MOLECULAR AND CELLULAR BIOLOGY, Sept. 1990, p. 4912–4919
0270-7306/90/094912-08$02.00/0

Vol. 10, No. 9

Upstream Elements Repress Premature Expression of an *Aspergillus* Developmental Regulatory Gene

THOMAS H. ADAMS†* AND WILLIAM E. TIMBERLAKE
Department of Genetics, University of Georgia, Athens, Georgia 30602

Received 30 March 1990/Accepted 11 June 1990

The *Aspergillus nidulans abaA* gene regulates intermediate steps in asexual reproductive development and is itself developmentally regulated. An 822-base-pair DNA fragment from the *abaA* 5′-flanking region is sufficient to drive developmentally appropriate expression of the *Escherichia coli lacZ* gene. Deletion analysis showed that this fragment contains elements that repress transcription in vegetative cells and immature conidiophores and that activate transcription later during development. A 45-base-pair region encompassing the major and minor *abaA* transcription initiation sites contains directly repeated sequences related to the mammalian initiator (Inr) element (S. T. Smale and D. Baltimore, Cell 57:103–113, 1989). This element or sequences in the untranslated leader were sufficient for correct transcription initiation and for measurable developmental induction. Similar elements were present at or near the initiation sites of other developmentally regulated genes. We propose that the temporal and spatial specificity of expression of these genes results from modulation of the activity of Inr elements.

Aspergillus nidulans conidiation is a useful system for investigating processes controlling development (40). Differentiation of the multiple cell types that make up the *A. nidulans* asexual reproductive apparatus, the conidiophore, involves sequential activation of several hundred genes (6, 10, 21, 38, 40, 45). Several of these genes are required for normal conidiophore assembly or conidiospore differentiation (1, 6, 12, 23, 26), whereas others have apparently dispensable functions (4).

Much of the temporal and spatial specificity of gene expression during conidiophore development is controlled by three regulatory genes, *brlA*, *abaA*, and *wetA* (1, 6, 23). The order of appearance of the products of these genes and their relative concentrations in the various conidiophore cell types determine where and to what extent other developmentally controlled genes are expressed (23).

brlA is activated a few hours after development begins, at the time when elongating conidiophore stalks near their mature height (6). Stalks of *brlA* mutant strains grow indeterminately and fail to differentiate other specialized cell types (10). By contrast, forced expression of *brlA* in vegetative cells under conditions that normally suppress sporulation leads to cessation of hyphal elongation and to differentiation of hyphal tips into phialidelike cells that produce viable spores (1). *abaA* is activated after *brlA* when metulae and phialides appear on the conidiophore vesicle (6). *abaA* mutant strains form functionally deranged phialides that proliferate instead of forming G_1-arrested conidia (10). Forced expression of *abaA* in vegetative cells induces *brlA* and leads to dramatic cellular transformations but does not cause phialide or spore formation, indicating that *brlA* and *abaA* must be expressed in the correct order for conidiophore development to take place (23).

The biochemical mechanisms responsible for gene activation by *brlA*, *abaA*, and *wetA* are not known. *brlA* appears to encode a nucleic acid-binding protein that requires two zinc fingers for activity (1, 2). However, a specific site for BrlA binding has not been identified. Because *brlA* expression is necessary (6) and sufficient (1) for *abaA* induction, DNA sequences associated with *abaA* are potential sites for BrlA interaction. We have begun to look for such sites by systematically mutating *abaA* upstream sequences. Our results show that *abaA* is repressed in hyphae and that *brlA* is required for its derepression during development. We propose that upstream regulatory sequences exert their effects on a short repeated initiator element that is itself sufficient to provide a detectable level of developmental induction. Interestingly, this element is found near the initiation sites of other developmentally regulated genes, including *brlA*, and contains a core sequence that is nearly identical to the core sequence of the mammalian initiator (Inr) element, which has an analogous function (32).

MATERIALS AND METHODS

Construction of the *abaA*::*lacZ* gene fusions. Plasmid pTA57 (Fig. 1A), containing *abaA* upstream sequences (GenBank accession number J04850) through the initiation codon fused to an internal codon of *Escherichia coli lacZ*, was constructed in three steps. First, the 982-base-pair (bp) *Sal*I-*Nco*I *abaA* promoter/leader (pl) fragment was cloned into the *Sal*I and *Bam*HI sites of Bluescript KS$^-$ (Stratagene, La Jolla, Calif.) by using the partially complementary oligonucleotides (5′-GATGGTCGCGACTGCAGG-3′ and 5′-GATCCCTGCAGTCGCGAC-3′). Second, the 3.2-kbp *Bam*HI *lacZ* fragment from pULAC (15) was fused in frame with the ATG from *abaA* to give pTA53. Finally, the 4-kbp *Xba*I fragment from pAC1 (15), containing the *argB*::*CAT* fusion gene, was inserted into the unique *Xba*I site in pTA53 to yield pTA57.

5′ deletions of the *abaA* promoter were generated by digesting pTA57 with *Xho*I and *Kpn*I and treatment with exonuclease III (16). The resultant fragments were treated with S1 nuclease and religated in the presence of an 8-bp *Kpn*I linker (GGGATCCC). The endpoints of each deletion were determined by DNA sequencing.

Fragments with 3′ deletions of the *abaA* promoter were

* Corresponding author.
† Present address: Department of Biology, Texas A & M University, College Station, TX 77843-3258.

generated by the polymerase chain reaction (30). *Bam*HI-digested pTA57 (10 μg) was mixed with 200 pmol of the appropriate 5′ and 3′ primers, 40 mM deoxynucleoside triphosphate, 1× polymerase chain reaction buffer (10 mM Tris hydrochloride [pH 8.3], 50 mM KCl, 2 mM $MgCl_2$, 0.01% gelatin), and 2 U of Taq DNA polymerase. Samples were subjected to 40 amplification cycles of 1 min at 94°C, 1.5 min at 53°C, and 2.5 min at 72°C. The 3′ oligonucleotide primers used were as follows: 5′-GGTACC(−77)AATGT ATCCCCAGTAGTAGC-3′, 5′-GGTACC(−103)GGATCAC GCTGCTTGTTTGG-3′, 5′-GGTACC(−224)ACTGCTCTG TTGCAGCGAAT-3′, and 5′-GGTACC(−317)GAGATTTT TAGGGTGCGCAA-3′, each of which is complementary to sequences in the *abaA* promoter and contains a *Kpn*I site (underlined) at the 5′ end. The 5′ oligonucleotide primer in all cases was 5′-CACTAAAGGGAACAAAAGCT-3′ which is complementary to sequences in Bluescript just beyond the *Kpn*I site located in the Bluescript polylinker. Fragments generated with these primers were digested with *Kpn*I and cloned into the *Kpn*I site of Bluescript KS^-. Internal *abaA* promoter deletions were then constructed by moving these sequences as *Kpn*I fragments into the *Kpn*I site at the 5′ endpoint of the deletions described above (Fig. 3).

