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NOTE

Cell Cycle-dependent Expression of Chitin Synthase Genes in *Aspergillus nidulans*

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The transcription of the chitin synthase genes (*chs*s) was cell cycle-regulated in *Aspergillus nidulans* and the expression pattern was classified into two groups. Group one, containing *chsA* and *chsC*, showed decreasing transcription level upon entry into the S-phase and no further variation during the remainder of the cell cycle. However, group two, containing *chsB*, *chsD*, and *csmA*, showed a sharp decrease of mRNA level upon entry into the G2-phase and an increase during the M-phase. Our results suggested that the *chs*s, belonging to same group with the similar expression pattern during the cell cycle are functionally linked and that *chsD* may play a role in hyphal growth and development in *A. nidulans*.

Key words: *Aspergillus nidulans;* chitin synthase genes; cell cycle; Northern analysis

Chitin, a β-1,4-linked polysaccharide of *N*-acetylglucosamine, is a major structural component of fungal cell walls (1) and is polymerized by chitin synthase. Enzymatic aspects and physiological roles of chitin synthases have been well characterized in the budding yeast *Saccharomyces cerevisiae*, in which chitin is a minor cell wall component (6). The three chitin synthase isozymes are encoded by separate genes, namely, *CHS1* (4), *CHS2* (23), and *CHS3* (25), and the activity of each isozyme is temporally and spatially regulated in *S. cerevisiae* (20). In contrast to *S. cerevisiae*, chitin constitutes up to 40% of the cell wall and 10% of the cell mass in *Aspergillus nidulans* (24), and the life cycle of this filamentous organism is generally more complex than that of *S. cerevisiae*. Its increased chitin content and developmental complexity correlate with the fact that this fungus has a large number of chitin synthase genes (*chs*s). Five different *chs*s (*chsA*, *chsB*, *chsC*, *chsD* and *csmA*) have been isolated to date from *Aspergillus nidulans*, and each gene plays different roles during hyphal growth and development (3, 7, 10, 15, 17, 24).

The cell wall is a structure that constantly undergoes change through the cell cycle. In *S. cerevisiae*, chitin is deposited in a ring, at the site of bud emergence, late in the G1-phase of the cell cycle (22). The chitin in the lateral walls is also cell cycle-regulated, and is present, for the most part, during the later stages of the cell cycle (20).

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In *A. nidulans*, there is no direct evidence of a relationship between chitin biosynthesis and the cell cycle, but recent results have shown a possible link between these two developmental events. *bimG11* mutants, which carry a temperature-sensitive type I protein phosphatase involved in cell cycle regulation and are blocked in mitosis, are deficient in chitin content (2). In addition, Dutton *et al.* (8) and Ye *et al.* (26) showed that interaction between development and cell cycle regulation is essential for the normal morphogenesis of *A. nidulans*. Taken together, these results strongly indicate that there is a relationship between chitin biosynthesis and cell cycle regulation. Despite multiple *chs*s being isolated from *A. nidulans*, the study of their characteristics in relation to morphogenesis has been confined to the asexual development of this organism. Here we present, for the first time, direct evidence that the expression of *chs*s is regulated during the cell cycle of *A. nidulans*, which will contribute to the understanding of the regulation of gene expression for cell wall biosynthesis with respect to fungal morphogenesis.

A. nidulans strain FGSC A781 (SO7, *w*A2; *nim*A5) was grown in CM medium (0.15% yeast extract, 0.15% casamino acid, 1% glucose, 20 ml minimal salt stock solution, and 1 ml vitamin solution (5). Minimal salt stock solution and vitamin solution were prepared as described previously (13). *E. coli* strain, DH5α, was used for the propagation and preparation of recombinant plasmids and a standard LB medium (0.5% yeast extract, 1% bactotryptone, and 1% NaCl) was used for bacterial growth,

Table 1. Primers used in this study

Gene	GenBank Accession Number	Primer	Oligonucleotide sequence (5' to 3')	Position in the gene
H2A	M18258	sense/antisense	CAAATCTGGTGGCAAGGCTA/GGTCTTCTTGGGGAGGAGGT	310-821
chsA	D21268	sense/antisense	GACCTGCGATCCAGATGATT/CCAACAGCACTCTTGACGAA	1157-2128
chsB	D21269	sense/antisense	GTTACAACTTGCGTCCAGCA/TGAATTCAGGAGCACCTTCC	1124-2174
chsC	D38409	sense/antisense	GAAGCACACCATGACGCTTA/CCTTGATCTCACCACAAGCA	1737-2734
chsD	D83246	sense/antisense	TATGGAGGCCAAGATGGAAG/ACGTGAATCCTGAACCAAGC	2352-3353
csmA	AB000125	sense/antisense	ATTTTGACGGACGCTCTGAC/GACAACGAGGAAGGGAACAA	3354-4852
gpdA	M19694	sense/antisense	TCACAGCACGGTCAGTTCAA/TATCCTTTGTCCAGCCTTCG	1319-2303

supplemented with ampicillin (50 μg/ml) when necessary.

