Suppressor Mutations Bypass the Requirement of *fluG* for Asexual Sporulation and Sterigmatocystin Production in *Aspergillus nidulans*

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ABSTRACT

Asexual sporulation (conidiation) in the filamentous fungus *Aspergillus nidulans* requires the early developmental activator *fluG*. Loss of *fluG* results in the blockage of both conidiation and production of the mycotoxin sterigmatocystin (ST). To investigate molecular mechanisms of *fluG*-dependent developmental activation, 40 suppressors of *fluG* (SFGs) that conidiate without *fluG* have been isolated and characterized. Genetic analyses showed that an individual suppression is caused by a single second-site mutation, and that all *sfg* mutations but one are recessive. Pairwise meiotic crosses grouped mutations to four loci, 31 of them to *sfgA*, 6 of them to *sfgB*, and 1 each to *sfgC* and *sfgD*, respectively. The only dominant mutation, *sfgA38*, also mapped to the *sfgA* locus, suggesting a dominant negative mutation. Thirteen *sfgA* and 1 *sfgC* mutants elaborated conidiophores in liquid submerged culture, indicating that loss of either of these gene functions not only bypasses *fluG* function but also results in hyperactive conidiation. While *sfg* mutants show varying levels of restored conidiation, all recovered the ability to produce ST at near wild-type levels. The fact that at least four loci are defined by recessive *sfg* mutations indicates that multiple genes negatively regulate conidiation downstream of *fluG* and that the activity of *fluG* is required to remove such repressive effects.

ASEXUAL sporulation in *Aspergillus nidulans* is a continual progression from growth to development and is a precisely timed and genetically programmed event in the life cycle in response to internal and external cues. It involves formation of multicellular reproductive organs termed conidiophores, each of which produces thousands of mitotically derived spores (for review see Adams 1994; Adams *et al.* 1998). In previous studies, we showed that two antagonistic signaling pathways control growth and asexual development in *A. nidulans*. Growth signaling is mediated by FadA and SfaD, the α- and β-subunits for a heterotrimeric G protein, respectively. When FadA (Gα) is active, GTP-bound FadA and the heterodimer SfaD(Gβ):Gγ are dissociated and both signal to enhance proliferative growth, which in turn represses both asexual sporulation and sterigmatocystin (ST) production (Figure 1; Yu *et al.* 1996; Hicks *et al.* 1997; Rosen *et al.* 1999). Constitutive activation of FadA growth signaling results in the fluffy autolytic phenotype. Initiation of asexual development requires the activity of two major genes, *flbA* and *fluG*. FluG is responsible for the production of an extracellular factor and it stimulates both development-specific events and activation of FlbA, which in turn inactivates FadA signaling (Lee and Adams 1994b; Yu *et al.* 1996). FlbA is a regulator of G protein signaling (RGS) domain protein and, like other RGS proteins, is predicted to negatively regulate G protein signaling by facilitating the intrinsic GTPase activity of the Gα (FadA)-subunit (Lee and Adams 1994a; Berman *et al.* 1996; Koelle and Horvitz 1996; Yu *et al.* 1996; Hepler *et al.* 1997). Asexual development requires both inhibition of growth and activation of sporulation.

A key step in the formation of conidiophores is activation of the *brlA* gene, which encodes a C_2H_2 zinc finger transcriptional activator required for expression of sporulation-specific genes (Figure 1; Adams *et al.* 1988; Chang and Timberlake 1992). Genes affecting *brlA* expression have been identified and characterized and these include *fluG*, *flbA*, *flbB*, *flbC*, *flbD*, and *flbE*. Mutations in all of these genes result in undifferentiated fluffy colonies (Wieser *et al.* 1994). Two of the delayed conidiation loci, *flbC* and *flbD*, are predicted to encode DNA-binding proteins and represent potential direct activators of *brlA* expression (Wieser and Adams 1995). Because mutations in *flbC* and *flbD* have additive effects on development, it has been proposed that these genes control independent steps in a nonlinear pathway (Figure 1). The *flbB* gene is predicted to encode a bZip-like transcription factor and *flbE* does not have clear homologs in the available databases (J. Wieser and T. H. Adams, personal communication). By testing the genetic requirements for the inappropriate conidiation observed

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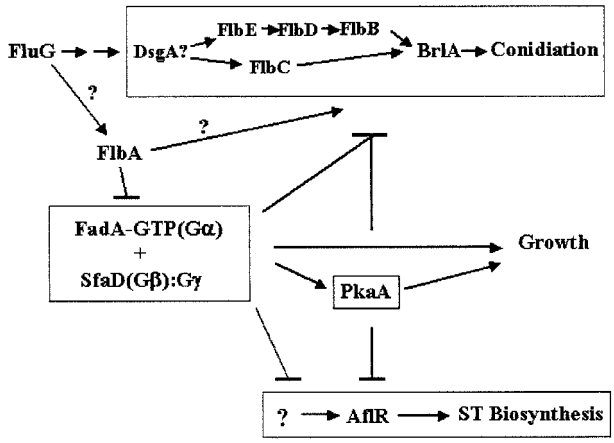

FIGURE 1.—Genetic model for growth and development in *A. nidulans*. In this model, the activities of two antagonistic signaling pathways control growth and asexual sporulation (and ST production). Growth signaling is mediated by a heterotrimeric G protein composed of FadA (Gα) and SfaD (Gβ):Gγ. Activated FadA-GTP and SfaD:Gγ transduce signals to downstream effectors that include PkaA (a catalytic subunit of protein kinase A; SHIMIZU and KELLER 2001). Activation of proliferative growth represses both asexual sporulation and ST biosynthesis. FlbA is an RGS protein that rapidly turns off FadA-mediated growth signaling likely by acting as a GTPase-activating protein (GAP) for FadA. At least partial inhibition of FadA-mediated growth signaling is required for conidiation and ST production. Activation of asexual sporulation requires the activities of FluG and other downstream developmental genes, *flbB*, *flbC*, *flbD*, *flbE*, and *brlA* (for review see ADAMS *et al.* 1998).

following overexpression of *fluG*, *flbA*, or *flbD*, the gene order, *fluG* ⟶ *flbE* → *flbD* → *flbB*, has been proposed (WIESER and ADAMS 1995). As *fluG* functions first in this regulatory network, *fluG* overexpression requires the activities of *flbC*, *flbA*, and *flbD* for activating *brlA* and causing conidiation in submerged culture (LEE and ADAMS 1996).

The *fluG* gene encodes a cytoplasmically localized 96-kD (864 amino acid) protein that is present at relatively constant levels throughout the life cycle (LEE and ADAMS 1994b). The C-terminal half of FluG shares ~30% identity with prokaryotic glutamine synthetase I whereas the N-terminal half of the protein shares no significant similarity with any functionally characterized proteins in the databases. Moreover, the entire N-terminal region could be deleted without affecting sporulation (D'SOUZA *et al.* 2001), indicating that FluG activity resides in the C-terminal half of the protein, which could be involved in constitutive synthesis of a small diffusible molecule related to glutamine or glutamate (LEE and ADAMS 1994b). Although FluG activity is known to be required for activation of conidiation and for post-transcriptional activation of FlbA (ADAMS *et al.* 1988; WIESER and ADAMS 1995; LEE and ADAMS 1996), specific molecular events responding to *fluG* activity and leading to the developmental switch from vegetative growth remain to be uncovered.

