Chitin synthase 1 plays a major role in cell wall biogenesis in *Neurospora crassa*

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In filamentous fungi, chitin is a structural component of morphologically distinct structures assembled during various phases of growth and development. To investigate the role of chitin synthase in cell wall biogenesis in *Neurospora crassa*, we cloned a chitin synthase structural gene and examined the consequences of its inactivation. Using degenerate oligonucleotide mixtures designed on the basis of conserved sequences of the *Saccharomyces cerevisiae* CHS1 and CHS2 polypeptides, a DNA fragment encoding a similar predicted amino acid sequence was amplified from *N. crassa* genomic DNA. This product was used to probe *N. crassa* libraries for a gene homologous to one of the yeast genes. Full-length genomic and partial cDNA clones were identified, isolated, and sequenced. The amino acid sequence deduced from a cloned 3.4-kb gene [designated chitin synthase 1 (*chs-1*)] was very similar to that of the *S. cerevisiae* CHS1 and CHS2 and the *Candida albicans* CHS1 polypeptides. Inactivation of the *N. crassa chs-1* gene by repeat-induced point mutation produced slow-growing progeny that formed hyphae with morphologic abnormalities. The *chs-1*^{RIP} phenotype was correlated with a significant reduction in chitin synthase activity. Calcofluor staining of the *chs-1*^{RIP} strain cross-walls, residual chitin synthase activity, and the increased sensitivity of the *chs-1*^{RIP} strain to Nikkomycin Z suggest that *N. crassa* produces additional chitin synthase that can participate in cell wall formation.

[Key Words: Cell wall biosynthesis; fungal morphology; multiple chitin synthases]

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Chitin is one of the most abundant natural polymers. The biosynthesis and deposition of chitin involves the sequential biotransformation of simple metabolites, polymerization of biosynthetic intermediates, and extrusion of the product outside the plasma membrane (Cabib 1987; Cohen 1987; Bartnicki-Garcia 1989). Although abundant in arthropods, some fungi, and other eukaryotes, chitin is absent from plant and mammalian species (Muzzarelli et al. 1986). Thus, biosynthesis of chitin is an attractive target for the development of anti-pest/anti-parasite drugs (Cohen 1987; Gooday 1989).

Chitin synthase (UDP-2-acetamido-2-deoxy-D-glucose : chitin 4- β -acetamidodeoxy-D-glucosyltransferase; EC 2.4.1.16) catalyzes the following reaction: 2n uridine-5'-diphospho-N-acetylglucosamine (UDPGlcNAc) $\rightarrow$ 2n UDP + [GlcNAc- β -(1 $\rightarrow$ 4)GlcNAc]_n.

Chitin synthase activity is associated primarily with the membranous fractions of the fungal cell (Gooday 1977) and has been partially purified from yeast and filamentous fungi (Bartnicki-Garcia et al. 1978; Selitrennikoff 1979; Kang et al. 1984; Leal-Morales et al. 1988). To date, two genes encoding chitin synthase polypeptides have been identified in *Saccharomyces cerevisiae* (Bulawa et al. 1986; Silverman et al. 1988). Although chitin is believed to be an essential component of the cell wall,

S. cerevisiae CHS1 has been shown to be dispensable (Bulawa et al. 1986; Cabib et al. 1989). CHS2 appears to be essential under certain conditions (Silverman et al. 1988). In some genetic backgrounds and environmental conditions, however, disruption of CHS2 results in abnormal morphology, not inviability (Bulawa and Osmond 1990). CHS1 and CHS2 of yeast have similar predicted amino acid sequences. Chitin synthase-like sequences have been deduced from the sequences of cloned fragments of other yeasts and several filamentous fungi (P. Robbins, pers. comm.), establishing the widespread existence of this enzyme in fungi. Since the pioneering work of Glazer and Brown (1957) on chitin synthesis in cell-free preparations of *Neurospora crassa*, the biochemical characteristics of fungal chitin synthases have been documented extensively (Cabib 1987; Cohen 1987; Gooday 1977; Gooday and Trinci 1980). Nevertheless, there is relatively little information on the genes responsible for chitin biosynthesis in filamentous fungi.

We have cloned, sequenced, and characterized a chitin synthase structural gene from *N. crassa* and designated it *chs-1*. We used the repeat-induced point (RIP) mutation process to inactivate the chromosomal *chs-1* locus. Strains lacking a functional CHS1 have an abnormal growth pattern and morphology, in addition to exhibit-

ing reduced levels of chitin synthase activity. Our findings suggest that *chs-1* expression is necessary for normal cell growth and that *N. crassa* produces at least two chitin synthases.

Results

Cloning of the *N. crassa chs-1* gene

Several pairs of degenerate oligonucleotide primers were designed on the basis of conserved amino acid sequences predicted from the *S. cerevisiae CHS1* and *CHS2* genes. These primers were used in polymerase chain reactions (PCRs) in an attempt to amplify corresponding regions of *N. crassa* genomic DNA. To allow amplification of sequences deviating slightly from the yeast consensus sequence, reactions were performed under low-stringency conditions (1.5 min at 92°C, followed by 35 cycles of 2 min at 50°C, 5 min at 60°C, and 1 min at 92°C). When the 1280 and 1281 primer combination was used as a control mixture (Table 1; see Fig. 4, below), a single abundant ~600-bp product was obtained. A product was unexpected, as both primers were designed to anneal to the same DNA strand. Nonetheless, the amino acid sequence predicted from the nucleotide sequence of the cloned ~600-bp product showed striking similarity to those of both yeast chitin synthases. Subsequently, it was discovered that primer 1280 is complementary (with several mismatches) to a nucleotide sequence from another region of the *N. crassa chs-1* gene (nucleotides

1390–1411; see Fig. 3, below), thus explaining the origin of the ~600-bp product.

