Molecular Characterization of the *Aspergillus nidulans yA* Locus

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ABSTRACT

We investigated the molecular organization of the region of *Aspergillus nidulans* chromosome I containing *yA*, a gene encoding the developmentally regulated enzyme conidial laccase. DNA fragments were identified that complemented the *yA2* mutation and were shown to correspond to *yA* by genetic mapping and gene disruption experiments. The molecular map of the region was oriented to the genetic map by testing DNA fragments for their ability to complement a mutation in the tightly linked *adE* gene. The *yA* gene codes for a 2200 nucleotide mRNA that is present at low levels in vegetative cells and mature conidia, but accumulates to high levels in sporulating cultures. *yA* mRNA appears shortly after differentiation of sporogenous phialide cells. It accumulates in two developmentally abnormal mutant strains that produce phialides but is absent from two mutant strains that do not produce phialides. Thus, *yA* transcription is probably restricted to phialides. This result is discussed in relationship to the physiological roles played by phialides in spore differentiation.

THE asexual spores (conidia) of *Aspergillus nidulans* are produced by complex, multicellular structures, termed conidiophores (reviewed by CLUTTERBUCK 1977; CHAMPE *et al.* 1981; TIMBERLAKE 1987). Conidia contain in their walls a dark green pigment of unknown composition. This pigment confers resistance to ultraviolet irradiation (WRIGHT and PATEMAN 1970; R. ARAMAYO, T. ADAMS and W. TIMBERLAKE, unpublished results) and is probably essential for extended survival of conidia in nature. Conidial pigment synthesis requires the activities of multiple genes, including *wA*, *yA* and *fwA*. The products of other genes (*e.g.*, *dilA*, *chaA*, *drkA*) modify the amount of pigment, its chemical properties or its physical presentation on the spore surface (PONTECORVO *et al.* 1953; CLUTTERBUCK 1974, 1977). wA^- mutants form white spores, whereas yA^- mutants form yellow spores. wA^- mutations are epistatic to yA^- mutations, suggesting that the *wA* and *yA* genes encode enzymes that sequentially catalyze pigment synthesis from a colorless precursor.

The product of the *wA* gene has not been identified. By contrast, CLUTTERBUCK (1972) showed that *yA* encodes a *p*-diphenol oxidase, or laccase, enzyme (EC 1.10.32) that is needed to convert the yellow pigment intermediate to the mature green form. Laccase enzyme activity can be detected only during conidiophore development and appears to be restricted to conidial cell walls. LAW and TIMBERLAKE (1980) showed that accumulation of laccase is accompanied by coordinate increases in the rate of laccase-specific protein synthesis and the accumulation of laccase protein. Results from experiments with inhibitors of RNA and protein synthesis indicated that *yA* expression is regulated at the level of gene transcription. *yA* is one of only a few genes selectively activated during asexual reproduction in *A. nidulans* that has a well defined physiological function in conidiophore development or spore differentiation (CLUTTERBUCK 1977; TIMBERLAKE 1987).

We are interested in the molecular basis of the temporal and cellular specificity of *yA* gene expression. For this reason we selected a clone from an *A. nidulans* cosmid bank that complemented the *yA2* mutation (YELTON *et al.* 1985). We demonstrate here that the selected cosmid contains the *yA* structural gene and that *yA* expression is regulated at the level of mRNA accumulation. We present evidence suggesting that *yA* mRNA accumulates specifically in the sporogenous phialide cells.

MATERIALS AND METHODS

Fungal and bacterial strains. *Escherichia coli* HB101 (BOYER and ROULLAND-DUSSOIX 1969) and JM105 (MESSING 1983) were used for routine propagation of plasmids and M13 bacteriophages. *Aspergillus nidulans* strains FGSC4 (Glasgow wild type), FGSC237 (*pabaA1, yA2; trpC801*) and FGSC377 (*yA2, adE20; riboA1*) were obtained from the Fungal Genetics Stock Center. The strain used in the gene deletion/disruption experiments (*PW1; biA1; argB2, methG*$^-$) was provided by P. WEGLENSKI, Department of Genetics, Warsaw University, Poland. Strain *UCD1* (*pabaA1, yA2, biA1; argB2, methG*$^-$; *trpC801*) was used as one of the recipient strains for yA^- complementation experiments. Strains AJC7.1 (*biA1; brlA1*), GO1 (*biA1; abaA1*) AJC1.1 (*biA1; apsA1*) and GO241 (*biA1; wetA6*) were provided by JOHN CLUTTERBUCK, Department of Genetics, Glasgow University, Scotland. *brlA1* strains produce conidiophore stalks but not sterigmata or conidia. *abaA1* strains form aberrant conidiophores that produce chains of abnormal cells instead of conidia. *wetA6* strains produce normal conidiophores at

