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Translational repression of *brlA* expression prevents premature development in *Aspergillus*

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The *Aspergillus nidulans brlA* developmental regulatory locus consists of two overlapping transcription units, *brlAα* and *brlAβ*, which encode functionally related polypeptides. We used translational fusions between each of the predicted *brlA* reading frames and the *Escherichia coli lacZ* gene to test the hypothesis that developmental regulation of *brlAα* and *brlAβ* expression occurs through different mechanisms. *brlAα* is transcriptionally controlled and a large portion of *brlAα*-directed β-galactosidase activity is regulated in a *brlA*-dependent manner. In contrast, *brlAβ* mRNA is constitutively transcribed but translation of the *brlA* polypeptide is prevented by the presence of a short open reading frame (μORF) present in the 5′ end of *brlAβ* mRNA. Removing the μORF initiation codon leads to deregulated *brlA* expression, resulting in an inappropriate activation of development. We propose that one mechanism for developmental induction in *A.nidulans* involves translational control.

Key words: Aspergillus nidulans/brlA/developmental genetics/fungi/translational control

Introduction

The asexual reproductive cycle of *Aspergillus nidulans* provides a well established model for examining developmental gene regulation and cellular differentiation in filamentous fungi (Timberlake, 1990). Multicellular spore-bearing structures, called conidiophores, are produced at a specific time in the fungal life cycle in response to environmental and developmental cues (Axelrod, 1972; Axelrod *et al.*, 1973; Champe *et al.*, 1981; Adams *et al.*, 1992). The nature of these signals and their mechanisms of action are poorly understood but they result in activation of a genetic regulatory cascade involving the coordinated control of hundreds of essential (Clutterbuck, 1969; Martinelli and Clutterbuck, 1971; Timberlake, 1980; Stringer *et al.*, 1991) and non-essential genes (Aramayo *et al.*, 1989). The sequential activation of three of these sporulation-specific genes, *brlA*, *abaA* and *wetA*, establishes the central regulatory network responsible for much of the temporal and spatial control of gene expression observed during conidiophore development (Clutterbuck, 1977; Adams *et al.*, 1988; Mirabito *et al.*, 1989; Marshall and Timberlake, 1991).

The best characterized regulatory gene for *A.nidulans* conidiophore development is *brlA*, which is predicted to encode a nucleic acid binding protein with TFIIIA-like zinc fingers (Miller *et al.*, 1985; Adams *et al.*, 1988, 1990). *brlA* mRNA is present at extremely low levels in hyphae and begins to accumulate shortly after induction of development (Boylan *et al.*, 1987; Prade and Timberlake, 1993). Control of *brlA* mRNA accumulation is regulated by unknown factors early in development and is required for a switch from polarized extension of the conidiophore stalk to nonpolar growth resulting in conidiophore vesicle formation. Developmental structures produced by *brlA* mutant strains initially resemble conidiophore stalks but they grow indeterminately and fail to differentiate other specialized cell types (Clutterbuck, 1969). By contrast, forced expression of *brlA* in vegetative cells under conditions that normally suppress conidiation results in a cessation of polar growth and development of reduced conidiophores which bear viable spores (Adams *et al.*, 1988). In addition, activation of *brlA* expression initiates a positive feedback regulatory loop (Mirabito *et al.*, 1989) and thus represents a commitment step towards spore formation.

Prade and Timberlake (1993) showed that the *brlA* locus is complex in that it consists of two overlapping transcription units designated *brlAα* and *brlAβ*. *brlAβ* transcription initiates ~1 kb upstream of *brlAα* transcription which begins within *brlAβ* intronic sequences (Figure 1). The *brlAβ* transcript encodes two open reading frames (ORFs) beginning with AUG, a short upstream μORF and a downstream ORF which is predicted to encode the same polypeptide as *brlAα* except that it includes 23 additional amino acids at its NH_2-terminus. Mutations that block expression of either transcript alone cause abnormal development but multiple copies of either *brlAα* or *brlAβ* can overcome the need for the other transcript in directing development. These results are consistent with the hypothesis that *brlAα* and *brlAβ* have evolved to create a requirement for different regulatory inputs in activating *brlA* expression to high enough levels to cause conidiophore development to proceed.

In this paper we describe experiments aimed at understanding the role that differential expression of *brlAα* and *brlAβ* plays in regulating development. We constructed translational gene fusions between each of the *brlA* ORFs and the *Escherichia coli lacZ* gene. Our results show that *brlAα* and *brlAβ* expression is developmentally regulated. However, β-galactosidase expression directed by the μORF is constitutive. Moreover, elimination of the μORF AUG allows deregulated expression of *brlA* leading to uncontrolled activation of development. We propose that the μORF negatively regulates translation of BrlA from the *brlAβ* mRNA and that removal of this block allows asexual development to initiate, stimulating transcription of *brlAα* and the downstream regulatory gene *abaA*.

Results

Developmental regulation of brlAα and brlAβ expression

The *brlA* gene encodes two mRNAs designated *brlAα* and *brlAβ* (Prade and Timberlake, 1993; Figure 1). The *brlAβ* transcript is predicted to encode a polypeptide that is identical to BrlAα except that 23 additional amino acids are present at the NH_2-terminus. We constructed developmentally wild type *A.nidulans* strains containing fusions (see Materials and methods) between the *brlAα* and *brlAβ* regulatory sequences up to and including each translational initiation codon (designated *brlAα* ATG and *brlAβ* ATG respectively; Figure 2A) and *lacZ*. Because the BrlA reading frames overlap, the *brlAα* ATG::*lacZ* fusion is expected to report expression from both *brlAα* and *brlAβ*. In contrast, the *brlAβ* ATG::*lacZ* fusion is expected to monitor expression from *brlAβ* exclusively. Expression of each fusion gene was measured in extracts from synchronously conidiating cultures (Adams and Timberlake, 1990b).

Figure 2B shows β-galactosidase activity measured at various times after inducing development in strains TRG27.1.c (*brlA*$^+$; *brlAα* ATG::*lacZ* fusion), TRG30.1.c (*brlA*$^+$; *brlAβ* ATG::*lacZ* fusion), and TTA11, a control strain containing an integrated plasmid lacking *brlA* and *lacZ* sequences. β-galactosidase activity was not detectable in uninduced cultures of TRG27.1.c or TRG30.1.c, but began to increase between 2 and 4 h post-induction (PI) in each strain. After this time, β-galactosidase activity increased steadily in TRG27.1.c until it reached a level of ~180 units by ~12 h PI. By contrast, β-galactosidase activity peaked at ~40 units by 6 h PI in the *brlAβ*ATG::*lacZ* fusion strain (TRG30.1.c). These results are in agreement with *brlAα* and *brlAβ* transcript levels measured by RNA blot analyses (Boylan *et al.*, 1987; Prade and Timberlake, 1993). No β-galactosidase activity was ever observed in TTA11.

