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Genomic Organization of *Penicillium chrysogenum chs4*, a Class III Chitin Synthase Gene

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Class III chitin synthases in filamentous fungi are important for hyphal growth and differentiation of several filamentous fungi. A genomic clone containing the full gene encoding Chs4, a class III chitin synthase in *Penicillium chrysogenum*, was cloned by PCR screening and colony hybridization from the genomic library. Nucleotide sequence analysis and transcript mapping of *chs4* revealed an open reading frame (ORF) that consisted of 5 exons and 4 introns and encoded a putative protein of 915 amino acids. Nucleotide sequence analysis of the 5' flanking region of the ORF revealed a potential TATA box and several binding sites for transcription activators. The putative transcription initiation site at -716 position was identified by primer extension and the expression of the *chs4* during the vegetative growth was confirmed by Northern blot analysis. Amino acid sequence analysis of the Chs4 revealed at least 5 transmembrane helices and several sites for post-translational modifications. Comparison of the amino acid sequence of Chs4 with those of other fungi showed a close relationship between *P. chrysogenum* and genus *Aspergillus*.

Key words **:** *Penicillium chrysogenum*, chitin synthase gene, screening, genomic structure, transcript mapping

Chitin, a β(1-4)-linked homopolymer of *N*-acetylglucosamine, is a major component of the cell walls of filamentous fungi, contributing to their shape and mechanical strength (13). Decrease in chitin content in the cell wall by treatment with chitin synthase specific inhibitors, such as polyoxin D and nikkomycin or by defects in glucosamine synthesis results in swelling and bursting of the hyphal tip (12, 21). These results suggest that chitin synthase is essential for hyphal growth.

The conserved region of genes for chitin synthase (Chs) from several filamentous fungi have been identified, establishing the widespread distribution of the enzymes in chitinous fungi (5, 26). Analyses of DNA fragments from taxonomically diverse fungal species have shown that most fungi have three to six chitin synthase genes (8, 9, 20, 27, 29, 30). Chitin synthases can be divided into five classes by sequence homology of deduced amino acids (5, 41). Expression of the chitin synthase gene should be tightly regulated in temporal and spatial ways during conspicuous morphological changes accompanied by the remodeling of the wall structure. Identification and characterization of the genes for chitin synthase, therefore, should facilitate molecular analysis of the function of these genes in fungal differentiation.

Functional analyses of chitin synthase genes from filamentous fungi, however, have not been studied as comprehensively as those of yeasts. Thus, there is little information on the function of the gene products during growth of filamentous fungi except for several fungi for which tools of molecular genetics are well established (3, 4, 16, 41). Interestingly, class III chitin synthases have been found only in filamentous fungi, suggesting that they may play unique roles in hyphal growth and differentiation. The phenotype of a *chs-1* disruptant of *Neurospora crassa* (46) and that of a *chsB* disruptant of *Aspergillus nidulans* (4) support this hypothesis.

Previously, we have cloned four PCR fragments encoding the conserved region of chitin synthases, *chs1, chs2, chs3*, and *chs4* from *Penicillium chrysogenum*, and described the phylogenetic relationship between *Penicillium* and ascomycetous fungi through comparison of deduced amino acid sequence data (30, 31). In this paper, we report cloning and sequence analysis of a genomic clone containing a 3'-flanking region, a 5'-flanking region and the entire ORF of the *chs4* that encodes a class III chitin synthase in *P. chrysogenum*.

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Materials and Methods

Strains and media

Penicillium chrysogenum KCTC6052 was used as the source of DNA for the genomic library. *Escherichia coli* strain XL1-blue was used for propagation of the genomic library constructed with Lambda EMBL3 vector (Stratagen, USA) and subcloning experiments. *P. chrysogenum* strain was maintained on potato dextrose agar (Difco, USA) medium at 25°C. Maintenance and transformation of the *E. coli* strain were done by the standard procedure described previously (36).

Construction of P. chrysogenum genomic library

High molecular weight *P. chrysogenum* DNA was isolated as described (18). For further purification, the isolated DNA was subjected to CsCl density gradient ultracentrifugation. The purified DNA was partially digested with *Sau*3A1 (100 μg/1,227 units) to give a mixture of fragments with an average size of 9~23 kb. The digestion mixture was layered on top of a 10 to 40% sucrose gradient, and centrifuged for 22 h at 22,000 rpm (Beckman, USA). The gradient was fractionated and analysed by agarose gel electrophoresis. The fractions containing fragments from 9 to 23 kb were pooled, dialysed against TE buffer (10 mM TrisCl, 1 mM EDTA, pH 8.0) and concentrated by ethanol precipitation. The fractionated DNA fragments were ligated into the pre-digested Lambda EMBL3 vector (Stratagene, USA). *In vitro* packing of the ligation mixture was performed using Packagene kit (Promega, USA) following the manufacturer's instruction.

