

Aspergillus nidulans *apsA* (Anucleate Primary Sterigmata) Encodes a Coiled-Coil Protein Required for Nuclear Positioning and Completion of Asexual Development

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Abstract. Many fungi are capable of growing by polarized cellular extension to form hyphae or by isotropic expansion to form buds. *Aspergillus nidulans* anucleate primary sterigmata (*apsA*) mutants are defective in nuclear distribution in both hyphae and in specialized, multicellular reproductive structures, called conidiophores. *apsA* mutations have a negligible effect on hyphal growth, unlike another class of nuclear distribution (*nud*) mutants. By contrast, they almost completely block entry of nuclei into primary buds, or sterigmata (bud nucleation), produced during development of conidiophores. Failure of the primary sterig-

mata to become nucleated results in developmental arrest and a failure to activate the transcriptional program associated with downstream developmental steps. However, occasionally in mutants a nucleus enters a primary bud and this event relieves the developmental blockage. Thus, there is a stringent developmental requirement for *apsA* function, but only at the stage of primary bud formation. *apsA* encodes a 183-kD coiled-coil protein with similarity to *Saccharomyces cerevisiae* NUM1p, required for nuclear migration in the budding process.

ORGANELLAR movement is essential for appropriate positioning of nuclei, mitochondria, chloroplasts, and vesicles within eukaryotic cells (Schliwa, 1984; Alexandre et al., 1989; Takenaka et al., 1990; Wagner and Grolig, 1992). For example, many secretory processes are mediated by the targeted delivery of vesicles containing enzymes, hormones, neuronal transmitters, or cell wall precursors to the plasma membrane. Fusion of the vesicles with the plasma membrane delivers their contents to the cell surface or extracellular space (De Camilli and Jahn, 1990). In a reverse process extracellular compounds are endocytosed and transported within the cell (Rodman et al., 1990). The mechanisms controlling vesicle movement are fairly well understood. Vesicles become attached to cytoplasmic microtubules and move along, driven by motor proteins. Kinesin as plus end-directed and cytoplasmic dynein as minus end-directed motors are the two most important mechanochemical enzymes involved in vesicle translocation. They hydrolyze ATP and use the chemical energy to produce force (Vale et al., 1985; Vale and Goldstein, 1990; Vallee and Shpetner, 1990; Pierre et al., 1992; Svoboda et al., 1993; Walker and Sheetz, 1993).

By contrast, much less is known about the mechanisms controlling movement of other organelles. Two fungi, *Aspergillus nidulans* and *Saccharomyces cerevisiae*, are suitable for studies of organellar movement, particularly nuclear movement, because mutants defective in nuclear movement have been isolated, making it possible to clone and characterize genes whose products are required for the process (Osmani et al., 1990; Kormanec et al., 1991). In *S. cerevisiae*, nuclei migrate to the budding neck before a localized mitosis, providing bud and mother cell with a nucleus (Byers, 1981). Several components have been shown to be involved in nuclear migration. Besides intact cytoplasmic microtubules (Jacobs et al., 1988; Huffacker et al., 1988), the functions of *MYO1* (Watt et al., 1987), *BIK1* (Berlin et al., 1990), *CIN1*, *CIN2*, *CIN4* (Hoyt et al., 1990), *SPA1* (Snyder and Davis, 1988), *TCPI* (Ursic and Culbertson, 1991, 1993), *DYN1* (Eshel et al., 1993), *JNMI* (McMillan and Tatchell, 1994), and *NUM1* (Kormanec et al., 1991; Revardel and Aigle, 1993) are required for proper bud nucleation. Binucleate mother cells were found frequently in these mutant strains. The corresponding proteins interact with microtubules and some are involved in their stabilization. Kormanec et al. (1991) suggested a role of NUM1p in the positioning of the G2 nucleus at the budding neck of a dividing yeast cell. Interaction of NUM1p with microtubules has not yet been demonstrated, although a function in polymerization or stabilization of microtubules has been proposed (Revardel and Aigle, 1993).

Similarly, in *A. nidulans*, nuclear movement is dependent

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on microtubules (Oakley and Morris, 1980; Oakley and Rinehart, 1985; Oakley et al., 1987). In addition, a class of temperature sensitive non-tubulin mutations have been isolated where nuclear distribution (= *nud*) in germlings was inhibited at restrictive temperature (Morris, 1976). *nudC* has been cloned and sequenced but the function has not yet been clarified (Osmani et al., 1990). *nudA* was identified as the motor protein dynein (Xiang et al., 1994). Recently cytoplasmic dynein and an actin-related protein (*Arp1*) were shown to be required for nuclear movement in *Neurospora crassa* (Plamann et al., 1994). Continued study of genes required for nuclear migration in *A. nidulans*, *N. crassa*, and *S. cerevisiae* can be expected to identify the essential components for this process and to determine how they interact.

During the growth of *A. nidulans* hyphae, nuclei are transported for a period of time but then become more or less fixed in one position, a process that leads to a fairly uniform distribution of nuclei along the axis of the cell. This observation implies that molecular signals exist to control the onset and cessation of nuclear movement, thereby providing a mechanism for nuclear positioning within the cell. The *A. nidulans* *apsA* gene is required for appropriate nuclear distribution along the hyphal axis, but not for nuclear migration per se, suggesting that its product participates in specifying nuclear position. *apsA* mutants were first identified by their nearly aconidial phenotype (Clutterbuck, 1969) and later found to have a defect in hyphal nuclear distribution (Clutterbuck, 1994) that has little or no effect on growth. We cloned the *apsA*⁺ gene and used gene disruption experiments to demonstrate that the original mutant phenotypes are due to loss of gene function. In *apsA*⁻ mutants, the primary buds, or sterigmata, of the conidiophore rarely become nucleated, because nuclei in the conidiophore vesicle are imprecisely positioned relative to the bud necks. Failure of the primary sterigmata to become nucleated leads to developmental arrest and a failure to activate development specific genes. However, occasionally nuclei enter primary sterigmata even in null mutants and these cells proceed to form sporogenous phialide cells and normal conidia. Thus, entry of nuclei into the primary buds (bud nucleation) could represent a developmental checkpoint (Losick and Shapiro, 1993) that serves to ensure that two separate morphogenetic events, bud formation and bud nucleation, have been successfully completed before initiating downstream events. Overcoming of this checkpoint could involve specific nuclear-cytoplasmic interactions. *apsA* encodes a 183-kD protein with a putative coiled-coil domain and structural similarities with the *S. cerevisiae* NUM1 protein which is also required for nuclear separation in that species.

Materials and Methods

Aspergillus nidulans Cultures and Transformation

A. nidulans strains (Table I) were grown at 37°C in appropriately supplemented minimal medium with 1% D-Glucose as carbon and NO₃⁻ as nitrogen source (Käfer, 1977) in liquid culture with shaking (300 rpm), or on with agar-solidified media in a humid incubator. *apsA*⁺ cultures were inoculated with conidia at a rate of 10⁶/ml. With the aconidial *apsA*⁻ strains, petri plates were inoculated by using a flat toothpick (20–30 cross-like streaks per plate). After incubation for 20–26 h at 37°C, the hyphae were scraped off the surface with a spatula in the presence of 10 ml of growth media. A hyphal suspension from 10 plates was used to inoculate 500 ml

Table I. *A. nidulans* Strains Used in This Study

Strains	Genotype	Source
AJC1.1	(<i>biA1</i> ; <i>apsA1</i>)	Clutterbuck (1969)
DRF54	(<i>pabaA1</i> , <i>γA2</i> ; Δ <i>argB</i> :: <i>trpCΔB</i> ; <i>apsA</i> :: <i>argB</i> ; <i>veA1</i> , <i>trpC801</i>)	this study*
DRF60	(<i>pyrG89</i> ; <i>wA3</i> ; Δ <i>apsA</i> :: <i>pyr4</i> , <i>pyroA4</i>)	this study†
FGSC26	(<i>biA1</i> ; <i>veA1</i>)	Fungal Genetics Stock Center
GR5	(<i>pyrG89</i> ; <i>wA3</i> ; <i>pyroA4</i>)	G. May, Houston, TX
SRF1	(<i>pabaA1</i> , <i>γA2</i> ; <i>wA3</i> ; <i>apsA1</i> ; <i>veA1</i> , <i>trpC801</i>)	this study‡
NK002	(<i>pabaA1</i> , <i>γA2</i> ; <i>wA3</i> ; <i>veA1</i> , <i>trpC801</i>)	Mayorga and Timberlake (1990)
RMSO11	(<i>pabaA1</i> , <i>γA2</i> ; Δ <i>argB</i> :: <i>trpCΔB</i> ; <i>veA1</i> , <i>trpC801</i>)	Stringer et al. (1991)
SAA21	(<i>biA1</i> ; <i>acrA1</i> ; Δ <i>argB</i> :: <i>trpCΔB</i> , <i>galA1</i> ; <i>pyroA4</i> ; <i>facA303</i> ; <i>SB3</i> ; <i>nicB8</i> ; <i>riboB2</i>)	A. Andrianopoulos, Athens, GA

* Obtained by transformation of RMSO11.

† Obtained by transformation of GR5.

‡ Obtained by crossing AJC1.1 to NK002.

medium. Protoplasts were prepared and transformed as described by Yelton et al. (1984).

Nucleic Acids and Recombinant DNA

RNA and DNA were isolated from liquid cultures or from conidiating cultures as described by Timberlake (1986). Standard recombinant DNA techniques (Sambrook et al., 1989) were used to construct the plasmids given in Table II. A λ gt10 cDNA library (*XLI*-blue as host) provided by G. May (Houston, TX) was screened with the 1.2-kb XhoI fragment near the 5' end

Table II. Plasmids Used in This Study

Plasmids	Construction and characteristics	Source
CRF1	cosmid, pKBY2 as vector, 40 kb genomic <i>Aspergillus</i> DNA insert covering the <i>apsA</i> gene, <i>trpC</i> from <i>Aspergillus</i> as selective marker	Yelton et al. (1984)
pRF7	10.5-kb BamHI subfragment of CRF1 cloned into <i>pBluescript KS</i> ⁻	this study
pRF2	3.8-kb XhoI subfragment of pRF7 cloned into <i>pBluescript KS</i> ⁻	this study
pDC1	<i>argB</i> gene of <i>Aspergillus</i> in pIC20R vector	Aramayo et al. (1989)
pRF9	1.8-kb XhoI-EcoRI fragment of pRF2 cloned into pDC1	this study
pRG1	<i>pyr4</i> gene containing plasmid, <i>pyr4</i> of <i>Neurospora crassa</i>	Waring et al. (1989)
pRF15	8.6-kb BamHI-SmaI fragment from pRF7 cloned into PUC18 and internal 5-kb XhoI fragments substituted by the <i>pyr4</i> gene obtained from pRG1	this study

of the *apsA*-coding region (see Fig. 2 A). Positive clones were plaque purified three times. The genomic BamHI–SmaI fragment and the *apsA* cDNAs were sequenced on both strands by chain termination (Sanger et al., 1977) with oligonucleotide primers.

