

The *Schizosaccharomyces pombe* *cwg2*⁺ gene codes for the β subunit of a geranylgeranyltransferase type I required for β -glucan synthesis*

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The product of the *Schizosaccharomyces pombe* *cwg2*⁺ gene is involved in the biosynthesis of β -D-glucan. When grown at the non-permissive temperature, *cwg2-1* mutant cells lyse in the absence of an osmotic stabilizer and display a reduced (1–3) β -D-glucan content and (1–3) β -D-glucan synthase activity. The *cwg2*⁺ gene was cloned by the rescue of the *cwg2-1* mutant phenotype using an *S.pombe* genomic library and subsequently verified by integration of the appropriate insert into the *S.pombe* genome. Determination of the nucleotide sequence of this gene revealed a putative open reading frame of 1065 bp encoding a polypeptide of 355 amino acids with a calculated M_r of 40 019. The *cwg2*⁺ DNA hybridizes to a main transcript, the 5' end of which maps to a position 469 bp upstream of the predicted start of translation. The sequence between the transcription and the translation start sites is unusually long and has several short open reading frames which suggest a translational control of the gene expression. Comparative analysis of the predicted amino acid sequence shows that it possesses significant similarity to three *Saccharomyces cerevisiae* proteins, encoded by the *DPR1/RAM1*, *CDC43/CAL1* and *ORF2/BET2* genes respectively, which are β subunits of different prenyltransferases. When grown at 37°C, *cwg2-1* mutant extracts were specifically deficient in geranylgeranyltransferase type I activity, as measured *in vitro*. Multiple copies of the *CDC43* gene can partially suppress the growth and (1–3) β -D-glucan synthase defect of the *cwg2-1* mutant at the restrictive temperature. In a similar manner, the *cwg2*⁺ gene can partially suppress the *cdc43-2* growth defect. These results indicate that *cwg2*⁺ is the structural gene for the β subunit of geranylgeranyltransferase type I in *S.pombe* and that this enzyme is required for (1–3) β -D-glucan synthase activity. The functional homology of Cwg2 with Cdc43, which has been implicated in the control of cell polarity, suggests a link between two morphogenetic events such as establishment of cell polarity and cell wall biosynthesis.

Key words: cell wall/fission yeast/glucan synthase/GTP-binding proteins/prenyltransferase

Introduction

The major structural component of the yeast cell wall, β -glucan, is a glucose homopolymer linked through either (1–3) β or (1–6) β -D-glycosidic bonds. Biosynthesis of (1–3) β -linked glucose polymers is mainly attributed to (1–3) β -D-glucan synthase activity (Shematek *et al.*, 1980) which is localized to the inner part of the fungal plasma membrane and is stimulated *in vitro* by nucleoside triphosphates, mainly guanosine derivatives (Shematek *et al.*, 1980). This seems to be a general feature possessed by fungal (1–3) β -D-glucan synthases (Pérez *et al.*, 1981; Szaniszló *et al.*, 1985). Dissociation of the activity into two proteinaceous components (Kang and Cabib, 1986) has provided evidence for the existence of a membrane-bound fraction which seems to contain the catalytic centre of the enzyme and a detergent solubilized GTP-binding fraction. It has been proposed that this second component may play an important role in the regulation of fungal cell wall glucan biosynthesis (Szaniszló *et al.*, 1985; Kang and Cabib, 1986). In order to get additional information about the mechanisms of yeast β -glucan biosynthesis, several genetic approaches have recently been developed. In some cases, the aim has been the isolation of mutants defective in β -glucan synthesis to enable the characterization of genes physiologically relevant for this process (Ribas *et al.*, 1991). In other cases, mutants have been selected due to their resistance to antifungal agents, such as Aculeacin A, that act on the cell wall (Font de Mora *et al.*, 1992) or to the *Saccharomyces cerevisiae* K1 killer toxin which possesses affinity for non-branched (1–6) β -D-glucan (Boone *et al.*, 1990). Genes affected in these mutants have been cloned and sequenced. One of them, *KRE6*, encodes a membrane protein that seems to be required for the synthesis of both (1–6) β - and (1–3) β -D-glucans (Roemer and Bussey, 1991). The specific activity of the (1–3) β -glucan synthase is reduced to 50% in *kre6* null mutants and therefore it was suggested that *KRE6* is a structural gene for this enzyme. If this is the case, functionally redundant (1–3) β -glucan synthases should exist in *S.cerevisiae* as has been shown for the chitin synthases (Cabib *et al.*, 1992; Shaw *et al.*, 1991). Finally, other genes have been isolated which may indirectly affect the cell wall β -D-glucan. Mutations affecting different protein kinases have been described (Levin and Bishop, 1990; Lee and Levin, 1992; Levin and Bartlett-Heubusch, 1992; Torres *et al.*, 1992), that cause a temperature-sensitive cell lysis defect suppressible by osmotic stabilizing agents. It has not been established yet if these kinases control biosynthetic or metabolic pathways for cell wall components.

In a previous paper (Ribas *et al.*, 1991) we reported the

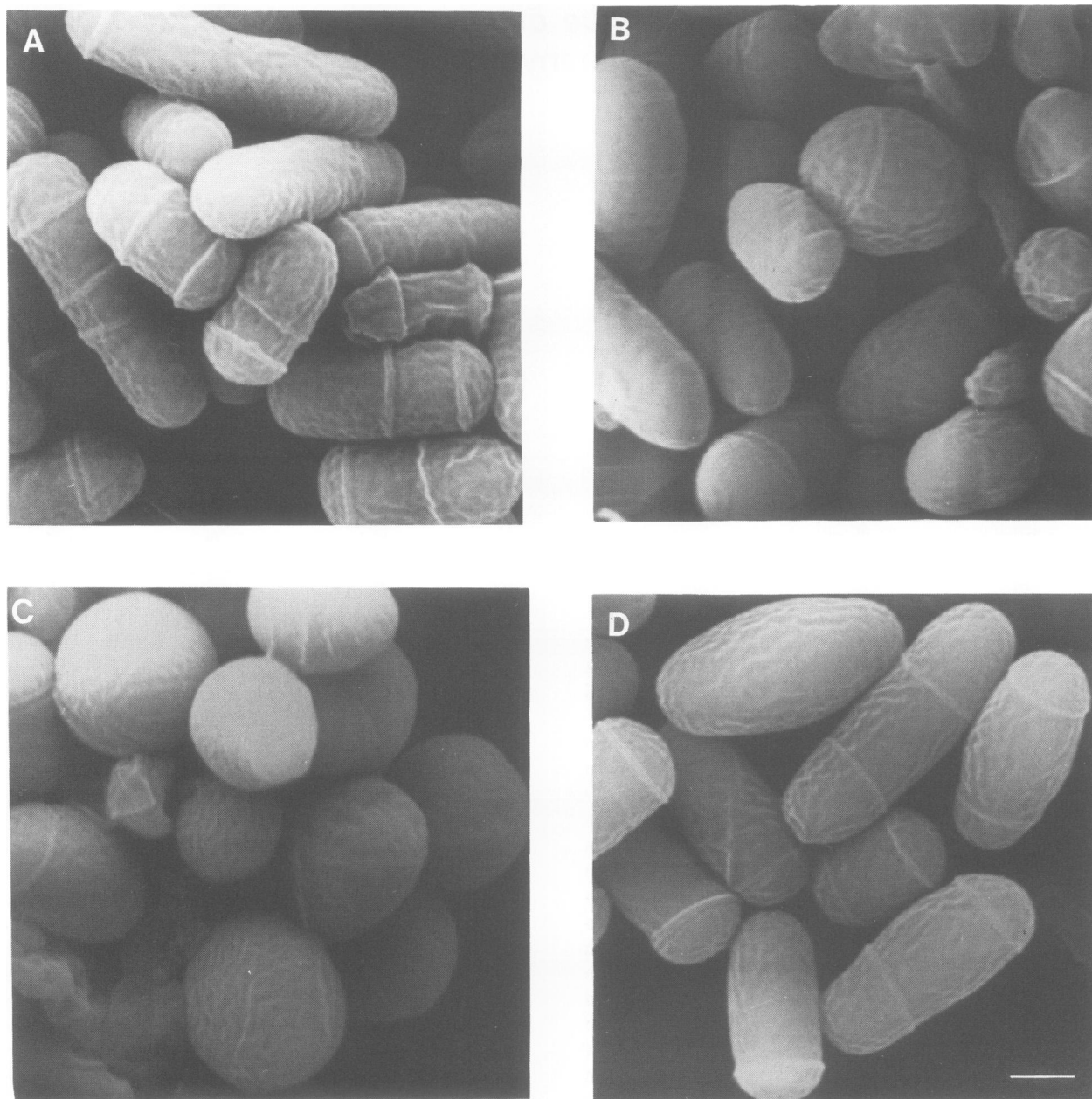


Fig. 1. Electron micrographs of *S.pombe* cells grown in YED medium at 37°C. Wild type (A); *cwg2-1* (B); *cwg2-1* mutant transformed with the plasmid pDB262 (C) and *cwg2-1* mutant transformed with the plasmid pMD7 (D). The bar corresponds to 2 μ m.

