

Sequence of the Clathrin Heavy Chain from *Saccharomyces cerevisiae* and Requirement of the COOH Terminus for Clathrin Function

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Abstract. The sequence of the clathrin heavy chain gene, *CHC1*, from *Saccharomyces cerevisiae* is reported. The gene encodes a protein of 1,653 amino acids that is 50% identical to the rat clathrin heavy chain (HC) (Kirchhausen, T., S. C. Harrison, E. P. Chow, R. J. Mattaliano, R. L. Ramachandran, J. Smart, and J. Brosius. 1987. *Proc. Natl. Acad. Sci. USA.* 84:8805–8809). The alignment extends over the complete length of the two proteins, except for a COOH-terminal extension of the rat HC and a few small gaps, primarily in the globular terminal domain. The yeast HC has four prolines in the region of the rat polypeptide that was proposed to form the binding site for clathrin light chains via an α -helical coiled-coil interaction. The yeast protein also lacks the COOH-terminal Pro-Gly rich segment present in the last 45 residues of the rat HC, which were proposed to be involved in the noncovalent association of HCs

to form trimers at the triskelion vertex. To examine the importance of the COOH terminus of the HC for clathrin function, a HC containing a COOH-terminal deletion of 57 amino acids (HCA57) was expressed in clathrin-deficient yeast (*chcl-Δ*). HCA57 rescued some of the phenotypes (slow growth at 30°, genetic instability, and defects in mating and sporulation) associated with the *chcl-Δ* mutation to normal or near normal. Also, truncated HCs were assembled into triskelions. However, cells with HCA57 were temperature sensitive for growth and still displayed a major defect in processing of the mating pheromone α -factor. Fewer coated vesicles could be isolated from cells with HCA57 than cells with the wild-type HC. This suggests that the COOH-terminal region is not required for formation of trimers, but it may be important for normal clathrin-coated vesicle structure and function.

CLATHRIN-COATED vesicles (CV)¹ are involved in the transfer of membranes and membrane associated components in the endocytic and secretory pathways (for review see Brodsky, 1988). In particular, clathrin serves to concentrate and sort selected receptors and other proteins from the general flow of membrane transport during receptor-mediated endocytosis, regulated secretory granule formation, and sorting of acid hydrolases to the lysosome. The surfaces of coated membranes and CV display a distinctive polyhedral lattice (Crowther et al., 1976) composed of clathrin triskelions, which contain three heavy chains (HC) of $M_r \sim 180,000$ and three light chains (LC) of M_r 30,000–40,000 (Pearse, 1976; Kirchhausen and Harrison, 1981; Ungewickell and Branton, 1981). The three HCs associate noncovalently to form a vertex with arms extended radially ~ 450 Å in a pinwheel-like structure (Ungewickell and Branton, 1981). Each arm has a globular terminal domain at the tip and an apparent bend or joint about halfway along its length that defines regions ~ 200 Å long proximal and distal to the

bend. LCs, which have a series of heptad repeats typical of proteins that associate with other polypeptides in an α -helical coiled-coil configuration (Kirchhausen et al., 1987a), bind noncovalently along the proximal region of each arm (Ungewickell, 1983). (See Fig. 1 for triskelion model.)

EM of triskelions assembled in cage lattices indicate that each HC extends from a vertex along two polyhedral edges (see Pearse and Crowther, 1987 for review). The terminal domains reside near the second vertex from the trimer center, pointing inward toward the center of the lattice (Vigers et al., 1986a). Thus each facet edge of the lattice is formed by the interactions of two proximal and two distal arm segments, and near each vertex resides a trimer center, three terminal domains, and three HC joint regions. In CV the terminal domains contact another major CV component (Vigers et al., 1986b), referred to as clathrin-associated proteins (Kirchhausen et al., 1989), assembly proteins (Zaremba and Keen, 1983), or adaptins (Pearse, 1988). It has been proposed that the clathrin-associated proteins provide the specific link between the clathrin coat and the cytoplasmic domains of receptors destined for entrapment in CV (Pearse, 1988).

1. *Abbreviations used in this paper:* CV, clathrin-coated vesicle; HC, heavy chain; LC, light chain; ORF, open reading frame.

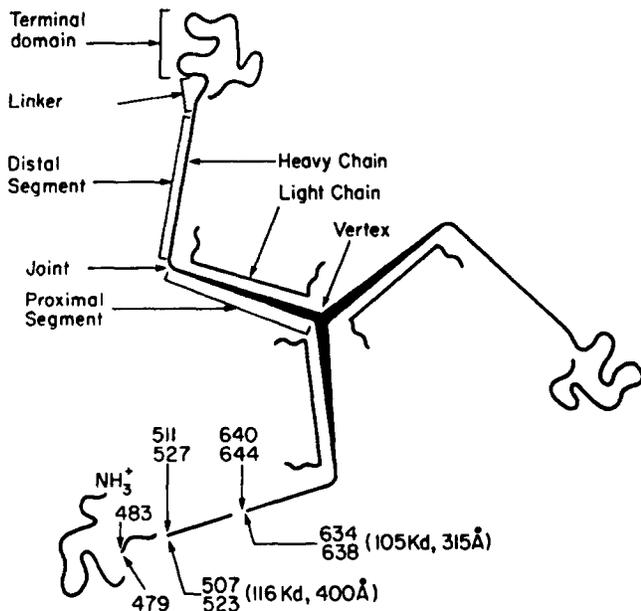


Figure 1. Triskelion model. The triskelion is shown with its three heavy chain arms extending radially from the vertex and the associated light chains. The lower arm of the triskelion shows the amino terminal residues of several of the bovine HC peptides sequenced by Kirchhausen et al. (1987b). Residue 479 corresponds to the position of the linker fragment; 507/523 corresponds to a fragment of 116 kD, which extends ~ 400 Å from the trimer center; 634/638 corresponds to the mammalian fragment of 105 kD, which extends ~ 315 Å from the trimer center. (The fragment sizes indicated are SDS-PAGE estimates.) Also shown are the amino acid numbers from the yeast clathrin HC sequence (residues 483, 511/527, and 640/644, respectively) as they align with the mammalian HC proteolytic fragments. (Drawing adapted from Fig. 4. Kirchhausen et al. [1987b]).

Recently Kirchhausen et al. (1987b) deduced the amino acid sequence of the rat clathrin HC from cDNA clones. The protein contains 1,675 amino acids and has a molecular weight of 191,569. The globular terminal domain is encoded by the amino-terminal one-third of the protein and the carboxy terminus of the HC is oriented towards the trimer vertex (Fig. 1). The rat HC has a Pro-Gly rich segment in the COOH terminus of the protein, which was postulated to be involved in the non-covalent association of the three HCs at the triskelion vertex (Kirchhausen et al., 1987b).

Our laboratory has been studying clathrin in the yeast *Saccharomyces cerevisiae*. Overall, the yeast and mammalian clathrins share many physical properties, including (a) size and structure; (b) ability of clathrin coats to assemble and disassemble in vitro under similar buffer conditions; (c) patterns of peptide fragmentation upon proteolysis of cage lattices (Mueller and Branton, 1984; Payne and Shekman, 1985; Lemmon et al., 1988). Here we report the nucleotide sequence of the yeast clathrin HC gene with its deduced amino acid sequence and show that the physical similarity of clathrin from distantly related organisms extends to the primary sequence of the HCs. One of our major goals is to take advantage of the yeast system to investigate the function of particular HC regions in determining clathrin/CV structure and in mediating vesicular transfer. In this article we exam-

ine the importance of the COOH terminus of the HC. We show that the last 57 amino acids of the yeast protein are not required for triskelion formation but that this region plays a role in coated vesicle function.

Materials and Methods

Media

YEPD and synthetic media for yeast cultures were prepared and used as described in Jones and Lam (1973). For YEPG plates, 5% glycerol replaced the glucose in YEPD. PSP sporulation medium contained 1% potassium acetate, 0.8% nutrient broth, 1% yeast extract (Difco Laboratories, Detroit, MI). KAC sporulation medium contained 1.5% potassium acetate, 0.25% yeast extract and 0.1% dextrose. Agar (1.4%) (Sigma Chemical Co., St. Louis, MO) was added to PSP or KAC media for plate sporulation. Minimal KAC contained 1.5% potassium acetate. Canavanine medium was arginine omission synthetic medium with 60 mg/liter canavanine sulfate added.

Strains and Genetic Methods

A list of strains used in this study is shown in Table I. Procedures for routine mating, tetrad dissection, and scoring of nutritional markers were essentially as described by Mortimer and Hawthorne (1969). Yeast strains were grown at 30°C except where noted. Diploid cells were sporulated on PSP or KAC plates for 5–7 d at 30°C for routine generation of asci for dissection. Two liquid sporulation protocols, which optimized sporulation of diploid strains in this study, were used. In one method, diploids were inoculated from an overnight YEPD culture into liquid KAC at $\sim 1 \times 10^7$ cells/ml, rotated on a roller drum at 30°C and examined after 4–5 d for asci formation using a phase-contrast light microscope. In the second protocol, diploids from an overnight culture grown in YEPD broth were inoculated at ~ 0.4 – 0.5×10^7 cells/ml PSP medium. After 2 d incubation on a roller drum at 30°C, PSP cultures were inoculated into minimal KAC at $\sim 1 \times 10^7$ cells/ml. Cells were examined after 2–3 d further incubation at 30°C.

The canavanine resistance (Can^r) test used to monitor increased genome content was performed by the "indirect" Can^r test as described previously (Lemmon et al., 1990). Briefly, each canavanine-sensitive (Can^s) strain to be examined was mass mated to a haploid Chc⁺ Can^r strain of the opposite mating type (BJ2549 or BJ2651), and prototrophic colonies were selected on minimal medium. Approximately 30 prototrophic colonies from each mass mating were tested on canavanine plates for their ability to give Can^r papillae by mitotic recombination. Since canavanine sensitivity is dominant to canavanine resistance, only zygotes formed from mating of a 1n Can^s cell to the 1n Can^r tester papillated extensively. If the Can^s cell that mated was $\geq 2n$, then no or few Can^r derivatives were obtained from the zygote, since ≥ 2 recombinatorial events would be required to become Can^r.

Quantitative Mating Tests

The mating assay was based on that of Michaelis and Herskowitz (1988). Strains to be tested were grown in YEPD overnight to a density of 1 – 5×10^7 cells/ml. 5 ml of each culture were sonicated two times for 5 s each at 55 W using a Branson sonifier equipped with a microprobe to disperse clumps. This was most important for analysis of Chc⁻ strains, which grow in small aggregates (Lemmon et al., 1990). Approximately 1×10^7 cells were pelleted, resuspended in 1.0 ml fresh YEPD, and diluted in YEPD. Tester strains (BJ3039 or BJ3250) were grown overnight to 2 – 5×10^7 cells/ml in YEPD. Cells were pelleted and resuspended in fresh YEPD at 5×10^8 /ml. 100 μ l of diluted cells ($\sim 10^2$ – 10^7) were combined with 200 μ l tester cells of the opposite mating type ($\sim 1 \times 10^8$) and plated directly on minimal medium. Prototrophic colonies were counted after 3 d incubation at 30°C for most matings; however for mating of Chc⁻ to Chc⁻ strains, colonies were counted after 4 d incubation. Viability counts were obtained from platings on YEPD or complete. Cell counts were identical on either medium. Duplicate samples for matings and viability were averaged and mating frequency calculated as a ratio of matings per viable cells plated.

Mating Pheromone Secretion

Production of α -factor was monitored by a halo assay (Michaelis and Herskowitz, 1988). Patches of cells to be tested were replicated onto YEPD plates spread with 10^6 cells of the MATa tester strain, RC669. RC669

