ami1, an Orthologue of the Aspergillus nidulans apsA Gene, Is Involved in Nuclear Migration Events Throughout the Life Cycle of Podospora anserina

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Abstract
The Podospora anserina ami1-1 mutant was identified as a male-sterile strain. Microconidia (which act as male gametes) form, but are anucleate. Paraphyses from the peritheciun beaks are also anucleate when ami1-1 is used as the female partner in a cross. Furthermore, in crosses heterozygous for ami1-1, some crozier cells are uninucleate rather than binucleate. In addition to these nuclear migration defects, which occur at the transition between syncytial and cellular states, ami1-1 causes abnormal distribution of the nuclei in both mycelial filaments and asci. Finally, an ami1-1 strain bearing information for both mating types is unable to self-fertilize. The ami1 gene is an orthologue of the Aspergillus nidulans apsA gene, which controls nuclear positioning in filaments and during conidiogenesis (at the syncytial/cellular transition). The ApSA and AMI1 proteins display 42% identity and share structural features. The apsA gene complements some ami1-1 defects: it increases the percentage of nucleate microconidia and restores self-fertility in an ami1-1 mat+ (mat−) strain. The latter effect is puzzling, since in apsA null mutants sexual reproduction is quite normal. The functional differences between the two genes are discussed with respect to their possible history in these two fungi, which are very distant in terms of evolution.

Nuclear migration and positioning are fundamental and ubiquitous processes. Numerous observations have shown that nuclear movements are essential for growth and development in both lower and higher eukaryotes. For instance, the migration of the male pronucleus within the animal oocyte after fertilization, together with movements of the female pronucleus, are essential events for nuclear fusion. Similarly, nuclear movements are required in yeasts after mating to achieve caryogamy. Later in development, nuclear positioning is especially important for asymmetric cell division in plant and animal embryos. Similarly, in Saccharomyces cerevisiae, migration of the mother cell nucleus to the bud neck before mitosis ensures that the daughter cell will contain one of the two sister nuclei. An interesting scenario is observed in early development of insects, the paradigm of which is Drosophila: after several mitotic divisions of the zygotic nucleus (without cytokinesis), there is a massive migration of nuclei from the center of the embryo to a subcortical position at which cellularization occurs. This situation, which requires a transition between a syncytial and a clearly cellular state, is not specific to insect development: it is also encountered during vegetative and sexual development in some plants and in filamentous fungi.

In the last decade, many studies associating cytological, biochemical, and genetic approaches have been devoted to nuclear movements. In this research area (as in others), yeasts and filamentous fungi have played key roles as model systems. Several genes required for proper nuclear distribution during vegetative growth have been characterized in Aspergillus nidulans and Neurospora crassa (Morris et al. 1995; Fischer 1999 for reviews). With respect to their functions, these genes (which have been named nud in A. nidulans and ro in N. crassa) can be grouped into three classes. Some encode components of cytoskeletal motors nudA (Xiang et al. 1994), nudG (Beckwith et al. 1998), ro1 (Plamann et al. 1994), ro3 (Tinsley et al. 1996), and ro4 (Plamann et al. 1994; Robb et al. 1995). Others probably fulfill regulatory roles: for instance, nudC and nudF (Osmani et al. 1990; Xiang et al. 1995). Still others embody poorly understood functions: for instance ro2 (Vierula and Mais 1997), ro10, and ro11 (Minke et al. 1999). Some of these genes have orthologues in higher eukaryotes. The role that filamentous fungi can fulfill in the elucidation of complex processes in higher eukaryotes, and in a comprehensive view of the evolutionary history of eukaryotes, can be illustrated by nudF, orthologues of which have been found in Drosophila and mammals (Reiner et al. 1993; Xiang et al. 1995; Cunniff et al. 1997; Morris et al. 1997). One remarkable point is that nudF, essential for vegetative growth in A. nidulans, has been identified as a gene required in a mammalian developmental program. In fact, mutations in LIS1 (the human orthologue of nudF) lead to diseases characterized by malformations in the cerebral cortex (Reiner et al. 1993). The conventional explanation of these defects

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assumes a problem in neuronal migration (Barth 1987). However, Morris et al. (1998) propose that LIS1 might be involved (as it is A. nidulans nudF orthologue) in the control of nuclear movements that occur early in cerebral development and have been implicated in cell fate determination (Morris et al. 1998 and references therein).

Although the nud and ro mutations can impair asexual and sexual development, the primary role of the relevant genes is at the level of vegetative growth. Other genes are more specifically involved in nuclear migration events linked to development. In A. nidulans, Clutterbuck (1994) identified two genes, apsA and apsB, required for conidiation (asexual sporulation) at a very precise stage of conidiophore development, corresponding to the transition between the syncytial and cellular states. In the mutants, the structures that are normally uninucleate do not (with rare exceptions) contain a nucleus. The apsA and apsB genes were cloned and null mutants were constructed: they show the same phenotypic properties as do the original mutants, i.e., clustering of nuclei in the filaments and a specific defect of nuclear migration during the conidiation process (Fischer and Timberlake 1995; Suelmann et al. 1998). The ApsA protein was localized at the cytoplasmic membrane and the ApsB protein was localized in the cytoplasm (Suelmann et al. 1997, 1998). ApsB shows sequence similarity to trichohyalin, which is thought to interact with intermediate filaments in mammals (Tarcsa et al. 1997 and references therein). apsA has a structural orthologue in S. cerevisiae, NUM1, whose mutations impair nuclear migration to the bud before (but not after) mitosis (Kormanec et al. 1991; Revardel and Aigle 1993; Farkasovsky and Kuntzel 1995).

Although the data as a whole focus on a regulatory role of ApsA and ApsB in nuclear migration/positioning processes, their precise functions remain unclear. In this study, we identify and analyze ami1 (anucleate microconidia), the orthologue of apsA in Podospora anserina, a filamentous ascomycete very distant from A. nidulans in terms of evolution. While apsA seems to be involved mainly in nuclear migration/positioning processes during conidiation, ami1 exhibits broader functions, especially at all steps requiring nuclear movements during sexual reproduction.