Microscopy

For examination of germlings, 25-ml liquid cultures were inoculated with 10^5 conidia or ascospores per ml in a petri dish and sterile coverslips were placed at the bottom of the plate. Conidia/ascospores attached to the coverslips and germinated. After 12 h of growth at 30°C, the coverslips were removed and cells were fixed and immunostained as described (Oakley et al., 1990). For digestion of the cell walls we used Novozyme (Lot No. 3897; InterSpex Products, Inc., Foster City, CA) at a concentration of 80 µg/ml PEM buffer (50 mM Pipes, pH 6.9; 25 mM EGTA, 5 mM MgSO₄) mixed 1:1 with filtered egg white immediately before use. A digestion time of 50 min at room temperature was found to be optimal. The antitubulin antibody DM1A (Sigma Chem. Co., St. Louis, MO) was diluted 1:500 in TBS (20 mM Tris/HCl, pH 7.6; 137 mM NaCl; 0.1% Tween 20, and 3% BSA). For actin we used the mouse antiactin C 4 antibody (ICN Biomedicals, Inc., Costa Mesa, CA) with the same dilution. As secondary antibody we applied the FITC-labeled goat anti-mouse IgG antibody (GIBCO BRL, Gaithersburg, MD) in a 1:100 dilution. Incubation times were 1 h for each antibody.

For examination of conidiophores, we point inoculated microscope slides, which were covered with a thin film of media (solidified with 0.7% agarose), and grew the cultures at 30°C for 1–2 d in a petri dish with 25 ml of media not covering the microscope slide but making contact to the agarose film. During the fixation procedure with 8% formaldehyde in PEM buffer the colonies came off the slide and floated on the surface. They were transferred to the different washes and antibody incubations without further fixation to a support material. Incubation of the primary antibody was overnight at 4°C and the secondary antibody was applied for 2 h at room temperature. Mounting of the specimens was in 0.1 M Tris/HCl, pH 8; 50% glycerol; 1 mg/ml phenylendiamine; and 0.1 µg/ml DAPI (4,6-diamino-2-phenylindole).

For examination of nuclei in hyphae and conidiophores, *Aspergillus* was grown on microscope slides (see above) on complete media (minimal media supplemented with 1 g yeast extract, 1 g NZ-amine and 2 g peptone per liter; 0.5% agar). Nuclei were stained with DAPI without prior fixation. Fluorescence microscopy was performed with a Zeiss Axiophot microscope with the appropriate filter combinations.

Results

Molecular Cloning of *apsA*

A. nidulans anucleate primary sterigmata (*aps*) mutants were originally isolated by Clutterbuck (1969) who identified two *aps* loci, *apsA* and *apsB*, that when mutated produced a nearly aconidial phenotype. Development of wild-type and *apsA1* conidiophores is compared in Fig. 1. In the wild type, conidiophore stalks cease apical extension and the tips swell to form a globose vesicle (Fig. 1 A). Metulae are then produced by budding, become nucleated, and bud to produce sporogenous phialide cells (Mims et al., 1988; Fig. 1, B and C). Phialides produce long chains of conidia by a specialized budding process (Sewall et al., 1990; Fig. 1 D). In *apsA1* mutants, development of the conidiophore is normal up to the stage of metula formation (Fig. 1, E and F). However, as noted by Clutterbuck (1969), nuclei typically fail to enter the metulae and development arrests as the anucleate metulae begin to bud (Fig. 1 F). Occasionally, nuclei enter metulae and these go on to produce functional phialides and normal chains of spores (Fig. 1 G) or hyphal-like structures (Fig. 1 H). Thus, *apsA*⁺ function is needed primarily for production of nucleated metulae.

We cloned the *apsA* gene as a first step toward understanding the biochemical function of its product. First, we constructed an *apsA1*; *trpC801* strain (SRF1; Table I) as a transformation recipient. We then transformed this strain with

DNA from an *A. nidulans* genomic library in the cosmid vector pKBY2 (Yelton et al., 1985), selecting for tryptophan independence. 14 of 900 prototrophic colonies produced normal conidiophores. We isolated total DNA from four of these, recovered two classes of cosmids in *E. coli* and retested the cosmids for their ability to complement the *apsA1* mutation. Only one class recomplemented and we chose one cosmid from this class (CRF1) for further analysis. We found that a 10.6-kb BamHI fragment (pRF7; Table II) from CRF1 complemented the *apsA1* mutation as efficiently as the intact cosmid and this fragment was used for subsequent experiments. A restriction map of this fragment is given in Fig. 2 A. This fragment hybridized with three transcripts 1.2, 1.8, and 6.5 kb in length (result not shown). We observed no difference in the levels of these transcripts in hyphae, conidiating cultures or mature conidia (results not shown). A 3.8-kb restriction fragment (Fig. 2 A) complemented the *apsA1* mutation at low frequency (less than 1% conidiating transformants in a cotransformation experiment in comparison to 30–40% with pRF7). The 1.8-kb XhoI–EcoRI fragment indicated in Fig. 2 A hybridized only with the 6.5-kb transcript (Fig. 2 B), suggesting that this transcript arose from *apsA*. To test this hypothesis, we cloned the 1.8-kb XhoI–EcoRI restriction fragment predicted to be internal to the proposed *apsA* transcription unit into pDC1 (Aramayo et al., 1989) containing the *argB* selective marker. We then used the resultant plasmid to transform strain RMS011, a strain lacking *argB* (Stringer et al., 1991; Table I), and obtained transformants displaying the *apsA* phenotype. Fig. 3 shows a Southern blot analysis of one transformant (DRF54), confirming that the plasmid had integrated at the site of homology thereby disrupting the targeted transcription unit.

We tested for linkage of the inserted *argB*⁺ allele in DRF54 to *apsA* in two genetic crosses. First, we crossed DRF54 with an *apsA1* strain (AJC1.1; Table I). Of 227 colonies, 225 displayed the *aps* phenotype. Second, we crossed DRF54 with a strain containing an *argB* deletion (SAA21; Table I). All of 225 progeny displaying the *apsA*⁺ phenotype were arginine independent whereas all of 192 conidiating progeny were arginine dependent. Thus, the inserted plasmid was tightly linked to the *apsA* locus strongly supporting the hypothesis that the 6.5-kb transcription unit represents *apsA*. We were unable to produce a diploid derivative of DRF54 and an *apsA1* strain to test for noncomplementation. However, we did obtain heterokaryons and found that the original mutation and the gene disruption were noncomplementing. These data strongly support the hypothesis that the 6.5-kb transcription unit represents *apsA*.

Sequence of *apsA*

Fig. 4 shows the sequence of an 8.6-kb BamHI–SmaI fragment from the *apsA*⁺ genomic region. To determine the transcriptional structure of the gene, we isolated three cDNA clones from a λgt10 library with insert lengths >6 kb. We determined the complete sequence of one clone and the sequences of the 5' and 3' ends of the other two and the results are shown in Fig. 4. We detected multiple polyadenylation sites and a single 65-bp intron. We used primer extension analysis to map the 5' end of the *apsA* transcription unit (data not shown) and detected four potential transcription start sites consistent with the 5' ends of the cDNA clones. Transla-

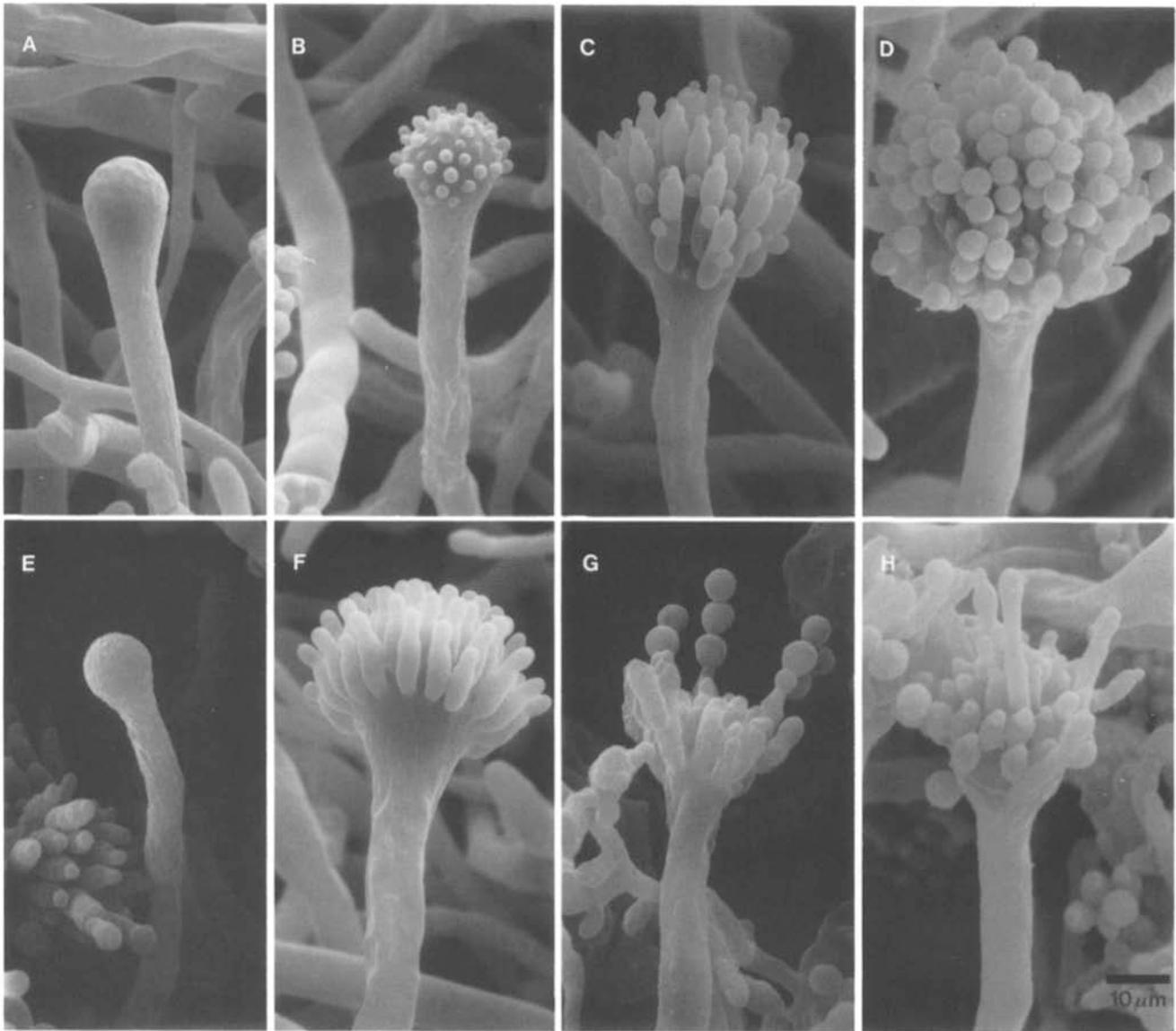


Figure 1. SEM study of conidiophore development of an *A. nidulans* wild type (FGSC26; Table I) (A–D) and an *apsA* mutant strain (AJC1.1; Table I) (E–H).

tional reading frame analysis revealed the presence of a single long reading frame initiated by AUG (Fig. 4) which we take to represent the *apsA* product, ApsA. The inferred polypeptide consists of 1,676 amino acid residues ($M_r = 183,000$). This result implies that the *apsA* transcript contains a 1,155-nt 5' non-translated region within which occur three AUG-initiated short (2–59 amino acid residues) reading frames (Fig. 4). The 3' non-translated region similarly contains six AUG-initiated short reading frames with 4–83 amino acid residues (Fig. 4).

