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Isolation and Physical Characterization of Three Essential Conidiation Genes from *Aspergillus nidulans*

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We cloned and characterized three genes from *Aspergillus nidulans*, designated *brlA*, *abaA*, and *wetA*, whose activities are required to complete different stages of conidiophore development. Inactivation of these genes causes major abnormalities in conidiophore morphology and prevents expression of many unrelated, developmentally regulated genes, without affecting the expression of nonregulated genes. The three genes code for poly(A)$^+$ RNAs that begin to accumulate at different times during conidiation. The *brlA*- and *abaA*-encoded RNAs accumulate specifically in cells of the conidiophore. The *wetA*-encoded RNA accumulates in mature conidia. Inactivation of the *brlA* gene prevents expression of the *abaA* and *wetA* genes, whereas inactivation of the *abaA* gene prevents expression of the *wetA* gene. Our results confirm genetic predictions as to the temporal and spatial patterns of expression of these genes and demonstrate that these patterns are specified at the level of RNA accumulation.

The mitotically derived spores, or conidia, of the ascomycetous fungus *Aspergillus nidulans* are produced on multicellular structures called conidiophores (14). Scanning electron microscope images of developing conidiophores are shown in Fig. 1. Under appropriate conditions, certain hyphal elements (Fig. 1A) differentiate into thick-walled foot cells and produce aerial stalk initials (Fig. 1B). The stalks grow away from the substratum for a defined period of time, growth stops, and a multinucleated vesicle forms by swelling of the stalk apex (Fig. 1C). A layer of primary sterigmata, or metullae, is formed on the surface of the vesicle by budding (Fig. 1D), and a single nucleus enters each cell. The metullae undergo a single division to produce a layer of secondary sporogenous sterigmata, or phialides (Fig. 1E). Conidia are formed following mitotic divisions of the phialide nucleus (Fig. 1F). After each division, one daughter nucleus is retained within the phialide, where it continues to undergo mitoses. The other daughter nucleus enters the tip of the phialide which buds off to form a conidium. As this process continues, previously formed conidia are displaced by newly formed conidia, producing a long chain of clonally derived spores at various stages of maturity. We have previously shown that conidiophore development in *A. nidulans* involves the stage- and cell-type-specific expression of approximately 1,000 different genes (15).

The genetic mechanisms that control the differentiation and spatial organization of conidiophore cells have been investigated by Clutterbuck and co-workers, who isolated a variety of mutant strains with altered conidiophore morphologies (1, 2, 4, 10). Many of the mutations they studied are conidiophore specific, having little or no detectable effect on hyphal growth or sexual reproduction. The mechanisms by which the genes identified by these mutations bring about the orderly assembly of the asexual reproductive apparatus are unknown.

Mutations in three genes cause major developmental defects and thus are of particular interest. Their phenotypes are shown in Fig. 2. The *brlA1* mutation does not interfere with initiation of conidiogenesis, but mutant strains produce conidiophore stalks that continue to elongate rather than forming apical vesicles, metullae, phialides, and conidia. A number of interesting leaky *brlA* alleles have been identified (1). The *abaA1* mutation does not interfere with the earlier stages of conidiophore development but causes the formation of abnormal phialides that proliferate by apical growth instead of switching to the budding of conidia. The *wetA6* mutation has little or no effect on the formation of the conidiophore proper but causes the spores to autolyse during the final stages of differentiation.

In this study, we describe the physical isolation and molecular characterization of the *brlA*, *abaA*, and *wetA* genes. Our results confirm genetic predictions as to the temporal and spatial patterns of expression of these loci and demonstrate that these patterns are specified at the level of mRNA accumulation.