MATERIALS AND METHODS

P. anserina strains and media: P. anserina is a heterothallic filamentous ascomycete whose life cycle and general methods for genetic analysis have been described (Rizet and Engelmann 1949; Escher 1974). All strains are derived from the wild-type S strain (Rizet 1952). We also used another wild-type strain called s, which differs from the S strain in the allele present at the locus (Bernet 1965) later named hës (Del eu et al. 1993). The 193 mutant strain exhibits white mycelium, perithecia, and ascospores (Picard 1971). The incA mutation (first named for incoloris) is highly pleiotropic (see results; Rizet and Engelmann 1949; Prillinger and Esser 1977; Bernet 1986, 1988). The cro1-1 mutation leads, when homozygous, to giant plurinucleate croziers (Simonet and Zickler 1978; Ber teaux-Lecellier et al. 1998). Mutants of the FM R1 and SM R2 (mat−) genes were described by Arnaise et al. (1997) and the Δmat (mat−) strain was described by Coppin et al. (1993). The culture and spor germination media have been reviewed recently by Ber teaux-Lecellier et al. (1995).

Counting of microconidia and perithecia: The relevant strains were grown on petri dishes containing minimal synthetic medium (M2) and incubated at 27° in the dark. The microconidia were recovered at different times (from 5 to 30 days) by washing the surface of the mycelia with 1.5 ml of sterile water. This permitted the recovery of 1 ml of microconidial suspension, which was counted by microscope with a hemacytometer. To test their fertilization ability, 1 ml of microconidial suspension (after dilutions when required) was spread on wild-type mycelia used as female partners, which were previously grown on M2 medium at 27° for 3 days in the light to allow formation of female organs. Perithecia were counted 5 days after fertilization.

Cosmids, plasmids, and bacterial strains: Cosmids N9 and N10 are taken from a mat− library (Picard et al. 1991). Cosmid U1 is from a mat+ library constructed by Turcq et al. (1990). The ami1 gene was sequenced using either cosmids or subclones in pUC18, pHblesscript SK (+), or KS (+) (Stratagene, La Jolla, CA). Cosmid and plasmid preparations were performed in Escherichia coli DH5α (Hanahan 1983) or CM5α (Camonis et al. 1990). The pMCosX plasmid was used in cotransformation experiments; it carries the bacterial hygromycin-resistant gene under the control of the cpc1 promoter of N. crassa (Orbach 1992). The plasmid carrying the A. nidulans apsA gene (a kind gift of Dr. R. Fischer) is pRF7, which has been described in Fischer and Timberlake (1995).

Transformation procedures: Transformation experiments were performed as previously described (Picard et al. 1991), except that the protoplasts were made with Glucanex (Novo Nordisk Ferment AG) rather than with Novozym. With cosmids N9 and N10, the recipient strain was amo1-1 leu1-1, since auxotrophy for leucine due to the leu1-1 mutation is suppressed by the su8-1 tRNA suppressor carried by these cosmids (Debuchy and Brygoo 1985). In all other cases (cotransformation experiments with pMCosX), the recipient was the amo1-1 strain and transformants were selected as hygromycin-resistant clones.

Physical localization of the amo1 gene: The gene was first localized in the vicinity of the mat locus through complementation of the amo1-1 mutant by cosmids encompassing this region. A precise localization of the gene on the N9 cosmid was then obtained according to the procedure developed by Turcq et al. (1990). Briefly, the cosmids was digested with one of several restriction enzymes. Each restriction mixture was used to transform the amo1-1 strain using the pMCosX reporter vector as a selective marker. Hygromycin-resistant transformants were tested for their ability to fertilize the wild-type strain used as the female partner. This method demonstrates which enzymes inactivate or fail to inactivate the gene.

Genetic and molecular attempts to obtain an amo1 mutation in the mat− context: The amo1 gene is tightly linked to the mat locus (~10 kb), and recombination events between the amo1 mutation and the mat locus were not obtained (among >100 asci analyzed). More importantly, cloning of the entire amo1 gene on a DNA fragment whose size would have permitted deletion of the gene and then obtaining a null mutant through gene replacement could not be achieved. In fact, in all cases the expected fragments were rearranged. Lastly, since amo1-1 is a suppressor of the incA mutant female sterility, UV mutageneses on an incA mat− strain were performed, without
success. This failure might be due to the fact that the incA mutation is dominant and that incA nuclei dominate any nuclei leading to an incA- phenotype.

Sequencing: The ami1 gene was sequenced on both strands with an automatic sequencing machine (373A DNA sequencer, Applied Biosystems, Foster City, CA) by the method of DyeDeoxy terminator cycle sequencing kit (Applied Biosystems). Universal primer and synthetic oligonucleotides were used to start the reactions. The sequence was performed either on cosmids or subcloned fragments of the ami1 gene. PCR amplification on genomic DNA from a mutant strain provided the templates for sequencing reactions. Total DNA was extracted by a miniprep method (Lecellier and Silar 1994). The amplified fragments were prepared for direct sequencing according to Rosenthal et al. (1993).

Northern (RNA) blot analysis: Total RNA was isolated from mycelia as described by Lecellier et al. (1987), glyoxal treated, and separated on a 1% agarose gel (Sambrook et al. 1989). Northern blots were performed using standard techniques, with a probe of a 4.6-kb EcoRI fragment containing the central part of the gene. Poly(A) RNAs were obtained with a QUIAGEN kit (oligotex mRNA minikit) and then treated with DNase and used as templates for reverse transcriptase (RT)-PCR experiments (TITAN one tube RT-PCR kit, Boehringer Mannheim, Mannheim, Germany). cDNA amplification was performed using synthetic oligonucleotides. The amplified fragments were prepared for direct sequencing as described above.

