The EMBO Journal vol.12 no.6 pp.2439-2447, 1993

The *Aspergillus nidulans brlA* regulatory locus consists of overlapping transcription units that are individually required for conidiophore development

Rolf A.Prade and William E.Timberlake[1]

Department of Genetics, University of Georgia, Athens, GA 30602, USA
[1]Corresponding author

Communicated by C.J.Leaver

The *Aspergillus nidulans brlA* locus controls conidiophore development in conjunction with the products of several other regulatory loci. In this paper, we show that the *brlA* locus consists of overlapping transcription units, designated α and β, with α transcription initiating within β intronic sequences. The predicted BrlA polypeptides differ by 23 amino acid residues at their N-termini. Targeted mutations specifically eliminating either the α or β transcript led to developmental abnormalities similar to those produced by previously identified hypomorphic mutants, showing that both transcripts have essential functions for normal development. However, provision of additional doses of α in a β^- strain or of β in an α^- strain remediated the developmental defects, indicating that the polypeptides have redundant functions. It is likely that differential regulation of α and β expression in the wild type is important for the initiation and temporal regulation of development.

Key words: *Aspergillus nidulans/brlA* /fungal sporulation/ gene regulation

Introduction

Asexual reproduction or conidiation in the filamentous ascomycete *Aspergillus nidulans* entails development of a complex, multicellular reproductive apparatus called the conidiophore (Oliver, 1972; Mims *et al.*, 1988). Numerous developmentally relevant genes have been identified by mutation. Many of these direct the synthesis of enzymes or structural proteins needed for the specialized activities of differentiated conidiophore cells (Clutterbuck, 1977; Law and Timberlake, 1980; O'Hara and Timberlake, 1989; Aramayo and Timberlake, 1990; Birse and Clutterbuck, 1990; Clutterbuck, 1990; Mayorga and Timberlake, 1990; Tilburn *et al.*, 1990; Stringer *et al.*, 1991). Others appear to be responsible for spatiotemporal coordination of the expression of the hundreds of genes that are selectively activated or inactivated during conidiation (Clutterbuck, 1977; Timberlake, 1980, 1990, 1991b; Zimmermann *et al.*, 1980; Miller *et al.*, 1991, 1993). The latter group of genes includes *stuA, medA, brlA, abaA* and *wetA*. Mutations in these genes are pleiotropic at the morphological and molecular levels, leading either to an arrest of development at a specific stage or gross alterations in conidiophore morphology and to major changes in the patterns of expression of numerous other developmentally regulated genes (Clutterbuck, 1969; Martinelli, 1979; Boylan *et al.*, 1987; Timberlake, 1990; Miller *et al.*, 1991, 1993). Many of the potential regulatory genes have been cloned and subjected to varying degrees of analysis (Johnstone *et al.*, 1985; Boylan *et al.*, 1987; Adams *et al.*, 1988, 1990; Mirabito *et al.*, 1989; Adams and Timberlake, 1990a,b; Marshall and Timberlake, 1991; Miller *et al.*, 1991, 1993).

Bristle (*brlA*) is the most extensively characterized regulatory gene. Several types of evidence indicate that *brlA* is of central importance in controlling the steps of conidiophore formation. First, even though *brlA* null mutants initiate development, they produce only rudimentary conidiophores consisting of stalks that elongate indeterminately and fail to form any other differentiated cell types (Clutterbuck, 1969). These mutants do not activate most developmentally regulated genes, including putative downstream regulatory genes (Boylan *et al.*, 1987). Hypomorphic *brlA* alleles permit more extensive, although abnormal, development and modify the expression patterns of genes encoding development-specific enzymes (Birse and Clutterbuck, 1991). Secondly, *brlA* activity is continuously required for the completion of all developmental steps from vesicle formation to spore differentiation (Mirabito *et al.*, 1989). Thirdly, *brlA* encodes a zinc finger protein, suggesting that BrlA is involved directly in regulating the transcription of other developmentally important genes (Adams *et al.*, 1988, 1990). The ability of *brlA* to transactivate transcription in yeast of a reporter gene fused to a minimal yeast promoter and putative *brlA* response elements supports such a role (Chang and Timberlake, 1993). Finally, induced transcription of *brlA* in *A.nidulans* hyphae under non-sporulating conditions leads to activation of numerous other sporulation-specific genes, including proposed downstream regulatory genes, induction of sporulation, growth arrest and major metabolic alterations (Adams *et al.*, 1988; Mirabito *et al.*, 1989; Adams and Timberlake, 1990b). Thus, *brlA* expression is sufficient to drive the core developmental program leading to spore formation. It is therefore apparent that elucidation of the mechanisms regulating *brlA* expression is essential for understanding how this locus controls the steps of conidiophore development.

In initial characterizations of the cloned *brlA* gene, results from S1 nuclease protection studies suggested that the gene was transcribed in alternate patterns (Boylan *et al.*, 1987). However, subsequent primer extension studies failed to confirm the existence of multiple transcription initiation or polyadenylation sites or to identify alternative introns (Adams *et al.*, 1988). In selecting *brlA* cDNA clones we obtained a class of plasmids that could not be explained by the proposed simple transcriptional structure of *brlA*. In this paper we show that *brlA* is a compound gene, consisting of overlapping transcription units, designated α and β, with α transcription initiation sites residing in a β intron. Mutations that specifically interfere with either α or β transcription cause formation of abnormal conidiophores, reminiscent of

those produced by previously identified hypomorphic mutants, showing that both transcript classes are required for normal development. However, additional copies of α are able to restore β mutants to wild type and additional copies of β are able to restore α mutants to wild type. Thus, although each transcription unit is essential for normal development, their products have redundant functions. Results described in Han *et al.* (1993) indicate that expression of *brlA*α and *brlA*β is controlled by different mechanisms. The data suggest that *brlA*β is instrumental in initiation of development, whereas *brlA*α is more important for continued development once it is initiated.

Results

brlA consists of two overlapping transcription units

The molecular structure of the *brlA* locus was initially determined by Boylan *et al.* (1987) and Adams *et al.* (1988). Their results indicated that *brlA* consists of a single intronless transcription unit. Subsequently, three lines of evidence suggested that the locus might have a more complex organization: (i) 3.5 kb of DNA 5′ of the assigned mRNA cap site were required to complement *brlA* null alleles *in trans* (Johnstone *et al.*, 1985; T.H.Adams, personal communication), even though < 1 kb of upstream DNA is usually required for *trans*-complementation with *A.nidulans* genes; (ii) two *brlA* transcripts (2.1 and 2.5 kb) were occasionally detected in RNA blots (unpublished results); and (iii) *brlA* cDNA clones were obtained with 5′ ends corresponding to sequences upstream of the proposed *brlA* mRNA cap site (see below). To re-evaluate transcription of *brlA*, blots of gels with RNA from hyphae and from developing cultures containing immature conidiophores were hybridized with DNA fragments from the *brlA* chromosomal region (Figure 1). A 0.7 kb *Bam*HI–*Hin*dIII fragment (−404 to +247) that contains the assigned *brlA* mRNA cap site (+1) and 5′-untranslated sequences, and a *Hin*dIII–*Sal*I fragment (+247 to +2085) that contains the *brlA* coding region, hybridized to a 2.1 kb transcript (designated α) and to a less abundant 2.5 kb transcript (designated β). An *Apa*I–*Bam*HI fragment (−1290 to −404) hybridized only to the 2.5 kb transcript. Other fragments from the region either showed no hybridization or hybridized to transcripts appearing to be unrelated to *brlA*. These results supported the existence of an overlapping transcription unit with initiation sites in the −1290 to −404 region (see Figure 1).

