

The *Aspergillus nidulans swoCI* Mutant Shows Defects in Growth and Development

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ABSTRACT

Previous work identified *swoCI* as a single-gene mutant with defects in polarity establishment. In this study *swoCI* was shown to have defects in endocytosis, compartmentation, nuclear distribution, and conidiation. Temperature-shift experiments showed that the *swoCI* mutant establishes multiple random sites of germ tube emergence. Surprisingly, these experiments also showed that even a slight delay in polarity establishment causes defects in later vegetative growth and asexual reproduction. The *swoC* gene was mapped to the centromere of chromosome III and cloned by complementation of the temperature-sensitive phenotype. The predicted SwoCp is homologous to rRNA pseudouridine synthases of yeast (Cbf5p) and humans (Dkc1p). However, neither rRNA pseudouridine synthesis nor rRNA processing appears to be affected in the *swoCI* mutant. The *swoCI* mutation occurs in the putative RNA-binding domain upstream of the C terminus, leaving the N-terminal TRUB catalytic domain intact. Interestingly, while deletion of the *swoC* gene was lethal in *A. nidulans*, the C terminus, including NLS, microtubule-binding, and coiled-coil domains, was dispensable for growth. SwoCp likely plays an important role in polar growth and nuclear distribution in *A. nidulans*, functions not yet described for its homologs.

IN filamentous fungi, spores break dormancy and expand isotropically before switching to polar tip growth. Further growth occurs exclusively at the hyphal tip (MOMANY and TAYLOR 2000). Germ tube emergence in wild-type *Aspergillus nidulans* is sequential and usually occurs in a bipolar pattern (HARRIS *et al.* 1999; MOMANY and TAYLOR 2000). Along the hyphae, septa are laid down at regular intervals and nuclei are evenly distributed (FIDDY and TRINCI 1976; TRINCI and MORRIS 1979). The temperature-sensitive *swoCI* mutant of *A. nidulans* was isolated on the basis of its isotropic (swollen) phenotype in a screen to identify genes involved in polar growth (MOMANY *et al.* 1999).

Polarity genes in filamentous fungi are proposed to fall into two categories (MOMANY *et al.* 1999; MOMANY 2002): those responsible for establishing a location where a germ tube will emerge (polarity establishment genes) and those responsible for maintaining this directed polar growth (polarity maintenance genes). Previous work showed that the *swoCI* mutant is not able to establish polarity, but is able to maintain polarity at restrictive temperature and thus is a polarity establishment mutant (MOMANY *et al.* 1999). The genetic analysis of polarity in yeast and filamentous fungi has shown that the cytoskeleton, Bud proteins, and members of G protein and mitogen-activated protein kinase signaling pathways play major roles in polarity generation (RAS-

MUSSEN *et al.* 1992; ROBERTS and FINK 1994; BACHEWICH and HEATH 1998; CHANT 1999; ROZE *et al.* 1999; PRUYNE and BRETSCHER 2000; WENDLAND and PHILIPPSSEN 2001; MOMANY 2002).

Surprisingly, we found that the *swoC* polarity establishment gene encodes a homolog of *Saccharomyces cerevisiae* Cbf5p. Originally discovered by its affinity to the yeast centromere, Cbf5p is the pseudouridine synthase responsible for isomerization of uridine to pseudouridine in rRNA (LAFONTAINE *et al.* 1998; WATKINS *et al.* 1998; ZEBARJADIAN *et al.* 1999; WATANABE and GRAY 2000). In eukaryotes, rRNA is transcribed by RNA polymerase I and undergoes a series of modifications and cleavages to produce 18S, 5.8S, and 28S mature rRNAs. Isomerization of uridine to pseudouridine is one of the most abundant post-transcriptional modifications and mostly occurs in the large subunit of ribosomal RNA (reviewed by CHARETTE and GRAY 2000).

The biological role of pseudouridine is not yet clear. Recent evidence suggests that pseudouridine is not essential for ribosomal functions. Yeast depleted of virtually all pseudouridine residues in rRNA are viable (BOUQUET-ANTONELLI *et al.* 1997). Nonetheless, deletion of *CBF5* is lethal (GANOT *et al.* 1997), suggesting that Cbf5p must have functions other than pseudouridine synthesis. Indeed, yeast Cbf5p has been shown to associate with microtubules (JIANG *et al.* 1993), Pol I transcription factor (CADWELL *et al.* 1997), and snR30, which is involved in pre-rRNA endonucleolytic processing (LAFONTAINE *et al.* 1998). The human homolog Dkc1p binds hTR (the RNA component of human telomerase complex) and plays an important role in telomere maintenance (MARCINIAK *et al.* 2000; DEZ *et al.* 2001). Muta-

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tions of *DKC1* cause the X-linked recessive diseases dyskeratosis congenita (HEISS *et al.* 1998) and Hoyeraal-Hreidarsson syndrome (YAGHMAI *et al.* 2000). Patients with these diseases have a rare bone-marrow failure disorder and early mortality.

MATERIALS AND METHODS

Strains and media: Strains used in this study were: A104 (*ya2; adE20; AcrA1; phenA2; pyroA4; lysB5; sB3; nicB8; coA1*), A457 (*proA1; biA1; galE9; sC12; diA1; phenA2; choA1*), A773 (*pyrG89; wA3; pyroA4*), A852 (*biA1; ΔargB::trpCΔB; methG1; veA1; trpC801\pabaA1 ya2; ΔargB::trpCΔB; veA1; trpC801*), AGA22 (*swoC1; pabaA*), and AXL8 (*swoC1; pyrG89; wA3; pyroA4*). All strains were obtained from the Fungal Genetics Stock Center (Department of Microbiology, University of Kansas Medical Center) except AGA22 and AXL8, which were constructed for this study. Identification of the temperature-sensitive *swoC1* mutant and verification that it is a single-gene mutation have been previously described (MOMANY *et al.* 1999). Media used were as previously reported (HARRIS *et al.* 1994). Strain construction and genetic analysis were by standard *A. nidulans* techniques (KAFFER 1977; HARRIS *et al.* 1994).

Growth conditions and microscopic observation: *Vegetative growth:* Conditions for vegetative growth and preparation of cells were as previously reported (MOMANY *et al.* 1999). Briefly, conidia were inoculated on coverslips in liquid medium and incubated in a petri dish. Cells were fixed, nuclei were stained with Hoechst 33258 (Sigma, St. Louis), and septa were stained with calcofluor (American Cyanamid, Wayne, NJ). Microscopic observations were made using a Zeiss (Thornwood, NY) Axioplan microscope and digital images were acquired using an Optronics (Goleta, CA) digital imaging system. Images were prepared using Photoshop 5.5 (Adobe, Mountain View, CA).

Conidiation: Conidia were inoculated on the edges of a small square of agar medium placed on top of a coverslip, which was placed in a petri dish containing solidified agar to keep it moist. Another coverslip was placed on top of the agar square after inoculation. Plates were sealed with parafilm, incubated inverted at 42° for 9 hr, and then shifted to 30° for 2 days. For observation of conidiophore structure, coverslips with aerial hyphae and conidiophores attached were dipped into 100% ethanol, mounted on slides, and observed microscopically. Otherwise, cells attached to coverslips were fixed and stained as described for vegetative growth.

