StuAp is a sequence-specific transcription factor that regulates developmental complexity in *Aspergillus nidulans*

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The *Aspergillus nidulans* Stunted protein (StuAp) regulates multicellular complexity during asexual reproduction by moderating the core developmental program that directs differentiation of uninucleate, terminally differentiated spores from multinucleate, vegetative hyphae. StuAp is also required for ascosporeogenesis and multicellular development during sexual reproduction. StuAp is a member of a family of fungal transcription factors that regulate development or cell cycle progression. Further, StuAp characterizes a sub-family possessing the conserved APSES domain. We demonstrate for the first time that the APSES domain is a sequence-specific DNA-binding domain that can be modeled as a basic helix–loop–helix (bHLH)-like structure. We have found that StuAp response elements ($A_TCGCGT/AN^A/C$) are located upstream of both critical developmental regulatory genes and cell cycle genes in *A.nidulans*. StuAp is shown to act as a transcriptional repressor in *A.nidulans*, but as a weak activator in budding yeast. Our data suggest that the differentiation of pseudohyphal-like sterigmatal cells during multicellular conidiophore development requires correct StuAp-regulated expression of both developmental and cell cycle genes in *A.nidulans*. The budding pattern of sterigmata may involve processes related to those controlling pseudohyphal growth in budding yeast.

Keywords: APSES domain/cell cycle/development/Mbp1/pseudohyphal growth

Introduction

Production of asexual spores (conidia) by an elaborate conidiophore in *Aspergillus nidulans* is the culmination of cellular differentiation determined by complex spatio-temporal gene interactions (Miller, 1993; Timberlake, 1993; Busby *et al.*, 1996). Following germination of uninucleate conidia, multinucleate vegetative hyphae grow by apical extension. The developmental program can be activated by environmental cues after the mycelium has attained developmental competence during a period of vegetative growth (Axelrod *et al.*, 1973; Champe *et al.*,

1981; Mooney and Yager, 1990; Lee and Adams, 1994). During development of the multinucleate conidiophore vesicle, growth by apical extension ceases and a transition occurs to an apolar or budding mode of growth. This is followed by the formation of two layers of uninucleate reproductive cells, the metulae and phialides. Chains of conidia arise from repeated interstitial budding of the phialides. Development of uninucleate cells from the multinucleate vesicle requires strict coordination between the nuclear division cycle and cytokinesis (Mirabito and Osmani, 1994).

Analysis of aconidial mutants and mutants that develop aberrant conidiophore morphology has revealed genetic interactions required for multicellular development (Clutterbuck, 1969; Martinelli and Clutterbuck, 1971). *bristle* (*brlA*) null mutants fail to differentiate beyond the formation of aerial hyphae. *abacus* (*abaA*) mutants do not complete terminal differentiation to form conidia but develop chains of reiterating phialide-like cells. *brlA* and *abaA* encode transcription factors that form a linear pathway activating expression of many sporulation-specific genes (Boylan *et al.*, 1987; Adams *et al.*, 1988; Mirabito *et al.*, 1989). A *brlA*–*abaA* positive feedback regulatory loop promotes conidiation. Morphological modifiers encoded by *stunted* (*stuA*) and *medusa* (*medA*) modulate expression of the BrlAp–AbaAp pathway. *stuA* and *medA* null mutants exhibit dramatically altered conidiophore complexity but conidia formation is not prohibited (Miller *et al.*, 1992; Busby *et al.*, 1996).

The Stunted protein (StuAp) is an important regulator of multicellular development in *A.nidulans*. *stuA* null mutants form aerial hyphae that are much shorter than those of the wild-type. Reduced conidiophores lack the uninuclear cell types, the metulae and phialides. Limited numbers of single, abnormally shaped spores differentiate either from short peg-like structures or directly from buds upon the vesicle. StuAp is also required for the sexual reproductive cycle. *stuA* null mutants fail to initiate ascosporeogenesis or to develop any of the cell and tissue types characteristic of the *A.nidulans* sexual cycle. During the *A.nidulans* life cycle *stuA* transcription is regulated primarily at competence. StuAp-reporter protein is distributed uniformly in vegetative hyphae. Both transcriptional and translational control of StuAp expression then occurs following developmental induction. During conidiation, elevated reporter expression is restricted to the periphery of the conidiophore vesicle, the metulae and phialides (Miller *et al.*, 1992). Both AbaAp- and BrlAp-reporter proteins are spatially mislocalized in *stunted* mutants, being expressed uniformly throughout induced *stunted* hyphae and reduced conidiophores (Miller *et al.*, 1992; Aguirre, 1993). By contrast, increased *stuA* gene dosage in *medA*[−] strains represses both *brlA*α and *abaA* transcription. This results in the inhibition of conidiation

Fig. 1. Multiple alignment of the 97 amino acid APSES domain of the *A. nidulans* Stunted protein with similar domains from other fungal proteins. Boxed residues indicate identity. Homologous residues are shaded. α -Helical regions predicted for StuAp are represented by shaded bars (Miller *et al.*, 1992 and this work).

and the extensive reiteration of metulae-like cells that resembles pseudohyphal growth in budding yeast (Busby *et al.*, 1996).

Limited but significant amino acid similarity was initially reported between the APSES domain of StuAp and the DNA-binding domain of Mbp1p (Koch *et al.*, 1993; Busby *et al.*, 1996). Mbp1p is a member of a family of fungal transcription factors that are the DNA-binding component in heterodimeric complexes regulating periodic cell cycle gene transcription (Andrews and Herskowitz, 1989; Caligiuri and Beach, 1993; reviewed by Koch and Nasmyth, 1994). StuAp also defines a family of proteins that regulate fungal development. Members of this family possess the APSES domain and include the proteins Phd1p and Sok2p from *Saccharomyces cerevisiae*, Efg1p from *Candida albicans* and Asm1p from *Neurospora crassa* (Gimeno and Fink, 1994; Stoldt *et al.*, 1997; Ward *et al.*, 1995; Aramayo *et al.*, 1996). Asm1p has a role in spore maturation in *N. crassa*. Phd1p and Efg1p have been shown to enhance pseudohyphal growth in diploid *S. cerevisiae*. By contrast, Sok2p appears to repress the transition from unicellular growth to pseudohyphae in yeast (Ward *et al.*, 1995).

