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Viability of Clathrin Heavy-Chain-Deficient *Saccharomyces cerevisiae* Is Compromised by Mutations at Numerous Loci: Implications for the Suppression Hypothesis

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The gene encoding clathrin heavy chain in *Saccharomyces cerevisiae* (*CHC1*) is not essential for growth in most laboratory strains tested. However, in certain genetic backgrounds, a deletion of *CHC1* (*chc1*) results in cell death. Lethality in these *chc1* strains is determined by a locus designated *SCD1* (suppressor of clathrin deficiency) which is unlinked to *CHC1* (S. K. Lemmon and E. W. Jones, Science 238:504–509, 1987). The lethal allele of *SCD1* has no effect on cell growth when the wild-type version of *CHC1* is present. This result led to the proposal that most yeast strains are viable in the absence of clathrin heavy chain because they possess the *SCD1* suppressor. Discovery of another yeast strain that cannot grow without clathrin heavy chain has allowed us to perform a genetic test of the suppressor hypothesis. Genetic crosses show that clathrin-deficient lethality in the latter strain is conferred by a single genetic locus (termed *CDL1*, for clathrin-deficient lethality). By constructing strains in which *CHC1* expression is regulated by the *GAL10* promoter, we demonstrate that the lethal alleles of *SCD1* and *CDL1* are recessive. In both cases, very low expression of *CHC1* can allow cells to escape from lethality. Genetic complementation and segregation analyses indicate that *CDL1* and *SCD1* are distinct genes. The lethal *CDL1* allele does not cause a defect in the secretory pathway of either wild-type or clathrin heavy-chain-deficient yeast. A systematic screen to identify mutants unable to grow in the absence of clathrin heavy chain uncovered numerous genes similar to *SCD1* and *CDL1*. These findings argue against the idea that viability of *chc1* cells is due to genetic suppression, since this hypothesis would require the existence of a large number of unlinked genes, all of which are required for suppression. Instead, lethality appears to be a common, nonspecific occurrence when a second-site mutation arises in a strain whose cell growth is already severely compromised by the lack of clathrin heavy chain.

Membrane vesicles play an integral role in the transfer of proteins between various cellular membranes and membrane-bounded compartments, including those of the secretory and endocytic pathways (16, 22). Acting as shuttles, the vesicles collect cargo proteins as they bud from donor compartments and deliver their contents by fusion to specific recipient membrane compartments. Studies of the yeast secretory pathway demonstrate that vesicle formation and fusion are necessary for cell growth (24). Clathrin, a protein complex composed of three heavy-chain molecules (180 kDa) and three light-chain molecules (30 to 40 kDa) associated in a three-legged structure called a triskelion, is intimately involved in the formation of endocytic transport vesicles and has also been implicated in the formation of transport vesicles and secretory granules from the Golgi complex in animal cells (2, 4, 6, 21). Two principal functions have been attributed to clathrin coats during this process of transport vesicle formation. First, assembly or rearrangement of triskelions could drive invagination of the membrane. Second, by collecting specific transmembrane receptor proteins, the clathrin coat could bestow selectivity to the process of packaging cargo proteins into transport vesicles.

The role of clathrin in intracellular protein transport has been studied in vivo in the yeast *Saccharomyces cerevisiae*. *S. cerevisiae* possesses clathrin heavy and light chains (15, 19, 27) encoded by single genes, *CHC1* and *CLC1*, respectively (19, 27). In most strains examined, elimination of *CHC1* results in viable, slow-growing cells (18, 19). These clathrin heavy-chain-deficient mutants (*chc1*) transport newly synthesized proteins through the secretory pathway to the cell surface and to the lysosomelike vacuole but mislocalize a Golgi membrane protein (Kex2p) to the cell surface (17, 20). These results led to the conclusion that clathrin is not essential for the formation of secretory pathway transport vesicles and therefore for cell growth.

Discovery of a yeast strain unable to grow in the absence of clathrin heavy chain led to the identification of a genetic locus which influences the viability of *chc1* strains but is phenotypically cryptic in *CHC1* strains (12). This locus, unlinked to *CHC1*, was designated suppressor of clathrin deficiency (*SCD1*). The interpretation was put forth that clathrin-deficient yeast cells are viable only if they harbor a suppressor allele of *SCD1*. It has been demonstrated in previous work that viability is not due to a suppressor allele arising during the propagation of diploids heterozygous for *chc1* (12, 18). This work could not exclude the possibility, however, that a suppressor could preexist in laboratory strains of *S. cerevisiae*. Given the paucity of strains that are inviable when *CHC1* is disrupted, the suppressor allele would have to be widespread. The significance of *SCD1* is unclear. In one view, the existence of a suppressor has important consequences for interpretations of the phenotypes of viable *chc1* cells. For example, the suppressor could encode a functional homolog of clathrin heavy chain which is capable of suppressing, in part, secretory pathway defects caused by the absence of clathrin heavy chain. Low-strin-

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gency hybridizations did not reveal genes with structural similarity to *CHC1* (18), but a functional substitute need not display sufficient similarity for detection by these procedures. On the other hand, the fragility of viable *chc1* strains suggests an alternative hypothesis. The debilitated state of strains harboring *chc1* increases the sensitivity of cells to traumatic growth conditions. Low pH, high temperature, or low levels of a toxic drug (the arginine analog canavanine) that are not lethal to *CHC1* cells can inhibit the growth of *chc1* cells (18). This finding raises the possibility that perturbation of a process unrelated to clathrin function could impose sufficient stress to prevent *chc1* cell growth. In this way, the lethal allele of *SCD1* could result in *chc1* cell lethality even though it plays no role in intracellular protein transport or any other aspect of clathrin function.

We describe here experiments designed to test whether lethality in some clathrin heavy-chain-deficient yeast strains is indicative of genes encoding products functionally related to clathrin or whether this lethality reflects nonspecific effects on cells whose growth is already retarded by *chc1*. Our results argue strongly that lethality is a consequence of nonspecific effects and the suppression hypothesis need not be invoked to explain the phenotypes of clathrin-deficient yeast strains.

MATERIALS AND METHODS

Media and strains. Yeast strains were grown in YP medium, which contains 1% yeast extract (Difco Laboratories, Detroit, Mich.) and 2% Bacto-Peptone (Difco) supplemented with 2% dextrose (YPD) or 2% galactose (YPG) as appropriate. For growth of Ade$^-$ strains, this medium was supplemented with 0.0025% (wt/vol) adenine hydrochloride. Minimal medium plates used for assessing nutritional requirements were prepared as described by Sherman et al. (25). All solid media included 2% agar. Cell densities in liquid culture were measured in a 1-cm quartz cuvette, using a Beckman Instruments DU-62 spectrophotometer. One A_{600} unit is equivalent to a density of 10^7 cells per ml. The genotypes of the various strains used are shown in Table 1.

Plasmids. Plasmid pCHC1-G, which is a pEMBLYex4-derived plasmid containing the *CHC1* gene under the control of the *GAL10* promoter, was constructed as follows. A derivative of pEMBLYex4 (1) that lacks the 2μm autonomous replication sequence was made by deletion of the 1.8-kb *Bst*EII-*Hin*dIII fragment. A 932-bp *Xmn*I-*Kpn*I fragment from *CHC1* was isolated from an *Xmn*I partial digest and *Kpn*I complete digest of a plasmid containing only the 2.2-kb *Bam*HI-*Kpn*I fragment of *CHC1*. The *Xmn*I site lies 36 bp upstream of the ATG initiation codon of *CHC1*. The complete *CHC1* gene was then reassembled in pUC119 by using the *Xmn*I-*Kpn*I fragment and a 6.5-kb *Kpn*I-*Sal*I fragment to generate pCHCXS. The *CHC1* gene was then isolated on a 7.3-kb *Sma*I-*Sal*I fragment from pCHCXS and inserted at the *Sma*I and *Sal*I sites in the polylinker of pEMBLYex4 Δ*Bst*EII-*Hin*dIII such that the direction of transcription is in the same sense as in the *GAL10* promoter. This plasmid is pCHC1-G (Fig. 1). Digestion of pCHC1-G with *Stu*I cuts within the *URA3* gene on this plasmid and directs insertion of the DNA to the chromosomal *URA3* locus.

Plasmid pchc1-Δ10, used for single-step gene transplacements (23), was described in Payne et al. (18) and carries the *LEU2* gene inserted such that only 178 bp at the 3′ end of the *CHC1* gene remain. For transplacement, pchc1-Δ10 was treated with *Hin*dIII.