Fig. 5 A shows that the deduced ApsA polypeptide is hydrophilic, with an overall predicted pI of 5.0. The amino-terminal portion (residues 1–1200) is acidic interspersed by two basic regions (residues 100–200 and 400–500). The carboxyl-terminal portion (residues 1201–1676) is very basic with a predicted pI of 11.1. A PH domain was found at the carboxy terminus of the protein (residues 1398–1499; align-

ment score 4030 [alignment not shown]) (Musacchio et al., 1993; Gibson et al., 1994). Fig. 5 B shows secondary structure predictions for ApsA. The amino-terminal region contains 25 heptad repeats in two stretches, yielding a probability of >0.93 for coiled-coil formation (Lupas et al., 1992). The intervening sequences and the remainder of the polypeptide had a non-significant probability for coiled-coil formation. The occurrence of 38% leucine residues at the a positions and 50% leucine residues at the d positions of the heptad repeats suggests that the polypeptides dimerize (Harbury et al., 1993; Oas and Endow, 1994). Fig. 5 C shows that ApsA contains three directly repeated sequences in the central, acidic region. These sequences are ~60% identical and contain 23% acidic amino acids.

Fig. 6 shows that ApsA has significant similarity to *S. cerevisiae* NUM1p, a gene product needed for nuclear migration into emerging buds (Kormanec et al., 1991). A

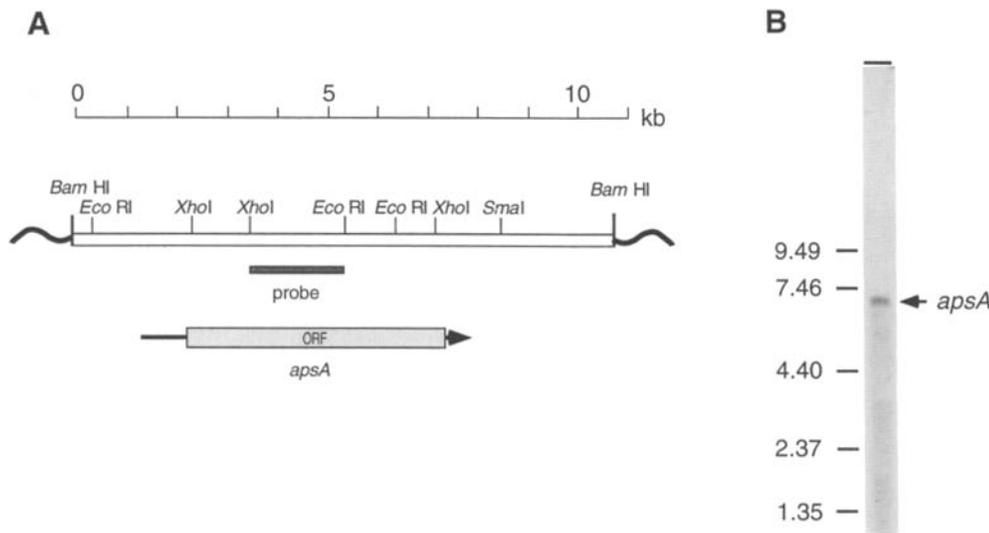


Figure 2. (A) Restriction map of the 10.6-kb BamHI fragment (pRF7) which complemented the *apsA* mutation. The location of the *apsA* transcript and the open reading frame are indicated. (B) RNA analysis of total RNA (10 µg) from hyphae using the probe indicated in A.

458-amino acid residue region near the carboxyl termini of the polypeptides shared 73% similarity and 27% identity. In addition to this limited, but significant, sequence similarity, the two polypeptides share some structural similarities with respect to their charge distributions and possession of heptad repeats at the NH₂ terminus and a PH domain at the COOH terminus of the protein. Moreover, NUM1p, like ApsA, contains directly repeated sequences. However, NUM1p contains 12 copies of a 64-amino acid residue sequence in comparison to the three shorter ApsA sequences. The repeated sequences are dissimilar in the two polypeptides. Interestingly the number of repeats in NUM1p can vary between 1 and 24 among different yeast strains without an influence on the function of the protein (Revardel and Aigle, 1993).

Construction and Characterization of an *apsA* Null Strain

We wished to confirm that the *apsA1* allele and the insertion allele in strain DRF54 represented null alleles. We therefore replaced the two internal XhoI fragments (see Fig. 2) with

the *pyr4* gene from pRG1 yielding pRF15 (Table II). We then transformed GR5 (Table I) with the gel-isolated linear BamHI fragment of pRF15. Transformants were scored for *apsA*⁻ phenotype and genomic DNA subsequently analyzed by Southern blot analysis. In one transformant the fragment had integrated with a double cross-over event thereby deleting 96% of the *apsA*-coding region (DRF60; Table I). Preliminary examination of DRF60 failed to reveal any significant differences in phenotype from the previously studied mutants, indicating that all displayed the null phenotype.

We characterized the phenotype of DRF60 by DAPI staining of nuclei and immunocytochemical staining of tubulin and actin in both hyphae and developing and mature conidiophores. Fig. 7A shows that in the wild-type strain nuclei are nearly uniformly distributed along the hyphae. By contrast, in DRF60 nuclei typically occurred in clusters separated by large gaps containing no nuclei (Fig. 7B). Thus, the loss of *apsA* results in a defect in nuclear distribution. However, unlike other nuclear distribution mutants (*nud* mutants; Morris, 1976), the *apsA* mutant grew at the wild-type rate

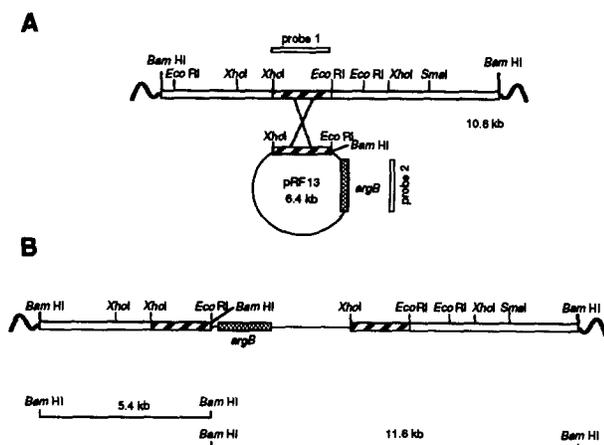


Figure 3. Disruption of the *apsA* gene. The *A. nidulans* *apsA*⁺ strain RMSO11 was transformed with the plasmid pRF9. Transformants were scored for *apsA*⁻ phenotype. A single genomic integration of pRF13 into *apsA* as depicted in A duplicates the XhoI-EcoRI fragment and introduces the plasmid sequences as shown in B. (C) Genomic DNA of RMSO11 and of a disruptant (DRF54) were digested with BamHI and subjected to a Southern blot analysis with the XhoI-EcoRI fragment (probe 1) as probe. The membrane was stripped and reprobated with the *argB* gene (probe 2).

