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HYP1, a Hydrophobin Gene from *Aspergillus fumigatus*, Complements the *rodletless* Phenotype in *Aspergillus nidulans*

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Aspergillus fumigatus **produces conidia that are highly dispersable and resistant to degradation. We have sought to analyze these properties by studying the rodlets which form the outer spore coat protein. Degenerate primers based on hydrophobins in other fungi were applied to genomic DNA from *A. fumigatus* in PCR. A product of this reaction with similarity to an *Aspergillus nidulans* gene as judged by Southern hybridization was chosen for further study. Cloning and sequencing revealed a gene with two introns which encodes a protein of 159 amino acids. Structural characteristics consistent with those of other fungal hydrophobin genes, especially conserved cysteine residues, are present. The expression of the gene is limited to the developmental stages in which maturing conidiophores are present. This *A. fumigatus* gene, *HYP1*, was used to transform a mutant strain of *A. nidulans* that lacks rodlets. Transformants with a single copy of *HYP1* expressed a rodlet layer on their conidia as observed by freeze-fracture electron microscopy.**

Aspergillus fumigatus is a major cause of invasive infection in the immunocompromised host (12). The predominance of *A. fumigatus* over other *Aspergillus* species may be due to specific virulence factors or more simply to the abundance of this species in the human environment. Conidia, which are the specialized structures of *Aspergillus* spp. that carry the determinants of dispersability, have been the subject of intensive and productive work with *Aspergillus nidulans* in the past several years (4, 22). Conidia are also the first form of the pathogen to encounter the host site from which invasive infection begins, and each stage of germination may have its own interaction with host cells (18). The recent cloning of the gene (*RODA*) encoding the spore coat protein of *A. nidulans* and analysis of its stage-specific regulation (20) have suggested a new avenue by which to investigate the roles of various conidial components. This spore coat protein, a layer of regularly arranged rodlets, is insoluble and is responsible for or intimately associated with conidial hydrophobicity of *Aspergillus* spp. and other fungi (3, 5). The extreme resistance of rodlets to chemical degradation has suggested a role for them in resistance to phagocytic defenses (6, 7). These observations indicate that rodlets could play a pivotal role in initiating infection by *A. fumigatus* and may provide an avenue by which to understand the relationship between dispersability and the ability of *A. fumigatus* to cause disease. In line with these observations, we have cloned and sequenced the *HYP1* gene (for hydrophobin) from *A. fumigatus* and expressed it in a *rodA* mutant of *A. nidulans* to complement the *rodletless* phenotype.

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MATERIALS AND METHODS

Strains, media, and library construction. *A. fumigatus* wild-type strain B-5233 is a clinical isolate maintained in our culture collection. Genomic DNA and poly(A)$^+$ RNA were extracted as described below, and library construction was provided commercially (Stratagene, La Jolla, Calif.). *A. nidulans* FGSC26 (*biA1*; *veA1*) is from the Fungal Genetics Stock Center (Kansas City, Kans.). *A. nidulans* RMS025 (*suA1 adE20*, *yA2*, *adE20*; *ΔargB*::*trpcΔB*, *ΔrodA*::*ArgB*; *pyroA4*; *ribo B2*, *veA1*) is a mutant with *RODA*, the gene encoding the hydrophobic, outermost spore coat protein of *A. nidulans* (20), deleted. All strains were grown on appropriately supplemented minimal media containing $NaNO_3$, glucose, salts, and trace elements (11).

Isolation, processing, and analysis of nucleic acids. All nucleic acid, including RNA from a developmental culture, was isolated as described by Timberlake (21). For developmental cultures, minimal broth was inoculated with 10^9 resting conidia, and the submerged culture was allowed to develop overnight in a shaking incubator at 30°C. The culture was filtered, and the harvested mycelia (the zero hour sample) were divided, plated on minimal agar, and allowed to grow to time points appropriate for *A. fumigatus* (5, 8, 11, and 14 h). Conidiophores and conidial chains were detectable by light microscopy 14 h after induction. Nucleic acid digestion, fractionation on agarose gels, and transfer to nylon membranes for hybridization were performed according to standard procedures (15). Random hexamer priming was used to label the DNA probes (Prime-it II; Stratagene). Enrichment for poly(A)$^+$ RNA was performed with Dynabeads (Dynal AS, Oslo, Norway). Poly(A)$^+$ RNA for construction of the cDNA library was extracted from overnight submerged cultures and from cultures of hyphae plated on solid media for 14 h after overnight submerged growth. The region of the genomic clone