Plasmid YCpCHCTRP consists of an 8.4-kb *Bam*HI-*Sal*I fragment containing the entire *CHC1* gene inserted into YCpR+CΔ*Bam*HI between the *Bam*HI and *Sal*I sites. The YCpR+CΔ*Bam*HI vector, which was constructed from YCpR+C (a gift from D. Mooney) by deletion of the *Bam*HI fragment containing the *ADH1-ADH2* gene fusion, contains *CEN3*, *ARS1*, the *TRP1* gene as a yeast selectable marker, and pBR322 sequences for replication in *Escherichia coli* K-12.

YCpchc1-521 is a derivative of YCpCHCTRP which carries *chc1-521* (a temperature-sensitive allele of *CHC1*). YCpchc1-Δ10 is YCpchc1-521 which has had the *chc1-521* gene replaced by *chc1-Δ10*. Isolation of the temperature-sensitive allele of *CHC1* will be described in a separate report.

pCHCc102 contains the complete wild-type *CHC1* gene on an 8.4-kb *Bam*HI-*Sal*I fragment inserted into the centromere plasmid YCp50 (*URA3*). pBP6 was constructed by inserting a 5.4-kb *Bam*HI-*Sac*I fragment of pCHCc102 (containing the amino-terminal end of the wild-type *CHC1* gene) and a 3.0-kb *Sac*I-*Sal*I fragment of YCpchc1-521 (containing the mutant carboxy-terminal end of the temperature-sensitive *CHC1* gene) into YCp50.

Nucleic acid manipulations. Large-scale plasmid purification from *E. coli* K-12 was performed using a Qiagen plasmid purification kit (Qiagen, Inc., Studio City, Calif.). Plasmid construction and restriction analysis were carried out essentially as described by Maniatis et al. (13), using restriction endonucleases and T4 DNA ligase supplied by Boehringer Mannheim Biochemicals (Indianapolis, Ind.) or Promega Biotec (Madison, Wis.).

Yeast genomic DNA was isolated as described by Payne and Schekman (19). Physical characterization of the yeast genomic DNA was carried out by the method of Southern as described by Maniatis et al. (13). Radioactive probes were generated by isolation from a SeaPlaque agarose gel (FMC BioProducts, Rockland, Maine) of the 971-bp and 3.26-kb *Eco*RI fragments internal to the *CHC1* gene and random priming of 50- to 100-ng aliquots of these fragments with a Boehringer Mannheim random priming kit and 50 μCi of [α-^{32}P]dATP (3,000 Ci/mmol; ICN Radiochemicals, Irvine, Calif.). Labeled DNA was separated from unincorporated nucleotides on a Biospin 30 chromatography column (Bio-Rad Laboratories, Richmond, Calif.). Prehybridization of nitrocellulose filters (Schleicher & Schuell, Keene, N.H.) bearing blotted yeast genomic DNA was performed in a solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0), 10× Denhardt's solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin), 5 mM sodium pyrophosphate, 0.5% sodium dodecyl sulfate (SDS), and 100 μg of heat-denatured salmon sperm DNA per ml at 45°C for 1 to 3 h. Hybridizations of radiolabeled fragments of the *CHC1* gene to prehybridized filters was carried out in the same solution, except with 50% formamide and no salmon sperm DNA, at 55°C for 16 h. The radiolabeled yeast *CHC1* fragments (100 ng each) were added to approximately 3×10^6 cpm/ml. After hybridization, filters were washed with 6× SSC at 50 to 60°C three times, dried, and then exposed to Kodak XAR5 X-ray film (Eastman Kodak Co., Rochester, N.Y.) in a cassette containing intensifying screens at −70°C for 16 h.

Preparation of protein extracts, SDS-PAGE, and immunodetection with specific antisera. Cells were grown in YPG or YPD for at least nine doublings at 30°C to mid-logarithmic phase. Approximately 10 A_{600} of cells was harvested, and cell extracts were prepared by lysis with 2% SDS as de-

TABLE 1. Yeast strains

Strain	Genotype	Source
W303-1A	*MATa leu2-3,112 ura3-1 his3-11,15 ade2-1 trp1-1 can1-100 cdl1*	R. Rothstein
W303-1B	*MATα leu2-3,112 ura3-1 his3-11,15 ade2-1 trp1-1 can1-100 cdl1*	R. Rothstein
W303	*MATa/MATα* diploid homozygous for markers in W303-1A and -1B	R. Rothstein
BJ3473	*MATa/MATα leu2/leu2 ura3-52/ura3-52 his1/HIS1 ade6/ADE6 gal2/(gal?) scd1/scd1*	S. Lemmon and E. W. Jones
GPY55-10B	*MATα leu2-3,112 ura3-52 trp1-289 prb1 gal2*	This study
GPY142-1C	*MATα leu2-3,112 ura3-52 his4 trp1-289 can1*	This study
GPY137-3A	*MATa leu2 ura3-52 his1 ade6 scd1 gal2*	This study
GPY150.2	*MATα leu2-3,112 ura3-52 his4 trp1-289 can1 URA3::GAL10-CHC1 chc1-Δ10::LEU2*	This study
GPY170	GPY55-10B/YCpchc1-521 (*TRP1*)	This study
GPY176	*MATα leu2-3,112 ura3-52 trp1-289 prb1 gal2 chc1-Δ10::LEU2*/YCpchc1-521 (*TRP1*)	This study
GPY177	*MATα leu2-3,112 ura3-52 trp1-289 prb1 gal2*/YCpchc1-Δ10::LEU2 (*TRP1*)	This study
GPY185.1	*MATα leu2-3,112 ura3-52 his1 ade6 scd1 chc1-521*	This study
GPY200	See GPY205-1A	This study
GPY205	GPY150.2 × GPY137-3A diploid	This study
GPY205-1A	*MATα leu2 ura3-52 his*	This study
GPY205-1D	*MATa leu2 ura3-52 his1 his4 ade6 trp1-289 URA3::GAL10-CHC1 chc1-Δ10::LEU2*	This study
GPY205-16A	*MATα leu2 ura3-52 his4 trp1-289 scd1 URA3::GAL10-CHC1 chc1-Δ10::LEU2*	This study
GPY206	GPY137-3A × GPY205-16A diploid	This study
GPY207	GPY205-16A × GPY205-1D diploid	This study
GPY207-12B	*MATa leu2 ura3-52 his4 ade6 trp1-289 scd1 URA3::GAL10-CHC1 chc1-Δ10::LEU2*	This study
GPY207-12C	*MATα leu2 ura3-52 his4 ade6 trp1-289 URA3::GAL10-CHC1 chc1-Δ10::LEU2*	This study
GPY207-14A	*MATa leu2 ura3-52 his4 ade6 trp1-289 URA3::GAL10-CHC1 chc1-Δ10::LEU2*	This study
GPY207-17C	*MATα leu2 ura3-52 his4 ade6 trp1-289 scd1 URA3::GAL10-CHC1 chc1-Δ10::LEU2*	This study
GPY212	GPY207-12B × GPY207-17C diploid	This study
GPY213	GPY207-12C × GPY207-14A diploid	This study
GPY214	W303 *chc1-Δ10::LEU2/CHC1* diploid	This study
GPY215	BJ3473 *chc1-Δ10::LEU2/CHC1* diploid	This study
GPY222	GPY214 *URA3::GAL10-CHC1/ura3-1* diploid	This study
GPY222-5A	MATα *leu2-3,112 ura3-1 his3-11,15 ade2-1 trp1-1 can1-100 cdl1 URA3::GAL10-CHC1 chc1-Δ10::LEU2*	This study
GPY222-1B	*MATa leu2-3,112 ura3-1 his3-11,15 ade2-1 trp1-1 can1-100 cdl1 URA3::GAL10-CHC1 chc1-Δ10::LEU2*	This study
GPY226	GPY205-1D × GPY222-5A diploid	This study
GPY227	GPY205-16A × GPY222-1B diploid	This study
GPY230	GPY222-5A × GPY222-1B diploid	This study
GPY246	GPY185.1 × W303-1A diploid	This study
GPY249	GPY246 *chc1-Δ10::LEU2/chc1-521* diploid	This study
GPY257	Trp^+ revertant of GPY205-1D	This study
GPY292	W303-1B × GPY1103 diploid	This study
GPY322	GPY214/pBP6 (*chc1-521 URA3*)	This study
GPY322-2A	*MATα leu2-3,112 ura3-1 his3-11,15 ade2-1 trp1-1 can1-100 cdl1 chc1-Δ10::LEU2*/pBP6 (*chc1-521 URA3*)	This study
GPY323	GPY214/pCHCc102 (*CHC1 URA3*)	This study
GPY323-2B	*MATα leu2-3,112 ura3-1 his3-11,15 ade2-1 trp1-1 can1-100 cdl1 chc1-Δ10::LEU2*/pCHCc102 (*CHC1 URA3*)	This study
GPY1103	*MATa leu2-3,112 ura3-52 his4-519 trp1 can1 chc1-Δ8::LEU2*	This study

