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The *Aspergillus nidulans yA* gene is regulated by *abaA*

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The developmentally regulated *Aspergillus nidulans yA* gene encodes a *p*-diphenol oxidase that is needed for synthesis of green conidial pigment. We subjected the *yA* 5′ flanking region to mutational analysis in *A.nidulans* and *Saccharomyces cerevisiae* to identify DNA sequence elements involved in its transcriptional control, and identified two functionally distinct elements. Element I contained potential BrlA binding sites and was required for full level *yA* transcription, but not for developmental regulation in the presence of element II. Element II contained putative TEF-1 binding sites flanking a CCAAT element and was sufficient for developmental regulation of transcription. Mutation of the TEF-1 binding sites eliminated developmental regulation, whereas mutation of the CCAAT element led to elevated levels of transcription. Element II was also sufficient to induce transcription in *S.cerevisiae* when the *A.nidulans* developmental regulatory gene *abaA* was expressed from the *GAL1* promoter. As AbaA and TEF-1 possess similar DNA binding domains, the *abaA*−*yA* interaction in yeast is probably direct. Thus, *abaA* appears to be a direct activator of *yA*, but *yA* regulation may also involve interactions with BrlA and a member of the CCAAT class of DNA binding proteins.

Key words: Aspergillus nidulans/conidiophore formation/development/transcriptional regulation

Introduction

Development in multicellular organisms requires interactions between the products of regulatory genes and their structural gene targets. These interactions can be readily studied during conidiophore development in the ascomycetous fungus *Aspergillus nidulans* due to its highly developed molecular genetic system and simple life cycle (Timberlake and Marshall, 1988, 1989; Clutterbuck and Timberlake, 1992). Much of the temporal and spatial specificity of gene expression during conidiophore development is controlled by the regulatory genes *brlA*, *abaA* and *wetA*, which may encode transcription factors (Boylan *et al.*, 1987; Adams *et al.*, 1988; Mirabito *et al.*, 1989; Marshall and Timberlake, 1991). *brlA* is activated in conidiophore stalk cells early during development and is required for expression of *abaA* and *wetA* and structural genes such as *rodA*, *yA* and *wA* (Boylan *et al.*, 1987; O'Hara and Timberlake, 1989; Mayorga and Timberlake, 1990; Stringer *et al.*, 1991). Forced expression of *brlA* in vegetative cells (hyphae) activates *abaA*, *wetA*, *rodA*, *yA* and other sporulation-specific genes, causes cessation of hyphal growth and initiates cellular transformations resembling those that occur during normal conidiophore development (Adams *et al.*, 1988). *abaA* directs the differentiation of sporogenous phialide cells and is required for maintenance of their function. Loss-of-function mutations in *abaA* result in formation of aberrant conidiophores that fail to produce conidia (Clutterbuck, 1969; Sewall *et al.*, 1990). Forced expression of *abaA* in hyphae is insufficient to direct spore formation, although it does lead to *brlA*, *wetA* and *yA* activation, and to dramatic cellular transformations (Mirabito *et al.*, 1989). The existence of putative DNA binding domains in their polypeptide products, C_2H_2 Zn(II) binding motifs in BrlA and an ATTS (*a*baA, *T*EC1, *T*EF-1, *s*calloped) (TEA) motif in AbaA (Adams *et al.*, 1988; Mirabito *et al.*, 1989; Andrianopoulos and Timberlake, 1991; Bürglin, 1991; Campbell *et al.*, 1992) supports the idea that these genes encode developmentally regulated transcriptional activators.

yA is a well characterized, developmentally regulated gene of known function. It encodes a *p*-diphenol oxidase (or laccase) that accumulates in conidial walls and converts a yellow pigment intermediate to the mature green form (Clutterbuck, 1972; Law and Timberlake, 1980; Aramayo and Timberlake, 1990). Thus, loss-of-function mutations in *yA* result in the formation of yellow spores. *yA* transcripts are not present in hyphae or mature conidia, but accumulate during conidiophore development, indicating that expression is localized in conidiophore cells (O'Hara and Timberlake, 1989). Efforts to identify pathway-specific regulators of *yA* by mutation have been unsuccessful. Forced expression studies with *brlA* and *abaA* showed that activation of either gene alone is sufficient to induce *yA* transcription; activation of *brlA* in an *abaA*$^-$ strain or activation of *abaA* in a *brlA*$^-$ strain led to *yA* transcript accumulation (Adams *et al.*, 1988; Mirabito *et al.*, 1989). Direct regulation of *yA* by *brlA* and *abaA* could explain the inability to identify pathway-specific regulators, because *brlA* and *abaA* are required for the expression of numerous other developmentally regulated genes. Loss-of-function *brlA* and *abaA* mutants are morphologically abnormal and asporogenous and therefore incapable of displaying the *yellow conidia* phenotype and gain-of-function *brlA* and *abaA* mutants are vegetative lethals.

To examine the possibility that *yA* is under direct control by *brlA* and/or *abaA*, we subjected its 5′ flanking region to mutational analysis in *A.nidulans* and the budding yeast *S.cerevisiae*. We identified two functional *cis*-acting regulatory elements (I and II): element I is required for full level *yA* transcription in *A.nidulans*, but not for developmental regulation; and element II is required for developmental regulation and sufficient to confer regulation on a minimal, heterologous *A.nidulans* promoter. Element II contained two putative binding sites for the ATTS class of DNA binding

proteins that were required for activity. Element II was also sufficient to mediate transcriptional activation in yeast strains expressing *abaA* and this response depended on the TEF-1 binding sites. It is therefore likely that element II is a direct target for AbaA, which can act as a transcriptional activator in *A.nidulans* or yeast. Element I contains potential BrlA binding sites that may confer *brlA* responsiveness in *A.nidulans*. The data further indicate that other DNA binding proteins may be involved in modulating *yA* expression.

Results

Construction of a yA(p/l)::lacZ reporter fusion gene

yA mRNA is absent from hyphae and accumulates during conidiophore development (O'Hara and Timberlake, 1989). We fused the *yA* ATG in-frame to an internal codon of the *Escherichia coli lacZ* gene to investigate the role of upstream sequences of *yA* in controlling *lacZ* gene expression. The resultant fusion gene, *yA*(p/l)::*lacZ*, contained the 52 bp *yA* untranslated leader and 861 bp upstream of the major mRNA cap site. The construct was inserted into *A.nidulans* transformation plasmid pRA42(8) (Figure 1A) and integrated at *argB* by forced homologous recombination (Figure 1B) to produce *A.nidulans* strain TRA42. As a control, plasmid pPM6 (Mirabito *et al.*, 1989) lacking *yA* and *lacZ* fragments, was integrated at the same locus to produce TRA-ARG. β-galactosidase was measured in TRA42 and TRA-ARG at various times following developmental induction. Figure 2A shows that β-galactosidase activity was not detectable in hyphae of either TRA42 or TRA-ARG, but became detectable 18 h after inducing development in strain TRA42, increasing rapidly thereafter. TRA-ARG produced only low β-galactosidase levels, presumably due to expression of an endogenous β-galactosidase gene (Fantes and Roberts, 1973). The kinetics of β-galactosidase accumulation by TRA42 were similar to those for accumulation of *yA*-encoded laccase (Law and Timberlake, 1980) and *yA* mRNA (O'Hara and Timberlake, 1989).