Heterologous hybridizations: Genomic DNA samples from Ascomobolus immersus, A. nidulans, S. cerevisiae, Schizosaccharomyces pombe, Arabidopsis thaliana, and Homo sapiens were kindly provided, respectively, by A. Grégoire, B. Felenbock, M. Bolotin-Fukuhara, M. Sipizck, M. Kreis, and J. Levilliers. Sordaria macrospora is the St. Ismier FGSC 4818 strain. DNAs were digested and separated on a 0.7% agarose gel. The transfer was performed on a nylon membrane (Amersham Corp., Piscataway, NJ). After hybridization at low stringency (37°C), using the procedure described by Church and Gilbert (1984), the membrane was washed at 45°C, in 2× SSC. Two 2-kb PCR fragments corresponding to the 5’ and 3’ regions of the ami1 gene were used as probes, except for S. macrospora DNA, which was hybridized only with the 3’ fragment. Control experiments were performed using the P. anserina AS4 gene encoding the evolutionarily well-conserved eEF1A elongation factor (Silar and Picard 1994).

Light microscopy preparations: Nuclei were visualized through 4,6-diamidino-2-phenylindole (DAPI) staining (0.5 μg/ml). Mitochondria were stained with the mitochondrion-specific dye 2-(4-dimethylaminostyryl)-1-methylpyridinium iodide (DASPMI; Sigma, St. Louis) using the procedure described by McConnell et al. (1990) and adapted for P. anserina by Jamet-Vierny et al. (1997). Microtubules were visualized, according to Thompson-Coffe and Zickler (1992), with a monoclonal anti-β-tubulin (Amersham, France) at a dilution of 1:1200. As secondary antibody we applied the FITC-labeled goat anti-mouse IgG antibody (Jackson Immunoresearch, West Grove, PA) at 1:100. Rosettes of asci were fixed in fresh Lu’s (1967) fixative (butanol, propionic acid, and 10% aqueous chromic acid; 9:6:2 volume). After a 10-min hydrolysis in 1 N HCl at 70°C, asci were stained in one drop of ferric acetate solution mixed on the slide with two drops of 2% hematoxylin.

RESULTS

The ami1-1 mutant differentiates anucleate microconidia: The ami1-1 mutant was serendipitously obtained during the systematic analysis of mat+ transgenic strains carrying a cosm id encompassing the mat- region (Picard et al. 1991). It was initially characterized as a male-sterile mutant, according to the following procedure: when two strains of opposite mating types are grown on the same petri dish, both produce male gametes (microconidia) and female organs (protoperithecia) but are self-sterile. To fertilize both strains with compatible microconidia, 1 ml of sterile water is poured on the mycelia and the dish is gently shaken to remove microconidia and distribute them over the surface of the cultures. As shown in Figure 1, this simple test defines the ability of a strain to be a male and/or a female partner. The ami1-1 mutant clearly exhibits a female-fertile but male-sterile phenotype (compare Figure 1, A and B). Dozens of such crosses were performed: the number of perithecia observed on the female partner of ami1-1 was
Figure 2.—The ami1-1 mutant differentiates anucleate microconidia and exhibits abnormal distribution of nuclei in the mycelium (stained by DAPI). (A) Wild type. In Podospora, conidia are formed directly from the mycelium (arrow). (B) incA mycelia produce especially large numbers of conidia from each cell (arrow points to a nucleus). (C) Double mutant incA ami1-1 mycelium with anucleate conidia (arrow, compare with B). (D) ami1-1 transgenic strain carrying the A. nidulans apsA gene [ami1-1 (apsA-1)], showing a mixture of nucleate (arrow) and anucleate conidia. (E) Wild-type mycelium with regularly spaced nuclei. (F) ami1-1 mycelium with irregular distribution of the nuclei (arrow). Bar, 5 μm.

mainly 0 and never exceeded 3 or 4, while >1000 perithecia were formed in control crosses.

The ami1-1 defect could have two possible explanations: either the mutant is aconidial, i.e., unable to differentiate macroconidia, or mutant microconidia are nonfunctional. Cytological analyses of ami1-1 cultures showed that the mutant was able to form microconidia, but that almost all were anucleate (Figure 2): less than $10^{-3}$ contained a nucleus. Moreover, the distribution of nuclei throughout mycelial filaments was also abnormal: while uniformly distributed along the length of the filaments in wild-type mycelium, they were mainly clustered in the mutant, leaving large gaps in the mycelium (Figure 2). However, mitoses are normal in the mutant. Although the same clustering of nuclei was observed in the female organs, their development was not hindered. Mutant trychogynes do not differ from those of the wild type: in both cases, these specialized filaments contain two to three nuclei (data not shown). To determine if nuclear clustering corresponded to a possible general defect in organelle distribution in the mutant, we stained the mitochondria with DASPMI and followed their distribution in growing mycelium and microconidia. The mutant mitochondria do not significantly differ from the wild-type organelles with respect to size, number, and distribution. Furthermore, anucleate microconidia contain the normal average number of mitochondria, i.e., from one to three (data not shown).

The rare perithecia observed when ami1-1 is used as a male partner could be explained by its very weak leakiness (production of $10^{-3}$ nucleate microconidia). However, perithecia could also arise from mycelial fragments resulting from ami1-1 cultures and acting as male gametes. To ascertain whether or not ami1-1 filaments could act as male gametes, two tests were performed. First, we crossed ami1-1 and the 193 mutant strain, which develops white perithecia, by confronting their mycelia on a petri dish. As shown in Figure 3A and B, the presence of white perithecia all along the confrontation line (where the two mycelia meet) shows that ami1-1 is able to act as a male partner. To ensure that this could not be explained by complementation of the mutant through heterocaryosis in the confrontation area, we performed a second set of crosses. When mycelial filaments of the wild-type strain meet those of the S strain (from which ami1-1 was issued), incompatibility results in the failure to form heterocaryotic filaments via hyphal fusions: fusion between incompatible hyphae leads to death of the heterocaryotic cells and causes the formation of a “barrage” or barrier between the two strains (Rizet 1952). As shown in Figure 3A, the confrontation of ami1-1 S and ami1-1+ S strains gives rise to a barrier, on both sides of which perithecia have developed. This confirms that ami1-1 filaments can indeed act as male gametes in a cross. In fact, in this case, heterocaryosis is prevented and the ami1-1 strain cannot be complemented for production of functional microconidia. If ami1-1 filaments were unable to act as male gametes, perithecia would have been observed on the ami1-1 strain but not on its ami1-1+ female partner.

