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Developmental Decisions in *Aspergillus nidulans* Are Modulated by Ras Activity

TAPAN SOM* AND VENKATA S. R. KOLAPARTHI†

Department of Microbiology and Immunology, Jefferson Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

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To better understand how Ras controls development of multicellular organisms, we have chosen *Aspergillus nidulans* as a model system. When grown on solid medium, this fungus follows a well-defined program of development, sequentially giving rise to several cell types which produce three distinct structures: vegetative hyphae, aerial hyphae, and the conidiophore structure. Here we describe a *ras* homolog found in this fungus (*Aras*) and demonstrate that it is an essential gene that regulates the ordered program of development. We created dominant alleles of this gene and expressed them to different levels in order to vary the ratio of GTP-bound (active) to GDP-bound (inactive) A-Ras protein. When the amount of active Ras is large, nuclear division proceeds, but further development is inhibited at the early step of germ tube formation. At an intermediate level of active Ras, aerial hypha formation is inhibited, while at a low level, conidiophore formation is inhibited. Maintenance of an even lower level of the active Ras is essential for initiation and progression of conidiophore formation, the final stage of development. When the level of active Ras is artificially lowered, each stage of development is initiated prematurely except germination, the initial stage of development. Therefore, the progression of the ordered developmental pathway of *A. nidulans* is dependent upon an initial high level of active Ras followed by its gradual decrease. We propose that several concentration thresholds exist, each of which allows development to proceed to a certain point, producing the proper cell type while inhibiting further development.

The importance of *ras* was first shown when a particular series of mutations in this gene were found to be associated with human malignancy (3). The wild-type gene product exists in two different forms in the cell membrane. In response to a variety of signals, the inactive GDP-bound form exchanges GDP for GTP and becomes active. In this active state it associates with cytoplasmic target proteins and transduces external signals across the cell membrane (26). The binding of these cytoplasmic proteins initiates a series of reactions leading to growth and differentiation. The mutations associated with malignancy lock the Ras protein in the active state, resulting in constitutive production of signals.

Ras has been found in all eukaryotes tested. In the yeast *Saccharomyces cerevisiae*, a signal from this gene product is essential for growth, while constitutive expression of this signal inhibits both growth and sporulation (19). In mammalian cells, the Ras protein is involved in transducing a variety of signals in different types of cells. For example, platelet-derived growth factor, epidermal growth factor, and other growth factor-mediated signals are transduced through Ras protein (15, 32). p21ras is necessary for the nerve growth factor-, fibroblast growth factor-, and v-Src-induced PC12 cell differentiation (20, 42). Ras is also involved in signaling through a T-cell receptor CD3 complex (11). In *Drosophila melanogaster*, Ras is involved in adult eye development (38) and also in the development of the terminal structures of the embryo (30). It is also involved in the development of the vulva in *Caenorhabditis elegans* (17). However, in both *D. melanogaster* and *C. elegans*, null mutations are lethal to larvae, suggesting that the gene products are involved in many other processes early in the life cycle.

The fungus *Aspergillus nidulans* is a multicellular organism that differentiates into various well-defined cell types. Genetically, this organism has been studied extensively and is ideal for the study of differentiation. This fungus has several modes of growth: asexual, parasexual, and sexual. Normally, *A. nidulans* reproduces asexually (Fig. 1). When the spores are grown in liquid, the nucleus replicates while the spore swells and germinates, giving rise to germ tubes. New cells arise by apical growth from the tip of the germ tube and produce mycelia (vegetative growth). Each mycelium consists of a linear array of cells separated by septa. When grown on solid medium, the spores initially germinate to produce vegetative hyphae. At a definite point in the life cycle, some cells in the hyphae differentiate into a foot cell that gives rise to a stalk that extends in the air vertically from the solid surface. A stalk grows to a definite length when its tip swells and forms a vesicle. Three different types of cells appear sequentially on the vesicle: metulae, phialides, and a chain of conidia (spores) (43). The entire structure is called a conidiophore. Besides conidiophores, vegetative hyphae also produce aerial hyphae which grow to an indefinite length and finally cover the entire colony. For a detailed description of the *A. nidulans* life cycle, see references 7 and 43.

An approach was initiated to determine if there is a Ras homolog in *A. nidulans* and if so, whether it has any role in the developmental processes. We found that there is one *ras* homolog (*Aras*) in *A. nidulans*. Our results indicate that *Aras* regulates the developmental decisions in this organism; at different stages of development, the level of active A-Ras determines the progression through the next stage of development.

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Jefferson Cancer Institute, Thomas Jefferson University, 1020 Locust St., Philadelphia, PA 19107. Phone: (215) 955-4725. Fax: (215) 923-1098. Electronic mail address: som@calvin.jci.tju.edu.

† Present address: Cancer Institute of New Jersey, Center for Advanced Biotechnology and Medicine, Piscataway, NJ 08854.

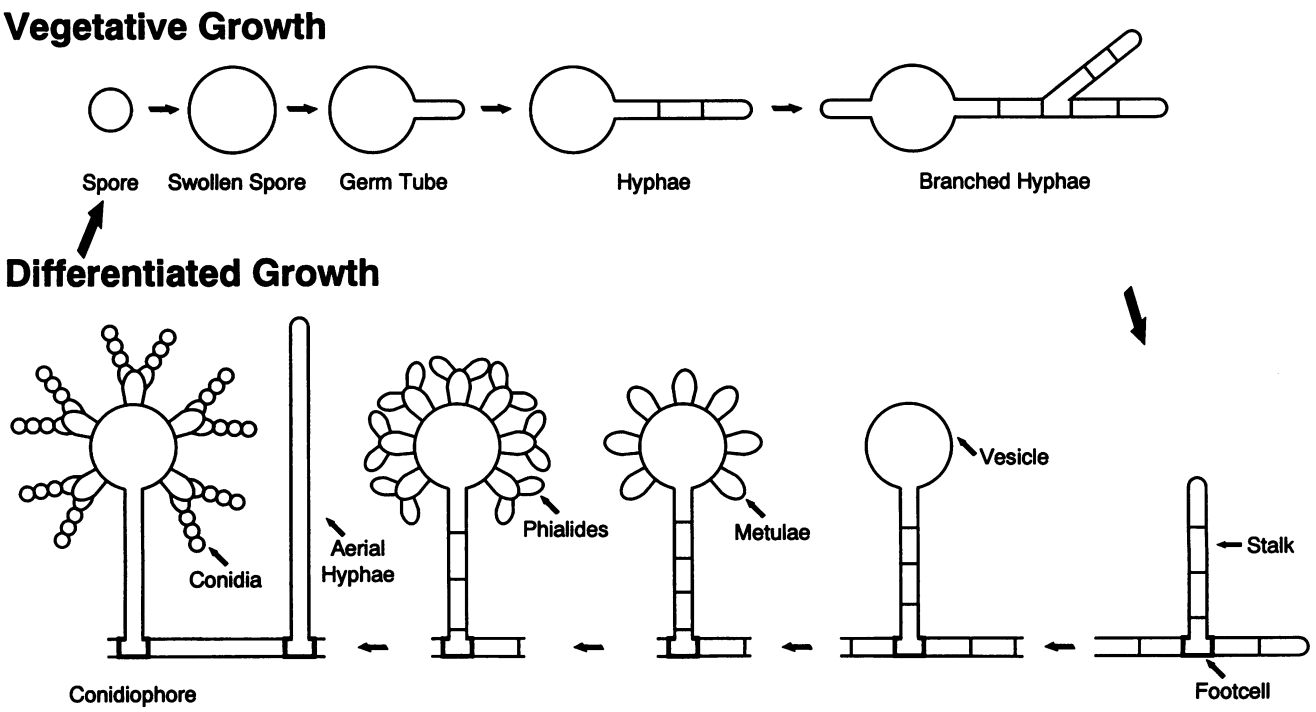

FIG. 1. Asexual cycle of *A. nidulans*.

MATERIALS AND METHODS

***Aspergillus* strains, growth conditions, and genetics.** The *A. nidulans* strains used in this study are described in Table 1. YG (0.5% yeast extract, 2% glucose, trace elements) and MMG (minimal salt, 2% glucose, trace elements) were used for growth in liquid (18). When required, glucose was replaced by 2% fructose (YF or MMF), 200 mM ethanol (YE or MME), or 50 mM threonine (YT or MMT) (9). For growth on a solid surface, these media were supplemented with 1.5% agar. The compositions of other media and general growth conditions were as described previously (18). In certain experiments, cells were first grown on cellophane-overlaid medium and then transferred to a second medium by a published procedure (27).

TABLE 1. *A. nidulans* strains used in this study

Strain	Genotype	Source or reference
FGSC4	Glasgow wild type (*veA*$^+$)	FGSC[a]
FGSC89	*biA1, argB2, veA1*	FGSC
AST27	*biA1, argB*$^+$::P$_{alcA}$:*Aras*^{G17V}, *veA1*	This study
AST29	*biA1, argB*$^+$::P$_{alcA}$:*Aras*^{S22N}, *veA1*	This study
AST30	*biA1, argB*$^+$::P$_{alcA}$:*Aras*, *veA1*	This study
AST32	*biA1, argB*$^+$::*Aras:lacZ*, *veA1*	This study
AST33	*biA1, veA1*	This study
RMS010	*biA1*, Δ*argB*::*trpC*ΔB,[b] *methG1*, *veA1*, *trpC801*	41
RMS011	*pabaA1, yA2*, Δ*argB*::*trpC*ΔB, *veA1*, *trpC801*	41
RMS012 (diploid)	*biA1*, Δ*argB*::*trpC*ΔB, *methG1*, *veA1*, *trpC801* *pabaA1, yA2*, Δ*argB*::*trpC*ΔB, *veA1*, *trpC801*	41

[a] FGSC, Fungal Genetics Stock Center.
[b] *trpC*ΔB, the single *Bam*HI site in the *trpC* gene is inactivated by in vitro mutagenesis (23).

Standard *A. nidulans* molecular genetic procedures were used (7, 8, 31). *A. nidulans* protoplasts were used for transformation according to a method described previously (28). Diploids were haploidized by a published procedure (41). Benomyl was a gift from E. I. DuPont. Growth rates in liquid were determined by inoculating 50 ml of medium with 1.5×10^5 spores per ml and measuring the dry weights of samples collected at different time points. Three samples were taken for each time point, and the dry weights were averaged. The growth rates on solid medium were determined by the diameters of single colonies at different time points. The relative rates of nuclear division during sporulation were determined by comparing the average number of nuclei in spores of AST27 with that of the control strains, AST30 and AST33, at a time just before germ tube formation in the control strains. Similarly, the relative rate of nuclear division in AST27 mycelia was determined by counting the number of nuclei in mycelial cells 5 h after germination and comparing the average number with that of the control strains. The number of nuclei per cell should reflect the rate of nuclear division for each of these strains at this time in development, since the overall growth rates are similar (as determined by dry weight [see above]) and the sizes of cells are also similar up to about 12 h of development (see Fig. 7).

Aspergillus extracts were prepared according to the following procedure. The strains were grown in liquid culture, the mycelia were collected on a filter and washed with water, and the excess liquid was squeezed out between filter papers. The cells were frozen immediately in liquid nitrogen and ground in a mortar with a pestle. The powdered cells were extracted with phenol and used for isolation of DNA or total RNA. For a protein extract, the powdered cells were suspended in extraction buffer (50 mM Tris-HCl [pH 7.4], 50 mM NaCl, 50 mM NaF, 20 mM sodium PP_i, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 10 μg of leupeptin

per ml, and 10 μg of pepstatin A per ml). β-Galactosidase was assayed as described before (39). Southern blot and Western blot (immunoblot) analyses and other general molecular biology methods were performed as described previously (34). In vitro mutagenesis was done by a published procedure (36).

Wild-type A-Ras and A-Ras^{G17V} proteins (see below for explanation of designations) were expressed in *Escherichia coli* JM101 containing plasmid pAST31 or pAST18, respectively, in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG) and were purified to homogeneity by a published procedure (16).