To confirm the existence of the upstream transcription unit, poly(A)$^+$ RNA from hyphae and developing cultures was subjected to primer extension analysis with an oligonucleotide from −737 to −717. Figure 2 shows a strong primer extension product ending at −851 and weaker products ending at −857 and −848. The −851 product was detected with 1 or 10 μg of either hyphal or developmental RNA, whereas the weaker products were reliably detected only with 10 μg of RNA. The generation of primer extension products with hyphal RNA was unexpected, because transcript was not observed in RNA blots of hyphal RNA hybridized with a −1290 to −404 probe (see Figure 1). Low levels of *brlA*α transcript were also detected by primer extension of hyphal poly(A)$^+$ RNA, even though no transcript was detected by RNA blot analysis (data not shown). Further confirmation of the existence of two transcription units was provided by cDNA clones. Comparison of genomic sequence with the sequences of β cDNA clones showed that the β transcription unit contained an intron from −99 to +292. Both α and β cDNAs possessed similar 3′ ends. These results showed that the *brlA* locus consists of two overlapping transcription units (Figure 3A).

The sequence of the *brlA* chromosomal region from −1289 to +460 is presented in Figure 3B. The 2.1 kb *brlA*α transcript initiates at +1 and +8 with the first ATG at +349 initiating a 432 codon open reading frame (ORF) (Adams *et al.*, 1988). The 2.5 kb *brlA*β transcript initiates at −857, −851 and −848 and contains an intron from −99 to +292. The first ATG occurs at −790 and initiates a 41 codon reading frame, designated μORF, with a second ATG at −112 followed by an ORF of 22 codons fused to the *brlA*α

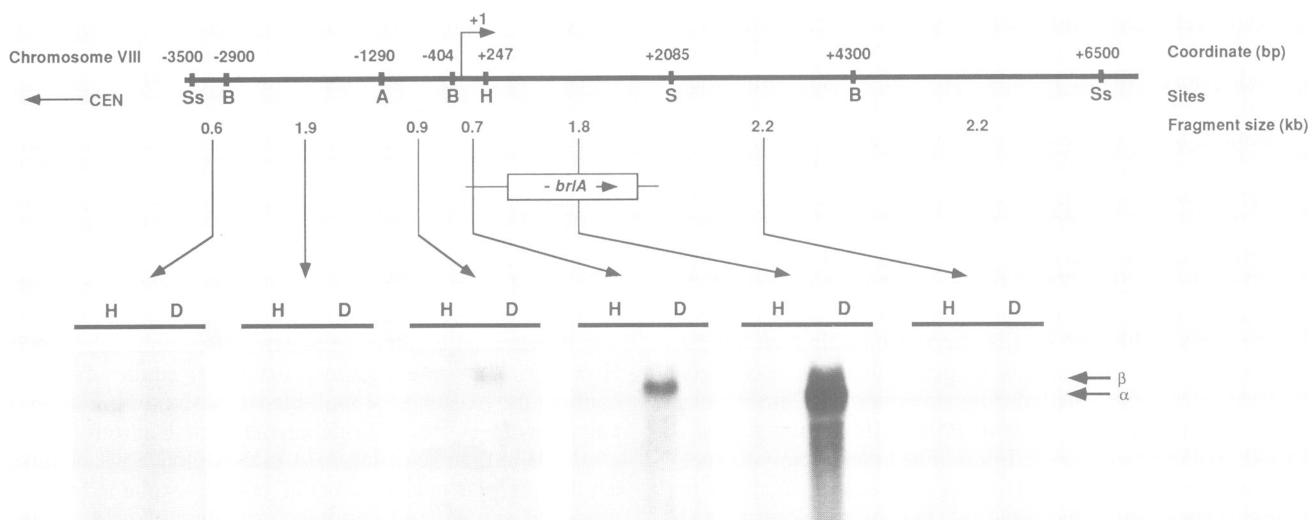


Fig. 1. Identification of transcripts encoded at the *brlA* chromosomal region. RNA was isolated from hyphae (H) or cells that had been induced to develop for 10 h (D) and gel blots were hybridized with radiolabeled DNA fragments from the *brlA* chromosomal region as indicated. The *brlA* transcript (α) and a second developmentally regulated transcript (β) mapping to the same region are indicated. Restriction sites are: Ss, *Sst*I; B, *Bam*HI; A, *Apa*I; H, *Hin*dIII; S, *Sal*I. All sites are not shown for B, A, H and S.

ORF at the α ATG. Thus, *brlA*β is predicted to encode a polypeptide that differs from BrlAα only by the addition of 23 N-terminal amino acids.

Construction of brlAα^- *and* β^- *mutants*

To assess the roles of *brlA*α and *brlA*β in regulation of development, we produced mutants that were blocked in either α or β transcription (Figure 4 and Materials and methods). Briefly, mutants shown in Figure 4A were constructed by gene replacement at the *brlA* locus. Mutants shown in Figure 4B were made by co-transformation of a strain (TRPΔ31N) completely deleted for *brlA*. Multiple, independent transformants were examined and exhibited identical phenotypes. Mutants shown in Figure 4C were constructed by targeted integration at *brlA* and *trpC*. In strain TRP44L (*brlA*$\alpha^+\beta^-$; Figure 4A) the *Apa*I–*Bam*HI −1290 to −404 fragment was replaced with a fragment containing the *A.nidulans argB* gene, thereby eliminating the β initiation sites and ~450 bp of 5′ and 3′ flanking sequence, but leaving 404 bp of normal sequence 5′ of the α initiation sites. In strain TRP62N (*brlA*$\alpha^-\beta^-$) the β intron was further deleted to eliminate the α transcription initiation sites. In strain TRPΔ31N (Δ*brlA*) a further deletion of a *Sma*I fragment from −299 to +1569 eliminated the entire *brlA*α,β coding region.

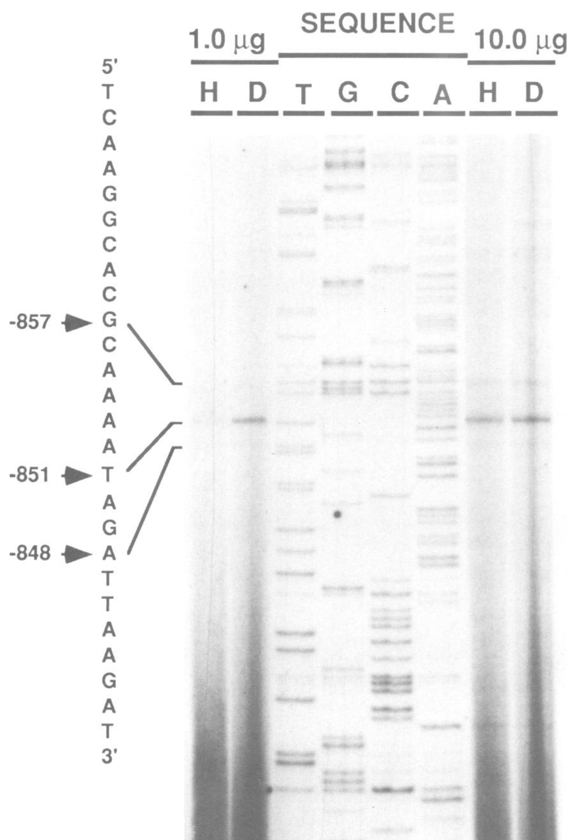


Fig. 2. Detection of an mRNA cap site upstream of *brlA*. Poly(A)$^+$ RNA was isolated from hyphae (H) or cells that had been induced to develop for 10 h (D). 1 or 10 µg of RNA were used as a template for extension with reverse transcriptase of a ^{32}P-labeled primer complementary to *brlA* sequences at −737 to −717 (see Figure 3C). A dideoxynucleotide chain termination sequence reaction generated with the same primer and an appropriate template is displayed such that the sequence of the mRNA-like strand is read 5′–3′ from top to bottom of the gel.