permissive temperature (30°) but produce autolytic conidia at restrictive temperature (37°). *apsA1* strains produce sterigmata but nuclei fail to migrate into them and phialide and spore formation abort.

***A. nidulans* transformation:** *A. nidulans* cells were transformed as described (YELTON, HAMER and TIMBERLAKE 1984). Cotransformation with gel-isolated DNA fragments was as described (TIMBERLAKE *et al.* 1985). Transplacement of homologous DNA sequences was done by using linear DNA fragments as described (MILLER, MILLER and TIMBERLAKE 1985).

DNA manipulations: Plasmids were constructed by using standard recombinant DNA techniques (DAVIS, BOTSTEIN and ROTH 1980; MANIATIS, FRITSCH and SAMBROOK 1982; MESSING 1983). Transcriptional polarity of *yA* was determined by using strand-specific probes derived from clones containing restriction fragments in opposite orientations in M13 vectors. Hybridization probes were prepared as described by BURKE (1984).

Nucleic acid isolation and gel blotting: DNA from *A. nidulans* transformants was prepared as described (YELTON, HAMER and TIMBERLAKE 1984). DNA samples were digested with restriction enzymes, fractionated in 0.8% agarose gels and transferred to nylon membranes. RNA was isolated as described (TIMBERLAKE 1986). Blots were hybridized with nick-translated DNA fragments isolated from agarose gels by use of the procedure of TAUTZ and RINZ (1983).

Genetic techniques: Standard *A. nidulans* genetic techniques were employed (PONTECORVO *et al.* 1953; CLUTTERBUCK 1974; KÄFER 1977).

RESULTS

Cosmid *yA1* contains the *yA* structural gene: YELTON *et al.* (1985) obtained two cosmid clones containing DNA inserts from the *A. nidulans* wild-type strain that genetically complemented the *yA2* mutation. However, they did not show formally that the cosmids contained the yA^+ gene. To do so, we constructed a restriction map of one of the cosmids, designated Cos yA1 (Figure 1). We then tested individual, gel-isolated fragments for their ability to complement the *yA2* mutation. *Bam*HI fragment "D" and *Sst*II fragment "B" (Figure 1) complemented the mutation thereby defining a ~2-kb region that contained the complementing activity.

We constructed a more detailed restriction map of the 0–16-kb region and tested additional cloned restriction fragments for their ability to complement the *yA2* and *adE20* mutations (Figure 2). *adE* is tightly linked to *yA* (<0.1 cM) and is centromere distal (KÄFER 1958). Figure 2 shows that the *yA2*-complementing activity resides in a 2.2-kb region (coordinate 8.3–10.5) and that a fragment to the right of this region efficiently complements the *adE20* mutation. These results support the hypothesis that the 2.2-kb region contains a wild-type copy of the *yA* gene and indicate that the centromere of chromosome *I* is leftward in Figure 2.

To substantiate further that the cloned DNA fragments contained the *yA* structural gene, a 3.3-kb fragment containing the *A. nidulans argB* gene was inserted into the *Sma*I site of a *Bam*HI fragment from coordinates 8.3–16.0. The resultant *Bam*HI fragment was gel isolated and used to transform a yellow-spored $argB^-$ strain (*pabaA1, yA2, biA1; argB2*) to arginine independence. Green-spored transformants were colony purified and their DNA was subjected to restriction digestion, electrophoretic fractionation and gel blot hybridization with *argB* and Cos yA1 probes. A green-spored transformant was identified in which the *argB*-containing, putative yA^+ fragment had precisely replaced the target chromosomal fragment. Thus, $argB^+$ and the complementing DNA fragment are physically linked in this strain.