Cloning. The cDNA library was obtained from Ronald Morris (29). The library was screened with an 1,110-bp ^{32}P-labeled *Hpa*I fragment as a probe; this probe contained the entire yeast *RAS2* gene (from the yeast *RAS2* clone pRAS2, provided by Jim Broach [19]). The filters were washed with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 55°C. Several positive clones were obtained. Because all the clones appeared to contain parts of the same mRNA, the largest clone (both strands of a 1,457-bp *Eco*RI fragment) was sequenced by the dideoxy method (35). This fragment was then used as a probe to screen an *A. nidulans* genomic library (obtained from the Fungal Genetics Stock Center, Kansas City, Kans. [6]). A 4.3-kb *Eco*RI fragment containing the *ras* homology region was subcloned (pAST33) and sequenced as described above.

Plasmids. Two vectors containing either an *Xba*I or an *Eco*RI site were constructed from pBR322. For this purpose, pBR322 was digested with *Eco*RI and *Pvu*II, an *Xba*I linker was added, and the resulting fragment was ligated. A *Taq*I fragment containing the single *Pst*I site was then replaced by a corresponding fragment from pUC19, resulting in the vector pST80. pST82 is a derivative of pST80 that contains one *Eco*RI site in place of *Xba*I.

The *Aras* clone from the genomic library was digested with *Eco*RI, and a 4.3-kb fragment was subcloned into pST82 to produce pAST33. For chromosomal disruption of *Aras*, the two *Bam*HI fragments containing most of the *Aras* open reading frame were replaced by a *Bam*HI-linked *Xba*I fragment from pM006 (obtained from Peter Mirabito) (23) containing the promoter and all of the open reading frame of *argB*, producing plasmid pAST47 (with the Δ*Aras:argB* construct).

For mutagenesis, the 1.4-kb *Eco*RI fragment containing the cDNA sequence of *Aras* was first cloned into M13mp18 to generate φAM1. Mutations in three different places in the *Aras* sequence were introduced by in vitro mutagenesis (36). φAM5 contains an *Nco*I site at the translation initiation site (codes for wild-type A-Ras). φAM3 is a derivative of φAM5 that codes for A-Ras^{G17V} (a Gly at position 17 is replaced by Val). φAM12 is another derivative of φAM5 that codes for A-Ras^{S22N} (a Ser at position 22 is replaced by Asn). The *Nco*I-*Eco*RI fragment of φAM5 was then used to replace the *Nco*I-*Eco*RI region of pUC12N (pUC12 with an *Nco*I site at the initiating ATG of *lacZ*; provided by Steve Huge) to obtain pAST31. A similar plasmid, pAST18, in which the *Nco*I-*Eco*RI fragment was obtained from φAM3, was constructed. These constructs, pAST31 and pAST18, were put into *E. coli* JM101 and when induced with IPTG produced A-Ras and A-Ras^{G17V}, respectively, without additional amino acids.

An *Nco*I site was introduced at the initiating ATG of *alcA* in pALB11 (the clone containing the promoter region of the alcohol dehydrogenase gene, P_{alcA}, of *A. nidulans* [45]), and the corresponding plasmid was called pAST30. Three plasmids were made by replacing an *Nco*I-*Hin*dIII fragment of pAST30 with *Nco*I-*Hin*dIII fragments containing the *Aras* regions of φAM5, φAM3, and φAM12. The plasmids obtained were pAST40, pAST39, and pAST62, containing *Aras*, *Aras*^{G17V}, and *Aras*^{S22N}, respectively. In these clones the different *Aras* genes are under control of P_{alcA} and when induced would produce proteins without additional amino acids.

As a first step to introduce the P_{alcA}:*Aras* constructs stably into *A. nidulans*, these hybrids were excised as *Eco*RI fragments and introduced in the middle of an *argB* clone such that the gene is inactivated. For this purpose the *Xba*I fragment containing *argB*, as mentioned above, was cloned in pST80, and then the single *Bgl*II site in the middle of *argB* was converted to an *Eco*RI site (pAST22). The *Eco*RI cassettes were introduced into this site, and the resulting clones pAST43, pAST41, and pAST64 contained P_{alcA}:*Aras*, P_{alcA}:*Aras*^{G17V}, and P_{alcA}:*Aras*^{S22N}, respectively. These constructs were used to transform FGSC89, and the arginine prototrophs obtained were tested for proper integration events at the *argB* locus by Southern blot analysis.

An *Aras:lacZ* hybrid was constructed in the following way. The untranslated upstream region in the *Aras* cDNA clone, pAST2 (1.4-kb *Eco*RI fragment in pST82), was replaced with a 1.3-kb upstream region containing the promoter of the genomic clone (pAST33) to produce pAST66. The *lacZ* gene from pYST173 was excised as an *Eco*RI-*Hin*dIII fragment, the ends were filled, and the product was ligated into the (filled) single *Bam*HI site of the *Aras* gene such that the *lacZ* open reading frame was in frame with that of *Aras* after amino acid 181. The resulting plasmid was called pAST70. The *Aras:lacZ* construct was excised from pAST70 as an *Eco*RI fragment and cloned into the *Eco*RI site of pAST22 to generate pAST72. FGSC89 was transformed, and the arginine prototrophs obtained were tested for proper integration of pAST72 at the *argB* locus as mentioned above. pYST173 was constructed by replacing the single *Bal*I site with a *Hin*dIII site downstream of the *lacZ* gene in pMLB1034 (37).

RNase protection. RNase protection was performed as described previously (22, 39). The RNA probes used were prepared in the following way. For determination of the transcription initiation site, first an *Nco*I site was created at the first ATG of the *Aras* open reading frame (position 325 [see Fig. 3]) in the *Aras* genomic clone. A 0.9-kb filled *Nco*I fragment containing the *Aras* promoter region was cloned into the *Hin*cII site of pGEM3, such that transcription from the T7 promoter synthesized an antisense RNA, resulting in clone pAST74. A 325-base fragment was protected. For determination of the transcription termination sites, a 711-bp *Bam*HI fragment from coordinate 1115 to 1826 (see Fig. 3) was cloned into pGEM4 such that transcription from the T7 promoter synthesized an antisense RNA. Four fragments of 261, 397, 489, and 514 bases were protected. In order to determine the expression of P_{alcA}:*Aras* transcripts, a 219-bp *Sau*3AI fragment encompassing the hybrid junction from pAST40 was cloned into pGEM3. When linearized with *Hin*dIII, transcription from the T7 promoter yielded a 284-base antisense RNA. A series of fragments ranging from 63 to 78 nucleotides in length were protected by the endogenous *alcA* transcripts, an 88-nucleotide fragment was protected by the endogenous *Aras* transcript, and a series of fragments of 153 to 168 nucleotides in length were protected by the hybrid transcripts.

In vitro assays. Bovine GTPase-activating protein (GAP) was obtained from Jay Gibbs. The GTPase assay was performed as described previously (14). For β-galactosidase assays, the extracts were prepared by grinding quickly frozen mycelia grown overnight in YG under liquid nitrogen. Assays were performed with CPRG as a substrate (39). Under the assay conditions used, 0.15 U of β-galactosidase gave 1.0 optical density unit per min.

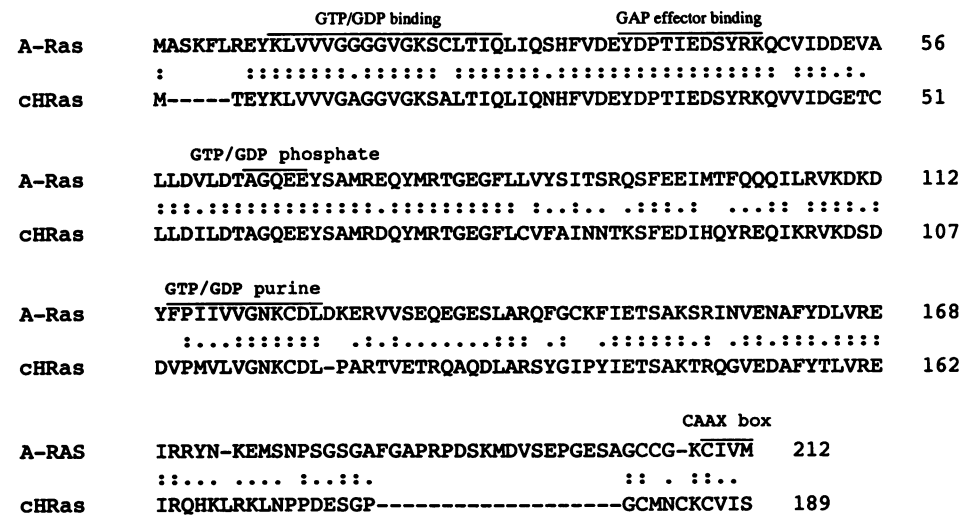

FIG. 2. Comparison of the protein sequence of A-Ras with that of cHRas. Identical sequences are indicated by colons, and conservative changes are indicated by dots. Gaps are denoted by dashes. Several conserved domains important for Ras activity are indicated (3).

Microscopy and photography. Light microscopy was done with a Nikon Optiphot-2. Conidiophores were viewed in situ on agar medium. Samples of submerged cultures were viewed and photographed without fixation. Septa were stained by incubating hyphal samples in 20 μg of Calcofluor White M2R (chitin specific; from Sigma) for 5 min at room temperature, and fluorescence microscopy was conducted by using epifluorescence optics. Staining of nuclei with the DNA-specific dye 2,4-diamidino-2-phenylindole (DAPI) was performed as described previously (27). Immunofluorescence was conducted by a published procedure (27). Briefly, FGSC4 was grown on MMG agar plates. A piece of agar containing conidiophores was placed on a microscope slide, fixed, washed, and treated with antibodies as described previously (27). The primary and secondary antibodies used were Y13-259 (13) and fluorescein isothiocyanate-conjugated goat anti-rat, respectively.

Nucleotide sequence accession number. The GenBank accession numbers for the cDNA and genomic sequences of *Aras* reported in this paper are U03023 and U03025, respectively.

RESULTS

Identification and cloning of the single *ras* gene homolog in *A. nidulans*. In order to determine if there is a *ras* homolog in *A. nidulans*, Southern blot analysis was performed with a yeast *RAS2* probe. One strong and several weak bands were detected, indicating the presence of at least one *ras* gene homolog (data not shown). The same probe was used to search a cDNA library (29), and several positive clones were found. Because all of these clones appeared to represent the same mRNA, the longest clone was sequenced and was found to contain an open reading frame. The corresponding *Aspergillus* gene (*Aras*) codes for a 212-amino-acid protein (A-Ras) with extensive homology to the chicken H-Ras (cHRas) protein (Fig. 2). All the known domains conserved in other Ras proteins are included. The A-Ras protein has 5 extra amino acids near the amino terminus and 19 extra amino acids in the nonconserved region near the carboxyl terminus, relative to the cHRas protein. The *Aras* cDNA was used to probe an *A. nidulans* genomic library (6), and a corresponding genomic clone was identified. A 4.3-kb *Eco*RI fragment was subcloned and sequenced. The genomic and cDNA sequences are shown in Fig. 3. *Aras* has three introns. The genomic clone containing *Aras* had already been assigned to chromosome 8 by the laboratory of W. E. Timberlake, in which the library was made. The chromosomal location of *Aras* was confirmed by probing chromosomes separated by pulsed-field gel electrophoresis (data not shown). The initiation site of the transcript and poly(A) addition sites as determined by RNase protection experiments are indicated in Fig. 3. Using the *Aras* cDNA as a probe, we found that *A. nidulans* has a single copy of the *ras* gene homolog (data not shown).

Biochemical properties of the A-Ras protein. The A-Ras protein was expressed in *E. coli* and purified to homogeneity as described previously (16). In vitro studies indicate that, as with the H-Ras protein, A-Ras has a GAP-susceptible GTPase activity that hydrolyzes bound GTP to GDP, thereby converting the active A-Ras to an inactive form. Also like H-Ras, A-Ras is always present in the active form in the presence of Mn^{2+} (Table 2). A mutant, $Aras^{G17V}$, with a Gly→Val mutation in position 17, which corresponds to position 12 in H-Ras, was constructed in vitro, expressed in *E. coli*, and purified. Such a mutation is expected to lock Ras in the activated state, and we have shown that A-Ras^{G17V} is not susceptible to the action of bovine GAP (Table 2), similar to the case with v-Ras. These data suggest that A-Ras is biochemically similar to the H-Ras protein.

