Asm-1+, a Neurospora crassa Gene Related to Transcriptional Regulators of Fungal Development

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ABSTRACT

This report describes the identification, cloning, and molecular analysis of Asm-1+ (Ascospore maturation 1), the Neurospora crassa homologue of the Aspergillus nidulans stuA (stunted A) gene. The Asm-1+ gene is constitutively transcribed and encodes an abundant, nucleus-localized 68.5-kD protein. The protein product of Asm-1+ (ASM-1), contains a potential DNA-binding motif present in related proteins from A. nidulans (StuA), Candida albicans (EFGTF-1), and Saccharomyces cerevisiae (Phd1 and Sok2). This motif is related to the DNA binding motif of the Swi4/Mbp1/Res family of transcription factors that control the cell cycle. Deletion of Asm-1+ destroys the ability to make protoperithecia (female organs), but does not affect male-specific functions. We propose that the APSES domain (ASM-1, Phd1, StuA, EFGTF-1, and Sok2) defines a group of proteins that constitute a family of related transcription factors involved in the control of fungal development.

Neurospora crassa is an excellent organism in which to study sexual development. It offers many of the experimental advantages of fungi like Saccharomyces cerevisiae and Aspergillus nidulans, but unlike them, Neurospora develops large multicellular structures (perithecia) that result from the fertilization of protoperithecia (female elements, formed in response to nitrogen deprivation) by a male element (macroconidia, microconidia or vegetative cells). After fertilization, the male-derived nuclei co-exist in a heterokaryotic tissue and are transported into the developing perithecium. Inside this multicellular apparatus, nuclear fusion (karyogamy), Meiosis I, Meiosis II, and mitosis occur and ascospore maturation takes place. Sexual development culminates in the ejection of mature ascospores from the mature perithecium (RAJU 1980, 1992).

To identify regulatory genes of sexual development, we explored the possibility that genes from a related organism like A. nidulans might be evolutionarily conserved and play similar roles in Neurospora crassa. For this we selected the stuA (stunted A) gene (MILLER 1990; MILLER et al. 1991, 1992). Our choice was based on the fact that, unlike other development mutants controlling conidiosephore development in A. nidulans, stuA mutants are in addition self-sterile, i.e., they fail to form sexual reproductive structures (cleistothecia) (TIMBERLAKE 1987, 1991). CLUTTERBUCK (1969), found stuA in a collection of mutants that he isolated on the criteria that they had a qualitative effect on conidiosephore development but had no significant alterations in either vegetative growth rate or nutritional requirements, and he predicted that these would encode regulatory genes (CLUTTERBUCK 1969; MARTINELLI 1979; MARTINELLI and CLUTTERBUCK 1971). The molecular structure of stuA, its homology to other transcription factors, and its pattern of developmental regulation have largely confirmed CLUTTERBUCK’s prediction (MILLER et al. 1992; KOCH et al. 1993; GIMENO and FINK 1994).

Here we report the cloning, molecular analysis, and deletion of Asm-1+ (Ascospore maturation 1), the N. crassa homologue of stuA. The gene was named for the postkaryogamic phenotype of the loss of function mutant (ARAMAYO and METZENBERG 1996b), which was obtained through the use of a “sheltered disruption” procedure. Although nonlethal, disruption of Asm-1+ caused a pronounced vegetative phenotype, and it is questionable whether the mutant would have been isolated without the sheltering procedure. The protein product of the Asm-1+ gene, ASM-1, is an abundant, nuclear-localized protein that is required for protoperithecial formation. ASM-1 has a region of homology with other proteins known to be involved in cell cycle control and fungal development. This region of homology is particularly conserved between ASM-1 and those proteins implicated in fungal development. We propose to call this motif APSES (ASM-1, Phd1, StuA, EFGTF-1, and Sok2), and we suggest this DNA binding domain may define a family of transcription factors involved in fungal development.

MATERIALS AND METHODS

Strain construction: Strains of N. crassa used are listed in Table 1. FGSC indicates the Fungal Genetics Stock Center,
TABLE 1

Fungal strains

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGSC 2489</td>
<td>(A)</td>
</tr>
<tr>
<td>FGSC 2490</td>
<td>(a)</td>
</tr>
<tr>
<td>RLM 25-15</td>
<td>(mep+ his-3; mtrα; pan-2 A)</td>
</tr>
<tr>
<td>RLM 80-21</td>
<td>(mep+ his-3; mtrα; Asm-1α::mtrβ; pan-2 A)</td>
</tr>
<tr>
<td>RLM 83-01</td>
<td>(mep+ his-3'::Asm-1'; mtrα; Asm-1β::mtrβ; pan-2 A)</td>
</tr>
<tr>
<td>RLM 83-05</td>
<td>(mep+ his-3'::Asm-1'; mtrα; Asm-1β::mtrβ; pan-2 A)</td>
</tr>
<tr>
<td>RLM 25-16</td>
<td>(mep- his-3; mtrα; pan-2 A)</td>
</tr>
<tr>
<td>RLM 80-23</td>
<td>(mep- his-3; mtrα; Asm-1α::mtrβ; pan-2 A)</td>
</tr>
<tr>
<td>RLM 83-03</td>
<td>(mep- his-3'::Asm-1'; mtrα; Asm-1β::mtrβ; pan-2 A)</td>
</tr>
<tr>
<td>RLM 83-07</td>
<td>(mep- his-3'::Asm-1'; mtrα; Asm-1β::mtrβ; pan-2 A)</td>
</tr>
<tr>
<td>RLM 77-01</td>
<td>(his-3 cyh-1' al-1; mtrα; int1 A)</td>
</tr>
<tr>
<td>RLM 77-06</td>
<td>(his-3 cyh-1' al-1; mtrα; int1 A)</td>
</tr>
</tbody>
</table>

Allele numbers or designations are al-1 (34508), cyh-1' (KH52r), his-3 (1-254-723), int1 (89601), mep+ (10), mtrα (deletion SR92), and pan-2 [B3(Y153M96)]. The construction of Asm-1α::mtrβ, his-3'::Asm-1', and his-3'::Asm-1' alleles are described in MATERIALS AND METHODS.

University of Kansas Medical Center, Kansas City, and RLM indicates strains constructed in this study. Strains RLM 25-15, 25-16, 77-01, and 77-06 have been deposited in the FGSC under FGSC numbers 7505, 7506, 7507, and 7508, respectively.

Allele Asm-1α::mtrβ was constructed by deleting the promoter and part of the coding region of Asm-1α as described in RESULTS.

