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Genetic and Biochemical Characterization of Clathrin-Deficient *Saccharomyces cerevisiae*

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Clathrin is important but not essential for yeast cell growth and protein secretion. Diploid *Saccharomyces cerevisiae* cells heterozygous for a clathrin heavy-chain gene (*CHC1*) disruption give rise to viable, slow-growing, clathrin heavy-chain-deficient meiotic progeny (G. Payne and R. Schekman, Science 230:1009–1014, 1985). The possibility that extragenic suppressors account for growth of clathrin-deficient cells was examined by deletion of *CHC1* from haploid cell genomes by single-step gene transplacement and independently by introduction of a centromere plasmid carrying the complete *CHC1* gene into diploid cells before eviction of a chromosomal *CHC1* locus and subsequent tetrad analysis. Both approaches yielded clathrin-deficient haploid strains. In mutants missing at least 95% of the *CHC1* coding domain, transcripts related to *CHC1* were not detected. The time course of invertase modification and secretion was measured to assess secretory pathway functions in the viable clathrin-deficient cells. Core-glycosylated invertase was converted to the mature, highly glycosylated form at equivalent rates in mutant and wild-type cells. Export of mature invertase from mutant cells was delayed but not prevented. Abnormal vacuoles, accumulated vesicles, and Golgi body-derived structures were visualized in mutant cells by electron microscopy. We conclude that extragenic suppressors do not account for the viability of clathrin-deficient cells and, furthermore, that many standard laboratory strains can sustain a *CHC1* disruption. Clathrin does not appear to mediate protein transfer from the endoplasmic reticulum to the Golgi body but may function at a later stage of the secretory pathway.

Vesicular membrane traffic is thought to mediate transport of proteins between secretory organelles in eucaryotic cells. Clathrin-coated membranes and vesicles have been implicated as key intermediates in the process of transport vesicle formation. A cogent demonstration of this association is provided by studies of receptor-mediated endocytosis in mammalian cells (reviewed in references 14 and 15). Initially, receptor-bound ligands are concentrated in indented domains (pits) of the plasma membrane which are decorated on the cytoplasmic surface with a polygonal protein lattice. The principal constitutent of the lattice is clathrin. Clathrin-coated pits invaginate and bud, forming clathrin-coated vesicles which carry receptors and associated ligands. The newly formed vesicles quickly shed their coats before fusion with the subsequent membrane compartment along the endocytic pathway.

In vitro, clathrin undergoes cycles of disassembly and assembly which may reflect the in vivo events described above. The polyhedral lattice on purified clathrin-coated vesicles depolymerizes to yield three-legged clathrin "triskelions" composed of clathrin heavy chain (180 kilodaltons [kDa]) and clathrin light chains (usually two species varying in size from 30 to 40 kD) (17). Triskelions can assemble to form empty lattice cages or rebind to vesicles previously stripped of their clathrin coats (17, 40). Additional proteins have been identified that facilitate disassembly or assembly (31, 43).

Taken together, the in vitro and in vivo properties of clathrin form the basis for a model in which polymerization of clathrin triskelions into polyhedral cages drives formation of coated vesicles from coated membrane regions (17, 29). Membrane-associated clathrin has also been proposed to tether, either directly or indirectly, receptors and bound ligands to membrane domains engaged in vesicle formation (15, 27). A number of investigations suggest that these hypotheses can be extended to encompass transport of newly synthesized proteins through the secretory pathway. Clathrin-coated membranes and vesicles have been associated with protein traffic between the endoplasmic reticulum (ER) and the Golgi body (1, 30), the Golgi body and lysosomes (13), and the Golgi body and the cell surface (26). However, on the basis of immunocytochemical examinations, Wehland et al. (41) have argued that clathrin-coated membranes are not involved in membrane glycoprotein export.

We initiated a genetic approach, using *Saccharomyces cerevisiae*, to assess directly the role of clathrin in intracellular protein transport (28). Our strategy was predicated on the observation that protein secretion is essential for yeast cell growth (38). Thus, if clathrin is required at any stage of the secretory pathway, a mutation which eliminates clathrin function will prevent protein export and thereby prove lethal to the cell. A molecular clone of the yeast clathrin heavy-chain gene (*CHC1*) was isolated and used to disrupt, in vivo, the single chromosomal copy of *CHC1*. Surprisingly, viable cells devoid of clathrin heavy chain and clathrin-coated vesicles were obtained. These results argue that clathrin-coated vesicle formation is not essential for protein transport through the secretory pathway.

Several possibilities could explain the viability of clathrin-deficient yeasts and belie our previous conclusions. (i) Extragenic suppressors of clathrin-deficient lethality unlinked to *CHC1* could accumulate in populations of clathrin-deficient cells or the heterozygous diploid progenitors of the mutants; (ii) residual clathrin heavy-chain gene sequences

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Source or reference
GPY1100	*MAT***a** *leu2-3 leu2-112 ura3-52 his4-519 trp1 can1*	Strain TD4 from G. R. Fink
GPY1101	*chc1-Δ8::LEU2* transformant of GPY1100	27
GPY1103	*chc1-Δ8::LEU2* transformant of GPY1100	This study
GPY1110	*MATα leu2-3 leu2-112 ura3-52 ade6*	Strain SF838-5Aα; this laboratory
GPY1114	*chc1-Δ8::LEU2* transformant of GPY1110	This study
GPY55-15B	*MATα leu2-3 leu2-112 ura3-52 his4-519 trp1 prb1*	This study
GPY68	*chc1-Δ10::LEU2* transformant of GPY55-15B	This study
GPY60	*MATα leu2-3 leu2-112 ura3-52 his4-519 trp1 prb1 pep4::URA3*	This study
PBY425A	*MAT***a** *ura3-52 suc2-Δ9*	P. C. Bohni
TBY100-3	*MAT***a** *ura3-52 suc2-Δ9 CHC1::*YIp5-TH (*URA3*)	This study
TBY50-41B	*MATα leu1 trp5 cyh2 met13 aro2 lys5 ade5 ura3-52*	This study
GPYD1004	*MAT***a***/MATα leu2-3 leu2-112/leu2-3 leu2-112 ura3-52/ura3-52 his4/his4 trp1/TRP1 can1/CAN1 mnn4-1/MNN4 pep4-3/PEP4*	28
RC634	*MAT***a** *sst1 rme1 ade2 his6 met1 ura1*	10

present in the gene disruptions may encode heavy-chain fragments capable of partial function; (iii) a gene partly divergent from *CHC1* could produce a clathrin heavy-chain functional analog.

Here we report experiments that address each of these issues and lend further support to our original interpretations. On the basis of these conclusions we have begun to monitor protein export and cell structure to identify transport stages perturbed by the absence of clathrin heavy chain.

MATERIALS AND METHODS

Strains and media. Yeast strains used in this study are listed in Table 1. Cells without plasmids were grown on YPD medium (1% yeast extract [Difco Laboratories, Detroit, Mich.], 2% Bacto-Peptone [Difco], 2% glucose). Solid medium also contained 2% agar. Diploids were sporulated on 2% agar plates containing 0.3% potassium acetate and 0.022% raffinose. Minimal medium plates used for assessing nutritional requirements were prepared as described by Sherman et al. (39). Cells with plasmids were grown in Wickerham's minimal liquid medium (42) with 2% glucose. For $^{35}SO_4^{2-}$ labeling, sulfate salts were replaced by chloride salts and ammonium sulfate was added to the desired concentration. The absorbance of dilute cell suspensions was measured in a 1-cm cuvette at 600 nm in a Zeiss PMQII spectrophotometer.