***Aras* is an essential gene.** Ras has been shown to be involved in signal transduction in many organisms. To test the impor-

TABLE 2. GTPase assay with purified Ras proteins in the presence and absence of bovine GAP

Protein	% GTP→GDP conversion with:			
	Mg^{2+}		Mn^{2+}	
	Without GAP	With GAP	Without GAP	With GAP
A-Ras	0.0	97.0	0.0	1.0
A-Ras^{G17V}	0.0	0.4	0.0	0.2
H-Ras	0.0	92.0	0.0	1.0
v-Ras	0.0	2.0	0.0	2.0

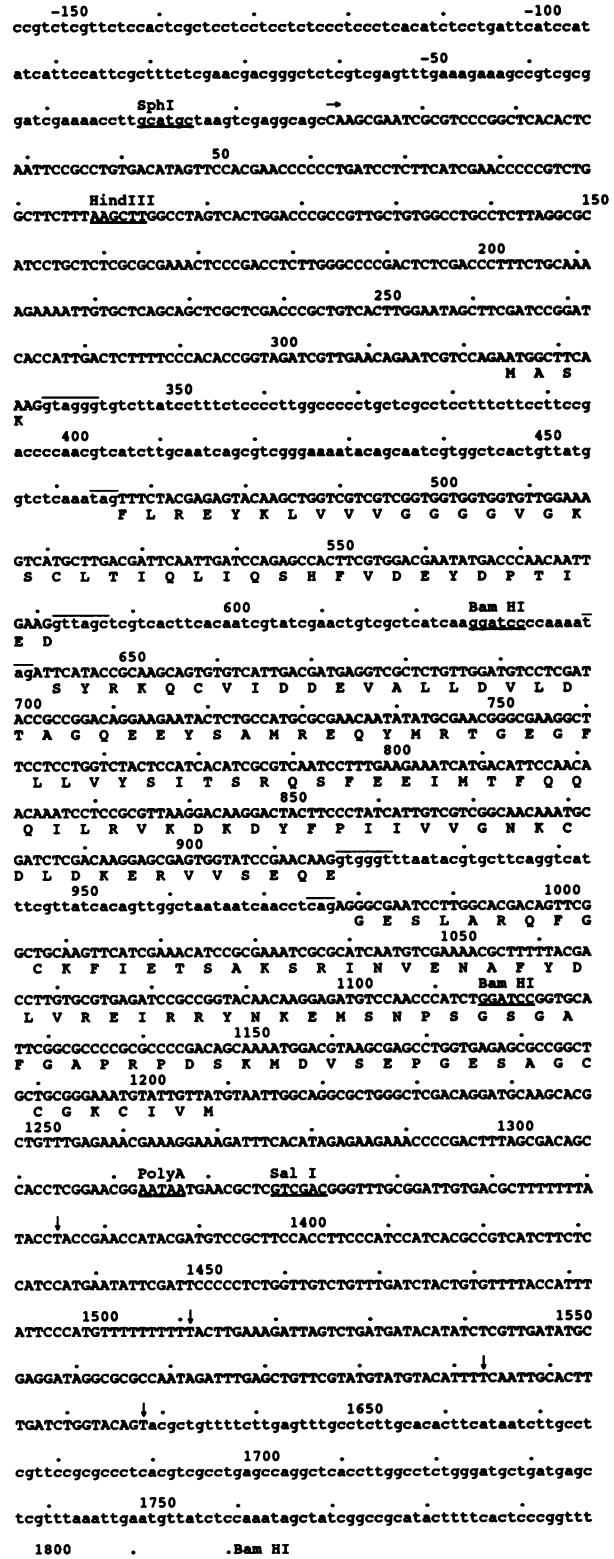

FIG. 3. Genomic sequence of *Aras*. The cDNA sequence is shown in uppercase. The transcription initiation site (→) and the poly(A) addition sites (↓) determined by RNase protection (22, 39) are indicated. Splice acceptor and donor sites are indicated by a line above the sequence.

tance of such signals in the life cycle of *A. nidulans*, we studied the effects of disruption of the gene.

Because of the possibility that a null *Aras* mutation might prove lethal in a haploid, we used a diploid (RMS012) to disrupt the *Aras* gene. This diploid has both copies of the *argB* locus deleted, resulting in an Arg^- phenotype (41), and produces spores with the wild-type green color. This strain was made from two haploids, RMS010, which also produces green spores, and RMS011, which produces yellow spores because of a recessive mutation (*yA2*). Most of the open reading frame in the cloned *Aras* gene was replaced in vitro by the $argB^+$ gene (44). The structure of the resulting construct, Δ*Aras*:*argB*, contained in the plasmid pAST47, is shown in Fig. 4A. The diploid strain was transformed with either a 6.2-kb *Eco*RI fragment or a 5.9-kb *Bst*EII-*Eco*RI fragment, both containing the Δ*Aras*:*argB* construct isolated from pAST47, and Arg^+ transformants were selected. A recombination event that replaces the wild-type gene with the disrupted construct will result in a null *Aras* genotype (Fig. 4B). Arg^+ diploid transformants in which one copy of the *Aras* gene had been replaced by the disrupted allele were obtained. Twenty such transformants were obtained by using the *Eco*RI fragment, and six were obtained by using the *Bst*EII-*Eco*RI fragment. Results of Southern blot analyses of five of the transformants obtained by using the *Eco*RI fragment are shown in Fig. 4C. Similar results were obtained with the *Eco*RI-*Bst*EII fragment. All 26 diploids were independently made haploid on benomyl plates as described previously (41), and 80 yellow colonies (presumably haploids) were purified and scored. Of these, 35 were Arg^- and 45 were Arg^+. Several of these were analyzed further for both the presence of the disrupted allele and the absence of the endogenous *Aras* gene. All the Arg^- strains produced only one band on Southern blot analysis, namely, the expected endogenous 4.3-kb band corresponding to the uninterrupted *Aras* gene (data not shown). The markers segregated as expected. All the isolates carried *pabaA1* and were prototrophic for biotin, while half were methionine requiring. However, all the Arg^+ yellow colonies produced two bands, one corresponding to endogenous *Aras* and the other corresponding to the disrupted Δ*Aras*:*argB* fragment. We did not find any yellow Arg^+ colonies that produced only one band corresponding to the 6.2-kb disrupted fragment. The fact that we obtained 20 haploids containing only the endogenous *Aras* gene but no haploids with only the disrupted Δ*Aras*:*argB* gene suggests that *Aras* is an essential gene at some point in the life cycle (see Discussion). Although all the isolates carried *pabaA1*, none of them were auxotrophic for methionine, suggesting that they might be diploids. In order to confirm that these yellow Arg^+ strains were in fact diploids, we determined the sizes of the spores of both the parental haploid and diploid strains and the yellow progeny (diploid spores are bigger than haploid spores). For accuracy, the sizes were measured on a television screen with the microscopic image enlarged. The diameters of both of the parental haploid strains and all of the Arg^- yellow strains were between 10 and 11 mm, while those of the parental diploids and all of the yellow Arg^+ strains were between 13 and 15 mm (10 spores of each strain were measured). This indicates that all of the yellow Arg^+ strains were probably diploids.

Expression of *Aras* under alcohol dehydrogenase promoter control can be varied to different levels by different inducers. Two different types of Ras mutants have been described for yeast and mammalian systems: constitutively active mutants and dominant-negative mutants. When expressed, these Ras mutants exert effects that are dependent on the particular cell type. In order to determine the role of A-Ras, we expressed the *Aras* gene in *A. nidulans* under control of the promoter of the

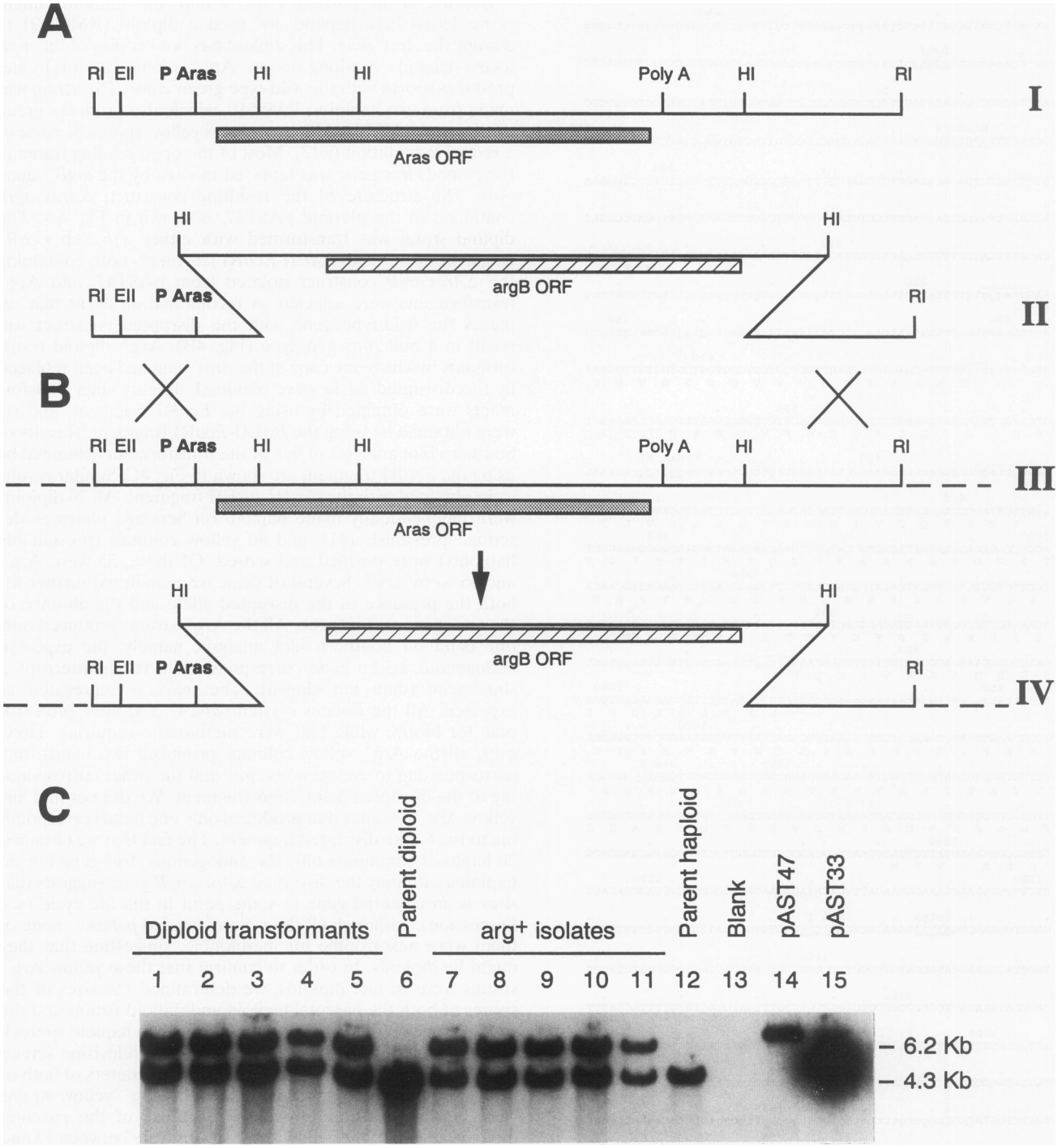

FIG. 4. Disruption of the *Aras* gene. (A) In vitro construct used to replace the *Aras* open reading frame with the selectable marker *argB*. I, A 4.3-kb *Eco*RI fragment containing genomic *Aras*; II, two *Bam*HI fragments containing most of *Aras* were replaced by a *Bam*HI fragment containing *argB* (Δ*Aras*:*argB* in pAST47). (B) In vivo replacement of the *Aras* gene with Δ*Aras*:*argB* converts an arginine auxotroph to a prototroph. III, *Aras* in chromosome 8; IV, structure of the disrupted genome. (C) Southern blot analysis of Δ*Aras*:*argB* diploids and haploids. When probed with *Aras*, the *Eco*RI-digested chromosomal DNA from the parental haploid (RMS011) and diploid (RMS012) strains produced the 4.3-kb endogenous *Aras* band. The Arg^+ diploid transformants and the Arg^+ isolates produced both 4.3-kb endogenous and 6.2-kb disrupted Ras bands. RI, *Eco*RI; EII, *Bst*EII; HI, *Bam*HI; P Aras, *Aras* transcription start site; Poly A, poly(A) addition signal. The *Aras* open reading frame depicted here contains four exons (see Fig. 3).