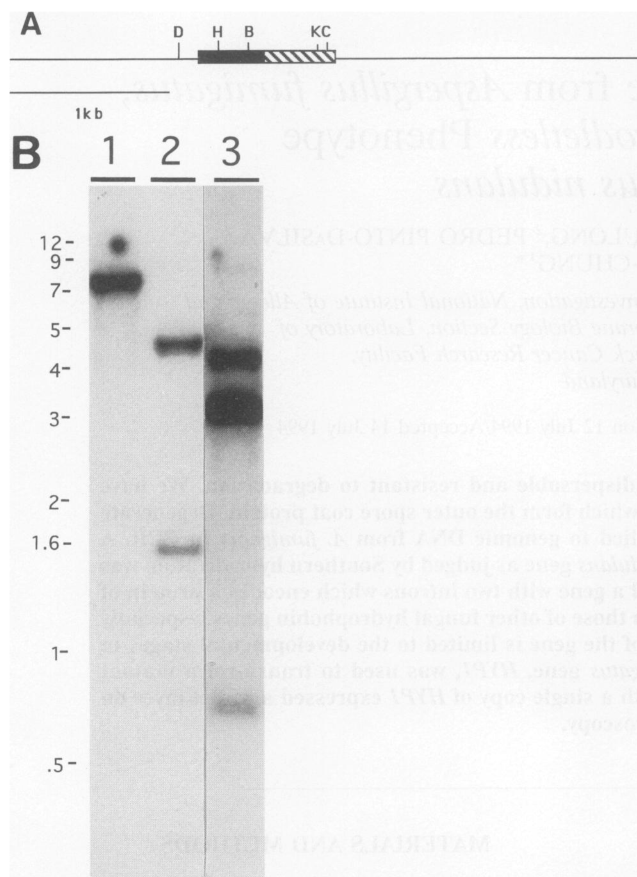

FIG. 1. (A) Restriction map of p453. The 4-kb insert isolated from a genomic library is shown. The 400-bp PCR product is shown by the black box; the rest of the open reading frame is indicated by the hatched box. B, *Bam*HI; C, *Cla*I; D, *Hin*dIII; H, *Hpa*I; K, *Kpn*I. (B) Genomic structure of the *HYP1* gene, showing Southern blotting of genomic *A. fumigatus* DNA digested with *Sal*I (lane 1: 4-h exposure), *Sal*I-*Bam*HI (lane 2; 4-h exposure), and *Hpa*I-*Cla*I (lane 3; 18-h exposure) and hybridized with p453. *Bam*HI, *Cla*I, and *Hpa*I each cut the genomic fragment once, and *Sal*I did not cut at all. The 700-bp *Hpa*I-*Cla*I fragment was used to indicate the presence of *HYP1* in transformants. The size markers are in kilobases.

containing *HYP1* was sequenced along both strands by using oligonucleotide primers in standard dideoxynucleotide chain-terminating reactions (16). Three cDNA clones were sequenced from their 5′ termini to determine the start of the message. Polyadenylation was used to identify the 3′ ends of the cDNA clones. Intron positions were inferred by comparison of the genomic and cDNA sequences. The predicted protein sequence of *HYP1* was compared with that of *RODA* by using The University of Wisconsin Genetics Group program.