To examine the requirements for *brlA* on expression of the *brlAα* ATG and *brlAβ* ATG::*lacZ* fusions we constructed Δ*brlA* strains (RSH1 and RSH2) containing the appropriate *lacZ* fusions. Figure 2B shows that β-galactosidase activity directed by the *brlAα* fusion is lower in the Δ*brlA* strain than in the wild type while β-galactosidase activity directed by the *brlAβ* fusion was unaffected. These data show that the expression of *brlAα*, but not that of *brlAβ*, is modulated by *brlA*.

brlAβ expression is regulated by a translational mechanism

The *brlAβ* transcript contains a 41 codon μORF near its 5′ end. To determine if the μORF initiation codon is recognized by *Aspergillus* ribosomes we constructed strain TRG29.1.b (*brlA*$^+$; *brlAβ* μORF ATG::*lacZ*) containing a gene fusion consisting of *brlA* upstream regulatory sequences up to and including the translational initiation site for the μORF (Figure 2A) fused to the *E.coli lacZ* gene. Figure 2C shows that ~30 U of β-galactosidase accumulated in strain TRG29.1.b before developmental induction. Nearly constant β-galactosidase levels were maintained as development continued. These results indicate that the μORF is translated constitutively and are consistent with the observations of Prade and Timberlake (1993) that *brlAβ* mRNA is present in uninduced hyphae.

A number of cases have been described where translation of downstream coding sequences is regulated by the activity of an upstream μORF (Werner *et al.*, 1987; Abastado *et al.*, 1991; Kozak, 1991). To determine if the *brlAβ* μORF influences *brlA* translation, we constructed a *brlA*$^+$ *Aspergillus* strain (TRG31.1.1) containing a *brlAα* ATG::*lacZ* fusion in which the μORF AUG was converted to an AUC, effectively eliminating the μORF. Figure 2C shows that β-galactosidase activity was detectable in TRG31.1.1 before developmental induction, indicating that removal of the μORF AUG resulted in derepressed translation of the downstream reading frame. Following induction, levels of β-galactosidase paralleled those observed in TRG27.1.c (Figure 2B). Because the μORF mutation specifically affects *brlAβ* mRNA, we interpret the observed derepression to be due to translation of *brlAβ* mRNA. Frameshift mutations in the μORF that leave the initiation codon intact did not result in derepressed β-galactosidase expression indicating that the sequence of the μORF polypeptide is probably not important to its function (data not shown).

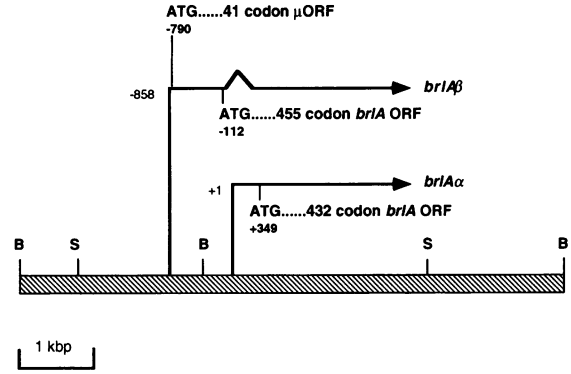

Fig. 1. Organization of the *brlA* locus. The *brlAα* and *brlAβ* transcripts are indicated by the arrows and the *brlAα* transcription start site is designated (+1). The three translational reading frames examined are shown in the upper part of the diagram with ATGs indicating the predicted translation start sites. Restriction sites are abbreviated: B, *Bam*HI; S, *Sal*I.

Elimination of the brlAβ μORF leads to premature development

Although *brlAβ* is transcribed in submerged culture (Prade and Timberlake, 1993), development is completely suppressed in wild type cells grown under these conditions. One explanation for this observation is that the ability of the μORF to block *brlAβ* translation prevents premature development. If this were the case, removal of the initiation codon for the *brlAβ* μORF would allow translation of *brlAβ* and might result in inappropriate conidiophore development during submerged growth.

To determine if the *brlAβ* μORF blocks premature development, we constructed a plasmid containing the AUG → AUC μORF mutation described above, upstream of a temperature sensitive allele of *brlA* (*brlA42*^{ts}), and introduced it by integration into the *trpC* locus of a *brlA*$^+$ *A.nidulans* strain, thus providing an altered copy of *brlA in trans* to the wild type gene (see Materials and methods). Several transformants were isolated that grew normally as colonies on the surface of agar medium at 37°C (restrictive temperature), but had severely inhibited growth when incubated at 28°C (permissive temperature). Analysis of genomic DNA from the transformants showed that 1–3

