CAL1, A Gene Required for Activity of Chitin Synthase 3 in *Saccharomyces cerevisiae*

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Abstract. The *CAL1* gene was cloned by complementation of the defect in Calcofluor-resistant *cal*^{R}*1* 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 *cal*^{R}*1* 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 synthases 1 and 2 from *S. cerevisiae* and chitin synthase 1 from *Candida albicans. cal*^{R}*1* mutants have been found to be defective in chitin synthase 3, a trypsin-independent synthase. Transformation of the mutants with a plasmid carrying *CAL1* restored chitin synthase 3 activity; however, overexpression of the enzyme was not achieved even with a high copy number plasmid. Since Calcofluor-resistance mutations different from *cal*^{R}*1* 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 *CAL1* gene was not lethal, indicating that chitin synthase 3 is not an essential enzyme for *S. cerevisiae.*

The antifungal effect of Calcofluor White (33, 34), a substance that binds specifically to chitin in vivo (10), was used to isolate and characterize several *Saccharomyces cerevisiae* mutants resistant to this fluorochrome (35). The most interesting feature of these mutants was a defect in chitin synthesis in vivo not only during the vegetative cycle but also in response to the pheromone α-factor (35). A defect in spore maturation in the homozygous condition was observed (35). The possibility that these mutants could define physiologically relevant genes involved in chitin synthesis was apparent; therefore, the cloning and sequencing of the gene corresponding to one of them, *cal*^{R}*1*, was undertaken. In this report we show that the protein sequence encoded by the cloned gene (*CAL1*) has significant similarities with those of chitin synthase 1 (9) and chitin synthase 2 (42, 43). Recently, Bulawa and Osmond (8) detected the presence of a third chitin synthase (chitin synthase 3 or Chs3[1]) in strains defective in chitin synthase 1 and 2. We report here that *cal*^{R}*1* mutants are deficient in chitin synthase 3, an enzymatic activity that, in contrast to synthases 1 and 2, does not require proteolytic activation.

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 CR1, ECY33-2A, and ECY33-18A were used as recipient strains in transformations with plasmids carrying the *CAL1* gene. The diploid strain HVY28 was the recipient in the *CAL1* gene disruption experiment. *S. cerevisiae* AP1 (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 (~10^6 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-

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[1] *Abbreviations used in this paper*: Chs1-3, chitin synthase 1-3; canChs1, chitin synthase 1 from *Candida albicans.*

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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 counting the cells.

Plasmids and Transformations

The yeast genomic library constructed in the plasmid YCp50 (18) and the additional yeast vectors YEp352 (16), YEp13 (7), and YIp5 were provided by F. del Rey (Instituto de Microbiología 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 diam 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 CAL1 Gene

The *CAL1* gene was cloned by complementation of the Calcofluor resistance phenotype. A *cal*^{R}*1, 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 nonselective conditions (YED medium) simultaneously lost both Ura$^+$ and Cal^S 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 *cal*^{R}*1, ura3* recipient strain. The new transformants were found to be Cal^S. The 8.4-kb-long DNA insert cloned in the pHV1 plasmid was used for subcloning and characterization of the *CAL1* 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 XhoI site and the right hand BamHI site could not effect reversion of the Cal^R 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	*MATα cal*^{R}*1 ade1 his3*	ATCC 64941
CRY1-15D	*MAT***a** *cal*^{R}*1 ura3 his3*	Derived from CR1
CR4217A	*MATα cal*^{R}*2 ura3*	Derived from ATCC 64944
HV2324	*MATα cal*^{R}*3 ura3*	ATCC 64948
HV2627B	*MATα cal*^{R}*5 ura3*	ATCC 64945
HVY28	*MAT***a***/MATα cal*^{R}*1/Cal*^{S} *ura3/ura3 leu2/LEU2 his3/HIS3*	This study
ECY33-2A	*MAT***a** *chs1-23 leu2-3,112 ura3-52*	This study
ECY33-18A	*MAT***a** *chs1-23 cal*^{R}*1 leu2-3,112 trp1-1 ura3-52*	This study
ECY19Δ2	*MAT***a***/MATα chs1-23/chs1-23 chs2::URA3/CHS2 leu2-3,112/leu2-3,112 ura3-52/ura3-52 trp1-1/trp1-1*	This study
ECY19Δ2-5B	*MATα chs1-23 chs2::LEU2 leu2-3,112 ura3-52 trp1-1*	From sporulation and dissection of ECY19Δ2
ECY36-3A	*MATα chs1-23 leu 2-3,112 ura3-52 trp1-1*	From cross ECY19Δ2-5B × ECY33-18A
ECY36-3C	*MAT***a** *chs1-23 chs2::LEU2 leu2-3,112 ura3-52 trp1-1*	Same tetrad as ECY36-3A
ECY36-3D	*MAT***a** *chs1-23 cal*^{R}*1 leu2-3,112 ura3-52 trp1-1*	Same tetrad as ECY36-3A
X2180-1A	*MAT***a** *suc2 mal1 gal2 cup1*	ATCC 26786

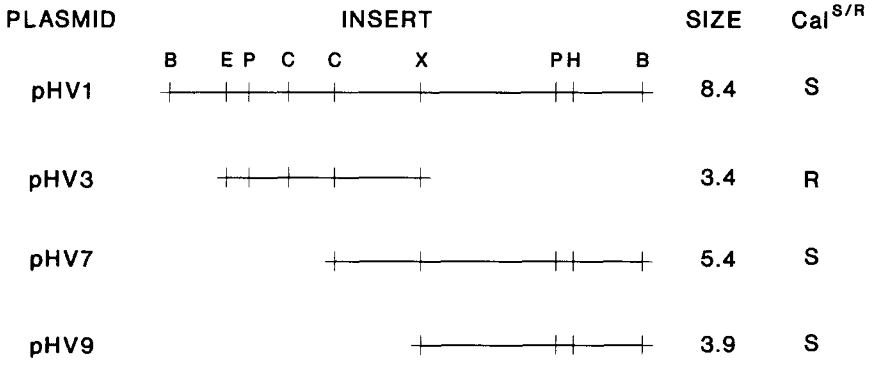

Figure 1. Structure of different plasmids constructed to subclone the *CAL1* gene. *B*, BamHI; *E*, EcoRI; *P*, PstI; *C*, ClaI; *X*, XhoI; *H*, HindIII. Sizes are given in kilobases. Cal$^{S/R}$ phenotype refers to the sensitivity or resistance to Calcofluor of the corresponding transformants. pHV1-pHV7 plasmids were constructed in the YCp50 vector; the pHV9 plasmid was constructed in the YEp352 vector.