Screening of chs4 clone

To select a phage clone containing *chs4* gene, phage library screening was performed using a combined method of PCR and plaque hybridization (19). Briefly, for primary PCR screening, the genomic library of phage DNAs and a host strain were mixed in NZY soft agar, poured onto three NZY plates (150 × 15 mm) to give 20,000 pfu per plate and incubated at 37°C overnight to allow plaque formation. After plaque formation, total phage DNAs from each plate were collected by gentle agitation with SM buffer (0.58% NaCl, 0.2% $MgSO_4H_2O$, 50 mM Tris-HCl, pH 7.5, 0.02% gelatin). Each preparation of the phage DNAs was subjected to PCR amplification with the primers (primer 1, 5'-CTG AAG CTT ACC ATG TAC AAC GAG GAT-3'; primer 2, 5'-GTT CTC GAG TTT GTA CTC GAA GTT CTG -3') specific for the *chs4* gene fragment previously identified. Then the PCR products from each reaction were subjected to agarose gel electrophoresis and followed by Southern hybridization with ^{32}P-labeled *chs4* gene fragment previously cloned (30) to identify the plate containing positive clones. For secondary PCR screening, phage DNAs from the positive plates of the primary screening were collected, diluted and plated onto ten of NZY plates to give 2 × 10^3 pfu per plate. After identification of a positive plate by PCR amplification and Southern hybridization, phage DNAs from the plaques on the positive plate were collected, top-agar plated onto three of the NZY plate to give 2 × 10^2 pfu per plate, and incubated. After plaque formation, the phage plaques were subjected to plaque hybridization using ^{32}P-labelled *chs4* DNA fragment. Finally, the insert was excised from the positive phage DNA by *Eco*RI digestion, size-fractionated by agarose gel electrophoresis, purified with the QIAquik kit (QIAGEN, Germany) and ligated into the pBluescript KS(+) vector (Stratagene, Germany) according to the manufacturer's instruction.

Subcloning and DNA sequencing

After constructing a restriction map of the insert from the positive phage DNA, fragments from the *Sal*I digestion of the insert DNA were subcloned into the pBluescript KS(+) vector. The recombinant vectors were introduced into *E. coli* strain XL1-blue by the method previously described (36). Plasmid DNAs from the transformants were isolated using Wizard Plus SV Minipreps DNA Purification System (Promega, USA). Nucleotide sequences of the *chs4* were determined using Top™ DNA Sequencing Kit and Silverstar™ Staining Kit (BIONEER, Korea) according to the manufacturer's instruction with the standard sequencing primers (T3, T7; Stratagene) and synthetic oligonucleotide primers (BIONEER, Korea). The nucleotide sequences of the fragments were assembled with the SeqEditor program (24).

The complete nucleotide sequence for the *chs4* was registered under GenBank accession number AF173559.

Northern blot analysis

For the preparation of total RNAs from the mycelia of *P. chrysogenum*, approximately 10^6 spores prepared with 0.05% Tween 80 were inoculated to 100 ml of potato dextrose broth and grown for 14 h at 25°C. Mycelia were harvested by filtration through a nylon filter and disrupted by a mortar in the presence of liquid nitrogen. Total RNAs were isolated by ultracentrifugation of cell extracts according to the method of Chirgwin *et al.* (7). Twenty micrograms of total RNAs were resolved by 1.2% agarose gel electrophoresis containing 2.2 M formamide (w/v). The gel was blotted onto a nylon membrane and hybridized for 20 h with a ^{32}P-labelled PCR fragment containing the coding region of the *chs4*. The radioactive signals were detected by exposing X-ray film for 40 h.

RT-PCR analysis

First-strand cDNA was prepared using the SuperScript Preamplification System (Gibco/BRL, USA) according to the manufacturer's instructions. After denaturing for 3 min at 92°C, aliquots of the first-strand cDNA were amplified for 25 cycles using the following conditions: at 94°C for

30 s, at 60°C for 30 s, and at 72°C for 1 min, followed by a final elongation step for 7 min at 72°C. Ten μl of the reaction products were analyzed on 1% agarose gel. The PCR primer sets used for the identification of the intron were as follows: Int I, 5'-GTT TCT CTC TGA CCC TGG-3'(P1) and 5'-CTC TTG GCC GGA ATA AAC-3'(P2); Int II, 5'-GGA AAG TCA AGC TAG TGC-3'(P3) and 5'-GGA TTT CTT CAG GTT GAC-3'(P4); Int III, 5'-ATG GTT ACA ACC TAC GCC-3'(P5) and 5-TTA AAG AGC CAA CGA TGC-3'(P6); In IV, 5'-GAC TAC AAA GGA CGA GGG-3'(P7) and 5'-CAA GGA ACC AAC AGC ATC-3'(P8).

Determination of transcription initiation site

To determine the actual transcript start position, primer extension was performed as described (36). Twenty μg of total RNAs were hybridized with 5 pmoles of the 5' end-labeled primer Ext1 (5'-CTT TTC CTT TCT CTT CTT CTT TGA ACT TTG TCC TC-3', 2 × 10^5 cpm) in a buffer containing 40 mM PIPES (pH 6.4), 400 mM NaCl, 1 mM EDTA, and 80% formamide at 37°C for 12 h after heating at 85°C for 10 min. After phenol extraction, RNA-primer hybrids were precipitated and dissolved in nuclease free water. The first-strand cDNA was synthesized at 37°C for 1 h in a buffer containing 200 units of M-MLV reverse transcriptase (Gibco/BRL, USA), 2 mM dNTP, 40 units of RNasin, 3 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.3), and 75 mM KCl. The product was analyzed on a 6% polyacrylamide gel along with appropriate dideoxynucleotide sequencing samples as size markers.

