Curr Genet (1994) 27: 62–69 © Springer-Verlag 1994

ORIGINAL PAPER

Jenny Wieser · Bee Na Lee · John W. Fondon III
Thomas H. Adams

Genetic requirements for initiating asexual development in *Aspergillus nidulans*

Received: 15 April 1994 / 9 June 1994

Abstract Conidiation in the filamentous ascomycete *Aspergillus nidulans* requires activation of *brlA*, a well-characterized transcriptional regulator of genes that are induced specifically during asexual development. We have isolated and characterized developmental mutations in six loci, designated *fluG*, *flbA*, *flbB*, *flbC*, *flbD*, and *flbE*, that result in defective development and reduced *brlA* expression. These mutants grow indeterminately to produce masses of aerial hyphae resulting in the formation of cotton-like colonies with a "fluffy" morphology. The results of growth and epistasis tests involving all pairwise combinations of fluffy mutations indicate complex hierarchical relationships among these loci. We discuss these genetic interactions and propose that there are multiple mechanisms for activating *brlA*.

Key words Conidiation · Fungi · *brlA*
Microbial development

Introduction

Asexual reproduction in the filamentous fungus *Aspergillus nidulans* involves the formation of complex multicellular structures called conidiophores that produce multiple chains of uninucleate spores called conidia (Timberlake 1990). The molecular genetic events controlling the onset of conidiation are poorly understood but ultimately lead to activation of *brlA*, a well-characterized early regulator of *Aspergillus* development (Boylan et al. 1987; Adams et al. 1988). *brlA* is predicted to encode a nucleic acid-binding protein with TFIIIA-like zinc fingers and its expression is required to direct a switch from polarized extension of the conidiophore stalk apex to nonpolar swelling, resulting in the formation of the conidiophore vesicle (Adams et al. 1988, 1990). *brlA*$^-$ mutant strains fail to produce conidiophore vesicles and instead form structures resembling conidiophore stalks, except that they grow indeterminately and fail to differentiate the other specialized cell types required for sporulation (Clutterbuck 1969). By contrast, inappropriate activation of *brlA* in vegetative cells causes polar growth to stop and initiates abnormal development leading to the formation of reduced conidiophores bearing viable spores (Adams et al. 1988). These results support the notion that activation of *brlA* expression early in conidiophore development represents a major control point for activating the sporulation pathway.

Several sporulation mutants that are unable to initiate development, and fail to make the switch from undifferentiated vegetative growth to conidiophore development, have been described (Dorn 1970; Yager et al. 1982; Tamame et al. 1983, 1988; Adams et al. 1992). One estimate suggested that 83% of sporulation mutants are altered in their ability to initiate development; that is, they are blocked in developmental stages occurring before vesicle formation and *brlA* activation (Martinelli and Clutterbuck 1971). One common phenotype observed among such early conidiation mutants has been described as "fluffy". *A. nidulans* fluffy mutants grow as undifferentiated masses of vegetative hyphae that form large cotton-like colonies. Fluffy mutant phenotypes can differ, but all these mutants are presumably defective in some aspect of the growth-development transition preceding activation of *brlA*. We showed previously that mutations in one fluffy locus, *fluG*, result in loss of *brlA* expression and formation of extremely fluffy colonies lacking conidiophores (Adams et al. 1992). We demonstrated that the *fluG* mutant phenotype is suppressed when *fluG* mutants are grown next to wild-type colonies, even if the two strains are separated by a dialysis membrane having a 6000–8000 dalton pore size, suggesting that FluG is required for the production of an extracellular signal that initiates a programmed sporulation pathway (Lee and Adams 1994 a). We also showed that this

J. Wieser · B. N. Lee · J. W. Fondon III · T. H. Adams (✉)
Department of Biology, Texas A & M University,
College Station, TX 77843, USA

Communicated by O. C. Yoder

requirement for the *fluG*-derived signal can be partially overcome when growth is limited nutritionally, resulting in sparse conidiation and detectable *brlA* expression (Adams et al. 1992). This result raises the possibility that *brlA* might be activated by more than one mechanism. In wild-type *A. nidulans*, the major pathway for conidiation requires *fluG* and results in programmed activation of development independent of nutrient status. Without *fluG*, development can be activated by a mechanism that senses growth rate or nutritional status directly, bypassing the need for *fluG*.

In order to understand the role of *fluG* and other fluffy genes in controlling initiation of *A. nidulans* conidiophore development, we have isolated 122 fluffy mutants following mutagenesis of a wild-type *A. nidulans* strain containing the *brlA* promoter fused to the *E. coli lacZ* gene. These fluffy mutants were screened to determine whether the developmental block affected *brlA* activation. We have focused on a group of 76 fluffy mutants, defining six unique loci, that have the most severe effects on *brlA* expression (FLB for **f**luffy **l**ow ***b****rlA* expression) when compared to wild-type (see Fig. 1). We describe the isolation of these mutants and the characterization of genetic interactions between different mutant loci.