Standard molecular cloning techniques, such as the isolation of plasmid DNA, restriction enzyme digestion, ligation, transformation, agarose gel electrophoresis, the extraction of target DNA from agarose gel, and RT-PCR were performed as described by Sambrook *et al.* (21).

Homogenous cell cycle phase-specific cultures were prepared using *A. nidulans* strain A781 as described previously (16). *A. nidulans* FGSC A781 strain carries the temperature-sensitive cell cycle mutation *nimA5*, which causes a specific arrest in the late G2-phase at a restrictive temperature (42°C). If *nimA5* is blocked for more than one doubling time and then shifted from restrictive to permissive temperature (32°C), nuclei immediately enter synchronous mitosis within a few minutes (18, 19). Briefly, the conidia of A781 were inoculated into 100 ml of CM to a final concentration of 2.0×10^6/ml, and initially grown for 16 h at the permissive temperature (32). This initial culture was used to obtain arrest/release-induced synchronous mitosis (19). G1-phase culture was prepared by shifting the initial culture to 42°C for 4 h and then transferring to 32°C for 15 min. S-phase culture was prepared by shifting the initial culture to 42°C for 4 h and then transfered to a medium containing 20 mM hydroxyurea for 90 min at 32°C. G2-phase culture was prepared by shifting the initial culture to 42°C for 4 h. M-phase culture was prepared by shifting the initial culture to 42°C for 4 h and then transfered to a medium containing 5 μg/ml benomyl at 32°C for 30 min. For arrest experiments, CM at 72°C was added to bring the temperature rapidly to 42°C. Incubation was continued at 42°C for 4 h before addition of CM at 4°C to bring the temperature rapidly to 32°C for the release experiment.

Cultured A781 cells were harvested by filtration through a Miracloth (Calbiochem-Novabiochem, San Diego, CA, USA) and then frozen rapidly with liquid N_2, and ground to a powder with a mortar and pestle. Total RNA was isolated by the modified guanidine thiocyanate/CsCl density gradient ultracentrifugation method (11). Poly $(A)^+$ RNA was isolated using an Oligotex mRNA purification kit (Qiagen. GmbH, Hilden, Germany) according to the manufacturer's instructions. Three μg of mRNA per lane was separated on 1% formaldehyde agarose gel as described previously (9). RNA was transferred to a Hybond-N membrane (Amersham Pharmacia Biotech, Buckinghamshire, England), according to the manufacturer's instructions and cross-linked with a UV crosslinker (Hoefer Pharmacia Biotech, San Francisco, CA, USA). Northern hybridization was performed in the presence of 50% formamide as described previously (9). Probes were prepared from gel-purified RT-PCR fragments synthesized with primers specific for each gene (Table 1), and then radiolabeled with [α-^{32}P]dCTP using a random primed DNA labeling kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Blots were washed at 42°C with decreasing concentrations of SSC, down to 0.1 × SSC, in the presence of 0.1% SDS, and exposed on Kodak BIOMAX MS film (Eastman Kodak, Rochester, NY, USA).

While the mechanisms controlling nuclear division in *A. nidulans* are in many ways similar to those which regulate cell cycle progression in yeast, significant differences do exist. Unlike yeast cells, *A. nidulans* hyphal cells growing under optimal conditions spend a considerable amount of time in each stage of the nuclear division cycle. In this respect, the *A. nidulans* nuclear division cycle bears considerable resemblance to that of animal cells. In the multicellular vegetative mycelium of *A. nidulans*, only tip growing cells contain actively dividing nuclei, which have a synchronous mitotic cycle. After formation of septa along vegetative hyphae, reentry into the duplication cycle is delayed in newborn subapical cells (For review, see Ref. 12). Because the level of the *H2A* transcripts is accumulated exclusively during the S-phase of *S. cerevisiae*, *H2A* has often been used as an indicator of the S-phase (14). In *A. nidulans*, the level of *H2A* transcript follows a pattern similar to that of *S. cerevisiae* and shows marked fluctuation through the nuclear division cycle: highest level in the S-phase, rapid decrease in the G2-phase, and increase through remainder of the cell cycle (19). In order to verify cell cycle-phase specificity of cultures used in this study, mRNAs isolated from the cell cycle phase-specific cultures were analyzed on RNA blot by hybridization with radiolabeled probes specific to *H2A* and *gpdA*. As shown in Fig. 1A, the level of mRNA hybridizing to the *gpdA* probe remains fairly constant but