Using the ~600-bp fragment (designated pCS12, Fig. 1) as a probe, a single genomic and several overlapping cDNA clones were identified and isolated from *N. crassa* genomic (Orbach et al. 1986) and cDNA (Orbach et al. 1990) libraries. From the genomic clone, 11-kb *ApaI* and 4.3-kb *BamHI* fragments hybridizing to pCS12, containing the entire *chs-1* gene, were isolated and subcloned. These clones, designated pOYA5 and pOY30, respectively (Fig. 1), were used in further analyses.

Sequence of *chs-1*

A 4.4-kb segment of pOYA5, containing the *chs-1* gene, was sequenced completely on both strands. The molecular organization of the genomic segment containing *chs-1* is presented in Figure 2. The complete nucleotide sequence of *chs-1* and the predicted amino acid sequence of the *CHS1* polypeptide are presented in Figure 3. The *chs-1*-coding region was located by comparing the predicted amino acid sequence with those of the two *S. cerevisiae* chitin synthases, using codon preferences of *N. crassa*. The nucleotide sequence immediately surrounding an initiation codon is believed to be important for efficient translation (Kozak 1986). The presumed *chs-1* start codon segment (GCAACCATGG) is slightly different from the *N. crassa* consensus (A/GTCAA/CAATGG) compiled for 20 genes (Roberts et al. 1988), but matches the sequence (GCCA/GCCATGG) conserved in higher eukaryotes (Kozak 1987). Two intervening sequences were tentatively identified on the basis of consensus 5'- and 3'-splice junction sequences (Orbach et al. 1986; Hager and Yanofsky 1990). The nucleotide sequence of a partial cDNA clone spanning 2.2 kb of the 3' end of the gene and the untranslated region verified the nucleotide sequence of a major portion of the genomic clone, as well as the position and boundaries of the 3' intron. The position and boundaries of the 5' intron were verified by analyzing the sequence of two reverse transcriptase-polymerase chain reaction (RT-PCR) products obtained with primer combinations 2049–2050 and 2013–2049 (Table 1). A CCAAT box and polyadenylation signal sequence (Proudfoot and Brownlee 1976) are present (Figs. 2 and 3). The 5' end of the *chs-1* transcript (Fig. 3) was determined (data not shown) by primer extension techniques (Sambrook et al. 1989), using the primers shown in Table 1. The 3'-end designation was based on the common poly(A) addition site in two independent cDNA clones.

Chromosomal localization of *chs-1*

Restriction fragment length polymorphism (RFLP) analyses were used to map *chs-1*. Of several restriction enzymes used (*BamHI*, *EcoRI*, *HindIII*, *KpnI*, *PstI*, and *XhoI*), only *XhoI* and *PstI* revealed polymorphisms in the vicinity of *chs-1* in *N. crassa* of Oak Ridge and Mauriceville backgrounds. *PstI* was used to digest DNA from progeny of the "small cross" (Metzenberg et al. 1985).

Table 1. Synthetic oligonucleotides

Oligo number ^a	Use	Sequence ^b
1280	PCR	<div style="text-align: center;"> <u>SacI</u> TCTAGAGCTCAA TATGTA TC T C G C G A G A T A G <u>XbaI</u> C CT T T </div>
1281	PCR	<div style="text-align: center;"> <u>SpeI</u> GTCGACTAGTC T T A T C G A A A G T T C T G <u>SaI</u> C T </div>
1990 (nucleotides 697–673)	Primer extension	CTAGTGGATCCATCATAGCCGTCGC <u>BamHI</u>
2013 (nucleotides 675–698)	PCR	GACGGCTATGATGGATCCACTAGT <u>BamHI</u>
2049 (nucleotides 844–816)	PCR Primer extension	GTTTTCGGAGAGGAAGGGACGTTGCCAT <u>EcoRI</u>
2050 (nucleotides 647–669)	PCR	CGCAACCATGGCGTACCACGGTC

^aNumbers in parenthesis designate nucleotide number and direction (based on the genomic nucleotide sequence presented in Fig. 3). Primers 1280 and 1281 were designed on the basis of conserved amino acid sequences, as marked in Fig. 4. Amino acid residues predicted to be encoded at the 5' and 3' ends of the product amplified using 1280 and 1281 (pCS12) are underlined in Fig. 4.

^bMultiple lettering indicates oligonucleotide mixtures. Restriction sites for cloning PCR products are marked.

Figure 1. Clones and constructs used in this study. pCS12 is a cloned *N. crassa* genomic DNA product amplified by low-stringency PCR. pOYA5 is an *Apa*I fragment obtained from the DNA of an individual plaque from the Orbach λ J1 *N. crassa* genomic library, identified after probing with pCS12. pOY30 is a 3.6-kb *Bam*HI subclone of pOYA5, identified by hybridization with pCS12. It was used for construction of pOY301. pOY301 is pOY30 containing a 4.3-kb *Pvu*II–*Sma*I fragment of pDH25, consisting of the hygromycin phosphotransferase gene driven by the *A. nidulans trpC* promoter region (Cullen et al. 1987).

Subsequent probing with a hexamer-labeled *Pst*I digest of pOY30 located *chs-1* between *Fsr-9* and *am*, on linkage group V (data not shown).

CHS1 amino acid homology

chs-1 encodes a predicted 961-residue polypeptide with a calculated mass of 107 kD and a pI of 8.8. The predicted amino acid sequence of *N. crassa* CHS1 is 37%, 39%, and 40%, identical to those of *S. cerevisiae* CHS1 and CHS2 and *Candida albicans* CHS1, respectively (Fig. 4). The calculated pI of the entire *N. crassa* CHS1 polypeptide is similar to those calculated for *S. cerevisiae* CHS2 (pI = 9.1) and *C. albicans* CHS1 (pI = 8.7) but differs from the value calculated for the *S. cerevisiae* CHS1 polypeptide (pI = 5.8). Nonetheless, the calculated pI value of the amino-terminal end 230 residues of the *N. crassa* CHS1 polypeptide (pI = 5.3) resembled more closely the calculated value for the amino-terminal region of the *S. cerevisiae* CHS1 polypeptide (pI = 4.5) than that of the same region of CHS2 (pI = 10.4). Hydrophilicity analysis predicts a hydrophilic region at the amino terminus (spanning residues 1–230) and a hydrophobic region at the carboxyl terminus (residues 560–961 of the polypeptide; Fig. 5). The presence of several putative membrane-spanning domains near the carboxyl

terminus is consistent with the membrane association of chitin synthase activity (Duran et al. 1975; Peberdy and Moore 1975). Two potential N-glycosylation sites are present (Pless and Lennarz 1977) at amino acid residues 544 and 788 (Fig. 3).