TRPΔ31N was used to construct the strains described in Figure 4B by transformation with either wild type (TRPΔ3125; *brlA*$\alpha^+\beta^+$) or mutant SstI (−3400 to +6500) fragments (see Materials and methods). In strain TRPΔ31342 (*brlA*2$\alpha^+\beta^-$) the β ORF ATG was converted to TAG so that α and β transcription should be unaffected, but translation should initiate at the α AUG with both mRNAs. Thus, some BrlAα polypeptide will be produced under *brlA*β control. In strain TRPΔ31396 (*brlA*$\alpha^-\beta^+$) the β ORF ATG was left intact but the β intron was deleted to eliminate α transcription. To determine whether the intronless version of β was functional, strain TRP2L14 was constructed containing a *brlA*$\alpha^+\beta^-$ fragment at the *brlA* locus and the intronless *brlA*$\alpha^-\beta^+$ fragment inserted *in trans* at the *trpC* locus (Figure 4C).

The effects of the introduced mutations on *brlA*α and *brlA*β transcription were assessed by RNA gel blot analysis (Figure 5). RNA was isolated from the parental strain (RMS011) and mutants at times after inducing conidiation, and duplicate gel blots were hybridized with α- and β-specific probes (Figure 3A). The α transcript accumulated in parallel and to similar levels in strains RMS011 ($\alpha^+\beta^+$) and TRP44L ($\alpha^+\beta^-$), whereas the β transcript was not detected in TRP44L. Neither the α nor β transcripts were detected in strains TRP62N or TRPΔ31N ($\alpha^-\beta^-$), whereas both were detected in strains TRPΔ3125 ($\alpha^+\beta^+$) and TRPΔ31342 ($2\alpha^+\beta^-$). Only the β transcript was detected in strain TRPΔ31396 ($\alpha^-\beta^+$). Both transcripts were detected in strain TRP2L14 ($\alpha^+\beta^-$ at *brlA*; $\alpha^-\beta^+$ at *trpC*). Thus, the transcription patterns exhibited by these strains were consistent with predictions based on the nature of the introduced mutations.

*Both brlA*α *and* β *are required for normal conidiophore development*

The effects of *brlA*α and *brlA*β mutations on conidiophore development were investigated by scanning electron microscopy of conidiophores (Figure 6). In all cases, several independent transformants were examined and found to produce consistent phenotypes. Strain RMS011 produced wild type conidiophores possessing normal vesicles, metulae, and phialides that produced chains of spores by successive budding (Figure 6A). Strain TRP44L ($\alpha^+\beta^-$), in contrast, produced conidiophores with abnormal sterigmata that failed to produce conidia (Figure 6B and C). A fraction of the sterigmata of nearly all primary conidiophores re-differentiated to form abnormal, secondary conidiophores. The two $\alpha^-\beta^-$ strains (TRP62N, TRPΔ31N) produced only conidiophore stalks that grew indeterminately and never produced sterigmata or conidia (Figure 6D and E). Strain TRPΔ31396 ($\alpha^-\beta^+$) formed nearly normal stalks and vesicles but abnormal sterigmata (Figure 6F and G). Some of the terminal cells produced single conidia, but conidial chains were never observed. Strains TRPΔ31342 ($2\alpha^+\beta^-$; Figure 6H and I) and TRP2L14 ($\alpha^+\beta^-$ at *brlA*, $\alpha^-\beta^+$ at *trpC*; Figure 6J) produced essentially wild type conidiophores. In addition, several transformants related to TRPΔ31396, but containing multiple integrated copies of an $\alpha^-\beta^+$ mutant construct ($n > 3$), also produced essentially wild type conidiophores (data not shown).

Discussion

The data presented in this paper permit three conclusions to be drawn concerning the *A.nidulans brlA* developmental

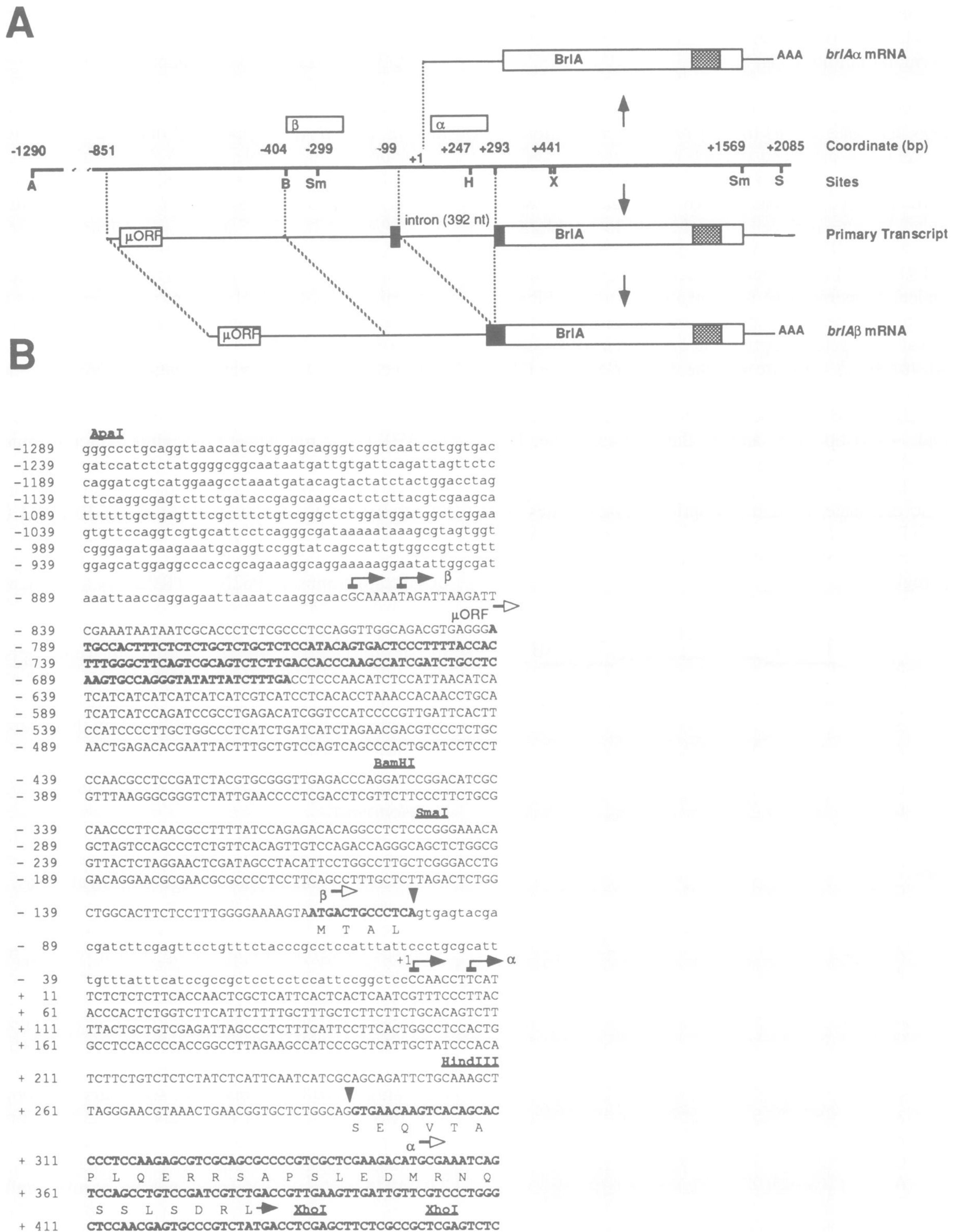
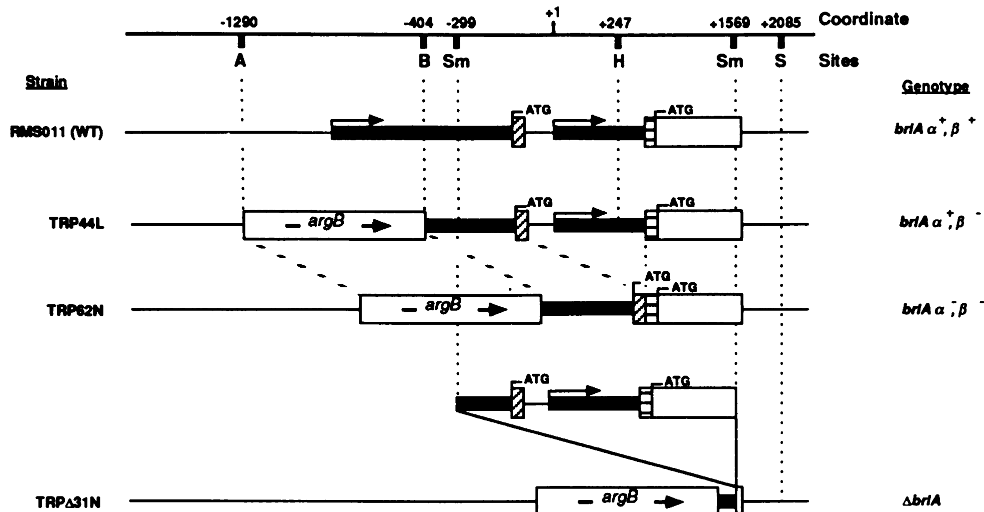