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 *cal*^{R}*1* mutation and that we had cloned the *CAL1* gene.

Mapping of CAL1

When a Southern blot of *S. cerevisiae* chromosomes separated by pulsed field electrophoresis (11) was probed with *CAL1* DNA, a signal was observed from chromosome II (results not shown). As a control the same blot was probed, after extensive washing, with *PHO5* 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 *CAL1* gene is on chromosome II.

Characteristics of the cal^{R}1 Mutation and Its Reversion by Transformation with Plasmid pHV9

The *cal*^{R}*1* 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 asci and ascospores were normally produced and that their viability was similar to that observed in a diploid control strain; however, micromanipulation of the ascospores was extremely difficult. Electron micrographs of sectioned asci 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 *cal*^{R}*1* 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 *cal*^{R}*2*, *cal*^{R}*3*, and *cal*^{R}*5* 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 *CAL1* 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 CRY1-15D mutant strain with a monocopy or multicopy plasmid carrying the *CAL1* gene restored all the wild type characteristics. Therefore, the *CAL1* locus appears to be responsible for all those characteristics.

DNA Sequence of CAL1 and Characterization of mRNA

The nucleotide sequence of the 3.9-kb fragment carrying the *S. cerevisiae CAL1* gene was determined following the strategy outlined in Fig. 2. The DNA sequence (Fig. 3) contains

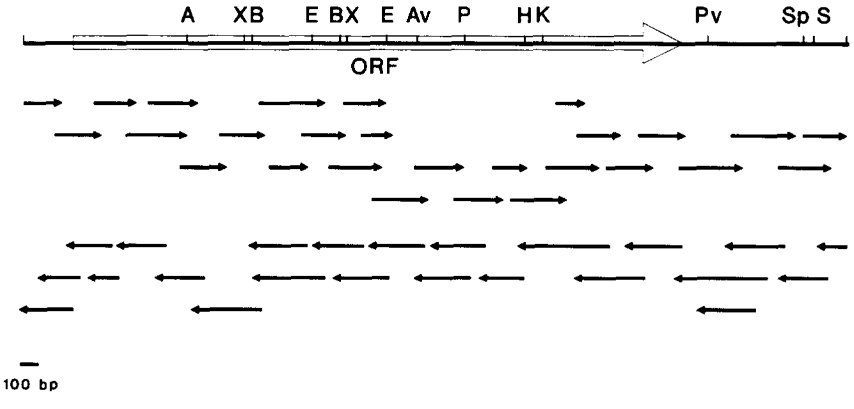

Figure 2. Partial restriction endonuclease map of the *CAL1* gene. *A*, ApaI; *X*, XbaI; *B*, BglII; *E*, EcoRV; *Av*, AvaI; *P*, PstI; *H*, HindIII; *K*, KpnI; *Pv*, PvuII; *Sp*, SphI, and *S*, SalI. Open reading frame (*ORF*) of *CAL1*. Arrow points to the direction of transcription. The sequenced fragments are indicated by black arrows.

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-278   CC TCT AGA GTC GAG TAT TTT TGG TGT AGG TCC CAT TTT CTT CAA AGG   -230
-231  TCC TGT TTA GAC TAT CCG CAG GAA AGA AAT TAG AAT GAC CGG CTT GAA   -184
-183  TGG AGA TGA TCC TGA TGA CTA CTA TCT GAA CCT TAA TCA AGA TGA AGA   -136
-135  GTC TCT ACT TAG GTC AAG ACA CAG TGT CGG CTC AGG AGC ACC TCA TAG    -88
 -87  ACA AGG CTC TTT AGT GCG GCC CGA AAG AAG CCG ACT GAA CAA TCC TGA    -40
 -39  TAA TCC ACT TTT TAT TAT GCG CAG AAA ACG CAG GAG CAG ATG AAT CAC      9
   1                                                  Met Asn His      3

  10  CTG GAT GTT TTA CCA TCA AGT ACC GGT GTA AAC CCA AAT GCA ACT CGT     57
   4  Leu Asp Val Leu Pro Ser Ser Thr Gly Val Asn Pro Asn Ala Thr Arg     19

  58  CGG AGT GGC TCC CTG CGC TCC AAA GGC TCA GTG AGA AGC AAA TTT AGT    105
  20  Arg Ser Gly Ser Leu Arg Ser Lys Gly Ser Val Arg Ser Lys Phe Ser     35

 106  GGC CGC GAA ACG GAT AGC TAT CTT TTA CAA GAT ATG AAT ACT ACT GAC    153
  36  Gly Arg Glu Thr Asp Ser Tyr Leu Leu Gln Asp Met Asn Thr Thr Asp     51

 154  AAG AAG GCT TCC GTT AAA ATA AGT GAT GAA GGT GTT GCG GAA GAC GAA    201
  52  Lys Lys Ala Ser Val Lys Ile Ser Asp Glu Gly Val Ala Glu Asp Glu     67

 202  TTT GAT AAA GAT GGT GAT GTG GAC AAT TTC GAA GAA AGC TCC ACG CAG    249
  68  Phe Asp Lys Asp Gly Asp Val Asp Asn Phe Glu Glu Ser Ser Thr Gln     83

 250  CCC ATA AAT AAG TCT ATC AAA CCA TTA AGA AAG GAA ACG AAT GAT ACA    297
  84  Pro Ile Asn Lys Ser Ile Lys Pro Leu Arg Lys Glu Thr Asn Asp Thr     99

 298  TTG TCA TTT TGG CAG ATG TAC TGT TAT TTC ATT ACG TTT TGG GCA CCT    345
 100  Leu Ser Phe Trp Gln Met Tyr Cys Tyr Phe Ile Thr Phe Trp Ala Pro    115

 346  GCT CCA ATT CTT GCT TTC TGC GGG ATG CCA AAG AAG GAA AGA CAA ATG    393
 116  Ala Pro Ile Leu Ala Phe Cys Gly Met Pro Lys Lys Glu Arg Gln Met    131

 394  GCG TGG AGA GAA AAG GTT GCT TTA ATT TCT GTC ATC TTG TAC ATT GGT    441
 132  Ala Trp Arg Glu Lys Val Ala Leu Ile Ser Val Ile Leu Tyr Ile Gly    147

