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Polarity-Defective Mutants of *Aspergillus nidulans*

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Osherov, N., Mathew, J., and May, G. S. 2000. Polarity-defective mutants of *Aspergillus nidulans*. *Fungal Genetics and Biology* 31, 181–188. We have identified two polarity-defective (*pod*) mutants in *Aspergillus nidulans* from a collection of heat-sensitive lethal mutants. At restrictive temperature, these mutants are capable of nuclear division but are unable to establish polar hyphal growth. We cloned the two *pod* genes by complementation of their heat-sensitive lethal phenotypes. The libraries used to clone the *pod* genes are under the control of the bidirectional *niaD* and *niiA* promoters. Complementation of the *pod* mutants is dependent on growth on inducing medium. We show that rescue of the heat-sensitive phenotype on inducing media is independent of the orientation of the gene relative to the *niaD* or *niiA* promoters, demonstrating that the intergenic region between the *niaD* and the *niiA* genes functions as an orientation-independent enhancer and repressor that is capable of functioning over long distances. The products of the *podG* and the *podH* genes were identified as homologues of the α subunit of yeast mitochondrial phenylalanyl–tRNA synthetase and transcription factor IIF interacting component of the CTD phosphatase. Neither of these gene products would have been predicted to produce a *pod* mutant phenotype based on studies of cellular polarity mutants in other organisms. The implications of these results are discussed.

Index Descriptors: Aspergillus nidulans; cell polarity; enhancer; gene expression.

Cellular polarity is a fundamental property of all cells. The factors that determine cell polarity have been the subject of extensive investigation in a variety of experimental organisms (Drubin and Nelson, 1996). The most extensive investigations of determinants of cell polarity have been conducted in the budding yeast *Saccharomyces cerevisiae* (Pruyne and Bretscher, 2000a,b) and the fission yeast *Schizosaccharomyces pombe* (Arellano *et al.*, 1999). The extensive investigations of cell polarity in these two yeast have implicated a variety of proteins of the actin cytoskeleton and the small G protein Cdc42p signaling pathway in determining the site of polar cell growth and its maintenance (Adams *et al.*, 1990). These elements are also important in determining cellular polarity in all eukaryotes, pointing to the ancient origin of cell polarity.

The pattern of growth exhibited by filamentous fungi is an extreme example of cell polarity. Vegetative growth in filamentous fungi is exclusively by apical extension at hyphal tips. Surprisingly, there have been few systematic genetic studies of polarity in filamentous fungi (Harris *et al.*, 1999; McGoldrick *et al.*, 1995; Momany *et al.*, 1999). Though this is the case, it seems likely that those genes that determine and maintain cell polarity in other eukaryotes will be found to function the same way in filamentous fungi. The results of our study of two polarity-defective (*pod*) mutants in the filamentous fungus *Aspergillus nidulans* suggest that genes other than those already identified can produce the *pod* mutant phenotype. The results also suggest the need for caution in the interpretation of a mutant phenotype.

MATERIALS AND METHODS

***A. nidulans* strains and growth media.** The strains used in this study were GR5 (*pryG89; wA3; pyroA4*),

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R153 (*wA3; pyroA4*), podE3 (*pyrG89 riboA1; pyroA4; podG3*), podF40 (*pyrG89; pyroA4; podH40*), and mpr16 (*pyrG89; wA3; pyroA4; myoA*^{mpr16}). The *pod* mutants were obtained from a collection of heat-sensitive mutants whose isolation was previously described (Osherov and May, 2000). MAG (2% malt extract, 0.2% peptone, 1% dextrose, trace elements (Cove, 1977), 1 μg/ml pyroxidine, 8.8 μg/ml riboflavin, and 2% agar for solid medium) was used for routine propagation of strains. Minimal medium was composed of 70 mM $NaNO_3$, 7 mM KCl, 4 mM $MgSO_4$, 12 mM KPO_4, pH 6.8, trace elements, and 1% (w/v) glucose. This medium was supplemented with 5 mM uridine, 10 mM uracil for *pyrG89* mutant strains. Genetic methods and other media employed were those described previously (Kafer, 1977; Pontecorvo *et al.,* 1953).

