StuA is required for cell pattern formation in *Aspergillus*

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The stunted (stuA) gene product is required for the orderly differentiation and spatial organization of cell types of the *Aspergillus nidulans* conidiophore. Expression of the stuA gene is complex. Two transcripts, stuAα and stuAβ, are initiated from separate promoters. Transcription of both RNAs increases ~50-fold during the establishment of developmental competence. Induction-dependent transcriptional and post-transcriptional regulatory mechanisms further enhance expression 15-fold. Consistent with the latter observation, both transcripts have structural features characteristic of RNAs subject to translational control. Conidiophore morphogenesis requires regulatory interactions between the products of the stuA, bristle (brlA), and abacus (abaA) genes. Enhanced stunted expression is cell type specific and dependent on a functional BrlA protein. StuA affects the spatial localization of AbaA by acting directly, or indirectly, to repress abaA expression.

[Key Words: *Aspergillus nidulans*; conidiation; spatial regulation; pattern formation]

Received January 1, 1992; revised version accepted July 1, 1992.

The asexual reproductive cycle (conidiation) of the filamentous ascomycete *Aspergillus nidulans* is characterized by a coordinated temporal and spatial pattern of cell differentiation (for review, see Miller 1990; Timberlake 1990). The mature conidiophore is a multicellular structure consisting of foot cell, aerial hypha, terminal vesicle, and single tiers of metulae and phialides. The latter two cell types are uninucleate cells with the phialide undergoing repeated mitotic divisions to yield chains of conidia [Mims et al. 1988; Sewall et al. 1990]. The genetic hierarchy controlling conidiation can be divided into preinduction and postinduction loci (Clutterbuck 1969, 1977; Axelrod et al. 1973; Pastushok and Axelrod 1976; Champe et al. 1981; Yager et al. 1982; Butnik et al. 1984). After conidium germination, preinduction loci determine biochemical events that allow the vegetative mycelia to acquire reproductive competence. Phenomena associated with competence can be experimentally separated from those of induction. Mycelia maintained as submerged cultures remain in a competent but undifferentiated state. Synchronous induction, by exposure to an air interface, results in sequential activation of ~1200 stage-specific RNAs [Timberlake 1980].

Correct cell pattern formation requires the functions of at least five genes: abacus (abaA); bristle (brlA); medusa (medA); stunted (stuA); and wet-white conidia (wetA) [Clutterbuck 1969, 1977; Martinelli and Clutterbuck 1971]. Epistatic relationships suggest that these postinduction loci define two separate genetic pathways with some interaction, or overlap, in function [Martinelli 1979]. The BRISTLE group of genes [brlA → abaA → wetA] define a linear path of differentiation that culminates in the formation of the conidium. Null mutations in members of this group are aconidial, with development being blocked at conidiophore vesicle, phialide, and mature conidium formation, respectively [Clutterbuck 1969]. Molecular analysis has demonstrated a linearly dependent regulatory relationship for members of the BRISTLE group [Boylan et al. 1987; Adams et al. 1988; Mirabito et al. 1989; Marshall and Timberlake 1991]. Ectopic expression of the transcriptional activator BrlA resulted in the cessation of vegetative growth. Hyphal tips acquired phialide-like features and produced conidia [Adams et al. 1988]. Therefore, BrlA expression is necessary and sufficient to direct the linear transition from vegetative hyphae to conidia. Normal patterns of cell differentiation, however, require additional regulatory phenomena [Clutterbuck 1977; Martinelli 1979].

Genes of the STUNTED group [stuAA → medA] have been classified as developmental modifiers. They affect patterns of cell differentiation and the spatial organization of the conidiophore [Clutterbuck 1969, Martinelli 1979, Miller et al. 1991]. stuA null mutants have greatly shortened conidiophores and lack normal metulae and phialides. Abnormal conidia frequently differentiate directly from buds formed on the conidiophore vesicle. medA mutations result in reiterated metulae and secondary conidiophore structures. Both stuA and medA mutants are also self-sterile, failing to form sexual reproductive structures (cleistothecia). Previously, we have described the physical isolation of the stuA gene [Miller et al. 1991] and have recently cloned the medA gene (K. Miller and B. Miller, in prep.). Although StuA is not required for vegetative growth, we found that stuA was transcriptionally activated during the acquisition of developmental competence. Transcriptional activation
was induction independent and under the control of unknown genetic loci (Miller et al. 1991).

Here, we report the nucleotide sequence and transcriptional organization of the \textit{stuA} gene. Structural features of the two alternate \textit{stuA} mRNAs and the temporal and spatial expression of a \textit{stuA[p/l]}::\textit{lacz} reporter gene suggest that induction-dependent, cell-type-specific expression is subject to both transcriptional and translational regulatory mechanisms that require a functional BrlA protein. Furthermore, we have observed that the ability of BrlA to activate transcription of \textit{abaA} is apparently modulated by the transient and spatially restricted repression of \textit{abaA} expression by StuA. Therefore, conidiospore morphogenesis in \textit{A. nidulans} requires complex interactions between functions of AbaA, BrlA, and StuA.

\textbf{Results}

Two \textit{stuA} mRNAs are produced by the use of alternate promoters and RNA splicing patterns

The structural features of the \textit{stuA} gene are summarized in Figure 1 and in the DNA sequence shown in Figure 2. Transcriptional organization was determined by S1 nuclease protection, primer extension, and DNA sequence analysis of PCR-generated cDNA clones, as described in Materials and methods. Two mRNAs, designated \textit{stuAa} and \textit{stub}, are similar in size (3335 and 3470 nucleotides, respectively) but are generated by alternate processing of RNAs initiated from different transcriptional start sites. Three introns common to both \textit{stuAa} and \textit{stub} transcripts are small (45, 47, and 46 nucleotides) and typical of fungal genes. The first intron of the \textit{stub} transcript, however, is 497 nucleotides in size and is the largest intron that has been reported for a fungal gene. Transcription of \textit{stuAa} is initiated 635 bp downstream of the \textit{stuA} RNA start sites, within DNA sequences specifying this large intron.

