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The *fluffy* Gene of *Neurospora crassa* Is Necessary and Sufficient to Induce Conidiophore Development

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ABSTRACT

The *fl* (*fluffy*) gene of *Neurospora crassa* encodes a binuclear zinc cluster protein that regulates the production of asexual spores called macroconidia. Two other genes, *acon-2* and *acon-3*, play major roles in controlling development. *fl* is induced specifically in differentiating tissue during conidiation and *acon-2* plays a role in this induction. We examined the function of *fl* by manipulating its level of expression in wild-type and developmental mutant strains. Increasing expression of *fl* from a heterologous promoter in a wild-type genetic background is sufficient to induce conidiophore development. Elevated expression of *fl* leads to induction of development of the *acon-2* mutant in nitrogen-starved cultures, but does not bypass the conidiation defect of the *acon-3* mutant. These findings indicate that *fl* acts downstream of *acon-2* and upstream of *acon-3* in regulating gene expression during development. The *eas*, *con-6*, and *con-10* genes are induced at different times during development. Morphological changes induced by artificially elevated *fl* expression in the absence of environmental cues were correlated with increased expression of *eas*, but not *con-6* or *con-10*. Thus, although inappropriate expression of *fl* in vegetative hyphae is sufficient to induce conidial morphogenesis, complete reconstitution of development leading to the formation of mature conidia may require environmental signals to regulate *fl* activity and/or appropriate induction of *fl* expression in the developing conidiophore.

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NEUROSPORA *crassa* grows rapidly to colonize the local environment. Following initial colonization, the mat of fungal mycelium gives rise to aerial hyphae that produce asexual spores called macroconidia. The macroconidia provide a means for rapid wind and splash dispersal to new sites of colonization. In the laboratory, macroconidiation (hereafter, conidiation) is induced when the fungus becomes desiccated and when it is starved for carbon or nitrogen (Turian and Bianchi 1972; Turian 1973).

Several genetic loci that are specifically required for conidiation have been identified. Two mutants, *aconidiate-2* (*acon-2*) and *fluffyoid* (*fld*), produce aerial hyphae but fail to initiate the transition from filamentous to budding growth due to an early block in development (Matsuyama *et al.* 1974; Springer and Yanofsky 1989). Two additional mutant strains, *aconidiate-3* (*acon-3*) and *fluffy* (*fl*), are blocked during the transition to budding growth (Lindegren 1933; Matsuyama *et al.* 1974). The fl^{Y} allele is partially active and displays a phenotype similar to *fld*. The conidial separation mutants, *csp-1* and *csp-2*, produce chains of budded cells but these do not mature to release free conidia (Selitrennikoff *et al.* 1974).

In addition to these loci that are involved in conidiophore development, other genes that are preferentially expressed during conidiation have been identified (Berlin and Yanofsky 1985; Roberts *et al.* 1988; Roberts and Yanofsky 1989; Springer and Yanofsky 1992). The functions of one class of these genes (*con* genes) are unknown, but it appears that in some cases they encode stress-response proteins (Lee and Ebbole 1998). The *eas* gene is also regulated in a complex manner and encodes a hydrophobin that is found on the outer surface of aerial hyphae and conidia. In addition to developmental control, many of these genes are regulated directly by other factors such as light, the circadian clock, and nutrient availability (Lauter and Yanofsky 1993; Corrochano *et al.* 1995; Bell-Pedersen 1998; Lee and Ebbole 1998).

The *eas*, *con-6*, and *con-10* genes also are repressed during vegetative mycelial growth. This repression is mediated by *rco-1* (Yamashiro *et al.* 1996). *rco-1* is a homolog of *Saccharomyces cerevisiae TUP1*, a gene involved in transcriptional repression of several classes of genes (Keleher *et al.* 1992). *rco-1* mutants are female sterile, grow more slowly than the wild type, and have a conidial maturation defect similar to that of *csp-1* and *csp-2* mutants.

fl is the only regulatory gene of those described above that has been characterized. *fl* encodes a binuclear zinc cluster protein that resembles the Gal4 class of transcription factors (Bailey and Ebbole 1998). *fl* is induced

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during synchronous development caused by nitrogen starvation or by transfer of the mycelium from liquid culture to air. Induction occurs at the time corresponding to the transition from filamentous to budding growth (Bailey and Ebbole 1998).

To better assess the genetic requirements for *fl* expression, we examined expression of *fl* in different tissues and in developmental mutants. *fl* is expressed at a basal level in vegetative hyphae and expression is induced during conidiation specifically in differentiating conidiophores. Induction of *fl* expression was observed in the developmental mutants *fld* and *acon-3* under nitrogen-starvation conditions. These induced levels are not seen in the *acon-2* and *fl*^{L} (null allele) strains. A heterologous promoter was used to elevate expression of *fl* in wild-type and developmental mutant strains. Wild-type strains expressing high levels of *fl* mRNA from a constitutive promoter sporulated in the absence of environmental cues inducing development. We measured the expression patterns of *con* genes in developmental mutants expressing *fl* to clarify the previously developed time line relating gene expression with stages of morphogenesis (Lauter and Yanofsky 1993). This study supports the view that *acon-2* precedes *fl* and that *acon-3* acts downstream of *fl* to regulate gene expression and morphogenesis.

