The *Neurospora crassa* chs-2 gene encodes a non-essential chitin synthase

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INTRODUCTION

Chitin is a structural component of morphologically distinct structures assembled during various phases of growth and development in filamentous fungi. In *Neurospora crassa*, at least three different DNA fragments related to chitin synthase have been identified. In this study we cloned, sequenced and characterized the chitin synthase 2 structural gene (designated chs-2). The amino acid sequence deduced from the cloned chs-2 genomic DNA fragments is very similar to that of chitin synthase genes isolated from other fungi. Inactivation of the *N. crassa* chs-2 gene by repeat-induced point (RIP) mutation produced progeny which under standard growth conditions were indistinguishable from the wild-type. However, a significant reduction in chitin synthase activity and increased sensitivity to the phosphatidylcholine biosynthesis inhibitor edifenphos are characteristic of the chs-2<sup>min</sup> strain.

Keywords: *Neurospora crassa*, cell-wall biosynthesis, multiple chitin synthases, polyoxin D

Chitin, a linear polymer of N-acetylglucosamine (GlcNAc) residues joined by (1→4)-β linkages, is one of the most abundant natural polymers. Its biosynthesis and deposition involve the sequential biotransformation of simple metabolites, polymerization of biosynthetic intermediates, and extrusion of the product outside the plasma membrane (Bartnicki-Garcia, 1989; Cabib, 1987; Peberdy, 1990; Ruiz-Herrera, 1992). Though abundant in arthropods, fungi and other eukaryotes, chitin is absent from plants and mammals (Muzzarelli et al., 1986). Thus, biosynthesis of this polymer is an attractive target in the development of anti-pest/anti-parasite drugs (Cohen, 1987; Gooday, 1990).

Chitin synthase activity is mainly associated 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; Gooday, 1977; Kang et al., 1984; Leal-Morales et al., 1988; Selitrennikoff, 1979). The genetics and molecular biology of chitin synthase in fungi were recently reviewed by Bulawa and Robbins (1992; Bulawa & Osmond, 1990; Bulawa et al., 1986; Cabib et al., 1989; Chen-Wu et al., 1992; Duran et al., 1993; Shaw et al., 1991; Silverman et al., 1988; Sudoh et al., 1993). Chitin-synthase-like sequences have been deduced from cloned DNA fragments isolated from several filamentous fungi (Bowen et al., 1992), establishing the widespread occurrence of these genes in the fungal kingdom. However, the analysis of chitin synthase genes in filamentous fungi has not been as comprehensive as in yeast. To date, full-length chitin synthase gene sequences from three filamentous species are available in databases [*Aspergillus nidulans* (N. Kojima, unpublished), *Rhizopus oligosporus* (T. Motoyama, unpublished), *Neurospora crassa* (Yarden & Yanofsky, 1991)], but only the *N. crassa* chs-1 gene has been subjected to functional analysis (Yarden & Yanofsky, 1991).

In this study we cloned, sequenced and characterized a second chitin synthase structural gene from *N. crassa*, and designated it chs-2. We then used the repeat-induced point mutations (RIP) process (Selker, 1990) to inactivate the chromosomal chs-2 locus. Strains lacking a functional CHS2 have reduced chitin synthase activity (as measured in vitro), which is not accompanied by a reduction in cell-wall chitin content. CHS2 activity is apparently not essential in vivo.

**Abbreviations:** GlcNAc, N-acetylglucosamine; RFLP, restriction fragment length polymorphism; RIP, repeat-induced point mutation.

The EMBL accession number for the sequence reported in this paper is X77782.
METHODS

Strains and media. Wild-type *N. crassa* strain 74-OR23-1A was used in all experiments. Procedures used in growth studies, crosses and other manipulations are described by Davis & de Serres (1970). Cultures were maintained on 1.5% (w/v) agar slants containing Vogel’s minimal medium N (Vogel, 1956). When appropriate, the medium was supplemented with hygromycin B (CalBiochem), polyoxin D or Hinosan (edifenphos; Serres (1970). Cultures were maintained on 1.5% (w/v) agar slants containing Vogel’s minimal medium N (Vogel, 1956). Growth studies, crosses and other manipulations are described by Davis & de Serres (1970). Cultures were maintained on 1.5% (w/v) agar slants containing Vogel’s minimal medium N (Vogel, 1956). When appropriate, the medium was supplemented with hygromycin B (CalBiochem), polyoxin D or Hinosan (edifenphos; Serres (1970). Cultures were maintained on 1.5% (w/v) agar slants containing Vogel’s minimal medium N (Vogel, 1956).