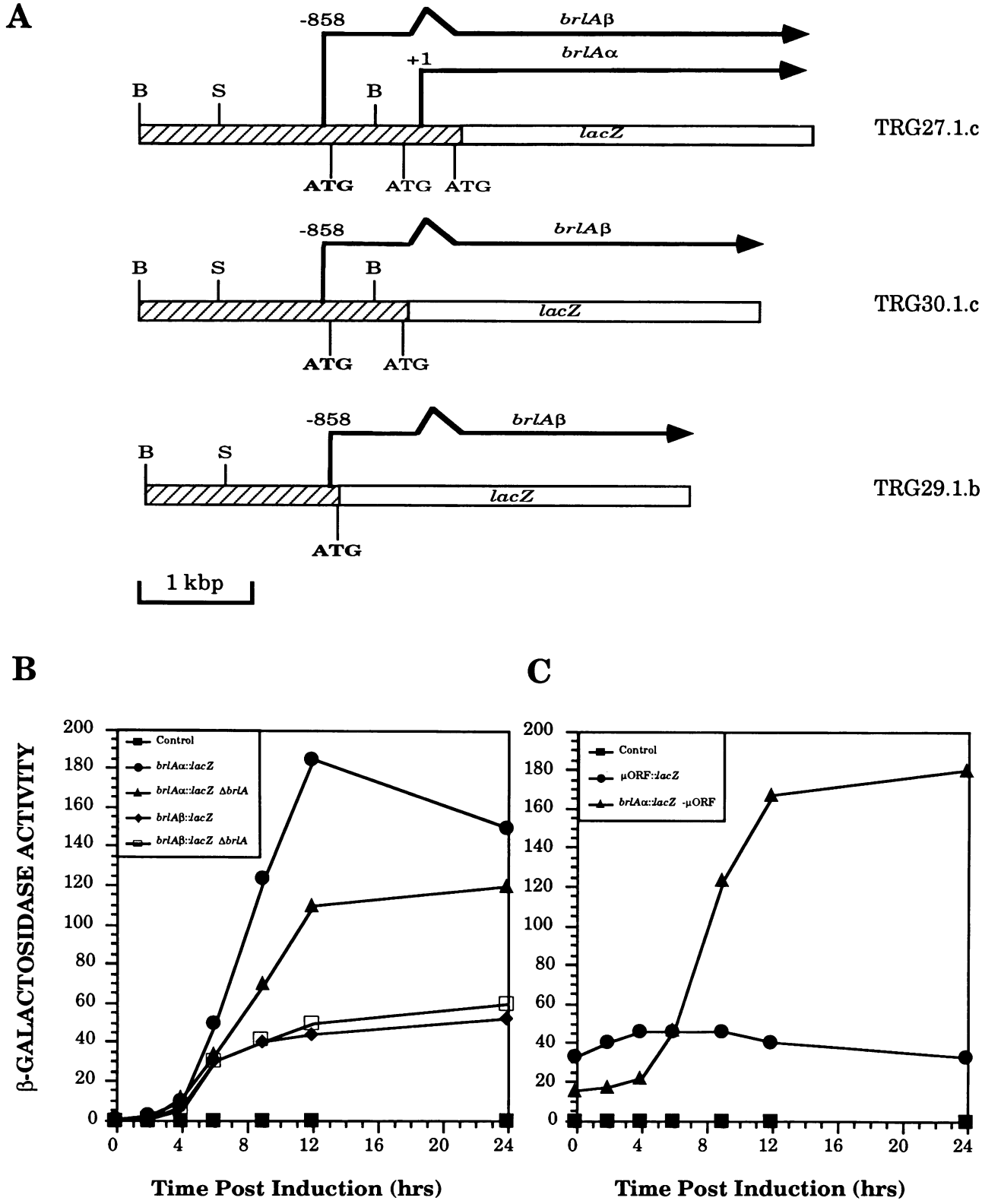

Fig. 2. Developmental regulation of *brlA* expression. (**A**) *brlA*::*lacZ* gene fusions. Translational fusions between the *E.coli lacZ* gene and each of the three *brlA* reading frames were constructed and *Aspergillus* strains containing the fusions integrated at the *trpC* locus were isolated as described in Materials and methods. Arrows indicate direction of transcription. ATGs indicate translational start sites for the μORF, the *brlAβ* BrlA ORF, and the *brlAα* BrlA ORF respectively. Restriction sites are abbreviated: B, *Bam*HI; S, *Sal*I. (**B**) Wild type (*brlA*$^+$) and *brlA* deletion strains containing the *brlAα* ATG::*lacZ* or *brlAβ* ATG::*lacZ* translational fusions shown in (A) integrated at the *Aspergillus trpC* locus were grown in liquid culture for 20 h and then induced to develop by exposing to an air interface (see 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. The control strain (TTA11) contains plasmid sequences integrated at *trpC* but lacks additional *brlA* or *lacZ* sequences. (**C**) Wild type (*brlA*$^+$) *Aspergillus* strains containing either a *brlAβ* μORF ATG::*lacZ* fusion or a *brlAα* ATG::*lacZ* fusion lacking the *brlAβ* μORF were grown vegetatively for 20 h and then induced to development. Samples were analyzed as above.

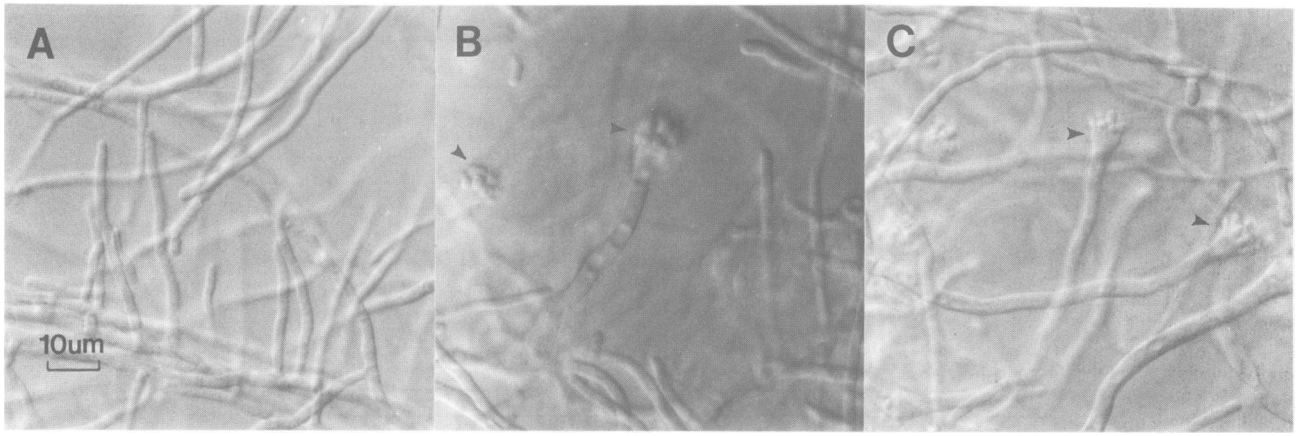

Fig. 3. The *brlAβ* μORF is essential to block premature development. Strains (**A**) TTA11 (*brlA*$^+$), (**B**) TRG44-13 (*brlA*$^+$, *trpC::brlA42*^{ts}/μORF$^-$) and (**C**) TA150 (Δ*brlA*; *trpC::brlA42*^{ts}/μORF$^-$) were grown in submerged culture for 16 h at 37°C and then transferred to 28°C and hourly microscopic observations were made. Micrographs shown were taken 9 h after the temperature shift. Characteristic conidiophore-like structures are indicated by arrows.

copies of the plasmid DNA had integrated as tandem repeats at the targeted genomic site (see Materials and methods; data not shown). The more copies of the plasmid that integrated, the more severely growth was inhibited. Conidia from a strain containing a single copy of the *brlA42*^{ts}/μORF$^-$ construct (TRG44f) were used to inoculate liquid cultures, incubated at 37°C for 16 h and then transferred to 28°C. As shown in Figure 3, many of the hyphae from strains containing the *brlA42*^{ts} allele lacking the μORF differentiated conidiophores under these conditions whereas a congenic *brlA*$^+$ control strain did not. Because the transformed strains contain a wild type copy of *brlA* as well as the altered *brlA42*^{ts} allele, it was possible that the development observed resulted from having multiple copies of *brlA* rather than from inactivation of the μORF. We tested this possibility by crossing a transformed strain having a single copy of the altered *brlA42*^{ts} allele (TRG44f) with a *brlA* deletion mutant (rM31.8) and isolated a recombinant (TA150) in which the only *brlA* gene present contained the μORF mutation. This strain behaved exactly as the strains containing both the wild type and mutant *brlA* alleles (Figure 3). In addition, strains containing a second wild type *brlA* allele integrated at the *trpC* locus grow only as hyphae in liquid medium (data not shown).