FM4-64 staining: Complete liquid medium with proper supplements was inoculated with $1-5 \times 10^4$ conidia/ml, poured into a petri dish containing a glass coverslip, and incubated as indicated. Coverslips with adhering cells were labeled with 20 μM FM4-64 (Molecular Probes, Eugene, OR) solution for 30 min at the indicated temperature. NaZ_3 (10 mM) was added to stop the labeling. A 20 μM FM4-64 solution with 10 mM NaZ_3 was used as control.

DNA and RNA isolation: DNA was isolated from *A. nidulans* using previously described methods (HARRIS *et al.* 1994). Total RNA from *A. nidulans* was extracted using Trizol reagent according to the manufacturer's instructions (GIBCO BRL, Grand Island, NY).

Cloning by complementation and plasmid rescue: A random genomic plasmid library carrying a *pyrG* marker provided by Greg May (University of Texas, M. D. Anderson Cancer Center, Houston; OSHEROV and MAY 2000) was transformed into protoplasts of the *swoC1* mutant AXL8 by standard *A. nidulans* protocols (YELTON *et al.* 1984). Transformants with *pyrG* prototrophy restored to wild-type growth at restrictive temperature (42°) were selected. The complementing plasmids were res-

cued by transformation of *Escherichia coli* XL1-blue with total DNA purified from the *A. nidulans* transformants. Restriction mapping showed that all the complementing plasmids contained the same fragment with overlapping genomic DNA inserts (data not shown). The smallest complementing plasmid, p8c1, was chosen for further study.

Confirmation of complementation by map-based cloning: *Mitotic mapping:* The *swoC1* mutant strain AGA22 (*swoC1; pabaA*) was fused with mitotic mapping strain A104 (*ya2; adE20; AcrA1; phenA2; pyroA4; lysB5; sB3; nicB8; coA1*) by standard methods (MA and KAFFER 1974; KAFFER 1977). Diploid conidia were incubated on solid complete medium containing 60 μg/ml benomyl for 2 days and transferred to complete medium without benomyl for 2 weeks. Genotypes of the resulting haploid sectors were scored. Of 300 haploid sectors, only 28 showed *swoC1* ts- phenotype, which may be caused by reduced viability of the ts- strain. The percentages of haploid sectors with the *swoC1* ts- phenotype segregated in repulsion to markers *adE20*⁻ (chromosome I), *acrA*⁻ (chromosome II), *phenA2*⁻ (chromosome III), *pyroA4*⁻ (chromosome IV), *lysB5*⁻ (chromosome V), *sB3*⁻ (chromosome VI), and *nicB8*⁻ (chromosome VII) were 57, 43, 100, 54, 78, 75, and 54%, respectively. The chromosome VIII marker in the mitotic mapping strain A104 has a ts- phenotype (compact morphology), which makes it impossible to score at the same time as *swoC1* (ts-).

Meiotic mapping: The *swoC1* mutant strain AGA22 (*swoC1; pabaA*) was crossed with meiotic mapping strain A457 (*proA1; biA1; galE9; sC12; diA1; phenA2; choA1*). Ascospores ($n = 350$) released from individual cleistothecia were plated on selective media to test genotypes, and map units between *swoC1* and other markers were calculated on the basis of recombination frequency of ts- strains. Only 59 of the progeny were ts-, which may be caused by reduced viability of the ts- strain. Map units between *swoC1* and *choA1*, *galE9*, *sC12*, and *phenA2* were 50, 30.5, 25.4, and 0, respectively.

Cloning of swoC: The *swoC* gene is tightly linked to the *phenA* marker, which is located near the centromere of chromosome III. On the basis of the physical map of *A. nidulans* (<http://gene.genetics.uga.edu/>), 20 cosmids near the centromere from the chromosome-specific genomic library (Fungal Genetics Stock Center, <http://www.fgsc.net/>) were chosen to transform into the *swoC1* mutant. Only cosmid W21H06 restored the *swoC1* mutant to wild-type growth at restrictive temperature. Southern blotting experiments (SAMBROOK *et al.* 1989) showed that cosmid W21H06 contains the same fragment as p8c1 and the two other complementing plasmids (data not shown).

Identification and sequencing of the swoC gene by transposon tag: Transposons (GPS-1 system; New England Biolabs, Beverly, MA) were randomly inserted into the complementing plasmid p8c1. Each of the resulting plasmids contains one copy of the transposon at random sites. Forty-eight plasmids were sequenced using primers unique for the transposon ends on an ABI 3700 DNA Analyzer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

The sequences were assembled and analyzed using the Phred (version 0.000925c), Phrap (version 0.990319), and Consed (version 11.0) computer programs (<http://depts.washington.edu/ventures/collabtr/direct/ppcombo.htm>). All sequences have at least fourfold redundancy with a quality rating of at least 20. The assembled contig was used to search the National Center for Biotechnology Information (NCBI) databases (www.ncbi.nlm.nih.gov), using the Blast program to identify open reading frames (ORFs). Only one ORF was found. Plasmids with transposons inserted within the ORF were transformed into the *swoC1* mutant. Plasmids that failed to rescue the *swoC1* mutant at restrictive temperature were assumed to have transposon insertions disrupting the complementing gene. The genomic sequence of the *swoC* gene, its

intron locations, and the predicted protein sequence were deposited in GenBank (accession no. AY057454).

Sequencing of the *swoC1* mutant allele: The *swoC1* mutant allele was amplified by three independent PCR reactions using the Expand high-fidelity PCR system (Roche Diagnostics, Indianapolis) and cloned into the pGEM-T vector system (Promega, Madison, WI). Genomic DNA from *swoC1* mutant strain AXL8 was used as the template. Primers used for PCR amplifications were 5' GAATGTTTACGCAGGTGG and 5' GTGGC TTGTGATGATGCGG. Three clones from each reaction (nine in total) were sequenced using the transposon strategy described above. All sequences showed an identical change in base 1220 (G to T) of *swoC1*. The sequences obtained were compared with the wild-type allele using GeneDoc (version 2.6.001) (www.psc.edu/biomed/genedoc) with default parameters.

Protein alignment: Sequences of SwoCp homologs were obtained from GenBank (<http://www.ncbi.nlm.nih.gov/>). Intron locations in *swoC* were based on alignments with homologs and presence of consensus splice sites (BALLANCE 1986). Protein sequences were aligned using GeneDoc (version 2.6.001) with default parameters.

Pseudouridine analysis by HPLC-MS: Enzymatic digestion of 100 μ g total RNA by nuclease P1 (Sigma), phosphodiesterase I (Sigma), and alkaline phosphatase (Sigma) was conducted as previously described (POMERANTZ and MCCLOSKEY 1990). The pseudouridine level of the hydrolytes was analyzed by HPLC-mass spectrometry (MS) basically as previously described (POMERANTZ and MCCLOSKEY 1990) by the Chemical and Biological Sciences Mass Spectrometry Facility, University of Georgia (<http://www.uga.edu/mass-spec/>). Standards (adenosine, cytidine, guanosine, pseudouridine, and uridine) were used to determine the optimal gradient conditions for separation. Peaks were identified by their retention time and mass spectrum. The experiment was repeated three times with the same results.

