Identification of Aspergillus *brlA* Response Elements (BREs) by Genetic Selection in Yeast

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ABSTRACT

The *brlA* gene of *Aspergillus nidulans* plays a central role in controlling conidiophore development. To test the hypothesis that *brlA* encodes a transcriptional regulator and to identify sites of interaction for the BrlA polypeptide, we expressed *brlA* in *Saccharomyces cerevisiae* (yeast) strains containing Aspergillus DNA sequences inserted upstream of a minimal yeast promoter fused to the *Escherichia coli lacZ* gene. Initially, a DNA fragment from the promoter region of the developmentally regulated *rodA* gene was tested and shown to mediate *brlA*-dependent transcriptional activation. Two additional DNA fragments were selected from an Aspergillus genomic library by their ability to respond to *brlA* in yeast. These fragments contained multiple copies of a sequence motif present in the *rodA* fragment, which we propose to be sites for BrlA interaction and designate *brlA* response elements (BREs). DNA fragments containing BREs upstream of a minimal Aspergillus promoter were capable of conferring developmental regulation in Aspergillus. Deletion of BREs from the upstream region of *rodA* greatly decreased its developmental induction. Multiple copies of a synthetic oligonucleotide with the consensus sequence identified among the BREs mediated *brlA*-dependent transcriptional activation in yeast. The results show that a primary activity of *brlA* is transcriptional activation and tentatively identify sites of interaction for the BrlA polypeptide.

THE filamentous Ascomycete *Aspergillus nidulans* produces complex multicellular structures, conidiophores, during its asexual reproductive cycle (OLIVER 1972; MIMS, RICHARDSON and TIMBERLAKE 1988). The *brlA* gene plays a key role in regulating conidiophore development (CLUTTERBUCK 1969; BOYLAN *et al.* 1987; ADAMS, BOYLAN and TIMBERLAKE 1988; MIRABITO, ADAMS and TIMBERLAKE 1989; TIMBERLAKE 1990, 1991). Null mutations in *brlA* block development at a very early stage and prevent expression of numerous genes that are specifically activated during conidiogenesis (CLUTTERBUCK 1969; BOYLAN *et al.* 1987). Hypomorphic *brlA* mutations permit more extensive, although abnormal, development and prevent expression of some, but not all, developmentally regulated genes (CLUTTERBUCK 1969; MARTINELLI 1979). Forced expression of *brlA* in vegetative cells (hyphae) inhibits growth (ADAMS and TIMBERLAKE 1990b) and leads to cellular differentiations that mimic several aspects of normal development, including production of viable conidia (ADAMS, BOYLAN and TIMBERLAKE 1988). The morphological changes induced by artificial activation of *brlA* are accompanied by expression of many conidiation specific genes, including the downstream regulatory genes *abaA* and *wetA* (MIRABITO, ADAMS and TIMBERLAKE 1989). Interactions among the products of *brlA*, *abaA*, *wetA* and two auxiliary regulatory loci, *stuA* and *medA*, appear to provide the spatiotemporal controls of gene expression required for normal conidiophore development (TIMBERLAKE 1990).

Although it has been apparent for some time that *brlA* is of central importance in regulating conidiophore development (CLUTTERBUCK 1977), the biochemical function(s) of its polypeptide product, BrlA, have not been determined. The conceptual BrlA polypeptide contains two C_2H_2 zinc finger motifs near its carboxyl terminus, similar to those found in numerous eukaryotic transcriptional regulatory proteins (MILLER, MACLACHLAN and KLUG 1985; ADAMS, BOYLAN and TIMBERLAKE 1988; JACOBS and MICHAELS 1990). Cysteine to serine mutations in either zinc finger abolish the capacity of ectopically expressed *brlA* to induce conidiation and associated changes in gene expression, suggesting that BrlA is indeed a transcriptional activator (ADAMS, DEISING and TIMBERLAKE 1990). However, attempts in our laboratory to detect specific binding of BrlA polypeptide to putative DNA targets have been unsuccessful, perhaps due to the low abundance of BrlA in Aspergillus complicated by proteolysis during preparation of cell extracts and insolubility or inactivity of BrlA produced in *Escherichia coli* (Y. C. CHANG, J. MOONEY and W. E. TIMBERLAKE, unpublished results).

To circumvent experimental difficulties associated with *in vitro* DNA binding assays, we adopted an

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alternative *in vivo* approach utilizing *Saccharomyces cerevisiae* (yeast) as a heterologous expression system. In this paper we show that *brlA* is capable of activating transcription from a minimal yeast promoter to which upstream sequences from the developmentally regulated *A. nidulans rodA* gene (STRINGER *et al.* 1991) had been fused. Additional random genomic DNA fragments mediating *brlA*-dependent gene activation were identified by their activities in the yeast system. The DNA sequences of these fragments were used to infer a short consensus for *brlA* response elements (BREs). Synthetic oligonucleotides containing this consensus also responded to *brlA* in yeast. Moreover, BREs identified in yeast were sufficient to direct developmental gene regulation in Aspergillus. Thus, the results strongly support the hypothesis that a primary activity of BrlA is as a transcriptional regulator. Implications of the existence of BREs in the promoters of developmentally regulated genes are discussed.

MATERIALS AND METHODS

Fungal strains and growth conditions: *S. cerevisiae* strain YPH500 (*ura3-52, lys2-801^{amber}, ade2-101^{ochre}, trp1-Δ63, his3-Δ200, leu2-Δ1*) was constructed by SIKORSKI and HIETER (1989). Yeast cells were grown in synthetic or YEP medium supplemented with 2% glucose, 2% glycerol/lactate, or 2% galactose. Cells were transformed by either lithium acetate treatment (ITO *et al.* 1983) or by electroporation (BECKER and GUARENTE 1991).