MATERIALS AND METHODS

Fungal strains: From the Fungal Genetics Stock Center (FGSC), Department of Microbiology, University of Kansas Medical Center, we obtained strains *acon-2 a* (FGSC no. 3263), *acon-3 a* (FGSC no. 5074), *fld a* (FGSC no. 7023), *fl*^{L} *a* (FGSC no. 46), *csp-1 a* (FGSC no. 2555), 74OR23-1V*A* (FGSC no. 2489), and 74-OR6*a* (FGSC no. 4200). The *acon-2* allele is temperature sensitive (conidiating at 25° but not at 34°). The *rco-1* strain (RCO353) has been described previously (Lee and Ebbole 1998).

Growth conditions: To examine gene expression during aerial development, Vogel's minimal medium N with 1.5% sucrose as carbon source (Davis and de Serres 1970) was inoculated with 1×10^5 conidia/ml of strain 74-OR23-1V*A*. After 20 hr growth at 34° (200 rpm), the culture was harvested onto 90-mm diameter circles of Whatman no. 1 filter paper. The harvested pads were inverted on top of a second filter paper on medium N with 0.45% agar. The cultures were placed at 34° in the light. The mycelium must first grow through filter paper to gain exposure to air, and aerial hyphae are first observed 7 hr after harvesting. Synchronous conidiation is obtained under these conditions and budding growth occurs 6 hr after aerial hyphae are first observed (13 hr after harvesting). The upper filter paper layer was peeled away and the aerial growth was harvested from the surface of the filter paper with a razor blade. The remaining mycelium was harvested separately for RNA isolation as described (Springer *et al.* 1992).

To examine nitrogen-starvation-induced development, strain 74-OR23-1V*A* was inoculated at a concentration of 1×10^5 conidia/ml into four 250-ml flasks containing 100 ml of modified medium N with 50 mM NH_4Cl as the nitrogen source and grown for 20 hr. The cultures were then washed twice and each 100-ml culture was divided among three flasks containing medium N either without an added nitrogen source for nitrogen starvation or with 50 mM NH_4Cl or 50 mM KNO_3 as the nitrogen source. Mycelia from each of the different media were collected after 1, 6, 12, and 24 hr for RNA extraction.

Mycelial fragments were used as inoculum in experiments involving aconidial strains. Initial cultures were inoculated with a loop of aerial hyphae and grown for 20 hr prior to fragmention in a Waring blender (two times 15 sec). Twenty-milliliter aliquots were transferred into 50 ml of fresh medium, grown for 20 hr, and this step was repeated to increase the mycelial mass. Cultures were then washed twice with sterile water, harvested, and divided into six equal portions. One of the portions was frozen immediately, and the remaining five portions were used to inoculate five 125-ml flasks containing 50 ml of medium N without nitrogen source. Samples were harvested after 4, 6, 8, 10, and 12 hr of incubation, frozen with liquid nitrogen, and used for RNA extraction.

RNA extraction and analysis: RNA extraction and Northern blot analyses were performed as previously described (Madi *et al.* 1994). A 1.5-kb *Spe*I fragment located within the coding region of the *fl* gene was used as the probe in these experiments. Probes for *eas*, *con-10*, and *con-6* were prepared from cDNA fragments isolated from plasmids pEAS, pBW100, and pCON6-6, respectively (Madi *et al.* 1994). A cosmid containing the rRNA repeat region was used to prepare a probe to detect ribosomal RNA. The *N. crassa* actin cDNA (Tinsley *et al.* 1998) was provided by M. Plamann (University of Missouri, Kansas City). Because actin mRNA is subject to significant regulation at the mRNA level, we used actin and ribosomal RNA as a means to assess RNA quality and as a rough guide to assess RNA loading for RNA blot experiments. For this reason we did not attempt to quantitate levels of induction of gene expression. However, all hybridizations for a given probe were performed at the same time and the membranes were exposed to film for the same lengths of time and qualitative estimates of gene expression levels were consistent in replicate experiments.

Plasmid construction and transformation: A plasmid containing the *fl* gene under the control of the *cross-pathway control-1* (*cpc-1*) promoter and the *trp*C terminator was used to examine the effect of increased *fl* expression. This plasmid was constructed in three steps. First, pLBS5 was constructed by ligating an ~0.95-kb *Eco*RI-*Hin*dIII fragment containing the *trp*C terminator from pCZT (Madi *et al.* 1994) into pCB1004 (Carroll *et al.* 1994) at the *Eco*RI-*Hin*dIII site. An *N. crassa cpc-1* promoter fragment was amplified from genomic DNA using a primer (5′-TTGGCACATGGAATGGAC-3′) that is upstream of an *Nhe*I site and a primer (5′-GTAAGCTAGCTTG ATGGATGCTTCTCG-3′), containing an incorporated *Nhe*I site, that is downstream of the transcriptional start site of *cpc-1*. The 1.2-kb amplified fragment containing the *cpc-1* promoter was digested with *Nhe*I and then cloned into the *Spe*I site of pLBS5. This fragment was sequenced to verify that no mutations were introduced into the DNA during amplification. The resulting plasmid was named pLBS6 and was used as the control plasmid in these experiments. The *fl* fragment was also amplified from genomic DNA using a primer (5′-CTC TCCCGGGCTTCCAGGTTGCTTCAGA-3′) upstream of the translational start site (nucleotides 453–470) and a primer (5′-ACCGCCCGGGCAAATGCCTTAGGTGAAC-3′) downstream of the last codon (nucleotides 3269–3286; Bailey and Ebbole 1998). This fragment was cloned and sequenced to verify its integrity. The *fl* gene was excised by digestion with *Sma*I and then ligated into the *Sma*I site of pLBS6. The resulting plasmid, pLBS7, was used to overexpress *fl* in our experiments.

pLBS6 and pLBS7 were used to transform protoplasts of 74OR23-IV*A*, *acon-2*, *acon-3*, and *fl*^{L} strains (Vollmer and

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Yanofsky 1986). Homokaryotic strains were obtained by serial passage on medium containing 200 μg/ml hygromycin. The naming convention for overexpression strains designates the parental strain, the plasmid used for transformation, and the number of individual transformants (*e.g.*, 74-LBS7-15 is pLBS7 transformant no. 15 of strain 74OR23-IVA).