Functional analysis of *chs-1*

In view of the finding that CHS1 of *S. cerevisiae* is non-essential, we disrupted *chs-1* of *N. crassa*. The gene was inactivated by using the RIP process (Selker 1990), which results in many premeiotic GC → AT base-pair transitions in duplicated DNA sequences. A 4.3-kb fragment of *chs-1* was transformed into a wild-type strain of *N. crassa*. The DNA used for transformation [designated pOY301 (Fig. 1)] consisted of pOY30 interrupted at the 2258 *Bal*II site (Fig. 3) by insertion of a 4.1-kb *Pvu*II–*Sma*I fragment, encoding an expressed *hph* gene isolated from pDH25 (Cullen et al. 1987). This construction permitted selection of transformants based on hygromycin resistance. One transformant (T-36), containing a single ectopic copy of pOY301 (Fig. 6A), was crossed with another transformant (T-51), of the opposite mating type. Among the viable ascospores isolated from this cross, ~41% exhibited very slow growth. Southern blot analysis provided evidence that in these progeny *chs-1* had been subjected to RIP (Fig. 6A); restriction sites were missing from both the resident and the ectopic *chs-1* gene copies. One of the progeny cultures that had undergone RIP (*chs-1*^{RIP}) was chosen for further analysis. The absence of *chs-1* message, as determined by Northern blot analysis (Fig. 6B), provided additional evidence for the extensive alteration of the *chs-1* gene in this strain. The absence of the *chs-1* RNA was specific, as similar *tub-2* RNA levels were detected in RNA samples from both the wild-type and *chs-1*^{RIP} strains. When the *chs-1*^{RIP} strain was crossed with a wild-type strain, a 1 : 1 ratio of wild-type/*chs-1*^{RIP} progeny was observed; this is typical of single locus alterations. To demonstrate that the altered phe-

Figure 2. Molecular organization of *chs-1*. Schematic diagram of a portion of pOYA5 that includes the *chs-1* gene. CCAAT, translation start, stop, and polyadenylation sequences are shown. (Shaded bars) Coding region; (open bars) noncoding region.

Figure 3. Complete nucleotide sequence for the *N. crassa chs-1* gene and flanking regions and the predicted amino acid sequence of CHS1. The CCAAT sequence is underlined. The transcription initiation site is marked by an asterisk (*). Conserved intron boundary and presumed lar-iat sequences are underlined, as is the conserved polyadenylation signal. Two potential N-glycosylation amino acid sequences are underlined.

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N.c.	1	5	10	15	20	25	30	35	40	45	50
C.a.	-	-	-	-	-	-	-	-	-	-	-
CHS1	E	Q	P	N	N	N	N	S	G	N	I
CHS2	-	-	-	-	-	-	-	-	-	-	-
N.c.	51	55	60	65	70	75	80	85	90	95	100
C.a.	-	-	-	-	-	-	-	-	-	-	-
CHS1	Q	I	N	S	Y	L	G	R	N	G	E
CHS2	N	L	Q	E	S	P	K	R	A	G	E
N.c.	101	105	110	115	120	125	130	135	140	145	150
C.a.	-	-	-	-	-	-	-	-	-	-	-
CHS1	G	V	S	S	G	V	D	Q	G	Y	N
CHS2	-	-	-	-	-	-	-	-	-	-	-
N.c.	151	155	160	165	170	175	180	185	190	195	200
C.a.	-	-	-	-	-	-	-	-	-	-	-
CHS1	M	S	A	I	D	E	D	D	S	W	L
CHS2	-	-	-	-	-	-	-	-	-	-	-
N.c.	201	205	210	215	220	225	230	235	240	245	250
C.a.	-	-	-	-	-	-	-	-	-	-	-
CHS1	A	I	R	N	A	V	Q	P	K	Y	R
CHS2	-	-	-	-	-	-	-	-	-	-	-
N.c.	251	255	260	265	270	275	280	285	290	295	300
C.a.	-	-	-	-	-	-	-	-	-	-	-
CHS1	N	R	H	T	E	L	L	I	A	I	T
CHS2	-	-	-	-	-	-	-	-	-	-	-
N.c.	301	305	310	315	320	325	330	335	340	345	350
C.a.	-	-	-	-	-	-	-	-	-	-	-
CHS1	Q	K	I	V	V	C	L	V	F	D	G
CHS2	-	-	-	-	-	-	-	-	-	-	-
N.c.	351	355	360	365	370	375	380	385	390	395	400
C.a.	-	-	-	-	-	-	-	-	-	-	-
CHS1	T	S	Q	L	S	V	T	P	N	O	A
CHS2	-	-	-	-	-	-	-	-	-	-	-
N.c.	401	405	410	415	420	425	430	435	440	445	450
C.a.	-	-	-	-	-	-	-	-	-	-	-
CHS1	G	R	I	L	N	P	E	V	C	I	L
CHS2	-	-	-	-	-	-	-	-	-	-	-
N.c.	451	455	460	465	470	475	480	485	490	495	500
C.a.	-	-	-	-	-	-	-	-	-	-	-
CHS1	G	K	K	L	L	N	P	L	V	A	V
CHS2	-	-	-	-	-	-	-	-	-	-	-
N.c.	501	505	510	515	520	525	530	535	540	545	550
C.a.	-	-	-	-	-	-	-	-	-	-	-
CHS1	R	-	-	-	-	-	-	-	-	-	-
CHS2	-	-	-	-	-	-	-	-	-	-	-
N.c.	551	555	560	565	570	575	580	585	590	595	600
C.a.	-	-	-	-	-	-	-	-	-	-	-
CHS1	A	K	A	G	Q	K	W	H	L	S	Y
CHS2	-	-	-	-	-	-	-	-	-	-	-
N.c.	601	605	610	615	620	625	630	635	640	645	650
C.a.	-	-	-	-	-	-	-	-	-	-	-
CHS1	G	R	M	Y	K	S	G	H	N	I	V
CHS2	-	-	-	-	-	-	-	-	-	-	-
N.c.	651	655	660	665	670	675	680	685	690	695	700
C.a.	-	-	-	-	-	-	-	-	-	-	-
CHS1	T	P	V	T	A	S	S	S	A	E	H
CHS2	-	-	-	-	-	-	-	-	-	-	-
N.c.	701	705	710	715	720	725	730	735	740	745	750
C.a.	-	-	-	-	-	-	-	-	-	-	-
CHS1	G	S	K	W	T	Y	I	T	S	F	F
CHS2	-	-	-	-	-	-	-	-	-	-	-
N.c.	751	755	760	765	770	775	780	785	790	795	800
C.a.	-	-	-	-	-	-	-	-	-	-	-
CHS1	S	L	F	G	G	S	G	S	A	G	V
CHS2	-	-	-	-	-	-	-	-	-	-	-
N.c.	801	805	810	815	820	825	830	835	840	845	850
C.a.	-	-	-	-	-	-	-	-	-	-	-
CHS1	I	N	I	L	M	I	Y	A	F	N	N
CHS2	-	-	-	-	-	-	-	-	-	-	-

