**CALI, A Gene Required for Activity of Chitin Synthase 3 in Saccharomyces cerevisiae**

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**Abstract.** The CALI gene was cloned by complementation of the defect in Calcofluor-resistant calR1 mutants of *Saccharomyces cerevisiae*. Transformation of the mutants with a plasmid carrying the appropriate insert restored Calcofluor sensitivity, wild-type chitin levels and normal spore maturation. Southern blots using the DNA fragment as a probe showed hybridization to a single locus. Allelic tests indicated that the cloned gene corresponded to the calR1 locus. The DNA insert contains a single open-reading frame encoding a protein of 1,099 amino acids with a molecular mass of 124 kD. The predicted amino acid sequence shows several regions of homology with those of chitin synthase 1 and 2 from *S. cerevisiae* and chitin synthase 1 from *Candida albicans*. calR1 mutants have been found to be defective in chitin synthase 3, a trypsin-independent synthase. Transformation of the mutants with a plasmid carrying CALI restored chitin synthase 3 activity; however, overexpression of the enzyme was not achieved even with a high copy number plasmid. Since Calcofluor-resistance mutations differ from calR1 also result in reduced levels of chitin synthase 3, it is postulated that the products of some of these CAL genes may be limiting for expression of the enzymatic activity. Disruption of the CALI gene was not lethal, indicating that chitin synthase 3 is not an essential enzyme for *S. cerevisiae*.

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**Materials and Methods**

**Strains and Media**

The *S. cerevisiae* strains used in this study are listed in Table I. Standard methods were used for genetic crosses (41). Dissection of spores containing a disruption of *CHS2* was carried out on minimal medium (8). *S. cerevisiae* strains CRI, ECY33-2A, and ECY33-18A were used as recipient strains in transformations with plasmids carrying the *CALI* gene. The diploid strain HVY28 was the recipient in the *CALI* gene disruption experiment. *S. cerevisiae* API (12) was a source of RNA for RNA blots.

**Escherichia coli** JM101, JM109, or DH1 were used for transformation and plasmid preparation. *S. cerevisiae* strains were grown in YED (1% glucose and 1% Bacto yeast extract), YEPD (1% Bacto yeast extract, 2% peptone, 2% glucose) or minimal medium (2% glucose, 0.7% Difco yeast nitrogen base without amino acids), plus nutritional requirements. *E. coli* was grown in LB medium supplemented with 50 μg/ml ampicillin or 15 μg/ml tetracycline, where appropriate. Solid medium plates also contained 2% agar.

**Plate Assay for Calcofluor Resistance**

Calcofluor susceptibility was tested on fresh cultures by suspending a small number of cells (~10⁶ cells/ml) in sterile water and dropping 5 μl of each suspension on plates. A synthetic medium was most frequently used (2% glucose, 0.7% Bacto yeast nitrogen base, 0.2% of an amino acid solid mix-
ture containing 0.5 g adenine, 4 g PABA, 4 g leucine, 2 g uracil, and 2 g of all other amino acids) buffered with 50 mM sodium phthalate pH 6.2 (34) and supplemented with 1 mg/ml Calcofluor and 2 % agar. Growth in liquid medium in the presence of Calcofluor (0.5 mg/ml) was determined by count-
ing the cells.

Plasmids and Transformations

The yeast genomic library constructed in the plasmid YCp50 (18) and the additional yeast vectors YEp352 (16), YEp3 (7), and YEp5 were provided by F. del Rey (Instituto de Microbiologia Bioquímica, Salamanca, Spain). S. cerevisiae was transformed by the lithium acetate procedure (17). E. coli was transformed as described by Kushner (21) or Golub (15). Bluescript KS'/SK+ vectors (Stratagene, Inc., La Jolla, CA) were used to subclone restriction fragments for sequencing.

DNA and RNA Preparations and Blots

All manipulations of DNA and RNA were by established molecular biological methods (3, 13, 25, 36) with the following exceptions. Southern analyses were modified so that, instead of transferring DNA to nitrocellulose filters, hybridization was carried out on the gel itself as recently described for RNA (1). Double-stranded DNA sequencing was performed according to Zhang et al. (47), as modified by Riley (31).