Programs for sequence analysis

Sequence analysis of 5'-flanking region and 3'-flanking region was performed by the MatInspector program (34) and Hamming-Clustering method (http://125.itba.mi.cnr.it/~webgene), respectively. Determination of the transmembrane helical region was performed by the SOSUI system (15). The hydropathy profile of the Chs4 was analyzed using the Kyte-Doolitle method (23). Amino acid sequence similarity was determined by the BLAST program (1). Potential post-translational modification sites were identified by the PROSITE pattern search with the PPSEARCH program. Conserved amino acid sequences of the class III Chs were aligned using the multiple alignment program CLUSTAL X (42).

Results and Discussion

Screening and cloning of chs4 gene from the genomic library

After the first round of amplification of the originally packaged phage library, the titer of the amplified phage library was 3.7 × 10^6 pfu/ml. This value was quite sufficient to cover the entire genome of *P. chrysogenum*, which consisted of ca. 3.5 × 10^4 kb (37). Through the combined screening method of PCR sib-selection and plaque hybridization, a positive clone containing a 16 kb of DNA insert with the *chs4* was identified (data not shown). After the restriction mapping and Southern analysis of the original insert, a 6.3 kb fragment sufficient to contain the entire ORF of the *chs4* gene was subcloned. The restriction map of the original insert from the positive phage clone and the subcloning strategy of the 6.3 kb fragment are shown in Fig. 1.

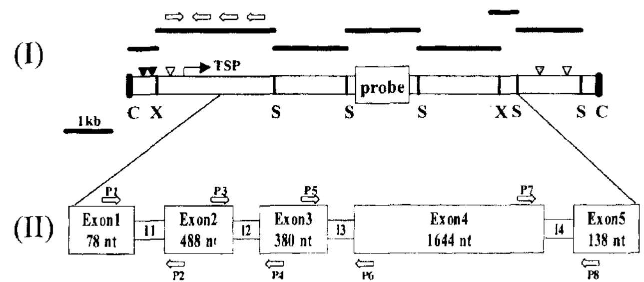

Fig. 1. The restriction map and subcloning strategy of the 6.3 kb of the genomic fragment containing the full gene of the *chs4* gene. Subclones are shown as the bars over the restriction map (I). Diagram for Exon-Intron organization is presented (II). Restriction sites for *Cla*I (C), *Xho*I (X), and *Sal*I (S) are shown. Shadowed arrows indicate the sets of primers for the reverse transcription PCR, and open arrows indicate synthetic primers for direct sequencing. TSP is the transcription start point. Closed triangles (▼), open triangles (▽), and shaded triangles indicate putative PacC and StuA binding sites, TATA-like box, and poly(A) tailing signal-like sequence, respectively.

Sequence analysis of chs4 flanking regions

In order to characterize the genome organization of the *chs4*, the nucleotide sequence of the 4.7 kb region being expected to contain the entire coding region, the 5' non-coding, and 3' non-coding region of the *chs4* were determined. A total 1,518 bp of the *chs4* upstream sequence was analyzed for the presence of the characteristic regulatory motif (Fig. 2). A putative TATA box (5'-gaTATAAac-3') was located at -847 bp from the putative translation initiation codon AUG. Interestingly enough, several consensus sequences for protein-binding sites, exclusively for transcription activators, were also identified. Two potential binding sites of the carbon catabolite repressor, CreA (22), were found. These sites (C1 and C2) match the consensus sequence-specific site for CreA, 5'-RYGGRG-3'. One of these sites, C2, is oriented in an antisense direction. Ten potential binding sites for the nitrogen regulatory protein NRE of *P. chrysogenum* (14), a member of the GATA family of transcription factors, were found. Among these, six sites match the consensus sequence for 5'-GATA-3', while four sites match the consensus sequence for 5'-GATT-3'. Four potential binding sites of transcriptional activator AbaA acting as a genetic switch to control development in *Aspergillus nidulans* (2) were also found. All of these sites (B1, B2, B3, and B4) match the