Overexpression experiments: Minimal medium was inoculated with conidia of the transformed wild-type strains (74-LBS7-5, 74-LBS7-7, 74-LBS7-15, and 74-LBS6-1) and the transformed *acon-2⁻* strains (ACON2-LBS7-4, ACON2-LBS7-10, ACON2-LBS7-13, and ACON2-LBS6-4). These strains were grown for 16 hr, harvested, and washed with 1× medium N salts without nitrogen source. The cultures were then divided, and half of the sample was transferred into fresh medium N and the other half was transferred to medium N without nitrogen source. These cultures were grown for 12 hr, harvested, and frozen with liquid nitrogen for RNA preparation.

Overexpression experiments with aconidial strains were performed by inoculating minimal medium with the transformed fl^{L} strains (FL-LBS7-2, FL-LBS7-12, and FL-LBS6-13) and the transformed *acon-3* strains (ACON3-LBS7-1, ACON3-LBS7-3, and ACON3-LBS6-1). These cultures were grown for 20 hr and then blended with a Waring blender. Ten milliliters of the blended culture was used to inoculate fresh minimal medium. These cultures were grown for 16 hr, harvested, washed, and divided into medium N and medium N without nitrogen source as described for the wild-type and *acon-2* transformants.

RESULTS

***fl* expression during aerial development and nitrogen starvation:** We showed previously that expression of *fl* is induced at approximately the time that budding growth initiates during conidiation (Bailey and Ebbole 1998). Chains of conidia develop at the tips of aerial hyphae and not within the mycelial mat from which these aerial hyphae emerge. Therefore, we examined the spatial pattern of *fl* expression to determine if the increase in *fl* mRNA occurs specifically in aerial hyphae.

Aerial hyphae and the underlying mycelium were harvested separately at different stages of development (see materials and methods). Basal levels of *fl* mRNA were maintained in the mycelial samples throughout development (Figure 1). Induction of *fl* mRNA in the aerial hyphae was observed early in development, when the majority of the tips of aerial hyphae had not yet differentiated and only a fraction of these initiated budding growth. *con-6* and *con-10* expression was also limited to the developing aerial hyphae (Figure 1). These results are consistent with those previously observed with *con-10* (Springer *et al.* 1992). Relatively high levels of *con-6* expression were reached by 13 hr, a time when budding growth is just commencing. *con-10* expression was still limited at this time but is found in greater abundance at later time points and when production of free conidia is observed. Thus, induction of *fl* expression is conidiation specific in the aerial hyphae where conidiation-specific genes are expressed. Actin mRNA is typically used as a control for a gene with constant expression levels. However, steady-state actin mRNA levels are regulated during aerial development with mRNA levels de-

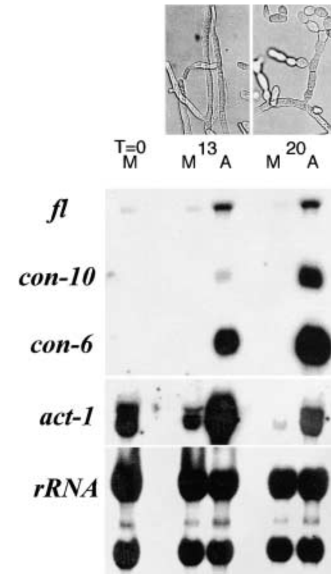

Figure 1.—Localization of *fl*, *con-10*, and *con-6* induction to aerial hyphae. *N. crassa* was grown for 20 hr and harvested to induce synchronous conidiation. Aerial hyphae (A) and mycelium (M) samples were harvested separately for RNA analysis. Photographs were taken of aerial hyphae samples at two stages during development. Samples taken early in development (13 hr) had swollen tips with limited initiation of budding growth. Samples taken late in development (20 hr) contained chains of budding cells and free conidia. $T = 0$ designates the time of harvest from liquid cultures. Actin (*act-1*) mRNA and rRNA levels are shown to assess RNA quality and loading.

clining in the mycelium and rising in the rapidly growing aerial hyphae. Although actin is appropriate as a loading control for aerial development in this experiment, the combination of actin and rRNA provides a good estimate of RNA loading and quality.

Uniform conidiation was also observed to commence in liquid cultures 6 hr after transfer to medium lacking a nitrogen source (not shown) and a strong induction of *fl* mRNA was observed by 6 hr (Figure 2A). *fl* expression was detected in low abundance in samples that were grown in media containing ammonium or nitrate as the nitrogen source. Examination of the time course of gene expression in medium lacking a nitrogen source showed that *fl* mRNA levels begin to rise by 2 hr and are strongly induced by 4 hr (Figure 2B). No increase in *fl* mRNA was detected at 1 hr of incubation; however, *eas* mRNA levels increased significantly by 1 hr. *con-6* was induced by nitrogen-starvation-induced development starting ∼4 hr after inoculation and *con-10* was induced by 6 hr (Figure 2B). Induction of *eas*, *con-6*, and *con-10* was dependent on *fl* (see below) and was not a response to nitrogen starvation.