-1047 GGATCCAGGCTATTCTGGGATGCTGGGAACAAGCTGGCAGACAAGCTTGCAGAAAGCTTG
 -987 GGTCTCTATATACAGCCCTAACATCCCCCATCCCCGTCATACCTACAAAGGGAGACAA
 -927 AACAGTGGCTTGCACAGAGACATATACAGCATATATAGTAAAGCACCACAAGCCCTACA
 -867 AGACCTGGGATATCAGACCCCATACAAAGGAAAGCGCACCCCGAGCACAAGCTTCCCC
 -807 GTTGGGTACTTGGCCGACTCGTCCGCCCGCGTACAGGCCACGGAGACTTTATGGCATAAC
 -747 ACCAGCGCTTCAACCACTCAGACTACCTGGAGAGCTGCTCTTGTGGTAGGACCAAGACCC
 -687 CAGTGTACTTCTTCTGCCCCATACACCAGAAAGTCTGGAAGATAGATAGAGATATA
 -627 TAAGGGACAGCCCGTCAAAAATATAGACTGGCTCTTAAGTACAGCTGCCGGGCTGAAG
 -567 AATTCAGCTGTATACACAGAATATCTCTTCAAGGATATATGCCCGAAGCTGGGCCCC
 -507 GCCGGAGCGCTTGTAGTGGCAGTCCACACATCTACTCTGGATAAAGGGTCCGGCCCT
 -447 CCCCCAATCTATAGGTAGTGAAGCGGCATCTGCCCTCAAAGACCTGCCAGGGCAGC
 -387 GCCGGGTCTTCTGCTTATTTCCAACATATATGTCATAGTGTGCTTCAAACT
 -327 GTATCTAGCTAGTCTAGGCACTTCTGTTTAGGTAGCAGCTCCAGATGCCCCCTGGGAG
 -267 GCCGAGATCAGCTGGGCCCGTATCCGCGAGTACGCTTAAATATAAAACCAAACCA
 -207 AACCAAACCAAACTTACTTAAAGGGTACTTAAAGACCAAGCAAAGGAGACTGGGAGA
 -147 CCACACAGCGGGATCAGACCACTGATCATCTCGACTCCGCAAACTTGGAGCCTGACTC
 -87 GCGTCTCAGGCTTTCAGGCTTTCGCTGACGATGGATGACGCCAGTAAACCGTGAACC
 -27 TTATTTGGTTCATGTTTGTAGCTGCACCTGAGATCAGTGTAGACTGTTAGACTGCCAT
 34 ATAACGCTGATTGCT ATG AACTGCTTCTGTTTCAATGCTGTTGTCGCTATTTCCTGTCT
 94 ATCAGATCGATTAAATG GGCCAT TCCCATAGACTAGTGTAGTACTA ATGTCG ATG CTT
 154 CAGCCCCATCAGCCCTGCTCGGTGCCCTGCTAGCTTCTCAGGCTCCAGCACTCGTCT
 214 TCTGCTCCTCGTCCGCTCCTCCCTTCCCTTCTTCCCTTCTGCTCCCGCCCAACGAG
 274 CGTCTCCTGCTCGTGTGCAACTGGCATCCTTCTTACTTATCTCTTATTACTTCCC
 334 CTCTCTTCCCTCCCT ATCCCTCTCTTCCCTACA GTCCCTTGTCTGTTTATATTTCTCC
 394 CTCACCTTAAATCCCTTCAATTTCCCATTCCTCCGTCGAGCTTTCCTTGTGCTATTCTGTCT
 454 TTCTGTCTATTCCCTAACTTATAAACCCTCTCAAACCTTAAATGACGCTGCTGTATG GG
 514 TAGCTGACTCCGGCATCCGGCTTACTCGTTCGCTACCGTATGATTTCTGGATCCGCCA
 574 GCTGCAGCTCTGCTTGCACCCGGACACTGAGCGCTTGTCCCTCCGTGTTGGCCCGCA
 634 TCCCGGTCCGACCGCATCCCAAGGTTACCAATAACTCTCCACCAACCGGTTGTTATTG
 694 TCCATCGGTCTGCTCCGAGCGGGTTTTACGCTATCTTTCATCTCCCTTCTAGCTACA
 754 CCGAAGCAAGATCCGAGATTCCTTTGGTGTCTGCTTCCCTCCCTAGCTCGGCTTTT
 814 GCGCTGCCCTTCCAGAGCTGACCGGTTGAACGGCTTACCTGGCGGATTACTACCGGACC
 874 GACCGCTGTACCCATACAGCAGCTGCTCCACCAACCGGCTTCCACTCACCOCGAGCCG
 934 TGGAAATCATGTAAGTCTGAGTCTGCTGCTGCGCCAGCTAAGGACCGTTTCAGGAGAC
 994 AGCTGGAGAAATGTCACAGTGAATAATGAACTGG ATG CCGAATAAGTACTGTTGGTTGA
 1054 TCCGGACA CACAGATAGACGAGCCACGAGCTTAAAAATCCAGCTGCAGTAAATCGTGG
 1114 TTTCAAG AACAAACACAGTTTACCACATATAAACTAAAGTATGGAGGATTCAACAGGAG
 1
 M E D S Q R G
 1174 GAAAC GCGCTCGATGATCCATGATGGATGACCTTTCGTTGTCAGCCGAGAAGGGGCGC
 8
 N A S M M S M M D D P F V V S P E G A R
 1234 GAGAC CCCCATCAACGAACCAATATCAACATTCGATCCGCAACTGTTCAATCTGGATG
 28
 D P P S T N Q Y S T F D A Q L P N L D A
 1294 CATCGA CTCCTCCGCAAGCAAAAGGGCTCTGAGGCCATCTGGCGGAAACCGAGCGCC
 48
 S T P A Q A K R A L E A H L A E T E R R
 1354 GACTT GAGGAAGCGTGAAGCTGGTACTGCGCTGCTGCAACACGGAAGGACCTGGAGG
 48
 L E E A S K L G T A L V E Q R K D L E D
 1414 ACAAG CTCAGAGAAGTGAACAACAGCAGGAGGAGGCTAAATCGGTGAGGAGCTTCGTC
 88
 K L R E V E Q Q Q E E G Q I G E E L R R
 1474 GGAACT GCGGATTTGGAGGCTGAATACAAGAAATAGGCCAGGAGACGGCGGAGCGT
 108
 K L A D L E R E Y N E I G Q E T A R A F
 1534 TCCTTG CACCAAGCGCTTTCGTTGGAGATGATGTCACCTAGGCACACCTCTATGG
 128
 L A P K R L A G G D D G H L G T P S M D
 1594 ACCAGA G GTTT GCTTTTAGCCCGCACAGCGATTAAACGCCAGAAACAGATGCTAAAT
 148
 Q K
 1654 TCTCCG ACTCTAG TCGCCGCTCAATTCAGCACTATTTCGCCGCGCAAGCTACCAATTCGCC
 150
 S P L H S A L F A G Q A T N S P
 1714 TAGCAA AGTGGCGTCCCGTCCGCAAACTCGGAAACCAAGTCAACCGCGCTCCATGATAT
 166
 S K V S V P S R K S R N Q S N R V H D I
 1774 CGAGT TTGCCACCAAACTTCGAGCTGCTACTTGCACAAGTACGCCAGCTCGACTGATT
 186
 E F A T E I S T S L A Q V R Q L Q S L
 1834 GCTCGC CGAAGCGAAGACACTGAAAACCGTGAACCTGGAGAAGTCCGACTGGAGCT
 206
 L A E R E E T L K T V N L E K S R L E L
 1894 AGAGC CGGAGGTTATGCAACGAATCCCGCTTTGGACGAGAGCGAAGCGTATAA
 226
 E A E G Y A Q R I R A L D E S E E R Y K
 1954 GGACG AGAATCGGGCTAGAAACCAAGATCCATGAGCTGATGGCTGCCGTGAAAGATGT
 246
 D E N W A L E T K I H E L M A A V K D V
 2014 CACAGC CTGAGACGAACTAACTAGCAGTCTGGGGCGCCATCGCGGAGAAAGCGC
 266
 T D R E T K L T S S L G A A T A E K S A
 2074 CATGG ACGGGAGCTGGAGGACTGAAACCAAGCAAGCAAGCAACTTATGGAGCACTAC
 286
 M E R E L E D L K Q A N A K I E D H T
 2134 TGGGCT CAGAAGCCACGATGCTGAGATTAATCTTTACGCCGGAACCTCAGTCCCGG
 306
 A A Q K A N D A E I N T L R R N L S A G
 2194 TGATC CGGAGGCTCACACTTCAGCGGAAGCTTGGAGATATGAACCTCAGAATCAAG
 326
 D A E R L T L Q R K L E D M N T Q N Q E
 2254 GCTCGA AAAAGCAGTTGCCATGCGATTCGGCAAGCAAGCGAGCTTACGAGGGAAGT
 346
 L A K A V A M R L R Q Q E A E S T R E V
 2314 TGTCG CCCACACGATTCGGAAGCAAGAGCAAGCCAGCCAGAGAACTCTCTCCACC
 366
 V R P H D S E D E E Q A T P E N S P P P
 2374 GTCGCC GAAACAAATTCACCCCTCGTCATAACCACTTGGAGACTGAGACTTTCGGGAGCTC
 386
 S P N K F T P R H N H L E T L R R S
 2434 GCTTGC CACGCTCATCGGATGATCCAGAACCTCGCAAGTCAATCCAGCTGAGAGAC
 406
 L G H A H R M I Q N L R S T I H R E K T
 2494 TGAGA AGATCGAGCTGAAGCGCATCTCAAGAGCTCGGATGAGGTTGAGGAGCGCGC
 426
 E K I E L K R M L Q E A R D E V E Q R R
 2554 CCGTG ACTCGGTGCTGCCAATGGACTTACCAACAGCAGCAAAAGCAAAAGCCGAGAC
 446
 R D S V A A N G P T N K R Q K T K A E T
 2614 GCGCAA CCGTGCAGCTCCGACCTGTTGGTGGCGTAGAAGAAGCGGAGGTCGAAT
 466
 R K P A R P D L L G A G R K K A E V E I
 2674 TCACG ACTCGGACTGGAACTCAATGCTGGGACATCAGCCGACTCAAAAGCGCTTAA
 486
 H D S D W E S N A G D I S P T H K A S N
 2734 TGATTC CGCGATTCGCGTGGAGACCGCGATTCGATGACCGTATGATCTTACCTAC
 506
 D S R D R R G D Q P I D D R I D S D A Y H T
 2794 TGCGA CAGAGGCTGATGATCCCTTCGAGACCGCAATGAAAGGAGGAGCCAAACCGAAG
 526
 A T E A D D P F E T A N E R E T T E S
 2854 TGAAG CTTCCAGACCGGCTGGAGGATGCGGCGACAGCAGGATGATGTAAGT
 546
 E A F Q T G V E S M A G D S T S R D E L
 2914 TACTG AGACCGAAGATCGTTCAAAGGACTCTCCGCGAGAGTCTCTCCATGACTTT
 566
 T E T E D R V Q R T P F R G R V S S M T L
 2974 GGCAAG CCCGCTGACGAACTTACTATAGCAGCCGATCCACTCTCCGATGAGG
 586
 A K A R D R T T Y Y S T A S T S A D E G
 3034 TGACG ATCAGATCTCGAAGCCGCTTACCTAGCCGATGATGATGATGATGATGATGATG
 606
 D S T D P G T P S I S P T P R Y R L
 3094 GCGAA AAGAGGAGCGTTTTCGCAAGATTCGCGCTCCGCGAGGCTCCGATGGCCCT
 626
 R K K R S V L R K I R P S G E A P M A F
 3154 CAATG CCGACCTCAAGTCTAGGAACTCCCGTCGACTGCTTCACTCGGATACATC
 646
 N S R P S S A R E S P S T S F T R D T S
 3214 CGCGC TCTGAAAGTCAAACTCTTTCGACGACTTCCGAAAGTGCATGGGATGAAGA
 666
 A A P E G Q S L F A E L A E V D G D E D
 3274 TGATTC GGTCCACCGATGACGATTCGAGGCGGCTTCCCTCAACCCGCGATGCTTC
 686
 D F G P P M Q F E A A S P S T P R M L P
 3334 AGGAT TTGATTCAGGAGACCGTCCGAGTACCTGAGGATTCCTCCGAAACCCGATM
 706
 G F D S R R P S A V T V E L P S K P D A
 3394 GTTGAC TCCGCGCTATGACTGACCTTGGGAGCCCACTTCACTTCGCTTTCAGAC
 726
 V D S G V M T D P W E P N L H L A S Q T
 3454 TGACG ATGAAAGGCTCATAGTCTTCCCTGTAAGCGCTGATAAGCAACATGTCGAGCC
 746
 D D E T V I S V P V T P D K P T M S D A
 3514 CAGTAC TGGATGATGTTGGAGTACCAGCTTAGTTCATCTTCAACCCGATGGAG
 766
 S T G M D V V E S P S L T L V H S S T G W T
 3574 TCCGT GAAGCCTAATGCCGAAACAGTACGATATGCTTCTGAGTGTGCTACGCCACC
 786
 P L K P N A E T S D D H V L S V P T P P
 3634 AAAGT GGCATGGAGCGGCGAGACTCTCAEATGAGAAACGGAAGTGCATCTCCCGACAG
 806
 K M A W D G Q T L N E R K A V D I P D S
 3694 CCCTA CAACCGAGGAGTTGAATATATCTCTGATCTTTTGAAGAGACAGAACCGGT
 826
 P T T Q R E L N I S S V S F E E T E P V
 3754 GGCTCC CAGCTTCCAGAGCTGAGAACGCTTCTTCTGTTGGGAAGCAGCAAGCCGCT
 846
 A P S F P E L R T A F P V G S T T E P V
 3814 TGCGC TCCGCTCCCTGTCGCGCAGAGGTAGCTTGTCCGCAATCTCGTCAAAACC
 866
 A A P V P V P P E V A L S T G C T T
 3874 TCAAC CCGAGCGCCGCTATCTCTGCTCCGCGGAGCCGAAACCAATATATGACCGGA
 886
 Q P T E P V I P A P P E P E P I Y V P E
 3934 GATGG CATTTCTCAGATCTCTGGAGGATACGCTGCCATCTTGCCTAAGCTTCTGTA
 906
 M A F S Q I L V E D T L P I L A K L P E
 3994 GCCTG CGCGTGAACCGGTTTTCGCGCAAGGAGCAAGTACAGATATGCGGAGGTTTC
 926
 P A P E R V F A E O G T S T D I A D V S
 4054 AGTTG CGCTATATCATCAGAAACAACTGAGCCGCTTGGAGCGGTTTATGAGCAAAAGCA
 946
 V S A I S S E O T E P V E P V Y E P K Q
 4114 AGAGT CGCATCTGGCTGAGCTGTCCGAAAGCCGCTATCTCTGTTGAACAGG
 966
 D V A I V A E A V R E G P L S F V E O G
 4174 GACAA ATACAGCAGCGTGGAGATTTCGTTCCCGCATCTCCCTAGCTGAAACTGAAC
 986
 T N T D D V E I S F P A I S S V E T E R P

Figure 4.