Table I. *Saccharomyces cerevisiae* Strains

Strain	Genotype*	Notes, source, or reference†
BJ2649	<i>MATα lys2 trp5 Can^r</i>	
BJ2651	<i>MATα lys2 trp5 Can^r</i>	
BJ3038	<i>MATα lys2 trp5</i>	
BJ3039	<i>MATα lys2 trp5</i>	
BJ3119	<i>MATα/MATα <i>chc1</i>-Δ::<i>LEU2/CHC1 leu2/leu2 ura3-52/URA3 trp1/TRP1 his1/HIS1 ade6/ADE6 scd1^S/SCD1^{NS}</i></i>	Lemmon and Jones, 1987
BJ3133	<i>MATα/MATα <i>chc1</i>-Δ::<i>LEU2/CHC1 leu2/leu2 ura3-52/ura3-52 trp1/TRP1 his1/HIS1 ade6/ADE6 scd1^S/SCD1^{NS}</i></i>	—§
BJ3223	<i>MATα CHC1 leu2 ade6 SCD1^{NS}</i>	Segregant of BJ3119
BJ3235	<i>MATα <i>chc1</i>-Δ::<i>LEU2 leu2 trp1 ade6 scd1^S</i></i>	Segregant of BJ3119
BJ3243	<i>MATα CHC1 leu2 ura3-52 SCD1^{NS}</i>	Segregant of BJ3119
BJ3247	<i>MATα <i>chc1</i>-Δ::<i>LEU2 leu2 trp1 his1 ade6 scd1^S</i></i>	Segregant of BJ3119
BJ3250	<i>MATα <i>chc1</i>-Δ::<i>LEU2 leu2 ura3-52 scd1^S</i></i>	Segregant of BJ3119
BJ3324	<i>MATα <i>chc1</i>-Δ::<i>LEU2 leu2 ura3-52 trp1 his1 scd1^S pSL6</i></i>	Segregant of BJ3358
BJ3358	BB3133 transformed with pSL6	
BJ3529	BJ3530 without pSL6 (obtained by plasmid loss)	
BJ3530	<i>MATα/MATα <i>chc1</i>-Δ::<i>LEU2/CHC1 leu2/leu2 ura3-52/ura3-52 his1/HIS1 ade6/ADE6 SCD1^{NS}/SCD1^{NS} pSL6</i></i>	Lemmon and Jones, 1987
BJ3556	<i>MATα <i>sst1-2 ade2-1 his6 met1 cyh2 rme1 ura1 can1</i></i>	RC665; Chan and Otte, 1982
BJ3579	<i>MATα <i>chc1</i>-Δ::<i>LEU2 leu2 ura3-52 his1 SCD1^{NS} pSL6</i></i>	Segregant of BJ3530
BJ3583	<i>MATα <i>chc1</i>-Δ::<i>LEU2 leu2 ura3-52 his1 SCD1^{NS} pSL6</i></i>	Segregant of BJ3530
BJ4045	<i>MATα <i>chc1</i>-Δ::<i>LEU2 leu2 ura3-52 his1 SCD1^{NS} pΔ57</i></i>	Segregant of BJ4326
BJ4046	<i>MATα <i>chc1</i>-Δ::<i>LEU2 leu2 ura3-52 trp1 ade6 scd1^S</i></i>	Segregant of BJ4315
BJ4053	<i>MATα <i>chc1</i>-Δ::<i>LEU2 leu2 ura3-52 trp1 his1 scd1^S pΔ57</i></i>	Segregant of BJ4315
BJ4054	<i>MATα <i>chc1</i>-Δ::<i>LEU2 leu2 ura3-52 his1 SCD1^{NS} pΔ57</i></i>	Segregant of BJ4315
BJ4055	<i>MATα CHC1 leu2 ura3-52</i>	Segregant of BJ4315
BJ4057	<i>MATα CHC1 leu2 ura3-52 trp1 his1 pΔ57</i>	Segregant of BJ4315
BJ4315	BJ3133 transformed with p Δ 57	
BJ4317	<i>MATα/MATα <i>chc1</i>-Δ::<i>LEU2/CHC1 leu2/leu2 ura3-52/ura3-52 trp1/TRP1 ade6/ADE6 scd1^S/scd1^S</i></i>	—
BJ4326	BJ3529 transformed with p Δ 57	
BJ4328	<i>MATα <i>chc1</i>-Δ::<i>LEU2 leu2 ura3-52 his1 SCD1^{NS} pΔ57</i></i>	Segregant of BJ4315
BJ4333	<i>MATα <i>chc1</i>-Δ::<i>LEU2 leu2 ura3-52 his1 SCD1^{NS} pΔ57</i></i>	Segregant of BJ4315
BJ4606	BJ4317 transformed with p Δ 57	
SL174	<i>MATα <i>chc1</i>-Δ::<i>LEU2 ura3-52 ade6 scd1^S pΔ57</i></i>	Segregant of BJ4606
SL176	<i>MATα <i>chc1</i>-Δ::<i>LEU2 ura3-52 trp1 ade6 scd1^S pΔ57</i></i>	Segregant of BJ4606
SL180	<i>MATα <i>chc1</i>-Δ::<i>LEU2 leu2 ura3-52 his1 ade6 SCD1^{NS} pΔ57</i></i>	Segregant of BJ4326

* *SCD1^{NS}* and *scd1^S* are indicated only where known or inferred. Haploid genotypes of *Chc^r* strains do not reflect possible changes in genome copy number (Lemmon et al., 1990). Except for BJ2649, BJ2651, BJ3038, BJ3039, and BJ3556, all other strains are derivatives of BJ3068 (Lemmon and Jones, 1987; Lemmon et al., 1990), which is congenic to X2180-1B (*MAT α gal2 SUC2*).

† Except where indicated, strains are from this study or this laboratory.

§ BJ3133 is a *ura3-52/ura3-52* derivative of BJ3119 obtained by the 5-fluoro-orotic acid selection procedure of Boeke et al. (1984).

|| BJ4317 was made by crossing a *CHC1 scd1^S* spore segregant from BJ3119 to a *chc1*- Δ ::*LEU2 scd1^S* pSL6 spore segregant from BJ3358, after which pSL6 was lost from the diploid by growth on nonselective medium.

(BJ3556) carries the *sst1-2* mutation, which renders it supersensitive to α -factor.

Construction of Plasmids

YCp50-*CHC1* (pSL6), which contains the complete clathrin HC gene (*CHC1*) from *S. cerevisiae* has been described previously (Lemmon and Jones, 1987). pAP4, a *TRPI-CEN4* vector containing the complete HC gene, was made by cloning the 8.15 kb Pvu II-Sal I *CHC1* fragment from pSL6 into the Sma I-Sal I sites of the polylinker of pUN30 (Elledge and Davis, 1988). p Δ 57 contains *CHC1* with a deletion of all sequences downstream of the 3' Eco RI site in the open reading frame (ORF) of the HC gene. This deletion results in truncation of the 57 COOH-terminal amino acids of the HC. p Δ 57 was made by first cloning the 0.95-kb Eco RI fragment from the COOH-terminal region of the ORF into the unique Eco RI site of YCp50 (Kuo and Campbell, 1983) to generate pCF1. The orientation of the *CHC1* reading frame in the insert was opposite from the direction of transcription of the the tetracycline resistance gene. pCF1 was digested with

Bam HI and the 6.2-kb Bam HI *CHC1* fragment was inserted to reconstruct the 5' *CHC1* sequences. The resulting plasmid (p Δ 57) includes \sim 1.4 kb of sequences upstream of the AUG translational start site and encodes 1,596 amino acids of the ORF. Fusion to vector sequences at the 3' Eco RI site generated an immediate translational UGA stop codon.

DNA Sequencing

Four overlapping DNA fragments that include the region encoding the clathrin HC were subcloned from pSL6 into M13mp18 and M13mp19 (Yanisch-Perron et al., 1985). A nested deletion set for each subclone was made according to the method of Dale et al. (1985). The region subcloned started from a 5' Hind III site \sim 0.92 kb upstream of the ORF to a 3' Hind III site \sim 1.2 kb downstream of the termination codon. DNA sequencing was performed by the dideoxy chain termination procedure (Sanger et al., 1977) using the Sequenase sequencing kit from U.S. Biochemical Corp. (Cleveland, OH) and [α -³⁵S]dATP from New England Nuclear (Boston, MA). The sequence shown in Fig. 2 was obtained on both strands from the dele-

tion set. Payne et al. (1987) sequenced a portion of the 5' region of the gene and identified the same translational start site. Additional partial sequence upstream of *CHCI* indicated that the nearest ORF begins ~650 nucleotides 5' of the *CHCI* initiation AUG and is divergently transcribed (not shown). This ORF encodes the peroxisomal enzyme fatty acyl-CoA oxidase (*POXI* gene product) which maps between *CHCI* and *KEXI* (Dmochowska et al., 1990). *KEXI* is 4–5 kb centromere proximal to *CHCI* (Lemmon and Jones, 1990). *POXI* is not essential for viability of yeast and disruption deletion mutants display normal growth on glucose medium (Dmochowska et al., 1990).

The yeast HC amino acid sequence was compared with the sequences deposited in the National Biomedical Research Foundation protein data bank and the Swiss Protein Database. Alignment of the rat HC with the yeast HC protein sequence was performed using the program of Wilbur and Lipman (1983) with a K-tuple size of 1 and a gap penalty of 2.

Radiolabeling and Immunoprecipitation

Cells were grown at 30°C to midlogarithmic phase in Wickerham's minimal medium (Wickerham, 1946) containing 0.1 mM MgSO₄. Cells (2 × 10⁷) were washed twice, resuspended in 1.0 ml Wickerham's minimal medium lacking sulfate salts and containing 1.0 mg/ml BSA. After preincubation at 30°C for ~30 min, cells were pulse labeled for 5 min with 0.5–1.0 mCi of carrier-free ³⁵SO₄²⁻ (ICN Biochemicals, Irvine, CA) and chased for various times with 3 mM (NH₄)₂SO₄ and 0.01% each of cysteine and methionine. To terminate the chase, samples (0.25 ml) were removed and combined with 0.25 ml of a stop solution (Wickerham's minimal medium containing the chase mixture, 1.0 mg/ml BSA, and 0.04% sodium azide) and placed on ice. Cells and culture medium were separated by centrifugation and the cell pellet was resuspended in 0.5 ml of stop solution. Samples were adjusted to a final concentration of 5% TCA and subjected to the double immunoprecipitation procedure of Klionsky et al. (1988). 1 μl of α-factor antiserum (Rothblatt and Meyer, 1986) was used in immunoprecipitations. Samples were run on 12.5% SDS gels, which were fixed, treated with Autofluor (National Diagnostics), dried and exposed to x-ray film at -70°C. A laser densitometer (LKB 2400 Ultrascan XL; LKB Instruments, Gaithersburg, MD) was used for analysis of autoradiograms to quantitate α-factor processing and secretion.

Biochemical Procedures

Total yeast extracts were prepared for immunoblot analysis by glass bead homogenization from cells grown in YEPD to 1–5 × 10⁷ cells/ml as described previously (Lemmon et al., 1988). 40 μg of protein from extracts were fractionated on 7.5% SDS polyacrylamide gels and transferred to nitrocellulose (Schleicher & Schuell, Keene, NH). Filters were stained with Ponc-5 (Sigma Chemical Co.). Immunoblots were developed using a pool of eight antiyeast clathrin HC mAbs as probes (Lemmon et al., 1988) and the Vectastain anti-mouse immunostaining kit from Vector Laboratories (Burlingame, CA) or alkaline phosphatase goat anti-mouse IgG (Zymed Laboratories).

For analysis of triskelions, yeast cells were grown overnight in YEPD to 1–5 × 10⁷ cells/ml. Approximately 6 × 10⁹ cells were harvested by centrifugation and washed once with water and once with triskelion extraction buffer which contains a 1:1 volume ratio of 1.0 M Tris-HCl, pH 7.0; buffer A (0.1 M MES, pH 6.5, 0.5 mM MgCl₂, 1.0 mM EGTA, 0.2 mM DTT, 0.02% NaN₃, 0.6 mM PMSF). The final cell pellet was resuspended in 1–2 ml extraction buffer and homogenized three minutes with 0.45 μm glass beads using a Braun MSK homogenizer with small sample adapter (Needleman and Tzagoloff, 1975). The homogenate was centrifuged at 27,000 g and the resultant supernatant was spun at 100,000 g for 1 h. A sample (0.5–0.75 ml) of the 100,000 g supernatant was applied to a Sepharose CL-4B gel filtration column (0.7 × 28 cm) and eluted with triskelion extraction buffer by gravity flow at ~3 ml/h. 30-drop fractions were collected and absorbance at 280 nm was read. Samples (40 μl) of fractions were subjected to SDS-PAGE, which was followed by transfer to nitrocellulose for immunoblot analysis as described above.

Other Methods

Routine cloning and DNA manipulations were performed by standard procedures essentially as described in Maniatis et al. (1982). Yeast cells were transformed by the method of Ito et al. (1983) with minor modifications. Coated vesicles and purified yeast clathrin trimers were prepared as described previously (Lemmon et al., 1988). Protein assays were performed using the pro-

tein assay kit from Bio-Rad Laboratories (Richmond, CA). For growth studies in liquid culture and estimates of doubling time, YEPD broth was inoculated at 1–2 × 10⁵ cells per ml from a log phase preculture grown at 30°C.

Results

Sequence Analysis of *CHCI* and Comparison of the Yeast and Rat HCs

CHCI has one long ORF of 4,959 nucleotides and encodes a protein of 1,653 amino acids (187,232 D) with a predicted pI of 4.91 (Fig. 2). There are two AUG codons (codons 1 and 15) near the beginning of the ORF. The first AUG is the likely start of the ORF, since a polypeptide beginning at this codon gives the best alignment with the rat HC (Fig. 3).

The yeast and the rat clathrin HC (Kirchhausen et al., 1987b) amino acid sequences are 50% identical, with >70% similarity if conservative amino acid changes are considered. There are some stretches of extensive homology (>70% identical), which are presumably structurally and/or functionally important. As with the rat HC, no internal repeats were identified and a search of sequence data bases revealed no significant homologies to other proteins. The alignment of the rat and yeast proteins extends over their complete length, except for a COOH-terminal extension of the rat HC. The rat HC is 1,675 amino acids long and has a predicted molecular weight of 191,569. The smaller calculated size of the yeast HC was a surprise, since the yeast protein migrates on SDS gels with a larger apparent molecular weight than both the bovine (Mueller and Branton, 1984) and rat HCs (not shown). Anomalous migration on gels could be caused by posttranslational modification of the HC. However, the only report to date of a HC modification (other than NH₂-terminal blockage) was the phosphorylation of HCs in chicken embryo fibroblast cells transformed by Rous sarcoma virus (Martin-Perez et al., 1989), and there were no obvious changes in the electrophoretic pattern of the HC in SDS gels in those studies. Thus, the cause of the unexpected migration pattern remains unknown, but it is most likely a gel artifact caused by the polypeptide sequences themselves.