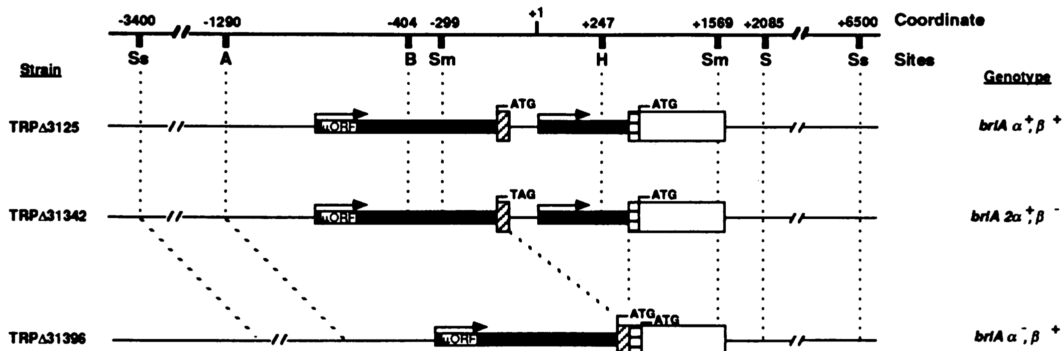
Fig. 3. Transcriptional organization of the *brlA* locus. (**A**) Summary of *brlAα* and *brlAβ* transcription units. The map was deduced from primer extension analysis (Figure 2) and sequence analysis of a genomic clone and 13 and four independent α and β cDNA clones, respectively (see Materials and methods). Splicing of the *brlAβ* primary transcript removes the *brlAα* 5′-untranslated sequences and adds 23 amino acids (■) to the common reading frame (□). The C_2H_2 zinc finger region (Adams *et al.*, 1988) is indicated (▩). A short ORF initiated by AUG (μORF) present near the 5′ end of *brlAβ* is shown. Positions of α- and β-specific hybridization probes are indicated. Restriction sites are: A, *Apa*I; B, *Bam*HI; H, *Hind* III; Sm, *Sma*I; X, *Xho*I; S, *Sal*I. (**B**) Sequence of the *brlA* 5′ region. Lowercase, uppercase and bold uppercase sequences represent non-transcribed, transcribed and translated sequences, respectively. Transcription initiation sites (↱), putative translation initiation sites (−▷), amino acid sequence (one letter code), and splice sites (▼) are indicated. Restriction sites shown in panel B are indicated.

regulatory locus. First, the locus consists of two overlapping transcription units, which we designate α and β, with α transcription initiating in β intronic sequences (Figure 3B and C). The *brlAβ* transcript contains two open reading frames beginning with AUG, an upstream μORF and a downstream reading frame identical to *brlAα* except for the

A



B



C

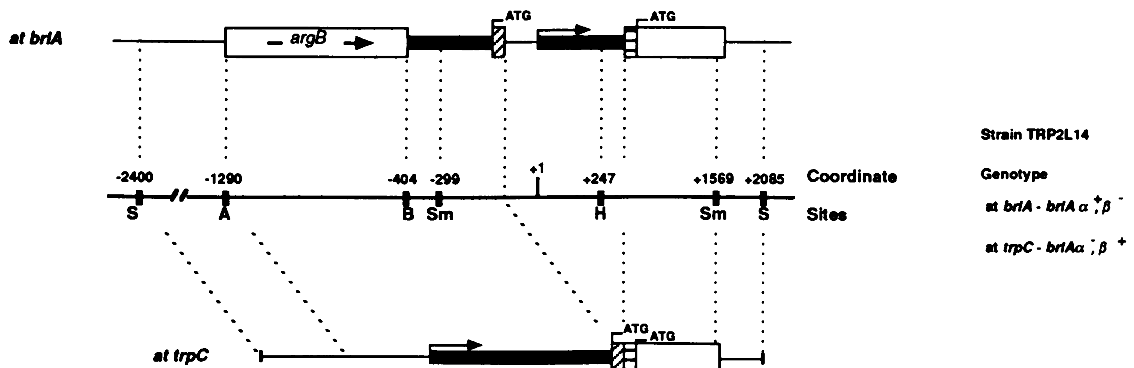


Fig. 4. Construction of *brlA* mutants. The following symbols are used: *argB* box, functional *argB* gene; closed boxes, 5'-untranslated region; open and dashed boxes, ORFs; arrows, major transcription start sites; ATG, translation initiation sites; TAG-mutated translation initiation site: A, *Apa*I; B, *Bam*HI; H, *Hin*dIII; S, *Sal*I; Sm, *Sma*I; and Ss, *Sst*I. (**A**) Inactivation of *brlA* transcription units. The structure of the wild type (WT) locus is shown for strain RMS011, which contains a deletion of *argB* on chromosome III. Strain TRP44L, lacking the *brlA*β transcription initiation sites, was made by replacing the *Apa*I–*Bam*HI fragment with *argB*. Strains TRP62N and TRPΔ31N are TRP44L derivatives lacking the *brlA*α and *brlA*β transcription initiation sites (constructed with a *brlA*β cDNA fragment) or the entire *brlA* locus (by deleting the *Sma*I fragment). (**B**) Reconstruction of the *brlA* locus. The constructs shown were made by transformation of strain TRPΔ31N (see panel A and Materials and methods). Strain TRPΔ3125 contains *brlA*α and β transcription and translation initiation sites. Strain TRPΔ31342 contains both transcription initiation sites, but the β ATG was converted to TAG by *in vitro* mutagenesis. Strain TRPΔ31396 contains α and β translation initiation sites but lacks the β intron and thus α transcription initiation sites. (**C**) *Trans*-complementation of a *brlA*$\alpha^+\beta^-$ allele with a *brlA*$\alpha^-\beta^+$ allele. A derivative of TRP44L (A) was transformed to introduce a processed version of *brlA*β at the *trpC* locus (see Materials and methods).