scribed previously (15). Various amounts of cell extract were then loaded onto a 10% SDS-polyacrylamide gel and subjected to electrophoresis (PAGE) as described by Laemmli (11). Electroblotting to nitrocellulose and visualization of clathrin heavy chain with a specific antiserum were carried out essentially as described by Burnette (3). The polyclonal antiserum used in these studies was that used by Payne and Schekman (19); the antiserum was preadsorbed three times with intact yeast cells and then used at a 1/400 dilution. The secondary antibody was goat anti-rabbit immunoglobulin G coupled to alkaline phosphatase (Bio-Rad) and was used at a 1/1,000 dilution. The blot was developed with a solution consisting of 330 μl of nitroblue tetrazolium (50 mg/ml; Sigma), 165 μl of 5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml; Sigma), and 50 ml of alkaline phosphatase buffer (0.1 M Tris-HCl [pH 9.5], 0.1 M NaCl, 5 mM $MgCl_2$).

Yeast genetic techniques and transformation. Yeast mating, sporulation, and tetrad analyses were conducted as described by Sherman et al. (25). DNA transformation of yeast strains was done by the lithium acetate procedure of Ito et al. (9).

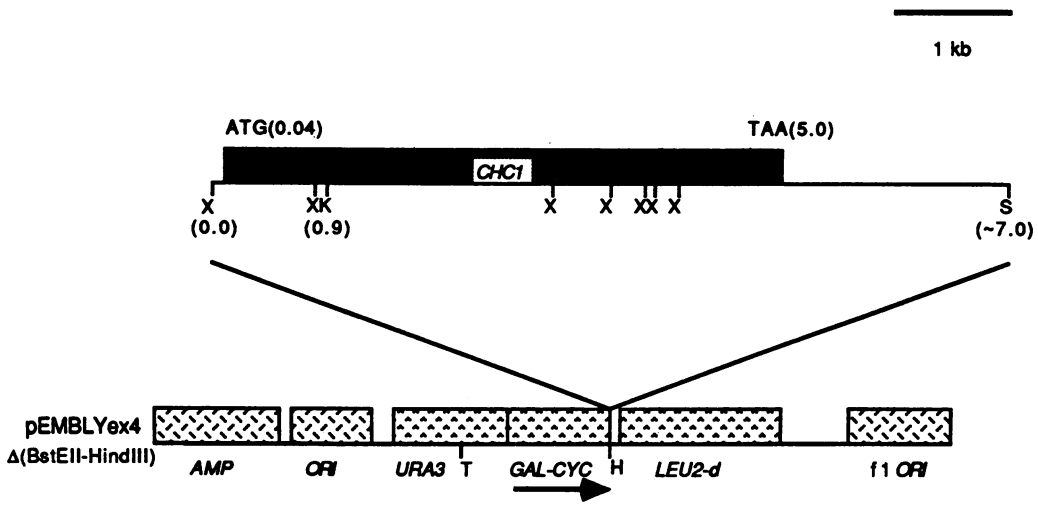

FIG. 1. Plasmid pCHC1-G carrying the *CHC1* gene under the control of the *GAL10* promoter. The arrow indicates the direction of transcription from the *GAL10* promoter. Restriction sites: K, *Kpn*I; X, *Xmn*I; S, *Sal*I; H, *Hin*dIII; T, *Stu*I.

To generate a strain in which *CHC1* expression is regulated by the *GAL10* promoter, pCHC1-G was digested with *Stu*I and introduced into GPY142-1C by lithium acetate-mediated transformation. A Ura$^+$ transformant was selected, and *CHC1* in this transformant was transplaced with *chc1-Δ10* (23). Leu$^+$ transformants were tested for their growth rates on glucose- and galactose-containing media. DNA from a transformant that grew at wild-type rates on galactose-containing medium but slowly on glucose-containing medium was subjected to analysis by the method of Southern to establish that the *CHC1* gene driven by the *GAL10* promoter is present at the *URA3* locus and that the *chc1-Δ10* allele is present at the *CHC1* locus. This strain is GPY150.2 (Table 1).

Haploid *cdl1 chc1-Δ10 CHC1-G* strains were prepared by sporulation of a diploid strain (GPY222) homozygous for *cdl1* and heterozygous for *chc1-Δ10* and *CHC1-G*. GPY222 was derived from W303 by transformation first with pchc1-Δ10 (to generate GPY214) and then with pCHC1-G. The genotype of GPY222 was confirmed by characterization of its genomic DNA by the method of Southern. Two Leu$^+$ and Ura$^+$ segregants from GPY222 were chosen, and their genotypes were confirmed by analysis of their genomic DNA by the method of Southern. These *cdl1 chc1-Δ10 CHC1-G* strains are GPY222-5A and GPY222-1B.

EMS mutagenesis and mutant screen. Mutagenesis of yeast cells was carried out by using ethyl methanesulfonic acid (EMS; Sigma Chemical Co., St. Louis, Mo.). Cells were grown with aeration in YPG at 30°C to stationary phase. Aliquots containing 10 A_{600} of cells were dispensed into glass tubes, and the cells were washed with 2 ml of phosphate buffer (100 mM sodium phosphate, pH 7). The cells were resuspended in phosphate buffer, divided into two 5-A_{600} (0.75-ml) aliquots, and brought to a final volume of 1.75 ml; 10 μl of EMS was then added to one of the two samples. The cells were incubated with shaking at 30°C for 1 h, at which time the EMS was quenched by the addition of 1 ml of a 12% aqueous solution of sodium thiosulfate. This treatment results in a 40 to 50% reduction in the number of viable cells. The cells were then sedimented, resuspended in 2 ml of YPG, and allowed to recover at 30°C with aeration for 1 h. Serially diluted samples were spread onto YPG plates, and colonies were allowed to form at 30°C.

To identify colonies that grow only when *CHC1* is expressed, YPG-grown colonies were replica plated onto YPD plates and then onto fresh YPG plates. The plates were incubated at 30°C, and colonies that grew on the YPG plates but not the YPD plates were saved for further characterization. Since several doubling times on glucose-containing medium are required before residual clathrin heavy-chain mRNA and protein disappear from the cells, in some cases the YPD plates containing the mutagenized colonies were replica plated a second time onto YPD and the colonies were allowed to grow at 30°C. Colonies that were unable to grow or grew at much reduced rates on the second YPD replica plates were saved for further investigation.

Metabolic labeling and immunoprecipitation of α-factor in *CHC1* and *chc1-521*(Ts) yeast strains. The *cdl1*(L) strains GPY322-2A and GPY323-2B were constructed by first transforming the diploid strain GPY214 [*cdl1* (L)/*cdl1* (L) *CHC1*/*chc1-Δ10 ura3-1*/*ura3-1*] with pCHCc102 (*URA3 CHC1*) and with pBP6 [*URA3 chc1-521*(Ts)] to create strains GPY323 and GPY322, respectively, sporulating these diploids, and then isolating *MAT*α haploids which inherited both *chc1-Δ10* and the plasmid. [We refer to the lethal allele of *CDL1* as *cdl1*(L) and the viable allele of *CDL1* as *CDL1*(V).] The *CDL1*(V) strains GPY176 and GPY177 were isolated by transforming GPY55-10B with YCpchc1-521 to create the strain GPY170 and then replacing one copy of *CHC1* in GPY170 with *chc1-Δ10::LEU2* by transformation with pchc1-Δ10. In GPY176 the chromosomal copy of *CHC1* was disrupted (leaving *chc1-521* intact), and in GPY177 the plasmid-borne copy of *chc1-521* was disrupted (leaving *CHC1* intact).