To better understand the ami1-1 conidiation defect, we constructed double mutant strains with the incA mutation, known to increase the number of microconidia by a factor of 1000 as compared to young wild-type cultures (Table 1). A massive production of microconidia can be observed after a few days of culture of the incA mutant while, with wild-type strains, several weeks are required to produce similar amounts (Table 1). The incA mutant is also female sterile and displays a defect in mycelial pigmentation (Figure 1C). The phenotypic properties of the ami1-1 incA double mutant were examined using the tests previously performed for the two single mutants. As shown in Figure 1D and Table 1, the double mutant strain is male sterile: the number of perithecia observed for the female partner is similar to the number observed with the single mutant ami1-1.
The *ami1* gene is involved in sexual reproduction:

Crosses between *ami1* and the wild type are fertile, although ascospores are expelled with a slight delay (~1 day) compared to wild type × wild-type crosses (data not shown). However, 36–38% of the asci formed in those heterozygous crosses are abnormal: the nuclei formed after meiosis and postmeiotic mitosis are more or less randomly distributed in the asc, leading to abnormal ascospore delimitation and/or formation. Thus, *ami1* is semidominant. We examined the microtubule (MT) cytoskeleton organization during both ascus development and sporulation. The densities of the cortical array organized by the apical microtubule organizer center during the growth period of the ascus, as well as the nuclear-envelope-associated MTs, suggested to serve in positioning nuclei before and after division (Thompson-Coffe and Zickler 1992), are both normal when compared to wild-type crosses. Spindle pole bodies, spindle elongation, and astral MTs are also normal.

During wild-type postmeiotic mitosis (PMM), the four spindles are in two widely separated pairs: thus, extensive nuclear movements occur before ascospores are formed. In ~40% of the asci formed in the *ami1* × wild-type crosses, the four PMM spindles are either located in the middle of the ascus or randomly spaced, hindering the proper migration of the nuclear pairs before ascospore formation (data not shown). We also noticed a maternal effect of the mutation in all crosses involving *ami1*. The paraphysal hyphae, which are formed between the asci and at the base of the perithecium, show an abnormal nuclear distribution. This is especially striking in the beak paraphysae, which are formed from the asci and at the base of the perithecial beak, show an abnormal nuclear distribution. This is especially striking in the beak paraphysae, which are normally formed by 5–10 uninucleate, short, and regularly sized cells emerging from basal plurinucleate cells (Figure 4A). In the mutant perithecium, these cells are irregular in size. Moreover, those cells located near the basal cell of the paraphysae maintain a plurinucleate and massive production of microconidia. In fact, as seen in Table 1, the number of microconidia produced by the *ami1* *incA* double mutant increases with the time of culture, but never reaches that observed in both *incA* and wild-type strains.

Moreover, the double mutant microconidia remain anucleate (Figure 2). Thus, the *incA* mutation does not suppress the *ami1* defect. In contrast, the *ami1* mutation acts as a suppressor of the *incA* mutation with respect to female sterility (compare Figure 1, C and D) and massive production of microconidia. In fact, as seen in Table 1, the number of microconidia produced by the *ami1* *incA* double mutant increases with the time of culture, but never reaches that observed in both *incA* and wild-type strains.

To complete this analysis, we attempted a search for suppressors of the male sterility phenotype of the *ami1* mutant. However, this was hampered by the spontaneous appearance of mutations leading to an incoloris-like phenotype able to partially suppress the mutant defect (S. Arnaise, personal communication).

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To further examine the possible role of the *ami1* gene in sexual reproduction sensu stricto, crosses in which the *ami1* mutation was homozygous were required. The original mutation was obtained in a mat+ context and genetic and molecular attempts to obtain a mat− strain bearing an *ami1* mutation have as yet been nonproductive (see materials and methods). We thus attempted to circumvent this problem in two ways. First, we examined the effect of the *ami1* mutation when heterozygous in crosses involving particular genetic backgrounds. Second, we constructed a strain allowing crosses in which all genes (including the *ami1* allele)
were artificially homozygous, with the exception of the mating-type genes.

With respect to the initial purpose, we employed the cro1-1 mutation and mutations in the mat- genes. An homozygous cro1-1 mutation leads to a defect in dicaryotic cell formation preceding meiosis; rather than binucleate, the crozier cells are multinucleate, as though the cro1-1 mutation hampers the transition between syncytial and cellular stages (Simonet and Zickler 1978; Berteaux-Lecellier et al. 1998). It is not clear whether this default is an initial failure in nuclear migration, or a defect in septum formation after cellularization. To determine if the ami1-1 mutation (when heterozygous) could enhance or suppress cro1-1 effects, a double mutant strain was constructed and the cro1-1 ami1-1 x cro1 cro1 ami1-1 cross was compared to a cro1-1 x cro1-1 cross. The differences between the two crosses appeared to be very slight. However, one difference was noticeable: ascii with four spores were more common in the ami1-1 cross than in the control cross (data not shown).

The mat- F. R. R1 and SM R2 genes are required for proper recognition of mat+ and mat- nuclei during dicaryotic cell formation: in crosses between a mat+ tester strain and a strain bearing a mutation in FM R1 or SM R2 the progeny is diminished and produces mainly uninucleate crozier and uniparental ascii containing markers of the mat- mutant partner (Zickler et al. 1995; Arnaise et al. 1997). The quantitative and qualitative characteristics of the progeny were similar when the relevant mat- mutants were crossed to either an ami1-1 or an ami1+ strain (data not shown).