A. nidulans strains MH1055 (*biA1; wA3; gatA312; niiA4*) was obtained from Dr. M. J. HYNES and RMS025 (*suA1 adE20, yA2, adE20; ΔargB::trpCΔB, ΔrodA::argB; pyroA4; riboB2*) was obtained from M. A. STRINGER. All strains were maintained on appropriately supplemented minimal medium (KÄFER 1977). Cells were transformed by protoplast fusion (YELTON, HAMER and TIMBERLAKE 1984). Developmental cultures were grown as described by ADAMS and TIMBERLAKE (1990a).

Clone construction: Yeast plasmid pLGΔ178 was constructed by GUARENTE and MASON (1983). The *Xho*I site in pLGΔ178 was replaced with a polycloning site (*Sma*I-*Kpn*I-*Bgl*II-*Xho*I) to yield pYC7. Yeast plasmid pYC5 was constructed as follows. An *Nde*I site was created at the first ATG codon of *brlA* (ADAMS, BOYLAN and TIMBERLAKE 1988) by *in vitro* mutagenesis (KUNKEL 1985). The *Nde*I-*Sal*I fragment containing the *brlA* coding region was cloned into the *Nco*I-*Sal*I site of pMTL, a YCp50-derived plasmid (ROSE *et al.* 1987) containing the *LEU2* selective marker and the *GAL1/GAL10* promoter/leader adjacent to a polylinker region (C. N. GIROUX, personal communication). Clones containing cysteine to serine mutations of BrlA (pYC9, pYC10, pYC11) were constructed by exchanging the *Sph*I-*Sal*I fragment of pYC5 with *Sph*I-*Sal*I fragments of pTA59, pTA60, pTA68, respectively (ADAMS, DEISING and TIMBERLAKE 1990).

The polymerase chain reaction (PCR) (SAKAI *et al.* 1988) was used to subclone the smaller BREs. All PCR subclones were verified by DNA sequencing (SANGER, NICKLEN and COULSON 1977). An oligonucleotide pair containing the proposed BRE, 5′-MRAGGGR-3′ (5′-GATCTCAAGGGGTACCA-3′ and 5′-GATCTGGTACCCCTTGA-3′), was annealed and cloned into the *Bgl*II site of pYC7 in different orientations and copy numbers, which were determined by DNA sequencing.

pLIT14, a derivative of an *amdS-lacZ* fusion plasmid, pLIT1 (DAVIS, COBBETT and HYNES 1988), was obtained from M. J. HYNES. pYC30 was constructed by inserting an 800-bp *Hin*dIII-*Xho*I fragment from the coding region of *amdR* into the *Kpn*I site of pLIT14. The pYC30-derived plasmids were transformed into MH1055 and selected on medium containing γ-aminobutyric acid (GABA) supplemented with L-alanine as a nitrogen source. This selection scheme was based on the method developed by A. ANDRIANOPOULOS and M. J. HYNES (personal communication). In brief, *amdR* is a positive regulatory gene that controls the expression of genes involved in the catabolism of certain amides, lactams, and ω-amino acids (HYNES and PATEMAN 1970; ARST 1976; KATZ and HYNES 1989; ANDRIANOPOULOS and HYNES 1990). Homologous integration of pYC30 derivatives at the *amdR* locus disrupts *amdR*. The *gatA* gene codes for an ω-amino acid transaminase and is required for growth on GABA (ARST 1976). A *gatA*$^-$, *amdR*$^+$ strain does not grow on medium containing GABA supplemented with L-alanine as its sole nitrogen source due to the toxicity of GABA. A *gatA*$^-$, *amdR*$^-$ strain survives on the same selective medium due to the fact that this strain does not take up GABA.

rodA-containing plasmids were obtained from M. A. STRINGER. A 2.3-kb *Bgl*II-*Kpn*I fragment from pPL1, containing the *riboB* gene (OAKLEY *et al.* 1987), was cloned into the downstream *Sal*I site of *rodA* plasmid, pYC20, to yield pYC24. The 150-bp ROD2B fragment was removed from the 5′-flanking region of *rodA* by *in vitro* mutagenesis (KUNKEL 1985) to yield pYC23.

β-Galactosidase activity assays and RNA analysis: Yeast β-galactosidase activity assays were performed according to AUSUBEL *et al.* (1987). For the results given in Figures 2, 3, and 7, ten independent transformants were mixed and grown to stationary phase in medium containing glucose as carbon source. The cells were diluted 1:100 into medium containing galactose as carbon source and grown to an A_{600} of 0.1–0.3. *A. nidulans* protein extracts for β-galactosidase activity assays were prepared from lyophilized samples of developmental cultures as described by VAN GORCOM *et al.* (1985). Protein concentrations were determined by the BRADFORD (1976) method.

RNA was isolated from developmental cultures as described by TIMBERLAKE (1986). RNA was electrophoretically fractionated in formaldehyde agarose gels and transferred to nylon membranes (Hybond-N, Amersham Corp., Arlington Heights Illinois). Hybridization to ^{32}P-labeled probes was performed as recommended by the membrane manufacturer. Random hexamer priming was used to label the DNA probes to specific activities $> 1 \times 10^8$ dpm/μg (FEINBERG and VOLGELSTEIN 1983).

RESULTS

The strategy used to detect DNA sequences mediating *brlA* transcriptional activation in yeast was analogous to that used to detect glucocorticoid response elements (SCHENA and YAMAMOTO 1988). The *brlA* coding region was fused at the ATG codon downstream of the yeast *GAL1/GAL10* promoter/leader region [*GAL*(p/l)] in a *LEU2, CEN4* plasmid to yield pYC5. Yeast transformants containing pYC5 (TYC5) or a control plasmid lacking *brlA* sequences (TMTL) were grown under repressing (glucose) and inducing

(galactose) conditions to test if expression of *brlA* had adverse effects on growth. *brlA* mRNA was readily detectable in blots of total RNA from TYC5 grown with galactose as carbon source, but was not detectable in TYC5 grown with glucose as carbon source or in TMTL grown with either carbon source (data not shown). However, BrlA polypeptide was not reliably detectable by immunoblotting in either strain under repressing or inducing conditions (data not shown), suggesting that BrlA did not accumulate to high levels. The growth rates and morphologies of TYC5 and TMTL cells were similar with either carbon source. Thus, in contrast to the growth inhibitory effects of forced *brlA* expression in Aspergillus (ADAMS, BOYLAN and TIMBERLAKE 1988), expression of *brlA* in yeast had no obvious effects on growth.