isolation and characterization of two *S.pombe* thermo-sensitive mutants, *cwg1-1* and *cwg2-1*, which are defective in (1-3) β -D-glucan synthase activity and require osmotic stabilization when growing at the non-permissive temperature (37°C). β -D-glucan synthase activity from the *cwg2-1* mutant strain grown at 37°C was diminished, as measured *in vitro*, relative to the wild type strain. Detergent dissociation of (1-3) β -D-glucan synthase into a soluble and a particulate fraction and subsequent reconstitution, proved that the *cwg2-1* mutant activity was affected in the soluble component which binds GTP (J.Ribas, unpublished results).

In the current paper we report on the cloning and nucleotide sequencing of the *cwg2+* gene. Amino acid sequence comparison revealed that the Cwg2 protein is structurally related to the Dpr1/Ram1 (Goodman *et al.*, 1988), Cdc43/Cal1 (Johnson *et al.*, 1990; Ohya *et al.*, 1991)

and Bet2/Orf2 (Rossi *et al.*, 1991) proteins from *S.cerevisiae*. All these proteins are homologous (Kohl *et al.*, 1991) and possess activities related to protein prenylation (Finegold *et al.*, 1991). Dpr1 is the β subunit of an *S.cerevisiae* cytosolic farnesyltransferase (FT) which farnesylates proteins such as Ras (Goodman *et al.*, 1988) and the *MATa* mating factor of *S.cerevisiae* (Chen *et al.*, 1991). Cdc43 is part of a geranylgeranyl transferase type I (GGT-I) (Finegold *et al.*, 1991) that has been implicated in the modification of Cdc42, a Ras-like protein involved in bud positioning and the control of cell polarity in yeast (Johnson and Pringle, 1990). The *BET2* gene product is a component of a second type of geranylgeranyl transferase, GGT-II (Kohl *et al.*, 1991), that allows the membrane attachment of Ypt1 and Sec4, two Ras-like GTP-binding proteins that regulate the vesicular traffic at different stages

of the secretory pathway (Rossi *et al.*, 1991). Protein prenylation in *S.pombe* has recently been studied following the incorporation of [³H]mevalonic acid (Giannakourous *et al.*, 1992). HPLC analysis of total *S.pombe* protein-bound isoprenoids revealed the existence of both geranylgeraniol and farnesol in similar amounts. However, up to now, no prenyltransferases have been identified in that yeast. Here we demonstrate that the *cwg2⁺* gene product is required for GGT-I activity in *S.pombe*. We also present genetic evidence of a functional homology between *cwg2⁺* and *CDC43/CAL1* which corroborates this result. We conclude that the *cwg2⁺* gene product is the β subunit of a GGT-I, and we propose that this activity modifies the GTP-binding component of the (1-3) β -D-glucan synthase.

Results

Isolation of a plasmid containing the *cwg2⁺* gene

The *S.pombe* strain *cwg2-1 leu1-32 h⁻* is a leucine auxotrophic, temperature-sensitive *cwg2-1* mutant. It has less (1-3) β -D-glucan in its cell wall than does the wild type and, when grown at 37°C in the absence of an osmotic stabilizer, it becomes rounded before eventually lysing (Figure 1B).

This strain was transformed with an *S.pombe* genomic DNA library made in the plasmid pDB262 (Wright *et al.*, 1986). Out of 30 000 transformants which could grow in the absence of leucine at 30°C, only one clone was found to grow at the non-permissive temperature (37°C); its morphology was similar to that of the wild type strain (Figure 1D and A). The phenotype was mitotically unstable and leucine prototrophy was found to cosegregate with the ability to grow at 37°C. DNA was prepared from the transformant clone and used to transform the *Escherichia coli* DH1 strain. Plasmids isolated from the transformed *E.coli* were able to rescue the wild type phenotype after retransformation of the *cwg2-1 leu1-32 h⁻* strain.

The pDB262-derived plasmid was called pMD1 and contained a 7.2 kb insert, the restriction map of which is shown in Figure 2A. In order to define the region responsible for the complementing activity, the 7.2 kb insert was shortened by using the restriction sites present on the fragment. The different plasmids obtained were tested for the ability to complement the *cwg2-1* mutation and to restore growth at 37°C in the absence of osmotic stabilizer. As shown in Figure 2A, this activity was present in the plasmid pMD7 which contains a 2.0 kb *PstI*-*Bam*HI fragment of the original pMD1 insert. Southern blot analysis using this fragment as a probe indicated that no rearrangement of the DNA structure occurred during the cloning and that the *cwg2⁺* gene exists as a single copy in the yeast genome (data not shown).

To establish the identity of the cloned DNA, we constructed the pMD71 plasmid containing the *cwg2-1* mutant complementing sequence from pMD7 inserted in the plasmid pDW232 which has the *S.pombe* selectable marker *ura4⁺*. pMD71 was linearized using the unique *Cla*I site and was used to transform an *S.pombe cwg2-1 ura4-d18* strain. To determine if the plasmid was integrated into the *S.pombe* genome, Southern blot analysis was performed with *Hind*III-cut genomic DNA from 12 transformants and a wild type *S.pombe* strain and using the *PstI*-*Bam*HI fragment of the *cwg2⁺* gene as a probe. All transformants' DNAs gave rise to two bands, a 3.2 kb band which was also present

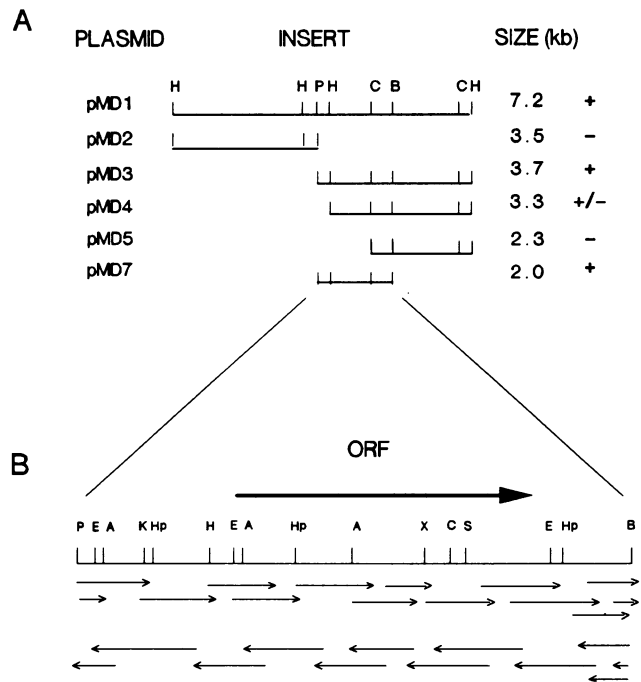


Fig. 2. Restriction map, subcloning (A) and sequencing strategy (B) used in determination of the nucleotide sequence of the *cwg2⁺*-containing fragment. The ability of subclones to rescue strain *cwg2-1* grown at 37°C without sorbitol is indicated. The position of the *cwg2⁺* gene is shown as a bold arrow indicating the direction of transcription. Thin arrows indicate the direction and the extent to which the sequence was determined. Abbreviations for restriction endonuclease sites are: A, *Ava*I; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hind*III; Hp, *Hpa*I; K, *Kpn*I; P, *Pst*I; S, *Sph*I; X, *Xba*I.