Our second purpose was to perform crosses in which the ami1-1 mutation was artificially homozygous. This situation can be obtained by the following procedure:

1. Wild type
2. incA
3. ami1-1
4. ami1-1 incA
5. ami1-1 (apsA-1)
6. ami1-1 (apsA-12)

**TABLE 1**

Microconidia production by the ami1-1 mutant and other relevant strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>No. of microconidia</th>
<th>No. of perithecia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 days</td>
<td>20 days</td>
</tr>
<tr>
<td>Wild type</td>
<td>≤10⁴</td>
<td>5 x 10⁴</td>
</tr>
<tr>
<td>inC</td>
<td>6 x 10⁷</td>
<td>8 x 10⁷</td>
</tr>
<tr>
<td>ami1-1</td>
<td>≤10⁴</td>
<td>4 x 10⁵</td>
</tr>
<tr>
<td>ami1-1 incA</td>
<td>≤10⁴</td>
<td>1.5 x 10⁶</td>
</tr>
<tr>
<td>ami1-1 (apsA-1)</td>
<td>≤10⁴</td>
<td>3 x 10⁵</td>
</tr>
<tr>
<td>ami1-1 (apsA-12)</td>
<td>≤10⁴</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not determined.

(apsA-1) and (apsA-12) correspond to two integration events of the A. nidulans apsA gene.

Microconidia were recovered at different times (from 5 to 30 days) by washing the surface (~60 cm²) of one petri dish of each culture. They were counted using a hemacytometer chamber. The numbers correspond to 1 ml of suspension. Perithecia were counted after fertilization of the wild-type strain used as the female partner with 1 ml of microconidia suspension of the relevant strain. The numbers are the mean values of three to four petri dishes with, in parentheses, the dilution factor of the microconidia suspension (standard deviation).

Values in the range of 10⁴ (and less) cannot be reliable since they correspond to the lowest limit of the chamber. Otherwise, we did not have confidence in concentrated microconidia suspensions obtained by centrifugation because they gave variable results from one strain to the other: in particular, it was observed that in strains carrying the ami1-1 mutation, the pellets contained highly enriched populations of nucleate microconidia.

Undiluted.
The number above each fragment gives its size in kilobases. Superposed numbers indicate the size of unordered fragments. Cosmid inserts end with either EcoRI (N9 and N10) or Sau3A (U1) sites. The arrow shows the position and orientation of the ami1 gene.

**nidulans apsA gene:** The ami1 gene appeared tightly linked to the mat locus: no recombination was observed in crosses between the wild-type mat− and ami1-1 mat+ strains among >100 uninucleate ascospores examined. Three cosmids encompassing the mating-type region (Figure 5) were used to transform an ami1-1 recipient and all three complemented the mutant with respect to its male-sterile phenotype (data not shown). A precise localization of the gene on the N9 cosmid was obtained according to the procedure described by Turcq et al. (1990). Since a subclone containing the whole ami1 gene was not obtained, it was sequenced using several subcloned fragments and the cosmid N9 (Figure 5).

Analysis of the nucleotide sequence identifies an open reading frame of 1882 codons, interrupted by one putative intron. Sequence comparisons revealed a high similarity between the putative protein AMI1 and the *A. nidulans* ApsA protein (Fischer and Timberlake 1995). As shown in Figure 6, the *A. nidulans* and *P. anserina* proteins display 42% identity and 59% similarity in a 1676-amino-acid overlap. The cDNA was also completely sequenced and the presence of a single 56-bp intron (see asterisk in Figure 6) was confirmed; it is located at exactly the same position as the apsA intron.

All structural features reported for ApsA (Fischer and Timberlake 1995) are also found in AMI1. Both proteins are hydrophilic, with an overall pl of 5.2 for AMI1 (5.0 for ApsA). Three imperfect repeats of ≈25 amino acids (for AMI1) are found in the central and most acidic region of the two proteins (residues 900–1400 in AMI1). In both cases, the N-terminal portions of the proteins show a high probability of coiled-coil formation, along with the presence of several heptad repeats (5 in AMI1 and 25 in ApsA). Both proteins contain a PH domain (Gibson et al. 1994 for a review) in their C-terminal parts (residues 1603–1715 in AMI1).

In addition, in AMI1, this region (residues 1840–1880) also displays a high probability (0.93) of coiled-coil formation (Lupas et al. 1991), a feature not exhibited by the C-terminal part of ApsA.
Figure 6.—Comparison of the amino acid sequences deduced from the P. anserina ami1 gene (top line) and the A. nidulans apsA gene (bottom line). Similar and identical amino acids are shown, respectively, in gray and black boxes. The localization of the stop codon in the ami1-1 mutant is designated by an arrow. The asterisk above the sequence shows the intron position. The alignment was obtained with Pileup algorithm, with a gap penalty of 1.000 and a length penalty of 0.100. The figure was obtained using the Boxshade program. The DDB/EMBL/GenBank accession number for the ami1 nucleotide sequence is AJ271779 PAN271779.
Sequence comparison also revealed a significant similarity between AMI1 and an even stronger signal with DNA from A. immersus. RIP-like process will be described elsewhere. We also examined the possible effects of the three proteins, including the PH domain. However, structural similarities are observed between the three proteins: Num1p also displays heptad repeats in its N terminus, direct repeats in its central part, and a PH domain in its C terminus (Kormanec et al. 1991; Fischer and Timberlake 1995; this study).

Northern blot analyses, using the 4.6-EcoRI fragment (Figure 5) as a probe, revealed a 6.5-kb transcript whose abundance was similar in mat+ and mat− mycelia (data not shown).

Heterologous Southern hybridization experiments were performed using two probes corresponding to the 5′ and 3′ regions of ami1. In both cases we observed a clear signal with DNA from A. nidulans and an even stronger signal with DNA from A. immersus. There were no signals with the DNA from the other organisms tested, even after long exposure of the blot, despite positive controls with the eEF1A probe (data not shown).

The ami1-1 mutant was obtained by a repeat-induced point mutation (RIP)-like mechanism. Until now, RIP has been described only in N. crassa (Selker et al. 1987; Selker 1997 for a review). The characteristics of the P. anserina RIP-like process will be described elsewhere (F. Graña, O. Lespinet, R. Rimbault, E. Coppin, M. Dequard-Chablat and M. Picard, unpublished results). With respect to the present purpose, the first 2 kb of one of the two ami1 copies present in the tandem duplication was sequenced after loss of the second copy by a recombination/excision (looping out) process. This revealed numerous G:C to A:T mutations, one of which created a nonsense codon 391 bp after the initiation codon (the localization of the mutation is designated by an arrow in Figure 6). Although the status of the second ami1 copy remains unknown, the stringency of the ami1-1 phenotype strongly suggests that the two copies are inactivated in the strain used in this study.

