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Aspergillus Asexual Reproduction and Sexual Reproduction Are Differentially Affected by Transcriptional and Translational Mechanisms Regulating *stunted* Gene Expression

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The Stunted protein (StuAp) is a member of a family of transcription factors that regulate fungal development and cell cycle progression. Regulated *stuA* gene expression is required for correct cell pattern formation during asexual reproduction (conidiation) and for initiation of the sexual reproductive cycle in *Aspergillus nidulans*. Transcriptional initiation from two different promoters yields overlapping mRNAs (*stuA*α and *stuA*β) that upon translation yield the same protein. Here we show that multiple regulatory mechanisms interact to control (i) developmental competence-dependent expression of both transcripts and (ii) induction-dependent expression of *stuA*α, but not *stuA*β, by the conidiation-specific Bristle (BrlAp) transcriptional activator. Quantitative levels of both mRNAs are further modulated by (i) an activator(s) located at a far-upstream upstream activation sequence, (ii) feedback regulation by StuAp, and (iii) positive translational regulation that requires the peptide product of a micro-open reading frame unique to the *stuA*α mRNA 5′ untranslated region. Gradients in *stuA*α expression were most important for correct cell and tissue type development. Threshold requirements were as follows: metula-phialide differentiation < ascosporogenesis < cleistothecial shell-Hülle cell differentiation. Altered *stuA* expression affected conidiophore morphology and conidial yields quantitatively but did not alter the temporal development of cell types or conidiophore density. By contrast, the sexual cycle showed both temporal delay and quantitative reduction in the number of cleistothecial initials but normal morphogenesis of tissue types.

The life cycle of the filamentous ascomycete *Aspergillus nidulans* is marked by three important developmental events. Germination of haploid, uninucleate conidia (spores) is followed by a period of multinucleate hyphal growth during which the mycelium acquires developmental competence (17, 18). This is the first identifiable development-specific event. The time at which competence is established (T_c) is genetically determined (12). T_c is affected by temperature and cell density but appears to be independent of nutrient status (12, 75). Competent cultures exposed to inductive signals, which include an air interface, light, or nutrient starvation, initiate the asexual reproductive cycle, and conidiation (41, 56, 64). Conidiation requires a transition from the polarized growth pattern of multinucleate vegetative hyphae to apolar budding of uninuclear cells. This is accomplished through spatial organization of differentiated cell types—foot cell, aerial hyphae, vesicle, metulae, and phialides—into a reproductive structure, the conidiophore. Pigmented conidia are produced by repeated interstitial budding of phialides (46, 52, 71). Initiation of the sexual reproductive cycle and differentiation of three sexual tissue types take place shortly after conidiation begins (18). Fusion of vegetative hyphae forms the cleistothecial primordium and ultimately a hard, red pigmented shell at maturity. Thick-walled globose cells (Hülle cells) develop by budding at the tips of specialized hyphae. Hülle cells envelop the developing cleistothecium and may serve as nurse cells (36). An intertwined network of specialized dikaryotic hyphae develops within the cleistothecial shell. Nuclear fusion, meiosis, and ascosporogenesis occurs within the dikaryon (36, 38).

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Conidiation requires the intimate coordination of cell-specific gene expression with progressive alterations in cell growth pattern and cell cycle regulation (14, 28, 53). The products of at least four major regulatory genes (*abacus*, *bristle*, *medusa*, and *stunted*) interact genetically to control conidiophore morphogenesis in *A. nidulans* (21, 23). Both Northern blot analysis and *lacZ* reporter genes have been used to show that overlapping spatiotemporal gradients in *abacus* (*abaA*), *bristle* (*brlA*), and *stunted* (*stuA*) expression are established during conidiation (3, 5, 13, 50, 51, 60). *brlA* and *abaA* constitute the central regulatory pathway required for conidium differentiation (2, 8, 13, 54, 60). *stuA* and *medusa* (*medA*) are developmental modifiers that affect morphogenesis during conidiation by regulating *brlA* and *abaA* expression (14). *stuA* is required for normal patterns of cell differentiation during conidiation. *stuA* null mutants form short conidiophores with reduced vesicles that fail to proliferate, or accumulate, normal numbers of nuclei. The *stuA* mutant also lacks intermediate cell types, the metulae and phialides. These elongated, uninuclear cells are formed by a budding pattern that is highly reminiscent of pseudohyphal growth in budding yeast (14, 32, 40). StuAp is required for correct spatial expression of *brlA* and *abaA*, suggesting that the *stunted* phenotype is most likely the consequence of aberrant expression of these regulatory proteins (6, 14, 51). *stuA* mutants are also sexually sterile, failing to form Hülle cells, cleistothecia, or ascospores (21, 46). However, sexual reproduction is *brlA* independent and can take place in the absence of conidiation (21, 46). Therefore, StuAp may have independent functions in the two reproductive cycles.

The complex structure and regulated expression of the *stuA* gene may reflect its multiple roles during the life cycle of *A. nidulans*. *stuA* is transcriptionally regulated 50-fold during the establishment of developmental competence and is both transcriptionally and translationally regulated 12- to 15-fold during

conidiation (50, 51). Two differentially regulated RNA species (*stuA*α and *stuA*β) are formed by initiation from different start sites and by alternative splicing (51). Expression of both transcripts is competence dependent, while *stuA*α, but not *stuA*β, RNA abundance is also regulated by developmental induction. A gradient in *lacZ* reporter gene expression was observed during development, with greatest activity in the periphery of the conidiophore vesicle, metulae, and phialides. No expression was observed in conidia (51). Both *stuA* mRNAs encode the same protein, a member of a family of transcription factors consisting of cell cycle regulators in budding and fission yeasts (Swi4, Mbp1, Pct1/Res1, and Sct1/Res2) and regulators of fungal development (Phd1 and Sok2 from budding yeast, Efg1 from *Candida albicans*, and Asm1 from *Neurospora crassa* [7, 10, 15, 32, 39, 43, 55, 61, 66, 69, 74, 77]). The latter proteins constitute the Stunted subfamily, which is characterized by a 100-amino-acid domain with 67 to 87% identity and 80 to 96% similarity. This APSES (Asm1, Phd1, StuA, Efg1, Sok2) domain is the sequence-specific DNA-binding domain of StuAp (29).

Here we report the functional organization of DNA and RNA sequences required for correct transcriptional and translational regulation of the *stuA* gene. We show that 2.4 kb of upstream sequence relative to the *stuA*α RNA start site (1.7 kb relative to *stuA*β +1) are required for correct spatiotemporal expression. Four functional regions containing potential *cis*-acting regulatory elements are required for transcriptional regulation. The *lacZ* gene was translationally fused to the *stuA* open reading frame (ORF), and reporter gene constructs were used to show that deletion of these regions variously affect quantitative levels of vegetative expression, developmental expression, or both. Normal reporter gene expression also required positive regulatory functions of both StuAp and BrlAp. It has been proposed that both transcriptional and translational regulatory mechanisms play important roles in *A. nidulans* development (4, 35, 51, 70). A 41-codon micro-ORF (μORF) present in the 5′ untranslated region (UTR) of the *A. nidulans brlA*β transcript has been shown to be essential for correct developmental regulation of *brlA* gene expression (35). Here we show that a μORF unique to the 5′ leader of the *stuA*α RNA is translated and that the peptide product is required for positive translational regulation of a *stuA*::*lacZ* reporter gene. These same deletions, when placed upstream of a gene capable of expressing functional StuAp and transformed into a *stuA* null strain, differentially affected temporal development and morphogenesis of the asexual and sexual reproductive cycles and resulted in novel phenotypes. We propose a model in which complex transcriptional and translational mechanisms establish a spatiotemporal gradient in StuAp, and we suggest that StuAp variously affects different aspects of the developmental program through a concentration-dependent mechanism.

MATERIALS AND METHODS

Plasmid construction. The vector pBR329(K) was constructed by inserting a *Kpn*I adapter at the *Pvu*II site of pBR329. The 6.8-kb *Kpn*I fragment containing the *stuA* gene was cloned into pBR329(K) to generate plasmid pK6.8 (Fig. 1).

pStuA5′ was constructed in four steps. pK6.8 was partially digested with *Sac*II; ends were blunted with *Escherichia coli* Klenow fragment and religated. After transformation, *E. coli* HB101 colonies were screened for those with plasmids lacking the first *Sac*II site (Fig. 1, $ScII_1$). This plasmid, pK6.8(Δ$ScII_1$), was restricted with *Bam*HI and *Sac*II, and the fragment containing the *Kpn*I-*Sac*II 5′ sequences of *stuA* (Fig. 1, K_1 to $ScII_2$) was gel purified. This fragment was cloned into *Bam*HI/*Sac*II-digested pBS(ΔSmK) to generate pStuA5′. pBS(ΔSmK) was a derivative of the phagemid pBluescript KS^- (Stratagene, La Jolla, Calif.), which was constructed by digestion with *Kpn*I and *Sma*I, blunting ends with mung bean nuclease, and then religating.