Strains carrying allele his-3'::Asm-1' were constructed by transforming RLM 80-21 and RLM 80-23 with pRAUW123 to generate RLM 83-01 and RLM 83-03, respectively. Similarly, strains carrying allele his-3'::Asm-1β were constructed by transforming RLM 80-21 and RLM 80-23 with pRAUW124 to generate RLM 83-01 and RLM 83-03, respectively. These alleles were generated by a double crossover event involving the integration of the left flank, insert (Asm-1α or Asm-1β) and right flank segment of pRAUW123 or pRAUW124 at the his-3 locus, as described in RAMAYO and METZENBERG (1996a).

Neurospora was transformed using procedures described by RAMAYO and METZENBERG (1996a).

**Growth conditions:** For production of mycelia and conidia, strains were grown in 1 ml of appropriately supplemented Vogel's Medium N with 2% sucrose, or in 20 ml batches solidified with 1.5% agar (in 250 ml Erlenmayer flasks) (Davies and DE SERRES 1970). When supplements were appropriate or required, they were generally used at the following concentrations: L-histidine, 1 mM or 0.5 mM, as indicated; L-arginine, 20 mM; inositol, 50 pg/ml; Ca-+ pantothenate, 10 μg/ml; benzyladenine, 1 μg/ml; 6-methyl purine, 1 mM; 3-fluoro-DL-phenylalanine (80 μg/ml or 15 μg/ml). All platings were done on Vogel's salts with the sugar mixture of BROCKMAN and DE SERRES (1963) to induce colonial growth. Strains were grown for 2 day at 34°C (dark) and 1 day at room temperature (light).

To determine female fertility/sterility, the strain in question was inoculated onto WESTERGAARD's medium (WESTERGAARD and MITCHELL 1947) and incubated at room temperature for 7 days. At this time, female-fertile strains show abundant protoperithecia. Plates were then flooded with either 5 ml of a heavy suspension of wild-type conidia of the opposite mating type in 1x Vogel's salts, or with a control suspension of the same mating type. The high NH₄⁺ concentration of Vogel's salts prevents the de novo formation of protoperithecia. Plates were then incubated at room temperature for another 7 days, during which the young perithecia darken and enlarge greatly as they mature.

For the developmental time course experiment, wild type A was inoculated at a density of 1 × 10⁶ conidia/ml into
minimal Vogel’s medium containing ampicillin and chloramphenicol each at 25 μg/ml to allow relaxation of sterile technique, and shaken at 250 rpm at 50° for 15 hr. Aliquots of cells (200 ml) were harvested onto 7 cm Whatman no. 1 filter papers by vacuum filtration. Each filter paper was transferred to a Petri dish containing minimal Vogel’s medium. Covers were replaced, and the dishes were incubated at 22° in a humid environment with constant light. Samples (one Petri dish per time point) were taken at 0, 3, 8, 12, 16, 24, 30, and 55 hr.

**Plasmid construction:** Plasmid pGDMtr, generously furnished by T. Randall, consists of the 2.9-kb HindIII-EcoRI fragment from pN816 (Koo and Stuart 1991; Stuart et al. 1988) inserted into the HindIII and EcoRI sites of pGEM9Zf(+) (Promega, Madison, WI).

The following plasmids were constructed using standard procedures: pRAUW43 contains the 8.3-kb EcoRI fragment (coordinates 4.5–12.5, Figure 1) from cosmid csm17-7A (Orbach and Sachs 1991) inserted into the EcoRI site of pBC KS(+)(Stratagene, La Jolla, CA). The direction of transcription of Ast-1 and lacZ are the same in this plasmid. pRAUW44 contains the 12.2-kb HindIII fragment (coordinates 0–12.2, Figure 1) from cosmid csm17-7A inserted into the HindIII site of pBC KS(+)(Stratagene). The direction of transcription of Ast-1 and lacZ are the same in this plasmid. pRAUW50 and pRAUW55 both contain the 3.5-kb SmaI fragment (coordinates 6.5–10, Figure 1) from pRAUW43 inserted into the SmaI site of pBluescript II KS(+)(Stratagene) but in opposite orientations. The direction of transcription of Ast-1 and lacZ are the same in pRAUW50 and opposite in pRAUW55. pRAUW51 contains the 0.38-kb EcoRI-SmaI fragment (coordinates 4.5–4.8, Figure 1) from pRAUW43 inserted into the EcoRI-Smal sites of pBC KS(+)(Stratagene). pRAUW52 contains the 2.5-kb SmaI fragment (SmaI-EcoRI fragment, coordinates 10–12.5, Figure 1) from pRAUW43 inserted into the SmaI site of pBluescript II KS(+)(Stratagene). pRAUW53 contains the 1.44-kb SmaI fragment (coordinates 5.5–6.5, Figure 1) from pRAUW43 inserted into the SmaI site of pBluescript II KS(+)(Stratagene). The direction of transcription of Ast-1 and lacZ are the same in the pRAUW53, pRAUW54 contains the 0.43-kb SmaI fragment (coordinates 4.8–5.3, Figure 1) from pRAUW43 inserted into the SmaI site of pBluescript II KS(+)(Stratagene). The direction of transcription of Ast-1 and lacZ are the same in pRAUW54 and opposite in pRAUW55. pRAUW56 and pRAUW64 both contain the 3.5-kb EcoRI fragment from pRAUW44 (coordinates 1–4.5, Figure 1) inserted into the EcoRI site of pBC KS(+)(Stratagene), but in opposite orientations. The direction of transcription of Ast-1 and lacZ are the same in pRAUW64 and opposite in pRAUW65. pRAUW65 contains the 3.5-kb EcoRI fragment from pRAUW64 (coordinates 1–4.5, Figure 1) inserted into the EcoRI site of pGDMtr. The direction of transcription of Ast-1 and minA are the same in this plasmid. pRAUW66 contains the 3.5-kb Nol-HindIII fragment from pRAUW50 (SmaI-Smal fragment, coordinates 6.5–10, Figure 1) inserted into the Nol and HindIII sites of pRAUW65. pRAUW110 contains 0.91-kb NeoI-SmaI fragment from pRAUW50 inserted into the Nol and the SacI site of pGSTAG (Smith and Johnson 1988). pRAUW122 has been described by Araya and Metzenberg (1996a). pRAUW123 contains the 12.2-kb HindIII fragment from pRAUW44 inserted into the XbaI site of pRAUW122. To clone this fragment, we half-fill-in the insert of pRAUW123 with DNA Polymerase I-Klenow fragment in the presence of dNTP’s (Asm-1 site (insert) coordinates 6.5–10, Figure 1) inserted into the HindIII site of pGEM9Zf(+) (Stratagene). A frameshift mutation in amino acid residue no. 189 of Asm-1, causing the chain to terminate at amino acid residue no. 193. This allele was called Ast-1P. pRAUW124 contains the 12.2-kb modified fragment from pRAUW113 inserted into the XbaI site of pRAUW122. This fragment was cloned by the half-fill-in strategy described for pRAUW123. The direction of transcription of Ast-1P and his-3F are convergent in this plasmid.