MATERIALS AND METHODS

***A. nidulans* strains and genetic techniques.** The strains used for RNA isolation were FGSC 4 (Glasgow wild type), obtained from the Fungal Genetics Stock Center; and AJC7.1 (*biA1 brlA1*), GO1 (*biA1 abaA1*), and GO241 (*biA1 wetA6*), which were kindly provided by John Clutterbuck, Department of Genetics, Glasgow University, Scotland. *A. nidulans* UCD7 (*pabaA1 yA2 abaA1 trpC801*; this study) and 472.2 (*pabaA1 yA2 wetA6 trpC399*; kindly provided by John Clutterbuck) were used as transformation recipients to identify mutation-complementing subclones. FGSC 237 (*pabaA1 yA2 trpC801*) was obtained from the Fungal Genetics Stock Center and used as the transformation recipient in the *abaA* disruption experiments; the strain used as the transformation recipient for the *wetA* disruption experiments (*biA1 argB2 methG*) was kindly provided by P. Weglenski, Department of Genetics, Warsaw University, Poland. Strains 461.1 (*pabaA1 yA2 trpC801 abaA14*) and 472.2, obtained from John Clutterbuck, were used to construct diploids with the mutants generated by gene disruption. Standard *A. nidulans* genetic techniques were used (3, 13).

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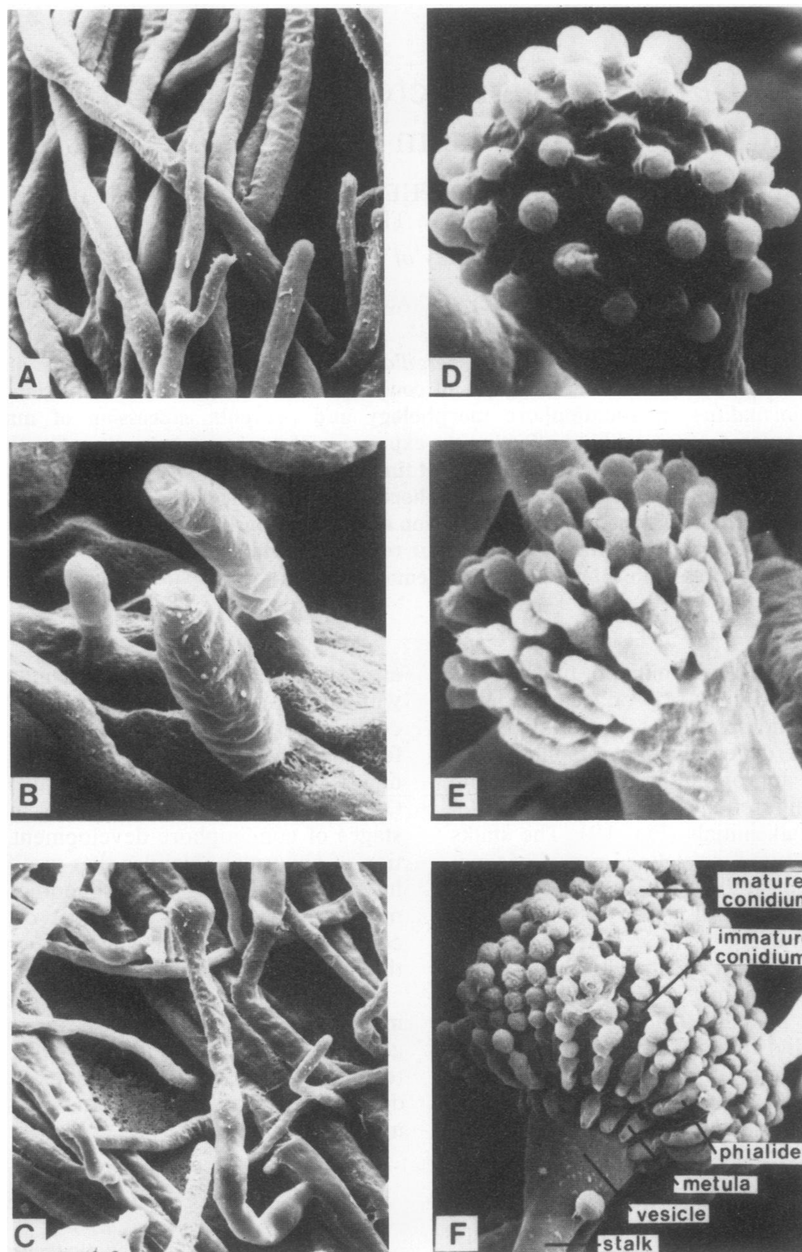