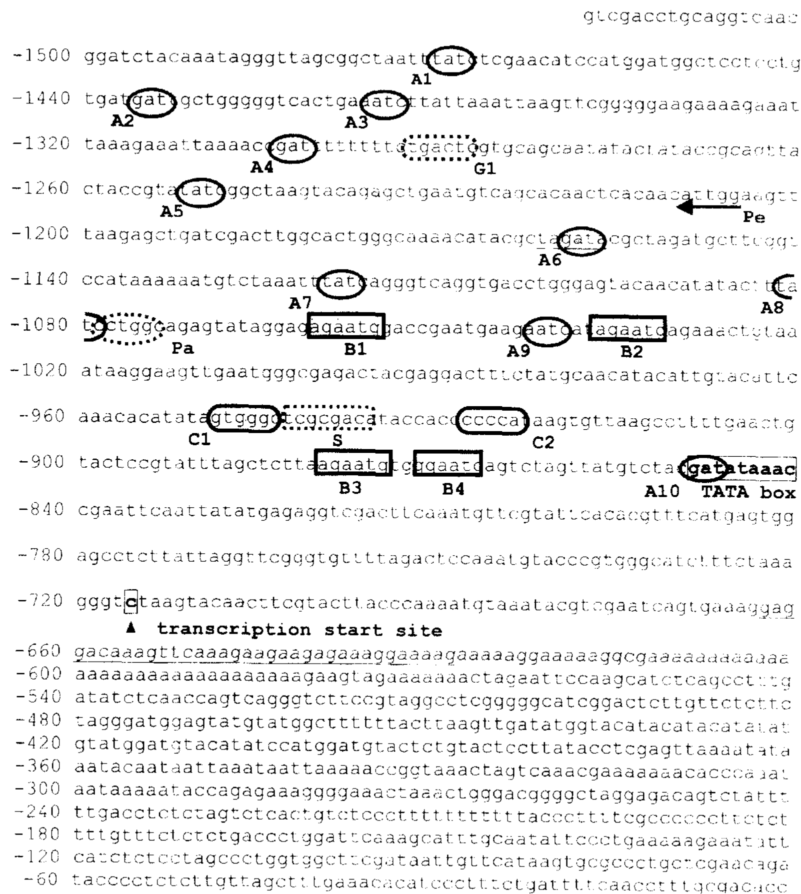

Fig. 2. Nucleotide sequence analysis of 5- flanking regions. Shaded boxes indicate a potential TATA box. Primer extension site is underlined. Putative binding sites for transcription factors are indicated. A; AreA (5'-GATT-3'/5'-AATC-3', 5'-GATA-3'/5'-TATC-3'), B; AbaA (5'-CATTCY-3'/5'-RGAATG-3'), C; CreA(5'-RYGGRG-3'/5'-CYCCRY-3'), S; StuA (5'-A/TCGCGT/ANA/C-3'), Pa; PacC (5'-GCCARG-3'/5'-CY TGGC-3'), G; GCN4/ Cpc1 (5'-TGACTC-3'), Pe; PENR1 (5'-CCAAT-3').

consensus sequence-specific site for AbaA, 5'-CATTCY-3' in an antisense direction. One each of the potential binding sites for various transcriptional activators were also identified: a site for StuA (5'-A/TCGCGT/ANA/C-3'), a modifier necessary for proper cell patterning and spatial organization of conidiophore in *A. nidulans* (10); a site for PacC (5'-GCCARG-3'), a zinc finger transcription factor mediating regulation of pH-dependent gene expression (43); a site for Cpc1/GCN4 (5'-TGACTC-3') (11), a general control response element bZIP protein; and a site for PENR1, a CCAAT-binding protein (25).

The existence of binding sites for AbaA and StuA is noteworthy, which are regulators and modifiers, respectively, for the asexual differentiation in *A. nidulans*. We do not have experimental evidence showing the interaction of any of these proteins with the potential regulatory sequences. It cannot be excluded, however, that the expression of *chs4* gene can be regulated by a similar mechanism for the regulation of *AnchsB* expression during asexual differentiation in *A. nidulans*. Although rigorous confirmation of this issue would demand further experimental evidence, our postulation is supported by the following; Chs4 showed higher amino acid identity (86.6 %) with ChsB of *A. nidulans* and a close evolutionary relationship with *Aspergillus* species exists (30, 31, 39). Our recent results also indicated the presence of a putative StuA binding site in the promoter region of *AnchsB* (unpublished result). It is also known that genes from *P. chrysogenum* can act functionally in *A. nidulans* (33).

A computer-assisted search of the 3' non-coding region revealed the presence of several putative poly(A) tailing signal-like sequences, among which a sequence located at position 3,254 (5'-tcAATATAtc-3') was most probable (Fig. 3). However, a perfect match for the polyadenylation signal, AATAAA, was not observed as is the case with many fungi (44).

Transcript mapping and northern analysis

The sequence analysis revealed that the coding region was interrupted by four introns. The introns are short and contain the perfect consensus 5' splice donor site (GT) and the 3' splice acceptor site (YAG), respectively as well as