4234 TGTTGCCCGCTTCGAGAGACCAAGGATGACGTTCTCGAGCCGGTCTGTCCTTACCGA
1006 V A P V R E T K D D V P E F V L S L T E

4294 GCAAGGAACAAGCAGGATCTGTGGAAATTCCTCACTTTCGAGCATTTCTCGGAAGAGAC
1026 Q G T S T D T V F S V S S I S S E E T

4354 CGAGCGTGTGAGCCATCCGTCGAGGCAAGGAAGACGGCGCTGTGGATGATGTGGC
1046 E P V E P I R E A K E E A A A V D D V A

4414 CTCAGAATCCACTCATCCGCTATTGAGCATCTTCTCCACCCCTCCGCATACACCGAGCC
1066 S E S T H P F V L S I P L T P F A Y T E P

4474 AACTGCGCAAGCTCCAGAAGCCGTTATTCGGCAGCAGCGAGTTGGCGTTGTCTAC
1086 T A P K L Q E A V C V I P P A P Q L A L S T

4535 GGTAGCTCAGTTGAAACACCCGCTGTCCAAATATACTCCGGATGTACTAATCTGCCAC
1106 V S R A R Q V G S A K T T T D R A S V R P L S P

4594 GCCCCGGCTCTCGAGAGAACACTCCCCCTAGTGTAAATGGCCAGCAGCTGCTAAAGCAAC
1126 P P A L D E N T P P S V M A S T A K A T

4654 AAAGAGTCCCCACCGCTTGTGTGGATGACACACCGGACAGGCTACTGCTGATGG
1146 P S A P P L V D V V D N T D Q A D K G T A D G

4714 GTTGGTTACCCAGCAGAAGCGTGTGACACTTCTTGGGTGCGATCTCTGGAATGCGGC
1166 L V T Q Q N G V T L P L G A I S G N A A

4774 TCCCGCTCGCAAGTTCGGGCTCGTCCAACCCAGCCGACAGGGTGTCTCAGACCTTT
1186 P R R A R S N Q A D Q G A D Q A T I L

4834 GTCATCCAAAGCAATCGATCACTCCTTATTGACCGAGCTTCTGTCCAGACCACTTTCTCC
1206 S S K Q I D Q L L I D R A S V R P L S P

4894 TCCTGATAGCGACAAGCTCAACGATGAGCACTCACCATTGCGCACACCAAGGCGCG
1226 P D S D K L E M S N S P F A T P K A R

4954 ATCGCGCCCTGTTCGCCAGCTTCCAATGCCCTTTACACAGAGCCCTGGAAGCGCCG
1246 S R P V P Q A S N A S L H K R P G S A A

5014 GAGCAAGCATCAAGTGTGAGATTCATCTCCATTCCTGACCATTAAGAAGCAT
1266 S Q A S S V Q I H P P L P A D H K E A I

5074 CATGGCTCGGAGAAGAAATCCATCGATCAACGCCCGCTGTCTGTGTTGATGGGCC
1286 M A A E K K S I I D Q R P A S A G L M G P

5134 GCCACTTGCCACAGCTTCGCGAGTACGAGGAGCTCACAGCAACCGCTAGGACGCCGAA
1306 P L A P A S A V R A S S Q Q R P R T P N

5194 TGAGTCGCTTCTCAAGTGGCTCTGCGAAGACAACATCACAGAGCCAGCGTGGAGAG
1326 E S A L Q V G S A K T T T S R A S L V R R

5254 CGATAGCCATATGCTCGAGCATCTCAGTTTCTTCAATTTGCTCCGAGCTGGAAGAGCG
1346 D S H M S R R S S V S S F A S E L E E R

5314 TTTCAACATGACGCCAATCCCGCTTTGCCCAACAGGATCTCCACGGGAAGTACCC
1366 F N M Q P N P P F A P Q G Y S T G D P

5374 TCGAATGATCCAAAGCAATCACAAACCATGATTGGTGAATCTCTGGAATAACTGCG
1386 R M I Q A I T Q T M I G E F L W K Y T R

5434 CAGAGCGTCTCTGAGAAATATCCAACACCCAGACATCGCAGATATTTCTGGGTCCACC
1406 R A V S G E I S N T R H R R Y F W V H P

5494 GTACACCGCAGTTATCTGAGGAGCAGTACCCGAGTCTGCTGGAAGAGCGAGGG
1426 Y T R T L Y W S E H D P Q S A G K S E G

5554 ACGGACAAAGAGTGTTCATTTGAAGCTGTCAAGGTGGTGGCGGACGACAAACCATACCC
1446 R T K S V S I E A V R V V A D D N P Y P

5614 ACCAGGGCTTCACTGCAAGAGCTTGGAGGTGTGAGCCCGCGGTGAGATCAGGTTTAC
1466 P G L H C K S L E V V S P G R R I R F T

5674 TGCCACTACAAGCCAAAGACATGAGACATGGTTCAACGCTCTTTCTTACCTGCTGGTACG
1486 A T T S Q R H E T W F N A L S Y L L V R

5734 GAACGGTCCGGAGGATGAAGAAGCAAAAATGGTGTACCGTGGATGACATTGACGAGTT
1506 N G P E D E E A E N G V T L D D I D E F

5794 CAACCCGGATTCGGCTCAGCTCCGCCAAACCGCACGATGTCAGTSTCTCCCTGCCCA
1526 N P G F R S R S R Q T A R M S V S S S Q

5854 GAGTCGGGAGCGGGGTCTACCGAAGCAACGGTCAAGGCTCAGCATGTCTTTACGGCC
1546 S R G T R G L P K Q R S G S A M S L R P

5914 CAGTGTGACACTGGCCGTCATCCCGTACCCCGTCCGCTACTCGGACCGAGCAAG
1566 S V T P F R A S P Y P P S H Y S D Q A R

5974 GCAGGCTTCGCTCGGTTGAGTACCTCTCAACTCGACAATCAAGGATCTTTTGG
1586 Q A S S S R L S T I F N S T I K G S F G

6034 ACGAAAAGCCCGTACGGCGCTTCCAGTCTAAATGAAGATAGCATCCATAACACGACGA
1606 R K G P Y A A S S L N E D S I H N H D D

6094 TAGTGTGAAGATCTTCGGCAGATGAGACAGGGCGATGATGTGGACCGAGCTTAGAA
1626 S V E D L R H M M D R G D D V D R L E N

6154 TGTCCGTGCTGTGCGATGGCAAGCAGATGTCAGCTCACTTCGAGGACGAGCGCTA
1646 V R A C C D G K H D V S S L S R T S R Y

6214 CAGCCCTCGCCCAACCGAATCCACTCCCATCAATTTCTGGAGTGGTGTGTGTTA
1666 S P R A N R I H S H

6274 AAAGTCTGTTATTAGCGGTCTCGCCCTCCACCTGTCATCCAGGAAGTAGCTGCTTAC
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6334 CTCTTGGCTCCATGTTTCAATTTCTTGTGGACGGTCAATCCAGCAAGTGGCGTCAAT
1706 C T C T T G G C T C C A T G G T T T C A T T T T G T G G A C G G T C A T C C C A G C A A G T G G C G T A T T

6394 TCCAGCTCGGTATTGATTCGTGCGTCCCTGCTTACAATCTGCATTTCTGCAACTT
1726 T C C A G C T C G G T A T T G A T T C G T G G C C T T G C T T C A C A A T C T G C A T T T C T G C A A C T T

6454 G A T G T T A T T T C T T T T T C T T A T C C A T C A T T A T C A C G C A T G A A A T T C A T T T G T T C T T A A T

6514 TATTGCTTGTGAGACATACCAGGCCACTGCTTCAATGAGCGCGTGAATCTCTCTT
1746 T A T T G C T T G T T A G G A C A T A C C A G G C C A C T G T T C A T T A T G C C G C T G A A T C T C T C T T

6574 ACCAACATATGCGGTACAAGGCCCTGTACTTTGACTTTAACCTTGGTATTGGTTTGA
1766 A C C A A C A T A T G C G G T A C A A G G C C C T G T A C T T T G A C T T T A A C C T T G G T A T T G G T T T G G A

6634 TCTTGATGACCGCGCTTGTAGGTGTAACCGAGGCTATGTTGGTGTCTGGATATCC
1786 T C T T G A T G A C C G C G T T G T A G G T G T A A C C G A G G C T A T G T T G G T G T C T G G A T A T C C

6694 TGGCCGAGATTGATCTATAGGCAAGTCAATGAGTAAATATCTCTTGAACCTTGTGACG
1806 T G G C C G A G A T T G A T C T A T A G G C A G T C A T T A T A T C T C T T G A A C T C T T G T G C A C G T

6754 AGTCGCTTTGCCATCTCATGGCATATCCGCTTTCGATGTGGAGTGGACTGCACACATC
1826 A G T C G C T T T G C C A T C T A T G G C A T A T C C G C T T T C G A T G T G G A G T G G A C T G C A C A C A T C

6814 TTAGCTGTATAAGTACTGTAGACGAGCTTGGTGTAGTATCCCCACATTTGAACCC
1846 T T A G C T G T A T A A G T A C T G T A G A C G A G C T T G G T G T A G T A T C C C C A C A T T T G A A C C C

6874 CAACTATAAAGTCTCCGTGGTGTCTCCGGAAGCCGTTATCCGCCACACCTCCCTACA
1866 C A A C T A T A A A G T C T C C G T G G T T G T C T C C G A A G C C G T T A T C G C C C A C A C C T C C C T C A C A

6934 TTTCAAGTACGTTTGAATGGCCAGGACGACCTCACAAACCGGCAATGGCTCTGGCT
1886 T T T C A A G T A C G T T T G A A T G G C C A G G A C G A C C T C A C A A C C G G C A A T G G C T C T G G C T

6994 CTGCTGGAGCCGCCAAAATTTGACATGGGCTATGCTTGTGACGCTCCACTTACTACC
1906 C T G C T G G A G C C G C C A A A T T T G A C A T G G G C T A T G C T T G T A C G T C C A C T T A C T A C C

7054 TAGAATCAATTTGTACTGTATCTCTCATTTGTCTACTATATGTTTGAACCTTGATGATG
1926 T A G A A T C A A T T T G T A C T G T A T C T C T C A T T T G T C T A C T A T A T G T T T G A A C C T T G C A T G A T G

7114 CTATGATTAGCCAGCTAACATGAACACAGACTACGTCGGCCCAATTTTAAAGCCGTT
1946 C T A T G A T T A G C C A G C T A A C A T G A A C A C A G A C T A C G T C G G C C C A A T T T T A A G G C C G T T

7174 CTCAGCGGACGCTCTTAGCAGCCATCTTAATGCCCATCAGCTTGTGACGAGCAAGCATT
1966 C T C A G C G G A C G C T C T T A G C A C C C A T C C T T A A T G C C C A T C A G C T T G T G A C G A A G C A T T C

7234 GGGAAATCTGGGTGCGATTAGTGTATGTCGCGCTTTCATCCCTTTGCAACCTCTCTTCT
1986 G G G A A T C T G G G T G C G A T T A G T G T A T G T C G C G C T T T C A T C C C T T T G C A A C C T C T C T T T C T

7294 ATCTCAATACAGTGCACAAACCAAGCAACCCGTTGAAGAACCCATCGACAGCACCC
2006 A T C T C A A T A C A G T G C A C A A A C C A A G C A A C C C G T T G A A G A A C C C A T C G A C A G C A C C

7354 TCTACCCCGGACACAGGACCAACAGGACCAACAGGACCAACAGCGCCAGCAAAAGTC
2026 T C T A C C C G G A C A C A G G A C C A A C A G G A C C A A C A G G A C C A A C A G C G C C A G C A A A A G T C

7414 CATTCCTCATCTCTTATCTAGCTCTGCAAAAGGAAGCAAAAGCAAAAGCACTACAAT
2046 C A T T C C T C A T C T C T T A T C T A G C T C T G C A A A G G A A G A G C A A A A A G C A A A G C A C T A C A A T

7474 CATCTGACCTGATCCCCCAATATCTCGCAAGCAACCGGTGTGCTCTTACGCTGGCC
2066 C A T C T G A C C T G A T C C C C C A A T A T C T C G C A A G C A A C C G G T G T G C T C T T G A C G T G G C C

7534 CGGG

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Figure 4. Nucleotide sequence, transcriptional organization, and deduced amino acid sequence of the *apsA*⁺ gene. The 8.6-kb BamHI–SmaI genomic DNA fragment shown in Fig. 2 was sequenced on both strands as was a 6.2-kb cDNA. Comparison of the cDNA sequence with the genomic sequence revealed one 65-bp intron (underlined) containing the *A. nidulans* consensus splice signals. The transcription start sites obtained in a primer extension experiment using the primer (underlined) are indicated (*). Three polyadenylation sites (*) were deduced from three cDNAs. Open reading frame analysis within the cDNA sequence revealed one 1,676–amino acid polypeptide starting with an ATG. The amino acid sequence is given in the one letter code below the nucleotide sequence. Three ATG-initiated 2–59 amino acids long ORF were found in the 5'-leader, and six ATG-initiated 4–83 amino acids long peptides in the 3'-tail (ATGs are underlined). Three direct repeats in the 1,676–amino acid polypeptide are also underlined. The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under the accession number X82289.

and had a normal colonial morphology. Moreover, nuclear division and migration were essentially as in the wild type; only nuclear positioning was severely affected by the mutation. Fig. 7 C shows staining of microtubules in DRF60. Microtubules formed long intracellular cables as previously observed (Osmani et al., 1988) and no differences were detected between wild type and mutant. Similarly, staining of actin filaments revealed no difference between wild type and mutant (Fig. 7 D). Thus, the defect in nuclear distribution in the *apsA*⁻ strain appears not to be due to defects in microtubules or actin filaments.

