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

Adi Beth Din and Oded Yarden

Author for correspondence: Oded Yarden. Tel: +972 8 481298. Fax: +972 8 466794. e-mail: YARDEN@HUJIAGRI.BITNET.AC.IL

Department of Plant Pathology and Microbiology, Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot 76100, Israel

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*^{RIP} strain.

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

INTRODUCTION

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 (1993). Several genes encoding chitin synthase polypeptides have been identified in the yeasts *Saccharo-*

myces cerevisiae and *Candida albicans*, and their roles studied (Au-Young & Robbins, 1990; Bulawa, 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.

Fig. 1. Clones and constructs used in this study. pOY2 is a *N. crassa* genomic DNA product amplified by PCR and cloned into a Bluescript vector (the insert is from the NCCH51 M13 clone provided by P.W. Robbins, Center for Cancer Research, Massachusetts Institute of Technology, USA). pOY633 is an EcoRV fragment obtained from a cosmid (pOY633) isolated from the Orbach/Sachs *N. crassa* genomic library, identified by hybridization with pOY2. pAB22 and pAB23 contain additional cloned fragments from the same cosmid. pAB1 is a 4 kb *chs-2* fragment (identical to pOY633) cloned into pCSN43, a vector carrying the hygromycin phosphotransferase gene driven by the *A. nidulans trpC* promoter region. In all constructs shown, the Bluescript vector sequences have been omitted.

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; *O*-ethyl-*S,S*-diphenylphosphorothioate, ICI) at appropriate concentrations. The drugs were filter-sterilized and added to sterile media at approximately 50 °C. DNA transformations of *N. crassa* were carried out as described by Orbach *et al.* (1986).

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 μ g RNase A ml⁻¹. Following 1 h of incubation at 37 °C, 100 μ g 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-

aration of various constructs. pCSN43 (Staben *et al.*, 1989) was used as a vector for *N. crassa* transformation. It carries the *hph* gene, encoding hygromycin phosphotransferase, which confers hygromycin resistance. This gene is driven by the *A. nidulans trpC* promoter region, and was used as a dominant selectable marker in the isolation of *N. crassa* transformants.

Mapping of *chs-2* was carried out by restriction fragment length polymorphism (RFLP) analysis of the 'small cross', according to the procedure of Metzenberg *et al.* (1985).

DNA sequencing and computer analysis. Double-stranded DNA was sequenced by the dideoxy chain-termination method (Sanger *et al.*, 1977) with the aid of the *Taq* DyeDeoxy Terminator Cycle Sequencing kit and a 373A DNA Sequencer (Applied Biosystems). Purified DNA (Magic Miniprep; Promega) was used as template. Clones used for sequencing were generated by insertion of restriction fragments into the Bluescript vector. Oligonucleotide primers for sequencing portions of the gene were synthesized on an Applied Biosystems DNA synthesizer. Programs of The University of Wisconsin Genetics Group were used for analysis of nucleic acid and protein sequences (Devereux *et al.*, 1984). The PAUP software package (Laboratory of Molecular Systematics, Smithsonian Institution) was used for maximal parsimony analysis among predicted chitin synthase polypeptides.

Fluorescent microscopy and chitin content determination.

Samples were viewed with a Zeiss epifluorescence microscope. For chitin visualization, a drop of Calcofluor (Fluorescent Brightener 28, Sigma; 10 μ g ml⁻¹) was applied to fungal samples prespotted on a microscope slide. The filter combination used was 380–425 nm excitation, 430 nm dichroic mirror, and 450 nm barrier.

Chitin content was determined by a slight modification of the procedure described by Bulawa *et al.* (1986). Washed mycelium (2.5–5 mg dry weight) was lyophilized, suspended in 1 ml 6% (w/v) KOH and heated for 90 min at 80 °C. Following the addition of 0.1 ml glacial acetic acid, the insoluble material was collected by centrifugation, washed twice with water, resuspended in 0.5 ml sodium phosphate buffer (pH 6.3) and sonicated. A portion of 40–100 μ l of a partially purified (15% protein by weight) *Streptomyces* spp. chitinase suspension (5 mg ml⁻¹) was added, and the tubes were incubated at 37 °C for 2–20 h. Following centrifugation, 450 μ l supernatant was treated with 40 μ l 50 mg β -glucuronidase type H-5 (Sigma) ml⁻¹ for 2 h at 37 °C. Portions (0.1 ml) of each sample were removed and assayed for GlcNAc content (Reissig *et al.*, 1955). In subsequent experiments, chitinases from *Serratia marcescens* (Sigma) or *Aeromonas hydrophila* (kindly provided by Y. Inbar and I. Chet, The Hebrew University of Jerusalem) were used.