Figure 4. (See facing page for legend.)

Figure 5. Hydropathicity plot for the predicted amino acid sequence of the *N. crassa* CHS1 polypeptide. Peaks above the axis are hydrophilic regions; those below the axis are hydrophobic. Note the hydrophobic segments beyond residue 550.

nototype of the *chs-1*^{RIP} strain was the result of inactivation of *chs-1*, we performed a complementation experiment with pOYA5 (containing the genomic copy of *chs-1*) and pMP6 (for transformant selection). Among 60 hygromycin-resistant transformants analyzed, 28 had a morphology that was indistinguishable from that of wild type. This result provides evidence that the transformation with pOY301, as well as the RIP process, did not affect other sequences that are involved in the determination of hyphal morphology.

chs-1^{RIP} morphology

Growth of the *chs-1*^{RIP} strain on both solid and liquid media was significantly slower than that of wild type. To quantitate the difference in hyphal elongation, the rate of radial growth was measured from a mycelial disc placed in the center of a 150-mm-diam. petri dish. Within 24 hr (at 34°C), the wild-type strain had grown to a 64-mm radius, covering most of the dish. During the same period, the *chs-1*^{RIP} strain had grown to only a 14-mm radius. Even after 1 week of growth, the *chs-1*^{RIP} colony had not equaled the radial growth of the 24-hr-old wild-type strain (Fig. 7). Furthermore, the density of hyphal growth was sparse, and there was little evidence of conidiation. Thus, *chs-1*^{RIP} lacked the confluent mycelial mat that is typical of wild-type growth. Supplementing either solid or liquid medium with 1 M sorbitol as an osmotic stabilizer had no effect on *chs-1*^{RIP} growth (data not shown).

Microscopic examination of cultures grown in liquid medium revealed extensive hyphal swelling and other hyphal abnormalities in the *chs-1*^{RIP} strain, when compared with wild type (Fig. 8A–E). In some instances, severe swelling of the hyphae of germinating conidia was observed (Fig. 8D). When grown on solid media, long thin aerial hyphae were apparent, and frequently, the hyphal tips had deteriorated (Fig. 8E). However, subjecting *chs-1* to RIP did not affect cross-wall formation or abundance. This was demonstrated by Calcofluor staining (Fig. 8F,G). When *chs-1*^{RIP} cultures grown on solid media matured to the conidiation phase, microscopic examination revealed that conidia, as well as major and minor conid-

iophore constrictions, were morphologically similar to those of wild type (data not shown).

Chitin synthase activity in the *chs-1*^{RIP} strain

Chitin synthase enzyme activity was measured as the incorporation of UDP[1-¹⁴C]GlcNAc into an insoluble product. The enzyme activity was measured in cell-free extracts prepared from wild-type and *chs-1*^{RIP} strains. Chitin synthase activity in the *chs-1*^{RIP} extracts was found to be 7- to 20-fold lower than in wild-type extracts (Fig. 9). Variation among duplicate samples prepared from each extract source did not exceed 12%. Attempts to determine the fractions of chitin synthase that were susceptible to activation by proteolysis were inconclusive, as activation was too variable from sample to sample. Our findings are consistent with the wide range of variation in the effect of proteases on the activity of *N. crassa* chitin synthase activity, which has been reported previously (Bartnicki-Garcia et al. 1978; Arroyo-Begovich and Ruiz-Herrera 1979). Residual chitin synthase activity was observed in all *chs-1*^{RIP} extracts, suggesting that other chitin synthases are active in the *chs-1*^{RIP} strain. To confirm that the *chs-1*^{RIP} strain was deficient in chitin synthesis, we determined the sensitivity of the *chs-1*^{RIP} strain to Nikkomycin Z, a competitive inhibitor of chitin synthase (Gow and Selitrennikoff 1984). When grown in the presence of the inhibitor, the *chs-1*^{RIP} strain was significantly more sensitive to the drug than was wild type (Fig. 10A). To establish that the hypersensitivity of the *chs-1*^{RIP} strain was specific to Nikkomycin, the comparative sensitivity of wild-type and *chs-1*^{RIP} strains to the microtubule inhibitor Benomyl was tested. Both strains were equally sensitive to Benomyl (Fig. 10B).