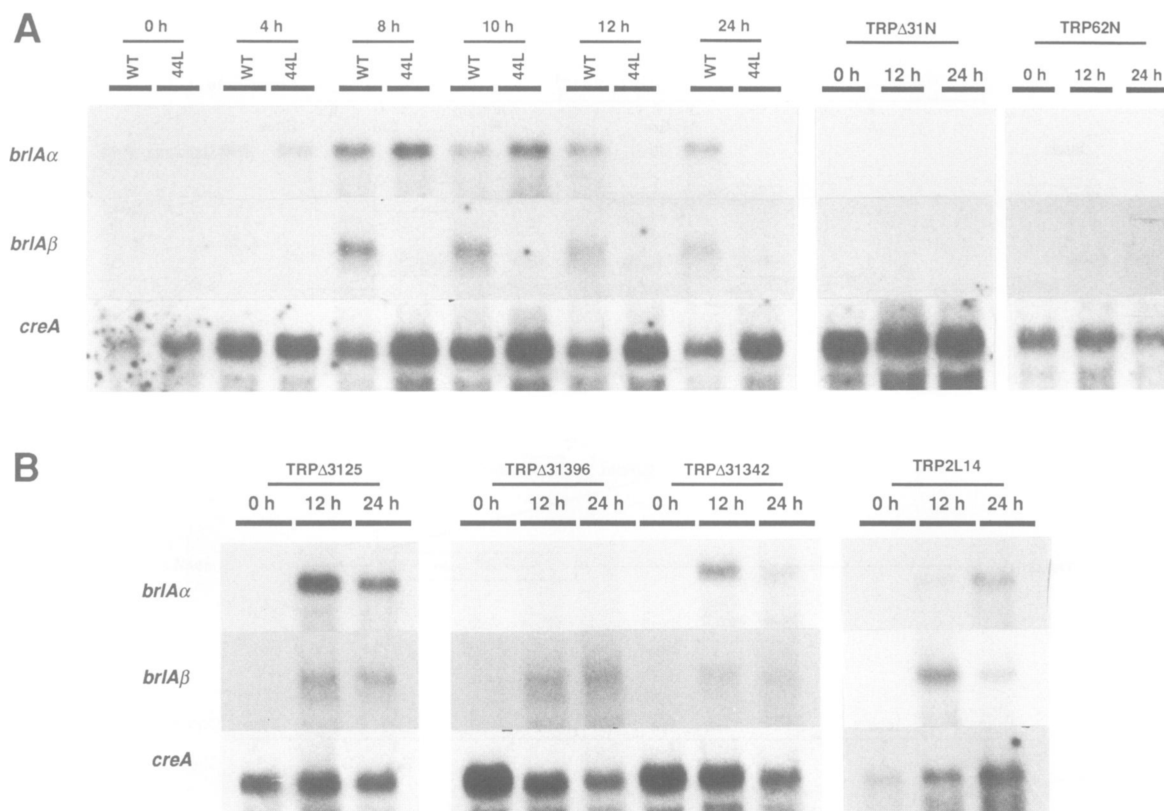


Fig. 5. Accumulation of *brlAα* and *brlAβ* mRNAs in mutants. RNA was isolated from hyphae (0 h) and at times following developmental induction. Radiolabeled α- and β-specific DNA fragments (see Figure 3A) were hybridized to gel blots. Blots were stripped and rehybridized with an *A.nidulans creA* internal *Eco*RV fragment as a loading control.

addition of 23 N-terminal amino acids. Searches of the GenBank and EMBL data bases failed to reveal significant similarities between the appended 23 amino acids and other polypeptides. We obtained no evidence for the existence of additional *brlA* transcript variants. The data presented by Han *et al.* (1993) indicate that the α, β and μORF reading frames are translated. The patterns of *brlAα* and β mRNA accumulation during development, as determined by RNA blot analysis, are similar and neither α nor β transcript was detected in RNA from hyphae. However, primer extension analysis permitted detection of *brlA* 5′ ends (Figure 2) in hyphal RNA, showing that the locus is transcribed during vegetative growth. The inability to detect *brlA* mRNAs by blot analysis was probably due to the low transcript levels and the relative insensitivity of the assay. Translation of the μORF fused to *lacZ* was detected in hyphae (Han *et al.*, 1993), confirming that β mRNA accumulates to some extent prior to developmental induction. It is likely that translation of the μORF prevents translation of the β ORF and is essential in preventing premature initiation of development (see Han *et al.*, 1993).

The existence of overlapping transcription units raised the question of whether the α and β gene products are individually required for conidiophore development. We therefore constructed mutant strains that were defective in transcription of α or β or both. As expected, elimination of both α and β expression resulted in the null *brlA* phenotype, production of conidiophore stalks that grow indeterminately. Elimination of β expression alone, however, led to formation of conidiophores that develop more extensively, but show distinct abnormalities. The *brlA*$\alpha^+\beta^-$ strain produced conidiophores that vesiculate and produce metulae that elongate but fail to bud and form phialides. Frequently the sterigmata develop into secondary, abnormal conidiophores, reminiscent of the phenotype of *medA* mutants (Clutterbuck, 1969). This result is of interest because *medA* appears to augment the activity of *brlA*; the asexual defects of *medA* mutants can be suppressed by supplying one or more extra copies of *brlA* (T.Toennis, K.Miller and B.Miller, in preparation). Thus, the phenotypes of both *brlA*$\alpha^+\beta^-$ and *medA* mutants may be explained by a reduction in *brlA* activity. The re-initiation of conidiophore development by some conidiophore sterigmata in these mutants suggests that full level expression of *brlA* is required both to terminate early developmental events and to initiate later developmental events.

A potential problem of interpretation of these experiments is that the deletion/insertion mutation introduced into strain TRP44L could have influenced expression of *brlAα*. This appears not to be the case because *brlAα* transcript accumulation was apparently unaffected (Figure 5) and an intronless version of *brlAβ* ($\alpha^-\beta^+$) fully complemented the mutation *in trans* (Figures 4C and 6J).