A *stuA*$(p/l)_{ORF}$::*lacZ* reporter gene carrying potential upstream regulatory sequences, *stuA*α and *stuA*β promoters, 5′ untranslated mRNA leader sequences, and the first 22 codons of the *stuA* ORF was constructed in four steps. First, the 5-kb *Kpn*I fragment (Fig. 1, K_2 to K_3) containing the *stuA* gene was cloned into pBR329(K) to yield plasmid pK5. pK5 was digested with *Nde*I and *Xho*I and then blunt ended with mung bean nuclease. The large fragment containing vector sequences plus the 5′ and 3′ portions of the *stuA* gene was gel purified. Second, plasmid pMC1871 (16) containing the *E. coli lacZ* gene was digested with *Bam*HI, and blunt ends were generated with Klenow fragment. The 3-kb *Bam*HI fragment containing the *lacZ* gene was gel purified and ligated to the gel-purified *Nde*I/*Xho*I fragment from pK5. The resultant plasmid, pK5lac, contains the *stuA*$(pl)_{ORF}$::*lacZ* reporter gene in which the *lacZ* gene is fused in frame to the 22nd codon of the *stuA* ORF. The junction of the fusion gene was confirmed by DNA sequencing. The 4.2-kb *Xho*I fragment containing the *A. nidulans trpC* gene from pJW2 was cloned into the *Sal*I site of pK5lac to generate the *A. nidulans* transforming plasmid pK5lacT. pK5lacT formed the basis for experiments investigating the effects of upstream deletions on *lacZ* reporter gene expression in *A. nidulans.*

Plasmid pK5T formed the basis for experiments investigating the morphological effects of upstream deletions on *stuA* gene expression in *A. nidulans*. This plasmid was constructed by inserting the *trpC* 4.2-kb *Xho*I fragment from pJW2 into the *Sal*I site of pK5.

Construction of *stuA* 5′ deletions. Deletions of *stuA* 5′ sequences were generated by digesting pStuA5′ first with *Kpn*I and then with either *Bgl*II, *Sal*I, *Kpn*I, or *Nar*I. These fragments were blunt ended with mung bean nuclease and religated to generate plasmid pStuA5′(ΔBg), pStuA5′(ΔS), pStuA5′(ΔK), or pStuA5′(ΔNr), respectively. The *Bam*HI/*Sac*II fragment containing the 5′ deletion was gel isolated from each of these constructs and used to replace the *Bam*HI/*Sac*II fragment of pK5lacT. The resulting *A. nidulans* transforming plasmid was pStuA5′(ΔBg)lacT, pStuA5′(ΔS)lacT, pStuA5′(ΔK)lacT, or pStuA5′(ΔNr)lacT, respectively. Internal *stuA* promoter deletions were generated by digesting pStuA5′ with *Eco*RI and *Nar*I or with *Nar*I and *Xba*I to generate the plasmid pStuA5′(Δβ) or pStuA5′(Δα), respectively. The *Bam*HI/*Sac*II fragments containing the Δβ and Δα deletions were subcloned into pK5lacT to yield *A. nidulans* transforming plasmids pStuA5′(Δβ)lacT and pStuA5′(Δα)lacT. Plasmid pStuA5′(ΔNr2.1) was constructed by deleting the internal 2.1-kb *Nar*I fragment (Fig. 1, $NarI_1$ to $NarI_2$) from pStuA5′ through digestion with *Nar*I and religation at dilute DNA concentration. The *Bam*HI/*Sac*II fragment from pStuA5′(ΔNr2.1) was subcloned into *Bam*HI/*Sac*II-digested pK5lacT to generate the *A. nidulans* transforming plasmid pStuA5′(ΔNr2.1)lacT.

These plasmids containing the *stuA*::*lacZ* fusion gene were used to transform *A. nidulans* FGSC 237. This strain has a wild-type *stuA* gene and therefore normal asexual and sexual reproductive cycles. The *Bam*HI/*Sac*II deletion fragments described above were also subcloned into pK5T to generate pStuA5′(ΔBg)T, pStuA5′(ΔS)T, pStuA5′(ΔK)T, pStuA5′(ΔNr)T, pStuA5′(Δβ)T, pStuA5′(Δα)T, and pStuA5′(ΔNr2.1)T. These plasmids all have an intact *stuA* ORF and were used to transform the *A. nidulans stuA* deletion strain UI 70.1.

Construction of the *stuA*α-specific μORF1::*lacZ* reporter gene. The 5′ untranslated leader of the *stuA*α transcript has a 24-codon μORF (μORF1) that is not present in the *stuA*β transcript (51). The pLP1lacT construct contains the *stuA*$(p/l)_{μORF1}$::*lacZ* fusion gene in which the *lacZ* gene is fused in frame to the last codon of μORF1. pLP1lacT was constructed in four steps. First, pK6.8 was digested with *Xba*I and *Xho*I and blunt ended with mung bean nuclease. The large fragment containing vector sequences and the internally deleted *stuA* gene was gel purified. The Casadaban plasmid pMC1871 was restricted with *Sal*I and blunt ended with Klenow fragment. The 3.0-kb *lacZ* fragment was gel purified and ligated to the aforementioned pK6.8 fragment to generate plasmid pLP1lac. The junction of the fusion gene was confirmed by DNA sequencing. Finally, the *A. nidulans trpC* 4.2-kb *Xho*I fragment was ligated to pLP1lac which had been partially digested with *Sal*I.

Site-directed mutagenesis of μORF1. Plasmid pBS/K-RV was generated by ligating the upstream 1.5-kb *Kpn*I/*Eco*RV fragment from pK5 to *Kpn*I- and *Eco*RV-digested phagemid Bluescript KS. pBS/K-RV was then digested with *Bam*HI and *Bst*XI; the ends were blunted with mung bean nuclease and religated

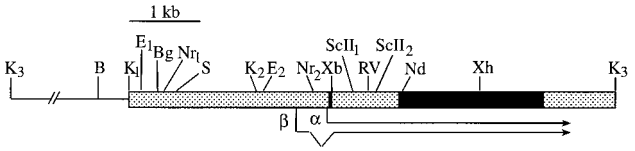

FIG. 1. Restriction map of pK6.8. Constructs described in Materials and Methods were derived from pK6.8. Lines represent pBR329 vector sequences. Boxes indicate *A. nidulans* sequences. Stippled box, noncoding sequences; black boxes, *stuA*α-specific μORF and the StuAp ORF common to both transcripts. The *stuA*α and *stuA*β transcriptional units are indicated below boxes. Three small introns interrupting the StuAp ORF are not shown (see reference 51). Restriction sites discussed in Materials and Methods: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; K, *Kpn*I; RV, *Eco*RV; Nd, *Nde*I; Nr, *Nru*I; ScII, *Sac*II; Xb, *Xba*I; Xh, *Xho*I.

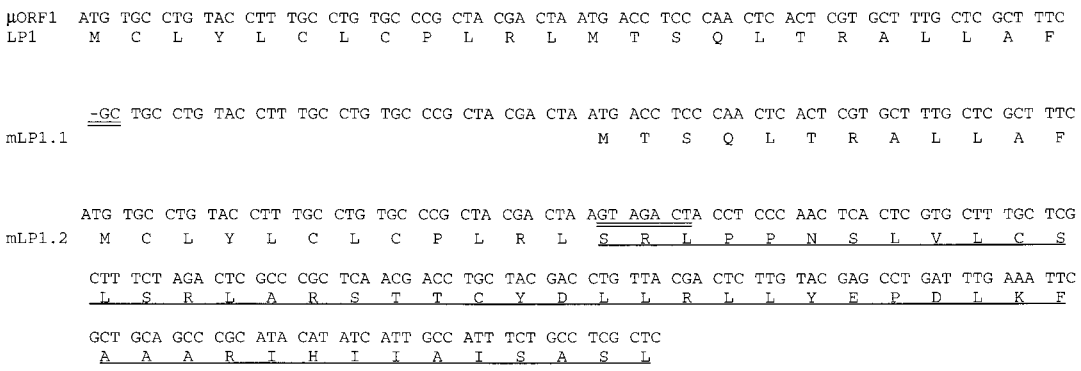

FIG. 2. Sequences of the *stuA*α-specific μORF and μORF mutations. Amino acid sequences for the leader-encoded peptide (LP1) and potential mutant peptides are also shown. Nucleotides altered by mutagenesis are indicated by double underlining. Novel amino acids in the potential peptide generated by the frameshift in the mLP1.2 mutation are underlined.