Plasmids and bacteriophage. λ7 contains most, but not all of *CHC1* (clathrin heavy chain) in λgt11 as described before (28). pCHC73 and pCHC74 carry the 1-kilobase-pair (kbp) *Eco*RI fragments from the 5′ and 3′ ends of *CHC1*, respectively (Fig. 1). pCHCc100 was isolated as described below and consists of the yeast centromere vector YCp50 (20) plus *CHC1* DNA sequences extending from the left-most *Bgl*II site shown in Fig. 1 to approximately 1 kbp beyond the *Sal*I site. pCHCc102 and pCHCe200 have the fragment shown in Fig. 1 inserted in YCp50 and the 2μ-based multicopy vector YEp24 (4), respectively. These plasmids were constructed by ligating the *CHC1 Bam*HI-to-*Cla*I fragment to the *CHC1 Cla*I-to-*Sal*I fragment and then inserting the reconstructed gene into YEp24 or YCp50 cleaved with *Bam*HI and *Sal*I. The probe fragments labeled A, B, C, and D were introduced into the polylinker in pGEM1 (fragments A and C) or pGEM2 (fragments B and D) (Promega Biotec, Madison, Wis.). An 867-base-pair (bp) *Bgl*II-to-*Eco*RI fragment, marked with a dot in Fig. 1, was inserted into pUC118 and pUC119 (gift from J. Viera, University of Minnesota) to

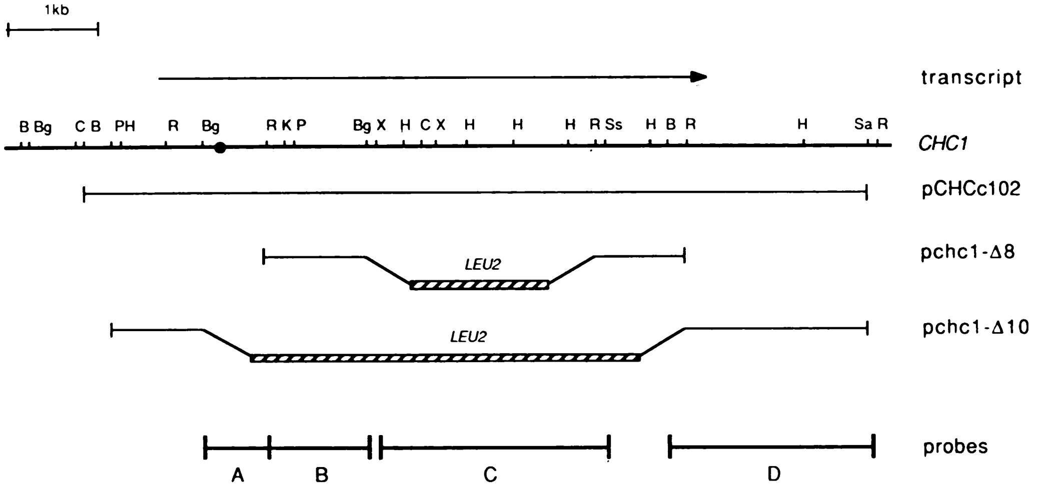

FIG. 1. *CHC1* gene structure, gene disruptions, and regions used for probe preparation. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; R, *Eco*RI; Sa, *Sal*I; Ss, *Sst*I; X, *Xba*I. The gap in each disruption is placed beneath segments of *CHC1* DNA that were removed. In both cases, DNA encoding *LEU2* (lined bars, not drawn to scale) was inserted in place of the deleted *CHC1* sequences. Antisense RNA probes used in the experiment described in Fig. 5 were prepared from regions indicated by probes A to D. The dot indicates the putative translation initiation site for *CHC1*. kb, Kilobase.

generate pUC118-12 and pUC119-12. pchcl-Δ8 was described previously (28). pchcl-Δ10 carries the yeast *LEU2* gene replacing the *CHC1* sequences between the *Bgl*II and *Eco*RI sites as shown in Fig. 1. Plasmid YIp5-TH was constructed by placing the *CHC1* 3′ *Eco*RI-to-*Sal*I fragment in the integrating vector YIp5 (3). pRB58 (9) carries the *SUC2* gene encoding invertase on YEp24. Plasmid pTS15 used to disrupt *PEP4* in strain GPY60 is reported in reference 32. All DNA manipulations were done as described by Maniatis et al. (22).

Nucleic acid manipulations. Plasmid isolation, nick translation, Southern transfer, and DNA-to-DNA hybridizations were performed by the methods of Maniatis et al. (22). Yeast DNA was prepared as described before (28). Polyadenylated RNA purification was reported previously (2). RNA was electrophoresed through formaldehyde agarose gels and transferred to nitrocellulose membranes (22). Antisense RNA probes were prepared from pGEM1 and pGEM2 vectors carrying fragments A to D by using T7 polymerase (Promega Biotec) as described by the supplier. RNA-to-RNA hybridization was done in 30% formamide–6× SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) · KOH (pH 7.4)–2 × Denhardt solution (11)–0.5% sodium dodecyl sulfate (SDS)–100 μg of salmon sperm DNA per ml for 16 to 24 h at 65°C. The filters were washed four times, 15 min each, with 0.3× SSC–0.5% SDS at 65°C.

To isolate a molecular clone of the complete *CHC1* gene, duplicate nitrocellulose replicas of bacterial colonies (*Escherichia coli* HB101) harboring a library of yeast DNA in YCp50 (generously provided by M. Rose, Princeton University) were probed with nick-translated *CHC1* fragments from pCHC73 or pCHC74. Colonies carrying plasmids which annealed to both probes were isolated, and plasmid DNA was prepared. Restriction endonuclease analyses were used to position *CHC1* in the yeast inserts, and pCHCc100 was chosen for further study.

To determine the nucleotide sequence of the *Bgl*II-to-*Eco*RI fragment that is dotted in Fig. 1, we subjected pUC118-12 and pUC119-12 to *Bal* 31 (International Biotechnologies, Inc., New Haven, Conn.) exonuclease treatment to generate an ordered deletion series. The deleted fragments subcloned in pUC118 were subjected to DNA sequence determination by the dideoxy chain termination method (34).

Genetic techniques and transformation. Methods of yeast mating, sporulation, and tetrad analysis were as described by Sherman et al. (39). Diploid cells were grown overnight on YPD plates before sporulation. Spheroplast transformation was used to insert pRB58 into GPY1100, -1101, -55-15B, and -68 (18). All other transformations used the lithium acetate procedure described by Ito et al. (19). For single-step gene transplacement (33), pchcl-Δ8 was cleaved with *Hin*dIII and *Pst*I and pchcl-Δ10 was cleaved with *Hin*dIII.

Mapping *CHC1*. Hybridization of a pCHC74 probe to chromosomes separated by orthogonal field alteration gel electrophoresis (8) positioned *CHC1* on either chromosome 7 or 15 (filters kindly provided by Jules O'Rear, University of California). Tetrad analysis of diploids formed between a *chc1-Δ8* strain and cells carrying genetic markers on chromosome 7 or 15 indicated that *CHC1* was linked to *ade5* on the left arm of chromosome 7. However, because of the growth defect, the genotypes of *chc1-Δ8* segregants often were difficult to establish confidently. To circumvent this problem and precisely map *CHC1*, the *URA3* gene was integrated adjacent to *CHC1*. YIp5-TH was cut with *Hin*dIII to target integration of the plasmid immediately 3′ to *CHC1*. *ura3-52* strain PBY425A was transformed with linear YIp5-TH, and Ura^+ colonies were isolated. The integration of *URA3* next to *CHC1* was confirmed by restriction enzyme and Southern hybridization analysis applied to DNA prepared from Ura^+ colonies (data not shown). One such Ura^+ strain, TBY100-3, was then mated to strain TBY50-41B which includes *ade5* and *lys5* mutations on the left arm of chromosome 7. Tetrad analysis of the resulting diploid is summarized in Table 2. *CHC1* mapped between *ade5* and *lys5* on the left arm of chromosome 7, approximately 23 centimorgans centromere proximal to *ade5*.