Yeast strains GPY176, GPY177, GPY322-2A, and GPY323-2B (Table 1) were grown in SD with complete supplements except uracil (for GPY322-2A and GPY323-2B) or with complete supplements except tryptophan (for GPY176 and GPY177) and 0.2% yeast extract. The cells were grown with shaking to 10^7 cells per ml at 24°C, diluted to 5×10^6 cells per ml in fresh medium, and shifted to 37°C. The cultures were maintained at 37°C for 3 h, until the growth of GPY322-2A [*cdl1*(L) *chc1*] had attained its minimum rate. At this time, a sample of 2×10^7 cells was taken from each culture, and the cells were sedimented and washed twice in 1 ml of the same medium (prewarmed to 37°C) except without yeast extract

and were taken up in 1 ml of the same medium. They were then preincubated for 10 min at 37°C. Then 100 μCi of [^{35}S]methionine-cysteine was added, and the cells were labeled for 20 min at 37°C. Each culture was divided into two; 0.5 ml was placed on ice and azide (10 mM final concentration) was added to prevent further α-factor secretion, and the other half was incubated for 20 min at 37°C after addition of 5 μl of chase solution (0.3% cysteine, 0.3% methionine, 0.3 M ammonium sulfate). Following the chase period, samples were harvested as described above, and then all samples were separated into cell and medium fractions by centrifugation. A 350-μl sample of medium was taken, 10 μl of 10% SDS was added, and the sample was then immediately heated to 100°C for 3 min and stored on ice. The cells were washed in 1 ml of phosphate-buffered saline (PBS) containing 10 mM azide and then lysed by adding 50 μl of 2% SDS and 0.2 g of glass beads, vortexing the cells vigorously for 90 s, and then immediately heating the cells to 100°C for 3 min.

α-factor was immunoprecipitated from aliquots of cell lysate and medium equivalent to 10^6 cells in 1 ml of a PBS solution containing 1% Triton X-100, 10 mM sodium azide, and 0.1% SDS (PTS). Then 5 μl of antiserum raised against α-factor was added to each sample, and the immunoprecipitations were carried out as previously described (20).

The immunoprecipitated α-factor was subjected to electrophoresis on a 15% polyacrylamide-SDS gel. After electrophoresis, the gel was immediately treated with Amplify (Amersham) and dried under vacuum. The labeled α-factor was visualized by autoradiography.

RESULTS

The *chc1*-Δ*10* disruption causes inviability in spores derived from strain W303. During the course of our studies of yeast clathrin, we discovered a diploid strain, W303, whose haploid progeny cannot grow if they harbor a disruption of *CHC1*. This property of W303 mimicked that of strains homozygous for the lethal version of *SCD1* [*scd1*(L)] described by Lemmon and Jones (12). [We refer to the lethal allele of *SCD1* as *scd1*(L) and the viable allele of *SCD1* as *SCD1*(V).] For direct comparison, the *chc1*-Δ*10*::*LEU2* gene disruption was introduced by transformation into strain BJ3473, which is homozygous for *scd1*(L) (Table 1), and W303 (see Materials and Methods). Examination of chromosomal DNA from selected transformants by the method of Southern established that the strains were heterozygous for the *CHC1* gene disruption (data not shown). After induction of sporulation of the BJ3473-derived transformant GPY215 and the W303-derived transformant GPY214, the meiotic progeny were dissected into tetrads (14) and incubated at 30°C on solid YPD medium to allow growth. In both strains, only two spores from each tetrad gave rise to colonies (Fig. 2). The surviving spores were all Leu$^-$, indicating that cells which inherited the *chc1*-Δ*10* allele were inviable. In both strains, spores predicted to carry the *chc1*-Δ*10* disruption gave rise to microcolonies consisting of roughly 4 to 32 cells. This result argues that mitotic growth, not spore germination, is defective in the *chc1* spores. A difference between GPY214 and -215 was noted when the sporulation and germination were carried out at 24°C instead of 30°C. Whereas tetrads obtained from GPY215 [derived from the *scd1*(L)/*scd1*(L) strain] were indistinguishable at the two temperatures, tetrads derived from GPY214 at 24°C gave rise to predicted *chc1* colonies just visible to the unaided eye (data not shown). Thus, the ability of *chc1* cells with the W303 genetic background to grow appears to be sensitive to temperature.

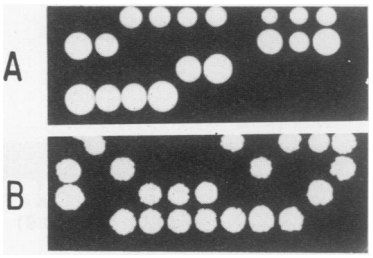

FIG. 2. Tetrads derived from strains in which *chc1* is lethal. (A) GPY214 [*cdl1*(L)/*cdl1*(L) *chc1*-Δ10/*CHC1*]; (B) GPY215 [*scd1*(L)/*scd1*(L) *chc1*-Δ*10*/*CHC1*]. Spores were germinated on YPD agar.

The lethality of the *CHC1* gene disruption in W303 is due to a single locus which is unlinked to *CHC1*. To determine whether, like *scd1*(L), a single gene in W303 is sufficient for *chc1* cell lethality, a diploid strain (GPY292) was generated by mating W303-1B (*CHC1*) with a viable *chc1* haploid (GPY1103). Suppose that W303-1B contains a single gene responsible for the clathrin heavy-chain-deficient lethality [which we will call *cdl1*(L)] so that the diploid GPY292 is heterozygous for both *chc1* and *cdl1*. If these two genes are unlinked, then independent assortment during meiosis would predict a defined ratio of three tetrad types (26; Table 2). In addition to two *CHC1* segregants, one class of tetrads would contain two slow-growing *chc1* segregants, a second class would contain one slow-growing and one inviable *chc1* segregant, and the third class would contain two inviable *chc1* segregants. If *CHC1* and the gene causing lethality were unlinked, the ratio of the three tetrad types would be 1:4:1 (Table 2) (26). In contrast, if two genes must be present for lethality in addition to *chc1*, then the expected ratio would be 19:16:1 (this ratio can be derived by assuming independent assortment of the three genes and considering the phenotype associated with each of the spores in the 36 possible tetrads). Meiotic products derived from GPY292 gave tetrad types in the ratio of 1:4:1. This analysis indicates

TABLE 2. Segregation of *chc1*-Δ*8* and *cdl1*(L) in tetrads derived from a diploid (GPY292)[a] heterozygous for each allele

Determination[b]	No. of given tetrad type		
	2 Chc$^+$: 2 Chc$^-$	2 Chc$^+$: 1 Chc$^-$: 1 dead[c]	2 Chc$^+$: 2 dead[c]
Expected no.			
Case 1 (n = 40)	7	26	7
Case 2 (n = 40)	21	18	1
Observed no.	7	27	6

[a] The full genotype of GPY292 can be found in Table 1.

[b] Case 1 assumes that one gene is required in addition to *chc1*-Δ*8* for lethality, and case 2 assumes that two unlinked genes in addition to *chc1*-Δ*8* are required. The 1:4:1 ratio of tetrad types expected from case 1 is explained in reference 26, and the 19:16:1 ratio of tetrad types expected from case 2 can be derived by assuming that *CHC1* and the other two genes are independently segregating and considering the phenotypes of all spores in each of the 36 possible types of tetrads.

[c] In this genetic background, most (35 of 39) of the presumptive *chc1 cdl1* spores were not as inviable as those derived from GPY214 shown in Fig. 2. After extended incubations, they grew to form microcolonies just visible to the eye. The microcolonies were impossible to score for nutritional markers. These spores were classified as dead.

that W303 harbors a single genetic locus, unlinked to *CHC1*, which affects the viability of *chc1* segregants.

The discovery of a single gene in the W303 background which is sufficient for *chc1* lethality provides a genetic means to address the idea that the viability of *chc1* strains is due to the presence of a suppressor gene. The lethal versions of *SCD1* and the gene that we have designated *CDL1* can each alone cause *chc1* cells to be inviable. Consequently, if *CDL1* is distinct from *SCD1*, and *scd1*(L) *chc1* strains carry *CDL1*(V) or *cdl1*(L) *chc1* strains carry *SCD1*(V), then strains would require the suppressor versions of both *SCD1* and *CDL1* in order to survive in the absence of clathrin heavy chain. Since *chc1* cell viability is common, the possibility of two independent suppressors in most laboratory strains is remote, and the result would argue against the suppressor hypothesis. We have investigated whether *CDL1* and *SCD1* are distinct by both genetic complementation and segregation experiments.