In this article, we describe the isolation, characterization, and genetic analyses of 40 suppressors of *fluG* (SFGs) that bypass the need of *fluG* in conidiation and production of ST in *A. nidulans*. Such second site mutations will be extremely useful to further dissect early regulatory mechanisms of asexual sporulation. As the *fluG*-dependent asexual sporulation is independent of and parallel to FadA- and SfaD:Gγ-mediated growth signaling, no mutations in FadA or SfaD were able to suppress Δ*fluG* (YU *et al.* 1996; ROSEN *et al.* 1999). Thus, these SFGs will likely identify genes that specifically function in the conidiation regulatory pathway downstream of FluG. The fact that at least four loci are defined by 39 *sfg* mutations and all but 1 of the *sfg* mutations are found to be recessive strongly indicates that genes defined by *sfg* mutations likely function as negative regulators of conidiation. Moreover, all SFGs are found to produce ST in the absence of *fluG* activity, suggesting that these *sfg* genes may also negatively affect *flbA* activity and mutations in *sfg* genes might cause at least partial inhibition of FadA growth signaling to allow ST production to occur. A new model for upstream regulation of asexual development and growth is also presented.

MATERIALS AND METHODS

Aspergillus strains, media, growth conditions, and genetic analysis: The *A. nidulans* strains used in this study are listed in Table 1. Genotypes of SFGs are essentially the same (*pabaA1*, *yA2*; Δ*fluG*::*trpC*$^+$; *trpC801*, *veA1*; *sfg*^{S}) except for the *sfg* locus and mutant alleles. Standard culture and genetic techniques were employed (PONTECORVO *et al.* 1953; KÄFER 1977). The growth rates of SFGs were checked on minimal medium (MM) from 2 to 5 days. All liquid cultures were inoculated with 5×10^7 spores in 100 ml of liquid MM or MM with 0.1% yeast extract (YE) with 250 rpm shaking at 37°. Submerged development in each SFG was observed under a microscope at 1-hr intervals after an initial 18-hr growth period in liquid shake culture. Asexual developmental induction was performed as previously described (ADAMS *et al.* 1988) and samples for RNA isolation were collected at designated time points.

All SFGs were crossed with a developmentally wild-type strain, FGSC773. From these crosses, Δ*fluG;sfg*^{S} strains carrying the *pyrG89* allele were also isolated to carry out pairwise crosses to determine the number of loci and for future gene cloning (Table 1). The dominance or recessiveness of each *sfg* mutation was tested by generating diploid strains between each SFG strain (Δ*fluG;sfg*^{S}) and RJH128 (Δ*fluG;sfg*^{WT}) or RJA4.4 (Δ*fluG;sfg*^{WT}).

Mutagenesis and isolation of SFGs: A Δ*fluG* strain (TTA127.4) was point inoculated on supplemented solid MM and incubated at room temperature for 7–10 days and conidia were collected for mutagenesis. Approximately 10^8 conidiospores of TTA127.4 were treated with 1 mg/ml or 10 mg/ml of 4-nitroquinoline-1-oxide (4-NQO; BAL *et al.* 1977) for 0, 30, and 60 min, respectively, as previously described (WIESER *et al.* 1994). The mean survival rate of the 1-mg 4-NQO with a 30-min treatment was 67% and survivors of this condition were further screened.

ST extraction and TLC analysis: Spores (~10^6) of each SFG were inoculated into 2 ml liquid complete medium (CM) in 8-ml tubes and the stationary cultures were incubated at 37° for 7 days as previously described (YU and LEONARD 1995). ST was extracted from 7-day-old cultures by adding 1 ml of

$CHCl_3$ to 8-ml tubes and vortexing for ~1–2 min. The organic phase was transferred to 1.5-ml tubes and centrifuged at 500 × *g* for 5 min. The $CHCl_3$ layer was collected, dried, and resuspended in 50 μl of $CHCl_3$ and ~4–5 μl of each sample was applied onto a thin-layer chromatography (TLC) silica plate containing fluorescence indicator (Kiesel gel 60, 20 cm × 20 cm, 0.25 mm thick; Merck). ST standard was purchased from Sigma and ~5 μg was applied onto the TLC plate with other samples. The plate was then developed with toluene:ethyl acetate:acetic acid (80:10:10, v/v/v), where the R_f value of ST is ~0.65. At this step ST exhibits dark red color under the long-wave UV (320 nm). To enhance visibility and detection limit of ST, aluminum chloride (20% $AlCl_3 \cdot 6H_2O$ in 95% ethanol) is sprayed on to the TLC plate and the plate is baked at 80° for 5 min. The color of ST changes from red to exhibit bright light green by this process (Stack and Rodrick 1971). To compare ST production with *stcU* mRNA levels (presented in Figure 5), duplicate samples were prepared and collected from days 1–4, one for total RNA isolation and the other for ST extraction as described above.

Extracellular rescue of sporulation defect of Δ*fluG* by SFGs: The green conidial Δ*fluG* strains (RJA23.1 and RJA23.2) were point inoculated at the center of MM with 0.5% YE and three SFGs were inoculated both sides of each Δ*fluG* strain in duplicate. The strains were incubated at 37° for ~2–3 days and examined under a stereomicroscope for possible extracellular rescue as previously described (Lee and Adams 1994b).

Nucleic acid isolation and manipulation: Total RNA was isolated by adding 0.6 ml of silica/zirconium beads (Biospec, Bartlesville, OK) and 1 ml of Trizol (Invitrogen, San Diego) and homogenizing in a Mini Bead Beater (Biospec) for 2 min and then subsequently following manufacturer's instructions (Invitrogen). Total RNA (15 μg/lane) was separated by electrophoresis using a 1.1% agarose gel containing 6% formaldehyde and ethidium bromide and the nucleic acids were transferred to a MagnaProbe Nylon membrane (0.45 μm; Osmonics, Minnetonka, MN). Probes for *brlA* and *stcU* mRNA were prepared by amplifying coding regions of *brlA* and *stcU* from wild-type (FGSC4) genomic DNA. A 1.48-kb *brlA* and a 1.12-kb *stcU* (Brown *et al.* 1996) amplicon were labeled with ^{32}P-dCTP using a random-priming kit (Promega, Madison, WI) and used as probes for Northern blot analyses. Hybridization was carried out using modified Church buffer (1 mm EDTA, 0.25 m $Na_2HPO_4 7H_2O$, 1% hydrolysated casein, 7% SDS, adjusted to pH 7.4 with 85% H_3PO_4; Yu and Leonard 1995).

Genomic DNA of wild-type and SFG strains was isolated by adding ~0.3–0.5 ml of silica/zirconium beads, 0.5 ml of breaking buffer [2% Triton X-100, 1% SDS, 100 mm NaCl, 10 mm Tris-HCl (pH 8.0), 1 mm EDTA], and 0.5 ml of phenol:chloroform:isoamylalcohol (25:24:1) to mycelial samples followed by homogenizing in a Mini Bead Beater for 2 min. DNA in the aqueous phase was collected and ethanol precipitated. The purified genomic DNA in 50 μl of Tris-EDTA buffer was diluted 10 times for PCR reactions.