Proteolysis of clathrin cages results in cleavage of the HC arm between the terminal domain and the major portion of the extended arm (Schmid et al., 1982; Kirchhausen and Harrison, 1984). Kirchhausen et al. (1987b) sequenced several peptides that resulted from such proteolysis and mapped the terminal domain to the amino terminus of the HC with the COOH terminus oriented towards the trimer center. They also identified sequence positions defining a flexible connector region (linker) between the terminal domain and the extended arm, as well as the amino termini of arm fragments residing ~400 Å and ~315 Å from the trimer center (see Figs. 1 and 3). Yeast cages yield a similar proteolytic pattern after trypsinization (Lemmon et al., 1988). Although the comparable yeast peptide fragment have not been sequenced, it should be noted that rat HC Arg-523 (~400 Å from the trimer center) and Arg-638 (~315 Å from the trimer center) are conserved in the yeast protein (Arg-527 and Arg-644, respectively). Most of the gaps required to align the yeast and rat HCs reside in the globular terminal domain or arm tips. Strick linear conservation of sequence spacing may be more crucial in the extended region of the HC, which has extensive contacts with other triskelion arms in clathrin lattices.

Light chains bind noncovalently along the proximal region of the HC arm (Ungewickell, 1983). Sequence analysis of LC cDNA clones indicated the presence of a series of heptad repeats containing neutral or hydrophobic amino acids in positions 1 and 4 of each heptad (Kirchhausen et al., 1987a). These are signatures of an α -helix that associates with another α -helix in a coiled-coil configuration (Cohen and Parry, 1986). Homology to intermediate filament proteins, which are known to associate in such a coiled-coil structure, was also noted in the heptad repeat region of LCs (Jackson et al., 1987). A weak pattern of 11 repeats was identified in the rat HC (amino acids 1,107–1,184, Fig. 3) (Kirchhausen et al., 1987b), that was postulated to form the site of LC binding by an α -helical coiled-coil association. Yeast amino acids 1,113–1,190 correspond to the region that aligns with the rat heptad repeats. There are 34/78 amino acid matches (44% identity) between the sequences; however, the yeast protein contains prolines in heptads 3, 4, 7, and 9, which could cause breaks in the helix, and acidic residues in the fourth position of heptads 2 and 5. Only the proline in "heptad" 4 is conserved in the rat HC. Thus the yeast protein does not have a weak heptad repeat in this region of the HC. Examination of α -helical regions of the yeast HC predicted from secondary structure analysis programs (Chou and Fasman, 1974; Garnier et al., 1978) did not reveal other long heptad repeat regions.

The HC COOH-terminal region is oriented towards the trimer vertex (Kirchhausen et al., 1987b). The last 45 amino acids of the rat HC contains 10 prolines, 7 glycines, and no charged amino acids. This unusual sequence led to the hypothesis that this region is involved in trimerization of clathrin HCs, which have a strong noncovalent association (Kirchhausen et al., 1987b). However, the yeast and rat molecules show striking differences in this segment. The rat HC has a COOH-terminal extension of 22 amino acids relative to the yeast HC, even when a major gap in the yeast protein is permitted to allow the alignment of GQP-LML to the rat sequence GQPQLML. There are only two prolines and two glycines in this region of the yeast protein, although it has no charged amino acids. These findings suggested that the sequences required for yeast HC trimerization are different than those postulated for the rat HC.

Effect of Truncation of the HC COOH Terminus

To directly test the possibility that the COOH terminus is not required for yeast HC trimerization, we generated a mutant HC gene encoding a protein missing the last 57 amino acids. The truncated HC gene cloned into the *URA3-CEN4* plasmid YCp50 (p Δ 57) was introduced into heavy chain-deficient yeast to test for clathrin function. The test we used took advantage of our previous findings that an independently segregating gene, suppressor of clathrin deficiency (*SCD*), influenced the ability of cells carrying a deletion of *CHCI* to survive (Lemmon and Jones, 1987). In the presence of the *SCD*^{NS} (nonsuppressing) allele, cells lacking clathrin HCs are inviable; in the presence of the *scd*^S (suppressing) allele, clathrin-deficient cells are viable but grow poorly. p Δ 57 was introduced into diploid BJ3529 (*SCD*^{NS}/*SCD*^{NS}) and a Ura⁺ transformant (BJ4325) was selected. One HC gene of BJ3529 is wild type, while the other is the disruption deletion marked by insertion of the *LEU2* gene (*chcl*- Δ ::*LEU2* or *chcl*- Δ). Tetrads from BJ3529 yield two Leu⁻ Chc⁺ spores

and two dead (presumably Leu⁺ Chc⁻) spores (Lemmon and Jones, 1987). Leu⁺ Chc⁻ spores can be rescued from lethality if BJ3529 is transformed with YCp50-*CHCI* (Lemmon and Jones, 1987), but are not rescued with the parent plasmid, YCp50 (Lemmon, S., unpublished results). Therefore, if p Δ 57 encodes a functional HC, it would be expected to rescue Chc⁻ spores, and Leu⁺ Ura⁺ spore clones would be recovered from BJ3529 transformed with the plasmid.

In analysis of 10 tetrads from BJ4325 (BJ3529 with p Δ 57), 19 Leu⁻ Ura⁺; 11 dead; 0 Leu⁺ Ura⁻ and 10 Leu⁺ Ura⁺ segregants were recovered. 10 of the "dead" segregants germinated and died after 4–5 doublings. This is typical of *chcl*- Δ ::*LEU2 SCD*^{NS} spores, which probably obtain clathrin and/or *CHCI* mRNA from the diploid parent (Lemmon and Jones, 1987). One spore did not germinate and was presumably Leu⁻, since only 19 Leu⁻ spores were recovered. Preliminary examination of the Leu⁺ Ura⁺ (*chcl*- Δ p Δ 57) segregants indicated that they grew well at 30°C and formed colonies almost as large or as large as *CHCI* cells. The growth was clearly better than Chc⁻ (*chcl*- Δ *scd*^S) cells. These results indicated that the COOH terminus of the yeast HC could be deleted and retain clathrin function.

Phenotypic Analysis of Cells Carrying the Truncated HC

To determine whether the truncated HC (HC Δ 57) showed full complementation of the *chcl*- Δ mutation, we performed a number of phenotypic analyses on *chcl*- Δ *SCD*^{NS} p Δ 57 cells and a parallel set of *chcl*- Δ *scd*^S p Δ 57 strains. In all studies minor phenotypic difference of cells with p Δ 57 could not be attributed to the *SCD* genotype of strains.

Growth of Strains Carrying the Truncated HC

Doubling times of COOH-terminal deletion mutants grown in liquid YEPD at 30°C were 2–2 1/2 h (Fig. 4), which was almost as fast as cells containing wild-type clathrin (doubling times were 1 1/2–2 h for cells with *CHCI*) (Fig. 4) but much more rapid than *chcl*- Δ *scd*^S cells (doubling times of 4–8 h [Lemmon and Jones, 1987]).

Typically *chcl*- Δ p Δ 57 strains showed temperature sensitive growth in plate tests. Patches of *chcl*- Δ p Δ 57 strains grew as well as wild type at 30°C on YEPD, but poorly at 37°C (Fig. 5). When p Δ 57 strains were grown in liquid YEPD at 37°C, they underwent two to three more doublings after temperature shift before arresting growth (Fig. 4), which was consistent with the formation of sparse patches on plates at 37°C (Fig. 5). The COOH-terminal deletion mutants were clearly distinguished from Chc⁻ cells (*chcl*- Δ *scd*^S) by the reduced growth of the latter at 30°C on YEPD plates and almost complete lack of growth on YEPG at this temperature (see Fig. 5, spore 3C).

The temperature sensitivity of HC Δ 57 mutants could be rescued by transformation with the *CHCI* plasmid, pAP4 (Fig. 4), but not by parent *TRP1-CEN4* plasmid, pUN30 (not shown). Cells expressing both the wild-type and mutant protein grew as well as wild-type strains in liquid culture (Fig. 4) and in plate tests (Fig. 5, spore clones 3B and 3D) indicating that the 57-amino acid deletion mutation is recessive when present in single-copy dosage. We have not tested the effect of overexpression of the mutant HC. Occasionally a *chcl*- Δ p Δ 57 spore segregant showed only a modest tem-

-300	AAAGGAGGTTTATAATAAAATATTAGCACATTATAGGTTTAAAGTAAAAAATAATAGGGTAAATTTTATTACCTACCCTTTTGGTCGAGATC	-201
	TCGCATTTTTCGTCGCTAGGAATAAATCAAAAGAGAATTAAGAAAAGTAAAGTGGAACTGAAGGAATCTACAGAAATAAGGAGATATTTGATAGTT	-101
	GTTCAGAGTAACTTATTGAACCTCGACAATCCGGATCAGAGTTGAGAGAGAAAGAAGATATTCAACTATAAAAATAAATATCACGAAAGTTCTAACCA	-1
	ATGAGTGACCTACCCATTGAATTTACCGAATTTGGTCGATCTGATGTCTTAGGAATTTCCCTCAATTCCTTGACTTCAGATCAACTACTTTTCGAGAGTG	100
	M S D L P I E F T E L V D L M S L G I S P Q F L D F R S T T F E S D	34
	ACCACTTCGTCCTGTTAGAGAAACAAGGACGGTACAAACTCTGTTGCAATCGTGGATTTGGCCAAAGGCAATGAAGTGACAAGGAAGATATGGGGGG	200
	H F V T V R E T K D G T N S V A I V D L A K G N E V T R K N M G G	67
	TGATTCGCTATCATCCATCTCAGATGGTTATTTCCGTTAGAGCAAAATGGTACTACTCGTGKAGATATTTAATTTGGAACTAAGAGCAAGTTAAAG	300
	D S A I M H P S Q M V I S V R A N G T I V Q I F N L E T K S K L K	100
	TCTTTACTTTTAGATGAACCTGTTATTTTTGGAGGTTGAGTGAAACCACTTTGGGTTTGTACCAGCAAGATCCATCTGACCTCGAACCTGCTTTG	400
	S F T L D E P V I F W R W L S E T T L G F V T A R S I L T S N V F D	134
	ATGGTAAAGTAAATGCTAAGCCTCAATTTGCTTACCTGAGACACGCTAACTTGAACAATACCCAAATTAATTTTGTGGCTAACAAAACCTTGACTG	500
	G N V N A K P Q L L T L R H A N L N N T Q I I N F V A N K N L D W	167
	GTTCCGAGTGTGGTATCTTACAAGAAAATGGCCGATTTGGCCGTAGAATTAATTTCTCAAACAACGTAACATCTCCCAAGCTATCGACGGTCAT	600
	F A V V G I L Q E N G R I A G R I Q L F S K Q R N I S Q A I D G H	200
	GTTGCTATCTTACGAATATATTACTAGAGGGTAATGGCTCGACCCGGTACAAGTTTTCTGTACTGGTACAGAAATGCCACCACGGTGTGGAGAAT	700
	V A I F T N I L L E G N G S T P V Q V F V T G N R N A T T G A G E L	234
	TAAGAATTAACGAGATTGATCAGGATGCTTCAATTAACAAGGAAACTACCGATATTTCTTCCACCAGATGCAACGATGATTTCCCT	800
	R I I E I D H D A S L P S Q Y Q K E T T D I F F P P D A T N D F P	267
	AATCGCTGTTGAGGTGCTGAGAAATACGGCATAATTTACCTATTAACCAAGTATGGCTTCATCCATTTGTACGAATGGAAACCGGTACCAATTTATTCT	900
	I A V Q V S E K Y G I I Y L L T K Y G F I H L Y E L E T G T N L F	300
	GTTAATAGAATTACAGCAGAACTGTTTTACTGCAGCTCCGTACAATCAGAGAACGGTATTCATGCATCAATAAAAAAGGTCAGTTTAGCAGTAG	1000
	V N R I T A E S V F T A A P Y N H E N G I A C I N K K G Q V L A V E	334
	AGATTTCTACTCTCAAAATCGTTCCATATCTTGAATAAATATCTAATGTGGCACTGGCTTAATCGTGTCTACAAGAGGTGGCTTACCAGGTGCAGA	1100
	I S T S Q I V P Y I L N K L S N V A L A L I V A T R G G L P G A D	367
	TGATTTATTTCAAACAAGTGTGAATCTTTATTGTTACAACAACGATTATCAAATGCAGCTAAAGTTGCAGCTCTTCCACTTCTTAAAGGAACCAAAAC	1200
	D L F Q K Q F E S L L L Q N D Y Q N A A K V A A S S T S L R N Q N	400
	ACCATCAACAGGTTGAAAAACATTCAAGCACCACCCAGGCGCCATTTCTCCTATCCTACTTACTTTTCAACTATTGGATAAAGGGAAATGAATAAAG	1300
	T I N R L K N I Q A P P G A I S P I L L Y F S T L L D K G K L N K E	434
	AGGAACAATGAATGGCTAGACCAGTTCTACAACAAGCAGAAAACAAATGTTTCGAAAATGGTAAAGGAAGATAAAGTGAATGCTCAGAAGACT	1400
	E A T I E L A R P V L Q Q D R K L G F E K W L K E D K L E C S E E L	467
	AGGTGACATCGTTAAGCCTTTCGATACTACTTTAGCGCTAGCTTGCTACTTAAGAGCAGGTGCCATGCAAAAGTTATACTTGTCTTGGCCGAATTACA	1500
	G D I V K P F D T T L A L A C Y L R A G A H A K V I S C L A E L Q	500
	CAGTTGAAAAAATATTCTTACTGCCAAAAGGTTGGATATCAACCTAACTTTTTAGTGCTGATTTCTTCTTGGATTAGATCATCACCAGACAGAGCTT	1600
	Q F E K I I P Y C Q K V G Y Q P N F L V L I S S L I R S S P D R A S	534
	CTGAATTTGCGTATCTCTATTACAAAATCCGAAAACCTGCTTCTCAGATCGACATAGAAAATCGCAGATCTATTCTTTCCAAAACCATATCCAACA	1700
	E F A V S L L Q N P E T A S Q I D I E K I A D L F F S Q N H I Q Q	567
	AGGAACCTCTACTTCTAGACCGGTTAAAGGAGATACCCAGACCAAGGTCCTTGCAAAACCTCGTGTCTTGAAGTCAATCTACTACACGCCACCACA	1800
	G T S L L L D A L K G D T P D Q G H L Q T R V L E V N L L H A P Q	600
	GTTGCTGACGCCATATGGGAAACAACATCTTTTCTATTATGACAAAACCACTATTGCTTCTTATCCGAAAAGGCGGACTATACAGAGAGCTTTAG	1900
	V A D A I L G N N I F S H Y D K P T I A S L S E K A G L Y Q R A L E	634
	AAAATTACAACAGACATCAAAGATCAAGAGATGCGTTGTCACACCAATGCATTACCTATCGATTGGTGTAGTCGGATACTTTGGTAAAGTTGAACGTTGA	2000
	N Y T D I K D I K R C V V H T N A L P I D W L V G Y F G K L N V E	667
	ACAATCTTTGGCGTGCTTAAAGCTTAAATGGATAACAATATTACGGCTAACATCCAGACCGTAGTACAGGTGGCAACTAAATCTCTGATTGATCGGT	2100
	Q S L A C L K A L M D N N I Q A N I Q T V V Q V A T K F S D L I G	700
	CCATCTACTTTGATTAATATTTCGAAGATATAATGCTACCGAAGGTTTATACTATTACTTAGCCTCTTTAGTCAACCTAACCGAAGATAAAGATGTGG	2200
	P S T L I K L F E D Y N A T E G L Y Y Y L A S L V N L T E D K D V V	734
	TTTACAAATACATTGAAGCTGCTGCTAAAATGAAACAATACAGAGAAATGAAAGAATTGTGAAGGATAACAATGTCTATGATCCGAAAGAGTCAAGAA	2300
	Y K Y I E A A A K M K Q Y R E I E R I V K D N N V Y D P E R V K N	767
	TTTCTGAAGGATGCCAATCTAGAAGACCAGTTGCCTCTAGTCATAGTTTGTGACCGTTTCGACTTTGTCATGAAATGATTTTATATTTGTACAATCA	2400
	F L K D A N L E D Q L P L V I V C D R F D F V H E M I L Y L Y K S	800
	CAGAACTTAAATTTATTGAACTTACGTGCAGCAGGTTAACCTTCAAAGACTGCCAAGTGTGGCGCATTACTTGTATGGACTGTGATGAGGCAT	2500
	Q N L K F I E T Y V Q Q V N P S K T A Q V V G A L L D M D C D E A F	834