Preparation of Membranes and Enzymatic Assays

Membranes were isolated after disruption of intact cells with glass beads followed by differential centrifugation, essentially as described by Orlean (27). Final membrane pellets were suspended in 1.6 ml 50 mM Tris-chloride (pH 7.5), containing 5 mM magnesium acetate, per gram (wet weight) of yeast cells. Chitin synthase activity was measured as previously described (37). Chitinase activity was determined in cell extracts essentially according to Kuranda and Robbins (20), using 4-methylumbelliferyl-β-D-N,N',N"-triacetylchitotriose as a substrate.

Analytical Procedures

Measurement of chitin in vivo was performed as described by Bulawa et al. (9) using chitinase from Serratia marcescens, either purified in the laboratory (32) or obtained from Serva Biochemicals, Heidelberg, Germany. N-acetylglucosamine was assayed colorimetrically by the method of Reissig et al. (30). Protein was measured according to Lowry et al. (24).

UDP-GlcNAc levels were measured by HPLC chromatography (Waters Chromatography Div., Milford, MA). Logarithmic phase cells were mechanically homogenized in 50% ethanol. The extract was centrifuged at 46,000 g for 15 min. The supernatant was evaporated to dryness in a Speed Vac Concentrator (Savant Instruments, Inc., Farmingdale, NY) and suspended in 50% ethanol. A Novapack C18 column (18 ×100 mm) with 5-μm diameter particles and an isocratic elution at 100 mM monobasic ammonium phosphate, pH 2.85, were used. Data were obtained by measuring absorbance at 260 nm with appropriate standards.

Results

Cloning of the CALI Gene

The CALI gene was cloned by complementation of the Calcofluor resistance phenotype. A calR1, ura3 strain (CRY1-15D) was transformed with a yeast genomic library constructed in the centromere vector YCp50, which contains the URA3 gene. Approximately 4,000 prototrophs were selected and the restoration of sensitivity to Calcofluor was observed by plating them as indicated under Materials and Methods. A transformant was isolated that after growth under non-selective conditions (YED medium) simultaneously lost both Ura and CalR phenotypes, an indication that both were encoded by the plasmid DNA. DNA from the transformant strain was amplified in E. coli. A single plasmid (pHV1) was isolated and used again to transform the original calR1, ura3 recipient strain. The new transformants were found to be CalR. The 8.4-kb-long DNA insert cloned in the pHV1 plasmid was used for subcloning and characterization of the CALI gene (Fig. 1). Several deletions of the DNA insert were carried out to define the minimal sequence required for restoring sensitivity to Calcofluor. Deletion of the 4.4-kb fragment between the Xhol site and the right hand BamHI site could not effect reversion of the CalR phenotype. Further deletions were attempted after inserting the 5.4-kb ClaI–BamHI fragment of the pHV7 plasmid in the multicopy vector YEp352 (plasmid pHV8). Previously we noticed that the presence of that fragment in a high copy plasmid was not

Table I. Strains of S. cerevisiae Used in This Study

| Strain   | Genotype                               | Source                  |
|----------|----------------------------------------|-------------------------|
| CR1      | MATa cal1 ade1 his3                    | ATCC 64941              |
| CRY1-15D | MATa cal1 ura3 his3                    | Derived from CR1         |
| CR4217A  | MATa cal2 ura3                         | ATCC 64944              |
| HV2324   | MATa cal3 ura3                         | ATCC 64945              |
| HV2627B  | MATa cal5 ura3                         | This study               |
| HVY28    | MATa/MATa cal1/Cal5 ura3/ura3 leu2/LEU2 his3/HIS | This study               |
| ECY33-2A | MATa chs1-23 leu2-3,112 ura3-52         | This study               |
| ECY33-18A| MATa chs1-23 cal1 leu2-3,112 trp1-1     | This study               |
| ECY19A2  | MATa/MATa chs1-23/chs1-23 chs2::URA3/CHS2 leu2-3,112/ura3-52 ura3-52/trp1-1/ura3-52 trp1-1 | This study               |
| ECY19A2-5B| MATa chs1-23 leu2-3,112 ura3-52 trp1-1   | From sporulation and dissection of ECY19A2 |
| ECY36-3A | MATa chs1-23 leu2-3,112 ura3-52 trp1-1   | From cross ECY19A2-5B × ECY33-18A |
| ECY36-3C | MATa chs1-23 leu2-3,112 ura3-52 trp1-1   | Same tetrad as ECY36-3A |
| ECY36-3D | MATa chs1-23 cal1 leu2-3,112 ura3-52 trp1-1 | Same tetrad as ECY36-3A |
| X2180-1A | MATa suc2 mat1 gal2 cup1                | ATCC 26786              |

