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Hyphal Tip Extension in *Aspergillus nidulans* Requires the *manA* Gene, Which Encodes Phosphomannose Isomerase

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A strain of *Aspergillus nidulans* carrying a temperature-sensitive mutation in the *manA* gene produces cell walls depleted of D-mannose and forms hyphal tip balloons at the restrictive temperature (B. P. Valentine and B. W. Bainbridge, J. Gen. Microbiol. 109:155–168, 1978). We have isolated and characterized the *manA* gene and physically located it between 3.5 and 5.5 kb centromere distal of the *riboB* locus on chromosome VIII. The *manA* gene contains four introns and encodes a 50.6-kDa protein which has significant sequence identity to type I phosphomannose isomerase proteins from other eukaryotes. We have constructed by integrative transformation a null mutation in the *manA* gene which can only be maintained in a heterokaryotic strain with wild-type *manA*$^+$ nuclei. Thus, a *manA* null mutation is lethal in *A. nidulans*. The phenotype of the mutation was analyzed in germinating conidia. Such conidia are able to commence germination but swell abnormally, sometimes producing a misshapen germ tube, before growth ceases. The reason for the lethality is probably the lack of synthesis of mannose-containing cell wall polymers that must be required for normal cell wall development in growing hyphae.

The fungal cell wall is critical for providing support against osmotic forces and for the maintenance of shape in the variety of cell types found in fungi. The regulation of cell wall synthesis provides the means by which these cell types are formed. Although many of the individual components of fungal cell walls have been identified, their organization in the mature wall and mode of synthesis are not well understood. Many fungi contain chitin, β-1,3-glucan, and β-1,6-glucan, in various ratios depending on the species, as the major structural polysaccharides (24). Also often present is a glycoprotein component consisting of heavily mannosylated protein. In *Saccharomyces cerevisiae*, this has been studied extensively (32, 33) and is known to consist of a protein moiety to which carbohydrate, consisting of *N*-acetylglucosamine, D-mannose, and D-glucose residues, is linked to Asn and/or Ser/Thr (5, 33). This carbohydrate is added to proteins in the endoplasmic reticulum and Golgi complex, and the mature mannoproteins are then secreted (11, 18). The mechanism by which some mannosylated proteins are secreted into the milieu while others become part of the cell wall is unclear, but incorporation into the wall may be through the formation of cross-links to other wall components (38, 39).

Mutants of *Aspergillus nidulans* defective in D-mannose utilization have provided an insight into the role of this sugar in fungal cell wall biosynthesis (15, 36). The *manA1* mutant strain of *A. nidulans* was isolated by Valentine and Bainbridge (36) as a temperature-sensitive revertant of the *mnrA455* temperature-sensitive mutant strain. Strains carrying *mnrA455* fail to grow on minimal medium at the restrictive temperature of 43°C unless supplied with D-mannose as the sole carbon source and cannot grow on D-glucose–D-mannose mixtures. The *manA1 mnrA455* revertant was selected by its ability to grow on D-glucose–D-mannose mixtures in the ratio 9:1 at 43°C. The *mnrA455* mutation produces a thermolabile phosphomannomutase (PMM), and therefore *mnrA455* probably defines the structural gene for PMM. The activity of phosphomannose isomerase (PMI) was greatly reduced in the *manA1* mutant at the restrictive temperature, but because PMI activity was apparently not thermolabile, it was suggested that *manA* may not represent the structural gene for PMI but rather may be a regulator of PMI activity.

At the restrictive temperature, strains carrying either *mnrA455* or *manA1* develop swollen hyphal tips (balloons) and cease growth (15, 36). It was also shown by these authors that the *manA1* mutant incorporated [^{14}C]mannose specifically at the hyphal tip because of the inability of exogenously added D-mannose to enter the glycolytic pathway and be further metabolized. In addition, cell walls prepared from the *manA1* mutant had reduced levels of D-mannose compared with the wild type. The ballooning phenotype of *manA1* strains was therefore attributed to reduced synthesis at the hyphal tip of mannose-containing cell wall polymers due to low PMI activity. The precise role of these mannose-containing cell wall polymers in the development of a mature cell wall remains unclear.

PMI (EC 5.3.1.8) is required to catalyze the reversible isomerization of fructose-6-phosphate to mannose-6-phosphate. Mannose-6-phosphate is then converted to mannose-1-phosphate by PMM, which serves as the precursor for synthesis of GDP-mannose and dolichol-P-mannose (dol-P-man), which are the D-mannose donors for mannoprotein biosynthesis (23, 32, 33). Thus, mutants defective in D-mannose utilization can display alterations in cell wall biosynthesis and morphology.

We are interested in the role of D-mannose and mannoproteins in fungal cell wall synthesis and wished to determine the nature of the defect responsible for the ballooning phenotype of *A. nidulans manA1* mutants. To this end we have cloned, sequenced, and begun the characterization of the *manA* gene. In addition, we have made a null mutation in the *manA* gene by integrative transformation and analyzed its phenotype in germinating conidia.

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MATERIALS AND METHODS

Chemicals and media. Medium components were purchased from Difco. All other chemicals were purchased from Sigma or Fluka Biochemika and were of the highest grade obtainable.

Strains and culture conditions. All *A. nidulans* strains were obtained from John Clutterbuck, University of Glasgow. G191 (*pabaA1, pyrG89; fwA1, uaY9*) was used as the reference strain. The isolation and characterization of mutant G851 (*pabaA1, biA1; AcrA1; facA303; manA1*) have been described previously (36). Strain PIM3 (*pabaA1, pyrG89, manA1; facA1*) was constructed by using standard techniques (8) from a cross of G191 and G851. Strains were propagated on either complex ACM (malt extract [2%, wt/vol], Bacto Peptone [0.1%, wt/vol], D-glucose [2%, wt/vol], solidified with agar [2%, wt/vol] as required) or minimal AMM containing normal supplements (8) at 37°C or, in the case of temperature-sensitive strains, at 30°C. The restrictive temperature for temperature-sensitive strains was 43°C. Sugars as carbon sources were supplied at 1% (wt/vol) or as indicated in Results. *pyrG* mutants were supplemented with filter-sterilized uridine (20 mM). *Escherichia coli* DH1 (*hsdR17 supE44 recA1 endA1 gyrA96 thi-1 relA1*) or DH5α (*hsdR17 supE44 ΔlacU169* [Φ80 *lacZΔM15*] *recA1 endA1 gyrA96 thi-1 relA1*) were used for propagation of plasmids and cultured with standard techniques (26).