The spatial distribution of β-galactosidase was determined in developing cultures of TRA42 and TRA-ARG by *in situ* X-gal staining for enzymatic activity. Figure 2B shows that β-galactosidase was largely restricted to the metulae, phialides and immature conidia of TRA42. Figure 2C shows that β-galactosidase activity was not detected in conidiophores of TRA-ARG. These data demonstrate that the region of *yA* from −861 to +52 is sufficient to confer appropriate temporal and spatial controls on the *lacZ* reporter gene.

5′ deletion analysis of the yA(p/l)::lacZ fusion gene

To define more precisely which sequences were required for developmental regulation, the −861 fragment was

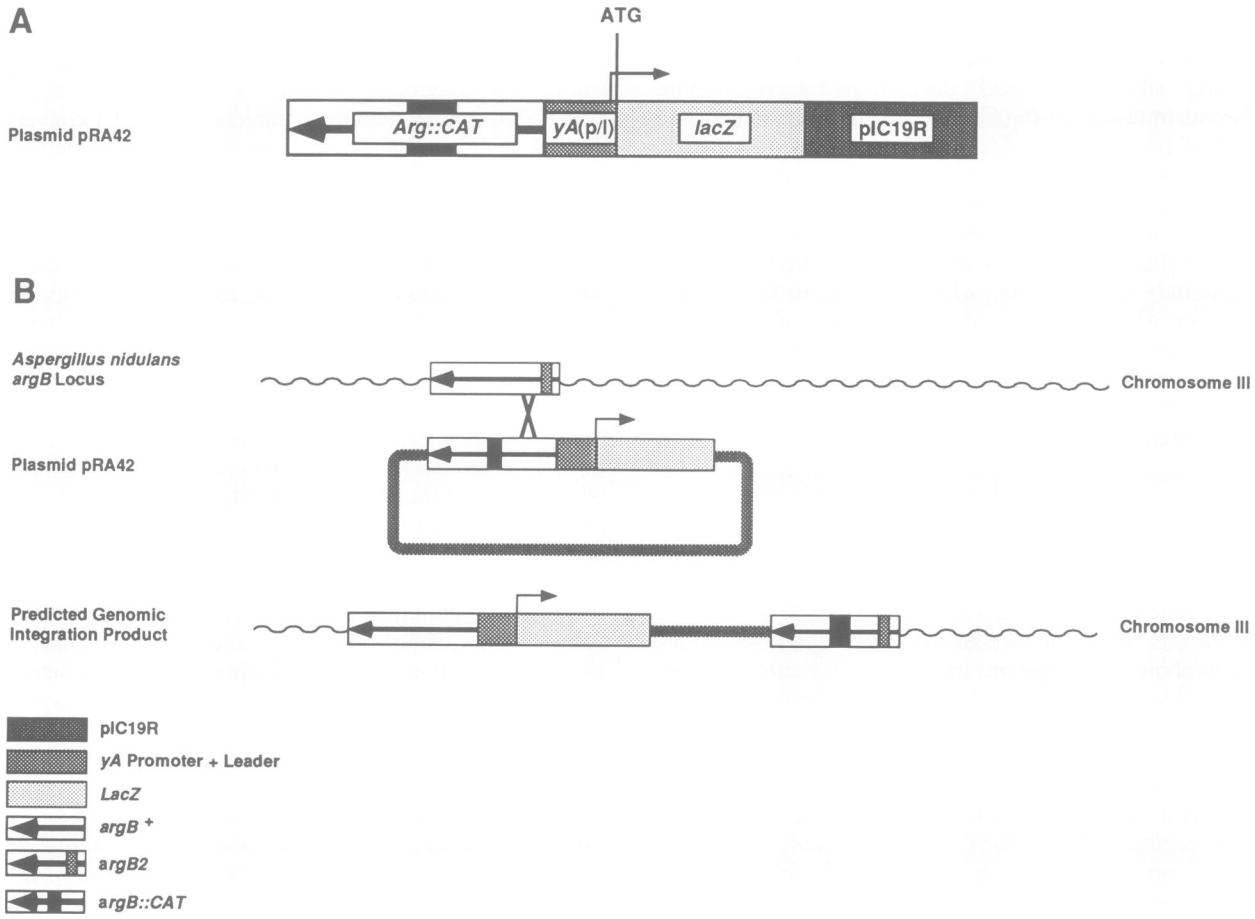

Fig. 1. Integration of *yA*(p/l)::*lacZ* fusion gene. **A.** Linear representation of plasmid pRA42(8). pRA42(8) was constructed to integrate the *yA*(p/l)::*lacZ* fusion gene at the *argB* locus on chromosome III (see Materials and methods). **B.** *A.nidulans* PW1 (*argB2*) was transformed with pRA42(8) to arginine-independence. pRA42(8) integration at *argB* was expected to generate the genomic structure shown. Transformants were checked for single integration events by Southern blot analysis and an appropriate strain (TRA42) was chosen.

subjected to 5′ deletion analysis. pRA42(8) derivatives with the 5′ end points shown in Figure 3A were transformed into *A.nidulans*, and strains containing single plasmid copies integrated at *argB* were obtained. β-galactosidase activities were then determined at various times after inducing conidiation. Figure 3B shows that deletions with end-points to −174 led to elevated β-galactosidase production during development, suggesting that upstream elements may repress transcription. The −159 deletion strain had essentially the same regulatory pattern as did the −861 (control) strain. The −146 strain had less than half the activity of the −861 strain, whereas the −66, −53, −45 and −26 strains produced very low enzyme levels and showed little or no regulation, equivalent to the negative control strain. These results show that sequences proximal to −159 are sufficient to confer appropriate developmental controls on the reporter gene. For convenience, we refer to the −159 to +1 region as the *yA* promoter.

Modular organization of the yA promoter

Figure 4 presents the sequence of the *yA* promoter and untranslated leader with several potentially interesting sequence elements indicated. The −161 to −115 region, required for full-level developmental induction (Figure 3B), contains three putative *brlA* response elements (5′-MRAGGG-3′; Chang and Timberlake, 1993). We designate the region from −115 to −161 element I. The region from −88 to +1 contains CCAAT and TATA boxes. In addition, two sites similar to the GT-IIC site (5′-GTGGAATGT-3′) for binding of the TEF-1 SV-40 enhancer factor (Davidson *et al.*, 1988; Fromental *et al.*, 1988; Xiao *et al.*, 1991) occur in inverted orientation flanking the CCAAT box. These sites were of particular interest, because *A.nidulans* AbaA belongs to the TEF-1 (ATTS) class of DNA binding proteins (Andrianopoulos and Timberlake, 1991; Bürglin, 1991). We designate the −88 to −60 region containing the putative TEF-1 sites element II.

To test elements I and II for function, sequences corresponding to elements I + II, element I alone, and element II alone were cloned upstream of the *A.nidulans amdS* promoter/leader, lacking upstream regulatory sequences, fused to the *E.coli* lacZ gene [*amdS*(p/l)::*lacZ*] (Davis *et al.*, 1988), as shown in Figure 5. Elements were inserted in both orientations at the*Bgl*II−*Bam*HI site located upstream of a potential TATA box and the resultant plasmids were integrated in single copies at the *argB* locus (Figure 1B). At various times after inducing development, transformants were harvested and β-galactosidase activities were determined. Figure 5 (right panel) shows the β-galactosidase induction profiles in the different strains. Elements I and II in the forward orientation produced strong developmental regulation, with β-galactosidase levels ~10-fold higher than those observed with TRA42 in a parallel, concurrent control experiment. Elements I and II in the inverse orientation also produced developmental regulation and β-galactosidase levels were similar to those observed with the TRA42 control strain. Element I alone did not produce significant developmental regulation in either orientation, whereas element II alone produced developmental regulation in both orientations. With element II in the forward orientation, β-galactosidase levels were similar to the relevant *yA*(p/l)::*lacZ* control strain (−88). In the inverse orientation, β-galactosidase levels were significantly higher. The *amdS*(p/l)::*lacZ* construct lacking added sequence elements produced negligible levels of β-galactosidase. These results show that element II alone is sufficient to confer developmentally regulated induction on a heterologous promoter, whereas element I is not. Element I stimulates transcription when it is upstream of element II, but not when it is downstream in reverse orientation.