In this report the APSES domain of StuAp is shown to be a sequence-specific DNA-binding domain with structural similarity to the mammalian basic helix-loop-helix (bHLH) proteins MyoDp, Maxp and E47p. StuAp response elements (StREs) are found upstream of important developmental and cell cycle genes in *A. nidulans*. StREs are related to cell cycle regulatory elements previously described for budding and fission yeast. We also demonstrate that StuAp can function as a transcriptional repressor in *A. nidulans*. These data suggest mechanisms by which StuAp can regulate the dramatic transformation of undifferentiated hyphal elements into a complex multicellular structure. Our results further suggest that in *A. nidulans* StuAp may play an important role in integrating the expression of critical developmental regulators, such as

abaA and *brlA*, with requisite changes in cell cycle regulation.

Results

The StuAp APSES domain is homologous to DNA-binding domains of fungal cell cycle regulators

Our initial searches of sequence databases revealed limited but significant amino acid similarity between StuAp and the *S. cerevisiae* cell cycle regulators Swi4p and Mbp1p. Homology was primarily restricted to the 97 amino acid StuAp APSES domain and the DNA-binding domains of the two yeast proteins. Subsequent reports and database submissions have shown that StuAp is a member of two closely related protein families that share this domain. The APSES family includes Asm1p, Phd1p, Efg1p and Sok2p which show 69% identity within this domain (Figure 1). The second family, represented by Mbp1p, Swi4p, Res1p/Sct1p, Res2p/Pct1p and Cdc10p (Tanaka *et al.*, 1992; Caligiuri and Beach, 1993; Koch *et al.*, 1993; Miyamoto *et al.*, 1994; Zhu *et al.*, 1994), are more closely related to each other but share significant homology to StuAp in regions of conserved secondary structure within the APSES domain (Figure 1). Members of this family control fungal cell cycle progression and include proteins that bind related MCB (ACGCGTNA) and/or SCB (CACGTAAA) DNA elements (Koch and Nasmyth, 1994).

APSES domain tertiary structure

Secondary structure predictions suggest that StuAp consists mostly of random coil with α -helical structure limited to the region delineated by the APSES domain (Miller *et al.*, 1992). A preliminary comparison of the APSES domain with known DNA-binding domains suggested similarity to the bHLH motif (Murre *et al.*, 1989). In particular, the positions of critical basic residues involved in DNA binding are conserved in the second and third

(B) Alignment of the bHLH domains of MyoD, E47 and Max with the theoretical model structure of the StuAp bHLH-like domain. Colored residues correspond to those marked with arrows in (A). A, white; B, orange; C, red; D, black; E, green; F, blue; G, purple.

Residues labeled B and E (Figure 2A) help define DNA-binding specificity in Max, MyoD and E47 (see below). Basic residues labeled A, C, D, F and G are conserved between the three solved bHLH structures and the theoretical model of this region of StuAp (Figure 2A). These residues all make DNA phosphate-backbone contacts in MyoD (Ma *et al.*, 1994). X-ray crystallography shows that the Maxp residues labeled F and G and the E47p residues labeled C, D and G also contribute to DNA binding through DNA phosphate-backbone contacts (Ferre-D'Amare *et al.*, 1993; Ellenberger *et al.*, 1994).

Similarity between the StuAp APSES domain and the DNA-binding domains of Mbp1p and Swi4p suggested that StuAp might bind MCB and/or SCB boxes. The APSES domain was purified using glutathione *S*-transferase (GST) fused to amino acids 129–226 of the StuA protein (StuAbd–GST). The MCB target probe used in gel retardation experiments was a radiolabeled DNA fragment containing three *Mlu*I sites (ACGCGT) (see Materials and methods). Figure 3A shows StuAbd–GST-dependent binding of the MCB fragment. Similar results were observed using GST fused to the whole StuA protein (data not shown). No complex was formed with GST

Fig. 3. Gel retardation analysis of StuAp DNA binding. (A) A radiolabeled fragment containing three MCB sites was incubated with purified protein extracts containing either GST alone or StuAp DNA-binding domain–GST fusion protein (StuAbd–GST). (B) Analysis of DNA-binding specificity of the StuAbd–GST fusion protein. A radiolabeled MCB fragment was incubated with 25 ng StuAbd as in (A) with the addition of specific unlabeled competitor DNA fragments in the molar excess shown.

protein alone. Complex formation was DNA sequence specific. Unlabeled MCB fragments, but not unlabeled MUT or SCB fragments, competed during complex formation with labeled MCB targets (Figure 3B). These fragments are similar to the MCB fragment except that the core motif is changed to ACTaGT (MUT) or CaCGT (SCB).

StuAp promotes transcription from MCB motifs in yeast

A *lacZ* reporter gene was used to determine if StuAp could bind and activate transcription from MCB elements (ACGCGT). In preliminary experiments we were unable to detect any effect of StuAp on reporter expression in the presence of functional Swi4p/Swi6p and Mbp1p/Swi6p heteromers. Results were confounded by significant cross-competition between Swi4p and Mbp1p for both SCB and MCB elements (data not shown), consistent with reports of cross-functionality between Mbp1p and Swi4p in binding to both MCB and SCB elements (Koch *et al.*, 1993). Therefore, we used a Swi6p-deficient yeast strain which has greatly reduced transcriptional activity of both Swi4p and Mbp1p (Dirick *et al.*, 1992; Lowndes *et al.*, 1992b). Modest but significant StuAp-activated expression of the MCB reporter was observed under these conditions (Table

I). StuAp does not have a strongly acidic domain, which might explain the modest transcriptional activity of StuAp in yeast (Miller *et al.*, 1992). StuAp did not promote expression from an identical reporter in which the core of the ACGCGT motif (pMCB) had been changed to ACTaGT (pMUT).