The reporter plasmid pYC7, a derivative of pLGΔ178 (GUARENTE and MASON 1983), contained the *CYC1* promoter [*CYC1*(p/l)], lacking UAS elements, fused to *E. coli lacZ* in a *URA3*, 2 μm plasmid. A polylinker was introduced upstream of the *CYC1*(p/l) to permit introduction of potential BREs. *brlA* mediated activation of the fusion gene was monitored by X-gal staining of yeast colonies or by assaying β-galactosidase activity *in vitro*. Negligible levels of β-galactosidase were produced by yeast strains carrying both pYC5 and pYC7 under inducing or repressing conditions (data not shown).

A well characterized potential target gene for *brlA* is *rodA*, which has been proposed to encode a protein component of the conidial rodlet wall layer (STRINGER *et al.* 1991). Developmental induction of *rodA* requires *brlA* expression, but not expression of known downstream regulatory genes, and forced expression of *brlA* is sufficient to activate *rodA* transcription (MIRABITO, ADAMS and TIMBERLAKE 1989; STRINGER *et al.* 1991). To test for the ability of *rodA* upstream sequences to respond to *brlA* in yeast, a DNA fragment containing the *rodA* cap site and 752 bp of 5′-flanking sequence was inserted into pYC7 in reverse orientation to produce pROD1. This plasmid was introduced into TYC5 and TMTL and transformants were tested for β-galactosidase production during growth on glucose or galactose. Figure 1 shows that the *rodA* fragment activated transcription only in TYC5 grown under inducing conditions, as evidenced by X-gal staining. To confirm that transcriptional activation was dependent on *brlA*, three additional yeast strains (TYC9-11) containing mutant versions of *brlA* in which zinc finger cysteines were converted to serines were tested. None of the mutant genes activated *lacZ* expression under inducing conditions (Figure 1), indicating that activation is dependent upon the primary sequence of the BrlA polypeptide.

The *rodA* upstream region was further characterized by testing smaller fragments for their ability to

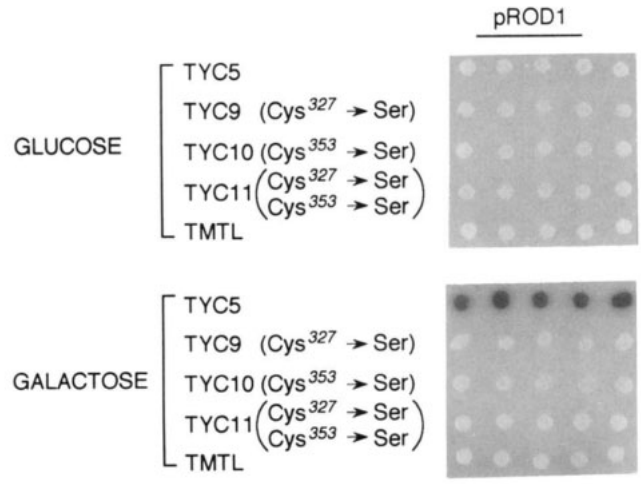

FIGURE 1.—*rodA* upstream elements respond to the *brlA* in yeast. An 899-bp fragment from the *A. nidulans rodA* promoter region was cloned in reverse orientation upstream of the *CYC1*(p/l)::*lacZ* fusion gene in pYC7 to yield pROD1. Yeast strains containing pYC5 (TYC5) [*GAL*(p/l)::*brlA*⁺], the *GAL*(p/l) vector alone (TMTL), or pYC5 derivatives containing *brlA* missense mutation alleles (TYC9-11) were transformed with pROD1. Five independent transformants were replicated onto medium containing X-gal and either glucose (top) or galactose (bottom) as carbon source. Plates were incubated for 3 days (glucose) or 4 days (galactose) at 30°.

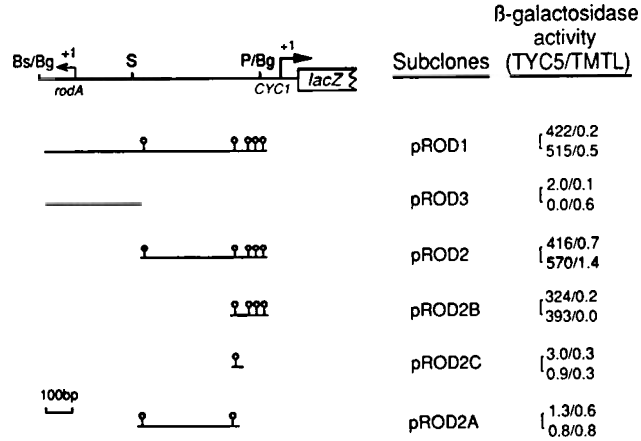

FIGURE 2.—Localization of *brlA* response elements upstream of *rodA*. The *rodA* upstream region was subdivided as indicated by cloning restriction endonuclease- or PCR-generated fragments into pYC7. The constructs were transformed into yeast strain TYC5 and TMTL and tested for galactose-inducible β-galactosidase production. β-Galactosidase activities were determined with permeabilized yeast cells and are given as $\Delta A_{420} \cdot 10^3 \cdot \text{min}^{-1} \cdot \text{ml}^{-1} \cdot A_{600}^{-1}$. Results from two independent experiments are shown with each value representing the average of two or three assays. Variability between assays was ≤10%. The sequence of the smallest fragment conferring *brlA* responsiveness is shown in Figure 6 (ROD2B). The arrows indicate the transcription direction of *CYC1* and *rodA*. The O symbols indicate the positions of consensus sequence elements described in Figure 6. Bg, *Bgl*II; Bs, *Bst*XI; P, *Pst*I; S, *Sst*I.