Figure 2. Nucleotide sequence of *CHC1* and derived amino acid sequence of the yeast clathrin heavy chain. The nucleotide sequence is shown with numbers starting from the initiator ATG. The derived amino acid sequence is in single letter code below the nucleotide sequence. The predicted protein (1,653 amino acids) has a molecular weight of 187,232. The two locations marked with Δ flank the region of the *CHC1* deletion disruption (*chc1- Δ :LEU2*) used in these and our prior studies (Lemmon and Jones, 1987; Lemmon et al., 1990). The deletion leaves intact only 102 amino acids of ORF at the COOH terminus of the HC. In frame termination codons (*underlined*) and potential TATA boxes (*overlined*) upstream of the open reading frame are indicated. These sequence data are available from EMBL/GenBank/DBJ under accession number X52900.

TTATTCAAAGTCTGCTACAATCAGTCTTAGGTCAGTCCCTATTAATGAATTAAGTACTGAGGTTGAAAAGAGAAATAGACTAAAATAATTGCTACCTTT I Q S L L Q S V L G Q V P I N E L T T E V E K R N R L K I L L P F	2600 867
CCTAGAACAAGCTTAAGTCAAGGTATCCAGGATCAAGCTGTTTACAATGCTTAGCAAATTTATATTGATTCCAATAATTCTCCTGAAAAGTTTTG L E Q S L S Q G I Q D Q A V Y N A L A K I Y I D S N N S P E K F L	2700 900
AAGGAAAACGACCAATATGACACCTTAGACGTTGGTCATTAAGTCCGAAAAGAGATCCCTACCTGCAATATATTGCGTATGAAAAGGTCAAAATGATG K E N D Q Y D T L D V G H Y C E K R D P Y L A Y I A Y E K G Q N D D	2800 934
ATGACTTGATAGAAATCACAATGAAAATAGCATGTACAAGTACCAGGCAAGATATTTGTTGGAACGTTCTGATCTCGATTTATGGAACAAAGTTTTGAA D L I R I T N E N S M Y K Y Q A R Y L L E R S D L D L W N K V L N	2900 967
TCAAGAAAATATTACAGAAGACAATTAATTGATTCGGTCACTCCGTTGGTATCCCTGAATTAAGTACTGCTGAACTGTTCCCTTAAGTCTCAAGCA Q E N I H R R Q L I D S V I S V G I P E L T D P E P V S L T V Q A	3000 1000
TTCATGACCAATGGATTAAAAGTGAAGTAAATGAGTGTAGAAAAATCATATTGGAACCTTCTCCTTCAACGAAAATGTGGCTTTACAAGGGTTGC F M T N G L K L E L I E L L E K I I L E P S P F N E N V A L Q G L L	3100 1034
TATTATTGCTGCTATTAAGTATGAACCAACTAAGGTTTCAAGCTATATCGAAAATAGATAACTACGACGCGGATGAAATTGCTCCAATTATGATTGA L L S A I K Y E P T K V S S Y I E K L D N Y D A D E I A P L C I E	3200 1067
ACATGATTGAAGGAGGAAGCTTTTGAATCTATGACAAAATGAAATGTACGGTAAAGCTTAAAGGTTTAAATCGAAGACATCATGCTTTAGATAGA H D L K E E A F E I Y D K H E M Y G K A L K V L I E D I M S L D R	3300 1100
GCTGCTTCTTATGCTGATAAATCAATACTCCCGAATTTGGTCTCAAATTTGGTACGGCTCAGTTAGATTGATTAAGAATCCCAGATGCTATTGAGTCTT A A S Y A D K I N T P E L W S Q I G T A Q L D G L R I P D A I E S Y	3400 1134
ATATTAAGCAGAAACCCATCAAATACGAAAATGTAATTTGATAGCCGGAACCGGTAATACGAGAATTAATCCATTTTACTAATGGCTAG I K A E D P S N Y E N V I D I A E Q A G K Y E E L I P F L L M A R	3500 1167
AAAGACTTTGAAAGAACCAAGATCGACGGTGCCTTAATCTTGGCCTATGCTGAACATAAACAAGATTCAATGAAATGAGAACTTGTGGCAGGTTCCAA K T L K E P K I D G A L I L A Y A E L N K I H E I E N L L A G S N	3600 1200
GTTGCAAACTTAGATCATGTTGGTATAAATATTTCGAGAATAAAGAGTATAAAGCGGCCAGGCTATGTTATTCTGCTGTTTCTAATTACTCCAAGCTTG V A N L D H V G D K L F E N K E Y K A A R L C Y S A V S N Y S K L A	3700 1234
CTTCTACCTTGGTTTACTGGGAGATTATCAGGCTGCTGTTGACACTGCTAGAAAGGCTTCAAACATTAAGTTTGGAAAAGTGGTAAACGACGCTTGTAT S T L V Y L G D Y Q A A V D T A R K A S N I K V W K L V N D A C I	3800 1267
TGAAAAGAAAAGAAATCAAGCTGGCTCAAATTTGTTGGGTTGAACCTGATCGTTACGCTGAAAGATTAGATGAACTGGTGGAAAGGATGAATCAAACGGC E K K E F K L A Q I C G L N L I V H A E E L D E L V E R Y E S N G	3900 1300
TATTTGAAAGAACTGATTTCAATTGTTTGAAGCTGGTTTGGGACTTGAAGAGCTCATATGGGCATGTTCACTGAATTGGCCATTTTGTATCCAAATATG Y F E E L I S L F E A G L G L E R A H M G M F T E L A I L Y S K Y E	4000 1334
AACCAGACAAGACCTTCGAACACTGAAAGCTATTCTGGTCCGAATAAATATTCCAAAGGTTATTAGAGCTGTCGAACAAGCTCACCTCTGGTGGGAAT P D K T F E H L K L F W S R I N I P K V I R A V E Q A H L W S E L	4100 1367
GGTCTTTTATATGCTCACTATGATGAATGGGATAATGCTGCTTAACTAATCGAAAATCAACCAAAGACTTGGATCACGCCATTTCAAAGAAGTT V F L Y A H Y D E W D N A A L T L I E K S T K D L D H A Y F K E V	4200 1400
GTTGTCAAAGTTTCTAACTTGAAGTCTAATACAAGGCCATTAATTTCTACGTCAAATTTACCCATCATTAAGTGTATCTTTTACCTCATTGACAC V V K V S N L E I Y Y K A I N F Y V K F H P S L L V D L L T S L T P	4300 1434
CAAGATTAGATATCCAAAGACTGTCAAATTTTCTCGAAATCCGACAACCTTGCTTTAATCAAGCCTTCTTGTATCAATGCTTACCAAAAAATAACTC R L D I P R T V K I F S K S D N L P L I K P F L I N V L P K N N S	4400 1467
AGTTGTCAAAGCCTACCAGTCTGATGATTGAAGAAGAAGTATAAAGCTTTGCAAGACGCTGTTGATTCTTATGCAAAATTTGACCAATTGGGA V V N Q A Y H D L M I E E E D Y K A L Q D A V D S Y D K F D Q L G	4500 1500
TTGGCATCTGTTTGGAACTCATAAGCTAATTTTTTCAAGAAAATAGGAGCCCTGTTATACCGTAGAAAATAAGAAGTGGCAAGAGTCTTTCAATCT L A S R L E S H K L I F F K K I G A L L Y R R N K K W A K S L S I L	4600 1534
TGAAGGAAGAAAAGCTATGGAAGGACGCTATTGAGACGGCAGCAATCTCAGGATCCAAAGGTAGTGGAGGCTCTACTCACATATTTGTTGAAACCGG K E E K L W K D A I E T A A I S Q D P K V V E A L L T Y F V E T G	4700 1567
TAACAGAGAAGGCTTTGTTGCTTTGCTATATGCTGCTTACAACCTTAGTGAGAATTGAATTTGTTTGAATAATCATGGATGAATCTTTGGAGGACTAC N R E G F V A L L Y A A Y N L V R I E F V L E I S W M N S L E D Y	4800 1600
ATTAAGCCATTTGAAATCTCCATAAAAAAAGAGCAGAATGATTCATTAAGAAAATCACCGAAGAACTCGCTAAAAAGTCCGGATCTAATGAAGAGCAC I K P F E I S I K K E Q N D S I K K I T E E L A K K S G S N E E H K	4900 1634
AAGATGGCCAACTTTGATGCTGATGAACAGCGGATGAACGTTCAACCCACAGGATTTTAAAGACGTGGATAAAATAATTCGTTTGGTGTACCCCATCGT D G Q P L M L M N S A M N V Q P T G F *	5000 1653
GTATTTTTTTTTTTTTTACCGTTATTATTTGTTTGTAGCATCTTTGATTTATCTGAGGGGATTTCAAATGCCAATAAAAAGACCTTATGAGTTCTGT ATTTTTCTTAATTTTTCGAGAGAATTTCCCAACTATAAA	5100 5140