**Nucleic acid manipulations:** DNA was isolated as follows:

20 μl of appropriately supplemented minimal medium in a 125-ml Erlenmeyer flask was inoculated with conidia and incubated without shaking at 34° for 36–48 hr. Cultures were agitated briefly by hand every 12 hr to keep the mycelia dispersed and prevent condensation. Mycelia were harvested, drained on paper towels, and gently pressed. Each damp-dry mycelial mat was transferred to a 1.7 ml Eppendorf tube, and the tubes were vacuum-desiccated for 24–36 hr. Each lyophilized mycelial pad was broken up into a fine powder with a stainless steel spatula and suspended in 1 ml of freshly prepared extraction buffer [50 mM EDTA, pH 8.0; 0.2% SDS; 1.5 μl/ml of diethyl pyrocarbonate (DEPC)]. After vigorous mixing (vortex), the suspensions were heated at 68° for 30 min with occasional mixing. Samples were spun in a microfuge for 15 min. Supernatants were poured into fresh Eppendorf tubes, and 45 μl of cold 8 M potassium acetate, pH 4.2, was added to each. Samples were mixed by inversion, kept on ice for 15 min and centrifuged for 5 min at room temperature. The supernatants (800 μl each) were transferred to clean Eppendorf tubes and iso-propyl alcohol (500 μl) was added. After thorough mixing, the samples were centrifuged for 5 min at room temperature. The pellets were washed with 80% ethanol and dissolved in 500 μl of 1× TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) containing 100 μg/ml of RNase A. After 5 min of incubation at 37°, the samples were extracted once with phenol:chloroform:iso-amyl alcohol and once with chloroform:iso-amyl alcohol. To the aqueous phase was added 1 ml of 95% ethanol. Samples were shaken and centrifuged for 5 min at room temperature. The pellet was washed with 80% ethanol, dried under vacuum, and dissolved in 150 μl of 1× TE. Usually 20–25 μl of this DNA was used per restriction enzyme digest.

*N. crassa* vegetative RNA was isolated essentially as described by Timberlake (1986). Other nucleic acid manipulations followed standard procedures (Ausubel et al. 1987; Sambrook et al. 1989). For blots, *N. crassa* DNA or RNA samples isolated as described above were electrophoretically fractionated in agarose (DNA) or formamide-agarose (RNA) gels, transferred to Nytran (Schleicher and Schuell, Keene, NH) following procedures recommended by the manufacturer, and hybridized to DNA fragments labeled with °P by random primer extension as described (Ausubel et al. 1987; Sambrook et al. 1989)

**Low stringency hybridizations:** Filters carrying DNA corresponding to an *N. crassa* cosmid library (Orbach and Sachs 1991) were probed with a radiolabeled 1.9-kb XhoI-XbaI DNA fragment containing most of the coding region of the *stuA* gene from *A. nidulans* (Miller et al. 1992). Hybridization was at 45° for 20 hr in 6× SSC, 5× Denhardt’s; 0.2% SDS; 50 μM Na phosphate buffer, pH 6.0, followed by a wash at 42° for 1 hr in 3× SSC; 0.1% SDS; 2 mm EDTA, pH 8.0, followed by a second wash at 42° for 1 hr in 1× SSC, 0.1% SDS; 2 mm EDTA, pH 8.0. The Denhardt’s solution used was as follows: 2.22% each in PVP 360,000, Ficoll 400,000, and BSA.
Isolation and analysis of cDNA clones: cDNA clones of Asm-1 were isolated by screening 1 × 10^6 plaques from two ZAP II libraries (nitrate induced and glutamine repressed) cDNA libraries constructed by R. H. Garrett (University of Virginia, Charlottesville, VA) and available through the FGSC. For a probe we used a 1.9-kb gel isolated fragment (coordinates 5–25, Figure 1). Seven positive cDNAs were identified and purified using phage procedures described by Ausubel et al. (1987) and Sambrook et al. (1989). Phagemids were rescued using ExAssist helper phage (Stratagene) following procedures recommended by the manufacturer. The two longest cDNAs were selected for further analysis (cDNA 21 and

**FIGURE 2.—**Structure of the Asm-1 gene. The positions of the main restriction sites present in the region are indicated above the DNA sequence. The names, location and orientation of the oligonucleotides used are indicated below the DNA sequence. The 5' end of the gene was determined by primer extension using ORAUW46 and it is indicated by an arrow above the nucleotide sequence (position 67). The 5' and 3' ends of the sequenced cDNAs (21 and 25) are indicated above the nucleotide sequence. Comparison of the cDNA sequences with the genomic sequence revealed no differences. The boxed nucleotides indicate a putative polyadenylation signal. Two polyadenylation sites were deduced from sequencing the 3' ends of the cDNAs. Open reading frame analysis revealed one polypeptide of 643 amino acid residues starting with a methionine. The amino acid sequence is given in one-letter code below the nucleotide sequence. Boxed amino acid residues indicate the conserved protein domain. Underlined amino acid residues represent identity between ASM-1 and the StuA protein from Aspergillus nidulans (JOTUN-HEIN alignment. MegaAlign, DNASTAR Inc., Madison, WI). The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under the accession number U51117.
cDNA25, both isolated from the glutamine-repressed library), and their sequences determined using standard procedures.

The template for the reverse transcriptase-polymerase chain reaction (RT-PCR) experiments was prepared using the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA), and poly(A)^+ RNA isolated from vegetative growing cells, following procedures recommended by the manufacturer. p39-42 was constructed by cloning the PCR amplification product obtained using oligonucleotides ORAUW39 and ORAUW42 (Figure 2) and the products of the first strand synthesis as a template. PCR products were digested with Smal and cloned into the Him11 site of pGEM3Z+(Promega). The direction of transcription of Asm-1 and lac-Z are the same in this plasmid. The conidial and perithecial A ZAP I1 cDNA libraries were constructed and provided by S. KANG (The University of New Mexico).