Northern hybridization: Total RNA was isolated from wild-type A773 and *swoC1* mutant AXL8 cultured in complete medium at 42°. RNA was separated on a 1.2% formaldehyde agarose gel and transferred to a nylon membrane by standard methods (SAMBROOK *et al.* 1989). The following probes were generated on the basis of *A. nidulans* 18S, ITS1, ITS2, and 25S rRNA sequences, respectively: probe 1, CTCCCCGCCG AAGCAACAGTG; probe 2, GAGCCATTCGCAGTTTCACAG; probe 3, GACGACGACCCAACACACAAGC; and probe 4, CACTCTACTTGTGCGCTATCGGTC. Probes were labeled at the 5' end by T4 polynucleotide kinase (Roche) according to the manufacturer's instructions.

Construction of *swoC* knock out: Flanking sequences from upstream and downstream of the *swoC* gene (1.1 kb each) were amplified by high-fidelity PCR. Primers used to amplify the 5' flanking sequence of *swoC* were 5' CCGCTCGAGGGT GAATGTTTACGCAGGTGGTTTTGG (with the addition of the *XhoI* restriction site) and 5' GGAATCCGGCCATTGC GACTGTTATTGAG (with the addition of the *EcoRI* restriction site). Primers used to amplify the 3' flanking sequence of *swoC* with the addition of *SacI* restriction sites were 5' TCCCCGCG GCGACCAATGGAAGTCCGAATAC and 5' CCCC GCGGG GACTAACGTCAAGTGTGGCGAGTG. After double digestion with *EcoRI* and *XhoI*, the 5' flanking sequence was inserted into pargBC-1 (MOMANY and HAMER 1997) bearing the *argB* gene as the selectable marker. The resulting plasmid pargBC-5F was then ligated with the 3' flanking sequence after digestion with *SacI*. Correct insert direction of the 3' flanking sequence was confirmed by restriction mapping and PCR. The plasmid pargBC-5F-3F was then digested with *Bss*HIII and separated on a 1% agarose gel. The 4.2-kb fragment containing *argB* with *swoC* flanking sequences (5F-argB-3F) was purified using the QIAGEN (Valencia, CA) gel purification kit.

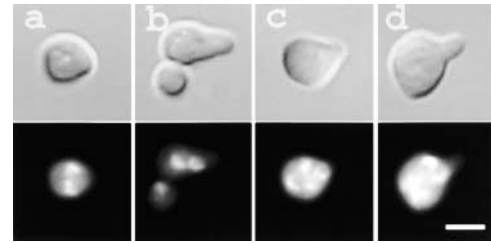


FIGURE 1.—Polarity establishment is delayed in the *swoC1* mutant. Conidia of wild-type A773 and *swoC1* mutant AXL8 were incubated for 6 hr (a and b) and 8 hr (c and d), respectively, at permissive temperature (30°), fixed, and stained with Hoechst 33258. (a and b) wild type; (c and d) *swoC1* mutant. The top and bottom rows show differential interference contrast (DIC) and fluorescent images of the same field. Bar, 5 μ m.

The linear fragment containing 5F-argB-3F was transformed into *A. nidulans* diploid strain A852. Transformants were selected for growth on minimal medium lacking arginine. Genomic DNA of 72 transformants was isolated and digested with *KpnI*. Southern blot was performed with random-labeled 3' flanking sequence as the probe. Putative homologous integrants were confirmed by Southern blot with *Bam*HI and *KpnI* double digestion. A single heterozygous diploid transformant with *argB* replacing *swoC* on one chromosome and a wild-type *swoC* on the other was treated with benomyl to induce haploidization as described for mitotic mapping. In total, 206 haploid sectors were scored for genotype. None of the haploid colonies grew on minimal medium without arginine supplement.

Construction of the *swoC* C-terminal deletion allele: The construction of the C-terminal deletion allele was essentially the same as the null allele except that the truncated *swoC* gene lacking the final 294 bases was inserted into pargBC-1 (MOMANY and HAMER 1997) after double digestion with *EcoRI* and *PstI*. This *swoCC*-terminal deletion fragment was amplified by high-fidelity PCR using primers 5' CTGCAGTGATGGTC AGGACTGG and 5' AACTGCAGAACCAATCCATTGGGGC TGGGGTGGCTTCGTTGG (with addition of the *PstI* site). The resulting plasmid pargBC-N-swoC was ligated with the 3' flanking sequence as described above. The linear fragment containing truncated-swoC-argB-3F was transformed into *A. nidulans* diploid strain A852. Screening and confirmation were performed essentially as described for the null mutant construct.

RESULTS

Phenotypic characterization of the *swoC1* mutant

Nuclear division is uncoupled from polarity establishment in the *swoC1* mutant: Previous work has shown that uninucleate spores switch from isotropic to polar growth after the first round of mitosis in wild-type *A. nidulans* (MOMANY and TAYLOR 2000) (Figure 1, a and b). In the current study, 80% of *swoC1* mutant spores did not switch to polar growth until the second round of mitosis at 30° (Figure 1, c and d). This observation suggests that nuclear division and polar growth are uncoupled in the *swoC1* mutant even at permissive temperature.

Previous work has shown that the *swoC1* mutant grows

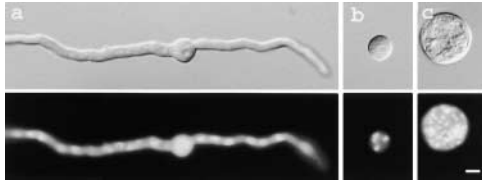


FIGURE 2.—The *swoCI* mutant continues isotropic growth with extended incubation at restrictive temperature. Conidia of (a) wild-type A773 and (b and c) *swoCI* mutant AXL8 were incubated at restrictive temperature (42°) for (a and b) 12 hr or (c) 24 hr, fixed, and stained with Hoechst 33258. The top and bottom rows show DIC and fluorescent images of the same field. Bar, 5 μ m.

isotropically at restrictive temperature (42°) for 13 hr and accumulates six nuclei on average (MOMANY *et al.* 1999). To determine if the *swoCI* mutant arrests isotropic growth upon extended incubation, we observed the growth of *swoCI* after 24 hr incubation at restrictive temperature. We found that the *swoCI* mutant grew into giant, balloon-like cells and accumulated a large number of nuclei (Figure 2, b and c). Some cells were at least 15 times larger than a wild-type conidium and contained >30 nuclei. Even after 53 hr at restrictive temperature, about half of the population survived (data not shown), indicating that the mutant is viable and nuclear division is not blocked by the polar growth defect. This supports our previous assertion that polar growth and nuclear division are carried out by two independent pathways in *A. nidulans* (MOMANY and TAYLOR 2000).