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   1 ATG GCC TAC CAC GGC TCT GGC TCG CAC TCA CCA AGC TAT GAT GAT AGT CAT CAT CTG CAG
     M   A   Y   H   G   S   G   S   H   S   P   S   Y   D   D   S   H   H   L   Q     20
  61 GAT GTG CCA GCT TCG CAG gtatgtgtgtggagaggcggtgaactagtcgcgagagctgacccggtaa
     D   V   P   A   S   Q                                       Intron 1            26
 130 atttttttttctcttag TAT CGC GAG GAT GAG GAT GCA GCC CGT GGA TTA TTG TCC CAA CAG
                       Y   R   E   D   E   D   A   A   R   G   L   L   S   Q   Q     41
 193 CAG GGT CCA TTT GCG ACT CCT TTT GAC GAC CCC CAC TCG CGC GGT GTT TCT CCC CAA CGT
     Q   G   P   F   A   T   P   F   D   D   P   H   S   R   G   V   S   P   Q   R     61
 253 CCG TCT TCT GGG TAC AGC TTG ACG GAG ACC TAT GCA ACG GAC ACC GCC CCT TCT TAC CAC
     P   S   S   G   Y   S   L   T   E   T   Y   A   T   D   T   A   P   S   Y   H     81
 313 GAC CCA TAC AGT ACT GGC ACC GTT TAT TCC GGC CAA GAG GAA AGT ACT GCG GCG GCC TTT
     D   P   Y   S   T   G   T   V   Y   S   G   Q   E   E   S   T   A   A   A   F    101
 373 GGT GTC CCC GGC CGA GTT GCA TCG CCC TAT GCC CGC AGC GAG ACC TCC TCC ACG GAG GCT
     G   V   P   G   R   V   A   S   P   Y   A   R   S   E   T   S   S   T   E   A    121
 433 TGG CGC CAG CGA CAG GCC CCC GGA GGC GCC TCT GGT GGT GGT GGT GGC GGN CGC TTG CGC
     W   R   Q   R   Q   A   P   G   G   A   S   G   G   G   G   G   X   R   L   R    141
 493 AGA TAT GCT ACT AGG AAA GTC AAG CTA GTG CAG GGT TCC GTC TTG AGC GTC GAC TAC CCG
     R   Y   A   T   R   K   V   K   L   V   Q   G   S   V   L   S   V   D   Y   P    161
 553 GTG CCG AGT GCC ATC CAG AAT GCC ATC CAA GCC AAA TAT CGC AAC GAC GTG GAA GGC GGA
     V   P   S   A   I   Q   N   A   I   Q   A   K   Y   R   N   D   V   E   G   G    181
 613 AGC GAA GAG TTT ACC CAC ATG CGA T  gtacgtgtggaccagacgaccaatatacggctctaagaga
     S   E   E   F   T   H   M   R   Y                                Intron 2        190
 678 aaaactaatgagccttcactcttcatag  AC ACC GCA GCC ACT TGT GAC CCT AAC GAA TTT ACC
                                      T   A   A   T   C   D   P   N   E   F   T    201
 741 CTC CAC AAT GGT TAC AAC CTA CGC CCC GCA ATG TAC AAC CGT CAT ACA GAG CTG CTC ATC
     L   H   N   G   Y   N   L   R   P   A   M   Y   N   R   H   T   E   L   L   I    221
 801 GCG ATT ACC TAC TAC AAC GAG GAT AAG AAT CTC ACG GCC CGT ACC TTG CAC GGT GTG ATG
     A   I   T   Y   Y   N   E   D   K   N   L   T   A   R   T   L   H   G   V   M    241
 861 CAG AAC ATT CGC GAT ATT GTC AAC CTG AAG AAA TCC GAG TTC TGG AAT AAG GGT GGA CCC
     Q   N   I   R   D   I   V   N   L   K   K   S   E   F   W   N   K   G   G   P    261
 921 GCG TGG CAG AAG ATT GTC GTG GCA CTC GTT TTT GAC GGT ATT GAC CCT TGC GAC AAG GAT
     A   W   Q   K   I   V   V   A   L   V   F   D   G   I   D   P   C   D   K   D    281
 981 ACT CTT GAC GTC CTT GCG ACC ATC GGT ATC TAC CAG GAT GGT GTC ATG AAG CGT GAC GTT
     T   L   D   V   L   A   T   I   G   I   Y   Q   D   G   V   M   K   R   D   V    301
1041 GAC GGG AAG GAG ACT TTG GCA CAT ATC gtaagcggatggcaggataggcctcccaaaggaaacagcg
     D   G   K   E   T   L   A   H   I                                Intron 3        310
1108 ctaactgaatttccag   TTC GAG TAT ACG ACC CAG CTT TCG GTC ACA CCA AGT CAG CAG CTC