Molecular biology techniques. Small- and large-scale plasmid isolation from *E. coli*, restriction enzyme digestion, *E. coli* transformation, Southern and Northern (RNA) blotting onto Hybond N (Amersham International, Zürich, Switzerland) membranes, plasmid construction, and agarose gel electrophoresis were performed according to standard techniques (26). Isolation of DNA fragments from agarose gels was performed with a Geneclean II kit (Bio 101, La Jolla, Calif.) as instructed by the manufacturer. DNA probes were prepared by using an Amersham Multiprime DNA labelling kit and [α-^{32}P]dCTP at ~3,000 Ci/mmol (Amersham). Southern and Northern blots were hybridized with labelled probes under conditions described in the text in a Hybaid standard hybridization oven (Hybaid Ltd., Teddington, United Kingdom). Autoradiography was performed with intensifying screens on Amersham Hyperfilm MP. Isolation of DNA from *A. nidulans* was by the following miniprep method. Mycelium from a 100-ml overnight culture was lyophilized, and ~100 mg was transferred to a microcentrifuge tube and ground to a powder with a pipette tip. Five hundred microliters of lysis buffer (Tris-HCl [200 mM, pH 8.5], NaCl [250 mM], EDTA [25 mM], sodium dodecyl sulfate [SDS; 0.5%, wt/vol]) was added to resuspend the mycelial powder, then 500 μl of phenol-chloroform-isoamyl alcohol (25:24:1) was added, and the tube was vortexed briefly. Following a 20-min centrifugation in a microcentrifuge, the aqueous phase was reextracted as described above and the nucleic acid was precipitated with isopropanol. The pellet was washed in 70% (vol/vol) ethanol, dried briefly, and resuspended in 50 μl of Tris-EDTA plus RNase A (50 μg/ml). Total RNA was prepared from liquid N_2 ground mycelium by the method of Cathala et al. (6). *A. nidulans* transformation was done by established procedures (1) from ACM-grown germinating conidia generated after 15 h of growth at 28°C. Plasmid DNA was prepared for sequencing by previously described methods (29) and sequenced with a Sequenase version 2.0 kit (United States Biochemical Corp., Cleveland, Ohio) as instructed by the manufacturer. Oligonucleotide primers for sequencing and PCR were synthesized on an Applied Biosystems model 394 DNA/RNA synthesizer or obtained commercially from Microsynth (Zürich, Switzerland). Perkin-Elmer Cetus GeneAmp PCR reagent kits (RNA or Standard) were used to generate cDNA or genomic fragments under conditions recommended by the manufacturer, which were then amplified in a Perkin-Elmer Cetus DNA Thermal Cycler. To sequence PCR-generated fragments, they were first purified by agarose gel electrophoresis to remove the amplifying primers and then sequenced according to the protocol described above except for a primer annealing time of 2 min. A 3′ rapid amplification of cDNA ends (RACE) technique was used to determine the location of the polyadenylation site in the *manA* gene. A cDNA synthesis reaction was performed on 300 ng of G191 poly(A)$^+$ RNA by using a Stratagene (La Jolla, Calif.) ZAP cDNA synthesis kit with the included oligo(dT) adapter primer. A portion of the cDNA reaction mixture was then amplified by PCR using the *manA*-specific primer CA GATTCGCTTCTTTATG and the adapter primer used in the cDNA synthesis reaction. The resulting ~500-bp fragment was subcloned into pUC18 and sequenced by using universal forward and reverse primers. The locations of the 5′ ends of the *manA* transcript were determined by primer extension mapping of G191 poly(A)$^+$ RNA, using a ^{32}P-labelled oligonucleotide (TTGGAGACGTAGAAGCGGTACCTGC) from positions +2 to +26 of *manA*, using standard methods for the primer extension reactions (26). The products of the primer extension reaction were run on a sequencing gel, using a sequencing ladder generated with the same oligonucleotide on a *manA*-containing plasmid as size markers. A control primer extension experiment with mouse 1B2 hybridoma total RNA was performed in parallel.

Computing. Computer programs supplied as part of the Genetics Computer Group version 7.0 sequence analysis package (10) running on a VAX 4000 were used for all sequence assembly, analysis, and comparisons.

Nucleotide sequence accession number. The nucleotide sequence data reported in Fig. 2 are in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession number M85239.

RESULTS

Isolation of the *A. nidulans manA* gene. The *manA1* and *riboB2* mutations of *A. nidulans* have been reported to be close together on linkage group VIII, with a recombination frequency of 0.8 (36). Although recombination frequency and physical map distance do not always correspond, such close linkage suggested that it should be possible to isolate a contiguous piece of cloned DNA containing both genes. The *riboB* gene has been isolated (21), and six cosmid clones containing *A. nidulans* genomic DNA inserts in the vector pWE15 or pLORIST2, which had previously been identified as containing the *riboB* gene (3a), were therefore tested for the ability to complement the temperature-sensitive growth defect of a *manA1* mutant strain. The six cosmid clones were introduced into the *manA1 pyrG89* strain PIM3 by cotransformation with plasmid pCAP2. This plasmid contains the *pyr-4* gene of *Neurospora crassa* and is able to complement *pyrG89* (35). The resulting PIM3 *pyrG*$^+$ transformants were allowed to grow for 20 h at the nonrestrictive temperature of 30°C before transfer to the restrictive temperature of 43°C. PIM3 cotransformants obtained with cosmid W7H05 were found to be able to grow at 43°C.

The method of Timberlake et al. (34) was used to identify restriction fragments of cosmid W7H05 which could complement *manA1*. A 20-kb *Not*I fragment isolated from the ~30-kb *A. nidulans* DNA insert of W7H05 was introduced into PIM3 by cotransformation and was able to complement the *manA1* mutation, permitting growth of PIM3 at 43°C. Smaller restric-

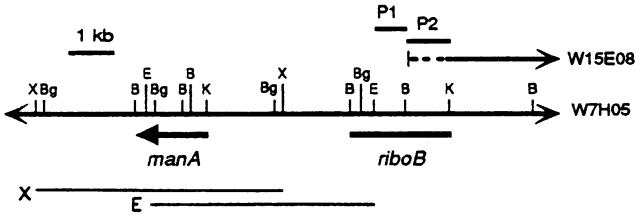

FIG. 1. Restriction map of the *A. nidulans manA* gene and surrounding DNA. The arrow represents the direction of transcription of the *manA* gene. The horizontal bars marked X and E indicate the *manA1*-complementing *Xba*I and *Eco*RI restriction fragments. The position of the *riboB* gene is marked. P1 and P2 represent the probe fragments 1 and 2 used for mapping the position of *manA* relative to *riboB* and the centromere. The approximate end of the *A. nidulans* DNA insert of cosmid W15E08 is indicated by the dashed line. X, *Xba*I; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; K, *Kpn*I.

