC CTA GTC TTC TTC CAA ACG TTT GGC GAT ATC GCA AAC AAA GAT GAT 1509
S   A   L   V   P   L   V   F   F   Q   T   F   G   D   I   A   N   K   D   D   503
ATT AGA ATG AAG AAT AGT GAA TAC GAA CAA CGT GAA TTA AAA CGA GCC AAT TCA GAT ATA 1569
I   R   M   K   N   S   E   Y   E   Q   R   E   L   K   R   A   N   S   D   I   523
GTT AGT CTG ATT ATG AGA CAT ATC TCG GTT CAG TCC CCA CTA ATG GCA AGT TTT ACG GAT 1629
V   S   L   I   M   R   H   I   S   V   Q   S   P   L   M   A   S   F   T   D   543
AGA AAA TCG TTA ATC TTT GAA ATA CTA CCA TAT CTA GAT TCG ATG ATC TCT TCC GAT TTT 1689
R   K   S   L   I   F   E   I   L   P   Y   L   D   S   M   I   S   S   D   F   563
AAT AAA ATA AGG AAC CTG AAA CTC AAA CAA GCC ATC ATG GAG GAA TTA GTT CAA TTG CTG 1749
N   K   I   R   N   L   K   L   K   Q   A   I   M   E   E   L   V   Q   L   L   583
AAA AGC TTT CAA CTG AAT CTA ATC CAA AAT CGG TCA GAA GGA TTT GAT GTA AGG GGT GGC 1809
K   S   F   Q   L   N   L   I   Q   N   R   S   E   G   F   D   V   R   G   G   603
CTA ACA ATC GAT CCC CCA ATC GAT GAA GTC GTA TTG TTA AAT CCT AAA CAT ATT AAC GAA 1869
L   T   I   D   P   P   I   D   E   V   V   L   L   N   P   K   H   I   N   E   623
GTC CAA CAT AAA CGG GCT AAT AAT TTG AGT TCA CTG TTA GCA AAG ATT GAG GAA AAC CGG 1929
V   Q   H   K   R   A   N   N   L   S   S   L   L   A   K   I   E   E   N   R   643
GCC AAG AAA AGG CAT ATA GAC CAA GTG ACT GAG GAT AGA CTA CAG TCA CAG GAA ATG CAT 1989
A   K   K   R   H   I   D   Q   V   T   E   D   R   L   Q   S   Q   E   M   H   663
AGC AAA AAA GTC AAA ACT GGG TTA AAT TCT TCC TCA AGT ACA ATC GAC TTT TTC AAG AAT 2049
S   K   K   V   K   T   G   L   N   S   S   S   S   T   I   D   F   F   K   N   683
CAG TAC GGA TTA TTG AAG CAA ACT CAG GAA TTG GAA GAG ACA CAA AAA ACT ATT GGA TCA 2109
Q   Y   G   L   L   K   Q   T   Q   E   L   E   E   T   Q   K   T   I   G   S   703
GAC GAA ACC AAC CAA GCA GAT GAC TGC AAT CAA ACG GTT AAA ATA TGG GTG AAA TAC AAT 2169
D   E   T   N   Q   A   D   D   C   N   Q   T   V   K   I   W   V   K   Y   N   723
GAG GGG TTC TCT AAC GCT GTC AGG AAA AAT GTG ACT TGG AAT AAC CTG TGG GAA TAA atg 2229
E   G   F   S   N   A   V   R   K   N   V   T   W   N   N   L   W   E   *       741
acg taa gat ata tac gca gtc tct ctt aag aag cat act tgt ata tgg acc agc agc tta 2289
cat aga aat cta aaa gaa gta aat aaa gaa att agc aat caa tcc tgc cgg tag tcg caa 2349
cag ctc cga ttt tgt cac gca gcg ttc gtt ttt tat tcg tga tcc                     2394
```

FIGURE 3.—Nucleotide sequence of the *CHL12* gene and flanking DNA. The predicted amino acid sequence, using the single-letter code, is given below the nucleotide sequence. Numbers above and below each line indicate nucleotide and amino acid residues, respectively. The degenerate forms of the *Mlu*I sequence are underlined.