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. 1 filter paper on a Büchner 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%, w/v, SDS, 1%, w/v, β-mercaptoethanol) containing 25 μg RNase A ml−1. Following 1 h of incubation at 37°C, 100 μg proteinase K (Boehringer Mannheim) ml−1 was added to the solution and incubation was continued for 1 h at 65°C. Two phenol:chloroform (1:1, v/v) extractions were followed by a single chloroform extraction, an isopropanol precipitation and a 75% (v/v) ethanol wash. The DNA pellet was dried and dissolved in TE buffer (10 mM Tris/ HCl, pH 8; 1 mM EDTA, pH 8.0).

The Orbach/Sachs *N. crassa* genomic library (Fungal Genetics Stock Centre) was screened as described by Benton & Davis (1977). Southern analysis was carried out on nylon membranes (MagnaCharge, MSI) as described by Sambrook et al. (1989), as were all other DNA modification and cloning procedures. Bluescript SK— (Stratagene) was used for cloning and prep-
Fig. 2. Complete nucleotide sequence of the N. crassa chs-2 gene and flanking regions and the predicted amino acid sequence of CHS2. The presumed CAAT, TATA, transcription start and polyadenylation signal sequences are shown in bold print. Conserved intron boundary and presumed lariat sequences are underlined.
with 1.5 × 10⁻⁷ M UDP[¹⁴C]GlcNAc [300 mCi mmol⁻¹ (11.1 GBq mmol⁻¹), Amersham, providing approximately 100 000 d.p.m per mixture]. Portions of the fungal cell-free extract used in each reaction mix contained 80 μg protein. Cell-free extracts were trypsin activated; 2 μl 0.2 mg trypsin (EC 3.4.21.4; Sigma) ml⁻¹ was added to the extracts, which were then incubated for 15 min at 30 °C. Soybean trypsin inhibitor was then added (2 μl 0.3 mg ml⁻¹ solution) to each reaction tube prior to the addition of the chitin synthase assay cocktail. Chitin synthase assay mixtures were incubated at 30 °C and reactions were terminated by the addition of 25 μl glacial acetic acid. Reaction products were separated by paper chromatography using Whatman No. 1 paper and 4:1 (v/v) ethanol:1 M acetic acid as solvent. Air-dried chromatograms were scanned and analysed with a Fuji Bioimaging BAS1000 analyser.

RESULTS

Cloning of the N. crassa chs-2 gene

A HindIII–SacI fragment (approximately 500 bp) from a N. crassa PCR-amplified chitin-synthase-related sequence originally cloned in M13 (NCCHS1; Bowen et al., 1992) was cloned into a Bluescript SK—vector and designated pOY2.

With the ~ 500 bp pOY2 insert used as a probe, a genomic clone was identified and isolated from the Orbach/Sachs N. crassa genomic library. From the genomic cosmid clone (pOY63), a 4 kb EcoRV fragment, hybridizing to pOY2, was isolated, subcloned and designated pOY633 (Fig. 1). Two more overlapping fragments containing the entire chs-2 gene were isolated from the same cosmid and subcloned (pAB22 and pAB23).

Sequence of chs-2

A 4 kb segment of pAB22 and pAB23 containing the chs-2 gene was sequenced completely on both strands. The molecular organization of the gene, the complete nucleotide sequence and the predicted amino acid sequence of the CHS2 polypeptide are shown in Fig. 2. The closest match to a polyadenylation signal was identified on the basis of consensus 5'- and 3'- splice junction sequences, characteristic of N. crassa introns (Bruchez et al., 1993a).

Nucleotide sequences resembling putative CAAT and TATA boxes (CTAAAT and TATAA, respectively) were identified 241 and 172 nucleotides upstream of the tentative translation start site; also present is a TCATCATA element, which is highly conserved in N. crassa as a transcription start signal (Bruchez et al., 1993b; Fig. 2). The closest match to a polyadenylation signal present in eukaryotes (ATAAA; Proudfoot & Brownlee, 1976) was an ATTA segment at nucleotides 3421–3424 (Fig. 2). One 57 bp intron was tentatively identified on the basis of consensus 5'- and 3'- splice junction sequences, characteristic of N. crassa introns (Bruchez et al., 1993a).