Microscopy and nuclear staining. Conidia were inoculated at 10^6 conidia/ml into petri dishes containing liquid medium and sterile coverslips. After incubation, cells were fixed using 4% freshly prepared paraformaldehyde and 0.1% Triton X-100 in 10 mM KPO_4, pH 6.8, for 10 min. Cells were washed twice in distilled water and stained for 15 min in 4′,6-diamidino-2-phenylindole (DAPI; 100 ng/ml). Cells were then washed twice in phosphate-buffered saline (PBS) and mounted in 50% (v/v) glycerol, 0.5% (w/v) *n*-propyl gallate in PBS. Cells were viewed using a Nikon Microphot-SA microscope. Images were captured into Adobe Photoshop using a Model C2400 camera, a SCSI adapter, and twain driver.

Cloning and identification of pod genes. We previously described the construction of the plasmid pRG3-AMA1 and a genomic library in the vector (Osherov and May, 2000). We changed the polylinker of pRG3-AMA1 to contain a *Not*I restriction site to facilitate the recovery of inserts by the addition of a double-stranded adapter composed of the oligonucleotides NotI top GATCCGCGGCCGCGCATG and NotI bottom CGCGGCCGCG, ligated to pRG3-AMA1 previously digested with *Bam*HI and *Sph*I to create pRG3-AMA1-NotI. The intergenic region between the *niiA* and the *niaD* genes was polymerase chain reaction (PCR)-amplified from cosmid W30C7 (available from the Fungal Genetics Stock Center, Kansas City, KS) using the oligonucleotides Kpn5′ AGCTGGTACCCATTGTGAGAGTATGGGA and Kpn3′ AGTCGGTACCCATGATGGCGGGCGCGGT. The product of the PCR was digested with *Asp*718 and cloned into pRG3-AMA1-NotI digested with *Asp*718 and dephosphorylated with calf intestinal alkaline phosphatase. Two plasmids were obtained differing only in the orientation of the intergenic *niiA-niaD* region. Libraries were constructed using *Sau*3AI partially digested *A. nidulans* genomic DNA from strain R153 that was size-fractionated (4–12 kb) and ligated into *Bam*HI-cut and phosphatase-treated vectors. Two libraries were constructed, one for each orientation of the intergenic *niaD-niiA* region. The libraries consisted of enough independent clones with an average insert size of 5 kb to contain 20 genome equivalents. Transformations were performed as described previously except that transformant colonies were selected for on minimal medium containing vitamins and 1 M sucrose to osmotically stabilize the protoplasts (May, 1989). Transformants (approximately 5000 *pyrG*$^+$ transformants/plate) were incubated at the restrictive temperature (42°C) for 2–3 days, yielding approximately one to five rescued transformants per plate. Genomic DNA was prepared from these transformant colonies and used to transform electrocompetent *Escherichia coli* cells. For each gene, 20 ampicillin-resistant colonies were analyzed for the recovery of the plasmid.

The complementing gene within a cloned insert was identified by randomly mutagenizing the rescuing plasmid with a transposon (GPS-1 system; New England Biolabs, Beverly, MA). Individual transposon-tagged clones were transformed into the *pod* mutant of interest. Plasmids that failed to rescue the mutant at the restrictive temperature were assumed to have insertions disrupting the complementing gene. These were sequenced using primers unique for the transposon ends. The *podG* and *podH* genes were identified and partially sequenced this way. The sequences derived from the transposon ends were used to search the DNA databases using the Blast program at the NCBI WEB site.

RESULTS

From a collection of heat-sensitive mutants, we identified candidate polarity-defective mutants (Osherov and May, 2000). The heat-sensitive and *pod* mutant phenotypes segregated as a single gene mutation in the *podG3* and *podH40* mutant strains when crossed to the strain GR5. Wild-type *A. nidulans* growth is characterized by an initial phase of isotopic growth or spore swelling followed by polar hyphal growth coupled with nuclear division (Fig. 1). The *podG3* and *podH40* mutants each fail to establish polar hyphal growth at restrictive temperature (Fig. 1). They do not have a nuclear division cycle defect as demonstrated by the presence of multiple nuclei in a spherical cell (Fig. 1). The mean number of nuclei per swollen spore was 2.60 for *podG3* (n = 15) and 2.58 for *podH40* (n = 30). The extent of nuclear division in both mutants indi-