The *swoCI* mutant sends out multiple germ tubes simultaneously at random sites: Previous work concluded that the *swoCI* mutant does not establish polarity at restrictive temperature on the basis of failure to send out germ tubes within 2 hr (just over one cell cycle) after shift from restrictive to permissive temperature (MOMANY *et al.* 1999). To determine if the *swoCI* mutant loses the competence for polar growth after sustained isotropic expansion, the *swoCI* mutant was incubated at restrictive temperature for 4, 10, and 24 hr (Figure 3, a–c) and then shifted to permissive temperature for 10 hr (several cell cycles). The *swoCI* mutant sent out germ tubes in all cases (Figure 3, d–f). Even after 53 hr at restrictive temperature, mutant cells sent out germ tubes upon temperature shift (data not shown). This suggests that the competence for polar growth either is retained in the *swoCI* mutant at restrictive temperature or can be established after release of the temperature block. Interestingly, the longer the incubation at restrictive temperature, the more germ tubes the *swoCI* mutant sent out after shift to permissive temperature (Figure 3). To determine if these multiple polarity sites are activated simultaneously after the temperature block is released, we incubated the *swoCI* mutant at restrictive

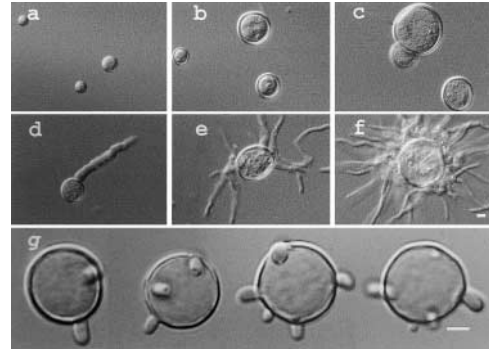


FIGURE 3.—The *swoCI* mutant sends out multiple germ tubes after extended incubation at restrictive temperature. Conidia of *swoCI* mutant AXL8 were incubated at restrictive temperature (42°) for (a) 4 hr, (b) 10 hr, and (c) 24 hr and shifted to permissive temperature (30°) for 10 hr (d, e, and f, respectively). (g) Conidia of *swoCI* mutant AXL8 were incubated at restrictive temperature (42°) for 20 hr and shifted to permissive temperature (30°) for 3 hr. Bars, 5 μ m.

temperature for 20 hr and shifted to permissive temperature for 3 hr (more than one cell cycle). We found that many germ tubes emerged at random sites (Figure 3g). Because these germ tubes were all the same length after 3 hr growth at permissive temperature, we assume that they emerged simultaneously. In contrast, in wild-type *A. nidulans*, germ tubes emerge sequentially, usually in a bipolar pattern (Figure 2a; HARRIS *et al.* 1999; MOMANY and TAYLOR 2000). Our results show that more than one polarity apparatus may form simultaneously in the *swoCI* mutant and suggest that polarity establishment may be tied to membrane volume.

The *swoCI* mutant is defective in endocytosis: One possible explanation for a connection between increased membrane volume and increased germ tube emergence in the *swoCI* mutant would be a failure to remove accumulated polarity establishment markers from the plasma membrane during isotropic expansion. Indeed in *S. cerevisiae*, an endocytic block causes isotropic cell growth, similar to the *swoCI* mutant swollen phenotype (reviewed by PRUYNE and BRETSCHER 2000). To investigate endocytosis of the *swoCI* mutant we used FM4-64, a membrane-selective dye that is internalized from the plasma membrane to internal membranes of organelles by endocytosis (VIDA and EMR 1995) and has been used to investigate endocytosis and vesicle trafficking in filamentous fungi (FISCHER-PARTON *et al.* 2000). Wild type and the *swoCI* mutant were incubated at restrictive temperature, and cells were labeled with FM4-64 for 30 min. In the presence of NaZ₃, the metabolic inhibitor, FM4-64 labeled only the plasma membrane of wild type and the *swoCI* mutant (Figure 4, b and d). In the absence of NaZ₃, FM4-64 labeled plasma membrane and internal organelles of wild type during both isotropic growth and polar growth (Figure 4c).

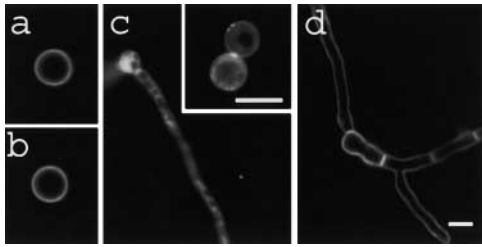


FIGURE 4.—The *swoC1* mutant is defective in endocytosis. Conidia of wild-type A773 and *swoC1* mutant AXL8 were incubated at 42° for 16 hr and treated with 20 μM FM4-64 or 20 μM FM4-64 with 10 mM NaZ₃ for 30 min. (a) *swoC1*; (b) *swoC1* with NaZ₃; (c) wild type (inset: wild type growing isotropically at 42° for 4 hr); (d) wild type with NaZ₃. Bars, 5 μm.

Under the same conditions, FM4-64 labeled only the plasma membrane of the *swoC1* mutant (Figure 4a), showing that the *swoC1* mutant is defective in endocytosis at restrictive temperature.

Early delay in polarity establishment causes a nuclear distribution defect during vegetative growth and conidiation in the *swoC1* mutant: To determine whether an early delay in polarity establishment affects later vegetative growth, we incubated wild type and *swoC1* at restrictive temperature for 9 hr (the *swoC1* mutant averaged four nuclei) and shifted to permissive temperature for 15 hr. We measured the length and nuclear number of subapical compartments ($n = 120$). Subapical compartment length and nuclear number were relatively uniform in the wild type and in the *swoC1* mutant grown at permissive temperature for the duration of the experiment (Figure 5 and data not shown). However, subapical compartment length and nuclear number varied greatly in the *swoC1* mutant after temperature downshift (Figure 5). Occasionally, we observed compartments without nuclei in the mutant (Figure 5A,c). Empty compartments were never observed in wild type. In wild

type, the majority of compartments were between 10 and 60 μm with 60% falling between 30 and 40 μm and 23% falling between 40 and 50 μm (Figure 5B). In the *swoC1* mutant after temperature downshift, subapical compartment length ranged from <10 μm to >110 μm with only 26% falling between 30 and 40 μm, and 20% falling between 40 and 50 μm. In wild type, nuclear number per compartment was between 1 and 7, with 87% of compartments containing two to four nuclei (Figure 5C). In *swoC1*, however, nuclear number per compartment ranged from 1 to 20, with only 47% of compartments containing two to four nuclei. The compartment length variation in the *swoC1* mutant probably reflects nuclear position variation since in this filamentous fungus, the site of septation is determined by nuclear position (WOLKOW *et al.* 1996).