PCR. Degenerate oligonucleotide primers Hp7 (TCGT[CT]GCT[CT]TC[GC]CCGCC[TC][CT]CG) and Hp4 (TT[GC]T[GC]GCAGCAG[GA]CA[GA]T[GC]T) were used to amplify a fragment of *HYP1* from *A. fumigatus* genomic DNA. PCR was performed with *Taq* DNA polymerase (Boehringer-Mannheim, Mannheim, Germany) in a 100-μl total reaction volume in 10 mM Tris-HCl–1.5 mM $MgCl_2$–50 mM KCl with 0.3 μg of each primer for 40 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min. To clone PCR products, the appropriate fragments were gel isolated (GeneClean II; Bio101, La Jolla, Calif.) and cloned into the *Sma*I site of pBluescript II SK(+) (Stratagene).

Clone construction and transformation of *A. nidulans*. pMS10 was a gift of M. Stringer and contains *RODA* from *A. nidulans* (20). p453 contains *HYP1* in a 4-kb fragment from a genomic library of *A. fumigatus* B-5233, and pMP7 contains the same genomic fragment as in p453 cloned into the *Sma*I site of pPL1 (13), a pUC19-based plasmid which carries the element responsible for complementation of the *riboB* mutation. pMP2 contains the 400-bp PCR fragment of *HYP1* cloned into the *Bgl*II site of pAN7-1, which contains the element responsible for hygromycin resistance (14). pMP4 contains 2 kb of the 5′ flanking region and 1.5 kb of the 3′ flanking region of *HYP1* cloned into the *Bgl*II and *Hin*dIII-*Xba*I sites, respectively, of pAN7-1. Transformation of RMS025 was performed by standard techniques (24) with 4 μg of linearized DNA per 10^7 protoplasts of pMP7. After 3 days of growth posttransformation, conidia from single colonies were isolated and plated on pyridoxine-containing medium. These colonies were grown to confluence and used for the analysis of complementation.

Phenotypic analysis and electron microscopy. Hydrophobicity was assessed by dropping 8 μl of distilled water on 3-day-old *Aspergillus* colonies. *A. fumigatus* (B5233) and *A. nidulans* (FGSC26) colonies shed water instantly, while RMS025, the *rodletless* strain of *A. nidulans*, absorbs water droplets, and colonies of this strain complemented with *HYP1* regain the ability to shed water. For electron microscopy, conidia were fixed in 2.5% glutaraldehyde (in phosphate-buffered saline [PBS], pH 7.4) for 2 h at room temperature, washed in PBS buffer four times in 1 h, and impregnated stepwise for 40 min in a 30% glycerol buffer solution. After 1 h in 30% glycerol and mounting on double gold discs, the conidia were quickly frozen in liquid-solid nitrogen slush and freeze-fractured at −130°C (Balzers Freeze-etching unit 301). Replication was in platinum-carbon at 45°C with reinforcement by carbon cast at 90°C. Replicas were floated in 5.25% hypochlorite bleach to remove the attached tissues, washed thoroughly in distilled water, picked up in formvar- and carbon-coated copper grids, and photographed by Philips EM 410 transmission electron microscopy.

RESULTS

Amplification of *HYP1* by PCR. Primers Hp4 and Hp7 (see Fig. 2) were constructed from areas of strong conservation in the published sequences of other fungal hydrophobins (*SC1*, *SC3*, *SC4*, and especially *RODA*) (9, 19, 20). These degenerate primers applied to *A. nidulans* and *A. fumigatus* genomic DNAs in PCR revealed bands of the predicted size on agarose gels stained with ethidium bromide. After Southern analysis of these PCR products, pMS10, which contains *RODA*, hybridized with an *A. fumigatus* product of approximately 400 bp under moderately stringent conditions. The PCR product was used as a probe to screen genomic and cDNA libraries of *A. fumigatus*.