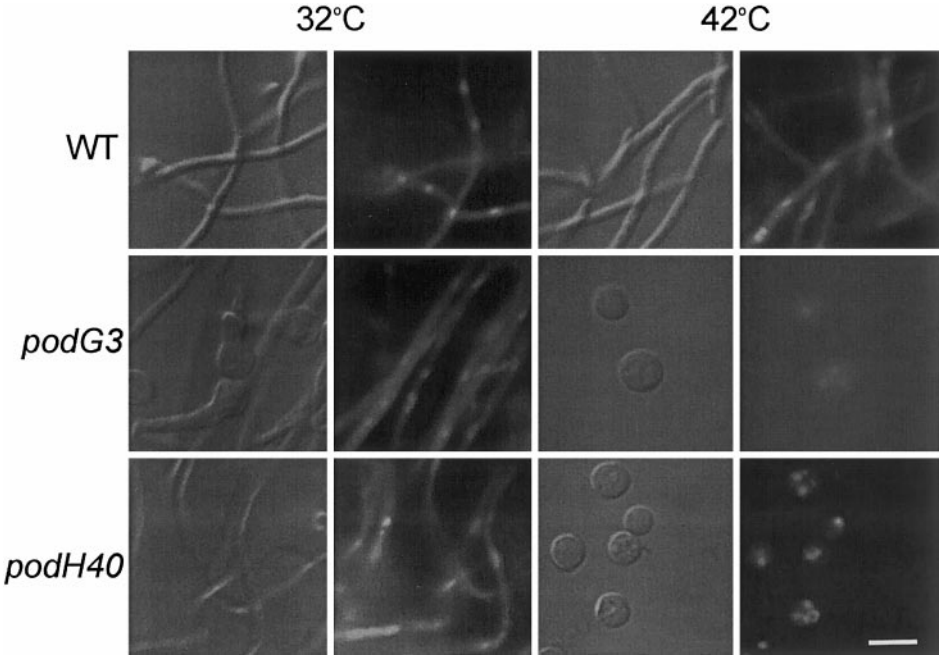

FIG. 1. Paired differential interference contrast (DIC) and DAPI fluorescence micrographs showing fungal cell morphology and nuclear number and distribution. The left half of the figure shows wild-type (WT) and *podG3* and *podH40* mutant strains grown at permissive temperature (32°C) for 14 h. The right half of the figure shows the same strains grown at restrictive temperature (42°C) for 14 h. The scale bar in the lower right-hand panel is 5 μm.

cates that they typically undergo one to two mitotic cycles before arrest.

We next investigated whether the *podG3* and *podH40* mutants would lose polarity once they had established it or regain polarity after germination at restrictive temperature in temperature shift experiments (Fig. 2). In the first experiment, wild type, *podG3,* and *podH40* conidia were allowed to germinate overnight in liquid medium at room temperature and then were shifted to restrictive temperature and incubated for 7 h. Following germination at permissive temperature, all the strains had established polar hyphal growth. After 7 h at restrictive temperature, the wild type continued to grow normally but both the *pod* mutant strains displayed abnormal hyphal morphology. The *podG3* mutant had increased hyphal width and some tip swelling, and the *podH40* mutant had swollen hyphal tips and increased numbers of intracellular vacuoles. Both mutant strains stopped growing after 8–12 h of incubation at the restrictive temperature (not shown). Thus, both mutants appear to lose the ability to establish and maintain polarity after the shift to the restrictive temperature. Next, we incubated the strains overnight at restrictive temperature and then shifted them to room temperature and allowed them to grow for 7 h. As expected, the wild-type strain grew into a mycelium after incubation overnight and continued to grow at room temperature. In contrast, the *podG3* and *podH40* mutants remained spherical after incubation overnight at restrictive temperature and both mutants were able to send out short germ tubes after 7 h at room temperature. Thus, the mutants appear to retain the ability to establish polarity and initiate hyphal growth after having been prevented from doing so for an extended period. The failure to establish completely normal hyphal growth after germination overnight is most likely the result of extended incubation at restrictive temperature. Based on their growth defects and the fact that the nuclear division cycle continued in the absence of polar hyphal growth, we consider these mutations good candidate genes that specifically function in establishing polar hyphal growth.