Chromosomal localization of chs-2

RFLP analyses were used to map chs-2. Of several restriction enzymes used (ApaI, BamHI, EcoRI, HindIII, KpnI, PstI and XhoI), only KpnI revealed polymorphisms in the vicinity of chs-2 in N. crassa of Oak Ridge and Mauriceville backgrounds (FGSC numbers 4411–4430). KpnI was used to digest DNA from progeny of the ‘small cross’ (Metzenberg et al., 1985). Subsequent probing with a hexamer-labelled HindIII–SacI insert of pOY2 located chs-2 between nlt-3 and 00031 on linkage group IV. The chs-2 polymorphism pattern was identical to that of 5S rRNA gene Fsr-4 (data not shown). GCAG repeats as well as a TC-rich region very similar to sequences of 5S RNA genes (Selker et al., 1981) were found downstream of the 3’ end of chs-2 (Fig. 2). This, together with the fact that RFLP mapping showed chs-2 to be linked to Fsr-4, raises the possibility that these two genes may be adjacent, even though the mapping procedure provides only relatively crude evidence with regard to physical distances.

CHS2 polypeptide and amino acid homology

The chs-2 gene encodes a predicted 945-residue polypeptide with a calculated mass of 107 kDa and a pI of 8.0. The predicted amino acid sequence of N. crassa CHS2 is 39%, 41%, 49%, 61% and 63% identical to those of N. crassa CHS1, A. nidulans CHSB, R. oligosporus CHS2 and CHS1 and A. nidulans CHSA, respectively. The most pronounced regions of predicted amino acid identity span amino acids 280–650 and 840–890 (Fig. 3). N. crassa CHS2 is also very similar to the S. cerevisiae and C. albicans zymogen-type chitin synthases (41–52% identity with the predicted polypeptides). Based on the Bowen et al. (1992) nomenclature, the CHS2 polypeptide is a class I chitin synthase. The similarity in predicted amino acid sequence is also reflected in similar calculated pl values and molecular masses. Hydrophilicity analysis predicts a hydrophilic region at the amino terminus of the poly-

Fig. 3. Multiple sequence alignment of six predicted zymogen-type chitin synthase polypeptides. The comparison was of N. crassa CHS2 (NcCHS2) to N. crassa CHS1 (NcCHS1), two A. nidulans polypeptides (AnCHSA and AnCHSB) and two R. oligosporus polypeptides (RoCHS1 and RoCHS2). Periods (.) mark gaps; dashes (−) mark amino acid residues identical to the N. crassa CHS2 polypeptide. The A. nidulans and R. oligosporus sequences were predicted on the basis of unpublished nucleotide sequences available from GenBank (submitted by N. Kojima and T. Motoyama, respectively).
peptide. The presence of several putative membrane-spanning domains near the carboxyl terminus is consistent with the membrane association of chitin synthase activity.

**chs-2 gene inactivation**

In an attempt to analyse the function of chs-2 we altered the gene by the RIP process, which brings about heavy cytosine methylation and many premeiotic GC → AT base-pair transitions in duplicated DNA sequences (Selker, 1990). The 4 kb EcoRV–SauI fragment (flanked by the Bluescript vector HindIII–SauI sites) from pOY633 was cloned into pCSN43 (a vector carrying a hygromycin resistance cassette). This clone (pAB1) was transformed into a wild-type strain. Two transformants, both containing a single copy of pAB1, were each crossed with a wild-type strain of the opposite mating type. Ascospores from these crosses gave rise to what appeared to be indistinguishable from the wild-type. Initially, DNA from 20 random progeny were analysed to determine if RIP had occurred. Southern analysis revealed a change in hybridization pattern of at least one of the progeny (Fig. 4). The change, consisting of the appearance of novel HpaII bands larger than the bands present in the parental strains, points to the inability of the methylation-sensitive restriction enzyme to cleave the original HpaII sites. Such changes are characteristic of RIP. Analysis of 20 additional random progeny provided evidence for at least two additional progeny in which chs-2 had undergone RIP. The chs-2\RIP progeny were further examined macroscopically and microscopically for possible morphological consequences of chs-2 gene inactivation. No differences between these progeny and the wild-type were observed with respect to germination rate, hyphal elongation, conidiation or hyphal and conidial morphology. In addition, no apparent effect on protoperithecia formation or completion of the sexual phase of the life cycle was observed in the chs-2\RIP strain. The chs-2\RIP strain that was initially designated progeny 2-13 (Fig. 4) was stained with Calcofluor white [α(1 → 4)-β] linkage polysaccharide dye and analysed by fluorescent microscopy. No changes in fluorescence intensities of hyphae, crosswalls or conidial septa staining were observed when compared to the wild-type, indicating that the chitin-rich structures were not affected by chs-2 alteration. The 2-13 chs-2\RIP strain was used in all subsequent analyses.