Materials and methods

Aspergillus strains and genetic techniques. All *A. nidulans* strains used in this study are listed in Table 1. Genotypes are designated as in Clutterbuck (1973). Standard genetic (Pontecorvo et al. 1953) and transformation techniques (Yelton et al. 1984) were used. All possible two-way combinations of mutant fluffy loci were constructed using one of two methods. In the first method double-mutant strains were isolated as meiotic progeny from crosses between two single mutants. The presence of two fluffy mutations in selected strains was confirmed by analyzing meiotic progeny of crosses between proposed double mutants and wild-type strains. In each case at least 200 colonies were examined, and ratios of 3 mutants to 1 wild-type were taken as evidence for the presence of both mutations. In the second method, a plasmid (pBN39; Lee and Adams 1994b) containing the *argB* gene inserted into the coding sequence of *flbA*, or a plasmid (pFM5; Adams et al. 1992) containing the *argB* gene inserted into the coding sequence of *fluG*, was used to transform *argB2* auxotrophs of $flbB^-$, $flbC^-$, $flbD^-$, or $flbE^-$ mutants. The $argB^+$ transformants were analyzed by Southern blots to confirm that the plasmid had integrated and disrupted either the *flbA* or *fluG* gene.

Media and growth conditions. Minimal medium was prepared as described by Käfer (1977). Complete medium is standard minimal medium containing 5 g of yeast extract per l. Mutants were isolated on complete media with 40 µl/l Triton X-100 added to reduce colony size. Developmental induction of liquid cultures was performed as described in Han et al. (1993). Radial growth rates of the mutants and wild-type colonies were determined as in Adams et al. (1992). All strains were incubated at 37 °C except when temperature-sensitive *fluG* mutant strains were used in which case incubation was at 30 °C for the permissive temperature or 42 °C for the restrictive temperature.

Mutagenesis. Approximately 10^8 conidia of either TRG27.1.C or FGSC26 were suspended in 1 ml of 0.1 M potassium phosphate in six 15-ml polycarbonate tubes. One microliter of 4-nitro-1-quinoline oxide (NQO) at a concentration of 10 mg/ml was added to three of the tubes and samples were incubated for 0, 30, and 60 min before the NQO was inactivated by the addition of 4 ml of 5% sodium thiosulfate to each tube (Bal et al. 1977). The other three tubes were similarly treated, except that 1 µl of a 1-mg/ml NQO solution was added. 10^3, 10^4, and 10^5 conidia from each of the treatments were plated on complete media with Triton X-100 to determine the survival rate. In three separate experiments using TRG27.1.C a total of 150 000 surviving colonies were screened and 112 fluffy and 43 other aconidial mutants, designated MJW001 to MJW155, were identified. In one experiment using FGSC26, an additional 8000 surviving colonies were screened and ten fluffy and ten other aconidial mutants, designated MBN001 to MBN020, were identified. The survival rate for each mutagenesis was between 1 and 10%.

Determination of β-galactosidase activity. Fluffy mutants isolated following mutagenesis of TRG27.1.C were examined for *brlA* expression levels during development by measuring β-galactosidase activity resulting from expression of the *brlA (p) : : lacZ* fusion. Fluffy mutants derived from the mutagenesis of FGSC26 were crossed with TRG27.1.C to obtain fluffy mutant progeny containing the *brlA (p) : : lacZ* fusion, and β-galactosidase levels were then measured after inducing development. Developmental cultures for examining *brlA (p) : : lacZ* expression levels were grown in two ways. In the first method, liquid cultures were inoculated from spores or macerated hyphae (for aconidial strains) and allowed to grow for 20 h after inoculation. The cultures were then induced to develop by harvesting them onto filters that were then placed on solid media as described (Han et al. 1993). Samples were taken 0, 5, 10, and 24 h after inducing development and their β-galactosidase activities were determined (Adams and Timberlake 1990). In the second method, spores were inoculated in the center of a piece of cellulose membrane on the surface of an agar plate containing either minimal or complete media. Complete colonies were harvested from the membrane 48, 72, or 96 h after inoculation and their β-galactosidase activities were determined.

Results

Isolation of fluffy mutants

Strains TRG27.1.C and FGSC26 were mutagenized as described in Materials and methods and visually screened to identify mutants with a fluffy phenotype. About 160 000 survivors of the mutagenesis were examined and 122 fluffy mutant colonies were isolated. All of the mutant colonies produced abundant aerial mycelia; however, some mutants also produced conidiophores following prolonged incubation. As described below, most fluffy mutants isolated had radial growth rates approximating that of wild-type (see Table 3).

Each of the 122 fluffy mutants was crossed to a wild-type strain (PW1 or FGSC237) and meiotic progeny were analyzed to determine if the fluffy phenotype resulted from a single mutation. More than 100 progeny were analyzed in each cross and, in all but one case, the fluffy mutations segregated from their wild-type alleles in a 1 : 1 ratio as expected if the fluffy phenotypes result from mutations at single loci. The single exception involved MBN014 which did not originally have a fluffy phenotype but was instead "flat" (Martinelli and Clutterbuck 1971). However, progeny from the meiotic cross between MBN014 and FGSC237 segregated in the ratio 1 wild-type: 2 flat: 1 fluffy indicating that MBN014 most likely had two mutations, one resulting in flat growth and the other resulting in fluffy

Table 1 *A. nidulans* strains used in this study. Strains listed in this table are the parental strains of the mutants mentioned by name in the paper or recombinants used in specific experiments in the paper