                         F   E   Y   T   T   Q   L   S   V   T   P   S   Q   Q   L    325
1169 ATT CGG CCC ACG GAC GAT GGC CCG AGC ACA CTA CCG CCT GTG CAG ATG ATG TTC TGT CTG
     I   R   P   T   D   D   G   P   S   T   L   P   P   V   Q   M   M   F   C   L    345
1229 AAA CAA AAG AAC AGT AAG AAG ATC AAT TCG CAT CGT TGG CTC TTT AAC GCT TTC GGC CGT
     K   Q   K   N   S   K   K   I   N   S   H   R   W   L   F   N   A   F   G   R    365
1289 ATT TTG AAC CCC GAG GTC TGT ATC CTG CTG GAT GCC GGT ACG AAA CCT GGC CCC AAG TCC
     I   L   N   P   E   V   C   I   L   L   D   A   G   T   K   P   G   P   K   S    385
1349 TTG TTA TAC CTC TGG GAG GCA TTC TAT AAC GAT AAG GAT CTC GGC GGT GCC TGT GGT GAA
     L   L   Y   L   W   E   A   F   Y   N   D   K   D   L   G   G   A   C   G   E    405
1409 ATC CAC GCC ATG TTG GGA AAG GGA TGG CGG AAC TTG ATC AAC CCT CTC GTG GCA GCC CAG
     I   H   A   M   L   G   K   G   W   R   N   L   I   N   P   L   V   A   A   Q    425
1469 AAC TTT GAG TAC AAG ATC AGT AAT ATC TTG GAT AAG CCA CTG GAG AGT TCG TTC GGC TAC
     N   F   E   Y   K   I   S   N   I   L   D   K   P   L   E   S   S   F   G   Y    445
1529 GTT AGT GTC TTG CCC GGT GCT TTC TCG GCC TAC CGA TTC CGT GCA ATC ATG GGT CGA CCC
     V   S   V   L   P   G   A   F   S   A   Y   R   F   R   A   I   M   G   R   P    465
1589 CTT GAG CAA TAT TTC CAC GGT GAG CAC ACG CTG TCT AAG CAA CTT GGA AAG AAG GGT ATT
     L   E   Q   Y   F   H   G   E   H   T   L   S   K   Q   L   G   K   K   G   I    485
1649 GAG GGT ATG AAC ATT TTC AAA AAG AAC ATG TTC TTG GCC GAG GAT CGA ATT CTC TGT TTC
     E   G   M   N   I   F   K   K   N   M   F   L   A   E   D   R   I   L   C   F    505
1709 GAG TTG GTC GCT AAG GCA GGC TCT AAG TGG CAC TTG TCT TAC GTT AAA GCC TCC AAG GGT
     E   L   V   A   K   A   G   S   K   W   H   L   S   Y   V   K   A   S   K   G    525
1769 GAA ACT GAC GTG CCC GAA GGT GCC GCC GAG TTT ATC TCC CAG CGT CGC CGT TGG CTC AAC
     E   T   D   V   P   E   G   A   A   E   F   I   S   Q   R   R   R   W   L   N    546
1829 GGT TCC TTT GCA GCA GGA ATC TAC TCG CTG ATG CAC TTT GGC CGT ATG TAT AAG AGC GGT
     G   S   F   A   A   G   I   Y   S   L   M   H   F   G   R   M   Y   K   S   G    565
1889 CAC AAC ATC ATT CGC ATG TTC TTC CTT CAC ATC CAG ATG TTG TAT AAC ATC TTC AAC ACT
     H   N   I   I   R   M   F   F   L   H   I  [Q   M   L   Y   N   I   F   N   T]   585
1949 TTC CTC ACC TGG TTC TCG TTG GCT TCA TAC TGG CTG ACC ACC ACT GTC ATC ATT GAC CTT
    [F   L   T   W   F   S   L   A   S   Y   W   L   T   T]  T   V   I   I   D   L    605
2009 GTC GGT ACT CCA AGC CCC AGC AAC GGA GAT ACC GCT TTC CCC TTT GGC AGG NAC GCT ACG
     V   G   T   P   S   P   S   N   G   D   T   A   F   P   F   G   R   X   A   T    625
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Fig. 3. Nucleotide sequence and deduced amino acid sequence of *chs4*. The presumed ATG initiation codon and its nucleotide are designated +1. Intron sequences are in lower-case letters, in which consensus acceptor site, donor site and internal lariat sequence are underlined. Putative trans-membrane domains are indicated by the boxed area. Potential *N*-glycosylation site is indicated by underlined capital letters. Putative poly-A sequences are bold-faced.