Table I. (1-3) β -D-glucan synthase activity in extracts from *S.pombe* wild type 972 *h⁻*, mutant *cwg2-1 h⁻* and *cwg2-1 h⁻* transformed with different plasmids (all grown at 37°C)

Strain	Specific activity ^a	%
<i>h⁻ 972</i>	3.2 ± 0.1	100
<i>h⁻ cwg2-1</i>	1.1 ± 0.1	35
<i>h⁻ cwg2-1, leu1-32 + pDB262</i>	1.0 ± 0.1	32
<i>h⁻ cwg2-1, leu1-32 + pMD1</i>	3.6 ± 0.3	110
<i>h⁻ cwg2-1, leu1-32 + pMD4</i>	2.0 ± 0.3	63
<i>h⁻ cwg2-1, leu1-32 + pMD7</i>	3.0 ± 0.2	93
<i>h⁻ cwg2-1, ura4-d18 + pMD71-lin^b</i>	2.8 ± 0.1	88

^a Expressed as milliunits per mg of protein.

^b Plasmid pMD71-lin was integrated into the *S.pombe* genome as detected by Southern blot analysis.

in the wild type DNA plus an extra 8.6 kb band expected if the plasmid were integrated at the *cwg2* locus (data not shown). The identity between the integrated *cwg2⁺* gene and the altered gene in the *cwg2-1* mutant strain used for the integrative transformation was analyzed by crossing the transformants with a *cwg2⁺ leu1-32 h⁻* strain. In 10 tetrads analyzed, the four ascospores were *cwg2⁺*. These results indicated that the integration is very closely linked to the mutated *cwg2-1* gene.

(1-3) β -D-glucan synthase activity of *cwg2-1* mutant and transformant strains

In order to ascertain whether the (1-3) β -D-glucan synthase activity was restored in the transformant strains, *S.pombe* 972 *h⁻*, *cwg2-1* mutant, the multicopy transformants

-549	CTCATAAGTATCTTCATTTTCTCATCAATTCGTCCTCAAAGCCATTGAATTCGGATTCTGATGATATAGATGCTAAAT	↓	CTTCATTAG	-461
-460	CGGGTCCATTCTCTCCAATTTTGTATCTCTTTTCTCTTTTGAAGTAGCACTCCTAGCTTCTCTTCTATGCTCGGACGTGTAAG			-369
-368	AAAGGATCAGACATGCTTTCTCTAAGGGTACCAACGGTTCGTAATTTCTGTATCTTCAAACACCTATATGGTATACTGCCGGTGACTTAAT			-277
-276	TTTATTCTCCGAGCATACTCCACAAAAGTCCGGTGATCCATACAAGAAATTTCCGAAGTTTCCGAGCTAATTCACCGAATTTCTTTTCAT			-185
-184	GAACAAATCATTACAGAAATATATCTGCTCATTACTATAAATCAAATACCTTGTGCGTTTTTTGTTTTTAAGCTAAAATAATTATCGT			-93
-92	TCTTGACCAATTTTGTTCGATAGATTTTTAAGACTTACTATTCTATTGACATCTATAGAATTCGTATCGGCGCATCTATTTATGGTCCA			-1
1	ATG GAA TTA ACA AGA GCT AAA CAT ATT GCT TTC TTT AAG AGA CAT CTT ATA CTA TTT CCT ACT CCA TAC			69
1	Met Glu Leu Thr Arg Ala Lys His Ile Ala Phe Phe Lys Arg His Leu Ile Leu Phe Pro Thr Pro Tyr			23
70	GAA GAA CAC GAT TGC GAA AGA ACA GTG TTA GCA TTC TTT TGT TTA TTA GGA TTA GAC TTG CTA AAT GCA			138
24	Glu Glu His Asp Cys Glu Arg Thr Val Leu Ala Phe Phe Cys Leu Leu Gly Leu Asp Leu Leu Asn Ala			46
139	TTG AAC ACC ATA GAT GAT GAC GAT AAA AAA AGT TGG ATC GAG TGG ATC TAC AAA AAC TAT GTA ACG AAA			207
47	Leu Asn Thr Ile Asp Asp Asp Lys Lys Ser Trp Ile Glu Trp Ile Tyr Lys Asn Tyr Val Thr Lys			69
208	GAA TCT AAA GGA ATC AAA TAC TCC GGT TTT CAG GCG TAT AGA ACT GGC ATT CAA CCC ATC TCA TTC GAA			276
70	Glu Ser Lys Gly Ile Lys Tyr Ser Gly Phe Gln Ala Tyr Arg Thr Gly Ile Gln Pro Ile Ser Phe Glu			92
277	CAG GAG CCG CAA TTA GCT GGG ACT GTG TTT TCA ATT TGT TGC CTT TTA TTT CTT GGC GAT AAT CTT TCT			345
93	Gln Glu Pro Gln Leu Ala Gly Thr Val Phe Ser Ile Cys Lys Leu Phe Leu Gly Asp Asn Leu Ser			115
346	CGT ATC GAC CGC GAT TTA ATT AAG AAT TTT GTG GAG TTG TGC AAA ACT TCG CAG GGT CAT TTT AGA AGT			384
116	Arg Ile Asp Arg Asp Leu Ile Lys Asn Phe Val Glu Leu Cys Lys Thr Ser Gln Gly His Phe Arg Ser			138
385	ATT GCT GTG CCA TCA TGT TCG GAC CAA GAC ATG CGA CAG TTA TAT ATG GCT ACG ACT ATT GCT TCG CTT			483
139	Ile Ala Val Pro Ser Cys Ser Asp Gln Asp Met Arg Gln Leu Tyr Met Ala Thr Thr Ile Ala Ser Leu			161
484	CTT GAT TTC TCT CTG TCT GAC CCT TTA TGT TCT ATC CAA TAT ATT AAA AGC TGT CAA CGC TAT GAA GGC			552
162	Leu Asp Phe Ser Leu Ser Asp Pro Leu Cys Ser Ile Gln Tyr Ile Lys Ser Cys Gln Arg Tyr Glu Gly			184
553	GGA TTT TCT TTG TTA CCA TAC GGC GAA GCC CAT GCT GGA GCT ACA TTT TGC GCG TTA GCA TCA TGG TCT			621
185	Gly Phe Ser Leu Leu Pro Tyr Gly Glu Ala His Ala Gly Ala Thr Phe Cys Ala Leu Ala Ser Trp Ser			207
622	TTA ATA TTG AAA ATG ATT CCA AAT TCC TCT TTG AAT ACT TCT AAT CAA TCA TAT AAC TTA ATG GAT TGT			690
208	Leu Ile Leu Lys Met Ile Pro Asn Ser Ser Leu Asn Thr Ser Asn Gln Ser Tyr Asn Leu Met Asp Cys			230
691	GTG CCT AAA GTA GAA CGC CTT ATC AGA TGG TTA GCT TCT AGA CAA TTA TCA TCT GGA GGA TTA AAC GGA			759
231	Val Pro Lys Val Glu Arg Leu Ile Arg Trp Leu Ala Ser Arg Gln Leu Ser Ser Gly Gly Leu Asn Gly			253
760	AGG ACG AAT AAA GAC GTT GAC ACC TGT TAT GCT TAT TGG GTT TTG AGT TCA TTA AAA CTC CTT GAT GCT			828
254	Arg Thr Asn Lys Asp Val Asp Thr Cys Tyr Ala Tyr Trp Val Leu Ser Ser Leu Lys Leu Leu Asp Ala			276
829	CTG CCT TTT ATC GAT GGG GGT GAG TTG GAA AAA TAT TTA TTA TTG CAT GCT CAA CAT GCT TTG GGC GGA			897
277	Leu Pro Phe Ile Asp Gly Gly Glu Leu Glu Lys Tyr Leu Leu Leu His Ala Gln His Ala Leu Gly Gly			299
898	TTT TCT AAA ACA CCA GGT GAG TTT CCT GAT GTT TTA CAT TCT GCA CTT GGT CTA TAT GCA ATG GCT TAT			966
300	Phe Ser Lys Thr Pro Gly Glu Phe Pro Asp Val Leu His Ser Ala Leu Gly Leu Tyr Ala Met Ala Tyr			322
967	CAA GAT GAT AAG TCT TTT CCT AAA GTA AAT GCA GAT ATA CAT ATG ACT TCC AAG TAT ATA AAC ATT TGT			1035
323	Gln Asp Asp Lys Ser Phe Pro Lys Val Asn Ala Ser Ile His Met Thr Ser Lys Tyr Ile Asn Ile Cys			345
1036	AGA GAC TGT ATT CAA GCA GCC AAG GGG AAA TGATAACTATATTATGATGATGAATTGTGCAAGAAAACCTAAACAATAGACTA			1673
346	Arg Asp Cys Ile Gln Ala Ala Lys Gly Lys ***			355
1674	TTAACTTAGAAACAACGTGATCAATCACTTGACTATAAAACCTAAAGACACTCTAAC <u>ATATA</u> AATTTGACTCAGTAACTTTAATTTCTC			1765
1766	TATTCTGTTTTTAGAAAGTGAATTTCGATTTGCTCGTCTTTTCATTCTCGCTTTCCGGCTTTTATGGAAGCCTCTTCTTCGAAGGACTG			1857
1858	GAGAAGCTAGATTTATGGACACTATCACTTTTCTCTCATCTCCATCTGTCCACTATCCGTATCTTCGGACGATCGAGGTGCTCTTGC			1949