Very low levels of clathrin heavy chain can rescue viability of cells containing the lethal alleles of either *SCD1* or *CDL1*. Since the lethal versions of *SCD1* and *CDL1* can be recognized only in the absence of clathrin heavy chain, we introduced a copy of *CHC1* under control of the *GAL10* promoter into strains carrying the lethal alleles of either *SCD1* or *CDL1*. In these strains, *CHC1* expression can be induced by growth on galactose-containing medium and repressed by growth on glucose-containing medium. The strains harboring *GAL10*-controlled *CHC1* were used to determine whether *scd1*(L) and *cdl1*(L) are recessive to the viable alleles and whether they define separate complementation groups. During characterization of the strains, we unexpectedly found that very low levels of clathrin heavy chain appear to rescue cell growth in strains with *scd1*(L) or *cdl1*(L) alleles.

In the case of *scd1*(L), a haploid *CHC1* strain with *scd1*(L) (GPY137-3A) was conjugated to a viable haploid *chc1-Δ10* strain harboring the *GAL10* promoter-controlled *CHC1* gene (*CHC1-G*) integrated at the *URA3* locus (GPY150.2; see Materials and Methods). We sought, from the meiotic segregants of this diploid, strains with *chc1-Δ10*, *CHC1-G* and *scd1*(L) allele. Since *chc1-Δ10* was marked with the *LEU2* gene and *CHC1-G* was marked with *URA3*, we tested Leu^+ Ura^+ segregants for their ability to grow after *CHC1* expression was repressed by glucose-containing medium. Since 50% of these segregants were predicted to carry *scd1*(L), half of the segregants should not have grown on the glucose-containing medium, while the other half (carrying the viable *SCD1* allele) should have yielded slow-growing cells. Initially, the Leu^+ Ura^+ isolates conformed to these expectations; however, after extended incubations, the isolates that initially appeared inviable produced very small colonies. Figure 3 shows the relative growth on galactose- and glucose-containing media of a *CHC1* strain (GPY200), a *CHC1-G* strain with the *SCD1* viable allele (GPY205-1D), and a *CHC1-G* strain with the putative *scd1* lethal allele (GPY205-16A).

The identification of *scd1*(L) initially relied on the inviability of *chc1* segregants after meiosis (Fig. 2). Yet, although 50% of the *CHC1-G* segregants should have carried the lethal *scd1* allele, none were completely inviable on glucose-containing medium. It seemed possible that *CHC1-G* somehow rescued viability, even under repressing conditions. To confidently assign *scd1*(L) to the extremely poorly growing strains, it was necessary to show that a single genetic locus could be recovered that causes inviability of meiotic *chc1* segregants. Accordingly, a diploid strain was formed from

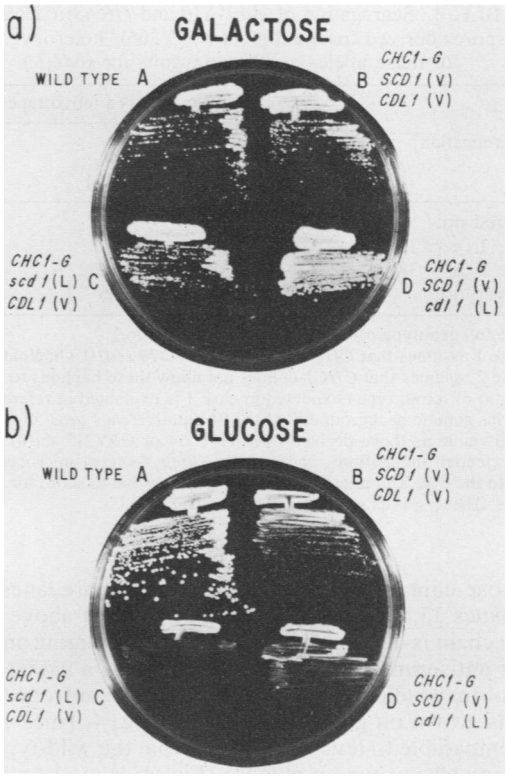

FIG. 3. Growth of strains carrying *CHC1* under the control of the *GAL10* promoter on YPG (galactose) or YPD (glucose) agar.

GPY205-16A and a *CHC1* strain carrying the *scd1*(L) allele. If GPY205-16A harbors *scd1*(L), then the diploid should be homozygous for this allele. Also, the diploid is heterozygous for *chc1-Δ10*::*LEU2* and heterozygous for *CHC1-G*. Tetrad analysis of such a strain dissected onto glucose-containing medium would be predicted to show that two members of each tetrad carry the *chc1-Δ10* allele and that each of these also carries *scd1*(L). On average, one of the *chc1-Δ10* segregants should also carry the *CHC1-G* allele. If *CHC1-G* rescues viability, then these segregants will form very small colonies like those in Fig. 3. Three tetrad types should be observed: 2 wild-type (WT):2 dead; 2 WT:1 very small:1 dead; and 2WT:2 very small. Assuming that *chc1-Δ10* and *CHC1-G* are unlinked, the ratio of the tetrad types is predicted to be 1:4:1 (26). Table 3 presents the results of the tetrad analysis, showing that the predicted outcome was observed. Furthermore, if *CHC1-G* rescues viability, then all very small colonies should consist of cells carrying both *chc1-Δ10* and *CHC1-G* and should therefore be Leu^+ Ura^+. This expectation was also confirmed (Table 4). This experiment indicates that GPY205-16A carries the *SCD1* lethal allele and also that *CHC1-G* can apparently rescue viability during both spore germination and vegetative growth even in the presence of glucose.

The ability of *CHC1-G* to rescue viability in the presence of the lethal version of *SCD1* suggests that repression of *CHC1-G* expression on glucose-containing medium may not be complete. We examined this possibility by conducting immunoblot analysis (Fig. 4) of extracts from *chc1-Δ10 CHC1-G* cells. When cells are grown in glucose-containing medium, the level of clathrin heavy chain is approximately 50-fold lower than that found in the same cells grown in

TABLE 3. Segregation of *chc1-Δ10* and *URA3::CHC1-G* in spores derived from a diploid (GPY206)[a] heterozygous for these alleles and homozygous for *scd1*(L)

Determination[b]	No. of given tetrad type		
	2 Chc$^+$: 2 Chc$^-$	2 Chc$^+$: 1 Chc$^-$: 1 dead[c]	2 Chc$^+$: 2 dead[c]
Expected no.			
Case 1 (n = 38)	6	26	6
Case 2 (n = 38)	0	0	38
Observed no.	5	26	7

[a] The full genotype of GPY206 is shown in Table 1.
[b] Case 1 assumes that *CHC1-G* allows *chc1-Δ10 scd1*(L) haploids to grow, and case 2 assumes that *CHC1-G* does not allow these haploids to grow. The 1:4:1 ratio of tetrad types expected in case 1 is explained in reference 26.
[c] In this genetic background, 7 of the 34 putative *chc1 ura3-52* spores were not as inviable as those derived from GPY214 or GPY215 shown in Fig. 2. After extended incubations, these spores grew to form microcolonies just visible to the eye. The microcolonies were impossible to score for nutritional markers. These spores were classified as dead.

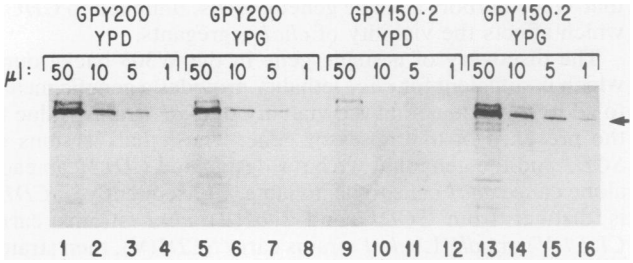

FIG. 4. Immunoblot analysis of clathrin heavy chain expressed from the *GAL10* promoter. Strains GPY200 (*CHC1*) (lanes 1 to 8) and GPY150.2 (*CHC1-G chc1-Δ10* (lanes 9 to 16) were grown on glucose-containing (lanes 1 to 4 and 9 to 12) or galactose-containing (lanes 5 to 8 and 13 to 16) medium for approximately nine doubling times. Protein extracts were prepared, and various amounts, indicated above each lane, were subjected to SDS-PAGE. After transfer to nitrocellulose, clathrin heavy chain was detected with a polyclonal antiserum. The arrow indicates clathrin heavy chain. The trace of clathrin heavy chain visible in lane 12 is due to spillover from the adjacent lane 13.

galactose-containing medium (Fig. 4; compare lanes 9 to 12 with lanes 13 to 16). The band that appears above clathrin heavy chain is due to a low level of contaminating antibodies in our antiserum. This reactivity serves as a useful internal control for loading. The levels of expression from *CHC1-G* in cells grown on galactose medium (Fig. 4, lanes 13 to 16) are comparable to levels produced from the wild-type *CHC1* locus in cells grown on glucose (Fig. 4, lanes 1 to 4). From this analysis, it appears that *CHC1-G* is not completely repressed on glucose-containing medium. This low-level expression, only about 2% of wild-type clathrin levels, apparently can rescue growth of cells carrying the *SCD1* lethal allele. We have noted a similar phenomenon in *chc1-Δ10 CHC1-G* cells carrying the lethal allele of *CDL1* (Fig. 3).