Structure and sequence analysis of *HYP1*. The entire genomic sequence of *HYP1* is contained on a 4-kb insert from our genomic library (Fig. 1A). Southern blotting of genomic DNA digested with *Sal*I (no restriction site in the gene), *Sal*I-*Bam*HI (one asymmetric site in the *Sal*I fragment), and *Hpa*I-*Cla*I (which generates a 700-bp fragment that encom-

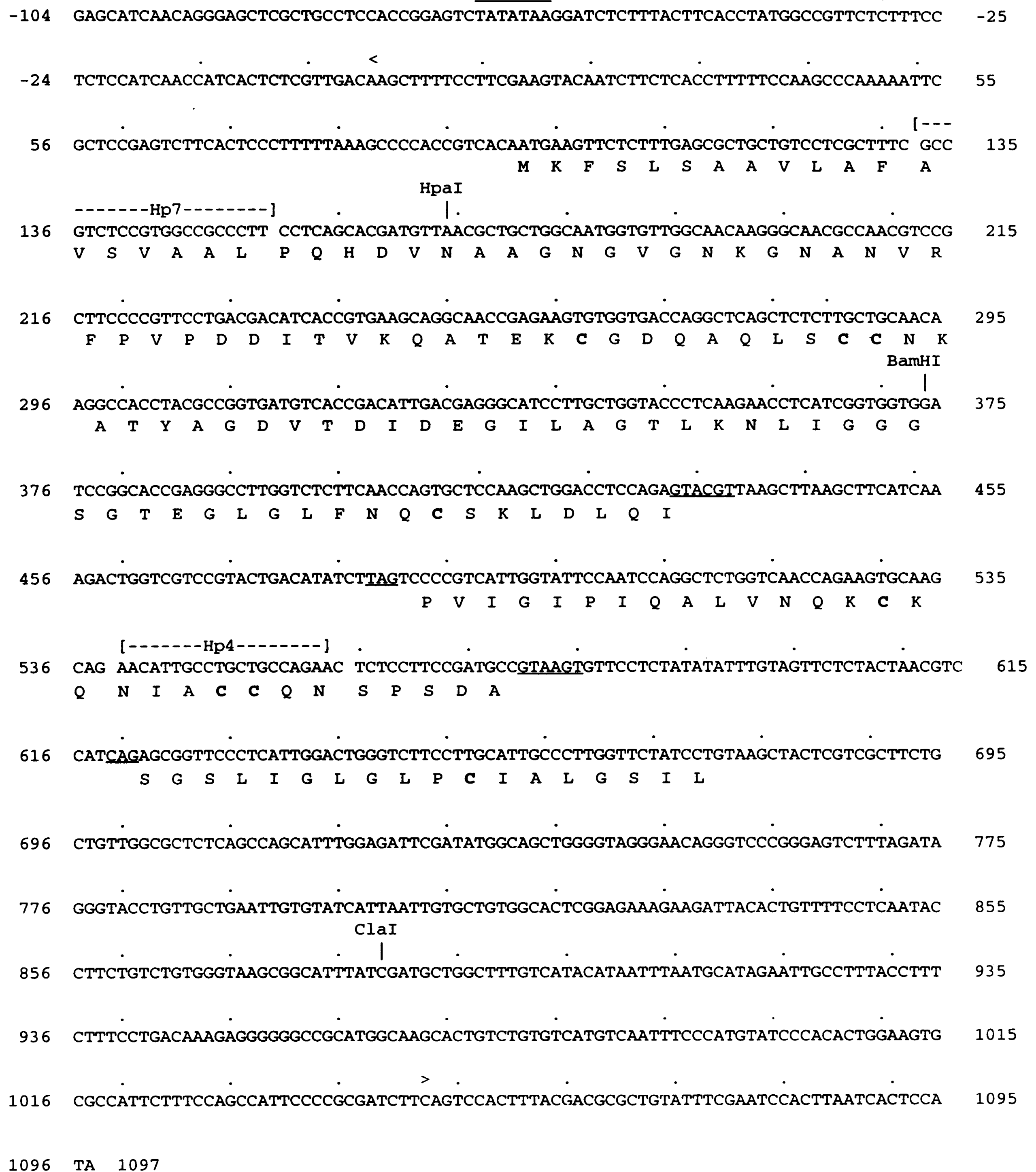

FIG. 2. Sequence of the *HYP1* gene. The PCR product amplified by using primers Hp4 and Hp7 (each bracketed with a broken overline) was used to isolate genomic and cDNA clones. Comparison of the genomic and cDNA sequences revealed two introns; these contain consensus splice signals (underlined) (2). The proposed amino acid sequence is in single-letter code. The eight cysteines characteristic of fungal rodlet proteins are in boldface (20). The transcription start (<) and poly(A)$^+$ addition (>) sites and a potential TATA box (overline) are noted. Restriction sites (*Bam*HI, *Hpa*I, and *Cla*I) are noted with vertical bars.