Fig. 3. Nucleotide sequence of the *S.pombe cwg2+* gene. The deduced amino acid sequence of the encoded protein is indicated. Transcription start site is indicated by an arrow. The sequence corresponding to a consensus polyadenylation signal is underlined.

cwg2-1 + pMD1, *cwg2-1* + pMD7 and *cwg2-1* + pMD4, and the integrative transformant *cwg2-1* + pMD71-lin were grown in minimal medium supplemented with sorbitol at the non-permissive temperature and (1–3) β -D-glucan synthase activity was determined *in vitro*. The results (Table I) indicated that the specific activity of the membrane preparation was similar for the wild type and the transformant strains whereas it was considerably reduced in the *cwg2-1* mutant (35% of the control). The presence of high copy number plasmids (pMD1 and pMD7) did not significantly increase the (1–3) β -D-glucan synthase activity above the control levels. Plasmid pMD4 only restored the activity partially (63% of the control).

Nucleotide sequence and transcription of the *cwg2+* gene

The nucleotide sequence of the 2.0 kb fragment from pMD7 carrying the *cwg2+* gene was determined in both DNA strands according to the strategy outlined in Figure 2B. The DNA sequence (Figure 3) contains a single long open reading frame (ORF) of 1065 bp and no consensus signals for intron splicing. The predicted encoded protein has 355 amino acids (Figure 3) with an M_r of 40 019. Leucine is the most abundant amino acid (13%). It is a relatively hydrophilic protein with a similar number of acidic and basic amino acids and a calculated isoelectric point of 6.52. The Kyte–Doolittle hydropathy plot of the product (Kyte and

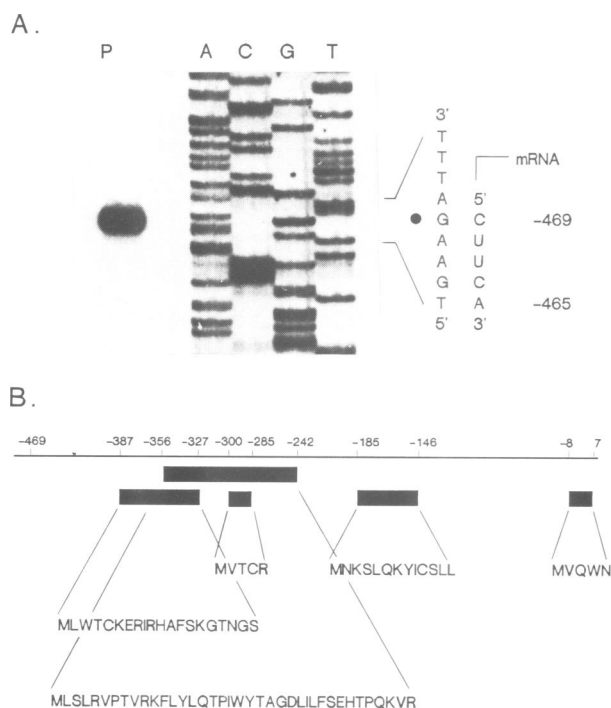


Fig. 4. (A) Determination of the 5' end of the *cwg2⁺* mRNA transcript by primer extension. The sequences shown correspond to the minus strand of the genomic DNA; they were determined using as primer the same oligonucleotide used to extend the mRNA with reverse transcriptase. The numbers indicate the nucleotide location with respect to the first base of the protein coding sequence. (B) Locations and amino acid sequences of the small ORFs in the 5' flanking region.

Doolittle, 1982) revealed no regions of marked hydrophobicity characteristic of N-terminal signal peptide sequences. There are two potential membrane-spanning domains (amino acids 31–47 and 95–111), four potential N-glycosylation sites (amino acids 113, 215, 219 and 222) and a potential site for tyrosine kinase phosphorylation at amino acid 63.

Evidence for the expression of the encoded ORF came from Northern blot analysis of *S.pombe* mRNA using the *EcoRI*–*XbaI* fragment containing part of the *cwg2⁺* as a probe. The size of the mRNA transcripts was 1.3–1.5 kb and the level of transcription was very poor (data not shown).

The 5' flanking sequence of the coding region has several TATA-like elements. To see which of them was functioning as a promoter, we determined the 5' initiation site of transcription by primer extension of an oligonucleotide complementary to the +83 to +63 nucleotides downstream of the ATG of the ORF in the sequence. One major transcript (Figure 4A) was detected 469 bp upstream of the ATG. A second faint transcript starting at +231 bp was always detected. Surprisingly, upstream of the major transcription start site there is not a clear TATA or CAGTCACA sequence. It is worth mentioning that in the 5' leader region of the main mRNA there are five small ORFs, shown in Figure 4B (nucleotides –8 to 7; –185 to –146; –300 to –285; –356 to –242; and –387 to –327). These small ORFs suggest that the gene is subject to translational control (Kozak, 1989). From the distribution of the codons used in the *cwg2⁺* gene, a codon bias index of –0.057 can be calculated (Russell, 1989) which suggests a poorly expressed gene. The 3' noncoding region contains one of the proposed (Miyake and Yamamoto, 1990) polyadenylation consensus

sequences, AATATA, 107 bp downstream of the ORF stop codon.

Homology between the Cwg2 protein and prenyltransferases

A search of the GenBank and EMBL databases revealed a significant resemblance of the *cwg2⁺*-encoded protein to three homologous *S.cerevisiae* proteins. Cwg2 showed 32% identity and 43% similarity with Cdc43/Cal1, 25% identity and 37% similarity with Orf2/Bet2 and 27% identity and 40% similarity with Dpr1/Ram1 which is a homolog to the β subunit of an FT purified from rat brain (Chen *et al.*, 1991). An alignment of the most similar regions of the five proteins is shown in Figure 5. The region corresponding to amino acids 250–275 in Cwg2 is highly conserved among the five proteins. Within the region shown, Cwg2 exhibits maximal similarity with Orf2.

Prenyltransferases in *S.pombe*

In order to ascertain if Cwg2 was functionally related to prenyltransferases, we assayed the three activities described so far (FT, GGT-I and GGT-II) in extracts of *S.pombe* 972h⁻, *cwg2-1* and integrative transformant *cwg2-1* + pMD71-lin grown at 37°C in YED plus 1.2 M sorbitol. A dramatic decrease was observed in GGT-I activity of *cwg2-1* extracts with a significantly reduced incorporation of [³H]geranylgeranylpyrophosphate radioactivity as compared with the integrative transformant (Figure 6B). By contrast, FT and GGT-II levels were similar in both mutant and integrative transformant strains (Figure 6A and C). The prenyltransferase activities of wild type strain 972h⁻ were similar to those detected with the integrative transformant (data not shown).