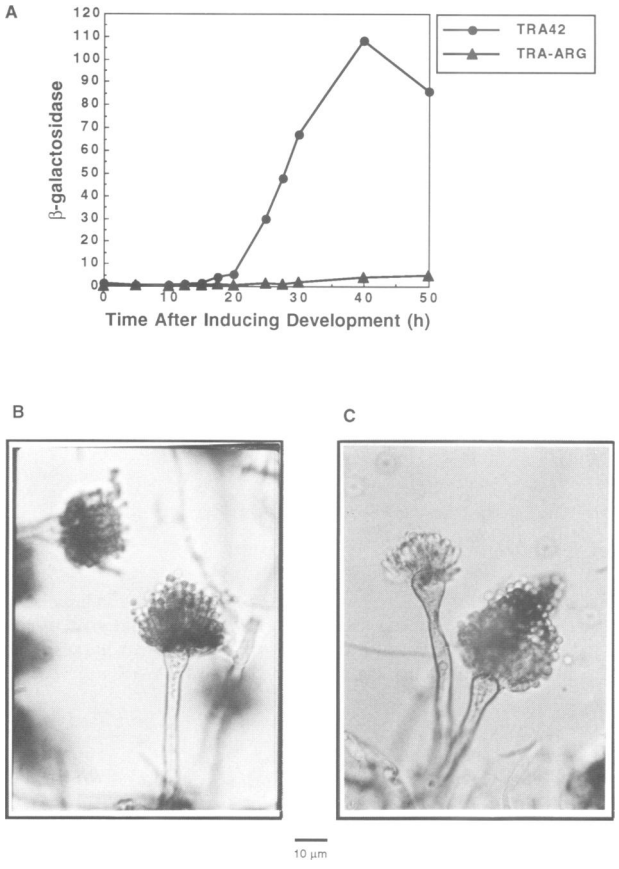

Fig. 2. The *yA*(p/l) regulates β-galactosidase. **A.** Strains TRA42 (Figure 1) and TRA-ARG [a control strain lacking the *yA*(p/l)::*lacZ* fusion gene] were grown and induced to develop as described in Materials and methods. Samples were taken at the times indicated. Cell extracts were assayed for β-galactosidase activity, which is expressed as nmol ONPG hydrolysed/min/mg protein (Miller, 1972). **B** and **C**. Strains TRA42 (B) and TRA-ARG (C) were inoculated onto agar-solidified medium and grown until mature conidiophores were present. Colonies were permeabilized with chloroform and stained with X-gal. Samples were photographed by using bright field microscopy.

Mutational analysis of element II

Element II contains a CCAAT box and two potential TEF-1 binding sites. To investigate the involvement of these elements in developmental control, synthetic oligonucleotides corresponding to element II were synthesized (see Materials and methods). In mutant elements 1 and 3, both TEF-1 sites were altered. In mutant elements 1 and 2, the CCAAT box was altered. These synthetic elements were inserted upstream of the *amdS*(p/l)::*lacZ* fusion gene and tested for their ability to produce developmental regulation in *A.nidulans*. Figure 6 shows that the wild-type element produced a pattern of developmental regulation similar to the element II *yA*(p/l)::*lacZ* positive control strain (−88) analysed in parallel. Mutation of both TEF-1 sites dramatically reduced, but did not eliminate, developmental regulation. Mutation

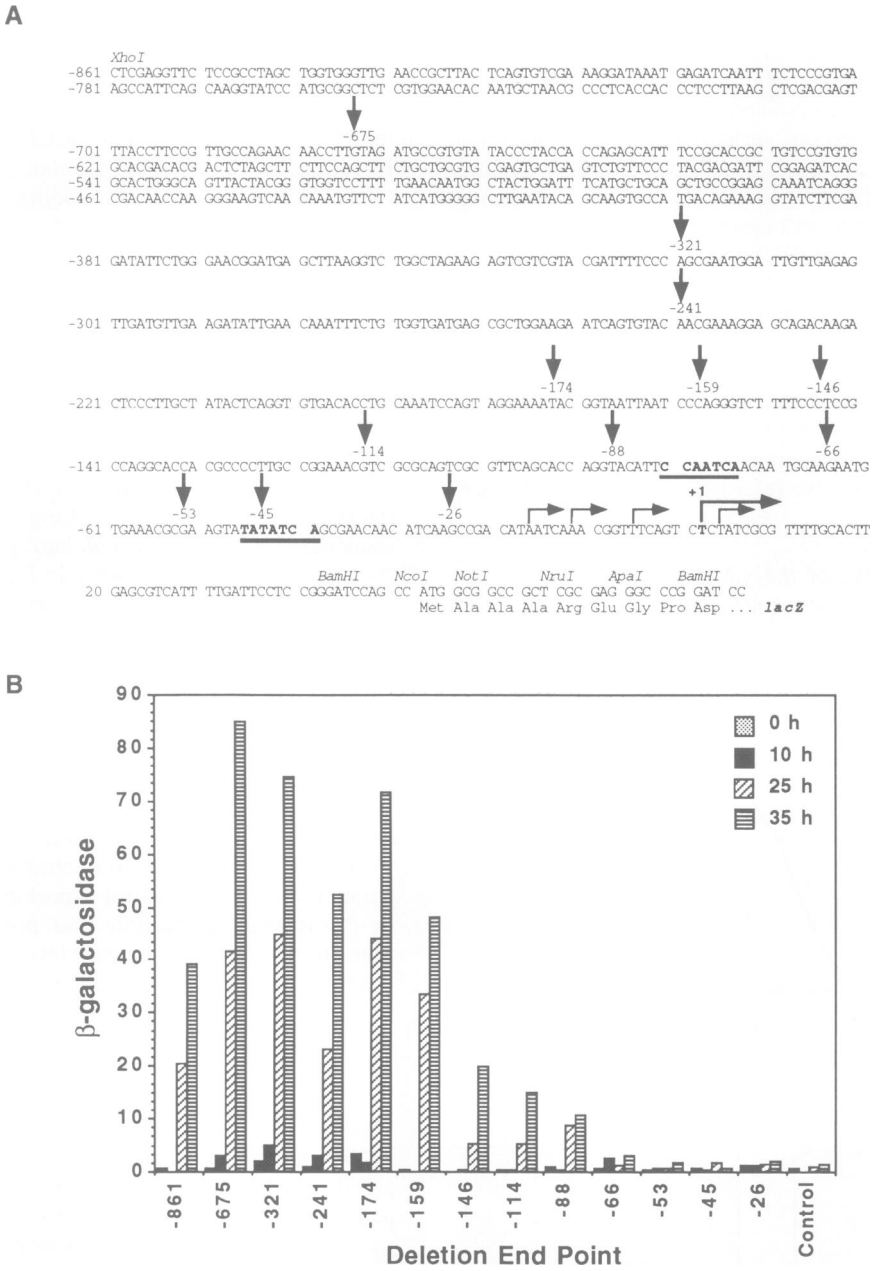

Fig. 3. Deletion analysis of the *yA* promoter. **A.** The sequence of the *yA* promoter and leader fused to *lacZ* is given and landmarks are indicated as follows: deletion end points, downwards arrow; putative CCAAT and TATA boxes, thick line; minor and major (+1) transcription start sites, bent arrow. **B.** *A.nidulans* PW1 was transformed with pRA42(8) deletion derivatives and transformants were assayed for β-galactosidase activity at the developmental times indicated. β-galactosidase activity is expressed as nmol ONPG hydrolysed/min/mg protein (Miller, 1972). TRA-ARG served as a negative control.