Strain	Genotype	Source
FGSC26	*biA1*; *veA1*	FGSC[a]
RBN127	*biA1*, *flbA*: : $argB^+$; *veA1*	Lee and Adams 1994b
TTA127.4	*pabaA1*; *fluG*: : $trpC^+$; *veA1*	Lee and Adams 1994a
TTA127.2	*pabaA1*; *fluG*: : $trpC^+$; *veA1*	Adams et al. 1992
FGSC237	*pabaA1*, *yA2*; *trpC801*, *veA1*	FGSC[a]
TU35	*pabaA1*, *yA2*; $fluG684^{ts}$; *pyrG89*, *pyroA4*	Adams et al. 1992
RBN130	*biA1*, *flbA*: : $argB^+$; *fluG*: : $trpC^+$; *veA1*	Lee and Adams 1994b
RBN137	*pabaA1*, *yA2*; *glnA1*; *fluG*: : $trpC^+$; *veA1*	Lee and Adams 1994b
TRG27.1.C	*pabaA1*, *yA2*; *veA1*, *trpC*: : *brlA*: : *lacZ*	Han et al. 1993
FGSC288	*suA1adE20*, *adE20*, *yA2*; *wa3*; *galA1*; *pyroA4*; *facA303*; *sB3*; *nicB8*; *riboB2*	FGSC[a]
TU11	$fluG684^{ts}$; *pyroA4*; *veA1*	Adams et al. 1992
WIM81	*pabaA1*, *yA2*; $fluG102^{ts}$; *veA1*	Adams et al. 1992
RM23	*biA1*; *wA3*; *argB2*; *trpC801*, *veA1*	J. Marhoul
PW1	*biA1*; *argB2*; *methG1*; *veA1*	Weglenski
MJW007	*pabaA1*; *flbB7*	This study
MJW021	*pabaA1*; *flbB21*	This study
MBN013	*biA1*; *flbA13*	This study
RJW012	*biA1*; *flbB7*	This study
RJW027	*pabaA1*; *argB2*; *flbB7*	This study
MBN008	*biA1*; *flbC8*	This study
RBN026	*pabaA1*; *flbC8*	This study
RJF001	*biA1*; *argB2*; *methG1*; *flbC*	This study
RJF003	*pabaA1*; *argB2*; *flbC*	This study
RBN021	*pabaA1*; *flbD14*	This study
MBN014	*biA1*; *flbD14*	This study
RBN070	*biA1*; *argB2*; *flbD14*	This study
MJW058	*pabaA1*; *flbE58*	This study
RJW120	*flbE58*; *methG1*	This study
RJW181	*pabaA1*, *flbE58*; *argB2*; *methG1*	This study
MJW013	*pabaA1*; *fmb13*	This study
MJW010	*pabaA1*; *fhb10*	This study
TRJW177	*pabaA1*, *flbA*: : $argB^+$; *flbB*	RJW027[b]
TRJW178	*pabaA1*, *flbA*: : $argB^+$; *flbC8*	RJF003[b]
TRJW179	*biA1*, *flbA*: : $argB^+$; *flbD14*	RBN070[b]
TRJW180	*pabaA1*, *flbE58*, *flbA*: : $argB^+$ *methG1*	RJW181[b]
RJW159	*pabaA1*; *flbC8*; *flbB21*	MJW021[c]×RJF001[c]
RJW161	*pabaA1*; *flbB21*; *flbD14*	MJW021[c]×RBN070[c]
RJW162	*pabaA1*, *flbE58*; *flbB21*, *methG1*	MJW021[c]×RJW120[c]
TRJW168	*pabaA1*; *fluG*: : $argB^+$; *flbB7*	RJW027[d]
RJW163	*pabaA1*; *flbC8*; *flbD14*	RBN026[c]×RBN070[c]
RJW166	*flbE58*; *flbC8*; *methG1*	RJF001[c]×MJW058[c]
TRJW169	*pabaA1*; *flbC8*, *fluG*: : $argB^+$	RJF003[d]
RJW167	*pabaA1*, *flbE58*; *flbD14*	RBN021[c]×RJW120[c]
TRJW172	*biA1*; *fluG*: : $argB^+$; *flbD14*	RBN070[d]
TRJW175	*pabaA1*, *flbE58*; *fluG*: : $argB^+$; *methG1*	RJW181[d]

[a] Fungal Genetics Stock Center
[b] Parental strain for transformation with pBN39, and *flbA* deletion plasmid
[c] Parental strains in meiotic cross
[d] Parental strain for transformation with pFM4, a *fluG* disruption plasmid

growth. The fluffy mutant progeny of MBN014 were analyzed further and shown to result from a recessive mutation in a single locus.

Analysis of *brlA* expression in fluffy mutants

All 122 fluffy mutants were analyzed to determine *brlA* expression levels following developmental induction. Because TRG27.1.C contains a gene fusion between *brlA* regulatory sequences and the *E. coli lacZ* gene [*brlA (p)*: : *lacZ*], *brlA* expression levels could be measured immediately by determining β-galactosidase activity. Mutants isolated from FGSC26 were first crossed with TRG27.1.C and fluffy progeny containing the *brlA (p)*: : *lacZ* fusion were identified by Southern-blot analysis.