Elimination of *brlAα* expression resulted in developmental abnormalities that were distinct from those exhibited in the *brlA*$\alpha^+\beta^-$ strain. The *brlA*$\alpha^-\beta^+$ strain produced multiple layers of branched metulae and occasional phialide-like cells that formed single conidia, but not conidial chains. Conversions of sterigmata to conidiophores did not occur in this mutant. Thus, the second conclusion of this study is that *brlAα* and *brlAβ* are individually essential for formation of morphologically normal conidiophores, but expression of

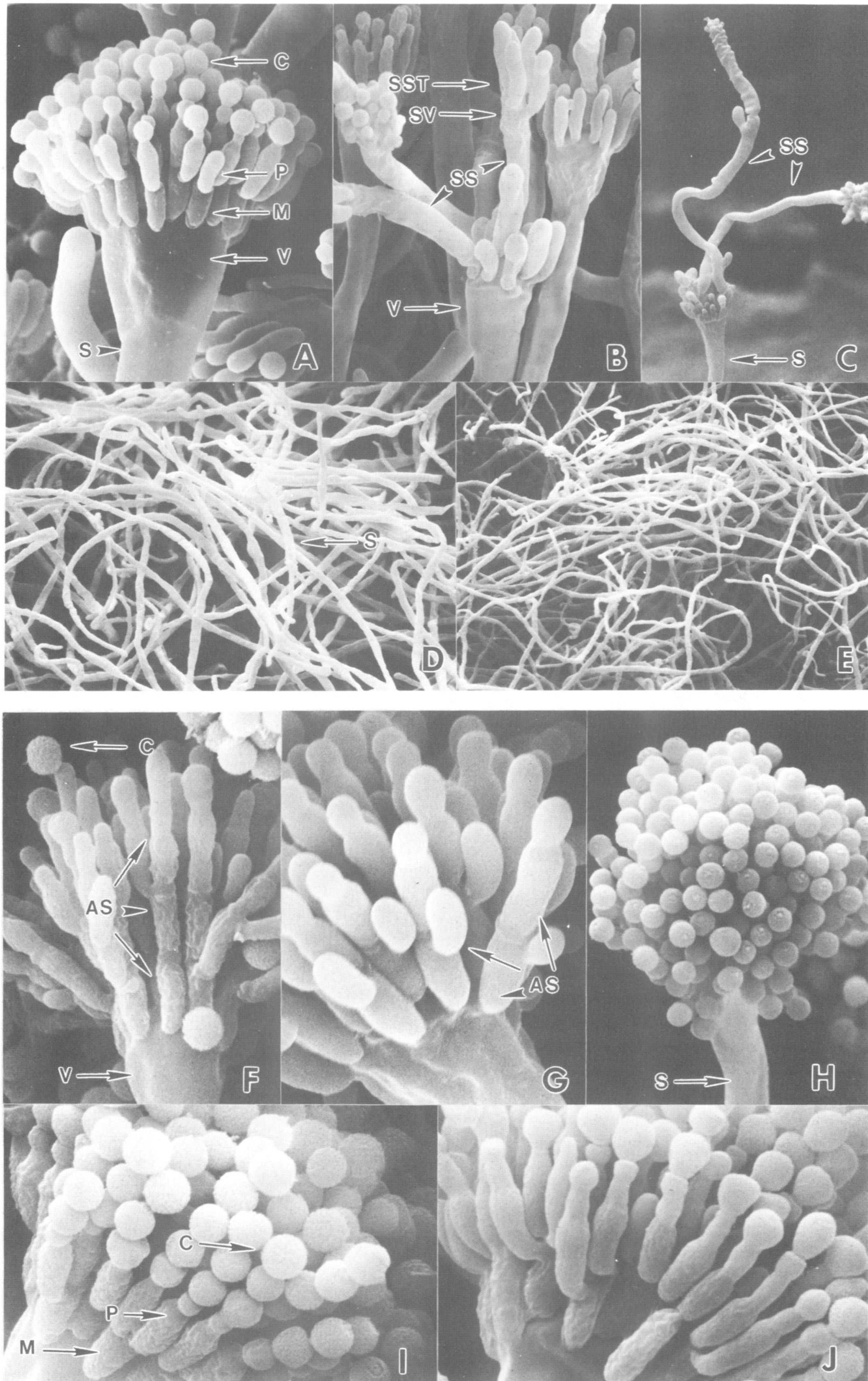


Fig. 6. Phenotypes of wild type and *brlA* mutant conidiophores. Samples were prepared for SEM as described by Mims *et al.* (1988). Representative conidiophores are shown. Abbreviations are: S, stalk; V, vesicle; M, metula; P, phialide; C, conidium; SS, secondary stalk; SV, secondary vesicle; SST, secondary sterigma; AS, abnormal sterigma. (**A**) strain RMS011 (*brlA*$\alpha^+\beta^+$); (**B** and **C**) strain TRP44L (*brlA*$\alpha^+\beta^-$); (**D**) strain TRP62N (*brlA*$\alpha^-\beta^-$); (**E**) strain TRPΔ31N (Δ*brlA*); (**F**) and (**G**) TRPΔ31396 (*brlA*$\alpha^-\beta^+$); (**H** and **I**) strain TRPΔ31342 (*brlA2*$\alpha^+\beta^-$); (**J**) strain TRP2L14 (*brlA*$\alpha^+\beta^-$ at *brlA*, *brlA*$\alpha^-\beta^+$ at *trpC*).

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

Strain	Genotype	Source
RMS011	*pabaA1*, *yA1*, *ΔargB* :: *trpC*, *trpC801*, *veA1*	Stringer *et al.* (1991)
FGSC237	*pabaA1*, *yA1*; *trpC801*, *veA1*	FGSC[b]
FGSC26	*biA1*; *veA1*	FGSC[b]
TRP44L	*pabaA1*, *yA1*; *ΔargB* :: *trpC*; *brlA44L*(α^+,β^-) :: *argB*, *trpC801*, *veA1*	This study[c]
TRP62N	*pabaA1*, *yA1*; *ΔargB* :: *trpC*; *brlA62N*($\alpha^-\beta^-$) :: *argB*, *trpC801*, *veA1*	This study[c]
TRPΔ31N	*pabaA1*, *yA1*; *ΔargB* :: *trpC*; *brlAΔ31* :: *argB*, *trpC801*, *veA1*	This study[c]
TRPΔ3125	*pabaA1*/*pabaA*$^{+a}$, *yA1*; *ΔargB* :: *trpC*; *brlAΔ31*, *brlA25* (α^+,β^+), *trpC801*, *veA1*	This study[d]
TRPΔ31342	*pabaA1*/*pabaA*$^+$, *yA1*; *ΔargB* :: *trpC*; *brlAΔ31*, *brlA342*($2\alpha^+,\beta^+$), *trpC801*, *veA1*	This study[d]
TRPΔ31396	*pabaA1*/*pabaA*$^+$, *yA1*; *ΔargB* :: *trpC*; *brlAΔ31*, *brlA396*(α^-,β^+), *trpC801*, *veA1*	This study[d]
TRP2L14	*pabaA1*, *yA1*; *ΔargB* :: *trpC*; *brlA44L* :: *argB*, *trpC* :: *brlA396*, *veA1*	This study[e]

[a]*pabaA*$^+$ integration pattern not known.
[b]Fungal Genetic Stock Center.
[c]Obtained by transformation of RMS011.
[d]Obtained by co-transformation of TRPΔ31N.
[e]Obtained by transformation of strain TRP44L, *pabaA1*,*trpC801*.

either permits much more extensive development than in *brlA* null strains. The phenotypes of the individual mutants are similar to those of *medA* mutants and previously identified hypomorphic *brlA* mutants (Clutterbuck, 1977). Fine structure mapping of the hypomorphic *brlA* alleles indicates that the mutations occurred within or near the regions of the gene encoding the zinc fingers (Adams *et al.*, 1988; Clutterbuck *et al.*, 1992) and are thus expected to affect both *brlA*α and β. These mutations differentially affect expression patterns of genes encoding development-specific enzymes (Birse and Clutterbuck, 1991) and anonymous developmentally regulated genes (unpublished results), consistent with changes in target site binding affinities. Thus, one possibility is that increasing levels of *brlA* products are responsible for sequentially filling target sites and activating gene expression according to a hierarchy established by differing target site binding affinities. In this model, alterations either in the absolute concentrations of wild type BrlA protein or in the affinity of BrlA for its target sites would be expected to produce similar effects.