 442  GCG ATT GTG GCT TTC CTG ACT TTT GGT TTC ACT AAA ACC GTT TGT AGT    489
 148  Ala Ile Val Ala Phe Leu Thr Phe Gly Phe Thr Lys Thr Val Cys Ser    163

 490  AGT TCG AAA CTA CGT TTG AAA AAC AAC GAA GTA TCA ACA GAA TTT GTC    537
 164  Ser Ser Lys Leu Arg Leu Lys Asn Asn Glu Val Ser Thr Glu Phe Val    179

 538  GTA ATT AAC GGT AAG GCT TAT GAA TTG GAT ACT TCC TCG CGT TCC GGT    585
 180  Val Ile Asn Gly Lys Ala Tyr Glu Leu Asp Thr Ser Ser Arg Ser Gly    195

 586  ATA CAA GAC GTT GAA GTA GAT TCA GAC ACC CTT TAT GGG CCC TGG TCA    633
 196  Ile Gln Asp Val Glu Val Asp Ser Asp Thr Leu Tyr Gly Pro Trp Ser    211

 634  GAT GCT GGT AAA GAT GCT TCG TTC TTG TTT CAA AAT GTG AAT GGT AAC    681
 212  Asp Ala Gly Lys Asp Ala Ser Phe Leu Phe Gln Asn Val Asn Gly Asn    227

 682  TGT CAT AAC CTT ATA ACT CCA AAG AGT AAT TCT TCC ATT CCC CAT GAC    729
 228  Cys His Asn Leu Ile Thr Pro Lys Ser Asn Ser Ser Ile Pro His Asp    243

 730  GAT GAT AAT AAT TTA GCA TGG TAT TTT CCT TGT AAG TTA AAG AAT CAA    777
 244  Asp Asp Asn Asn Leu Ala Trp Tyr Phe Pro Cys Lys Leu Lys Asn Gln    259

 778  GAT GGC TCT TCG AAG CCA AAC TTC ACA GTT GAA AAT TAC GCA GGA TGG    825
 260  Asp Gly Ser Ser Lys Pro Asn Phe Thr Val Glu Asn Tyr Ala Gly Trp    275

 826  AAC TGT CAT ACG TCT AAA GAA GAT AGG GAC GCA TTT TAC GGT TTA AAG    873
 276  Asn Cys His Thr Ser Lys Glu Asp Arg Asp Ala Phe Tyr Gly Leu Lys    291

 874  TCG AAA GCT GAT GTT TAC TTC ACT TGG GAT GGT ATA AAG AAC TCT TCT    921.
 292  Ser Lys Ala Asp Val Tyr Phe Thr Trp Asp Gly Ile Lys Asn Ser Ser    307

 922  AGA AAC TTG ATT GTT TAT AAT GGC GAC GTT TTG GAT TTA GAT CTT CTT    969
 308  Arg Asn Leu Ile Val Tyr Asn Gly Asp Val Leu Asp Leu Asp Leu Leu    323


 970  GAT TGG TTG GAA AAG GAT GAC GTT GAC TAT CCC GTT GTA TTC GAT GAC   1017
 324  Asp Trp Leu Glu Lys Asp Asp Val Asp Tyr Pro Val Val Phe Asp Asp    339

1018  TTG AAG ACT TCA AAT TTA CAA GGT TAT GAT CTT TCG TTG GTT TTG TCA   1065
 340  Leu Lys Thr Ser Asn Leu Gln Gly Tyr Asp Leu Ser Leu Val Leu Ser    355

1066  AAT GGG CAT GAA AGA AAA ATT GCG AGA TGT TTG AGC GAA ATT ATT AAA   1113
 356  Asn Gly His Glu Arg Lys Ile Ala Arg Cys Leu Ser Glu Ile Ile Lys    371

1114  GTT GGT GAA GTA GAC TCC AAA ACC GTC GGT TGT ATT GCC TCT GAT GTC   1161
 372  Val Gly Glu Val Asp Ser Lys Thr Val Gly Cys Ile Ala Ser Asp Val    387

1162  GTT TTG TAT GTT TCT CTG GTA TTT ATT CTT TCA GTG GTG ATA ATT AAA   1209
 388  Val Leu Tyr Val Ser Leu Val Phe Ile Leu Ser Val Val Ile Ile Lys    403

1210  TTC ATA ATT GCC TGC TAC TTC CGT TGG ACT GTA GCT AGG AAA CAA GGT   1257
 404  Phe Ile Ile Ala Cys Tyr Phe Arg Trp Thr Val Ala Arg Lys Gln Gly    419

1258  GCA TAT ATC GTG GAC AAT AAA ACA ATG GAT AAA CAC ACA AAC GAT ATC   1305
 420  Ala Tyr Ile Val Asp Asn Lys Thr Met Asp Lys His Thr Asn Asp Ile    435

1306  GAG GAT TGG TCT AAT AAT ATT CAA ACA AAA GCT CCT CTA AAG GAA GTA   1353
 436  Glu Asp Trp Ser Asn Asn Ile Gln Thr Lys Ala Pro Leu Lys Glu Val    451

1354  GAT CCT CAT TTG AGG CCA AAG AAA TAC TCA AAA AAG TCG TTG GGA CAC   1401
 452  Asp Pro His Leu Arg Pro Lys Lys Tyr Ser Lys Lys Ser Leu Gly His    467

1402  AAG CGT GCT TCA ACC TTT GAC TTG CTG AAA AAA CAC AGC TCC AAA ATG   1449
 468  Lys Arg Ala Ser Thr Phe Asp Leu Leu Lys Lys His Ser Ser Lys Met    483

1450  TTT CAA TTT AAC GAA TCT GTG ATA GAT CTA GAC ACC TCC ATG AGC AGT   1497
 484  Phe Gln Phe Asn Glu Ser Val Ile Asp Leu Asp Thr Ser Met Ser Ser    499

1498  TCA CTA CAA TCT TCT GGT TCA TAC AGA GGA ATG ACA ACA ATG ACC ACT   1545
 500  Ser Leu Gln Ser Ser Gly Ser Tyr Arg Gly Met Thr Thr Met Thr Thr    515

1546  CAA AAT GCT TGG AAA CTC TCG AAT GAA AAC AAA GCT GTA CAT TCC CGT   1593
 516  Gln Asn Ala Trp Lys Leu Ser Asn Glu Asn Lys Ala Val His Ser Arg    531