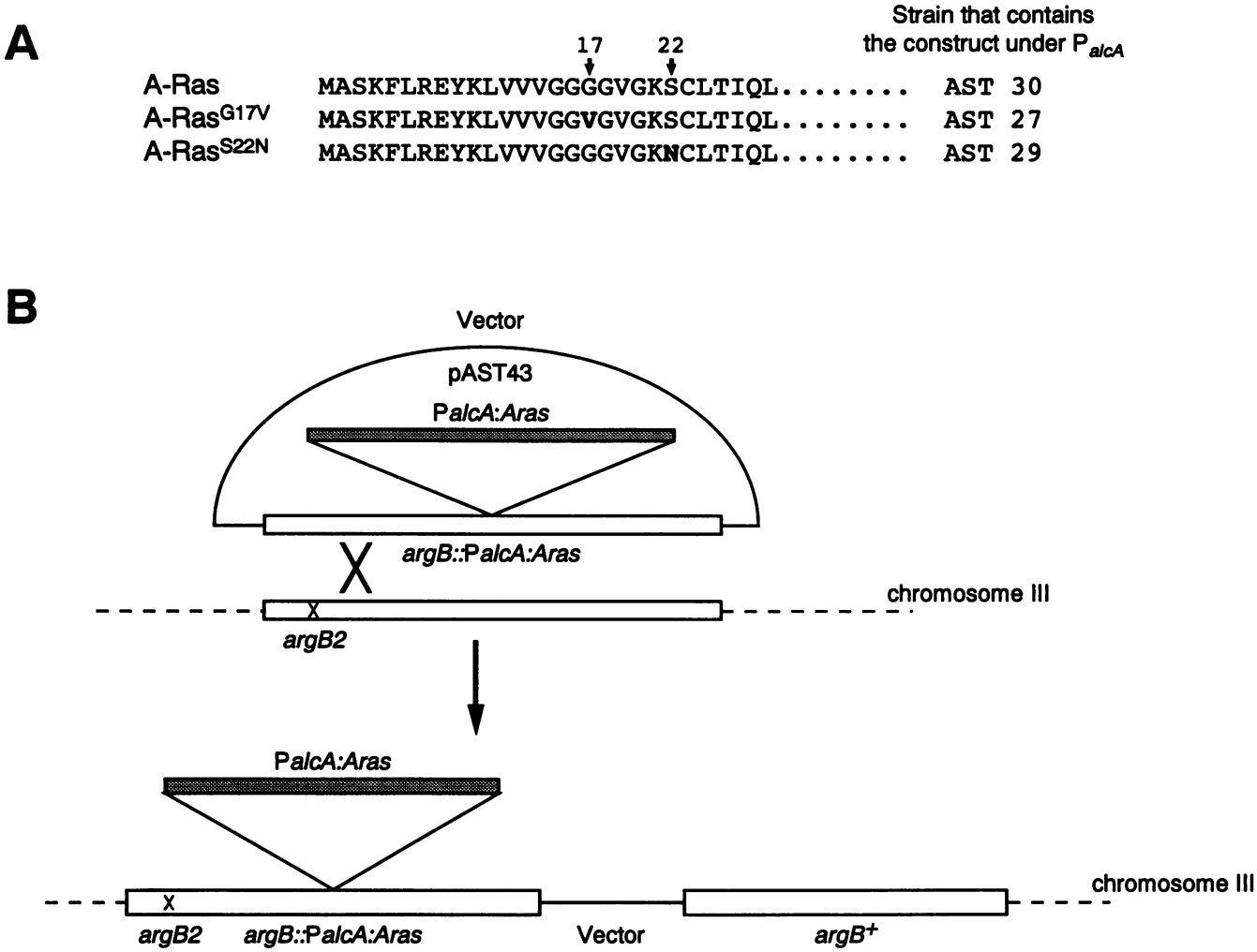

FIG. 5. Integration of P_{alcA}:*Aras* constructs at the *argB2* locus. (A) Sequences of the wild-type A-Ras and A-Ras mutants and the strains containing the corresponding constructs under *alcA* promoter control. (B) Generation of arginine prototrophs (*argB*$^+$) by integration of pAST43, containing the P_{alcA}:*Aras* construct in the *argB* gene, at the *argB2* locus in chromosome 3.

alcohol dehydrogenase gene (P_{alcA}) of *A. nidulans* (21). This inducible promoter has been used in *A. nidulans* to express several heterologous genes (25, 45). One of the useful properties of this promoter is that it can be induced to different levels by different inducers (9). We first made a P_{alcA}:c*Aras* hybrid and introduced it into *A. nidulans* at the *argB* locus. For this purpose the hybrid gene was cloned in vitro into the middle of the *argB* open reading frame (Fig. 5B). The entire construct was used to transform an *argB2* strain, and non-arginine-requiring strains were selected and tested for the integration event. An arginine prototroph can be generated by integration of the in vitro construct at a specific region in the *argB2* locus (Fig. 5B). Southern blot analysis of these transformants identified several strains in which such an event had occurred. All these strains behaved similarly upon further analysis; one of the strains, AST30 (Fig. 5A), is described here.

With different growth conditions in liquid culture, the level of expression of the *alcA:Aras* hybrid message from the P_{alcA} promoter in strain AST30 was first estimated by using an RNase protection assay (22, 39). The RNA probe was made from the junction of the *alcA*:c*Aras* hybrid gene. This probe detected the hybrid *alcA:Aras* message as well as the endogenous *Aras* and *alcA* messages, producing different lengths of protected fragments (Fig. 6A). When mycelia were grown in medium containing yeast extract and glucose (YG) or fructose (YF), no expression from the *alcA* promoter was observed (repressing conditions; Fig. 6B, lanes 4 and 5). Expression also was not detected when the cultures were first grown in YG and then induced for 3 h in medium containing yeast extract and ethanol (YE) or threonine (YT) (Fig. 6B, lanes 6 and 7). However, an increased level of expression was observed in mycelia induced overnight in similar media (intermediate inducing conditions; Fig. 6B, lanes 8 and 9). In minimal medium containing glucose (MMG) or fructose (MMF), a very low level of expression was observed (low inducing conditions; Fig. 6B, lanes 12 and 13). In contrast, a very high level of expression was observed in cultures that were first grown in MMG and then induced for 3 h in minimal medium containing either ethanol (MME) or threonine (MMT) (high inducing conditions; Fig. 6B, lanes 14 and 15). An increased level of the corresponding protein was detected by Western blot analysis of cultures grown under high inducing conditions (data not shown).

Overexpression of wild-type A-Ras has no apparent phenotypic effect. The effects of overexpression of wild-type A-Ras on cell growth and morphology were determined in liquid and on solid medium. No changes in cellular morphology or growth rate were observed when AST30 (containing P_{alcA}:*Aras*, which overexpresses wild-type A-Ras upon induction) was grown either in liquid or on solid medium under either repressing or inducing conditions, compared with the control strain AST33, an arginine prototroph of the recipient strain FGSC89 (not containing a P_{alcA} construct). The times required for spores to germinate and to induce different cell types were similar in the two strains in several different media, although these times varied from one medium to another. This suggests that the amount of A-Ras present is not limiting at any stage of the *A. nidulans* life cycle, indicating that *Aras* activity is not regulated at the level of expression, at least with regard to its role in regulating the developmental pathway (also see below).

Development is inhibited by expression of active A-Ras. Ras is a small G protein that can bind either GDP or GTP. It has been shown that the GTP-bound form of Ras is the active form in signal transduction. In order to determine the role of *Aras* in the life cycle of *A. nidulans*, a constitutively active mutant of A-Ras that is locked in the GTP-bound form was expressed in vivo. A mutation which changes a Gly to Val at position 17 was introduced in vitro in the *Aras* gene ($Aras^{G17V}$; Fig. 5A). Such a mutation in the corresponding position (amino acid 12 from the N terminus) of v-*Hras* abolishes its internal GTPase activity and confers dominant effects through constitutive activity. The lack of GAP-sensitive GTPase activity of $A\text{-}Ras^{G17V}$ was confirmed in vitro (Table 2).

When AST27 (containing P_{alcA}:$Aras^{G17V}$) was grown in liquid medium under either repressing conditions or low inducing conditions, no change in growth rate was observed (data not shown). In addition, the appearance of cells in these cultures was similar to that of the control cells (AST30 and AST33). However, under intermediate inducing conditions, the rate of growth of AST27 was inhibited after 12 h compared with that of the control cells under similar growth conditions (Fig. 7). Before 12 h, both growth rates and rates of nuclear division were normal (see Materials and Methods). Although the spores swelled to the same extent as those of the control strains and the time of germination was unaltered, most of the AST27 mycelia, while normal up to 12 h, subsequently became much wider and shorter, with large vacuoles (Fig. 8a and c). The cell walls also appeared thicker, and the septa between cells were prominent. These cells stained more heavily than normal for chitin, indicating either an increased presence of chitin in the cell wall or a decreased presence of glucan, which is known to cause increased chitin staining (Fig. 8b) (4). Most of these cells had up to 5 to 10 times as many nuclei as the control strains after 30 h, as determined by staining with DAPI (Fig. 8c and d). The control strains have the normal number of two (in some cells up to eight nuclei [33]) per hyphal cell throughout mycelial growth. This suggests that an intermediate level of active A-Ras hinders cell division but does not inhibit nuclear division. Only a few such cells arose when AST27 was grown under low inducing conditions or repressing conditions or when the control cells were grown under any conditions.

When AST27 was grown under high inducing conditions, the spores swelled excessively and, depending upon the length of growth, become about 5- to 20-fold larger than a swollen wild-type spore (we call such spores giant swollen spores) (Fig. 8e). None of the giant swollen spores were found to produce germ tubes even after 48 h. During the swelling stage, the nucleus of a wild-type spore divides to produce two or three nuclei. When the germ tube appears, continuing nuclear

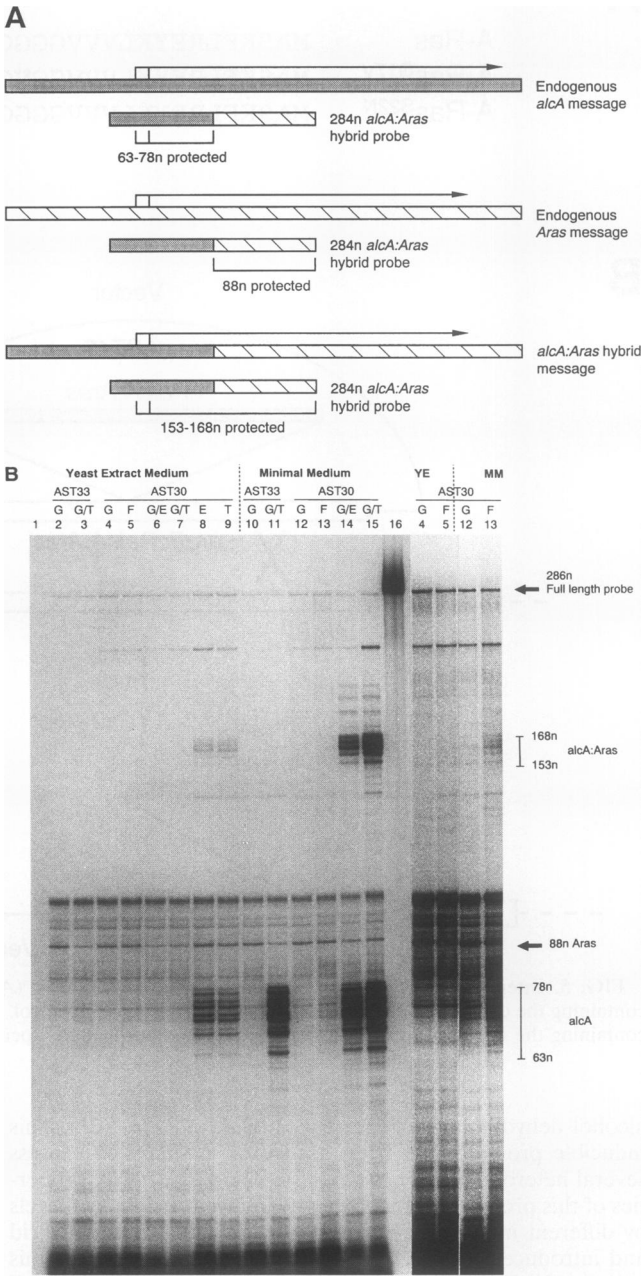

FIG. 6. Levels of induction of the *alcA* promoter as estimated by RNase protection. (A) Structure of the probe used and the expected protected fragment lengths. (B) Protected fragments separated on a 5% sequencing gel. Lane 1, probe only; lane, 16, full-length probe. Spores were grown for 19 h on yeast extract containing glucose (G) (lanes 2 and 4), fructose (F) (lane 5), ethanol (E) (lane 8), or threonine (T) (lane 9) or for 16 h on glucose and then for 3 h on ethanol (lane 6) or threonine (lanes 3 and 7). Cells were grown for 40 h on minimal medium containing glucose (lanes 10 and 12) or fructose (lane 13) or for 37 h on minimal glucose and then for 3 h on minimal ethanol (lane 14) or minimal threonine (lanes 11 and 15). RNA was extracted, and RNase protection was performed as described in Materials and Methods. The presence of several bands indicates multiple initiation sites of *alcA* transcripts. The four lanes on the right are longer exposures of the indicated lanes on the left. n, nucleotides.