We cloned the *podG* and *podH* genes by complementation of the heat-sensitive lethal mutant phenotypes of the *podG3* and *podH40* mutants, respectively, using novel expression vectors that we developed (Fig. 3A). The vectors consisted of pRG3-AMA1 (Osherov and May, 2000; Waring *et al.,* 1989) with a modified polylinker and the intergenic promoter sequences found between the *niaD* and the *niiA* genes cloned in two orientations. To test whether a gene downstream of the *niaD* or *niiA* promoters could rescue a conditional mutant phenotype, we cloned

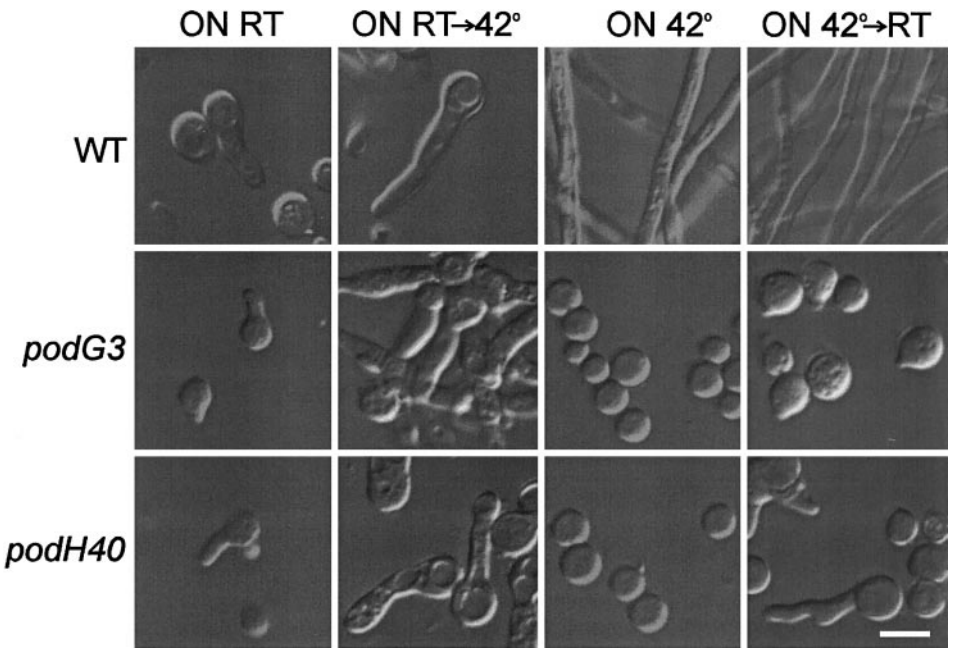

FIG. 2. Effect of temperature shifts on fungal cell growth and morphology for wild-type (WT), *podG3*, and *podH40* strains viewed by DIC microscopy. The left half of the figure shows the effect of germinating conidia overnight at room temperature and then 7 h after shifting the germlings to restrictive temperature. The right half of the figure shows the effect of germinating conidia overnight at restrictive temperature and then 7 h after shifting the germlings to room temperature. The scale bar in the lower right hand panel is 5 μm.

wild-type *myoA* in front of the *niiA* promoter construct and transformed it into a cold-sensitive *myoA* mutant, mpr16. The mpr16 mutant strain grows on YAG medium at 37°C, the permissive temperature (Fig. 3B). Growth of mpr16 transformed with the empty vector or niiA-myoA expression vector was then tested at restrictive temperature (20°C) on nitrate (inducing) or ammonium (repressing) minimal media (Fig. 3B). The mpr16 strain transformed with niia-myoA grew at restrictive temperature only on the inducing-media plates. Similar results are obtained when *myoA* is downstream of the *niaD* promoter (data not shown). This demonstrates that this vector set can be used to conditionally rescue a mutant phenotype by choice of nitrogen source in the medium.

Having demonstrated that the *niaD-niiA* intergenic region could rescue a cold-sensitive *myoA* mutant and was dependent on the nitrogen source in the medium, we tested genomic DNA libraries made in the two inducible vectors and one in pRG3-AMA1 for complementation of the *podG3* and *podH40* mutant phenotypes. *podG* and *podH* transformant colonies were selected for at restrictive temperature on minimal medium nitrate (inducing) plates that would drive expression from both the *niaD* and the *niiA* promoters. All three libraries produced transformant colonies that complemented the heat-sensitive mutant phenotype. The two inducible libraries produced transformant colonies that complemented the heat-sensitive growth phenotype of the mutants in a nitrogen source-dependent manner (Table 1 and Fig. 3C). The number of transformant colonies obtained for each of the mutants varied because of the reduced transformation efficiency for the *podH40* mutant strain. DNA was prepared from eight independent rescued transformants of *podG* and four of *podH* and used to transform electrocompetent *E. coli* cells. For both the *podG3* and the *podH40* mutant strains the AMA1-library plasmids recovered from each of these transformant colonies was shown to be overlapping because the genomic inserts cross-hybridized. Therefore, although additional analysis is necessary to formally conclude that we have cloned the mutated gene and not suppressors, we have reason to believe, based on our repeated recovery of a single gene from multiple transformants, that we have cloned the genes of interest.