CTPAYT, the putative internal consensus sequence for lariat formation (38) (Fig. 3).

In order to confirm the location of the putative introns, reverse transcription (RT) PCR was performed with the sets of primers complementary to the sequences flanking both sides of the putative introns. The nucleotide sequences of the RT-PCR products matched well with the cDNA sequence predicted (data not shown). For the primer extension experiment to determine the transcription initiation site, radiolabeled oligonucleotides complementary to the sequences in the 5' non-translated region were annealed to total RNAs. The results from the primer extension suggested that the actual transcription initiation site is located at 716 bases upstream from the putative translation initiation codon AUG (Fig. 4). Northern blot analysis showed a single transcript of 3.5 kb sufficient for the Chs4 (Fig. 5). This result indicated that *chs4* gene was expressed during the vegetative mycelia growth of *P. chrysogenum*.

Amino acid sequence analysis of the chs4 product

The *chs4* encoded a predicted polypeptide of 915 amino

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2069  CCC ATT ATC AAC ACA ATT GTG AAG TAC GGA TAC CTC GCT TTC CTA TTG TTG CAG TTC ATT
       P   I   I   N   T   I   V   K   Y   G   Y   L   A   F   L   L   Q   F   I    645
2129  CTC GCT CTT GGT AAC AGA CCC AAG GGT TCC AAG TTC TCA TAC CTC GCC TCG TTT GTG GTC
       L   A   L   G   N   R   P   K   G   S   K   F   S   Y   L   A   S   F   V   V    665
2189  TTC GGT CTC ATT CAG GTG TAT ATT GTG GTC GAT TCG CTC TAC TTG GTA GTT CGC GCG TTC
       F   G   L   I   Q   V   Y   I   V   V   D   S   L   Y   L   V   V   R   A   F    685
2249  AGC GGT GGC GCA CCC ATG GAC TTT GTT ACC GGC NAA GGC CTG AAA GCC TTC CTC GAC TCT
       S   G   G   A   P   M   D   F   V   T   G   X   G   L   K   A   F   L   D   S    705
2309  TTC TTT TCG TCG ACG GGC GCT GGT ATC ATT ATC ATT GCC TTG GCT GCC ACA TTC GGT CTT
       F   F   S   S   T   G   A   G   I   I   I   I   A   L   A   A   T   F   G   L    725
2369  TAC TTC GTT GCC TCA TTC ATG TAC GCC GAC CCA TGG CAC ATG TTC ACC TCC TTC CCT GCC
       Y   F   V   A   S   F   M   Y   A   D   P   W   H   M   F   T   S   F   P   A    745
2429  TAT ATG GTT GTC CAG TCG TCC TAT ATC AAC ATC CTG AAC GTC TAT GCC TTC AGC AAC TGG
       Y   M   V   V   Q   S   S   Y   I   N   I   L   N   V   Y   A   F   S   N   W    765
2489  CAC GAT GTC TCC TGG GGT ACC AAG GGT TCT GAC AAG GCC GAT GCA TTN CCG TNA GCC ACG
       H   D   V   S   W   G   T   K   G   S   D   K   A   D   A   X   P   X   A   T    785
2549  ACT ACA AAG GAC GAG GGT AGC AAG GAA GCA GTN ATA GAA GAA ATC GAC AAG CCC CAA GCG
       T   T   K   D   E   G   S   K   E   A   X   I   E   E   I   D   K   P   Q   A    805
2609  GAT ATC GAC AGC CAG TTC GAG GCA ACT GTG AAG CGT GCG CTC ACT CCA TTC GTT GCC CCC
       D   I   D   S   Q   F   E   A   T   V   K   R   A   L   T   P   F   V   A   P    825
2669  GTG GAA CAG AAC GAA AAG ACA CTC GAG GAC TCT TAC AAG AGT TTC CGT ACC CGG CTC GTC
       V   E   Q   N   E   K   T   L   E   D   S   Y   K   S   F   R   T   R   L   V    845
2729  ACC TTT TGG ATC TTC AGT AAT GCG GCC ATG GCA GTA TAC ATC ACC AGC GAG TCC GTC GAT
       T   F   W   I   F   S   N   A   A   M   A   V   Y   I   T   S   E   S   V   D    865
2789  AAG GCG GCT TCA CAN gtaagctttcactcttcttcaactctttctgactagaccacgactaacatcatca
       K   A   A   S   Q                                      Intron 4              870
2859  tag AAT ACC GCC ACC ACC CGC ACA GCC CGT TTC TTC CAG GCA CTC TTG TGG TCC AAT GCC
           N   T   A   T   T   R   T   A   R   F   F   Q   A   L   L   W   S   N   A    889
2919  GCT GTG GCA TTG ATC CGT TTC ATT GGA TGC TGT TGG TTC CTT GGT CGT ACT GGT ATC AAG
       A   V   A   L   I   R   F   I   G   C   C   W   F   L   G   R   T   G   I   K    909
2979  TGC TGC TTT GCA CGT CGG TAG
       C   C   F   A   R   R   *                                                      915
3000  ttccccaggacaaaatgtgtacccttttcatgacccccctgacccaattttctattctcttccttttatttcccatacc
3080  gtgatctgtattctacctaaagatgcccgactttgttctcttttgattttgcaatgccagccagtatgtattcgcacggt
3160  tttacctttgacggttactgtggtcactttttttgtcggatcagccatgcaacgggatcgcattcggagcgctatgatta
3240  tgtacttcatattcAATATAtcttttttccccctttgagatttattgagtagaaagatatatgtcatgtttcccataga
3320  ctgtacatgcatagtagagagtccccgggtccaatacgtcaactggaaggcggagaggcgataaaggcgattgctgatct
3400  gagaccccccgcaagagggctaccctttcactctgtttgtcatccttgtcacccgttgatcacgtcctctaaaatccttc
3480  ctttctgtctctcttttttattatctgatacctctagagtggttaccttgcctcctactcactctccccaaacctccat
3560  tccaccaggtggggtggtgactcccgaaatgttgaaaatctggtcgatggtagggattcatctggcttttgaaaaaatg
3640  ccgtatgattaactttctgtgcagaaacaaaagcagcaggaggctgagaatgccgagggtgctgcaggcaagaagaagaa
3720  gaaggtcacagcggcgcaattacgcgtgcaacgaggtgagtgactttcccgtagtggacagtacaactcgcagtacttac
3800  tggatcgttgtcatatggatctctaacctcttatatctttagaccttcaagaactcacctcggcagacgatgaaaatgtc
3880  tttcccaaaccccgacgacatcctcaacttcaccttgaccatcgaacccgacgagggcatgtataagggtggtgtcttca
3960  attgctttagctccgtcaaccagaacttccctcatgatcctcccaaggtcaaatgcacacaaaagatctatcatcccaat
4040  atcgacttggagggtaacgtttgCTTAAAcattctacgagaggactggaagcccgtgttgaatttgaatgctgtcattgt
4120  gggcatgcaggtctgttgatttatctattctgactggtcattgatttcgctgacaggtattccactgcagttcctttcc
4200  tcgagccaaacgcctccgaccctcttaacaaggacgctgcggaagatcttcgcgctactcgtgacatcttcaagaagaac
4380  gtacgcaactccatggccggcgggtccgtccgaggcatatcgtttgagcgggtgttacggtgaatgctagctaggatttc
4360  ttcacacttgcagttttccatcggttcccatatccggactccgcccataccccacggcgacgagcagatacgggatatc
4440  gat
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Fig. 3. Continued.

acids with a calculated mass of 101 kDa (Fig. 3). The deduced amino acid sequences from the *chs4* showed higher level of similarities with those of the order Eurotiales in Ascomycota such as *Aspergillus* (*Emericell*) *nidulans*. A BLASTP similarity search of the complete sequence of the Chs4 with other Chs proteins gave an overall identity of 87% with *Aspergillus fumigatus* ChsG, 86% with *A. nidulans* ChsB, and 68% with *N. crassa* Chs3, all of which belong to class III chitin synthase. These results are in good agreement with our previous results from a similarity search performed with the PCR amplified *chs4* gene fragment for the conserved region of the chitin synthase (30, 31).