Analysis of RNA from strains carrying the derepressed *brlA42*^{ts} allele showed that *brlAβ* transcripts could be detected during growth in liquid medium even at the restrictive temperature (Figure 4). *brlAβ* levels increased steadily for 9 h after switching to the permissive temperature. In addition, *brlAα* transcripts were detected after prolonged incubation at 28°C. As expected, no *brlA* mRNA was detected in the wild type control strain before the temperature shift. However, trace amounts of *brlAβ* transcript were observed 9 h after shifting to 28°C (Figure 4).

Overexpression of brlAβ activates development

Activation of *brlAα* expression to high levels in vegetative cells has previously been shown to cause a cessation of normal growth and to lead to the development of reduced conidiophores that produce viable spores (Adams *et al.*, 1988). We constructed *Aspergillus* strains carrying fusions between the inducible *alcA* promoter and *brlA* sequences to allow vegetative overexpression of either wild type *brlAβ*

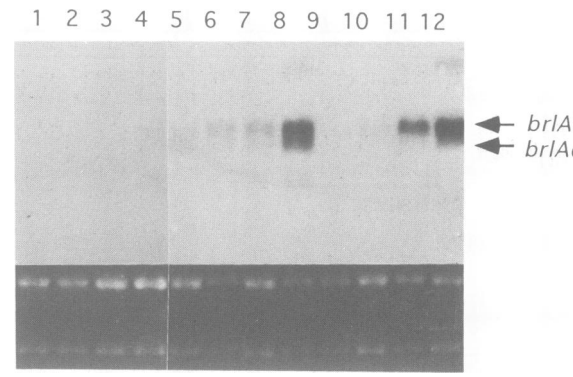

Fig. 4. Elimination of the *brlAβ* μORF results in accumulation of *brlAβ* message in vegetative cells. Strains TTA11 (lanes 1–4; *brlA*$^+$), TRG44-13 (lanes 5–8; *brlA*$^+$, *trpC::brlA42*^{ts}–μORF) and TA150 (lanes 9–12; Δ*brlA*; *trpC::brlA42*^{ts}–μORF) were grown for 16 h at 37°C and then transferred to 28°C and grown for an additional 9 h. 5 μg of total RNA isolated from these strains at the time of the temperature shift (lanes 1, 5 and 9) and 3 h (lanes 2, 6 and 10), 6 h (lanes 3, 7 and 11) and 9 h (lanes 4, 8 and 12) later was separated electrophoretically and then hybridized with *brlA*-specific DNA probe. *brlAα* and *brlAβ* transcripts are indicated by the arrows. The lower panel shows a photograph of the ethidium bromide stained RNA gel demonstrating approximately equal loading.

transcript (TSH54.b) or *brlAβ* transcript lacking the μORF (TSH55.c). These strains were grown for 12 h in minimal medium containing glucose and then transferred to *alcA*-induction medium; microscopic observations were then made at various times. As shown in Figure 5, overexpression of *brlAβ* with or without the μORF resulted in development like that observed when *brlAα* was activated in vegetative cells. However, the development observed after inducing *brlAβ*/μORF$^+$ expression was typically delayed by ~1 h when compared with the development induced by *brlAβ*/μORF$^-$ or *brlAα* (not shown).

Discussion

The *A.nidulans brlA* developmental regulatory locus is composed of two overlapping transcription units designated *brlAα* and *brlAβ*, which encode polypeptides that differ by

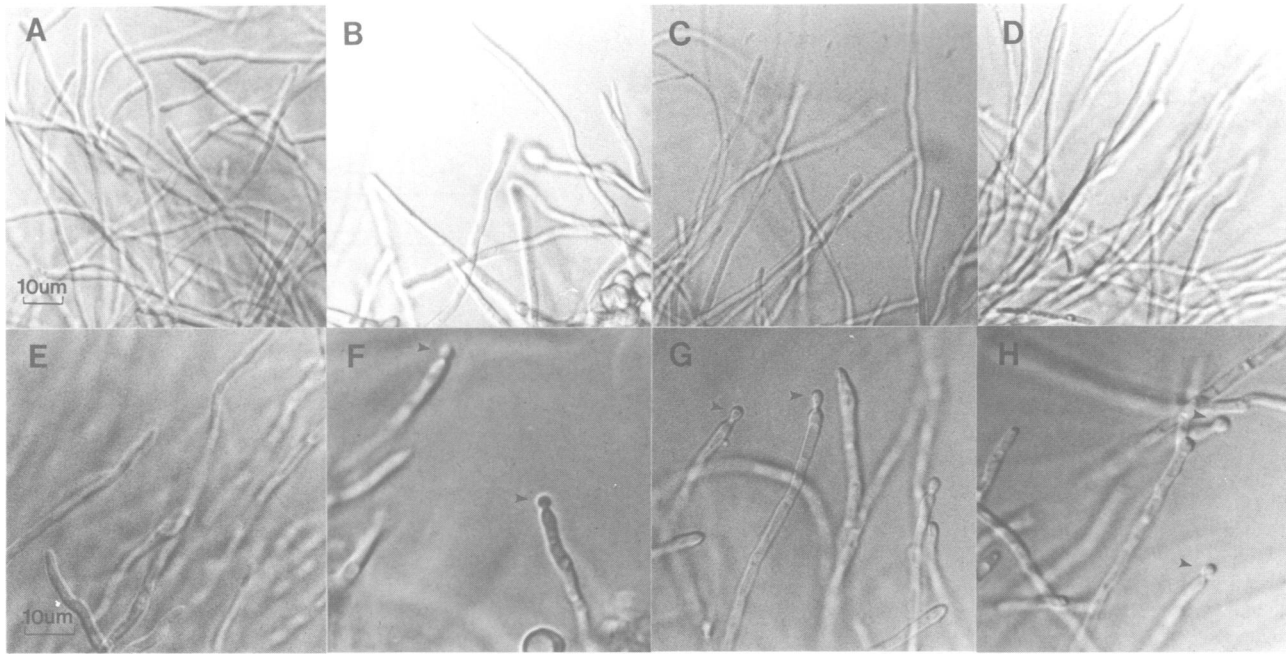

Fig. 5. Overexpression of *brlAβ* in vegetative cells causes development. Strains TTA11 (**A** and **E**), TTA292 (**B** and **F**; *brlA*$^+$; *alcA*[p]::*brlAα*), TSH55 (**C** and **G**; *brlA*$^+$; *alcA*[p]::*brlAβ*/μORF$^-$) and TSH54 (**D** and **H**; *brlA*$^+$; *alcA*[p]::*brlAβ*/μORF$^+$) were grown for 12 h in liquid minimal glucose medium (A−D), and then transferred to minimal threonine medium and allowed to grow for an additional 4 h (E−H). Characteristic spores forming at the tips of hyphae are indicated by arrows. The scale bar in panel A applies to panels A−D; that in panel E applies to panels E−H.