1594  AAT CCA TCT ACT TTG TTG CCT ACA TCC TCG ATG TTT TGG AAT AAA GCG   1641
 532  Asn Pro Ser Thr Leu Leu Pro Thr Ser Ser Met Phe Trp Asn Lys Ala    547

1642  ACT TCC TCT CCT GTA CCA GGA TCA TCG CTG ATT CAG AGT CTT GAT TCG   1689
 548  Thr Ser Ser Pro Val Pro Gly Ser Ser Leu Ile Gln Ser Leu Asp Ser    563

1690  ACG ATT ATA CAT CCC GAT ATC GTT CAA CAA CCA CCA CTG GAT TTT ATG   1737
 564  Thr Ile Ile His Pro Asp Ile Val Gln Gln Pro Pro Leu Asp Phe Met    579

1738  CCA TAC GGG TTC CCA TTG ATT CAT ACT ATC TGT TTT GTT ACT TGT TAT   1785
 580  Pro Tyr Gly Phe Pro Leu Ile His Thr Ile Cys Phe Val Thr Cys Tyr    595

1786  TCT GAG GAT GAA GAG GGT TTA AGA ACC ACT TTA GAC TCT CTT TCT ACC   1833
 596  Ser Glu Asp Glu Glu Gly Leu Arg Thr Thr Leu Asp Ser Leu Ser Thr    611

1834  ACA GAT TAT CCA AAT TCC CAT AAA CTA CTG ATG GTT GTT TGT GAT GGT   1881
 612  Thr Asp Tyr Pro Asn Ser His Lys Leu Leu Met Val Val Cys Asp Gly    627

1882  TTA ATT AAG GGC TCG GGC AAC GAT AAG ACT ACT CCA GAG ATA GCG TTA   1929
 628  Leu Ile Lys Gly Ser Gly Asn Asp Lys Thr Thr Pro Glu Ile Ala Leu    643

1930  GGA ATG ATG GAC GAC TTT GTC ACC CCA CCT GAT GAA GTT AAA CCT TAC   1977
 644  Gly Met Met Asp Asp Phe Val Thr Pro Pro Asp Glu Val Lys Pro Tyr    659

1978  TCC TAT GTG GCA GTG GCA TCA GGC TCT AAA AGA CAC AAT ATG GCC AAG   2025
 660  Ser Tyr Val Ala Val Ala Ser Gly Ser Lys Arg His Asn Met Ala Lys    675

2026  ATA TAT GCG GGT TTT TAC AAA TAT GAC GAT TCT ACA ATT CCA CCA GAA   2073
 676  Ile Tyr Ala Gly Phe Tyr Lys Tyr Asp Asp Ser Thr Ile Pro Pro Glu    691

2074  AAT CAA CAA CGT GTC CCA ATC ATT ACA ATT GTG AAG TGC GGT ACT CCT   2121
 692  Asn Gln Gln Arg Val Pro Ile Ile Thr Ile Val Lys Cys Gly Thr Pro    707

2122  GCA GAG CAG GGG GCC GCC AAA CCC GGT AAC AGA GGT AAG CGT GAT TCT   2169
 708  Ala Glu Gln Gly Ala Ala Lys Pro Gly Asn Arg Gly Lys Arg Asp Ser    723

2170  CAA ATT ATT CTG ATG TCC TTT TTA GAA AAA ATA ACA TTT GAT GAA AGA   2217
 724  Gln Ile Ile Leu Met Ser Phe Leu Glu Lys Ile Thr Phe Asp Glu Arg    739

2218  ATG ACT CAA TTG GAA TTT CAG CTT TTA AAA AAT ATT TGG CAG ATT ACG   2365
 740  Met Thr Gln Leu Glu Phe Gln Leu Leu Lys Asn Ile Trp Gln Ile Thr    755

2266  GGG CTA ATG GCA GAC TTC TAC GAA ACG GTA CTT ATG GTT GAT GCT GAT   2313
 756  Gly Leu Met Ala Asp Phe Tyr Glu Thr Val Leu Met Val Asp Ala Asp    771

2314  ACT AAA GTC TTT CCC GAT GCT TTA ACT CAT ATG GTC GCT GAA ATG GTT   2361
 772  Thr Lys Val Phe Pro Asp Ala Leu Thr His Met Val Ala Glu Met Val    787

2362  AAA GAT CCT TTG ATT ATG GGT CTT TGT GGT GAG ACC AAG ATC GCT AAT   2409
 788  Lys Asp Pro Leu Ile Met Gly Leu Cys Gly Glu Thr Lys Ile Ala Asn    803

2410  AAG GCA CAA TCT TGG GTA ACT GCA ATT CAA GTG TTT GAG TAC TAT ATT   2457
 804  Lys Ala Gln Ser Trp Val Thr Ala Ile Gln Val Phe Glu Tyr Tyr Ile    819

2458  TCG CAT CAT CAG GCT AAA GCT TTT GAA TCT GTC TTC GGT TCG GTA ACT   2505
 820  Ser His His Gln Ala Lys Ala Phe Glu Ser Val Phe Gly Ser Val Thr    835

2506  TGT TTG CCG GGA TGT TTC TCA ATG TAT CGT ATA AAA TCT CCT AAA GGT   2553
 836  Cys Leu Pro Gly Cys Phe Ser Met Tyr Arg Ile Lys Ser Pro Lys Gly    851

2554  TCA GAT GGT TAT TGG GTA CCT GTA TTG GCA AAT CCA GAT ATT GTT GAA   2601
 852  Ser Asp Gly Tyr Trp Val Pro Val Leu Ala Asn Pro Asp Ile Val Glu    867

2602  AGA TAT TCG GAT AAT GTT ACA AAC ACT TTG CAT AAG AAG AAC TTA TTA   2649
 868  Arg Tyr Ser Asp Asn Val Thr Asn Thr Leu His Lys Lys Asn Leu Leu    883

2650  TTA CTT GGT GAA GAT AGA TTT TTA TCT TCA TTA ATG TTA AAG ACT TTC   2697
 884  Leu Leu Gly Glu Asp Arg Phe Leu Ser Ser Leu Met Leu Lys Thr Phe    899

2698  CCT AAG AGA AAG CAA GTA TTT GTT CCA AAA GCT GCT TGT AAA ACT ATT   2745
 900  Pro Lys Arg Lys Gln Val Phe Val Pro Lys Ala Ala Cys Lys Thr Ile    915

2746  GCC CCT GAT AAA TTC AAA GTC TTA CTT TCC CAG CGT CGA AGA TGG ATT   2793
 916  Ala Pro Asp Lys Phe Lys Val Leu Leu Ser Gln Arg Arg Arg Trp Ile    931