StuAp does not enhance pseudohyphal growth in yeast

Previous reports have shown that ectopic expression of the StuAp homologs Phd1p from budding yeast and Efg1p from *Candida albicans* significantly enhance pseudohyphal growth in diploid *S.cerevisiae* (Gimeno and Fink, 1994; Stoldt *et al.*, 1997). Sok2p, another StuAp homolog from budding yeast, represses Phd1p-promoted pseudohyphal growth (Ward *et al.*, 1995). Surprisingly, given the degree of similarity to Phd1p and Efg1p, expression of StuAp in *S.cerevisiae* using either the constitutive *adc1* promoter or the inducible *gal1* promoter did not promote or enhance pseudohyphal growth (data not shown). Neither did StuAp expression suppress pseudohyphal growth caused by overexpression of Phd1p. Ectopic expression of StuAp did, however, induce flocculation and the formation of large round, vacuolated cells in both haploid and diploid *S.cerevisiae* cultures.

StuAp binds MCB-like elements in promoters of essential *A.nidulans* developmental regulatory and cell cycle genes

Both AbaAp and BrlAp are spatially mislocalized in the absence of StuAp. Analysis of the *abaA* promoter identified a functional region required for spatially restricted *abaA* expression (Adams and Timberlake, 1990). This region is encompassed by the AbaII fragment and contains a single MCB box (Figure 4). The StuAbd–GST fusion protein formed a specific complex with the AbaII fragment (Figure 5A). Competition for complex formation was observed with addition of unlabeled AbaII and MCB fragments but not MUT or SCB fragments. The *abaA* upstream sequences also contain two other consensus StREs at –651 and –718 (Figure 4). We have previously shown that overexpression of *stuA* represses *brlA* α , but not *brlA* β expression (Busby *et al.*, 1996). StuAbd–GST also formed a specific complex with DNA sequences from the *brlA* α promoter (Figure 5B) The BrlII fragment (Figure 4) contains two tandem MCB-like motifs (ACGCGA/ACGCGC). This mobility shift was abolished by addition of unlabeled MCB fragments but unaffected by the addition of MUT fragments. Homology to yeast cell cycle regulators suggested that StuAp might also regulate cell cycle genes in *A.nidulans*. We tested *A.nidulans* homologs of two cell cycle genes regulated at the G₁/S phase transition in budding yeast. We determined that StuAbd could bind MCB-like elements found in promoters of the *A.nidulans* genes *nimE*^{cyclinB} and *nimO*^{Dbf4p} (O'Connel *et al.*, 1992; S.James, personal communication) (Figures 4 and 5). These fragments compete with the binding of StuAp to a labeled MCB fragment but MUT fragments do not (Figure 5C).

StuAp represses transcription of the *abaA* developmental regulatory gene in *A.nidulans*

During normal development, correct spatiotemporal expression of *abaA* requires both functional StuAp and

Table I. Expression from MCB and SCB motif reporters in *S.cerevisiae*

Strain	StuA	Swi4	Mbp1	Swi6	β -Galactosidase specific activity ^a			
					pYC7	pSCB	pMCB	pMUT
BY600	–	+	+	–	1.5 $\pm$ 0.3	3.0 $\pm$ 0.2	3.5 $\pm$ 0.9	–
BY600pStu97	+	+	+	–	3.5 $\pm$ 0.7	6.8 $\pm$ 0.2	11.7 $\pm$ 0.4	2.7 $\pm$ 0.2

^aValues are the mean result of at least nine independent measurements.

the *AbaII* fragment described above. The ectopic expression of *brlA α* in vegetative hyphae activates *abaA* transcription and initiates the formation of conidia directly from hyphal tips (Adams *et al.*, 1988). When the strain UI76 was shifted from glucose to threonine media, expression of the *alcA(p)::brlA α* fusion gene was induced, which in turn activated *abaA* expression (Figure 6). Strain UI123 has a single copy of both the *alcA(p)::stuA* and *alcA(p)::brlA α* fusion genes, allowing ectopic expression of both *StuAp* and *BrlAp* in vegetative tissue. Following transfer of UI123 to inducing media, ectopic expression of *brlA α* was similar to UI76. However, in contrast to UI76, simultaneous ectopic *stuA* expression repressed *abaA* transcription (Figure 6).

Expression of the developmentally regulated *A.nidulans* *Awh11* gene is repressed by *StuAp*

Boylan *et al.* (1987) described the isolation and characterization of a set of developmentally regulated *A.nidulans* cDNAs, the pCAN library. Expression of these cDNAs varied both temporally and in different developmental mutants. We selected one, designated here as *Awh11*, for further study. The expression of *Awh11* is highly modified in a *stuA1* strain. Total RNA from hyphal and developmental tissues (7.5 h post-induction) from wild-type and *stuA1* strains was probed with the *Awh11* cDNA probe. This developmental time point coincides with the differentiation of metulae and phialides. A developmentally induced 450 nt transcript (Figure 7A) was observed in both strains. However, the *Awh11* transcript was detected in hyphal tissue of the *stuA1* strain but not wild-type. In addition, developmental *Awh11* expression was greatly derepressed in the *stuA1* mutant compared with that in the wild-type. Ectopic expression of *StuAp* in the *stuA1* strain was sufficient to re-establish hyphal repression of the *Awh11* transcript (Figure 7B). The *Awh11* cDNA clone was used to isolate a genomic clone from an *A.nidulans* λ library. *StuAp* interacted directly with the *Awh11* promoter (Figure 7C). Gel retardation assays show *StuAbd* binds the *Awh11A* promoter fragment that contains two MCB-like elements (Figure 4). *StuAbd* had no detectable affinity for the *Awh11B* promoter fragment that contains no MCB-like elements.

Fig. 4. Position of potential MCB-like *StuAp* response elements (StREs) in promoters of *A.nidulans* genes. Transcript start sites are indicated by the bent arrow. Positions of PCR-generated fragments used in this study are illustrated by shaded bars.