to yield plasmid pBS/K-RV.1. This plasmid was used to make single-stranded DNA for oligonucleotide-directed mutagenesis. Two oligonucleotides, LP1.1 (5′ CGTCCCTTCAGCTGACCTCCC 3′) and LP1.2 (5′ CGACTAATGTAGTA CTACCTCCC 3′), were used to introduce mutations into μORF1, using a T7 in vitro mutagenesis kit (United States Biochemical Corp., Cleveland, Ohio). The 24-codon μORF1 has two ATG codons, at positions 1 and 12 (51). Oligonucleotide LP1.1 was used to remove the first ATG codon. Translation of this mutant μORF1 (mLP1.1 [Fig. 2]) would result in a shorter leader-encoded peptide of 12 amino acids initiated at the second ATG. Oligonucleotide LP1.2 was used to remove the second ATG of μORF1 and to introduce a frameshift (Fig. 2). This μORF1 mutation (mLP1.2 [Fig. 2]) could give rise to a leader-encoded peptide of 63 amino acids consisting of the original first 12 amino acids plus 51 novel amino acids. The *Kpn*I/*Eco*RV fragments containing altered μORF1 were gel isolated from pBS/K-RV.1 after mutagenesis and ligated to *Kpn*I- and *Eco*RV-digested pStuA5′ to create pStuA5′(mLP1.1) and pStuA5′(mLP1.2). Finally, the *Bam*HI/*Sac*II fragments containing these mutations were isolated from pStuA5′(mLP1.1) and pStuA5′(mLP1.2) and subcloned into pK5lacT to generate the *A. nidulans* transforming plasmids pStuA5′(mLP1.1)lacT and pStuA5′(mLP1.2)lacT. The latter plasmids were used to test the effects of the μORF1 mutations on *stuA*(p/l)$_{ORF}$::*lacZ* reporter gene expression. Alternatively, the *Bam*HI/*Sac*II fragments were subcloned into pK5T to yield *A. nidulans* transforming plasmids pStuA5′(mLP1.1)T and pStuA5′(mLP1.2)T. These plasmids were used to test the morphological effects of μORF1 mutations.

Growth and genetic manipulation of *A. nidulans*. Minimal medium was prepared as described by Käfer (37). Rich medium, YG-MTV, was prepared as described by Miller et al. (51). Liquid medium, YG-MTV, was inoculated with liquid conidial stocks to give a final concentration of 5.0×10^6 conidia per ml. Cultures were grown as shake cultures for 17 h at 37°C and 250 rpm. Synchronous development was induced by exposing competent, undifferentiated hyphae to an air interface as described by Miller et al. (51). Tissue samples were collected at desired postinduction time intervals and quickly frozen in liquid nitrogen.

Genetic manipulations of *A. nidulans* were performed by using standard methods (22, 37, 59). The genotypes of the strains used in this study are given in Table 1. Strains designated FGSC were obtained from the Fungal Genetics Stock Center, University of Kansas Medical Center; strains designated UI were constructed at the University of Idaho.

DNA-mediated transformation of *A. nidulans* was performed as previously described (47, 76). *A. nidulans* genomic DNA was isolated from transformants by using previously described methods (47, 48). Single-copy integrants at the *trpC* locus were confirmed by Southern blot analysis (48, 65).

Plasmid p2Klac/Trp, containing all 2,373 bp upstream of the *stuA*α RNA start site, has been previously described as p2Klac/Trp (51). The *lacZ* reporter plasmids p2Klac/Trp, pStuA5′(ΔBg)lacT, pStuA5′(ΔS)lacT, pStuA5′(ΔK)lacT, pStuA5′(ΔNr)lacT, pStuA5′(Δα)lacT, pStuA5′(Δβ)lacT, pStuA5′(mLP1.1)lacT, pStuA5′(mLP1.2)lacT, and pStuA5′(ΔNr2.1)lacT were used to transform strain FGSC 237. The resultant strains were UI 38.1, UI 38.4, UI 38.2, UI 38.0, UI 38.3, UI 38.5, UI 38.6, UI 38.8, UI 38.7, and UI 38.9, respectively (Fig. 3; Table 1). Plasmids pStuA5′(ΔBg)T, pStuA5′(ΔS)T, pStuA5′(ΔK)T, pStuA5′(ΔNr)T, pStuA5′(Δα)T, pStuA5′(Δβ)T, pStuA5′(mLP1.1)T, pStuA5′(mLP1.2)T, and pStuA5′(ΔNr2.1)T contain deletions upstream of the intact *stuA* ORF and were used to transform the *stuA* deletion strain UI 70.1 (Table 1). The resultant strains, UI 95, UI 94, UI 91, UI 90, UI 92, UI 96, UI 97 and UI 98, respectively, were used to determine the effects of promoter or leader mutations on asexual and sexual reproduction. Sexual crosses between UCD 1 (Table 1) and UI 90, UI 92, UI 94, UI 95, UI 96, and UI 98 were used to generate UI 90.1, UI 92.1, UI 94.1, UI 95.1, UI 96.1, and UI 98.1.

Determination of conidial yields and conidiophore density. Conidia were center point inoculated onto 1.2% agar plates containing appropriately supplemented minimal medium. Colony diameters were measured, and areas were determined after growth at 37°C for 3 to 4 days. Ten to 15 ml of 0.01% Tween 80 was added to each plate, and conidia were harvested by using a spatula or glass rod. Conidia were counted in a Petroff-Hausser counting chamber. Conidial yields reported are means of three replica counts of conidia collected from three

TABLE 1. *Aspergillus* strains used in this study

Strain	Genotype[a]
FGSC 26	*biA1*
FGSC 237	*pabaA1 yA2*; *trpC801*
UCD 1	*pabaA1 yA2 biA1*; *methG2 argB2*; *trpC801*
UI 38.0–UI 38.9[b]	*pabaA1 yA2*; *stuA*(Δp/l)$_{ORF}$::*lacZ* (*trpC*$^+$/*trpC801*)
UI 42	*pabaA1 yA2*
UI 61.1	*pabaA1 yA2*; *stuA*(pl)$_{μORF1}$::*lacZ* (*trpC*$^+$/*trpC801*)
UI 70.1	*adE20 riboA1* Δ*stuA*::*argB*$^+$; *biA1 anA1 pabaA1 yA2*; *argB2*; *trpC801*
UI 76	*pabaA1 yA2*; *alcA*(pl)::*brlA* (*argB*$^+$/*argB2*); *stuA*(pl)::*lacZ* (*trpC*$^+$/*trpC801*)
UI 90–UI 98[c]	*adE20 riboA1* Δ*stuA*::*argB*$^+$; *biA1 anA1 pabaA1 yA2*; *argB2*; *stuA*(Δp/l) (*trpC*$^+$/*trpC801*)
UI 90.1–UI 98.1[d]	Δ*stuA*::*argB*$^+$ *biA1 pabaA1 yA2*; *argB2*; *stuA*(Δp/l) (*trpC*$^+$/*trpC801*)
UI 108	*pabaA1 yA2*; (*alcA*(p)::*abaA*$^+$/*argB2*); *argB stuA*(pl)::*lacZ* (*trpC*$^+$/*trpC801*)
UI 111	*stuA1 pabaA1 biA1*; *stuA*(p/l)::*lacZ* (*trpC*$^+$/*trpC801*)
UI 112	*adE20 riboA1 biA1 anA1 pabaA1 yA2*

[a] All strains are *veA1*.
[b] Has *stuA*(Δp/l)$_{ORF}$::*lacZ* fusion gene deletions integrated at the *trpC* locus. See Materials and Methods and Fig. 3 for details.
[c] Has *stuA* genes with upstream promoter or leader deletions integrated at the *trpC* locus. See Materials and Methods and Fig. 3 for details.
[d] Progeny from crosses of UCD 1 with UI 90 to UI 98.