The *abaA*::*trpC*::*lacZ* fusion clones (pTA61 and pTA62; Fig. 2A) were constructed by exchanging an *Sst*I-*Cla*I fragment from either pJHΔ47 or pJHΔ11 (containing a deletion in the *trpC* promoter and the amino-terminal portion of the *trpC*::*lacZ* fusion; 15) with a similar fragment from pTA57, containing sequences from −45 of the *abaA* promoter to the same point in *lacZ*.

***Aspergillus* strains, growth, and genetics.** *A. nidulans* PW1 (*biA1 argB2 methG1 veA1*) was provided by P. Weglenski, and all of the strains shown in Fig. 1 through 4 were constructed by transformation of PW1 and pTA57 or various *abaA*(pl) deletion plasmid derivatives with selection for arginine prototrophy. *brlA* deletion strains carrying *abaA* (pl)::*lacZ* fusions are progeny from crosses between the transformant strains described above [*biA1 abaA*(pl)::*lacZ methG1 veA1*] and TA046 (*biA1 pyroA4 veA1 ΔbrlA*). All of the strains analyzed had the genotype *biA1 abaA*(pl)::*lacZ pyroA4 veA1 ΔbrlA*. Diploid strains containing both the *alcA*(p)::*brlA* and the *abaA*(pl)::*lacZ* gene fusions were selected from heterokaryons of TA023 [*biA2 pabaA2 alcA*(p)::*brlA veA1*] and transformants carrying the appropriate *abaA*(p/l)::*lacZ* fusion gene. Standard *A. nidulans* genetic (11, 27) and transformation techniques (39, 44) were used.

All strains were grown in appropriately supplemented minimal medium (18). Developmental cultures were grown as described previously (19) with modifications. Conidia (2×10^5/ml) were inoculated into supplemented minimal medium and incubated for 22 h at 37°C and 300 rpm. Samples were harvested onto filter paper disks, placed on 1.2% agar plates containing the same medium, and incubated at 37°C for the times indicated (Fig. 1 and 2). The timing of appearance of various developmental structures was as described previously (19). Cultures of *brlA* deletion strains were started with ascospores from self-fertilized cleistothecia. Induction of the *alcA*(p)::*brlA* fusion gene was achieved by transfer to *alcA*-inducing medium as described (1).

In situ staining for β-galactosidase activity. Material for in situ staining of β-galactosidase activity was prepared by inoculating conidia onto supplemented minimal medium (containing 1% agarose and 2% glucose) that was solidified on the surface of a microscope slide. Samples were incubated at 37°C in moist chambers until mature conidiophores were observed. Cells were fixed by treatment with chloroform vapors for 15 min at room temperature. Enzyme activity was detected by submerging the slides in a solution of 50 mM sodium phosphate (pH 7.5), 0.01% Tween 20, 0.1% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and incubating at 37°C for 1 to 2 h. Sometimes, darker staining was obtained by incubating the slides for an additional 12 h at 4°C. No staining was observed for the control strain under either condition.

β-Galactosidase assays. Protein extracts were prepared from lyophilized samples of vegetative and conidiating cultures of *A. nidulans* as described (41). The assay for β-galactosidase activity with *o*-nitrophenol-β-D-galactopyranoside (ONPG) substrate was done as described by Miller (22). To assure repeatability of the assays, a time course was done with each strain by taking samples every 5 h after development was induced and enzyme activity was measured at each time. Protein concentrations were determined by the Bradford (7) procedure.

RESULTS

***abaA* upstream elements confer developmental control.** *abaA* mRNA is not detectable in hyphae and accumulates during conidiophore development (6). To investigate the role of sequences 5′ of the *abaA* coding region in controlling gene expression, we fused the *abaA* ATG in frame to an internal codon of *E. coli lacZ*. The resultant fusion gene *abaA* (pl)::*lacZ*, coding for the 156-nucleotide *abaA* untranslated leader and containing 726 bp upstream of the major mRNA cap site, was inserted into an *Aspergillus* transformation vector (Fig. 1A) and integrated at *argB* by forced homologous recombination (1, 15) to obtain *Aspergillus* strain TTA573. Figure 1B shows that β-galactosidase activity was not present in TTA573 hyphae but became detectable 10 h after inducing development and increased thereafter. The onset of β-galactosidase accumulation coincided with the appearance of metulae and phialides and paralleled accumulation of *abaA* mRNA (6). Low levels of endogenous β-galactosidase (13) were detected at 30 h postinduction in a control strain (TTAARG [Fig. 1B]).

We determined the spatial distribution of β-galactosidase in developing cultures of TTA573 and TTAARG by fixing and staining for enzymatic activity. Figure 1C shows that β-galactosidase was largely restricted to the metulae, phialides, and immature conidia of TTA573. No staining of vesicles prior to phialide formation was observed. However, faint staining of vesicles was occasionally detected in mature conidiophores. Figure 1D shows that β-galactosidase activity was not produced in the conidiophores of TTAARG.