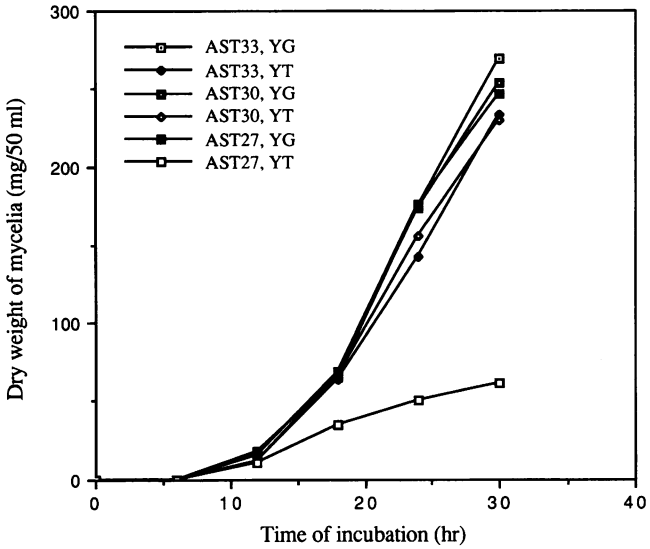

FIG. 7. Intermediate levels of expression of A-Ras^{G17V} inhibit growth. Medium was inoculated with 1.5×10^5 spores per ml, samples were taken at the indicated times, and dry weight was determined. AST33 contains no P_{alcA} construct, AST30 contains P_{alcA}:*Aras*, and AST27 contains P_{alcA}:*Aras*^{G17V}.

division produces more nuclei, which become distributed into the newly formed cells produced by apical growth of the germ tube (Fig. 8f and g). In giant swollen spores, nuclear division continues without germ tube formation, giving rise to cells with at least 16 nuclei after 48 h, as determined by DAPI staining (Fig. 8h and i). The rate of nuclear division appeared to be normal, at least until 12 h (see Materials and Methods). Thus, a high level of active A-Ras inhibits the first step of development, the initiation of apical growth (germ tube formation), without inhibiting nuclear division. These giant swollen spores stain heavily for chitin, and the staining occurs all around the periphery. A wild-type swollen spore stains very lightly for chitin (data not shown).

The effects of overexpression of active A-Ras on development were further assessed by growing AST27 on solid medium under different inducing conditions. Wild-type spores grown on solid medium swell and sequentially produce germ tubes, vegetative hyphae, aerial hyphae, and conidiophore structures (Fig. 1). When spores from control strains were plated on agar medium and grown under either repressing or inducing conditions, their developmental programs were normal. However, when plated on ethanol plates, they produced larger quantities of aerial hyphae, which is characteristic of growth on ethanol plates. Even with these larger quantities of aerial hyphae, many conidiophore structures were visible microscopically after 36 h, buried under the aerial hyphae. When AST27 was plated under repressing conditions, both its growth rate and developmental program were indistinguishable from those of control strains. However, when AST27 was grown on

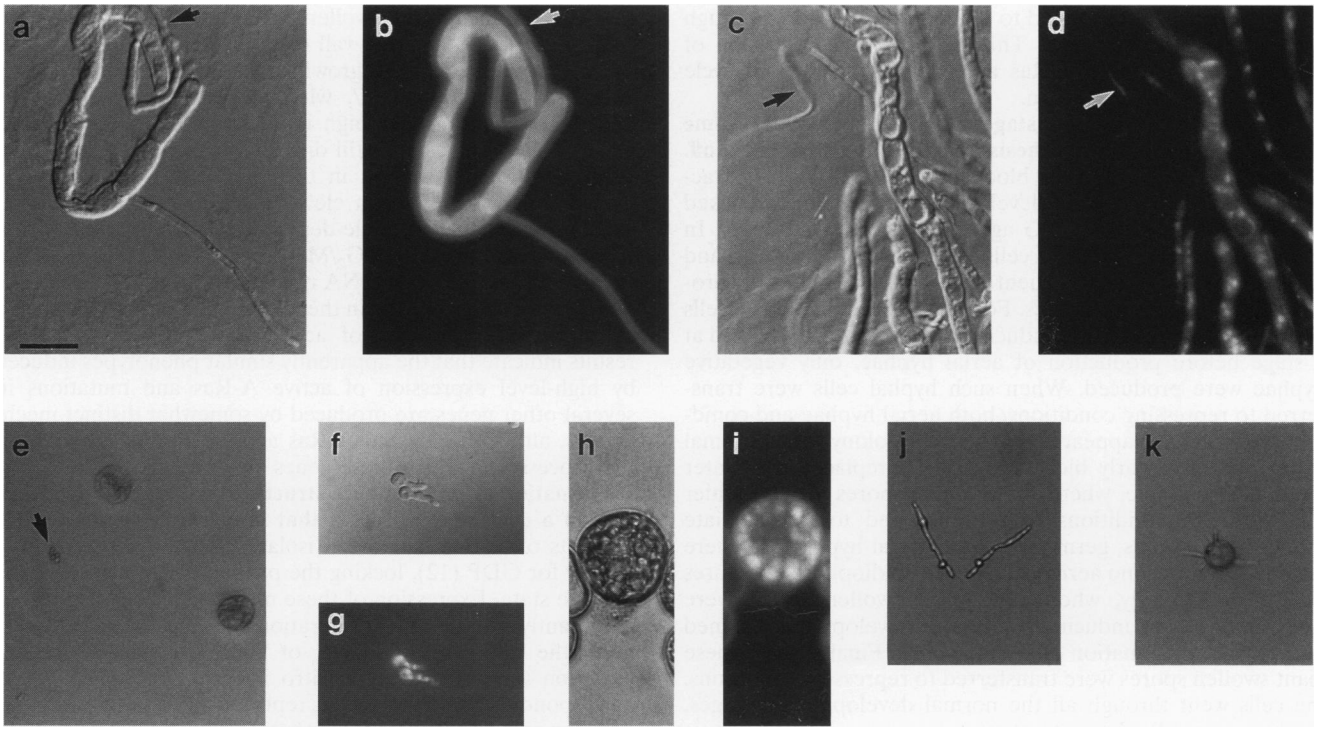

FIG. 8. Phenotypes resulting from expression of constitutively active A-Ras. (a to d) AST33 (➡) and AST27 mycelia grown under intermediate inducing conditions (YT) and mixed for comparison: a and c, Nomarski; b, Calcofluor; d, DAPI. (e) AST33 and AST27 grown under high inducing conditions (MMT) and mixed for comparison, producing normal swollen spores (➡) and giant swollen spores, respectively. (f and g) AST33 spores with germ tubes: f, Nomarski; g, DAPI. (h and i) Giant swollen spores: h, Nomarski; i, DAPI. (j) AST33 cells swollen under high inducing conditions (MMT) and transferred to repressing conditions (YG) produce a second germ tube when the first one is relatively long. Similar results were obtained with AST30. (k) Giant swollen spores produce multiple germ tubes when transferred to repressing conditions (YG). Bar: a through d, 6 μm; e, j, and k, 12 μm; f through i, 1.25 μm.

MMG plates (low inducing conditions), only aerial hyphae were produced (Fig. 9; the strain AST29 presented in this figure is described below). No conidiophore structures were found even after 3 days of incubation. In contrast, only vegetative growth was observed when AST27 was grown on YE or YT plates (intermediate inducing conditions; Fig. 9). Only a few aerial hyphae appeared after 3 days. Under all of these conditions, the germination time and growth rate of AST27 remained unchanged, as did those of both of the control strains, as determined by the diameters of single colonies (data not shown), although the growth appeared thinner under intermediate inducing conditions (Fig. 9). When observed under the microscope, the AST27 cells grown under intermediate inducing conditions looked similar to ones grown in liquid under intermediate inducing conditions. Compared with the control cells, these cells were shorter and wider with thick cell walls, were highly septate, and contained large vacuoles.

On MME or MMT agar plates (high inducing conditions), giant swollen spores that failed to germinate were produced, similar to the phenotype observed when AST27 is grown in liquid under high inducing conditions, except that the swollen spores were much larger on the agar plates. These spores also contained a large number of nuclei as visualized by DAPI staining. No change in phenotype was observed in the control strains (AST30 and AST33) under these growth conditions (Fig. 9).

Expression of active A-Ras at any stage of the life cycle blocks further development. In order to test the effect of overexpression of active A-Ras at different stages of the life cycle, AST27 was grown on YG agar (repressing conditions), and cells were allowed to grow to different stages of development. Upon transfer (27) to YT agar (intermediate inducing conditions), these cells failed to differentiate further, although mycelial growth continued. This indicates that induction of higher levels of active A-Ras at any stage of the life cycle blocks further differentiation.

Cells blocked at different stages of development can resume normal development if synthesis of active A-Ras is turned off. In order to determine if the block induced by A-Ras hyperactivity at different stages of development is reversible, blocked cells were transferred to YG agar (repressing conditions). In every instance the blocked cells were able to reinitiate and traverse the normal subsequent developmental stages to produce conidiophore structures. For example, when AST27 cells grown under intermediate inducing conditions were blocked at a stage before production of aerial hyphae, only vegetative hyphae were produced. When such hyphal cells were transferred to repressing conditions, both aerial hyphae and conidiophore structures appeared, producing a colony with a normal appearance. An early block can also be replaced by a later block. For example, when giant swollen spores formed under high inducing conditions were transferred to intermediate inducing conditions, germ tubes and lateral hyphal cells were produced, but still no aerial hyphae or conidiophore structures appeared. Similarly, when these giant swollen spores were transferred to low inducing conditions, development resumed but only up to formation of aerial hyphae. Finally, when these giant swollen spores were transferred to repressing conditions, the cells went through all the normal developmental stages, producing conidiophore structures.

The time taken to initiate formation of hyphae in a giant swollen spore was compared with that for the control strains. When the control strains AST30 and AST33 were grown in liquid medium under high inducing conditions, germ tubes appeared in about 11 h. Spores of the control strains and of AST27 were grown under high inducing conditions for 10 h and then transferred to YG medium (repressing conditions). In the control strains, germ tubes appeared in about 1 h, while AST27 took about 5 h to produce germ tubes. This suggests that the inability to produce a germ tube from a giant swollen spore is not a block that can be reversed immediately. Rather, additional time is necessary either to deplete the repressor (lower the level of active A-Ras) or to synthesize a positive factor(s) necessary for germ tube formation, synthesis of which was inhibited under such growth conditions. During germination, most of the spores of the control strains initially produced only one germ tube. Only after the first germ tube had grown to at least 10 times the diameter of the spore did a second germ tube appear, this one directly across the spore from the first germ tube (Fig. 8i). However, the giant swollen spores of AST27 produced multiple germ tubes simultaneously (Fig. 8j). During germination, a wild-type spore retains most of its cell wall components and produces one germ tube at the pole. The giant swollen spores of AST27 probably have altered amounts of some cell wall components, as discussed above, judging by the intensity of chitin-specific staining, possibly resulting in a loss of polarity that causes the simultaneous initiation of multiple germ tubes.