of the CCAAT box increased β-galactosidase levels by ~2-fold in comparison to the wild-type, synthetic element II. In addition, β-galactosidase began to accumulate earlier during development in this mutant than in the relevant control strain.

abaA activation of element II in yeast

The results described above raised the possibility that AbaA binds directly to element II and induces transcription. As an initial test of this possibility, we determined whether expression of *abaA* in yeast was sufficient to activate transcription from a minimal yeast promoter to which element II had been fused. The experimental strategy is outlined in Figure 7A. An intronless version of *abaA* was placed under the control of the *GAL1* promoter in the *CEN* plasmid pAA54 to yield pAA35 (A.Andrianopoulos and W.E.Timberlake, unpublished results). Wild-type and mutant versions of element II were inserted upstream of a minimal *CYC1*(p/l)::*lacZ* fusion gene in the 2 μm plasmid pYC7. Yeast strains that had been co-transformed with the plasmids were tested for β-galactosidase production on X-gal plates and supplemented minimal liquid medium containing either glucose or galactose as carbon source. *CEN* plasmids lacking *abaA* or containing the *abaA100* frameshift allele (see Materials and methods) were included as controls. Figure 7B and Table I show that element II in either

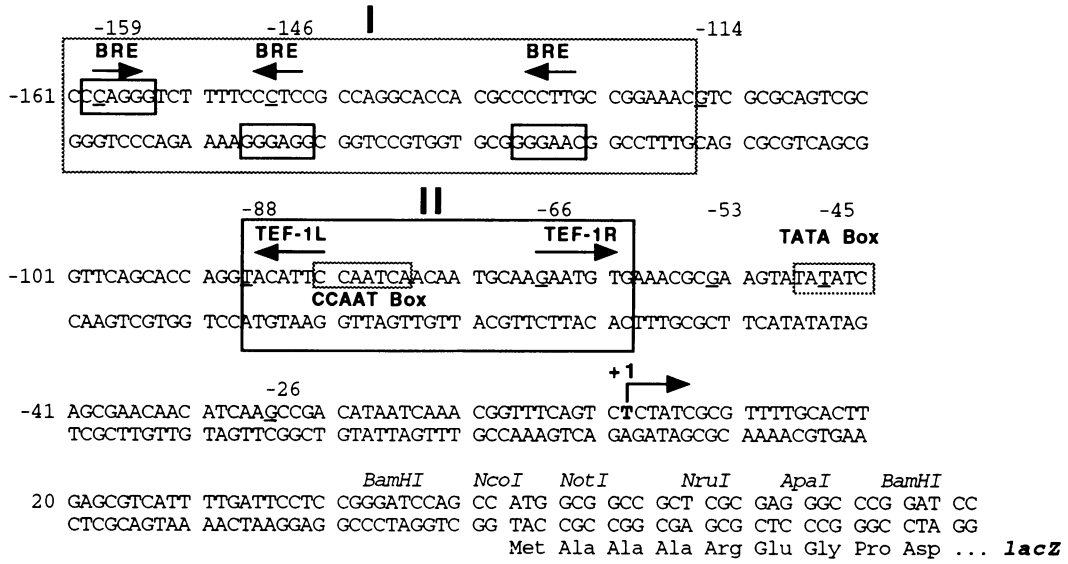

Fig. 4. Modular organization of the *yA* promoter. The sequence of the *yA* promoter and leader fused to *lacZ* is given. Element I (−161 to −115) contains three sequences with five or six of six identities with the putative *brlA* response element (BRE) (5′-MRAGGG-3′) (Chang and Timberlake, 1993) at positions −160, −148 and −128. Element II (−88 to −60) contains two potential GT-IIC-like TEF-1 binding sites (5′-GAATGT-3′) surrounding a potential CCAAT box. A potential TATA box and the major transcriptional start site (+1) are indicated. Numbers above underlined bases indicate the positions of the deletion end points shown in Figure 3.

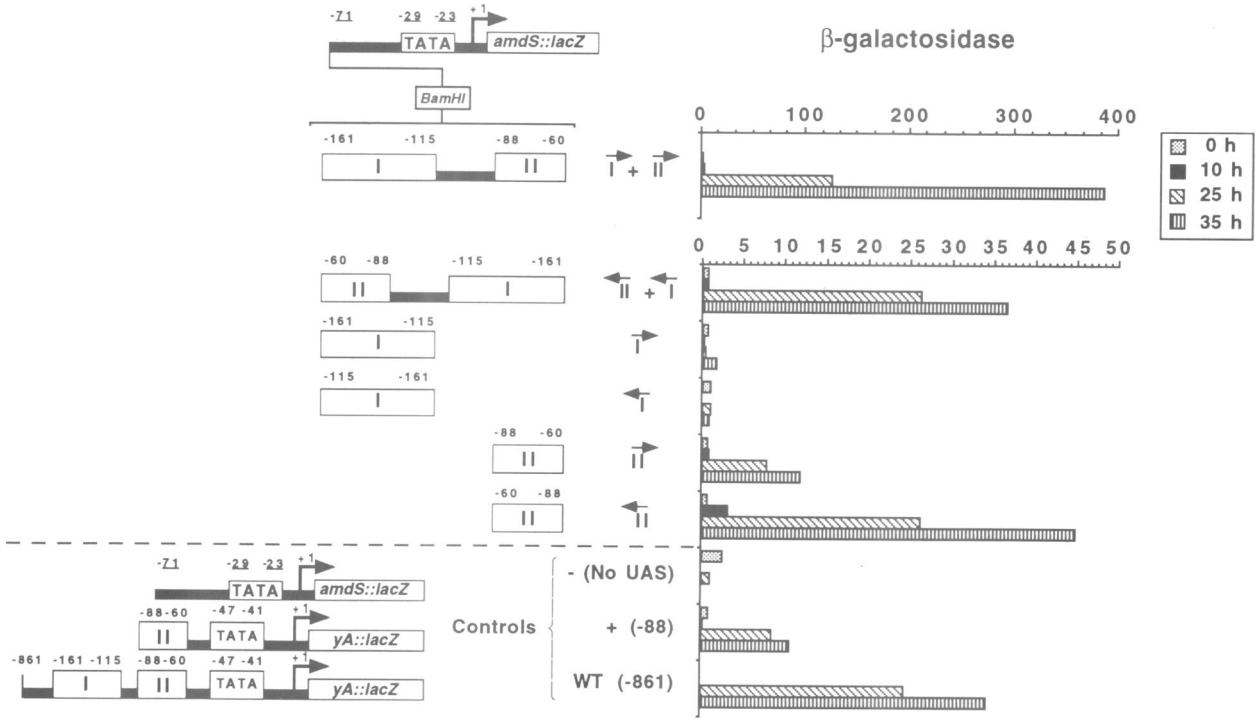

Fig. 5. Elements from the *yA* promoter confer developmental expression on an *A.nidulans* heterologous promoter lacking UASs. The left panel shows a schematic representation of the *A.nidulans amdS* promoter, lacking UASs, fused to *lacZ*. Elements I + II, I and II were inserted upstream of the minimal promoter as indicated. Position of the potential *amdS* TATA box, relative to the cap site, is indicated by underlined numbers. Negative (no UAS), positive (−88) and wild-type (−861) control constructs are shown underneath the broken line. β-galactosidase activities of strains containing the elements indicated are presented in the right panel. β-galactosidase activity is expressed as nmol ONPG hydrolysed/min/mg protein (Miller 1972).