23 amino acids at their NH_2-termini. The predicted BrlAα and BrlAβ polypeptides appear to be functionally redundant because either of them is capable of directing normal conidiophore development when present in multiple copies (Prade and Timberlake, 1993). The results presented in this paper indicate that one role for the two transcription units is to provide different mechanisms for controlling *brlA* expression during development. Activation of *brlAβ* expression can apparently occur by modulation of the choice of translational initiation codons to determine whether or not BrlAβ protein is synthesized. On the other hand, positive feedback regulation of *brlAα* transcription (Mirabito *et al.*, 1989) provides a means to obtain higher levels of BrlA expression as development proceeds.

A model accounting for the coordination of *brlAα* and *brlAβ* expression during conidiophore development is presented in Figure 6. Based on results from three types of experiments, we propose that one mechanism for activating *brlA* expression following developmental induction involves a change in the translational machinery allowing ribosomes to begin translation at the internal *brlAβ* ATG. First, we showed that a μORF present near the 5′ end of the *brlAβ* mRNA is recognized by *Aspergillus* ribosomes to direct constitutive translation of a fused *lacZ* gene. Furthermore, mutations that eliminate the μORF ATG resulted in derepression of β-galactosidase expression from a *brlA*::*lacZ* fusion. Because these mutations alter *brlA*::*lacZ* expression in strains that contain a wild type *brlA*$^+$ gene, these results imply that *brlAβ* is transcribed in vegetative cells but that translation of BrlA is blocked by a *cis*-active regulatory function of the μORF. If the μORF polypeptide was able to repress *brlA* expression *in trans*, the effect of this mutation could only be observed in *brlA*$^-$ strains. Prade and Timberlake (1993) found that the 5′ end of *brlAβ* mRNA could be detected by primer extension analysis using RNA isolated from vegetative cells supporting our conclusion that *brlAβ* is transcribed in uninduced cultures.

Small ORFs present in the 5′ ends of several eukaryotic mRNAs have been shown to play a role in regulating translation from downstream AUGs (see Kozak, 1991 for review). In some cases, this regulatory activity requires a specific amino acid sequence in the regulatory peptide whereas in others the presence of an AUG is sufficient to regulate downstream translation (Hinnebusch, 1984; Mueller and Hinnebusch, 1986; Werner *et al.*, 1987; Abastado *et al.*, 1991). Frameshift mutations in the *brlAβ* μORF did not affect regulation of *brlA*::*lacZ*, but mutation of the AUG allowed derepressed BrlA expression. Therefore, we consider it likely that translation of the *brlAβ* μORF alone is required to regulate translation of BrlA. Given that there are apparently no specific amino acid sequence requirements for the μORF in imposing translational inhibition, it may be that the proposed change in translational initiation is a general response that occurs during developmental induction and is not specific to *brlA*. In fact, at least one other *Aspergillus* developmental regulatory gene, *stuA*, has small ORFs present upstream of the predicted initiation codon, suggesting that translation may play a more general role in regulating *Aspergillus* development (Miller *et al.*, 1992).

The best characterized example of translational regulation by the activity of upstream ORFs occurs in the *Saccharomyces cerevisiae GCN4* gene. *GCN4* encodes the transcriptional activator for general control of amino acid biosynthesis (Hinnebusch, 1984; Mueller and Hinnebusch, 1986). Small ORFs present in a long transcribed leader repress *GCN4* translation during growth in rich media. During amino acid starvation, this repression is overcome through a mechanism that involves phosphorylation of the α subunit of eIF2 (Dever *et al.*, 1992). Although nutritional status is not known to regulate *Aspergillus* conidiophore

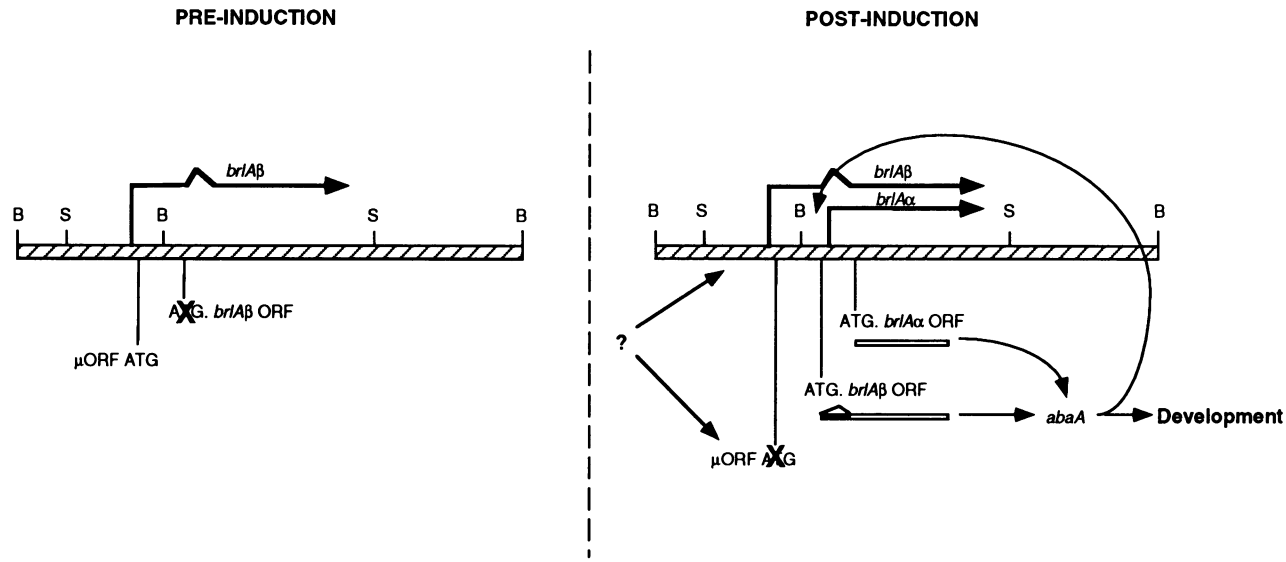

Fig. 6. Model for differential control of *brlAα* and *brlAβ* during conidiophore development. The *brlAβ* message is transcribed by vegetative cells before developmental induction but translation of the μORF represses translation of BrlA. The peptide sequence of the μORF is apparently not important for this regulatory activity. Following induction unknown regulatory factors result in activation of BrlA translation from *brlAβ* by removing the block imposed by the μORF, increasing transcription of *brlAβ*, or both. Activation of BrlA translation leads to transcription of abaA and other downstream regulatory proteins. This in turn activates a positive feedback regulatory loop and leads to high levels of *brlAα* expression to cause further developmental changes.

development directly, it remains possible that translation of *brlAβ* could be controlled by a related mechanism. In support of an involvement of nutritional status in developmental induction, we have identified a mutant strain that has lost the ability to conidiate as a programmed event during the life cycle when grown on rich medium but develops when exposed to growth limiting conditions (Adams *et al.*, 1992).