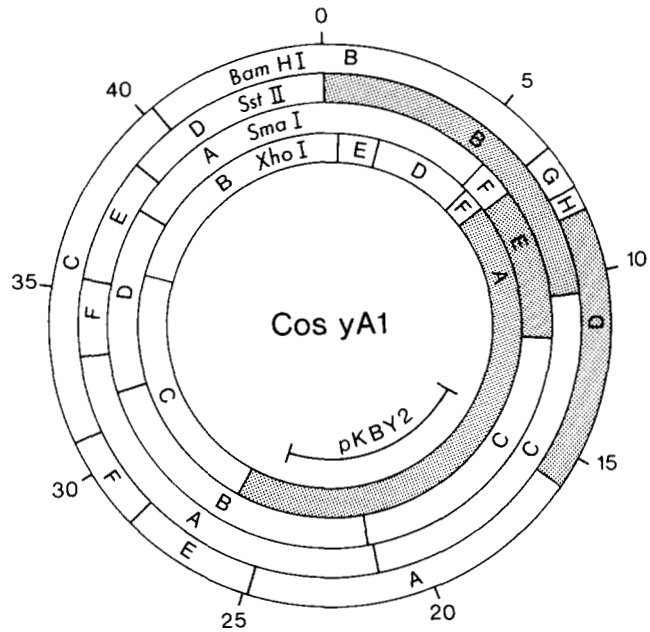

FIGURE 1.—Restriction map of the *yA2*-complementing cosmid Cos yA1. The cosmid consists of a ~35-kb fragment of *A. nidulans* nuclear DNA cloned into the *Bam*HI site of the vector pKBY2 (YELTON *et al.* 1985). Fragments are labeled according to size (kb) with "A" designating the largest fragment. *yA2*-complementing fragments are shaded.

We crossed the transformant to a strain that was $pabaA^+$, *yA2*, biA^+; *argB2* and determined the genotypes of 714 progeny (Figure 3). Recombination frequencies over the *pabaA-yA-biA* region were consistent with published values. $argB^+$ showed nearly complete linkage to *yA*; only one yA^+, $argB^-$ recombinant was obtained and no *yA2*, $argB^+$ recombinants were obtained.

We performed a final experiment to confirm that we had obtained the *yA* structural gene. A clone was constructed containing an NruI restriction fragment (coordinates 4.3–10.2, Figure 2) from which the two internal BamHI fragments had been deleted (coordinates 6.6–8.3; deletion 1 in Figure 4). A 1.8-kb DNA fragment containing the $argB^+$ gene was inserted into the *Bam*HI site. The NruI restriction fragment was gel isolated and used to transform a yA^+; $argB^-$ strain