Functional relationship between CDC43 and *cwg2⁺* genes

Since Cwg2 was required for GGT-I activity in *S.pombe*, we examined whether the prenyltransferase mutants from *S.cerevisiae* also were affected in (1–3) β -D-glucan synthase activity. *dpr1*, *cdc43-2* (thermosensitive) and *orf2-1* (thermosensitive) mutants and the corresponding wild type strains, KMY5–2A, TD1 and W303 1A, were grown at 24°C. Then the *orf2-1* and *cdc43-2* cultures were shifted for 2 and 7 h respectively at the non-permissive temperature, and the corresponding activity was measured *in vitro*. As shown in Table II, the specific activity of (1–3) β -D-glucan synthase from *dpr1* mutant was close to that of the wild type strain, while both *cdc43-2* and *bet2* mutants had a considerably reduced activity, 47% and 32% respectively, as compared with their wild type strains. We decided, therefore, to analyze the *cwg2-1* phenotype complementation by the *CDC43/CAL1* or *BET2/ORF2* genes. These genes were cloned in the autonomously replicating inducible expression plasmid, pREP1, under the control of the thiamine repressible *nut1* promoter (Maudrel, 1993), strongly transcribed in minimal medium. As shown in Figure 7A, the plasmid pREPCDC43, carrying the *CDC43/CAL1* gene, was able to prevent the lysis and restore the growth of the *cwg2-1*, *leu1-32* mutant at 37°C in minimal medium without osmotic stabilizer. pREPCDC43 was also able to complement partially the morphological phenotype of the *cwg2-1*, *leu1-32* mutant and when the cultures were supplemented with 20 mM CaCl₂, the morphological complementation was

cgw2	143	S C S D Q D M R Q L Y M A T T I A S L L D F S L S
CAL1	159	S V D S D D L R F C Y I A V A L Y I C G C R S K E D F D E
BET2	131	. F G E V D T R F V Y T A L S A L S I G E L T S E
DPR1	205	. V G E V D T R G I Y C A L S A T L N I L T E E
RATFTβ	196	. G G E V D V R S A Y C A A S V A S L I N I I I P D
cgw2	168	. . D P L C S I Q Y I K S C Q R Y E G G F S L L P Y G E A
CAL1	189	Y I D T E K L L G Y I M S Q Q C Y N G A F G A H . . . N E P
BET2	156	. V V D P A V D F V L K C Y N F D G G F G C P N A . E S
DPR1	230	. . L T E G V L N Y L K N C O N Y E G G F G S C P H V D E A
RATFTβ	221	. . L F E G T A E W I A R C O N W E G G I G G V P . G M E A
cgw2	195	H A G A T F C A L A S W S L I L K H I P N S S L N T S N Q S
CAL1	216	H S G Y T S C A L S T L A L L S S L
BET2	183	H A A Q A F T C L G A L A I A N K L
DPR1	258	H G G Y T F C A T A S L A I L R S M
RATFTβ	248	H G G Y T F C G I A A L V I L K K E
cgw2	225	Y N L M D C V P K V E R L I R W L A S R Q L
CAL1	234	E K L S D K F . . . K E D T I T W L L H R Q V S S H G C M K F
BET2	201	D M L S D D Q . . . L E E I G W L C E R Q L P
DPR1	276	D Q I N V E K L L E W S S A R Q L Q
RATFTβ	266	R S I N L K S L L Q W V T S R Q M R
cgw2	247 S S G G L N G R T N K D V D T C Y A Y
CAL1	262	E S E L N A S Y D Q S D D G G F Q G R E N K F A D T C Y A F
BET2	222 E G G L N G R P S K L P D V C Y S W
DPR1	293 E E R G F C G R S N K L V D G C Y S F
RATFTβ	283 F E G G F Q G R C N K L V D G C Y S F
cgw2	266	W V L S S L K L L D A L P F I D G
CAL1	292	W C L N S L H L L T K D W K M L C Q T
BET2	240	W V L S S L A I I G R D W I N Y
DPR1	312	W V G G S A A I E A F G Y G C Q F N K
RATFTβ	302	W Q A G L L P L L H R A L H A Q G D P A L S M S H W M F H Q
cgw2	284	G E L E K I L L L H A Q H A L G G F S K T P G E F P D V L
CAL1	311	E L V T N Y L L D R T Q K T L T G G F S K N D E E D A D L Y
BET2	257	E K T E F I L . . K C Q D E K K G G I S D R P E N E V D V F
DPR1	332	H A L R D Y I I Y C C O E K E Q P G L R D K P G A H S D F Y
RATFTβ	332	Q A L Q E Y I L M C C Q . C P A G G L R D K P G K S R D F Y
cgw2	312	H S A L G L Y A M A
CAL1	339	H S C L G S A A L A
BET2	286	H T V F G V A G L S
DPR1	361	H T N Y C L L G L A
RATFTβ	361	H T C Y C L S G L A

Fig. 5. Alignment of the *cgw2*⁺ predicted amino acid sequence with those of *CAL1*, *DPR1*, *BET2* and *RATFTβ* at the region of maximal similarity. Gaps were introduced where necessary to optimize the alignment. Identical (*) or conserved (.) amino acid residues in the five proteins are indicated.

slightly improved (data not shown). The plasmid pREP^{BET2} could not suppress the growth defect of *cgw2-1* at 37°C (Figure 7A). However, when the cells were grown at 35°C, some morphological complementation was observed. At this temperature, the *cgw2-1* mutant was able to grow but the cells were still rounded (Figure 7B, panel 4) while pREP^{BET2} transformant cells were rod-shaped like the wild type (Figure 7B, panel 3). The cells transformed with pREPCDC43 were always larger than the control (Figure 7B, panel 2).

Phenotypic complementation of *cgw2-1* with *CDC43/CAL1* or *BET2/ORF2* was also analyzed by measuring the (1–3)-β-D-glucan synthase activity of those transformants grown in minimal medium supplemented with sorbitol and 20 mM CaCl₂ at the non-permissive temperature (Table III). The activity was partially restored in the mutant transformed with pREPCDC43 (68% of the control), whereas it remained considerably reduced in the *cgw2-1* mutant transformed with pREP^{BET2} or pREP1 (18% and 23% of the control).

Complementation of the *dpr1*, *cdc43-2* or *orf2-1* mutations by *cgw2*⁺ was assayed by cloning the gene in an *S.cerevisiae* expression plasmid, p2HG, under the constitutive GPD

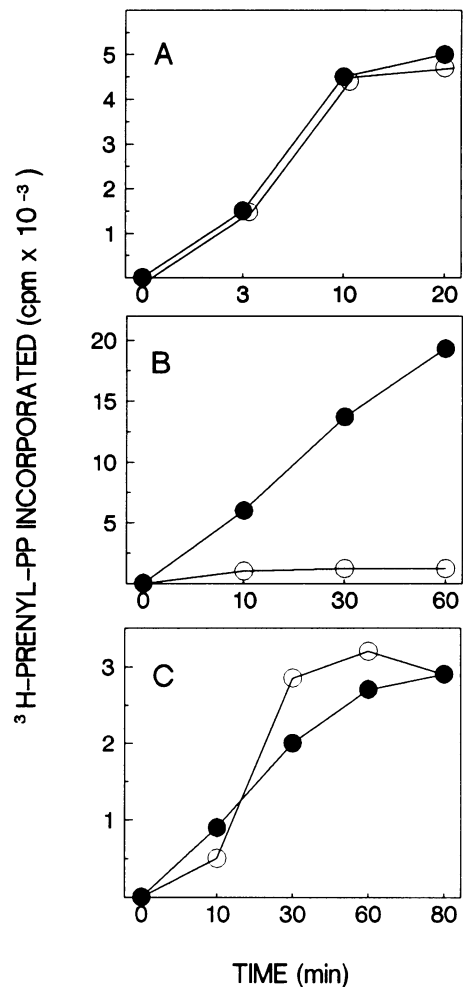


Fig. 6. Detection of FT (A), GGT-I (B) and GGT-II (C) activities in *S.pombe*. Soluble yeast extracts (160 μg) of *cgw2-1 ura4-d18 h*⁺ (●) or *cgw2-1 ura4-d18 h*⁺ [*cgw2*⁺ *ura4*⁺] (○) cells were incubated with the substrates: GST-CIIS (A), GST-CIIL (B) or YPT1 (C) in the presence of [³H]farnesyl pyrophosphate (A) or [³H]geranylgeranyl pyrophosphate (B and C) at 37°C for the time indicated and 10 μl were assayed for incorporation of radioactivity into protein by a filter-binding assay.