2794  AAT TCT ACG GTA CAT AAC CTT TTT GAA TTA GTT CTA ATC AGA GAC TTA   2841
 932  Asn Ser Thr Val His Asn Leu Phe Glu Leu Val Leu Ile Arg Asp Leu    947

2842  TGT GGC ACT TTC TGT TTT TCC ATG CAA TTT GTG ATT GGT ATT GAA TTG   2889
 948  Cys Gly Thr Phe Cys Phe Ser Met Gln Phe Val Ile Gly Ile Glu Leu    963

2890  ATT GGT ACT ATG GTA CTG CCG TTA GCC ATT TGC TTT ACT ATT TAT GTC   2937
 964  Ile Gly Thr Met Val Leu Pro Leu Ala Ile Cys Phe Thr Ile Tyr Val    979

2938  ATT ATT TTT GCC ATT GTA TCA AAA CCT ACA CCC GTA ATC ACT TTA GTT   2985
 980  Ile Ile Phe Ala Ile Val Ser Lys Pro Thr Pro Val Ile Thr Leu Val    995

2986  TTA CTG GCA ATT ATT CTT GGT CTG CCC GGC TTA ATT GTT GTT ATA ACT   3033
 996  Leu Leu Ala Ile Ile Leu Gly Leu Pro Gly Leu Ile Val Val Ile Thr   1011

3034  GCT ACG AGA TGG TCG TAC CTA TGG TGG ATG TGC GTA TAT ATT TGT GCT   3081
1012  Ala Thr Arg Trp Ser Tyr Leu Trp Trp Met Cys Val Tyr Ile Cys Ala   1027

3082  TTG CCT ATT TGG AAT TTC GTA CTA CCT TCA TAT GCG TAC TGG AAA TTT   3129
1028  Leu Pro Ile Trp Asn Phe Val Leu Pro Ser Tyr Ala Tyr Trp Lys Phe   1043

3130  GAT GAC TTC TCA TGG GGT GAT ACG AGA ACT ATT GCG GGA GGT AAT AAA   3177
1044  Asp Asp Phe Ser Trp Gly Asp Thr Arg Thr Ile Ala Gly Gly Asn Lys   1059


3178  AAG GCA CAA GAC GAG AAT GAA GGT GAA TTT GAT CAC TCA AAG ATT AAA   3225
1060  Lys Ala Gln Asp Glu Asn Glu Gly Glu Phe Asp His Ser Lys Ile Lys   1075

3226  ATG AGG ACA TGG AGG GAA TTT GAA AGG GAA GAT ATT CTC AAT CGG AAG   3273
1076  Met Arg Thr Trp Arg Glu Phe Glu Arg Glu Asp Ile Leu Asn Arg Lys   1091

3274  GAG GAA AGT GAC TCC TTC GTT GCA TAG ACA GTA TGA AAA TAT TTT TAC   3321
1092  Glu Glu Ser Asp Ser Phe Val Ala                                   1099

3322  TGT GAT ACT TAC AAG TTG ATA TAT GGT TGT GTG TAA CTT ATT TAT TTG   3369
3370  AGA GGT ATT TTA ACA CAC CTT AGA ACT AAA ACT TAA TAA ATA AAT ATT   3417
3418  TCT CTA TCT TTA AAG GCA CAT ATT ACG TGG CTA AGG CAA TTA CAG CTG   3465
3466  ATA TAC TGT AAA ACT CAT GTC GCC ACT AAA TTC TTC TAA CAC GCG TTC   3513
3514  TGT CTC TTT CCA AGG GAC TCC GAA TAT GCC ACT ATT TAT CTG TGG CAT   3561
3562  TTC CAA TTT ATA TTC CCC TAT TGG GTA TTT GAT GTG GCC GTT TAA ATA   3609
3610  GTC ACC GAT TGA ATC TTC ACT TGT TCG AGT TTT GTC TTT TGC TTC TCT   3657
3658  AAA GGT CTT CAA TTT ATC TAA AGC AAG TTT TGT ATA ATT CAA AAT ACT   3705
3706  TTG CTT TTC TCC ATG ACT TGA ACC TCC AAA TGA TGA TGT AAA CAA GCA   3753
3754  ACA AAT CAG CAG ATC                                               3768
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Figure 3. Nucleotide and predicted amino acid sequences of *CAL1*. The open reading frame (3,297 bp) contained within the pHV9 plasmid is shown along with the predicted amino acid sequence (1,099 amino acids). These sequence data are available from EMBL/GenBank/DDBJ under accession number X57300.

Table II. Effects of Transformation with a CAL1-carrying Plasmid

Strain		Plasmid	Calcofluor resistance	Thick septa	Chitin	Mature ascospores
					%	
X2180-1A	(Cal^{S})		–	+	100	+
X2180-1A	(Cal^{S})	[pHV9]	–	+	ND	+
CRY1-15D	(*cal^{R}1*)		+	–	10	–
CRY1-15D	(*cal^{R}1*)	[pHV7]	–	+	112	+
CRY1-15D	(*cal^{R}1*)	[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.

a single, long open-reading frame of 3,297 bp and no consensus signals for intron splicing (23). Starting from the first ATG in the open reading frame, the *CAL1* locus encodes a protein of 1,099 amino acids with a molecular mass of 124,023 daltons.

The Kyte and Doolittle (22) hydropathy plot (Fig. 4) of the product inferred from the *CAL1* 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 *CAL1* gene a bias index of 0.16 can be calculated (5) which suggests a poorly expressed gene.

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 *CAL1* locus. The consensus heptameric repeating 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 *CAL1* 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 CAL1 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 *CAL1* locus. Despite the fact that little similarity exists between the nucleotide sequences, a remarkable degree of amino acid sequence homology was observed when the *CAL1* 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 Cal1, and the other, a stretch of five identical residues from 926 to 930 (note that this identity extends over nine amino acids between Cal1 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 Cal1.