Microscopy: Photomicrographs were taken using an Olympus BH2 compound microscope with the Kodak MDS290 digital imaging system. All other photographs were taken using a SONY digital camera (DSC-F707).

RESULTS

Isolation of suppressors of *fluG*: To gain further insights into the molecular events arising from *fluG* activity and leading to activation of developmental switch, we took an unbiased approach involving the isolation of second-site mutations that overcome the sporulation defects of a loss of *fluG* function. Unlike the previous study where a homozygous Δ*fluG* diploid strain was employed to specifically look for dominant suppressor mutations (D'Souza *et al.* 2001), we used a Δ*fluG* haploid strain (TTA127.4), expecting that various recessive and/or dominant suppressor mutants would be isolated. We visually screened ~125,000 survivors from 4-NQO mutagenesis and isolated 40 putative suppressors of Δ*fluG* (SFGs) that restored conidiation to clearly distinguishable levels. These 40 SFG mutants show varying levels of conidiation recovery and on the basis of the phenotypes on solid MM, the mutants were tentatively grouped into three classes: 9 high sporulators (HS), 18 wild-type level sporulators (W), and 13 delayed sporulators (DS). Delayed sporulators initially resemble the Δ*fluG* mutant for ~2–3 days, but most of them achieve wild-type sporulation levels within 5 days. Representative SFG strains are presented in Figure 2. To be consistent, we designate a mutant strain with SFG#, a mutant allele with *sfg*#, suppressor mutations collectively with sfg^S, and a wild-type allele with sfg^{WT}.

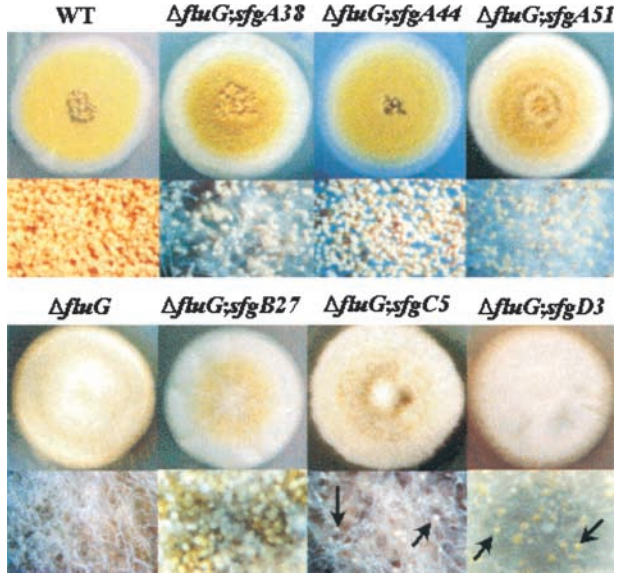

Figure 2.—Phenotypes of SFGs. Each top row shows the colonies of wild type, Δ*fluG*, and designated SFG strains. The bottom shows the close-up views of the colonies (40× magnification). Note that a Δ*fluG* strain (TTA127.4) shows undifferentiated hyphal mass (fluffy) whereas SFG strains show varying levels of restored conidiation. Most of the delayed sporulators restore conidiation to near wild-type levels within 5 days after inoculation on solid medium. Photographs show colonies that were point-inoculated onto MM with 0.1% YE and incubated for 3 days at 37°. Arrows indicate conidiophores in delayed sporulators.

Individual suppression is caused by a single second-site mutation unlinked to *fluG*: As a first step in genetic analyses, each SFG strain was meiotically crossed with a developmentally wild-type strain (FGSC773) and all of the crosses generated matured cleistothecia. If suppression was caused by a single second-site mutation, segregation of the relevant genotypes (with corresponding

phenotypes) of progeny would be Δ*fluG;sfg*^{S} (conidiating), *fluG*$^{+}$*;sfg*^{S} (probably conidiating like wild type), Δ*fluG;sfg*^{WT} (fluffy due to Δ*fluG*), and *fluG*$^{+}$*;sfg*^{WT} (wild type), thus generating 25% fluffy progeny. All of our successful crosses showed ~25% recovery of Δ*fluG* (fluffy phenotype) progeny, indicating that a single gene mutation, not linked to *fluG*, caused individual suppression. In addition, no new phenotypes among the conidiating progeny were evident, indicating that individual *sfg*^{S} mutation does not cause readily detectable morphological changes with respect to wild-type FluG function. In these crosses, we have also isolated multiple recombinant SFG strains carrying the auxotrophic marker *pyrG89* and these were used to determine the number of *sfg* loci (see below). Isolation of Δ*fluG*; *sfg*^{S}; *pyrG89* strains was accomplished by examining ~10 independent uracil-requiring conidiating progeny from each cross for the Δ*fluG* pattern by genomic DNA PCR. Conidial strains with the Δ*fluG* PCR pattern are expected to be Δ*fluG* with *sfg*^{S} (Table 1).

Dominance and recessiveness of *sfg*^{S}: As a prerequisite of the SFG gene identification we tested whether each *sfg*^{S} is dominant or recessive to its wild-type allele by generating diploids that are homozygous for Δ*fluG* and heterozygous for *sfg* (*sfg*^{S}; *sfg*^{WT}) by fusing an individual SFG strain with another Δ*fluG* strain, RJH128 or RJA4.4. Diploid strains of 39 SFGs exhibited a fluffy phenotype like Δ*fluG*, indicating that these *sfg*^{S} mutations are recessive to their wild-type alleles (Table 2). One diploid strain (dSFG38) sporulated to wild-type level, suggesting that *sfg38* defines a dominant (interfering) mutation, which was later found to be an allele of recessive mutants (see below).

At least four loci are defined by *sfg* mutations: In an attempt to determine the number of genes defined by *sfg* mutations, each primary SFG strain was meiotically crossed with recombinant SFG strains carrying different auxotrophic markers (Δ*fluG*; *sfg*^{S}; *pyrG89*; and/or *pyroA4*). While pairwise crosses of two allelic (or tightly linked) *sfg*^{S} mutations would produce only conidial progeny, crosses of nonallelic *sfg*^{S} mutations are expected to generate ~25% fluffy progeny due to Δ*fluG* (*sfg*^{wt1}*;sfg*^{wt2}*;*Δ*fluG*). The results of pairwise crosses showed that 39 *sfg*^{S} mutations represented at least four linkage groups (*sfgA* ~ *D*), where 31 mapped to *sfgA*, 6 mapped to *sfgB*, and 1 each mapped to *sfgC* and *sfgD*, respectively (Tables 1 and 2). Although SFG8 could not be assigned to a specific linkage group due to extreme difficulty in sexual crosses, it was found not to be an allele of *sfgA* or *sfgC*. Isolation of 31 suppressors mapped to *sfgA* (or a tightly linked locus) presents a strongly biased distribution of suppressor mutations in *sfgA*. Moreover, mutations in the *sfgA* locus seem to result in varying ranges of recovered conidiation (Figure 2; Table 2; HS, W, and DS) indicating that partial loss of *sfgA* function might be sufficient to cause suppression of Δ*fluG*.