The phenotype caused by high-level expression of active A-Ras is different from that caused by defects in other genes. Several mutations that affect cell wall biosynthesis also cause production of giant swollen spores. Conditional mutations in four different genes, *tsE*, *orlA*, *orlB*, and *gcnA*, cause production of low levels of chitin and giant swollen spores at an elevated temperature (5). As mentioned above, we have found that the giant swollen spores produced by high-level expression of active A-Ras in fact show a higher level of chitin in immunofluorescence studies. On the other hand, *orlC* and *orlD* mutants produce giant swollen spores that are deficient in β-1,3-glucan, another cell wall component (5). However, this defect can be overcome by growing the spores in an osmotically balanced medium. AST27, when grown in an osmotically balanced medium under high inducing conditions, produced giant swollen spores and still did not germinate.

A conditional mutation in the *bimG* gene also produces giant swollen spores at an elevated temperature. The *bimG* gene product is a cell cycle-dependent phosphatase that becomes active during the G_2/M transition (10). However, in *bimG* mutants, although DNA replication proceeds, the nuclei do not separate as they do in the giant swollen spores produced by high-level expression of active A-Ras (Fig. 8h). These results indicate that the apparently similar phenotypes induced by high-level expression of active A-Ras and mutations in several other genes are produced by somewhat distinct mechanisms, although elevated A-Ras activity may offset some of the processes in which these genes are involved.

Formation of conidiophore structures is induced by expression of a derivative of A-Ras that preferentially binds GDP. Mutants of p21ras have been isolated that have preferential affinity for GDP (12), locking the protein constitutively in the inactive state. Expression of these mutants has been shown to dominantly inhibit cell proliferation (40). In order to determine the effect of inhibition of A-Ras activity, a similar mutation was introduced in vitro into the *Aras* gene in the corresponding position. Ser was replaced by Asn at position 22, corresponding to position 17 of p21 (Fig. 5A). The mutant gene was put under control of P_{alcA} and introduced into *A. nidulans* at the *argB* locus by using the strategy described above (Fig. 5B). Two strains were obtained, both of which behaved similarly; strain AST29 is discussed.

When grown in liquid for 30 h, AST29 and the control strains (AST30 and AST33) were indistinguishable regardless

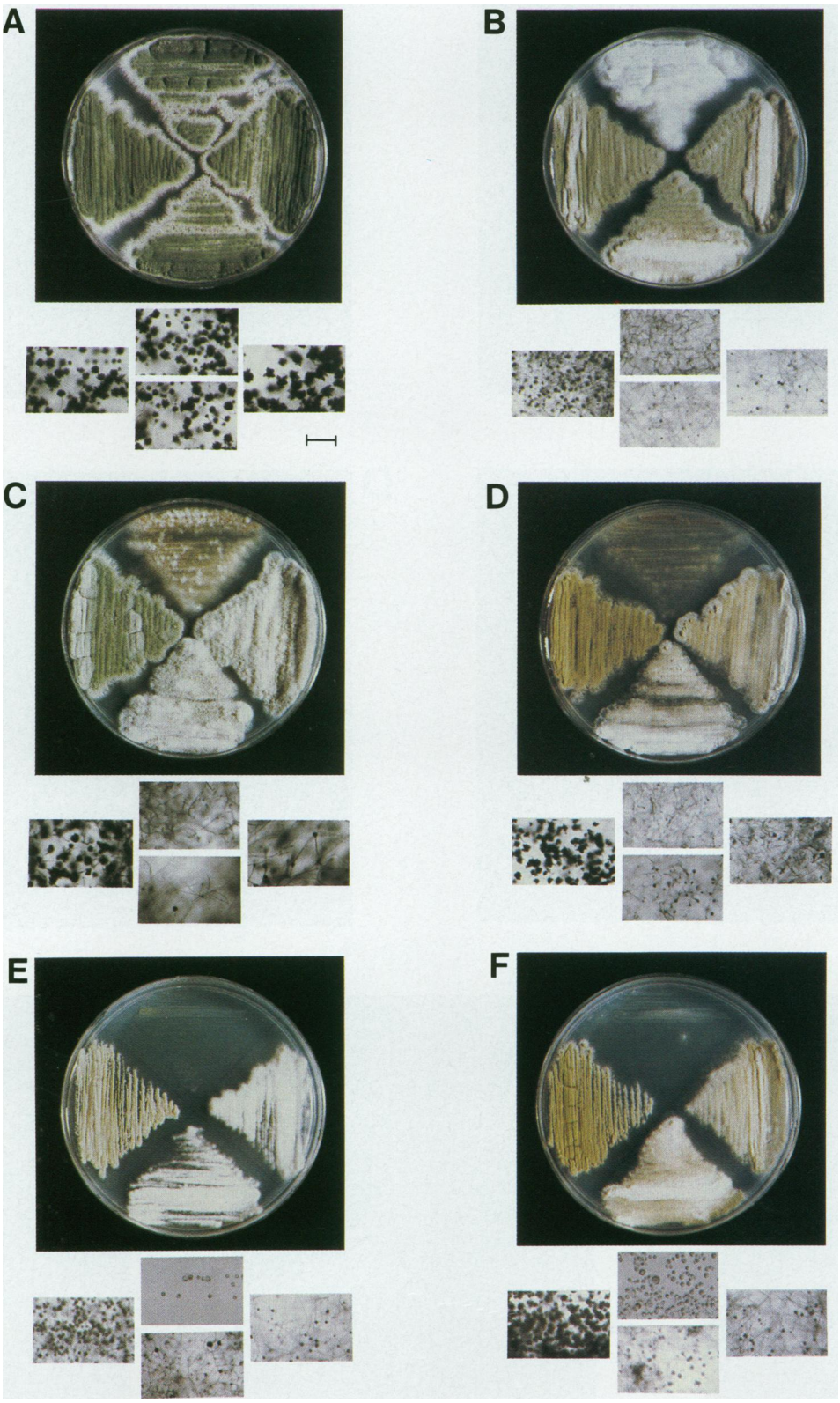

FIG. 9. Growth of *Aspergillus* strains on agar under different inducing conditions. (A) YG (repressing conditions); (B) MMG (low inducing conditions); (C and D) YE and YT, respectively (intermediate inducing conditions); (E and F) MME and MMT, respectively (high inducing conditions). On each plate the strains streaked are as follows: top, AST27 (containing P_{alcA}:*Aras*^{G17V}); left, AST29 (containing P_{alcA}:*Aras*^{S22N}); bottom, AST30 (containing P_{alcA}:*Aras*); right, AST33 (an arginine prototroph of the host strain, FGSC89). A close-up of each patch is shown at the corresponding position at the bottom of the plate. The green color is produced by conidia. Bar, 40 μm.

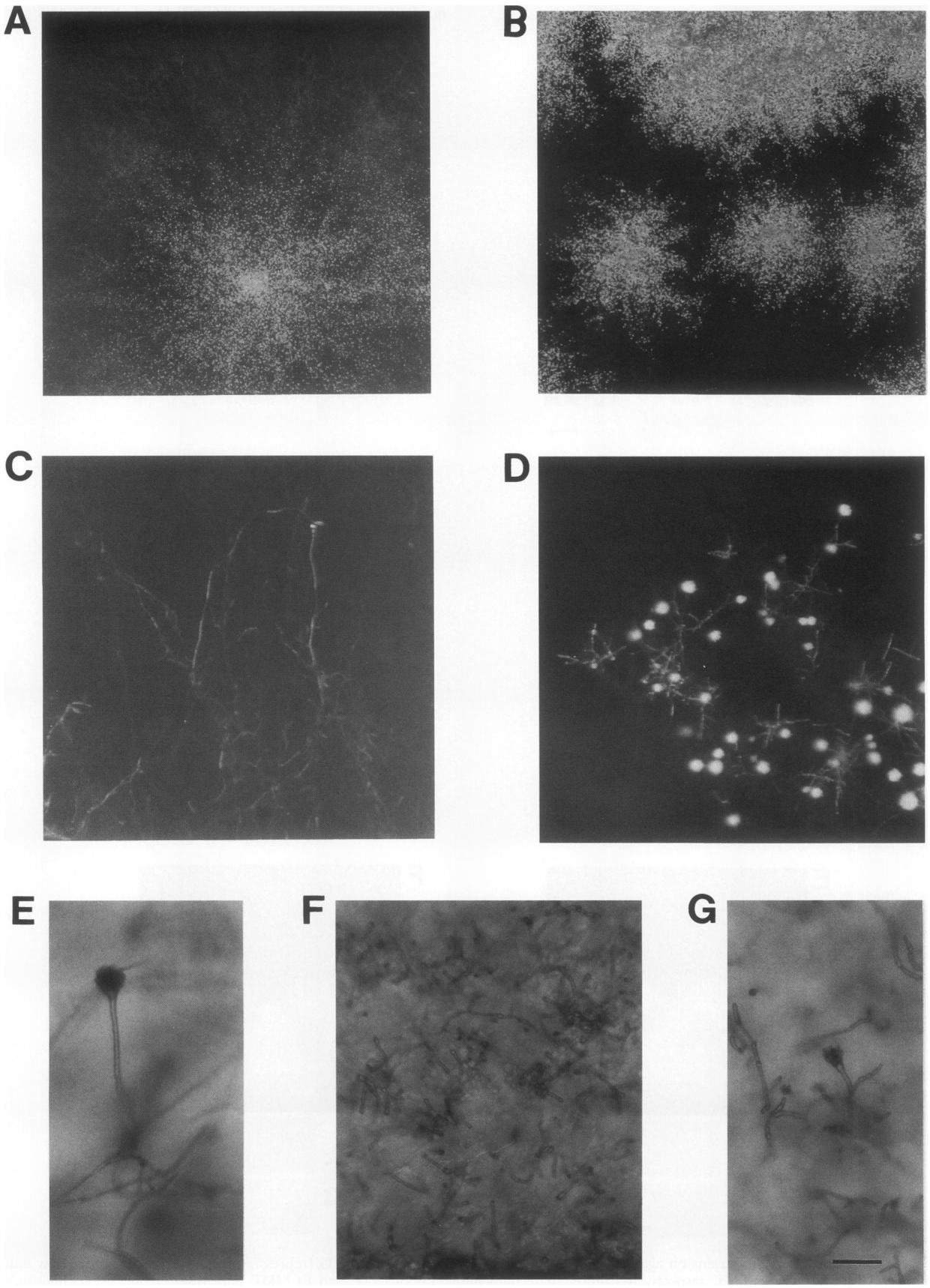

FIG. 10. Phenotype due to expression of dominant-negative A-Ras. Growth was on MMT (high inducing conditions). (A and B) Colony size: A, AST30; B, AST29. (C and D) Edge of the colony: C, AST30; D, AST29. (E) Conidiophore of AST30. (F and G) Stem, vesicle, and conidiophore of AST29. Bar, C and D, 50 μm; E through G, 12 μm.

of growth conditions, except that AST29 took longer to germinate (14 h under high inducing conditions as opposed to 11 h for AST30 and AST33 under similar growth conditions). To assess the effects of overexpression of GDP-bound mutant A-Ras on development beyond formation of vegetative hyphae, AST29 was grown on solid agar medium. When AST29 was grown under repressing conditions, its program of development was identical to that of the control strains (Fig. 9A). However, when grown under either low or intermediate inducing conditions, this strain produced more conidiophores than normal, with a conspicuous absence of aerial hyphae (Fig. 9B and C). The number of conidiophore structures was increased about three- to fourfold under intermediate inducing conditions. This was determined by counting the conidiophores in a microscopic field and comparing this number with the number of conidiophores appearing when the same strain was grown under repressing conditions. This result was even more pronounced when AST29 was grown on MME agar (high inducing conditions) (Fig. 9E). The two control strains produced large amounts of aerial hyphae, characteristic of a wild-type strain on ethanol plates. In contrast, AST29 produced very few if any aerial hyphae on such a plate (see enlarged picture in Fig. 9). Thus, this dominant-negative phenotype is the opposite of that produced by the constitutively active mutant. This result suggests that the formations of these structures are mutually exclusive and that aerial hyphae probably are the precursors of conidiophores. An aerial hypha normally initiates from a vegetative hypha and grows to a definite length. At that point it may either continue growth as an aerial hypha or differentiate into a vesicle. Our results suggest that this is determined by the level of active A-Ras protein. When this level is higher than a critical value, protruding hyphae continue growth as aerial hyphae (as in the case of low-level expression of constitutively active A-Ras protein [Fig. 9B]). Conversely, when this level falls below a critical value, the dividing cell at the tip of the hypha differentiates into a vesicle and follows the pathway toward conidiophore formation (as in the case of low- or medium-level expression of dominant-negative A-Ras protein [Fig. 9B, C, and D]).