\textbf{Figure 1.} Structure of the \textit{stuA} gene. Schematic drawing of the transcriptional unit and DNA sequences required for complementation of both asexual and sexual reproductive deficiencies of a \textit{stuA} null mutant. Arrows indicate transcriptional initiation sites and the direction of transcription of the \textit{a} and \textit{b} transcripts. Stippled boxes represent transcribed, nontranslated mRNA sequences, solid boxes indicate the major \textit{stuA} ORF that begins with an AUG codon. Mini-ORFs found within nontranslated leader sequences are indicated by solid triangles and are designated mORF 1–3 (from left to right). mORF 1 is unique to the \textit{a} transcript. A caret (\textasciicircum) indicates the position of an additional AUG codon found in the nontranslated leader. [H] \textit{HindIII}, [K] KpnI, [Nd] NdeI, [P] PstI, [R1] EcoRI, [RV] EcoRV, [S] SalI, [X] Xhol, [Xb] XbaI.

\textit{A. nidulans} \textit{stuA} gene

\textit{stuAa} and \textit{stub} mRNAs possess long nontranslated leaders with multiple mini-ORFs

The first AUG codon of the \textit{stuA} open reading frame (ORF) is located 1088 nucleotides downstream of the \textit{a} cap site and 1221 nucleotides downstream of the \textit{b} cap site (Figs. 1 and 2). These leader sequences are the longest that have been described for the filamentous fungi. Both \textit{a} and \textit{b} leaders have two common AUG-initiated mini-ORFs (mORFs 2 and 3) of 8 and 3 amino acids, respectively. There is also a common AUG codon followed immediately by a stop codon. A third mini-ORF (mORF 1) of 24 amino acids is found at the extreme 5' end of the \textit{a} leader, in sequences unique to this transcript (Figs. 1 and 2). The stop codon of mORF 1 is also the 3' splice junction of the large \textit{stub} intron. Therefore, processing of the \textit{b} precursor RNA removes mORF 1. Long nontranslated leaders containing mini-ORFs are characteristic of mRNAs from a number of genes in which expression has been demonstrated to be under translational control (Hinnebusch 1984; Thireos et al. 1984; Werner et al. 1987; Garret et al. 1989).

\textit{stuA} gene encodes a 63.5-kD polypeptide

Both the \textit{stuAa} and \textit{stub} mRNAs potentially encode the same 590-amino-acid, 63.5-kD polypeptide. \textit{StuA} has a predicted pi of 8.48 and consists of 12% serine, 11% proline, and 11% basic amino acids (Fig. 2). The majority of the basic residues are found in two domains. An amino-terminal domain (residues 148–215) consists of 24% basic amino acids and is largely defined by exon 3 of the \textit{a} RNA (exon 4 of the \textit{b} transcript). A carboxy-terminal domain (residues 545–570) contains two clusters of amino acids, KRKR and KRRK, separated by an 18-residue spacer. This 26-amino-acid domain closely resembles the bipartite nuclear targeting signal described for mammalian nucleoplasm and a number of mammalian transcription factors (Robbins et al. 1991). Secondary structure predictions (Garnier et al. 1978; Gascuel and Golmard 1988) suggest that StuA consists almost entirely of extended or random coil conformation. The limited \alpha-helical structure consists of two helices (residues 134–150 and 205–217) that lie on either side of the amino-terminal basic domain and a third helix (residues 180–187) that lies within this domain. There are two Pro/Gln-rich regions that could function as transcriptional activation domains (Mitchell and Tjian 1989). One region (residues 39–129) is 23% proline, 10% glutamine, and 17.5% serine, whereas the second (residues 253–395) is 20% proline, 12% glutamine, and 16% serine. A search of the current nucleotide and protein data bases did not reveal any gene products with significant sequence similarities to StuA.

Major changes in \textit{stuA} RNA abundance are associated with acquisition of developmental competence rather than induction

The \textit{stuAa} and \textit{stub} transcripts are barely detectable in mature conidia or in mycelia that are not developmen-
tally competent. A 50-fold increase in the relative abundance of both transcripts, however, coincides with the establishment of developmental competence (Miller et al. 1991). After induction, the relative abundance of the α and β transcripts increased approximately fivefold and twofold, respectively (Fig. 3A). These increases occurred 5–7.5 hr postinduction, during conidiophore vesicle, metulae, and phialide differentiation. Semiquantitative
A. nidulans stuA gene

2615

ATC CTC AAG AGT GAA AAG GTC CAG AAT GTG GAA ATT GGC CCG ATG CAT CTG AAG GGC GGT TC gtaagcttcctccc
2693
cctgatttttccataaatatcgtaaccaagtag A GTT CCC TTC CAG GCG GCC GGT CCA GAA TTC GCC AAC AGG GAA GAG YAC
2775