The results of staining of nuclei, tubulin, and actin during conidiophore development in the wild type and mutant strains are shown in Fig. 8. In the wild type, nuclei under- went several synchronized mitoses in the elongating conidiophore stalk and expanding vesicle (Fig. 8, A and B). Once metular initials were formed, gradually all metulae received nuclei. We observed only very rarely (three times in ~100 conidiophores where spindles were visible) nuclear divisions localized to the bud neck (Fig. 8, C and D) with one daughter entering the metula and the other being retained in the conidiophore vesicle. This suggested that nuclei enter

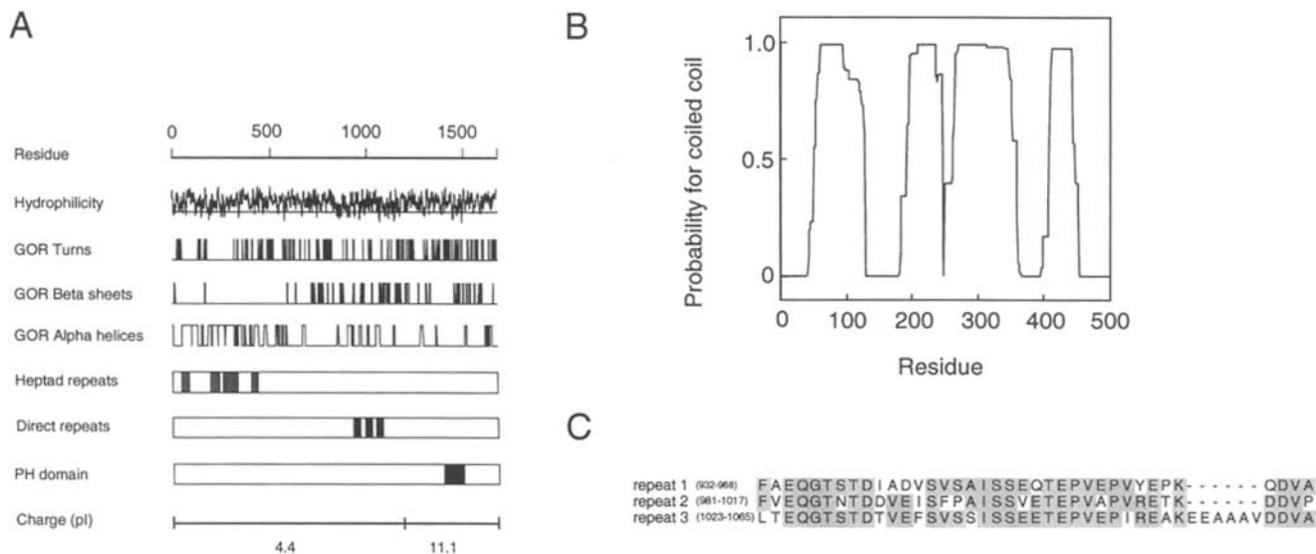


Figure 5. (A) Properties of the ApsA polypeptide. Secondary structure predictions for ApsA were performed with the GCG programs PEP-TIDESTRUCTURE and PLOTSTRUCTURE, the COILS program of Lupas et al. (1992) and the GCG program REPEAT. A PH domain was identified in a profile search (Gibson et al., 1994). The charge (pI) of the regions indicated in the last line was calculated with the IG program pI. (B) Result of the heptad repeat analysis. The probability of each residue for the involvement in a heptad repeat formation is shown for the first 500 amino-terminal amino acids of ApsA. In the heptad repeat regions depicted in A (stippled boxes), the probability is higher than 93%. For the amino acids 500–1676, the probability is smaller than 1% (not shown). (C) The three direct repeats are aligned and identical residues are highlighted with the stippled background. The exact location of the repeats in the protein sequence is noted in brackets.

metula probably by migration of a postmitotic nucleus rather than via a localized mitosis. Metulae then divided to form phialides (Fig. 8, E and F). Mitoses in metulae and phialides were not synchronous (Fig. 8 G). Division of nuclei in phialides resulted in production of uninucleate conidia (Fig. 8 H). In the *apsA*⁻ strain, as in the wild type, multiple nuclear divisions occurred during stalk elongation and vesicle expansion and metular buds were produced (Fig. 8, I–L). However, we failed to observe localized mitoses at the budding necks of the metulae which remained anucleate (Fig. 8 M). Anucleate metulae never proceeded to produce phialides. However, rarely a nucleus entered a metula (Fig. 8 N) after which phialides immediately differentiated, and these began to produce conidia as in the wild type. Occasionally, the latter stages of development were abnormal and hypha-like structures were produced instead of phialides and conidia (Fig. 8 P). In older colonies, many conidiophore heads contained some chains of conidia and hypha-like structures. Many metulae and phialides from conidiophores in these older cultures were multinucleated (Fig. 8, O and P) and some conidia were binucleate, which we never observed in the wild type.

Fig. 9 shows analysis of microtubules and actin filaments in the conidiophores of the wild type and the *apsA*⁻ mutant. Microtubules formed a network in the conidiophore stalk and vesicle. Most of the fibers were oriented longitudinally with some cables extending from the vesicle into the metulae (Fig. 9, A and B). Actin occurred in patches with a concentration at the growing tips of differentiating metulae (Fig. 9, C and D). We observed no differences between the wild type and the mutant.

Discussion

A. nidulans conidiophores develop from an ordered series of cell differentiations finally leading to formation of the sporogenous phialide cells. Phialides repeatedly produce conidia that can be dispersed and germinate, thereby completing the life cycle (Timberlake, 1990, 1991; Clutterbuck and Timberlake, 1992; Timberlake, 1993). Many steps of conidiophore development are controlled by the concerted activities of five regulatory genes, *brlA*, *abaA*, *wetA*, *stuA*, and *medA*, all or most of which encode transcription factors (Boylan et al., 1987; Timberlake, 1987; Adams et al., 1988; Mirabito et al., 1989; Adams et al., 1990; Sewall et al., 1990; Marshall and Timberlake, 1991; Miller et al., 1991, 1992; Aramayo and Timberlake, 1993; Prade and Timberlake, 1993; Andrianopoulos and Timberlake, 1994). Loss-of-function mutations in these genes result in pleiotropic defects in development leading to formation of highly abnormal conidiophores. Several genes, for example *wA*, *yA*, *rodA*, and *dewA*, whose products contribute directly to the specialized forms and functions of conidiophore cells have been identified, cloned, and demonstrated to be under the direct or indirect control of the regulatory genes (Aramayo and Timberlake, 1990; Mayorga and Timberlake, 1990; Stringer et al., 1991; Mayorga and Timberlake, 1992; Aramayo and Timberlake, 1993; Andrianopoulos and Timberlake, 1994; Stringer and Timberlake, 1994). One class of developmentally defective mutants, called anucleate primary sterigmata (*aps*) mutants, have not previously been investigated in detail. In *aps* mutants nuclei fail to enter primary sterigmata, or metulae, and development ceases at the stage of phialide bud initiation (Clutterbuck, 1969, 1994) (Figs. 1 and 8). In