Protein expression and affinity purification of antibodies: Amino acid residues no. 277 through no. 578 of ASM-1 were expressed as a glutathione-Transferase (GST) fusion protein in Escherichia coli dh5α-mcr cells ( Gibco-BRL, Life Technologies, Inc., Gaithersburg, MD) using the vector pR AUW110 (for details see plasmid construction). The thrombin-cleaved fragment was purified as described (Guam and Dixon 1991). Rabbit polyclonal antiserum was raised against the purified fragment of ASM-1. Primary immunizations with the fragment (0.5 mg per rabbit) were performed in Freund's complete adjuvant. Boost immunizations with 0.1 mg per rabbit were performed at 4-wk intervals in Freund's incomplete adjuvant. Sera were removed 14 day after each boost. IgG was purified on a protein A-Sepharose column. Polyclonal antibodies were affinity-purified on a column to which the fragment was coupled using the Immunopure kit (Pierce, Rockford, IL). The purified antibodies reacted only with ASM-1 in a test immunoblot.

Preparation of cell extracts and Western analysis: N. crassa strains grown in shake-flasks on supplemented Westergaard's medium (Westergaard and Mitchell 1947) for 24 hr at room temperature were harvested on Whatman no. 1 filter paper and the mycelia were resuspended in 2 ml of 20 mM Tris-HCl, pH 7.5; 50% (v/v) glycerol; 0.2 mM PMSF; 1 mM EDTA; 0.1 mM DTT. Mycelia were homogenized for 90 sec at 4°C with a motor-driven teflon homogenizer. Cell debris were removed by centrifugation at 4°C. The clear supernatant was diluted 1:1 to 2X SDS-PAGE sample buffer and boiled. Samples (25 µg each) were subjected to SDS-PAGE and the proteins transferred to nitrocellulose membranes (Schleicher and Schuell). Immunodetection of ASM-1 using the ECL kit (Amersham, Arlington Heights, IL) was carried out with affinity-purified antibodies (1:600 dilution).

Preparation of cells for confocal immunolocalization: Conidia were germinated at 25°C and 180 rpm in 25 ml of Westergaard's medium (Westergaard and Mitchell 1947), supplemented with t-histidine (1 mM) and Ca2+ pantothenate (2.5 µg/ml), for ~6 and 12 hr for strains RLM 25-15 and RLM 80-21, respectively. After this time, ~90% of the conidia corresponding to each strain had germinated. The following protocol was modified from Oakey et al. (1990). The cultures were adjusted to 4% formaldehyde [from a freshly prepared 40% stock made by heat depolymerization of EM grade paraformaldehyde (Polysciences, Inc., Warrington, PA)]; and 0.25% glutaraldehyde [from a freshly opened ampoule of 70% glutaraldehyde (EMS, Fort Washington, PA)]. Cells were harvested by brief centrifugation at 4°C and resuspended in 2 ml of freshly made 4% formaldehyde; 0.25% glutaraldehyde; 50 mM K+PIPES, pH 6.5; 25 mM EGTA; 1% DMSO; 5 mM MgSO4. Cells were transferred to an Eppendorf tube, and incubated with gentle agitation for 90 min at room temperature. Cells were then harvested by centrifugation at 15,000 rpm for 10 sec and washed three times with 50 mM K+PIPES, pH 6.5; 1 mM EGTA; 1 mM MgSO4 (PEM) buffer. The washed cells were resuspended in 2 ml of PEM supplemented with 1 M sorbitol, 1% bovine serum albumin (PEMSB), and protease inhibitor cocktail (PIC). The PIC contains aprotinin, chymostatin, pepstatin, and APMSF, each at 5 µg/ml in DMSO. The PIC was diluted 1:100 into PEMS immediately before use. To 1.8 ml of resuspended fixed cells, 0.2 ml of 1% Novozyme 234 (Lot # PPM 3944; Interspec Products, Inc., Foster City, CA) in PEMS was added. The cells were digested for 70 min at 30°C with gentle agitation, harvested by centrifugation, washed three times as described before with PEMS, and resuspended in 0.5 ml of PEM with PIC.

Confocal immunofluorescence microscopy: All steps were done at room temperature. Samples were not allowed to dry between washing steps. All washes were done with 150 µl of the indicated solution. A suspension of fixed cells (20–50 ml) was pipetted into polylysine-coated wells (Pringle et al. 1991). After 10 min, the buffer was carefully removed by aspiration and the fixed cells were washed twice in PEMS. The cells were then incubated for 10 min in 150 µl of permeabilization solution (50 mM K+PIPES, pH 6.5; 1 mM EGTA; 10% DMSO; 1 mM MgSO4; 0.2% Nonidet P-40). The solution was removed by aspiration and the cells were washed once with PEM followed by two washes with TTBS (30 mM Tris-HCl, pH 8.0; 200 mM NaCl; 0.1% Tween-20). To reduce background fluorescence, cells were incubated for 15 min in 0.25 mM trithionolamine, pH 7.5; 1 mM EGTA; 1 mM MgSO4, after which the buffer was replaced with fresh buffer for another 15 min. The cells were then washed twice for 2 min each with TTBS and incubated in TTBS supplemented with 10% goat serum for 30 min in a humid chamber. The cells were then washed twice with TTBS as described above and incubated for 1 min in TBS with 1% SDS. After washing the cells as described before, they were incubated with primary antibodies (1:50 dilution) in TTBS plus 1% bovine serum albumin and 0.04% NP40 with PIC (incubation buffer) for 1 hr in a humid chamber. Next, cells were washed five times with TTBS for 3 min each and incubated with the secondary antibodies (1:20 dilution; Molecular Probes, Inc., Eugene, OR) in incubation buffer for ~1 hr in a humid chamber in the dark. Before use, the secondary antibodies were diluted 1:20 in incubation buffer and incubated for 45 min on ice with aceton powder of RLM 80-21 grown in supplemented Vogel's medium. After incubation, the mixture was centrifuged at 15,000 rpm for 1 min, and the supernatant was recovered. After incubation with the secondary antibody, the cells were washed as before; wells were covered with a mounting medium containing 90% glycerol; 10% PBS, pH 9.0; 1 mg/ml of p-phenylenediamine. A coverslip was added and sealed with clear nail polish as described (Pringle et al. 1991). To delineate the nuclei, cells were incubated with 2 nM YOYO-1 (Molecular Probes) for 20 min in the dark as described. Eugene supplier, except than 100 µg/ml of RNAse was present during the incubation. Controls included no antibodies, and primary or secondary antibodies alone. Slides were viewed with a Biorad MRC600 Confocal microscope.