S.c.	MSDLPIEFTELVDLMSLGISPQFLDFRSTTTFESDHFVTVRETKDGTNSVAIVDLAKGNEVTRKMGGSAIMHPSQMVIVSVRANGTIVQIFNLETSKSL	99
Rat	MAQILPIRFQEHLLQNLGINPANIGFSTLTMSDFKICIREKVGEOAQQVVIDMNDPSNPIRRPIADSAIMNPASKVIALKAGKTL-QIFNIEKSKM	99
	↳Terminal domain	
	KSFTLDEPVI FWRWLETTLGFVTARSILTSNVFDGNVNAKQQLTLRHLNNTQIINFVANKNLDWFAVVGILQENGRIAGRIQLFSKQRNISAIDG	199
	KAHTMTDDVTFWKWISLNTVALVTDNAVYHWSMEGESQPVKMFDRHSSLAGCQIINYRTDAKQKWLTLGISAQQNRVVGAMQLYSVDRKVSQPIEG	196
	HVAIFTNILLEGNG--STPVQVFTGNRNATTGAGELRIEIDHDASLPSQYQKETTDFPPDATNDFPIAVQVSEKYGIYLLTKYGFHLYELETGT	297
	HAASFAQFKMEGNAEESTLFCFAVRGQAG-----GKLHIIEVGTPTGNQPPFKKAVDVFPPPEAQNDFPVAMQISEKHDVVFLITKYGYHLYDLETGT	291
	NLFVNRIATAESVFTAAPYNHENGIACINKKQVLAVEISTSQIVPYILNKL SNVALALIVATRGGLPGADDL FQKQFESLLQNDYQNAAKVAASST--	395
	CIYMNRI SGETIFVTAPHEATAGIIGVNRKQVLSVCVEENIIPYITNVLQNPDLALRMAVRNLAGAEELFARKFNALFAQQNYSEAAKVAANAPKGI	391
	LRNQNTINRLKNIQAPPGAISPIILLYFSTLLDKGKLNKEETIELARPVLQQDRKQLFEKWLKEDKLECESEELGDIVKPFDTTLALACYLRAGAHAKVISC	495
	LRTPTDIRRFQSVPAQPGQTSPLLYQYFGLLDQQLNKYESLELCRPVLQQGRKQLLEKWLKEDKLECESEELGDLVKSVDPTLALSVYLRANVPNKVIQC	491
	↳Linker	
	LAELQQFEKIIPYQKQVGYQPNFLVLISSLRSSPDRASEFAVSLQNPETASQIDIEKIADLFFSQNHIIQQGTSLLLDALKGDTPDQGHGLQTRVLEVNL	595
	FAETGQVQKIVLYAKKVGYPDWIFLLRNVMRISPDQGGQFAQMLVQDEEPLA--DITQIVDVFMEYNLIQQCTAFLLDALKNNRPSGEPQLTRLEMLN	589
	↳400Å (133/116 kD)	
	LHAPQVADAILGNMIFSHYDKPTIASLSEKAGLYQRALENYTDIKDKRCVVHTNALPIDWLVGYFGKLNVEQSLACLKALMDNNIQANIQTVVQVATKF	695
	MHAPQVADAILGNMFTHYDRAHIAQLCEKAGLLQRALEHFDLYDIKRAVVHTHLLNPELVNLYFGSLSVEDSLECLRAMLSANIRQNLQIIVQVASKY	689
	↳315Å (120/105 kD)	
	SDLIGPSTLIKLFEDYNATEGLYYLASLVNLTEDKDVVYKIEAAAKMKQYREIERIVKDNVYDPERVKNFLKDNANLEQLPLVIVCDRDFVHEMIL	795
	HEQLSTQSLIELFESFKSFEGLFYFLGSIVNFSQDPDVHFYKIQAACTGQIKEVERICRESNCYDPERVKNFLKEAKLTDQLPLIIVCDRDFVHDLVL	789
	YLYKSQNLKFIETYVQVNPSTKAQVVGALLDMDCDEAFIQSLLQSVLGGVPINELTTEVEKRNRLKILLPFLEQSLSQIGDQAVYNALAKIYIDSNN	895
	YLYRNSLQYIEIYVQVNPSPRLPVVIGGLDQVDCSEVDIKMLILVVRGQFSTDELVAEVEKRNRLKLLLPLEARIHGCEEPATHNALAKIYIDSNN	889
	PEKFLKENDQYDLDVGHYCEKRDOPYLAIYAYEKQGNDDDLIRITNENSMYKYQARYLLERSDLDLWNVLNQENIHRRLQIDSVISVGIPELTDPEPVS	995
	PERFLRENPPYDSRVVGYCEKRDPHLACVAYERGQCDELELINVCMENSLFKSLRYLVRKQPELWGSVLLSNPYRPLIDQVQVQTALSETQDPEEVS	989
	LTVQAFMTNGLKLELIELEKIILEPSPFNENVALQGLLLSAIKYEPTKVSSYIEKLDNYDAEIAPLCIEHDLKEEAFEIYDKHEMYGKALKVLIEDI	1095
	VTVKAFMTADLPNELIELEKIVLDNSVFSEHRNLQNLILITAIKADRTRVMEYINRLDNYDAPDIANIAISNELFEEFAIFRKFVNTSAVQVLEIHI	1089
	MSLDRAASYADKINTPELWSQIGTAQLDGLRIPDAIESYIKAEDPNSYENVIDIAEQAGKYEELIPFLLMARKTLKEPKIDGALILAYAELENKHEIENL	1195
	GNLDRAYEFAERCNEPAVWSQLAKAQLKGMVKEAIDSYIKADDPSSYMEVVAANTSGNWEELVKYLQMARKKARESYVETELIFALAKTNRLAELEEF	1189
	LAGSNVANLDHVGDKLFENKEYKAARLCYSAVSNYSKLASTLVYLGDYQAAVDTARKASNIKVWKLVDACIEKKEFKLAQICGLNLIVHAEDELVER	1295
	INGPNNAHIQQVGDRCYDEKMYDAKLLYNNVSNFGLASTLVHLGEYQAAVDGARKANSTRTWKEVCFACVDGKEFRLAQMCGLHIVVADELEELINY	1289
	YESNGYFEELISLFEAGLGLERAHMGMTLAILYSKYEPDKTFEHLKLFWSRINIPKVIQRAVEQAHLWSELVFLYAHYDEWDNAALTLIEKSTKDLDA	1395
	YQDRGYFEELITMLEAALGLERAHMGMTLAILYSKFKPQKMRHLELFWSRVNIQKVLRAEQAHLWAEVLVFLYDYKYEEDNAIITMMNHPTDAWKEG	1389
	YFKEVVVKSNIYYKAINFYVVFHPSLLVDLLTSLTPRLDIPRTVKIFSKSDNLPKIPFLINVLPKNNSVNVQAYKDLMIIEEEDYKALQDAVDSYDK	1495
	QFKDIITKVANVELYKAIQFYLEFKPLLLNDLLMVLSPRLAHTRAVNYFSKVKQLPLVKPYLRSVQNHNNKSVNESLNNLFIITEEDYQALRTSIDAYDN	1489
	FDQLGLASRLESHKLIFFKKIGALLYRRNKKWAKSLSILKEEKLWKDAIETAASQDPKVEALLTYFVETGNREGFVALLYAAYNLVRIEFVLEISWMN	1595
	FDNISLAQRLEKHELIEFRRIAAYLFGNRRWQSVLCKKDSLYKQDAMQYASESKDTAEELLQWFLQEEKRECFGACLFTCYDLLRPDVVLETAWRH	1589
	↳Δ57	
	SLEDYIKPFEISIKKEQNSIKKITEELAKKSGSNEEHKDRGQ-LMLMNSAMNVQPTGF 1653	
	NIMDFAMPYFIQVMKEYLTKVDKLD--ASESLRKEEQATETQPIVYGQPLMLTAGPSVAVPPQAPFGYGYTAPPYQGPQPGFGYSM 1675	

Figure 3. Alignment of the yeast and rat clathrin HC protein sequences. The deduced protein sequence of the yeast HC is shown on the upper line and beneath is the rat HC sequence (Kirchhausen et al., 1987b). Colons indicate identical residues and dashes indicate gaps introduced to generate an optimal alignment. Domains of the rat HC identified by Kirchhausen et al. (1987b) from sequence analysis of proteolytic digestion fragments of bovine clathrin HCs are noted beneath the sequences: 1, terminal domain; 2, linker region (flexible connector) between the terminal domain and the extended arm; and 3, amino termini of two major HC arm fragments that extend ~400 Å and ~315 Å from the trimer center. In parentheses next to the 400- and 315-Å locations are the peptide molecular weights: 1, calculated from the amino acid sequence beginning at the amino terminus of the peptide and proceeding through the COOH terminus of the HC; 2, the respective SDS-PAGE estimates (Kirchhausen et al., 1987b). The proposed heptad repeat/LC binding region is indicated by brackets under the sequences. Prolines and acidic residues in the heptad repeat region are marked by overlines. Δ57 indicates the COOH-terminal segment of the yeast HC that was truncated HCΔ57. Also marked by overlines are the prolines near the COOH termini of the two proteins. (See text for further discussion.)

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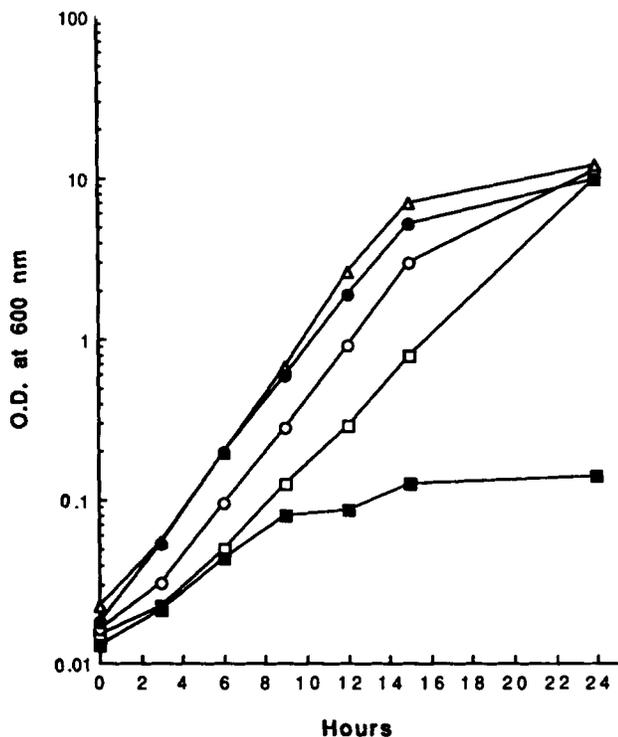


Figure 4. Growth of strains with the HC truncation mutation at 30° and 37°C. Log phase precultures ($\sim 2 \times 10^7$ cells/ml) grown in YEPD at 30°C were used to inoculate YEPD at $\sim 2 \times 10^5$ cells/ml (OD_{600} of ~ 0.01 – 0.02). Cultures were grown at 30° or 37°C and samples taken at the times indicated for absorbance readings at 600 nm. 30°-C cultures (*open symbols*); 37°-C cultures (*closed symbols*). Strains and their relevant genotypes were: BJ3324, *CHC1* control (Δ); SL176, *chcl-Δ pΔ57* ($Ura^+ Trp^-$), (\square, \blacksquare); SL176 transformed to *CHC1* with pAP4, *chcl-Δ pΔ57 pAP4* ($Ura^+ Trp^+$), (\circ, \bullet). Approximately 7–8% of cells in the SL176 + pAP4 culture lost pΔ57 and were therefore $Ura^- Trp^+$. A Ura^- derivative (lost pΔ57) of SL176 + pAP4 was isolated and growth was also assessed at 30° and 37°C. This strain gave identical results to the SL176 derivative containing both pΔ57 and pAP4 (data not shown).

perature-sensitive phenotype at 37°C on YEPD; however, the temperature sensitive phenotype on glycerol was always present (not shown). We do not know the basis for these subtle strain differences.

Strains with the Truncated HC Are Genetically Stable and Sporulate

Clathrin deficient yeast are genetically unstable (Lemmon et al., 1990). *chcl-Δ scd1^s* spore clones, which initially form small slow growing colonies, often given large and small colony isolates upon restreaking (Lemmon and Jones, 1987; Lemmon et al., 1990). In addition, in the collection of strains used in these studies, Chc^- cells with increased genome numbers arise at a high frequency or are selected due to a growth advantage (Lemmon et al., 1990). In contrast, strains of *chcl-Δ pΔ57* genotype were genetically stable. Colonies were uniform in size and early passage colonies appeared identical in morphology to later passage colonies. In addition, cells with HCΔ57 did not show increases in genome number, which was assessed by a canavanine resistance test (Table II), and segregational analysis in crosses (not shown).

Chc^- diploids did not sporulate under any of the conditions we tested (see Materials and Methods) and typically were either lysed and collapsed or were extremely clumped and had a granulated appearance in sporulation media. In two out of three cases examined, *chcl-Δ/chcl-Δ* diploids sporulated well after transformation to Chc^+ with pSL6. Wild-type diploids showed normal asci with $\geq 50\%$ sporulation. *chcl-Δ/chcl-Δ* diploids with pΔ57 also sporulated. Approximately 10–30% of cells formed asci with visible spores and remaining unsporulated cells were not lysed or granulated. A number of the asci had a normal appearance and four spores, but many had a slightly abnormal appearing ascus containing only two or three spores. Thus, HCΔ57 was able to rescue the absolute sporulation defect of Chc^- diploids, although not quite as well as the wild-type HC.

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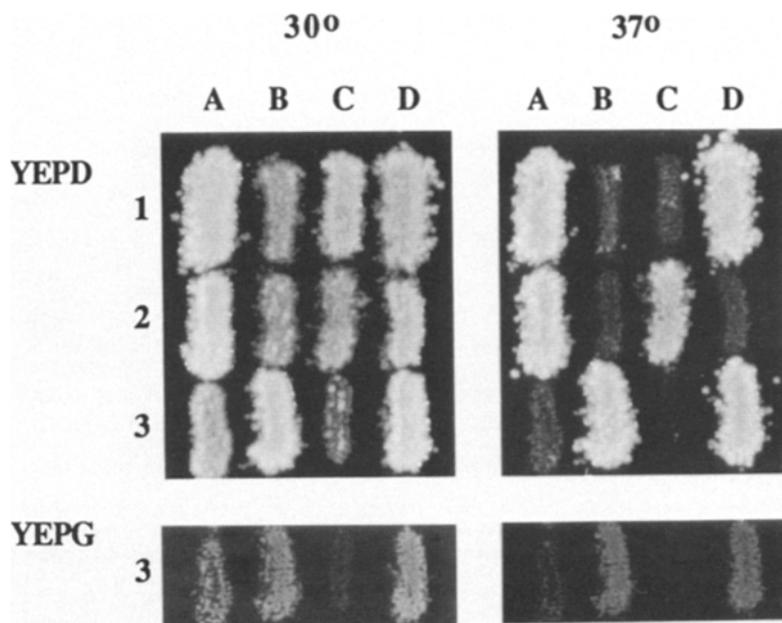


Figure 5. Temperature sensitivity of *chcl-Δ pΔ57* strains. Sample tetrads derived from *chcl-Δ::LEU2/CHC1 pΔ57* diploids were replicated to YEPD and YEPG plates and grown at 30 or 37°C. Tetrad 1 was from BJ4328 (*SCD1^{NS}/SCD1^{NS}*) and tetrads 2 and 3 were from BJ4606 (*scd1^s/scd1^s*). Genotypes of spores are: 1A and 1D, *CHC1 SCD1^{NS} pΔ57*; 1B and 1C, *chcl-Δ SCD1^{NS} pΔ57*; 2A and 2C, *CHC1 scd1^s pΔ57*; 2B and 2D, *chcl-Δ scd1^s pΔ57*; 3A, *chcl-Δ scd1^s pΔ57*; 3B, *CHC1 scd1^s*; 3C, *chcl-Δ scd1^s (Chc^-)*; 3D, *CHC1 scd1^s pΔ57*. Note that spore 1B is SL180 and spore 3A is SL176.