orientation was sufficient to mediate an *abaA* response that was dependent on galactose induction, the presence of a non-mutant version of *abaA* and the presence of the TEF-1 elements and CCAAT box. Figure 7C and Table I further show that the TEF-1 elements and CCAAT box were necessary for the response. Thus, *yA* element II is capable of responding to *abaA* in the heterologous yeast system.

Discussion

The data presented in this paper support the hypothesis that the *A.nidulans yA* gene, which encodes conidial laccase, is directly regulated by *abaA* at the transcriptional level. In addition, they indicate that *yA* regulation involves the participation of additional factors, perhaps BrlA and a member of the CCAAT class of DNA binding proteins.

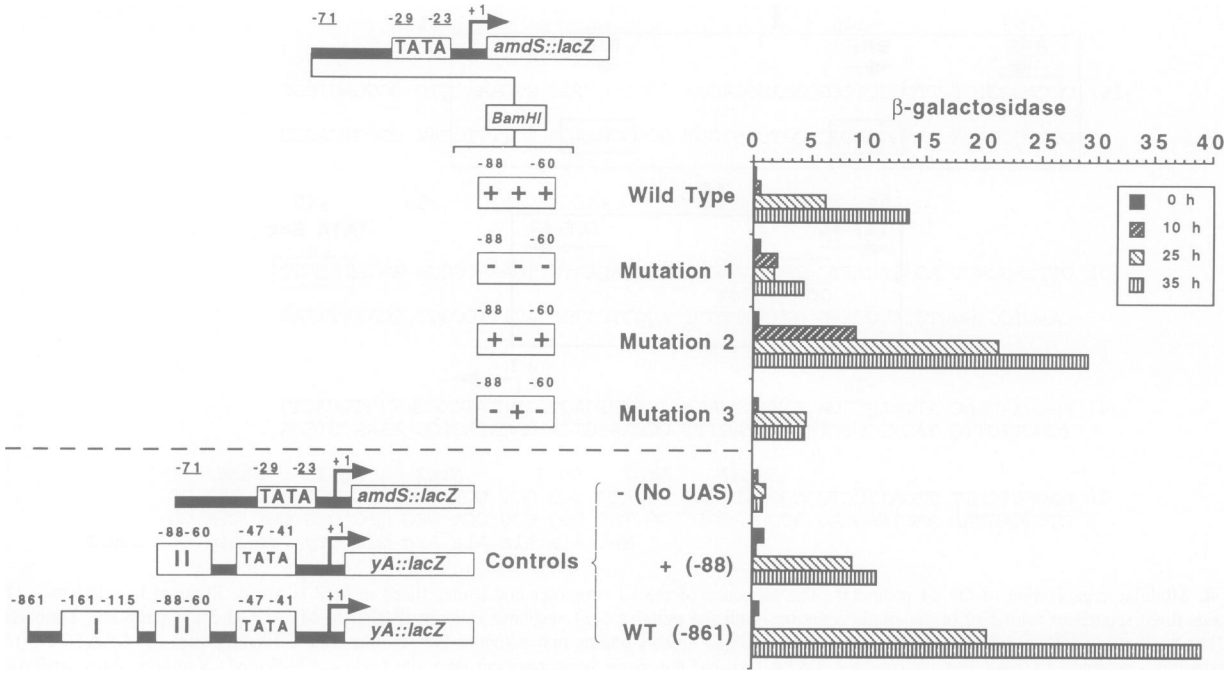

Fig. 6. Mutagenesis of element II. The left panel shows a schematic representation of the *A.nidulans amdS* minimal promoter fused to *lacZ*. A wild-type element II and mutant elements, in which either TEF-1 or CCAAT sites were eliminated (see Materials and methods), were inserted upstream of the minimal promoter. The position of the potential TATA box of *amdS* is indicated by underlined numbers. β-galactosidase activities of strains containing the elements indicated are presented in the right panel. β-galactosidase activity is expressed as nmol ONPG hydrolysed/min/mg protein (Miller, 1972). Controls, shown below the dashed line, were as described in Figure 5.

Transcriptional regulation of *yA* during asexual development is mediated by at least two *cis*-acting regulatory elements, designated I and II. Element I contains three potential *brlA* response elements (BREs) (Chang and Timberlake, 1993), suggesting that *yA* expression is in part controlled by the *brlA* regulatory gene. However, deletion of element I led to a reduction in *yA* transcription without significantly affecting developmental regulation. Furthermore, element I did not confer developmental regulation on a minimal, heterologous promoter. On the other hand, *brlA*$^+$ *abaA*$^-$ strains accumulate nearly wild-type levels of *yA* transcript during development (O'Hara and Timberlake, 1989), indicating that *brlA* alone is sufficient to activate *yA* transcription. The lack of dependency on element I for developmental regulation in the experiments described here may be explained by the existence of proximal element II, which is sufficient for developmental activation by *abaA* (see below). The results of Chang and Timberlake (1993) indicate that three or more BREs are needed for *brlA*-induced transcription in yeast, and probably in *A.nidulans*. Only one of the three potential BREs in element I fit the consensus sequence precisely. Thus these elements may be insufficient to mediate the *brlA* response without interaction with other elements or factors. A fourth consensus BRE (5′-CCCTTG-3′) is present upstream of element I at position −219 (see Figure 3) and could provide an additional, required site for BrlA interaction. Alternatively, BrlA bound at element I could interact with other factors bound to the *yA* promoter outside element I. Such interactions are implied by the ability of element I, upstream of element II, to enhance element II-mediated developmental induction.

Element II alone is capable of conferring developmental regulation on a heterologous promoter. We noted that element II contains two potential TEF-1 binding sites (Davidson *et al.*, 1988; Fromental *et al.*, 1988; Xiao *et al.*, 1991) in inverted orientation around a CCAAT box. AbaA and TEF-1 belong to the ATTS class of DNA binding proteins (Andrianopoulos and Timberlake, 1991; Bürglin, 1991), which also includes the *S.cerevisiae* TEC1 (Errede *et al.*, 1987; Company and Errede, 1988; Laloux *et al.*, 1990) and *Drosophila melanogaster* scalloped (Campbell *et al.*, 1992) proteins. The M-CAT binding factor (Mar and Ordahl, 1990) recognizes a similar sequence element (Mar and Ordahl, 1988). It therefore seemed likely that these sequences were the sites of AbaA binding to the *yA* promoter. Consistent with this hypothesis, mutation of the putative AbaA binding sites significantly reduced the ability of element II to mediate developmental induction. In addition, element II was sufficient to mediate *abaA*-induced transcription in yeast and induction was eliminated by mutation of the putative AbaA binding sites or the AbaA polypeptide. The direct interaction of AbaA and the proposed binding sites has been confirmed by results from *in vitro* footprinting experiments with AbaA protein produced in *E.coli* (A.Andrianopoulos and W.E.Timberlake, unpublished results). Thus, the results of *in vivo* experiments done with *A.nidulans* and yeast and *in vitro* experiments show that AbaA binds to element II and activates transcription.