Scanning electron microscopy (SEM): Sample preparations for SEM observations were carried out by the procedure of Springer and Yanofsky (1989) with minor modifications. Strains used for vegetative morphology studies were spotted onto 1.5% agar containing Westergaard's salts + sucrose and incubated for 4 day at room temperature (22°C). Agar blocks (~1 cm³) were removed and incubated for 2 hr at room temperature in a fixative solution of 2% glutaraldehyde and 1% formaldehyde (the latter freshly prepared from paraformaldehyde) in 0.1 M sodium phosphate buffer (pH 7.2). Samples were rinsed three times with 0.1 M sodium phosphate
buffer, postfixed for 1 hr with 1% OsO₄, in the same buffer, washed five times with water, and dehydrated through a series of ethanol concentrations (30, 50, 70, 90 and 100%, 30 min each step). Critical-point drying was performed using a Bal-Tec model Bal-190 dryer. Samples were mounted on metal stubs, sputter-coated with gold, and viewed with a Hitachi (model S570) scanning electron microscope.

RESULTS

Cloning of Asm-1*: Preliminary experiments indicated the presence in Neurospora of a gene homologous to stuA from A. nidulans (data not shown). To clone this homologue, we used a radionucleated DNA corresponding to the coding region of stuA to probe filters under low stringency carrying DNA corresponding to Neurospora cosmids library (see MATERIALS AND METHODS). Two positive cosmids were identified (X17:7A and X19:5C), and one of them (X17:7A) was chosen for further analysis. Using low stringency hybridization, we localized the stuA-homologue to a 8.3-kb EcoRI fragment (coordinates 4.5–12.5, Figure 1) and to a 12.2-kb HindIII fragment (coordinates 0–12.2, Figure 1). Both fragments were subcloned to generate plasmids pRAUW43 and pRAUW44, respectively (Figure 1).

Location and structure of Asm-1*: The region was mapped by restriction fragment length polymorphism analysis to linkage group V, near the al-3 locus (Metzenberg et al. 1984).

The location of the putative stuA homolog in plasmids pRAUW43 and pRAUW44 was determined by subcloning fragments of plasmid pRAUW43 to generate plasmids pRAUW50 through pRAUW54 (see MATERIALS AND METHODS). These plasmids were individually used to probe Northern blots of gel-fractionated RNA from vegetative cultures of wild-type strains. Plasmids pRAUW54, pRAUW53, and pRAUW50 hybridized to an abundant 3-kb RNA (data not shown). In contrast, plasmids pRAUW43 and pRAUW44 did not recognize any transcripts and they consequently established outer limits for transcription of the homologue (Figure 1).

The region containing Asm-1* (a 4-kb DNA fragment—coordinates 4.3–8.3, Figure 1) was sequenced on both strands following standard procedures. The precise location of the 5'-end of the transcript was determined by primer extension to position 770 (Figure 2), in agreement with previous RT-PCR experiments that mapped the 5'-end of the gene somehow between the sequences corresponding to oligonucleotides ORAUW43 and ORAUW44 (Figure 2; data not shown). The DNA sequence of the region showed an open reading frame starting with an ATG (position 986, Figure 2), interrupted by a potential intron with several canonical 5'- and 3'-consensus splice sites (positions 1128 and 1149 for the 5'-splice signal and positions 1325 and 1337 for the 3'-splice signal; Figure 2). This potential intron was followed by a second long open reading frame. To test for the expected splicing of this potential intron, we cloned two partial cDNAs from phage libraries (cDNAs 21, and 25; Figure 2) and sequenced them. We used oligonucleotides ORAUW39 and ORAUW42 (Figure 2) to PCR amplify a small overlapping region of the transcript, using reverse transcribed poly(A)+ RNA isolated from vegetative growing cultures as template (RT-PCR; see MATERIALS AND METHODS). The PCR products generated with oligonucleotides ORAUW39 and ORAUW42 were cloned and sequenced. Comparison of the cDNA sequences (cDNAs 21, and 25) and PCR clone (p3942; MATERIALS AND METHODS) with the genomic DNA sequence surprisingly revealed no processing out of the intron in the region (data not shown). To investigate whether this potential intron was being processed in a developmental stage-specific manner, we used oligonucleotides ORAUW39 and ORAUW42 to PCR amplify the region from cDNA libraries constructed using conidial and perithelial poly(A)+ RNA as templates (asexual and sexual development, respectively). In both cases, we obtained a 1.1-kb DNA fragment, as predicted for the nonprocessing of the intron in the region (see Figure 2 and MATERIALS AND METHODS). The next ATG, which is preceded by a good translational initiation consensus sequence (Rambosek and Leach 1987; Cavener and Ray 1991) is located at position 1514 and generates an open reading frame whose predicted peptide is composed of 643 amino acid residues. The deduced structure of the gene is summarized in Figure 2. The promoter region in general resembles fungal promoters of constitutive genes and lacks defined CCAAT and TATA boxes (Rambosek and Leach 1987; Ward 1991). The transcript of the gene contains a long leader of 743 bp and two polyadenylation sites that were inferred from the cloned cDNAs. These sites are preceded by a potential polyadenylation signal (AA-TACG; Bruchez et al. 1993) located ~300 bp downstream from the stop codon of the gene (Figure 2). The predicted length of the mRNA is 3096 nucleotides, in good agreement with the size estimated by denaturing gel electrophoresis (3000 nucleotides, data not shown).