Table II. *Can*⁺ Test for Genome Copy Numbers in *chc1*-Δ Cells Carrying *pΔ57*

Can ⁺ strain	Relevant genotype	Colonies not papillating to Can ⁺	Inferred genome numbers
		total scored	
BJ3243	<i>CHC1 SCD1</i> ^{NS}	0/32	1 <i>n</i>
BJ3324	<i>chc1</i> -Δ <i>scd1</i> ^S pSL6	0/31	1 <i>n</i>
BJ3579	<i>chc1</i> -Δ <i>SCD1</i> ^{NS} pSL6	0/32	1 <i>n</i>
BJ3583	<i>chc1</i> -Δ <i>SCD1</i> ^{NS} pSL6	0/32	1 <i>n</i>
BJ4045	<i>chc1</i> -Δ <i>SCD1</i> ^{NS} pΔ57	0/28	1 <i>n</i>
BJ4053	<i>chc1</i> -Δ <i>scd1</i> ^S pΔ57	0/30	1 <i>n</i>
BJ4054	<i>chc1</i> -Δ <i>SCD1</i> ^{NS} pΔ57	0/32	1 <i>n</i>
BJ4328	<i>chc1</i> -Δ <i>SCD1</i> ^{NS} pΔ57	0/28	1 <i>n</i>
BJ3235	<i>chc1</i> -Δ <i>scd1</i> ^S	26/26	≥2 <i>n</i>
BJ3250	<i>chc1</i> -Δ <i>scd1</i> ^S	31/32	≥2 <i>n</i>
BJ3247	<i>chc1</i> -Δ <i>scd1</i> ^S	31/31	≥2 <i>n</i>

Mass matings were performed by mating strains listed (*CANI*⁺) to haploid Can⁺ tester strains of the opposite mating type (BJ2649 or BJ2651). Prototrophs resulting from matings were tested for their ability to papillate to Can⁺ by mitotic recombination on canavanine plates. Since *CANI*⁺ is dominant to *can1*⁺, mating of a haploid Can⁺ to the tester strain results in extensive papillation to Can⁺, but mating of a Can⁺ cell of higher genome copy number to the Can⁺ strain does not result in significant papillation.

HCΔ57 Rescues the Mating Defect of *Chc*⁻ Strains to Near Normal

In a patch mating test on plates we noted that *chc1*-Δ *scd1*^S strains mated less efficiently than wild type strains. Reduced mating ability of *Chc*⁻ strains has also been observed by Payne and Schekman (1989). In order to assess to conjugation competence of *chc1*-Δ pΔ57 cells, quantitative mating assays were performed. A study of a series of *MATα* strains

of different *CHC1* genotypes is shown in Table III. Mating tests of cells with HCΔ57 gave mating frequencies that were usually at least 50% of wild type when the *MATα* tester strain was *CHC1*, which was two to five-fold more efficient than the mating of *chc1*-Δ strains. Mating of HCΔ57 strains with the *Chc*⁻ tester often yielded frequencies that were 20–50% of *CHC1* matings to the *Chc*⁻ tester. This was much more efficient than was observed for *Chc*⁻ × *Chc*⁻ pairs, which

Table III. Efficiency of Mating*

<i>MATα</i> strain	Relevant genotype	Relative mating frequency in			
		Exp. 1	Exp. 2	Exp. 3	Exp. 4
A. Mating to <i>MATα</i> <i>CHC1</i> tester[†]					
BJ3223	<i>CHC1 SCD1</i> ^{NS}	0.93	—	1.0	0.84
BJ3324	<i>chc1</i> -Δ <i>scd1</i> ^S pSL6	1.07	1.0	—	1.16
BJ4045	<i>chc1</i> -Δ <i>SCD1</i> ^{NS} pΔ57	0.57	0.72	0.49	0.70
BJ4328	<i>chc1</i> -Δ <i>SCD1</i> ^{NS} pΔ57	0.58	—	—	—
BJ4333	<i>chc1</i> -Δ <i>SCD1</i> ^{NS} pΔ57	0.40	—	—	—
SL174	<i>chc1</i> -Δ <i>scd1</i> ^S pΔ57	—	—	0.43	0.85
SL180	<i>chc1</i> -Δ <i>SCD1</i> ^{NS} pΔ57	—	—	0.26	0.78
BJ3235	<i>chc1</i> -Δ <i>scd1</i> ^S	0.089	0.18	—	—
BJ3247	<i>chc1</i> -Δ <i>scd1</i> ^S	0.15	0.19	0.091	—
B. Mating to <i>MATα</i> <i>chc1</i>-Δ <i>scd1</i>^S tester[‡]					
BJ3223	<i>CHC1 SCD1</i> ^{NS}	0.82 (0.098)	—	1.07 (0.23)	—
BJ3324	<i>chc1</i> -Δ <i>scd1</i> ^S pSL6	1.18 (0.14)	1.0 (0.25)	0.93 (0.20)	1.0 (0.23)
BJ4045	<i>chc1</i> -Δ <i>SCD1</i> ^{NS} pΔ57	0.35 (0.042)	0.18 (0.044)	0.16 (0.034)	0.48 (0.11)
BJ4328	<i>chc1</i> -Δ <i>SCD1</i> ^{NS} pΔ57	0.076 (0.0091)	—	—	—
JB4333	<i>chc1</i> -Δ <i>SCD1</i> ^{NS} pΔ57	0.035 (0.0042)	—	—	—
SL174	<i>chc1</i> -Δ <i>scd1</i> ^S pΔ57	—	—	0.51 (0.11)	0.52 (0.12)
SL180	<i>chc1</i> -Δ <i>SCD1</i> ^{NS} pΔ57	—	—	0.16 (0.035)	0.40 (0.093)
BJ3235	<i>chc1</i> -Δ <i>scd1</i> ^S	0.0025 (0.0003)	0.008 (0.002)	—	0.0043 (0.001)
BJ3247	<i>chc1</i> -Δ <i>scd1</i> ^S	0.0059 (0.0007)	0.002 (0.0005)	—	0.013 (0.003)

* Mating tests were performed as described in Materials and Methods. Mating frequencies were calculated as the ratio of number of prototrophs that the *MATα* strain formed with the *MATα* tester strain to the number of viable *MATα* cells plated.

[†] In A (mating to the *MATα* *CHC1* tester strain, BJ3039), relative frequency of mating was calculated as a ratio of the mating frequency of the *MATα* strain divided by the mating frequency of the *MATα* strain carrying a wild-type HC gene (BJ3223 and/or BJ3324). In cases where both BJ3223 and BJ3324 were tested, their mating frequencies were averaged and relative mating frequencies were calculated using this value, which was near 1.0. Thus the reported relative mating frequencies were essentially the same as the actual frequencies of diploid formation for A.

[‡] In B (mating to the *MATα* *chc1*-Δ tester strain, BJ3250), the mating frequency of the *MATα* strain to the *chc1*-Δ tester was divided by the average of the mating frequencies of the *MATα* *CHC1* and *MATα* *chc1*-Δ pSL6 strains to the *chc1*-Δ tester strain, to obtain the relative mating frequency. In parentheses are the actual frequencies of diploid formation.

had mating frequencies that were reduced by 10^2 – 10^3 fold as compared with the mating of $\text{Chc}^- \times \text{Chc}^+$ pairs. However, mating frequencies of $\text{HC}\Delta 57$ strains to Chc^- showed some variability (for example, compare $\text{HC}\Delta 57$ strains $\times \text{Chc}^-$, Table III, exp. 1).

It is interesting to note that in our mating studies of Chc^- strains to Chc^+ , we observed no difference in the mating ability of $\text{MAT}\alpha \text{ chcl-}\Delta$ and $\text{MAT}\beta \text{ chcl-}\Delta$ strains. Both mating frequencies were reduced to 10–20% of $\text{Chc}^+ \times \text{Chc}^+$ pairs. These results are slightly different than those of Payne and Schekman (1989), who found a greater defect in the mating of $\text{MAT}\alpha \text{ chcl-}\Delta$ to CHCI strains than in the mating of $\text{MAT}\beta \text{ chcl-}\Delta$ to wild type. The most likely explanation for this discrepancy is that we used a different mating assay than was used in the prior studies. In the assay used by Payne and Schekman (1989), conjugation is allowed to take place for only 6 h, whereas conjugation time is not limiting in the assay we used. Nevertheless, we found a similar severe sterility for $\text{Chc}^- \times \text{Chc}^-$ matings (10^{-3} – 10^{-4} as compared to Chc^+ pairs), which indicates that both $\text{MAT}\alpha$ and $\text{MAT}\beta \text{ chcl-}\Delta$ strains are mating deficient and there is a strong bilateral mating defect.

Alpha Factor Secretion Is Defective in Cells with Truncated HCs

$\text{MAT}\alpha$ cells secrete the mating pheromone α -factor, which binds to its receptor on $\text{MAT}\beta$ cells causing growth arrest and other responses associated with mating (Herskowitz, 1989). $\text{MAT}\alpha \text{ chcl-}\Delta$ cells secrete an inactive precursor form of α -factor. This results from mislocalization of the membrane-bound KEX2 endoprotease from an intracellular compartment, presumably the late Golgi, to the cell surface (Payne and Schekman, 1989). Maturation of α -factor is normally initiated by the KEX2 protease (see Fuller et al., 1988 for review). We examined whether strains with the truncated HC produce biologically active α -factor by a halo assay. Patches of $\text{MAT}\alpha$ cells were replicated onto a newly seeded lawn of $\text{MAT}\beta$ cells. When α -factor is secreted by the $\text{MAT}\alpha$ cells, growth arrest of the $\text{MAT}\beta$ lawn surrounding the patch appears as a clear zone of nongrowing cells (halo). As expected, wild-type cells ($\text{MAT}\alpha$, CHCI or $\text{MAT}\alpha \text{ chcl-}\Delta$ pSL6) caused the formation of a large halo (Fig. 6, 3B and 4B), while $\text{MAT}\alpha \text{ chcl-}\Delta \text{ scd1}^S$ strains did not elicit a surrounding halo of growth-arrested $\text{MAT}\beta$ cells (Fig. 6, 1B and 2B). $\text{chcl-}\Delta$ p $\Delta 57$ strains also did not generate a halo (Fig. 6, 1A–4A), and, therefore, appear to be defective in α -factor secretion. The defect was associated with the clathrin HC truncation mutation, since transformation with a plasmid containing the intact HC gene resulted in correction of the α -factor secretion defect (not shown). When a comparable series of $\text{MAT}\beta$ strains were examined for their ability to cause growth inhibition of a $\text{MAT}\alpha$ lawn, all strains caused a similar sized halo regardless of the CHCI genotype (not shown). This confirmed previous studies that secretion of α -factor, which does not exit the cell via the secretory pathway (Kuchler et al., 1989; McGrath and Varshavsky, 1989), is essentially normal in $\text{MAT}\beta$ cells carrying a clathrin HC gene deletion (Payne and Schekman, 1989).

A pulse–chase analysis was used to examine in more detail the defect in secretion of biologically active α -factor from cells with $\text{HC}\Delta 57$ (Fig. 7). After a 5-min pulse with $^{35}\text{SO}_4^{2-}$, CHCI strains (Fig. 7 A) accumulated mature low

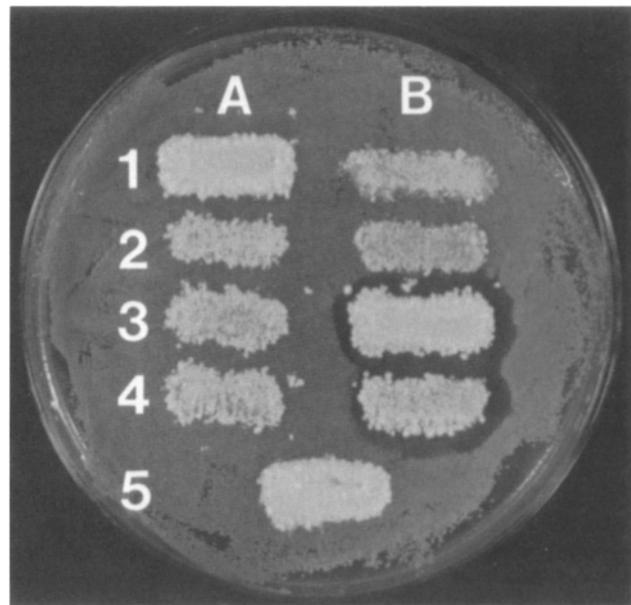


Figure 6. Alpha factor production in $\text{chcl-}\Delta$ p $\Delta 57$ strains. Patches of strains were replicated onto a lawn of the $\text{MAT}\beta \text{ sst1}$ strain, RC669 (BJ3556), and incubated at 30°C for 48 h. Secretion of bioactive α -factor prevents growth of the surrounding lawn, resulting in a clear zone (halo). p $\Delta 57$ strains tested were BJ4045 ($\text{chcl-}\Delta \text{ SCD1}^{\text{NS}}$ p $\Delta 57$), 1A; SL180 ($\text{chcl-}\Delta \text{ SCD1}^{\text{NS}}$ p $\Delta 57$), 2A; SL174 ($\text{chcl-}\Delta \text{ scd1}^S$ p $\Delta 57$), 3A; and SL176 ($\text{chcl-}\Delta \text{ scd1}^S$ p $\Delta 57$), 4A. Chc^- ($\text{chcl-}\Delta \text{ scd1}^S$) strains tested were BJ3247, 1B; BJ3235, 2B. Cells with the wild-type HC gene were BJ3324 ($\text{chcl-}\Delta \text{ scd1}^S$ pSL6), 3B; BJ3223 ($\text{CHCI} \text{ SCD1}^{\text{NS}}$), 4B. All strains were $\text{MAT}\alpha$ except for the one in row 5, which is a $\text{MAT}\beta \text{ CHCI}$ strain (BJ3039). No halo is expected since $\text{MAT}\beta$ strains secrete α -factor, to which the $\text{MAT}\beta$ lawn is not responsive.