FIG. 1. Morphological changes during conidiation. Conidiating colonies (FGSC 4) were examined by scanning electron microscopy, and conidiophores at various stages of development were identified. (A) Undifferentiated hyphae. (B) Young, aerial stalks. (C) Vesiculated conidiophore. (D) Metullae budding from the vesicle. (E) Phialide formation. (F) Mature conidiophore.

***A. nidulans* transformation.** *A. nidulans* protoplasts were prepared, transformed, and regenerated as previously described (20), except that hyphal macerates (16) rather than conidia were used to inoculate liquid cultures of the aconidial strains.

RNA preparation and RNA blot hybridizations. Undifferentiated *A. nidulans* hyphae, conidiating cultures, and purified conidia were prepared as previously described (15). Total and poly(A)$^+$ RNAs were isolated by using standard procedures (15, 17). RNA was analyzed by electrophoresis on formaldehyde-agarose gels and transferred, without any pretreatment, to Hybond-N (Amersham Corp., Arlington Heights, Ill.) by using 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The blots were hybridized with ^{32}P-labeled nick-translated probes by using procedures recommended by the membrane manufacturer.

S1 nuclease mapping. S1 nuclease mapping was performed with single-stranded M13 DNA subclones as previously described (19). Transcriptional polarities were assigned by cloning fragments in opposite orientations in the vectors M13mp18 and M13mp19 (18). S1 nuclease-resistant fragments were analyzed by electrophoresis on native and alkaline agarose gels (8) with *Hae*III-digested φX174 and *Hin*dIII-digested λ DNA as markers.

Differential hybridization screening of the cDNA library. An *A. nidulans* cDNA clone bank was prepared by McKnight et al. (1), using poly(A)$^+$ RNA isolated from conidiating cultures grown for 25 h (7). This cDNA library,