In wild-type *A. nidulans*, asexual reproduction produces uniform uninucleate conidia on a reproductive structure called the conidiophore (TIMBERLAKE 1991; ADAMS *et al.* 1998). Conidiophore development starts as aerial hyphae elongate and swell at the tip to form a vesicle (Figure 6a). From the vesicle forms a layer of primary sterigmata called metulae (Figure 6b). The metulae bud twice to form a layer of uninucleate phialides (Figure 6c). The phialides produce chains of uninucleate conidia after repeated mitotic division and cytokinesis (Figure 6d). Mitosis, nuclear migration, and cytokinesis must be tightly coordinated to ensure normal conidiation. To determine whether an early delay in polarity establishment affects asexual reproduction in the *swoC1* mutant, we incubated wild type and the *swoC1* mutant at restrictive temperature for 9 hr, shifted to permissive temperature for 2 days, and observed conidiophores. After this slight delay in polarity establishment, all mutant conidiophores ($n > 50$) appeared to be missing cell layers (Figure 6, g and h). About 5.5% of *swoC1* conidia contained more than one nucleus and

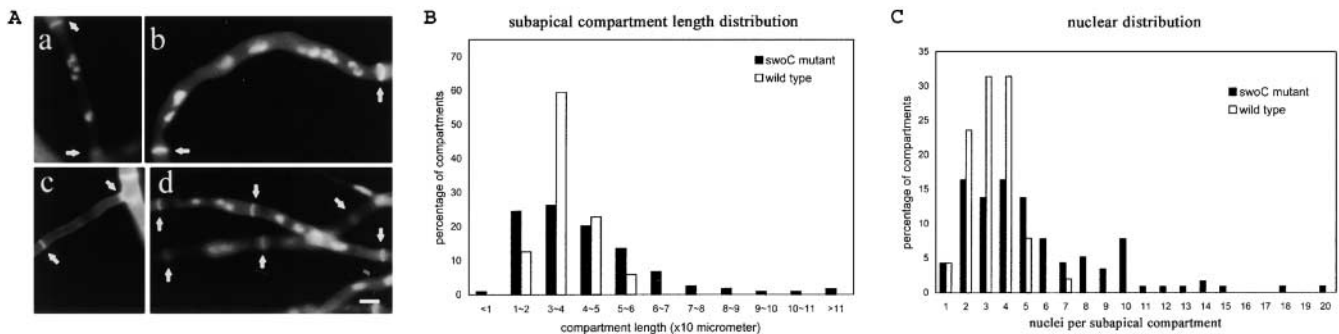


FIGURE 5.—The *swoC1* mutant shows variation in subapical compartment length and nuclear number after a delay in polarity establishment. Conidia of wild-type A773 and *swoC1* mutant AXL8 were incubated at restrictive temperature (42°) for 9 hr and shifted to permissive temperature (30°) for 15 hr. Cells were fixed and stained with Hoechst 33258 and calcofluor. Nuclear number per compartment and compartment length were measured ($n = 120$). (A) Micrographs of *swoC1* (a–c) and wild type (d): (a) *swoC1* compartment with crowded nuclei; (b) *swoC1* compartment with 10 nuclei; (c) *swoC1* compartment with no nuclei; (d) wild-type compartments with relatively uniform length and two to four nuclei. Arrows point to septa. Bar, 5 μm. (B) Compartment length. (C) Number of nuclei per compartment.

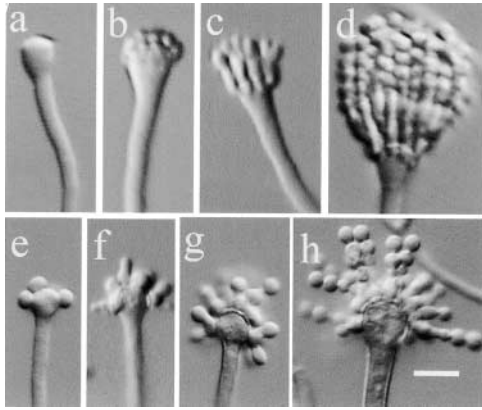


FIGURE 6.—The *swoCI* mutant shows abnormal conidiation after a delay in polarity establishment. (a–d) Wild-type A773 and (e–h) *swoCI* mutant AXL8 were grown at 42° for 9 hr and shifted to 30° for 2 days. Cells were fixed in 100% ethanol. (a) Vesicle; (b) metulae; (c) phialides; (d) chains of conidia; (e) metulae; (f–h) chains of conidia. Bar, 10 μ m.

4.5% contained no nucleus ($n = 200$). The *swoCI* mutant incubated only at permissive temperature showed lower levels of these morphological defects with 1% of conidia containing more than one nucleus or no nucleus (data not shown).

The *swoC* gene encodes rRNA pseudouridine synthase

The *swoC* gene maps near the centromere of chromosome III: To identify the chromosome on which the *swoC* gene lies, we took advantage of the parasexual cycle in *A. nidulans* (KAFFER 1977). A heterozygous diploid was made between the *swoCI* mutant strain AGA22 and the mitotic mapping strain A104, which has a marker on each chromosome. The diploid was treated with the microtubule-destabilizing drug benomyl to stimulate chromosome loss until a stable haploid state was reached. The chromosomes in each haploid sector are expected to represent a random mixture derived from either the *swoCI* mutant or the mitotic mapping strain. The genotypes of all haploid sectors were scored. The *phenA2* marker on chromosome III segregated 100% in repulsion to the *swoCI* temperature-sensitive phenotype in these haploid sectors. Therefore the *swoC* gene is on chromosome III.

To define the position of the *swoC* gene on chromosome III, the *swoCI* mutant strain AGA22 was crossed with the meiotic mapping strain A457. The distance between the *swoCI* allele and other markers on chromosome III was determined by recombination frequency. The *swoCI* allele was tightly linked to the *phenA2* marker near the centromere of chromosome III.

The *swoC* gene encodes a homolog of rRNA pseudouridine synthase: On the basis of the physical map of *A. nidulans* (<http://gene.genetics.uga.edu/>), 20 cosmids near the centromere of chromosome III were chosen

from the chromosome-specific library and transformed into the *swoCI* mutant. Only cosmid W21H06 complemented the *swoCI* ts⁻ phenotype.

In a separate experiment, three autonomously replicating plasmids from a random plasmid library were also found to restore the *swoCI* mutant to wild-type growth at restrictive temperature. Restriction mapping showed that these three plasmids contained overlapping genomic DNA inserts (data not shown).

Southern blotting indicated that the three high-copy plasmids shared a common fragment with the W21H06 cosmid (data not shown), showing that the plasmids contain the authentic *swoC* gene, rather than a suppressor of the *swoCI* mutation. The smallest plasmid, p8c1, was chosen for sequencing using a transposon-tagging strategy. Only one ORF was identified in the 8-kb *A. nidulans* genomic DNA insert in plasmid p8c1. This is not surprising since the *swoC* gene localizes to the gene-poor centromere-proximal region. Using the NCBI Blast program, the predicted SwoCp was found to be 70% identical with *S. cerevisiae* Cbf5p, 71% identical with *Kluyveromyces lactis* Cbf5p, and 63% identical with *Homo sapiens* Dkc1p (Figure 7). All are members of a highly conserved family of eukaryotic rRNA pseudouridine synthases. Like other members of this family, SwoCp contains several predicted domains: a pseudouridylate synthase domain (TruB), an RNA-binding domain (PUA), a microtubule (MT)-binding domain, a nuclear localization signal (NLS), and a coiled-coil protein-protein interaction domain (Figure 7).

Sequencing of the *swoCI* mutant allele amplified from strain AXL8 by PCR showed a G-to-T mutation at base 1220, which results in a valine-to-phenylalanine substitution at amino acid 338 in the predicted PUA domain.