TABLE 2. Mapping data

Gene pair	Segregation (no. of tetrads)[a]			Map distance[b]
	PD	NPD	TT	
ADE5, CHC1::URA3	58	1	38	23
LYS5, CHC1::URA3	39	1	57	33
ADE5, LYS5	28	2	67	41

[a] PD, Parental ditype; NPD, nonparental ditype; TT, tetratype.

[b] Map distances (centimorgans) were calculated by using equation 3 of Mortimer and Schild (24). No corrections were made to the long map distances.

Immunoblotting. Cells were grown in minimal medium plus 2% glucose to the mid-logarithmic phase. A sample (10 units at an optical density of 600 nm [OD_{600}]) was centrifuged, and cells were washed once with minimal medium and then lysed by agitation with glass beads and 2% SDS for 90 s on a vortex mixer. Lysates were heated immediately at 100°C for 5 min, and then Laemmli sample buffer was added (2). The liquid was removed from the beads and centrifuged at 12,000 × *g* in a microcentrifuge (Fisher Scientific Co., Pittsburgh, PA.) for 10 min. Samples of the supernatant fraction corresponding to 0.5 OD_{600} equivalents (approximately 40 μg of protein) were electrophoresed through SDS-polyacrylamide gels (21) and evaluated by immunoblotting (6). Preparation and characterization of antiserum specific for yeast clathrin heavy chain was reported before (28). Bound antibody was detected with ^{125}I-labeled *Staphylococcus aureus* protein A.

Radiolabeling, preparation of lysates, and immunoprecipitation. Cells were grown to the mid-logarithmic phase at 30°C in minimal medium plus 2% glucose and 200 μM ammonium sulfate. Cells were harvested by centrifugation, washed once with minimal medium, and then suspended at 1.0 OD_{600} unit per ml in minimal medium plus 0.1% glucose and 20 μM ammonium sulfate. After 30 min of incubation at 30°C, cells were sedimented, washed as above, suspended at 2 OD_{600} units per ml in minimal medium with 0.1% glucose without ammonium sulfate, and placed at 30°C. Five minutes later, labeling was initiated by the addition of 200 μCi of $^{35}SO_4{}^{2-}$ (1,200 Ci/mmol; ICN Pharmaceuticals Inc., Irvine, Calif.) per OD_{600} unit. After 5 min of labeling, ammonium sulfate (3 mM), cysteine (0.01%), and methionine (0.01%) were added. At designated time intervals, samples (1 to 2 OD_{600} units) were removed and mixed on ice with an equal volume of 20 mM sodium azide. Upon completion of the time course, cells were collected by sedimentation and treated with lyticase to degrade cell walls. Spheroplast and periplasmic fractions were separated, spheroplasts were lysed with Triton X-100, and invertase was immunoprecipitated as described by Schauer et al. (36). Precipitated invertase was resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. Autoradi-

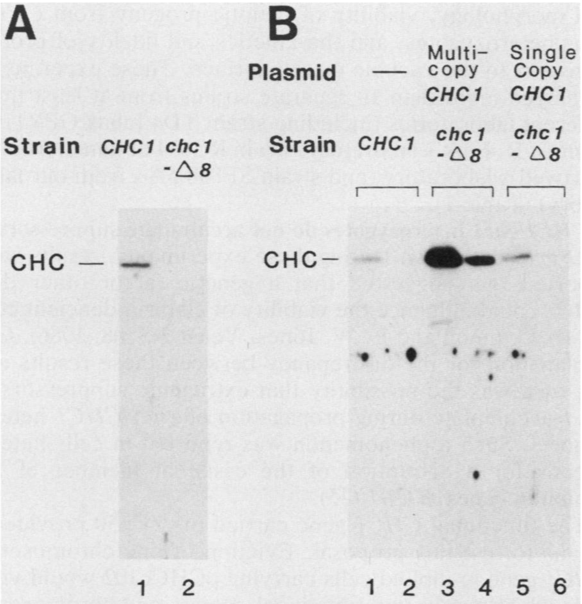

FIG. 2. Immunoblot analysis of clathrin heavy chain in clathrin-deficient strains with or without plasmid-borne *CHC1*. Cell extracts were prepared and analyzed by immunoblotting with antiserum specific for yeast clathrin heavy chain (see Materials and Methods). (A) Lane 1, Proteins from *CHC1* strain GPY1100; lane 2, proteins from *chc1-Δ8* strain GPY1101. (B) Proteins from: lanes 1 and 2, strain GPY1100; lanes 3 and 4, strain GPY1101 carrying pCHCe200; lanes 5 and 6, strain GPY1101 carrying pCHCc102. Each lane in panel A received 0.5 OD_{600} equivalents of cell extract. The odd-numbered lanes in panel B contained five times more cell extract (0.5 OD_{600} equivalents) than the even-numbered lanes (0.1 OD_{600} equivalents).

ograms were quantified by scanning with a Kratos model SD3000 spectrodensitometer coupled to a Kratos SDS300 density computer (Kratos Analytical Instruments, Ramsey, N.J.) and Hewlett-Packard 3380A integrator (Hewlett-Packard Co., Palo Alto, Calif.). To control for variations in the immunoprecipitations, values for internal core-glycosylated and mature forms of invertase were normalized to the value obtained for the cytoplasmic species. Cytoplasmic invertase was stable during the time course of these experiments.

Electron microscopy. Thin-section electron microscopy was performed on samples prepared by the method of Byers and Goetsch (7).

RESULTS

Single-step gene transplacement in haploid cells yields clathrin-deficient strains. Initially, our strategy for eliminating *CHC1* was based on the possibility that a clathrin deficiency would be lethal. Consequently, we disrupted one of the two *CHC1* alleles in diploid cells (28). The resulting heterozygous *CHC1/chc1* diploids were induced to sporulate and then dissected into tetrads to generate *chc1* haploid cells. When the *chc1* haploid cells proved to be viable, it became reasonable to consider disrupting *CHC1* directly in a haploid strain. Using the single-step gene transplacement protocol (33), the *chc1-Δ8::LEU2* disruption (Fig. 1) was introduced into *leu2* haploid strains. Two types of colonies appeared on medium lacking leucine after transformation with the altered gene. Normal size colonies appeared about 2.5 days posttransformation, and small colonies arose after about 3.5 days at 30°C. The number of large and small colonies varied from experiment to experiment depending on the strain and the particular DNA construction used for gene disruption. Commonly, severalfold more small colonies were observed. Restriction enzyme and Southern hybridization analyses of DNA obtained from the large colonies showed only the wild-type *CHC1* gene. These colonies likely arose owing to gene conversion of the *leu2-3 leu2-112* mutations by the *LEU2* sequences present in the *chc1-Δ8::LEU2* DNA. Similar analyses indicated that the small colonies were composed of cells that harbored the *chc1-Δ8* disruption (data not shown). Immunoblotting experiments with antiserum specific for yeast clathrin heavy chain confirmed that the *chc1-Δ8* disruption eliminated the expression of clathrin heavy chain (Fig. 2A).