***abaA* upstream elements modulate transcription from a heterologous promoter.** We fused *abaA* 5′ sequences to a heterologous promoter to determine whether they were sufficient to provide transcriptional control. The *trpC* promoter was chosen because its promoter has been subjected to a detailed functional analysis (15). *trpC* is active in hyphae but is induced to higher levels during development (43). We fused the *abaA* −726 to −45 fragment to two *trpC*::*lacZ* fusion genes, one containing 11 bp 5′ of the mRNA cap site (yielding pTA62 and TTA621; Fig. 2A and B), the other containing 47 bp (yielding pTA61 and TTA612; Fig. 2A and B). The −47 *trpC* construct has been shown to have full activity, whereas the −11 construct has reduced activity (15). Figure 2C shows that addition of the *abaA* fragment to

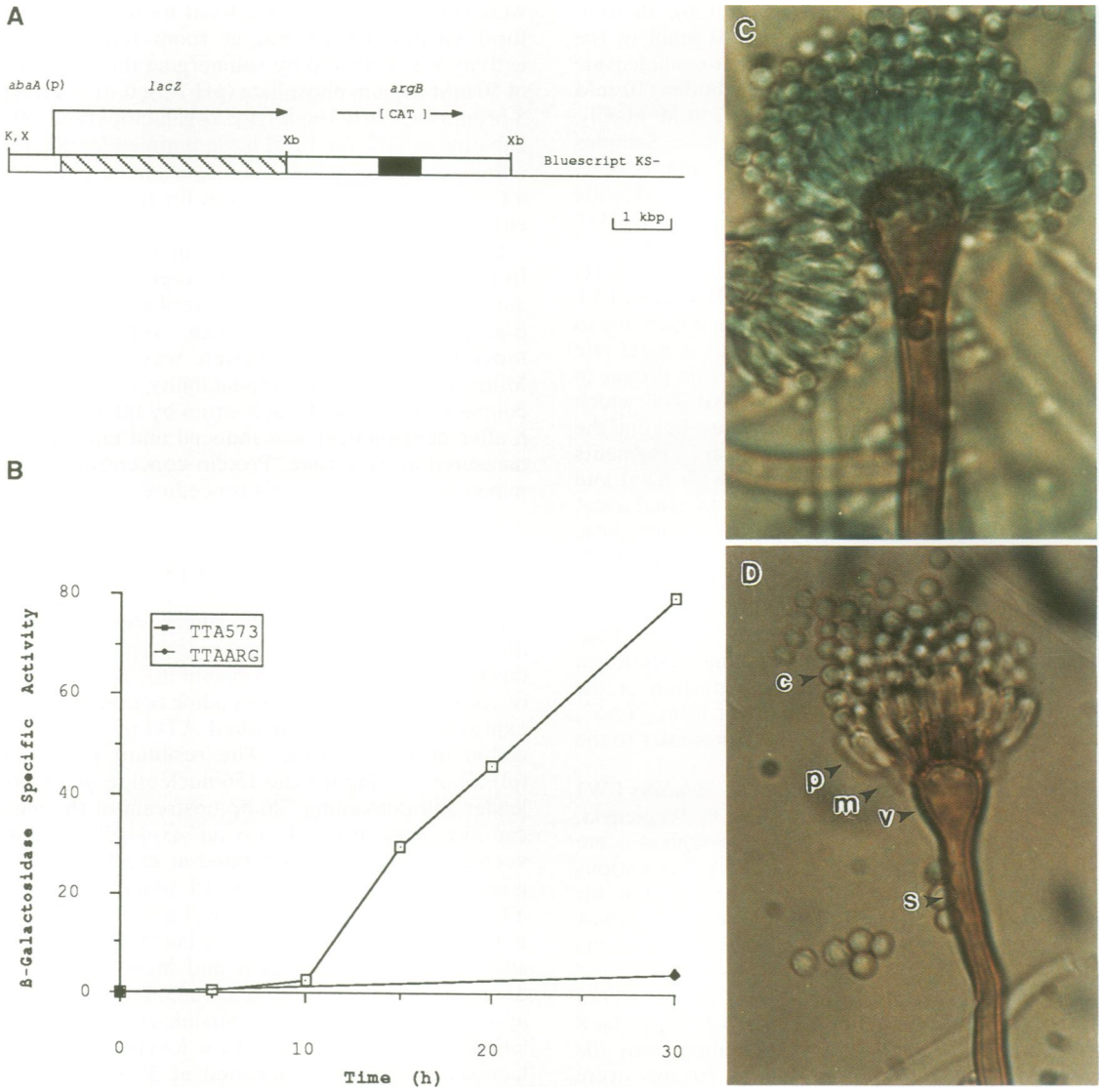

FIG. 1. Developmental regulation of *abaA*::*lacZ* fusion gene. (A) pTA57 was constructed as described in Materials and Methods by fusing the *abaA* promoter/leader region *abaA*(p/l) to *lacZ*. Arrows indicate directions of transcription. Restriction site abbreviations: K, *Kpn*I; X, *Xho*I; Xb, *Xba*I. (B) *Aspergillus* strain TTA573, containing pTA57 integrated at *argB*, and strain TTAARG, containing a similar plasmid that lacks the fusion gene (1), were grown vegetatively for 22 h and then induced to develop at 0 h. Samples were taken at the times indicated, and cell extracts were assayed for β-galactosidase activity. Activity is expressed as nanomoles of ONPG hydrolyzed per minute per microgram of protein. (C and D) TTA573 (C) and TTAARG (D) conidia were inoculated onto agar medium on the surface of a microscope slide, grown for 36 h, fixed, and stained for β-galactosidase activity as described in Materials and Methods. Photographs were taken by using bright-field microscopy. Conidiophore structures indicated are as follows: stalk (s), vesicle (v), metula (m), phialide (p), and conidium (c).

the −47 *trpC*::*lacZ* fusion gene completely repressed hyphal *lacZ* expression and partially repressed developmental expression (compare JHΔ47 and TTA612). Addition of the *abaA* fragment to the −11 construct further reduced the already low level of hyphal expression but slightly stimulated developmental expression up to the same level observed for the −47 fusion (compare JHΔ11 and TTA621). With both constructs, the final β-galactosidase levels were fivefold lower than with the *abaA*(−726 to +156)::*lacZ* fusion gene (Fig. 1B). Nevertheless, β-galactosidase specific activities increased >100-fold during development.

Multiple regulatory elements control *abaA* expression. To determine which *abaA* sequences are required for hyphal repression and developmental induction, we made sequential 5′ deletions and tested the resultant mutant constructs for activity following integration at *argB*. Figure 3 shows that deletion of sequences from −726 to −224 had little effect on the developmental timing or extent of *lacZ* induction. Deletion to −104 or −103 dereppressed *lacZ* expression in hyphae without affecting β-galactosidase levels during development. Further deletion to −84 or −77 reduced hyphal expression two- to threefold relative to that of −104 or −103 mutants, while increasing developmental β-galactosidase to levels threefold higher than those in the undeleted control (TTA573). Additional deletions to −36, −26, or −13 resulted in patterns of *lacZ* expression that were similar to those observed for the −103 or −104 deletions. Finally, a deletion extending 3′ of the major and minor mRNA cap sites (+100)