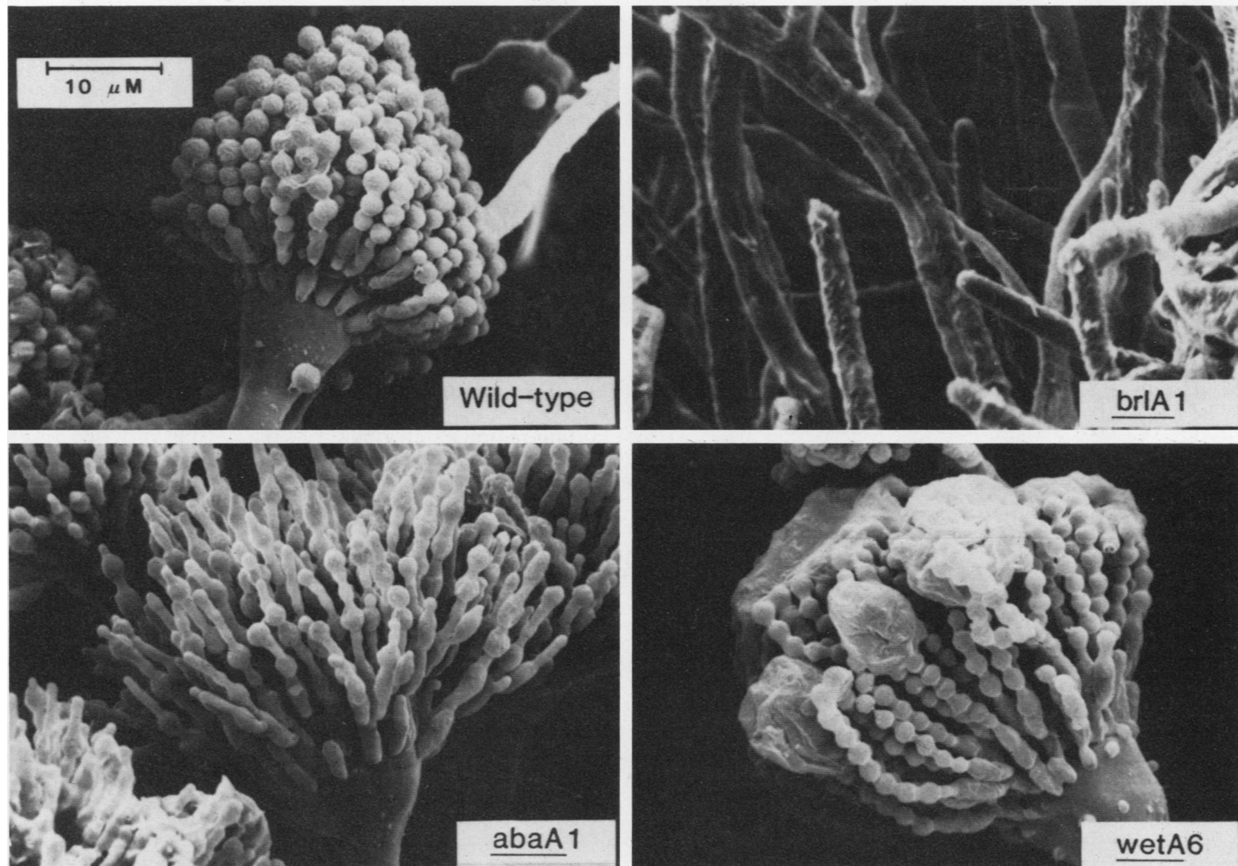

FIG. 2. Morphology of wild-type and mutant conidiophores as visualized by scanning electron microscopy.

kindly provided by G. McKnight, was plated onto nitrocellulose filters, and replica filters were screened in duplicate with ^{32}P-labeled cDNAs synthesized by using poly(A)$^+$ RNA isolated from either undifferentiated *A. nidulans* hyphae or conidiating cultures (8, 15). Clones which hybridized with the conidiation cDNA probe but not with the hyphal cDNA probe were chosen for further analysis.

Scanning electron microscopy. Samples were fixed in 4% glutaraldehyde–0.5% yeast extract–2% glucose for 6 h at 25°C. They were then treated with 4% OsO_4 for 12 h, dehydrated in increasing concentrations of ethanol, and transferred to amyl acetate. Samples were dried in a critical-point drier, coated with gold, and examined with a scanning electron microscope.

RESULTS AND DISCUSSION

Effects of *brlA*, *abaA*, and *wetA* mutations on gene expression. We showed previously that about 1,000 genes are preferentially activated during conidiation in *A. nidulans*. Because *brlA*, *abaA*, and *wetA* mutations are pleiotropic at the morphological level (4), we were interested in determining if they were also pleiotropic at the molecular level. We therefore investigated the effects of the *brlA1*, *abaA1*, and *wetA6* mutations on gene expression by isolating cDNA clones corresponding to mRNAs that accumulate preferentially during conidiation. Clones were selected encoding RNAs that begin to accumulate at different times after conidiation was induced. Total RNA was isolated from wild-type cultures that had been induced to conidiate for various times, and gel blots were hybridized with either the cDNA clones or with a clone of the *A. nidulans argB* gene, which has been shown to be expressed at constant levels throughout development (19) (Fig. 3A). In addition, total RNA was isolated from *brlA1*, *abaA1*, and *wetA6* strains 25 h after the induction of differentiation, and gel blots were hybridized with the same probes (Fig. 3B). None of the mutations reduced the levels of three moderately regulated RNAs (pCAN34, -63, and -44) or of the *argB* mRNA. Indeed, they enhanced the levels of pCAN44 mRNA. By contrast, the mutations had various effects on the accumulation of the other RNAs examined. As *brlA1*, *abaA1*, and *wetA6* are loss-of-function mutations (see below), we conclude that *brlA*$^+$, *abaA*$^+$, and *wetA*$^+$ activities are required for the expression of other unrelated, developmentally regulated genes. Results from similar experiments conducted with bacteriophage clones containing developmentally regulated genes confirmed this conclusion (5, 22; C. R. Zimmermann, Ph.D. thesis, University of California, Davis, 1986). We do not yet know how the products of the *brlA*, *abaA*, and *wetA* genes affect expression of these genes.