GAT CTT TTA TAC CCT TGG TTC GAA CAA GAC ATC AGC AAC ATG CTG TAC CAG CCG GCC AAC AGT CAG AGG AAC
2850

ATG ACA GTG CCA TGA AGT CCA GGT GCC CCC CAA CCC GCT GGC GGT CCA GAC ATG CCA AAC ACC CAG GCA CAG GCC
2925

AAC CTA CAC CAG CTC CTT CCT AAG CCA CAG GGA GCC AGT GCC TCG AAG CTC CAA CAG CAG CAG CAG CAG CAG CAG
3000

GCC GCA CAT AGC TTT CCT AGC CCA CTT GCC AGT GCC TCG AGC GTC CAG CAA CAG CAG CAG CAG CAG CAG CAG CAG
3075

AGT TTG AGT AAT GCA CGA TCA AT(: CCC ACA ACC CCT GCT ACG ACG CCA CCT GCC TCG AGC GTT CCC AAC ACC CAG CTC
3150

GCC CGC CAT ACG TTT CCT AGC CCA CTT GCC AGT GCC TCG AGC GTC CAG CAA CAG CAG CAG CAG CAG CAG CAG CAG
3225

ACC GCT GCA AAG ACC GAG ACC CAG GAG CAG GAG TAT GCC GAG CAG AGT GCC AAG GAG ACC CAG TAT GCT GAG ATG
3300

TCC TAC CAG CCT GCC ACC AAG AGT CAG GCC AGC GCG AGC GTC TAT GGC CAC TCT ATG CCA ACG AGC TCG GAT GAT TAC
3375

ACC CCT CCT GCC CCC GCC GCT GGC AGT AGT CTG TAC AAC ATT GTC ATG GAC AGC GGA AGC GTA GCC AAC CAA ACC GTC
3450

TVA KAP TRA PPA AA AAS L SLY N T M A G T A G D Y T T G R G
3525

TGG TTA TAA CAA TCT GCC CCA GCT GCG ACC GAG ACG TCA ATG GCC AGC TCG ACC AGC AGC AGC AGC AGC AGC AGC AGC
3600

SYP Q Q N S G M T P R T S N T P A Q W A P
3675

ACC CCT CCT GCC CCC GCC GCT GGC AGT AGT CTG TAC AAC ATT GTC ATG GAC AGC GGA AGC GTA GCC AAC CAA ACC GTC
3750

TVA KAP TRA PPA AA AAS L SLY N T M A G T A G D Y T T G R G
3825

CGC CGG ACC TTT ACA GAC ACC GCG CCT GGT GGT GGT GGT CCA GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC
3900

gttcgtctcgctgtaatgtgagacagactcataatcaaaaaaagaaaaaaaatgtgctgccgccccagaggacagacagagccggggg
3993

Figure 2. Nucleotide sequence of the stuA gene. Numbers at left of each line indicate nucleotide positions; numbers at right indicate amino acid residues. Transcription initiation sites for stuA and stuAβ are marked > and *, respectively. Exon sequences of the β transcript are uppercase letters and introns are lowercase. [Note that the first 100 nucleotides of the α transcript are also in lowercase letters, indicating that initiation is within the first intron of stuAβ.] Mini-ORFs are underlined. Potential TATA and CAAT sequences are indicated by dots above sequences. A potential nuclear targeting sequence is double-underlined. The 3’ ends of both α and β transcripts are identical and are indicated by #. The GenBank accession number for this sequence is M83569.
analysis with scanning densitometry of X-ray films from either Northern blot or primer extension experiments yielded similar results. The constitutively expressed argB gene was used as an internal control to standardize RNA sample loadings and for direct comparison of stuA signals between lanes [see Materials and methods].

Spatially localized changes in StuA expression are dependent on both induction and BrlA

Strain UI38.1 is a transformant that carries a single copy of the stuA[p/l]/::lacZ translational fusion gene integrated at the trpC locus on chromosome VIII. All genetic loci affecting conidiphore and conidia morphogenesis (including stuA) in this strain are wild type. This strain expressed very low levels of StuA:α-Gal activity (1-2 units sp. act.) prior to developmental competence. We use the abbreviations StuA:α-Gal, AbaA:β-Gal, and BrlA:β-Gal to distinguish β-galactosidase expressed from the stuA[p/l]/::lacZ, abaA[p/l]/::lacZ, and brlA[p/l]/::lacZ reporter genes. Expression increased ~50-fold [to 120 units sp. act.] in developmentally competent, but undifferentiated, vegetative hyphae (Fig. 4). StuA is required throughout conidiophore morphogenesis but not for vegetative growth. Therefore, it was possible that StuA:α-Gal expressed in uninduced mycelia was localized in specific cell types, such as foot cell initials or cleistothecial initials. In situ localization indicated that this was not the case, the fusion protein being uniformly distributed throughout undifferentiated hyphae [data not shown].

We constructed two additional strains to confirm the temporal and spatial patterns of reporter gene expression observed for UI38.1. One strain, UI37.1, was a transformant in which the plasmid p2KLAC/TRP [Fig. 3B] had undergone homologous integration at the stuA locus on chromosome I. In this strain, the fusion gene has all chromosomal sequences normally found 5' of the stuA α/β start sites. The temporal and spatial pattern of stuA α/β RNAs, stuA[p/l]/::lacZ RNAs, and StuA:β-Gal activity in UI37.1 was identical to UI38.1, indicating that the fusion gene construct had all sequences necessary for correct regulated expression [data not shown]. A second strain, UI83, was constructed to determine whether UI38.1 possessed any secondary mutations that had resulted from either protoplast preparation or transformation and that might affect reporter gene expression. UI83 was a progeny of a genetic cross with UI38.1 as one of the parental strains. We observed that temporal and spatial expression of the reporter gene was identical to UI38.1 [data not shown].