tion fragments of the 20-kb *Not*I fragment were then isolated and tested for the ability to complement *manA1* in the same manner. In this way, we identified a 6.2-kb *Xba*I fragment which was able to repair the temperature-sensitive phenotype due to the *manA1* mutation with a transformation frequency of ~10 transformants per μg of fragment. In addition, we identified a 5.5-kb *Eco*RI fragment which could also complement but with a lower transformation frequency of ~0.5 transformants per μg of fragment. We inferred from this finding that the *Xba*I and *Eco*RI restriction fragments contained at least part of a gene able to repair the genetic lesion in the mutant *manA1* allele. Restriction enzyme mapping (Fig. 1) indicated that the 5.5-kb *Eco*RI fragment and the 6.2-kb *Xba*I fragment overlapped by 3.0 kb, suggesting that at least part of the complementing DNA was located within this region, which is shown in Fig. 1. The lower transformation frequency to growth at 43°C obtained with the *Eco*RI fragment suggested that it may not contain all of the wild-type gene but rather contains the portion of it that corresponds to the genetic lesion in *manA1* mutant allele. At least one end of the *Eco*RI fragment would therefore be predicted to encode the *manA* gene. DNA sequence information obtained from both ends of the *Eco*RI fragment revealed, at one end, an open reading frame (ORF) with significant identity to PMI from *S. cerevisiae* (31). The *manA1* temperature-sensitive mutation results in greatly reduced PMI activity at the restrictive temperature. The isolation of *manA1*-complementing DNA fragments containing an ORF encoding PMI which, as expected from genetic data, were closely linked to the *riboB* locus suggested that we had isolated the *manA* gene.

The *manA* gene. The complete DNA sequence of a 2,045-bp region in the overlapping, *manA1*-complementing, *Eco*RI and *Xba*I fragments from cosmid W7H05 was determined on both strands by subcloning suitable DNA fragments into pUC19. DNA sequence at the ends of these fragments was obtained by using M13 universal and reverse sequencing primers, and the sequence thus generated allowed the synthesis of novel oligonucleotide primers capable of initiating second-strand synthesis on previously unsequenced DNA (Fig. 2).

Analysis of the DNA and predicted protein sequence corresponding to the *manA* gene revealed four regions which either introduced breaks in the ORF or, when translated, had no homology to PMI from *S. cerevisiae*. This finding indicated that they probably contained intron sequences. Further examination of these regions showed the presence of consensus filamentous fungal intron splice sites bordering them (14). The exons contained a single ORF of 1,383 bp which was com-

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-385 CGGTGCTGGGACGCATTTGACGGGCCACGGCCGATGATTGGATGATTCCATGTTGATCAC  -326
-325 GGGGTCAGCTTATCCGTGACGGATGCGGTGCGATGCGGTGGCCGAATCAGGCAAGTCTAA  -266
-265 TGATTTAGGTCTTGTTTATTTCGAGGGGTAGAAGACAAGCTTAGCCAGCTCTGCTAATTC  -206
-205 TACCAGTCCAGATAAAAGGCAGACAGCTTCTGTACGCAGGAGAAACACAGAGGCGGCGTC  -146
-145 AAGAGTAAAAAACAACATATTTGAGCAGCTGCGAGGCAATTACCAGAAATCCACGCAGTC   -86
 -85 AATTGCCATCAGATACCCCGCTCTTGAGCTCATTCATCTACACAACACAGCTCGGTGTGT   -26
 -25 GCAGTAGCTTCTTGTCTTCGTGAAAATGCAGGTACCGCTTCTACGTCTCCAATGTGgtat    34
                                M  Q  V  P  L  L  R  L  Q  C  G
  35 gtctgttctctcgttctctcttttgagttgacgctaatgaatgtgtctcatagGCGTAA    94
                                                          V  N
  95 ATAGCTACGACTGGGgtaaggtccctccgttcgaatcgccgtttcatgtacatgtctaag   154
       S  Y  D  W  G
 155 ctcccacagGCAAGGTTGGCCCCGAGTCAGCAGCTGCAAAATATGCAGCGGCCACGGCGC   214