Identification and physical characterization of the *brlA*, *abaA*, and *wetA* genes. We previously reported the isolation of cosmid clones that complement the *A. nidulans brlA1*, *abaA14*, and *wetA6* mutations (16, 21). To determine whether these clones contain the corresponding wild-type alleles for these genes, we localized the complementing activities to small restriction fragments by using standard *A. nidulans* transformation procedures (10, 16). The results indicate that the *brlA1*-, *abaA14*- and *wetA6*-complementing

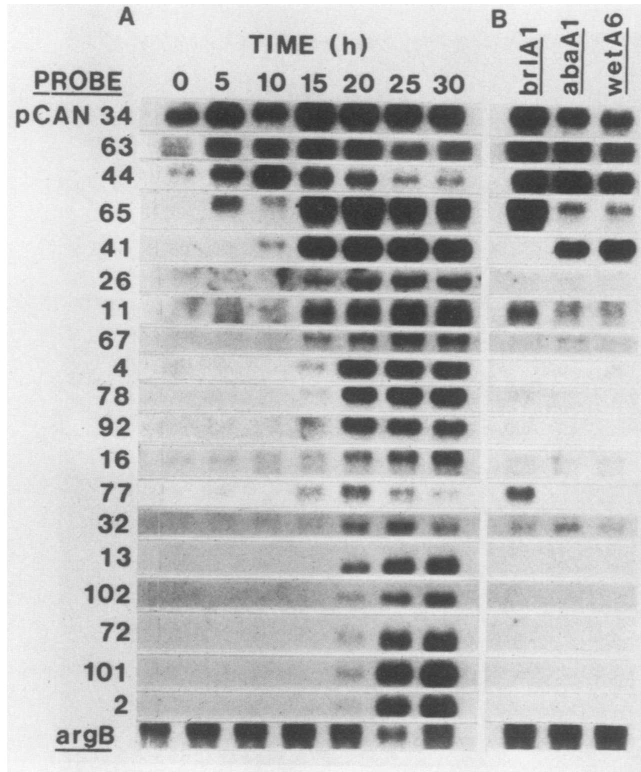

FIG. 3. Developmental expression of transcripts encoded by the cDNA clones. (A) Temporal pattern in wild-type cultures. Total RNA was isolated from wild-type (FGSC 4) cultures at 5-h intervals following the induction of conidiation. Conidiophore development at these various time points as follows: 0 h, undifferentiated hyphae; 5 h, aerial stalks present; 10 h, stalks had begun to vesiculate; 15 h, metullae and phialides present; 20 h, immature conidia; 25 h, mature, dark-green conidia; and 30 h, mature, dark-green conidia. Gel blots, containing 3 μg of RNA per time point, were hybridized with ^{32}P-labeled cDNA probes (pCAN34 through pCAN2) or with ^{32}P-labeled plasmid pMA2 (16) containing the *A. nidulans argB* gene. (B) Effects of the *brlA1*, *abaA1*, and *wetA6* mutations. Total RNA was isolated from cultures of *brlA1*, *abaA1*, and *wetA6* strains 25 h after they had been induced to differentiate. RNA samples (3 μg per lane) were electrophoresed, blotted, and hybridized in parallel with the samples shown in Fig. 3A.

activities reside on 4.5-kilobase (kb) *Bam*HI, 6.0-kb *Sal*I, and 7.5-kb *Eco*RI fragments, respectively (Fig. 4).