The reporter gene was used to determine whether expression of the stuA gene might include post-transcriptional regulatory mechanisms, as suggested by the struc-

![Figure 3](https://genesdev.cshlp.org/)

Figure 3. Expression of stuAα and stuAβ transcripts during induction of wild-type and brlA mutant strains. (A) Northern blot of poly[A]+ RNAs isolated from the wild-type control strain FGSC 26. End-labeled oligonucleotide 395 was used for the stuAβ-specific probe; oligonucleotide 974 was used for the stuAα-specific probe. Northern blots were reprobed with a random-primed argB probe for internal standardization of RNA samples. [Lane 1] Uninduced vegetative hyphae; [lanes 2-6] cultures isolated 2.5, 5, 7.5, 10, and 14 hr after induction. (B) Northern blot of poly[A]+ RNAs isolated from either wild-type strains UI38.1 and UI83, the null brlA mutant strain UI58, or the conditional brlA42 mutant strain UI68. UI68 was grown at the nonpermissive temperature for these experiments. Blots were probed simultaneously with a random-primed argB-specific probe and the internal XbaI-XhoI stuA-specific probe. Duplicate samples were used for the in vitro assays of β-galactosidase activity shown in Fig. 4. All strains possess the stuA[p/l]/::lacZ fusion gene integrated at the trpC locus. [stuA::lacZ] The fusion gene transcript. [Lanes 1,4,7,10] Uninduced hyphae; [lanes 2,5,8,11] 7.5-hr developmental cultures; [lanes 3,6,9,12] 14-hr developmental cultures. p2KLAC/TRP is the plasmid used for construction of UI38.1 (see Materials and methods). Thin lines are pBR329 sequences. (K1-K3) The three KpnI sites shown in Fig. 1. [Sm] Smal; (S/X) SalI-XhoI fusion.
Figure 4. Temporal expression and spatial localization of the \textit{stuA}[p/1]:\textit{lacZ} reporter gene in induced wild-type and \textit{brlA} mutant strains. Cultures of indicated strains were isolated at various time intervals after induction and in vitro StuA-\(\beta\)-galactosidase specific activity determined in crude extracts. (Specific activity) Units of ONPG hydrolyzed/min per mg of total protein. Duplicate samples were used for the Northern blot analysis shown in Fig. 3B. Strains UI83 and UI68 (not shown) had temporal and spatial patterns of expression identical to UI38.1 and UI58, respectively. Morphological landmarks are indicated by arrows. Photomicrographs are conidiophores which have been stained with the chromogenic \(\beta\)-galactosidase substrate X-Gal. (A) Control strain UI42; (B) strain UI38.1.

A.

B.

A. \textit{nidulans} \textit{stuA} gene

Synchronously induced conidiation resulted in a 15-fold increase in in vitro StuA-\(\beta\)-Gal activity during the 14 hr required to complete the asexual cycle (Fig. 4). After an initial lag, the maximum increase in activity occurred during morphogenesis of the metulae and phialides. Vegetative hyphae maintained in an undifferentiated state for an additional 14 hr exhibited only a slight increase in StuA-\(\beta\)-Gal activity (Fig. 4), which was distributed uniformly throughout the mycelium (data not shown).

Two observations suggested that \textit{BrlA} may enhance \textit{StuA} expression. First, the temporal pattern of the induction-dependent increase in StuA-\(\beta\)-Gal expression was similar to that of \textit{BrlA} expression (Boylan et al. 1987;
Aguirre et al. 1990]. Second, in situ localization with the chromogenic substrate X-gal showed that these elevated levels of fusion protein were spatially restricted to the apex of the conidiophore vesicle, metulae, and phialides [Fig. 4]. Only negligible amounts of StuA:β-Gal were detected in conidia. Therefore, except in immature conidia, the spatial localization of StuA:β-Gal overlapped that of BrlA:β-Gal [Aguirre et al. 1990]. Strains UI58 and UI68 have the stuA[p/1]:lacZ reporter gene and either the brlA1 null allele or the brlA42 allele, respectively. The loss of brlA function resulted in the failure of StuA:β-Gal expression to respond normally to inductive stimuli [Fig. 4]. At 14 hr postinduction, expression of StuA:β-Gal in UI58 was only 25% that of UI38.1 [Fig. 4] and was uniformly distributed throughout the elongated aerial hyphae characteristic of brlA null mutants (data not shown). We did detect a fourfold increase in StuA:β-Gal activity in induced UI58 cultures that was induction dependent and brlA independent. This increase, however, was delayed by 2.5–3 hr [Fig. 4]. Similar results were observed for UI68 induced under nonpermissive conditions.

The relative abundance of the fusion RNAs was significantly greater than the wild-type stuA RNAs in either UI38.1 or UI83. This was particularly true for competent, undifferentiated hyphae [Fig. 3B]. Whereas total wild-type stuA RNA increased about fivefold after induction, the change in fusion RNA was much smaller, about twofold. Furthermore, the presence of either brlA1 or brlA42 mutations resulted in a reduction in induced levels of fusion RNA in UI58 and UI68 but had only a slight, or no, effect on levels of wild-type stuA RNA [Fig. 3B]. The explanation for this observation is unknown but may result from differences in the stability of fusion and wild-type RNAs after induction. Ectopic expression of BrlA results in both activated transcription of conidia-specific genes and transcriptional and post-transcriptional repression of metabolic genes (Adams et al. 1988; Adams and Timberlake 1990a). Therefore the stability of the stuA wild-type and fusion RNAs may be differentially affected when BrlA is expressed during normal conidiation.