The clear similarity between the P. anserina AMI1 and the A. nidulans ApsA proteins strongly suggested that the two genes could be functional homologues. To test this hypothesis, the ApsA gene (kindly provided by Dr. R. Fischer) was introduced in the ami1-1 strain through cotransformation with a plasmid carrying a selectable marker, the bacterial hygromycin resistance gene (materials and methods). Among the hygromycin-resistant primary transformants, some exhibited a male-fertile phenotype. Two of these were purified through crosses with a mat− tester strain. They correspond to two transgenic strains carrying the ApsA gene integrated in the P. anserina genome and called (apsA-1) and (apsA-12). As shown in Figure 1, one transformant, ami1-1 (apsA-1), exhibited an efficient complementation of the mutant phenotype with respect to male fertility while the other, ami1-1 (apsA-12), displayed partial complementation. Cytological observations showed that some microconidia were indeed nucleate but that their percentage was very low in both transformants: about 3–4% in ami1-1 apsA-1 and <1% in ami1-1 apsA-12, compared to 0.1% in ami1-1 strains. These results were confirmed by functional tests (Table 1). Interestingly, in spite of the poor complementation with respect to conidia nucleation, the apsA-1 transgene decreases the amount of abnormal ascis when present in crosses between ami1-1 and the wild type: 20–25% [ami1-1 (apsA-1) × ami1+] vs. 36–38% ([ami1-1 × ami1+]). Moreover, it is able to restore self-fertility when present in mat+ (mat−) strains carrying the ami1-1 mutation (Figure 3C). However, the perithecia issued from a cross between this strain and a mat− tester strain remain mostly barren, although in this case the mat− strain was used as the female partner: the ami1-1 (apsA-1) mat+ (mat−) is male fertile and its self-fertility does not permit its use as a female partner in a cross. Cytological analyses did not show significant differences between the two crosses: ami1-1 mat+ (mat−) × ami1+ mat− (see above) and ami1-1 mat+ (mat−) × ami1+ mat−. These data lead to two conclusions: first, the apsA transgene does not complement this defect in the P. anserina mutant; second, this defect is not due to a maternal effect of the ami1-1 mutation.

We also examined the possible effects of the apsA-1 transgene in an ami1+ background. The relevant transgenic strains did not exhibit any particular phenotype with respect to microconidial nucleation and sexual reproduction in crosses bearing the transgene in a homozygous or heterozygous state (data not shown).

DISCUSSION

Genetic control of nuclear migration events required for transition to the cellular state in filamentous ascomycetes: As emphasized in the Introduction, nuclear migration events are common to all eukaryotes; however, the genetic control of these processes is still incompletely understood, even in systems amenable to classical and molecular genetics. Nevertheless, systematic studies, especially those performed in filamentous ascomycetes (Fischer 1999 for a review), have designated two classes of genes implicated in these processes: those that are essential or limiting for cell survival and have pleiotropic roles in both vegetative growth and development, and those that function primarily at the level of developmental processes (see Introduction). We will focus here on the second class, because of the remarkable ability exhibited by filamentous ascomycetes to achieve syncytial/cellular transitions at several steps of their life cycle.

Many genes controlling conidiophore development have been characterized in A. nidulans (Adams et al.,
Among them, apsA and apsB (Clutterbuck 1994) are required at the transition between the syncytial state (which is maintained in the conidiophore stalk) and the initial uninucleate state corresponding to the metula cells (Adams et al. 1998). apsA and apsB mutants grow quite normally, while displaying abnormal nuclear distribution in the mycelial filaments. The most conspicuous phenotype of these mutants is their very poor efficiency in nuclear transmission to the metulae, such that these cells are mostly anucleate, blocking further development. Interestingly, when a metula receives a nucleus, conidia are formed as in the wild type. Thus, the apsA and apsB genes are required at a very precise stage of conidiophore development (Clutterbuck 1994; Fisher and Timberlake 1995).

The main difference between apsA and apsB mutants concerns sexual reproduction, which also requires a transition from a syncytial to a cellular state (see below). While sexual development is quite normal in apsA mutants (Krüger and Fisher 1996) with the exception of one of the original mutants (which is sterile), all apsB mutants give low yields of ascospores (Clutterbuck 1994) but the step altered in these mutants remains unknown.

In N. crassa, orthologues of apsA and apsB have not yet been described (Springer 1993; Maheshwari 1999 for reviews). One can postulate that these genes are not involved in the macroconidiation pathway: macroconidia are uninucleate and their formation does not require a loss of the syncytial state. In contrast, microconidia are uninucleate and their nucleation might require apsA and apsB orthologues.

In P. anserina, which is closely related to N. crassa and evolutionarily very distant from A. nidulans, a systematic search for genes involved in nuclear migration events has not yet been undertaken. However, in this species, several genes required for a proper transition between the syncytial and the cellular states during sexual reproduction have been characterized. This transition occurs when pairs of nuclei must migrate from the sexual uninucleate cells into the ascogenous hyphae and croziers, to form the dicaryotic stage required for caryogamy, meiosis and ascospore formation (Zickler 1973). The cro1 gene is required for this syncytial/cellular transition. Croziers formed in a cro1-1 mutant are mainly uninucleate (Simonet and Zickler 1978). We do not yet know if the role of the cro1 gene is situated at the nuclear migration process necessary for exit from the uninucleate state and/or if it is involved after this transition, to control the coordination between mitoses and cell divisions required for maintenance of the dicaryotic state. The cro1-1 mutant grows slowly but nuclear distribution is normal in vegetative filaments (Bertheaux-Lecellier et al. 1998). The mating-type genes are also required for a proper transition to the dicaryotic state because they ensure nuclear recognition between mat+ and mat− nuclei at this critical step of sexual reproduction. Cytological and genetic data have shown that inactivation of one of these genes results in croziers that are mostly uninucleate and to a diminished progeny, mainly uniparental, with the genotype of the mat mutant partner. Our hypothesis is that the loss of mating-type expression in the mutant nuclei disturbs nuclear migration such that ascogenous hyphae are mostly populated by the mutant nuclei (Zickler et al. 1995; Arnaise et al. 1997; Debuchy 1999). Interestingly, in both cases (as mentioned above for the aps mutations), the mutations do not preclude further development. Plurinucleate (cro1-1) and uninucleate (mat mutants) cells can undergo caryogamy, followed by polyploid (cro1-1) or haploid (mat mutants) meiosis, and, when meiosis does not abort, ascospores are produced (Zickler et al. 1995; Bertheaux-Lecellier et al. 1998).