If the developmental defects of *brlA*$\alpha^+\beta^-$ and *brlA*$\alpha^-\beta^+$ mutants are due to reduced levels of functionally similar proteins, then it might be possible to remediate the defects by providing multiple copies of either *brlA*$\alpha^+\beta^-$ or *brlA*$\alpha^-\beta^+$ allele, and this expectation was met. A strain (TRPΔ31342; Figure 4B) containing a copy of the *brlA* locus in which the *brlA*β ATG was converted to TAG produces both α and β mRNAs (Figure 3) and essentially wild type conidiophores (Figure 6H and I). Presumably translation of β mRNA begins at the α AUG and the increased level of α product compensates for the absence of the β product. Similarly, integration of multiple (≥ 3) copies of an intronless version of *brlA*β (*brlA*$\alpha^-\beta^+$) in a *brlA*$^-$ strain is sufficient for normal development, implying that the α product is dispensable. Thus, the third conclusion of this study is that although *brlA*α and β are individually essential for normal development, one can substitute for the other; the products appear to be functionally redundant. In the case of TRPΔ31342 (Figure 4B), alteration of the *brlA*β ATG to TAG did not affect patterns of mRNA accumulation so that *brlA*α product was expected to be produced under direction of the *brlA*β regulatory system. Thus, the requirement for transcription of *brlA*α and β may relate to the developmental timing of gene expression. On the other hand, strains containing multiple copies of a *brlA*$\alpha^-\beta^+$ construct also formed wild type conidiophores, even though no *brlA*α mRNA was produced. This result indicates that generalized over-expression of *brlA* can compensate for loss of one transcript class. Nevertheless, the α and β genes are not equivalent, because $\alpha^+\beta^-$ and $\alpha^-\beta^+$ mutants have clearly distinguishable phenotypes. These phenotypic differences could be due to subtle differences in the activities of the α and β polypeptides or differences in the regulatory properties of the genes, or both. In an accompanying paper, Han *et al.* (1993) present evidence indicating that *brlA*α and β expression is differentially regulated, with initial regulation of β occurring at the translational level.

Materials and methods

A.nidulans procedures

Standard *A.nidulans* genetic techniques (Pontecorvo *et al.*, 1953; Clutterbuck, 1974; Käfer, 1977) and transformation procedures and approaches (Yelton *et al.*, 1984; Timberlake, 1991a) were employed. Genotypes of strains used in this study are given in Table I. Synchronous sporulating cultures were prepared according to Law and Timberlake (1980). DNA was isolated from frozen or lyophilized cells as described by Yelton *et al.* (1984). RNA was isolated according to Timberlake (1986).

Molecular techniques

Primer extension reactions utilized poly(A)+ RNA from vegetative cultures and cultures that had been induced to sporulate for 10 h, after which time conidiophore vesicles had formed. A molar excess of 5′ end-labeled primer (5′-GAGACTGCGACTGAAGCCCA) was added to reaction mixtures containing 1 or 10 μg of RNA and extended with AMV reverse transcriptase. Alkaline-treated extension products were analyzed on 6% acrylamide sequencing gels.

Total RNA was fractionated by electrophoresis in formaldehyde–agarose gels, transferred to Hybond-N (Amersham) and hybridized with ^{32}P-labeled *brlA*α- or *brlA*β-specific DNA probes. The probes were synthesized by PCR utilizing pRP05 (see below) as template and primer pairs 5′-GGGATAG-CAATGAGCGGGATG/5′-AATCGTTTCCCTTACACCA (*brlA*α-specific) and 5′-GAGCAAGGCCAGGAAT/5′-GGATCCGACATCGCGTTTAA-GGC (*brlA*β-specific) and labeled by primer extension with random hexamers.

Construction of brlA mutant strains

A 10.5 kb *Sac*I fragment containing a wild type copy of *brlA* flanked by 3.5 kb of 5′ and 4.5 kb of 3′ sequence was subcloned from cosmid W6E8 (Brody *et al.*, 1991) into Bluescript KS(−) to produce pRP05. An upstream, 2.5 kb *Bam*HI fragment (Figure 1) was subcloned to produce pRP07. An upstream 0.86 kb *Bam*HI–*Apa*I fragment (Figure 1) was subcloned to produce pRP11. pRP07 was digested with *Apa*I, an *Apa*I–*Eco*RI adaptor (5′-GAATTCGGCC) was added and an *Eco*RI fragment from pDC1 (Aramayo *et al.*, 1989) containing the *argB*$^+$ gene was cloned into the *Eco*RI site to produce pRP15. A *Bam*HI fragment containing *brlA* sequences ligated to *argB* was isolated from pRP15 and inserted into the *Bam*HI site of pBS25 containing a *brlA* *Bam*HI–*Sal*I fragment (Adams *et al.*, 1988; see Figure 1) to produce pRP17 (*brlA*$\alpha^+\beta^-$). pRP16 is a derivative of pBS25 in which a genomic *Sma*I fragment encompassing the *brlA*β intron was replaced by the equivalent *Sma*I fragment from a cDNA clone (see below). pRP16 was used to construct pRP18 (*brlA*$\alpha^+\beta^-$; Δ intron)

(analogous to pRP17) by adding the pRP15 *Bam*HI fragment. pRP19 (Δ*brlA*) was created by deleting the *Sma*I fragment from pRP17. pRP17, 18 and 19 were used to transform *A.nidulans* RMS011 (Table I) and transformants were selected by Southern blot analysis. Selected strains were crossed with FGSC237 (*brlA*$^+$) and progeny were checked for appropriate segregation of mutant versus wild type conidiophore morphologies.

The 10.5 kb *brlA Sac*I fragment was cloned into a Bluescript KS(−) derivative in which sequences between the *Sma*I and *Eco*RV sites were deleted to produce pRP25. Mutations were introduced into the *brlA Sma*I fragment (Figure 3B) cloned in Bluescript and the modified fragments were exchanged into pRP25. In pRP34, the putative *brlA*β initiation codon was converted to TAG by *in vitro* mutagenesis (Kunkel, 1985). In pRP39, the *brlA*β intron was removed by exchange with the *Sma*I fragment from a cDNA clone.

A.nidulans strain TRPΔ31N was derived by transformation with pRP19 and used as recipient for co-transformation with pRP25, 34 and 39 and a *pabaA*$^+$ cosmid. Transformants with altered developmental phenotypes were checked by Southern blot analysis for integration of single plasmid copies. Three transformants were selected [TRPΔ3125 (pRP25), TRPΔ31342 (pRP34) and TRPΔ31396 (pRP39)] and characterized further by blot and segregation analysis. Each was determined to contain a single plasmid copy integrated at a heterologous chromosomal site. Additional, independent transformants containing single copies of pRP25 (*brlA*$^+$) all produced wild type conidiophores, indicating that the *brlA Sac*I fragment was sufficient to complement the *brlA* deletion in TRPΔ31N *in trans* from many chromosomal sites.