respond to *brlA* induction. The results of these experiments are summarized in Figure 2. Two plasmids containing fragments encompassing 150 bp from the *CYC1*-proximal region of the pROD1 insert (pROD2 and pROD2B) responded to *brlA* activation. The frag-

TABLE 1

β-Galactosidase activity comparison in selected yeast transformants

Strain	TYC5 (*GAAL1*(p)::*brlA*)		TMTL (vector)	
	Glycerol/lactate	Galactose	Glycerol/lactate	Galactose
pLC2	996 ± 347	1159 ± 643	632 ± 150	601 ± 25
pLC8	47 ± 12	69 ± 11	35 ± 5	26 ± 3
pLC11	22 ± 7	64 ± 24	16 ± 2	11 ± 3
pLC13	305 ± 62	1239 ± 79	239 ± 78	198 ± 22
pLC18	1306 ± 367	809 ± 181	855 ± 171	441 ± 43
pLC20	29 ± 6	1942 ± 332	29 ± 9	26 ± 1
pLC28	1492 ± 118	1704 ± 100	881 ± 330	944 ± 74
pLC43	109 ± 10	238 ± 16	83 ± 16	91 ± 12
pYC7	Nil	Nil	Nil	Nil

Yeast TYC5 transformants (~30,000) with pYC7 derivatives containing random *A. nidulans Mbo*I fragments inserted upstream of the *CYC1*(p/I) were selected on X-gal plates containing either glucose or galactose as carbon source. Plasmids of putative positives were transferred to a yeast strain lacking pYC5 but containing pMTL. All transformants and a negative control strain (pYC7) were grown in liquid culture containing glycerol/lactate or galactose. Protein extracts were prepared and assayed for β-galactosidase activity, which is reported as units/mg protein. pLC13 and pLC20 were selected for further analysis.

ments in pROD2A and pROD2C, overlapping with the pROD2B 150-bp fragment, failed to activate gene expression.

The number of known genes that are good candidates for direct induction by *brlA* is limited (BOYLAN *et al.* 1987; ADAMS, BOYLAN and TIMBERLAKE 1988). We therefore decided to select *brlA* responsive sequences by screening for their activity in the yeast system. An *A. nidulans* library was constructed by digesting nuclear DNA to completion with *Mbo*I and inserting the fragments into the multiple cloning site of pYC7. Plasmid DNAs were electroporated into yeast strain TYC5. Transformants were screened by replica plating them onto X-gal plates containing glucose or galactose as a sole carbon source. Of ~30,000 transformants screened, 44 produced higher levels of β-galactosidase on galactose plates than on glucose plates. These transformants were tested further by recovering the plasmids in *E. coli* and transforming them into TYC5 and TMTL. Eight transformants appeared to show *brlA*-dependence in plate tests, and these were subjected to quantitative analysis by preparing protein extracts from cells grown with glycerol/lactate or galactose as carbon sources and measuring β-galactosidase activity. The results are presented in Table 1. Two plasmids, pLC13 and pLC20, showed clear galactose inducibility and *brlA* dependence and were thus chosen for further characterization.

The DNA fragments cloned in pLC13 and 20 mediated *brlA*-dependent gene expression in yeast as did the *rodA* upstream fragment (data not shown). pLC20 was selected for further analysis, because it conferred the highest β-galactosidase activity. The insert in

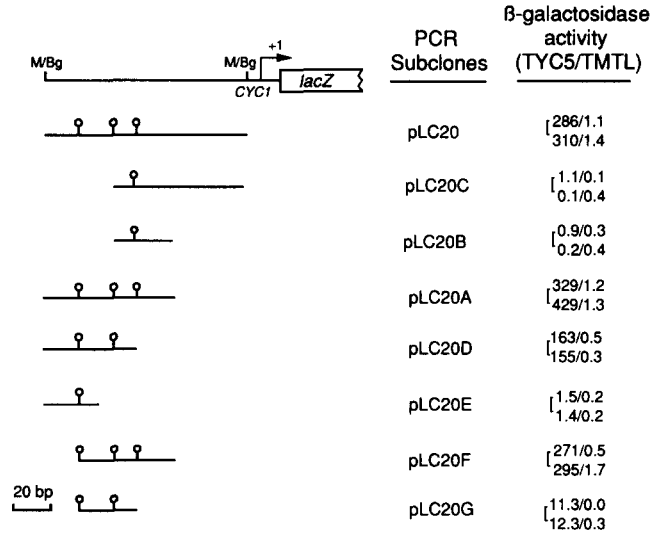

FIGURE 3.—Localization of *brlA* response elements in LC20. The LC20 fragment was subdivided as indicated by cloning PCR-generated fragments into pYC7. These constructs were transformed into yeast strain TYC5 and TMTL. Transformants were tested for galactose-inducible β-galactosidase production as described in the legend to Figure 2. The O symbols indicate the positions of consensus sequence elements described in Figure 6. Bg, *Bgl*II; M, *Mbo*I.

pLC20 was subdivided into several overlapping regions that were tested individually for *brlA*-dependent activation. The results presented in Figure 3 show that a 54-bp fragment contained in pLC20F had as much activity as did the original fragment, whereas the overlapping fragment in pLC20D had reduced activity and the nested fragment in pLC20G had low activity.

The 54-bp insert of pLC20F (LC20F) was used to determine the dependency of gene activation on orientation and spacing relative to the *CYC1* promoter. The fragment was equally active in either orientation (data not shown). However, insertion of a 950-bp fragment from the *A. nidulans yA* upstream region between the LC20F sequence and the *CYC1* promoter abolished activity, whereas insertion of the same fragment upstream of LC20F had no effect on activity (data not shown).