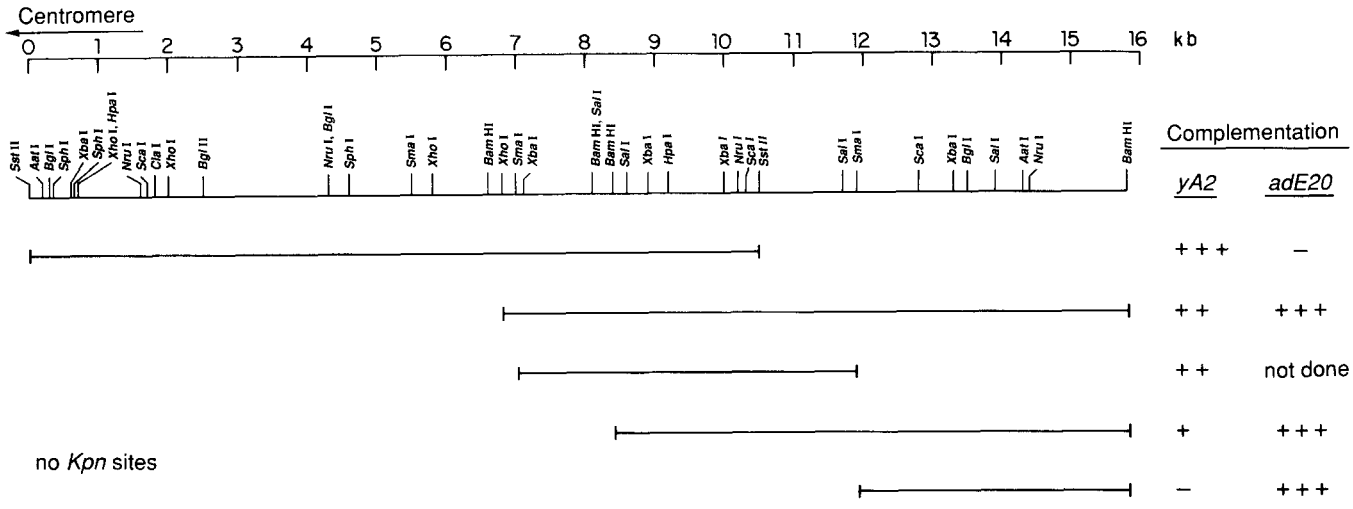

FIGURE 2.—Cos yA1 DNA fragments complement both the *yA2* and *adE20* mutations. The *A. nidulans* DNA fragments shown were subcloned into plasmid vectors and tested for their ability to complement either the *yA2* or *adE20* mutations. Complementation is indicated by +. For *yA2*, +++ indicates that ~80%, ++ ~15% and + ~0.1% of the transformants selected for the presence of the $argB^+$ or $trpC^+$ marker genes were also green-spored. – indicates that no green-spored transformants were detected (<0.01%). For *adE20*, the fragments were used to select directly for adE^+ transformants. Fragments scored as "+++" complemented at a frequency of ~20 transformants/μg DNA. The direction of the centromere was inferred from the complementation data and the *A. nidulans* genetic map.

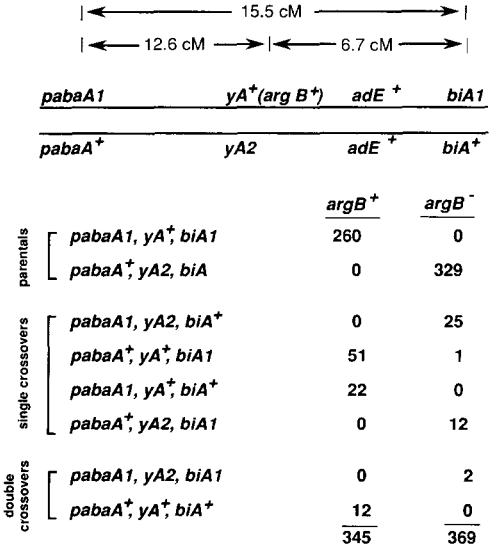

FIGURE 3 —The *yA2*-complementing DNA fragment corresponds to the *yA* genetic locus. The parent indicated at the top of the figure was constructed by transplacement of the *yA* region with a DNA fragment containing the $argB^+$ gene as described under Results and was crossed with the strain indicated. Random ascospores were isolated, grown and tested for all markers segregating in the cross.

to arginine independence. Thirty percent of arg^+ transformants were yellow-spored, and gel blot analysis showed that all yellow-spored transformants tested (5) lacked the two *Bam*HI fragments and contained the $argB^+$ marker inserted into the region of chromosome *I* under investigation, whereas the green-spored transformants tested (2) did not (data not shown). We infer that the selective marker disrupted the *yA* structural gene. A second clone was constructed containing the *Nru*I fragment from which a 1 kb *Xho*I fragment (coordinates 5.8–6.8; deletion 2 in Figure 4) was removed from the parental clone and the resulting *Nru*I restriction fragment was gel isolated and used in cotransformation experiments with $argB^+$ as the selective marker. Sixteen percent of the arginine prototrophs were also yellow-spored, and gel blot analysis of DNA from three of these strains demonstrated that the 1-kb *Xho*I fragment had been eliminated (data not shown).

***yA* mRNA levels are developmentally regulated:** We characterized the transcriptional patterns of the *yA-adE* region by hybridizing RNA gel blots containing samples from vegetative cells (hyphae), from conidiating cultures (containing hyphae, conidiophores, and conidia) and from purified conidia with radiolabeled restriction fragments from coordinates 4.3 kb to 16.0 kb (Figure 4). Numerous transcripts having different regulatory properties hybridized with the fragments (data not shown). DNA blot analyses indicated that this region is unique in the genome at the hybridization criterion employed in these experiments (data not shown). Thus, the RNAs identified almost certainly arose from transcription of this chromosomal region. The transcription patterns in the region are complex, with some transcription units apparently overlapping others. Ten transcripts showing various regulatory patterns were detected. We have not attempted to elucidate the details of transcription in this region.