Two previously reported mutations, *sfdA15* and *sfdB38*, which were originally isolated as suppressors of Δ*flbD*, were found to suppress Δ*fluG* (Kellner and Adams 2002). Thus, it was of interest to see whether these *sfd* mutations could be mapped to any of four *sfg* loci. REK65.13 and REK88.23 were meiotically crossed with SFG strains from each linkage group and *sfdA15* was found not to be linked to *sfgA*, *sfgB*, or *sfgC*. Meiotic crosses between *sfdB38* mutant and selected SFG strains or *sfdA15* with *sfgD* mutants failed to generate matured cleistothecia.

Mutations in *sfgA* and *sfgC* cause submerged conidiation: One of the phenotypic characteristics of hyperactive conidiation is the formation of conidiophores in liquid submerged culture, conditions under which wild-type strains do not sporulate. To test whether some SFGs show hyperactive conidiation even in the absence of FluG activity, we examined an individual SFG strain's ability to form conidiophores in liquid shake culture and found that 14 SFGs elaborated conidiophores in submerged culture within 25 hr, where 13 and 1 belong to the linkage groups A and C, respectively. Particularly, SFG43 (Δ*fluG*; *sfgA43*) and SFG44 (Δ*fluG*; *sfgA44*) began to form vesicles at 18 hr and produced complete conidiophores within 20 hr (Table 2; Figure 3). SFG5 (Δ*fluG*; *sfgC5*) produced complete conidiophores within 22 hr in liquid MM with 0.1% YE, but not in liquid MM alone (Figure 3). SFG5 exhibited delayed conidiation phenotype with enhanced growth (25% more than wild type) on solid MM (Figure 2 and Table 2). Addition of YE to SFG5 cultures, however, caused reduced growth (Table 2), slightly increased conidiation on solid medium (not shown), and inappropriate conidiation in liquid culture (Figure 3). None of the mutants belonging to linkage group B or D produced conidiophores in submerged culture.

We selected four SFG mutants, SFG5, SFG38, SFG44, and SFG51, and examined accumulation of *brlA* transcript at various stages of the life cycle. As presented in Figure 4, a wild-type strain (FGSC26) does not show *brlA* transcript accumulation during vegetative growth phase or before 8 hr post-asexual induction. However, SFG5, which elaborated conidiophores within 22 hr of vegetative growth, accumulated *brlA* transcript at 24 hr in liquid culture and at 12 and 24 hr post-asexual developmental induction (Figure 4). Almost identical *brlA* accumulation patterns were observed for SFG44 (not shown). Both SFG5 and SFG38 strains exhibit an ~4-hr delay of *brlA* (and *stcU*, see below) transcript accumulation. SFG51 (a delayed sporulator) accumulated detectable levels of *brlA* transcript at 24 hr postinduction (not shown). These data show that the timing and levels of *brlA* transcript accumulation upon asexual developmental induction are closely related to phenotypes of SFGs on solid medium. Unlike SFG5 and SFG44, SFG38 and SFG51 do not form conidiophores in liquid submerged culture.

Most *sfg* mutations affect hyphal growth: Asexual de-

TABLE 1

***A. nidulans* strains used in this study**

Strain	Genotype[a]	Source
FGSC26	*biA1*	FGSC[b]
FGSC4	*veA+*	FGSC
FGSC773	*pyrG89;wA3;pyroA4*	FGSC
RKH51.117	*pabaA1, yA2*	K.-H. Han and J.-H. Yu (unpublished data)
TTA127.4	*pabaA1, yA2;*Δ*fluG::trpC*$^+$*;trpC801*	Lee and Adams (1994a)
RJH128	*biA1;methG1,*Δ*fluG::trpC*$^+$*;alcA(p)::aflR::trpC*$^+$	J. K. Hicks and N. P. Keller (unpublished data)
RJA4.4	*pyrG89, yA2;*Δ*fluG::trpC*$^+$	This study
RJA23.1	*pabaA1;*Δ*fluG;hisJ122*	This study
RJA23.2	*pabaA1;*Δ*fluG;hisJ122*	This study
REK65.13	*biA1;methG1;sfdA15*	Kellner and Adams (2002)
REK88.23	*biA1;sfdB38;methG1*	Kellner and Adams (2002)
HDCD15.1	Δ*fluG::trpC*$^+$; *dsgA1*; *hisJ122*	C. A. D'Souza and T. H. Adams (unpublished data)
SFG44[c]	*pabaA1,yA2;*Δ*fluG::trpC*$^+$*;trpC801;sfgA44*	This study
SFG7[d]	*pabaA1,yA2;*Δ*fluG::trpC*$^+$*;trpC801;sfgB7*	This study
SFG5	*pabaA1,yA2;*Δ*fluG::trpC*$^+$*;trpC801;sfgC5*	This study
SFG3	*pabaA1,yA2;*Δ*fluG::trpC*$^+$*;trpC801;sfgD3*	This study
SFG8[e]	*pabaA1,yA2;*Δ*fluG::trpC*$^+$*;trpC801;sfg8*	This study
RSFG2.21	*pyrG89;*Δ*fluG::trpC*$^+$*;sfgA2*	This study
RSFG4.2	*wA3;*Δ*fluG::trpC*$^+$*, pyroA4; sfgA4*	This study
RSFG5.4	*pyrG89;*Δ*fluG::trpC*$^+$*;sfgC5*	This study
RSFG6.4	*pyrG89;*Δ*fluG::trpC*$^+$*;sfgA6*	This study
RSFG12.24	*pyrG89;*Δ*fluG::trpC*$^+$*,pyroA4;sfgA12*	This study
RSFG13.6	*pyrG89,yA2;*Δ*fluG::trpC*$^+$*;sfgA13*	This study
RSFG14.16	*pyrG89;wA3;*Δ*fluG::trpC*$^+$*,pyroA4;sfgA14*	This study
RSFG15.21	*pyrG89;*Δ*fluG::trpC*$^+$*,pyroA4;sfgA15*	This study
RSFG22.2	*pyrG89,yA2;*Δ*fluG::trpC*$^+$*;sfgA22*	This study
RSFG23.1	*pyrG89;*Δ*fluG::trpC*$^+$*;sfgA23*	This study
RSFG26.16	*pyrG89;wA3;*Δ*fluG::trpC*$^+$*;sfgA26*	This study
RSFG27.17	*pyrG89;*Δ*fluG::trpC*$^+$*,pyroA4;sfgB27*	This study
RSFG28.22	*pyrG89;*Δ*fluG::trpC*$^+$*;sfgB28*	This study
RSFG30.6	*pyrG89,yA2;*Δ*fluG::trpC*$^+$*;sfgA30*	This study
RSFG34.14	*pyrG89;*Δ*fluG::trpC*$^+$*,pyroA4;sfgA34*	This study
RSFG37.6	*pyrG89;wA3;*Δ*fluG::trpC*$^+$*,pyroA4;sfgA37*	This study
RSFG38.3	*pyrG89;*Δ*fluG::trpC*$^+$*,pyroA4;sfgA38*	This study
RSFG44.16	*pyrG89;*Δ*fluG::trpC*$^+$*;sfgA44*	This study
RSFG45.1	Δ*fluG::trpC*$^+$*,pyroA4;sfgA45*	This study
RSFG47.2	*pyrG89,yA2;*Δ*fluG::trpC*$^+$*;sfgA47*	This study
RSFG51.4	*pyrG89;wA3;*Δ*fluG::trpC*$^+$*;sfgA51*	This study
RSFG52.28	*pyrG89;*Δ*fluG::trpC*$^+$*;sfgB52*	This study
RSFG53.12	*pyrG89,yA2;*Δ*fluG::trpC*$^+$*;sfgB53*	This study
DSFG[f]	$\frac{+,+,biA1;methG1,\Delta fluG::trpC^+;\ alcA(p)::aflR::trpC^+;+}{pabaA1,yA2,+;+,\Delta fluG::trpC^+;trpC801;sfg\#}$	This study