Discussion

chs-1 from *N. crassa*, a member of a chitin synthase gene family, has been cloned and characterized. We have shown that *chs-1* is dispensable, yet its product is required for normal cell growth. The similarity between the predicted amino acid sequence of CHS1 of *N. crassa*

Figure 4. Multiple sequence alignment of four predicted chitin synthase polypeptides. The comparison was of residues 1–780 of *N. crassa* CHS1 (N.c.), residues 200–975 of *S. cerevisiae* CHS1 (CHS1), residues 50–815 of *S. cerevisiae* CHS2 (CHS2), and residues 1–741 of *Candida albicans* CHS1 (C.a.). Asterisks (*) mark amino acid residues identical to the *N. crassa* polypeptide; dashes mark gaps. Regions used for the design of degenerate oligonucleotide primers 1280 and 1281 (Table 1) are indicated. Amino acid residues predicted to be encoded at the 5' and 3' ends of the PCR product obtained (pCS12, Fig. 1) are underlined.

Figure 6. Molecular analysis of *chs-1* DNA and transcript in wild type, pOY301-transformed, and *chs-1*^{RIP} strains of *N. crassa*. (A) Southern blot analysis of *Bam*HI–*Eco*RV digests of genomic DNA isolated from wild type (wt), two isolates that were transformed with pOY301 (T-36, T-51), and two *chs-1*^{RIP} progeny (S-1, S-2) from a cross between T-36 and T-51, exhibiting slow growth and abnormalities in hyphal morphology. The probe used was a hexamer-labeled *Bam*HI digest of pOY30. (B) Northern blot analysis of total RNA from wild type (wt) and the S-1 slow-growing progeny from the T-36–wild-type cross. The blot was probed with a hexamer-labeled *Bam*HI digest of pOY30 (for *chs-1*) and a 1.2-kb *Sac*I digest of pBT3 (for *tub-2*). The large arrow indicates the position of the *chs-1* message; the small arrow indicates the position of the *tub-2* message.

and those of the yeast chitin synthases permitted us to detect and clone the *N. crassa* gene.

Hydropathy plots suggest that the chitin synthase polypeptides of *N. crassa*, *S. cerevisiae*, and *C. albicans* have similar characteristics throughout their length. However, Silverman (1989) has found a significant difference between the calculated pI values of the amino-terminal regions of the two *S. cerevisiae* chitin synthase polypeptides. We performed a similar comparison between the *N. crassa* and yeast chitin synthases. Because the similarities and differences between the various polypeptides, on the basis of calculated pI values, are not

uniform throughout the length of the polypeptides, additional criteria must be applied before we can determine which synthases perform the same functions.

The RIP phenomenon was used to inactivate *chs-1*. The slow growth and abnormal swelling of *chs-1*^{RIP} hyphae demonstrate that *chs-1* plays a major role in hyphal growth. Conidia do not seem to be affected by *chs-1* disruption; therefore, an additional chitin synthase may be primarily responsible for chitin deposition during sporulation. Reduction in the chitin content of the primary cell wall is probably responsible for the gross hyphal alterations observed in the *chs-1*^{RIP} strain. Lack of the primary chitin building block (although it constitutes only 10% of the fully assembled cell wall) could lead to alteration of the architecture of the fungal cell wall, resulting in the observed morphologic abnormalities. Nonetheless, lack of CHS1 activity does not render the organism inviable. Using Calcofluor staining of the *chs-1*^{RIP} strain, what appears to be normal deposits of chitin were detected in cross-walls, where chitin is the major component (Hunsley and Gooday 1974), and in conidial septa. This finding is supported by our detection of residual chitin synthase activity in this strain (which is also hypersensitive to the chitin synthase inhibitor Nikkomycin Z) and is in agreement with the detection of chitin synthase activity and chitin deposition in chitin synthase mutants of *S. cerevisiae* (Bulawa et al. 1986; Sburlati and Cabib 1986; Orlean 1987; Cabib et al. 1989; Silverman 1989; Bulawa and Osmond 1990).

Differential involvement of chitin synthase isozymes in distinct cell-wall biosynthetic functions may provide one explanation for the absence of an effect of inactivation of *chs-1* by RIP on formation of cross-walls and conidia. This possibility is supported by a recent report describing the different functions of *S. cerevisiae* CHS2 and CHS3, which are specific for primary septum formation and the chitin ring at the bud emergence location, respectively (Shaw et al. 1991). Another possibility is that separate chitin synthase isozymes have the ability to perform the same functions. The activity of the other chitin synthases, however, is not adequate for the normal rate of assembly of the hyphal cell wall, yet it suf-

Figure 7. Comparative growth of wild-type (left; 30 hr) and *chs-1*^{RIP} (right; 7 day) strains on solid Vogel's N medium. Growth was at 34°C for the times indicated. A difference in hyphal biomass as well as limited conidiation in the *chs-1*^{RIP} strain is apparent.

Figure 8. Microscopic analysis of hyphae of wild type (A,F) and *chs-1*^{RIP} (B–E,G) strains. (A) Hyphae of a wild-type strain grown in liquid medium. (B) Abnormal swelling of *chs-1*^{RIP} strain hyphae grown in liquid culture. Swelling is evident at hyphal tips, as well as along the entire filament. Occasionally, spherical swellings are evident. (C) Hyphal swelling is not uniform, although the general filamentous nature of the mycelium is maintained. (D) Gross abnormalities are sometimes observed in germinating conidia (dark-field settings). (E) Degenerated aerial hyphal tips and enhanced constrictions in cultures grown on solid medium. (F,G) Calcofluor staining of wild-type (F) and *chs-1*^{RIP} (G) for visualizing chitin deposition in cross walls. Bars, 50 μ m in A–G; 20 μ m in D.

lices for formation of apparently normal cross walls and conidia.

Two chitin synthase genes have been isolated and characterized from *S. cerevisiae* (Bulawa et al. 1986; Silverman 1989). Two *chs* PCR products, different from *chs-1*, have been isolated from *N. crassa* (P. Robbins, pers. comm.). Thus, it is likely that each fungal species produces several chitin synthases. Although *chs-1* is not essential, it is tempting to speculate that the different chitin synthases are primarily responsible for chitin deposition in one or more of the following processes: hyphal elongation, branching, cross-wall and/or septa formation, conidiation, and/or repair of ruptured cell walls.