Chitin synthase assays. Chitin synthase was assayed in fungal cell-free extracts. Extracts were prepared from 1.5–5-h-old germinating conidia, which were harvested by filtration (GF/C filter; Whatman). Following a water wash, the germlings were placed in a 2 ml screw-cap tube (Sarstedt) containing 1.4 g 0.5 mm-diameter zirconium 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 (Biospec Products), using two 1 min pulses. The tubes were centrifuged for 10 min (4 °C) and the supernatants collected. Protein concentration was determined by the method of Sedmak & Grossberg (1977).

Chitin synthase activity was monitored in 25 μ l reaction mixtures containing 100 mM HEPES, pH 7.8, 1 mM MgCl₂, 32 mM GlcNAc, 1 mM UDP-GlcNAc and supplemented

	1				50					100
NcCHS2			M	ESRISNRLS.	SSATR	TVRAFRNRCH	ARPGTP....	GSSYG	NARRPLPSAP	APLHYNPSR
AnCHSA	MPLRDRVLCH	IMDCQNGRRA	NRTVRFARTA	--Y-E-Y-Y	EYDPEETLS-	AAPSM--APT	IP-P-ASGAD	EMRYTA-RPA	SPA--WSPTR	-ADWVRP--A
RoCHS2										
RoCHS1										
NcCHS1							M	AYHGR-DG-D	GSTSYKILRW	-QPGDQHDDA
AnCHSB								MTAIS	S-TSHTLIPY	EEEA
	101				150					200
NcCHS2	AASHYPRYH.		GGYADDVTVS	MGPDD..DRT	DIFGEETDLS	ETRLHNDAYG	FRSSQITLSE	DPHG....T	HARSR..YDD	EDDVS....T
AnCHSA	---Y-E-ADI	NGSPRPGTPS	SR-GGSPRRP	LP-APLFSKP	GTTTQD-KID	IGDGEE-PF-	GGGRT-SSRH	G-Q-SVQSF-	SESTF..IA-	-T-LEKVDLD
RoCHS2	MY	PEGPKPEHDQ	FPLQ-TQFSN	QP-VHRSPFE	-PYPEDQPHF	DKQP-LTSPA	YPPT-YPT-P	P-PNFPGSPA	VQQPYPPFNN	NPSVPSPGVP
RoCHS1		M	YP-NNSNNN-	PM-SARLPSR	PRY-GGEEYP	MQQVSFNNS	PPPPPPPS-P	NDT-FYDND	VQ-PL..LVN	NGSTASSVTL
NcCHS1	QRPFLESENPM	PY.....D	NDRLGTD-PP	VR-VSAYSL-	ESYA-GAGTT	RAGVAVNPTP	PPHGGYGGGG	VSS-VDQGYN	YG.....	G
AnCHSB	SHGLLSSQQS	PFAGPFDDPH	QQRGLTASPV	QR-TSGYSL-	ESYA-DAAYH	P-S	ANQ-VYSGH.	SENPAAAF	VP.....	G
	201				250					300
NcCHS2	TYSSNTGTSA	SGVDKFEHYG	PIPEEGKHER	RGVRPPQMSR	KEVQLINGEL	V.LECKIPTI	LYSFLPRR..	DEVEF	THMRYTAVTC	DPDDFV.ARG
AnCHSA	E-EEESNETK	-M--PNL---	-A---QS-	----NA-AK	-----V---	I.....	-H-----	DR-	-----	-----T.Q-
RoCHS2	A-FNPA....	PPSPNM...	..HY-QAP-	-QP-RFKTT-	Q.-K-TK-N-	..-D-PV--A	YLNVDV-IK..	GK--	-----A--	---K-A.SD-
RoCHS1	NNGNGGNVRF	TNAKSNMIG-	GPDNV-IAP-	KQT-RYKTI-	R.-K-TG-N-	..-DNPV-SK	YLQRVK-K..	KE-	-----A--	---A-E.REN
NcCHS1	D-ATDPAYRM	-AI-EDDSWL	RRQQPNAAPT	G-LKRYAT..	RK-K-VQ-SV	LS-DYVP-SA	IRNAVQPKYR	DE.EGNN-	FK-----	---N--TLKN-
AnCHSB	RVA..SPYAR	-ETSST-AWR	RRQAGARRGG	N-L-RYAT..	RK-K-VQ-SV	LSVDYVP-SA	IQNAIQAKYR	NDLEGGSE-	-----A--	---NE-TLHN-
	301				350					400
NcCHS2	YKLRQNIGRT	ARETELFICV	TMVNEDEFGF	TRTMHAYMKN	ISHFCSRNSK	..RTWGADGW	QKIVVCVUSD	GREIIHPRTL	DALAAMGVYQ	HGIAKNFVNQ