We divided the 122 fluffy mutant strains into three groups based on the level of *brlA* expression relative to the wild-type parent. Figure 2 shows a graph of β-galactosidase levels observed in mutant strains that represent each of the three groups. Strain MJW007 is an example of a strain in the first class, designated FLB for **f**luffy **l**ow ***b****rlA* expression. Even after prolonged incubation, the 76 mutants in this class never expressed greater than 5% of wild-

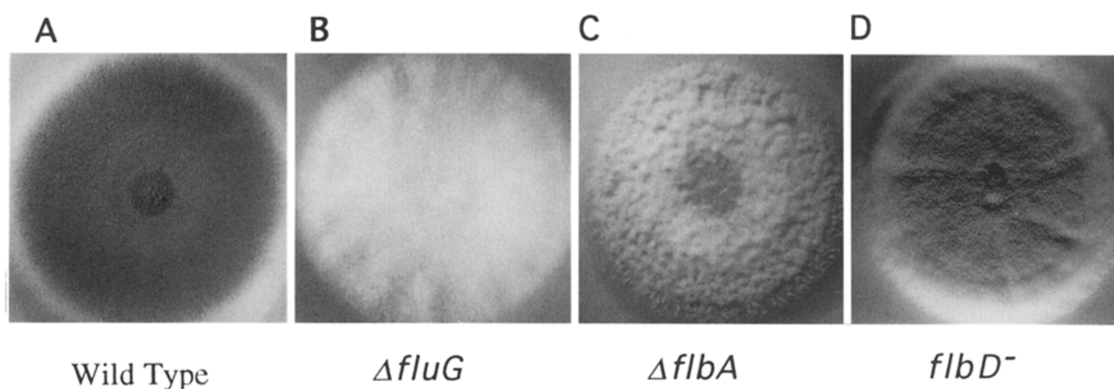

Fig. 1 A–D Phenotypic classes of fluffy mutant strains. A wild-type strain (**A**; FGSC26), a *fluG* deletion mutant (**B**; TTA127.4), a *flbA* deletion mutant (**C**; RBN127), and a delayed conidiation mutant (**D**; RBN021) were point inoculated onto complete medium and allowed to grow for 4 days. The initial stages of colony autolysis can be seen in the center of the *flbA* deletion mutant colony (**C**)

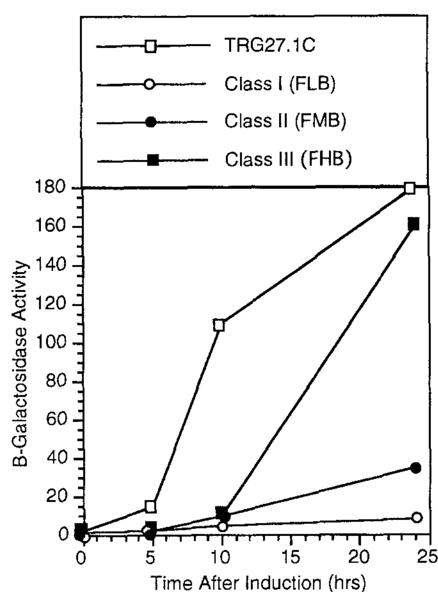

Fig. 2 Regulation of *brlA* expression in fluffy mutant strains. Wild-type (TRG27.1.C) and fluffy mutant strains from each *brlA* expression class containing a *brlA(p)::lacZ* gene fusion were grown in liquid culture for 20 h and then induced to develop as described in the Materials and methods. Samples were taken at the times indicated and cell extracts were assayed to determine β-galactosidase levels; β-galactosidase activity is expressed as nmol ONPG hydrolyzed per min per mg protein. For each of the strains shown experiments were repeated more than five times and the results never differed by more than 10% among experiments

type β-galactosidase activity, although some conidiophores were observed in delayed conidiation mutants by 24 h post-induction. Strain MJW013 is an example of a mutant in the second class, designated FMB for **f**luffy **me**dium *brlA* expression. The 28 FMB mutants never expressed greater than 25% of wild type β-galactosidase levels, but all mutants in this class produced some conidiophores by 24 h post-induction. Finally, strain MJW010 is an example of a mutant in the third class, designated FHB for **f**luffy **h**igh *brlA* expression. Although *brlA* activation may be somewhat delayed in FHB mutants as compared to wild-type, after longer incubation times, β-galactosidase levels in the 18 FHB strains approached wild-type levels, and all produced conidiophores by 24 h post-induction. Because FLB mutants are the most drastically altered in their ability to activate *brlA* expression, we have concentrated our initial analysis of the role of fluffy genes in early development by characterizing the 76 mutants in this group.

Phenotypic description and complementation analysis of FLB mutants

The 76 FLB mutants were grouped into three categories based on differences in their developmental phenotypes (Fig. 1; Table 2). The first group included 20 mutants all of which had phenotypes closely resembling that resulting from mutation in a previously characterized locus, *fluG* (Adams et al. 1992; Lee and Adams 1994 a). This group of mutants was distinguished from other fluffy mutants in three ways. First, they were fully aconidial on complete media, but conidiated to a small degree on minimal media. Second, they could be induced to conidiate at the margin of the colony when grown beside wild-type or other fluffy mutants, but not beside *fluG* mutants. Third, they produced a metabolite that turned the media beneath the colony orange. Of the 20 *fluG*-like mutants, ten have been tested for allelism with *fluG* by crossing with a known *fluG⁻* strain (TTA127.2, WIM81, or TU11) and then examining the progeny. The fluffy mutations in all ten strains tested were shown to be either allelic to *fluG* or else very closely linked, with recombination frequencies of less than 0.1%. The radial growth rates for a *fluG* deletion mutant (Table 3) and for all other *fluG* mutants characterized (data not shown) were slightly less than wild-type.