molecular weight α -factor ($\sim 65\%$ of total α -factor label incorporated) and a precursor form of ~ 28 kD ($\sim 35\%$), which corresponds to the ER form of pro- α -factor (Julius et al., 1984). The ER form was rapidly processed to mature α -factor, which was chased into the medium. As shown previously (Payne and Schekman, 1989), after 5 min of labeling, $\text{chcl-}\Delta$ cells (Fig. 7 B) had accumulated primarily the ER form (20%) and a highly glycosylated precursor (>100 kD) (60–70%), which corresponds to pro- α -factor that has been transported to and further glycosylated in the Golgi complex (Julius et al., 1984). A small amount of mature α -factor and other intermediate low molecular weight species (3–8 kD) were also found in $\text{chcl-}\Delta$ cells, which represented $\sim 6\%$ of the total labelled immunoprecipitable protein. After 2 min of chase, the ER form had virtually disappeared, while the high molecular weight glycosylated precursor was the major species remaining and was gradually chased into the medium. Strains with $\text{HC}\Delta 57$ showed a processing and secretion pattern similar to that found in $\text{chcl-}\Delta$ cells. After 5 min of labeling, the ER form (15%) and significant amounts of the highly glycosylated precursor (60–65%) had accumulated (Fig. 7 C). Although more pro- α -factor was processed to the low molecular weight 3–8 kD α -factor species ($\sim 20\%$ of total incorporated label) than for $\text{chcl-}\Delta$ cells, only $\sim 7\%$ of this corresponds to a band comigrating with authentic mature α -factor (lowest molecular weight

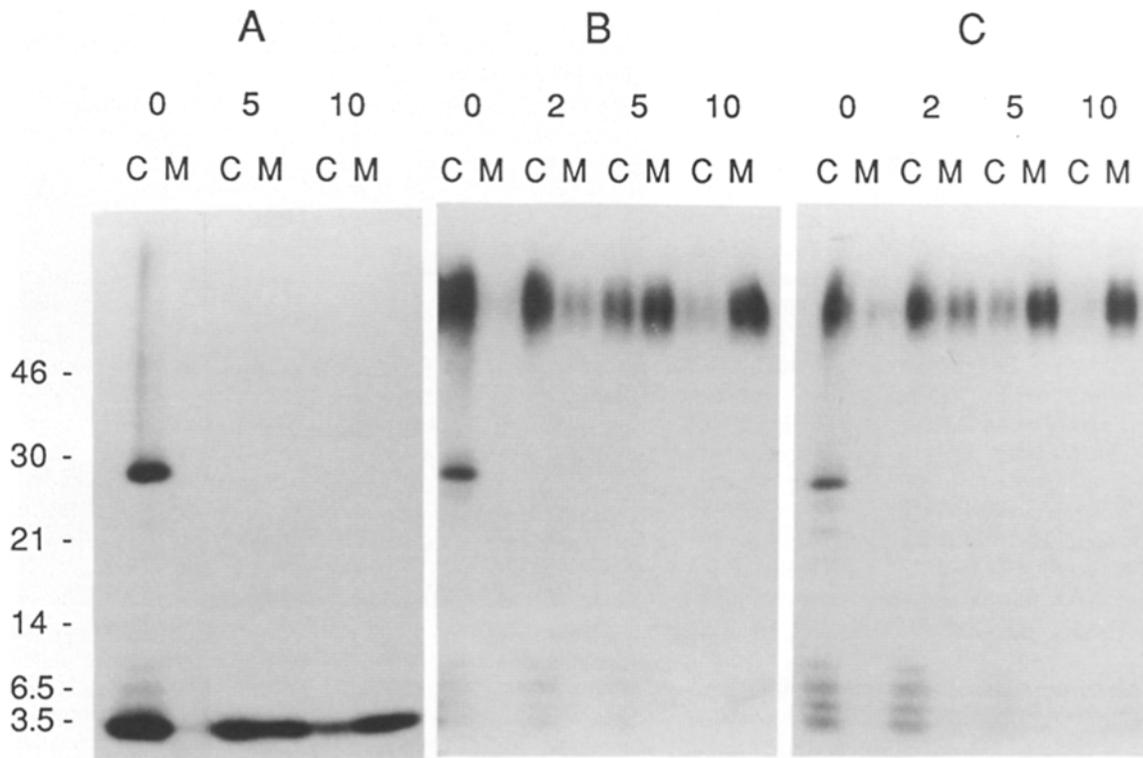


Figure 7. Alpha factor processing in HCA57 strains. Pulse-chase labeling with $^{35}\text{SO}_4^{2-}$ was performed as described in Materials and Methods. Labeling was for 5 min and then samples were chased for 0, 2, 5, or 10 min as shown above each pair of lanes. Labeling reactions were separated into cell (C) or medium (M) fractions and immunoprecipitated with α -factor antibodies and analyzed by SDS-PAGE and autoradiography. Strains examined: (A) *CHCI*, BJ3223; (B) *chcl-Δ*, BJ3247; (C) *chcl-Δ pΔ57*, BJ4045. Molecular weight markers in kilodaltons are indicated on the left.

band). In addition, as was found for *chcl-Δ* cells, there was no further processing of the high molecular weight "Golgi" precursor to the low molecular weight forms and the high molecular weight form was secreted into the medium. In both *chcl-Δ* and HCA57 strains, the smaller processed forms seen at early times in cells disappeared and very little appeared in the medium. We do not know the cause or location of this apparent turnover of the low molecular weight species, but this appears to be a general phenomenon since there was some decrease in the net mature α -factor secreted into the medium of *CHCI* cells as well. As observed previously (Payne and Schekman, 1989), the overall kinetics of transit throughout the secretory pathway was similar for *CHCI* and mutant strains. We conclude that, like *chcl-Δ* cells, the lack of mature α -factor and the predominance of the Golgi precursor in the medium of HCA57 strains accounts for the lack of halo formation in the biological assay.

Biochemical Characterization of HCA57

To biochemically characterize the clathrin in cells with HCA57 we first performed immunoblot analysis on whole yeast extracts probing with a pool of antiyeast clathrin HC mAbs (Lemmon et al., 1988) (Fig. 8). The HCA57 migrated on the gel slightly faster than the wild-type protein consistent with its reduced size (also compare Fig. 9, lanes C and L). The amount of mutant HC in strains of *chcl-Δ pΔ57* genotype (Fig. 8, lanes 3 and 4) was similar to the amount of HC made by cells with the full-length protein (lanes 2 and 6).

This indicated that the observed phenotypes in mutant cells were not due to reduced expression of the truncated HC. HCA57, however, appeared more susceptible to proteolysis, as evidenced by the accumulation of lower molecular weight degradation products in extracts from *chcl-Δ pΔ57* strains. When the wild-type and mutant HCs were coexpressed, the

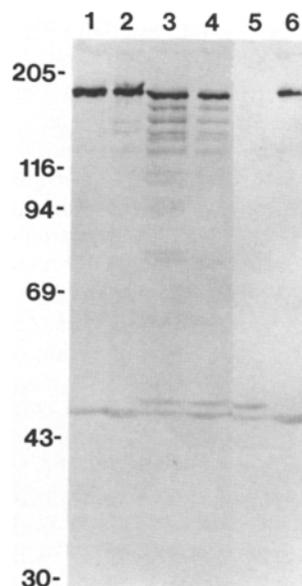


Figure 8. Expression of HCA57. Whole cell extracts from yeast cultures were prepared and samples (40 μg protein) were subjected to SDS-PAGE and immunoblot analysis as described in Materials and Methods. A pool of antiyeast heavy chain mAbs was used as a probe. Samples analyzed were: lane 1, BJ4057 (*CHCI pΔ57*); lane 2, BJ4055 (*CHCI*); lane 3, BJ4054 (*chcl-Δ SCD1^{NS} pΔ57*); lane 4, BJ4053 (*chcl-Δ scd1^S pΔ57*); lane 5, BJ4046 (*chcl-Δ scd1^S*); lane 6, BJ3133 (*chcl-Δ/CHCI*). The 48-kD band that appears in lanes with HCA57 and *chcl-Δ* extracts is an artifact of the Vectastain immunoblotting kit.

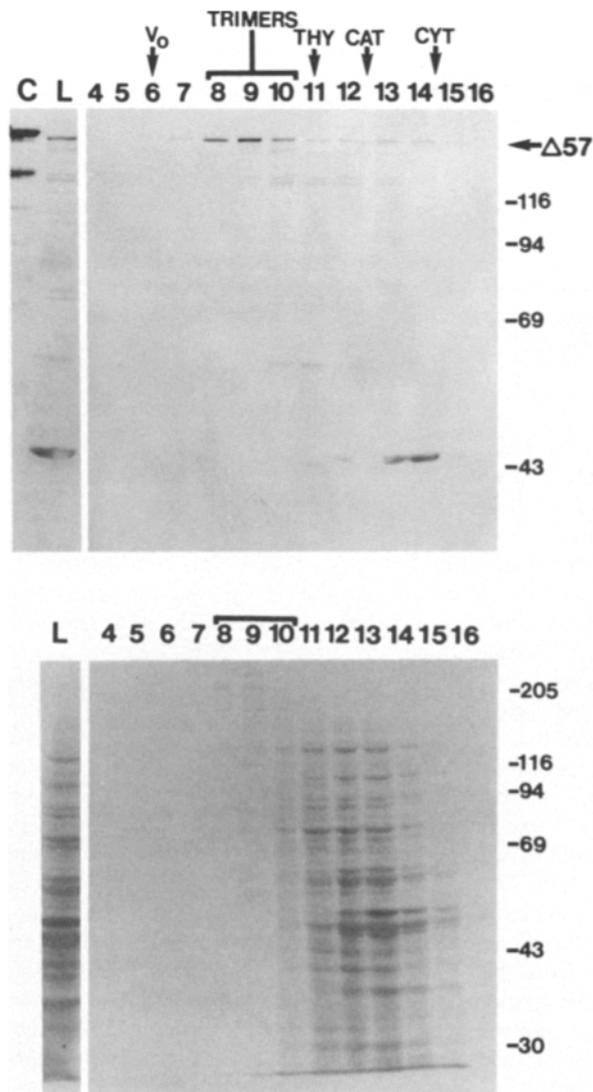


Figure 9. HCA57 fractionates as a trimer in gel sizing columns. A cell extract from BJ4053 (*chcl-Δ scd1^S pΔ57*) was prepared for triskelion analysis on a Sepharose CL-4B column (see Materials and Methods). Aliquots of column samples were subjected to SDS-PAGE and gels were transferred to nitrocellulose for immunoblot analysis. (*Bottom*) Ponceau S stain of total protein transferred to nitrocellulose; (*top*) immunoblot probed with antiyeast clathrin HC antibodies. Samples lanes were: C, purified clathrin triskelions; L, column load (20 μ l); lanes 4–16, column fraction numbers. The elution positions of protein size standards are indicated with an arrow: thyroglobulin (THY), 669 kD; catalase (CAT), 232 kD; and cytochrome C (CYT) 14.5 kD. The major peak fractions of triskelion elution are indicated in brackets, which was assessed by independent analysis of a *CHCI* strain and is consistent with the elution pattern of purified clathrin on Sepharose CL-4B. The prominent \sim 44-kD band in several lanes of the immunoblot is an artifact of the immunoblotting kit.

accumulation of degradation products was similar to that of *CHCI* cells without p Δ 57 (Fig. 8, lanes 1 and 2). Although the bands were not clearly resolved in this immunoblot, in other coexpression experiments HCA57 appeared to be reduced in amount relative to the wild-type protein. These

results indicate that the truncated HC is less stable than the wild-type protein and degradation products are turned over more efficiently in *CHCI* cells.

To investigate whether the COOH terminus of the HC is required for the noncovalent association of the three HCs at the trimer vertex, we examined whether HCA57 formed stable triskelions. Cell lysates from *chcl-Δ pΔ57* cultures grown at 30°C were prepared in a buffer that released all of the clathrin into the soluble fraction. A 100,000 g supernatant from the extract was applied in an analytical Sepharose CL-4B column and aliquots of eluted fractions were analyzed by immunoblotting (Fig. 9). The majority of the truncated HC eluted in fractions 8–10 before the thyroglobulin molecular weight marker, as expected for triskelions (Lemmon et al., 1988). Independent analysis of a *CHCI* strain indicated that wild-type triskelions also eluted in this region (not shown). There appeared to be smaller amounts of HC in fractions 11–14 as well. Some of this was probably due to trailing of the major trimer peak, which has been observed with these analytical columns in experiments using *CHCI* extracts. A small amount of monomeric HC (fraction 13) was also present in this experiment, but it was a minor portion of the clathrin compared with that eluting in the trimer fraction. Most of the minor degradation products of HCA57 eluted in the middle fractions of the column (primarily fractions 10–11 with lesser amounts in 12–13). These could represent partially proteolyzed trimers or some dimers. Overall these studies indicate that HCA57 is primarily in trimeric form in yeast, and, therefore, the 57 COOH-terminal amino acids are not required for trimerization of yeast clathrin HCs.