Deletion of the *swoC* gene is lethal in *A. nidulans*: Even though elimination of all detectable pseudouridine residues in rRNA from *S. cerevisiae* has no phenotype (BOUSQUET-ANTONELLI *et al.* 1997), deletion of the *CBF5* gene is lethal in yeast (GANOT *et al.* 1997). rRNA pseudouridine synthase knock-out mutants have not yet been described for other organisms. Because we expected that deletion of *swoC* was likely to be lethal in *A. nidulans*, we constructed a heterozygous diploid wherein one copy of the *swoC* gene was replaced with the *argB* marker by homologous integration (Figure 8). We induced haploidization of the heterozygous diploid and scored haploid sectors (KAFFER 1977). None of the 206 haploid sectors recovered was *argB*⁺, indicating that only haploids with the intact *swoC* gene survived. Therefore, the *swoC* gene is essential in *A. nidulans*.

The C terminus of SwoCp is dispensable: To determine which portion of the *swoC* gene is required to complement the *swoCI* mutation, we took advantage of the transposon inserts in plasmid p8c1 created for sequencing *swoC*. Two p8c1 plasmids with transposons in the first exon and the third exon (p8c1-A11) of the gene were not able to restore the *swoCI* mutant to wild-

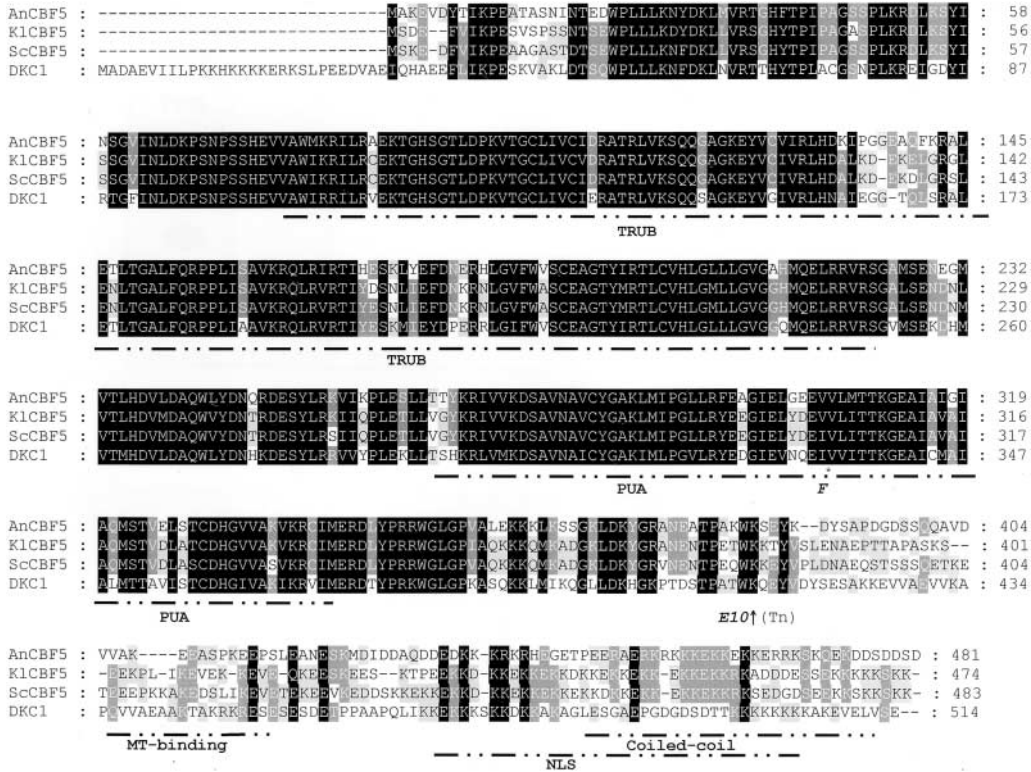


FIGURE 7.—Alignment of rRNA pseudouridine synthases. SwoC1p is from *A. nidulans* (accession no. AY057454), K1Cbf5p is from *K. lactis* (accession no. O13473), ScCbf5p is from *S. cerevisiae* (accession no. NP_013276), and Dkc1p is from *H. sapiens* (accession no. O60832). Black background indicates identical or highly similar residues. Dark and light gray backgrounds indicate 75 and 50% shared similar residues, respectively. Position of E10 Tn insertion is indicated by an arrow below the sequence. The *swoC*I V338F mutation is indicated by an asterisk and an F below the sequence. The positions of predicted motifs are indicated by the broken line beneath the sequence and labeled as follows: TRUB, pseudouridylylase synthase domain; PUA, RNA-binding domain; MT-binding, microtubule-binding domain; NLS, nuclear localization signal; coiled-coil, protein-protein interaction domain.

type growth at restrictive temperature (Figure 9c and data not shown). The p8c1 plasmid with a transposon in the first intron (p8c1-H07) also did not complement the *swoC*I phenotype (Figure 9b), likely because *A. nidulans*, which usually has 40- to 100-bp introns, could not properly process the 5-kb intron resulting from transposon insertion. Most interestingly, the plasmid p8c1 with a transposon in the C terminus (p8c1-E10) complemented the *swoC*I phenotype (Figure 9a). The E10 insertion is predicted to remove 98 amino acids from the C terminus of the encoded protein including the MT-binding domain, NLS, and coiled-coil domain. Distribution of nuclei and compartment length were similar in *swoC*I mutant strains transformed with either the intact p8c1 or p8c1-E10 (data not shown). Two explanations are possible: either the C terminus is not essential for SwoCp or the truncated *swoC* gene product can function together with the *swoC*I mutated gene product to restore the wild-type phenotype. To distinguish between these explanations, we constructed a heterozygous diploid wherein the C terminus of one copy of the *swoC* gene was replaced with the *argB* marker by homologous integration (Figure 8). We induced haploidization of the heterozygous diploid and scored haploid sectors (KAFFER 1977). Of 380 haploid sectors recovered, 327

haploid sectors were *argB*⁺, indicating that the C terminus is, indeed, dispensable.

The *swoC*I mutant shows no detectable defect in pseudouridine synthesis or rRNA processing: Pseudouridination is one of the two most abundant nucleic acid modifications and occurs predominantly in rRNA (MADEN and HUGHES 1997; OFENGAND 2002). In eukaryotes, ~1% of rRNA nucleic acids are pseudouridines (reviewed by CHARETTE and GRAY 2000; OFENGAND 2002). Evidence suggests that the box H/ACA small nuclear ribonucleoprotein particles (snRNPs) may be the universal complex responsible for all eukaryotic rRNA pseudouridine synthesis using boxH/ACA snRNAs as a guide and Cbf5p as the key enzyme in the complex (BOUSQUET-ANTONELLI *et al.* 1997; PECULIS 1997; LAFONTAINE *et al.* 1998; WATKINS *et al.* 1998; ZEBARJADIAN *et al.* 1999; CHARETTE and GRAY 2000; WATANABE and GRAY 2000; YANG *et al.* 2000; PIENKOWSKA and SZWEYKOWSKA-KULINSKA 2001; OFENGAND 2002). To determine if *A. nidulans* uses the same box H/ACA snRNP complex for rRNA pseudouridine synthesis, we searched *A. nidulans* databases and found homologs of other genes predicted to encode box H/ACA snRNP proteins (Gar1p, Nhp2p, and Nop10p) in addition to SwoCp. The presence of these highly conserved proteins