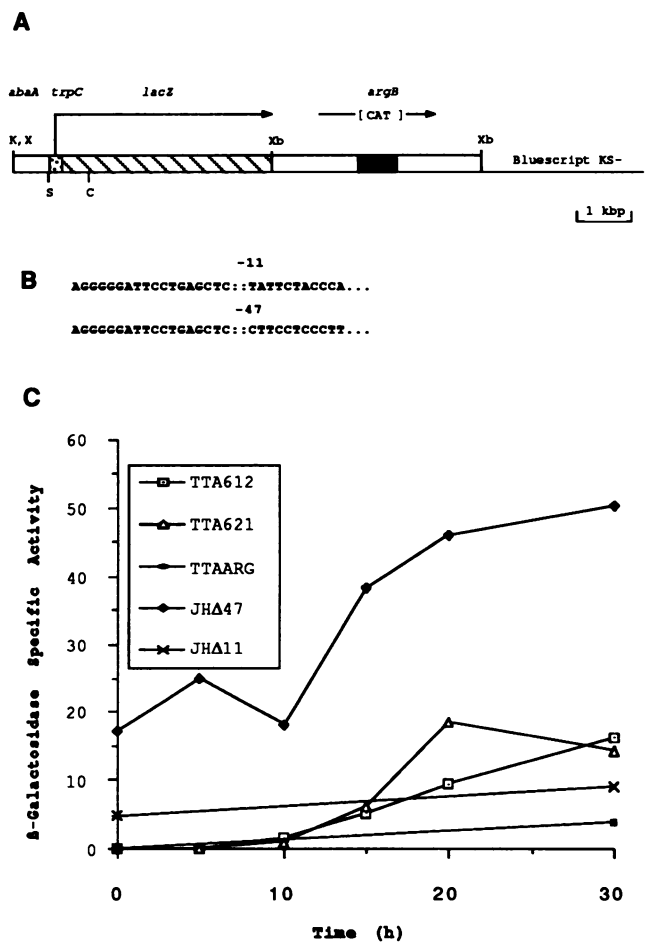

FIG. 2. Repression of hyphal expression of a *trpC*::*lacZ* fusion by *abaA* sequences. (A) pTA61 [*abaA*::*trpC*(Δ47)::*lacZ*] and pTA62 [*abaA*::*trpC*(Δ11)::*lacZ*] were constructed as described in Materials and Methods. Arrows indicate directions of transcription. Restriction site abbreviations: K, *Kpn*I; S, *Sst*I; C, *Cla*I. (B) Sequences at the junctions of the *abaA-trpC* fusions. (C) Strains TTA612 [*abaA*::*trpC*(−47)::*lacZ*], TTA621 [*abaA*::*trpC*(−11)::*lacZ*], JHΔ47 [*trpC*(−47)::*lacZ*], JHΔ11 [*trpC*(−11)::*lacZ*], and TTAARG (control) were grown vegetatively for 22 h and induced to develop at 0 h. Samples were taken at the times indicated, and cell extracts were assayed for β-galactosidase. Activity is given as nanomoles of ONPG hydrolyzed per minute per milligram of protein.

resulted in low β-galactosidase levels equivalent to those in the TTAARG control strain.

These results indicated that the *abaA* upstream region consists of functionally distinct elements. To confirm the existence of these elements and to map their positions more precisely, we determined the activities of constructs containing internal deletions. The results from these experiments (Fig. 3) were consistent with the existence of five classes of elements, which we designated A through E. Deletion of D (TTA1043; −77 to −13) had little effect on *lacZ* expression, but a partial deletion of D (TTA871; −77 to −26) caused a fourfold reduction in developmental β-galactosidase levels. Deletion of C (TTA1095; −103 to −77) led to somewhat derepressed hyphal levels of β-galactosidase and to a 3.5-fold increase in developmental levels, while deletion of C and D (TTA1101; −103 to −13) resulted in normal regulation. Deletion of B2 (TTA842; −224 to −103) or B1 (TTA1023; −317 to −224) had little effect on *lacZ* expression, but deletion of both elements (TTA916; −317 to −103) resulted in derepressed hyphal expression. Finally, deletion of B1, B2, C, and part of D (TTA1081; −317 to −26) resulted in derepressed hyphal expression and a 3.5-fold increase in developmental β-galactosidase activity. Existence of the A and E elements was inferred from these results and those from the 5′ deletions described immediately above.

***brlA*-Independent *abaA* expression.** To ascertain which aspects of *abaA* deregulation were *brlA* dependent, we determined β-galactosidase levels for the 5′ deletion mutants in a *brlA* mutant background. Table 1 shows that *brlA* was required for developmental induction of the −726 and −317 constructs. By contrast, a significant proportion of the developmentally induced β-galactosidase activity observed with the −103 through −13 deletion mutants was independent of *brlA*, the effect being most pronounced in the −84 and −77 mutants, whose activities increased ~40-fold during development in the *brlA* mutant strain. As predicted from the fact that *brlA* is inactive in vegetative cells (6), its deletion had no effect on hyphal expression of any *abaA* promoter mutants.

To test whether these constructs retained their ability to respond to *brlA*, we constructed diploids containing selected *abaA*::*lacZ* fusion genes integrated at *argB* on chromosome III and an *alcA*(p)::*brlA* fusion gene (1) integrated at the same locus on the homologous chromosome. *brlA* expression was initiated by transferring hyphae to growth medium that induces the *alcA* promoter but normally represses sporulation. Spore formation occurred beginning 2 h after induction, as expected (1). Table 1 shows that in the strains tested, *brlA* activation induced *abaA*::*lacZ* expression more than fivefold after 4 h.

5′ deletions affect temporal and spatial regulation. A deletion of element C that left D intact (TTA57-130; Fig. 3) led to slight hyphal derepression and to a 3.5-fold increase in expression during development. Figure 4 shows that temporal and spatial control of *lacZ* expression in this mutant is also abnormal. β-Galactosidase levels in TTA57-130 increased detectably by 5 h after developmental induction, as opposed to the −726 fusion strain (TTA573) in which enzyme levels did not become detectable until 10 h (Fig. 4A). In addition, in situ staining for β-galactosidase showed that immature conidiophore stalks and vesicles as well as the vesicles, metulae, phialides, and young conidia of mature conidiophores of this deletion mutant had detectable β-galactosidase levels (Fig. 4B and E). Developmental control was also abnormal for mutants containing deletions extending beyond −77 in that conidiophore stalks and vesicles of a −13 deletion had detectable β-galactosidase levels (Fig. 4C), whereas the same structures in the undeleted control did not have detectable levels (Fig. 1C and 4D).