Complementation tests with lethal alleles of *SCD1* and *CDL1*. The extremely poor growth of the *chc1-Δ10 CHC1-G* strains containing the lethal *scd1* or *cdl1* allele can easily be distinguished from the growth of similar strains carrying the viable version of each gene (Fig. 3). These strains were therefore used to investigate the recessive or dominant nature of each lethal allele and also to test whether the two lethal alleles were capable of complementation. To determine whether the lethal alleles are recessive to the viable alleles, diploid strains homozygous for *chc1-Δ10* and *CHC1-G* but heterozygous for the lethal allele of either *SCD1* or *CDL1* were placed on glucose-containing medium. For comparison, we also tested growth of a strain homozygous for the viable alleles of both *SCD1* and *CDL1* and growth of strains homozygous for the lethal alleles of either *SCD1* or *CDL1*. Figure 5 shows that cells heterozygous for the lethal alleles of *SCD1* or *CDL1* (sectors D and B) grow at rates commensurate with rates of cells homozygous for the viable alleles (sector A). In contrast, the strains homozygous for either lethal allele are clearly much retarded in growth (sectors E and C). By convention, the lethal allele of each gene will be designated with lowercase letters. The diploid strain prepared by mating one haploid carrying *scd1* with a second haploid carrying *cdl1* was tested for growth on glucose-containing medium to determine whether the two mutations could complement each other. The growth rate of this strain on glucose approaches that of the strain homozygous for both viable alleles, although complementation is not quite complete (compare sector F with sector A). This result suggests that *cdl1* and *scd1* define two different complementation groups, suggesting that they are different genes.

Segregation analysis of *scd1* and *cdl1*. To confirm that *SCD1* and *CDL1* are distinct, we tested the ability of the

TABLE 4. Numbers of spores of each genotype obtained from GPY206[a]

Determination[b]	No. of spores of given genotype				
	CHC1 URA3::CHC1-G	*CHC1 ura3-52*	*chc1-Δ10 URA3::CHC1-G*	*chc1-Δ10 ura3-52*[c]	Total
Expected no.					
Case 1	38	38	38	0	114
Case 2	38	38	0	0	76
Observed no.	34	42	35	1[d]	112

[a–c] See Table 3, footnotes *a* to *c*.
[d] This isolate grew very slowly, but its genotype could be determined. It may be a partial phenotypic revertant of *scd1*(L).

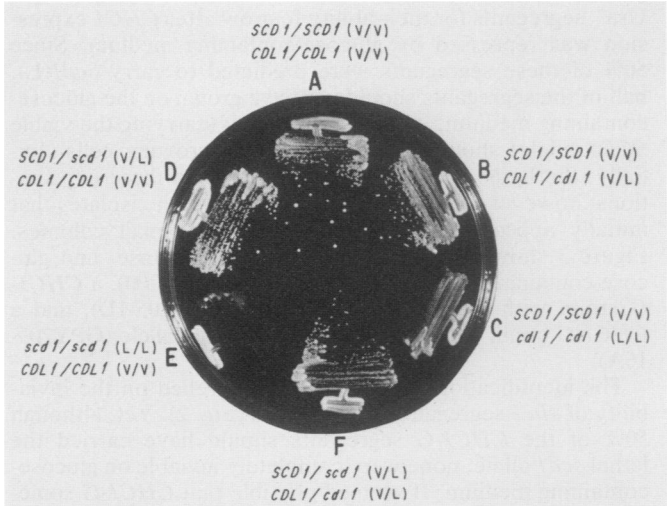

FIG. 5. Complementation test of *scd1*(L) and *cdl1*(L). Strains with the designated genotypes were streaked onto YPD (glucose) agar. (A) GPY213; (B) GPY226 [*cdl1*(L)/*CDL1*]; (C) GPY230 [*cdl1*(L)/*cdl1*(L)]; (D) GPY207 [*scd1*(L)/*SCD1*]; (E) GPY212 [*scd1*(L)/*scd1*(L)]; (F) GPY227 [*scd1*(L)/*cdl1*(L)].

TABLE 5. Segregation of *chc1*-Δ*10*, *scd1*(L), and *cdl1*(L) in tetrads derived from a diploid strain (GPY249)[a] heterozygous for each allele[b]

Determination[c]	No. of given tetrad type		
	2 Chc$^+$: 2 Chc$^-$	2 Chc$^+$: 1 Chc$^-$: 1 dead[d]	2 Chc$^+$: 2 dead[d]
Expected no.			
Case 1 (n = 36)	1	16	19
Case 2 (n = 36)	0	0	36
Observed no.	0	13	23

[a] The full genotype of GPY249 is shown in Table 1.

[b] GPY249 carries a temperature-sensitive *CHC1* allele (*chc1-521*) in place of *CHC1*, but the diploid sporulation and spore germination were carried out at permissive temperatures for this mutation.

[c] Case 1 assumes that *SCD1* and *CDL1* are distinct, unlinked genes, and case 2 assumes that they are alleles of the same gene. The 1:16:19 ratio of tetrad types expected from case 1 can be derived by assuming that *SCD1*, *CDL1*, and *CHC1* are independently assorting and considering the phenotypes of all the spores in each of the 36 possible types of tetrads.

[d] In this genetic background, 9 of the 59 presumptive *chc1 scd1* or *chc1 cdl1* spores were not as inviable as those derived from GPY214 or GPY215 shown in Fig. 2. After extended incubations, these spores grew to form microcolonies just visible to the eye. The microcolonies were impossible to score for nutritional markers. This was similar to the result with the GPY292 dissection, where most of the presumptive *chc1 cdl1* spores grew to form microcolonies but were impossible to score. These spores were classified as dead.

lethal alleles to segregate during meiosis. A diploid strain was generated by mating two *CHC1* strains, one harboring *cdl1* and the other harboring *scd1*. In fact, the *scd1* strain carries a temperature-sensitive allele of *CHC1*, but since all experiments were carried out at the permissive temperature, the strain was effectively Chc$^+$. One *CHC1* allele was disrupted by gene transplacement, and the resulting heterozygous genotype of one transformant (GPY249) was confirmed by physical characterization of the chromosomal DNA by the procedure of Southern (13) (data not shown). The results of tetrad analysis of GPY249 are presented in Table 5. Of 44 tetrads, 36 gave two *CHC1* segregants. We have consistently noted a degree of nonspecific spore inviability in BJ3473-derived strains, and this accounts for the loss of a few *CHC1*, and presumably *chc1*, segregants. If *SCD1* and *CDL1* are independently assorting genes, then the tetrads should fall into three classes. All tetrads should have two *CHC1* members. The first class will have two viable *chc1* segregants (containing neither *scd1* nor *cdl1*), the second will have one viable and one inviable *chc1* segregant (one with either *scd1*, *cdl1*, or both and one with neither lethal allele), and the third will have two inviable segregants (both with *scd1*, *cdl1*, or both). Since *scd1* and *cdl1* can each lead to inviability, independent assortment of the two genes should yield tetrad types in a ratio of 1:16:19 (this ratio can be derived by assuming that *CHC1*, *SCD1*, and *CDL1* are independently assorting and considering the phenotype of each spore in all of the 36 possible tetrad types). In total, 25% of the Chc$^-$ spores should be viable and 75% should be inviable. To compare, if *SCD1* and *CDL1* are the same gene, then all tetrads should be class III; that is, no viable Chc$^-$ spores should be obtained. The data obtained from 36 tetrads containing two viable *CHC1* segregants clearly indicates that *scd1* and *cdl1* are alleles of different genes (Table 5).