                   K  V  G  P  E  S  A  A  A  K  Y  A  A  A  T  A  P
 215 CCTCCGATTTCACTATTGAAGCAGACAAACCTTATGCAGAAgtaggtgttctaggatgcc   274
       S  D  F  T  I  E  A  D  K  P  Y  A  E
 275 cgtagtcgacacaatgcagtggacgagaagctgatccatgatagCTGTGGATGGGAACCC   334
                                                L  W  M  G  T  H
 335 ATCCCTCTCTTCCCTCGAAGGATGTTGAAACGCAGCGAACCCTGCTCGATATGGTCCAGG   394
       P  S  L  P  S  K  D  V  E  T  Q  R  T  L  L  D  M  V  Q  D
 395 ATAACTTGGCTTTGATGTCTCCGGAAGTTAGCGAACGATATGGTGGCAAGCTTCCATTCC   454
       N  L  A  L  M  S  P  E  V  S  E  R  Y  G  G  K  L  P  F  L
 455 TCTTCAAGGTGCTGTCGATCCGTAAAGCTCTCAGCATTCAGGCCCATCCGAACAAAAAGC   514
       F  K  V  L  S  I  R  K  A  L  S  I  Q  A  H  P  N  K  K  L
 515 TTGCGGAAGCTCTTCATGCCCGGGATCCCCGAAACTACCCCGgtaggtaaatcctctcta   574
       A  E  A  L  H  A  R  D  P  R  N  Y  P  D
 575 tgtggttctccttaattgaccttatcaattcagATGACAACCACAAGCCAGAGATGACCA   634
                                     D  N  H  K  P  E  M  T  I
 635 TTGCAATCACGCCTTTTGAAGGCCTGTGTGGCTTCCGACCTCTGGCTGAGATTGTCCATT   694
       A  I  T  P  F  E  G  L  C  G  F  R  P  L  A  E  I  V  H  F
 695 TTCTTAAAGCCGTTGCGCCCCTTCGTTACCTCATCGGCGTACAAACTGCAACTGACTTTG   754
       L  K  A  V  A  P  L  R  Y  L  I  G  V  Q  T  A  T  D  F  E
 755 AGAATGCGGTACGCGGCTTTGAAAACACCGAGGATCCTGAACAGACAAAGAAAAATAAAG   814
       N  A  V  R  G  F  E  N  T  E  D  P  E  Q  T  K  K  N  K  V
 815 TCGCGCTGCGAACTCTCTTTACCTCATTGATGCAATCTGCCTCCGAAAACATCGAGCAAG   874
       A  L  R  T  L  F  T  S  L  M  Q  S  A  S  E  N  I  E  Q  A
 875 CCGCGAGAGAGCTCGTTGCAGCTGCTCAGAGCTCCCCAGAGACGTTCGCATCGCTGGTCA   934
       A  R  E  L  V  A  A  A  Q  S  S  P  E  T  F  A  S  L  V  N
 935 ATGCTCCTGATACGAACCCTACTAACGCCGCTGAGTTGGCATCAATCATAATTCGACTAA   994
       A  P  D  T  N  P  T  N  A  A  E  L  A  S  I  I  I  R  L  N
 995 ACGAGCAGTTCCCCAATGATATTGGCTTGTTCGTCTTCTTCTTCCTTAACTTTGTTCGTC  1054
       E  Q  F  P  N  D  I  G  L  F  V  F  F  F  L  N  F  V  R  L
1055 TCGAGCCCGGAGAGGCTATGTTCCTCAAGGCCGACGATATTCACGCCTACATCTCCGGCG  1114
       E  P  G  E  A  M  F  L  K  A  D  D  I  H  A  Y  I  S  G  D
1115 ATATTATCGAATGCATGGCATCGTCCGACAATGTTGTTCGTGCGGGATTTACACCCAAGT  1174
       I  I  E  C  M  A  S  S  D  N  V  V  R  A  G  F  T  P  K  F
1175 TTAAGGACGTGGACACTCTCACTGAGATGTTGACGTACTCGTATGCGCCAATTGACGAGC  1234
       K  D  V  D  T  L  T  E  M  L  T  Y  S  Y  A  P  I  D  E  Q
1235 AGAAGCTGCAGCCTACGGACTATCCATACACGGTGCTGAATGCTGCGGCGTACTCGAGTG  1294
       K  L  Q  P  T  D  Y  P  Y  T  V  L  N  A  A  A  Y  S  S  A
1295 CTTCAGATTCGCTTCTTTATGATCCTCCAATTGAGGAATTCAGCGTGGTTAAGACGTCCC  1354
       S  D  S  L  L  Y  D  P  P  I  E  E  F  S  V  V  K  T  S  L
1355 TCCGCCGCACAGGGGCGAAAGCGACATTTGATCCGCTGACTGGGCCAAGTATTTTGATTT  1414
       R  R  T  G  A  K  A  T  F  D  P  L  T  G  P  S  I  L  I  C
1415 GCACTGGGGGTACTGGTAAGATCTCGGTAGGTCACAAGACAGAGGAAGTCAAAGAGGGGT  1474
       T  G  G  T  G  K  I  S  V  G  H  K  T  E  E  V  K  E  G  Y
1475 ATGTGTTCTTTGTAGGCGCAAATGCCGAATGCATCATTGAGAATACCGGAACTGGGTCGG  1534
       V  F  F  V  G  A  N  A  E  C  I  I  E  N  T  G  T  G  S  D
1535 ATGAGGAGAATGTTTTTACTACATTCAAGGCTTTTTGTGACTTGACAGGAAAGGAGGATA  1594
       E  E  N  V  F  T  T  F  K  A  F  C  D  L  T  G  K  E  D  M
1595 TGGCCAACGGACACTAGGGCTACAACTGCACGTATGAACAAAATTTAACGAATTCTGTTT  1654
       A  N  G  H  *
1655 GCTTCAAAAAAAAAAAAAAAAAA  1677
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FIG. 2. Sequences of the *A. nidulans manA* gene and protein. Intron sequences are indicated in lowercase letters in which conserved splicing elements are shown underlined. The poly(A) site as determined by 3′ RACE PCR is shown in italics. The position of the major transcription start site at −113 is indicated by a #, and minor sites are shown with a @ over the initiating nucleotide.