It could be argued that the cells carried an unlinked suppressor of lethality generated by the mutagenic nature of the transformation protocol. To test this possibility, we mated one *chc1-Δ8::LEU2* strain obtained by this approach to a *leu2 CHC1* strain. The resulting heterozygous diploid was sporulated and dissected into tetrads. If the *chc1* haploid parent harbored a suppressor unlinked to the *CHC1* locus, then the tetrads should show a 1:4:1 ratio of 4 viable:3 viable:2 viable segregants (24). However, each tetrad consisted of two normal size colonies and two small colonies (Fig. 3). The large colonies were Leu$^-$, and the small colonies were Leu$^+$. The other markers in the cross, *pep4* (chromosome 16) and *MAT* (chromosome 3), segregated 2:2. Since high spore viability was observed and the genetic markers segregated normally, it appeared that the clathrin-deficient cells did not carry an unlinked suppressor nor did they suffer substantial chromosome imbalances or polyploidy (24). In other crosses the number of tetrads containing 4, 3, or 2 viable segregants varied, but tetrads with four viable spores always made up the majority. An alternative interpretation of these results postulates that the parental diploid cells, heterozygous at both *CHC1* and putative suppressor loci, became homozygous for the suppressor allele during propagation. This hypothesis is unlikely in light of the experiments described in the following sections which independently argue that suppressors are not responsible for clathrin-deficient cell viability. Thus, it is possible to generate clathrin-deficient strains by single-step gene transplace-

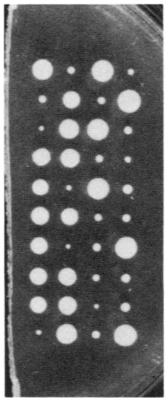

FIG. 3. Tetrads from a diploid formed by mating haploid-derived *chc1-Δ8* strain GPY1103 to *CHC1* strain GPY60. Each small colony was subsequently scored Leu$^+$. Cells in the large colonies were Leu$^-$.

ment in haploid cells. This finding allows the generation of sets of congenic *CHC1* and *chc1* partners.

Isolation of complete *CHC1* gene. The original molecular clone of *CHC1* was isolated from a library of yeast DNA inserted in λgt11 (28). *CHC1* DNA carried by the recombinant phage extended beyond the 5′ limit of the gene but did not include the 3′ end (data not shown). This constrained the size of deletions that could be introduced into *CHC1*; each disrupted version of the chromosomal *CHC1* gene retained at least the 1 kbp of 3′ coding information which was not present in the cloned DNA (Fig. 1, pchc1-Δ8). A molecular clone of the complete *CHC1* gene would allow construction of more extensive deletions and would further the definition of *CHC1* gene structure. Probes prepared from DNA fragments representing both ends of the λgt11 *CHC1* insert were used to screen a yeast genomic DNA library carried by the yeast centromere vector YCp50. Plasmids from positive colonies were purified and tested for the ability to restore normal growth rates to *chc1-Δ8* cells (data not shown). From one plasmid which fulfilled these criteria, the *Bam*HI-to-*Sal*I DNA fragment shown in Fig. 1 was subcloned into YCp50 yielding pCHCc102 and into the multicopy vector YEp24 to create pCHCe200. Two experiments indicated that this DNA fragment contained the entire *CHC1* gene. First, these plasmids eliminated the slow growth phenotype of *chc1* cells. Second, the immunoblotting experiment shown in Fig. 2B confirmed that each plasmid encoded clathrin heavy chain. Antiserum specific for yeast clathrin heavy chain detected the 190-kD heavy chain in wild-type cell proteins (Fig. 2A, lane 1; 2B, lane 1), but not in *chc1-Δ8* cell extracts (Fig. 2A, lane 2). *chc1-Δ8* cells with pCHCc102 exhibited quantities of 190-kD heavy chain equivalent to that seen in *CHC1* cells (compare lanes 1 and 5, Fig. 2B). Relative to these strains, clathrin heavy chain was overproduced about 30-fold in *chc1-Δ8* cells containing pCHCe200. Notably, these cells formed wild-type-size colonies, suggesting that this degree of overproduction was not deleterious to cell growth.

The complete clone was used to identify additional restriction endonuclease sites (Fig. 1) and to map the *CHC1* locus to the left arm of chromosome 7 (see Materials and Methods) (Table 1). DNA fragments from the gene were used to position approximately the transcript and coding boundaries through a combination of sequencing, Northern blot (RNA blot) hybridization experiments, and analysis of gene fusions constructed to produce immunoreactive hybrid proteins with the *E. coli trpE* gene (28). The *CHC1* transcript did not extend beyond the distal *Hin*dIII site shown in Fig. 1. A fusion between *trpE* and the distal *Eco*RI-to-*Hin*dIII fragment produced a 43-kD hybrid protein that reacted with clathrin antibody. Since the normal *trpE* protein electrophoresed as a 38-kD species, *CHC1* coding sequences extended less than 150 bp beyond the *Eco*RI site. A putative N-terminal translation initiation site was identified by DNA sequence analysis of the *Bgl*II-to-*Eco*RI fragment that is dotted in Fig. 1. A single open reading frame started with an ATG codon 202 bases after the first base of the *Bgl*II site and extended through the rest of the fragment. This ATG position is indicated by a dot on the restriction map in Fig. 1.

With the complete *CHC1* gene, a new, more extensive disruption (pchc1-Δ10, Fig. 1) was generated. This alteration removed more than 95% of the *CHC1* coding domain including the putative initiator codon. Haploid cells carrying the *chc1-Δ10* deletion behaved identically to *chc1-Δ8* strains, thus reducing the possibility that an undetectable C-terminal fragment of clathrin accounted for the viability of *chc1-Δ8* strains. The parameters examined included growth rates, cell morphology, viability of meiotic progeny from *CHC1*/*chc1* heterozygotes, and the kinetics and fidelity of protein transport to the vacuole or cell surface. These experiments employed more than 10 separate strains from at least three different laboratories (including strain TD4 [alias GPY1100] from G. R. Fink's laboratory, strain RC634 obtained from L. Hartwell's laboratory, and strain SF838-5Aα from our laboratory) (Table 1).

***CHC1*/*chc1* heterozygotes do not accumulate suppressors of *chc1* growth defects.** During these experiments, results were reported that suggested that a genetic factor other than *CHC1* could influence the viability of clathrin-deficient cells (S. K. Lemmon and E. W. Jones, Yeast 2:S208, 1986). One explanation for the discrepancy between these results and our own was the possibility that extragenic suppressors of *chc1* accumulate during propagation of *chc1*/*CHC1* heterozygotes. Such a phenomenon was reported in cells heterozygous for a disruption of the essential member of the α-tubulin genes, *TUB1* (35).

The functional *CHC1* gene carried on YCp50 provided a means to test this proposal. Eviction of one chromosomal *CHC1* gene in diploid cells carrying pCHCc102 would yield cells which retain two functional copies, one chromosomal and one plasmid linked. Under these conditions, populations of heterozygous cells should not experience any selective pressure that favors the growth of cells with suppressors. If selection of suppressors in the heterozygous diploids accounted for *chc1* cell viability, then, after sporulation and dissection of plasmid-bearing diploids into tetrads, segregants with a chromosomal *chc1-Δ10* allele but lacking pCHCc102 should be inviable (Fig. 4A)—that is, Leu$^+$ Ura$^-$ spores should not be observed. If this model was not correct, then *chc1-Δ10* cells without pCHCc102 would form small Leu$^+$ Ura$^-$ colonies.

Six transformants derived by single-step gene transplacement of *chc1-Δ10* into cells carrying pCHCc102 were subjected to sporulation and dissection. Two transformants gave rise to tetrads with four normal size colonies. Genetic characterization suggested that the plasmid-borne copy of *CHC1* was disrupted in these cells (data not shown). This was substantiated in one case by Southern hybridization analysis of total DNA prepared from the diploid cells. The other four transformants produced primarily tetrads with four viable spores that gave rise to either normal or small colonies. An example is shown in Fig. 4B. The few spores that appeared to be inviable actually gave rise to small colonies. Tables 3 and 4 presents the results of tetrad characterization. All but two of the small colony segregants that could be scored were Leu$^+$ Ura$^-$. The other two were Leu$^-$ Ura$^-$ and most likely arose by delayed germination of wild-type spores. Such small colonies were observed at a similar frequency in tetrads of the original *CHC1*/*CHC1* diploid carrying pCHCc102. These small colonies grew normally when replated onto fresh medium. *chc1* cells replated as small colonies.