```
DROGLTFAC      421  WVDKHKPTSIKEIVGQAGA 439
128kD hRFC     591  WVDKYKPTSLKTIIGQQGD 609
37kD hRFC       40  WVEKYRPKCVDEVAFQEPN  58
37kD yRFC       27  WVEKYRPKNLDEVTAQDPH  45
 40 kD yRFC     15  WVEKYRPETLDEVYGQNPH  33
40kD hRFC       38  WVEKYRPVKLNEIVGNEPN  56
CHL12          116  WVEKWRPKKFLDLVGNEKT 134

Consensus           WVEKYRP--L-E++GQ---


DROGLTFAC      480  ALLSGPPGIGKTTTATLVVKEL......GFDAVEFNASDTRSKRLLKDEVSTLLSN   528
128kD hRFC     647  ALLSGPPGVGKTTTASLVCQEL......GYSYVELNASDTRSKSSLKAIVAESLNN   697
37kD hRFC       74  LLFYGPPGTGKTSTILAAARELFGPELFRLRVLELNASDERGIQVVREKVKN.ICN   128
37kD yRFC       61  MLFYGPPGTGKTSTILALTKELYGPDLMKSRILELNASDERGISIVREKVKNLICN   116
130kD yRFC      49  LLFYGPPGTGKTSTIVALARELYGKN.YSNMVLELNASDDRGIDVVRNQIKDVLAN   103
40kD hRFC       74  IIIAGPPGTGKTTSILCLARALLGA..LKDAMLELNASNDRGIDVVRNKIK.MACN   124
CHL12          179  LLLHGPPGIGKTSVAHVIAKQ......SGFSVSEINASDERAGPMVKEKIYNLLFN   228

Consensus           +L+-GPPG-GKT+T+-++--EL---------++ELNASDER+--+V+++V---+-N


DROGLTFAC      595  CYDLRFQRPRLEQIKGKIMSICFKEKVKISPAKVEEIIAA.TNNDIRQSINHIA   648
128kD hRFC     767  CFDLRFQRPRVEQIKGAMMSIAFKEGLKIPPPAMNEIILG.ANQDIRQVLHNLS   820
37kD hRFC      194  CSKFRFKPLSDKIQQQRLLDIAKKENVPIS.HRGIAYLVKVSEGDLRKAITFLQ   246
37kD yRFC      184  CSKFRFKALDASNAIDRLRFISEQENVKCD.DGVLERILDISAGDLRRGITLLQ   236
130kD yRFC     161  CTRFRFQPLPQEATERRIANVLVHEKLKLSPNAEKALLIELSNGDMRRVLNVLQ   214
40kD hRFC      184  CAVLRYTKLTDAQILTRLMNVIEKERVPYTDDGLEAIIFT.AQGDMRQALNNLQ   236
CHL12          312  CEIIAVKRPSDTTLLERLNLICHKENMNIPIKAIND.LIDLAQGDVRNCINNLQ   364

Consensus           C--+RF+-+-------RL--I--KE-+----------++--++GD+R+-++-LQ
```

FIGURE 4.—Domains of amino acids similarity between CHL12 and RF-C predicted proteins. Multiple alignment of three regions from CHL12 (present work); *D. melanogaster* protein DROGLTFAC (GenBank accession no. L17340); 37-kD subunit of human RF-C (GenBank accession no. M87339); 40-kD subunit of human RF-C (GenBank accession no. M87338); 128-kD subunit of human RF-C (GenBank accession no. L23320) and 40- and 37-kD subunits of yeast RF-C (LI and BURGERS 1994; and NOSKOV *et al.* 1994) is presented. Conservative substitutions denoted as "++" in consensus sequence.

teins (for a review, see HIGGINS *et al.* 1988), beginning at amino acid 183 in CHL12. However, we were unable to identify any other conserved sequences associated with ATP- or GTP-binding proteins in the CHL12 sequence.

Analysis of the region 5′ to the CHL12 open reading frame revealed the presence of two degenerative *Mlu*I motifs (ACGCGT) at positions −84 bp (ACACGT) and −105 bp (TCGCGT). Such motifs are characteristic of almost all yeast genes controlling DNA replication or the synthesis of nucleotide precursors (WHITE *et al.* 1987). Nuclear targeting signal, KKRKKKR, (DINGWALL and LASKEY 1991) was found in positions 279–285.