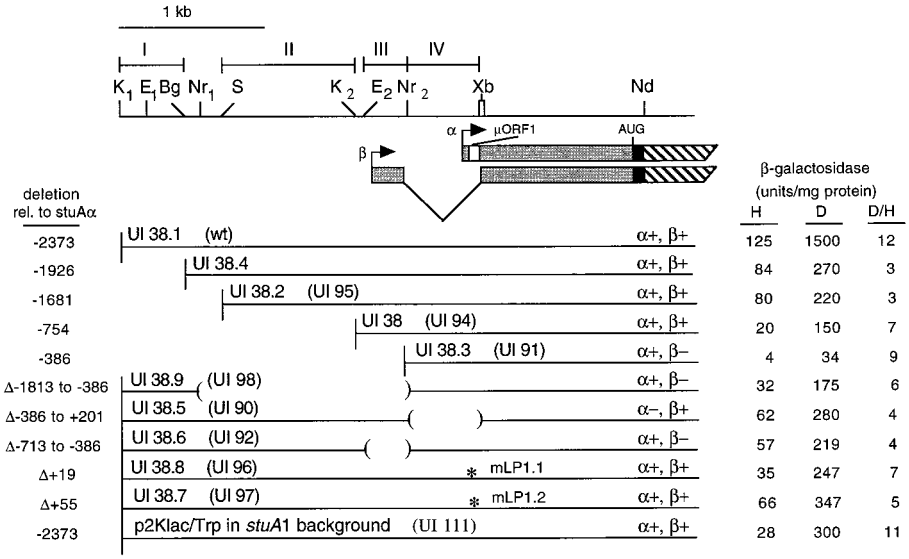

FIG. 3. Deletion analysis of the *stuA* gene. Strains UI 38.0 to UI 38.9 have a wild-type *stuA* gene on chromosome I and the indicated upstream deletion in a *stuA*::*lacZ* reporter gene integrated at the *trpC* locus. Strain UI 38.1 contains the control plasmid with intact 5′ sequences, p2Klac/Trp, described in reference 51. The corresponding member of strains UI 90 to UI 98 is also indicated by parentheses for each deletion. Each of these strains has a *stuA* null mutation on chromosome I and the indicated upstream deletion in a *stuA* gene integrated at the *trpC* locus. Strains UI 90 to UI 98 were used for the functional studies described in the text. Regions I to IV (discussed in the text) are indicated above the restriction map. On each line, α and β refer to the presence (+) or absence (−) of the respective *stuA* RNA start sites and transcriptional units. Replicates were within ~10% of the mean for each of the strains and treatments shown. Arrows, *stuA*α and *stuA*β transcriptional start sites; open box, μORF; gray box, 5′ untranslated leader; black box, StuAp ORF; slashed box, lacZ; *, position of μORF mutations. H, competent, undifferentiated hyphae; D, 14-h developmental cultures. Bg, *Bgl*I; E, *Eco*RI; K, *Kpn*I; Nd, *Nde*I; Nr, *Nru*I; Xb, *Xba*I.

replica plates. The conidial yield ([11.1 ± 0.46] × 10^6 conidia/cm^2) of the morphologically wild-type strain UI 112 represents 100%.

Conidiophore density was estimated by using bright-field microscopy and photographing cultures grown as described above. Slides were projected onto a screen containing a grid, and conidiophores were counted directly from the screen. Magnification (enlargement) values for both the microscope and slide projector were factored into area calculations.

Determination of Hülle cell, cleistothecium, and ascospore formation. Conidia were center point inoculated onto 1.2% agar plates containing appropriately supplemented minimal medium and grown at 37°C. Plates were sealed 24 h after inoculation to promote sexual sporulation. The formation of Hülle cells and cleistothecia was monitored microscopically for 8 to 10 days. Ascospore production was determined by cleaning four or five cleistothecia on 3.0% agar plates containing only water, crushing them individually in Eppendorf tubes containing water, and examining them microscopically. Ascospore viability was determined by cleaning three individual cleistothecia and crushing them in Eppendorf tubes containing water. An aliquot of the ascospores was inoculated onto solid minimal medium and incubated at 37°C. Reproducibility was problematic with strains UI 90 to UI 98, apparently due to the combined effect of the *adE20*, *riboA1*, and *anA1* mutations. Therefore, UI 42 was used as the control strain and strains UI 90.1 to UI 98.1 were used to determine the effect of upstream deletions on the sexual cycle.

β-Galactosidase activity assays. Tissue from each treatment was collected, split in half, and frozen in liquid N_2. One half of the sample was used for RNA extraction (see below), and the other half was used for β-galactosidase assays. Cell extracts from *A. nidulans* were prepared from uninduced hyphae and induced cultures by using standard procedures (33, 73). Specific activity of β-galactosidase was determined by using *o*-nitrophenyl-β-D-galactopyranoside as the substrate (9, 34, 49). Replicates were within ~10% of the mean for each treatment. Protein concentration in extracts was determined by using the Bio-Rad Laboratories (Richmond, Calif.) protein assay reagent.

Nucleic acid hybridization. (i) Southern hybridization. DNA from *A. nidulans* strains was isolated by using miniprep procedures (47, 76). Miniprep DNA was digested with the restriction endonuclease *Xho*I, separated by 1% agarose gels, and transferred onto Hybond-N membranes (Amersham Corp.). Blots were hybridized with either a *trpC*-specific probe or a *stuA*-specific probe to confirm single-copy integration events at either the *trpC* locus or the *stuA* locus (50, 51).

(ii) Northern hybridization. Total RNA was isolated from *A. nidulans* cultures and poly(A)$^+$ as previously described (50, 51). Each lane was loaded with 10 μg of poly(A)$^+$ RNA based on reading of optical density at 260 nm. RNA was electrophoresed under denaturing conditions, using formaldehyde and formamide, and blotted onto Hybond-N membranes (Amersham). Blots were hybridized with either a *stuA*α-specific (stuA974 [5′-GAGTTGGGAGGTCATTAG TCGTAGCGGGCA-3′]) or a *stuA*β-specific (stuA395 [5′-GTCTGTCTGAG ACGGATGTAGCTGCAAGGC-3′]) end-labeled oligonucleotide.

Expression of the wild-type *stuA* gene present in *lacZ* reporter strains UI 38.0 to UI 38.9 is unaffected by reporter deletions. The wild-type *stuA*β mRNA abundance is minimally affected by developmental induction (51) and was used as an internal measure of sample loading.

DNA sequencing. Single-stranded DNA sequencing was performed by using a Sequenase kit (version 2; United States Biochemical). The endpoints of some *stuA* promoter deletions were determined by sequencing double-stranded templates (20), using chain termination (63). The sequence of junction regions of all *lacZ* fusion genes was confirmed by double-stranded DNA sequencing using the *lacZ*-specific primer 5′-GGTGCCGGAAACCAG-3′.

RESULTS

***stuA* gene expression requires multiple upstream regulatory elements.** Complementation of both asexual and sexual reproductive defects of a *stuA* null mutation required 2,373 bp of DNA sequences 5′ to the *stuA*α mRNA start site (1,738 bp relative to the *stuA*β mRNA start site) (50, 51). A single copy of a *lacZ* reporter plasmid with essential *stuA* 5′ and 3′ DNA sequences, p2Klac/Trp, was integrated at the *trpC* locus of strain UI 38.1 (Fig. 3 and reference 51). Significant changes in StuAp–β-galactosidase (StuAp:β-Gal) activity occurred at two developmental landmarks. Specific activity was 1 to 2 U in hyphae prior to competence, ~100 U in developmentally competent vegetative hyphae, and ~1,500 U in fully differentiated cultures. Expression of *stuA*α::*lacZ* and *stuA*β::*lacZ* mRNAs was similar to that of the native *stuA*α and *stuA*β mRNAs, with *stuA*α transcription being developmentally regulated and *stuA*β transcription showing minimal change during development (Fig. 4 and reference 51).

Deletion of 447-bp region I, −1926 to −2373 relative to *stuA*α +1, caused a moderate reduction of StuAp:β-Gal activity in vegetative hyphae (67% of the wild-type level) but greatly reduced expression in developmental cultures (18% of the wild-type level; Fig. 3, UI 38.4). Deletion of an additional 260

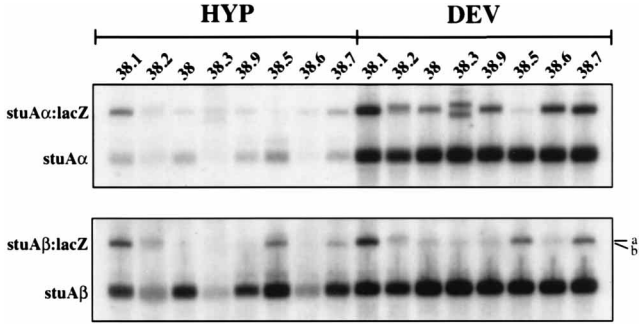

FIG. 4. Effects of upstream deletions on *stuA*α::*lacZ* and *stuA*β::*lacZ* RNA expression. *stuA*α- and *stuA*β-specific probes were used in Northern blot analysis of RNAs expressed in undifferentiated vegetative hyphae (HYP) and 14-h developmental cultures (DEV) of deletion strains UI 38.0 to UI 38.9. Numbers refer to the specific UI strain used in each lane. Each lane was loaded with 10 μg of poly(A)$^+$ RNA based on readings of optical density at 260 nm. Expression of the wild-type *stuA* gene present in these strains is unaffected by reporter deletions. Wild-type *stuA*β mRNA abundance is minimally affected by developmental induction (51) and was used as an internal measure of sample loading. A faint ghost band (b) of unknown origin was detected just below the *stuA*β transcript (a) in the *stuA*β deletion strain UI 38.6. A similar band was also observed in the *stuA*α deletion strain UI 38.5.

bp of DNA had no additional effect on reporter expression (Fig. 3, UI 38.4 versus UI 38.2). Northern blot analysis was used to determine if the decrease in StuAp:β-Gal activity was the result of reduced transcription of *stuA*α, *stuA*β, or both. UI 38.2 had significantly reduced *stuA*α::*lacZ* and *stuA*β::*lacZ* mRNA abundance compared to control strain UI 38.1, particularly in developmental cultures (Fig. 4). These results suggest that one or more upstream activation sequences (UASs) in region I enhance expression from both *stuA*α and *stuA*β promoters and, more importantly, promote induction-dependent expression from the *stuA*α promoter.