An increased number of conidiophore structures appeared with only a small increase in expression of the dominant-negative mutant. This suggests that small changes in the level of active A-Ras at the aerial hyphal tip are sufficient to control the decision to differentiate.

Expression of dominant-negative A-Ras protein alters the timing but not the sequence of the program of development. The program of development in *A. nidulans* is stringently controlled, so that in a wild-type cell, different cell types appear at specific times of the life cycle. When the control strains (AST30 and AST33) were grown on minimal agar under high inducing conditions, germ tubes appeared in about 11 h, aerial hyphae appeared after an additional 4 h (in about 15 h), and vesicles appeared after another 8 h (in about 23 h). New spores appeared in about 30 h. Although strain AST29 grown under the same conditions (capable of overproducing the dominant-negative mutant) produced all the cell types except undifferentiated aerial hyphae, the times of appearance of each cell type differed. Germ tubes appeared later than usual, in 14 h. Once the germ tubes were formed, most other cell types appeared sooner than in the control cells. Aerial hyphae (all of which differentiated into conidiophores) appeared within 2 h (total, 16 h). The vesicles appeared in another 4 h (total, 20 h), 4 h earlier than usual. The spores appeared in 24 h. In addition, aerial hyphae formed on shorter vegetative hyphae and at a higher density along the hyphae. Similarly, vesicles appeared on shorter aerial hyphae (Fig. 10; compare panel E with panels F and G). As a result, AST29 colonies are much smaller than those of the control strains (e.g., 6 mm in diameter at 36 h on an MMT plate [high inducing conditions] compared with 18 to 20 mm for AST30 [Fig. 10; compare panels A and B]). The edge of a control colony consists of mycelium and aerial hyphae, while that of AST29 contains conidiophores but no aerial hyphae (Fig. 10; compare panels C and D). This suggests that in the presence of excess dominant-negative A-Ras protein, germination is inhibited, while further differentiation is accelerated, but the sequence of appearance of cell types is not altered. The delay in germ tube formation without the inhibition of growth observed with the dominant-negative mutant indicates that this mutant does not completely block formation and function of active A-Ras from the endogenous *Aras* gene. Nevertheless, the delay in germ tube formation indicates that during germination, the concentration of active A-Ras normally reaches a threshold level that triggers germ tube formation. After germination, expression of the dominant-negative mutant reduces the time required for subsequent differentiation, suggesting that the concentration of active A-Ras must fall before further differentiation (germ tube formation) can occur. It should be noted that since the concentration of inducer in the conidiophore cannot be independently determined, we have assumed that these compounds can freely move throughout the reproductive structures and therefore are present at concentrations similar to those in vegetative hyphae.

As previously mentioned, AST29 does not produce conidiophores or spores in liquid cultures even under high inducing conditions. This contrasts with results with *brlA* (the "master control gene" for conidiophore formation), which, when overexpressed, causes spore production even in liquid culture (1). Since *brlA* affects only the final stages of development, it appears to be a downstream factor that implements one of the determinations affected by A-Ras activity. These results suggest that other factors specific for each cell type are involved downstream of A-Ras and are responsible for the ordered appearance of the cell types.

A-Ras is expressed in all cell types. We have shown that expression of dominant-negative A-Ras protein, which causes a reduction in the levels of active A-Ras, induces development. For example, it accelerates the timing of conidiophore formation. Since GDP-bound Ras has generally been found to be functionally inactive, a sequential reduction in the level of active A-Ras probably occurs during the normal life cycle of the fungus. This could result from a reduction in the level of expression of the *Aras* gene or from subsequent regulatory steps or both. As a first step in testing these possibilities, we determined whether the *Aras* gene is expressed at all stages of the life cycle, particularly in the conidiophore structure, where the lowest level of active *Aras* is required.

In order to determine whether the level of expression of the gene is regulated during development, we placed the bacterial *lacZ* gene under control of the *Aras* promoter, introduced the construct into *A. nidulans*, and examined the levels of β-galactosidase protein in different cell types. For these studies, the bacterial *lacZ* gene was fused in frame to the *Aras* open reading frame. The resulting hybrid contains about 1.3 kb of *Aras* upstream region (from the transcription start site, as shown in Fig. 3). This region should contain the complete *Aras* promoter, because *Aspergillus* promoters are normally contained within several hundred bases of the transcription start site. This hybrid construct was then cloned into the middle of the *argB* gene and integrated at the *argB* locus by using the strategy described in Fig. 5B. The resulting strain (AST32) was tested for the expression of β-galactosidase in different cell types during development. Expression of β-galactosidase was

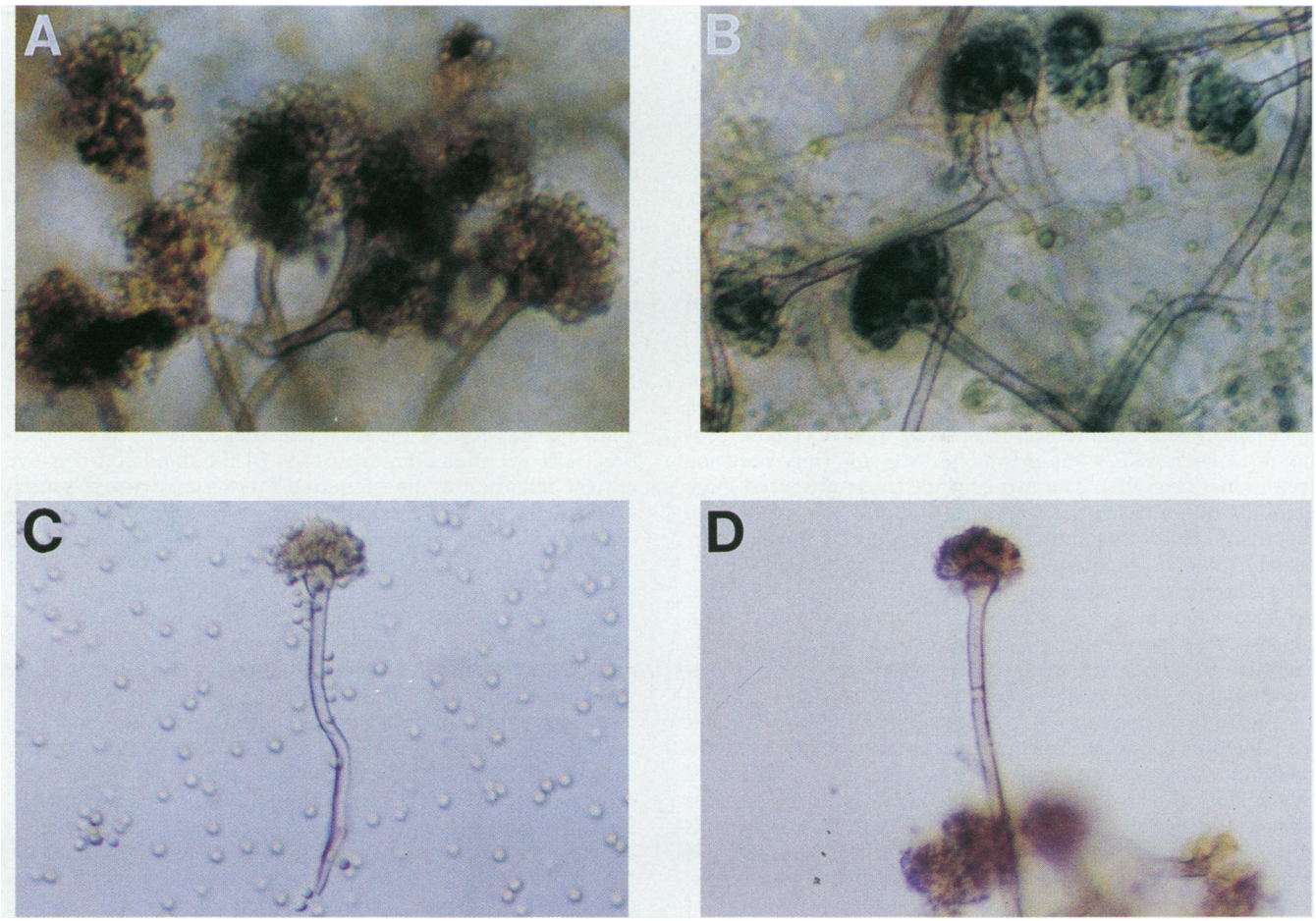

FIG. 11. In vivo expression of *Aras*. Cultures were grown and color was developed on solid medium. (A and B) *Aras* expression monitored in an *Aras:lacZ* hybrid. Color was developed as described previously (2, 24). (A) AST33; (B) AST32. (C and D) *Aras* expression monitored by immunofluorescence in FGSC4 without (C) and with (D) Ras antibody Y13-259.

first determined by assaying the enzyme activity (39) in extracts of mycelia grown in liquid culture (where *A. nidulans* grows only vegetatively). A high level of β-galactosidase activity in AST32 (0.0025 U/min/μg of protein) compared with that in the control strain AST33 (0 U) indicated successful expression of β-galactosidase driven by *Aras* in mycelia. Expression from the *Aras* promoter in conidiophores was determined by spraying 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) on colonies grown on agar plates as described previously (2, 24). As shown in Fig. 11B, development of a deep blue color in all parts of conidiophores of AST32 indicated expression of *Aras* in every cell type. Similar results were obtained when the Ras antibody Y13-259 (13) was used to stain wild-type *A. nidulans* FGSC4 (Fig. 11). We have shown above that overexpression of wild-type A-Ras has no phenotypic effect. These results, taken together, suggest that A-Ras activity is not regulated by the level of A-Ras expression but is probably regulated solely at the posttranslational level, as in other systems.

DISCUSSION

Ras has been shown to be involved in many different signaling pathways in different mammalian cell types. In *S. cerevisiae*, it is essential for growth (19); in *D. melanogaster*, it is involved in the development of the adult eye (38), as well as of terminal structures of the embryo (30); while in *C. elegans* (17), it is involved in vulval development. However, little is known about its role in determining the progression through different developmental stages in an organism. For this purpose, we have used *A. nidulans* as a model system. In this paper we have shown that A-Ras has two different roles in the life cycle of this organism: an essential role and a regulatory role. In the absence of a functional gene, the organism fails to propagate, while the decision to differentiate at each stage of development is affected by the level of active A-Ras. With a progressive increase in the level of constitutively active A-Ras, *Aspergillus* cells go through progressively fewer steps of their normal developmental program. At the highest level of expression, cells fail to initiate the first morphological step in development, production of a germ tube. Instead, the cells swell and continue nuclear division, producing giant swollen spores. However, when this high level of active A-Ras is allowed to fall to the normal level (maintained by the endogenous *Aras*) by transfer of the giant swollen spores to an appropriate medium, normal growth and development resume. Similarly, when active A-Ras is expressed at an intermediate level, cells are blocked in vegetative growth, but they can resume further development when transferred to repressing conditions. This suggests that particular levels of active A-Ras allow development to proceed to certain points while inhibiting

further development. This led us to the following model. The spores are in G_0 of the cell cycle. When placed in a growth medium, the concentration of the active A-Ras increases to a high level, which is necessary for nuclear division, nuclear migration, and swelling but which keeps a check on cell division. During this time, germ tube formation and subsequent steps are inhibited while all the components necessary for germination are synthesized. As the concentration of active A-Ras falls below a critical level, inhibition of germ tube formation is relieved. At this intermediate concentration, active A-Ras still inhibits formation of aerial hyphae, but vegetative growth can continue. In certain mycelial cells the concentration of active A-Ras drops further, below another critical level, allowing foot cell and aerial hypha formation but inhibiting vesicle formation. Thus, the concentration of active A-Ras continues to drop until spores are formed.