Accurate transcription initiation is maintained in minimal promoters. Expression of the *abaA*::*lacZ* fusion gene was derepressed in hyphae and induced during development even when deletions extended to within 13 bp of the major *abaA* transcription initiation site (Fig. 3, TTA57-411; Fig. 4C). To determine whether transcription initiated at the correct sites, hyphal RNA from derepressed mutants was subjected to primer extension analysis. Figure 5 shows that no transcription was detectable from either start site with hyphal RNA of a strain lacking an *abaA*::*lacZ* fusion gene (TTAARG) or in a strain with the −726 *abaA*(p) fused to *lacZ*. However, promoter deletions to −26 or −13 allowed derepressed hyphal *lacZ* expression and accurate transcriptional initiation from both of the normal *abaA* start sites.

STRAIN	DELETION	ß-GALACTOSIDASE ACTIVITY H	D
TTA573	-726	0.1	80
TTA57-5	-317	nil	84
TTA57-287	-224	nil	81
TTA57-132	-104	13.0	87
TTA57-30	-103	9.0	107
TTA57-133	-84	4.0	227
TTA57-130	-77	2.5	283
TTA57-368	-36	14.5	79
TTA57-423	-26	11.5	80
TTA57-411	-13	11.5	78
TTA57-339	+100	nil	6
TTA1041	-77 → -13	0.4	103
TTA871	-77 → -26	nil	23
TTA1095	-103 → -77	1.1	281
TTA1101	-103 → -13	nil	139
TTA842	-224 → -103	nil	49
TTA1023	-317 → -224	nil	53
TTA916	-317 → -103	8.0	133
TTA1081	-317 → -26	1.6	260
TTAARG		nil	4

FIG. 3. Multiple regulatory elements in *aba* promoter. The boxed regions A through E indicate presumptive functional elements based on data described in text. The two *abaA* transcriptional initiation sites are indicated by the arrow. 5′ deletions are indicated by a single number representing the deletion endpoint to which vector (Bluescript KS$^-$) sequences were fused. Internal deletions are specified by positions of the deletion endpoints. β-Galactosidase activity is given as nanomoles of ONPG hydrolyzed per minute per milligram of protein. All constructs were integrated in single copy at the *argB* locus. H and D refer to hyphal and developmental, respectively.

With the −77 deletion, we detected only transcription from the upstream initiation site.

DISCUSSION

abaA transcription initiates from a single site within each of two directly repeated 22-bp elements (Fig. 5 and 6; 23). A deletion that leaves all but 7 bp of the first repeat and the entire second element permits accurate transcription initiation from both start sites and developmental induction. For many eucaryotic genes, transcription is initiated at a specific site downstream from a TATA homology (9, 36, 42). Mutations in TATA either decrease initiation frequency or result in initiation from heterogeneous sites (14, 42). The single potential *abaA* TATA (TATAA, −39 to −34) was removed in several deletion mutants without affecting initiation (Fig. 3 and 5). Vector DNA joined to the deletion endpoints contained two potentially active TATAs. However, insertion of *abaA* 5′ fragments lacking any TATA-like sequences between −13 and the vector did not affect transcriptional activity (Fig. 3). There are no TATA-like sequences on either strand in the 156 bp corresponding to the *abaA* untranslated leader (23). We infer that transcription from the minimal *abaA* promoter does not require TATA.

TABLE 1. *brlA*-Independent *abaA*::*lacZ* expression

5′ Deletion[a]	β-Galactosidase sp act[b]					
	brlA$^+$		Δ*brlA*[c]		*alcA*(p)::*brlA*[d]	
	0 h	30 h	0 h	30 h	0 h	4 h
−726	0.1	80.0	0	1.4	0	9.7
−317	0.0	84.0	0	1.6	–	–
−103	9.1	107.0	4.0	21.0	3.2	18.4
−84	4.0	227.0	2.0	84.0	–	–
−77	3.0	283.0	2.0	80.0	2.0	11.0
−26	11.4	78.0	9.3	21.0	–	–
−13	11.4	78.0	5.1	9.2	–	–
TTAARG	0	4.0	–	–	0	0

[a] Each number represents the 5′ *abaA* promoter deletion endpoint.
[b] Expressed as nanomoles of ONPG hydrolyzed per minute per milligram of protein.
[c] Strains contain *brlA* deletions (1). –, Not done.
[d] *brlA* expression was activated in hyphae by induction of the *alcA*(p)::*brlA* fusion gene (1).

Smale and Baltimore (32) characterized an independent initiator element (Inr) for lymphocyte-specific TdT. Inr responds to TATA and other upstream regulatory elements but does not require them for activity. Figure 6 shows that sequences within the *abaA* 22-bp repeats, near the transcription initiation sites, closely resemble Inr. These core *abaA* Inr-like sequences are left intact in the *abaA* promoter deletions that extend into the 22 bp-repeats but remain transcriptionally active. Inr-related sequences also occur in association with the *brlA* and *trpC* transcription initiation sites (Fig. 6; 1, 15, 24). We propose that these *Aspergillus* elements are functionally related to the mammalian Inr and