The pCHCc102 plasmid (Ura$^+$) was retained in every instance (95 of 95) that cells harboring *chc1-Δ10* on the chromosome (Leu$^+$) formed a large colony. This demonstrates that *CHC1* on YCp50 complements the growth abnormality associated with a *chc1-Δ10* disruption. As a further control, diploid cells carrying YCp50 were subjected to *chc1-Δ10* gene transplacement. Tetrads from such transformants were similar to those in Fig. 3. In this case (Table 5), the plasmid segregated independently of colony size. Tables 4 and 5 show that the plasmid segregated similarly in both experiments.

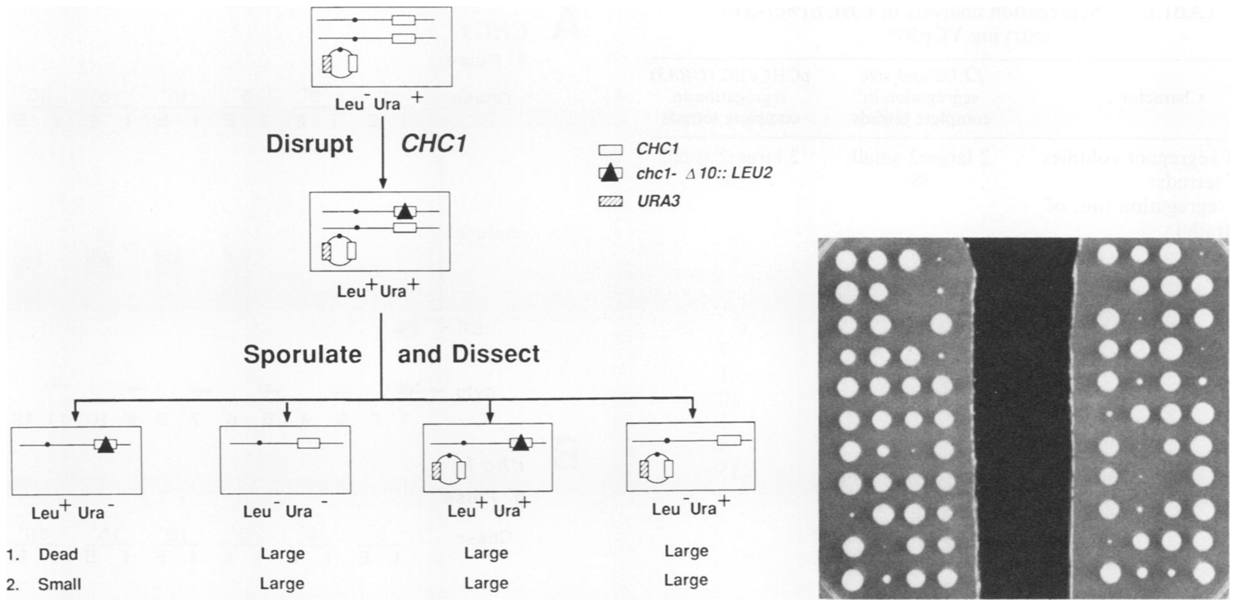

FIG. 4. Clathrin heavy-chain gene disruption in a diploid strain carrying *CHC1* on a centromere plasmid. (A) Strategy used to test the possibility that clathrin-deficient meiotic progeny survive owing to suppressors that accumulate during propagation of the heterozygous diploid. When one of the two chromosomal *CHC1* alleles is evicted, four classes of spores are expected as shown in the third row of squares. Each class has a distinct phenotype when assessed for leucine or uracil auxotrophy. Prediction 1 follows from the suppressor hypothesis. If clathrin-deficient progeny are viable in the absence of suppressors, then prediction 2 is expected. See the text for details. (B) Tetrad analysis of GPYD1004 carrying pCHCc102 and a chromosomal *chc1*-Δ*10* allele. Small colonies were Leu$^+$ Ura$^-$ in accordance with prediction 2 in panel A.

These results argue strongly that *chc1* haploid cell viability was not due to suppressors that arose among the *CHC1*/*chc1* heterozygotes.

No *CHC1*-homologous transcripts are present in *chc1*-Δ*10* strains. The construction of *chc1*-Δ*10* haploid strains permitted assessment of the possibility that a homologous gene might substitute for *CHC1*. Previous Southern hybridization experiments suggested that *CHC1* was unique in the yeast genome (28). The more sensitive technique of RNA-RNA hybridization (23) was applied to determine whether any transcripts homologous to *CHC1* were present in *chc1*-Δ*10* cells. Four antisense RNA probes (A to D, Fig. 1) which span most of *CHC1* were prepared and annealed to RNA preparations from wild-type and *chc1*-Δ*10* cells (Fig. 5). Under conditions of low stringency (30% formamide, 6× SSC, 65°C), no transcripts homologous to *CHC1* were observed even though polyadenylated RNA from *chc1*-Δ*10* cells (Fig. 5, odd lanes) was present at levels five times that of wild-type-cell polyadenylated RNA (Fig. 5, even lanes). Other experiments indicated that the 1-kilobase RNA detected by probe D probably originated from sequences beyond the 3′ end of *CHC1* that were not affected by the *chc1*-Δ*10* deletion. At the exposure lengths and low hybridization stringency used in these studies, weak diffuse signals were occasionally obtained (for example, see Fig. 5, lane 1). The absence of discrete bands and variability from experiment to experiment suggest that these signals represent a small degree of nonspecific hybridization. The integrity of RNA in each lane was established by detection of an independent large mRNA (*SEC7*, 6 kilobases). It therefore seems unlikely that a *CHC1* homolog exists in yeasts.

Invertase secretion is delayed but not blocked in *chc1* cells. Transport kinetics of newly synthesized invertase was monitored to assess the functional integrity of the secretory pathway. The *SUC2* gene encodes two forms of invertase (9). The cytoplasmic form is made constitutively, whereas synthesis of the secreted form is derepressed when cells are placed in medium with low glucose concentrations. The ER transit form of secreted invertase migrates in SDS-polyacryl-

TABLE 3. Segregation analysis of *CHC1*/*chc1*-Δ*10* strain carrying pCHCc102: *LEU2* and size segregation in complete tetrads[a]

Size of segregant colonies	No. of tetrads	*LEU2* segregation (no. of tetrads)			No. of small colonies	No. of Leu$^+$ Ura$^-$ small colonies	No. of small colonies with other genotypes
		2:2	1:3	NS[b]			
4 large:0 small	31	31	0	0	0	0	0
3 large:1 small	36	31	1	4	32	31	1[c]
2 large:2 small	12	9	0	3	18	17	1[c]

[a] Viability in tetrads (number of tetrads): 4 alive:0 dead, 79; 3 alive:1 dead, 6; 2 alive:2 dead, 1.

[b] Some colonies were too small to assess their growth in the absence of leucine or uracil. Tetrads with such colonies are listed in this column.

[c] Colonies contain cells with genotype *leu2 ura3*.

TABLE 4. Segregation analysis of *CHC1*/*chc1*-Δ*10* strain carrying pCHCc102: pCHCc102 segregation in complete tetrads[a]

Size of segregant colonies	No. of tetrads	pCHCc102 (*URA3*) segregation (no. of tetrads)					
		4:0	3:1	2:2	1:3	0:4	NS[b]
4 large:0 small	31	19	1	11	0	0	0
3 large:1 small	36	0	5	26	1	0	4
2 large:2 small	12	0	0	5	0	4	3

[a] See Table 3, footnote *a*.