By contrast, deletion of all sequences upstream of −754 bp relative to the *stuA*α start site (regions I and II) had little additional effect on expression in developmental cultures but reduced expression in competent, uninduced hyphae to 16% of wild-type levels (Fig. 3, UI 38.0 versus UI 38.4). *stuA*β::*lacZ* mRNA was not detectable in either vegetative hyphae or developmental cultures of UI 38.0 (Fig. 4). The *stuA*α::*lacZ* mRNA could still be detected in hyphae, and its abundance in developmental cultures did not appear to be significantly affected by the additional deletion of region II (Fig. 4). A comparison of StuAp:β-Gal activities and *stuA*α/β::*lacZ* mRNA abundances in strains UI 38.0, UI 38.2, and UI 38.4 suggests that factors at elements in region II predominately affect *stuA*β transcription. UI 38.0 still has 118 bp upstream of *stuA*β +1, which includes a potential TATA sequence (51).

The reporter gene in UI 38.3 has only *stuA*α promoter sequences (Fig. 3, region IV) plus the *stuA*α::*lacZ* transcriptional unit. Reporter expression was significantly reduced in both vegetative and developmental tissues (3 and 2%, respectively, of wild-type levels). Further reduction in reporter expression in UI 38.3 compared to UI 38.0 may be due to residual transcription of *stuA*β::*lacZ* mRNA in UI 38.0. Alternatively, there may exist elements within the *stuA*β intron that affect *stuA*α transcription. UI 38.3 expressed two aberrant-size *stuA*α RNAs (Fig. 4). However, primer extension with the *stuA*α-specific oligonucleotide showed transcript initiation from the normal +1 site, and S1 nuclease protection assays did not indicate altered splicing events (data not shown). Therefore, one transcript must result from correct initiation and the other must result from aberrant initiation at a cryptic site. The latter transcript probably does not yield functional StuAp. Based on RNA size, the latter transcript would be initiated downstream of μORF1, which is required for positive translational regulation of the *stuA*α RNA (see below). Notably, both StuAp:β-Gal activity and *stuA*α mRNA abundance in UI 38.3 show normal developmental regulation. Induction-dependent expression of the *stuA*α transcriptional unit is probably mediated through multiple BrlAp response elements (BREs) located upstream of the *stuA*α RNA start site (see below).

TABLE 2. Expression of the *stuA*(pl)$_{ORF}$::*lacZ* reporter is regulated by ectopic *brlA* expression

Strain	Fusion gene	StuAp:β-Gal activity (U/mg of protein)[a]		Fold increase
		G→G	G→T	
UI 38.1		143 ± 9	169 ± 16	1.2
UI 76	*alcA*(p)::*brlA*	44 ± 8	186 ± 21	4.4
UI 108	*alcA*(p)::*abaA*	39 ± 4	58 ± 9	1.5

[a] Cells were shifted from glucose to glucose medium (G→G) or from glucose to threonine medium (G→T).

StuAp:β-Gal activity was enhanced five- to eightfold in both vegetative and developmental tissues when region I was placed directly upstream of the developmentally regulated *stuA*α promoter (Fig. 3, UI 38.9 versus UI 38.3). This result supports the observation that the UAS(s) in region I enhances competence-dependent expression in vegetative hyphae and, in particular, induction-dependent expression in developmental tissue. The presence of the UAS(s) in UI 38.9 also restored correct transcriptional initiation at *stuA*α +1 (Fig. 4).

Induction-dependent *stuA* expression is mediated by BREs upstream of the *stuA*α promoter. Reporter expression in vegetative tissue from *brlA* mutants was similar to that in the wild type. However, expression in developmental cultures was only ~25% of wild-type levels (Table 3 and reference 51). We previously proposed that induction-dependent, *brlA*-dependent *stuA* expression was primarily through regulated *stuA*α transcription (51). Figure 5 indicates nine BREs (NYTCCCY [19]) located between −55 and −354 relative to *stuA*α +1. These are the only BREs within the 2,373 bp of upstream sequences required for normal *stuA* expression. We tested the direct effect of BrlAp on *stuA* expression by using the glucose-

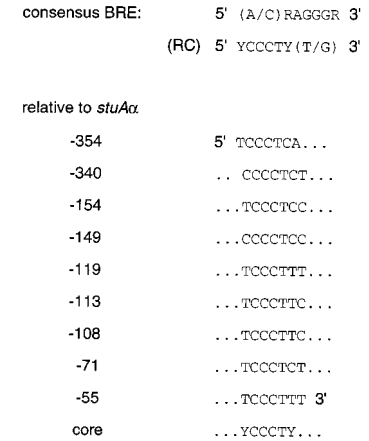

FIG. 5. Positions of BREs upstream of the *stuA*α mRNA start site. RC, reverse complement.

repressible, threonine-inducible *alcA* promoter [*alcA*(p)] to drive ectopic *brlA* expression in vegetative hyphae (2). Induction of the *alcA*(p)::*brlA* fusion in UI 76 resulted in a 4.4-fold increase in reporter expression, compared to control strain UI 38.1, when vegetative hyphae were shifted from glucose to threonine medium (Table 2). This increase is similar to the fourfold effect of BrlAp on reporter expression during normal development (51). Two additional observations indicate that enhanced reporter expression in UI 76 was not indirect, through BrlAp-activated *abaA* expression. Ectopic *brlA* expression in an *abaA* null strain gave results identical to those for UI 76 (data not shown), and threonine-induced expression of an *alcA*(p)::*abaA* fusion gene did not lead to increased reporter expression (Table 2, UI 108). Taken together, these results suggest that during normal development, regulated *stuA* expression results from BrlAp-activated transcription from the *stuA*α promoter.

***stuA*α and *stuA*β transcriptional units contribute equally to vegetative but not developmental *stunted* gene expression.** UI 38.6 expresses only the *stuA*α::*lacZ* mRNA because the reporter includes the *stuA*α promoter and regions I and II but lacks region III, containing the promoter and first exon of *stuA*β (Fig. 3 and 4). Reporter expression in vegetative tissue of UI 38.6 was ~50% of wild-type levels (Fig. 3). UI 38.5 expresses only the *stuA*β::*lacZ* mRNA because the first and second *stuA*β exons of the reporter gene have been fused, thereby deleting the *stuA*α promoter, transcriptional start sites, and sequences specifying the first 201 nucleotides (nt) of the *stuA*α RNA's nontranslated leader (Fig. 3 and 4). Reporter expression in vegetative tissue of UI 38.5 was also ~50% of wild-type levels.

Developmental expression in UI 38.5 was ~15% of wild-type levels (Fig. 3). This result was not unexpected, as *stuA*β transcription normally shows only minor developmental regulation (Fig. 4, UI 38.1; reference 51). If contributions of the two transcriptional units were simply additive during development, reporter expression driven by the *brlA*-regulated *stuA*α promoter in UI 38.6 would be predicted to be ~85% of wild-type levels. Unexpectedly, expression in UI 38.6 was only 15% of wild-type levels. Thus, summation of the individual contributions of *stuA*α::*lacZ* mRNA and *stuA*β::*lacZ* mRNA to developmental expression was only ~1/3 of wild-type levels. It is possible that regulatory elements necessary for *stuA*α transcription lie within the first intron of the *stuA*β transcriptional unit, sequences deleted in UI 38.5 (Fig. 3, region IV). Alternatively, a positive feedback regulatory loop may explain this result.

***stuA* regulates its own expression through a positive feedback loop.** Plasmid p2Klac/Trp was integrated at the *trpC* locus of a *stuA1* null strain. Reporter expression in strain UI 111 was approximately 20% of wild-type levels in both hyphae and developmental cultures (Fig. 3). Thus, a positive feedback regulatory loop enhances *stuA* expression. The effect of any deletion that reduces expression from either the *stuA*α or *stuA*β promoter would be compounded through this mechanism.

Positive translational regulation of *stuA* expression requires a *stuA*α mRNA-specific leader-encoded peptide. We previously proposed that both transcriptional and translational regulatory mechanisms control *stuA* gene expression (51). The unique 24-amino-acid μORF present in the untranslated 5′ leader of the *stuA*α transcript was translationally fused to *lacZ* (Fig. 2 and 3). Expression of the μORF1::*lacZ* fusion in UI 61.1 was developmentally regulated ~5- to 7-fold (Table 3). However, unlike that of the $stuA_{ORF}$::*lacZ* fusion in UI 38.1, μORF1::*lacZ* expression was independent of the allelic state of *brlA* (Table 3).