To test for the ability of the DNA fragments characterized in yeast to mediate developmental regulation in Aspergillus, the ROD2B and LC20F elements were substituted for a *Bgl*II-*Bam*HI restriction fragment upstream of the developmentally nonregulated *A. nidulans amdS* promoter fused to *E. coli lacZ* (DAVIS, COBBETT and HYNES 1988), as indicated in Figure 4A. The plasmid in addition contained an internal fragment from the *amdR* gene to provide a direct selection for transformants containing integrated plasmids at the *amdR* locus (ANDRIANOPOULOS and HYNES 1990). A *gatA*$^-$, *amdR*$^+$ *A. nidulans* strain was transformed with circular plasmid DNA, selecting for resistance to γ-aminobutyric acid resulting from inactivation of *amdR* (see MATERIALS AND METHODS).

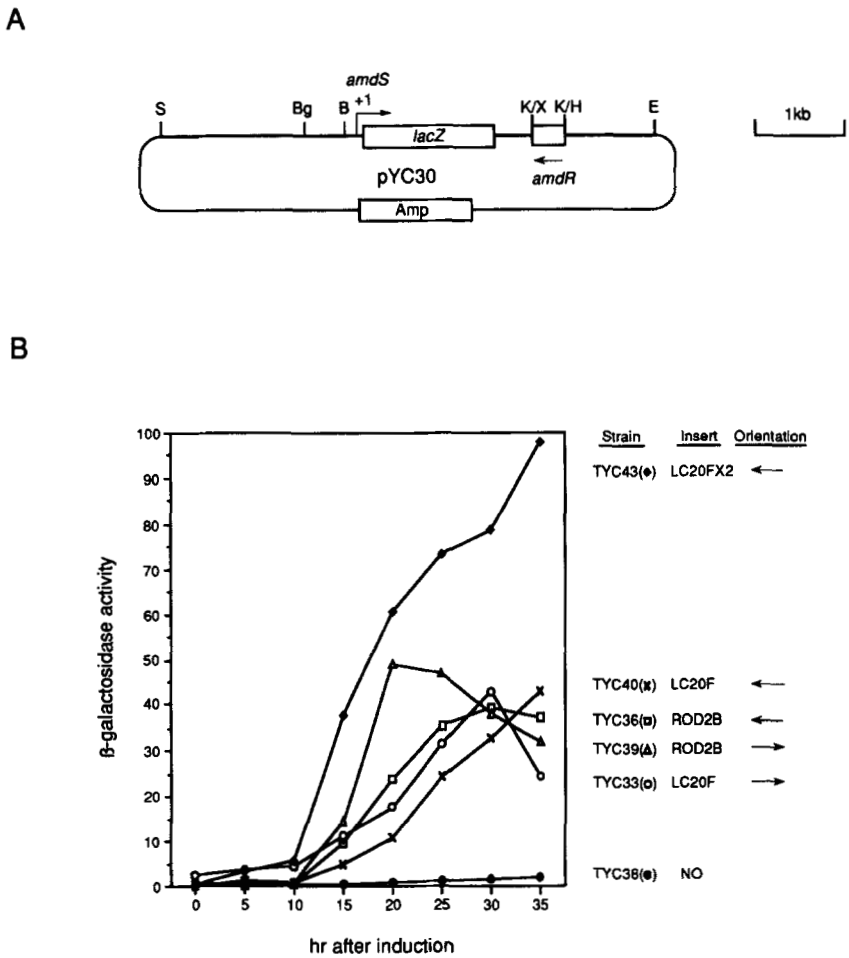

FIGURE 4.—*brlA*-responsive elements identified in yeast mediate developmental regulation in *A. nidulans*. (A) Construct employed to assay *brlA*-responsive elements in *A. nidulans*. The construction of pYC30 is described in MATERIALS AND METHODS. The *E. coli lacZ* gene was used as a reporter. An internal fragment of *amdR* was inserted in the 3′-flanking region of the *amdS* gene as a site-directed selective marker (ANDRIANOPOULOS and HYNES 1990). BREs were substituted for the *Bgl*II-*Bam*HI fragment shown. Circular plasmids of pYC30 derivatives containing the entire *Sal*I-*Eco*RI fragment were used to transform *A. nidulans* strain MH1055. Homologous integration at *amdR* leads to loss of *amdR* function, alleviating the toxicity associated with growth on media containing γ-aminobutyric acid. Bg, *Bgl*II; B, *Bam*HI; E, *Eco*RI; K/H, *Kpn*I-*Hin*dIII fusion; K/X, *Kpn*I-*Xho*I fusion; S, *Sal*I. The arrows indicate the transcription direction of *amdS* and *amdR*. (B) Developmental regulation of *BRE::amdS*(p/l)::*lacZ* fusion genes. *A. nidulans* transformants containing single integrated copies of plasmids were subjected to developmental induction and samples were harvested at 5-hr intervals. Protein extracts were prepared and assayed for β-galactosidase activity, which is reported as units/mg protein. The element in each strain is indicated. Orientations are given relative to the map in (A) and to the DNA sequences presented in Figure 6. TYC43 contains two tandem copies of LC20F. Data are averaged from at least two independent transformants for each strain.

Transformants containing single integrated plasmid copies were developmentally induced and samples were harvested at 5-hr intervals. Protein extracts were prepared and assayed for β-galactosidase activity. As shown in Figure 4B, a control strain (TYC38), containing a plasmid without the 530-bp *Bgl*II-*Bam*HI fragment of the 5′-flanking region of *amdS*, showed negligible β-galactosidase induction during conidiation. By contrast, strains containing plasmids with LC20F or ROD2B fragments in either orientation at the *Bgl*II-*Bam*HI site showed significant developmental regulation. The timing of β-galactosidase accumulation paralleled that of *brlA* RNA accumulation (BOYLAN *et al.* 1987) (also see Figure 5B). A strain containing a plasmid with two copies of LC20F at the *Bgl*II-*Bam*HI site (TYC43) produced approximately twice as much β-galactosidase activity as did the strains containing single copies of LC20F (TYC33 and 40). To test for dependence of activation on *brlA*, *brlA*$^-$ derivatives of the strains shown in Figure 4B were constructed. These strains produced negligible levels of β-galactosidase following developmental induction, similar to the TYC38 control strain (data not shown). Thus, these DNA fragments imparted *brlA*-dependent developmental regulation on a minimal *A. nidulans* promoter.