Approximate transcription maps were determined for these DNA restriction fragments by hybridizing gel blots of poly(A)$^+$ RNA isolated from hyphae, conidiating cultures (containing hyphae, conidiophores, and conidia), or purified conidia with the probes designated a through m shown in Fig. 4. The *brlA1*-complementing *Bam*HI fragment hybridized to a single 2.4-kb transcript that accumulated only in conidiating cultures (Fig. 5A). The orientation of transcription was determined by S1 nuclease protection experiments with asymmetrical M13 clones. Hybridization of probe a with poly(A)$^+$ RNA from conidiating cultures yielded a 0.8-kb S1-resistant fragment, as estimated by electrophoresis in nondenaturing agarose gels, and two fragments of 0.5 and 0.3 kb, as estimated by electrophoresis in alkaline agarose gels (data not shown), indicating that a small intron is present near the 5′ end of the transcription unit. Probe b was completely protected from S1 nuclease digestion, and probe c yielded a 0.4-kb S1-resistant fragment in native and denaturing agarose gels, defining the 3′ end of the transcription unit as indicated in Fig. 4. The temperature-sensitive *brlA42* allele was isolated independently by Johnstone et al. (6). This clone is known to correspond to the *brlA* structural gene because it can transform *brlA1* null strains to the temperature-sensitive *brlA42* phenotype. The 4.5-kb *Bam*HI fragment we isolated has a restriction map that is identical to that of the *brlA42* clone. We conclude that this fragment contains the *brlA* gene, which consists of two exons and a short intron.

The 6.0-kb *abaA14*-complementing *Sal*I fragment hybridized to 3.0- and 1.5-kb transcripts (Fig. 5A). The smaller transcript was more prevalent in hyphae and spores than in conidiating cultures, whereas the 3.0-kb transcript accumulated specifically in conidiating cultures. Transformation of an *abaA1* strain with subclones specific for each RNA-coding region established that the 4.3-kb *Sal*I-*Pst*I fragment coding for the 3.0-kb transcript complemented the *abaA14* mutation. The orientation of transcription was determined as described for the *brlA* gene. S1 nuclease protection studies using probes f and g (Fig. 4) showed that the transcription unit consists of two exons and a short intron. To determine if this transcription unit corresponds to the *abaA* gene, a plasmid was constructed containing the 1.5-kb *Cla*I-*Hin*dIII fragment from within the transcribed region and the *A. nidulans trpC* gene and used to transform an *abaA*$^+$ *trpC801* strain to tryptophan independence. Colonies were obtained that had the abacus phenotype, and DNA blot analyses indicate that the putative *abaA* transcription unit had been disrupted through homologous recombination with the circular plasmid containing the internal *Cla*I-*Hin*dIII restriction fragment (data not shown). One such strain was used to construct a diploid with a strain carrying the temperature-sensitive *abaA14* allele. The diploids were wild type at 25°C but showed the abacus phenotype at 37°C. This result demonstrates that the site-directed mutation was allelic with the *abaA14* mutation and that the abacus phenotype was caused by loss of gene function.

The *wetA6*-complementing *Eco*RI fragment hybridized to 2.6-, 2.4-, 1.7-, and 1.1-kb transcripts (Fig. 5A). The 2.6- and 2.4-kb transcripts are *trpC* mRNAs (19) that hybridized because a portion of the *Eco*RI fragment was derived from the part of the pKBY2 vector (21) containing the *A. nidulans trpC* gene. A subcloned 2.7-kb *Bgl*II-*Sma*I fragment hybridized only with the 1.8-kb transcript and complemented the *wetA6* mutation. The orientation of transcription was established as described for the *brlA* gene. S1 nuclease protection studies using probes j and k (Fig. 4) show that the transcription unit consisted of a single exon. The 1.1-kb transcription unit was not investigated in detail, but it is interesting that both the 1.1- and 1.8-kb transcripts accumulated specifically in conidia. Clustering of conidium-specific genes is common in *A. nidulans* (12). To determine if the 1.8-kb transcription unit corresponds to the *wetA* gene, a plasmid was constructed containing the 0.6-kb *Sal*I-*Pst*I fragment from within the transcribed region and the *A. nidulans argB* gene and used to transform a *wetA*$^+$ *argB2* strain to arginine independence. Colonies were obtained that had the wet-white phenotype, and DNA blot analyses show that the putative *wetA* transcription unit had been disrupted through homologous recombination with the circular plasmid containing the internal *Sal*I-*Pst*I restriction fragment (data not shown). One strain was used to construct a diploid with a strain carrying the temperature-sensitive *wetA6* allele. The diploids were wild type at 25°C but showed the wet-white phenotype at 37°C. This result indicates that the site-directed