**Chitin synthesis in the chs-2\RIP strain**

An in vitro chitin synthase assay was carried out to determine whether chs-2 alteration had influenced the rate of UDP-GlcNAc incorporation as measured in cell-free extracts. Following chromatographic separation of the reaction products, the intensity of insoluble radioactive product (concentrated at the chromatogram origin) was visualized and measured with the aid of a bioimager. The relative incorporation of UDP-GlcNAc into insoluble product was calculated to be significantly lower in the chs-2\RIP extracts than that in the wild-type control [8 nmol h⁻¹ (mg protein)⁻¹ and 31 nmol h⁻¹ (mg protein)⁻¹, respectively]. This was evident in several experiments in which measurements were carried out at different time points.

On the basis of these results we analysed the chitin content of the chs-2\RIP strain, in order to determine whether the reduction in enzymic activity (as determined above) had a measurable effect on cell-wall chitin content. Lyophilized mycelial powder samples of the wild-type and chs-2\RIP strains were hydrolysed in alkali and digested by chitinase and β-glucuronidase. The two samples were assayed for GlcNAc (Reissig et al., 1955). The GlcNAc content of the chs-2\RIP strain was calculated to be 1.93 ± 0.3% of the total dry weight, closely resembling the GlcNAc content calculated for the wild-type control (2.06 ± 0.2%) and in agreement with the mycelial glucosamine content reported by Mahadevan & Tarum (1965) and Schmit et al. (1975). Similar results were obtained in several experiments in which different bacterial chitinases and various incubation periods were tested.

We examined the sensitivity of the chs-2\RIP strain to competitive and non-competitive inhibitors of chitin synthase. When examined for colony growth, the chs-2\RIP strain was not found to be more sensitive to polyoxin D than the wild-type (EC₅₀ of both strains was approximately 100 mM), and no significant changes in dose-response curves were observed (data not shown). This is in contrast to the increased sensitivity to a related drug, Nikkomycin Z, conferred by chs-1 gene inactivation.
Chitin synthase 2 is not essential in \textit{N. crassa}

(Yarden \& Yanofsky, 1991). We examined the sensitivity of the \textit{cbs-2} RIP strain to the fungicide edifenphos, an organophosphorus phosphatidylethanolamine biosynthesis inhibitor that was shown to be a non-competitive inhibitor of chitin synthase in several fungal species (Binks \textit{et al.}, 1993; Robson \textit{et al.}, 1990). When grown on solid medium amended with Hinosan (250 mM active ingredient), hyphal proliferation was significantly delayed in the \textit{cbs-2RIP} strain compared to that of the wild-type. This was expressed in the area of the \textit{cbs-2RIP} strain colony, which after several days reached only 10-15\% of wild-type growth. Thus, even though no morphological abnormalities were observed under standard laboratory conditions, an altered \textit{cbs-2} gene may render the organism more sensitive to edifenphos, and perhaps also to other fungal growth inhibitors.

**DISCUSSION**

In this study, we present evidence for the presence of a non-essential chitin synthase gene in \textit{N. crassa}. The gene, \textit{cbs-2}, is the second zymogen-type chitin synthase from a filamentous fungus to be structurally and functionally analysed.