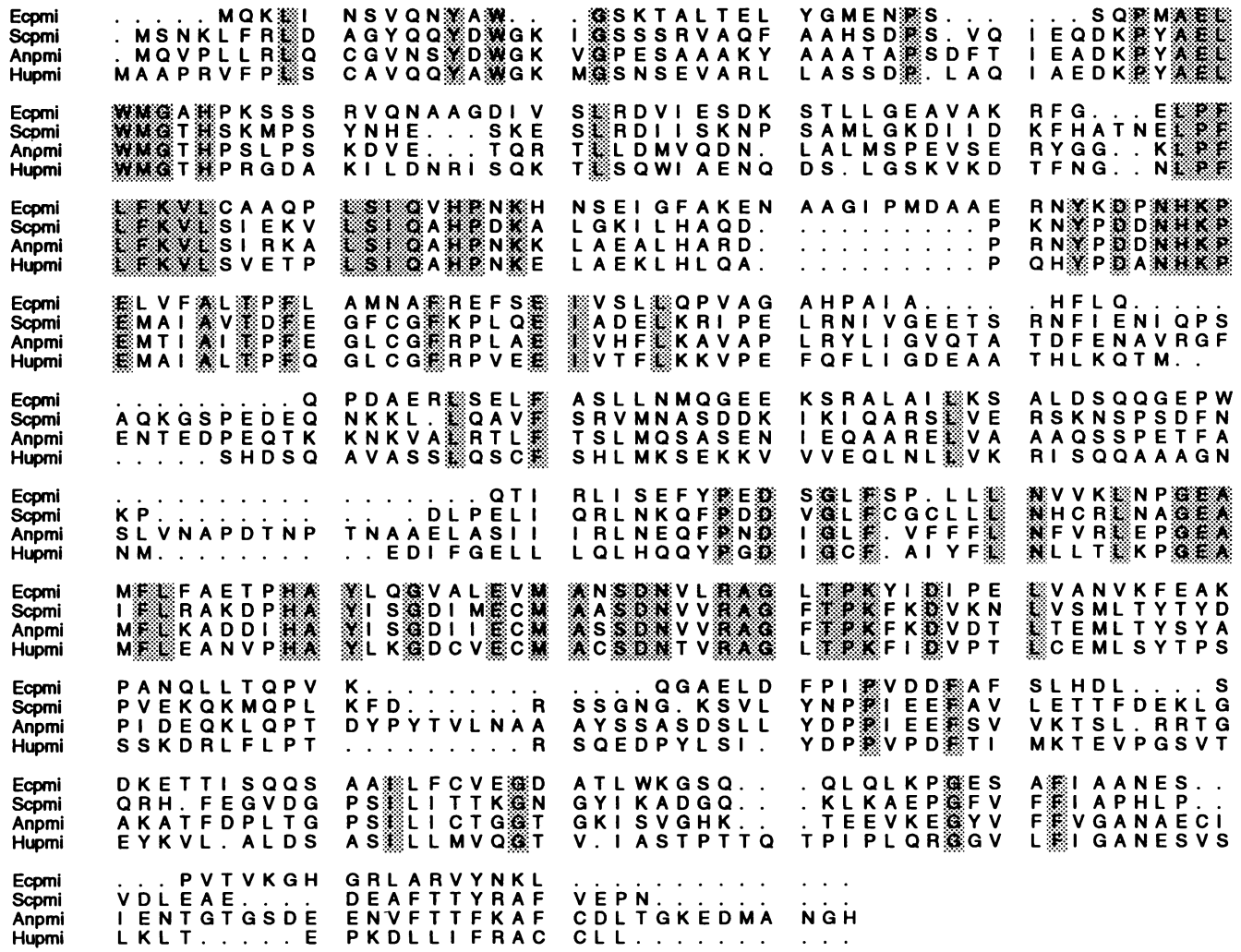

FIG. 3. Multiple alignment of PMI primary sequences. Ecpmi, *E. coli* PMI (9, 16); Scpmi, *S. cerevisiae* PMI (31); Anpmi, *A. nidulans* PMI; Hupmi, human PMI (25). The alignment was performed with the Genetics Computer Group program Pileup (12), using the default parameters. Identical residues conserved in all four proteins are shaded.

pletely contained within the *Xba*I fragment (Fig. 1) and encoded a 461-amino-acid protein of 50.6 kDa which had 36, 48, and 42% identity to PMIs from *E. coli* (16), *S. cerevisiae* (31), and humans (25), respectively (Fig. 3).

Additional evidence that the cloned gene corresponds to *manA* was obtained by sequencing on both strands a cloned *manA1* allele. As predicted from the complementation experiments, which demonstrated that a 5.5-kb *Eco*RI fragment containing a part of the wild-type *manA* gene was able to complement *manA1*, a single point mutation was found in the corresponding *manA1* allele *Eco*RI fragment (30). Southern hybridization of *A. nidulans* genomic DNA using a *manA* gene-specific probe indicated that the gene appears to be unique in the genome (data not shown).

The presence of introns in the *manA* gene was confirmed by using reverse transcription PCR on total RNA prepared from *A. nidulans* G191 to generate a partial cDNA fragment. Two PCR primers flanking all four putative introns were used and gave a DNA fragment of a size which indicated that it lacked intron sequences. This fragment was then sequenced directly by using internal sequencing primers corresponding to exon regions, and analysis of the sequence revealed that all of the putative intron sequences were absent.

The locations of the 5′ ends of the *manA* transcript were determined by primer extension transcript mapping of G191 poly(A)$^+$ RNA. We identified a major transcription start site at position −113 in addition to several minor sites between −159 and −75 (Fig. 2). Major and minor multiple transcription initiation sites are frequently found in fungal genes (14), and as in this case, a consensus TATA element need not be present upstream of them. To determine the location of the polyadenylation site of the *manA* gene, 3′ RACE PCR was performed as described in Materials and Methods. The resulting amplified fragment was subcloned, and sequence analysis of it showed a poly(A) sequence commencing at bp +1660 following a 48-bp untranslated region after the stop codon (Fig. 2).

Chromosomal location of the *manA* gene. The complementation of the *manA1* mutation by a cosmid clone containing the *riboB* gene as well as classical genetic analysis (36) shows that *riboB* and *manA1* are physically linked on linkage group VIII. Examination of the restriction map of the DNA region con-

taining *manA* showed similarity with a part of the restriction map of the *riboB* gene determined by Oakley et al. (21). Although the sequence of the *riboB* gene has not been determined or its position precisely mapped, it has been localized to the region of DNA indicated on the restriction map in Fig. 1. The *manA* gene is therefore located between 3.5 and 5.5 kb from *riboB*.

The *mipA* gene, encoding gamma tubulin, is also closely linked to *riboB* on chromosome VIII and has been determined by genetic techniques as being located centromere proximal to it (37). Examination of the restriction map of the *riboB* and *mipA* region reported by Oakley and Oakley (20) and Oakley et al. (21) showed that a 0.7-kb *Bam*HI-*Eco*RI fragment (probe 1) of the *riboB*-containing region was the centromere-distal fragment and the contiguous 1.1-kb *Bam*HI-*Kpn*I fragment (probe 2) was centromere proximal (Fig. 1). Probes 1 and 2 were used on Southern blots of *Bam*HI restriction digests of the *manA1*-complementing cosmid W7H05 and cosmid W15E08, which did not complement *manA1*. The centromere-distal probe 1 hybridized to a fragment of the predicted size in W7H05 but did not hybridize to any fragments in W15E08. The centromere-proximal probe 2 also hybridized to a fragment of the expected size in W7H05, but in W15E08 a fragment larger than expected hybridized, indicating that it was at one end of the cloned DNA and so fused to vector sequences. Thus, one end of the *A. nidulans* DNA insert of W15E08 terminates in the middle of probe 2 at the *riboB* locus and extends toward the centromere (Fig. 1). As W7HO5 is capable of complementing the *manA1* mutation and both probes 1 and 2 hybridized to it, then *manA1* must be situated centromere distal to *riboB*.

Analysis of *manA* gene transcription. In *S. cerevisiae*, the *PMI40* gene encoding PMI is transcriptionally regulated by the carbon source used for growth, and steady-state levels of *PMI40* mRNA are increased by growth on D-mannose as the sole carbon source (31). We wished to determine whether the *manA* gene of *A. nidulans* was also subject to such regulation. Three 25-ml shake flask starter cultures of *A. nidulans* G191 in AMM plus D-glucose, D-mannose, or glycerol were grown for 24 h and used to inoculate 200 ml of an identical fresh medium; these cultures were grown for 14 h, and the mycelia were harvested by filtration. A Northern blot of total RNA prepared from these mycelial samples was probed with a *Bam*HI-*Bgl*II internal fragment of the *manA* gene. A 1.8-kb transcript was detected, the levels of which did not significantly vary according to the carbon source used (data not shown). The equivalency of the RNA loading on the gel was verified by reprobing the filter with a fragment of the *A. nidulans* actin gene (13).

Disruption of the *manA* gene. We wished to determine the phenotypic effect of a complete absence of PMI activity due to the *manA* gene on hyphal extension and conidial germination in *A. nidulans* and so constructed a strain carrying a disrupted *manA* gene. We predicted that such a gene disruption would be lethal to *A. nidulans* but that the disruptant strain would require D-mannose for growth and should be able to grow on media supplemented with D-glucose and D-mannose in the ratio of 9:1 in a similar manner to *manA1* mutant strains.