A

```

(1114)
ApsA  V Q Y T P D V L I L P T P P A L D E N T P P S - V M A S T A
NUM1  V E Y G P D D P T F T V R Q L K K P A G D R N L I L T S R E
(2301)
ApsA  K A T K S A P P L V V D D N T - - D K G T A D G L V T Q Q
NUM1  K T L L S R D D N I M S Q N E A V Y G D D I S D S F V D E S

ApsA  N G V T L P L G A I S G N A A P R R R A R S G S S N Q A D Q G
NUM1  Q E I K N D V D I I K T Q A M - - K Y G M L C I P E S N F V

ApsA  A Q T I L S S K Q I D Q L L I D R A S V - R P L S P P D S D
NUM1  G A S Y A S A Q D M S D I V V L S A S Y Y H N L M S P E D M

ApsA  K L N E M S N S P F A T P K A R S R R P V P Q A S N A S L H K
NUM1  K W N C V S N E E L Q A - E V K K R G L Q I A L T T K E D K

ApsA  R P G S A A S Q A S S V Q I H P P L P A D H K E A I M A A E
NUM1  K Q A T A S K H E Y V S - H K L N N K T S T V S T K S G A

ApsA  K K S I D Q R P A S A G L M G P P L A P A S A V R S S Q Q
NUM1  K K G L A E A A A T T A Y E D S - - E S H P Q I E E Q S H R

ApsA  R P R T P N E S A L Q V G S A K T T T S R A S V R R D S H M
NUM1  T N H H K H H K R Q Q S L N S N S T S K T T H S S R N T P A

ApsA  S R R S S V S S P F A S E L E E R F N M Q P N P P F A P Q G Y
NUM1  S R R D I V A S P M S R A G S - - - - A S R T A S L Q T L

ApsA  S T G T D P R M I Q A I T Q T M I G E F L W K Y T R R A V S
NUM1  A S L N E P S I I P A L T Q T V I G E Y L F K Y Y P R L G P

ApsA  G E I S N T R H R R Y F W V H P Y T R T L Y W S E H D P Q S
NUM1  F G F - E S R H E R F F W V H P Y T L T L Y W S A S N P I L

ApsA  A G K S E G R T K S V S I E A V R V V A D D N P Y P P G L H
NUM1  E N P A N T K T K G V A I L G V E S A T D P N P Y B T G L Y

ApsA  C K S L E V V S P G R R I R F T A T T S Q R H E T N P N A L
NUM1  H K S I V V T T E T R T I K F T C P T R Q R H N I N Y N S L

ApsA  S Y L L V R N G P E D E E A E N G V T L D D I D E F N P G F
NUM1  R Y L L Q R N - - - - - M Q G I S L E D I A D - D E T D

ApsA  R S R S R Q T A R M S V S S S Q S R G T R G L P K O R S G S
NUM1  N M Y S G K I F P L P G E N T K S S S K R L S A S R R S V S

ApsA  A M S L R P S V T P G R
NUM1  T R S L R H R V P Q S R

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B

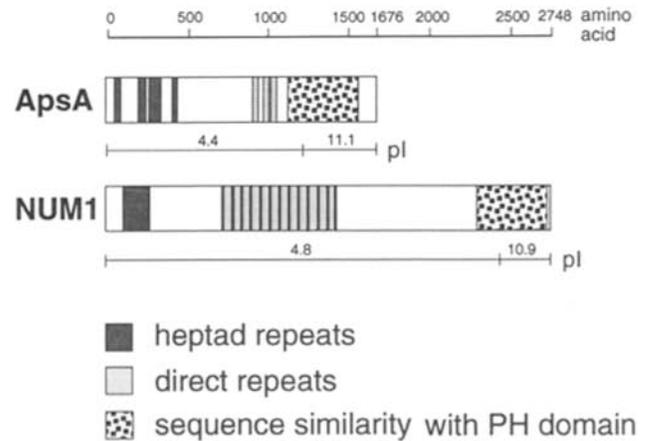


Figure 6. Comparison of *A. nidulans* ApsA with *Saccharomyces cerevisiae* NUM1p. (A) Significant similarity was found in the carboxy terminus of the two proteins. For the alignment, the Jotun Hein method (MegAlign program, LaserGene Navigator, DNASTAR Inc., Madison, WI) was used (Gap penalty 11, Gap length penalty 7). The region of homology starts at amino acid residue 1114 in the case of ApsA and at 2301 in the case of NUM1p. In both proteins a PH domain was found (location indicated with arrows). (B) Comparison of ApsA to NUM1 including sequence similarity, heptad repeats, direct repeats, PH domain, and charge distribution.

addition to developmental arrest, many conidiation-specific genes fail to become active in *apsA* mutants (Zimmermann, 1986), indicating either that the product of *apsA* is directly responsible for activating them or that an *apsA*-directed function is prerequisite for continuation of the transcriptional program.

We cloned and characterized the *apsA* gene to gain insights into the function of its product. Cosmid clone CRF1 was obtained which complemented the *apsA1* mutant allele at high frequency. Four lines of evidence showed that the clone contains the *apsA*⁺ gene: (1) A 10.6-kb BamHI subfragment of CRF1 was sufficient for complementation of the *apsA* defect in *trans*; (2) integrative mutation or deletion of the putative *apsA* transcription unit produced colonies with the *apsA* phenotype; (3) introduced mutations were tightly linked to the *apsA1* mutation; and (4) the introduced and *apsA1* mutations were noncomplementing in forced heterokaryons. The phenotype of the *apsA1* mutant raised the possibility that the mutation was not null; occasionally nuclei entered metulae and phialides and chains of conidia were then produced. However, the phenotype of null mutants obtained by deletion of essentially the entire transcription unit was indistinguishable from the phenotype of the *apsA1*

strain, showing that bud nucleation can occasionally occur in the absence of the *apsA* product (ApsA).

We detected *apsA* transcript in hyphae and in conidiating cultures at about the same levels (result not shown), raising the possibility that the gene has vegetative as well as reproductive functions. We confirmed this when *apsA* mutations resulted in a moderate, but readily detectable, phenotypic change in hyphae in addition to their dramatic effects on conidiophore development. In the mutants, hyphal nuclei occurred in punctate clusters whereas nuclei were evenly distributed in the wild type (Fig. 7, A and B; Clutterbuck, 1994), indicating that *apsA*⁺ is required for normal nuclear distribution. *A. nidulans* nuclear distribution (*nud*) mutants have been identified and characterized (Morris, 1976; Osmani et al., 1990; Xiang et al., 1994), but their vegetative defects distinguish them from *apsA* mutants. *nud* mutants were isolated as temperature sensitive for growth and possessing nuclei that failed to migrate out of the germinating spore body into the germ tube at the restrictive temperature. Mitosis continued in these mutants so that upon prolonged incubation, many nuclei accumulated in the spore body and left an anucleate hypha that stopped growing. Genetic mapping studies indicate that the *nud* and *aps* mutations identify

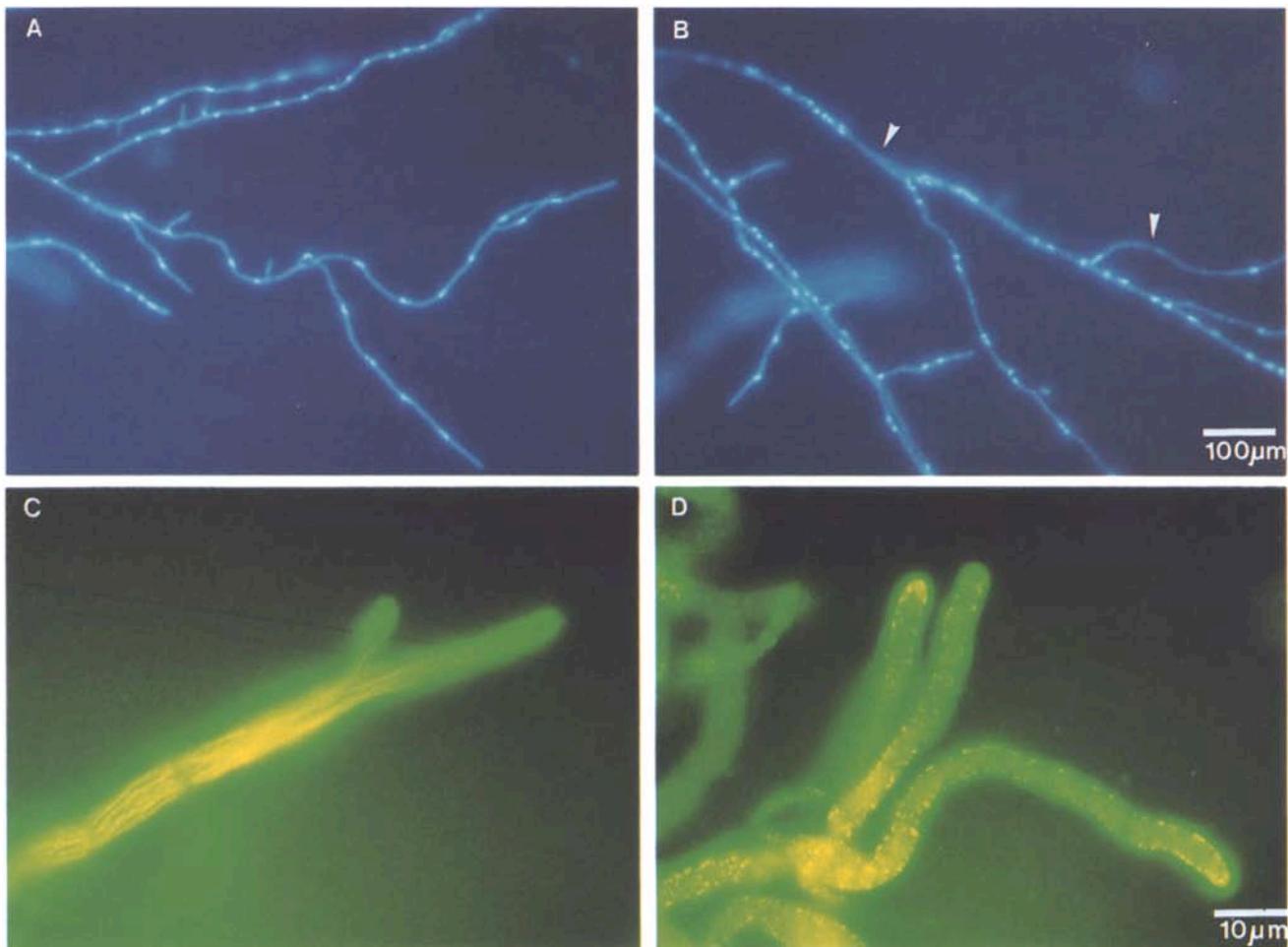


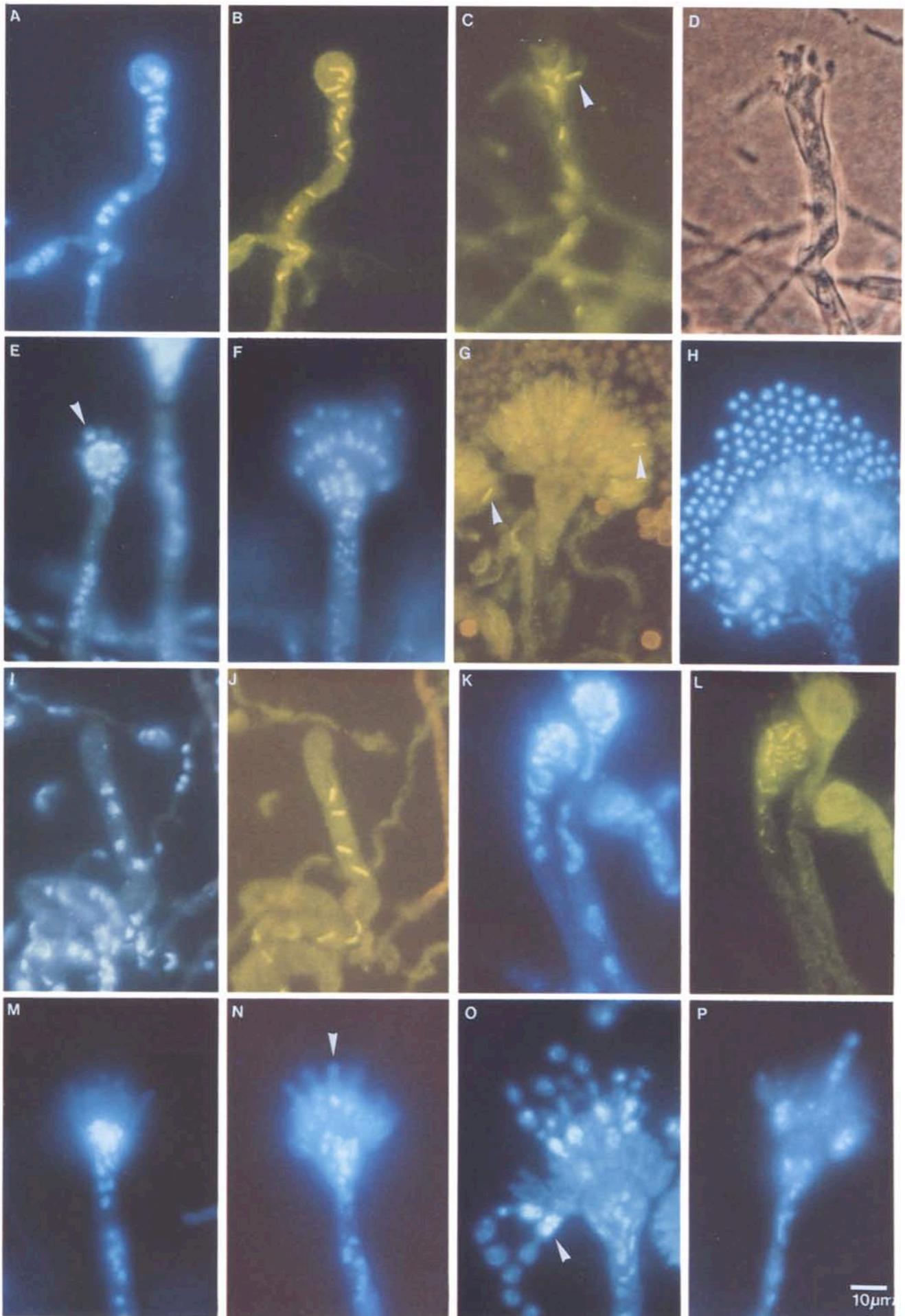
Figure 7. Nuclear distribution, cytoplasmic microtubules, and actin in hyphae of *A. nidulans*. (A) Nuclei in wild-type hyphae (FGSC26; Table I) are evenly distributed. (B) Clusters of nuclei are separated by large gaps (arrows) in mutant hyphae (DRF60; Table I). (C) Immunofluorescence of cytoplasmic microtubules and (D) actin in *apsA* mutant hyphae.

different loci (Morris, N. R., personal communication). Moreover, *apsA* mutants grow and branch almost normally and nucleation of germ tubes and hyphal tips is not affected. Thus, the nuclear translocation machinery remains largely unaffected by the absence of ApsA, even though the regular distribution of nuclei along the hyphae is disturbed.

These observations lead us to propose a “railway system” model for nuclear movement and distribution in fungi. In this model, the cell possesses a basic machinery for nuclear movement; microtubules, microtubule-associated proteins, and molecular motors (Oakley and Morris, 1980, 1981; Osmani et al., 1990; Xiang et al., 1994). Similarly, a railway

system has tracks and train cars with components to keep them on the tracks and to move them forward and backward. Mutations interfering with this basic machinery will often destroy the cell’s ability to translocate nuclei, resulting in a severe phenotype. By contrast, ancillary mechanisms instruct migrating nuclei where to stop, when to start, and when to travel in a new direction, just as a train car stops precisely at a given station, starts on schedule, and, when appropriate, moves in a different direction after separating from the remainder of the train. Mutations affecting this ancillary machinery would often not disrupt nuclear movement per se but would interfere with the cell’s ability to ensure that