The *yA* transcript was identified as follows. Complementation tests shown in Figure 2 demonstrated that the *yA2* mutation is to the right of the *Bam*HI site at coordinate 8.3 kb and to the left of the *Sst*II site at coordinate 10.5 kb (Figure 4). Conidial laccase has an

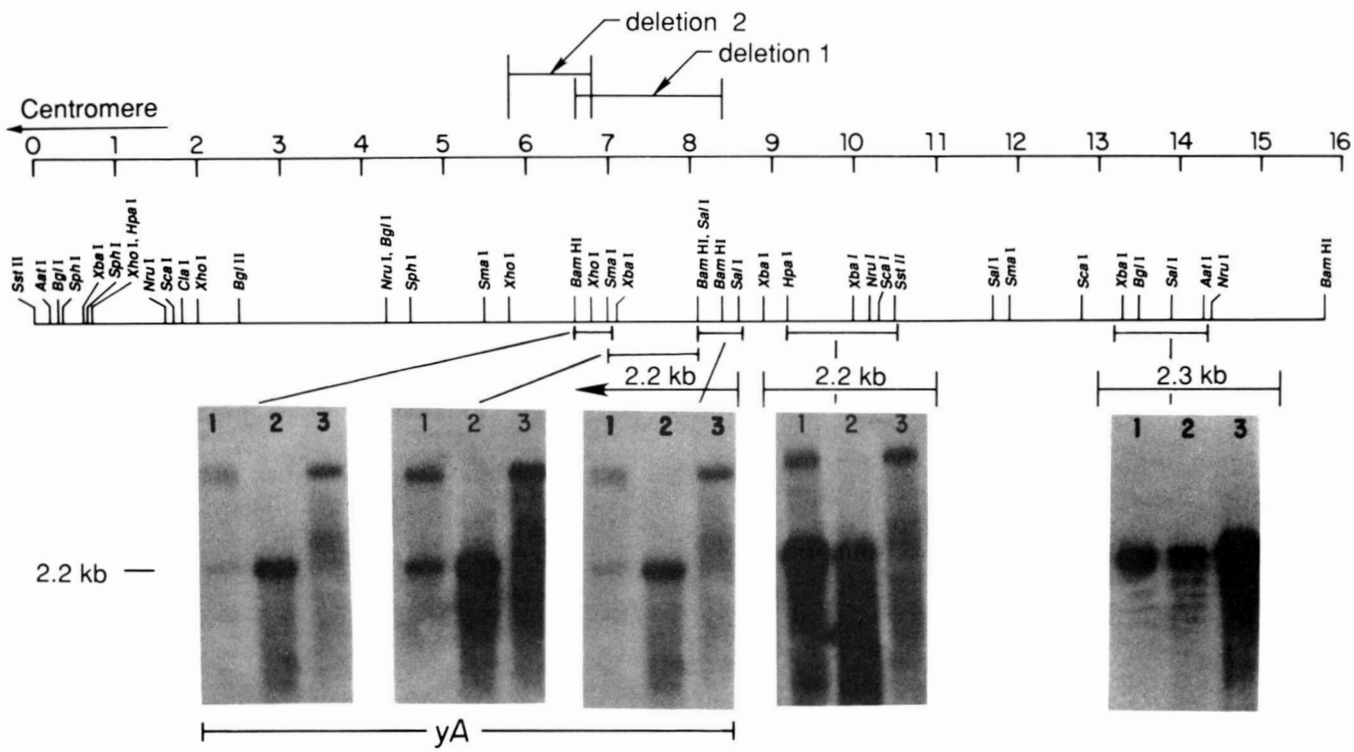

FIGURE 4.—Partial transcription map of the *yA* chromosomal region. The transcript map was deduced as described under Results. Deletion mutations that resulted in formation of yellow spores are indicated above the coordinates. Blots of gels containing RNA from hyphae (lane 1), conidiating cultures (lane 2) and purified conidia (lane 3) are shown and hybridization probes are indicated. The size estimate was made by comparison to *E. coli* and *A. nidulans* rRNA markers fractionated in the same gel.