AnCHSA	-----Q---	M-----I	-----TH-	-----G-Q-	-----S-	K-	K-----II-	--KKV---	N-----L---	E-----V---
RoCHS2	-----PM..L	G-K-----VL	-----VL-	A---G---	-A-L-T-DR-	PN-	E-V-----	--NK-NQ---	SV--LL----	D-----V-HG
RoCHS1	-K-----L..L	G-----VL-	-----VL-	C---NG---	V-L-----R-	PE-	K-V---I---	--TK-----	SV-----	E-M---I-EG
NcCHS1	-D--PRM...Y	N-H---L-AI	-Y----KVLL	S--L-S--T-	-RDIIVNLKK-	SFWNR-GPA-	-----L-F-	-LDKTDKNV-	-V--TI----	D-VI-KD-DG
AnCHSB	-N--PAM..Y	N-H---L-AI	-Y----KTLT	A--L-G--Q-	-RDIIVNLKK-	EFWNK-GPA-	-----L-F-	-IDPCDKD-	-V--TV-I--	D-VM-RD-DG
	401				450					500
NcCHS2	KAVQAHVYE.	YTTQVSLDSD	LMF.KGAEGK	...IVPCQMI	FCLKEKNQKK	LNSHRWFFNA	FGKALNPNCV	ILLDVGTRPG	GTSYLHLWKA	FDTDSNVAGA
AnCHSA	-Q-N-----	-----K-	-----V-	-----H---	-----	-----	--R-Q-I-	-----E	P-A-----	--Q-----
RoCHS2	-P-T--I---	-----V-PN	MEVK-AGS-N	...V---IL	-----	I-----	-Q-----	-PVID-HI-	V-I-----K-	-----I-----
RoCHS1	-P-T--I---T	H---L-I-A	MN-.R-D---	...V-V-IL	-----	I-----	-Q-----	-PL-K---	V-I---K-	-R-I-----C
NcCHS1	-ETV--IF-	--S-L-VTPN	QALIRPVDD-	PQTLV-V-F-	---Q-T---	I-----L-	--RI---E-	---A-K-S	PR--LA--EG	-YN-KDLG--
AnCHSB	-ETV--IF-	---L-VTPN	QQLIRPTDD-	PSTLP-V-M	---Q-S---	I-----L-	--RI---E-	---A-K-	PK--LY--E-	-YN-KDLG--
	501				550					600
NcCHS2	CGEIKAMKGR	FGNLLNPLV	ASQNFYKMS	NILDKPLESV	FGYITVLPGA	LSAYRYHALQ	NDETGHGPLS	QYFKGETLHG	Q.....H	ADVFTANMYL
AnCHSA	A-----S--K	NMLG-----	-----	-----	-----	-----FF-	--AE-N--N	-----	K.....D	-----
RoCHS2	---R--A-T	R-VA-----	-A-----	-----	-----S-	F-----	--VN-----E	K-L---Q-	G.....D	--I-----
RoCHS1	---L-P	G-VH-----	-----	-----	-----H-S-	F-----E	--VN-----E	K-L--KM-	S.....D	--I-----
NcCHS1	---H--L-K	G-KK-----	-V-----I-	-----DHTA	---VS---	F-----FR-IM	GR.....E	--H-DHTLS	KLLGKKGIEG	MNI-KK--F-
AnCHSB	---H--L-K	GKKK-----	-A-----I-	-----S	---VS---	F-----FR-IM	GR.....E	--H-DHTLS	KLLGKKGIEG	MNI-KK--F-
	601				650					700
NcCHS2	AEDRILCWEL	VAKRGERWVL	KYVKGCTGET	DVPDTPVEFV	SQRRRWLNGA	FFAAVYSLVH	FRQIWKTDHT	FMRKALLHVE	FLYHLLQLLF	TYFSLANFYL
AnCHSA	-----E---	RF--SAV---	-----SI--I	-----	-----	-----I-N	VK-L---S	LA--I--QI-	SV-Q-----	-----G-----
RoCHS2	-----Y-	---KEAH---	H-SSSY---	---K-D-I-	-----S	---G--A-Y-	W-KV-QS--S	YL--MMFM-	DI-NTYN-I-	SW-A-G---
RoCHS1	-----Y-	---K-AA---	H-CSNAY---	---A-I	-----S	---GI--M-	WSKV-SSN-N	IL-CIMFM-	D--Q-YN-I-	SW-AIG---
NcCHS1	-----F-	---A-QK-H-	S-IAAK---	---EGA--I	-----S	-A-SL---M-	-GRMY-SG-N	IV-MFFF--Q	LI-NIANVI-	-W---SYW-
AnCHSB	-----F-	---A-SK-H-	S---ASK---	---EGA--I	-----S	-A-GI---M-	-GRMY-SG-N	IV-MFF--LQ	M--NWFSTFL	-W---SYWL
	701				750					800
NcCHS2	AFYFIAGGLA	DPHVDPFNSD	G.....H	VARIIFNLR	YVCVLICTQ	FILSLGNRPQ	GAKRMYLASM	IIYAVIMVYT	TFATIFIVVR	QIQPSQKSD.