To characterize and elucidate the function of Chs4, the existence of transmembrane domains, Con1 and Con2 domains, in the putative catalytic domain of the chitin synthase (28), and of sites for post-translational modification were investigated by amino acid sequence analysis. High levels of similarity of Chs4 to other class III chitin synthase proteins is restricted to the 194 amino acid residues including the highly conserved central regions, in which the Con I and Con II micro-domain of Chs catalytic domain are located (5, 28, 45). The hydropathy profile

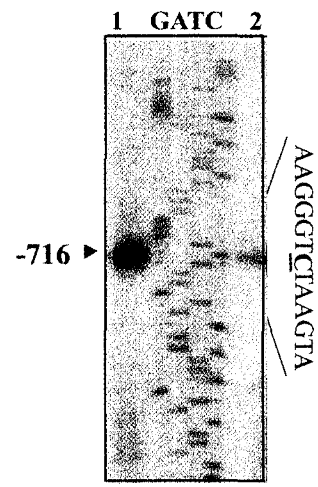

Fig. 4. Analysis of *chs4* transcription initiation site by primer extension. Primer extension reactions were performed using 35 base oligonucleotide primers and 20 μl of total RNA as described in Materials and Methods. The primer extension products, 5 μl (lane 1) and 1 μl (lane 2), were co-electrophoresed on a sequencing gel with a sequencing reaction with the same primer. The sequence surrounding the transcription initiation site is indicated alongside the autoradiogram. The nucleotide corresponding to the transcription initiation site is bold-faced.

(23) of the product inferred from the *chs4* nucleotide sequences revealed a protein with three major domains: a mostly hydrophilic region at the amino acid terminus, a neutral region at the central domain, and a hydrophobic region at the carboxy terminus (data not shown). This three-domain structure is typical for all Chs enzymes described to date (9, 40, 46). The hydropathy profile of the Chs4 and other chitin synthases was practically identical within the neutral central domains and hydrophobic C-terminal domain. At least five potential membrane-spanning regions, TM1 (amino acid 557 to 599), TM2 (627 to 649), TM3 (660 to 682), TM4 (700 to 731), and TM5 (883 to 905) were located throughout the central and C-terminal domains of Chs4 (Fig. 6A). Like chitin synthases in other fungi, Con I and Con II, which are essential for the catalytic activity of Chs, reside in central region and C-terminal region, respectively (Fig. 6B, C). Most interestingly, Con I and Con II domains are located

Fig. 5. Northern blot analysis of *P. chrysogenum chs4*. Total RNA was prepared from the mycelia as described in Materials and Methods. Fifty μg aliquot of total RNA was loaded in lane, and after electrophoresis, transferred onto nitrocellulose filter and hybridized with a radiolabeled PCR fragment of the *chs4* coding region. The positions of ethidium bromide-stained 28S and 18S rRNA are shown on the left-hand side.

Fig. 6. Highly conserved region of Chs4. (A) The highly conserved regions of Con1 and Con2 are located in the middle(Con1) and in the C-terminal half(Con2) of Chs4. TMs indicate five putative transmembrane domains. (B), (C) Comparison of the amino acid sequence of Con1(B) and Con2(C) with several chitin synthases. Amino acids conserved in all chitin synthases listed here are marked in bold. In the region of Con1, I~III represent subdomains, in which conserved amino acids appear at high frequences. ScChsp, *S. cerevisiae* chitin synthase; AnChsp, *A. nidulans* chitin synthase; CaChsp, *C. albicans* chitin synthase. AfChsp, *A. fumigatus* chitin synthase; WdChsp, *W. dermatidis* chitin synthase; NcChsp, *N. crassa* chitin synthase.

in the vicinity of the transmembrane domains and region of TM4 overlapped with the Con II domain. The topology of Chs4 is consistent with a model of vectorial synthesis for chitin synthesis, according to which the chitin synthase receives *N*-acetylglucosamine residues from UDP-*N*-acetylglucosamine at the cytoplasmic face of the membrane and transfers them vectorially to a growing chain of chitin that is concomitantly extruded. (6).

The peptide domain analysis of Chs4 revealed several putative sites for post-translational modification: for example, two possible sites (at amino acid residues 231 and 779) for *N*-glycosylation (Fig. 3) and several possible sites for *N*-myristoylation (data not shown). Membrane protein(s) and cell wall protein(s) are glycosylated during translocation from ER to Golgi complex for proper localization and assembly (32). For some proteins, the myristate moiety provides not only membrane binding, but also specific membrane targeting functions (35). These results strongly support the nature of Chs4, as a membrane protein, which resides in or is associated with the plasma membrane that synthesizes chitin polymer in fungal cell walls.

The results reported here showed that Chs4 is expressed during the vegetative growth of *P. chrysogenum* and suggests that expression of *chs4* could be modulated temporally and spatially by the transcripton factors such as StuA and AbaA equivalent in *P. chrysogenum* during its development. Further studies such as *chs4* gene disruption and gel-shift assay for promoter analysis will provide basic information which will contribute to understanding the function of chitin synthase in the development of an industrially important fungi, *P. chrysogenum*.

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