The Journal of Cell Biology, Volume 114, 1991 102
detrimental to the recipient strain and restored sensitivity to Calcofluor. The 1.5-kb Clai-XhoI fragment was not required for restoring sensitivity to Calcofluor (see plasmid pHV9) but any further deletion either by removing the 2.4-kb XhoI-PstI fragment or by controlled digestion with exonuclease III failed to restore Calcofluor sensitivity. Thus, the minimal sequence necessary for restoring sensitivity to Calcofluor is present in the 3.9-kb insert of pHV9. Southern blots with a radiolabeled pHV9 fragment as a probe showed hybridization to a single locus (results not shown). Allelic tests (see below) indicated that this fragment was closely linked to the calRI mutation and that we had cloned the CALI gene.

Mapping of CALI

When a Southern blot of S. cerevisiae chromosomes separated by pulsed field electrophoresis (11) was probed with CALI DNA, a signal was observed from chromosome II (results not shown). As a control the same blot was probed, after extensive washing, with PH05 DNA (kindly provided by A. Domínguez, Instituto de Microbiología, Salamanca, Spain), which was previously mapped on chromosome II (26). A signal was observed from exactly the same location, thereby confirming the result that the CALI gene is on chromosome II.

Characteristics of the calRI Mutation and Its Reversion by Transformation with Plasmid pHV9

The calRI genotype was described (35) as a result of a mutation displaying a pleiotropic phenotype, i.e., resistance to Calcofluor, absence of thick septa in the presence of Calcofluor, reduced cell wall chitin content and a peculiar sporulating defect in the homozygous condition. Further characterization of this defect indicated that asc and ascospores were normally produced and that their viability was similar to that observed in a diploid control strain; however, micro-manipulation of the ascospores was extremely difficult. Electron micrographs of sectioned ascii revealed the absence of the two outermost dark layers (results not shown) of which the inner one consists of chitosan (6).

Further characterization of the calRI mutant indicated that levels of yeast endochitinase and UDP-GlcNAc, the substrate for chitin synthesis, were similar to those of the wild type strain (results not shown); the same results were obtained with the calR2, calR3, and calR5 mutants. Activation of chitin synthesis after α-factor treatment (38) was almost null in the mutant strain (1.3-fold activation as compared to approximately fivefold in the case of the wild type strain).

The availability of a plasmid containing just the coding sequence of the CALI gene (see next section) allowed us to test whether or not the pleiotropic effects described above were the result of a single mutation. The results clearly indicate (Table II) that transformation of the CRYI-15D mutant strain with a monocopy or multicopy plasmid carrying the CALI gene restored all the wild type characteristics. Therefore, the CALI locus appears to be responsible for all those characteristics.

DNA Sequence of CALI and Characterization of mRNA

The nucleotide sequence of the 3.9-kb fragment carrying the S. cerevisiae CALI gene was determined following the strategy outlined in Fig. 2. The DNA sequence (Fig. 3) contains
Table II. Effects of Transformation with a CALI-carrying Plasmid

| Strain       | Plasmid | Calcofluor resistance | Thick septa | Chitin | Mature ascospores |
|--------------|---------|-----------------------|-------------|--------|-------------------|
| X2180-1A (Cal³) | -       | +                     | 100         | +      |                   |
| X2180-1A (Cal³) [pHV9] | -       | +                     | ND          | +      |                   |
| CRY1-15D (cal²¹) | +       | -                     | 10          | -      |                   |
| CRY1-15D (cal²¹) [pHV7] | -       | +                     | 112         | +      |                   |
| CRY1-15D (cal²¹) [pHV9] | -       | +                     | 102         | +      |                   |

Calcofluor resistance (+/-) refers to ability/inability to grow in the presence of Calcofluor. Thick septa (+/-) refers to the presence or absence of anomalous thick septa between mother and daughter cells when growing in the presence of Calcofluor. The amount of chitin in the cell walls is expressed as the percent of the level measured in a wild-type strain. Mature ascospores (+/-) refers to the presence or absence of the two outermost darker layers in the ascospore cell wall.