An *A. nidulans* transformation vector was constructed by inserting an *Eco*RI-*Sma*I fragment containing the *N. crassa pyr-4* gene from the vector pCAP2 (35) into the *Nde*I site of pUC19. The resulting plasmid, pGFV1, has no extensive sequences homologous with the *A. nidulans* genome and therefore abolishes all homologous recombination except that between *A. nidulans* DNA fragments subcloned into this vector and their genomic counterpart when used in *A. nidulans* transformation experiments.

An internal *Bam*HI-*Bgl*II fragment of the *manA* gene was inserted into pGFV1 to generate the *manA* gene disruption plasmid pANPMIΔ3 (Fig. 4). This plasmid was used to transform *A. nidulans* G191 to uridine prototrophy. Transformed protoplasts were plated on AMM without uridine supplemented with 0.9% D-glucose and 0.1% D-mannose. Conidia from the 44 resulting transformants were tested for the ability to grow on the same medium or one supplemented with 1% D-glucose alone. No transformants which were able to grow only in the presence of D-mannose were identified, suggesting either that a *manA* gene disruptant cannot grow even in the presence of D-mannose or that we had not disrupted the *manA* gene. The 44 transformants were then retested for the ability to grow on AMM without uridine when inoculated as a hypha-containing agar block (heterokaryotic inoculum) or as conidia (uninucleate inoculum). Of the 44 transformants tested in this way, we identified 5 for which conidia could not grow but the corresponding mycelial inoculum gave rise to viable colonies. On AMM containing uridine, both conidial and hyphal inoculums could grow. This is the phenotype predicted for a heterokaryotic strain with both transformed (*manA pyrG*$^+$) nuclei and untransformed (*manA*$^+$ *pyrG*) nuclei in which *manA* is an essential gene with a recessive phenotype (19, 22). The addition of 0.1% D-mannose and 0.9% D-glucose to AMM lacking uridine did not permit growth of the five putative heterokaryotic *manA* gene disruptants.

Genomic DNA was prepared from the five heterokaryotic transformants, parental strain G191, and one transformant with viable conidia on AMM lacking uridine, which would be predicted not to have undergone a *manA* gene disruption. A Southern blot of these *Bgl*II digested DNAs was probed with the 0.65-kb *Bam*HI-*Bgl*II fragment of *manA* DNA contained in the disruption plasmid pANPMIΔ3 (Fig. 4). This probe detects the presence of both the disrupting plasmid and the wild-type *manA* gene and showed that one of the five transformants, ΔPMI-F (Fig. 4, lane 1), had the expected 3.1-kb *manA* wild-type *Bgl*II fragment and an additional 8.1-kb fragment predicted from the single-copy integration of pANPMIΔ3 at the *manA* locus. The similar band intensities also suggested single-copy integration and that half of the nuclei were parental type and half were *manA* gene disruptants. Control DNA prepared from the untransformed parent strain G191 (lane 6) had only the 3.1-kb *manA* wild-type *Bgl*II fragment. Three of the remaining 4 heterokaryotic transformants (lanes 2, 3, and 5) had, in addition to the wild-type 3.1-kb *manA* band, larger bands which may be due to multiple integration of pANPMIΔ3 at the *manA* locus or integration by nonhomologous recombination at sites in an essential gene other than *manA*. In the remaining heterokaryotic transformant, ΔPMI-C (lane 4), two fragments of 2.5 and approximately 18 kb were found, suggesting that rearrangements of a disrupted *manA* gene may have taken place. The conidial viable strain PMI-A (lane 7) had the expected 3.1-kb *manA* fragment and a larger fragment, probably due to integration of pANPMIΔ3 at a nonhomologous and nonessential site.

Hyphal growth in a *manA* gene disruptant. To study the effect of lack of PMI activity on hyphal development in *A. nidulans*, conidia from the ΔPMI-F heterokaryon carrying wild-type and disrupted *manA* genes were used to inoculate 100 ml of AMM, and samples were removed at time intervals for microscopic analysis (Fig. 5). Germination of *manA*$^+$ *pyrG* conidia was suppressed by omitting uridine from the growth medium. Conidia from the parental strain G191 were used to inoculate a similar flask supplemented with uridine. Both G191 and ΔPMI-F conidia appear to begin the normal germination process by swelling slightly. After 8 h of incubation, G191