Awh11 encodes a protein homologous to a white phase-specific protein from *C.albicans*

An ORF of 79 amino acids was predicted from cDNA clones (the *Awh11* cDNA sequence has been assigned an accession number, AF016187). Database searches revealed that *Awh11p* shares 31–34% identity and 65–70% homology with a family of small heat shock proteins (Figure 8). These include *Wh11p*, a white phase-specific protein from *C.albicans* WO-1 (Srikantha and Soll, 1993).

Discussion

Stunted protein is necessary for complex cellular development

Sexual and asexual reproduction in *A.nidulans* require the transformation of undifferentiated hyphae into complex structures consisting of specialized cell types. Research

Fig. 5. Gel retardation analysis of StuAp binding to *A. nidulans* promoter fragments. (A) A radiolabeled 236 bp functional region of the *abaA* promoter (AbaII) containing a consensus MCB box was incubated with StuAbd-GST fusion protein. Lanes 2–4 contain the probe incubated with 5, 10 and 25 ng StuAbd. Lanes 5–12 contain 25 ng StuAbd incubated with the AbaII probe and specific unlabeled competitor DNA fragments in the molar excess indicated. (B) Analysis of StuAp binding to a region of the *brlAα* promoter (BrIII) with MCB-like motifs. Lanes 2 and 3 contain the BrIII fragment incubated with 20 and 40 ng StuAbd-GST. Lanes 4–7 contain the probe, 40 ng of protein and specific unlabeled competitor fragments in the molar excess indicated. (C) Analysis of binding of StuAbd-GST to promoter fragments from *A. nidulans* cell cycle genes. The binding of 25 ng StuAbd to a radiolabeled MCB fragment was challenged by the addition of unlabeled competitor fragments in the molar excess shown.

Fig. 6. StuAp represses transcription of *abaA*. *abaA* transcripts were detected in vegetative hyphae following induction of *alcA(p)::brlAα* alone, or simultaneously with *alcA(p)::stuA*. Total RNA was isolated from strains UI76 and UI123 that were grown in glucose and then transferred to either fresh glucose media or inducing threonine media for 10 h. Ten µg of total RNA was hybridized with ³²P-labeled probes specific for *abaA*, *brlA* and *argB*. *argB* and ethidium bromide-stained total RNA are included as controls.

in a number of developmental systems has addressed the formation of morphogen gradients that influence expression of target genes to enable cellular and tissue-specific differentiation (Courey and Huang, 1995). Similarly, during conidiophore development in *A. nidulans* many genes direct cellular differentiation. The ratio of these gene expressions must be correctly balanced for the full complexity of cell types to be produced (Busby *et al.*, 1996). In wild-type strains, layers of uninucleate sterigmatal cells (metulae and phialides) develop by budding from the multinucleate conidiophore vesicle. The elongated shape and polar budding pattern of these cells strongly resemble that of pseudohyphal cell division in *S. cerevisiae*. Metulae and phialides are absent in *stunted* null mutants. By contrast, branched chains of reiterated metulae are formed by *medA* mutants bearing an extra copy of *stuA* (Busby *et al.*, 1996). Furthermore, elongate, uninucleate, pseudohyphal-like cells proliferate when StuAp is over-expressed in *A. nidulans* strains ectopically expressing *brlA* (B. Miller, unpublished results). In this report we have identified StuAp as a sequence-specific transcription factor. We propose that StuAp influences morphogenesis by moderating expression of key regulatory genes such as *abaA* and *brlA* by interaction with StREs in promoter regions (Figure 9). BrlAp is an extremely potent transcriptional activator under strict transcriptional and post-transcriptional control (Han *et al.*, 1993; Prade and Timberlake, 1993). Ectopic BrlAp expression results in simple conidiophores similar to those of *stuA* nulls (Adams *et al.*, 1988). Therefore it is the parallel expression of StuAp and BrlAp during conidiophore development that establishes antagonistic spatiotemporal transcription factor gradients and allows complete aerial hyphae extension, nuclear proliferation and a controlled transition to budding growth necessary for differentiation of uninucleate sterigmatal cells. The expression of StuAp in competent uninduced hyphae may contribute to preventing premature conidiation in the absence of induction. StuAp-mediated repression of *abaA* and *brlAα* transcription may prevent inappropriate initiation of the *abaA*–*brlAα* feedback loop.

StuAp is related to known fungal transcription factors and binds similar DNA elements

The APSES domain of StuAp shares significant homology with DNA-binding domains of transcription factors con-

trolling the critical G₁/S phase cell cycle transition in both *S.cerevisiae* and *S.pombe*. Mbp1p, Swi4p, Sct1/Res1p and Pct1/Res2p are the DNA-binding component of heterodimeric transcriptional regulators that bind related MCB (ACGCGTNA) and SCB (CACGAAA) sequences (Andrews and Herskowitz, 1989; Lowndes *et al.*, 1991, 1992a; Caligiuri and Beach, 1993; Zhu *et al.*, 1994). The SCB-binding factor (SBF) containing Swi4p and Swi6p, regulates HO and G₁ cyclins in budding yeast. Mbp1p and Swi6p in *S.cerevisiae* and Res1/Sct1p and Cdc10p in *S.pombe* comprise the MCB-binding factor (MBF, also known as DSC1) (Tanaka *et al.*, 1992; Caligiuri and

Beach, 1993; Koch *et al.*, 1993). MBF regulates DNA synthesis and other cell cycle genes. In *S.cerevisiae*, genes under MBF control are repressed throughout most of the cell cycle and transcribed only at the G₁/S phase transition (Koch *et al.*, 1993; Breeden and Mikesell, 1994; Koch and Nasmyth, 1994). Mbp1p, although not essential for viability, moderates control of the cell cycle by binding to DNA and tethering the transcriptional regulator Swi6p to MCB boxes (Koch *et al.*, 1993). StuAp is dispensable during vegetative growth in *A.nidulans*. However, StuAp-mediated gene regulation is essential for correct cell differentiation, indicating that StuAp plays a moderating role during development. The family of known cell cycle regulators vary in their binding preference for MCB and SCB boxes. The StuAp sequence specificity is similar to that of Res2p/Pct1p that binds MCB but not SCB boxes, while Mbp1p and Swi4p can bind either (Koch *et al.*, 1993; Zhu *et al.*, 1994). The core CGCG of the MCB-like StRE (^A/T₁CGCGT/_AN^A/C) appears critical for binding by the StuAp APSES domain.