[b] Some colonies were too small to assess their growth in the absence of leucine or uracil. Tetrads with such colonies are listed in this column.

TABLE 5. Segregation analysis of *CHC1*/*chc1-Δ10* carrying YCp50[a]

Character	*LEU2* and size segregation in complete tetrads	pCHCc102 (*URA3*) segregation in complete tetrads
Size of segregant colonies	2 large:2 small	2 large:2 small
No. of tetrads	38	38
LEU2 segregation (no. of tetrads)		
2:2	28	
NS[b]	10	
pCHCc102 segregation (no. of tetrads)		
4:0		7
3:1		1
2:2		10
1:3		1
0:4		4
NS[b]		15
Total no. of small colonies	56	
No. of Leu$^+$ Ura$^-$ small colonies	28	
No. of Leu$^+$ Ura$^+$ small colonies	28	

[a] Viability in tetrads (number of tetrads: 4 alive:0 dead, 38; 3 alive:1 dead, 2; 2 alive:2 dead, 2).

[b] See Table 3, footnote *b*.

amide gels as a series of bands centered at 80 kD (12). On transport to the Golgi body, invertase is converted to a more highly glycosylated species that electrophoreses with extreme heterogeneity corresponding to molecular sizes in excess of 80 kD (12). This mature form is packaged into secretory vesicles without further apparent modification and delivered to the cell surface where it is retained in the periplasmic space by the cell wall. Similar rates of enzyme appearance at the cell surface were observed after transferring *chc1-Δ8* and wild-type cells to low-glucose medium (28). The data did not, however, exclude a reduced rate of invertase transfer from the ER to the Golgi body or from the Golgi body to the cell surface. Transit times were measured accurately by subjecting cells to a radiolabel pulse-chase regimen. The multicopy *SUC2* plasmid pRB58 (9) was introduced into *chc1-Δ8*, *chc1-Δ10*, and their congenic *CHC1* partners to facilitate detection of invertase secretory intermediates. Mutant and wild-type cells were pulse-labeled in low-glucose medium for 5 min with $^{35}SO_4{}^{2-}$, and then excess unlabeled $SO_4{}^{2-}$, cysteine, and methionine were added. Samples were collected at specified time intervals, and cells were harvested and separated into intracellular and periplasmic fractions. Invertase was immunoprecipitated from each fraction and displayed by SDS-polyacrylamide gel electrophoresis. Figure 6 shows the results of this analysis with wild-type (Fig. 6A) and *chc1-Δ8* mutant (Fig. 6B) cells. Comparing Fig. 6A with Fig. 6B, it appeared that the ER forms in wild-type and mutant cells became more highly glycosylated at similar rates. However, a marked accumulation of internal highly glycosylated invertase was apparent in the *chc1* cells (Fig. 6B, lanes 1, 3, 5, and 7). Also, *chc1-Δ8*

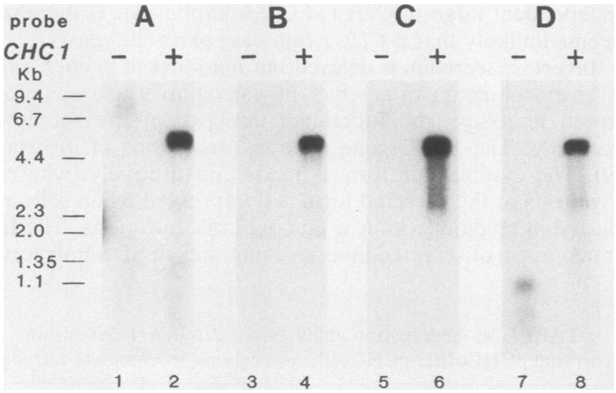

FIG. 5. Absence of transcripts homologous to *CHC1* in *chc1-Δ10* strain GPY68. Polyadenylated RNA was prepared from *CHC1* strain GPY55-15B and *chc1-Δ10* strain GPY68, subjected to formaldehyde agarose gel electrophoresis, and transferred to nitrocellulose. Odd-numbered lanes contained 10 μg of poly(A)$^+$ RNA from GPY68. Even-numbered lanes contained 2 μg of poly(A)$^+$ RNA from GPY55-15B. Separate nitrocellulose filters (A to D) were annealed to antisense RNA probes derived from sequences designated probes A to D in Fig. 1 under conditions of low hybridization stringency. kb, Kilobases.

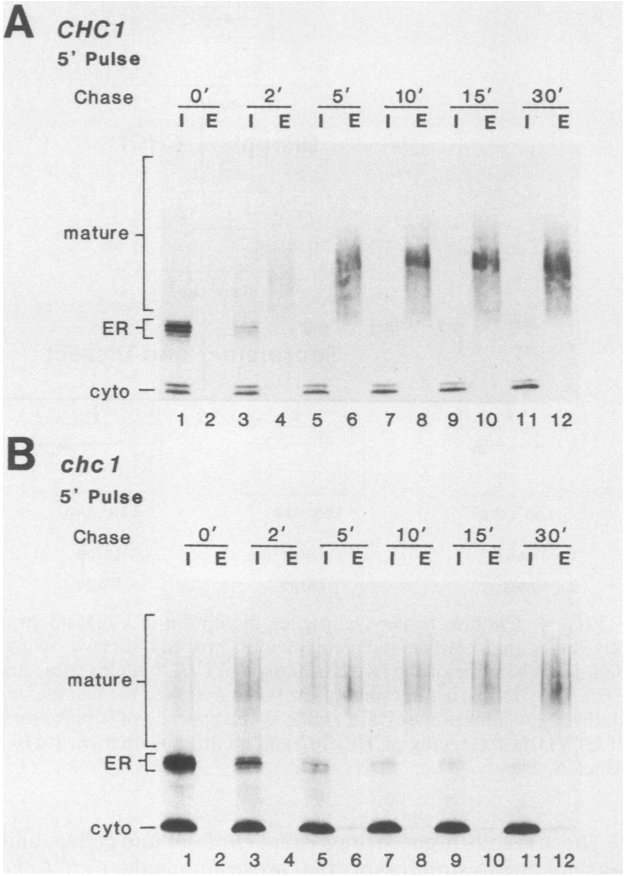

FIG. 6. Invertase export from clathrin-deficient cells is delayed but not blocked. *CHC1* strain GPY1100 (A) and *chc1-Δ8* strain GPY1101 (B), each carrying pRB58, were pulse-labeled for 5 min with $^{35}SO_4{}^{2-}$ and then subjected to a chase regimen. Samples were harvested at the designated times after initiation of the chase, fractionated into internal (I) and external (E, periplasmic) compartments, and invertase immunoprecipitated from each fraction. Precipitated invertase was resolved and visualized by polyacrylamide gel electrophoresis and autoradiography. Since the secreted form of invertase was not equally derepressed in wild-type and mutant cells, a longer exposure of the gel containing mutant-cell invertase was necessary to visualize highly glycosylated species. cyto, Constitutively expressed cytoplasmic invertase; ER, core-glycosylated invertase present in the ER; mature, highly glycosylated mature species.