Comparison of the Asm-1*-encoded polypeptide with sequences in the GenBank, EMBL, DDBJ, NBRF PIR, and SWISS-PROT databases revealed significant similarities to other fungal proteins (Altschul et al. 1990). The regions of homology were spread along the entire protein—coordinates 4.3–8.3, Figure 1) was sequenced on both strands following standard procedures. The precise location of the 5'-end of the transcript was determined by primer extension to position 770 (Figure 2), in agreement with previous RT-PCR experiments that mapped the 5'-end of the gene somewhere between the sequences corresponding to oligonucleotides ORAUW43 and ORAUW44 (Figure 2; data not shown). The DNA sequence of the region showed an open reading frame starting with an ATG (position 986, Figure 2), interrupted by a potential intron with several canonical 5'- and 3'-consensus splice sites (positions 1128 and 1149 for the 5'-splice signal and positions 1325 and 1337 for the 3'-splice signal; Figure 2). This potential intron was followed by a second long open reading frame. To test for the expected splicing of this potential intron, we cloned two partial cDNAs from phage libraries (cDNAs 21, and 25; Figure 2) and sequenced them. We used oligonucleotides ORAUW39 and ORAUW42 (Figure 2) to PCR amplify a small overlapping region of the transcript, using reverse transcribed poly(A)+ RNA isolated from vegetative growing cultures as template (RT-PCR; see MATERIALS AND METHODS). The PCR products generated with oligonucleotides ORAUW39 and ORAUW42 were cloned and sequenced. Comparison of the cDNA sequences (cDNAs 21, and 25) and PCR clone (p3942; MATERIALS AND METHODS) with the genomic DNA sequence surprisingly revealed no processing out of the intron in the region (data not shown). To investigate whether this potential intron was being processed in a developmental stage-specific manner, we used oligonucleotides ORAUW39 and ORAUW42 to PCR amplify the region from cDNA libraries constructed using conidial and perithelial poly(A)+ RNA as templates (asexual and sexual development, respectively). In both cases, we obtained a 1.1-kb DNA fragment, as predicted for the nonprocessing of the intron in the region (see Figure 2 and MATERIALS AND METHODS). The next ATG, which is preceded by a good translational initiation consensus sequence (Rambosek and Leach 1987; Cavener and Ray 1991) is located at position 1514 and generates an open reading frame whose predicted peptide is composed of 643 amino acid residues. The deduced structure of the gene is summarized in Figure 2. The promoter region in general resembles fungal promoters of constitutive genes and lacks defined CCAAT and TATA boxes (Rambosek and Leach 1987; Ward 1991). The transcript of the gene contains a long leader of 743 bp and two polyadenylation sites that were inferred from the cloned cDNAs. These sites are preceded by a potential polyadenylation signal (AA-TACG; Bruchez et al. 1993) located ~300 bp downstream from the stop codon of the gene (Figure 2). The predicted length of the mRNA is 3096 nucleotides, in good agreement with the size estimated by denaturing gel electrophoresis (3000 nucleotides, data not shown).

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Based on the overall identity between the conserved motifs and on the presence or absence of other regions of identity outside this conserved domain, the gene products related to *ASM-I* can be sub-divided in two subfamilies (Figure 3). One subfamily is represented by the products of the *N. crassa* *Asm-I* gene itself, the *A. nidulans* *stuA* gene, the *C. albicans* *EGF Tf-I* gene, and the *S. cerevisiae* *PHD1* and *SOK2* genes (MILLER et al. 1992; GIMENO and FINK 1994; WARD et al. 1995). This group of proteins shares what we propose to call the Swi4/Mbp1/Res DNA binding domain.

In addition to the *ASM-I* open reading frame, there is an uninterrupted open reading frame of 454 amino acid residues, beginning with a methionine, on the opposite strand (coordinates 3007-1643. Figure 2). This hypothetical protein has no recognizable homology to any protein in the current databases. Primer extension and probing of cDNA libraries both failed to detect any messages or clones corresponding to this hypothetical message, respectively. However, we cannot exclude the possibility that this opposite-strand sequence is transcribed and translated under conditions that we have not tested. In several instances in yeast, the existence of a plausible open reading frame on the opposite strand from a canonical gene has been described (BOLES and ZIMMERMANN 1994). As yet, no biological role is known for these genes, but their presence has been correlated with the high expression of the canonical gene, and this correlation is in harmony with the high level of expression of *Asm-I* (BOLES and ZIMMERMANN 1994).

**Transcriptional regulation of *Asm-I***: To determine the pattern of accumulation of *Asm-I* message during
velopment, we PCR amplified a region of Asm-l+ with an Asm-l+-specific probe. Asm-I+ transcript was readily detected from both vegetative cultures growing in liquid medium and from cultures developing at an air-interface (from young, undifferentiated to old, fully conidiating mycelia; data not shown). To investigate the role of Asm-l+ during sexual development, we isolated RNA at various times after inducing development (see MATERIALS AND METHODS) and hybridized a Northern blot of gel-fractionated RNA with an Asm-l+-specific probe. Asm-I+ transcript was readily detected (see above, data not shown). To investigate the role of Asm-I+ during sexual development, we PCR amplified a region of Asm-l+ with oligonucleotides ORAUW39 and ORAUW42 from a cDNA library constructed using N. crassa perithecial poly(A)+ RNA as a template. A DNA fragment of the predicted size was readily detected (see above, data not shown). Taken together, these results indicate that Asm-I+ codes for an abundant message that seems to be constitutively expressed during the mycelial, conidial, and perithecial stages of the life cycle.

**Deletion of Asm-I**: The role of Asm-l+ was investigated by deleting the promoter and most of the coding region of the gene (Figure 1, deleted region). Plasmid pRAUW66 was constructed by fusing DNA fragments from chromosomal regions adjacent to Asm-l± (left flanking, 3.5-kb EcoRI and right flanking, 3.5-kb Smal DNA fragments; Figure 1) to the selective marker mtr+ gene (STUART et al. 1988; KOO and STUART 1991), as described in MATERIALS AND METHODS. The composite Sfi-I Not1 DNA fragment from pRAUW66 (Figure 1) was isolated and used to transform mating type A and mating type a strains (RLM 25-15 and RLM 25-16, respectively, Table 1). The transplacement integration event was designed to evict 2260 bp of the Asm-I+ region, leaving it in its place an mtr+ allele (ROTHSTEIN 1983).

**Isolation of the deletion mutant**: Because it was quite possible that the deletion of Asm-l+ would be a vegetative-lethal event, we used a precautionary approach for deletion of our potentially essential gene. This approach can be generalized for the disruption of any such gene (see Figure 4). A newly transformed spheroplast invariably still contains many untransformed nuclei and transformed nuclei (class 1 and class 2, respectively). We will refer to transformants and to any other strain carrying two classes of nuclei as heterokaryons and strains with three classes of nuclei as trikaryons. Deletion of a gene essential for vegetative growth will be “sheltered” by the untransformed nuclei. Under selective conditions, the resulting transformant will persist as a balanced heterokaryon. Failure to obtain a class 2 homokaryon (in this case, homokaryotic for the deletion of Asm-l+ event) hints that the gene is essential, but fails far short of proving it, since even a viable but slow-growing homokaryon component would be extremely difficult to resolve. This dilemma can be circumvented by trading class 1 nuclei for a suitably chosen new nuclear type, class 3, a strategy that has been used for other purposes by BROCKMAN et al. (1969) and by GRANT et al. (1984). This trade involves making a nutritionally forced trikaryon intermediate in which class 3 is a “helper” strain with appropriate nutritional and other markers. The resulting trikaryon is then resolved by streaking it out under nutritional conditions that are permissive for growth of the new heterokaryon of (class 2 + class 3) nuclei. Once isolated, this new heterokaryon can be grown under conditions that tend to select against nuclei of class 3 because they contain the sensitive allele of an antimetabolite resistance marker for which class 2 carries the resistance allele. This succeeds because there will always be hyphae in which class 2 nuclei are highly predominant. If deletion of the gene in question is not lethal, a homokaryon of class 2 nuclei will be obtained, even if it is slow growing. In contrast, if deletion of the gene is lethal, the heterokaryon can not be resolved to give a fully antimetabolite-resistant isolate. Failure to resolve this heterokaryon thus indicates that deletion of the gene in question is extremely deleterious or lethal (Figure 4).