The second class of FLB mutants was distinguished by the fact that after 3-days growth the center of the colony began to disintegrate. In fact, by about 5 days after inoculation, the entire colony had undergone autolysis. The lo-

Table 2 FLB mutant classes

Class I (*fluG*-like)	Class II (*flbA*-like)	Class III (delayed conidiators)
MJW028 [a]	MBN013 [b]	MBN001 (*flbB1*)
MJW032 [a]	MJW067 [b]	MBN005 (*flbB5*)
MJW048	MJW087	MBN008 (*flbC8*)
MJW049	MJW089	MBN014 (*flbD14*)
MJW051 [a]	MJW091	MJW007 (*flbB7*)
MJW063 [a]	MJW092	MJW008 (*flbB8*)
MJW065	MJW093	MJW017 (*flbB17*)
MJW075 [a]	MJW094	MJW018 (*flbB18*)
MJW082 [a]	MJW095 [b]	MJW019 (*flbE19*)
MJW104	MJW096 [b]	MJW020 (*flbE20*)
MJW106	MJW097	MJW021 (*flbB21*)
MJW107 [a]	MJW098	MJW025 (*flbB25*)
MJW108	MJW099 [b]	MJW026 (*flbB26*)
MJW113	MJW100 [b]	MJW029 (*flbB29*)
MJW115 [a]	MJW101	MJW047 (*flbE47*)
MJW116	MJW103	MJW056 (*flbB56*)
MJW138 [a]	MJW117	MJW058 (*flbE58*)
MJW141 [a]	MJW120	MJW069 (*flbE69*)
MJW142	MJW125	MJW071 (*flbB71*)
MJW151	MJW127	MJW083 (*flbD83*)
	MJW130 [b]	MJW108 (*flbB108*)
	MJW131	
	MJW132 [b]	
	MJW133 [b]	
	MJW134	
	MJW135	
	MJW136	
	MJW137	
	MJW139	
	MJW140	
	MJW143 [b]	
	MJW144	
	MJW149	
	MJW153	
	MJW154 [b]	

[a] The fluffy mutation has been demonstrated to represent an allele of *fluG*
[b] The fluffy mutation has been demonstrated to represent an allele of *flbA*

Table 3 Radial growth rate of single-mutant strains. The radii of three different colonies were measured from the center of the colony of two different margins ($n = 6$) over 120 h of growth on minimal medium and the mean and standard deviation was calculated. The significance of the difference between wild-type and mutant growth rates was determined by calculating t (student's t-test, two-tailed)

Strain	mm/day (mm)
FGSC26	7.5±0.8
RBN127 (*flbA*⁻)	6.4±0.8*
MJW007 (*flbB*⁻)	7.1±0.8
MBN008 (*flbC*⁻)	7.4±0.3
MBN014 (*flbD*⁻)	7.0±1.6
MJW058 (*flbE*⁻)	6.8±1.1
TTA127.4 (*fluG*⁻)	6.1±1.3*

* Mutants having significantly different ($P < 0.05$) growth rates than wild-type. All other mutant strains had growth rates that were not significantly different ($P > 0.05$) than wild-type

cus identified by one of the mutants in this group was called *flbA* and the wild-type *flbA* allele was isolated by complementation (Lee and Adams, 1994b). The 35 *flbA*-like mutants isolated from our screens could not be put into complementation groups directly because heterokaryons formed between two *flbA*-like mutants lysed before mating took place. In order to determine if the mutations were alleles of *flbA*, diploids were constructed between *flbA*-like strains and either RM23 or PW1. The diploids treated with benomyl to promote the formation of haploid sectors and fluffy *argB2* mutant segregants were recovered. These strains were then cotransformed with one plasmid (pBN29) containing the *flbA*$^{+}$ gene and a second plasmid containing the *argB*$^{+}$ gene (pSalargB). For all 11 *flbA*-like strains tested to-date, developmentally wild-type transformants were recovered indicating that the mutations most likely correspond to *flbA* or a closely linked locus. Similar transformation experiments using a related plasmid (pBN39), which has the *argB* gene inserted into the coding sequence of the *flbA* gene, did not yield developmentally wild-type transformants supporting the notion that each of these mutants defines a *flbA* allele. The radial growth rates for a *flbA* deletion mutant (Table 3) and for all other *flbA* mutants characterized (data not shown) were slightly less than wild-type.

The third group of FLB mutants was distinguished based on the observation that all 21 mutants in this group made conidiophores in the center of the colony beginning 2–3 days after inoculation while the colony margin remained fluffy. We refer to this phenotype as delayed conidiation because the appearance of conidia is delayed at least 1 day relative to wild-type. These 21 mutants were shown to define four unique complementation groups by constructing all possible combinations of heterozygous diploids and by examining linkage in meiotic crosses. Thirteen of the mutants comprised a single complementation group designated *flbB*. Additional experiments showed that *flbB* was an allele of an independently isolated fluffy mutant called *vegA* (Aguirre et al. 1993). The other three loci were designated *flbC*, *flbD*, and *flbE* and included one, two, and five independent mutants respectively. The radial growth rates of all the delayed conidiation mutants were not significantly different from that of wild-type (for example see Table 3).