The 5'-flanking sequence of the coding region revealed the presence of two "TATA-like" elements at positions -40 (ATAAT) and -27 (TATTA). Other sequences functionally substituting for TATA boxes, as recently described (44), are not present in the 5' region of the CAlI locus. The consensus heptamer sequence TGAAACA (19), proposed as a part of the specific activating system by yeast pheromones, is not present either. The 3' noncoding region contains the proposed (29) polyadenylation consensus sequence AATAAA at positions 3,404-3,409 and the termination sequence TAC . . . TACTGT/TATGGT . . . TTT, slightly different from the consensus TGA . . . TA(T)GT . . . TTT proposed by Zaret et al. (46). The size of the CAlI mRNA, as determined by Northern blot analysis of poly(A)+ RNA (results not shown), was ~4 kb, i.e., similar to that described for CHS1 mRNA (2).

Similarity Between CAlI and Chitin Synthase Genes

A search for similarity to any known sequences was conducted by screening the EMBL and GENBANK DNA and protein databases with the nucleotide or derived protein sequence of the CAlI locus. Despite the fact that little similarity exists between the nucleotide sequences, a remarkable degree of amino acid sequence homology was observed when the CAlI predicted protein was compared with those of the chitin synthase 1 (CHS1; 9) and chitin synthase 2 (CHS2; 42) genes of S. cerevisiae and with that of CHS1 from Candida albicans (4). A region of 251 amino acids, located at the carboxyl terminal portion of the protein sequence, shared 22% identity with the Chs1 protein. In a region of 189 amino acids 25% identity was found with the Chs2 protein. There are two blocks of substantial identity among all four proteins (Fig. 5): one in which 17 out of 35 amino acids are identical from positions 811 to 845 of Call, and the other, a stretch of five identical residues from 926 to 930 (note that this identity extends over nine amino acids between Call and canChs1). It should be borne in mind that the degree of homology between Chs1 and Chs2 (42) or canChs1 and Chs1 (4) is much higher than that observed between any of the three and Call.

The Kyte and Doolittle (22) hydropathy plot (Fig. 4) of the product inferred from the CAlI nucleotide sequence shows a protein with three major domains, a mostly hydrophilic region at the amino terminus spanning residues 1–500 in which two hydrophobic regions are present (residues 100–150 and 300–400), a central neutral sequence from residues 550 to 940, and a carboxyl terminus in which a hydrophobic domain (residues 900–1,000) is followed by a hydrophilic domain (residues 1,000–1,099). The carboxyl terminus hydrophobic region contains several potential membrane-spanning domains. The predicted sequence carries three possible sites for N-glycosylation (39) at positions 48–50 (Asn-Thr-Thr), 86–88 (Asn-Lys-Ser), and 872–874 (Asn-Val-Thr). From the distribution of codons used in the CAlI gene a bias index of 0.16 can be calculated (5) which suggests a poorly expressed gene.
Figure 5. Comparison of predicted protein sequences for CALI, CHSI, CHS2, and C. albicans CHSI (canCHSI). Pairwise LFASTA (28) alignments among all four sequences were used to derive a consensus alignment of Call and Canchsl to the preexisting alignment of Chs1 and Chs2 given by Silverman (42). Gaps were introduced where necessary to hold the alignment of Chs1 and Chs2 constant. Shaded boxes indicate identity while shading only indicates similarity. Only those amino acids that are either identical or similar in all four proteins are so indicated. Limits of the sequences shown are, for Call, amino acids 518-1,099; for Chs1, 361-936; for Chs2, 213-770; for Canchsl, 10-584.
Disruption of the CAL1 Gene and Its Effect