Physical mapping of *CHL12*: The *CHL12* gene was mapped to chromosome *XIII* using the 2 μm DNA-chromosome destabilization method (FALCO and BOTSTEIN 1983). The physical map position of the *CHL12* gene on chromosome *XIII* was determined by hybridization of the *CHL12* probe with an array of λ bacteriophage clones (donated by M. OLSON) that completely covers chromosome *XIII* (OLSON *et al.* 1986). The *CHL12* probe hybridized with two λ clones, 6996 and 4000, both containing the *ADH3* gene, indicating that *CHL12* is physically linked to the *ADH3* gene on the right arm of chromosome *XIII*. Analysis of the sequence proximal to the promoter region of the *CHL12* revealed the beginning of an open reading frame identical to that of the *SEC14* gene. The *SEC14* gene encodes a cytosolic factor that is required for transport of secretory proteins from the yeast Golgi complex (BANKAITIS *et al.* 1989). Only 259 bp separate the coding regions of two genes.

Disruption of *CHL12*: To establish the role of the *CHL12* gene in mitotic chromosome transmission, we constructed the deletion *chl12-Δ2::LEU2 in vitro* in which the whole open reading frame of *CHL12* is eliminated (described in detail in MATERIALS AND METHODS). The *chl12-Δ2::LEU2* construct was introduced into the diploid strain YPH501 by a one-step gene replacement technique (ROTHSTEIN 1983). The derived strain YE102, in which one copy of *CHL12* was replaced with *chl12-Δ2::LEU2* and was sporulated, and tetrads were dissected. In virtually all of the tetrads examined all four spores gave rise to growing colonies, indicating that the deletion of the *CHL12* gene does not result in the loss of cell viability. Cells containing the null *chl12* mutation exhibited an increase in the rate of chromosome *III* loss and interchromosomal recombination not different from that in the original *chl12* mutants (Table 2). As well as in the original mutants the rate of spontaneous mutations was not increased in the null *chl12* mutant (Table 4). Analogous data were obtained for insertion mutation *chl12-Δ1::URA3*. We conclude that the *CHL12* gene product is not required for cell viability under standard laboratory growth conditions.

Several segregants carrying the *chl12-Δ2::LEU2* allele were tested for growth at various temperatures. All the segregants exhibited cold sensitivity (11°). Most of *chl12-Δ2* cells (YE105) incubated at 11° exhibited a dumbbell shape (Figure 6). In 70% of all large budded cells nuclei were localized near or in the isthmus (Figure 5). Under these conditions the percentage was 23% for the isogenic wild-type strain (YE104). (At least 350 cells were analyzed for each strain.) The same result was obtained for mutant cells carrying another cold sensitive allele *chl12-1* (data not shown). This terminal morphol-

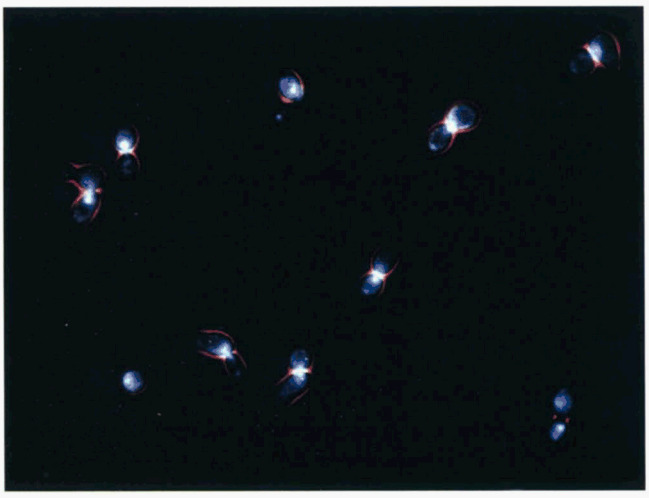

FIGURE 5.—The morphology of cells lacking the *CHL12* gene at a restrictive temperature (11°). Cells were fixed and stained as described in MATERIALS AND METHODS. The cells viewed with fluorescence optics.