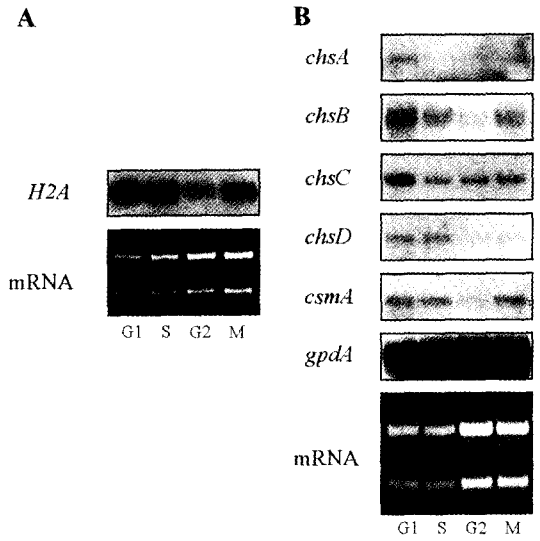

Fig. 1. Expression of the chitin synthase genes is cell cycle-regulated. A: *H2A* expression level was analyzed to verify synchrony of cultures. B: The expression levels of 5 chitin synthase genes. Glyceraldehyde-3-phosphate dehydrogenase gene (*gpdA*) expressed constantly was used as a positive control. Autoradiographies were established by exposing the filter for 24 h to X-ray film, except for *chsA* which was exposed for 3 days to X-ray film to determine its lower expression level.

to the *H2A* probe it shows a pattern identical to the one previously reported (19). These results indicated that the cell cycle-phase specific cultures were prepared properly.

The nucleotide sequences of *chs*s show a high level of similarity and some of them hardly show a clear difference in the predicted size of transcript, for example, 3.5 kb of *chsA*, 3.3 kb of *chsB*, and 3.4 kb of *chsC* transcript. Primers complementary to the highly variable N-terminal region of *chs*s were designed by sequence comparison and used to amplify cDNA probes specific to each of the *chs*s. When the mRNA expression level of the *chs*s in the cell cycle phase-specific cultures was analyzed using PCR-amplified *chs*-specific cDNAs as probes, a cell cycle-regulated expression of all *chs*s tested was revealed (Fig. 1B). *chsA* expression was highest during the G1-phase but decreased rapidly to a minimum level during the S-phase and maintained this level through the remainder of the cell cycle. The same pattern was observed for *chsC* expression, however, the expression level of *chsC* was higher than that of *chsA*. In contrast to *chsA*, whese expression was detected by exposing the mRNA blot for 3 days, the mRNA hybridizing to *chsA*-specific probe was detected by exposing the blot for 24 h. The *chsB* and *chsD* genes were expressed at a minimum level during the G2-phase, and were at a maximum during the G1-phase, but high mRNA levels were also observed during the S- and M-phases. *csmA* expression was slightly lower during the G1-phase than the S-phase, in which the expression level reached a maximum, and showed a minimum level during the G2-phase, as did those of *chsB* and *chsD*. These results indicated that the probes used for Northern analysis were specific enough to differentiate the expression pattern of *chs*s, whose expression can hardly be differentiated by their transcript size. Although a definitive answer will be given by further experiments such as Western analysis using antibodies specific to each of the *Chsp*s, these results suggest that chitin synthesis during vegetative growth is primarily regulated at the transcription level.

During the cell cycle, cells undergo constant morphogenetic changes. Hyphal cells of *A. nidulans* are primarily responsible for preparing materials for cell division, branch formation and lateral expansion during interphase. Division of cells by septum is dependent on mitiosis, nuclear positioning and attainment of a critical size. Reentry into the duplication cycle in subapical cells appears to occur at a time concomitant with the establishment of a new axis of polarized growth, which initiates branch formation (12). Recent reports indicated that interaction between developmental and cell cycle regulation is essential for normal morphogenesis in *A. nidulans* (8, 26). Although much is known about the function of *chs*s in relation to *A. nidulans* morphogenesis (3, 7, 10, 15, 17, 24), nothing is known about the relation between cell cycle and expression of *chs*s. To clearly demonstrate differences in the expression levels of each *chs* during the cell cycle, we quantified *chs* expression levels by densitometry and normalized against the amount of mRNA loaded onto the gel. As shown in Fig. 2, the expression patterns of *chs*s during the cell cycle can be classified into two groups. The expression pattern of *chsA* was similar to that of *chsC*, on the other hand, the expression pattern of *chsB* was similar to those of *chsD* and *csmA*.