A.nidulans strain TRP2L14 (*brlA*$\alpha^+\beta^-$ at *brlA*, *brlA*$\alpha^-\beta^+$ at *trpC*) was constructed as follows. The 4.5 kb *Sal*I fragment from pRP39 was ligated into an internal *Sal*I site of *trpC* in pTA113 (from T.H.Adams) to produce pRP27. *A.nidulans* strain TRP44L (*trpC*801, *brlA*$\alpha^+\beta^-$; see Figure 4A) was transformed with circular pRP27 with selection for tryptophan independence. This requires homologous integration of the plasmid at the *trpC* locus to generate a *trpC*$^+$ allele. Transformants were characterized by Southern blot analysis to confirm that the expected integration event had occurred and a representative strain (TRP2L14) was chosen for further characterization. Complementation was further confirmed in progeny from a cross between TRP44L and TRPΔ31396 (results not shown).

Isolation of cDNA clones

brlA cDNA clones were isolated from a λ-ZAP library made from poly(A)$^+$ RNA from cultures that had been induced to sporulate for 24 h (R.Aramayo and W.E.Timberlake, unpublished results). Seventeen clones were characterized by restriction mapping and sequencing of the 5′ and 3′ ends of the inserts. Two independent *brlA*β cDNAs were sequenced in their entirety for comparison with genomic sequence. The genomic sequence upstream of the −404 *Bam*HI site (Figure 3C) was determined from both strands. All DNA sequencing was done by the dideoxynucleotide chain termination procedure (Sanger *et al.*, 1977).

Electron microscopy

Cultures were prepared for SEM according to Sewall *et al.* (1990) and examined in a Phillips 505 SEM at 15 keV.

Acknowledgements

We thank Bruce Miller, Karen Miller, Tom Adams and John Clutterbuck for communicating results prior to publication; Alex Andrianopoulos and Yun Chang for critical reviews of the manuscript; our other colleagues in the lab for their many useful suggestions; and Kathy Vinson for expert assistance in preparation of the manuscript. We are especially grateful to Charles Mims and Beth Richardson for their assistance with SEM. This work was supported by National Institutes of Health grant GM37886 to W.E.T. R.A.P. was in part supported by grant 1334-89 (FAPESP, Brazil) and CEBIQ/FTI (Brazil).

References

Adams,T.H. and Timberlake,W.E. (1990a) *Mol. Cell. Biol.*, **10**, 4912–4919.
Adams,T.H. and Timberlake,W.E. (1990b) *Proc. Natl Acad. Sci. USA*, **87**, 5405–5409.
Adams,T.H., Boylan,M.T. and Timberlake,W.E. (1988) *Cell*, **54**, 353–362.
Adams,T.H., Deising,H. and Timberlake,W.E. (1990) *Mol. Cell. Biol.*, **1**, 1815–1817.
Aramayo,R. and Timberlake,W.E. (1990) *Nucleic Acids Res.*, **18**, 3415.
Aramayo,R., Adams,T.H. and Timberlake,W.E. (1989) *Genetics*, **122**, 65–71.
Birse,C.E. and Clutterbuck,A.J. (1990) *J. Gen. Microbiol.*, **136**, 1725–1730.
Birse,C.E. and Clutterbuck,A.J. (1991) *Gene*, **98**, 69–76.
Boylan,M.T., Mirabito,P.M., Willett,C.E., Zimmermann,C.R. and Timberlake,W.E. (1987) *Mol. Cell. Biol.*, **7**, 3113–3118.
Brody,H., Griffith,J., Cuticchia,A.J., Arnold,J. and Timberlake,W.E. (1991) *Nucleic Acids Res.*, **19**, 3105–3109.
Chang,Y.C. and Timberlake,W.E. (1992) *Genetics*, **133**, 29–38.
Clutterbuck,A.J. (1969) *Genetics*, **63**, 317–327.
Clutterbuck,A.J. (1974) In King,R.C. (ed.), *Handbook of Genetics*. Plenum Press, New York, pp. 447–510.
Clutterbuck,A.J. (1977) In Pateman,J.A. and Smith,J.E. (eds), *Genetics and Physiology of Aspergillus*. Academic Press, New York, pp. 305–317.
Clutterbuck,A.J. (1990) *J. Gen. Microbiol.*, **136**, 1731–1738.
Clutterbuck,A.J., Stark,M.S. and Gupta,G. (1992) *Mol. Gen. Genet.*, **231**, 212–216.
Han,S.T., Navarro,J., Greve,R.A. and Adams,T.H. (1993) *EMBO J.*, **12**, 000–000.
Johnstone,I.L., Hughes,S.G. and Clutterbuck,A.J. (1985) *EMBO J.*, **4**, 1307–1311.
Käfer,E. (1977) *Adv. Genet.*, **19**, 33–131.
Kunkel,T.A. (1985) *Proc. Natl Acad. Sci. USA*, **82**, 488–492.
Law,D.J. and Timberlake,W.E. (1980) *J. Bacteriol.*, **144**, 509–517.
Marshall,M.A. and Timberlake,W.E. (1991) *Mol. Cell. Biol.*, **11**, 55–62.
Martinelli,S.D. (1979) *J. Gen. Microbiol.*, **11**, 277–287.
Mayorga,M.E. and Timberlake,W.E. (1990) *Genetics*, **126**, 73–79.
Miller,K.Y., Toennis,T.M., Adams,T.H. and Miller,B.L. (1991) *Mol. Gen. Genet.*, **227**, 285–292.
Miller,K.Y., Wu,J., and Miller,B.L. (1992) *Genes Dev.*, **6**, 1770–1782.
Mims,C.W., Richardson,E.A. and Timberlake,W.E. (1988) *Planta*, **144**, 132–141.
Mirabito,P.M., Adams,T.H. and Timberlake,W.E. (1989) *Cell*, **57**, 859–868.
Oliver,P.T.P. (1972) *J. Gen. Microbiol.*, **73**, 45–54.
O'Hara,E.B. and Timberlake,W.E. (1989) *Genetics*, **121**, 249–254.
Pontecorvo,G., Roper,J.A.,Hemmons,L.M., MacDonald,K.D. and Bufton,A.W.J. (1953) *Adv. Genet.*, **5**, 141–238.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl Acad. Sci. USA*, **74**, 5463–5467.
Sewall,T., Mims,C.W. and Timberlake,W.E. (1990) *Dev. Biol.*, **138**, 499–508.
Stringer,M.A., Dean,R.A., Sewall,T.C. and Timberlake,W.E. (1991) *Genes Dev.*, **5**, 1161–1171.
Tilburn,J., Roussel,R. and Scazzocchio,C. (1990) *Genetics*, **126**, 81–90.
Timberlake,W.E. (1980) *Dev. Biol.*, **78**, 497–510.
Timberlake,W.E. (1986) In Timberlake,W.E. (ed.), *Biology and Molecular Biology of Plant–Pathogen Interactions*. Springer-Verlag, Berlin, H1, NATO ASI, pp. 343–357.
Timberlake,W.E. (1990) *Annu. Rev. Genet.*, **24**, 5–36.
Timberlake,W.E. (1991a) In Bennett,J.W. and Lasure,L. (eds), *More Gene Manipulations in Fungi*. Academic Press, Orlando, pp. 51–85.
Timberlake,W.E. (1991b) *Curr. Opin. Genet. Dev.*, **1**, 351–357.
Yelton,M.M., Hamer,J.E. and Timberlake,W.E. (1984) *Proc. Natl Acad. Sci. USA*, **81**, 1470–1474.
Zimmermann,C.R., Orr,W.C., Leclerc,R.F., Barnard,E.C. and Timberlake,W.E. (1980) *Cell*, **21**, 709–785.

Received on December 17, 1992; revised on February 11, 1993