The second experiment presented that addresses the role of the μORF in regulating *brlA* expression involved eliminating the μORF initiation codon upstream of the *brlA* gene. Strains containing one or more copies of the temperature sensitive *brlA42*^{ts} allele from which the μORF ATG had been eliminated produced conidiophores in submerged culture when grown at the permissive temperature, but did not at the restrictive temperature, for *brlA42*^{ts} (Figure 3). Thus, the μORF plays a functional role in preventing premature development. Again, this activity functions *in cis*, given that premature development occurs even in the presence of a wild type *brlA* locus. We propose this block is imposed primarily at the translational level.

Eliminating the μORF upstream of the *brlA* gene also resulted in accumulation of *brlAβ* mRNA at both restrictive and permissive temperatures for *brlA42*^{ts}. Wild type strains containing unaltered *brlA*$^{+}$ alleles grew strictly as vegetative hyphae and no full length *brlAβ* message could be detected under these conditions (Figures 3 and 4). These results could most easily be explained if translation of the *brlAβ* μORF prevented efficient translation of BrlA and this led to message instability. In support of this idea, frameshift mutations causing early translational termination frequently result in decreased mRNA levels and, in at least some cases, this has been demonstrated to result from changes in message stability (Losson and Lacroute, 1979; Pelsey and Lacroute, 1984; Corrick *et al.*, 1987). An alternative explanation for this observation is that the μORF mutation resulted in increased *brlAβ* transcription rates. These two possibilities cannot be distinguished from the experiments presented here. In either case, the mutation removing the initiation codon for the μORF resulted in measurable changes in *brlAβ* expression and misscheduled development.

The third experiment presented to ascertain the role of the *brlAβ* μORF involved overexpression of *brlAβ* mRNA in submerged cultures (Patemen *et al.*, 1983; Gwynne *et al.*, 1987; Adams *et al.*, 1988). Overexpression of either *brlAβ* or *brlAβ*/μORF^{-} in vegetative cells resulted in development of hyphal tips into reduced conidiophores. However, the development induced by overexpression of wild type *brlAβ* was delayed in comparison with that induced by overexpression of the *brlAβ* transcript without the μORF. The fact that overexpression of *brlAβ* resulted in delayed misscheduled development implies that the μORF only damps BrlA expression and cannot act as an absolute block of translation before induction. Thus, it is possible that increased transcription of *brlAβ* could be sufficient to cause developmental induction even without a change in the translational machinery. Alternatively, low level *brlAβ* translation could lead to cellular alterations that cause modification of translation (Adams and Timberlake, 1990a).

Regulation of *brlAα* expression differs from *brlAβ* control in at least two important respects. First, there is no evidence for regulation of *brlAα* translation. The first AUG present in *brlAα* mRNA initiates the BrlA reading frame and expression of β-galactosidase activity from the *brlAα* ATG::*lacZ* fusion closely parallels mRNA accumulation. Second, maximal expression from the *brlAα* ATG::*lacZ* fusion requires *brlA*$^{+}$ whereas *brlAβ* ATG::*lacZ* expression is unaltered in a Δ*brlA* strain. These data suggest that the positive feedback control from *abaA* works primarily through increased transcription of *brlAα* and not *brlAβ* (Mirabito *et al.*, 1989). However, increases in *brlAβ* transcript levels were observed both during early development and when strains carrying a *brlA42*^{ts} allele lacking the μORF were shifted from the restrictive to permissive temperature. These results could be interpreted to mean that *brlAβ* is also

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

Strains	Genotype	Source
TRG27.1.c	*pabaA1, yA2; veA1, trpC::brlAα ATG::*lacZ	this study
TRG29.1.a	*pabaA1, yA2; veA1, trpC::brlAβ* μORF ATG::lacZ	this study
TRG30.1.c	*pabaA1, yA2; veA1, trpC::brlAβ* ATG::*lacZ*	this study
TRG31.1.1	*pabaA1, yA2; veA1, trpC::brlAα* ATG::*lacZ*/μORF$^-$	this study[1]
TRPΔ31	*pabaA1, yA2; argB::trpC; trpC801, veA1, ΔbrlA::argB*	Prade and Timberlake, 1992
RM31.8	*biA1, yA2; ΔbrlA::argB; veA1*	this study
RSH1	*pabaA1, yA2; veA1, trpC::brlAα* ATG:*lacZ, ΔbrlA*	this study
RSH2	*pabaA1, yA2; veA1, trpC::brlAβ* ATG::*lacZ, ΔbrlA*	this study
TRG44.f	*pabaA1, yA2; veA1, trpC::brlA42*(1)/μORF$^-$	this study[1]
TRG44.g	*pabaA1, yA2; veA1, trpC::brlA42*(2)/μORF$^-$	this study[1]
TRG44.r	*pabaA1, yA2; veA1, trpC::brlA42*(3)/μORF$^-$	this study
TRG44.13	*pabaA1, yA2; veA1, trpC::brlA42*(2)/μORF$^-$	this study[1]
TSH46.d	*pabaA1, yA2; veA1, trpC::brlAα* ATG::*lacZ*/μORF$^-$	this study[2]
TSH47.c	*pabaA1, yA2; veA1, trpC::brlAα* ATG::*lacZ*/μORF$^-$	this study[3]
TSH54.b	*pabaA1, yA2; veA1, trpC::alcA*(p)::*brlAβ*	this study[1]
TSH55.c	*pabaA1, yA2; veA1, trpC::alcA*(p)::*brlAβ*/μORF$^-$	this study[1]
TTA11	*pabaA1, yA2; veA1, trpC801*	*this study*
TA150	*pabaA1, biA1; ΔbrlA::argB trpC::brlA42*/μORF$^-$	this study[1]
TTA292	*biA1; argB::alcA(p)::brlAα; methG1; veA1*	Adams *et al.*, 1988
FGSC26	*biA1;veA1*	FGSC[4]
FGSC237	*pabaA1, yA2; trpC801, veA1*	FGSC[4]

[1]μORF$^-$ mutation converts first codon ATG−>ATC
[2]μORF$^-$ mutation results from a +1 frameshift by addition of A after second codon
[3]μORF$^-$ mutation results from a −1 frameshift by converting TC−>A in twenty-fourth codon
[4]Fungal Genetics Stock Center

transcriptionally activated by the positive feedback loop. Alternatively, changes in stability of the *brlAβ* message may account for the observed increase in *brlAβ* transcript levels (see above).