Materials and methods

Strains and media

Wild-type *N. crassa* strains 74-OR23-1A (FGSC 987) and ORSa (FGSC 2490) were used in all experiments. Procedures used in growth studies, crosses, and other manipulations are described in Davis and de Serres (1970). Cultures were maintained on 1.5% agar slants containing Vogel's minimal medium N (Vogel 1956). When appropriate, the medium was supplemented with either hygromycin, Nikkomycin Z (both from Calbiochem), or Benomyl (DuPont) at appropriate concentrations. The drugs were filter-sterilized and added to sterile media at $\sim 50^{\circ}\text{C}$. DNA transformations of *N. crassa* were carried out as described by Orbach et al. (1986). Cotransformation was performed with a

Figure 9. Chitin synthase activity of wild-type (wt) and *chs-1*^{RIP} cell-free extracts. Reactions were carried out at 37°C in 40- μ l volumes containing 35 μ g of protein. Incorporation of UDP[1-¹⁴C]GlcNAc into an insoluble product was used as a measure of chitin synthesis.

6- μ g plasmid mixture consisting of a 1 : 1 ratio of pOYA5 and pMP6 (which contains the *N. crassa* *cpc-1* regulatory region lacking the two upstream open reading frames fused to the *hph* gene that confers hygromycin resistance; M. Plamann and C. Yanofsky, unpubl.). Hygromycin-resistant transformants were detected on regeneration medium containing sorbose as a carbon source, and single colonies were transferred to sucrose-containing minimal agar for further analysis of hyphal morphology.

Isolation and analysis of nucleic acids from *N. crassa*

Genomic DNA was isolated as follows: Mycelia from cultures grown in 25 ml Vogel's N medium were collected by filtration on Whatman No. 2 filter paper on a Buchner funnel. Samples were quick-frozen in liquid nitrogen and lyophilized. The dry samples were powdered by grinding and were suspended in an equal volume of lysis buffer [50 mM Tris-HCl (pH 8.0), 50 mM EDTA, 2% SDS, 1% β -mercaptoethanol] containing 25 μ g/ml of RNase A. After 30 min of incubation at 37°C, 100 μ g/ml of proteinase K was added to the solution and incubation was continued for 1 hr at 65°C. Two phenol-chloroform (1 : 1) extractions were performed, followed by a single chloroform extraction, an ethanol precipitation, and a 75% ethanol wash. The DNA pellet was dried and dissolved in TE buffer [10 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 8.0)].

Total RNA was isolated by a slight modification of the procedure of Orbach et al. (1990). Mycelia were harvested as described above. After a quick freeze in liquid nitrogen, 25 mg was transferred to a 2-ml screw-cap tube (Sarstedt) containing 480 μ l of extraction buffer [100 mM Tris-HCl (pH 7.5), 100 mM LiCl, 10 mM EDTA, 20 mM dithiothreitol], 420 μ l of phenol, 420 μ l of chloroform, 84 μ l of 10% SDS, and 2 grams of zirconium beads (Biospec Products Inc.). The samples were shaken twice for 30 sec in a mini-bead beater (Biospec Products Inc.). After 15 min of centrifugation in a microcentrifuge, the aqueous phase was transferred to a new tube and reextracted with phenol-chloroform (1 : 1). After an additional chloroform extraction, the RNA was precipitated, washed, dried, and dissolved in 10 mM Na-HEPES (pH 7.5) containing 1 mM EDTA. When RNA was to be used for RT-PCR or primer extension analyses, samples were treated with RQ1 DNase I (Promega), in the presence of 5 mM dithiothreitol and 2.5 units of RNasin (Promega).

N. crassa genomic DNA (Orbach et al. 1986) and cDNA (Orbach et al. 1990) libraries were screened as described by Benton and Davis (1977). Southern blot analysis was carried out on nylon membranes (Nytran, Schleicher & Schuell, Inc.) as described by Sambrook et al. (1989), as were all other DNA modification and cloning procedures. Bluescript (SK-) (Stratagene) was used for the cloning and preparation of various constructs. Northern blot analysis was performed as described by Orbach et al. (1990). *tub-2* RNA levels were determined by probing Northern blots with a hexamer-labeled 1.2-kb *SacI* fragment isolated from pBT3 (Orbach et al. 1986). pDH25 (Cullen et al. 1987) was the source of the *hph* gene, encoding hygromycin phosphotransferase, which confers hygromycin resistance. This gene is driven by the *Aspergillus nidulans* *trpC* promoter region and was used as a dominant selectable marker in the isolation of *N. crassa* transformants.

Mapping of *chs-1* was carried out by RFLP analysis of the small cross, according to the procedure of Metzenberg et al. (1985).

PCRs were carried out in 100- μ l reaction volumes in an Eri-comp thermal cycler. Two and one-half units of *Taq* polymerase (Perkin-Elmer Cetus) were used in each reaction tube containing ~2 μ g of genomic DNA as template in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, and 200 μ M of each dNTP. One microgram of each primer was used for each reaction. RT-PCR was carried out using the components of the Perkin-Elmer Cetus mRNA PCR kit in accordance with the manufacturer's instructions. Oligonucleotide primers were designed with restriction sites at the 5' end to facilitate cloning of PCR products (Table 1). Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer.

Primer extension analyses were performed to determine the

Figure 10. Effect of Nikkomycin Z (A) and benomyl (B) on growth of wild-type (open bar) and *chs-1*^{RIP} (hatched bar) strains of *N. crassa*. Inhibitors, at various concentrations, were incorporated in presterilized Vogel's N medium. Mycelial discs containing wild-type or *chs-1*^{RIP} hyphae were placed in the center of a petri dish containing either unsupplemented or drug-supplemented solidified medium. After 12 or 50 hr, colony surface area of wild-type and *chs-1*^{RIP} strains (respectively) grown on drug-containing medium was compared with growth area of the same strain on drug-free medium.

chs-1 transcription start site with two end-labeled oligonucleotide primers (Table 1), according to Sambrook et al. (1989).