**ami1** is the functional orthologue of apsA but has broader functions: Both genes encode proteins that display 42% identity and share all structural features previously reported for ApsA (Fisher and Timberlake 1995). Furthermore, apsA is able to complement, at least partially, some phenotypic defects of the ami1-1 mutant. However, the ami1-1 mutation is more pleiotropic than the apsA mutations and causes defects not only in conidiation, but also in sexual reproduction (summarized in Table 2).

Overall, the ami1-1 defects can be grouped in three classes: abnormal distribution of nuclei in plurinucleate cells, lack of nuclear transmission at the syncytial/cellular transition, and inability to self-fertilize. The first type of defect occurs in vegetative filaments (as in apsA mutants) but also in part of the asci, thus hampering correct ascospore formation. In wild-type asci, nuclei that cooperate in ascospore formation are placed in close proximity by the arrangement of the PMM spindles: after division, each nuclear pair remains linked by astral microtubules and they must migrate before binucleate ascospore formation (Thompson-Coffe and Zickler 1992). Cortical, astral, and spindle microtubules as well as spindle pole bodies appear normal in an ami1-1 × wild-type cross. Conidiophore microtubules and actin filaments were also found normal in the apsA mutant of A. nidulans (Fisher and Timberlake 1995). Thus, the role played by the ApsA/AMI11 protein in nuclear positioning remains unknown.

The second type of defect is observed during conidiation. This is also a property in common with apsA mutants, even though conidiation is far more complex in A. nidulans than in P. anserina. However, in the ami1-1 mutant, this type of defect is also encountered at two other stages of the life cycle involving a syncytial/cellular transition: the formation of the perithecial beak paraphyses and of the croziers. In the first case, the mutant shows a maternal effect, since these structures are issued from the perithecial envelope whose origin is maternal. Instead of forming regularly uninucleate cells as in the wild type, the mutant maintains the plurinucleate state in one or two cells and then forms cells that are normally septate but anucleate and misshapen. In the second
case, while wild-type croziers are always binucleate, ami1-1 perithecia yield a few uninucleate and abnormal croziers. This was observed in a particular genetic background in which the ami1-1 nucleus carries the two mating-type information. The reasons for which this context is more sensitive to the ami1-1 mutation (while heterozygous) and why only some croziers are abnormal remain unclear.

The third type of ami1-1 defect is the inability to self-fertilize. Since we could not perform ami1-1 mat+ × ami1-1 mat− crosses, we took advantage of the previous observation that strains carrying the two mat information in the same (haploid) nucleus displayed selfing (Picard et al. 1991). An ami1-1 mat+ (mat−) strain is unable to self-fertilize. This strain is able to act as a male partner (through filaments) in a cross and develops numerous protoperithecia (female organs) but only a few are available for fertilization by exogenous (wild-type) microconidia. Thus, the strain can act as a female partner, but to a limited extent. The simplest explanation of these observations is as follows: recognition between male and female structures indeed occurs in the ami1-1 mat+ (mat−) strain but the ami1-1 male nucleus is unable to migrate in the ami1-1 female trichogyne, thus hampering perithecial development. This block occurs so early that fertilized protoperithecia cannot be distinguished from those that are unfertilized. However, the signal caused by this pseudofertilization event would render impossible a true fertilization of the relevant female structures by an exogenous male gamete. Notably, this selfing defect is complemented by apsA, which also increases the percentage of nucleate microconidia but seems unable to complement other defects of ami1-1. These differences in the complementation abilities of apsA will be discussed below.

NUM1/apsA ami1: an example of the functional evolution of a gene: Both ApsA (Fischer and Timberlake 1995) and AMI1 (this article) proteins show striking structural similarities with the protein encoded by the NUM1 gene of S. cerevisiae (Kormanec et al. 1991), although primary sequence similarities are limited to the C-terminal portions. num1 mutant strains grow normally but exhibit abnormal distribution of nuclei in budded cells, with two nuclei in the mother cell. In fact, in the mutant the order of the nuclear migration and nuclear division events is reversed compared to the wild type. Normally, nuclear migration to the bud neck occurs before mitosis, while in the mutant this occurs after nuclear division (Kormanec et al. 1991; Reverdel and Aigle 1993). NUM1 shows a cell-cycle-dependent expression and its product associates with the cortex of mother cells, but not with the bud. Genetic and cytological data suggest that the NUM1 protein controls nuclear migration by affecting microtubule functions (Farkasovsky and Küntzel 1995). The ApsA protein was also localized at the cytoplasmic membrane along the mycelial filaments and at the septa (Suelmann et al. 1997). In contrast, the apsB gene encodes a cytoplasmic protein. The remarkable point is that, in the apsB null mutant, nuclei show increased chaotic movements, which may explain their clustering in filaments (Suelmann et al. 1998). The data overall led the authors to propose an attractive hypothesis for the developmental defect of the mutants (anucleate metulae), rather than the simple hypothesis based on a nuclear migration failure: the nuclei would migrate into metulae but, because of an increased motility, would exit the cells before septum formation. Thus, the aps genes would regulate nuclear positioning and not nuclear movements per se (Suelmann et al. 1997, 1998).