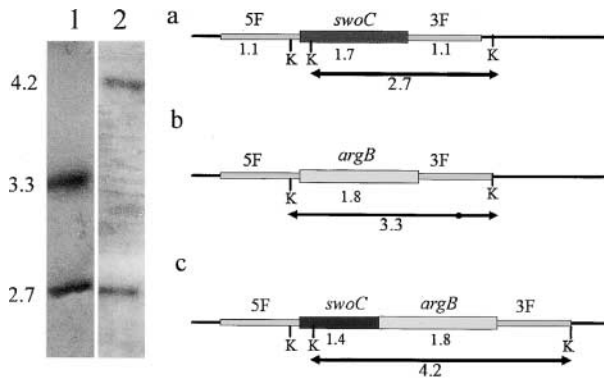


FIGURE 8.—Heterozygous diploid strains with *swoC* null allele and C-terminal deletion allele. Left, the Southern blot (1) heterozygous diploid with one copy of *swoC* replaced by *argB* and (2) the heterozygous diploid with *argB* replacing the C terminus of *swoC*. Genomic DNA was digested with *KpnI* and probed with randomly labeled 3' flanking sequence. Sizes (kilobases) based on molecular markers are shown at left. Right, the restriction map of (a) wild-type *swoC*, (b) *argB* replacement of *swoC*, and (c) *argB* replacement of *swoC* C terminus. Fragment sizes after restriction digestion are indicated at the bottom. K, *KpnI*; 5F and 3F, 5' and 3' flanking sequences, respectively.

makes it very likely that *A. nidulans* uses the same mechanism for rRNA pseudouridine synthesis.

Since >90% of total RNA is rRNA, we expected to see a gross decrease of pseudouridine in total RNA if the *swoCI* phenotype is due to loss of pseudouridine synthesis activity. Total RNA isolated from overnight cultures of wild type and the *swoCI* mutant grown at restrictive and permissive temperatures was enzymatically digested. We used HPLC coupled with MS to separate and identify pseudouridine. HPLC has long been used to detect modified nucleic acids (RUSSO *et al.* 1984; AMURO *et al.* 1988; POMERANTZ and MCCLOSKEY 1990; UMEGAE *et al.* 1990; PALMISANO *et al.* 1995; SHINGFIELD and OFFER 1999; PATTESON *et al.* 2001). Three independent experiments showed that pseudouridine levels were similar in the *swoCI* mutant and wild type at both temperatures (Figure 10 and data not shown). The other major modified nucleic acid, 2'-*O*-methylated adenosine, was also detected in both the *swoCI* mutant and wild type, indicating that our analysis was adequately sensitive (Figure 10). These results suggest that the *swoCI* mutation does not grossly affect pseudouridine synthesis *in vivo*.

In *S. cerevisiae*, certain mutations in *CBF5* cause defects in pre-rRNA processing and steady-state levels of mature cytoplasmic ribosomes (CADWELL *et al.* 1997). To determine if the *swoCI* mutant phenotype could be caused by defective rRNA processing, we isolated total RNA from the *swoCI* mutant and wild type and probed with oligonucleotides designed on the basis of 18S, ITS1, ITS2, and 25S sequences of *A. nidulans* (LAFONTAINE *et al.* 1998). The *swoCI* mutant and wild type showed

identical band size and intensity of mature rRNA products (data not shown) and accumulation of pre-rRNA was not detected. A Northern blot probed with ITS1-5.8S-ITS2 PCR product also showed no difference between wild type and the *swoCI* mutant cultured at restrictive temperature. These results suggest that rRNA processing is normal in the *swoCI* mutant, consistent with our observation that at restrictive temperature the *swoCI* mutant has sustained isotropic growth. Sustained growth is unlikely to occur if there is a severe defect in rRNA processing since breaking dormancy and growing isotropically both require protein synthesis (HERMAN and RINE 1997; WENDLAND 2001).

DISCUSSION

Polarity establishment cues persist or are reinitiated in *swoCI*: In *S. cerevisiae*, budding occurs once in each cell cycle. Sites for bud emergence are tagged by cortical markers laid down during the previous round of budding. Cdc42p is recruited to these sites, where it drives polar growth through interactions with the cytoskeleton and polarity maintenance apparatus (MADDEN and SNYDER 1992; ZIMAN *et al.* 1993; KRON *et al.* 1994; CHANT *et al.* 1995; GOODSON *et al.* 1996; SHAFATIAN *et al.* 1996; YABE *et al.* 1996; SANTOS and SNYDER 1997; MADDEN and SNYDER 1998; SHULMAN and ST. JOHNSTON 1999; SVOBODA *et al.* 2001). The identities of cortical polarity markers tagging the sites for germ tube emergence are not yet known in filamentous fungi. It is not clear whether the germ tube emergence marker is laid down during formation of the spore or after dormancy is broken (MOMANY 2002).

In the *swoCI* mutant, an extreme delay in polarity establishment did not cause loss of competence for polar growth, suggesting that the underlying polarity markers either persist or can be formed despite a delay. While polar growth could still occur when *swoCI* was shifted from restrictive to permissive temperature, the normal bipolar pattern of germ tube emergence was disrupted. We were especially surprised by the increase in the number of germ tubes with increased incubation time at restrictive temperature (Figure 3). Given the endocytosis defect of the *swoCI* mutant, it is possible that new polarity markers are synthesized during incubation at restrictive temperature and that failure to remove these markers leads to the emergence of multiple germ tubes in random positions after release of the temperature block. It is also possible that the increased number of nuclei that accumulate in the *swoCI* mutant at restrictive temperature triggers the synthesis of additional polarity markers. *A. nidulans nud* mutants also accumulate nuclei and send out multiple germ tubes when they polarize, though the mechanism is not clear (XIANG *et al.* 1999). In regenerating yeast protoplasts containing several nuclei, multiple buds emerged simultaneously at random

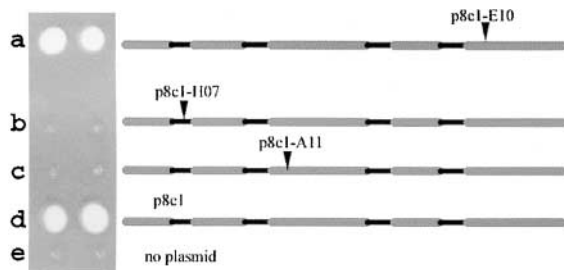


FIGURE 9.—Transposon insertions affect the ability of p8c1 to complement *swoCI*. The *swoCI* mutant carrying p8c1 with transposon insertions was grown at restrictive temperature (42°) for 2 days. The *swoCI* mutant was transformed with (a) p8c1-E10, (b) p8c1-H07, (c) p8c1-A11, (d) p8c1, and (e) no plasmid. Positions of insertions are indicated diagrammatically at right. Shaded bars, exons; solid lines, introns.

sites thought to be determined by the positions of the nuclei (SVOBODA *et al.* 2001).