A plasmid that complemented either the *podG3* or the *podH40* mutant was subjected to transposon mutagenesis. A collection of transposon-tagged plasmids was then used to transform the appropriate mutant strain. Those plasmids that did not rescue the heat-sensitive mutant pheno-

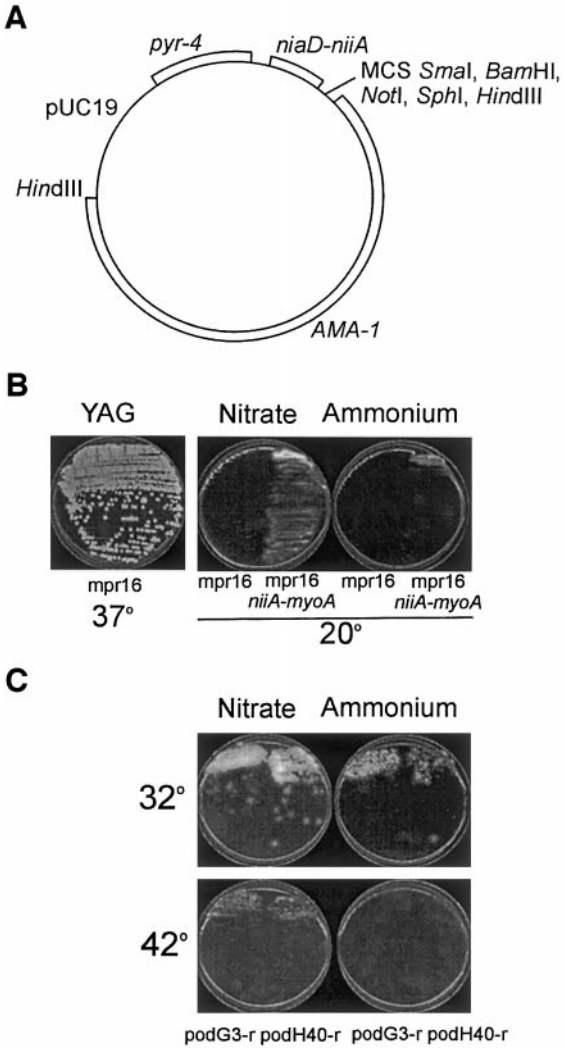

FIG. 3. (A) Map of the structure of the expression vectors using the *niaD-niiA* intergenic promoter. The two vectors differ only by the orientation of the promoter, the segment labeled *niaD-niiA*. (B) Rescue of the cold-sensitive mpr16 *myoA* mutant strain by the plasmid *niia-myoA* is dependent on growth on minimal nitrate medium at restrictive temperature. (C) Rescue of the heat-sensitive phenotypes of the *podG3* and *podH40* mutants at restrictive temperature is dependent on nitrate as the sole nitrogen source in the medium.

type were then sequenced from the two ends of the transposon and DNA sequences were derived. We obtained approximately 2.6 kb of sequence from the *podG3* mutant complementing plasmid (Accession No. AF265224) and identified the gene as a homologue of the α subunit of the yeast mitochondrial phenylalanyl–tRNA synthetase (Fig. 4). Similarly, we obtained approximately 2.5 kb of sequence from the *podH40* mutant complementing plasmid (Accession No. AF265225). The gene identified on this plasmid was a homologue of the yeast TFIIF interacting component of CTD phosphatase (Fig. 4).

To better understand how the expression libraries worked, we examined the structure of one *niiA* and one *niaD* library plasmid that complemented the *podG3* mutant strain by DNA sequencing and restriction mapping (Fig. 5). Interestingly, neither of the plasmids that we characterized had the complementing gene fused downstream of the *niaD* or *niiA* promoters as was expected. Instead, the gene was transcribed in the opposite direction and in the case of the *niaD* library plasmid was approximately 10 kb away from the promoter. These results, although surprising, are not without precedent. Similar results, showing gene activation at a distance, have been demonstrated in an AMA-1-containing vector containing a strong constitutively active promoter (Aleksenko *et al.*, 1996).