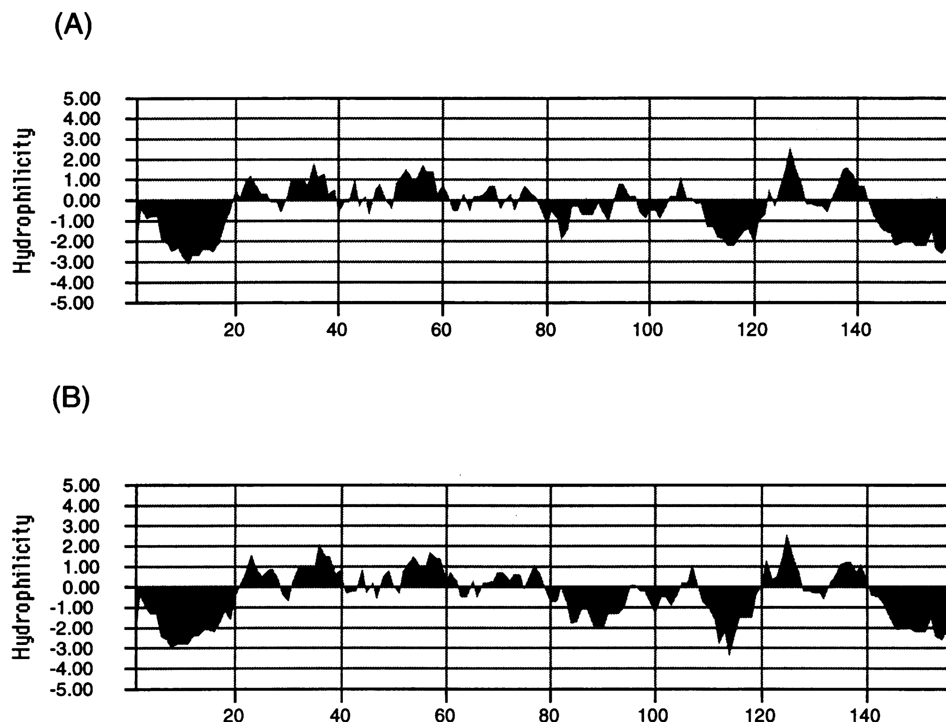

FIG. 3. Hydrophobicity comparisons of HYP1 (A) and RODA (B) Points above the center line represent hydrophilic regions. Plots were generated with the Kyte-Doolittle algorithm of the IBI MacVector 3.0 program (International Biotechnologies, Inc., New Haven, Conn.).

passes most of the coding region) suggested that only one copy of *HYP1* exists in the genome (Fig. 1B). Comparision of genomic and cDNA sequences revealed two introns (Fig. 2). The cDNA is 77% identical to *RODA* and encodes a putative protein of 159 amino acids. This polypeptide contains the arrangement of eight cysteine residues found in previous studies to be characteristic of this family of proteins (20), has a hydrophilicity plot comparable to that of RODA (Fig. 3), and is 86% identical to the RODA protein of *A. nidulans*.

Developmental expression. RNA was extracted from submerged cultures of *A. fumigatus* after overnight growth (zero hour) or from hyphal mats plated on minimal medium and allowed to grow for 5, 8, 11 or 14 h. Total RNA hybridized with the *HYP1* probe at all developmental time points revealed no expression of *HYP1* before 14 h, when conidial chains were first becoming apparent under the light microscope (data not shown). Northern (RNA) analysis of poly(A)$^+$-enriched RNA revealed no message at 0 h and a strongly positive signal at 14 h (Fig. 4).