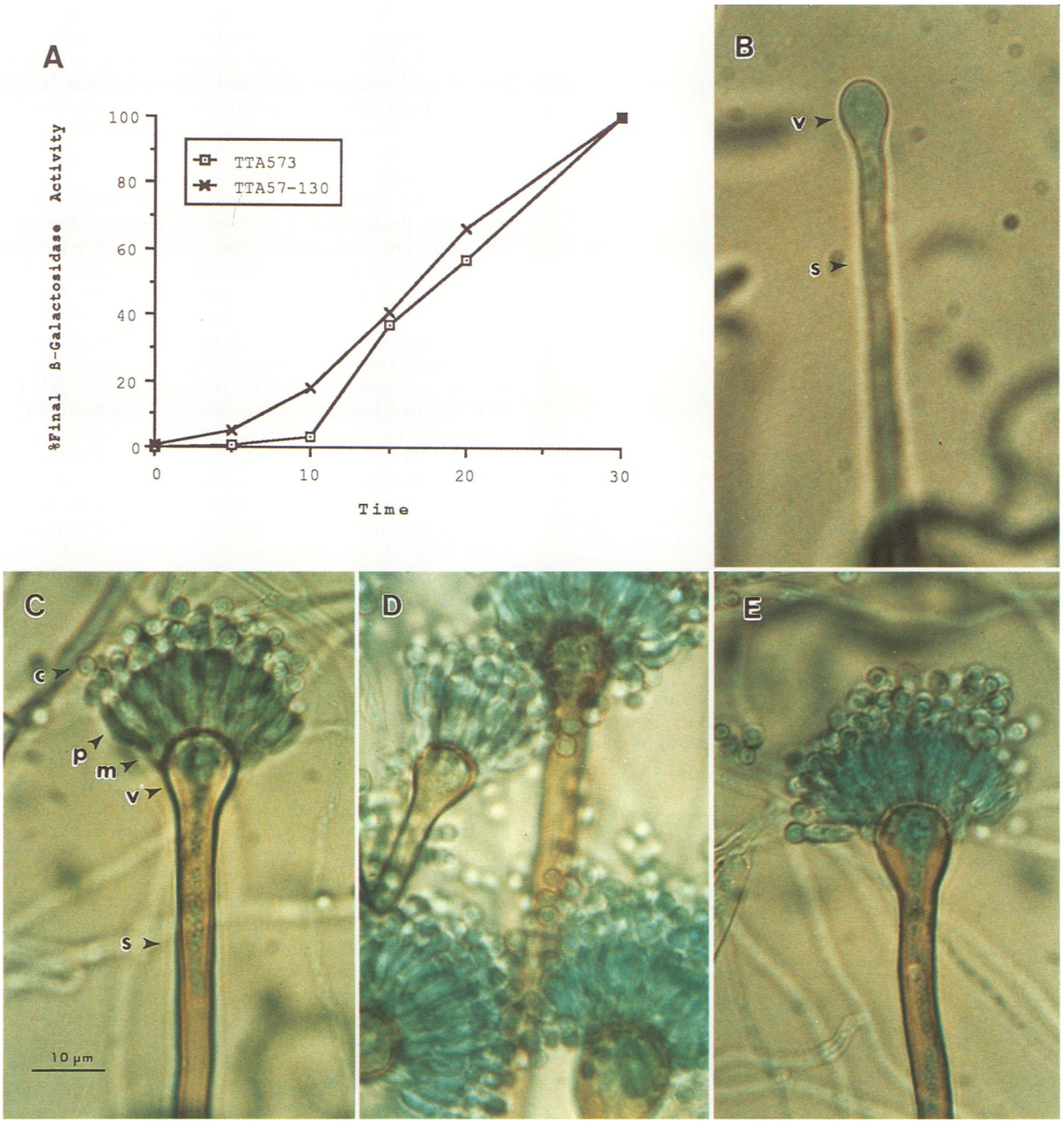

FIG. 4. Altered temporal and spatial control of *abaA* promoter deletion mutants. (A) Strain TTA573 (−726 deletion) and TTA57-130 (−77) were induced to develop at 0 h. Samples were taken at the times indicated, and β-galactosidase activity was determined and plotted as a percentage of the final β-galactosidase level at 30 h. (B through E) Spores from TTA57-130 (B and E), TTA57-411 (C), and TTA573 (D) were inoculated onto agar medium on the surface of microscope slides, incubated until mature conidiophores had developed, fixed, and stained for β-galactosidase activity as described in Materials and Methods. Photographs were taken by using bright-field microscopy. Conidiophore structures indicated are as follows: stalk (s), vesicle (v), metula (m), phialide (p), and conidium (c).

constitute a simple promoter. In contrast to the mammalian Inr, no specific base within the element is used for transcription initiation.

abaA promoter deletions containing only 13 bp upstream of transcription start are derepressed but nevertheless show approximately sevenfold induction during development. Thus, the *abaA* Inr or downstream sequences are sufficient for significant, albeit greatly reduced, developmental control. Similarly, the minimal *trpC* promoter, containing only 47 bp upstream and 11 bp downstream of the initiation site, was expressed in hyphae and induced approximately threefold during development (Fig. 2B, JHΔ47). Deletion of the *trpC* promoter to −11 disrupted the Inr and concomitantly decreased transcription levels, developmental induction, and accuracy of initiation (Fig. 2B; 15), indicating that the *A. nidulans* Inr may be necessary to direct transcriptional initiation for these developmentally regulated genes. We have not identified Inr elements at the initiation sites of several other *A. nidulans* promoters, including some that lack potential TATA homologies (for a recent review, see

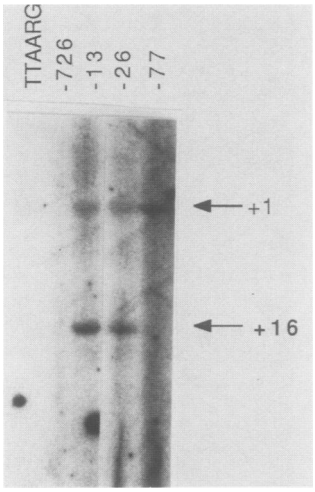

FIG. 5. Transcription from a minimal promoter element initiates appropriately. A radiolabeled oligonucleotide complementary to the *lacZ* sequence 5′-GTCACGACGTTGTAAAAC-3′ was used as a primer for extension reactions with 5 mg of poly(A)$^+$ RNA isolated from hyphal cultures of TTAARG, TTA573 (−726 deletion), TTA57-411 (−13), TTA57-423 (−26), and TTA57-130 (−77). The arrows correspond to the *abaA* transcription start sites identified previously (23).

reference 15). Thus, the Inr element appears to be restricted to a class of developmentally regulated *A. nidulans* genes.