AnCHSA	-F-----S-T	-EKI-----G	H.....N	MGKY--IV-	-A---VM-L-	-F-M-----	--KL--S-	-V-SIV-AY-	A-C-LYLI-L	ELMA...TG.
RoCHS2	T-FILTKA-G	HGVDGSTLT-	P....PSPD	TGETLHTVFN	-IYIV-VI-	-MA-----	-S-IA-TS-	VFF-IL--M	M-A-W-T-V	GVKTVVETS.
RoCHS1	V--IMTTSIN	SVPN	P....PFAKN	AGSVIHTV-N	-LY--LII-	I-IAM----	-Y-WA-FMII	VFF-IL-G-V	L-C-AW-TIE	GVKTAVETA.
NcCHS1	TTTV-MDLVG	T-VTASSS-A	EHHGWPFGDT	-TPFFNAV-K	-IYLAFLVL-	---A-----K	-S-WT-IT-F	FVPSL-QS-I	LVLSGYL-A-	AFSVPLDQQL
AnCHSB	TTTV-MDLVG	T....SS-N	-YTAPFPGKT	ATP--NTLVK	-IYLAFLVL-	---A-----K	-S-LS----F	VAFGI-QL-V	VVDALYL--	AFTGGAPM-F
	801				850					900
NcCHS2	..DKPDLE..	..LGNNVFT	NLIVSVASTL	GLYFMSFLY	LDPWHMFTSA	IQYFVLLPSY	ICTLQIYAF	NTHDVTWGK	GDNVMRTDLG	GA.IVKGSL
AnCHSA	..HDVPIT..	..MSDTL-V	-IV--LL--V	---FT--M-	-----S	A---A-----	-----C-----	-----	---TIN---	T-R-IN---
RoCHS2	GGQF	IEM-EQST-R	-I-I-LCA-Y	VM---S-MF	-----F	---IL-S---	TNV-N----	-----	---TVA---	VVKAK-DGSG
RoCHS1	..QHNQDAQF	TALIKQ-S-R	DV---C--Y	IM---S-L-F	-----L	-P-IFMS-	-NV-N----	-----S---	---GVS---	VVKST--KDG
NcCHS1	QL-NAKDAMA	SLFG-SGSAG	VIL-ALVTIY	---LA-M-	-----H-F	PY-ML-MST-	-NI-M---N	-W---S---	-SDK.AEA-P	S-NVS--EKD
AnCHSB	NT-DGIGAF	SSFF-SSGAG	II-IAL-ATF	---A-M-	-----F	PA-MAVQS-	-NI-NV---S	-W---S---	-SDK.ADA-P	S-KTTG-KGE
	901				950					1000
NcCHS2	..TVELEMP	DQLDIDSGYD	ECLRLNRDRV	MVPCRSLSED	QLQQDYKSKV	RTYVMV....	..LNMKGFRW	IKKKGWDADV	KGKFEGLGDR	AR
AnCHSA	..I--V---	E-----	AA-----L	E--DPGV--S	-Q-E--RA-	-----S...	..V-----	VV-----V		
RoCHS2	DLA--V-V-V	EEK--NAAFI	DAQVE-TKKI	EPEKSHRDAK	TK-E---R-F	---RL-L---	...A-IIS-	LA-VV-IANT	TVI.....	
RoCHS1	VHE--VDL-T	E-K-LNEQ-E	-ACMA-KVAP	IPEVTKRDAK	TK-E---F	---TL-T...	...C-IIS-	LI-VTIING	QEVWQWF...	
NcCHS1	EAV--.-IEK	P-E---QQFE	ATV-RALAPY	KEDETP..-P	KDLE-CTSL	EPCLSCLGCF	PTAS-RLLSP	VITLTHLASG	KPHPRELRGS	SSSFCSLLR
AnCHSB	EAVI--.-IDK	P-A---QFE	ATVKRALTPY	VP-EEK...E	KSLD-S--F	---RL-TL...	LFS-	GL--VCITSE	GLDKFGFTVC	FGCALSIIRS
	1001				1050					1100
NcCHS2	GDS	EIGDNFYLR	ILWAVAALAL	FRALGSTTFA	AINLV:ALVE	GRVRLR....	..LNMKGFRW	IKKKGWDADV	KGKFEGLGDR	AR
AnCHSA	VG	SS-T-V-AI	---S-V-I	I--I---AY-	VLYLIQKL-	-KAKFQAGNI	ASA-ASAAGS	SLGTSKNSVY	GS-GLNMT-	INETKWAIS-
RoCHS2	I	KGKASI--G	---S-G-SV	I-FT---LYL	IFKIFTG*..					
RoCHS1	-Y	-KRATV--S	---S-G-SA	I-FV--ILYL	FMKIFTG*..					
NcCHS1	SLSSALLASV	GSLAGR-YVL	LCPSLSMANP	KHVVGACTL	LTKRKEEGR	EKGYCFLLAA	RRHGRVVP..			
AnCHSB	ETDRIQONT-	TERTSRFFQA	L--SN-VV--	I-.FIGATWF	LGK.....T	GLLCCF..A	RR*.....			
	1101									
NcCHS2	GLAKR*....									
AnCHSA	-MQ-AMFWK	K*								
RoCHS2										
RoCHS1										
NcCHS1										
AnCHSB										