The CCAAT box in element II is also implicated in developmental regulation by the results presented in this paper. In *amdS*(p/l)::*lacZ* constructs containing a synthetic element II, mutation of CCAAT led to a 2- to 3-fold stimulation of *lacZ* expression relative to the wild-type control, indicating that CCAAT mediates transcriptional repression. The observation that the CCAAT mutation in element II returns expression levels nearly to those observed

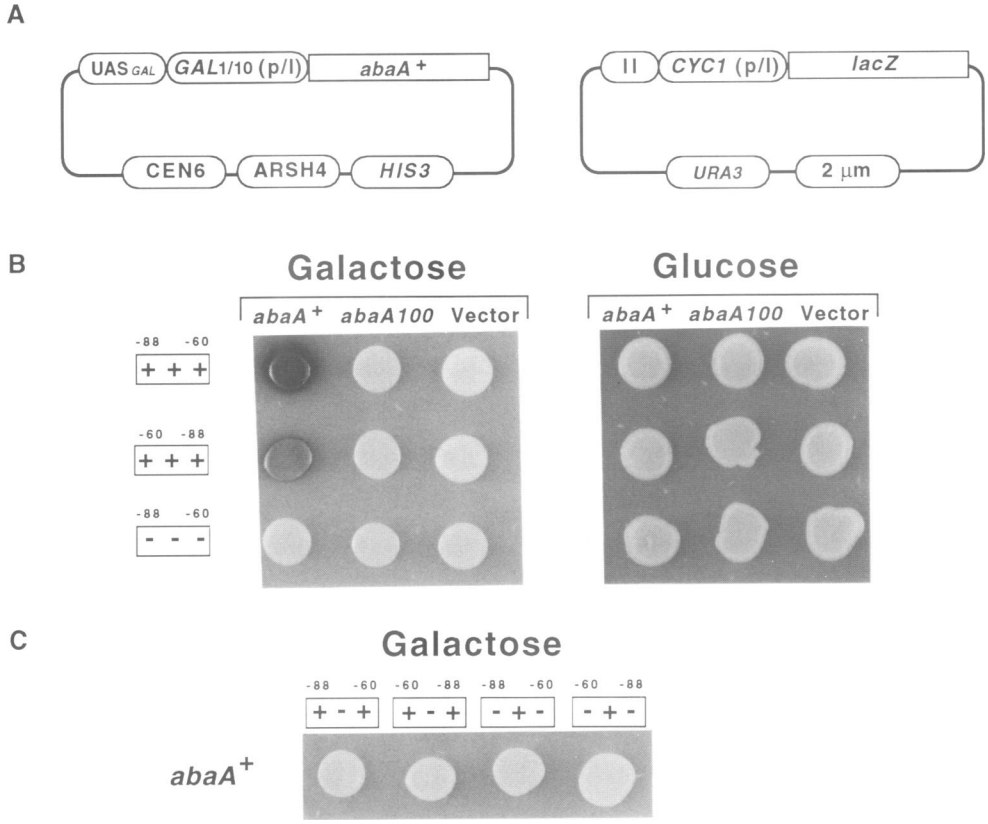

Fig. 7. *abaA*/element II-directed transcription in yeast. **A.** The *A.nidulans abaA* gene was placed under the control of the yeast *GA1* promoter [UAS_{GAL}-*GAL1*(p/l)] in a centromeric plasmid containing the *HIS3* selective marker. Element II was inserted in different combinations upstream of the yeast *CYC1*(p/l), lacking UASs, fused to *lacZ* in a 2 μm-based plasmid containing the *URA3* selective marker. Growth of yeast transformants containing both plasmids in glucose represses *abaA* expression, whereas growth in galactose induces *abaA* expression. Activation of the *CYC1*(p/l) leads to production of β-galactosidase and consequently blue color after X-gal staining. **B.** Yeast strains containing plasmids with element II inserted upstream of the *CYC*(p/l)::*lacZ* gene fusion were grown under inducing (galactose) and non-inducing (glucose) conditions. Plasmids containing a wild-type version of element II (−88 to −0) in both orientations and a mutant version of element II in a single orientation were introduced into cells containing either a wild-type copy of *abaA* (*abaA*+), a frameshift mutant copy of *abaA* (*abaA100*) or vector alone. Cells grown in liquid medium plus galactose were spotted onto appropriately supplemented solid medium containing X-gal and either glucose or galactose as carbon source. **C.** Strains containing a plasmid with a wild-type version of *abaA* and plasmids with mutant versions of element II inserted in different orientations upstream of the *CYC1*(p/l)::*lacZ* gene fusion were grown under inducing (galactose) conditions.

in element I + II constructs suggests that element I is involved in relieving CCAAT-mediated repression. A model to account for these results is presented in Figure 8. In hyphae, the *yA* promoter may interact with a CCAAT binding protein, which acts as a negative regulator (Figure 8A). *brlA* and *abaA* are not expressed in hyphae. Early during development, *brlA* becomes active and BrlA is proposed to bind to element I and stimulate low level transcription (Figure 8B). Later during development, *abaA* becomes active, AbaA binds to element II and, perhaps assisted by BrlA bound at element I, displaces (or neutralizes) CCAAT binding factor, leading to fully induced and derepressed transcription (Figure 8C). Interactions of BrlA and AbaA are also proposed in the control of *abaA* (A.Andrianopoulos and W.E.Timberlake, unpublished results) transcription. It should, however, be noted that the CCAAT sequence is required for *abaA* induction in yeast (Figure 7), indicating that it functions differently in the two systems. In this regard, our data imply that yeast factors may interact with AbaA and that these interactions may be essential for the response.

The direct involvement of *brlA*, *abaA* and possibly CCAAT binding factor, explains the inability to identify pathway-specific regulators of *yA* by mutational analysis.

Table I. *abaA*/element II-directed β-galactosidase expression in yeast

Construction	β-galactosidase activity[a]	
	Glucose[b]	Galactose[b]
abaA+/element II (→)	4.8	111
abaA100/element II (→)	0.8	0.6
vector/element II (→)	2.5	0.4
abaA+/element II (←)	1.7	15.1
abaA100/element II (←)	1.4	nil
vector/element II (←)	4.3	0.6
abaA+/mutation 1 in element II (→)	1.1	0.2
abaA100/mutation 1 in element II (→)	0.6	0.1
vector/mutation 1 in element II (→)	0.9	0.4
abaA+/mutation 2 in element II (→)	1.3	0.1
abaA+/mutation 2 in element II (←)	1.6	0.4
abaA+/mutation 3 in element II (→)	1.3	0.4
abaA+/mutation 3 in element II (←)	1.1	nil

[a]β-galactosidase activity is given as nmol *o*-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolysed per min per milligram of protein.
[b]Strains were grown, harvested and lysed as described in Materials and methods.