To test if a small DNA fragment mediating *brlA* induction in yeast and developmental regulation in Aspergillus was necessary for developmental gene activation, the sequences corresponding to ROD2B

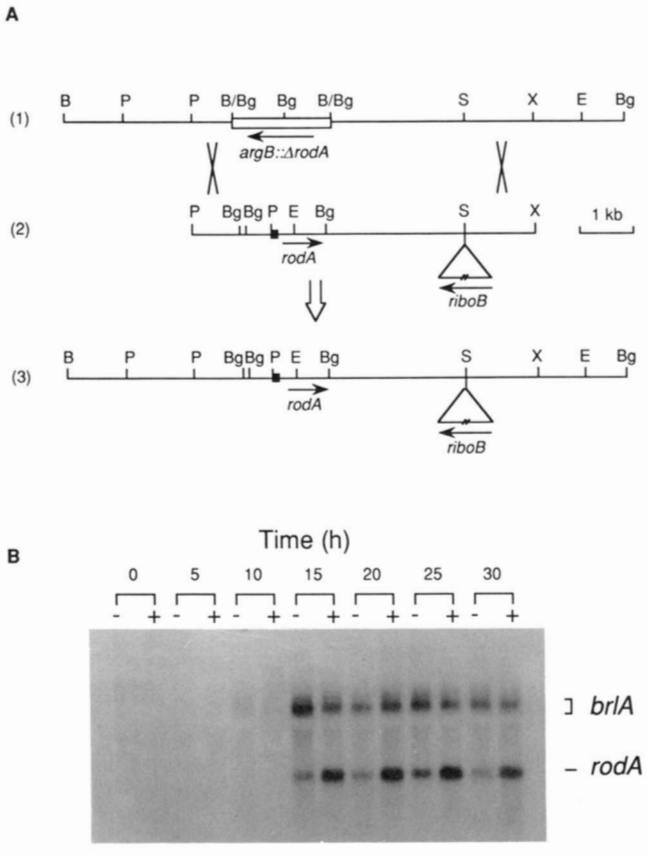

FIGURE 5.—ROD2B sequences are required for developmental induction of *rodA*. (A) Gene replacement strategy for ROD2B deletion. (1) The *rodA* gene has been deleted and replaced with the *argB* (open box) selectable marker in *A. nidulans* strain RMS025 (M. A. STRINGER, personal communication). (2) A plasmid (pYC23) was constructed in which the ROD2B sequence (black box) was deleted from the *rodA* 5′-flanking region and the *riboB* selectable marker (OAKLEY *et al.* 1987) was inserted into a downstream *Sal*I site. Another plasmid (pYC24), similar to pYC23 except that the ROD2B is intact, was used as a control. The plasmids were used separately to transform Aspergillus strain RMS025 to riboflavin-independence and arginine-dependence. Transformants containing single copies of the fragment inserted into the genome as shown in (3) were identified by Southern blot analysis (data not shown). The arrows indicate the transcription direction for *argB*, *rodA* and *riboB*. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; P, *Pst*I; S, *Sal*I; X, *Xho*I. (B) RNA blot analysis of the *rodA* transcripts. Transformants were subjected to developmental induction, samples were harvested at 5-hr intervals, and total RNA was isolated. RNA samples were fractionated electrophoretically, blotted onto a nylon membrane, and hybridized with a mixed *brlA*/*rodA* probe. Equal loading of the gel lanes was checked by ethidium bromide staining of rRNA prior to transfer (data not shown). "−," RNA from transformant containing ROD2B-deleted *rodA* (TYC23); "+," RNA from transformant containing wild-type *rodA* upstream sequences (TYC24).

were deleted from the *rodA* locus *in vitro* and the mutant construct was inserted at the *rodA* locus by the procedure shown in Figure 5A. Strains containing the desired substitution events were identified by Southern blot analysis (data not shown). To examine the effect of removal of ROD2B, RNAs were isolated from developmentally induced transformants and RNA gel blots were hybridized with *brlA* and *rodA* probes. As shown in Figure 5B, deletion of ROD2B sequences significantly decreased *rodA* RNA accumulation despite the presence of similar levels of *brlA* RNA during conidiation. Thus, this upstream region is required for full developmental induction of *rodA*.

The sequences of the smallest DNA fragments imparting *brlA* responsiveness in yeast are given in Figure 6. Computer-assisted searches for similarities among the sequences revealed that all of the DNA fragments contained multiple copies of the sequence 5′-CAAGGG-3′ and additional copies of the less conserved sequence 5′-(C/A)(G/A)AGGG(G/A)-3′. Ten copies of this element were present in the total of 358 bp shown in Figure 6. The probability of occurrence of this number of elements by random chance is $<1 \times 10^{-4}$. Several additional elements containing the core consensus sequence 5′-AGG(G/A)-3′ were also present. No other highly significant sequence similarities were detected among these three fragments.

To test the possibility that this repeated sequence element was responsible for *brlA*-dependent gene activation, a synthetic oligonucleotide containing the core sequence 5′-CAAGGGG-3′ was cloned in one or more copies upstream of the *CYC1*(p/l)::*lacZ* gene in pYC7 (see MATERIALS AND METHODS). One copy of the oligonucleotide failed to produce *brlA* responsiveness in yeast (data not shown). Two copies of the oligonucleotide in various orientations produced low or no *brlA* responsiveness in yeast (Figure 7). On the other hand, three or four copies of the oligonucleotide produced higher β-galactosidase activity in TYC5 than in TMTL. Low levels of β-galactosidase activity were detected in some of these transformants even when grown with glucose as carbon source, indicating that the elements in some combinations can have a generalized UAS activity in yeast.