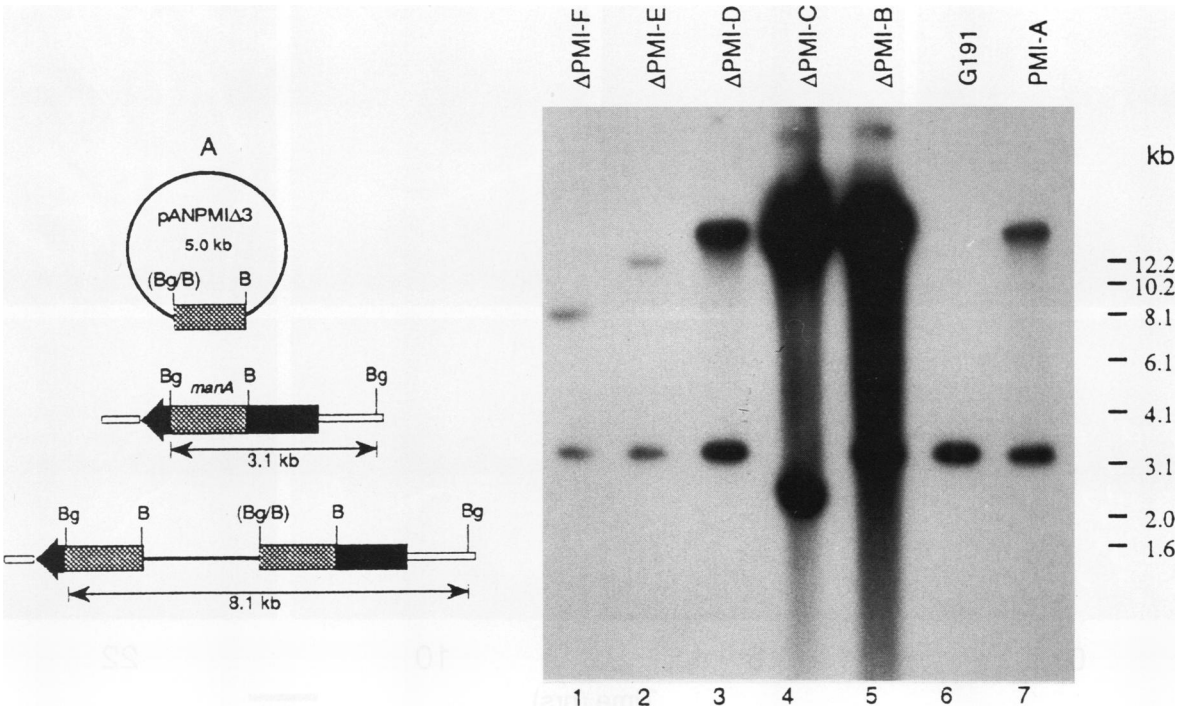

FIG. 4. Integration of pANPMIΔ3 at the *manA* locus generates two truncated copies of the *manA* gene and gives a predicted increase in a *manA* *Bgl*II fragment size from 3.1 to 8.1 kb. B, *Bam*HI; Bg, *Bgl*II; (B/Bg), destroyed *Bam*HI-*Bgl*II site. The Southern blot shows *Bgl*II-digested genomic DNA isolated from five heterokaryotic pANPMIΔ3 transformants with nonviable conidia on AMM without uridine (lanes 1 to 5), parental strain G191 (lane 6), and one pANPMIΔ3 transformant with viable conidia on AMM without uridine (lane 7). The probe was the 0.65-kb *Bgl*II-*Bam*HI *manA* DNA fragment present in the transforming plasmid pANPMIΔ3. Hybridization wash stringency was 0.1% (wt/vol) SDS–0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C for 2 h.

conidia have begun to develop germ tubes, whereas ΔPMI-F conidia have not and remain enlarged compared to dormant conidia. By 11 h, G191 has well-developed germ tubes but most ΔPMI-F conidia have become enlarged, misshapen, and vacuolated, with the vacuole appearing to occupy much of the cytoplasm. In some cases, a germ tube does emerge but can also be swollen, and ballooning of the emerging germ tube tip does not appear to occur. Growth of ΔPMI-F ceases by 22 h, and many cells appear grossly enlarged and misshapen, with some cells showing lysis (Fig. 5). In comparison, wild-type strain G191 at 22 h has produced many mature and branched hyphae forming a growing mycelium.

Strains of *A. nidulans* containing the temperature-sensitive *manA1* mutation can be repaired for growth only on AMM containing both D-glucose and D-mannose in the ratio of 9:1 (36) so as to satisfy the requirement for both D-mannose for the dol-P-man pathway and mannosylation and D-glucose for glycolysis. Conidia of the ΔPMI-F strain of *A. nidulans*, carrying a deletion of the *manA* gene, were tested for the ability to grow on AMM plus uridine with different D-mannose/D-glucose ratios at a constant 1% total carbohydrate concentration. The proportions varied between 1% D-glucose–0% D-mannose and 0% D-glucose–1% D-mannose in 0.1% increments. In contrast to strains with the *manA1* mutation, ΔPMI-F was unable to grow on any of these carbon source mixtures. The presence of an osmotic stabilizer (0.6 M KCl) did not permit growth on these carbon sources, suggesting that wall weakness was not responsible for the lack of growth.

DISCUSSION

We have isolated the *A. nidulans manA* gene encoding PMI by using its linkage to the *riboB* gene and complementation of a *manA1* mutation as a means to identify DNA clones containing it. Evidence that the cloned gene is *manA* is supported by the complementation data and close genetic linkage described here and the identification of a single point mutation in the corresponding *manA1* allele (30). The *manA* gene is closely linked to *riboB*, being between 3.5 and 5.5 kb centromere distal of it. Our evidence that *manA* encodes PMI is based first on the fact that the gene can complement a temperature-sensitive *manA1* mutant strain which has been shown to have greatly reduced PMI activity at the restrictive temperature (36). Second, there is significant sequence identity over the full length of the predicted protein sequence of the *manA* gene product with those of the *S. cerevisiae*, human, and *E. coli* PMIs, indicating that they are homologous enzymes, and the sequence of the whole PMI coding region has been determined. The locations of the *manA* 5′ transcription start sites and polyadenylation sites also strongly suggest that we have isolated the entire gene and its coding sequences. Previous work had suggested that *manA* may represent a regulator of PMI activity (36) because although PMI activity was greatly reduced in strains bearing the *manA1* temperature-sensitive mutation grown at the restrictive temperature, thermolabile PMI activity was not demonstrable. However, it appears that *manA* defines the structural gene for PMI in *A. nidulans*, and genomic DNA hybridizations indicate that the gene is probably unique in the *A. nidulans* genome.