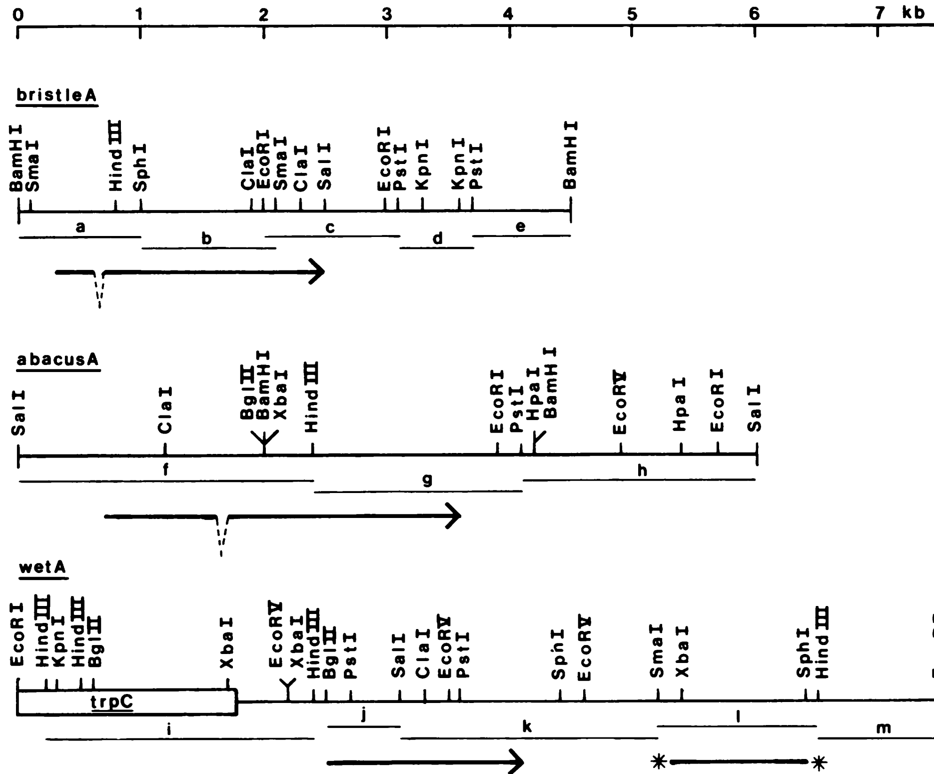

FIG. 4. Restriction maps of the *brlA*, *abaA*, and *wetA* genes. The complementing fragments of cosmid DNA were subcloned into pUC19 (18). The bars beneath the restriction maps labeled a through m indicate the various gel-eluted restriction fragments of the pUC19 inserts that were used as hybridization probes in RNA blotting experiments. Probes a, b, and c hybridized to the 2.4-kb *brlA* transcript; probes f and g hybridized to the 3.0-kb *abaA* transcript; probes j and k hybridized to the 1.8-kb *wetA* transcript. The arrows show the extent and orientations of these transcripts as determined by S1 nuclease experiments. S1-resistant products were analyzed electrophoretically, blotted, and hybridized with the 4.5-kb *Bam*HI fragment encoding the *brlA* locus, the 4.3-kb *Sal*I-*Pst*I fragment encoding the *abaA* locus, or the 2.7-kb *Hin*dIII-*Sma*I fragment encoding the *wetA* locus. The dashed lines indicate the approximate locations of the *brlA* and *abaA* introns. The line designated by the asterisks points out the approximate map position of the conidium-specific transcript adjacent to the *wetA* locus.