DISCUSSION

Expression of the *A. nidulans brlA* gene is necessary and sufficient for the induction of asexual reproduction (ADAMS, BOYLAN TIMBERLAKE 1988). It has been proposed that *brlA* encodes a transcriptional regulator responsible for activating downstream regulatory genes (MIRABITO, ADAMS and TIMBERLAKE 1989) and genes whose products contribute directly to conidiophore or spore morphogenesis (STRINGER *et al.* 1991). Direct *in vitro* tests of the hypothesis that BrlA is a transcriptional activator have thus far been unsuccessful, perhaps due to low cellular concentrations and proteolytic sensitivity of BrlA in *A. nidulans* and inactivity of BrlA produced in *E. coli* (Y. C. CHANG, J. MOONEY and W. E. TIMBERLAKE, unpublished results). Alternatively, negative results from DNA-binding studies with Aspergillus cellular extracts or with BrlA produced in *E. coli* could indicate that BrlA is not a DNA binding protein, but instead acts through

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                10        20        30        40        50        60
LC20F   CAAGGGGCAGCTGGGTTTGAAAGGGATGACTCAAGGGGCAATGTCCAACCTGAC
        GTTCCCCGTCGACCCAAACTTTCCCTACTGAGTTCCCCGTTACAGGTTGGACTG

LC13    TGAATAGTACCGATGAGATTTATGATAAGTTAGATGGGGAGGAAGGGGATTTGGCGCGGG
        ACTTATCATGGCTACTCTAAATACTATTCAATCTACCCCTCCTTCCCCTAAACCGCGCCC

        GACTCAAGGGAAGGCAAGGGGATGATAGGGAGAGGTTGCGGGTTGATGATGAAGAGAGGG
        CTGAGTTCCCTTCCGTTCCCCTACTATCCCTCTCCAACGCCCAACTACTACTTCTCTCCC

        TTGCGAGGGGCCCAGAGGAGATGGAGGCGGAAGC
        AACGCTCCCCGGGTCTCCTCTACCTCCGCCTTCG

ROD2B   CATGCTTTAGCCTGCAAGGGATGATTTAGACAAAGTCAACCCAGAAATCTAAAACACATA
        GTACGAAATCGGACGTTCCCTACTAAATCTGTTTCAGTTGGGTCTTTAGATTTTGTGTAT

        TGCACGGTTGGTCCCTCTCAACTCCTGTCCCTTGAAGCGGCAAGGGATGCGCGATCTCTC
        ACGTGCCAACCAGGGAGAGTTGAGGACAGGGAACTTCGCCGTTCCCTACGCGCTAGAGAG