Expression of *fl* in developmental mutants: Since *fl* mRNA levels are induced during development, we examined *fl* expression in developmental mutants to examine epistatic relationships. Six genes (*acon-2*, *fl*, *fld*,

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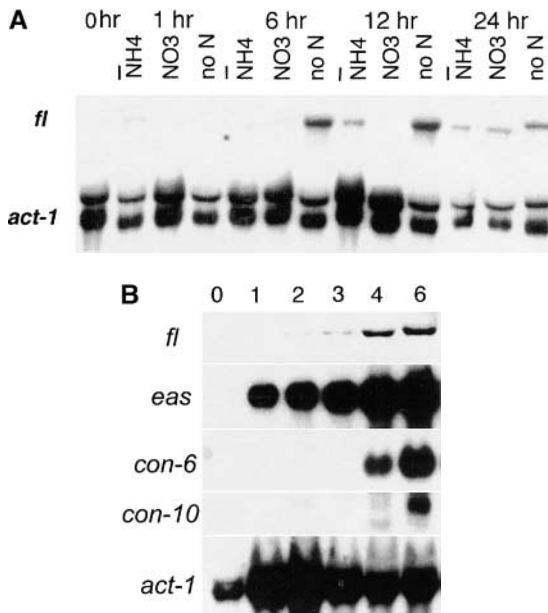

FIGURE 2.—Induction of gene expression in response to nitrogen source. (A) Expression of *fl* was determined in strains grown in medium N containing 50 mM ammonium chloride (NH_4), 50 mM potassium nitrate (NO_3), or no nitrogen source (no N). RNA for Northern blot analysis was isolated from samples taken 0, 1, 6, 12, and 24 hr after inoculation. *act-1* (actin) was used as a probe to assess mRNA loading. (B) Expression of *fl*, *eas*, *con-6* and *con-10*, and *act-1* at hourly intervals during the first 6 hr after transfer to nitrogen-starvation medium.

acon-3, *csp-1*, and *csp-2*) that are required for conidial development have been identified. Only *fl* has been characterized and is known to have null alleles (such as fl^L) and a partially active allele, fl^Y (Bailey and Ebbole 1998). Another regulator of conidiation-specific genes, *rco-1*, encodes a cell-type-specific repressor of *ccg-1* and *con-10*. Shortly after induction of development, repression of the *con-10* gene mediated by *rco-1* is no longer detected (Lee and Ebbole 1998).

The basal level of *fl* mRNA (0 hr) in the *acon-2* and fl^L mutants was similar to wild type (Table 1, Figure 3). After transfer to medium lacking a nitrogen source, induction of *fl* mRNA was reduced in the *acon-2* and fl^L mutants. This suggests that *acon-2*, and possibly *fl*, contributes to induction of *fl* mRNA. We note, however, that induction of *fl* mRNA in the fl^Y mutant was comparable to that in the wild type (Table 1). Since the *csp-1* mutation disrupts development at a very late stage of conidiophore maturation, it is not surprising that *fl* expression in this mutant was similar to the wild type (Table 1). The basal level of *fl* expression was not elevated in the *rco-1* mutant, indicating that *fl* is not subject to repression by *rco-1* and induction of *fl* was not markedly altered. Induction of *fl* was observed in the *fld* and *acon-3* strains, suggesting that these genes do not act to regulate *fl* transcription (Figure 3).

***con-6*, *con-10*, and *eas* expression:** We also assessed the roles of *acon-2*, *acon-3*, *fld*, *fl*, *csp-1*, and *rco-1* genes in regulating *eas* and *con* gene expression by Northern blot analysis. As expected for *csp-1*, the expression patterns of *eas*, *con-6*, and *con-10* resembled those of the wild type (Table 1, Figures 1 and 3). *eas*, *con-6*, and *con-10* expression is not appreciably induced in the fl^L or *acon-2* strains, although a small amount of *con-10* expression was detected in the fl^L strain in this experiment. Delayed expression of *eas* was observed in the fl^Y, *acon-3*, and *fld* strains (Table 1, Figure 3). *con-6* and *con-10* were not expressed in the *acon-3* strain and reduced levels of *con-6* and *con-10* mRNA were observed in the *fld* strain (Table 1, Figure 3). Expression of these genes in the wild type and of these developmental mutants was also assessed during aerial development (data not shown) and found to be similar both to previously published results (Roberts *et al.* 1988; Lauter *et al.* 1992; White and Yanofsky 1993) and to our results with nitrogen-starvation-induced cultures, with one exception. When we examined a time course of *eas* expression in cultures undergoing aerial development, we found that *eas* expression was induced in the *acon-3* strain similarly to the wild-type strain, whereas *eas* expression is delayed in the nitrogen-starved *acon-3* strain (Figure 3). Other than this exception, the epistatic relationships between these regulatory genes and the timing of *eas*, *con-6*, and *con-10* expression during nitrogen-starvation-induced conidiation are similar to those in studies examining aerial development.

In the absence of *rco-1*, a number of nutrient and environmental stress conditions were found to regulate *con-10* expression independently of developmental induction (Lee and Ebbole 1998). Basal levels of *eas*, *con-6*, and *con-10* transcripts were elevated in the *rco-1* sample (0 hr; Figure 3). Thus, *eas* and *con-6* also appear to be subject to *rco-1*-mediated repression. It is unknown why *con-6* expression declined after transfer to nitrogen-starvation conditions in the *rco-1* mutant.