Mutations at numerous loci can influence viability of *chc1* cells. The finding that *SCD1* and *CDL1* are two distinct genes prompted us to investigate the number of genes which, when mutant, can similarly affect the growth of *chc1* cells. For this purpose, we started with *CHC1-G chc1-Δ10 SCD1 CDL1* cells and searched for mutants on glucose-containing medium which displayed the extremely poor growth associated with *scd1* or *cdl1* cells when *CHC1* expression is repressed. Two strains of opposite mating type, GPY150.2 and GPY257, were chosen to facilitate complementation analysis. Each strain was mutagenized with EMS. Individual colonies were grown on galactose-containing medium and then tested for growth on glucose-containing medium by replica plating (see Materials and Methods). In total, 71 mutants having the desired phenotype were identified from among the roughly 12,000 colonies screened. The mutants could be divided into four classes on the basis of the severity of the growth defect on glucose-containing medium (Table 6). Class I mutants do not form colonies on glucose-containing medium, even after prolonged incubations; class II mutants grow on glucose-containing medium much more poorly than do *scd1* or *cdl1* strains; class III mutants grow similarly to *scd1* and *cdl1* strains; class IV mutants grow slightly better than *scd1* and *cdl1* strains but still markedly worse than the starting *CDL1 SCD1* strains. Most of the mutants grow as well as the parent strains on galactose-containing medium, although in some cases growth on galactose is also affected.

To determine the number of complementation groups represented in the collection, a set of 23 GPY150.2-derived mutants was crossed to a set of 33 GPY257-derived strains. In addition, the GPY257-derived strains were tested for their ability to complement *scd1* and *cdl1*. Of the 56 tested strains, 49 exhibited recessive growth phenotypes. Of these strains, only two pairs failed to complement. One pair defined a new complementation group, and the second consisted of a GPY257-derived mutant and the *cdl1* strain. The finding that almost all of the strains with recessive growth phenotypes carry mutations that complement each other suggests that most of the mutations have affected distinct genes.

The nature of the genetic screen does not immediately allow the distinction between mutations that cause growth defects because of the lack of *CHC1* expression and muta-

TABLE 6. Growth phenotypes of clathrin-requiring mutants when *CHC1* expression is repressed

Class	Phenotype on glucose	No. of mutants	No. of mutants unaffected on galactose	No. of mutants dominant (of 56 tested)
I	No growth	7	3	1
II	Very extreme growth defect [much worse than *scd1*(L) or *cdl1*(L)]	19	13	3
III	Strong growth defect [like *scd1*(L) or *cdl1*(L), or slightly worse]	36	30	3
IV	Moderate growth defect [slightly better than *scd1*(L) or *cdl1*(L)]	9	6	0
Total		71	52	7

tions that prevent glucose utilization regardless of the expression state of *CHC1*. To determine the frequency of each type of mutation in our collection, we introduced a plasmid (YCpCHCTRP) which allows constitutive expression of *CHC1* into 12 of the GPY150.2-derived mutants on galactose-containing medium. This plasmid should not affect the ability of mutants to grow on glucose-containing medium if the mutation affects glucose utilization. If the growth defect is due to the lack of *CHC1* expression, then the YCpCHCTRP plasmid should rescue growth on glucose-containing medium. In 11 of the mutants, growth was clearly restored by YCpCHCTRP. In one, growth was not restored. In each case, restoration of growth could be attributed to YCpCHCTRP, since loss of the plasmid resulted in the original growth phenotype. In summary, the phenotypes on glucose-containing medium of 11 of 12 mutants can be attributed to the absence of clathrin heavy chain, not the inability to utilize glucose.

The *cdl1* allele does not cause a general defect in secretory pathway function in *chc1* cells. Studies of yeast *sec* mutations have established that cell growth depends on a functional secretory pathway (24). Given this precedent, a block in secretion could be responsible for the lethality caused by combination of *chc1* with the mutant loci described above. To monitor the secretory pathway, secretion of the peptide mating pheromone α-factor was examined. During transport through the secretory pathway, α-factor undergoes a series of modifications which occur at specific stages of the pathway (10). Thus, if a secretory block occurs, the accumulated form of α-factor provides a signature for the affected compartment. α-Factor is synthesized as part of a larger precursor which is translocated into the lumen of the endoplasmic reticulum and core glycosylated to generate a 26.5-kDa endoplasmic reticulum-specific form. Transport to the Golgi complex results in further glycosylation, creating a molecular weight shift to about 125 kDa. Finally, in the late Golgi or in transport vesicles on the way to the cell surface, the high-molecular-weight form of α-factor is processed by a series of proteases to the mature 3.5-kDa form, which is secreted. In clathrin-deficient cells, this processing does not occur efficiently and highly glycosylated α-factor is secreted into the medium (20).

To assess secretion in *cdl1* strains, we made use of a temperature-sensitive allele of *CHC1* (*chc1-521*). Strains carrying both *chc1-521* and either *scd1* or *cdl1* grow normally at 24°C but are barely able to grow at 37°C (their growth rate is much slower at 37°C than that of *chc1-521* strains with *SCD1* or *CDL1*). The relative growth rates of *chc1-521 CDL1* (GPY176), *CHC1 CDL1* (GPY177), *chc1-521 cdl1* (GPY322-2A), and *CHC1 cdl1* (GPY323-2B) before and after a shift from 24 to 37°C are shown in Fig. 6A. After the shift to 37°C, the growth defect of the *chc1-521 cdl1* strain is apparent in 1 h and becomes maximal by 3 h. Secretion of α-factor was therefore monitored following incubation of the four strains at 37°C for 3 h.

The four strains described above were metabolically labeled with [^{35}S]methionine-cysteine after the 3-h incubation, and α-factor was immunoprecipitated from cell or culture supernatant fractions immediately after labeling or after a 20-min chase period. The *CHC1* strains secrete all of the labeled α-factor during the labeling period, and it appears in the medium as mature α-factor (Fig. 6B, lanes 5, 6, 13, and 14). In contrast, the *chc1-521* strains retain some highly glycosylated α-factor precursor after the labeling period (lanes 1, 2, 9, and 10), and this is only fully exported from the cells after the 20-min chase period (lanes 3, 4, 11, and 12).

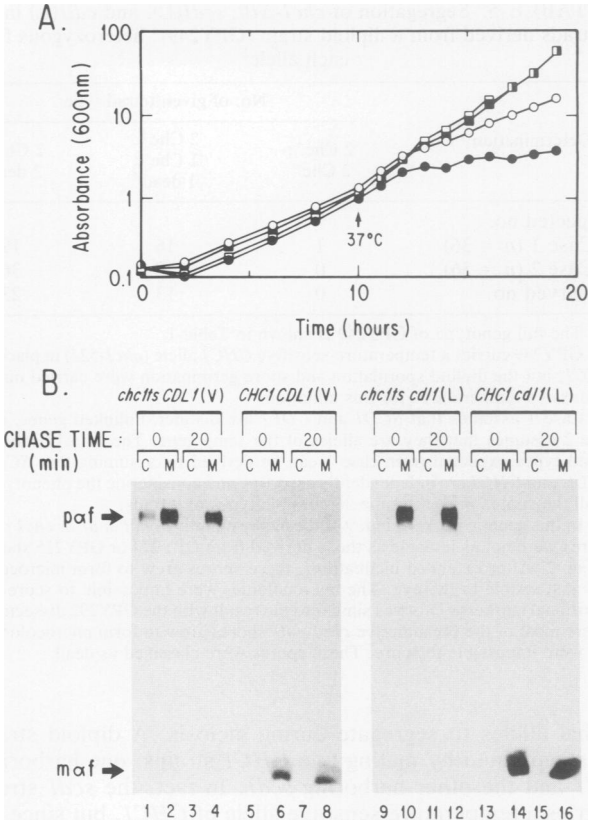

FIG. 6. (A) Growth of GPY177 [*CHC1 CDL1*(V)] (closed squares), GPY323-2B [*CHC1 cdl1*(L)] (open squares), GPY176 [*chc1-521*(Ts) *CDL1*(V)] (open circles), and GPY322-2A [*chc1-521*(Ts) *cdl1*(L)] (closed circles) strains before and after a shift in temperature from 24 to 37°C. Cells were inoculated to a starting density of 0.1 A_{600} in SD containing complete supplements except uracil (GPY322-2A and GPY323-2B) or complete supplements except tryptophan (GPY176 and GPY177) and 0.2% yeast extract and then grown with agitation at 24°C. When the A_{600} reached 1.0, the cultures were immediately placed at 37°C (indicated by the arrow), and incubation was continued. Samples of the cultures were taken every 2 h at 24°C and every hour at 37°C, and the A_{600} was determined. When the cultures reached an A_{600} of 2 to 4, they were diluted to an A_{600} of 0.1 to 0.2 and incubation was continued, but the readings taken after this were adjusted with the appropriate correction factor. (B) Secretion of α-factor in *CHC1* and *chc1-521*(Ts) strains carrying either *CDL1*(V) (lanes 1 to 8) or *cdl1*(L) (lanes 9 to 16) after a shift to the restrictive temperature. Cells were grown at 24°C and shifted to 37°C for 3 h. The cells were labeled with [^{35}S]methionine-cysteine at 37°C for 20 min and then either harvested immediately (lanes 1, 2, 5, 6, 9, 10, 13, and 14) or incubated for 20 min at 37°C with cold methionine and cysteine (lanes 3, 4, 7, 8, 11, 12, 15, and 16). Cells (C) were separated from medium (M) by centrifugation, cell extracts were prepared, and α-factor was immunoprecipitated from each fraction. Immunoprecipitates were resolved by SDS-PAGE and visualized by fluorography. The *chc1-521*(Ts) strains secrete highly glycosylated pro-α-factor (pαf) into the medium after growth at the restrictive temperature; this phenotype was previously observed in *chc1-Δ10* mutants (20). The *CHC1* strains secrete mature α-factor (mαf) into the medium.