**StuA is required for correct spatial expression of the abaA gene**

The abaA gene product is required for correct differentiation of the conidiogenic phialide and mature conidia [Clutterbuck 1969; Sewall et al. 1990]. It has been suggested that the conidiophore morphology of stuA mutants is a function of incorrect temporal or spatial expression of phialide-specific genes such as abaA. The result is a failure to differentiate metulae or phialides, and conidia are produced from buds on the conidiophore vesicle [Martinelli 1979; Miller et al. 1991]. Strain UI49 was constructed to test this hypothesis. This strain possesses both an abaA[p/1]:lacZ reporter gene integrated at the argB locus on chromosome III and a stuA1 null mutation.

The control strain TTA573 did not express AbaA:β-Gal activity in undifferentiated vegetative hyphae [Fig. 5; Adams and Timberlake 1990b]. After induction, there was a slight increase in AbaA:β-Gal activity during elaboration of the aerial hyphae, conidiophores, and metulae. There was a dramatic increase in activity, however, which began at the onset of phialide differentiation (~7.5 hr postinduction) and continued during conidia formation [Fig. 5]. AbaA:β-Gal was localized in metulae, phialides, and immature conidia and was absent from vegetative hyphae, foot cells, aerial hyphae, conidiophore vesicles, and mature conidia (Fig. 5A,B).

The temporal pattern of AbaA:β-Gal expression in strain UI49 was identical to that of TTA573 [Fig. 5]. The level of expression was also very similar, being only slightly reduced in UI49. Therefore, the absence of StuA did not appear to have a major effect on the temporal expression of the reporter gene or, by inference, the resident abaA gene. However, there were significant alterations in the spatial localization of AbaA:β-Gal expression. No AbaA:β-Gal activity was detected in somatic hyphae of strains TTA573 or UI49 prior to induction. After induction, however, activity was detected throughout somatic hyphae, foot cells, aerial hyphae, and conidiophore vesicles of immature conidiophores of UI49 [Fig. 5D]. AbaA:β-Gal expression was also observed throughout the fully differentiated conidiophores of UI49 [Fig. 5E,F].

**Discussion**

StuA is required during two critical stages in the *A. nidulans* life cycle. Shortly after the establishment of developmental competence, assexual reproduction is initiated. StuA is required at this time for normal patterns of cell differentiation during conidiophore morphogenesis. Sexual reproduction begins as conidiation is completed [Champe et al. 1992]. Meiosis and ascospore formation take place within specialized reproductive structures [cleistothecia] [Benjamin 1955]. Sexual reproduction can take place in the absence of conidiation and does not require BrlA [Clutterbuck 1969; B. Miller, unpubl.]. stuA mutants, however, are self-sterile and fail to form either cleistothecial primordia or associated nurse cells [Hulle cells] [Clutterbuck 1969; Miller et al. 1991]. The complex structure and regulated expression of the stuA gene may reflect multiple roles during the life cycle. In this respect, stuA is similar to complex genes in higher eukaryotes that are required for morphogenesis [Gehring 1987; Biggin and Tjian 1989; Coen et al. 1990; Hayashi and Scott 1990; Schwarz-Sommer et al. 1990; Drews et al. 1991].

Precompetent hyphae of strain UI38.1 expressed low levels of β-galactosidase [1–2 units of β-Gal/min per milligram of protein], whereas activity was ∼50-fold higher in competent, uninduced hyphae. Therefore, during this stage of development, the level of StuA:β-Gal activity closely paralleled changes in the relative abundance of stuA/β RNAs. Two lines of evidence indicate that a component of stuA expression includes induction-dependent, post-transcriptional regulation. Between 2.5
Figure 5. Temporal expression and spatial localization of the abaA[p/l]:lacZ reporter gene in induced wild-type and stuA mutant strains. Induced cultures were isolated at the same time intervals as in Fig. 4, and the specific activity of AbaA::galactosidase was determined. [Specific activity] Units of ONPG hydrolyzed/min per mg of protein. Photomicrographs were stained with X-Gal to determine the in situ localization of the fusion protein. [A] A young conidiophore of wild-type strain TTA573 that is beginning to form the terminal vesicle; [B] a mature TTA573 conidiophore; [C] control strain GO256 containing no fusion gene; [D] young conidiophore of UI49 at same stage as in A; [E,F] mature UI49 conidiophores.

and 7.5 hr postinduction, StuA::Gal specific activity in UI38.1 (or UI83) increased at a linear rate that was ~30 times that observed for cultures maintained vegetatively. One would predict a similar increase in total stuA [α + β] RNA if the translational initiation rate remained constant during conidiation and the change in β-Gal activity was directly related to changes in RNA abundance. Semiquantitation by scanning densitometry indicated
that total \textit{stuA} RNA abundance increased approximately three- to fourfold and total \textit{stuA}:\textit{lacZ} RNA increased about twofold during this time. We do not know whether these changes are the result of increased transcription or altered RNA stability. Although enhanced cell-specific expression of \textit{StuA}:\textit{β-Gal} may be partially explained by these small changes in RNA abundance, there was not a close correlation between \textit{stuA}:\textit{lacZ} RNA abundance and \textit{β-Gal} specific activity.

Both \textit{stuA} RNAs possess long 5' leaders with multiple mini-ORFs. These structural elements have been shown to be important for the post-transcriptional regulation of mRNAs from yeast [Hinnebusch 1984; Thireos et al. 1984; Mueller and Hinnebusch 1986; Werner et al. 1987; Tzamarias et al. 1989] and vertebrates [Kozak 1984, 1987; Garrett et al. 1989, Arrick et al. 1991]. Translation of the \textit{stuA} specific mORF 1 would yield a 2.8-kD peptide (LP1). We have found that translational fusion of the \textit{lacZ} gene to the end of mORF 1 results in expression of an LP1:\textit{β-Gal} fusion protein. The integrity of mORF 1 is required for the induction-dependent increase in \textit{StuA}:\textit{β-Gal} activity. J. Wu and B. Miller, in prep.). Therefore, translation of mORF 1 appears to have a regulatory function and suggests that the α and β transcripts may have different translational efficiencies. Functional analysis of 5’ RNA leader sequences will clarify the contribution of post-transcriptional regulatory mechanisms to \textit{stuA} expression.