[a] All strains are *veA1* except FGSC4.

[b] Fungal Genetics Stock Center.

[c] SFG1, -2, -4, -6, -11, -12, -13, -14, -15, -18, -22, -23, -26, -30, -32, -33, -34, -35, -36, -37, -38, -42, -43, -44, -45, -46, -47, -48, -50, -51, and -54 are isogenic except for *sfgA#* mutant alleles.

[d] SFG7, -27, -28, -41, -52, and -53 are expected to carry *sfgB#* mutant alleles.

[e] SFG8 is not mapped to a specific linkage group but is not linked to *sfgA* or *sfgC*.

[f] DSFG# represents a diploid strain generated by fusing each SFG with RJH128 or rJA4.4 (diploid genotype: *pyrG89*, +, *yA2*; Δ*fluG::trpC*$^+$; +;+/+, *pabaA1*, *yA2*; Δ*fluG::trpC*$^+$;*trpC801*;*sfg#*). Each diploid strain is isogenic except for the *sfg* number.

velopment and hyphal growth are antagonistic in that elevation of one process causes downregulation of the other process. The fact that SFGs bypass the requirement for *fluG* in asexual development and that some SFGs exhibit a hyperactive conidiation phenotype leads us to think that some *sfg* mutations might also affect hyphal growth, probably due to elevated asexual development. To test this, growth rates of each SFG strain

TABLE 2

Characteristics of SFGs

Strain	Linkage group	Phenotype[a]	Submerged conidiation[b]	Growth rate: mm/hr (% of WT)[c]
FGSC26	NA[d]	W	−	0.55 ± 0.03 (100)
TTA127.4	NA	F	−	0.53 ± 0.01 (96.4)
SFG1	A	HS	−	0.38 ± 0.12 (69.1)
SFG2	A	HS	−	0.56 ± 0.03 (101.8)
SFG3	D	DS	−	0.40 ± 0.09 (72.7)
SFG4	A	W	++	0.45 ± 0.09 (81.8)
SFG5	C	DS	++[f]	0.69 ± 0.007 (125)
SFG6	A	DS	−	0.48 ± 0.07 (87.3)
SFG7	B	DS	−	0.46 ± 0.10 (83.6)
SFG8	ND[e]	DS	−	0.32 ± 0.05 (58.2)
SFG11	A	W	++	0.39 ± 0.12 (70.9)
SFG12	A	HS	−	0.39 ± 0.11 (70.1)
SFG13	A	W	−	0.42 ± 0.09 (76.4)
SFG14	A	HS	−	0.43 ± 0.09 (78.2)
SFG15	A	DS	−	0.38 ± 0.12 (69.1)
SFG18	A	W	−	0.40 ± 0.11 (72.7)
SFG22	A	HS	−	0.37 ± 0.13 (67.3)
SFG23	A	W	−	0.53 ± 0.03 (96.4)
SFG26	A	DS	−	0.37 ± 0.09 (67.3)
SFG27	B	DS	−	0.27 ± 0.05 (49.1)
SFG28	B	DS	−	0.36 ± 0.08 (65.5)
SFG30	A	W	++	0.42 ± 0.09 (76.4)
SFG32	A	W	−	0.42 ± 0.09 (76.4)
SFG33	A	W	−	0.40 ± 0.11 (72.7)
SFG34	A	HS	−	0.46 ± 0.07 (83.6)
SFG35	A	HS	+	0.45 ± 0.07 (81.8)
SFG36	A	W	−	0.57 ± 0.02 (104)
SFG37	A	W	++	0.42 ± 0.11 (76.4)
SFG38	A	W	−	0.40 ± 0.11 (72.7)
SFG41	B	DS	−	0.43 ± 0.01 (78.2)
SFG42	A	HS	+	0.50 ± 0.07 (90.9)
SFG43	A	W	+++	0.42 ± 0.10 (76.4)
SFG44	A	HS	+++	0.38 ± 0.11 (69.1)
SFG45	A	W	++	0.42 ± 0.11 (76.4)
SFG46	A	W	+	0.56 ± 0.06 (102)
SFG47	A	W	++	0.42 ± 0.09 (76.4)
SFG48	A	W	++	0.43 ± 0.09 (78.2)
SFG50	A	W	++	0.59 ± 0.03 (107.3)
SFG51	A	DS	−	0.42 ± 0.10 (76.4)
SFG52	B	DS	−	0.28 ± 0.09 (50.9)
SFG53	B	DS	−	0.42 ± 0.05 (76.4)
SFG54	A	W	−	0.46 ± 0.06 (83.6)

[a] Each SFG mutant phenotype was assigned by growing SFGs on MM with 0.1% of YE at 37° and then measuring the number of conidia/cm^2 (data not shown), growth rate, and the degree of fluffy edge (data not shown). F, fluffy with no conidiation; HS, high sporulation; DS, delayed sporulation; W, wild-type level sporulation.

[b] Submerged conidiation was tested in liquid MM at 37° with 250 rpm shaking. +++, conidiation within 20 hr; ++, conidiation between 21 and 23 hr; +, conidiation between 24 and 25 hr; −, no conidiation observed until 25 hr.

[c] Growth rates were determined by measuring diameters of colonies point inoculated at the center of MM grown at 37°. Measurement was made at 2–5 days with 24-hr intervals. Two separate experiments were carried out in duplicate. The mean value, the standard deviation, and relative growth to wild type (%) are presented.

[d] Not applicable.

[e] Not determined.

[f] SFG5 produces conidiophores at 22 hr in MM with 0.1% of YE but not in MM alone (see text).