with 1.5×10^{-7} mM UDP[1- 14 C]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 μ g protein. Cell-free extracts were trypsin activated; 2 μ l 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 μ l 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 μ l 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 *Hind*III–*Sac*I 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 $\sim$ 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 *Eco*RV 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 *chs-2* coding region was located by comparison of the predicted amino acid sequence with those of *N. crassa chs-1* and chitin synthase polypeptides predicted on the basis of genes described from two other filamentous fungi, *R. oligosporus* and *A. nidulans* (T. Motoyama, 1992 and N. Kojima, 1993, unpublished submissions to GenBank). The compiled *chs-2* reading frame shows a high bias for codons preferred by *N. crassa* (data not shown). The presumed *chs-2* start codon segment is GAGACCATGG. This segment is similar to that found in *N. crassa chs-1* (Yarden & Yanofsky, 1991) and aligns well with the sequence conserved in higher eukaryotes (Kozak, 1987). These sequences vary slightly from the *N. crassa* translation initiation consensus (Bruchez *et al.*, 1993a).

Nucleotide sequences resembling putative CAAT and TATA boxes (CTAAT and TAGATA, 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 (AATAAA; 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 (*Apa*I, *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Pst*I and *Xba*I), only *Kpn*I revealed polymorphisms in the vicinity of *chs-2* in *N. crassa* of Oak Ridge and Mauriceville backgrounds (FGSC numbers 4411–4430). *Kpn*I was used to digest DNA from progeny of the 'small cross' (Metzenberg *et al.*, 1985). Subsequent probing with a hexamer-labelled *Hind*III–*Sac*I insert of pOY2 located *chs-2* between *nit-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 %, 51 % 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 pI 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).