Although clear epistatic relationships exist among members of either the \textit{BRISTLE} or \textit{STUNTED} group of genes, double mutant strains carrying a defective allele in both groups have complex morphologies with intermediate characteristics. Conidiophore morphology is also dependent on the specific alleles used [Martinelli 1979]. Martinelli interpreted these results as evidence for complex interactions and possible overlap in functions between the two groups of genes.

\textit{BrI}A is required for the transition from a vegetative growth pattern of apical extension to a budding pattern of growth and to direct conidia differentiation [Clutterbuck 1969; Boylan et al. 1987; Adams et al. 1988]. We have found that \textit{BrI}A is also required for the induction-dependent, spatially localized increase in \textit{StuA}:\textit{β-Gal} expression. There was a 75% reduction in \textit{StuA}:\textit{β-Gal} activity in the \textit{brlA}1 and \textit{brlA42}+ mutant strains. The fourfold increase in activity that did occur in UI58 or UI68 was delayed 2.5–3 hr relative to the wild-type strain UI38.1. Our interpretation of the exact quantitative effect of \textit{brlA} mutations is confused by apparent differences in the stability of \textit{stuA} and \textit{stuA}:\textit{lacZ} RNAs after induction. A comparison of temporal changes in the fusion RNA and \textit{StuA}:\textit{β-Gal} activity, however, reinforces earlier observations that there is not a strict correlation between RNA abundance and activity. \textit{BrI}A probably does not have a direct effect on \textit{StuA} expression. Ectopic expression of \textit{brlA} results in the inhibition of vegetative growth, the transcriptional activation of a large number of conidiation-specific genes, and the repression of certain catabolic genes at both the transcriptional and post-transcriptional level [Adams et al. 1988; Adams and Timberlake 1990a]. Our results suggest that \textit{BrI}A-induced events also result in the selective activation of important developmental genes, such as \textit{stuA}, at the post-transcriptional level.

Martinelli [1979] has suggested that the stunted phenotype could result from incorrect temporal or spatial expression of genes coding for phialide-specific functions. We considered the \textit{abaA} gene to be a likely candidate for such aberrant expression because of its central role in phialide differentiation. Furthermore, functional analysis of \textit{abaA} cis-acting regulatory sequences has revealed complex features that include elements required for spatial restriction of \textit{abaA} expression to metulae, phialide, and immature conidia [Adams and Timberlake 1990b]. \textit{StuA} null mutations do not appear to have a significant temporal or quantitative effect on the expression of the \textit{abaA} reporter gene. Loss of \textit{StuA} function, however, results in the uniform distribution of \textit{AbaA}:\textit{β-Gal} throughout the reduced conidiophore, including foot cells, aerial hyphae, and conidia. Our results suggest that \textit{StuA} is required to establish a spatial, cell-specific gradient of \textit{abaA} expression during conidiation. The precise molecular mechanisms involved are unknown; however, the effect may be indirect. \textit{BrI}A is necessary and sufficient to activate \textit{abaA} expression. It has been shown recently that \textit{StuA} is also required for correct spatial, but not temporal, expression of \textit{brlA} [J. Aguirre and W.E. Timberlake, pers. comm.]. Therefore, in a \textit{stuA} null mutant, \textit{brlA} expression is still activated in response to unknown induction signals, but the spatial gradient is not established. This, in turn, could result in incorrect spatial expression of \textit{abaA} and aberrant morphogenesis. The highly simplified conidiophores of a \textit{stuA} null mutant mimic the conidia-bearing structures formed during ectopic overexpression of \textit{BrI}A [Adams et al. 1988; Mirabito et al. 1989].

The temporal and spatial patterns of \textit{StuA} and \textit{BrI}A expression are similar during morphogenesis. Therefore, it is unlikely that \textit{StuA} is acting directly to repress \textit{brlA} expression. Although various interpretations are possible, the simplest model would involve repression of \textit{brlA} by a currently uncharacterized repressor, X. \textit{StuA} would, in turn, repress X expression or function. Thus, an increasing gradient in \textit{StuA} expression would be able to establish a spatial gradient in \textit{BrI}A expression. Maintenance of this gradient could be self-regulating by means of the positive effect of \textit{BrI}A (either direct or indirect) on \textit{StuA} expression. Alternatively, \textit{StuA} may be necessary for the expression of a factor that blocks the function of repressor X. Indirect evidence for the role of \textit{StuA} in transcriptional activation comes from observations that certain classes of RNAs expressed in induced cultures of wild-type strains are not expressed in \textit{stuA} mutant strains [Zimmermann 1986; K. Miller and B. Miller, unpubl.]. The function of \textit{StuA} in somatic hyphae is unknown but may be related to its role in the sexual reproductive cycle.