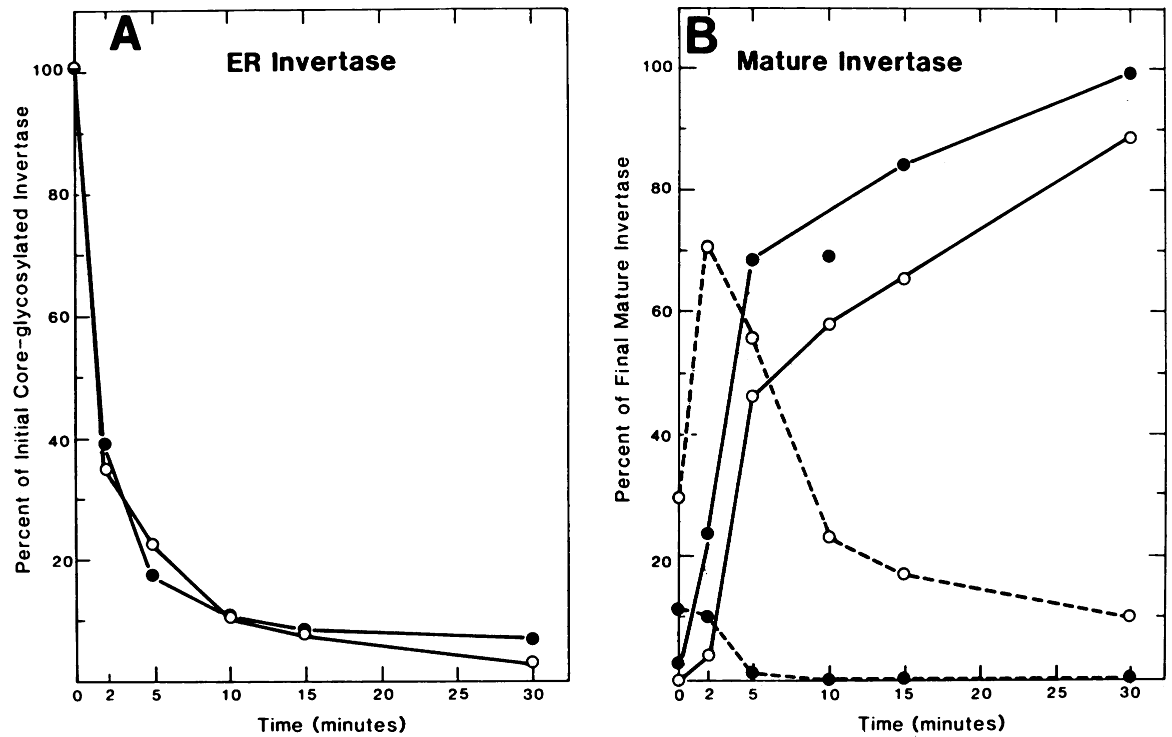

FIG. 7. Kinetics of invertase export from *chc1-Δ8* strain GPY1101 and *CHC1* strain GPY1100. Different autoradiographic exposures of the gels presented in Fig. 6 were quantified by densitometry. Symbols: ○, invertase from *chc1-Δ8* strain GPY1101; ●, invertase from *CHC1* strain GPY1100. (A) Rate of conversion of invertase ER forms. (B) Kinetics of mature invertase export. Dotted lines, Internal mature invertase; solid lines, external (periplasmic) invertase.

cells exhibited a slight delay in the external appearance of invertase (Fig. 6B, lanes 4, 6, and 8 compared with Fig. 6A, lanes 4, 6, and 8). These results were quantified by densitometric analysis of different autoradiographic exposures of the gels depicted in Fig. 6 (Fig. 7). Quantitation substantiated the impression that ER invertase was converted at identical rates in mutant and wild-type cells (Fig. 7A). In contrast, at 2 min postchase, nearly 75% of the newly synthesized invertase was retained within mutant cells as the highly glycosylated species. This was seven- to eightfold more than the peak levels of internal mature invertase in wild-type cells at the initiation of the chase period. The half-time of invertase external appearance was 3.6 min in *CHC1* cells and 6.4 min in *chc1-Δ8* cells, a delay of 1.8-fold. An analysis of congenic strains GPY68 (*chc1-Δ10*) and GPY55-15B (*CHC1*) produced similar results (data not shown). In both sets of congenic partners the mutant strains retained approximately 10% internal mature invertase at 30 min. This could represent a stable internal pool of mature enzyme, but more likely reflected a technical difficulty. Mutant cells clumped together, making it difficult to remove the cell walls completely and release the entire periplasmic contents.

These experiments revealed a transport delay in the secretory pathway at some stage after invertase reached the Golgi apparatus.

Morphological examination of mutant cells. Thin sections of wild-type and mutant cells were prepared and examined in the electron microscope. Examples of electron micrographs depicting a *chc1-Δ8* cell and a wild-type cell are presented in Fig. 8. Unlike mammalian cells, wild-type yeasts (Fig. 8A) exhibit only a low level of ER, Golgi bodies, and secretory vesicles consistent with the rapid transit times of secretory intermediates (37). Major organelles in the micrograph of the wild-type cell (Fig. 8A) are the vacuole (va) and the nucleus (n). The *chc1* cell shown in Fig. 8B was representative of the population examined in the electron microscope and presents evidence of a substantial accumulation of membrane-bounded organelles. Both vesicles (ve) and structures similar to Berkeley bodies (Bb) were apparent. Experiments with secretory mutant cells suggested that Berkeley bodies are an abnormal form of Golgi body membranes (34). ER was also apparent, but most mutant cells did not display more ER than wild-type cells. In addition, the vacuole appeared fragmented and multivesicular in cross-section (va). Similar profiles were seen with *chc1-Δ10* strain GPY68. These results were consistent with the interpretation of Fig. 6 and 7 which suggested a transport impediment late in the secretory pathway.

DISCUSSION

We demonstrated previously that cells incurring a clathrin heavy-chain gene disruption are viable but grow more slowly than wild-type cells (28). Since a functional secretory pathway is necessary for cell growth, the results argued against an obligate role for clathrin in the secretory process. This unexpected conclusion contradicted the prevailing view of clathrin function, although more recent studies of mammalian cell protein transport are consistent with our findings (16, 25).

Suppressors do not account for clathrin-deficient cell viability. The experiments presented in this report address several hypothesis raised to account for the viability of clathrin-deficient cells. A common feature of several proposals is that genes other than *CHC1* can, after alteration, suppress the