After transformation of N. crassa strains RLM 25-15 or RLM 25-16 (A and a mating type, respectively; Table 1) with pRAUW66, the resulting heterokaryons (untransformed class 1 nuclei + transformed class 2 nuclei) were selected on plates of 0.5 mM histidine, 20 mM arginine (typical mtr+ selection), and Ca2+-pantothenic acid (not part of the mtr+ selection but required by strains RLM 25-15, and RLM 25-16; Table 1). The rationale for this selection is as follows: at the pH of Vogel's medium, histidine is both a “basic” amino acid and “neutral.” The forward selection for mtr+ function occurs when a histidine-requiring mutant is required to grow on a relatively low concentration of histidine in the presence of a much larger concentration of argi-
nine, a basic amino acid. Arginine interferes with the transport of histidine through the basic amino acid permease and creates a requirement for the presence of the neutral permease. mtr" (the gene that codes for the neutral amino acid permease), provided via transformants, is therefore under positive selection. Colonies from the transformation plates were first picked into selective liquid medium with the same supplements as described above. Conidia, which were generally sparsely formed on this medium, were spot-tested onto plates with the same supplements plus p-fluoro-DL-phenylalanine. This toxic compound is also a substrate for the neutral amino acid permease and is used in a typical mtr" selection. Transformants containing a significant fraction of class 2 nuclei will therefore not grow on this medium (because they are mtr"), and only those that failed to grow were carried forward. This step eliminated the "false positive transformants" seen in the original transformation plates and selected for those heterokaryons containing a high ratio of class 2 nuclei to class 1 nuclei. Trikaryon intermediates were selected by mixing conidia in liquid medium with 0.5 mM histidine and 20 mM arginine (maintaining the mtr" selection), but without either Ca²⁺-pantothenate or inositol from the candidate heterokaryons (class 1 nuclei + class 2 nuclei; see Figure 4) with conidia from either helper strain RLM 77-01 or RLM 77-06. The choice of helper strain depended on the mating type—A conidia were mixed with A, and a conidia were mixed with a. Conidia from the resulting trikaryons were streaked onto similarly supplemented solid medium. Colonies were picked into liquid medium with 1 mM histidine and allowed to conidiate. The conidia can be expected to be a population that includes heterokaryons (class 1 + class 2) nuclei, (class 2 + class 3) nuclei, and unresolved trikaryons (class 1 + class 2 + class 3) nuclei. To distinguish between these, we streaked conidia to plates of histidine and Ca²⁺-pantothenate, p-fluoro-DL-phenylalanine, and 6-methyl purine. Under these conditions, heterokaryons (class 1 + class 2) nuclei or trikaryons (class 1 + class 2 + class 3 nuclei) will grow because of spontaneous resolution to give class 1 nuclei in the homokaryotic state, but heterokaryons containing only (class 2 + class 3) nuclei will not grow. We chose nongrowers and transferred them to liquid Vogel's sucrose medium with histidine and allowed them to conidiate. Conidia were streaked onto solid medium with histidine, Ca²⁺-pantothenate, and 6-methyl purine. Under these conditions homokaryons with class 2 nuclei will survive and grow if, and only if, the gene replacement event is not lethal. The presence of class 3 nuclei render a heterokaryon sensitive to 6-methyl purine. Colonies were picked and tested in three media: histidine, Ca²⁺-pantothenate, and inositol; histidine and Ca²⁺-pantothenate; and histidine and inositol. Results from this auxanography distinguished between homokaryons, which were indeed seen, and heterokaryons. The successful isolation of a class 2 homokaryon, albeit a slow growing one, demonstrates that deletion of Asm-1" is not a vegetatively lethal event. A representative homokaryon was streaked once more, and DNA was extracted from it and shown by Southern blot analysis to have the predicted integration event. The deletion strain was designated RLM 80-21 (Table 1). In all, 13 of the original 108 transformants of RLM 25-15 (mating type A) showed such a deletion event.

The resulting mutant strain had a complex phenotype. It was female-sterile but male-fertile (see below). When crossed as a male to any strain of the opposite mating type, it gave rise to perithecia and to an abundant crop of ascospores that were, however, almost all white, immature, and inviable (Araya and Metzenberg 1996b). To determine the phenotype of the deletion of the Asm-1" gene in the opposite mating type, we deleted Asm-1 in a mating type A background, since we clearly could not recover the mutant from outcrosses. Strain RLM 25-16 was transformed as described for RLM 25-15. Among 30 transformants analyzed by Southern blotting (see MATERIALS AND METHODS for details), 11 showed the expected integration event. One of them was processed as described above, resulting in the homokaryon designated RLM 80-23 (Table 1).

Asm-1" is required for rapid conidial germination, normal vegetative morphology, and protoperithecial formation: In addition to the mycelia growing slowly, conidia of Δ-Asm-1 homokaryons germinate abnormally slowly (≥12 hr later than their wild-type counterparts), and this is particularly marked in nitrogen-limiting medium. The aerial hyphae of the mutants are stunted, compared to those of wild type; consequently, they conidiate very close to the agar surface, giving the culture a mat-like appearance. Clutterbuck (1969) described an analogous phenotype for the Aspergillus siuA mutant. The effects of Δ-Asm-1 in conidiation have not been further investigated.