The data presented in this paper show that the *abaA* Inr is subject to negative regulation by upstream elements that are also capable of repressing transcription from a heterologous promoter. Repression of regulatory genes in cells in which they should not be expressed has emerged as a common theme in development (3, 5, 17, 20, 25, 28, 29, 33, 34). Negative controls may be needed to damp out positive feedback loops (1, 8, 23, 31, 33, 35, 37). During *A. nidulans* conidiophore development, *brlA* induces *abaA*, initiating a

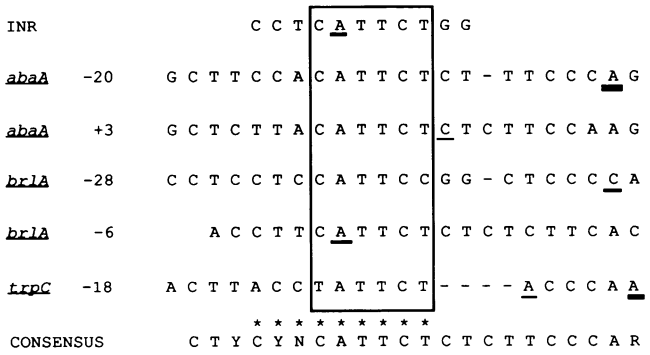

FIG. 6. Developmentally regulated Initiator sequence for *Aspergillus* genes. Two repeated sequences from the *abaA* transcription initiation region (23) are shown aligned with each other, with two similar sequences found at the *brlA* initiation region (1), with a similar sequence found at the *trpC* initiation site (24), and with the mammalian TdT Inr element (32). Underlined nucleotides indicate initiation sites, with the thickness of the lines representing relative frequency of initiation. A consensus initiator sequence for the *A. nidulans* genes is shown at the bottom of the figure. An asterisk indicates a match between the *A. nidulans* consensus and the minimal mammalian Inr element. Abbreviations: Y, pyrimidine; R, purine; N, any base; dash, space.

positive feedback loop that may serve to amplify the primary inductive signal (1, 23). Activation of this loop in vegetative cells by forced expression of either *brlA* or *abaA* stops growth and induces abnormal developmental transformations that are ultimately lethal. Negative control of developmentally regulated genes was previously unknown in this system (23) but could play a role in regulating the *brlA-abaA* feedback loop.

The *abaA* upstream regulatory region consists of multiple elements, designated A through E, having different activities. Deletion of A and B1 had no effect on regulation, yet their presence in some constructs had major effects on transcriptional activity. Deletion of B1 or B2 alone had no effect on transcription, but deletion of both led to derepression, indicating that they each repress transcription and are functionally redundant to each other. However, there are no obvious sequence similarities between B1 and B2. Deletion of C led to hyperactivation, whereas deletion of C and D together gave normal control, indicating that factors bound to C and D may interact. Partial deletion of D reduced the level of developmental induction. Additional *cis*-acting regulatory elements may exist that have not been resolved by our experiments. These results indicate that numerous proteins bind to the *abaA* upstream region and interact to control expression of this crucial developmental regulatory gene. The identities of these proteins and their modes of action are not known.

The only known pathway-specific gene required for *abaA* expression is *brlA* (12). *brlA* is necessary and sufficient for *abaA* expression (1, 6) and encodes a protein that requires two Zn fingers for activity (2). Thus, *brlA* has properties consistent with it encoding a transcriptional inducer of *abaA*. The results presented in this paper indicate that at least part of the demand for *brlA* is mediated by element C (Table 1). Deletion of C alone results in developmental hyperactivation, a large fraction of which is *brlA* independent, with only slight hyphal derepression. One possibility is that BrlA may displace a negative regulatory factor(s) bound to C. Some *brlA*-independent developmental activation was also observed with promoters lacking A, B1, and B2, but containing C, although no hyperactivation was seen. Thus, C may not be the only element that binds repressors that are displaced by BrlA. Promoters lacking A, B1, B2, and C show a significant level of *brlA*-dependent induction, consistent with the idea that BrlA also interacts with D or E to enhance gene expression. Alternatively, BrlA could act indirectly through other gene products to control *abaA* transcription.

Our observation that *abaA* is negatively regulated has significant implications concerning the identification of developmental regulatory genes in *A. nidulans* species. Previous screens for developmental mutants (10, 21) would probably have failed to identify lesions in *abaA* repressors. Loss of function mutations are expected to lead to activation of *abaA* in hyphae, which has been shown to be lethal (23). Rare, altered function mutations might be expected to yield phenotypes similar to those displayed by *abaA* mutants or, if the repressors are developmentally nonspecific, to be lethal. The availability of the cloned *brlA* and *abaA* genes and knowledge of their *cis*-acting regulatory elements makes mutant searches with refined sensitivities possible.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant GM37886 from the National Institutes of Health to William E. Timberlake.

We thank our colleagues in the laboratory for their many useful

contributions to this work and Bob Geever for his critical evaluation of the manuscript.