CVs were purified from *CHCI* or HCA57 strains using our standard cell fractionation procedures and Sephacryl S-1000 column chromatography (Lemmon et al., 1988). Immunoblot analysis of fractions from the S-1000 column indicated that clathrin eluted in the same region of the column for both the mutant and wild-type cells, but recovery of clathrin in the coated vesicle fraction was consistently reduced for cells with HCA57 (Fig. 10). When the clathrin containing fractions from the S-1000 columns were pooled and centrifuged at 100,000 g for one hour, similar amounts of the total clathrin in each pool (\sim 70%) were in the pellet fraction for both mutant and wild-type heavy chains (not shown). This indicated that the clathrin eluting from the column was still largely associated with membranes, even for the HCA57 strains.

Discussion

Previous studies have shown that mammalian and yeast clathrins have similar physical properties (Mueller and Branton, 1984; Payne and Schekman, 1985; Lemmon et al., 1988). In this report we show that the similarities extend to the primary amino acid sequences of the rat and yeast HCs, which are 50% identical. In general, the two ends of the polypeptide chain are less conserved between the species. There is 42% identity for the terminal domain segment up to yeast residue 437, 56% identity for the major body of the HC arm (yeast residues 438–1,520) and only 33% identity for the remaining COOH-terminal region. The major body of the extended arm also contains some highly conserved segments, such as that corresponding to rat residues 437–

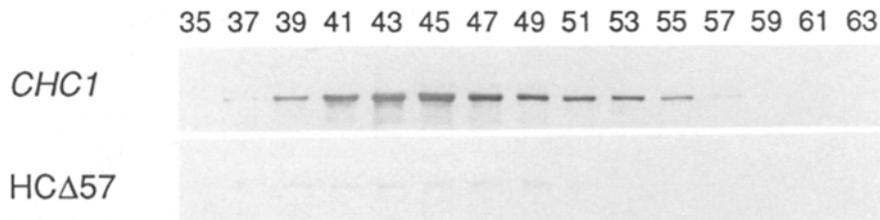


Figure 10. Coated vesicle purification from *CHC1* and *HCA57* strains. 6 liters of cells grown to 5×10^7 cells/ml in YEP-sucrose were harvested, lysed, and centrifuged as described previously (Lemmon et al., 1988). The 100,000-g pellet was resuspended in 10 ml buffer A, spun 5 min at 12,000 *g* and the supernatant was applied to a 2.5×95 cm Sephacryl S-1000 gel filtration column. 8-ml frac-

tions were collected and 40 μ l of each fraction indicated was subjected to SDS-PAGE. Gels were transferred to nitrocellulose for immunoblot analysis using yeast HC mAbs as probes. The 280-nm absorption profile was similar for both *CHC1* and *HCA57* strains. The column void volume was at fraction 27 and the total included volume was at fraction 63.

465, which is in the terminal domain adjacent to the linker or flexible connector (rat residues 479–523). This conserved region is likely to be positioned near the trimer vertices in the lattice, since the flexible connector is thought to allow the terminal domain to point inward towards the coated vesicle interior. These sequences could be required for specific terminal domain contacts at the vertex or association with clathrin associated proteins, which interact with the terminal domains in triskelions (Heuser and Keen, 1988) and clathrin lattices (Vigers et al., 1986b). Other studies indicated that the terminal domain is required by uncoating ATPase, a member of the 70-kD heat shock family of proteins (Unge-wickell, 1985; Chappell et al., 1986), for the promotion of lattice disassembly (Rothman and Schmid, 1986). Perhaps this highly conserved segment of the terminal domain is an important site for binding of the uncoating enzyme. Recently, Heuser and Steer (1989) showed that three molecules of uncoating ATPase actually bind near the vertex of the triskelion, one per HC. This association is consistent with models of lattice uncoating, where juxtaposition of a terminal domain and a site near the trimer center would be required for triskelion release (Rothman and Schmid, 1986). It is interesting to note that another highly conserved segment, including yeast residues 1,300–1,371, may be fairly close to the trimer center and could serve as a contact site for the conserved region of the terminal domain and/or for binding of uncoating ATPase.

Light Chain Binding Region

The yeast and rat HCs contain significant amino acid differences in the proposed region of LC binding (Kirchhausen et al., 1987b), which in the rat HC (amino acids 1,107–1,184) contains a heptad motif that may be involved in an α -helical coiled-coiled association with the heptad repeat region of the LC. The heptad motif is much weaker in the yeast HC primarily due to the presence of four prolines, which could create breaks in the helix. Since the yeast LC has similarities to mammalian LCb (Silveira et al., 1988) and since there are no gaps in the alignment of the rat and yeast HC's over most of the extended arm segment, we think it is likely that the LC binding region on the HC is the same for clathrin from different organisms.

Although it is possible that the LC binds to another region of the HC, two lines of immunological evidence suggest that the proposed region is important for LC/HC association. First, using protein fusions we found that a number of our antiyeast HC mAbs (Class C, see Lemmon et al., 1988) bind

to a region between amino acids 1,074–1,233 which encompasses the proposed LC binding site. These antibodies do not bind to triskelions with associated LC's (Lemmon, S. K., unpublished results) but do cross-react with denatured HCs on immunoblots (Lemmon et al., 1988). This result is consistent with the hypothesis that the HC epitopes are masked by LCs in the triskelion, but it does not exclude other interpretations. Second, Blank and Brodsky (1987) described an anti-bovine HC mAb (mAb X35) that has similar properties, binding preferentially to LC free trimers. Proteolysis studies showed that mAb X35 could bind to trimers with distal HC segments removed and containing residual arm fragments of 62 kD, but it could not bind to trimers containing 56-kD residual fragments. LCs were also unable to bind to trimers containing residual 56-kD arm fragments. These results are consistent with binding of mAb X35 and LCs to the proposed heptad region of the rat HC, since the deduced molecular weights of mammalian HC peptides whose NH₂ termini flank the proposed heptad region and end at the HC COOH terminus are 66,000 (amino acids 1,107–1,675) and 57,000 (amino acids 1,184–1,675). Nevertheless, how and where the LC binds to the HC and whether there are important species differences in this association remain unanswered questions that will require further investigation.

COOH-Terminal Region of the HC

The rat HC is extended at the COOH terminus by 22 amino acids (28 residues if the gaps in the COOH-terminal alignment are not allowed) relative to the yeast HC. The rat HC extension is also unusual due to its high proline and glycine content. This finding led to the hypothesis that this region is involved in trimerization. This was supported by the observation that a fragment beginning at residue 1,603 of the rat HC was released during proteolysis experiments that also resulted in release of the HC from the triskelion (Kirchhausen et al., 1987b). However, the yeast HC lacks the Pro-Gly rich segment and nonetheless trimerizes. Moreover, *HCA57*, which is missing 57 COOH-terminal amino acids of the yeast HC (corresponding to a truncation distal to yeast residue 1,596 or rat residue 1,590), still formed triskelions that allowed yeast to grow nearly as well as cells containing wild-type clathrin under standard cultivation conditions. In addition, cells with *HCA57* showed no apparent genetic instability typical of clathrin-deficient yeast and defects in mating and sporulation of clathrin-deficient strains were restored to near normal in strains with the truncated protein. Nevertheless, mutants with the truncated HC displayed some

interesting phenotypic differences from wild type cells. First, HCA57 cells were temperature sensitive for growth at 37°C. We considered the possibility that the truncation might result in destabilization of triskelions at the elevated temperature. However, we were unable to observe any significant differences in the fractionation pattern of the truncated HC on gel sizing columns when we compared extracts from cells incubated for up to 2 h at 37°C and cells retained at 30°C (not shown).

An alternative hypothesis is that the assembly of HCA57 into trimers is defective at 37°C, but trimers are stable at this temperature once they are formed. Consistent with this hypothesis is the fact that cells with the truncated HC's undergo two to three more doublings before growth arrest at 37°C (Fig. 4). This idea requires that clathrin turnover slowly in cells, which we have confirmed using a *GALI:CHCI* inducible promoter fusion. When *GALI:CHCI* cells are shifted from galactose to glucose medium to shut off denovo HC synthesis, growth continues at wild-type rates for several generations before endogenous clathrin is depleted and growth is arrested (Nelson, K., and S. K. Lemmon, unpublished results).

Another possibility is that deletion of 57 amino acids at the COOH terminus of the HC affects some other aspect of clathrin structure and/or function, such as lattice assembly or disassembly or other vertex associations in the lattice. HCA57 could be inactivated rapidly at 37°C, but the growth of cells for several generations might represent the time it takes for some other cellular process to be affected by the loss of clathrin function. Our observations indicate that there are fewer CV in cells with HCA57 or that they are not as stable during the isolation procedure as wild-type CV. This is consistent with the idea that the 57 COOH-terminal amino acids of the yeast HC do play a role in some other aspect of CV formation. This idea would include the possibility that, although, the 57 COOH-terminal amino acids are not absolutely required for yeast HCs to form trimers, the trimerization of the truncated HCs might not be completely normal, which could, in turn, affect the maintenance of normal amounts of coated vesicles.

The importance of the Pro-Gly-rich region of the rat HC remains unknown. Our studies do not address whether the requirements for trimerization by yeast and mammalian clathrins are different and whether in the mammalian clathrin these sequences are, indeed, important for forming trimers. Alternatively, the Pro-Gly-rich segment might be needed for specialized lattice structures or coated vesicle functions not required in yeast.

Mating Pheromone Secretion

In mammalian cells clathrin functions in both receptor-mediated endocytosis and the secretory pathway, including regulated secretion and sorting of acid hydrolases to the lysosome (see Brodsky, 1988 for review). In contrast, in yeast, clathrin is not required for processing and localization of hydrolases to the lysosome-like vacuole, and there is no evidence, thus far, for clathrin-mediated endocytosis (Payne et al., 1988). The only specific role identified for clathrin in yeast is its involvement in the retention of proteins in an intracellular membrane compartment, presumably the *trans*-Golgi (Payne and Schekman, 1989). For example, *MAT α*

clathrin-deficient yeast secrete primarily a biologically inactive precursor form of the mating pheromone α -factor. This results from mislocalization of *KEX2* protease, which initiates maturation of pro- α -factor (Fuller et al., 1988), to the cell surface from a late Golgi processing compartment (Payne and Schekman, 1989). The direct cause of the slow growth, genetic instability, and the mating and sporulation defects of *Chc⁻* yeast remains unknown, but all of these processes could be affected by a general defect in retention of proteins whose function is required in the intracellular/*trans*-Golgi compartment.

An interesting observation from our studies is that both HCA57 and *chcl- Δ scd1^s* strains showed a severe defect in secretion of biologically active α -factor, even though growth rates of strains with the truncated HC at 30°C were near that of *CHCI* cells and a number of other manifestations of the *chcl- Δ* mutation were near normal or fully corrected in the COOH-terminal deletion mutants. There are a number of ways to account for this separation of effects on pheromone processing from the effect of HC mutations on the overall growth of cells. One is that α -factor processing may be much more sensitive than overall cell growth to a slightly inefficient retention of proteins whose function is required in the Golgi complex. It is likely that in *CHCI* cells, as pro- α -factor traverses the secretory apparatus it is processed rapidly and efficiently in an appropriate ionic environment, since after a 5-min pulse labeling period with ³⁵SO₄²⁻ >50% of pro- α -factor is present in its mature form and the high molecular weight Golgi precursor is not observed (this study, Payne and Schekman, 1989). This rapid maturation might be very sensitive to any slight perturbation in the localization of the processing machinery, which involves at least the three proteases encoded by the *KEX2*, *KEX1*, and *STE13* genes (Fuller et al., 1988). Such a slight perturbation might be caused by the HCA57 mutation and result in the almost exclusive secretion of the high molecular weight Golgi precursor form. The presence in cells with mutant HCs of a number of lower molecular weight α -factor species (most of which would be biologically inactive) also supports this idea. These could be products of inefficient processing of the high molecular weight precursor, which rapidly exit the normal processing compartment before cleavages are completed.

An alternative explanation for the differential effect on growth and α -factor secretion in HCA57 cells is that in yeast, as in other eukaryotic cells, clathrin's sorting and localization functions are not limited to the Golgi compartment and the HCA57 mutation differentially affects these functions. Distinguishing these possibilities will await the identification of further specific processes in yeast that require clathrin's participation.

We thank J. Toyne and D. Meyer for the generous gift of α -factor antibodies; William Brown and Joyce Jentoft for help with protein structure analysis; Tomas Kirchhausen and David Thomas for providing results before publication; Martin Snyder, Vance Lemmon, Chris Bazinet, and Karen Nelson for critical reading of this manuscript; and our colleagues at Carnegie Mellon University and Case Western Reserve University for helpful discussions and comments. We are especially grateful to Dr. Elizabeth W. Jones for her continued support and interest and for providing her facilities to carry out major portions of this research.

C. L. Freund was a recipient of a National Science Foundation Research Experience for Undergraduates award (BBS8712919). This work was supported by U. S. Public Health Service grants GM-29713, AI-06884, and

GM-36995, a grant from the American Heart Association, Northeast Ohio Affiliate and an award from the Lucille P. Markey Charitable Trust to the Case Western Reserve University School of Medicine.

Received for publication 26 March 1990 and in revised form 19 September 1990.

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