The functional evolutionary relationship between apsA and ami1 can be discussed at two (overlapping) levels: the partial complementation of ami1-1 by apsA and the differences between phenotypic defects caused by null mutations in the two genes. Of the two defects shared by apsA and ami1 mutants (summarized in Table 2, first two rows), one is partly rescued by the apsA transgene, which increases 30-fold the percentage of nucleate microconidia produced by ami1-1. However, this corresponds only to 3% of the wild-type level. This

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**TABLE 2**

<table>
<thead>
<tr>
<th>Fungal structures or events</th>
<th>ami1-1</th>
<th>ami1-1 (apsA1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycelial filaments</td>
<td>Nuclei clustered</td>
<td>Nuclei clustered</td>
</tr>
<tr>
<td>Microconidia</td>
<td>0.1% nucleate</td>
<td>3% nucleate</td>
</tr>
<tr>
<td>Self-fertilization</td>
<td>Nulla</td>
<td>Restoredb</td>
</tr>
<tr>
<td>Beak paraphysae</td>
<td>Anucleate apical cells</td>
<td>Partly restored</td>
</tr>
<tr>
<td>Croziers</td>
<td>Some uninucleate</td>
<td>Some uninucleate</td>
</tr>
<tr>
<td>Postmeiotic nuclear distribution</td>
<td>Abnormal in 36–38% asci</td>
<td>Abnormal in 20–25% asci</td>
</tr>
</tbody>
</table>

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a In strains carrying both mating types in the same nucleus: ami1-1 mat+ (mat−) vs. ami1-1 (apsA1) mat+ (mat−).
b Maternal effect.
c In crosses: ami1-1 mat+ (mat−) × ami1+ mat− vs. ami1-1 (apsA1) mat+ (mat−) × ami1+ mat−.
d In ami1-1 or ami1-1 (apsA-1) × ami1+ crosses.
partial complementation (and the lack of detectable complementation of abnormal distribution of nuclei in the mycelium) can be explained in two (nonexclusive) fashions. First, the apsA gene might be underexpressed, due either to position effects in the two integration events analyzed, or to an inefficient utilization of its promoter in P. anserina. In A. nidulans, ApsA is a low-abundance protein (Suelmann et al. 1997) and in P. anserina, it might be produced below the threshold required for full complementation of the ami1-1 mutant. The second explanation for partial complementation relies upon the partners with which the ApsA protein must interact to be fully functional and that could be, at least partially, different in the two fungi. This second hypothesis is suggested by the differences in phenotypic defects displayed by the apsA and ami1 mutants. The most striking difference is seen at the level of sexual reproduction: ami1-1 mat+ (mat−) strains are unable to self-fertilize while sexual reproduction of apsA null mutants is quite normal (Krüger and Fischer 1996). Since P. anserina and A. nidulans are evolutionarily distant, the two genes may have evolved such that ami1 would play a general role, while the role of apsA would be restricted to the asexual part of the life cycle. However, several puzzling observations suggest that apsA might play some role in sexual reproduction. First, a mutant allele of apsA causes sterility (Clutterbuck 1994) but its precise defect is not known. Second, among the two analyzed extragenic suppressors of apsA, one (samA−) fails to produce fruiting bodies (cleistothecia) and the other (samB−) gives barren cleistothecia (Krüger and Fischer 1996). Deletion of the samB gene has shown that it is involved in morphogenesis rather than in nuclear distribution per se: the mutation causes premature hyphal ramifications and mislocalization of septa. Thus, its suppression effect upon apsA mutants may be very indirect (Krüger and Fischer 1998). However, its sexual defect has not been investigated precisely. The third and most amazing point concerning apsA and sexual reproduction is its ability to restore self-fertilization of ami1-1 mat+ (mat−) strains. There is, in fact, an impressive parallel between apsA/ami1 and the rca1/flbD genes. flbD is an A. nidulans gene that encodes a DNA-binding protein involved in initiation of conidial development (Adams et al. 1998 for a review). rca1 is a N. crassa gene that seems to be the functional orthologue of flbD since it fully complements the A. nidulans flbD mutant. The striking point is that deletion of rca1 in N. crassa does not cause any major effect on conidiation and sexual reproduction (Shen et al. 1998). As the authors conclude, if rca1 is involved in N. crassa development, its role is subtle or redundant. This conclusion might also be applied to apsA with respect to sexual reproduction. Interestingly, the A. nidulans flbD gene was the sole among six regulators of conidiation that crosshybridized with N. crassa DNA (Shen et al. 1998).

This heterologous hybridization approach has been used with two fragments of the ami1 gene. There is no signal with S. macrospora, which is closely related to P. anserina, while there is a clear-cut signal with A. nidulans and A. immersus, which are very distant from each other and from P. anserina. S. macrospora and P. anserina belong to the same family (Sordariacae) but S. macrospora is aconidal and homothallic. This does not explain, per se, the lack of hybridization since sexual development is similar in these two fungi. S. macrospora may, however, have an orthologue of apsA/ami1, which might have evolved significantly at the nucleotide sequence level. A. nidulans and A. immersus belong to the two other subgroups of filamentous ascomycetes: Plectomycetes and Discomycetes, respectively. A. nidulans is homothallic and differentiates conidia, while A. immersus is heterothallic and aconidal. It would be of great interest to clone the A. immersus apsA/ami1 orthologue to learn more about the functional evolution of these genes. It would also be interesting to identify a possible orthologue in Homobasidiomycetes and understand its putative role in nuclear migration at mating. In these organisms, the process is controlled by a pheromone/receptor control via one of the two mating-type loci (Wendland et al. 1995; Casselton and Olesnicky 1998 for a review). However, the genes acting downstream of this signal remain to be discovered.

The major role of apsA/ami1 is to regulate nuclear movements and/or positioning; these genes are thus essential for proper development at one or several steps that require a syncytial/cellular state transition. It is of the utmost importance, in terms of the functional evolution of this class of genes, to discover if they have orthologues in multicellular organisms, especially those that display syncytial/cellular transitions in their development. This concerns not only insect embryogenesis (see Schejter and Wieschaus 1993 for a review on Drosophila) but also those plants which require this type of transition for formation of their female gametes (Huang and Sheridan 1994 and references therein) or in their early embryonic development (Johansen 1950).

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