Analysis of transformants. Our first attempts at disrupting functional *HYP1* in *A. fumigatus* failed. Transformation with plasmids constructed to yield deletion (pMP4) or disruption (pMP2) (see Materials and Methods) of *HYP1* in *A. fumigatus* yielded multiple integrations at ectopic sites; yet, with the genomic fragment from p453 as a probe, Southern analysis of genomic DNAs from more than 200 transformants revealed no change in the native gene.

To determine if the *A. fumigatus HYP1* gene complements the *rodletless* phenotype in *A. nidulans*, strain RMS025 was transformed with pMP7 (Fig. 5A). Fully mature conidia of transformants were tested with water droplets to assess their relative hydrophobicities. DNAs of those isolates that demonstrated a phenotype of increased hydrophobicity, indicating complementation of the *rodletless A. nidulans* mutation, were subjected to Southern analysis. Plasmid Bluescript II SK(+) was used to detect integration of the plasmid sequence in transformants to assess copy number (data not shown). The clones which contained a single copy were further analyzed with p453 to detect the *A. fumigatus* gene. One transformant, TMP5, contains the 700-bp band that indicates the presence of intact *HYP1* (Fig. 5B). Freeze-fracture electron microscopy of wild-type *A. fumigatus*, *A. nidulans*, RMS025, and TMP5 demonstrates that the transformant has a conidial surface with rodlets which are not present

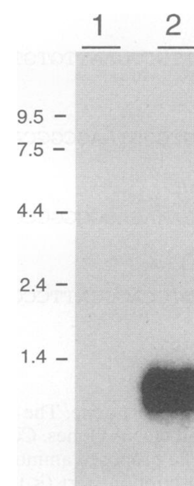

FIG. 4. Northern analysis of developmental cultures. RNA extracted at 0 h (lane 1) and 14 h (lane 2) was hybridized with a 400-bp fragment generated by PCR. The size markers are in kilobases.

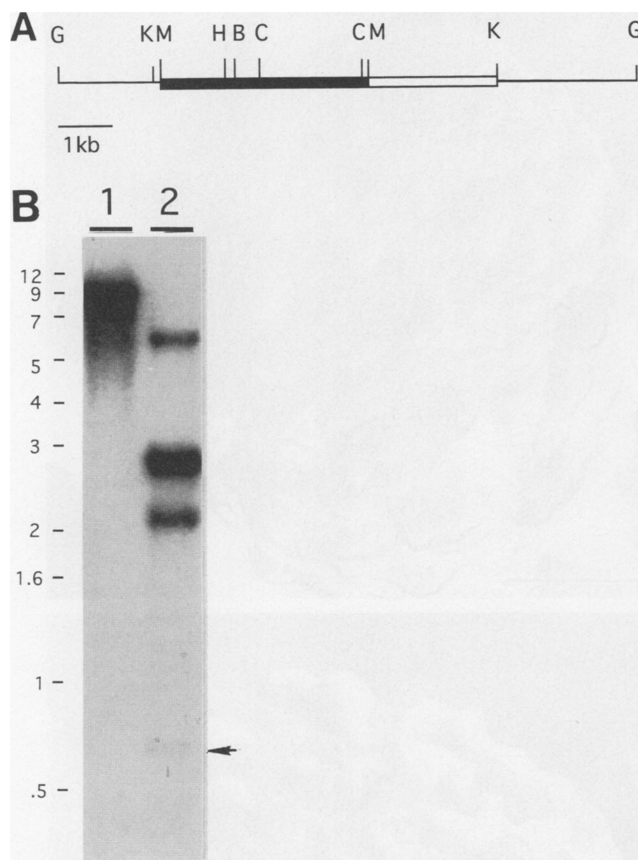

FIG. 5. (A) Restriction map of pMP7, showing pMP7, an 11-kb plasmid containing pUC19 (open box), *A. nidulans* DNA from pPL1 (straight line) (13), and the genomic sequence from *A. fumigatus* encoding *HYP1* (black box). The plasmid was linearized at the *Bgl*II site for transformation. The segment from the first *Bgl*II site to the black box is responsible for complementation of *A. nidulans riboB*. B, *Bam*HI; G, *Bgl*II; C, *Cla*I; H, *Hpa*I; K, *Kpn*I; M, *Sma*I. (B) Analysis of transformant TMP5. Genomic DNA was digested with *Bgl*II (lane 1) and *Hpa*I-*Cla*I (lane 2). Hybridization with p453 is shown and demonstrates the presence of the 700-bp fragment (arrow) which encompasses most of the coding region of *HYP1*. The size markers are in kilobases.

in the recipient strain before transformation (Fig. 6). We interpret the changes in TMP5 on Southern analysis and electron microscopy as expression of the product of the *A. fumigatus HYP1* gene.