	UB	SB	LB	AM
30°				
WT	38%	44%	18%	0
chl12-Δ2	44%	38%	16%	2%
chl12-Δ2 rad9	58%	25%	16%	1%
11°				
WT	43%	40%	16%	<1%
chl12-Δ2	10%	9%	78%	3%
chl12-Δ2 rad9	35%	47%	18%	0

FIGURE 6.—Quantitation of the morphologies of cells lacking the *CHL12* gene. Wild-type (WT) and *chl12* strains were analyzed after 24 hr at 25° and after 48 hr at 11°. Similar results were obtained for the 24-hr time point at a restrictive temperature. Percentages of cells with the indicated morphology: UB, unbudded; SB, small budded; LB, large budded; and AM, abnormal morphology such as multibudded. Strains: YE104 (wild-type), YE105 (*chl12-Δ2*), and YNK109 (*chl12-Δ2 rad9*).

ogy was observed in mutants defective in DNA replication or segregation under nonpermissive conditions (PRINGLE and HARTWELL 1981).

Cell cycle arrest of the *chl12* mutant requires the *RAD9* gene: DNA damage, such as that produced by X-ray or a temperature-labile DNA ligase and DNA polymerases, induces a *RAD9*-dependent cell cycle arrest (WEINERT and HARTWELL 1988, 1993). Therefore, we checked whether cell cycle arrest caused by *chl12* mutations was alleviated by *rad9*. We used two sets of criteria to test the cell cycle arrest of *chl12* and *chl12 rad9* cells after shift to a restrictive temperature (11°). A direct measure of cell cycle arrest comes from the cell morphology. A second criterion was the affect a *rad9* mutation on cell viability when cells were limited for *chl12* function. After 48 hr at the restrictive temperature most *chl12 RAD9* mutant cells remain arrested with a dumbbell shape morphology. Their nuclei were localized near or in the isthmus. In contrast, a *chl12 rad9* double mutant failed to arrest after shift to the restrictive temperature (Fig. 6). Remarkably, the viability of *chl12 rad9* cells, whether grown at the permissive temperature or following incubation at the restrictive temperature, was always the same as that of the *chl12 RAD9* cells (Table 5). Thus, inactivation of the *RAD9* gene caused a recovery from cell cycle arrest in *chl12* cells but had no detectable effect on cell viability. This phenotype is different from that observed in mutants defective in DNA replication. These temperature sensitive mutants exhibit reduction of cell viability under nonpermissive conditions when the *RAD9* gene is not functional (WEINERT and HARTWELL 1993).

The recombination-repair gene *RAD52* does not affect cell viability of the *chl12* mutant: The increase in mitotic recombination in the *chl12* mutants may be explained by the accumulation of chromosomal lesions in the absence of a functional gene product. If these lesions are double-strand breaks (DSBs), the viability of *chl12* mutant cells should be significantly decreased when cells are unable to repair DSBs. We constructed the double mutant strain YNK85 containing complete deletions of the *CHL12* and *RAD52* genes. The *RAD52* gene is implicated in mitotic and meiotic recombination and is required for recombinational repair of DSBs (RESNICK and MARTIN 1976; THOMAS and ROTHSTEIN 1989; BOUNDY-MILLS and LIVINGSTON 1993).

chl12-Δ2 is viable in combination with a deletion of the *RAD52* gene. The double mutant YNK85 was compared to the isogenic *rad52* strain YNK84 for sensitivity to γ-irradiation. Both strains displayed no significant difference in sensitivity (data not shown). However, the double mutant grew very poorly compared with the isogenic single mutant *chl12-Δ2* and *rad52-Δ1* strains. The doubling time of the double mutant was about 80 min higher then that of single mutants (Table 5). We determined the cell viability for YE104 (wild type), YE105 (*chl12-Δ2*), YNK84 (*rad52-Δ1*) and YNK85 (*rad52-Δ1 chl12-Δ2*) isogenic strains. Percentage of dead cells in the cultures of YE105 and YNK84 was approximately the same. Percentage of dead cells in the culture of the double mutant was approximately equal to the sum of those for single mutants (Table 5). Because there was no synergetic effect of the double mutation on cell viability, we conclude that the *RAD52* gene product is not important for cell viability in a *chl12* mutant background.