We cloned, mapped and sequenced \textit{cbs-2}. The predicted CHS2 polypeptide has a similar pI value and hydrophobicity pattern to that of other fungal (yeast and filamentous) chitin synthases that have been sequenced. When compared to other predicted chitin synthase polypeptides, \textit{cbs-2} most resembles the \textit{chsA} gene of \textit{A. nidulans}. Bowen \textit{et al.} (1992) suggested a chitin synthase class subdivision and presented a possible prediction of phylogenetic relationship on the basis of the difference among 32 tentative chitin synthase gene products of 15 fungal species. They pointed out that analysis of partial sequences limits the value of the calculations and they suggested extending the analysis to complete polypeptide sequences once they become available. We have carried out maximal parsimony similarity analysis of the ten available full chitin synthase polypeptides (Fig. 5). The obtained similarity tree is in close agreement with the dendrogram presented by Bowen \textit{et al.} (1992), suggesting that partial sequence analysis (even of highly conserved regions of the polypeptide) may be sufficient for initial chitin synthase classification.

The RIP phenomenon was used to study the role of \textit{cbs-2} in \textit{vivo}. In contrast to the consequence of \textit{cbs-1} inactivation (Yarden \& Yanofsky, 1991), progeny from crosses in which \textit{cbs-2} had undergone RIP did not exhibit any abnormal morphology. This was determined by both macroscopic (e.g. colony morphology, conidiation) and microscopic (hyphal morphology, Calcofluor-white staining) examination. Though a significant reduction (approximately 75\%) in \textit{in vitro} chitin synthase activity was observed in the \textit{cbs-2RIP} strain, no striking decrease of cell-wall chitin accompanied the reduction in enzyme activity when compared to that measured in the wild-type. As in the \textit{cbs-1RIP} strain, crosswalls and conidial septa, where chitin is the major component (Hunsley \& Gooday, 1974), appeared to be normal in the \textit{cbs-2RIP} strain. The fact that the reduction in chitin synthase activity did not affect the cell-wall chitin content raises the possibility that compensation for loss of CHS2 activity may occur \textit{in vivo}, presumably by another \textit{N. crassa} chitin synthase gene product(s). Once additional \textit{N. crassa} chitin synthase mutants (and multiple mutants) are obtained, the comprehensive analysis of this possibility can be carried out.

Even though there was no apparent morphological consequence of the RIP of the \textit{cbs-2} gene, it is possible that loss of \textit{cbs-2} may result in reduced fitness of the fungus. This is demonstrated by its increased sensitivity to the lipid-biosynthesis inhibitor edifenphos. On the other hand, in contrast to the \textit{cbs-1RIP} strain, which was shown to have increased sensitivity to nikkomycin Z (Yarden \& Yanofsky, 1991), no increase in sensitivity to polyoxin D (a structurally related competitive inhibitor of chitin synthase) was detected in the \textit{cbs-2RIP} strain. Thus, in contrast to the \textit{S. cerevisiae} chitin synthases (Bulawa, 1993), it seems that not all \textit{N. crassa} chitin synthases may be equally sensitive to the nikkomycin/polyoxin drug family. Alternatively, as the expression of different chitin synthase genes may be developmentally regulated, it is conceivable that our measurements, which were confined to mycelial growth on solid medium, have not exposed all sensitivities to this drug family. Further analysis of chitin-synthase-deficient mutants is being carried out to elucidate the differential sensitivity to chitin-biosynthesis inhibitors as well as to other antifungal compounds.

Even though RIP of \textit{cbs-2} was followed by a significant reduction in chitin synthase activity measured \textit{in vitro}, the function of CHS2 \textit{in vivo} has yet to be fully elucidated. So far, it is known not to be essential for normal growth and development under standard laboratory conditions. In \textit{S. cerevisiae}, \textit{CHS1} was shown to encode for non-essential chitin synthase activity (Bulawa \textit{et al.}, 1986) and its involvement in repair functions was suggested (Cabib \textit{et al.}, 1986). However, further analysis is needed to confirm this hypothesis.
al., 1989). It is possible that the N. crassa chs-2 gene may have a similar role.

We thank P. W. Robbins for the M13 NeCHS1 clone and Streptomyces sp. chitinase, and Y. Inbar and J. Chet for A. hydrophila chitinase. A. Beth Din was partially supported by a Gad Solovis Memorial Fellowship. This research was supported by the Wolfson Research Awards administered by the Israel Academy of Sciences and Humanities.


Chitin synthase 2 is not essential in *N. crassa*.


Received 7 March 1994; revised 19 May 1994; accepted 25 May 1994.