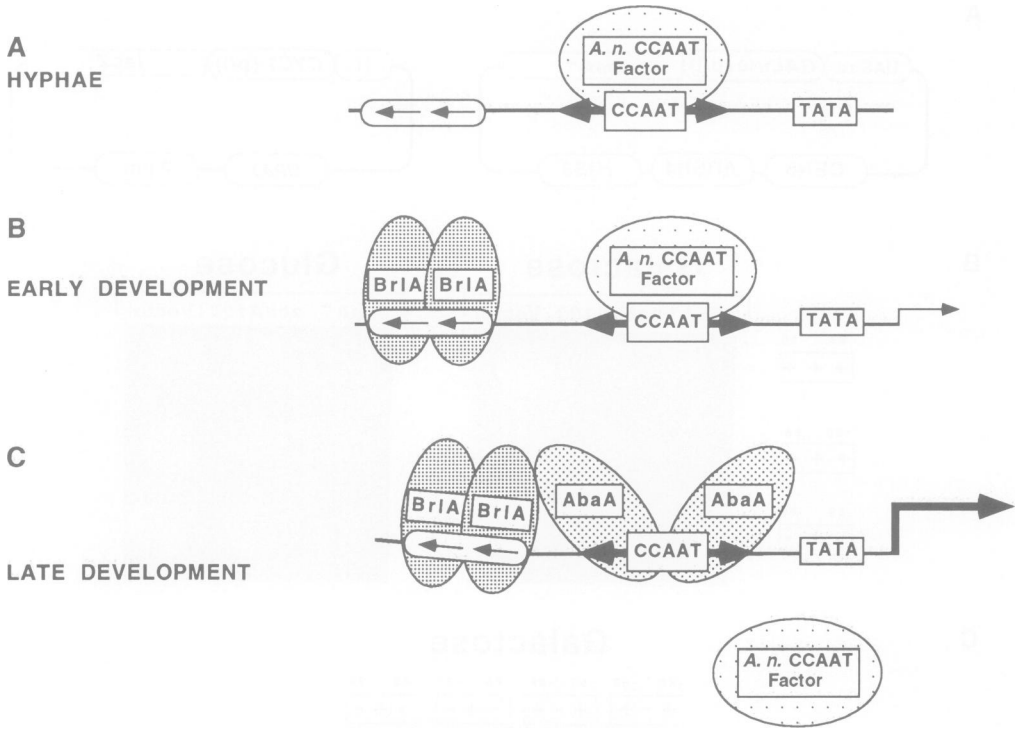

Fig. 8. Model for regulation of *yA* transcription.

Table II. Oligonucleotides used in this study

Name	Sequence
OY29	5′-(+42)GGATCCAGCCATGGACCTCTCCAC(+65)-3′
OY30	5′-CTAGAGGTACCTCGCGACCCGGGC-3′
OY31	5′-TCGAGCCCGGGTCGCGAGGTACCT-3′
OY32	5′-(+51)CATG(+54)GCGGCCGCTCGCGAGGGCCCG-3′
OY33	5′-GATCCGGGCCCTCGCGAGCGGCCG(+55)C(+56)-3′
OY35	5′-GGACCGTCTATTAC-3′
OY36	5′-GAGGTACC(−114)GTCGGCCAGTCGCGTTC(−98)-3′
OY37	5′−GAGGTACC(−66)GAATGTGAAACGCGAAG(−50)-3′
OY38	5′-GAGGTACC(−53)GAAGTATATATCAGCGA(−37)-3′
OY39	5′−CCCGGGATCCGGTACCI(−161)CCCAGGGTCTTTTCCCT(−145)-3′
OY40	5′-CCCGGGATCCIGGTACC(−115)GTTTCCGGCAAGGGGCG(−131)-3′
OY41	5′-CCCGGGATCCIGGTACC(−88)TACATTCCAATCAACAATGCAAGAATGTG(−60)-3′
OY42	5′-CCCGGGATCCIGGTACC(−60)CACATTCTTGCATTGTTGATTG(−81)-3′
OY43	5′-CCCGGGATCCGGTACC(−88)TAACGGCACCGCAACAATGCAAGCCGTTG(−60)-3′
OY44	5′-CCCGGGATCCIGGTACC(−60)CAACGGCTTGCATTGTTG(−77)-3′
OY45	5′-CCCGGGATCCIGGTACC(−88)TACATTCACCGCAACAATGCAAGAATGTG(−60)-3′
OY46	5′-CCCGGGATCCIGGTACC(−60)CACATTCTTGCATTGTTG(−77)-3′
OY47	5′-CCCGGGATCCGGTACC(−88)TAACGGCCAATCAACAATGCAAGCCGTTG(−60)-3′

Numbers in parenthesis indicate the relative positions of the oligonucleotide with respect to the cap site of *yA*. The following symbols are used: polylinker regions, dotted underlined; *Bam*HI restriction site, underlined and *Kpn*I restriction site, double underlined.

Mutations in these genes are highly pleiotropic, because their activities are required for induction of numerous developmentally regulated genes, including the developmental regulatory genes themselves (Timberlake, 1990, 1991). The observation that the two core developmental regulatory genes, *brlA* and *abaA*, directly regulate the gene whose product catalyses synthesis of conidial wall pigment, suggests that intermediate regulatory genes may be rare or absent in this system. Thus, much of the spatiotemporal specificity of gene expression apparent in *A.nidulans* conidiophore development may simply depend on the timing of expression and combinatorial interactions of a limited number of core regulatory genes.

Materials and methods

Nucleic acid manipulations

DNA manipulations followed standard procedures (Ausubel *et al.*, 1987; Sambrook *et al.*, 1989). *A.nidulans* DNA was isolated, electrophoretically fractionated in agarose gels and transferred to Nytran (Schleicher and Schuell, Keene, NH) following procedures recommended by the manufacturer. DNA fragments were labelled with ^{32}P by nick translation or random primer extension and hybridized to filters as described in Sambrook *et al.* (1989).

Double-stranded DNA sequencing utilized Sequenase 2.0 (United States Biochemical Corporation, Cleveland, OH), following the manufacturer's recommended procedures. *In vitro* mutagenesis was carried out as described by Kunkel (1985).

Construction of the yA(p/l)::lacZ gene fusion

pRA42(8) was constructed in four steps: (i) pRA9AP1, containing a 991 bp *Xho*I fragment encompassing the *yA* promoter, leader and translational start site (GenBank accession number X52552), was mutagenized *in vitro* to introduce an *Nco*I site at the ATG of *yA* (oligonucleotide OY29) to yield pRA36(1). (ii) The 3.0 kb *Bam*HI *lacZ* fragment from pMC1871 (Casadaban *et al.*, 1983) was cloned into the *Bam*HI site of pUC13CMR (obtained from T.Schmidhauser and D.Helinski, University of California, San Diego) to yield pRA35(6A). (iii) The 913 bp *Xho*I−*Nco*I fragment from pRA36(1) was ligated with the 3.0 kb *Bam*HI fragment from *lacZ* [removed from pRA35(6A)] in the presence of the *Nco*I−*Bam*HI linker (oligonucleotides OY32 and OY33) and *Xho*I- and *Bam*HI-digested pIC19R (Marsh *et al.*, 1984) to yield pRA37(27). (iv) The 4.2 kb *Xba*I fragment containing the *argB*::CAT fusion gene obtained from pPM6 (Hamer and Timberlake, 1987; Mirabito *et al.*, 1989) was ligated with the *Xho*I- and *Xba*I-digested pRA37(27) in the presence of an *Xba*I−*Xho*I linker (oligonucleotides OY30 and OY31) to yield pRA42(8). All critical regions were checked by DNA sequencing.