DISCUSSION

The conidia of *A. fumigatus*, a major cause of disease in immunocompromised hosts, are infectious, widespread, and resistant to chemical degradation (5, 12). We have sought to analyze these properties by studying the rodlets which form the outer spore coat protein. After cloning and sequencing *HYP1*, we analyzed cultures that synchronize conidiation and found that *HYP1* transcript is detectable only in cultures that contain mature conidiophores. This is consistent with the analysis of developmentally regulated expression of genes in *A. nidulans* (20) and is important in understanding how rodlets might be involved in disease processes. If the *HYP1* gene is related to disease caused by *A. fumigatus*, the developmental analysis here indicates that this would be in the interaction between the host and conidia but not hyphae.

Previous work on the interaction of fungal spores with human cells has indicated that there is a substantial difference in this interaction depending on the lineage of the host cell and the stage of spore development (17). In the normal human host and animal models, *Aspergillus* species are of low virulence and are rapidly eliminated (17). This may occur when pulmonary macrophages ingest and kill resting conidia (18). Resting conidia that are induced to germinate first swell and at some point lose their outer coat as they produce filaments. Hyphae from conidia that have escaped the pulmonary macrophage line then are susceptible to a combination of the oxidative and nonoxidative products of neutrophils (8).

Conidial development involves the production of spores that have physical properties that allow them to be efficiently dispersed and resistant to extreme conditions such as UV light and dehydration in vitro. This resistance apparently is also effective against the oxidative stress provided by the human neutrophil but fortunately not against that of the resident macrophage of the human respiratory tract (23). It has been known for some time that there is a layer of the spore coat that is extremely resistant to solubilization and chemical degradation, and this has been associated with the layer of the spore coat that is visualized on electron microscopy as a fascicular coat or "rodlet" layer (3, 5, 7). In addition to providing resistance to environmental stress, this protein coat has been demonstrated to be associated with conidial hydrophobicity in *A. nidulans* (20).

We have obtained a mutant strain of *A. nidulans* that now expresses the *A. fumigatus* rodlet protein (Fig. 6). This will allow us to examine the species-specific nature of the interaction of conidia with human cells from normal individuals and from hosts, such as patients with chronic granulomatous disease of childhood, specifically prone to infection with *Aspergillus* spp. This is particularly interesting in the case of the latter disease, since there seems to be an unusual propensity of *A. nidulans* to infect patients with chronic granulomatous disease of childhood. We are interested in determining the role of the hydrophobins in this interaction, a line of investigation that might be quite fruitful inasmuch as the specific defects in the cytochrome oxidase systems from these patients have been outlined in much more detail recently (1).

Transformations with pMP2 and pMP4, which were intended to yield disruption or deletion, respectively, of *HYP1*, yielded no homologous integration as assayed by Southern analysis. This is consistent with reports that homologous integration in *Aspergillus* spp. and other fungi can be species, strain, and gene specific (10). Whether this relates to modification of DNA introduced before integration or to peculiarities of the locus has not been determined; however, transformation in *A. nidulans* was accomplished with relative ease. During the complementation of the *rodA* mutation in *A. nidulans*, *HYP1* was introduced in a large fragment of *A. fumigatus* DNA that contains no *A. nidulans* regulatory regions. This implies that all the functional elements necessary for expression of the *A. fumigatus* gene are sufficient in *A. nidulans*, thereby providing a system in which genetic manipulation is possible.