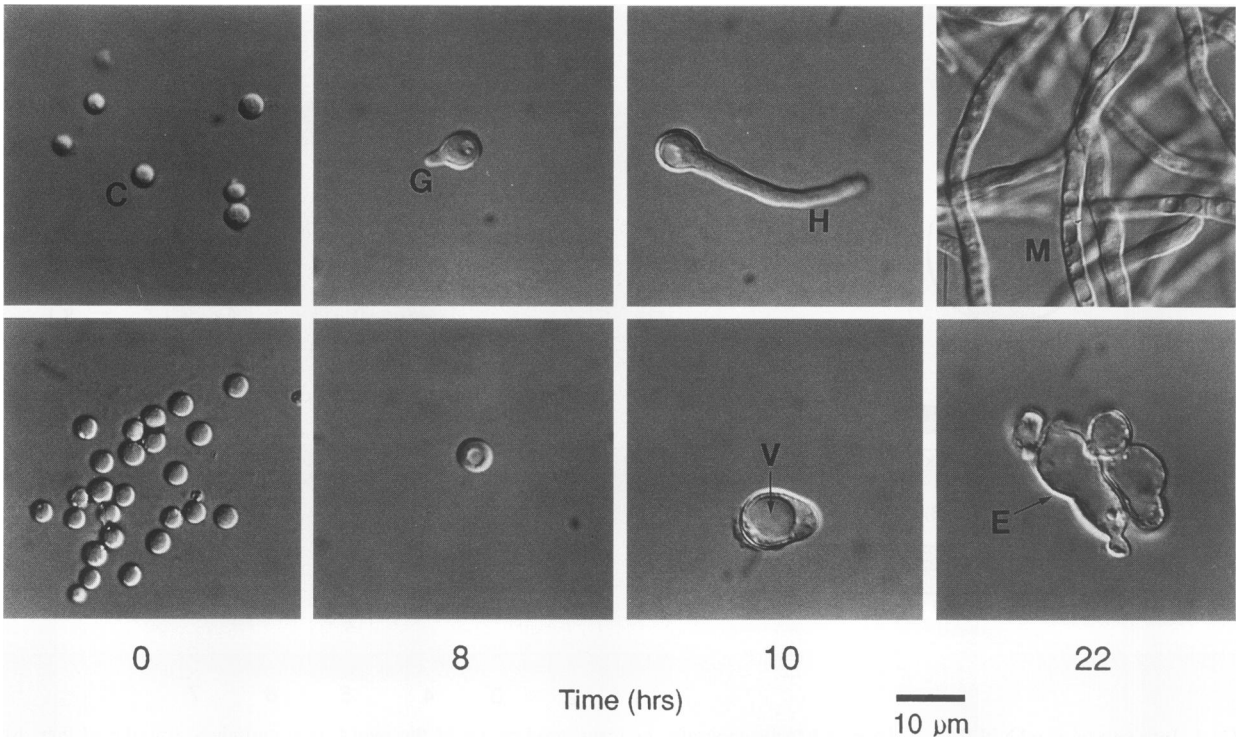

FIG. 5. Micrographs through interference contrast optics (Leitz) of the process of germination of wild-type *A. nidulans* G191 (top row) and the *manA* gene-disrupted heterokaryotic strain ΔPMI-F (bottom row). Conidia from ΔPMI-F were used to inoculate 100 ml of AMM, and samples were removed at the time intervals indicated for microscopic analysis. Germinating conidia were selected in the field of view; an equal number of ungerminated conidia which were presumed to be *pyrG* conidia of parental origin which are unable to germinate in this medium were seen. Conidia from the parental strain G191 were used to inoculate a similar flask supplemented with uridine and examined at the same time intervals. A consistent magnification (100× objective) was used throughout. C, conidium; G, germ tube; H, hyphum; M, mycelium; V, vacuolelike structure; E, enlarged and misshapen condium, perhaps lysed.

Enzymes with PMI activity have been identified in a number of eukaryotes and prokaryotes (7, 16, 25, 27, 28, 31), and on the basis of sequence similarity, it has been possible to divide them into three classes (25). The *A. nidulans* PMI clearly belongs to type I, having significant amino acid sequence similarity to other members of the same group. Thus far, all other eukaryotic PMIs are members of the type I class.

The structure of the *manA* gene is typical of filamentous fungal genes in that it possesses a number of short introns, four in the case of *manA*, with 5′ and 3′ splice sites conforming to the consensus established for filamentous fungi (14). Interestingly, the first intron of the *A. nidulans manA* gene shares identical position conservation with the single intron of the *PMI40* gene encoding PMI in *S. cerevisiae* (31). The intron was therefore probably present in a progenitor of these two ascomycetes and predates their divergence. The two introns are of different size, 58 bp for *A. nidulans* and 93 bp for *S. cerevisiae*, and share no significant sequence identity apart from those regions corresponding to the 5′ and 3′ splice junctions. As far as we are aware, the only other *S. cerevisiae* gene containing an intron for which an *A. nidulans* counterpart has been isolated is the actin gene. In this case, the intron position is not conserved (13, 17).

The *manA1* mutation leads to hyphal tip balloon formation in mature hyphae, probably as a result of loss of polarized growth rather than wall weakness since, in contrast to mutants deficient in cell wall chitin or glucan synthesis (3), the ballooning phenotype cannot be repaired by osmotic stabilizers (36). A disruption of the *manA* gene is lethal in *A. nidulans*, and the mutation can be maintained only in a heterokaryotic state with *manA*$^+$ nuclei. Expression of the mutant phenotype of the *manA* disruption in the uninucleate germinating conidium shows that *manA* is required for normal hyphal tip growth. Conidia are able to commence germination but are unable to complete the process by producing a normal germ tube. Conidia with a disrupted *manA* gene appear swollen and sometimes produce abnormal germ tubes before ceasing growth. Lysis of the swollen conidia can be seen after extended incubation. An osmotic stabilizer in combination with exogenously added D-mannose does not permit growth of a *manA* gene disruptant strain. Thus, the defect is not simply due to lack of integrity of the cell wall. The inability of a strain with a disrupted *manA* gene to grow on media containing D-mannose is unexpected, as strains with the *manA1* mutation are able to grow. It is possible that the aberrant development of the cell wall in the *manA* disruption strain hinders D-mannose uptake, perhaps by preventing a sugar transport system from operating correctly, whereas the *manA1* mutant may have sufficient residual PMI activity at the restrictive temperature to allow some normal development and allow D-mannose uptake.

It is apparent that PMI is required for normal germination and cell wall development in *A. nidulans*. Like other fungi, *A. nidulans* contains mannose as a minor but significant component of the cell wall (4, 36, 40), and it is likely that much of it is present in the form of highly mannosylated glycoproteins which, in yeast cells, have been termed mannoproteins and form an integral part of the cell wall. In *S. cerevisiae*, the mannosyl residues found in mannoproteins are derived from

the dol-P-man pathway (32, 33). Mannose-6-phosphate produced by PMI from glycolytic intermediates is required for dol-P-man synthesis, and it appears probable that *A. nidulans* produces dol-P-man and mannosylated proteins by a similar pathway.

It seems that in *A. nidulans*, the synthesis of dol-P-man and mannoproteins plays a crucial role in the development of a correct cell wall structure permitting growth of the hyphal tip. It has been suggested that a maturation process in the fungal cell wall occurs after its synthesis in which cross-links are formed between the various components making up the wall, thereby generating a more rigid structure (38, 39). The ballooning phenotype suggests that mannoproteins may play a critical role in this maturation process, their absence leading to uncontrolled expansion of newly synthesized wall in the germinating conidium or growing hyphal tip. Such an effect could be due to the absence or reduced level in the newly formed wall of a structural mannoprotein, between which cross-links are formed with other cell wall components such as β-glucan or chitin, or the lack of a mannosylated and secreted cross-linking enzyme required for the maturation process.

Mutants of PMI in *S. cerevisiae* are defective in secretion (23) because mannosylation is essential for the passage of proteins through the secretory pathway. It is therefore likely that *manA* mutants of *A. nidulans* are also defective in secretion of mannosylated proteins. *Aspergillus* species have been investigated for use as hosts for secretion of recombinant proteins which can sometimes be hyperglycosylated, and there is evidence that this occurs through hypermannosylation (2). It would be interesting to determine whether by controlling the level of PMI activity in such strains it would be possible to reduce the level of mannosylation without altering normal cell growth.

Further studies on PMI, the mannosylation pathway, and mannoprotein biosynthesis should lead to a greater understanding of the role that mannose plays in cell wall growth in fungi.

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