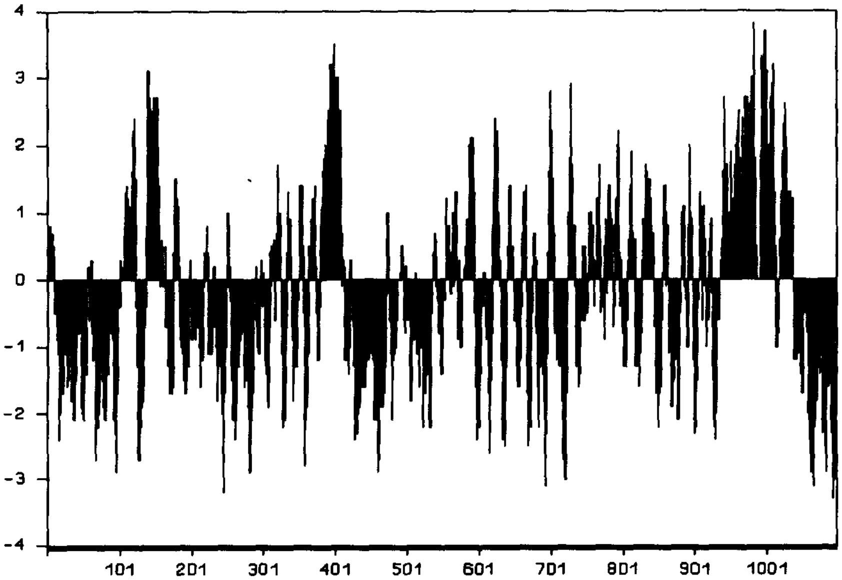
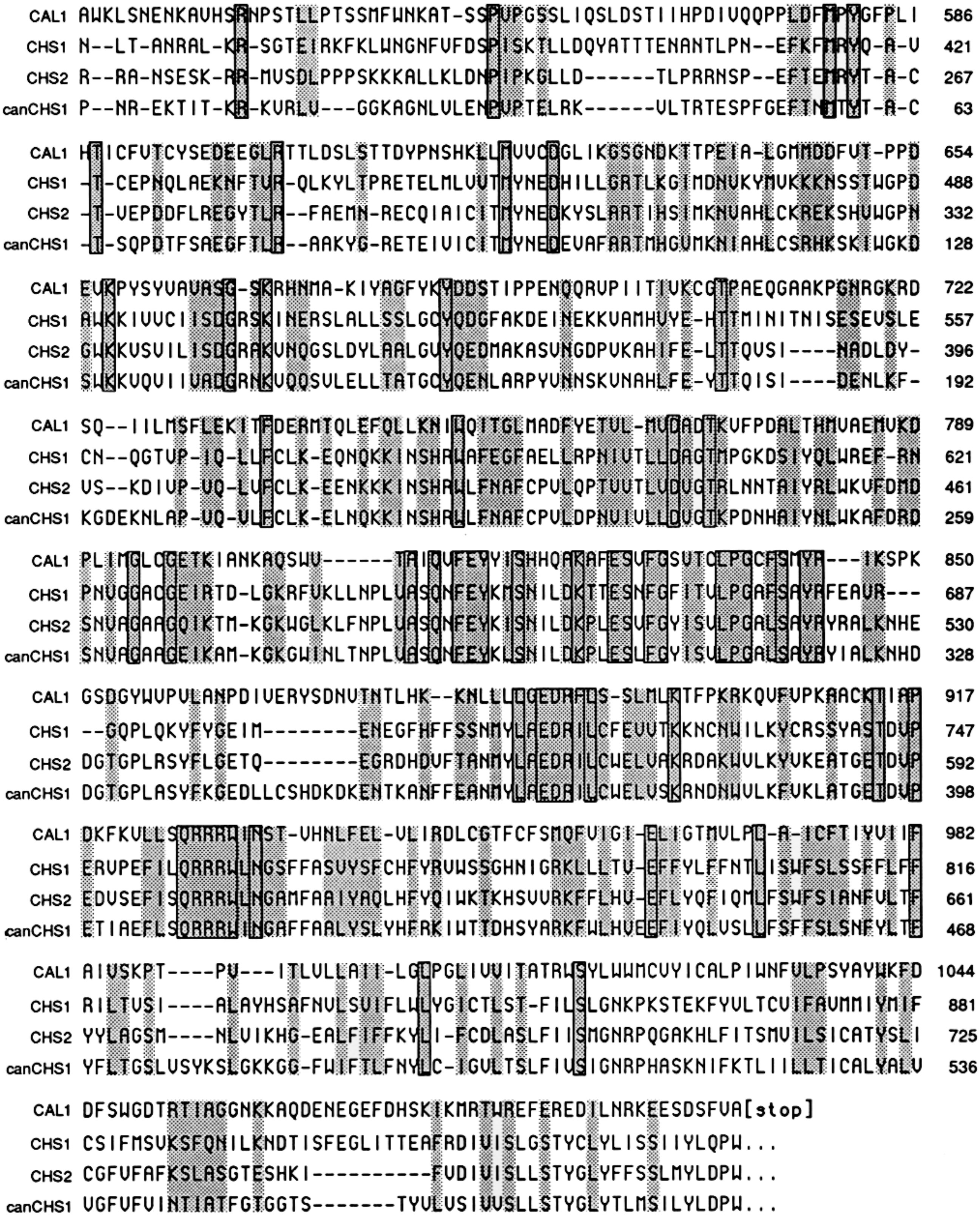