DISCUSSION

We have characterized two polarity-defective mutants, *podG3* and *podH40*. We have cloned the genes by complementation of their heat-sensitive lethal phenotype. *podG* codes for a homologue of the α subunit of the yeast mitochondrial phenylalanyl–tRNA synthetase and *podH* is a homologue of the yeast TFIIF interacting component of CTD phosphatase (Archambault *et al.*, 1997). Cloning of the *podG* and *podH* genes was facilitated using two new genomic libraries made in vectors containing the autonomously replicating AMA1 sequence (Aleksenko *et al.*, 1996). The two libraries differ only by the orientation of the *niaD-niiA* intergenic promoter region in each of the libraries. We developed these two libraries as inducible expression libraries to facilitate the cloning of high-copy or overexpression suppressors of conditional lethal mutants.

TABLE 1
Results of Transformation of *podE3* and *podF40* Mutant Strains

Mutant/library	Number of hs^+ transformant colonies	Number of colonies plasmids rescued from
podE3/AMA	25	2
podE3/niiA	11	6
podE3/niaD	8	6
podF40/AMA	2	2
podF40/niiA	1	1
podF40/niaD	1	1

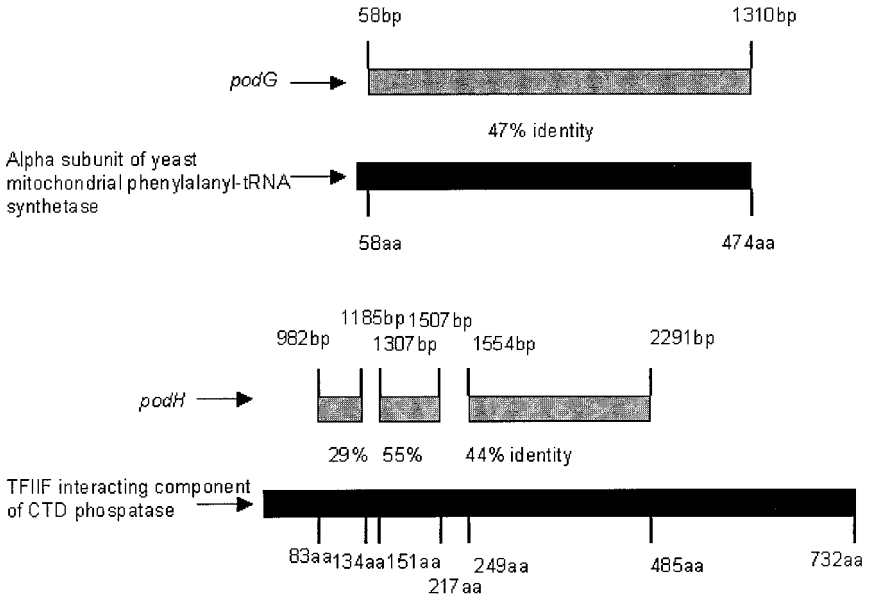

FIG. 4. Schematic representation of comparisons between *podG* and *podH* genes to known genes in the database. *podG* and *podH* were cloned by complementation and sequenced as described under Materials and Methods. Sequence data for *podG* and *podH* were used to run Blast database searches to identify possible *pod* gene homologues. Regions of significant sequence identity and their range are shown above the homologous gene. Accession Nos. for the *podG* and *podH* are AF265224 and AF265225, respectively.

The genetic analysis of cellular polarity in other systems has shown that elements of the actin cytoskeleton and components of the Cdc42 small G protein signaling pathway play major roles (Adams *et al.*, 1990). When we began our study, we anticipated that we would identify the homologues of these components in *A. nidulans.* It was surprising, therefore, to find that *podG3* and *podH40* mutations were in genes that encode proteins that are neither components of the actin cytoskeleton system nor components of the Cdc42 G protein signaling system. Why do mutations in the α subunit of the homologue of the yeast mitochondrial phenylalanyl–tRNA synthetase or a homologue of the yeast TFIIF interacting component of CTD phosphatase produce the *pod* mutant phenotype?