In our model (Figure 6), either *brlAβ* translation is initiated through a controlled change in choice of translational initiation sites from the μORF ATG to the BrlA ATG or increased *brlAβ* transcript levels provide sufficient template to overcome the inefficient translational block imposed by the μORF. This ultimately leads to an activation of other developmentally specific genes resulting in differentiation of conidiophore-specific cells. We propose that the major mechanism for activating expression of *brlAα* transcript occurs through the positive feedback loop that is initiated following activation of *abaA* (Mirabito *et al.*, 1989). This conclusion is supported by the fact that three AbaA binding sites occur upstream of the *brlAα* transcription initiation site whereas no AbaA binding sites occur within ~1 kbp upstream of the *brlAβ* transcription start site (A.Andrianopoulos and W.Timberlake, unpublished results). In addition, we found that forced activation of *brlAβ* leads to accumulation of *brlAα* transcript whereas *brlAα* expression does not activate *brlAβ* (S.T.Han and T.H.Adams, unpublished). Feedback activation of *brlAα* leads to greatly increased BrlA levels, resulting in further development.

The observation by Prade and Timberlake (1993) that *brlAα* mRNA accumulates in a strain that lacks the *brlAβ* transcriptional start (TRP44L) indicates that *brlAα* activation is not strictly dependent on *brlAβ*. Thus, both *brlAα* and *brlAβ* are somewhat responsive to signals that initiate development. Different controls for activation of *brlAα* and *brlAβ* may provide a means to separate responses both to the multiple signals that initiate development and to the endogenous events that result in commitment to the conidiation pathway.

Materials and methods

Aspergillus strains, growth and genetics

The *A.nidulans* strains used in this study are described in Table I. Standard *A.nidulans* genetic (Pontecorvo *et al.*, 1953; Clutterbuck, 1974) and transformation techniques (Yelton *et al.*, 1985) were used.

All *A.nidulans* transformant strains used in this study were generated by integration of plasmid sequences at the *trpC* locus. For *brlA::lacZ* gene fusion experiments, strains TRG27.1.c, TRG30.1.c, TRG29.1.a, TRG31.1.1, TSH46.d and TSH47.c were constructed by transforming *Aspergillus* strain FGSC237 with *brlA::lacZ* fusion plasmids pRG27, pRG30, pRG29, pRG31, pSH46 and pSH47, respectively. Strains TSH54.b and TSH55.c, containing the *alcA*(p) fused to *brlAβ*, were constructed by transforming FGSC237 with pSH54 and pSH55. Strains containing copies of the *brlA42*^{ts} allele lacking the *brlAβ* μORF integrated at *trpC* were constructed by transforming FGSC237 with pRG44. All transformants were analyzed by Southern blot analysis to verify that the predicted integration event had occurred at the *trpC* locus.

The *brlA* deletion strains TA150, RSH1 and RSH2 were constructed by using sexual crosses between RM31.8 and either TRG44f, TRG27.1.c or TRG30.1.c respectively. RM31.8 was constructed by crossing TRP31 (Prade and Timberlake, 1993) with PW1 (Adams *et al.*, 1988). *brlA*$^-$ recombinants were isolated and analyzed by Southern blot analysis to make certain that the gene fusions were unaffected by recombination.

All strains were grown in appropriately supplemented minimal medium (Käfer, 1977). Developmental cultures were grown as described previously (Law and Timberlake, 1980) with some modifications. Conidia (10^5/ml) were inoculated into appropriately supplemented liquid minimal medium and incubated for 20 h at 37°C and 300 r.p.m. Samples were harvested onto Whatman number 1 filter paper disks, placed on 1.2% agar plates containing the same medium and incubated at 37°C for the times indicated. Under these conditions, conidiophore vesicles first appeared 4 h after induction and spores began to be produced ~6 h later. Cultures of *brlA* deletion strains were inoculated using ascospores (3×10^5/ml) from self-fertilized cleistothecia. For the *alcA*(p)::*brlA* induction time course experiment (Figure 5), strains TTA11 (*brlA*$^+$), TSH54, TSH55 (*trpC::alcA*[p]::*brlAβ*) and TTA292 (*argB::alcA*[p]::*brlAα*; Adams *et al.*, 1988) were inoculated at a density of 2×10^6 spores/ml in minimal medium containing 50 mM glucose and shaken at 37°C for 12 h. Cells were harvested onto Miracloth (Calbiochem), washed once with minimal medium containing 100 mM L-threonine, transferred to this medium and incubated as above. Samples were taken hourly for microscopic observation. For the *brlA42*^{ts} induction time course experiment (Figures 3 and 4), spores were inoculated at a density of 2×10^6 spores/ml into appropriately supplemented minimal

media and shaken at 37°C for 16 h. 100 ml samples were taken and the *brlA42*^{ts} allele was induced by shifting the culture to 28°C. Samples were taken 3, 6 and 9 h after the temperature shift and subjected to microscopic observation and to RNA isolation as previously described (Adams *et al.*, 1988).