DISCUSSION

Previously we described the isolation of yeast *chl* (for *ch*romosome *l*oss) mutants using novel methods of selection (KOUPRINA *et al.* 1988, 1993). In the mutants, chromosome *III* is lost during mitotic growth several fold more frequently than in the isogenic wild-type strain. *chl* mutants are also incapable of stable maintaining circular centromeric plasmids. Among the collection, we have identified several genes that are poten-

tially involved in chromosome replication (KOUPRINA *et al.* 1992, 1993).

In this study we have analyzed one of the genes from this group, *CHL12*. The *CHL12* gene was also identified in separate screen (SPENCER *et al.* 1990) designed to identify genes involved in mitotic chromosome transmission. Recently the *CTF18* gene, which is identical to *CHL12*, has been cloned and sequenced independently (F. SPENCER and P. HIETER, personal communication). The analysis of phenotypes associated with mutations in this gene suggests that the protein encoded by *CHL12* functions in DNA metabolism.

Mutations in *CHL12/CTF18* lead to chromosome destabilization in mitosis. The increase in frequency of chromosome loss was observed for linear chromosomes (including a natural chromosome *III* and a test chromosome fragment derived from chromosome *III*) as well as for circular centromeric plasmids. An analysis of segregation of the circular artificial minichromosomes (KOUPRINA *et al.* 1993) showed that loss of these structures in mitotic division in *chl12* mutants is not accompanied by their accumulation in a subset of the cell population. Chromosome destabilization appears to be caused predominantly by loss of sister chromatids rather than by nondisjunction. It has been proposed that this mode of chromosome destabilization is consistent with the presence of a defect in DNA metabolism (HARTWELL and SMITH 1985; PALMER *et al.* 1990).

The observed increase in the frequency of mitotic recombination events in the *chl12* mutants also supports this conclusion. An increase in mitotic recombination has been described for several DNA metabolism mutants in yeast, including defects in DNA ligase, DNA polymerases and DNA replication accessory proteins (HARTWELL and SMITH 1985; KOUPRINA *et al.* 1992).

Analysis of the region 5′ to the *CHL12* open reading frame revealed the presence of two degenerative *Mlu*I motifs (ACGCGT) at positions −84 bp and −105 bp (ACACGT and TCGCGT). The *Mlu*I motif is found upstream of many yeast genes important for DNA metabolism. To date, more than 20 genes of this group have been identified, including the DNA-polymerase I, II, and III genes (BOULET *et al.* 1989; JOHNSTON *et al.* 1985; MORRISON *et al.* 1990; ARAKI *et al.* 1991), the DNA-ligase gene (WHITE *et al.* 1987), and other genes controlling DNA replication or the synthesis of nucleotide precursors (WHITE *et al.* 1987). Expression of these genes appears to be coordinately regulated, occurring near the boundary between G_1 and S phases (LOWNDES *et al.* 1991). TCGCGT and ACACGT sequences in the promoter region of *CHL12* differ from the canonical *Mlu*I site by a single nucleotide. Similar "near-fit" *Mlu*I sequences were found in the promoter region of three DNA synthesis genes, *PRI1*, *RFA3* and *DPB3* encoding DNA primase I (JOHNSTON *et al.* 1991), subunit C of replication factor A (BRILL and STILLMAN 1991) and subunit C of DNA polymerase II (JOHNSTON *et al.* 1991), respectively. The presence of the *Mlu*I motif in the promoter region of the *CHL12* gene suggests that this gene is a member of a family of genes involved in DNA metabolism. It is still unknown whether the *CHL12* gene is expressed near the G_1/S boundary, as are several other genes containing the *Mlu*I motif.

The open reading frame of *CHL12* encodes a predicted protein of 742 amino acids with a molecular mass of 84 kD. Analysis of the amino acid sequence reveals that the protein shares a low but significant degree of homology with the 37 kD, and 40 kD subunits of human activator 1 or human replication factor C (RF-C) (CHEN *et al.* 1992a,b).