Figure 8. Comparison of nuclear distribution and localization of nuclei undergoing mitoses via phase contrast microscopy, DAPI stain of nuclei (blue fluorescence), and anti-microtubule stain of mitotic spindles (green fluorescence). (A–H) Development of an *A. nidulans* *apsA*⁺ conidiophore (FGSC26; Table I). (A and B) Multiple nuclei undergo synchronized mitoses in stalk and vesicle of a young conidiophore. (C and D) One nucleus undergoes a localized mitosis at the budding neck between vesicle and metula (arrowhead). (E) One metula contains a nucleus (arrowhead), the others are still anucleate. (F) All metulae contain nuclei, phialides are partly nucleated. (G) Conidiophore with metulae and phialides. Mitoses occur asynchronous (arrowhead). (H) Fully developed conidiophore head with conidia. Each conidium contains a single nucleus. (I–P) Development of an *apsA*[−] conidiophore (DRF60; Table I). (I and J) Multiple synchronized mitoses in stalk and vesicle. (K and L) Multiple mitoses in the vesicle but not localized at the budding neck. (M) Vesicle contains many nuclei and metulae are fully developed, but anucleate. (N) One metula received a nucleus (arrowhead). (O) Conidiophore head with some single chains of conidia. Many metulae and phialides are multinucleate (arrowhead). (P) One hyphal-like metula is growing out of the conidiophore head.



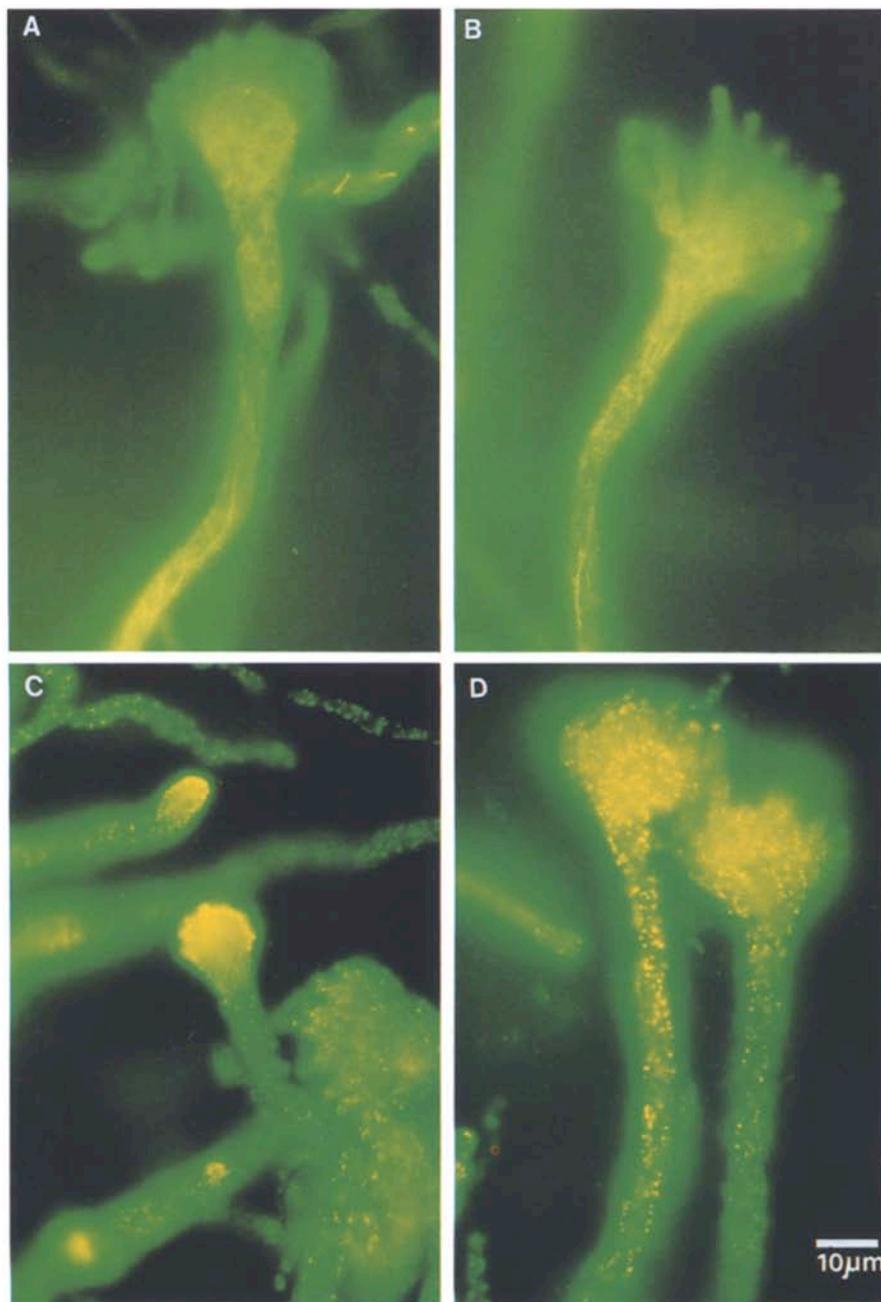


Figure 9. (A and B) Cytoplasmic microtubules and (C and D) actin distribution in conidiophores of the *apsA* deletion strain DRF60.

nuclei are correctly positioned, resulting in a more subtle phenotype. The phenotypes resulting from mutations in mechanistic and regulatory genes are expected occasionally to overlap. For example, partial defects in mechanistic functions could produce mild phenotypes, whereas defects in major regulatory functions could produce more severe phenotypes. The inferred functions of the products of *nud* genes are consistent with both mechanistic and regulatory roles (Morris, 1976; Osmani et al., 1990; Xiang et al., 1994; N. R. Morris, personal communication).

Why might there be a more stringent requirement for *apsA*⁺ function during one stage of conidiophore development than during vegetative growth or other stages of development? The answer may reside in the transition from the coenocytic to cellular state that occurs when metulae bud off

from the surface of the multinucleate conidiophore vesicle (Fig. 8). We rarely observed localized mitoses at the budding neck of metulae, suggesting that mitosis and bud nucleation are separable events, in contrast to the situation in *S. cerevisiae* where bud nucleation occurs as a direct result of an oriented mitosis (Byers, 1981). Furthermore, we observed no defects in the orientations or structure of mitotic spindles in the *apsA*⁻ mutants. Thus, it is likely that post-mitotic nuclei must be accurately positioned within the large volume of the vesicle in order to move to and pass through the narrow bud necks efficiently. ApsA may be involved in positioning by fixing the nuclei near the bud necks. However, there was no obvious difference between wild-type and *apsA*⁻ mutants in the arrangement of nuclei within the vesicle, indicating that fixation, if it occurs, is transient. More-

over, the requirement for ApsA for bud nucleation is not absolute, because in null mutants nuclei do occasionally enter metulae, perhaps as a result of random interaction with the nuclear translocation machinery. This is similar to the situation with *S. cerevisiae* *NUM1*⁻ mutants in which bud nucleation occurs much less frequently (Kormanec et al., 1991). Moreover, NUM1p and ApsA have significant sequence similarities, reinforcing the idea that the two proteins have related functions. Unfortunately, the predicted amino acid sequences of NUM1p and ApsA have not provided many clues to their functions, although aspects of their predicted structures (PH domain at the COOH terminus of ApsA and NUM1p; three direct repeats in ApsA and twelve repeats in NUM1p) might suggest that they interact with microtubules or membranes or both (Schneider et al., 1988; Morrow, 1989; Lux et al., 1990; Aizawa et al., 1991; West et al., 1991; Saleeba et al., 1992; Cravchik et al., 1994). Additional studies will be needed to clarify the precise functions of these proteins.

It is of interest that once nuclei enter metulae the requirement for *apsA*⁺ is largely relieved. Nucleated metulae proceed to divide to produce phialides and these phialides go on to produce normal spores. Only occasionally does this process fail in the mutant leading to formation of abnormal, hypha-like cells. This observation implies either that nuclear positioning is relatively unimportant for cell divisions occurring after differentiation of metulae or that other cellular components assume the functions of ApsA. In the divisions giving rise to phialides or conidia, bud nucleation probably occurs by a mechanism similar to that found in *S. cerevisiae* (Kozakiewicz, 1978; Byers, 1981). Thus *A. nidulans* potentially possesses a functional equivalent of *NUM1* that is different from *apsA*. However, it remains plausible that the positioning and orientation of nuclear divisions in metulae and phialides are constrained by the shape and size of the cells and that nucleation of the daughter cell can occur in the absence of either *apsA*-like or *NUM1*-like activities.

The stringent, but incomplete, requirement for *apsA* function in bud nucleation suggests that entry of the nucleus into the metula represents a developmental checkpoint (Losick and Shapiro, 1993). Numerous mRNAs that accumulate specifically during the later steps of development in the wild type are under-represented or undetectable in conidiating cultures of *apsA*⁺ mutants (Zimmermann, 1986). Yet when an *apsA*⁻ nucleus occasionally enters a metula the developmental program continues on, presumably accompanied by activation of the normal repertoire of conidiation-specific genes. This result indicates that there is communication between the nucleus and the cytoplasm of the metula leading to continuation of the developmental transcriptional program only after the nucleus has successfully moved from the vesicle into the bud. This interpretation of results further suggests that the cytoplasm of the vesicle and metulae differ from one another and implies cytoplasmic polarization during bud formation. The challenge will be to identify cytoplasmic components that are unique to metulae and to test for their involvement in regulating subsequent developmental steps.

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