The calR1 mutation was not deleterious for growth despite a considerable decrease in the biosynthesis of cell wall chitin and some morphological abnormalities. It was possible, however, that the mutation could be leaky and for that reason not lethal. To explore that possibility we obtained calR1 mutants by gene disruption of the homologous copy present in a wild type strain. A 0.5-kb BgIII-BglII fragment from within the CAL1 open-reading frame was cloned into the integrative plasmid Yip5 that contains the URA3 gene as a marker. This plasmid was used to transform a diploid Ura3- yeast strain (HVY28) and uracil prototrophs were selected. Integration by homologous recombination between the cloned CAL1 internal fragment and the corresponding chromosomal locus would generate URA+ calR mutant strains containing two defective CAL1 genes separated by the vector sequence. Several transformants were isolated; after sporulation, ascis were dissected. Of 33 ascis, 25 produced four viable ascospores; in all of them segregation of Ura+/Ura- was 2:2. The URA3 marker always cosegregated with the calR1 marker. A cross between a CalR, Ura+ disruptant, and a calR1 mutant strain originated diploids resistant to Calcofluor; after sporulation, the appearance of the ascis and ascospores was similar to that observed for the calR1/calR1 homozygous diploids mentioned above. Analysis of the progeny raised from 30 isolated ascis showed that all clones were resistant to Calcofluor. The same disruptant was crossed to a Ura+, Chs strain, and diploids sporulated. Genetic analysis of eight tetrads revealed 4:0, 3:1, and 2:2 segregation of Ura+/Ura- whereas all other markers segregated 2:2. These results confirmed that plasmid integration had not been produced at the URA3 locus. The structure of the integrated cal::URA3 gene was confirmed by Southern analysis of the chromosomal DNA from several disrupted haploids (results not shown). The phenotype of cal::URA3 strains was identical to that of calR1 mutants. It may be concluded that CAL1 is a nonessential gene in S. cerevisiae.

The CAL1 Gene Is Required for Activity of Chitin Synthase 3

The low chitin content of calI mutants and the sequence similarities between Cal1 and Chs1 and Chs2 suggested the possibility that the gene product of CAL1 may be involved in chitin synthase activity. Therefore, the synthase activity was measured under different conditions in strains carrying appropriate mutations. All strains were defective in Chs1, whose high activity would have obscured the results. Membranes were obtained by direct disruption of cells with glass beads, because this method preserved consistently both Chs3, i.e., trypsin-independent activity, and Chs2 (trypsin-stimulated activity), whereas in preparations from protoplasts usually only Chs2 was measured. The preparation from strain ECY36-3A (chsl CHS2 CALI) showed a decrease in activity after trypsin treatment in the presence of Mg2+ but an increase in the presence of Co2+ (Fig. 6), in agreement with the results of Orlean (27) for a strain of similar genotype. The ratio of activity with Co2+ to that with Mg2+ in the trypsin-treated enzyme was similar to that observed for Chs2 (37, 43). Strain ECY36-3C (chsl chs2::URA3 CALI) yielded an enzyme (Chs3) whose activity was decreased by trypsin both in the presence of Mg2+ and of Co2+ (Fig. 6).

Finally, the preparation from strain ECY36-3D (chsl CHS2 calI) had very low chitin synthase activity without trypsin but was stimulated by trypsin with either Mg2+ or Co2+, as expected for Chs2. The results are consistent with the presence of both Chs2 and Chs3 in strain ECY36-3A, of only Chs3 in strain ECY36-3C and of only Chs2 in strain ECY36-3D. Thus, calR1 strains appear to be specifically deficient in Chs3. In confirmation of these results, in a tetrad resulting from a cross between a CALI and a calI strain, both Calcofluor-resistant segregants showed a very low level of trypsin-independent activity compared to the CALI segregants (results not shown).

Transformation of calI strains with a plasmid containing the CAL1 gene (pHV9) resulted in restoration of trypsin-independent activity at a level somewhat higher than that of wild type (Fig. 7). The plasmid had little effect on the activity of membranes from a CALI strain (Fig. 7). The results were not very different whether a high-copy plasmid (Fig. 7) or a centromere plasmid (results not shown) was used. There-
fore, if CALI is the structural gene of Chs3, other factors needed for maximal activity are limiting in the preparations used.