Conidiation in \textit{A. nidulans} has been shown to require transcriptional activation of a complex regulatory hierarchy that is initiated by \textit{BrI}A and that includes at least
two feedback regulatory loops [Adams et al. 1988, Mira- 
bite et al. 1989]. Our results show that both transcrip-
tional and post-transcriptional regulatory mechanisms 
play important roles during conidiophore morphogene-
sis. We have also shown that normal patterns of cell 
differentiation in A. nidulans do not result from a simple 
linear progression of gene activation but require spatial 
that are critical to morphogenesis have been isolated and 
can now be subjected to detailed molecular genetic anal-
ysis to test regulatory models. Reproductive competence 
in A. nidulans is not well understood (Miller 1990; Tim-
berlake 1990). Therefore, an analysis of cis-acting ele-
ments and trans-acting factors required for competence-
dependent stuA transcription should provide important 
insights into the molecular genetic basis for this critical 
stage of the A. nidulans life cycle.

Materials and methods

Culture, developmental induction, and genetic 
manipulation of A. nidulans

Procedures for the preparation of supplemented minimal media 
and for the culture of A. nidulans were performed as described 
by Kafer (1977) and Pontecorvo et al. (1953). Standard genetic 
manipulations used in this study followed methods described 
by Clutterbuck (1974) and Pontecorvo et al. (1953). Strains 
AJC7.1 and GO256 were obtained from A.J. Clutterbuck [Uni-
versity of Glasgow, Scotland]. Strain TTA573 was received from 
T.H. Adams [Texas A&M University, College Station, TX]. 
Strains designated FGSC are available from the Fungal Genetics 
Stock Center [University of Kansas Medical Center, Kansas 
City]. Strains designated UI were constructed at the University 
of Idaho (Table 1).

Silanized flasks containing liquid YG-MTV media were inoc-
ulated with 5 x 10^6 conidia per milliliter. Shake cultures were 
grown for 17 hr at 37°C. Mycelia acquired developmental com-
petence after 13 hr but remained in a vegetative state [Miller 
et al. 1991]. Synchronous conidiation can be induced by exposing 
undifferentiated hyphae to an air interface. Hyphae were col-
lected onto nylon mesh and placed onto solid agar media. Foot 
cell initials cannot be detected microscopically but are believed 
to be formed shortly after induction. Aerial hyphae were ob-
served after 2.5 hr, vesicles and metabasal initials after 5 hr, 
metabasal and phialides after 7.5 hr, immature conidia after 10 hr, 
and mature pigmented conidia after 12-14 hr. Samples were col-
clected at desired time intervals and frozen quickly in liquid N_2.

Construction of A. nidulans strains

KpnI linkers were added to the PvuII site of the Escherichia coli 
plasmid pBR329. The two KpnI fragments encompassing the 
stuA gene [Fig. 1] were cloned into this vector. The resulting 
plasmid, pK6.8, was linearized with NdeI and blunt ends were 
generated with mung bean nuclease, restricted with XhoI, and 
ligated to a gel-purified Smal–SalI fragment containing the E. 
coli lacZ gene from pMC1871 [Casadaban et al. 1983]. In this 
construct, p2KLAC, lacZ is fused in-frame to codon 22 of the 
stuA ORF. Expression of β-galactosidase is therefore under the 
control of all of the upstream cis-acting DNA elements neces-
sary for correct stuAα and stuAβ expression. This includes any 
post-transcriptional regulatory mechanisms that involve ele-
ments of the 5' a and β leaders. Two fusion RNAs, stuAα-lacZ 
and stuAβ-lacZ, are expressed from this fusion gene. p2KLAC 
was linearized by SalI partial digestion and ligated to the gel-
purified 4.2-kb XhoI fragment containing the A. nidulans trpC 
gene. Plasmid p2KLAC/TRP [Fig. 3B] was used to transform A. 
nidulans strain FGSC237 to tryptophan prototrophy. Methods 
for DNA-mediated transformation of A. nidulans protoplasts 
were those of Yelton et al. (1984) and Miller et al. (1985). Mini-
prep DNA was isolated from transformants and Southern blot 
analysis was used to screen for single-copy integration events at 
the trpC locus [Miller et al. 1985, 1987]. Transformant UI38.1 
was selected for further studies. Strains UI58, UI68, and UI83 
were obtained as progeny from a sexual cross between UI38.1 
and AJC7.1, UI29, and TTA292, respectively. Strain UI49 was

Table 1. A. nidulans strains used in this study

| Strain | Genotype | Source |
|--------|----------|--------|
| FGSC 26 | biA1 | Fungal Genetics Stock Center |
| FGSC 237 | pabaA1, yA2, trpC801 | Fungal Genetics Stock Center |
| AJC 7.1 | biA1, brlA1 | Clutterbuck [1969] |
| GO 256 | stuA1, brlA1 | Clutterbuck [1969] |
| TTA 292 | biA1, methG1, alcA[p]: brlA [argB'/argB::CAT] | Adams et al. [1988] |
| TTA 573 | biA1, methG2, abaA[p]: lacZ [argB'/argB::CAT] | Adams and Timberlake [1990b] |
| UI 8 | stuA1, biA1, trpC801 | this study |
| UI 29 | biA1, brlA42*, trpC801 | this study |
| UI 37.1 | pabaA1, yA2, stuA[p]: lacZ [trpC'/stuA'] | this study |
| UI 38.1 | pabaA1, yA2, stuA[p]: lacZ [trpC'/trpC801] | this study |
| UI 42 | pabaA1, yA2 | this study |
| UI 49 | stuA1, biA1, methG2, abaA[p]: lacZ [argB'/argB::CAT] | this study |
| UI 58 | pabaA1, brlA1, stuA[p]: lacZ [trpC'/trpC801] | this study |
| UI 68 | pabaA1, brlA42*, stuA[p]: lacZ [trpC'/trpC801] | this study |
| UI 83 | brlA1, stuA[p]: lacZ [trpC'/trpC801] | this study |

Abbreviations: [arg] arginine, [bi] biotin, [meth] methionine, [paba] p-aminobenzoic acid, [trp] tryptophan, [abaA] abacus; [brlA] bristle; [stuA] stunted; [yA] yellow conidia; [lacZ] E. coli β-galactosidase. All strains are veA1: velvet morphology. [p/l] A translational fusion that places β-galactosidase expression under the control of potential cis-regulatory elements present in the promoter and the 5' RNA leader of the heterologous gene. [trpC'/trpC801] The trpC· transforming plasmid integrated by homology at the trpC locus.
Miller et al.

the result of a cross between TTA573 and UI8. During cultivation of these strains, ascospore progeny were replica plated onto solid agar media in glass petri dishes. After 2 days of growth at 37°C, the cultures were treated with chloroform vapors and the presence of the stuA[p1]:lacZ fusion gene was detected by in situ staining as described below.