This model is further supported by the phenotype observed with expression of a dominant-negative A-Ras mutant. By analogy with a similar mutant of $p21^{ras}$, expression of this protein inhibits the activation of wild-type A-Ras. Expression of this dominant-negative mutant results in a delay in germination, consistent with our proposal that the highest level of active A-Ras is essential to turn on germination-specific processes. These processes might include either nuclear division or the synthesis of components necessary for apical extension, or both. However, since nuclear division occurs normally at much lower levels of active A-Ras later in development, other processes that are germination specific are more likely to be those driven by a high Ras signal. This notion is consistent with the fact that a high level of constitutively active A-Ras, while preventing germination, does not accelerate nuclear division. Thus, it seems likely that an early process other than nuclear division requires a very strong Ras signal, but the signal must also drop to some extent to allow the completion of germination. Once germination is complete, one of functions of the Ras signal is to act as a brake on developmental progression, since expression of the dominant-negative mutant accelerates subsequent steps of differentiation.

Our disruption experiment shows that *Aras* is essential at some stage of the life cycle, while overexpression of the dominant-negative protein only delays germination. This suggests that this mutant protein does not block A-Ras function completely and that a complete block might prevent germination entirely. Following germination, a complete block might have one of three distinct consequences. It might simply accelerate developmental progression more than the dominant-negative mutant. Alternatively, a complete loss of *Aras* function at later stages might disrupt the normal ordered appearance of cell types or stop growth completely. We are currently testing these possibilities.

We have shown that *Aras* is expressed in every cell type, including the spores. This suggests that it is the fraction of the active A-Ras that controls differentiation, rather than differential expression of the gene at different stages.

Our results also indicate that the level of the active A-Ras is not the only factor regulating development. When cells are grown in liquid culture, reducing A-Ras activity by overexpression of dominant-negative A-Ras does not initiate conidiophore formation. This indicates that other factors not present in a submerged culture are necessary for differentiation beyond vegetative growth. Adams et al. (1) have shown that overexpression of *brlA* can initiate spore formation even in liquid culture, suggesting that additional factors are necessary in conjunction with an appropriately low level of active A-Ras for synthesis of a sufficient amount of BrlA protein.

For a stalk to differentiate and form a vesicle, it must switch from growth by apical extension to growth by budding (43). When the level of active A-Ras is increased modestly, the formation of conidiophore structures is suppressed, with a concomitant increase in aerial hyphae. However, when this level is lowered experimentally, no aerial hyphae are found, but conidiophore formation is enhanced. These results indicate that the formation of conidiophores and the formation of aerial hyphae are mutually exclusive and suggest that they have a common antecedent. The fate of a stalk is determined by the level of active A-Ras. Below a critical level, the apical growth mode is switched to the budding mode, allowing the cell at the growing tip to differentiate into a conidiophore. When higher levels are artificially maintained, apical growth continues indefinitely to form aerial hyphae. The decision to differentiate into a vesicle, the first step of conidiophore formation, may be made either in the cell at the growing tip of the stalk or in the foot cell (Fig. 1).

Low-level expression of either the constitutively active or the dominant-negative mutants alters the fate of conidiophore formation without affecting any other steps in the developmental program. This indicates that either the absence of or a relatively low level of active A-Ras is necessary during conidiophore formation. However, we have shown that A-Ras is expressed in this structure. Because no function has been attributed to GDP-bound Ras, different levels of active A-Ras probably regulate the various decisions between continued growth and further differentiation that we have described here. The level of active A-Ras, and therefore the timing of developmental decisions, may respond to either growth conditions, composition of the medium, or other environmental factors.

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REFERENCES

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. **Aguirre, J., T. H. Adams, and W. E. Timberlake.** 1990. Spatial control of developmental regulatory genes in *Aspergillus nidulans*. Exp. Mycol **14**:290–293.
3. **Barbacid, M.** 1987. ras genes. Annu. Rev. Biochem. **56**:779–827.
4. **Borgia, P. T.** 1992. Roles of the *orlA*, *tsE*, and *bimG* genes of *Aspergillus nidulans* in chitin synthesis. J. Bacteriol. **174**:384–389.
5. **Borgia, P. T., and C. L. Dodge.** 1992. Characterization of *Aspergillus nidulans* mutants deficient in cell wall chitin or glucan. J. Bacteriol. **174**:377–383.
6. **Brody, H., J. Griffith, A. J. Cuticchia, J. Arnold, and W. E. Timberlake.** 1991. Chromosome-specific recombinant DNA libraries from the fungus *Aspergillus nidulans*. Nucleic Acids Res. **19**:3105–3109.
7. **Clutterbuck, A. J.** 1974. *Aspergillus nidulans*, p. 447–510. *In* R. C. King (ed.), Handbook of genetics. Plenum Press, New York.
8. **Cove, D. J.** 1977. The genetics of *Aspergillus nidulans*, p. 82–95. *In* The British Mycological Society Symposium, vol. 1. Academic Press, New York.
9. **Creaser, E. H., R. L. Porter, K. A. Britt, J. A. Pateman, and C. H. Doy.** 1985. Purification and preliminary characterization of alcohol dehydrogenase from *Aspergillus nidulans*. Biochem. J. **225**:449–454.

10. **Doonan, J. H., and N. R. Morris.** 1989. The *bimG* gene of *Aspergillus nidulans*, required for completion of anaphase, encodes a homolog of mammalian phosphoprotein phosphatase 1. Cell **57:**987–996.
11. **Downward, J., J. D. Graves, P. H. Warne, S. Rayter, and D. A. Cantrell.** 1990. Stimulation of p21ras upon T-cell activation. Nature (London) **346:**719–723.
12. **Feig, L. A., and G. M. Cooper.** 1988. Inhibition of NIH 3T3 cell proliferation by a mutant *ras* protein with preferential affinity for GDP. Mol. Cell Biol. **8:**3235–3243.
13. **Furth, M. E., L. J. Davis, B. Fleurdelys, and E. M. Scolnick.** 1982. Monoclonal antibodies to the p21 products of the transforming gene of Harvey murine sarcoma virus and of the cellular *ras* gene family. J. Virol. **43:**294–304.
14. **Gibbs, J. B., M. D. Schaber, W. J. Allard, I. S. Sigal, and E. M. Scolnick.** 1988. Purification of ras GTPase activating protein from bovine brain. Proc. Natl. Acad. Sci. USA **85:**5026–5030.
15. **Gibbs, J. B., M. S. Marshall, E. M. Scolnick, R. A. Dixon, and U. S. Vogel.** 1990. Modulation of guanine nucleotides bound to ras in NIH 3T3 cells by oncogene, growth factors and GTPase activating protein (GAP). J. Biol. Chem. **265:**20437–20442.
16. **Gross, M., R. W. Sweet, G. Sathe, S. Yokoyama, O. Fasano, M. Goldfarb, M. Wigler, and M. Rosenberg.** 1985. Purification and characterization of human H-*ras* proteins expressed in *Escherichia coli*. Mol. Cell. Biol. **5:**1015–1024.
17. **Han, M., and P. W. Sternberg.** 1990. let-60, a gene that specifies cell fates during *C. elegans* vulval induction, encodes a ras protein. Cell **63:**921–931.
18. **Kafer, E.** 1977. Meiotic and mitotic recombination in *Aspergillus* and its chromosomal aberrations. Adv. Genet. **19:**33–131.
19. **Kataoka, T., S. Powers, G. McGill, O. Fasano, J. Strathern, J. Broach, and M. Wigler.** 1984. Genetic analysis of yeast *RAS1* and *RAS2* genes. Cell **37:**437–445.
20. **Kremer, N., G. D'Arcangelo, S. Thomas, M. DeMarco, J. Burgge, and S. Halegous.** 1991. Signal transduction by nerve growth factor and fibroblast growth factor in PC12 cells requires a sequence of src and ras actions. J. Cell Biol. **115:**809–819.
21. **Lockington, R. A., H. M. Sealy-Lewis, C. Scazzocchio, and R. W. Davies.** 1985. Cloning and characterization of the ethanol utilization regulon in *Aspergillus nidulans*. Gene **33:**137–149.
22. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient in vitro synthesis of biologically active DNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12:** 7035–7056.
23. **Miller, B. L., K. Y. Miller, and W. E. Timberlake.** 1985. Direct and indirect gene replacement in *Aspergillus nidulans*. Mol. Cell. Biol. **5:**1714–1721.
24. **Miller, K. Y., J. Wu, and B. L. Miller.** 1992. *StuA* is required for cell pattern formation in *Aspergillus*. Genes Dev. **6:**1770–1782.
25. **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:**859–868.
26. **Moodie, S. A., B. M. Willumsen, M. J. Weber, and A. Wolfman.** 1993. Complexes of Ras.GTP with Raf-1 and mitogen-activated protein kinase kinase. Science **260:**1658–1661.
27. **Osmani, S. A., D. B. Engle, J. H. Doonan, and N. R. Morris.** 1988. Spindle formation and chromatin condensation in cells blocked at interphase by mutation of a negative cell cycle control gene. Cell **52:**241–251.
28. **Osmani, S. A., G. S. May, and N. R. Morris.** 1987. Regulation of the mRNA levels of *nimA*, a gene required for the G2-M transition in *Aspergillus nidulans*. J. Cell Biol. **104:**1495–1504.
29. **Osmani, S. A., R. T. Pu, and N. R. Morris.** 1988. Mitotic induction and maintenance by overexpression of a G2-specific gene that encodes a potential protein kinase. Cell **53:**237–244.
30. **Perrimon, N.** 1993. The torso receptor protein-tyrosine kinase signaling pathway: an endless story. Cell **74:**219–222.
31. **Pontecorvo, G.** 1953. The genetics of *Aspergillus nidulans*. Adv. Genet. **5:**141–238.
32. **Qui, M. S., and S. H. Green.** 1991. NGF and EGF rapidly activate p21ras in PC12 cells by distinct convergent pathways involving tyrosine phosphorylation. Neuron **7:**937–946.
33. **Rosenberger, R. F., and M. Kessel.** 1967. Synchrony of nuclear replication in individual hyphae of *Aspergillus nidulans*. J. Bacteriol. **94:**1464–1469.
34. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
35. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
36. **Sayers, J. R., S. W. Schmidt, and F. Eckstein.** 1988. 5′-3′ exonucleases in phosphorothioate-based oligonucleotide-directed mutagenesis. Nucleic Acids Res. **16:**791–802.
37. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusion, p. 250. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Simon, M. A., D. D. Bowtell, G. S. Dodson, T. R. Laverty, and G. M. Rubin.** 1991. Ras1 and a putative guanine nucleotide exchange factor perform crucial steps in signaling by the sevenless protein tyrosine kinase. Cell **67:**701–716.
39. **Som, T., K. A. Armstrong, F. C. Volkert, and J. R. Broach.** 1988. Autoregulation of 2-micron circle gene expression provides a model for maintenance of stable plasmid copy levels. Cell **52:**27–37.
40. **Stacey, D. W., L. A. Feig, and J. B. Gibbs.** 1991. Dominant inhibitory *ras* mutants selectively inhibit the activity of either cellular or oncogenic *ras*. Mol. Cell. Biol. **11:**4053–4064.
41. **Stringer, M. A., R. A. Dean, T. C. Sewall, and W. E. Timberlake.** 1991. Rodletless, a new *Aspergillus* developmental mutant induced by directed gene inactivation. Genes Dev. **5:**1161–1171.
42. **Szeberényi, J., H. Cai, and G. M. Cooper.** 1990. Effect of a dominant inhibitory Ha-*ras* mutation on neuronal differentiation of PC12 cells. Mol. Cell. Biol. **10:**5324–5332.
43. **Timberlake, W. E., and M. A. Marshall.** 1988. Genetic regulation of development in *Aspergillus nidulans*. Trends Genet. **4:**162–169.
44. **Upshall, A., T. Gilbert, G. Saari, P. J. O'Hara, P. Weglenski, B. Berse, K. Miller, and W. E. Timberlake.** 1986. Molecular analysis of the *argB* gene of *Aspergillus nidulans*. Mol. Gen. Genet. **204:**349–354.
45. **Waring, R. B., G. S. May, and N. R. Morris.** 1989. Characterization of an inducible expression system in *Aspergillus nidulans* using *alcA* and tubulin-coding genes. Gene **79:**119–130.