Determination of linkage groups

Each of the FLB mutants was analyzed to determine chromsomal linkage. This was accomplished by constructing diploids between each mutant and a mitotic mapping strain (FGSC288) that has a mutation on each of its eight chromosomes. All of the diploids constructed were developmentally wild-type indicating that in each FLB mutant, the fluffy phenotype resulted from a recessive mutation. Haploid segregants were isolated following treatment of the diploid with benomyl and the assortment of each fluffy mutation with respect to known markers was followed. As shown in Table 4, *flbA* and *flbE* segregated with chromo-

Table 4 Linkage analysis of FLB mutants

Linkage group	Phenotypic marker	No. of haploid segregants									
		flbA13[a]		*flbB7*		*flbC8*[b]		*flbD14*[c]		*flbE58*[b]	
		–	+	–	+	–	+	–	+	–	+
I	Y^+	0	14	nd[d]	0	4	25	16	11	50	0
	Y^-	13	0	nd	34	33	20	18	8	0	17
II	W^+	nd	5	nd	21	nd	18	nd	8	nd	12
	W^-	nd	9	nd	13	nd	27	nd	11	nd	5
III	GAL^+	4	8	4	17	8	11	29	16	22	12
	GAL^-	9	6	18	17	29	34	5	3	28	5
IV	$PYRO^+$	7	6	22	0	20	14	22	10	11	11
	$PYRO^-$	6	8	0	34	17	31	12	9	39	6
V	FAC^+	1	10	8	22	16	25	26	11	25	14
	FAC^-	12	4	14	12	21	20	8	8	25	3
VI	S^+	2	1	10	14	18	28	19	7	40	13
	S^-	11	13	12	20	19	17	15	12	10	4
VII	NIC^+	12	13	7	22	26	33	34	19	41	14
	NIC^-	1	1	15	12	11	12	0	0	9	3
VIII	$RIBO^+$	6	9	11	17	19	37	34	2	39	16
	$RIBO^-$	7	5	11	17	18	8	0	17	11	1

[a] The *flbA* mutant was $biA1^-$, and $argB2^-$ which allowed linkage to chromosome I to be measured by following segregation of *biA* rather than *yA* which could not be scored in aconidial mutants; linkage to chromosome III was measured by following segregation of *argB*
[b] The *flbC* and *flbE* mutants were $pabaA1^-$ (chromosome I) and segregation of chromosome-I markers was scored as described for the *flbA* mutant
[c] The *flbD* was $pabaA1^-$ (chromosome I) and $argB2^-$ and segregation of chromosome-I and -III markers was scored as described for the *flbA* mutant
[d] Fluffy mutant strains were not scored for conidial color

Table 5 Epistatic interactions between *flb* mutant loci. The phenotype of the double mutant formed between any two of the mutants is given. Abbreviations are as follows: **A** phenotype resembles a *flbA* mutant. **EDC** extra delayed conidiation, double mutants conidiate in the center of the colony at least one day later than the single mutant strains. **DC** phenotype resembles the delayed conidiation mutant parent. **G** phenotype resembles the *fluG* mutant parent. **g** phenotype closely resembles a *fluG* mutant but does not accumulate the orange pigment characteristic of *fluG* mutants

	flbA	*flbB*	*flbC*	*flbD*	*flbE*	*fluG*
flbA	–	**A**	**A**	**A**	**A**	**A**
flbB		–	**EDC**	**DC**	**DC**	**g**
flbC			–	**EDC**	**EDC**	**G**
flbD				–	**DC**	**g**
flbE					–	**g**
fluG						–

some I, *flbB* with chromosome IV, and *flbD* with chromosome VIII. We were unable to assign a linkage group to *flbC* as the mutation failed to segregate with any one chromosome. *vegA* (*flbB*) was previously shown to be linked to *palC* (Aguirre et al. 1993) and this linkage was confirmed here (data not shown).

Epistatic interactions among *flb* mutants

We constructed double mutants between members of different complementation groups in order to assess possible interactions and determine the epistatic relationships among the *flb* genes (Table 5). In all cases, double mutants including an $flbA^-$ mutation phenotypically resembled the $flbA^-$ parent and produced autolytic colonies. However, while $flbA^-$ single mutants are partially remediated and produce conidiophores on high-osmolarity medium (0.8 M NaCl) all of the double mutants were aconidial under these conditions. Double-mutant combinations involving $flbB^-$, $flbD^-$, and $flbE^-$ have the same delayed conidiation phenotypes as observed in each single mutant, suggesting that *flbB*, *flbD*, and *flbE* function at the same step in a pathway. However, double mutants between $flbC^-$ and $flbB^-$, $flbD^-$, or $flbE^-$ have extra delayed conidiation phenotypes in which the onset of conidiation is delayed 1 day as compared to the single mutants, indicating an additive effect for mutations in *flbC* and those in other delayed conidiation loci. $fluG^-$ $flbB^-$, $fluG^-$ $flbD^-$, and $fluG^-$ $flbE^-$ double mutants have characteristics of both $fluG^-$ and delayed-conidiation mutants. Like $fluG^-$ strains, the double mutants were nearly completely aconidial under optimal growth conditions and produced very few conidia under any conditions, but unlike $fluG^-$ strains, the double mutants failed to accumulate the characteristic orange pigment in the media. $fluG^-$ $flbC^-$ double mutants phenotypically resembled $fluG^-$ mutants in all respects. Radial growth rates for most double mutants were somewhat slower than observed for wild-type (Table 6) although overall growth was probably not greatly diminished given the abundance of aerial mycelia in fluffy mutant strains.