Plasmid construction

The following plasmids were constructed using standard procedures: **pRG18**: A 3.0 kbp *Bam*HI *lacZ* fragment from pULAC (Hamer and Timberlake, 1987) inserted into the *Bam*HI and *Bgl*II sites of PIC-20H (Marsh *et al.*, 1984) in an orientation that leaves the 5′ *Bam*HI site intact. **pRG19**: A 3.0 kbp *Xba*I−*Xho*I *lacZ* fragment from pRG18 inserted into the *Xba*I and *Xho*I sites of Bluescript SK(−) (Stratagene, La Jolla, CA). **pRG20**: A 3.0 kbp *Bam*HI−*Kpn*I *lacZ* fragment from pRG19 inserted into the *Bam*HI and *Kpn*I sites of pK19 (Pridmore, 1987). **pEP3**: A 1.8 kbp *Sst*I−*Xho*I *trpC* fragment (Hamer and Timberlake, 1987) inserted into the *Sst*I and *Xho*I sites of PIC-20H. **pEP4**: A 1.8 kbp *Bam*HI−*EcoR*I *trpC* fragment from pEP3 inserted into the *EcoR*I and *Bam*HI sites of Bluescript KS(−). **pSH9**: A 1.8 kbp *Kpn*I *trpC* fragment from pEP4 inserted into the *Kpn*I site of pK19. **pTA133**: A 5.2 kbp *Sst*I−*Kpn*I *brlA* fragment inserted into the *Sst*I and *Kpn*I sites of Bluescript KS(−). **pRG21**: pTA133 with a *Bgl*II site introduced downstream from the *brlAα* initiation codon by using a partially complementary oligonucleotide (5′-CGGACAGGCTAGATCTGATTTC-GC-3′) for *in vitro* mutagenesis as described by Kunkel (1985). **pRG24**: The 3.0 kbp *Bam*HI−*Kpn*I *lacZ* fragment from pRG20 cloned into the *Bgl*II and *Kpn*I sites of pRG21. **pRG27**: pRG24 containing the 1.8 kbp *Kpn*I *trpC* fragment from pSH9 inserted into the single *Kpn*I site to give rise to the *brlAα*::*lacZ* fusion. **pTA134**: pTA133 with a *Bgl*II site introduced downstream from the *brlAβ* initiation codon by using a partially complementary synthetic oligonucleotide (5′-CTGAGGGCAGATCTTC-ATTACTTTTC-3′). **pTA137**: The 3.0 kbp *Bam*HI−*Kpn*I *lacZ* fragment from pRG20 inserted into the *Bgl*II and *Kpn*I sites of pTA134. **pRG30**: pTA137 containing the 1.8 kbp *Kpn*I *trpC* fragment from pSH9 inserted into the single *Kpn*I site. **pTA135**: pTA133 with a *Bgl*II site introduced downstream from the *brlAβ* μORF initiation codon by using a partially complementary synthetic oligonucleotide (5′-GCAGAGCAGATCTGAG-AAAGTC-3′) for *in vitro* mutagenesis. **pTA136**: The 3.0 kbp *Bam*HI−*Kpn*I *lacZ* fragment from pRG20 inserted into the *Bgl*II and *Kpn*I sites of pTA135. **pRG29**: pTA136 containing the 1.8 kbp *Kpn*I *trpC* fragment from pSH9 inserted into the single *Kpn*I site. **pRG31**: pRG27 with a mutation introduced to remove the AUG codon in the *brlAβ* μORF using the synthetic oligonucleotide (5′-GAGAAAGTGGATCCCTCACG-3′) for *in vitro* mutagenesis. **pTA111**: A 4.3 kbp *Sal*I *brlA* fragment cloned into the *Sal*I site of Bluescript KS(−). **pSH42**: pTA111 containing a *Bgl*II site inserted just downstream of the *brlAβ* transcriptional initiation site using the synthetic oligonucleotide (5′-ATTATTTCGAGATCTTAATCT-3′) for site-directed mutagenesis. **pSH51**: A 0.5 kbp *Sst*I−*Bam*HI fragment from pTA18 (Adams *et al.*, 1988) containing the transcription initiation site and 5′ regulatory sequences of *alcA* (Gwynne *et al.*, 1987) inserted into the *Sst*I−*Bam*HI sites in pK19. **pSH52**: A 0.5 kbp *Sst*I−*Bam*HI *alcA*(p) fragment from pSH51 inserted into the *Sst*I and *Bgl*II sites in pSH42. **pSH54**: pSH52 containing the 1.8 kbp *Sst*I *trpC* fragment from pSH9 inserted into the unique *Sst*I to give rise to the *alcA*(p)::*brlA* gene fusion. **pSH53**: pSH52 with a mutation introduced to remove the AUG codon in the *brlAβ* μORF using the synthetic oligonucleotide (5′-GAGAAAGTGGATCCCTCACG-3′) for *in vitro* mutagenesis. **pSH55**: pSH53 containing the 1.8 kbp *Sst*I *trpC* fragment from pSH9 inserted in the unique *Sst*I site to give rise to the *alcA*(p)::*brlA*-μORF fusion. **pTA33**: A 2.5 kbp *Bam*HI−*Sal*I fragment containing the *brlA*42 temperature sensitive allele (Johnstone *et al.*, 1985) inserted into the *Bam*HI and *Sal*I sites in Bluescript SK(−). **pRG43**: A 3.8 kbp *Sst*I−*Hin*dIII fragment from pRG31 inserted into the *Hin*dIII and *Sst*I sites of pTA33. pRG44: pRG43 containing the 1.8 kbp *Sst*I *trpC* fragment from pSH9 inserted into the unique *Sst*I site. **pSH13**: *Bam*HI−*Kpn*I *brlAα*::*lacZ* fusion fragment from pRG24 inserted into the *Bam*HI and *Kpn*I sites of pK19. **pSH16**: pSH13 containing a 1.8 kbp *Kpn*I fragment from pEP4 with the 5′ end of *trpC* inserted into a unique *Kpn*I site. **pSH43**: pTA133 with a +1 frameshift mutation introduced after the second codon in the μORF by using a partially complementary synthetic oligonucleotide (5′-GAGCAGAGATCTTGGC-ATCCC-3′) for *in vitro* mutagenesis. **pSH44**: pTA133 with a −1 frameshift mutation introduced after codon 24 in the μORF using a partially complementary oligonucleotide (5′-GGTCAAGATCTGCGACTGA-3′) for *in vitro* mutagenesis. **pSH46**: pSH16 containing a 2.5 kbp *Bam*HI fragment from pSH43 inserted in a unique *Bam*HI site. **pSH47**: pSH16 containing a 2.5 kbp *Bam*HI fragment from pSH44 inserted in a unique *Bam*HI site. The relevant portion of all plasmids made by site-directed mutagenesis was sequenced to make certain no unwanted mutations had occurred.

β-Galactosidase assays

β-galactosidase activity was measured using protein extracts prepared from lyophilized samples of vegetative and conidiating cultures of *A.nidulans* as described by van Gorcom *et al.* (1985) and Adams and Timberlake (1990b). The assay for the activity with *o*-nitrophenol-β-D-galactopyranosidase (ONPG) substrate was done as described by Miller (1972). Developmental time courses were done with each strain by taking samples at the times indicated after induction of development. Enzyme activity was measured using multiple samples from each time. Protein concentrations were determined by the Bradford procedure (Bradford, 1976). Less than 5% variation was observed between multiple assays for each strain in any given experiment.

Microscopy and photography

All light microscopy was done with an Olympus BH-2 microscope using differential interference contrast optics.

RNA preparation and analysis

Total RNA was isolated as previously described (Adams *et al.*, 1988), separated by electrophoresis on formaldehyde−agarose gels and transferred, without any pretreatment, to Hybond-N$^+$ membrane (Amersham Corp., Arlington Heights, IL), and hybridized to randomly ^{32}P-labeled probes according to procedures recommended by the membrane manufacturer. Plasmid pSH54, containing the 2.5 kbp *Bam*HI−*Sal*I fragment from the *brlA* gene, was used as a *brlA*-specific probe.

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