apparent M_r of 80–85,000 (LAW and TIMBERLAKE 1980; KURTZ and CHAMPE 1982) and the predicted size of its mRNA is thus ~2200 nucleotides. At least two candidate transcripts from this region are apparent in Figure 4. However, genomic insertion of foreign DNA into the HpaI site (coordinate 9.2 kb in Figure 4) did not produce yellow-spored mutants (data not shown). Thus, the transcript spanning the HpaI site almost certainly does not encode conidial laccase and its product has a dispensable function. The ~2200 nucleotide transcript spanning the HpaI site was present at readily detectable levels in wild-type hyphae and deletion mutant 1. The other 2200-nucleotide transcript mapping to the left of the *Bam*HI site at coordinate 8.3-kb (Figure 4) spans the region where deletions result in yellow-spored mutants. This transcript was not detectable in hyphae or conidiating cultures of the yellow-spored deletion 1 strain described above (data not shown), but accumulated in a wild-type strain during conidiation (Figure 5). The transcript was also not detectable in deletion mutant strain 2, which contained a larger (~4 kb) transcript not present in wild-type. Thus, the only transcript affected by deletions 1 and 2 was the 2.2-kb transcript hybridizing with DNA fragments from coordinates 6.6–8.5 kb. This 2.2-kb transcript was absent from both deletion strains. We conclude that it is the product of the *yA* gene. Additional RNA blotting experiments showed that the transcription unit is transcribed from right to left as shown in Figures 2 and 4. It is likely that a 2300 nucleotide transcript mapping to coordinates 13.0–15.0 kb (Figure 4) is the product of *adE* as restriction fragments from this region complemented the *adE20* mutation.

***yA* transcripts accumulate specifically during conidiophore development:** Total RNA was isolated from cultures that had been induced to conidiate as described by TIMBERLAKE (1980) and LAW and TIMBERLAKE (1980). Under these conditions, phialides appeared at 10–15 h and pigmented spores appeared at 15–20 h. Figure 5 shows that *yA* mRNA was undetectable in hyphae and mature conidia. It began to accumulate in cultures at about the time of phialide differentiation.

yA mRNA accumulation was also examined in several developmentally abnormal mutant strains (CLUTTERBUCK 1977) that had been induced to develop for 25 h (Figure 5; see MATERIALS AND METHODS). *yA* mRNA was detectable in *abaA1* and *wetA6* mutant strains, but not in *brlA1* or *apsA1* mutant strains.

DISCUSSION

The data presented in this paper show that we have cloned the *yA* gene of *A. nidulans* based on four observations: (1) in DNA-mediated transformation experiments fragments from the clone Cos yA1 comple-

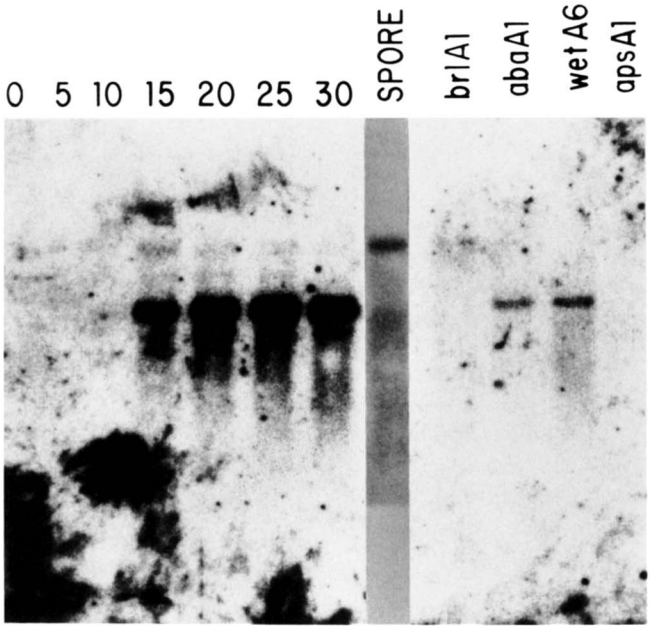

FIGURE 5.—Regulation of *yA* transcript levels in wild-type and developmental mutant strains. Total RNA from conidiating cultures of FGSC4 was fractionated by denaturing agarose gel electrophoresis, blotted and hybridized with a radiolabeled internal *yA* gene fragment (1.2-kb xho Bam fragment, coordinates 6.8–8.0). Times indicated are hours after induction of conidiation. The blot was washed and rehybridized with an *argB* probe to assure equal loading of the lanes (not shown). Total RNA was also isolated from cultures of developmentally abnormal strains, containing *brlA1*, *abaA1*, *wetA6* or *apsA1* mutant alleles (see MATERIALS AND METHODS), that had been induced to develop for 25 h and analyzed as for FGSC4.