The APSES domain of StuAp can be modeled as a bHLH domain

We have found significant sequence similarity between the APSES domain of StuAp and bHLH DNA-binding domains. Families of interacting bHLH proteins affect cellular transformation and proliferation (Amati and Land, 1994). StuAp plays a similar role in *A.nidulans*. Although some bHLH proteins may bind DNA as homodimers, target affinity is enhanced through heterodimerization mediated by the HLH domain (Blackwell and Weintraub, 1990; Blackwood and Eisenman, 1991). Basic regions in the first helix determine primary DNA sequence specificity with further preference established by loop residues and through partner dimerization. StRE specificity (^A/T₁CGCGT/_AN^A/C) is reflected in the residues of the StuAp APSES domain. StuAp lacks the glutamate residue (labeled B in Figure 2A) that is conserved in Max, MyoD and E47. This residue contacts the CA nucleotides of the E-boxes (AACAG_C^Y/rTG) recognized by these proteins (Ellenberger *et al.*, 1994; Ma *et al.*, 1994). However, the arginine at position 177 in StuAp (labeled E in Figure 2A) is conserved throughout class B bHLH proteins such as Max. This arginine is essential for the G₃ nucleotide contact and specifies binding to a CXCG sequence in preference to the CXGC of the class A bHLH proteins (Blackwell *et al.*, 1993). The size of the loop and conserved basic residue at position F in Figure 2A may also contribute to sequence specificity

Fig. 7. Expression of the developmentally regulated *Awh11* transcript is derepressed in a *stunted* mutant. (A) Hybridization of a radiolabeled *Awh11* cDNA probe with a 450 bp transcript in total RNA from hyphal and 7.5 h developmental tissue of strains FGSC26 (wild-type) and GO256 (*stuA1*). (B) Ectopic expression of StuAp represses hyphal expression of *Awh11*. Total RNA from *stuA1* strains with and without the threonine-inducible *alcA(p1)::stuA* construct was hybridized with a radiolabeled *Awh11* probe. RNA samples were prepared from uninduced glucose-grown hyphae and from hyphae 2.5 and 5 h following threonine induction. Duplicate blots were probed with *argB* as an internal control. (C) StuAbd-GST protein binds MCB-like elements in the *Awh11* promoter. Equal amounts of probes of the same specific activity were incubated with 25 and 50 ng of StuAbd. Probe AwhIIA is a 300 bp fragment of the *Awh11* promoter that bears MCB-like elements. AwhIIB is a 290 bp region of the *Awh11* promoter that has no MCB-like sequences.

Awh11	1	M	S	D	F	A	R	K	D	F	S	T	K	A	K	E	E	I	T	P	D	A	S	K	S	T	Q	E	R	V	K	E	T	V	T	D	T	T	D	R	I	S	R	G	V	Q	
Wh11	1	M	S	D	L	G	R	K	D	I	G	D	K	I	E	S	K	L	T	P	D	S	Q	K	S	T	P	E	Q	F	K	D	K	V	T	D	S	L	D	S	A	A	G	K	A	T	
Hsp12	1	M	S	D	A	G	R	K	G	F	G	E	K	A	S	E	A	L	K	P	D	S	Q	K	S	Y	A	E	Q	G	K	E	Y	I	T	D	K	A	D	K	V	A	G	K	V	Q	
Hsp9	1	M	S	D	P	A	R	K	S	F	T	E	Q	G	K	E	K	M	T	P	D	S	S	K	S	T	L	D	K	A	K	E	S	I	T	G	A	Y	D	K	V	A	S	A	F	T	
Awh11	46	P	D	D	Q	K	S	T	T	Q	Q	A	F	D	K	S	Q	R	V	S	D	R	E	G	H	G	S	T	P	S	L	L	A	I	R												
Wh11	46	S	E	N	D	K	S	F	V	Q	K	A	S	D	A	I	F	G	D	S																											
Hsp12	46	P	E	D	N	K	G	V	F	Q	G	V	H	D	S	A	E	K	G	K	D	N	A	E	G	Q	G	E	S	L	A	D	Q	A	R	D	Y	M	G	A	A	K	S	K	L	N	
Hsp9	46	S	D	E	D	K	S	T	S	Q	E	A	H	D	K	A	Q	R	F	V	D	D	K	L																							
Hsp12	91	D	A	V	E	Y	V	S	G	R	V	H	G	E	E	D	P	T	K	K																											

Fig. 8. Alignment of the amino acid sequence of Awh11p and members of a family of heat shock proteins. Boxed residues are identical. Wh11p is specific to white phase *C.albicans* WO-1 (Srikantha and Soll, 1993). Hsp12p and Hsp9p are from *S.cerevisiae* and *S.pombe* respectively (Praekelt and Meacock, 1990; Orlandi *et al.*, 1996).

(Ma *et al.*, 1994). In some cases, binding affinity of a bHLH protein may vary significantly from the canonical E box. For example, the budding yeast protein Rtg3 binds to the GGTCAG motif (Jia *et al.*, 1997). DNA binding specificity and transcriptional regulation of specific target genes by bHLH proteins are also determined by dimerization preferences, association with accessory proteins, affinity for non-canonical sequences and the position of the target sequence with respect to the transcript start. Thus, possession of a bHLH-like region could allow StuAp to bind target genes with the potential for diverse transcriptional regulation through possible dimerization with different partners. The role of the conserved first helix of the APSES domain remains unknown and will require studies using site-specific mutations and co-crystallographic studies of APSES domain and StRE.