Table 6 Radial growth rate of double-mutant strains. The radii of three different colonies were measured from the center of the colony to two different margins ($n = 6$) over 120 h of growth on minimal medium and the mean and standard deviation was calculated. The significance of the difference between wild-type and mutant growth rates was determined by calculating t (student's t-test, two-tailed)

Strain	mm/day (mm)
FGSC26	7.5 ± 0.7
TRJW177 (*flbA*$^-$, *flbB*$^-$)	5.1 ± 2.5 *
TRJW178 (*flbA*$^-$, *flbC*$^-$)	5.7 ± 1.1 *
TRJW179 (*flbA*$^-$, *flbD*$^-$)	6.0 ± 1.1 *
TRJW180 (*flbA*$^-$, *flbE*$^-$)	5.9 ± 0.9 *
RBN130 (*flbA*$^-$, *fluG*$^-$)	5.9 ± 0.6 *
RJW159 (*flbB*$^-$, *flbC*$^-$)	6.9 ± 0.5
RJW161 (*flbB*$^-$, *flbD*$^-$)	6.3 ± 1.2 *
RJW162 (*flbB*$^-$, *flbE*$^-$)	5.6 ± 1.2 *
TRJW168 (*flbB*$^-$, *fluG*$^-$)	6.1 ± 1.1 *
RJW163 (*flbC*$^-$, *flbD*$^-$)	6.9 ± 0.8
RJW166 (*flbC*$^-$, *flbE*$^-$)	7.2 ± 1.0
TRJW169 (*flbC*$^-$, *fluG*$^-$)	6.5 ± 1.7
RJW167 (*flbD*$^-$, *flbE*$^-$)	6.9 ± 0.9
TRJW172 (*flbD*$^-$, *fluG*$^-$)	6.4 ± 0.8 *
TRJW175 (*flbE*$^-$, *fluG*$^-$)	5.9 + 1.4 *

* Double-mutant strains having growth rates that were significantly different ($P < 0.05$) from wild-type. All other double-mutant strains had growth rates that were not significantly different ($P > 0.05$) from wild-type

Discussion

The molecular genetic mechanisms controlling initiation of asexual sporulation in the filamentous fungus *A. nidulans* are largely unknown but ultimately lead to activation of the development-specific transcriptional regulator *brlA*. We have isolated 122 *A. nidulans* mutants with defects in asexual sporulation leading to phenotypically fluffy colonies. Seventy-six of these mutants did not activate *brlA* and defined six complementation groups having at least three distinct fluffy phenotypes. Mutants from each of the six complementation groups grew vegetatively nearly as well as wild-type (Table 2) indicating that these mutants most likely have specific defects in development rather than general growth defects that somehow prevent (or delay) development.

The qualitative differences in the various fluffy phenotypes allowed us to investigate the hierarchical relationships among genes by constructing double-mutant strains. Double mutants that were *flbA*$^-$ and mutant in any of the other *flb* genes were autolytic, indicating that *flbA* was epistatic to the other *flb* mutations for colony development. The simplest explanation for this result is that *flbA* functions upstream of all the other *flb* loci in a genetic pathway leading to the activation of *brlA* and development. However, this might also result because *flbA* has effects on both growth and development. *flbA* mutants have an unusual hyphal growth morphology in which hyphae grow on top of one another in tight clumps instead of in the well-separated branching pattern observed for wild-type and other fluffy mutants. Thus, one possibility is that the epistasis observed for *flbA* with respect to the other fluffy mutants is due to its abnormal growth pattern and not to developmental epistasis. This interpretation is supported by the fact that *flbA*$^-$ single mutants, but not *flbA*$^-$ *flb*$^-$ double mutants, are able to produce conidiophores when grown on high-osmolarity medium (Lee and Adams 1994 b).

Double mutants that were *fluG*$^-$ and had delayed conidiation mutations (*flbB*$^-$, *flbC*$^-$ *flbD*$^-$, and *flbE*$^-$) exhibited phenotypes that most closely resembled *fluG*$^-$ strains. However, the interactions observed between *fluG*$^-$ and *flbC*$^-$ mutations were somewhat different from those observed between *fluG*$^-$ and other delayed conidiation mutations. *fluG*$^-$ was epistatic to *flbC*$^-$ in all respects indicating that *fluG* and *flbC* could function in the same linear pathway leading to activation of *brlA* and development with *fluG* acting first. Double-mutant strains involving *fluG*$^-$ and *flbB*$^-$, *flbD*$^-$, or *flbE*$^-$ also closely resembled *fluG*$^-$ mutants in that they formed very fluffy colonies that did not conidiate in the center. However, unlike *fluG*$^-$ single mutants or *fluG*$^-$ *flbC*$^-$ double mutants, these double mutants failed to accumulate the orange pigment in the medium that is characteristic of *fluG*$^-$ mutant strains. We interpret these results to indicate *flbB*, *flbD*, and *flbE* have both *fluG*-dependent and *fluG*-independent functions. This distinction between *flbC* and the other delayed-conidiation loci is also supported by the phenotypes observed for double-mutant strains with combinations of mutations in the delayed conidiation loci *flbB*, *flbC*, *flbD*, and *flbE*. All double-mutant strains including *flbC*$^-$ and a second delayed-conidiation mutation were fluffier and had a longer delay in conidiation than strains with either of the single mutations alone. In contrast, double-mutant strains with combined *flbB*$^-$, *flbD*$^-$ and *flbE*$^-$ mutations were no different phenotypically from strains with each single mutation, as expected if *flbB*, *flbD*, and *flbE* function at the same step in a single pathway.