**Discussion**

Genetic analysis of a cross between an integrative disruptant of the cloned CALI gene and a calA mutant strain indicates that the cloned gene corresponds to the calA locus. Accordingly, plasmids carrying the cloned gene corrected all the deficiencies of calA, i.e., resistance to Calcofluor, low chitin level and defective spore maturation. The sharp decrease in chitin content in calA mutants compared to wild type (35) suggested that CALI may be involved in chitin synthesis. This hypothesis was supported by the finding that the predicted amino acid sequence of the CALI gene product has significant homology with the predicted sequences of Chsl (9) and Chs2 (42) as well as with a chitin synthase gene from C. albicans (4). The homology is detected in the carboxyl terminal region, as was the case in the comparison between Chsl and Chs2. Furthermore, the predicted size of the CALI gene product is close to that of the CHSI and CHS2 products. The amino terminal region of the CALI predicted amino acid sequence contains two hydrophobic domains not present in Chsl and Chs2, included in a mostly hydrophilic region larger than the corresponding one of the other two proteins. The central neutral portion is similar in size to that of Chsl and Chs2, whereas the carboxyl terminal region is much shorter. As in the case of Chsl and Chs2, the CALI protein also has several potential membrane-spanning domains near the carboxyl terminus. A gene that appears to be identical to CALI, based on the restriction map, has been cloned by C. Bulawa (Massachusetts Institute of Technology) and also by M. Breitenbach and his co-workers (University of Vienna, Austria) (genes CSD2 and DIT10, respectively; personal communication of C. Bulawa).

Assays of chitin synthase activity were carried out in three strains from the same tetrad, all of them lacking Chsl: the strain with the genotype CHS2 CALI showed both trypsin-independent and trypsin-dependent activity; the chs2 CALI strain exhibited only trypsin-independent activity (chitin synthase 3; 8), whereas CHS2 calA had only trypsin-dependent activity (Chs2; 37, 43). Clearly, the calA strain is deficient in Chs3. Incidentally, these results indicate that the "chitin synthase II" preparations studied by Orlean (27) actually contained a mixture of Chs2 and Chs3, although most of his results dealt with the properties of Chs3.

The findings summarized above are consistent with the notion that CALI may be the structural gene for Chs3. Other results, however, do not support this possibility. Although transformation of calA strains with plasmids carrying the CALI gene resulted in restoration of Chs3 activity, overexpression of the enzyme was not obtained even with the use of a high-copy plasmid. Transformation of Schizosaccharomyces pombe with plasmids containing CALI did not lead to expression of trypsin-independent chitin synthase activity or to alteration of the natural resistance of this organism to Calcofluor (results not shown). The explanation of these results may reside in the need for more than one protein for Chs3 activity. In addition to calA, three other Calcofluor-resistant mutations have been identified (35), i.e., calB, calP3, and calP5 (calP4 has been reclassified as calP9). All of these mutants are deficient in chitin in vivo (35) and in Chs3 in vitro (results not shown). It is probable, therefore, that the corresponding gene products are required for expression of Chs3, possibly as subunits of the enzyme or activators. If some of these factors are in limiting amounts in the cell, overexpression of the CALI product will not result in increased activity of Chs3. When the genes corresponding to the other calA mutations are cloned, it will be possible to test this hypothesis by overexpressing them together with CALI.

If CALI is a structural gene for Chs3, it seems possible that the relatively few regions that show homology with CHSI and CHS2 may be crucial for synthase activity or regulation. Thus, the availability of the CALI sequence may facilitate the study of structure and function in chitin synthases and suggest the sequence of appropriate nucleotides to search for chitin synthase genes in other fungi by polymerase chain reaction amplification.

Disruption of CALI was not lethal. The phenotype was similar to that of chs2 strains. Therefore, Chs3 is not an essential enzyme. The availability of viable mutants deficient in Chs3 as well as in Chs2 (8) opens the possibility of ascertaining the function of each one of the two synthases. This is the topic of an accompanying report (40).

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