TABLE 3. Expression of *lacZ* reporters in wild-type and *brlA* mutant backgrounds

Strain/fusion gene	β-Galactosidase activity (U/mg of protein)			
	Wild type		*brlA1*/*brlA42*	
	Uninduced	Induced (10 h)	Uninduced	Induced (10 h)
UI 38.1 / $stuA(p/l)_{ORF}$::*lacZ*	123	1,500	134	362
UI 61.1 / $stuA(p/l)_{\mu ORF1}$::*lacZ*	76	352	78	572

The size of μORF1 suggested that, in addition to placing ribosomes into a reinitiation mode, the leader-encoded peptide, LP1, may also be required for mechanisms regulating *stuA*α mRNA translation. Two site-directed mutations, mLP1.1 and mLP1.2, were introduced into μORF1 of the $stuA(p/l)_{ORF}$::*lacZ* fusion gene, and constructs were integrated at the *trpC* locus. mLP1.1 eliminated the first AUG codon of μORF1, but translation could still generate a peptide product beginning with the ATG at codon 13 of the wild-type μORF and consisting of the last 12 residues of LP1 (Fig. 2). mLP1.2 introduced a frameshift at the internal AUG codon of μORF1, creating a 62-codon μORF with the first 12 codons identical to those in μORF1 and the remainder unique (Fig. 2). The mLP1.2 mutation did not significantly affect *stuA*α mRNA abundance (Fig. 4, UI 38.7 versus UI 38.1). However, StuAp: β-Gal activity was reduced approximately 50% in undifferentiated hyphae and 80% in developmental cultures (Fig. 2, UI 38.7). Similar results were observed for the mLP1.1 mutation (Fig. 3, UI 38.8).

Upstream deletions differentially affect temporal development and morphogenesis of the asexual and sexual reproductive cycles. An intact *stuA* gene with 2,373 bp of DNA sequence 5′ to the *stuA*α transcriptional start site complements both asexual and sexual reproductive defects of a *stuA* null strain when integrated ectopically (51). The deletions described above were used to replace upstream sequences of an otherwise intact *stuA* gene. Constructs were integrated at the *trpC* locus to generate *A. nidulans* UI 90 to UI 98 and UI 90.1 to UI 98.1 (Materials and Methods; Table 1). For comparison, strains from the UI 90 to UI 98 series and UI 38.0 to UI 38.9 (*lacZ* fusion) series with identical deletions are shown in Fig. 3.

Deletion of region I severely affected both asexual and sexual reproduction (Table 4). Conidiophores of UI 95 and UI 95.1 had aerial hyphae that were approximately half the height of those of the wild type, as well as having smaller conidiophore vesicles and reduced numbers of primary reproductive cells or metulae (Fig. 6). Conidial yields were reduced to 39% of wild-type levels (Table 4). However, induction of conidiation, temporal development of the various cell types of the conidiophore, and conidiophore density in these strains were the same as for the wild type. During sexual reproduction in the wild type, development of viable ascospores was closely linked temporally, with the appearance of mature cleistothecia having cleistothecial shells with dark purple pigmentation. By contrast, development of sexual reproductive structures and ascospores was significantly delayed and temporally unlinked in UI 95 and UI 95.1. Hülle cells and pigmented cleistothecia were observed 2 days later than in the case of the wild type, while formation of mature, viable ascospores was delayed by 7 days (Table 4). Cleistothecial shells, although normally pigmented, were very fragile. The number of cleistothecia formed was ~1% of that of the wild type, suggesting that induction of cleistothecial primordia was severely impaired. Deletion of

TABLE 4. Effects of upstream deletions on asexual and sexual reproductive cycles

Strain	*stuA*	Conidial yield (%)[a] during asexual cycle	Days postinduction in sexual cycle[b]		
			Hülle cells	Cleistothecia	Ascospores[c]
UI 112	α^+, β^+	100	4	5.5	5.5
UI 95	α^+, β^+	39	6[d]	7.5[d]	11.5[d]
UI 94	α^+, β^+	29	NO	NO	NO
UI 91	α^+, β^-	20	NO	NO	NO
UI 98	α^+, β^-	38	NO	NO	9.5[e]
UI 90	α^-, β^+	34	6.5	9	9
UI 92	α^+, β^-	76	5	6.5	6.5
UI 96	mLP1.1	84	5	6.5	6.5
UI 97	mLP1.2	79			
UI 70.1	Δ*stuA*	5	NO	NO	NO

[a] 100% conidial yield = 1.31×10^7 conidia/cm^2.
[b] Strains UI 90.1 to UI 98.1 were used for these experiments. See Materials and Methods. Specific cells or tissue types were observed on the days indicated. NO, none observed.
[c] Appearance of mature, viable pigmented ascospores.
[d] Only a limited number of Hülle cells and cleistothecia are formed, mostly near the point of inoculation. The number of cleistothecia is <1% of the wild-type number. Cleistothecial shells are very fragile.
[e] Masses of dikaryotic tissue and ascospores formed near the point of inoculation; no Hülle cells or cleistothecial shells are apparent.

both region I and region II had only a slight additional effect on asexual reproduction. Conidiophores of UI 94 were intermediate in size between those of UI 95 and UI 91 (Fig. 6). Conidial yields were reduced another 10%, from 39 to 29% (Table 4). However, the sexual reproductive cycle was completely eliminated with the additional deletion of region II.

Strain UI 91 contains a deletion of all sequences upstream of −386 relative to the *stuA*α RNA start site (regions I, II, and III) and therefore expresses only the developmentally regulated *stuA*α mRNA. Conidiophore morphology of UI 91 resembled that of a *stuA* null strain with greatly shortened aerial hyphae, varying from 1/4 to 1/10 of those of the wild type. Conidiophore vesicles were greatly reduced and had only a few (five to seven) buds on the surface (Fig. 6). However, unlike the *stuA* null strain, UI 91 differentiated metulae, phialides, and short conidial chains. Conidial yields were 20% of wild-type levels. Induction of the asexual cycle and morphogenesis of the various cell types in UI 91 were temporally the same as for the wild type, as in the other deletions described above. Similarly, conidiophore density was not significantly affected (data not shown). Therefore, the conidial yield/square centimeter and the yield of conidia/conidiophore decreased in similar proportions for all deletion strains (Table 4).

***stuA*α and *stuA*β transcriptional units do not contribute equally to development.** When region I was positioned directly upstream of the *stuA*α promoter region, there was a twofold increase in conidial yields compared to the *stuA*α promoter alone (Fig. 3, UI 98 versus UI 91; Table 4). A novel phenotype was observed for the sexual reproductive cycle. Unorganized masses of mature, viable ascospores were observed 9.5 days after developmental induction, approximately twice the time required for the wild type (Table 4). However, there was no apparent Hülle cell development, and the dikaryotic tissue and ascospores were not enclosed within cleistothecia.

Internal deletion of sequences from −713 to −386 in UI 92 places both regions I and II directly upstream of the *stuA*α promoter by eliminating the *stuA*β promoter and RNA start site (Fig. 3). Conidiophore morphology of UI 92 was not readily distinguishable from that of the wild type, and conidial yields (76%) were near wild-type levels (Table 4). The number of cleistothecia produced by UI 92 was also similar to that of the wild type (data not shown). Hülle cell, cleistothecium, and ascospore development were normal, but there was a 1-day delay relative to the wild type for all cell types (Table 4). Therefore, essentially normal development of both reproductive cycles occurred in the absence of *stuA*β transcription. By contrast, strain UI 90 cannot express the *stuA*α transcript because an internal deletion from −386 to +201 eliminates the