Table II. (1–3)-β-D-glucan synthase activity in *S.cerevisiae* strains

Strain	Relevant genotype	Specific activity ^a	%
W303 1A	<i>ORF2</i>	8.7 ± 0.1	100
YF1594	<i>orf2-1</i>	2.8 ± 0.1	32
TD1	<i>CDC43</i>	12.9 ± 2.1	100
CJ198-2B	<i>cdc43-2</i>	6.1 ± 2.3	47
KMY2-3A	<i>DPR1</i>	14.3 ± 1.4	100
KMY5-2A	<i>dpr1</i>	11.3 ± 2.4	79

All extracts were prepared from strains grown at 24°C and shifted for 2 h (W303 1A and YF1594) or 7 h (TD1 and CJ198-2B) at 37°C.

^a Expressed as milliunits per mg of protein. Values are the means and standard deviations calculated from three independent experiments.

promoter. Transformation of the *cdc43-2* mutant with the constructed pYSCWG2 plasmid did not suppress the phenotype at 37°C. However, partial suppression was observed at 36°C (Figure 8). Addition of 100 mM CaCl₂

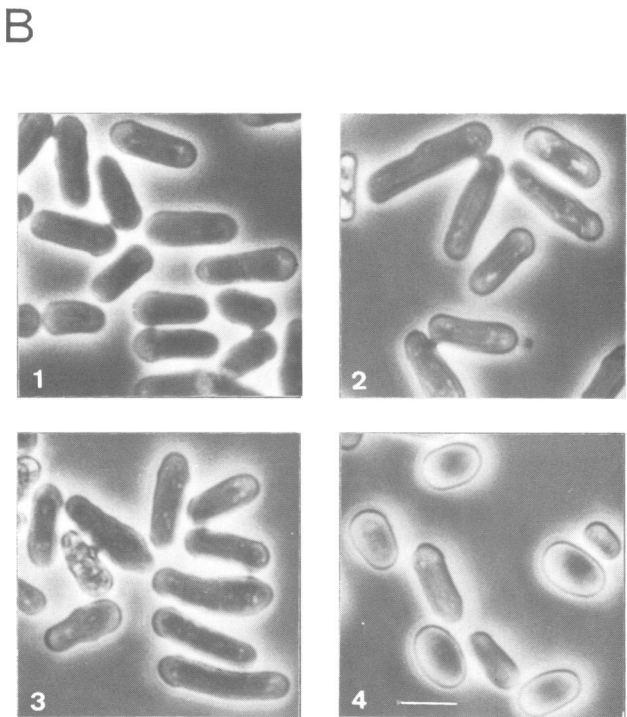
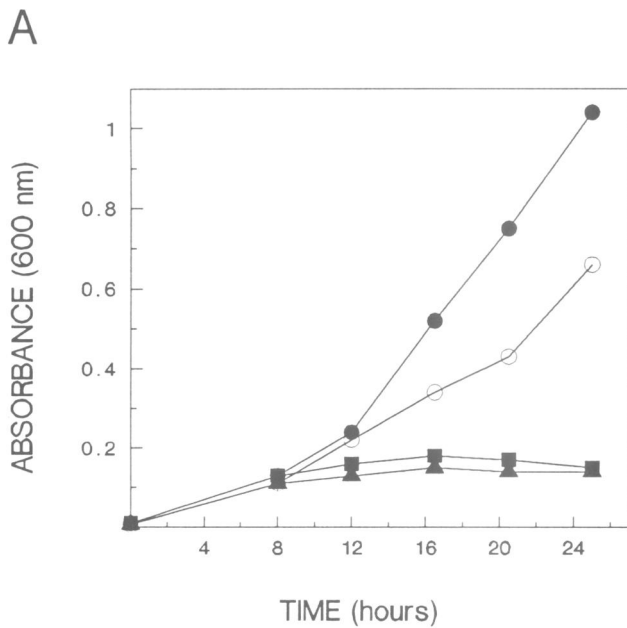


Fig. 7. Growth curves (A) and phase-contrast micrographs (B) of *S.pombe* strains: *leu1-32* (●, B1) and *cwg2-1, leu1-32* transformed with pREPCDC43 (○, B2), pREPBET2 (■, B3) or pREP1 (▲, B4). Cells exponentially growing at 28°C were diluted to the same optical density and further grown at 37°C (A) or 35°C (B) in minimal medium or minimal medium plus leucine for the *leu1-32* strain. Bar, 10 μ m.

to the medium had no effect on the complementation. pYSCWG2 plasmid was unable to complement the *orf2-1* or *dpr1* mutations (data not shown).

Discussion

Data from a previous paper indicate that the *cwg2+* gene was not the structural gene of (1–3) β -D-glucan synthase although it was directly related to its activity. The *cwg2-1*

Table III. (1–3) β -D-glucan synthase activity in *S.pombe* strains

Strain	Specific activity	%
<i>leu1-32, cwg2+</i>	4.4 \pm 0.1	100
<i>leu1-32, cwg2-1</i> + pREPCDC43	3.0 \pm 0.3	68
<i>leu1-32, cwg2-1</i> + pREPBET2	0.8 \pm 0.3	18
<i>leu1-32, cwg2-1</i> + pREP1	1.0 \pm 0.3	23

All extracts were prepared from strains grown at 37°C in MM supplemented with 1.2 M sorbitol and 20 mM CaCl₂. The *S.pombe leu1-32* culture was also supplemented with 2 mM leucine.

^a Expressed as milliunits per mg of protein. Values are the means and standard deviations calculated from three independent experiments.

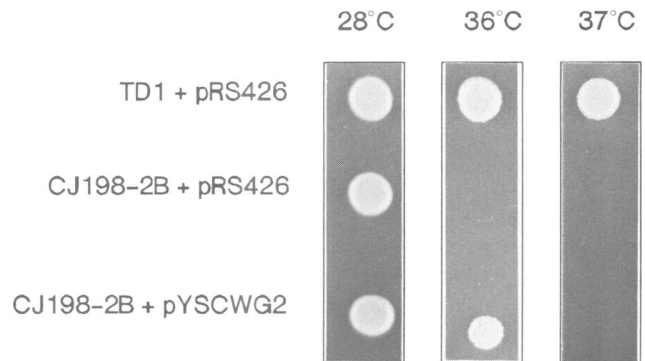


Fig. 8. Suppression of the *cdc43-2* mutation by multiple copies of *cwg2+* gene. Plasmids pRS426 and pYSCWG2 were transformed into TD1 or CJ198-2B (*cdc43-2*) *S.cerevisiae* strains. The cell suspensions of the transformants grown at 28°C were spotted on to minimal medium plates. Colony formation was analyzed following 2 days' incubation at 28, 36 or 37°C.

mutant strain showed lower *in vitro* levels of (1–3) β -D-glucan synthase activity than the wild type strain as measured after culturing at 37°C in YED supplemented with 1.2 M sorbitol. This lower activity level always cosegregated with the lytic morphological defect of this mutant and with a slightly lower content of (1–3) β -D-glucan in the cell wall. Cloning of the *cwg2+* wild type gene demonstrated that all the phenotypes caused by the *cwg2-1* mutation were due to the alteration of a single gene. There is an apparent discrepancy between the considerably lower level of (1–3) β -D-glucan synthase activity in the *cwg2-1* mutants, as measured *in vitro*, and the smaller reduction in their cell wall (1–3) β -D-glucan (Ribas *et al.*, 1991). A possible explanation is the existence in *S.pombe* of functionally redundant β -glucan synthase activities, as is the case for chitin synthesis in *S.cerevisiae* where at least three structural chitin synthases have been described (Shaw *et al.*, 1991; Valdivieso *et al.*, 1991). Disruption of *CHS1* (Bulawa *et al.*, 1986) or *CHS2* (Bulawa and Osmond, 1990) decreases the cell wall chitin content by <10%. The data obtained with the disruption of the *S.cerevisiae KRE6* gene (Roemer and Bussey, 1991), required for β -glucan synthesis *in vivo*, support this hypothesis.