DNA sequencing

DNA sequencing was performed by the dideoxy chain-termination method of Sanger et al. (1977) with α -³⁵S-labeled dATP. Sequencing reactions were electrophoresed on either 7 M urea–1× TBE [Tris-borate EDTA (Sambrook et al. 1989)] or 6% Long Ranger (AT Biochem)–0.6× TBE acrylamide gels by using a BRL sequencing gel apparatus.

Clones used for sequencing were generated either by insertion of restriction fragments into the Bluescript vector or by progressive deletions from one end of a DNA fragment. The deletions were produced by using exonuclease III, as described by Henikoff (1987).

Microscopy

Samples were viewed with a Nikon Microphot FX epifluorescence microscope. For cross-wall visualization, a drop of 10 μ g/ml of Calcofluor (Fluorescent Brightener 28, Sigma) was applied to fungal samples prespotted on a microscope slide. The filter combination used was 380- to 425-nm excitation, 430-nm dichroic mirror, and 450-nm barrier. Photographs were taken with Kodak Ektachrome ASA 400 film.

Chitin synthase assays

Chitin synthase assays were performed with total fungal extracts. Extracts were prepared from 1.5- to 4-hr-old germinating conidia that were harvested by filtration (GF/C filter, Whatman). After a water wash, the germlings were placed in a 2-ml screw-cap tube containing 1.4 grams of 0.5-mm-diam. glass beads (Biospec Products). The tube was then filled with cold 100 mM HEPES (pH 7.8). The germlings were disrupted in a mini-bead beater, using two 1-min pulses. The tubes were centrifuged for 10 min in a microcentrifuge, and the supernatants were collected. Protein concentration was determined by the method of Bradford (1976), with the Bio-Rad protein assay mixture.

Chitin synthase activity was monitored in 25- μ l reaction mixtures containing 100 mM HEPES (pH 7.8), 1 mM MgCl₂, 32 mM GlcNAc, and 1 mM UDP-GlcNAc and supplemented with 1.5×10^{-7} mM UDP[1-¹⁴C]GlcNAc (sp. act. 300 mCi/mmol) (Amersham), providing ~100,000 dpm/mixture. Aliquots of the fungal cell-free extract used in each reaction mix contained 25–40 μ g of protein. When trypsin activation of chitin synthase was examined, 2 μ l of a 0.2-mg/ml trypsin (EC 3.4.21.4, Sigma) solution was added to the cell-free extract before adding the other components of the assay mixture. After 15 min of digestion at 30°C, 2 μ l of a 0.3-mg/ml solution of soybean trypsin inhibitor was added to each reaction tube, followed by the components of the chitin synthase reaction mixture. Mixtures were incubated at 30°C, and reactions were terminated by adding 25 μ l of glacial acetic acid. Reaction products were separated by paper chromatography with Whatman No. 1 paper and water as solvent. Chromatographically immobile radioactivity (in chitin) was determined by liquid scintillation counting with Ready Safe (Beckman) cocktail mix and a Beckman LS3801 scintillation counter. To verify that the source of immobile radioactivity was chitin, we occasionally solubilized the product by treatment with chitinase, as described by Selitrennikoff (1979).

Computer methods

Programs of The University of Wisconsin Genetics Group were used for analysis of nucleic acid sequences (Devereux et al.

1984). The MacVector program (International Biotechnologies, Inc.) was used for Kyte and Doolittle (1982) hydrophilicity analysis. Multiple sequence alignments for comparison of predicted amino acid sequences corresponding to different chitin synthase genes were performed with the Tulla1 program (Subbiah and Harrison 1989).

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Note added in proof

The sequence data described in this paper were submitted to the Genbank/EMBL data libraries under accession number M73437.

References

- Arroyo-Begovich, A. and J. Ruiz-Herrera. 1979. Proteolytic activation and inactivation of chitin synthase from *Neurospora crassa*. *J. Gen. Microbiol.* **113**: 339–345.
- Bartnicki-Garcia, S. 1989. The biochemical cytology of chitin and chitosan synthesis in fungi. In *Chitin and chitosan* (ed. G. Skjak-Braek, T. Anthonsen, and P. Sandford), pp. 23–36. Elsevier Applied Science Press, London, England.
- Bartnicki-Garcia, S., C.E. Bracker, E. Reyes, and J. Ruiz-Herrera. 1978. Isolation of chitosomes from taxonomically diverse fungi and synthesis of chitin microfibrils *in vitro*. *Exp. Mycol.* **2**: 173–192.
- Benton, W. and R. Davis. 1977. Screening of lambda gt11 recombinant clones by hybridization to single plaques *in situ*. *Science* **196**: 180–182.
- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248–254.
- Bulawa, C.E. and B.C. Osmond. 1990. Chitin synthase I and synthase II are not required for chitin synthesis *in vivo* in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci.* **87**: 7424–7428.
- Bulawa, C.E., M. Slater, E. Cabib, J. Au-Young, A. Sburlati, W.L.J. Adair, and P.W. Robbins. 1986. The *S. cerevisiae* structural gene for chitin synthase is not required for chitin synthesis *in vivo*. *Cell* **46**: 213–225.
- Cabib, E. 1987. The synthesis and degradation of chitin. *Adv. Enzymol.* **59**: 59–101.
- Cabib, E., A. Sburlati, B. Bowers, and S.J. Silverman. 1989. Chitin synthase I, an auxiliary enzyme for chitin synthesis in *Saccharomyces cerevisiae*. *J. Cell Biol.* **108**: 1665–1672.
- Cohen, E. 1987. Chitin biochemistry: Synthesis and inhibition.