mented the null *yA2* mutation, (2) some overlapping or adjacent DNA fragments complemented a mutation in the tightly linked *adE* gene, (3) a copy of the $argB^+$ gene became tightly linked to *yA* when integrated into the genome at a position adjacent to the proposed *yA* transcription unit (see below), and (4) disruption of the putative *yA* transcription unit produced the yellow-spored phenotype characteristic of *yA* null mutations (CLUTTERBUCK 1972).

The *yA2* and *adE20* complementation data shown in Figure 2 were used to orient the physical map of the *yA* region with the genetic map. The results shown in Figure 3 are potentially inconsistent with the proposed orientation. The existence of one $pabaA^+$, yA^+, *biA1*; $argB^-$ recombinant implies that a crossover event occurred between the integrated $argB^+$ gene and *yA* and that $argB^+$ is centromere proximal to *yA*, even though we predicted from the *adE20* complementation data and the strategy used to insert $argB^+$ into chromosome *I* that $argB^+$ would be centromere distal. However, only one such recombinant was obtained in 714 progeny and we propose that it arose as the result of a spontaneous mutation or by gene conversion. We favor these alternatives because of the clarity of the *yA2*/*adE20* complementation results shown in Figure 2 and the certainty of the relative positions of the marker genes, including *adE* (KÄFER 1958).

CLUTTERBUCK (1972, 1977) and LAW and TIMBERLAKE (1980) showed that the product of the *yA* gene, conidial laccase, is not present in hyphae but accumulates during conidiation. The enzyme is loosely associated with conidial walls where it converts a yellow substrate to mature green pigment. LAW and TIMBERLAKE (1980) suggested that expression of *yA* is regulated at the level of gene transcription based on the results of inhibitor studies. The data presented in this paper support that conclusion and in addition indicate that *yA* is expressed preferentially in the sporogenous phialide cells. We showed that a DNA fragment from the *yA* locus hybridized to a developmentally regulated transcript that we infer corresponds to *yA* mRNA. *yA* mRNA was present at low or undetectable levels in hyphae but increased to readily detectable levels in conidiating cultures at a time corresponding to the appearance of phialides and some immature spores. Thus, *yA* expression is regulated at the level of RNA accumulation. Pulse labeling experiments indicate that *yA* is regulated at the level of transcription (T. H. ADAMS and W. E. TIMBERLAKE, unpublished results).

yA mRNA does not accumulate significantly in conidia implying that it accumulates in one or more of the differentiated conidiophore cells. We examined *yA* transcript accumulation in four developmentally abnormal mutant strains to obtain evidence for which cell(s) was the site of *yA* mRNA accumulation. *brlA1* and *apsA1* strains (CLUTTERBUCK 1977; TIMBERLAKE 1987) lack phialides and failed to produce detectable levels of *yA* mRNA whereas *abaA1* and *wetA6* strains have phialides and produced readily detectable levels of *yA* mRNA. These observations, along with the observation that appearance of phialides and *yA* mRNA in synchronously conidiating cultures are coincident, indicate that *yA* is preferentially expressed in phialide cells.

A. nidulans phialides are like stem cells in that they undergo asymmetrical divisions. One daughter nucleus enters the differentiating spore and becomes arrested in the G_0 phase of the cell cycle. The other daughter nucleus is retained in the phialide and undergoes additional mitoses (MIMS, RICHARDSON and TIMBERLAKE 1988). Our conclusion that *yA* mRNA occurs in phialides, and not in conidia, indicates that the phialide also acts as a "nurse cell." Conidial laccase is apparently synthesized in the phialide and secreted into the differentiating spore wall where it converts substrate to conidial pigment. Given this observation, it is reasonable to speculate that the phialide might secrete many or all of the substrates and enzymes for conidial wall formation. Thus, the conidial protoplast may play no or a limited role in conidial wall synthesis.

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