        TAGGTAATTTCCCAAGCAGGGATAGAAACC
        ATCCATTAAAGGGTTCGTCCCTATCTTTGG
```

FIGURE 6.—Sequence comparison of *brlA*-response elements. The sequences of LC20F, LC13 and ROD2B are given. The 5′-CAAGGG-3′ sequences are boxed whereas the less conserved 5′-(C/A)(A/G)AGGG(G/A)-3′ sequences are underlined. No other regions of highly significant similarity were present within the three sequences.

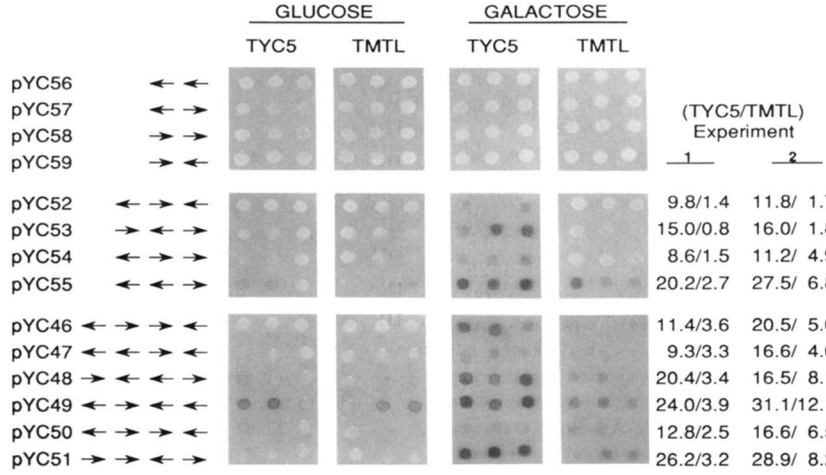

FIGURE 7.—A synthetic oligonucleotide confers β-galactosidase activity in yeast. Synthetic oligonucleotides containing 5′-CAAGGGG-3′ (see Materials and Methods) were cloned into the minimal promoter of *CYC1* in pYC7. The copy number and orientations of the oligonucleotides in each construct are indicated by arrows (5′-CAAGGGG-3, →; 5′-CCCCTTG-3′, ←). Plasmids were transformed into TYC5 and TMTL. Three independent transformants were replicated onto medium containing X-gal and either galactose or glucose as carbon source. Plates were incubated for 3 days (glucose) or 4 days (galactose) at 30°. Ten independent TYC5 and TMTL transformants containing 3 or 4 oligonucleotide copies were pooled and β-galactosidase activities were determined as described in the legend to Figure 2. The results from two independent experiments are shown, with each valve representing the average of three assays. Variability between assays was ≤10%.

a different mechanism, or that additional factors are required for DNA binding. We therefore used a heterologous system, *S. cerevisiae* (yeast), to test for the ability of *brlA* to activate transcription *in vivo* in the absence of Aspergillus-encoded factors. The experimental approach was similar to that used for the identification of steroid hormone (SCHENA and YAMAMOTO 1988) and NGFI-B (WILSON *et al.* 1991) response elements.

Three short *A. nidulans* DNA fragments were identified that led to *brlA*-mediated gene transcription when inserted upstream of a minimal yeast promoter. One of these originated from the upstream region of the *A. nidulans rodA* gene, whose developmental induction depends on *brlA*$^+$, but not on the activities of downstream regulatory genes (BOYLAN *et al.* 1987; STRINGER *et al.* 1991). The two others were anonymous DNA fragments that have not been shown to be associated with developmentally regulated genes. Transcriptional activation was independent of the orientation of the fragments, but dependent on spacing. Thus, these fragments behaved like some yeast UASs (for review see GUARENTE 1987), except that their activity was dependent upon expression of the *A. nidulans brlA* gene.

Sequence comparisons among these DNA frag-

ments revealed the presence of multiple copies of an element with the consensus sequence 5′-MRAGGGR-3′, which we propose to be the binding site for BrlA. In support of this hypothesis, three or more copies of a synthetic oligonucleotide containing the sequence 5′-CAAGGGG-3′ mediated a detectable *brlA* response in yeast. Furthermore, one of the BrlA zinc finger regions contains the FSRSD motif, found in a minority of C_2H_2 zinc finger proteins (NEHLIN and RONNE 1990). Many of the targets for this subset of proteins contain a G rich core (CROSBY *et al.* 1991; DOWZER and KELLY 1991, and references therein), as does the proposed BrlA binding site. Finally, two to five copies of the proposed BrlA binding site occur upstream of the transcription initiation sites of other *A. nidulans* genes that are at least in part under *brlA* control, including *brlA* itself (ADAMS, BOYLAN and TIMBERLAKE 1988), *abaA* (MIRABITO, ADAMS and TIMBERLAKE 1989; ADAMS and TIMBERLAKE 1990a), *stuA* (MILLER, WU and MILLER 1992), *wetA* (MARSHALL and TIMBERLAKE 1991), *wA* (M. MAYORGA and W. E. TIMBERLAKE, unpublished results), and *yA* (ARAMAYO and TIMBERLAKE 1990).

Although the simplest explanation for the results presented in this paper is that BrlA binds directly to 5′-MRAGGGR-3′ and interacts with the yeast transcriptional complex to stimulate transcription, it is possible that the observed effects are indirect; BrlA could interact with yeast cellular components that in turn interact with the BREs. Arguing against this possibility is the observation that induced expression of *brlA* does not detectably affect the growth rate or cellular morphology of yeast, a non-conidial Hemiascomycete, although induced expression of *brlA* in some conidial Euascomycetes that are more closely related to *A. nidulans*, for example *Penicillium chrysogenum*, has profound effects similar to those observed for *A. nidulans* (ADAMS, BOYLAN and TIMBERLAKE 1988; W. E. TIMBERLAKE, unpublished results). Thus, it is unlikely that BrlA has major effects on yeast metabolism. Furthermore, *brlA*-mediated gene activation is dependent upon the integrity of both putative zinc coordination sites, indicating that the effect is specific and not simply the result of high level expression of a foreign protein. Final confirmation of proposed interaction of BrlA and the BREs will require a direct demonstration of DNA binding. These experiments should be facilitated by identification of short DNA fragments that mediate the *brlA* response in yeast.

Regardless of the actual mechanism through which the BREs mediate gene activation in yeast, they have a similar activity in *A. nidulans*. Addition of the DNA fragments identified in yeast in either orientation upstream of a minimal Aspergillus promoter from a developmentally nonregulated gene, *amdS*, conferred developmental regulation in Aspergillus, and the pattern of developmental induction was consistent with *brlA* activation. Moreover, deletion of the BREs from the genomic copy of *rodA* significantly decreased, but did not eliminate, *rodA* mRNA accumulation during development. The residual accumulation of *rodA* mRNA may be explained by the existence of other developmental control elements. Although accumulation of *rodA* transcript during normal development is dependent on $brlA^+$ (BOYLAN *et al.* 1987), forced, high level expression of *abaA* in a $brlA^-$ strain nevertheless leads to accumulation of *rodA* mRNA (MIRABITO, ADAMS and TIMBERLAKE 1989), suggesting that the gene can be activated by either *brlA* or *abaA*. In addition, *abaA* binding sites exist in the *rodA* promoter (A. ANDRIANOPOULOS and W. E. TIMBERLAKE, unpublished results). The existence of multiple response elements upstream of *rodA* that are recognized by sequentially activated regulatory genes could ensure that the transcription rate of the gene rises appropriately as development proceeds.

brlA has long been known to be of critical importance for the control of the steps of conidiophore development (CLUTTERBUCK 1969). Null mutants produce only conidiophore stalks (aerial hyphae) that grow indeterminately and these strains fail to activate most conidiation-specific genes (CLUTTERBUCK 1977; TIMBERLAKE 1990). Hypomorphic *brlA* alleles permit more extensive development and activation of subsets of developmentally regulated genes (CLUTTERBUCK 1969; MARTINELLI 1979). Interactions of BrlA and the products of other developmentally regulatory genes, for example *abaA* and *medA* (CLUTTERBUCK 1977; TIMBERLAKE 1990, 1991; CLUTTERBUCK and TIMBERLAKE 1992), could further refine patterns of gene expression. Such interactions have been inferred from genetic analysis of development and are implied, for example, by the close association of putative BrlA and AbaA binding sites in the promoter regions of developmentally regulated genes such as *wA* and *yA* (ARAMAYO and TIMBERLAKE 1990; M. MAYORGA and W. E.TIMBERLAKE, unpublished results; R. ARAMAYO and W. E. TIMBERLAKE, manuscript in preparation; A. ANDRIANOPOULOS and W. E. TIMBERLAKE, manuscript in preparation). A detailed description of these interactions should lead to a better understanding of the molecular mechanisms controlling the temporal and spatial patterns of gene expression during conidiophore development.

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