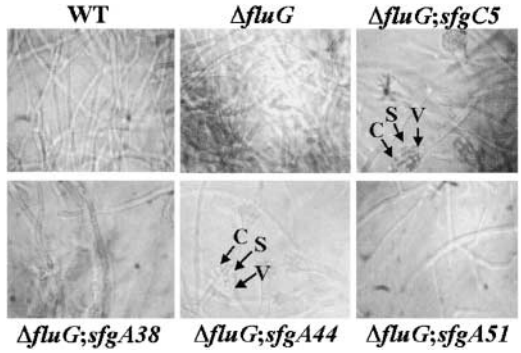

FIGURE 3.—Mutations in *sfgA* or *sfgC* result in hyperactive conidiation. SFG44 (Δ*fluG*; *sfgA44*) and SFG5 (Δ*fluG*; *sfgC5*) started to form vesicles (V) within ~18–20 hr and produced complete conidiophores after ~20–22 hr of incubation. Like most SFGs, SFG38 (Δ*fluG*; *sfgA38*) and SFG51 (Δ*fluG*; *sfgA51*) do not produce conidiophores in liquid submerged culture. Approximately 5×10^7 conidia were inoculated into 100 ml of liquid MM with supplements and 0.1% YE in 250-ml flasks and incubated at 37° with 250 rpm shaking. Photographs were taken at 22 hr of submerged culture. C, conidia; S, sterigmata; V, vesicle.

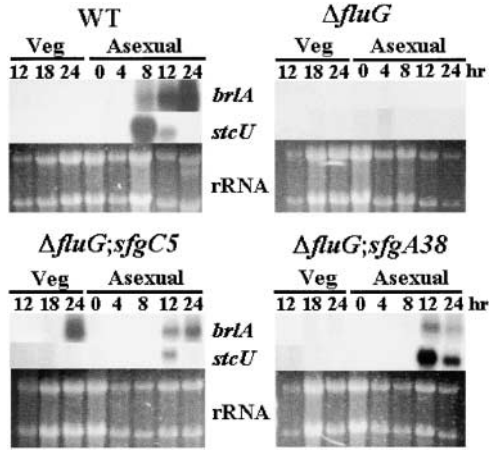

FIGURE 4.—SFGs restore accumulation of *brlA* and *stcU* transcripts. While *brlA* and *stcU* transcripts were readily detected in wild-type strains upon asexual developmental induction (Asexual), no *brlA* or *stcU* transcripts are detected at any time points of a Δ*fluG* strain. As SFG5 (Δ*fluG*; *sfgC5*) produces conidiophores within 22 hr postinoculation in submerged culture, it shows *brlA* mRNA accumulation at 24 hr of vegetative growth (Veg) as well as 12 and 24 hr post-asexual induction. SFG38 (Δ*fluG*; *sfgA38*) shows *brlA* mRNA accumulation at 12 and 24 hr postinduction but not in vegetative cultures. SFG44 and SFG51 show similar Northern blot results to SFG5 and SFG38, respectively (data not shown). While wild type shows the highest level of *stcU* transcript accumulation at 8 hr postinduction, SFG5 and SFG38 accumulate *stcU* in a delayed manner.

on solid MM and MM with 0.1% YE (not shown) were measured in triplicate and compared with those of a wild-type strain (FGSC26). As shown in Table 2, SFG mutants show varying levels of growth, ~49–125% of that of wild type, and all SFGs but five (SFG2, SFG5, SFG36, SFG46, and SFG50) show reduced hyphal growth compared to wild type. Particularly, two *sfgB* mutant alleles, *sfgB27* and *sfgB52*, caused reduction of growth rates to ~50% of wild type on MM. The fact that mutations in *sfgB* cause reduced growth yet have low levels of conidiation (delayed conidiation) suggests that *sfgB* might elucidate a new cross-talking network between growth and asexual development.

No *sfg* mutations extracellularly rescue the conidiation defect of Δ*fluG*: One of the phenotypic characteristics of Δ*fluG* strains is that the conidiation defect can be rescued by growing Δ*fluG* strains next to either wild-type or other developmental mutants (LEE and ADAMS 1994b). We tested whether any of 40 SFGs could extracellularly rescue the sporulation defect of Δ*fluG* strains. All primary SFGs originated from TTA127.4 (*pabaA1, yA2*; Δ*fluG*::*trpC*$^+$; *trpC801*, *veA1*) and produce yellow conidia due to the *yA2* mutation. Thus, for efficient rescue experiments, we generated two Δ*fluG* strains that produce green conidia (RJA23.1 and RJA23.2; see Table 1) and tested all SFGs in duplicate and found that no SFGs were able to rescue the conidiation defect of Δ*fluG* strains.

All SFG mutants regain the ability to produce ST: Previously, it was shown that FluG is required for the production of the mutagenic and carcinogenic mycotoxin ST in *A. nidulans*. It has been proposed that this requirement for FluG is via activating FlbA, which in turn inactivates FadA growth signaling (HICKS *et al.* 1997). To test whether SFGs regain the ability to produce ST, we initially examined 40 SFGs for ST production at 7 days of culture and found that all were able to produce ST at near wild-type levels without *fluG* activity (data not shown). Two developmentally wild-type strains, FGSC26 and RKH51.117 (an isogenic wild-type strain, Table 1), have been compared with SFG strains and both wild-type strains showed the same ST accumulation patterns. To confirm that ST recovery is through restored *stc* gene expression we examined the accumulation of ST and *stcU* mRNA in selected SFGs days 1–4 as previously described (YU and LEONARD 1995). As presented in Figure 5, while the Δ*fluG*; *sfg*^{WT} strain (TTA127.4) does not accumulate *stcU* transcript or ST, four selected SFGs, including dominant SFG38, show certain levels of restored *stcU* mRNA and ST accumulation. Although until day 4 some SFGs produced less ST than did wild type, they all produced ST at near wild-type levels at 7 days of culture. We also examined *stcU* transcript accumulation in SFG5, SFG38, wild-type, and Δ*fluG* strains at various stages of the life cycle. SFG5 does not show *stcU* transcript accumulation in liquid culture, even though it conidiates and accumulates *brlA* transcript (Figure 4). Upon induction of asexual development, however, both SFG5 and SFG38 show similar patterns of *brlA* and *stcU* transcript accumulation (Figure 4). Recovery of ST production is an important difference between *sfg*^{S} mutations and the previously reported