Figure 4. Hydropathic profile of amino acid sequence of *CAL1*. The abscissa indicates residue number and the ordinate relative hydropathic index.



```
CAL1    AWKLSNENKAVHSRNPSTLLPTSSMFWNKAT-SSPVPGSSLIQSLDSTIIHPDIVQQPPLDFMPYGFPLI   586
CHS1    N--LT-ANRAL-KR-SGTEIRKFKLWNGNFVFDSPISKTLLDQYATTTENANTLPN--EFKFMRYQ-A-V   421
CHS2    R--RA-NSESK-RR-MVSDLPPPSKKKALLKLDNPIPKGLLD------TLPRRNSP--EFTEMRYT-A-C   267
canCHS1 P--NR-EKTIT-KR-KVRLV---GGKAGNLVLENPVPTELRK------VLTRTESPFGEFTNMTYT-A-C    63

CAL1    HTICFVTCYSEDEEGLRTTLDSLSTTDYPNSHKLLMVVCDGLIKGSGNDKTTPEIA-LGMMDDFVT-PPD   654
CHS1    -T-CEPNQLAEKNFTVR-QLKYLTPRETELMLVVTMYNEDHILLGRTLKGIMDNVKYMVKKKNSSTWGPD   488
CHS2    -T-VEPDDFLREGYTLR--FAEMN-RECQIAICITMYNEDKYSLARTIHSIMKNVAHLCKREKSHVWGPN   332
canCHS1 -T-SQPDTFSAEGFTLR--AAKYG-RETEIVICITMYNEDEVAFARTMHGVMKNIAHLCSRHKSKIWGKD   128

CAL1    EVKPYSYVAVASG-SKRHNMA-KIYAGFYKYDDSTIPPENQQRVPIITIVKCGTPAEQGAAKPGNRGKRD   722
CHS1    AWKKIVVCIISDGRSKINERSLALLSSLGCYQDGFAKDEINEKKVAMHVYE-HTTMINITNISESEVSLE   557
CHS2    GWKKVSVILISDGRAKVNQGSLDYLAALGVYQEDMAKASVNGDPVKAHIFE-LTTQVSI----NADLDY-   396
canCHS1 SWKKVQVIIVADGRNKVQQSVLELLTATGCYQENLARPYVNNSKVNAHLFE-YTTQISI----DENLKF-   192

CAL1    SQ--IILMSFLEKITFDERMTQLEFQLLKNIWQITGLMADFYETVL-MVDADTKVFPDALTHMVAEMVKD   789
CHS1    CN--QGTVP-IQ-LLFCLK-EQNQKKINSHRWAFEGFAELLRPNIVTLLDAGTMPGKDSIYQLWREF-RN   621
CHS2    VS--KDIVP-VQ-LVFCLK-EENKKKINSHRWLFNAFCPVLQPTVVTLVDVGTRLNNTAIYRLWKVFDMD   461
canCHS1 KGDEKNLAP-VQ-VLFCLK-ELNQKKINSHRWLFNAFCPVLDPNVIVLLDVGTKPDNHAIYNLWKAFDRD   259

CAL1    PLIMGLCGETKIANKAQSWV------TAIQVFEYYISHHQAKAFESVFGSVTCLPGCFSMYA---IKSPK   850
CHS1    PNVGGACGEIRTD-LGKRFVKLLNPLVASQNFEYKMSNILDKTTESNFGFITVLPGAFSAYRFEAVR---   687
CHS2    SNVAGAAGQIKTM-KGKWGLKLFNPLVASQNFEYKISNILDKPLESVFGYISVLPGALSAYRYRALKNHE   530
canCHS1 SNVAGAAGEIKAM-KGKGWINLTNPLVASQNFEYKLSNILDKPLESLFGYISVLPGALSAYRYIALKNHD   328

CAL1    GSDGYWVPVLANPDIVERYSDNVTNTLHK--KNLLLQGEDRAQS-SLMLRTFPKRKQVFVPKAACKTIAP   917
CHS1    --GQPLQKYFYGEIM--------ENEGFHFFSSNMYLAEDRILCFEVVTKKNCNWILKYCRSSYASTDVP   747
CHS2    DGTGPLRSYFLGETQ--------EGRDHDVFTANMYLAEDRILCWELVAKRDAKWVLKYVKEATGETDVP   592
canCHS1 DGTGPLASYFKGEDLLCSHDKDKENTKANFFEANMYLAEDRILCWELVSKRNDNWVLKFVKLATGETDVP   398

CAL1    DKFKVLLSQRRRWINST-VHNLFEL-VLIRDLCGTFCFSMQFVIGI-ELIGTMVLPL-A-ICFTIYVIIF   982
CHS1    ERVPEFILQRRRWLNGSFFASVYSFCHFYRVWSSGHNIGRKLLLTV-EFFYLFFNTLISWFSLSSFFLFF   816
CHS2    EDVSEFISQRRRWLNGAMFAAIYAQLHFYQIWKTKHSVVRKFFLHV-EFLYQFIQMLFSWFSIANFVLTF   661
canCHS1 ETIAEFLSQRRRWINGAFFAALYSLYHFRKIWTTDHSYARKFWLHVEEFIYQLVSLLFSFFSLSNFYLTF   468

CAL1    AIVSKPT----PV---ITLVLLAII-LGLPGLIVVITATRWSYLWWMCVYICALPIWNFVLPSYAYWKFD  1044
CHS1    RILTVSI----ALAYHSAFNVLSVIFLWLYGICTLST-FILSLGNKPKSTEKFYVLTCVIFAVMMIYMIF   881
CHS2    YYLAGSM----NLVIKHG-EALFIFFKYLI-FCDLASLFIISMGNRPQGAKHLFITSMVILSICATYSLI   725
canCHS1 YFLTGSLVSYKSLGKKGG-FWIFTLFNYLC-IGVLTSLFIVSIGNRPHASKNIFKTLIILLTICALYALV   536

CAL1    DFSWGDTRTIAGGNKKAQDENEGEFDHSKIKMRTWREFEREDILNRKEESDSFVA[stop]
CHS1    CSIFMSVKSFQNILKNDTISFEGLITTEAFRDIVISLGSTYCLYLISSIIYLQPW...
CHS2    CGFVFAFKSLASGTESHKI----------FVDIVISLLSTYGLYFFSSLMYLDPW...
canCHS1 VGFVFVINTIATFGTGGTS-------TYVLVSIVVSLLSTYGLYTLMSILYLDPW...
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Figure 5. Comparison of predicted protein sequences for *CAL1*, *CHS1*, *CHS2*, and *C. albicans CHS1* (canCHS1). Pairwise LFASTA (28) alignments among all four sequences were used to derive a consensus alignment of Cal1 and Canchs1 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 Cal1, amino acids 518–1,099; for Chs1, 361–936; for Chs2, 213–770; for Canchs1, 10–584.

Disruption of the CAL1 Gene and Its Effect

The *cal*^{R}*1* mutation was not deleterious for growth despite a considerable decrease in the biosynthesis of cell wall chitin and some morphological abnormalities (40). It was possible, however, that the mutation could be leaky and for that reason not lethal. To explore that possibility we obtained *cal*^{R}*1* mutants by gene disruption of the homologous copy present in a wild type strain. A 0.5-kb BglII–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$^+$ *cal*^{R} mutant strains containing two defective *CAL1* genes separated by the vector sequence. Several transformants were isolated; after sporulation, asci were dissected. Of 33 asci, 25 produced four viable ascospores; in all of them segregation of Ura$^+$/Ura$^-$ was 2:2. The *URA3* marker always cosegregated with the *cal*^{R}*1* marker. A cross between a Cal^{R}, Ura$^+$ disruptant, and a *cal*^{R}*1* mutant strain originated diploids resistant to Calcofluor; after sporulation, the appearance of the asci and ascospores was similar to that observed for the *cal*^{R}*1*/*cal*^{R}*1* homozygous diploids mentioned above. Analysis of the progeny raised from 30 isolated asci showed that all clones were resistant to Calcofluor. The same disruptant was crossed to a Ura$^+$, Cal^{S} 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 *cal1::URA3* gene was confirmed by Southern analysis of the chromosomal DNA from several disrupted haploids (results not shown). The phenotype of *cal1::URA3* strains was identical to that of *cal*^{R}*1* 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 *cal1* mutants (35) 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 (8, 27) preserved consistently both Chs3, i.e., trypsin-independent activity, and Chs2 (trypsin-stimulated activity), whereas in preparations from protoplasts usually only Chs2 was measured (37). The preparation from strain ECY36-3A (*chs1 CHS2 CAL1*) showed a decrease in activity after trypsin treatment in the presence of Mg^{2+} but an increase in the presence of Co^{2+} (Fig. 6), in agreement with the results of Orlean (27) for a strain of similar genotype. The ratio of activity with Co^{2+} to that with Mg^{2+} in the trypsin-treated enzyme was similar to that observed for Chs2 (37, 43). Strain ECY36-3C (*chs1 chs2::URA3 CAL1*) yielded an enzyme (Chs3) whose activity was decreased by trypsin both in the presence of Mg^{2+} and of Co^{2+} (Fig. 6).

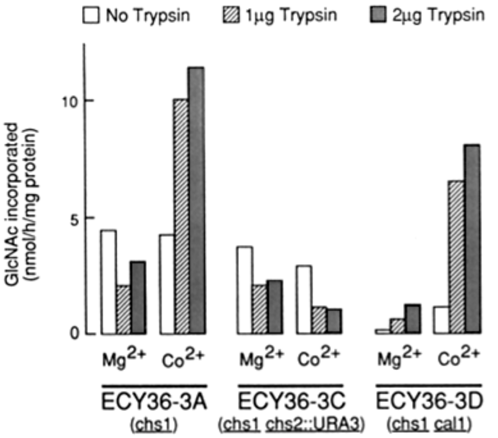

Figure 6. Chitin synthase activity in different strains. The indicated amounts of trypsin were added to each 50-μl assay mixture.

Finally, the preparation from strain ECY36-3D (*chs1 CHS2 cal1*) had very low chitin synthase activity without trypsin but was stimulated by trypsin with either Mg^{2+} or Co^{2+}, 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, *cal*^{R}*1* strains appear to be specifically deficient in Chs3. In confirmation of these results, in a tetrad resulting from a cross between a *CAL1* and a *cal1* strain, both Calcofluor-resistant segregants showed a very low level of trypsin-independent activity compared to the *CAL1* segregants (results not shown).

Transformation of *cal1* 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 *CAL1* 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-

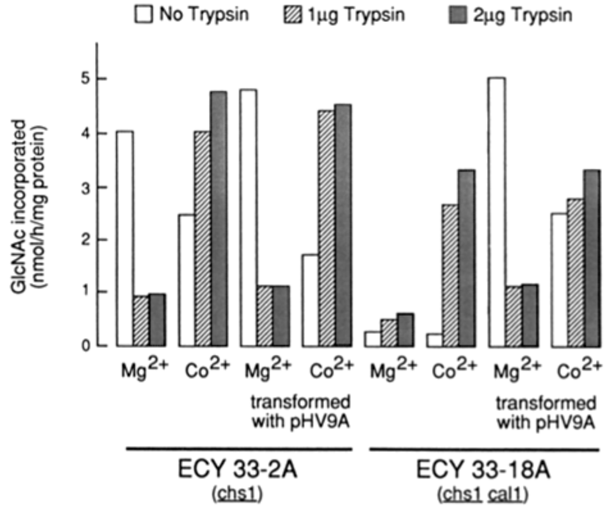

Figure 7. Effect of transformation with a *CAL1*-carrying plasmid on chitin synthase activity. The indicated amounts of trypsin were added to each 50-μl assay mixture.

fore, if *CAL1* 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 *CAL1* gene and a *cal*^{R}*1* strain indicates that the cloned gene corresponds to the *cal*^{R}*1* locus. Accordingly, plasmids carrying the cloned gene corrected all the deficiencies of *cal*^{R}*1*, i.e., resistance to Calcofluor, low chitin level and defective spore maturation. The sharp decrease in chitin content in *cal*^{R}*1* mutants compared to wild type (35) suggested that *CAL1* may be involved in chitin synthesis. This hypothesis was supported by the finding that the predicted amino acid sequence of the *CAL1* gene product has significant homology with the predicted sequences of Chs1 (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 Chs1 and Chs2. Furthermore, the predicted size of the *CAL1* gene product is close to that of the *CHS1* and *CHS2* products. The amino terminal region of the *CAL1* predicted amino acid sequence contains two hydrophobic domains not present in Chs1 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 Chs1 and Chs2, whereas the carboxyl terminal region is much shorter. As in the case of Chs1 and Chs2, the *CAL1* protein also has several potential membrane-spanning domains near the carboxyl terminus. A gene that appears to be identical to *CAL1*, 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 *DIT101*, 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 Chs1: the strain with the genotype *CHS2 CAL1* showed both trypsin-independent and trypsin-dependent activity; the *chs2 CAL1* strain exhibited only trypsin-independent activity (chitin synthase 3; 8), whereas *CHS2 cal*^{R}*1* had only trypsin-dependent activity (Chs2; 37, 43). Clearly, the *cal*^{R}*1* 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 *CAL1* may be the structural gene for Chs3. Other results, however, do not support this possibility. Although transformation of *cal1* strains with plasmids carrying the *CAL1* 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 *CAL1* 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 *cal*^{R}*1*, three other Calcofluor-resistant mutations have been identified (35), i.e., *cal*^{R}*2*, *cal*^{R}*3*, and *cal*^{R}*5* (*cal*^{R}*4* has been reclassified as *cal*^{R}*1*). 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 *CAL1* product will not result in increased activity of Chs3. When the genes corresponding to the other *cal*^{R} mutations are cloned, it will be possible to test this hypothesis by overexpressing them together with *CAL1*.

If *CAL1* is a structural gene for Chs3, it seems possible that the relatively few regions that show homology with *CHS1* and *CHS2* may be crucial for synthase activity or regulation. Thus, the availability of the *CAL1* 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 *CAL1* was not lethal. The phenotype was similar to that of *cal*^{R}*1* 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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