Elevated expression of *fl* induces conidiation: Because we observed elevated levels of *fl* mRNA in aerial hyphae during conidiophore formation, we wanted to determine if elevated expression of *fl* in vegetative hy-

TABLE 1

Gene expression patterns in developmental mutants

Gene	Strains						
	csp-1	*rco-1*	*acon-2*	fl^L	fl^Y	*fld*	*acon-3*
fl	+	+	−	−	+	+	+
eas	+	+M	−	−	+d	+d	+d
con-6	+	+M	−	−	+d	+d	−
con-10	+	+M	−	−	+d	+d	−

+, gene expressed simlarly to wild type; +M, gene expressed in vegetative mycelium; −, a gene that is not induced during nitrogen-starvation-induced development; +d, the gene is expressed with a delay relative to wild type.

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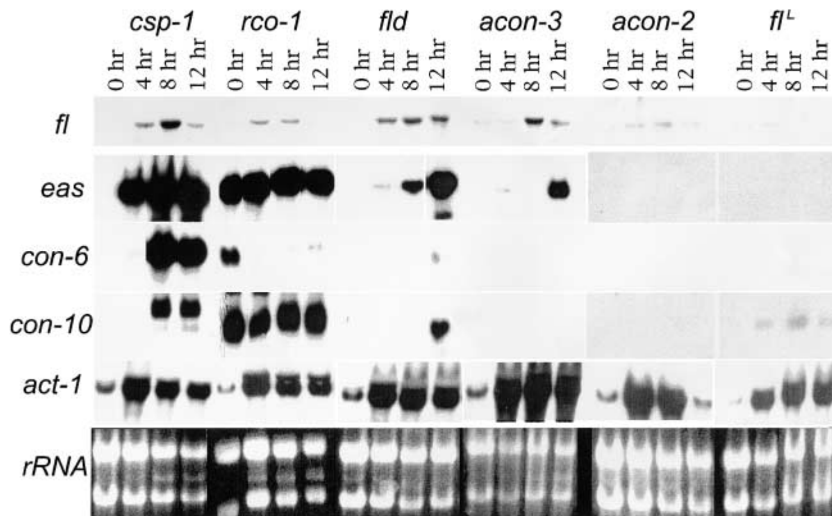

Figure 3.—Gene expression patterns in developmental mutants. Developmental mutants (*csp-1*, *rco-1*, *fld*, *acon-3*, *acon-2*, and *fl*) were inoculated into medium N for 20 hr. The mycelia were then harvested, washed, and transferred to medium N with no nitrogen source. Samples were harvested immediately (0) or incubated for 4, 8, and 12 hr prior to RNA isolation and Northern blot analysis using *fl*, *eas*, *con-6*, *con-10*, and *act-1* as probes. rRNA bands from ethidium-bromide-stained agarose gels are shown to assess RNA loading and quality.

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phae was sufficient to induce conidiation. We used the *cpc-1* promoter to drive expression of *fl* in these experiments. The *cpc-1* gene is moderately expressed during germination and mycelial growth with reduced levels of expression during conidiation (Ebbole *et al.* 1991; Paluh and Yanofsky 1991; Sachs and Yanofsky 1991). A 1.2-kb fragment of the *cpc-1* promoter was used to create a transcriptional fusion to drive expression of the *fl* gene. This plasmid, pLBS7, was used to generate a series of strains bearing single ectopically integrated copies of the plasmid. The vector lacking the *fl* gene was used as a control (pLBS6).

We examined 10 independent isolates of 74-ORS6*a* transformed with pLBS7. Of the 10 strains, 8 produced elevated levels of *fl* transcript (not shown), indicating integration of intact copies of the gene expressed from the *cpc-1* promoter. These eight pLBS7 transformants produced conidiophores in liquid culture without starvation. Conidiophore development was observed in minimal medium after a 10-hr period of incubation but was more pronounced with longer incubation or nitrogen starvation. None of the control strains transformed with pLBS6 conidiated in minimal medium and all conidiated in medium lacking nitrogen, as was the case for the wild type. Strains 74-LBS6-1 and 74-LBS7-15 are representative of these transformed wild-type strains (Figure 4). We noted no deleterious effects of *fl* expression on growth rate of the transformants.

The *fl*^{L} strain that was transformed with pLBS7, FL-LBS7-2, produced conidia on solid minimal medium, indicating that the *fl* mutation was complemented by the plasmid (not shown). Conidiophore development in the FL-LBS7-2 strain was also observed when it was grown in liquid minimal medium and nitrogen-starvation medium (Figure 5). In contrast to the 74-LBS7-15 strain, the conidiation in the nitrogen-starved culture resembled that of the minimal medium culture and free conidia were not present at 10 hr. The negative control strain, FL-LBS6-13, was aconidial on solid media and it produced only vegetative hyphae when it was grown in nitrogen-starvation medium (Figure 5).