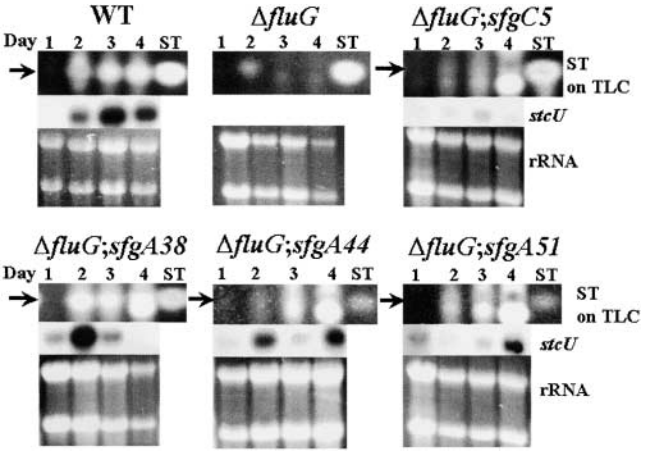

FIGURE 5.—Sterigmatocystin (ST) production and *stcU* mRNA accumulation in SFGs. Duplicates of wild type (FGSC26), TTA127.4 (Δ*fluG*), SFG5 (Δ*fluG*; *sfgC5*), SFG38 (Δ*fluG*; *sfgA38*), SFG44 (Δ*fluG*; *sfgA44*), and SFG51 (Δ*fluG*; *sfgA51*) were grown in stationary liquid CM for 1, 2, 3, and 4 days at 37°. One set was then used for ST analysis and the other was used for total RNA isolation. An isogenic wild-type strain (RKH51.117) was also examined and showed the same ST accumulation patterns as FGSC26 (not shown). The top shows ST analysis on TLC and arrows indicate the position of ST determined by the ST standard. Accumulation of ST was confirmed by (1) the migration distance (R_f value ~ 0.65), (2) dark red color of the spot before $AlCl_3$ spray, and (3) bright light green fluorescence after spraying $AlCl_3$ (see MATERIALS AND METHODS). SFGs with *sfgC5*, *sfgA44*, and *sfgA51* mutations accumulated relatively low amounts of ST until 4 days. Bright spots below and above ST shown in these SFGs, which have different R_f values and colors (light blue), are not ST but unknown compounds. Although low amounts of ST and increased accumulation of unknown compounds in these SFGs make it difficult to clearly view ST accumulation, they all produced ST at day 4 and even accumulated ST at near wild-type levels at 7 days of culture. Northern blot analyses of *stcU* mRNA are shown at the bottom. Equal loading of total RNA was evaluated by ethidium bromide staining of rRNAs.

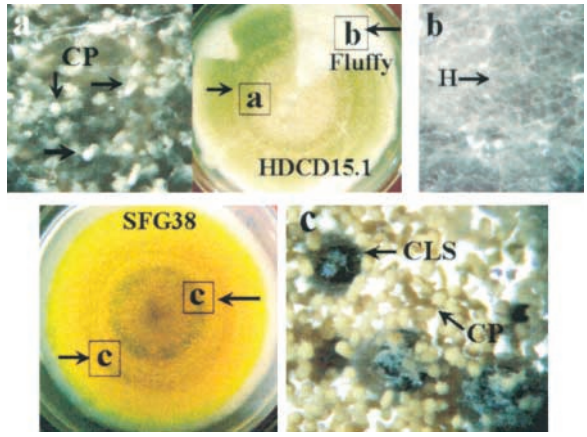

FIGURE 6.—Phenotypes of dominant *fluG* suppressor mutants. Colonies of two dominant *fluG* suppressors, HDCD15.1 (Δ*fluG*; *dsgA1*) and SFG38 (Δ*fluG*; *sfgA38*), grown on MM for 7 days at 37° are shown. Magnified images (40×) of conidial (a) and fluffy (b) regions of HDCD15.1 and a region of enriched cleistothecia (c) in SFG38 are also presented. CP, conidiophores; H, hyphae; CLS, cleistothecia.

dominant suppressor of *fluG*, *dsgA1*, because *dsgA1* cannot bypass the need for *fluG* in ST production (D'SOUZA *et al.* 2001). These results indicate that *sfg*^{S} mutations, even without FluG activity, can (at least partially) inhibit FadA growth signaling and allow ST biosynthesis to occur.

Dominant negative *sfgA38* is different from *dsgA1*: At present, only two (*sfgA38* and *dsgA1*) dominant suppressors of *fluG* have been isolated. As described, *sfg38* and 30 other recessive *sfg* mutations mapped to linkage group A, suggesting that *sfgA38* is likely a dominant interfering (negative) mutant allele. We attempted to test whether *dsgA1* can be mapped to any of the four *sfg* linkage groups by meiotic crosses between SFGs and a Δ*fluG*; *dsgA1* strain (HDCD15.1). While HDCD15.1 readily formed heterokaryons with most Δ*fluG*; *sfg*^{S} mutant strains, no matured cleistothecia were formed even under conditions that enhance sexual development (D'SOUZA *et al.* 2001).

Despite unsuccessful crosses between SFGs and HDCD-15.1, on the basis of their clear phenotypic differences, one can speculate that *sfgA38* and *dsgA1* might define different genes. As shown in Figure 6, HDCD15.1 readily generates fluffy sectors consistently whereas SFG38 never does that. The fluffy sector stays fluffy in subsequent generations, indicating that the dominant nature of the *dsgA1* mutation is somehow lost permanently in this sector. While SFG38 does not form fluffy sectors, it produces circles of enriched cleistothecia (Figure 6c). The interval and width of cleistothecia-enriched bands indicate that SFG38 likely undergoes elevated sexual development at 24-hr intervals, each interval lasting ~12 hr. Additional critical differences between *sfgA38* and *dsgA1* are: (1) while *sfgA38* restores ST production to wild-type level without *fluG*, *dsgA1* requires *fluG* for ST production; and (2) *sfgA38* does not cause conidiophore formation in liquid submerged culture whereas *dsgA1* causes formation of conidiophores in the absence of *fluG* (D'SOUZA *et al.* 2001).

DISCUSSION

The genus Aspergillus encompasses the most common fungi in our environment. Members of this genus reproduce asexually by forming long chains of conidiospores radiating from a central structure known as a conidiophore. One of the primary questions has been how such a complex structure differentiates from vegetatively growing hyphae. Previous studies in *A. nidulans* showed that FluG is required for this developmental switch by activating the conidiation process and indirectly regulating the G protein-mediated growth-signaling cascade via activating FlbA (LEE and ADAMS 1994a, 1996; YU *et al.* 1996). Balanced control of these two

parallel signaling pathways is essential for maintaining the genetically programmed life cycle. For the last decade, heterotrimeric G proteins and their signaling/regulatory mechanisms have been intensively studied and are found to be conserved from yeasts to humans. Such structural and functional conservancy of the G protein signaling components makes reverse genetics and functional genomics favorable approaches. However, genes functioning in the conidiation process and associated regulatory mechanisms are largely unknown primarily due to uniqueness of the conidiation processes in filamentous fungi. Moreover, like FluG, many genes are expected to be novel in their structures and functions. Therefore, we believe that unbiased genetic studies represent the most suitable way to further dissect upstream regulatory mechanisms of conidiation in *A. nidulans*.

FluG is required for activation of conidiation and it functions upstream of other developmentally specific genes including *flbE*, *flbD*, *flbC*, *flbB*, and *brlA* (see Figure 1). Previous genetic studies looking for developmentally defective mutants from a wild-type strain were specifically aimed at the identification of positive regulators of conidiation. A recent study of *fluG* describing the isolation of dominant suppressors of Δ*fluG* was also biased to the identification of activating components of conidiation (D'Souza *et al.* 2001). If negative elements were involved in the regulatory cascade of conidiation, previous studies would have missed these critical components. Thus, to investigate molecular mechanisms associated with the FluG-mediated activation of conidiation in an unbiased way, we have isolated and characterized a large number of SFGs, employing a haploid Δ*fluG* strain. Because the *fluG*-dependent initiation of asexual sporulation is independent of and parallel to FadA- and SfaD:Gγ-mediated growth signaling and no mutations in FadA or SfaD are able to suppress Δ*fluG* (Yu *et al.* 1996, 1999; Rosen *et al.* 1999), genes presented by suppressors of *fluG* are expected to specifically function in the conidiation pathway downstream of FluG.