chsA and *chsC* are non-essential and thus a single disruption of these genes does not reveal defects in growth and morphology, possibly because of functional redundancy among them (7, 10). However, defects in conidiophore development and loss of hyphal wall intergrity in *chsA* and *chsC* double disruption are apparent. It was suggested, therefore, that *ChsA* and *ChsC* share critical functions in hyphal wall integrity and development of asexual structure, such as the conidiophore (10). As shown in Fig. 2, the *chsA* and *chsC*, which were expressed mainly in the G1-phase, may contribute to the chitin subfraction necessary for maintenance of hyphal wall rigidity and for expansion of lateral cell walls after mitosis. However, differences between *chsA* and *chsC* in the levels of transcripts (Fig. 1B) suggest that *chsA* participates at a minimum level in chitin synthesis to hyphal wall expansion and wall rigidity. Actually *chsA* mRNA was mainly detected in the conidiophore rather than in growing hyphae (our unpublished data).

Disruption of either *chsB* (3) or *csmA* (15, 24) shows dramatic phenotypic changes indicating that *chsB* and *csmA* play the most important roles in hyphal growth and

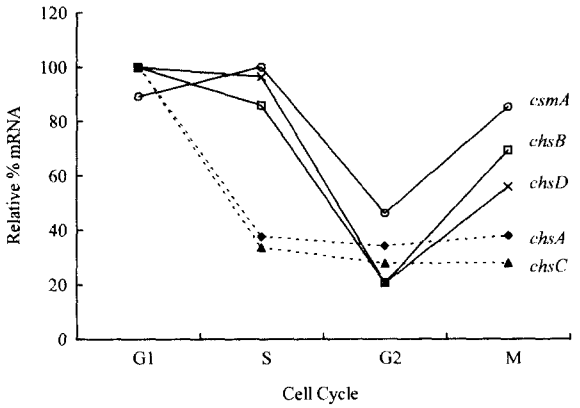

Fig. 2. Relative amount of mRNA of the chitin synthase genes during the cell cycle. All transcript levels were quantified by densitometry and normalized against the amount of RNA loaded on the gel, the highest value being taken as 100%.

hyphal development among the *chs*s identified in *A. nidulans*. The *chsB* disruption causes severe defects of growth, which are not remedied by the presence of osmotic stabilizer in the medium, such as slowly germinating conidia, hyphae with enlarged tips, a high degree of branching, and disorganized lateral walls, although the mycelium is not deficient in chitin content (3). Predominant expression of *chsB* during conidia germination was also observed (our unpublished data). These results indicate that chitin synthesized by ChsB does not substantially contribute to the rigidity of the cell wall but is necessary for normal hyphal growth and organization (3). *csmA* disruption also shows morphological abnormalities in tip growth and septum formation suggesting that the production of *csmA* has important roles in polarized cell wall synthesis for tip growth, maintenance of cell wall integrity, and septal distribution (15, 24). But abnormalities are predominantly seen in the old part of hyphae, such as balloon formation along hyphae, spatially irregular and structurally aberrant septum formation, small population of conidiophores, and intrahypha proliferation (15). Taken together, tight regulation of *chsB* and *csmA* expression in conjunction with cell division cycle could be postulated. Interestingly enough, *chsB* and *csmA* was expressed throughout the cell cycle, with the exception of the G2-phase (Fig. 2) suggesting that either one or both of the genes may be involved in chitin synthesis for septum formation as well as for maintenance of hyphal wall integrity.

Since no growth defect caused by *chsD* disruption has been reported (17, 24), except 30~40% reduction of the chitin content in disruptant hyphae (24), the function of *chsD* production is not known yet. However, our result showed cell cycle-regulated expression of the *chsD* suggesting involvement of *chsD* production in chitin synthesis for hyphal growth and development. Similar expression patterns of *chsD* to that of *chsB* and *csmA* may indicate that *chsD* is functionally linked with *chsB* and/or *csmA*. Although further experimentation will be needed to address this issue, it is noteworthy that some functional overlap between *csmA* and *chsD* (24) and between *chsA* and *chsD* (17) was previously observed.

Taken together, our results strongly suggest that the *chs*s, belonging to same group with similar expression patterns during the cell cycle are functionally linked and that *chsD* may play some role in hyphal growth and development in *A. nidulans*.

In this presentation, we show evidence for the first time, that the expression of chitin synthase genes is primarily regulated at the transcription level during hyphal growth, and that the expression of these genes are cell cycle-regulated. Our results presented here also suggest that the cell cycle-dependent regulation of chitin biosynthesis during vegetative growth is essential for the normal progression of morphogenesis in *A. nidulans*. In addition, chitin synthase genes with similar expression patterns may be functionally linked under certain developmental circumstances.

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