An interesting feature of the *cwg2+* gene is that the major transcript starts 469 nucleotides upstream of the long ORF while in *S.pombe* transcription generally starts within 200 nucleotides upstream of the ORF (Russell, 1989). Besides, in the *cwg2+* mRNA leader sequence there are five short ORFs. Such a long sequence between the promoter and the protein-coding ORF, very unusual in yeast, and the five short

ORFs might imply that the *cwg2⁺* gene is translationally controlled. In several instances it has been shown that multiple ORFs found in extended mRNA leaders, similar to that of the *S. cerevisiae* *GCN4* gene, are recognized as translational start sites and inhibit initiation at the downstream protein coding sequences (Kozak, 1989). The significance of the five short ORFs found in the *cwg2⁺* mRNA leader is currently under study. The presence of a second weak transcript 231 nucleotides upstream of the ATG could explain the partial recovery of the (1–3) β -glucan synthase activity in the transformant with the plasmid pMD4. In this plasmid the 5' flanking sequences have been partially deleted (see Figure 2), and it is possible that a less efficient promoter allows the transcription of *cwg2⁺* from this plasmid.

The *cwg2⁺* gene does not have a TATA sequence upstream of the major transcription start (nucleotide –469). Although RNA polymerase II promoters have not been well investigated in *S. pombe* (Russell, 1989), this organism seems to behave like higher eukaryotes. Transcripts of *S. pombe* are initiated within 25–45 nucleotides downstream of the TATA element. However, TATA boxes are less obvious in genes which have a low transcription efficiency as does the *cwg2⁺* gene. The codon bias index of this gene is negative (–0.057), indicating that it is expressed at low levels. This correlates well with the very low amount of transcript detected by Northern analysis of the mRNA. The *cwg2⁺* gene also lacks the CAGTCACA box, recently described in some 'TATA-less' *S. pombe* genes (Witt *et al.*, 1993). Some other element is presumably required for initiation of transcription of this gene.

The Cwg2 amino acid sequence is similar to those of Cdc43/Cal1, Bet2/Orf2, Dpr1/Ram1 and the β subunit of rat FT, a number of proteins related to prenyltransferase activities (Kohl *et al.*, 1991). The similarity is especially strong in the C-terminal part of the protein, previously noted as being the most conserved region. Protein prenylation plays a critical role in directing the modified proteins to their membrane destinations. A broad range of proteins controlling different physiological processes undergo this modification. Particularly, most Ras-related GTP-binding proteins, widely conserved in eukaryotes, seem to be subject to this modification (Clark, 1992). So far, no prenyltransferases have been described in *S. pombe*; however, protein prenylation in this organism has recently been studied (Giannakourous *et al.*, 1992) and prenylated Ras-like YPT proteins have been described (Newman *et al.*, 1992). Our results indicate that *cwg2⁺* encodes the β subunit of an *S. pombe* GGT-I. Genetic evidence also supports a functional homology between *cwg2⁺* and *CDC43/CAL1*, the gene coding for the β subunit of GGT-I in *S. cerevisiae*. Mutations in *CDC43* were identified on the basis of two independent phenotypes; *cal1-1* mutants arrest with a small bud at the G₂–M transition of the cell cycle unless grown in the presence of high levels of calcium (Ohya *et al.*, 1984), and *cdc43-2* mutants have a defect in the establishment of cell polarity (Adams *et al.*, 1990). Based on genetic data, three Rho-related proteins: Cdc42, Rho1 and Rho2, have been proposed as possible substrates of Cdc43 (Schafer and Rine, 1992). Cdc42 is required for bud formation (Johnson and Pringle, 1990) and it has been suggested that Rho1 functions in the targeting of vesicles to the bud via the polarized cytoskeleton (McCaffrey *et al.*, 1991). Genetic data also indicate a relationship of all these proteins with Cdc24

(Madden *et al.*, 1992), a guanine nucleotide exchange protein which might regulate the activity of Rho-related proteins (Downward, 1992). It seems therefore that GTP-binding proteins play a central role in the control of cell polarity in *S. cerevisiae* (Madden *et al.*, 1992).

The morphological phenotype of *cwg2* mutant cells is quite similar to that exhibited by *S. pombe* cells defective in growth polarity; they grow as spheres rather than as rods and also appear to have an altered cell wall composition (Levin and Bishop, 1990). Since the *cwg2-1* mutant is affected in the (1–3) β -D-glucan synthase activity (Ribas *et al.*, 1991) and more precisely in the detergent-soluble fraction, where the GTP-binding component of the enzyme has been found (Kang and Cabib, 1986; J. Ribas, unpublished results), it is tempting to propose that *cwg2⁺* is responsible for the geranylgeranylation of this GTP-binding component, allowing its attachment to the membrane in order to interact with and activate the catalytic component of the (1–3) β -D-glucan synthase activity. Whether this component is the homolog of Cdc42, recently described in *S. pombe* (Fawell *et al.*, 1992), a Rho protein, or a different substrate of the GGT-I remains unknown. Hence, further analysis of the function of the *cwg2⁺* gene in *S. pombe* appears to be critical, since it might be directly implicated in the specific regulation of the (1–3) β -D-glucan synthase or it might be involved in different steps of the establishment of cell polarity. It is reasonable to expect that mechanisms controlling the morphogenesis will also control the eventual localized growth of the cell surface and therefore the biosynthesis of β -glucan which must be restricted at the growing poles of the cell.

Materials and methods

Chemicals

Yeast extract, Yeast Nitrogen Base and agar were from Difco Laboratories (Detroit, MI). UDP-[U-¹⁴C]glucose (240 mCi/mmol), [γ -³²P]ATP (5000 Ci/mmol) and [α -³⁵S]dATP (3000 Ci/mmol) were from Amersham International plc (Amersham, UK). [³H]farnesyl pyrophosphate (20 Ci/mmol) and [³H]geranylgeranyl pyrophosphate (20 Ci/mmol) were from Du Pont/NEN (Boston, MA). Sorbitol and UDP-glucose were from Sigma Chemical Co. (St Louis, MO). Zymolyase 100T was from Seikagaku Kogyo Co. Ltd (Japan). Other reagents were of analytical grade.

Strains, growth conditions and genetic methods

S. pombe and *S. cerevisiae* strains used in this work are described in Table IV. They were derived from the heterothallic standard wild type strains 972 (mating type *h*[–]) and 975 (mating type *h*⁺). Yeasts were usually grown in YED medium (1% yeast extract, 1% glucose). Sporulation medium was made as described by Egel (1971). To check the different auxotrophies, minimal medium was used (1% glucose, 0.7% yeast nitrogen base without amino acids, 0.9 g/l KCl, 1 mg/l citric acid, 10 μ g/l biotin, 1 mg/l calcium pantothenate, 10 mg/l nicotinic acid and 10 mg/l *m*-inositol), supplemented with the necessary requirements. Incubations were carried out either at 30°C or at 37°C. Growth was monitored either by A₆₀₀ measurements or by cell number counting. Tetrad analysis was performed by ascus micro-manipulation; introduction of convenient auxotrophic markers was carried out as described previously (Kohli *et al.*, 1977).

E. coli JM101, JM109 or DH1 were used as transformation hosts and for propagation of plasmids. They were grown in LB medium (1% Bactotryptone, 0.5% yeast extract, 1% NaCl) supplemented with 50 μ g/ml ampicillin or 15 μ g/ml tetracycline when appropriate. Solid medium plates contained 2% agar.