Fig. 4. Molecular analysis of *chs-2* DNA in the wild-type strain, a pAB1-transformed strain, and progeny from a cross between a transformed parent and the wild-type. DNA samples from pAB1, wild-type (74A), transformed parent (2212) and 20 progeny (2-1–2-20) from the cross were digested with *Hpa*II, electrophoresed and blotted onto a nylon membrane. The blot was probed with the hexamer-labelled 4 kb *Eco*RV insert from pOY633. The strain 2-13 is a *chs-2*^{RIP} progeny.

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 *Eco*RV–*Sac*I fragment (flanked by the Bluescript vector *Hind*III–*Sac*I sites) from pOY633 was cloned into pCSN43 (a vector carrying a hygromycin B 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 colonies in which the growth rate and morphology were 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 *Hpa*II bands larger than the bands present in the parental strains, points to the inability of the methylation-sensitive restriction enzyme to cleave the original *Hpa*II 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 [a (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 & Tatum (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

(Yarden & Yanofsky, 1991). We examined the sensitivity of the *chs-2*^{RIP} strain to the fungicide edifenphos, an organophosphorus phosphatidylcholine biosynthesis inhibitor that was shown to be a non-competitive inhibitor of chitin synthase in several fungal species (Binks *et al.*, 1993; Robson *et al.*, 1990). When grown on solid medium amended with Hinosan (250 mM active ingredient), hyphal proliferation was significantly delayed in the *chs-2*^{RIP} strain compared to that of the wild-type. This was expressed in the area of the *chs-2*^{RIP} 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 *chs-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 *N. crassa*. The gene, *chs-2*, is the second zymogen-type chitin synthase from a filamentous fungus to be structurally and functionally analysed.

We cloned, mapped and sequenced *chs-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, *chs-2* most resembles the *chsA* gene of *A. nidulans*. Bowen *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 *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 *chs-2* *in vivo*. In contrast to the consequence of *chs-1* inactivation (Yarden & Yanofsky, 1991), progeny from crosses in which *chs-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 *in vitro* chitin synthase activity was observed in the *chs-2*^{RIP} 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 *chs-1*^{RIP} strain, crosswalls and conidial septa, where chitin is the major component (Hunsley & Gooday, 1974), appeared to be normal in the *chs-2*^{RIP} 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 *in vivo*, presumably by another *N. crassa* chitin synthase gene product(s). Once additional *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 *chs-2* gene, it is possible that loss of *chs-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 *chs-1*^{RIP} 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 *chs-2*^{RIP} strain. Thus, in contrast to the *S. cerevisiae* chitin synthases (Bulawa, 1993), it seems that not all *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 *chs-2* was followed by a significant reduction in chitin synthase activity measured *in vitro*, the function of CHS2 *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 *S. cerevisiae*, CHS1 was shown to encode for non-essential chitin synthase activity (Bulawa *et al.*, 1986) and its involvement in repair functions was suggested (Cabib *et*

Fig. 5. Maximal parsimony dendrogram showing fungal (An = *A. nidulans*; Nc = *N. crassa*; Ro = *R. oligosporus*; Ca = *C. albicans*; Sc = *S. cerevisiae*) chitin synthase grouping. The tree was calculated by the program PAUP from deduced amino acid sequences. Branch length is relative to the number of amino acid substitutions. Neither rigorous calculation of evolutionary distances nor phylogenetic relationship can be inferred with confidence from this tree. Polypeptide designations are on the basis of EMBL entries.

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 NcCHS1 clone and *Streptomyces* sp. chitinase, and Y. Inbar and I. 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.

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