StuAp regulates transcription in *A.nidulans*

We previously showed that spatiotemporal expression of the developmental regulator *abaA* was derepressed in a *stuA* null strain. In this report we have shown that *Awh11* transcription is also derepressed in *stuA* null strains. By contrast, ectopic expression of StuAp represses *abaA* transcription. The *abaA* promoter has three StREs, one of which lies within a functional region previously identified as necessary to repress hyphal expression of *abaA*. (Adams and Timberlake, 1990). *brlA* is a complex locus consisting of two overlapping transcriptional units (Prade and Timberlake, 1993). Like *abaA* and *Awh11*, *brlA* expression is derepressed in a *stuA* null strain. StRE motifs in the *brlA* promoter bound by StuAp in our experiments are situated above the *brlA* RNA start site. No StREs are found upstream of the *brlA* start site. It is the *brlA* transcript that is repressed in *medA*⁻ strains that overexpress *stuA* (Busby *et al.*, 1996). Therefore, we propose that StuAp binds to the MCB-like StREs upstream of the *abaA* and *brlA* RNA start sites and functions as a repressor to spatially restrict their expression.

Loss of StuAp results in the derepression of the developmentally regulated *Awh11* transcript in both hyphal and developmental tissues. Ectopic expression of StuAp was sufficient to restore repression in hyphae through StRE elements present in the *Awh11* promoter. Awh11p is a member of a family of small chaperone/heat shock proteins that includes Wh11p from *C.albicans* WO-1. This strain can readily switch between white and opaque cell phenotypes. White cells can undergo a bud-hyphae transition

		Tested Sites	Potential sites
SCB	CACGAAA	-	
MUT	AGACTAGTCT	-	
MCB	AGACGCGTCT	+	
<i>abaA</i>	GAACGCGTGA	+	-192bp
			ATACGCGTCC -651bp
			TTTCGCGATA -718bp
<i>nimE</i>	TTTCGCGACA	+	-84bp
	AATCGCGAGA	+	-199bp
<i>Awh11</i>	CTTCGCGAAC	+	+17bp
RC	CTTCGCGCAA	+	-110bp
<i>brlAa</i>	GAACGCGAAC	+	-193bp
	GAACGCGCCC	+	-187bp
			CATCGCGTTT -404bp
<i>nimO</i>			GCTCGCGCTC +187bp
			AGTCGCGTGC +156bp
RC	CACGCGTCC	+	-153bp
	AATCGCGTGT	+	-172bp
			CTCCGCGATT -416bp
	A _{1/2} CGCG T _{1/2} A N A _{1/2}		Consensus StuAp Response Element (StRE)
	A CGCG T N A		MCB box

Fig. 9. Sequence of the consensus StuAp response element (StRE). Sequences bound by StuAbd in this study are indicated (+). Underlined residues fit the consensus StRE sequence. The distance of tested and potential StREs from transcript start sites is shown. RC denotes reverse complement.

but the opaque cells cannot. The transitions from opaque to white budding cells and from hyphae to budding in white cells involve activation of *wh11* transcription (Srikantha and Soll, 1993). In *A.nidulans*, StuAp-mediated expression of *Awh11* coincides with the hyphae to budding transition during conidiophore development (*Awh11* is pCan44 in Mooney and Yager, 1990). Therefore, StuAp affects a pattern of gene expression in *A.nidulans* that is conserved in other fungi during homologous developmental events.

StuAp promotes pseudohyphal-like cell development in *A.nidulans*

In the absence of StuAp the transition to budded spores occurs with no pseudohyphal-like sterigmatal cells. However, increasing levels of StuAp expression promote increasing conidiophore complexity (Wu and Miller, 1997). In addition, overexpression of StuAp promotes extensive uninucleate polar budding and therefore proliferation of sterigmata at the expense of conidial differentiation. In the wild-type, these pseudohyphae-like sterigmata

represent an intermediate cell form between multinucleate, apically extending hyphae and spherical, budded conidia. Cytokinesis and progression of the nuclear division cycle are subsequently tightly coordinated during this stage of development (Mirabito and Osmani, 1994). Kron *et al.* (1994) suggest that the pseudohyphal cell shape and division pattern in *S.cerevisiae* is achieved by a G₂/M checkpoint that delays daughter cell mitosis until attainment of critical cell size. StuAp may promote pseudohyphal-like growth of metulae and phialides in part by regulating expression of cell cycle genes such as *nimO* and *nimE*. In delaying the transition to a spherical cell type and promoting a pseudohyphal, polar budding pattern, the consequence of StuAp expression in *A.nidulans* is analogous to the result of Phd1p overexpression in budding yeast (Gimeno and Fink, 1994). However, important differences in the regulation of these two processes are suggested by our observation that StuAp expression in budding yeast neither promotes nor blocks induction of pseudohyphal growth. By contrast, AbaAp promotes pseudohyphal growth in budding yeast but not sterigmatal development in *A.nidulans*. AbaAp, a homolog of the yeast transcription factor Tec1p, activates Tec1p target genes which include those involved in pseudohyphal growth (Gavrias *et al.*, 1996; Madhani and Fink, 1997).

Another intriguing parallel between the role of StuAp in *A.nidulans* and cell cycle control in *S.cerevisiae* is also suggested. An important function of Mbp1p during the budding yeast cell cycle is to activate transcription of the B-type cyclin genes *clb5* and *clb6* via MCB boxes. The increased pool of cyclins in concert with Cdk1p initiates chromosome replication (Schwob and Nasmyth, 1993). This in turn requires the activity of the Cdc7p/Dbf4p heterodimer (Dowell *et al.*, 1994). *nimE* encodes the *A.nidulans* B-type cyclin and *nimO* encodes a Dbf4p homolog (O'Connel *et al.*, 1992; S.James, personal communication). Moderation of the expression of these genes by StuAp may delay completion of DNA synthesis and mitosis to allow elongation of metulae and phialide cell types.