This result indicates that the *chc1-521* cells have a slight delay in secretion at a post-Golgi complex stage, as has been reported previously for strains carrying null alleles of *CHC1* (19). Closer examination of the data reveals that the presence of *CDL1* or *cdl1* does not appear to influence the

secretion of α-factor. For both the *CHC1* strains and the *chc1-521* strains, the kinetics of delivery of α-factor to the cell surface is the same in the presence of *cdl1* or *CDL1*. In other experiments, we have shown that delivery of the soluble vacuolar protease carboxypeptidase Y to the vacuole is normal in both *cdl1* and *CDL1* strains carrying *chc1-521* after 3 h at the restrictive temperature (data not shown). This finding indicates that the growth defect in clathrin heavy-chain-deficient cells carrying *cdl1* is not due to a blockage of the secretory pathway.

DISCUSSION

Discovery of the lethal *scd1* allele led to the hypothesis that the absence of clathrin heavy chain in yeast cells is lethal unless a suppressor gene is present (12). Mechanisms of suppression directly or indirectly related to clathrin function were postulated. We have taken advantage of a second strain which cannot grow in the absence of *CHC1* to assess the likelihood that *chc1* cell viability is due to genetic suppression. Our studies indicate that a single genetic locus, *CDL1*, is responsible for lethality in this second strain. Both genetic complementation and segregation tests show that *CDL1* is distinct from *SCD1*. To account for this finding, the suppressor hypothesis would require that viable strains carry two independent suppressor genes, an unlikely possibility. Pursuing this line of reasoning, our observation that many different genes can give *scd1*-like phenotypes makes the suppressor hypothesis untenable. A more likely interpretation is that, given the recessive character of *scd1* and *cdl1*, these lethal alleles represent mutations which reduce or abolish the activity encoded by genes found in wild-type yeast cells. In this scenario, a disruption of *CHC1* in wild-type cells results in slow-growing cells, but in cells with reduced or absent *SCD1* (or *CDL1*) activity, a *CHC1* disruption is lethal. This interpretation is supported by the demonstration that a mutation that fails to complement *cdl1* can be isolated from a population of chemically mutagenized viable *chc1* cells. Our results therefore argue that *scd1* and *cdl1* are mutant genes which are cryptic in otherwise wild-type cells but result in synthetic lethality when combined with *chc1*.

The significance of genes that display synthetic lethality with *chc1* is central to the evaluation of clathrin function. In some cases, synthetic lethality has been a meaningful prognosticator of functional interactions (see reference 8 for a discussion). For example, in *S. cerevisiae*, a cold-sensitive allele of one of the two α-tubulin genes displays lethality at all temperatures when combined with certain alleles of the second α-tubulin gene or the β-tubulin gene. In each of these cases, synthetic lethality occurred at a temperature that was permissive for at least one of the mutant alleles. This is not the case with *chc1*, which lacks clathrin heavy chain at all temperatures. Furthermore, it is unlikely that disruption of another member of clathrin coats would enhance the phenotype of cells lacking the principal coat component. Alternatively, synthetic effects have been proposed to signify parallel pathways rather than direct interaction (7). Applying this idea to *scd1* and *cdl1* raises the provocative possibility that the wild-type products of these genes normally perform functions similar to that of clathrin in parallel pathways and can functionally substitute for clathrin heavy chain in the *chc1* cells. To address this possibility, we conducted a systematic search for *scd1*-like genes, reasoning that only a limited number of genes could encode functional clathrin heavy-chain homologs. However, numerous mutations were obtained which caused growth defects equal to or greater than those caused by *scd1* and *cdl1* when clathrin heavy-chain expression was repressed. Of these mutations, one failed to complement *cdl1* and two others failed to complement each other. The remainder appear to define new genes, since each pair of mutants tested displayed complementation. We have not directly demonstrated that these mutations are lethal when present in a background completely devoid of clathrin heavy chain. However, since these mutations act similarly to *scd1* and *cdl1* in cells with *CHC1-G* grown on glucose-containing medium, and since both *scd1* and *cdl1* are lethal in *chc1* backgrounds, it seems likely that most of the mutations will also act as synthetic lethals in *chc1* cells. Given the large number of different genes that can yield *scd1*-like mutations, it is difficult to put forth a compelling argument that such mutations identify clathrin heavy-chain substitutes.

The large number of *scd1*- and *cdl1*-like mutations might be significant if, when clathrin is lacking, a second pathway could provide an alternative secretory route for proteins which normally would travel via clathrin-coated vesicles. Such a pathway may involve many proteins and thus give many targets for synthetically lethal mutations. If an alternative pathway is blocked by such mutations, then in combination with *chc1*, a blockage or delay in the transport of proteins to the cell surface might be anticipated. We have addressed this possibility by examining secretion of α-factor in strains carrying a temperature-sensitive *chc1* allele and *cdl1*. By 3 h following a shift to the nonpermissive temperature, the *chc1*(Ts) *cdl1* cells exhibit their maximum growth defect yet transport α-factor to the cell surface and deliver carboxypeptidase Y to the vacuole with the same kinetics as do the *chc1*(Ts) *CDL1* cells. Thus, in combination with *chc1*, *cdl1* does not cause a novel secretory defect. This evidence suggests that mutations which display synthetic lethality in combination with *chc1* do not affect a secretory pathway parallel to one involving clathrin-coated vesicles. Instead, the most plausible interpretation of our results is that minor perturbations in pathways unrelated to clathrin function can impose sufficient stress to kill a cell already debilitated by *chc1*. Our studies provide a strong argument that care must be exercised in interpreting synthetic lethal mutations when one mutation already causes a substantial growth defect.

Unexpectedly, cells harboring *scd1* or *cdl1* form slow-growing colonies when clathrin heavy-chain expression from the *GAL10* promoter is repressed on glucose-containing medium. The growth rate of the cells on glucose-containing medium is much slower even than that of *chc1 CDL1 SCD1* cells, but the viability clearly contrasts with the inability of cells carrying a disruption of *CHC1* and *scd1* or *cdl1* to form colonies. Genetic experiments attribute the viability to the *GAL10* promoter-driven *CHC1*, and we have found that under repressing conditions this construct is not completely off but expresses clathrin heavy chain at 2% of wild-type levels. These results suggest that very low levels of clathrin heavy chain can suffice to rescue the viability of *chc1 scd1* (or *cdl1*) cells. This seems surprising, since clathrin is thought to act in a structural rather than enzymatic fashion. Perhaps the cooperativity of clathrin assembly (5) allows the limited amount of heavy chain present in cells grown on glucose-containing medium to assemble into a sufficient number of functional coat structures to allow growth. On the other hand, the cause of the growth defect in viable or inviable *chc1* cells is not known, and it is conceivable that the small number of heavy-chain molecules may provide a previously unknown function independent of assembly

which rescues viability of the *scd1* cells. Regardless, the results indicate that growth of cells carrying *scd1* or *cdl1* offers a sensitive assay for clathrin heavy-chain function in vivo. Since a deletion of the clathrin light-chain gene (*CLC1*) in *scd1* cells yields slow-growing rather than inviable cells (27), our results would argue that clathrin heavy chain retains some function in the absence of clathrin light chain. The sensitivity of the *scd1* and *cdl1* cells to clathrin functions suggests that they will be good hosts to evaluate the effect of directed mutations in *CHC1*.

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