Delayed polarity establishment perturbs nuclear distribution and development in *swoCI*: In wild-type *A. nidulans* the isotropic-to-polar switch normally occurs just after the first mitosis, when spores have two nuclei. Polar growth and nuclear division continue, nuclei become distributed evenly along the germ tube, and the hypha is partitioned by evenly spaced septa (HARRIS *et al.* 1994; MOMANY and TAYLOR 2000). Previous work has shown that although nuclear division and polar growth are coordinated, they are not dependent (MOMANY and TAYLOR 2000). The *swoCI* mutant continues nuclear division in the absence of polar growth, consistent with the notion that nuclear division and polar growth employ independent pathways. Surprisingly, a slight delay in polarity establishment in the *swoCI* mutant resulted in abnormally spaced nuclei and septa and conidiophores with missing layers. It is possible that the abnormal septal spacing and conidiophore development resulted from the subtle nuclear distribution defect. Nuclear positioning has been shown to determine the septation site in *A. nidulans* (WOLKOW *et al.* 1996) and conidiation requires coordination of growth, nuclear distribution, and cytokinesis (ADAMS *et al.* 1998). However, it is also possible that the *swoCI* mutation affects an unidentified signal also involved in nuclear distribution, septation, and asexual development. Regardless of the mechanism, such clear morphological consequences of delayed polarity establishment suggest that spatial cues for development must be properly set up very early in hyphal growth.

The *swoCI* phenotype is likely related to cryptic function in the PUA domain: Surprisingly, the gene that complemented the *swoCI* ts⁻ phenotype was >60% identical with rRNA pseudouridine synthases from other eukaryotes. On the basis of our inability to detect any change in multiple activities associated with rRNA pseudouridine synthases, it is reasonable to propose that

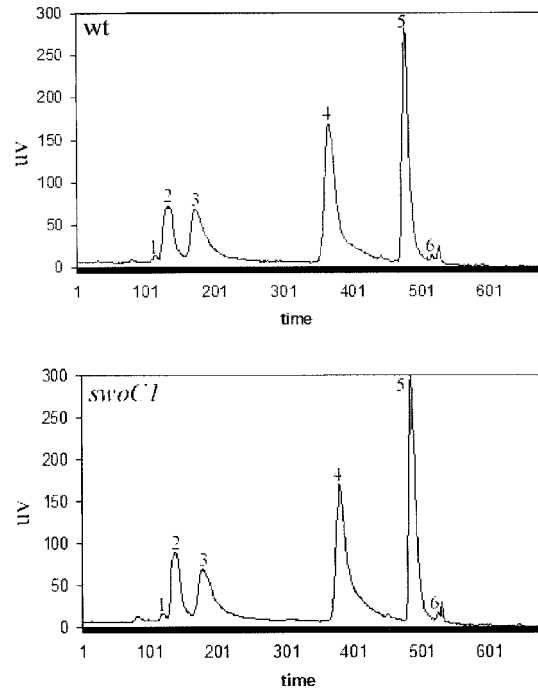


FIGURE 10.—The *swoCI* mutant shows normal pseudouridine level. Total RNA was isolated from wild type and *swoCI* incubated at 42° for 18 hr, enzymatically digested, and analyzed by HPLC-MS. Numbered peaks were identified by MS as follows: (1) pseudouridine, (2) cytidine, (3) uridine, (4) guanosine, (5) adenosine, and (6) 2'-*O*-methylated adenosine.

we cloned a suppressor rather than the authentic *swoC* gene. However, we genetically mapped the *swoCI* mutation to the centromere of chromosome III. Only cosmid W21H06 from the chromosome III centromere region complemented the *swoCI* ts⁻ phenotype. The rRNA pseudouridine synthase gene from complementing plasmids hybridized to the W21H06 cosmid. In addition, sequencing revealed a point mutation in the PUA domain in the *swoCI* mutant allele. Thus the rRNA pseudouridine synthase gene is the authentic *swoC* gene and not a suppressor.

If *swoC* encodes a pseudouridine synthase, why do we fail to detect any changes in pseudouridine levels or rRNA processing in the *swoCI* mutant? The most obvious explanation is that the mutation affects some function of SwoCp other than pseudouridine synthesis. Mutations in the TruB catalytic sites of *CBF5* inhibit pseudouridine synthesis and rRNA processing. Indeed, the TruB domain is intact in the *swoCI* mutant allele. This is consistent with work on yeast Cbf5p and the *E. coli* pseudouridine synthase RluD, implying that the pseudouridine synthesis function of the enzyme is not critical for cell growth while the protein itself is essential (BOUSQUET-ANTONELLI *et al.* 1997; GUTGSELL *et al.* 2001; OFENGAND 2002).

Our results suggest that the unknown essential function of SwoCp may require the PUA domain. The *swoCI*

mutation occurs in the PUA domain, a conserved RNA-binding domain found in both archaea and eukaryotes (BECKER *et al.* 1997; ARAVIND and KOONIN 1999). Interestingly, another pseudouridine synthase in yeast, which modifies cytoplasmic and mitochondrial tRNAs, does not contain a PUA domain, consistent with the idea that the PUA domain may contribute to binding of a specific RNA structure (BECKER *et al.* 1997). The *swc1* V338F mutation may disrupt the ability of Swc1p to bind certain RNA substrates, while retaining the pseudouridine synthase enzyme activity. In X-linked dyskeratosis congenita patients, many mutations of DKC1 occur in or around the PUA domain (KNIGHT *et al.* 1999). These mutations may alter the interaction of Dkc1p with telomerase hTR, which has a box H/ACA motif (MITCHELL *et al.* 1999). We did not detect any telomere length change in the *swc1* mutant after numerous replications at restrictive temperature (our unpublished observation).

It is possible that an RNA substrate of Swc1p other than box H/ACA snoRNA might be affected by the *swc1* mutation. Indeed, asymmetric distribution of RNA is critical for development in many organisms (MICKLEM 1995; STEPHEN *et al.* 1999; VAN EEDEN and ST. JOHNSTON 1999; STEBBINGS 2001). In *Drosophila* and *Xenopus*, mRNA localizes to opposite poles of the oocyte. Perhaps Swc1p participates in a similar asymmetric mRNA localization in *A. nidulans* development. It is also possible that the PUA domain might serve as a DNA-binding domain since yeast Cbf5p has been shown to bind centromere and kinetochore complexes *in vitro* (JIANG *et al.* 1993).

We cannot rule out the possibility that the *swc1* mutation does not directly cause the nonpolar phenotype. Many different mutations have been reported to result in a nonpolar phenotype. In yeast, defects in a 60S ribosomal subunit protein QSR1 (EISINGER *et al.* 1997), a C53 subunit of RNA polymerase (MANN *et al.* 1992), and a ubiquitin ligase SCF (PATTON *et al.* 2000) result in formation of large unbudded cells. In *A. nidulans*, two polarity-defective ts⁻ mutants (*podG* and *podH*) have defects in the α -subunit of mitochondrial phenylalanyl-tRNA synthase and transcription factor IIF interacting component of the CTD phosphatase, respectively (OSHEROV *et al.* 2000). This evidence suggests that polarity establishment requires coordination of multiple processes. The *swc1* mutation may perturb one or more of these processes, which in turn may block polarity establishment.

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