The *acon-2* transformant containing pLBS7, ACON2-LBS7-4, conidiated when it was grown on solid medium at the nonpermissive temperature, while the control strain (ACON2-LBS6-15) was aconidial (not shown). When ACON2-LBS7-4 was grown in minimal medium,

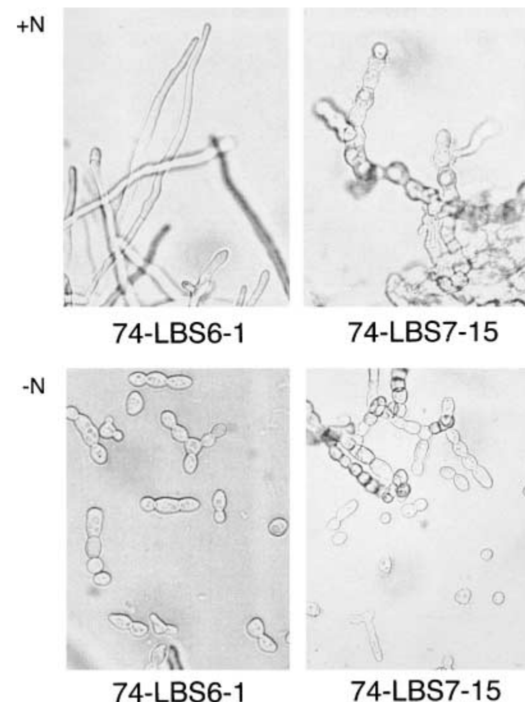

Figure 4.—Heterologous expression of *fl* induces conidiophore development in the wild-type genetic background. Transformants of strain 74-ORS6*a* containing pLBS6 (74-LBS6-1) and pLBS7 (74-LBS7-15) were transferred to medium N (+N) and medium lacking a nitrogen source (−N) for 10 hr. Cultures shown in Figures 4–7 were harvested for RNA preparation after 12 hr growth in minimal medium and subjected to Northern blot analysis using *fl*, *eas*, *con-6*, *con-10*, and *rRNA* probes.

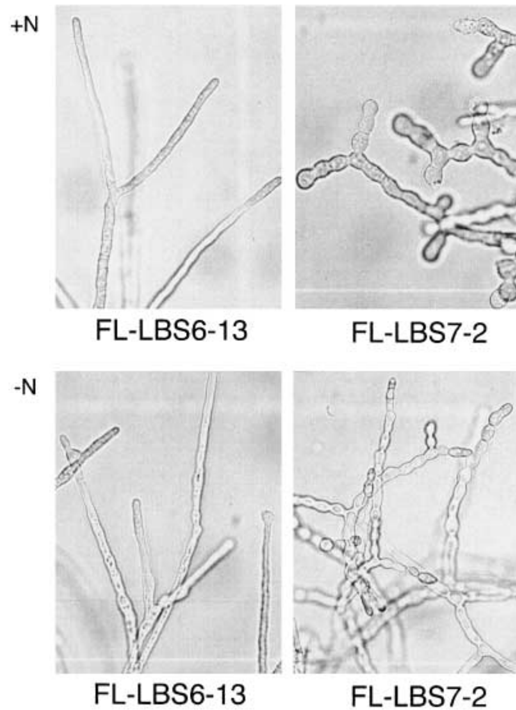

Figure 5.—Heterologous expression of *fl* in the *fl*^{L} genetic background. Transformants of the *fl*^{L} strain containing pLBS6 and pLBS7 were transferred to medium N (+N) and medium lacking a nitrogen source (−N) for 10 hr.

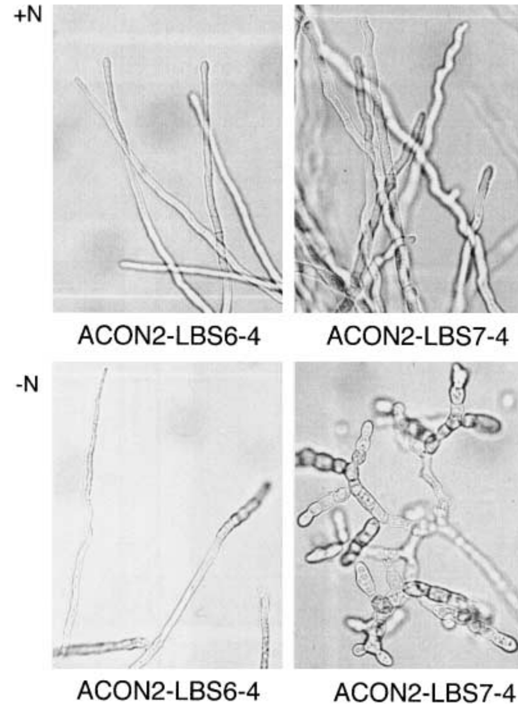

Figure 6.—Heterologous expression of *fl* in the *acon-2* genetic background. Transformants of the *acon-2* strain containing pLBS6 and pLBS7 were transferred to medium N (+N) and medium lacking a nitrogen source (−N) for 10 hr.

it did not produce conidiophores but displayed a slightly irregular morphology (Figure 6). The ACON2-LBS7-4 transformant produced conidiophores and free conidia when it was grown in the nitrogen-starvation medium. Thus, with exposure to air or nitrogen starvation the *acon-2* mutation could be bypassed by the *cpc-1-fl* fusion gene. The ACON2-LBS6-15 negative control strain grew as vegetative hyphae under these conditions (Figure 6).

The *acon-3* strain containing the *fl* expression plasmid (ACON3-LBS7-3) was indistinguishable from the control strain when it was grown in minimal medium. This was also typical of growth in nitrogen-starvation medium (not shown). ACON3-LBS7-3 did produce conidiophores on solid medium after a long delay (2–4 days; not shown), indicating that elevated expression of *fl* can eventually bypass this allele of *acon-3*. However, the conidiophores displayed a moderate conidial separation defect indicative of slow or incomplete maturation. The ACON3-LBS6-1 strain did not produce conidia under any condition.