We showed previously that the *fluG* mutant conidiation defect can be rescued extracellularly by both wild-type and non-*fluG*$^-$ fluffy mutant strains and we consider it likely that the primary function of *fluG* is to catalyze the production of an extracellular signal that activates a programmed sporulation pathway leading to *brlA* expression and development (Lee and Adams 1994 a). If this is indeed the case, other loci identified by fluffy mutations could function in the downstream response to the sporulation signal.

flbA is predicted to encode a 717 amino-acid polypeptide with 30% identity to the yeast *SST2* gene (Lee and Adams 1994 b). The precise role for *SST2* remains to be determined but it has been implicated in regulating the activity of the G-protein-mediated signal transduction pathway required for mating (Dietzel and Kurjan 1987; Kurjan 1992); *sst2*$^-$ mutants are supersensitive to mating pheromone produced by the opposite mating type. Thus, Sst2 activity is thought to allow unmated yeast cells to adapt to mating pheromone and resume growth following prolonged exposure. The similarity between *flbA* and *SST2* led us to suggest that FlbA could function directly in regulating the activity of a signal transduction pathway for responding to the putative *fluG* sporulation signal (Lee and

Adams 1994b). Whatever the role of *flbA*, we think that *flbA* has a direct function in activating development because forced expression of *flbA* in liquid culture, a condition that does not support conidiation, results in the activation of *brlA* and the production viable spores.

Genes identified by delayed-conidiation mutations apparently define separate branches of the pathway leading to developmental activation, with *flbB*, *flbD*, and *flbE* defining one branch and *flbC* defining the second. *flbC* and *flbD* have recently been isolated and the DNA sequences determined (our unpublished results). *flbD* is predicted to encode a 308-codon polypeptide that includes a region with significant identity to the DNA-binding domain in the human proto-oncogene c-*myb* (Luscher and Eisenman 1990). *flbC* is predicted to encode a 354-codon polypeptide that includes two C_2H_2 zinc-finger domains typical of a large group of DNA-binding proteins that includes the *A. nidulans brlA* gene (Miller et al. 1985; Adams et al. 1988). Thus, both *flbC* and *flbD* are likely to function as DNA-binding proteins and could control the transcriptional activation of other developmental regulators, like *brlA*, in response to sporulation signals.

Although each of the *flb* genes represents a potential developmental regulator, it is likely that additional genes are required to complete the pathway. Because we have identified multiple alleles for all *flb* loci except *flbC*, it seems likely that this screen is approaching saturation. However, additional fluffy mutants with lesser effects of *brlA* expression (FMB and FHB mutants) were isolated and might define additional pathway-specific genes. Because our screen concentrated on identifying fluffy mutants, we may have missed other genes required for the pathway if mutations in these genes cause phenotypes other than fluffy. For instance, other genes required for initiation of development could have related functions in essential cellular processes so that mutations in these genes are lethal. Likewise, if genes encoding other developmental regulators were redundant, mutations would not cause developmental defects. Finally, each of the fluffy mutants described will conidiate under some environmental conditions indicating that there must be other ways to activate *brlA* (Adams et al. 1992). Different classes of fluffy mutants might be isolated by varying the growth conditions in our screen.

Acknowledgements This work was supported by National Institutes of Health grant GM45252. We thank Dr. J. Aguirre for providing materials and our colleagues in the laboratory for many helpful discussions.

References

Adams TH, Timberlake WE (1990) Mol Cell Biol 10: 4912–4919
Adams TH, Boylan MT, Timberlake WE (1988) Cell 54: 353–362
Adams TH, Deising H, Timberlake WE (1990) Mol Cell Biol 10: 1815–1817
Adams TH, Hide WA, Yager LN, Lee BN (1992) Mol Cell Biol 12: 3827–3833
Aguirre J, Ortiz R, Clutterbuck J, Tapia R, Cardenas M (1993) Fungal Genet Newslett 40A: 68
Bal J, Kajtaniak EM, Pieniazek NJ (1977) Mutat Res 56: 153–156
Boylan MT, Mirabito PM, Willett CE, Zimmerman CR, Timberlake WE (1987) Mol Cell Biol 7: 3113–3118
Clutterbuck AJ (1969) Genetics 63: 317–327
Clutterbuck AJ (1973) Genet Res 21: 291–296
Dietzel C, Kurjan J (1987) Mol Cell Biol 7: 4169–4177
Dorn GL (1970) Genetics 66: 267–279
Han S, Navarro J, Greve RA, Adams TH (1993) EMBO J 12: 2449–2457
Käfer E (1977) Adv Genet 19: 33–131
Kurjan J (1992) Annu Rev Biochem 61: 1097–1129
Lee BN, Adams TH (1994a) Genes Dev 8: 641–651
Lee BN, Adams TH (1994b) Mol Microbiol (in press)
Luscher B, Eisenman RN (1990) Genes Dev 4: 2235–2241
Martinelli SD, Clutterbuck AJ (1971) J Gen Microbiol 69: 261–268
Miller J, McLachlan AD, Klug A (1985) EMBO J 41: 1609–1614
Pontecorvo G, Roper JA, Hemmons LM, MacDonald KD, Bufton AWJ (1953) Adv Genet 5: 141–238
Tamame M, Antequera F, Santos E (1988) Mol Cell Biol 8: 3043–3050
Tamame M, Antequera F, Villanueva JR, Santos T (1983) Mol Cell Biol 3: 2287–2297
Timberlake WE (1990) Annu Rev Genet 24: 5–36
Yager LN, Kurtz MB, Champe SP (1982) Dev Biol 93: 92–103
Yelton MM, Hamer JE, Timberlake WE (1984) Proc Natl Acad Sci USA 81: 1470–1474