In vitro assay and in situ localization of β-galactosidase activity

Specific activity of β-galactosidase in A. nidulans crude cell extracts was determined by use of the ONPG assay described by Miller (1972), as modified by Hamer and Timberlake (1987). Cultures were grown for in situ detection of fusion gene expression by point-inoculating conidia onto thin layers of minimal media contained in sterile chamber culture slides (Nunc, Inc., Naperville, IL). Cultures were allowed to grow for 2–3 days at 37°C. The chambers were then inverted over chloroform-soaked filter paper for 10–15 min to permeabilize the conidiating cultures. Chloroform vapors were allowed to dissipate. The chambers were flooded with buffered X-gal and incubated at 37°C for 30–60 min to allow color development (Adams and Timberlake 1990b). Aguirre et al. (1990). The chambers were drained, and bright-field photomicrographs were taken with a Zeiss Axioplan.

S1 nuclease protection, primer extension, and isolation of PCR-generated cDNA clones of stuAo and stuAb

The following synthetic oligonucleotides were used for S1 nuclease protection, primer extension, or RNA sequencing experiments: oligo 395, 5’-GTCTTGTCTGAGACGGA-TGTAGCTGCAACGGGAC-3’; oligo 974, 5’-GAGTTGGGAG-GTCCATTAGTGCTGAGCCGAGGCA-3’; oligo 1139, 5’-CCGC-ACCTGAGGACACAGAAGCAAGACACGGC-3’; oligo 1460, 5’-CAGCAAGACAGACAGCAGAAGCAGACACGGC-3’; oligo 1699, 5’-GACAAAA-UGGATTGCTGCCAGGCCCGGCTTGTA-3’; oligo 2000, 5’-TTCATCTTGCCACATC-3’; oligo 2480, 5’-CAGACCGTTC-GCTTC-3’; oligo 2640, 5’-TTCCAGACCTTTTCACCTTGA-GGATCCG-3’; oligo 2855, 5’-GTCATGTTCCTCTGATTCGATTTAGTCGTAGCGGGCA-3’; oligo 1139 yielded an ambiguous nucleotide ladder once the sequence of Figure 2A. The PCR-amplified products were cloned in Bluescript KS(+) phagemid vectors. The sequences represent the RNA-like strand, and the number designates the position of the 5’ nucleotide in the sequence of Figure 2A. The PCR-amplified products were cloned into Bluescript phagemid vectors.

Northern blot analysis, nucleic acid sequencing, and protein sequence analysis

Total and poly[A] + RNA was isolated from A. nidulans as described previously (Timberlake 1980; Miller et al. 1987). Methods used for Northern blot analysis were the same as those of Miller et al. (1991). In these studies, end-labeled oligonucleotides 974 and 395 were used for the stuAo-specific and stuAb-specific probes, respectively. Replicate blots were probed with both an argB-specific probe and an oligonucleotide 974 or 395. For total stuA (α + β) RNA determination, blots were probed simultaneously with the argB-specific probe and the internal XbaI–XhoI stuA fragment is shown in Figure 1. After determining that signal response was linear, different film exposure times were used to avoid film saturation and to maintain linearity in response. The intensity of bands was quantitated by using a Zeineh soft laser scanning densitometer. Peak areas for stuAo and stuAb from different developmental time points were standardized with the constitutively expressed argB RNA as an internal control. This allowed direct comparison from sample to sample and corrected for any sample loading variation.

To sequence the stuA gene, overlapping restriction fragments were subcloned into Bluescript KS (+) phagemids (Stratagene Cloning Systems, La Jolla, CA). Nested deletions were generated by ExoIII resection (Henikoff 1984). Single-stranded templates were sequenced with Sequenase (U.S. Biochemical Corp., Cleveland, OH). The PCGENE software package (IntelliGenetics, Mountain View, CA) was used for nucleotide and protein sequence analyses. Secondary structure predictions for the StuA protein were determined by use of the method of Garnier et al. (1978) and the Gascuel and Gombrich Basic Statistical Method (Gascuel and Gombrich 1988). Data base searches with either the nucleotide or amino acid sequences were provided by services offered through the Washington State University Visualization, Analysis and Design in the Molecular Sciences (VADMS) Laboratory (Pullman, WA).

Acknowledgments

We thank Drs. Jesus Aguirre and William E. Timberlake for sharing information prior to publication and Drs. Thomas H. Adams, Nancy A. Federspiel, Scott A. Minnich, and David J. Oliver for many useful discussions and critical review of the manuscript. We also thank Dr. Susan Johns and Steve Thompson at the Washington State University VADMS Laboratory for their assistance in data base searches. This work was supported by National Science Foundation grant DCB 8819219 to B.L.M.

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*Genes Dev.* 1992, 6:
Access the most recent version at doi:10.1101/gad.6.9.1770

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