- Annu. Rev. Entomol.* **32**: 71–93.
- Cullen, D., S.A. Leong, L.J. Wilson, and D.J. Henner. 1987. Transformation of *Aspergillus nidulans* with the hygromycin resistance gene, *hph*. *Gene* **57**: 21–26.
- Davis, R.H. and F.J. de Serres. 1970. Genetic and microbiological research techniques for *Neurospora crassa*. *Methods Enzymol.* **17A**: 79–143.
- Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12**: 387–395.
- Duran, A., B. Bowers, and E. Cabib. 1975. Chitin synthetase zymogen is attached to the yeast plasma membrane. *Proc. Natl. Acad. Sci.* **72**: 3952–3955.
- Glazer, L. and D.H. Brown. 1957. The synthesis of chitin in cell free extracts of *Neurospora crassa*. *J. Biol. Chem.* **228**: 729–742.
- Gooday, G.W. 1977. Biosynthesis of the fungal cell wall—Mechanisms and implications. *J. Gen. Microbiol.* **99**: 1–11.
- . 1989. Control and inhibition of chitin synthesis in fungi and nematodes. In *Chitin and chitosan* (ed. G. Skjak-Braek, T. Anthonsen, and P. Sandford), pp. 13–22. Elsevier Applied Science Press, London, England.
- Gooday, G.W. and A.P.J. Trinci. 1980. Wall structure and biosynthesis in fungi. *Soc. Gen. Microbiol. Symp.* **30**: 207–252.
- Gow, L.A. and C.P. Selitrennikoff. 1984. Chitin synthetase of *Neurospora crassa*: Inhibition by Nikkomycin, Polyoxin B and UDP. *Curr. Microbiol.* **11**: 211–216.
- Hager, K.M. and C. Yanofsky. 1990. Genes expressed during conidiation in *Neurospora crassa*: Molecular characterization of *con-13*. *Gene* **96**: 153–159.
- Henikoff, S. 1987. Unidirectional digestion with exonuclease III in DNA sequence analysis. *Methods Enzymol.* **125**: 157–165.
- Hunsley, D. and G.W. Gooday. 1974. The structure and development of septa in *Neurospora crassa*. *Protoplasma* **82**: 125–146.
- Kang, M.S., N. Elango, E. Mattia, J. Au-Young, P.W. Robbins, and E. Cabib. 1984. Isolation of chitin synthetase from *Saccharomyces cerevisiae*: Purification of an enzyme by entrapment in the reaction product. *J. Biol. Chem.* **259**: 14966–14972.
- Kozak, M. 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* **44**: 283–292.
- . 1987. An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.* **15**: 8125–8148.
- Kyte, J. and R.F. Doolittle. 1982. A simple method for displaying the hydropathy of a protein. *J. Mol. Biol.* **157**: 105–132.
- Leal-Morales, C.A., C.E. Bracker, and S. Bartnicki-Garcia. 1988. Localization of chitin synthetase in cell-free homogenates of *Saccharomyces cerevisiae*: Chitosomes and plasma membrane. *Proc. Natl. Acad. Sci.* **85**: 8516–8520.
- Metzenberg, R.L., J.N. Stevens, E.U. Selker, and E. Morzycka-Wroblewska. 1985. Identification and chromosomal distribution of 5S rRNA genes in *Neurospora crassa*. *Proc. Natl. Acad. Sci.* **82**: 2067–2071.
- Muzzarelli, R., C. Jeuniaux, and G.W. Gooday, eds. 1986. *Chitin in nature and technology*. Plenum Press, New York.
- Orbach, M.J., E.B. Porro, and C. Yanofsky. 1986. Cloning and characterization of the gene for β -tubulin from a benomyl-resistant mutant of *Neurospora crassa* and its use as a dominant selectable marker. *Mol. Cell. Biol.* **6**: 2452–2461.
- Orbach, M.J., M.S. Sachs, and C. Yanofsky. 1990. The *Neurospora crassa arg-2* locus; structure and expression of the gene encoding the small subunit of the arginine-specific carbamoyl phosphate synthetase. *J. Biol. Chem.* **265**: 10981–10987.
- Orlean, P. 1987. Two chitin synthases in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **262**: 5732–5739.
- Peberdy, J.F. and P.M. Moore. 1975. Chitin synthase in *Mortierella vinacea*: Properties, cellular location and synthesis in growing cultures. *J. Gen. Microbiol.* **90**: 228–236.
- Pless, D.D. and W.J. Lennarz. 1977. Enzymatic conversion of proteins to glycoproteins. *Proc. Natl. Acad. Sci.* **74**: 134–138.
- Proudfoot, N.J. and G.G. Brownlee. 1976. 3′ non-coding region sequences in eukaryotic mRNA processing intermediates. *Nature* **263**: 211–214.
- Roberts, A.N., V. Berlin, K.M. Hager, and C. Yanofsky. 1988. Molecular analysis of a *Neurospora crassa* gene expressed during conidiation. *Mol. Cell. Biol.* **8**: 2411–2418.
- Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci.* **74**: 5463–5467.
- Sburlati, A. and E. Cabib. 1986. Chitin synthetase 2, a presumptive participant in septum formation in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **261**: 15147–15152.
- Selitrennikoff, C.P. 1979. Chitin synthase activity from the slime variant of *Neurospora crassa*. *Biochim. Biophys. Acta* **571**: 224–232.
- Selker, E. 1990. Premeiotic instability of repeated sequences in *Neurospora crassa*. *Annu. Rev. Genet.* **24**: 579–613.
- Shaw, J.A., P.C. Mol, B. Bowers, S.J. Silverman, M.H. Valdivieso, A. Duran, and E. Cabib. 1991. The function of chitin synthases 2 and 3 in the *Saccharomyces cerevisiae* cell cycle. *J. Cell Biol.* **114**: 111–113.
- Silverman, S.J. 1989. Similar and different domains of chitin synthases 1 and 2 of *S. cerevisiae*: Isozymes with distinct function. *Yeast* **5**: 459–467.
- Silverman, S.J., A. Sburlati, M.L. Slater, and E. Cabib. 1988. Chitin synthase 2 is essential for septum formation and cell division in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci.* **85**: 4735–4739.
- Subbiah, S. and S.C. Harrison. 1989. A method for multiple sequence alignment with gaps. *J. Mol. Biol.* **209**: 539–548.
- Vogel, H.J. 1956. A convenient growth medium for *Neurospora crassa* (medium N). *Microb. Genet. Bull.* **13**: 42–43.

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