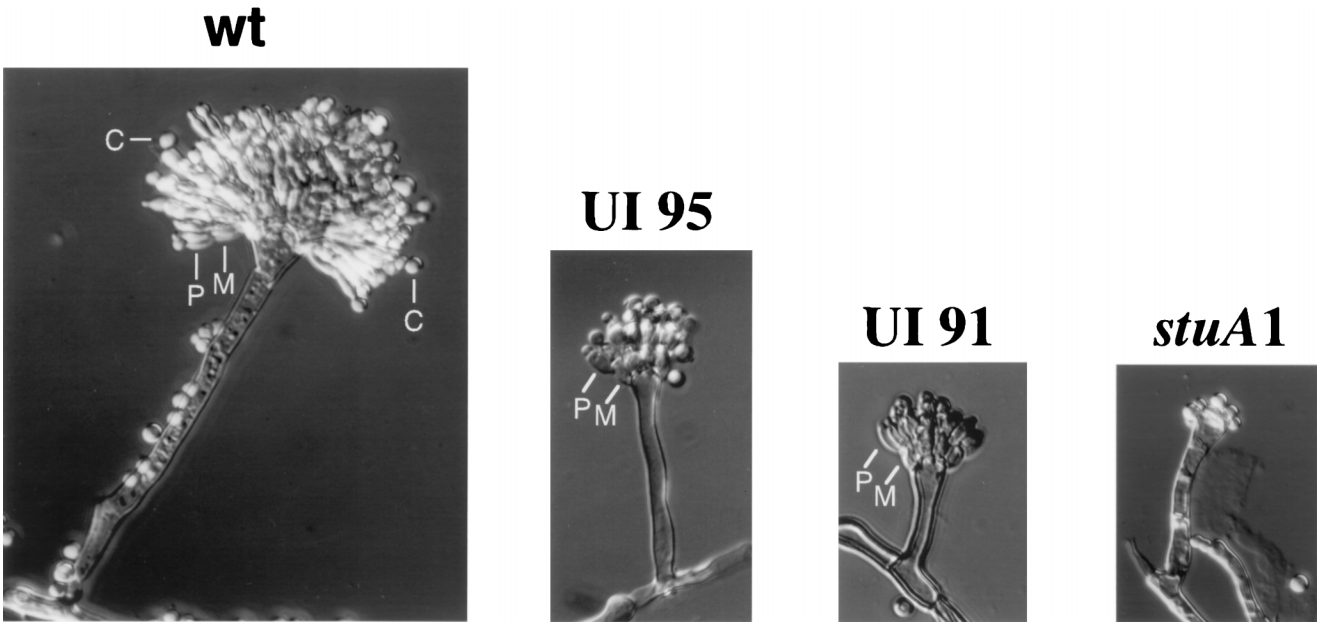

FIG. 6. Altered conidiophore morphology in upstream deletion strains. Differential interference contrast micrographs are shown for typical conidiophores from selected strains. Deletion strains not shown form a gradient of morphologies between those shown. Uninucleate sterigmata: M, metula; P, phialide; C, conidium. wt, wild type.

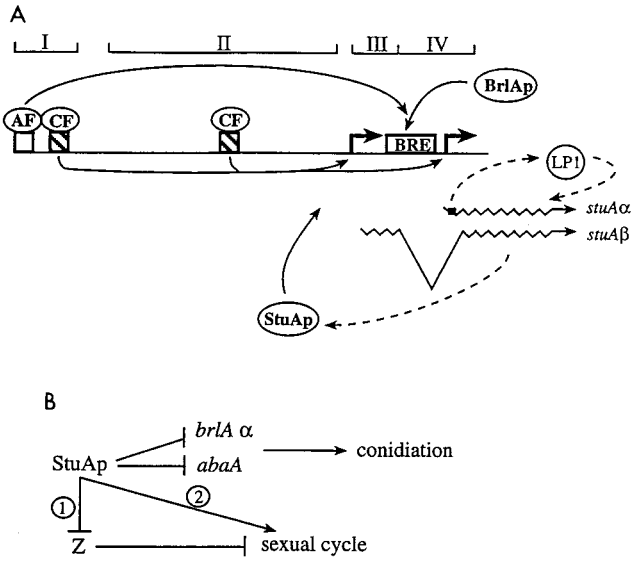

FIG. 7. Model of regulatory mechanisms controlling *stuA* gene expression. (A) Regions I to IV are described in the text. Only *stuA*β intron I is indicated. See Discussion for details. Open boxes indicate potential upstream response elements. AF, general activation factor(s); CF, competence factor(s); closed box, *stuA*α-specific μORF; solid arrows, transcriptional control; dashed arrows, translational control. (B) Z, hypothetical repressor.

*stuA*α promoter and the 5′ end of the *stuA*α transcript through fusion of the first and second *stuA*β exons (Fig. 3). Conidiophore morphology of UI 90 was readily distinguishable from that of the wild type, being similar to that of UI 95 (Fig. 6). Conidial yields were 34% of the wild-type yields. The number of cleistothecia formed by UI 90 and UI 90.1 appeared similar to the wild-type number (data not shown). However, Hülle cell development was delayed by ~2.5 days. Cleistothecium development and ascospore development were even more severely affected, being delayed by ~4 days (Table 4).

The *stuA*α leader-encoded peptide (LP1) is required for normal asexual and sexual reproduction. Strains UI 96 and UI 97 contain the μORF1 deletions mLP1.1 and mLP1.2, respectively. Both strains produced conidiophores with normal morphology, but conidial yields were reduced to 84 and 70% of wild-type levels for UI 96 and UI 97, respectively (Table 4). The mLP1.1 deletion had a greater impact on sexual reproduction than on asexual reproduction, with Hülle cell, cleistothecium, and ascospore development being delayed by 2 days relative to the wild-type strain (Table 4).

DISCUSSION

The *stuA* locus encodes two overlapping transcriptional units that are differentially regulated from the *stuA*α and *stuA*β promoters. Competence-dependent *stuA* expression is the earliest-identified transcriptional regulatory event of the *A. nidulans* developmental cycle and the only known molecular marker for the time at which developmental competence is established. Developmental induction results in an additional increase in *stuA*α RNA abundance, although not enough to account for the large increase in reporter expression observed during differentiation. This observation plus the long 5′ untranslated leaders of both *stuA* mRNAs led us to propose that translational regulatory mechanisms also play an important role in *stuA* expression (51). We have found that at least five mechanisms contribute to *stuA* expression during the *A. nidulans* life cycle (Fig. 7A). A determination of the specific quantitative contribution for any individual mechanism is confounded by their interdependence, as described below.

Multiple mechanisms control competence-dependent and induction-dependent expression from the *stuA*α and *stuA*β promoters. In precompetent vegetative hyphae, *stuA* reporter expression is negligible (1 to 2 U of specific activity), and neither *stuA*α nor *stuA*β transcripts are detected in Northern blots (50, 51). Strains expressing only the *stuA*α::*lacZ* or *stuA*β::*lacZ* reporter indicate that the *stuA*α and *stuA*β promoters contribute equally to expression in competent vegetative hyphae. However, *stuA*β transcription makes only a minor contribution to induction-dependent, *brlA*-dependent changes in *stuA* gene expression. These conclusions are based on several observations. The abundance of *stuA*β mRNA changes only about twofold after developmental induction (Fig. 4 and reference 51). Further, the developmental regulators BrlAp and AbaAp would not be expected to affect *stuA*β transcription because the *stuA*β promoter has neither BrlAp or AbaAp response elements (46a, 51). The small increase in StuAp:β-Gal activity during development in a strain expressing only *stuA*β::*lacZ* (UI 38.5) is consistent with our earlier observation of an induction-dependent, BrlAp-independent component to *stuA* regulation and represents the maximum contribution of the *stuA*β transcriptional unit during development. Induction-dependent *stuA* gene expression is primarily through *stuA*α expression. Only *stuA*α and *stuA*α::*lacZ* RNA abundance increased significantly after developmental induction. Most of this increase in reporter expression was BrlAp dependent, probably through the direct effect of BrlAp-activated transcription from the nine BREs located within 354 bp of the *stuA*α transcriptional start site (Fig. 7A). Direct regulation by BrlAp is indicated by the absence of AbaAp response elements upstream of either the *stuA*α or the *stuA*β RNA start site and enhanced reporter expression by ectopic *brlA*, but not *abaA*, expression.

Additional upstream DNA sequences function to enhance vegetative expression from both *stuA*α and *stuA*β promoters and developmental expression from the *stuA*α promoter. The 447-bp region I was critical for enhanced reporter expression in developmental tissue but not in competent, uninduced vegetative hyphae. Furthermore, region I enhanced developmental expression five- to eightfold when positioned directly upstream of the *stuA*α promoter (UI 38.9 versus UI 38.3). Because developmental regulation would be primarily through changes in *stuA*α::*lacZ* expression, we propose that enhanced reporter expression is mediated, at least in part, through synergistic interactions between a development-specific transcriptional activation factor at a UAS within region I and BrlAp located at BREs of the *stuA*α promoter (Fig. 7A). By contrast, a comparison of strains UI 38.0, UI 38.2, UI 38.5, and UI 38.6 and control strain UI 38.1 indicates that DNA sequences and associated factors within region II have a predominant role in regulating reporter expression in competent vegetative hyphae but do not significantly affect developmental expression. Furthermore, Northern blot analysis indicates that region II affects both *stuA*α::*lacZ* and *stuA*β::*lacZ* mRNA abundance. Therefore, we propose that region II includes response elements for competence factors that enhance expression from both *stuA*α and *stuA*β promoters (Fig. 7A).