5′ deletion analysis

5′ deletions of the *yA* promoter were generated by either exonuclease III treatment or PCR amplification of plasmid pRA42(8). Exonuclease III treatment was performed by first digesting pRA42(8) with *Xho*I and *Kpn*I and treating the linearized plasmid with exonuclease III (Henikoff, 1984, 1987). The resultant fragments were treated with S1 nuclease and religated in the presence of an 8 bp *Kpn*I linker (5′-GGGTACCC-3′). PCR deletions (Sakai *et al.*, 1988) were made as follows: pRA42(8) (10 ng) was mixed with 1 μM primers, 200 μM deoxynucleoside triphosphates, 1×amplification buffer (10 mM Tris−HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$ and 0.01% gelatin) and 2 U Taq DNA polymerase. Samples were subjected to the following amplification cycle: plasmid DNA, primers, buffer and water were heated for 5 min at 95°C and nucleotides plus enzyme were added. The samples were heated for 2 min at 92°C, allowed to hybridize for 2 min at 42°C and subjected to 30 cycles of extension (1 min at 72°C), denaturation (2 min at 92°C), and renaturation (1 min at 56°C). The end-points of each deletion were determined by DNA sequencing with OY35 as primer.

Insertion of elements I + II, I and II upstream of amdS (p/l):lacZ

Plasmid pRA91(L5−49) was constructed by cloning the *Bam*HI−*Kpn*I fragment from pLIT14 (Davis *et al.*, 1988), containing the inactive *A.nidulans amdS* promoter/leader translationally fused to the *E.coli* lacZ gene, into the *Bam*HI- and *Kpn*I-digested pPM6 plasmid (Mirabito *et al.*, 1989). pPM6, a pUC19-based plasmid, contains the *argB*::CAT fusion gene for integration at the *argB* locus by forced homologous recombination (Hamer and Timberlake, 1987). Elements I + II were obtained by PCR amplification of pRA42(8) with OY39 and OY42. Element I was obtained by PCR amplification of pRA42(8) with OY39 and OY40. Element II was obtained by annealing oligonucleotides OY41 and OY42, filling in the ends with Sequenase, digesting with *Bam*HI or *Kpn*I and inserting into either *Bam*HI-digested pRA91(L5−49) or *Kpn*I-digested pYC7 yeast plasmid (Chang and Timberlake, 1993).

Directed mutagenesis of element II

Mutations were introduced into element II to inactivate TEF-1L, CCAAT, TEF-1R (mutation 1), CCAAT alone (mutation 2) or to inactivate both TEF-1 binding sites leaving the CCAAT box intact (mutation 3). Mutations produced transversions in the 4 internal bases of the TEF-1 binding site (5′-ACATTC-3′ to 5′-AACGGC-3′) or CCAAT box (5′-CCAATCAA-3′ to 5′-CACCGCAA-3′). Mutant versions of element II were obtained by preparing inserts as described for element II with oligonucleotides OY43 and OY44 (mutation 1), OY45 and OY46 (mutation 2), and OY47 and OY44 (mutation 3).

Yeast plasmids

pYC7 was the recipient for element II and its derivatives. This plasmid contains the inactive *CYC1* promoter/leader fused to the *E.coli lacZ* in a 2 μm-based plasmid containing the *URA3* selectable marker (Guarente, 1987, and references therein; Chang and Timberlake, 1993). pAA54 contains the yeast *GAL1/GAL10* promoter [UAS_{GAL}-*GAL*(p/l)] in a centromeric plasmid (*CEN6 ARS4*) containing the *HIS3* selectable marker (A.Andrianopoulos and W.E.Timberlake, unpublished results). Plasmids containing relevant constructs are: *abaA*$^+$ (pAA35) and *abaA100* [pRA112(10)]. pAA35 is a derivative of pAA54. In this plasmid the *abaA*$^+$ gene is under the control of the *GAL1/GAL10* promoter region (Lohr, 1984; West *et al.*, 1984; A.Andrianopoulos and W.E.Timberlake, unpublished results). Plasmid pRA112(10) was constructed by linearizing pAA35 at the unique *Bst*EII site located in the *abaA*$^+$ coding region. DNA ends were filled in with Sequenase that often adds a non-templated base and ligated, creating a frameshift mutation at amino acid 207 of the AbaA polypeptide (*abaA100*). Plasmids lacking the *Bst*EII site were selected and confirmed by DNA sequencing.

Oligonucleotides

Oligonucleotide sequences are shown in Table II.

Fungal strains and transformation techniques

A.nidulans PW1 (*biA1 argB2 methG1 veA1*) (P.Weglenski, Department of Genetics, Warsaw University, Poland) and *S.cerevisiae* YPH500 (*ura3-52 lys2-801*^{amber} *ade2-101*^{ochre} *trp*-Δ63 his3-Δ200 leu2-Δ1) (Sikorski and Hieter, 1989) were used. Strains were grown in appropriately supplemented minimal media (Pontecorvo *et al.*, 1953; Käfer, 1977; Ausubel *et al.*, 1987). Standard *A.nidulans* and *S.cerevisiae* transformation procedures were employed (Yelton *et al.*, 1984; Timberlake, 1986; Ausubel *et al.*, 1987). For developmental cultures, exponentially growing cells were harvested onto Whatman I filter paper discs and placed in Petri dishes to induce conidiation (Law and Timberlake, 1980). Under these conditions, conidiophores develop synchronously over a 24 h period and continue to produce spores for at least 3 days.

In situ staining for β-galactosidase activity in A.nidulans

In situ staining for β-galactosidase activity was performed essentially as described by Adams and Timberlake (1990) and Aguirre *et al.* (1990).

Plate assay for β-galactosidase activity in yeast

Cells were grown for 30 h at 32°C in synthetic liquid medium plus 0.5% galactose and spotted onto Petri plates (6×10^5 cells) containing appropriately supplemented synthetic solid medium plus X-gal and either glucose or galactose as a carbon source. Plates were incubated at 32°C for 4 days and photographed. Media were prepared as described in Ausubel *et al.* (1987).

β-galactosidase assays

Protein extracts from *A.nidulans* were prepared from lyophilized samples as described by van Gorcom *et al.* (1985) and Gomez and Peñalva (1990). β-galactosidase activity assays were done as described by Miller (1972). A time-course was done with each strain by taking samples every 5 h after developmental induction up to 40 h. We observed week to week variation in the absolute levels of β-galactosidase in *A.nidulans* developmental cultures. Therefore parallel, concurrent controls were incorporated into all experiments. Variation was ≤10% for replicate samples in individual experiments. Protein concentrations were determined by the Bradford (1976) procedure. A single *S.cerevisiae* colony was pre-inoculated in 3 ml of appropriately supplemented synthetic medium containing 2% lactate plus 0.5% galactose, incubated at 32°C, 300 r.p.m. for 30 h. 50 ml of either glucose-containing or galactose-containing media were inoculated at the same density and incubated until reaching an OD_{600nm} of 1.2. After harvesting and washing, the cells were suspended in 400 μl Z buffer (60 mM Na_2HPO_4, pH 7.0, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ and 50 mM 2-β-mercaptoethanol) containing protease inhibitors + 800 μl cold glass beads (Sigma). Cells were disrupted by vortexing and the extracts were cleared by centrifugation. β-galactosidase activities and protein concentrations were determined essentially as described by Ausubel *et al.* (1987), Miller (1972) and Bradford (1976).

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