Plasmids and recombinant DNA methods

The *S. pombe* genomic library was constructed in pDB262 (Wright *et al.*, 1986). This vector contains the *S. cerevisiae* *LEU2* gene which complements *S. pombe* *leu1-32* and part of the 2 μ m circle which allows high frequency transformation. This gene library and the additional *S. pombe* plasmids,

Table IV. Strains of *S.pombe* and *S.cerevisiae* used in this study

Strain	Genotype	Source	Reference
<i>S.pombe</i>			
972	<i>h⁻</i>	P.Munz	Kohli <i>et al.</i> (1977)
975	<i>h⁺</i>	P.Munz	Kohli <i>et al.</i> (1977)
	<i>h⁻ leu1-32</i>	P.Munz	Kohli <i>et al.</i> (1977)
	<i>h⁺ leu1-32</i>	P.Munz	Kohli <i>et al.</i> (1977)
JCR5A	<i>h⁻ cwg2-1</i>	J.C.Ribas	Ribas <i>et al.</i> (1991)
JCR5B	<i>h⁺ cwg2-1</i>	J.C.Ribas	Ribas <i>et al.</i> (1991)
	<i>h⁻ ura4-d18</i>	S.Moreno	Grimm <i>et al.</i> (1988)
	<i>h⁺ ura4-d18</i>	S.Moreno	Grimm <i>et al.</i> (1988)
	<i>h⁻ cwg2-1 leu1-32</i>	This study	
	<i>h⁺ cwg2-1 ura4-d18</i>	This study	
	<i>h⁻ cwg2-1 ura4-d18</i>	This study	
<i>S.cerevisiae</i>			
KMY5-2A	<i>MATα dpr1 his3 leu2 ura3</i>		Goodman <i>et al.</i> (1988)
KMY2-3A	<i>MATα leu2 his3</i>		Goodman <i>et al.</i> (1988)
CJ198-2B	<i>MATα cdc43-2 ura3 trp1 gal2</i>	D.Johnson	Johnson <i>et al.</i> (1990)
TD1	<i>MATα ura3 his4 trp1 gal2</i>	D.Johnson	Johnson <i>et al.</i> (1990)
W303 1A	<i>MATα his3 leu2 trp1 ura3 ade2</i>	J.Friesen	Petersen-Bjorn <i>et al.</i> (1990)
YF1594	W303 1A <i>ORF2::LEU2/pYS133^a</i>	J.Friesen	Petersen-Bjorn <i>et al.</i> (1990)

^a Centromeric plasmid pYS133 contains *URA3*, a temperature-sensitive *orf2-1* allele, as the sole copy of the gene.

pREP1 (Maundrell, 1993) and pDW232, were kindly provided by Dr S. Moreno (Department of Biochemistry, University of Oxford) and Dr D. Weilguny (Institute of Genetics, University of Copenhagen). The *S.cerevisiae* plasmids used were pRS426 (Christianson *et al.*, 1992) and p2HG, which contains the constitutive glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter (Scheda *et al.*, 1991) and the *HIS3* gene as a prototrophic marker.

Plasmid pYSCWG2 was constructed by cloning the ORF of the *cwg2⁺* gene flanked by *Bgl*II and *Bam*HI under the control of the GPD promoter using the *Bam*HI site of the p2HG polylinker. The *URA3* gene was cut with *Hind*III from the pRS426 plasmid and introduced into the *Sal*I site of the pYSCWG2 plasmid by blunt end ligation.

pREPCDC43 and pREPBET2 were constructed by cloning the ORFs of *CDC43/CAL1* and *BET2/ORF2* in the REP1 polylinker sites *Nde*I + *Bam*HI and *Sal*I + *Bam*HI, respectively. The coding region of the *CDC43* and *BET2* were obtained by PCR amplification from *S.cerevisiae* genomic DNA using appropriate primers incorporating the restriction sites.

S.pombe and *S.cerevisiae* were transformed by the protoplast method (Sipiczki *et al.*, 1985) or the lithium chloride method (Ito *et al.*, 1983; Rose *et al.*, 1990). All manipulations of DNA and RNA were carried out by established methods (Ausubel *et al.*, 1989; Russell, 1989; Sambrook *et al.*, 1989; Rose *et al.*, 1990).

DNA sequencing and analysis of mRNA 5' termini

Determination of the nucleotide sequence was done by subcloning the 2.0 kb fragment carrying the *cwg2⁺* gene in Bluescript vectors (Stratagene Inc., La Jolla, CA). A series of overlapping deletions were created by unidirectional exonuclease III digestion of the gene cloned in both orientations in KS⁻ or KS⁺ vectors using the restriction enzymes *Pst*I and *Bam*HI. Single-stranded DNA was produced using M13K07 helper phage and both strands were sequenced using the dideoxy chain termination method (Sanger *et al.*, 1977). The DNA sequence was translated to the predicted amino acid sequence using the DNAsis program. The amino acid sequence was thereafter compared with the sequences available in the EMBL databases using the FASTA program. Alignment of the sequences was done with the CLUSTAL and PROFILE programs.

Analysis of mRNA 5' termini was carried out by primer extension essentially as described by Sambrook *et al.* (1989). Briefly, a synthetic oligonucleotide (sequence 5'-GCAATCGTGTCTTCGTATGG-3') complementary to nucleotides +83 to +63 of the *cwg2⁺* sequence was 5' end-labeled with [γ -³²P]ATP and polynucleotide kinase. Poly(A) RNA was isolated from strain 972*h⁻* and after denaturation was hybridized with an excess of the labeled complementary oligonucleotide. Reverse transcriptase RAV-2 (Amersham) was then used to extend the primer producing a labeled cDNA complementary to the RNA template. The radioactive cDNA was analyzed by electrophoresis on a 6% acrylamide/8.3 M urea sequencing

gel adjacent to the sequencing reactions of the *cwg2⁺* gene primed with the same oligonucleotide.

Nucleotide sequence accession number

The sequence of the gene *cwg2⁺* reported in this paper has been assigned the EMBL Data Library accession number Z12155.

Prenyltransferase assays

S.pombe strains *cwg2-1 ura4-d18* and *cwg2-1 ura4-d18 [cwg2⁺ ura4⁺]* were grown in YED supplemented with 1.2 M sorbitol at 37°C to late logarithmic culture phase. Cells from 100 ml cultures were resuspended in 200 μ l of buffer A (0.1 M MES-NaOH, pH 6.5; 0.1 mM MgCl₂; 0.1 mM EGTA; 1 mM mercaptoethanol) plus 6 μ l PMSF and broken with glass beads. After low speed centrifugation at 3000 r.p.m. for 10 min in a Sorvall HB4 rotor, the supernatants were subjected to high speed centrifugation at 36 000 r.p.m. for 1 h using a Ti70 rotor. The supernatants were saved as crude extracts. Protein concentration was usually 10 mg/ml.

Prenyltransferase assays were performed essentially as described before (Finnegold *et al.*, 1991; Moores *et al.*, 1991). A typical reaction contained (in a final volume of 30 μ l): 50 mM Tris-HCl, pH 7.4; 5 mM dithiothreitol; 10 mM MgCl₂; 1.2 μ M [³H]farnesyl pyrophosphate (20 Ci/mmol) for FT or 1.2 μ M [³H]geranylgeranyl (20 Ci/mmol) for GGT-I and GGT-II; 160 μ g crude extracts and 28.5 μ g substrate. The protein used as substrate was GST-CIIS for FT, GST-CIIL for GGT-I (Finnegold *et al.*, 1991) and pUC-YPT1 for GGT-II (Marshall *et al.*, 1989). All the assays were carried out at 37°C or 42°C. Aliquots were spotted on to filter paper at time intervals, and the radioactivity incorporated into TCA-precipitable protein was measured.

Electron microscopy

Exponentially growing *S.pombe* cells cultivated at 37°C in minimal medium supplemented with sorbitol were harvested and fixed in 3% glutaraldehyde for 1 h at room temperature followed by 1 h at 4°C in 2% OsO₄. Both fixatives were in 0.1 M sodium phosphate buffer, pH 6.8. After dehydration in graded acetone solutions, the cells were embedded with a 10 nm gold film in a Polaroid 5000 Metalizer. Cells were observed in a scanning electron microscope PSEM model 500 (Philips Electronic Instruments, Inc.).

Other methods

Cell-free extracts were prepared and (1-3) β -D-glucan synthase activities assayed and determined as described previously (Varona *et al.*, 1983; Ribas *et al.*, 1991).

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