Materials and methods

Media and growth manipulation

A.nidulans strains were grown in appropriately supplemented media as described by Pontecorvo *et al.* (1953) and Käfer (1977). Standard genetic

techniques were employed as described by Pontecorvo *et al.* (1953) and Clutterbuck (1974). *Aspergillus* strains were transformed by standard methods (Yelton *et al.*, 1984). Synchronous induction of conidiophore development was performed as described by Miller *et al.* (1992). Threonine induction of *alcA(p)::stuA* was carried out as follows. Minimal medium containing 2% glucose carbon source was inoculated with $\sim 2 \times 10^6$ spores/ml and grown with shaking (200 r.p.m.) for 17 h at 37°C. Half the hyphal tissue was then collected on Miracloth and washed twice with 100 ml distilled water and once with 100 ml minimal medium containing 100 mM L-threonine before being resuspended in threonine medium and incubated with shaking at 37°C. The remaining hyphae were washed with water and 2% glucose medium before resuspension in glucose media for continued incubation.

Standard *S.cerevisiae* methods and media were employed (Sherman *et al.*, 1991).

Strains

The *A.nidulans* strains used in this study were: UI 123 [*alcA(p)::stuA*; *alcA(p)::brlA*; *biA1*]. This strain was progeny from a cross between University of Idaho stock strains UI35.1 [*stuA1*, *adG14*; *alcA(p)::stuA*] and UI 76. UI76 [*yA2*, *pabaA1*; *alcA(p)::brlA*; *stuA(p)::lacZ*]. UI127 [*stuA1*, *biA1*; *argB2*; *alcA(p)::stuA*] is progeny from a cross between PW1 (*biA1*; *methG1*, *argB2*) (a gift from P.Weglenski) and UI35.1. GO256 (*biA1*, *stuA1*) (a gift from J.Clutterbuck). FGSC26 (*biA1*) was from the Fungal Genetics Stock Centre. The *S.cerevisiae* strains used in this study were: BY600 (*a swi6TRP1-197 ade2 ho::lacZ ura3 his 3 leu2-3 -112 trp1-1 can1-100 met*) (a gift from Linda Breeden); BY606 (*a swi4DLEU2-194 ade2 holacZ ura3 his3 leu2-3 leu2-112 trp1-1 can1-100 met*); CY128 (*a MBP1::HISG ade2-1 trp1-1 can1-100 leu2-112 his3-11 his3-15 ura3 GAL psi*) (a gift from Christian Koch).

Preparation of PCR products

PCRs were performed using Vent (New England Biolabs, Beverly, MA) and Taq Gold (Perkin Elmer Corporation, Foster City, CA) polymerases as described in the manufacturers' instructions. The primer pairs listed in Table II were used with the appropriate template. The primer sequences are written 5'→3' and the numbers designate nucleotide residues on the templates referenced. PCR products were resolved by 2% agarose gel electrophoresis and purified using Elutip columns (Schleicher and Schuell, Keene, NH).

Plasmids

Aspergillus nidulans. pRA91 minimal *amdS(p)::lacZ* reporter plasmid containing *Arg::CAT* (Aramayo and Timberlake, 1993); pRA-MCB, PCR fragment containing *MluI* sites from pMCB cloned into the *BamHI* site of pRA91. pRA91 constructs are targeted to the *argB* gene of *A.nidulans*.

Saccharomyces cerevisiae. pStu97, StuAp expressed from the constitutive pADC1 promoter in pPC97 (Chevray and Nathans, 1992); pYC7, 2 µm plasmid (pLGA178) with additional polylinker and containing minimal *CYC1 (p)::lacZ* fusion (Guarente and Mason, 1983; Chang and Timberlake, 1993); pMCB, 3× *MluI* sites (3× ACGCGT) cloned in pLGA178 (Lowndes *et al.*, 1992a); pMUT, 3× ACtAGT cloned in pLGA178

Table II. Oligonucleotides used in this study

Fragment	Oligonucleotides	Template	Reference
Mcb	URA: CTCACAAATTAGAGCTTC CYC: GTCAGCACTAAAGTTGCC	pMCB	Lowndes <i>et al.</i> , 1992a
Mut	URA and CYC	pMUT	Lowndes <i>et al.</i> , 1992a
Scb	URA and CYC	pSCB	Breeden and Nasmyth, 1985
AbaII	Aba-224: GCGGATCCTGTCTCGCTTTCTTT Aba-39: GCGGATCCGGGAGAGCTCAGGAA	<i>abaA</i> promoter	Mirabito <i>et al.</i> , 1989
BrIII	Brl-225: GGAAGATCTTTTACATTCCTCGC Brl-143: CCGGATCCCCAAAGGAGAAGT	<i>brl A</i> promoter	Adams <i>et al.</i> , 1988
AwhIIA	Awh300a: GTGTACGCCATCAGAG Awh500: TCGATGTGATTTTCAGTG	<i>AwhII</i> promoter	this study
AwhIIB	Awh108: CCGTTATCATGACGTCG Awh300b: CTACCCACGGAGTTTTG	<i>AwhII</i> promoter	this study
NimO	NimO350: GGAAGATCTAAGCAGCAGCATCG NimO475: CCGGATCCGTTCTGTTTGTGTTGC	<i>nimO</i> promoter	S.James, personal communication
NimE	NimE-248: GCGGATCCTAACCGCTGTTTGTGTTG NimE-1: CCGGATCCGATAGGCCGAAAGAAGG	<i>nimE</i> upstream region	this study

(Lowndes *et al.*, 1992a); pSCB, Swi4-binding motifs (CACGAA) cloned in pLGΔ178 (Breedon and Nasmyth, 1985)

β-Galactosidase assays

Growth of strains and preparation of cell-free extracts of *S.cerevisiae* for *in vitro* β-galactosidase assays were as described by Aramayo and Timberlake (1993). *In situ* localization of β-galactosidase activity in *A.nidulans* was performed by flooding chloroform-permeabilized colonies with buffered X-Gal (Miller *et al.*, 1992). Specific activity of β-galactosidase was determined using a chromogenic assay with the substrate ONPG (Miller, 1972; Hamer and Timberlake, 1987). Protein concentrations were determined by the method of Bradford (1976). β-Galactosidase activity in yeast was visualized by growing colonies on supported nitrocellulose filters placed on solid medium. The filters were frozen in liquid nitrogen and placed on Whatman 3MM filter paper saturated in buffered X-Gal and incubated at 37°C for 40 min.