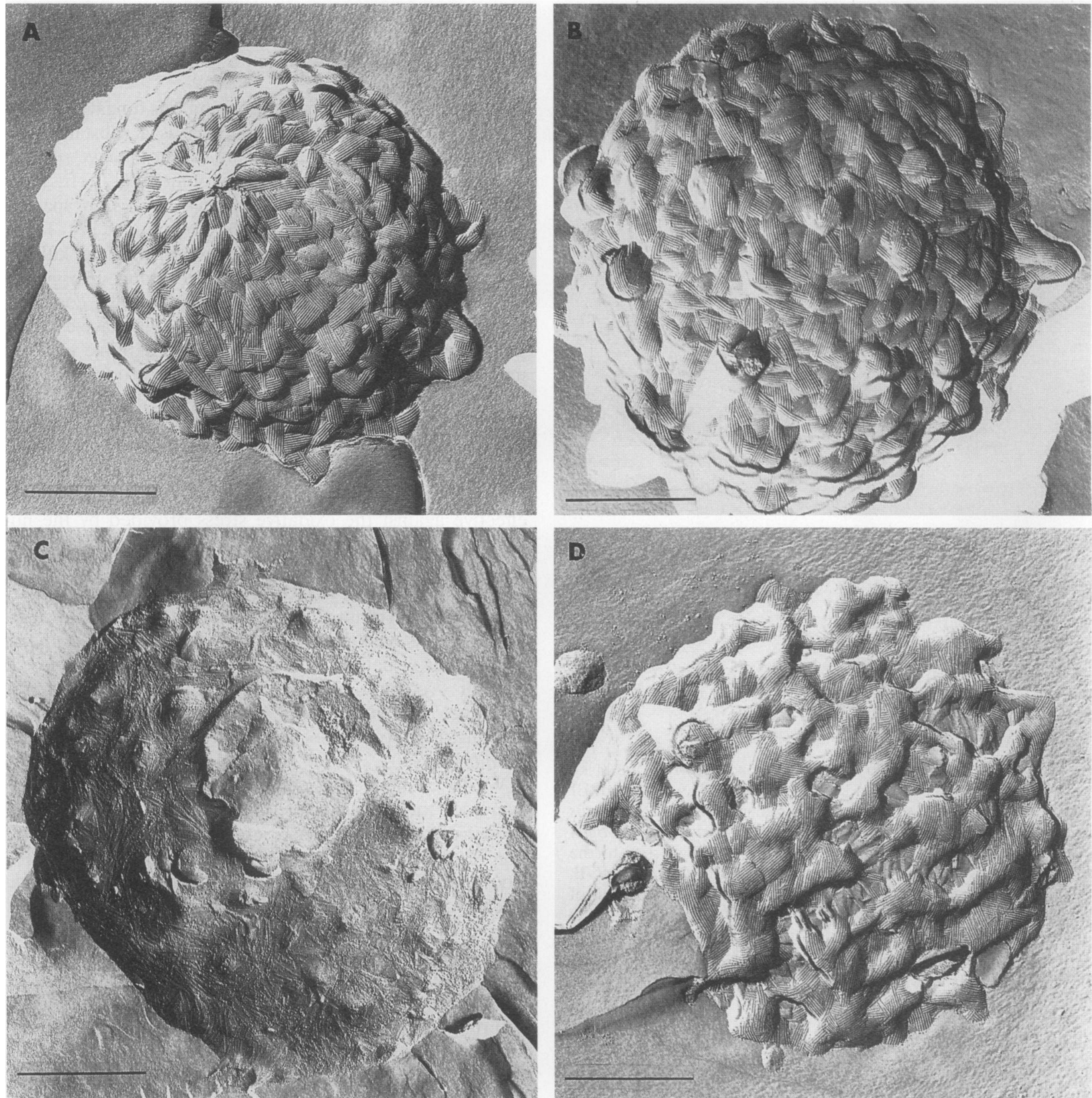

FIG. 6. Electron microscopy, showing replicas of wild-type *A. fumigatus* (A), wild-type *A. nidulans* (B), recipient strain RMS025 (C), and TMP5 (D). The rodlet layer present in the wild-type strains and TMP5 is absent in RMS025. Bars, 1 μm.

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