The level of reporter expression in competent vegetative hyphae of a strain having a construct with only the *stuA*α promoter (UI 38.3) is similar to that observed in precompetent tissue of the wild-type control strain. In the absence of region II, it was observed that region I also enhanced reporter expression from the *stuA*α promoter by five- to eightfold in vegetative

hyphae (UI 38.3 versus UI 38.9). This finding suggests that the activation factor(s) at region I may not be a development-specific factor but a general activator always present at the UAS after T_c. However, a comparison of strains UI 38.3, UI 38.9, UI 38.6, and UI 38.4 suggests that competence factor response elements may be present in both regions I and II and contribute in an additive fashion to *stuA* expression at T_c. Numerous palindromic and repeated sequences are located within regions I and II, some found only in region I and others common to both regions I and II (46a). Thus, it is possible that competence factors bound to response elements in both regions I and II interact with basal transcription apparatuses found at the *stuA*α and *stuA*β promoters (Fig. 7A).

We have found that *stuA* expression is enhanced by a regulatory loop in which StuAp positively regulates *stuA* expression (Fig. 7A). Therefore, the effect of any deletion that reduces either *stuA*α or *stuA*β transcription would be compounded through this mechanism, particularly during development. Positive feedback regulatory loops have also been described for the *A. nidulans* genes *abaA* and *brlA* (6, 8, 54). However, unlike the case for *abaA* and *brlA*, a direct feedback regulatory mechanism probably does not function for *stuA*. No StuAp response elements are found upstream of either the *stuA*α or the *stuA*β RNA start site (46a). Furthermore, StuAp is a DNA sequence-specific transcriptional repressor in *A. nidulans* (29).

The product of the *stuA*α mRNA 5′ UTR-encoded μORF1 is required for positive translational regulation of *stuA*α expression. Mutations in the 24-codon μORF1 affected StuAp:β-Gal activity but not *stuA*α::*lacZ* mRNA abundance. The 12-codon mutant μORF mLP1.1 maintained the same termination codon as the wild-type μORF1 and therefore the same scanning distance between the end of μORF1 and the start of the *stuA* ORF. Mutant μORF mLP1.2 would shorten this scanning distance by 114 nt, from 985 to 871 nt. This small change in scanning distance would not be expected to have a major effect unless translational reinitiation mechanisms are highly specific in *A. nidulans*. Taken together, these results suggest that the mere presence of a μORF in the *stuA*α 5′ UTR is insufficient for positive translational control and that LP1 is an essential regulatory component. This is in contrast to well-characterized mechanisms controlling GCN4 expression in budding yeast, where reinitiation at the downstream GCN4 ORF is controlled by the relative position of the μORFs, not by their peptide products (1, 27, 57, 73).

There are numerous examples from both prokaryotes and eukaryotes in which mRNA leader-encoded peptides are effector molecules required for *cis* regulation of the downstream ORF (44, 45; for a review, see reference 42). In all cases so far described, translation of leader-encoded μORFs attenuates translation of the downstream ORF. A number of these μORFs are related in sequence, having an Asp codon near the 3′ end of the coding region that is essential for function (42). By contrast, the *stuA*α μORF1 is required for positive *cis* regulation of the StuAp ORF, and the μORF1 sequence appears to be unique to the *stuA*α mRNA. This finding suggests that translational regulation of the *stuA*α mRNA may involve novel mechanisms. Notably, the five- to sevenfold developmental regulation of the $stuA(p/l)_{\mu ORF1}$::*lacZ* reporter was independent of the allelic state of *brlA* and therefore independent of BrlAp-enhanced *stuA*α transcription. Our results suggest that during development, there is an induction-dependent, BrlAp-independent alteration in the translational machinery and, further, that the ability to reinitiate translation at the downstream ORF of the *stuA*α mRNA is affected by an uncharacterized BrlAp-dependent mechanism.

Asexual reproduction and sexual reproduction have different threshold requirements for *stuA* expression. The regulatory mechanisms described above differentially affected the asexual and sexual reproductive cycles of *A. nidulans*. Translation of either *stuA*α or *stuA*β mRNA would yield identical proteins. Therefore, the phenotypes of strains with various structural gene deletions are a result of altered transcriptional and/or translational mechanisms controlling StuAp concentration, not synthesis of qualitatively different proteins. Internal promoter deletions indicate that the *stuA*α transcriptional unit has the most significant role during both reproductive cycles. *stuA*β expression appears to make a predominately quantitative contribution to the level of *stuA* expression and to development, rather than being essential. Interestingly, these same internal promoter deletions upstream of the *lacZ* reporter yielded quantitatively similar levels of β-galactosidase activity in both vegetative and developmental tissues. By analogy, this result would predict similar amounts of StuAp in UI 90/UI 90.1 and UI 92/UI 92.1. The distinct phenotypes of these mutants, however, suggest qualitative spatiotemporal differences in StuAp localization. Developmentally regulated, BrlAp-dependent *stuA* expression is spatially localized to the vesicle, metulae, and phialide. This is a consequence of induction-dependent *brlA* expression which is itself spatially localized to the conidiophore vesicle, metulae, phialides, and immature conidia. The major contribution to developmental *stuA* gene expression would be from changes in *stuA*α mRNA abundance and/or translational efficiency. Therefore, only deletion strains with the intact *stuA*α promoter would respond to BrlAp regulation.

Upstream regions I and II are both essential for normal development. Strains with only region I upstream of the *stuA*α promoter had dramatically reduced conidial yields, intermediate-size conidiophores, and a limited number of disorganized masses of ascospores (UI 98 and UI 98.1). Strains with only the *stuA*α promoter had minimal levels of expression and differentiated only a few normal metulae and phialides on a stunted conidiophore (UI 91). None of the sexual cycle tissue types were evident. The importance of regions I and II was also evident in strains having both *stuA*α and *stuA*β promoters. Deletion of region I had the greatest quantitative impact on both reproductive cycles. The sexual cycle was completely eliminated in strains lacking both regions I and II (UI 94 and UI 94.1). These results indicate that factors located in both regions I and II are required to provide sufficient levels of *stuA* expression for normal development of the two reproductive cycles. Notably, limited asexual and sexual reproduction is still observed even in the absence of developmentally regulated *stuA*α mRNA, as long as regions I and II are present to provide sufficient levels of *stuA*β expression (UI 90 and UI 90.1).

Reduced *stuA* expression differentially affected temporal development and morphogenesis of the two reproductive cycles. Conidiophore morphology was altered and conidial yields were quantitatively reduced as the level of *stuA* expression declined, but neither temporal development nor conidiophore density was affected. By contrast, the sexual cycle showed both a temporal delay and a quantitative reduction in the number of cleistothecia, while morphologies of the various cell and tissue types appeared normal. In only one example (UI 98) did ascosporogenesis occur in the absence of other sexual cell types. Although little is known about events initiating the sexual cycle, this result would suggest that the differentiation of dikaryotic tissue and ascosporogenesis are independent of cleistothecia and associated Hülle cell development. The response of the two reproductive cycles is likely due to the different roles that StuAp plays in these processes. Initiation of

asexual development and terminal differentiation of conidia are BrlAp driven. StuAp plays a modifying role by presumably repressing and spatially restricting *brlA*α and *abaA* transcription, thus allowing elaboration of the conidiophore (references 14 and 29; Fig. 7B). In contrast to conidiation, sexual reproduction is *stuA* dependent but *brlA* and *abaA* independent. This finding suggests that there are sexual cycle-specific StuAp target genes and/or that the sexual cycle requires different spatiotemporal gradients in StuAp concentration. StuAp may function as an activator of the sexual cycle (Fig. 7B). Alternatively, StuAp may block expression of a repressor of the sexual cycle (Fig. 7B) in a manner similar to the repression of RME (repressor of meiosis) by the a1/α2 repressor in diploid yeast (25, 26).

Because translation of either *stuA*α or *stuA*β mRNA yields the same protein, a comparison of specific mutations and their effects on both reporter expression and morphogenesis suggests that these multiple regulatory mechanisms are used to generate necessary threshold concentrations of StuAp. Our results suggest that threshold requirements for the asexual and sexual reproductive cycles are as follows: metula-phialide differentiation < ascosporogenesis < cleistothecial shell-Hülle cell differentiation. Once minimal threshold levels were met, there appeared to be a quantitative response in which increasing levels of StuAp resulted in increasing conidiophore size and conidial yields. Similarly, temporal delays in the sexual cycle were shortened and the number of cleistothecia increased. The structural organization of the *stuA* gene must reflect the regulatory mechanisms required to generate the spatiotemporal gradients in *stuA* expression that we have previously described. These results also suggest that StuAp likely regulates different classes of genes through concentration-dependent mechanisms. This relationship between gene structure and function and the formation of gradients in regulatory factors is similar to that found for other higher eukaryotic genes essential for morphogenesis (11, 24, 30, 31, 58, 62, 67, 68).

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