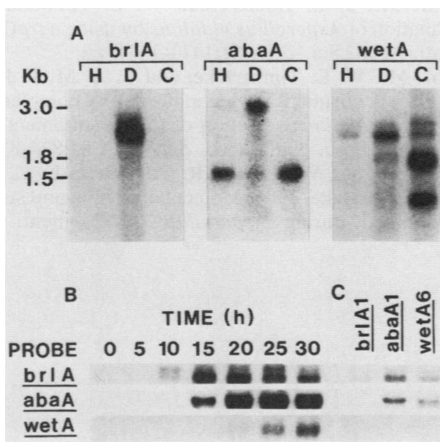

FIG. 5. Expression of *brlA*, *abaA*, and *wetA* RNAs. (A) Developmental regulation of expression. Northern blots (RNA blots) of equal amounts (2 μg) of poly(A)$^+$ RNA isolated from hyphae (H), conidiating cultures (D), and purified conidia (C) were hybridized with the 4.5-kb *brlA Bam*HI fragment, the 6.0-kb *abaA Sal*I fragment, or the 7.5-kb *wetA Eco*RI fragment. Indicated size markers are in kilobases. (B) Temporal pattern of expression. Northern blots of equal amounts (3 μg) of total RNA isolated at 5-h intervals during conidiation were hybridized with the probes described for panel A. (C) Expression in mutants. Northern blots of equal amounts (3 μg) of total RNA isolated from mutant cultures 25 h after the induction of differentiation were hybridized with the probes described for panel A. All blots were rehybridized with an *argB* probe to confirm that equal amounts of RNA had been loaded into the gel lanes.

mutation was allelic with the *wetA6* mutation and that the wet-white phenotype was caused by loss of gene function.

Epistatic relationships among the *brlA1*, *abaA1*, and *wetA6* mutations. At the morphological level, *brlA* mutations are epistatic to *abaA* and *wetA* mutations and *abaA* mutations are epistatic to *wetA* mutations (9). We determined that these epistatic relationships were manifested at the molecular level. Total RNA was isolated from cultures that had been induced to conidiate for various times, and gel blots were hybridized with *brlA*, *abaA*, or *wetA* probes. The *brlA* transcript began to accumulate at 10 h postinduction, at which time conidiophore stalks had formed and had begun to vesiculate (Fig. 5B). The *abaA* transcript began to accumulate at 15 h postinduction, at which time conidiophore vesicles, metullae, and phialides had formed. The *wetA* transcript began to accumulate at 20 h postinduction, at which time conidia had begun to form. Total RNA was also isolated from *brlA1*, *abaA1*, and *wetA6* strains that had been induced to differentiate for 25 h, and gel blots were hybridized with the same probes. The *brlA1* mutation blocked accumulation of all three RNAs (Fig. 5C). The *abaA1* and *wetA6* mutations caused reduced accumulation of the *brlA*

and *abaA* RNAs and blocked accumulation of the *wetA* RNA. Thus, the *brlA1* and *abaA1* mutations affected their own expression and the expression of one another. An interesting finding from these experiments is that the *wetA* transcript failed to accumulate in a *wetA6* (temperature-sensitive) mutant strain grown at the restrictive temperature. This result might imply that the *wetA* gene product is required for activation of the *wetA* gene; that is, the gene is autoregulatory. It is also possible that *wetA* RNA is rapidly degraded in autolysing conidia.

The results presented here show that we have isolated the *A. nidulans brlA*, *abaA*, and *wetA* loci. The genes were regulated during conidiation and when mutated prevented formation of normal conidiophores. Thus, they represent genes whose expression is both unique and necessary for asexual development. The existence of temperature-sensitive alleles for each of these loci implies that they are protein-coding genes. This is confirmed by our observation that they code for poly(A)$^+$ RNAs and by preliminary DNA sequence data showing that the genes have open translational reading frames. The biochemical functions of the *brlA*, *abaA*, and *wetA* proteins remain to be determined.

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