Nucleic acid manipulations

Standard DNA manipulations were performed as described by Sambrook *et al.* (1989). Hybridization probes were labeled with ³²P by random priming. Southern hybridization and Northern analysis were performed as described by Sambrook *et al.* (1989) and the manufacturers of Hybond N (Amersham, Cleveland, OH). DNA was sequenced as described by Miller *et al.* (1992) and by the manufacturers of Sequenase (United States Biochemical Corporation, Cleveland, OH).

Preparation of StuAp–GST fusion proteins

Expression vectors for GST fusion proteins were constructed using pGEX-2T (Pharmacia, Piscataway, NJ). Plasmid pStuAbd consists of the *stuA* gene sequence coding for amino acids 129–226 cloned in-frame in pGEX-2T. StuAbd–GST fusion protein expression in *Escherichia coli* was induced as described by Guam and Dixon (1991). Cells were lysed by sarkosyl treatment and sonication and the fusion protein affinity purified using glutathione–agarose beads. The fusion protein was eluted from the beads using reduced glutathione (Smith and Johnson, 1988) and dialyzed to remove excess glutathione before storage at –80°C (Peleg and Metzenberg, 1994).

Gel retardation assays

Probes for mobility shift assays were generated as PCR products and purified by Elutip columns (Schleicher and Schuell, Keene, NH). The fragments used are listed in Table I. The fragments were labeled with [³²P]dATP using T4 polynucleotide kinase (Gibco-BRL, Gaithersburg, MD). Binding reactions were performed essentially as described previously (Lowndes *et al.*, 1991; Peleg and Metzenberg, 1991). Briefly, a 20 ml reaction volume was used containing 20 mM HEPES–KOH, pH 7.9, 50 mM KCl, 2 mM MgCl₂, 0.3 mg/ml BSA, 10% glycerol, 0.1 mM EDTA, 1 mM DTT, 0.1 μg poly(dI–dC)/ml, 0.5–1 ng radiolabeled probe (~2×10⁴ c.p.m./ml, 2×10⁷ c.p.m./mg) and appropriate amounts of purified protein. Following addition of the probe the reactions were incubated at 25°C for 25 min. When required, unlabeled competitor fragments were included immediately prior to probe addition in the excess indicated. The complexes were resolved on a 6% polyacrylamide gel (29:1 crosslinking) with 0.5% TBE running buffer at 150 V for 3.5 h at 25°C. The gel was transferred to Whatman 3MM filter paper and dried before autoradiography.

Sequence analysis

Database searches and alignments of both DNA and protein sequences were performed with the aid of services provided by the National Center for Biotechnology Information.

Constructing a theoretical model of the StuAp APSES domain

The following signature pattern was developed from the alignment of StuAp and the three bHLH proteins for which tertiary structures have been solved (MyoDp, Maxp, and E47p): (k,r)x8(k,r,s)x(k,q,r,e)(r,v,l,i,n)xx(l,i,v,e)x{12,22}(k,r)x{1,8}(d,e,n,s,t,a,p,q)(k,r,d,i,s)(l,i,v,m,w,a,g,n,y,q)~(f,w,c,p,h,k,r)(a,e,l,i,v,t)(m,f,l,i,v)x2(s,a,t,v,r,k)(e,l,i,v,m,s,t,a,c)x(v,m,f,y,h,i)(l,i,v,m,t,a)~(p)~(p)(l,i,v,m,r,s). This pattern was tested against the SwissProt database for specificity. Multiple hits (86/59 021) for proteins containing bHLH domains were obtained.

A multiple sequence alignment was prepared of the APSES domain of the StuA protein and the bHLH sequences with known crystal structures (E47, Max and MyoD) using GCG sequence analysis software (GCG program manual, Genetics Computer Group, Madison, WI). This alignment showed a possible conserved basic amino acid pattern in the

helix around residues in E47, Max and MyoD known to make contact with DNA. The known HLH motif for the second helix was explored to find the extent to which this area of StuAp varied from the established functional motif. Ten of the 16 positions matched, another three could be made to match simply by either substituting a lysine for an arginine or expanding the accepted residues by using residues contained in similar sites within the pattern. A request to the Swiss-Model server (Peitsch, 1995, 1996) with the first part of the StuAp sequence and using MyoD and E47 as templates resulted in a theoretical helical model for residues RGRRDGILKSEKVR. Additional analysis on these coordinates by DSSP (Kabsch and Sander, 1993) and Define_s (Richards and Kundrot, 1988) showed that the segment was helical. The assigned secondary structures for the three known structures all show a helix extending to and in some cases beyond a basic residue at alignment position 26 or 27. PredictProtein (Rost and Sander, 1993) results on the StuAp region suggests that this helix would extend to alignment position 28. Since the known structures appear to prefer to end after a basic amino acid the StuAp first helix was modeled from positions 1–29 of the alignment. The HLH motif is for a second helical region as well. Therefore, the StuAp fragment was modeled as a helix from residues 163 to 191 and from 205 to 220. Helical segments were grown using MacroModel software version 3.0 (Mohamadi *et al.*, 1990) to correspond to these regions of the sequence. They were aligned on a MyoD structure trimmed to contain the alignment region. The starting point of the first helix and the ending point of the second were used as reference points and the helices were aligned to match the MyoD conformation. Five additional residues were inserted into the MyoD loop section to accommodate StuAp's greater loop length. The actual sequence of the StuAp loop section was then overlaid onto this region. The three sections of the structure were then connected and the final structure was minimized with the MM2 force field to eliminate side chain collisions. The resulting structure was again aligned with the MyoD structure and the data visualized through the use of Molscript software (Kraulis, 1991).

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