RF-C is the most recently identified replication factor required for *in vitro* replication of SV40 (TSURIMOTO and STILLMAN 1990, 1991a,b). The factor functions as an auxiliary protein for proliferating cell nuclear antigen (PCNA) in the elongation stage of DNA replication (TSURIMOTO and STILLMAN 1990). Yeast RF-C is multisubunit complex of polypeptides with molecular masses reported as 128, 86, 41, 40, 37 and 27 kD (YODER and BURGERS 1991). Purified RF-C proteins have DNA-dependent ATPase activity and, like PCNA, is believed to be an accessory protein for DNA polymerase δ (TSURIMOTO and STILLMAN 1990; LEE *et al.* 1991; BURGERS 1991).

The amino acid sequences of CHL12 and the 37-kD subunit of human RF-C are 27% identical and 50% similar over a 350-amino acid region. Clustering of amino acid sequence similarity occurred in three segments, which comprise 19, 50 and 53 residues. Surprisingly, that these segments are also present in two recently sequenced other subunits of human and yeast RF-C and the DROGLTFAC transcription factor of Drosophila (Figure 4). These regions may identify a functionally important domains of the proteins, perhaps mediating interactions of the subunits of RF-C.

The presence of a functional NTP binding domain has been demonstrated for the product of cloned genes encoding the 37- and 40-kD subunits of human RF-C (CHEN *et al.* 1992a,b) as well as the 37- and 40-kD subunits of yeast RF-C (NOSKOV *et al.* 1994; LI and BURGERS 1994). Computer analysis of *CHL12* displayed a sequence, GPPGIGKT, which meets the necessary requirements for a NTP binding domain in positions 183–190. It is interesting that among three regions of homology between CHL12 and subunits of RF-C, one contains a NTP binding domain (Figure 4). The significance of the homology between CHL12 and RF-C is not clear because the critical active site residues of RF-C have not yet been identified. Recently P. BURGERS (personal communication) has shown that RF-C from *chl12-Δ2* and *CHL12* cells contain the same polypeptides. However, this result does not exclude possibility that CHL12 could be a component of RF-C which is lost from the RF-C complex

during its purification from yeast cells. More data need to elucidate possible relationship between CHL12 and RF-C.

A strain containing a null allele of *CHL12* is viable under standard growth conditions and phenotype of the mutant cells is not different from that of original mutants (cold sensitivity, slow growth, an increased rate of chromosome loss and recombination). About 80% of cells carrying the *chl12* deletion exhibited an accumulation of large, budded cells with the nucleus near or in the isthmus at the nonpermissive temperature. Thus, *chl12* mutant cells exhibit a phenotype similar to that of cell division cycle (*cdc*) mutants that arrest in the cell cycle at a point either immediately preceding M phase or during S phase (PRINGLE and HARTWELL 1981). Flow cytometry analysis has shown that after shift to the restrictive temperature most of the mutant cells contained close to $2n$ DNA content (E. KROLL, unpublished data). While this result demonstrates that most chromosomal DNA has completed replication in the *chl12* cells at nonpermissive temperature, the method is not sufficient to determine whether 10% of the genome, for example, is unreplicated in the arrested cells. The unreplicated DNA (formally damaged DNA) could activate the *RAD9* checkpoint (WEINERT and HARTWELL 1988, 1993). Indeed, the *RAD9*-dependent cell cycle arrest of *chl12* cells has been observed. However, in contrast with known DNA replication *cdc* mutants, viability of the *chl12 rad9* double mutant was not reduced after shift to the restrictive temperature. This phenotype suggests that only a small amount of lesions is accumulated in DNA of the *chl12 rad9* mutant. It is likely that they can be efficiently repaired in subsequent cell divisions similar to that observed when a specific double strand break was introduced near the end of dispensable chromosome in a *rad9* mutant strain (SANDELL and ZAKIAN 1993).

The results presented here are in agreement with the proposed role of *CHL12* in DNA metabolism. The fact that *CHL12* is not essential for mitotic growth under normal conditions indicates either that this gene product performs an essential function but can be substituted for another gene product or bypassed in a parallel pathway, or that it is required for the fidelity of a process in DNA metabolism such as DNA synthesis, recombination or repair. Additional experiments are necessary to understand the function of the *CHL12* gene in DNA metabolism.

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