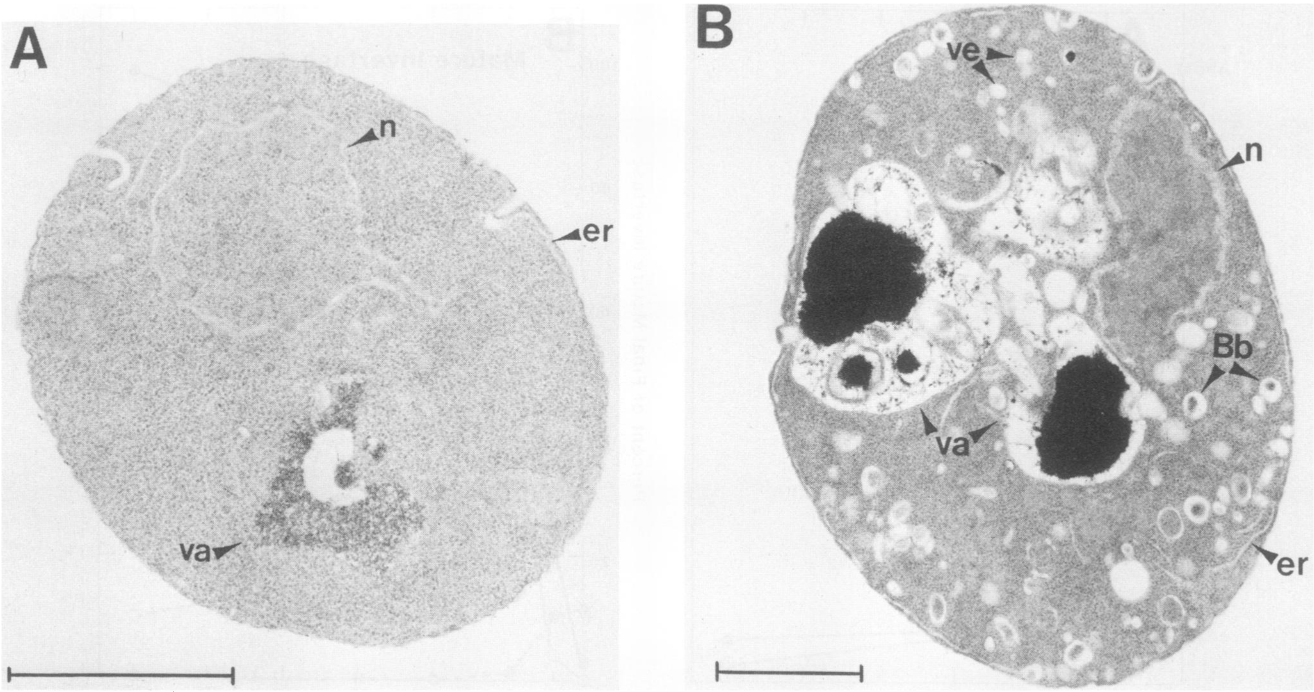

FIG. 8. Electron micrographs of thin sections cut through *CHC1* strain GPY1100 (A) and *chc1-Δ8* strain GPY1114 (B). n, Nucleus; er, endoplasmic reticulum; va, vacuole; ve, vesicles; Bb, Berkeley bodies. Bars, 1 μm.

lethality of a *chc1* lesion. Suppression could occur by mutations in, or amplification of, single genes or by chromosomal imbalances such as disomy. The first clathrin-deficient strains were obtained from the meiotic progeny of cells heterozygous for the *chc1* null allele. If these *chc1* haploid strains rely on extragenic mutations for growth, then the suppressors may arise during expansion of the heterozygous diploid cell population. For this to occur, heterozygous diploid cell growth must be somewhat retarded to allow cells with suppressors to become a dominant component of the population. Schatz et al. (35) have described diploid yeast cells heterozygous for a *TUB1* (the essential member of the two α-tubulin genes) null mutation which display compromised growth and accumulate chromosomal imbalances that rescue the growth defect. After sporulation, some haploid cells harboring the *TUB1* disruption are viable because they carry amplified copies of the nonessential *TUB3* gene. Although obvious phenotypes are not apparent in diploids carrying *chc1* null alleles, careful comparisons between heterozygous and homozygous wild-type strains have not been made.

To address the possibility that *chc1* strains carry suppressors, we performed two experiments. First, to alleviate the postulated selective pressure, a third copy of *CHC1* on a centromere vector was introduced into diploid cells before eviction of one chromosomal *CHC1* locus so that cells would retain two functional alleles. Tetrad analysis revealed slow-growing *chc1* segregants. Second, *chc1* strains were generated by direct disruption of *CHC1* in haploid cells. Thus, propagation of heterozygous diploids and induction of meiosis is not required to acquire viable *chc1*-bearing cells. Furthermore, a diploid strain formed from the mating of a haploid-derived *chc1* mutant strain and wild-type strain gave rise to complete tetrads displaying two large and two small colonies. This result indicates that the original *chc1* strain did not harbor an unlinked suppressor nor did it suffer substantial chromosome imbalance or polyploidy.

Lemmon and Jones (Yeast 2:S208, 1986) reported tetrad analyses of heterozygous *chc1* strains that implied the existence of an independently segregating gene that, in conjunction with *chc1*, affected spore viability. Several possibilities could account for the discrepancy with our data. First, the segregation pattern observed by Lemmon and Jones could result from a heterozygous suppressor of *chc1*-induced lethality arising during propagation of the *CHC1/chc1* diploid cells. Our results, summarized above, argue that *chc1* cell growth does not depend on such suppressors. Second, since they employed a slightly different *chc1* allele (elimination of the 4.5-kbp *Bgl*II fragment) from those presented here, their deletion may have generated a dominant phenotype that caused suppressor accumulation in the heterozygous diploid. This seems unlikely since we have not observed differences among *chc1* strains carrying deletions with four different endpoints, and the *Bgl*II fragment deletion removed only about 200 bp less than the *chc1-Δ10* disruption. Finally, a gene present in one of their haploid strains may have influenced the viability of *chc1* spores. Which genetic background then represents wild type: one that can sustain a *chc1* null mutation or one that cannot? Our findings are based on more than 10 strains from three different laboratories. Hence, it is unlikely that we examined an unrepresentative sample of genetic backgrounds. In addition, we found that *chc1* mutant cells are generally more sensitive to traumatic conditions such as high temperature or low pH. Perhaps the strain used by Lemmon and Jones harbors a mutation that does not alter the growth of *CHC1* cells but is lethal in the presence of a *chc1* null allele.

Sequence-related homologs of clathrin are not represented in mRNA from wild-type or *chc1-Δ10* cells. Our approach, using antisense RNA probes and low-stringency conditions of hybridization, was developed by others to detect *Drosophila* opsin gene transcripts with a bovine opsin gene probe (44). The *Drosophila* and bovine opsins display only 22% amino acid homology, and the genes share only two 45-bp stretches with about 75% nucleotide identity. Although our findings are negative, the yeast *CHC1* probes detected transcripts in HeLa cell polyadenylated RNA that are large enough to encode clathrin heavy chain (T. Hasson, unpublished data). Limited preliminary nucleotide sequence comparison (T. Kirchhausen, personal communication) shows 40 to 50% amino acid homology between yeast and rat heavy chains. Hence, if a functional analog of clathrin heavy chain exists, it cannot contain extensive nucleotide homology to *CHC1*. On the other hand, the topoisomerase I and II genes of yeasts, although sharing no homology, nevertheless perform partially interchangeable functions (5). Similarly, a functional replacement for clathrin heavy chain may exist.

Perturbation of the secretory pathway in clathrin-deficient mutants. Detailed investigation of intercompartmental protein transport in clathrin-deficient strains may provide clues to clathrin function in wild-type cells. Wild-type and mutant cells exhibit equal rates of outer chain carbohydrate addition to core-glycosylated invertase (Fig. 6 and 7). Since this modification requires transport of invertase from the ER to the Golgi body, traffic between these compartments must be clathrin independent. Evidence for a role of clathrin-coated vesicles in transport from the ER in mammalian cells is controversial (1, 30).

Transport of highly glycosylated invertase to the cell surface is delayed. This impediment could occur during transport through the Golgi membrane stacks, during packaging into secretory vesicles, or during transfer of the vesicles to the cell surface; analysis of invertase secretion does not allow distinction among these stages. Preliminary experiments suggest that mutant cells are deficient in proteolytic maturation of prepro-α factor (G. Payne, unpublished data). Processing normally occurs before α factor appears in mature secretory vesicles. This finding points to a defect late in transport through the Golgi body.

Cytological examination reveals accumulation of vesicles and Berkeley bodies in the clathrin mutant. Berkeley bodies appear to derive from Golgi membranes (38). The presence of vesicles and Berkeley bodies is consistent with a defect late in the secretory pathway. Vacuoles in mutant cells are abnormal, exhibiting multivesicular structure and apparent fragmentation in cross-section, possibly as a result of convolution of the normally smooth vacuole membrane. The significance of the vacuolar abnormality is not clear. Transport and localization of a vacuolar protease is normal in *chc1*-bearing strains (G. Payne, D. Baker, E. van Tuinen, and R. Schekman, submitted).

Both the measurement of invertase transport rates and the electron microscopic examination monitor characteristics of cells grown continuously in the absence of clathrin. With a complete molecular clone of *CHC1*, it should be possible to construct conditional mutations. Phenotypic characterization of conditionally mutant strains should more precisely define the immediate consequences of a clathrin heavy-chain deficiency.

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