Characterization and genetic analyses of 40 SFG mutants have been carried out. Because the *fluG* deletion mutant was used for the isolation of suppressor, all SFGs are expected to be extragenic and bypass suppressors of *fluG*. Genetic analyses of SFGs can be summarized as follows: (1) each SFG is derived from a single second-site mutation; (2) 39 *sfg* mutations are recessive to their wild-type alleles and only 1 (*sfgA38*) is dominant; (3) at least four loci are defined by *sfg*^{s} mutations; (4) 31, 6, 1, and 1 mutations are mapped to linkage groups *sfgA*, *sfgB*, *sfgC*, and *sfgD*, respectively; and (5) the dominant mutation *sfgA38* is an allele of *sfgA* and is different from *dsgA1*. The fact that at least four loci are defined by recessive *sfg* mutations indicates that multiple genes are involved in negative control of conidiation downstream of *fluG*, which supports our reason for using a haploid Δ*fluG* strain. In this study, the most interesting findings

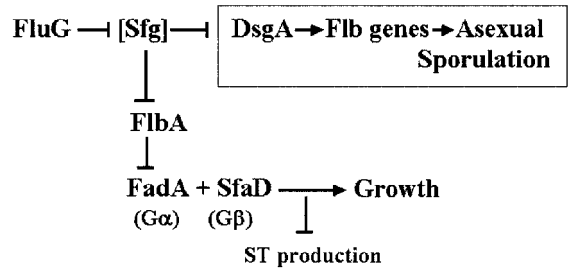

Figure 7.—A new model for upstream regulation of asexual sporulation. DsgA is positioned within the developmental-specific functions that do not affect FlbA-mediated ST remediation. FluG functions the most upstream and its primary role is to remove repressive effects imposed by downstream *sfg* genes. Elimination of Sfg-mediated negative regulation is necessary for activation of conidiation-specific functions and FlbA, which in turn confers ST production.

are that 31 SFGs including the dominant mutant SFG38 are mapped to the *sfgA* linkage group and that they show varying levels of recovered conidiation (Table 2), where 28 are wild-type level or hyperactive sporulators and 4 are delayed sporulators. These results strongly indicate that *sfgA* functions as a key negative regulator of conidiation and it might have multiple functional domains. Any mutations causing (at least partial) loss of *sfgA* function(s) may be sufficient to restore conidiation to certain levels. Depending on the levels of remaining functionality of the SfgA mutant products, varying ranges of suppression, *i.e.*, delayed sporulation to hyperactive sporulation, might result. On the other hand, incremental loss of *sfgA* function would result in elevated levels of restored conidiation and even hyperactive conidiation. Supporting this idea, a relatively large number (13 of 31) of *sfgA* mutations are found to cause submerged conidiation in the absence of *fluG* activity. Unlike *sfgA* mutations, however, most mutations in *sfgB* seem to result in delayed conidiation, suggesting that partial (or even complete) loss of *sfgB* function might not be sufficient to cause full recovery of conidiation. Furthermore, the fact that only one suppressor mutation each has been mapped to *sfgC* or *sfgD* after screening 125,000 survivors suggests that only specific mutation(s) in *sfgC* or *sfgD*, *e.g.*, a complete loss of function, might bypass *fluG* function. Similar to *sfgA*, a complete loss of *sfgC* function might be sufficient to cause hyperactive conidiation, but only with yeast extract (see Figure 3). However, regardless of levels of recovered conidiation, all SFG mutants regained the ability to produce ST to near wild-type levels, suggesting that all *sfg* mutations could cause (at least partial) activation of FlbA.

On the basis of our findings, a new genetic model for upstream regulation of asexual development in *A. nidulans* is proposed (Figure 7). In this model, *dsgA* is positioned in the box of conidiation-specific functions because *dsgA1* suppresses conidiation but not the ST production defects of Δ*fluG* (D'Souza *et al.* 2001). While

genetic interactions among four *sfg* genes need to be studied, the products of the *sfgA*, *sfgB*, *sfgC*, and *sfgD* genes, represented as [Sfg], negatively regulate conidiation-specific functions as well as certain FlbA functions, which are necessary for ST production through inhibition of FadA growth signaling. We propose that the primary role of FluG is to remove these repressive effects imposed by multiple *sfg* genes. In the proposed model, a complete loss of negative regulation by the *sfg* genes would cause a full elimination of repressive effects, resulting in hyperactive sporulation as seen in 13 *sfgA* and 1 *sfgC5* mutants. Conversely, partial functions of the *sfg* genes would maintain certain levels of negative regulation, which cause low levels of remediation of conidiation, *i.e.*, delayed conidiation.

However, removal of negative regulation is not sufficient to alter developmental competence. Previously, it has been shown that conidiation does not occur until cells have gone through a defined period of vegetative growth (~18 hr), during which cells acquire competence to respond to developmental signaling or induction (for review see Adams *et al.* 1998). Although many SFGs produced conidiophores in liquid culture in a relatively short time, no SFGs were able to shorten the proposed time (18 hr) for acquisition of developmental competence. Furthermore, no mutations in FadA or SfaD result in earlier than 20-hr conidiophore development in liquid culture (Yu *et al.* 1996, 1999; Rosen *et al.* 1999). These results are consistent with the observation that overexpression of *fluG* does not alter the time required for conidiophore development to begin in an air-exposed colony (Lee and Adams 1996). In contrast, *dsgA1* causes submerged conidiation between 9 and 11 hr of incubation even without FluG activity, indicating that time required for competence in a *dsgA1* mutant is greatly shortened (D'Souza *et al.* 2001). Collectively, acquisition of developmental competence may be defined by activation of downstream developmentally specific functions, which involve coordination of upstream positive and negative regulatory functions.

It has been proposed that FluG is required for the production of the extracellular sporulation factor. The fact that both FluG mRNA and protein are present at relatively constant levels throughout the life cycle implies that accumulation of the factor above a certain level (threshold) might be necessary to trigger the switch from vegetative growth to conidiation (Lee and Adams 1996; D'Souza *et al.* 2001). No SFG mutants were able to rescue the conidiation defect of Δ*fluG* by proximal growth, suggesting that suppression of Δ*fluG* is due to alterations in intracellular regulation, not through the recovery of the production of the extracellular sporulation factor(s). Overexpression of *fluG* results in inappropriate production of conidiophores that are remarkably similar to wild-type conidiophores and have all the cell types including stalks, vesicles, metulae, phialides, and conidia, indicating that activation of *fluG* results in activation of all the genes necessary to form a complete conidiophore (Lee and Adams 1996). Similarly, 14 SFGs produce complete conidiophores within 25 hr of liquid submerged culture, implying that *sfg* genes likely function immediately downstream of *fluG* (see Figure 3). Taken together, we propose that *sfg* genes act to repress conidiation during the early vegetative growth phase and that accumulation of the sporulation factor above certain levels serves as a signal to remove this negative regulation.

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