LITERATURE CITED

1. **Adams, T. H., M. T. Boylan, and W. E. Timberlake.** 1988. *brlA* is necessary and sufficient to direct conidiophore development in *Aspergillus nidulans*. Cell **54**:353–362.
2. **Adams, T. H., H. Deising, and W. E. Timberlake.** 1990. *brlA* requires both zinc fingers to induce development. Mol. Cell. Biol. **10**:1815–1817.
3. **Akerblom, I. E., E. S. Slater, M. Beato, J. D. Baxter, and P. L. Mellon.** 1988. Negative regulation by glucocorticoids through interference with a cAMP-response enhancer. Science **241**:350–353.
4. **Aramayo, R., T. H. Adams, and W. E. Timberlake.** 1989. A large cluster of highly expressed genes is dispensable for growth and development in *Aspergillus nidulans*. Genetics **122**:65–71.
5. **Biggin, M. D., and R. Tjian.** 1989. A purified *Drosophila* homeodomain protein represses transcription in vitro. Cell **58**:433–440.
6. **Boylan, M. T., P. M. Mirabita, C. E. Willett, C. R. Zimmerman, and W. E. Timberlake.** 1987. Isolation and physical characterization of three essential conidiation genes from *Aspergillus nidulans*. Mol. Cell. Biol. **7**:3113–3118.
7. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
8. **Chater, K. F., C. J. Bruton, K. A. Plaskitt, M. J. Buttner, C. Mendez, and J. D. Helmann.** 1989. The developmental fate of *S. coelicolor* hyphae depends upon a gene product homologous with the motility sigma factor of *B. subtilis*. Cell **59**:133–143.
9. **Chen, W., and K. Struhl.** 1985. Yeast mRNA initiation sites are determined primarily by specific sequences, not by the distance from the TATA element. EMBO J. **4**:3273–3280.
10. **Clutterbuck, A. J.** 1969. A mutational analysis of conidial development in *Aspergillus nidulans*. Genetics **63**:317–327.
11. **Clutterbuck, A. J.** 1974. *Aspergillus nidulans*, p. 447–510. *In* R. C. King (ed.), Handbook of genetics. Plenum Publishing Corp., New York.
12. **Clutterbuck, A. J.** 1977. The genetics of conidiation in *Aspergillus nidulans*, p. 305–317. *In* J. E. Smith and J. A. Pateman (ed.), Genetics and physiology of *Aspergillus*. Academic Press, Inc. (London), Ltd., London.
13. **Fantes, P. A., and C. F. Roberts.** 1973. Beta-galactosidase activity and lactose utilization in *Aspergillus nidulans*. J. Gen. Microbiol. **77**:471–486.
14. **Guarente, L.** 1987. Regulatory proteins in yeast. Annu. Rev. Genet. **21**:425–452.
15. **Hamer, J. E., and W. E. Timberlake.** 1987. Functional organization of the *Aspergillus nidulans trpC* promoter. Mol. Cell. Biol. **7**:2352–2359.
16. **Henikoff, S.** 1987. Unidirectional digestion with exonuclease III in DNA sequence analysis. Methods Enzymol. **155**:156–165.
17. **Johnson, A. D., and I. Herskowitz.** 1985. A repressor (MATa2 product) and its operator control expression of a set of cell type specific genes in yeast. Cell **42**:237–247.
18. **Käfer, E.** 1977. Meiotic and mitotic recombination in *Aspergillus* and its chromosomal aberrations. Adv. Genet. **19**:33–131.
19. **Law, D. J., and W. E. Timberlake.** 1980. Developmental regulation of laccase levels in *Aspergillus nidulans*. J. Bacteriol. **144**:509–517.
20. **Levine, M., and S. L. Manley.** 1989. Transcriptional repression of eukaryotic promoters. Cell **59**:405–408.
21. **Martinelli, S. D., and A. J. Clutterbuck.** 1971. A quantitative survey of conidiation mutants in *Aspergillus nidulans*. J. Gen. Microbiol. **69**:2261–2268.
22. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Mirabito, P. M., T. H. Adams, and W. E. Timberlake.** 1989. Interactions of three sequentially expressed genes control temporal and spatial specificity in *Aspergillus* development. Cell **57**:859–869.
24. **Mullaney, E. J., J. E. Hamer, K. A. Roberti, M. M. Yelton, and W. E. Timberlake.** 1985. Primary structure of the *trpC* gene from *Aspergillus nidulans*. Mol. Gen. Genet. **199**:37–45.
25. **Nasmyth, K., D. Stillman, and D. Kipling.** 1987. Both positive and negative regulators of HO transcription are required for mother-cell-specific mating-type switching in yeast. Cell **48**:579–587.
26. **O'Hara, E. B., and W. E. Timberlake.** 1989. Molecular characterization of the *Aspergillus nidulans yA* locus. Genetics **129**: 249–254.
27. **Pontecorvo, G., J. A. Roper, L. M. Hammons, K. D. Macdonald, and A. W. J. Bufton.** 1953. The genetics of *Aspergillus nidulans*. Adv. Genet. **5**:141–238.
28. **Remmers, E. F., J.-Q. Yang, and K. B. Marcu.** 1986. A negative transcriptional control element located upstream of the murine c-*myc* gene. EMBO J. **5**:899–904.
29. **Sakai, D. D., S. Helms, J. Carstedt-Duke, J.-A. Gustafsson, F. M. Rottman, and K. R. Yamamoto.** 1988. Hormone mediated repression: a negative glucocorticoid response element from the bovine prolactin gene. Genes Dev. **2**:1144–1154.
30. **Sakai, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and M. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostabile DNA polymerase. Science **239**:487–491.
31. **Schneuwly, S., R. Klemenz, and W. Gehring.** 1987. Redesigning the body plan of *Drosophila* by ectopic expression of the homoeotic gene antennapedia. Nature (London) **325**:816–818.
32. **Smale, S. T., and D. Baltimore.** 1989. The "initiator" as a transcription control element. Cell **57**:103–113.
33. **Sternberg, P. W., and H. R. Horvitz.** 1989. The combined action of two intercellular signaling pathways specifies three cell fates during vulval induction in *C. elegans*. Cell **58**:679–673.
34. **Sternberg, P. W., M. J. Stern, I. Clark, and I. Herskowitz.** 1987. Activation of the yeast HO gene by release from multiple controls. Cell **48**:567–577.
35. **Struhl, G.** 1985. Near-reciprocal phenotypes caused by inactivation or indiscriminate expression of the *Drosophila* segmentation gene fushi tarazu. Nature (London) **293**:36–41.
36. **Struhl, K.** 1987. Promoters, activator proteins, and the mechanism of transcriptional initiation in yeast. Cell **49**:295–297.
37. **Thayer, M. J., S. J. Tapscott, R. L. Davis, W. E. Wright, A. B. Lassar, and H. Weintraub.** 1989. Positive autoregulation of the myogenic determination gene *myoD1*. Cell **58**:241–248.
38. **Timberlake, W. E.** 1980. Developmental gene regulation in *Aspergillus nidulans*. Dev. Biol. **78**:497–510.
39. **Timberlake, W. E.** 1986. Isolation of stage- and cell-specific genes from fungi, p. 343–357. In J. Bailey (ed.), Biology and molecular biology of plant-pathogen interactions, NATO ASI Series, vol. H1. Springer-Verlag KG, Berlin.
40. **Timberlake, W. E., and M. A. Marshall.** 1988. Genetic regulation of development in *Aspergillus nidulans*. Trends Genet. **4**:162–169.
41. **van Gorcom, R. F. M., P. H. Pouwels, T. Goosen, J. Visser, H. W. J. van der Broek, J. E. Hamer, W. E. Timberlake, and C. A. M. J. J. van den Hondel.** 1985. Expression of *Escherichia coli* β-galactosidase gene in *Aspergillus nidulans*. Gene **40**:99–106.
42. **Wasylyk, B.** 1988. Transcription elements and factors of RNA polymerase B promoters of higher eukaryotes. Crit. Rev. Biochem. **23**:77–120.
43. **Yelton, M. M., J. E. Hamer, E. R. de Souza, E. J. Mullaney, and W. E. Timberlake.** 1983. Developmental regulation of the *Aspergillus nidulans trpC* gene. Proc. Natl. Acad. Sci. USA **80**:7576–7580.
44. **Yelton, M. M., J. E. Hamer, and W. E. Timberlake.** 1984. Transformation of *Aspergillus nidulans* by using a *trpC* plasmid. Proc. Natl. Acad. Sci. USA **81**:1470–1474.
45. **Zimmermann, C. R., W. C. Orr, R. F. Leclerc, E. C. Barnard, and W. E. Timberlake.** 1980. Molecular cloning and selection of genes regulated in *Aspergillus* development. Cell **21**:709–715.