Gene expression in pLBS7 transformants: Strain 74-LBS7-15 produced elevated levels of *fl* mRNA that exceeded the level found in 74-LBS6-1, even following induction by nitrogen starvation (Figure 7). In the wild-type strain transformed with either pLBS6 or pLBS7, we found that the *eas*, *con-6*, and *con-10* genes were induced in the nitrogen-starved cultures that displayed abundant conidiation (Figure 4). However, in the 74-LBS7-15 strain grown in minimal medium, *eas* was strongly induced, while *con-6* and *con-10* expression was not detected (Figure 7). This result is surprising since the culture appeared to have reached a morphological stage where *con-6* and *con-10* expression would be anticipated (Figures 1 and 4).

The FL-LBS6-13 control strain produced a basal level of *fl* expression that was not markedly elevated in nitrogen-starvation medium and, as expected, this *fl* mutant did not express any of the conidiation-induced genes. The level of *fl* expression in the FL-LBS7-2 cultures was significantly elevated over that in the FL-LBS6-13 strain (Figure 7). In FL-LBS7-2, *eas* was expressed under both culture conditions, but significant levels of *con-6* or *con-10* were not induced even with nitrogen starvation (Figure 7).

As expected for the control strain, ACON2-LBS6-4, there was no increase in *fl* mRNA levels during nitrogen starvation and development was not observed. *fl* mRNA levels were elevated in the ACON2-LBS7-10 strain (Figure 7). The requirement for *acon-2* to activate *eas* is completely bypassed by elevated *fl* mRNA levels. However, even with nitrogen starvation, little *con-6* or *con-10* expression was observed. Thus, gene expression patterns in ACON2-LBS6-4 and ACON2-LBS7-4 were very similar to those of the corresponding *fl* mutant strains (Figure 7).

For the ACON3-LBS6-1 control strain, nitrogen starvation induced *fl* and *eas* expression but not *con-6* or *con-10* expression. In the ACON3-LBS7-3 strain, expres-

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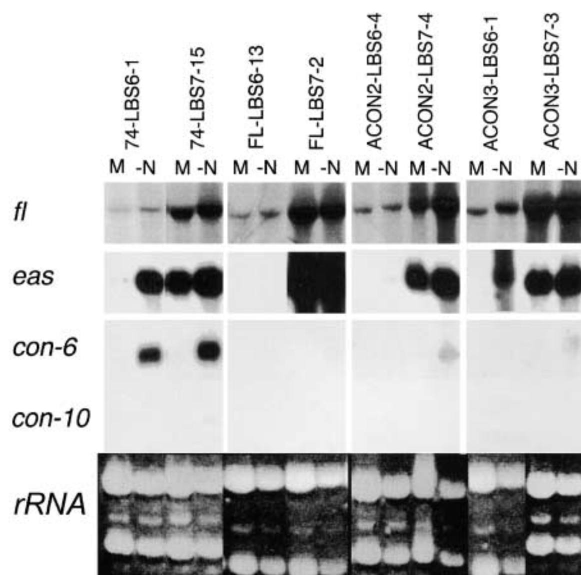

FIGURE 7.—Effect of heterologous expression of *fl* on gene expression in wild-type and developmental mutants.

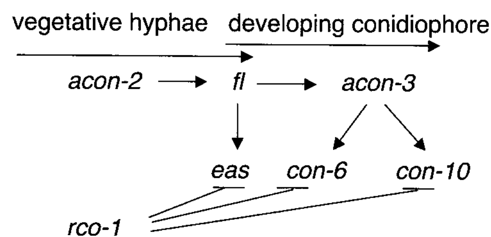

FIGURE 8.—Proposed role of regulators in development and gene expression. RCO1 (a homolog of *S. cerevisiae* Tup1p) functions in vegetative hyphae to repress expression of the developmentally regulated genes *eas*, *con-6*, and *con-10*. *acon-2* functions to increase *fl* activity by promoting transcription of the *fl* gene. Nitrogen-starvation medium triggers a rapid FL- and ACON2-dependent induction of *eas*. Increased expression of *fl* from a heterologous promoter is sufficient to induce *eas* expression in the absence of ACON2 or nitrogen starvation. *fl* expression triggers the switch to conidiophore development and budding growth. ACON3 is required for full entry into budding growth and is for induction of *con-6* and *con-10*. Tissue-specific induction of *fl* expression in developing conidiophores may be important for proper induction of *con-6* and *con-10* since wild-type levels of *con-6* and *con-10* gene expression are observed only when the endogenous *fl* gene is present in strains that are also undergoing nitrogen starvation.

sion of the *cpc-1/fl* fusion led to expression of *eas* in minimal medium and in nitrogen-starvation medium but expression of *con-6* and *con-10* was not observed (Figure 7).

DISCUSSION

fl is expressed at a low level in mycelia, but is induced in the developing aerial hyphae after the initiation of conidiation. Our current model supposes that FL is a transcription factor since the closest homologs are related to the Gal4p class of transcriptional activators. *fl* is required for the induction of *con-6* and *con-10* that occurs at the time at which *fl* mRNA levels are induced. Since *acon-3* is required for *con-6* and *con-10* induction, even in the presence of the *cpc-1-fl* fusion gene, *fl* either is required to activate *acon-3* or works with *acon-3* directly to induce *con-6* and *con-10*.