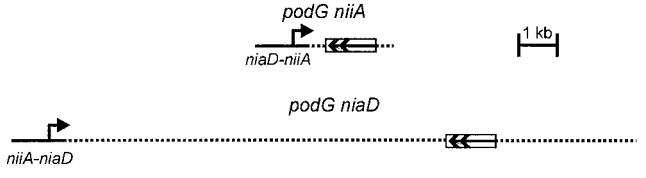

FIG. 5. Diagram of the plasmids *podG-niiA* and *podG-niaD* showing the *niaD-niiA* intergenic promoter region (solid black line with arrow), the *podG* gene (box with double arrowhead showing direction of transcription for the gene), and other genomic DNA sequences (broken line). The structure of *podG-niiA* was determined by DNA sequencing from the *niiA* promoter using a oligonucleotide primer and restriction mapping. That of *podG-niaD* was determined by restriction mapping.

First, it is important to note that not all the yeast genes whose inactivation results in a pod-like phenotype are directly related to the establishment of cell polarity. For example, defects in QSR1, a 60S ribosomal subunit protein (Eisinger *et al.*, 1997), RPC53, the C53 subunit of RNA polymerase (Mann *et al.*, 1992), SCF (Met30), a ubiquitin ligase (Patton *et al.*, 2000), and many others all result in the formation of large unbudded cells characteristic of the pod phenotype.

Second, conidial germination and the establishment of polar hyphal growth are distinct in many ways from the polarity determination processes seen in the more traditional rapidly proliferating systems (such as *S. cerevisiae*) used previously to study cellular polarity. Conidia are metabolically inactive or dormant cells designed to store the genetic information of the organism until the spore finds itself in an environment suitable for supporting vegetative growth. Conidia therefore represent a unique metabolic state in which nuclear, mitochondrial, and probably other organelles are placed in a kind of suspended animation or stasis. This state of stasis probably also requires that these organelles, certainly the case for the nucleus based

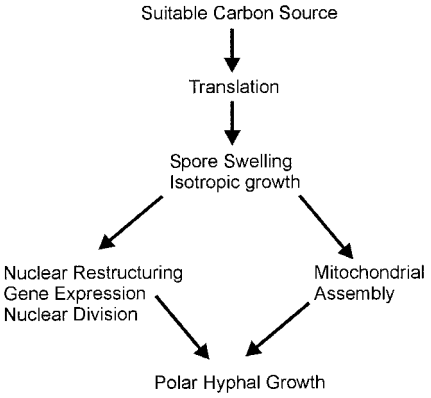

FIG. 6. Proposed pathway for the sequence of biochemical and morphological events of conidial germination in *A. nidulans* based on our previous studies of spore germination mutants (Osherov and May, 2000) and our present studies of the *podG3* and *podH40* mutants.

on purely morphological criteria, be restructured, leading to their inactivation. Recovery from this dormant state, the process of germination, requires that these organelles be reorganized into a state that supports their normal vegetative or proliferative functions. Therefore, failure to reestablish mitochondrial function, as in the case of the *podG3* mutant, or normal transcriptional regulation, as in the case of the *podH40* mutant, results in a *pod* mutant phenotype because the mutants block cellular proliferation late in spore germination relative to nuclear division but before cellular polarity and hyphal growth is established.

An alternative explanation may be found in the genetic screen used to identify the *podG3* and *podH40* mutants (Osherov and May, 2000). These heat-sensitive mutants were identified in a screen for spore germination-defective (*sgd*) mutants. These mutants were enriched by germination at restrictive temperature in the presence of the fungicide nystatin, which kills metabolically active germlings. The screen was designed to kill any hyphae that initiate active growth and enrich mutants defective in the early stages of spore germination. Perhaps this resulted in the loss of later-stage pod-like phenotypes that are more directly involved in the establishment of cell polarity.

The results reported in this paper, combined with those of our earlier study of spore germination, lead to a temporal model of events in early conidial germination and polar hyphal growth for *A. nidulans* (Fig. 6). We propose that conidial germination is a sequential, multistep process that begins when a suitable carbon source is detected by the conidium. This is followed by the initiation of translation and a period of spore swelling and isotropic growth. During the isotropic phase of growth, the condensed nucleus of the conidium is restructured into a normal interphase state containing a nucleolus and mitochondrial assembly and function is being established. It is also during this initial phase of isotropic growth that gene expression begins and the nuclear division or cell cycle is begun, as determined by the appearance of multiple nuclei. The final stage of this process is the initiation of polar hyphal growth.

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