Nitrogen-starvation-induced development was used in most of our experiments because of the strong synchrony of development. This also provided an opportunity to compare gene expression during nitrogen-starvation-induced development with gene expression during aerial development since expression of *con-6* and *con-10* in aerial conidiation has been characterized in developmental mutants previously (Roberts *et al.* 1988; White and Yanofsky 1993). The most significant difference was that *eas* expression was delayed in nitrogen-starved cultures of the *acon-3* mutant whereas no delay in *eas* expression was observed in the *acon-3* mutant during aerial development. This may reflect the interplay of developmental and environmental regulation of gene expression during conidiation.

fl is required for *eas* expression during development. However, *eas* mRNA levels increase more rapidly than those of *fl* mRNA, suggesting that the basal level of *fl* expression in vegetative hyphae is sufficient to induce *eas* in the presence of appropriate environmental cues. This suggests the possibility of a post-transcriptional step for regulating *fl* protein activity in vegetative hyphae. Since *acon-2* is also required for *eas* expression in wild-type cells, one possibility is that *acon-2* protein is responsible for activating the basal level of FL activity in response to environmental cues. The finding that *eas* is expressed in the *acon-2* mutant bearing the *cpc-1-fl* fusion is not inconsistent with this view since increased *fl* expression may increase total FL activity and bypass the requirement for *acon-2* (and environmental cues) with respect to *eas* expression. Several members of the binuclear zinc cluster family of transcription factors are responsive to nutritional conditions (Wang *et al.* 1999). Alternatively, a small but undetectable increase in *fl* mRNA may be responsible for induction of *eas* early during development.

Our data suggest a model in which *fl* is responsible for induction of *eas* early in development (Figure 8). Because of the rapid response of *eas* to developmental signals and the dependence on *fl* for expression, we suggest that *eas* is likely to be a direct target for the FL transcription factor. *acon-3* appears to act downstream of *fl* to regulate *con-6* and *con-10*. These genes are normally repressed in vegetative hyphae by *rco-1*. *acon-2* is required for full transcriptional activation of *fl*, but as discussed above, *acon-2* may also have other activities important for activation of the basal level of FL protein. The role of *fld* is unclear: it does not affect transcriptional induction of *fl*, but the delay in downstream target gene ex-

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pression suggests that it is required for efficient activation of gene expression or is possibly required for derepression of *eas*, *con-6*, and *con-10* (perhaps by affecting repression by *rco-1*).

Although our results confirm that *fl* plays a key role in developmental regulation, molecular analysis of the *acon-2*, *fld*, and *acon-3* genes is required to define the regulatory pathway further. Recent progress in genome sequencing (Galagan *et al.* 2003) should greatly facilitate cloning of these key genes. Preliminary restriction fragment length polymorphism mapping experiments using markers near to where *acon-2* has been previously mapped (*ro-2*, *phe-2*, *tyr-1*) show that *acon-2* lies ∼5 cM away (on the basis of 80 segregating progeny). Unfortunately, the adjacent supercontig in the current physical map displayed no linkage to *acon-2* (our unpublished observation).

There are aspects of *fl* function that we could not resolve with our experimental approach of expressing *fl* from the *cpc-1* promoter. In addition to the ectopic copy of *cpc-1-fl*, 74-LBS7-15 has a functional endogenous copy of *fl*. The *cpc-1* gene is expressed at lower levels during conidiation (Sachs and Yanofsky 1991), and thus the promoter does not provide elevated expression in developing conidiophores as does the endogenous *fl* promoter and this may explain why the development of FL-LBS7-2 appears to be significantly delayed relative to 74-LBS7-15 under nitrogen-starvation conditions. Additionally, some features of the normal developmental program may not be executed properly in the absence of appropriate environmental cues, for example, release of *rco-1*-mediated repression. This may explain why *con-6* and *con-10* expression is not observed in cultures that have reached a morphological stage at which *con-6* and *con-10* expresssion is always observed in wild-type strains.

In some respects *fl* plays a role in conidiation analogous to *Aspergillus nidulans brlA* (Adams *et al.* 1998). *brlA* is necessary for conidiophore morphogenesis and encodes a transcription factor that is induced during conidiation. Both *fl* and *brlA* are expressed constitutively at low levels and their induction is coincident with the onset of morphogenesis (Boylan *et al.* 1987). Forced expression of *brlA* using the *alcA* promoter in vegetative hyphae results in an incomplete form of conidiation where viable conidia bud from the tips of the hyphae grown in liquid cultures (Adams *et al.* 1988). This is similar to our observations in *N. crassa* with the *cpc-1-fl* fusion. However, overexpression of *brlA* causes a cessation of hyphal growth (Adams *et al.* 1988), while *fl* activates development without interfering with vegetative growth. *fl* expression does not, on its own, act as a switch between growth and development as is apparently the case for *brlA*.

Although there may be analogous roles for regulators of conidiation in these two fungal species, the regulators are not homologous. *brlA* encodes a Cys_2-His_2 zinc-finger protein in contrast to the binuclear zinc cluster encoded by *fl*. Furthermore, a homolog of *A. nidulans flbD* has been cloned from *N. crassa*, and this gene, *rca-1*, complements the sporulation defect of the *A. nidulans* mutant. However, the *rca-1* mutant of *N. crassa* is not defective in spore development and plays a different role in the biology of the organism (Shen *et al.* 1998). The lack of homology in the regulatory genes involved in conidiation in these two fungi points to independent evolutionary origins for this developmental process. It seems likely that during the evolution of *N. crassa* and *A. nidulans* different members of the available pool of regulatory genes in the common ancestor were recruited to regulate the emerging developmental processes resulting in asexual sporulation.

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