Mating type A and a mycelia from Δ-Asm-1 mutants growing on medium ordinarily used to induce mating did not form obvious protoperithecia. To see whether precursors of them or related structures were present, we used SEM (scanning electron microscopy). The Δ-Asm-1 mutant and wt-Asm-1 control strains (RLM 80-21

**Figure 5.—Asm-1" regulates protoperithecial development.** Representative protoperithecia of strains RLM 25-15 (A) and RLM 83-01 (B) were prepared for SEM as described in MATERIALS AND METHODS. Magnification is ×500. Strains are described in Table 1.
and RLM 25-15, respectively; Table 1) were inoculated onto nitrogen-limiting solid medium, incubated at room temperature, fixed, and prepared for SEM. We found neither mature nor incipient protoperithecia in the Δ-Asm-1 mutant (data not shown). Typical protoperithecial structures were observed in the wt-Asm-1 control (RLM 25–15; Figure 5A). However, it seemed possible that Δ-Asm-1 mutants might occasionally form female structures at a density low enough to have escaped detection by ordinary light microscopy and by SEM. To check this, we grew lawns of Δ-Asm-1 mutants (RLM 80-21 and RLM 80-23; Table 1) and then fertilized the lawns with wild-type conidia of the opposite mating type (see MATERIALS AND METHODS). Under these conditions the formation of even one protoperiteum would immediately be noticed. wt-Asm-1 control strains grown and fertilized in this way produced thousands of black perithecia per lawn. In contrast, Δ-Asm-1 mutants, used as prospective females, did not show any perithecia.

If deletion of Asm-1° causes female sterility, reinsertion of a wild-type copy of the gene at another location should restore the formation of protoperithecia in Δ-Asm-1 mutants and at the same time provide a proof of the functionality of that relocated copy. In contrast, insertion of a defective copy of the gene should not do so. An Asm-1° nonfunctional allele was constructed by artificially introducing a frameshift mutation (fs) inside the coding region of the gene at the BamHI site (Figure 1, coordinate 6). We cloned either an Asm-1° functional allele (pRAUW123; 12.20-kb HindIII DNA segment; Figure 1, coordinates 0–12.2) or the Asm-1° nonfunctional allele (pRAUW124; HindIII DNA fragment; Figure 1, coordinates 0–12.2) into plasmids capable of integrating these constructs at the his-3 chromosomal locus (AR-

FIGURE 6.—Immunoblot showing ASM-1 protein. Total protein extracts (25 μg) of strains RLM 25-15 (wt-Asm-1°) and RLM 80-21 (Δ-Asm-1) were electrophoresed in Tricine-SDS-polyacrylamide gel, transferred to nitrocellulose membranes and probed with anti-ASM-1 antibodies.

FIGURE 7.—Immunolocalization of ASM-1. Neurospora crassa strains RLM 25-15 and RLM 80-21 were incubated in WES-ERGAARD's medium for 6 and 12 hr, respectively. Cells were fixed and incubated with antibodies or dye as described in MATERIALS AND METHODS. Confocal images of the same field are shown. Whole cells of RLM 25-15 were double-stained to detect DNA and ASM-1. (A) Detection of chromosomal DNA was by YOYO-1 staining of RLM 25-15 (false yellow color). (B) Detection of ASM-1 was by indirect immunofluorescence staining of RLM 25-15 using affinity purified anti-ASM-1 and Texas Red conjugated goat anti-rabbit IgG (false red color). (C) The merged image in (C) was obtained by superimposing the images of (A) and of (B). The resulting orange color arises from the coincidence of anti-ASM-1 antibody staining (red) and of YOYO-1 staining (yellow). (D) As a control, fixed cells of Δ-Asm-1 (RLM 80-21) were treated with anti-ASM-1 and Texas Red conjugated goat anti-rabbit IgG. Magnification is ×600.

AMAYO and METZENBERG 1996a). Plasmids pRAUW123 (Asm-1°), and pRAUW124 (Asm-1°) were used to construct strains RLM 83-01, RLM 83-03, and RLM 83-5, RLM 83-07, respectively (Table 1) as described in MATERIALS AND METHODS.

We tested strains RLM 83-01, and RLM 83-03 [Δ-Asm-1 (his-3°::Asm-1°)] and strains RLM 83-05, and
Sequence of *Neurospora crassa* (Ne) ASM-1 protein compared with sequences of related proteins

<table>
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<tr>
<th>Protein</th>
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<th>Aspergillus nidulans</th>
<th>Schizosaccharomyces pombe</th>
<th>S. cerevisiae</th>
<th>K. lactis</th>
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<td>38.1</td>
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**Percent identity or similarity of the ASM-1 protein from *Neurospora crassa* with: An-StuA (*Aspergillus nidulans*); Ca-EFGTF (*Candida albicans*); Kl-Mbp1 (*Klebsiella pneumonia*); Sc-Mbp1 (*Saccharomyces cerevisiae*), and Sc-Swi4 (*S. cerevisiae*); and Sp-Res1/Sct1, Sp-Res2/Pct1, and (*Schizosaccharomyces pombe*). A-values generated from aligning complete polypeptides. B-values generated from aligning the conserved DNA binding domain of each protein. Sequence were aligned using the GAP method (DEVEREUX et al. 1984).**

RML 83-07 [Δ-Asm-1 (his-3::Asm-l管理体系)] for their ability to form protoperithecia. As expected, insertion of Asm-1+ at the his-3 locus restored female fertility (see Figure 5, Panel B), whereas insertion of Asm-l did not (data not shown).

**ASM-1 is abundant and nucleus-localized:** On the basis of its potential DNA binding domain, Asm-1+ might be expected to code for a nucleus-localized protein. We raised antibodies against an internal peptide (amino acid residues no. 277 to no. 578; see MATERIALS AND METHODS) and used them to detect ASM-1 in wild type and mutant strains of *N. crassa*.

When immunoblots of total cell extracts were probed with anti-ASM-1 antibodies (MATERIALS AND METHODS), we observed a single polypeptide of ~68,000 D in both mating type A (Figure 6) and mating type a (data not shown) strains (RLM 25-15 and RLM 25-16, respectively; see Table 1). As expected, this polypeptide was absent in cell extracts prepared from strains having a deletion in the *Asm-1* region [Δ-Asm-1, strains RLM 80-21 (Figure 6), and RLM 80-23 (data not shown)]. This polypeptide was more abundant in A than in a strains (data not shown).

We examined the localization of ASM-1 by confocal immunofluorescence microscopy. *N. crassa* germlings were fixed, converted to spheroplasts, and challenged with affinity-purified primary rabbit antibody followed by secondary antibody (goat anti-rabbit IgG conjugated to Texas Red). The same preparations were treated with YOYO-1 to localize bulk DNA, which we take as delineating nuclei (MATERIALS AND METHODS). A predominantly nuclear signal for ASM-1 was observed in wild type cells [RLM 25-15; Figure 7A, DNA (synthetic yellow), and Figure 7B, ASM-1 (synthetic red); Figure 7C is a superimposition of A and B (synthetic orange)]. As expected, the nuclear signal for ASM-1 was absent in the deletion strain (RLM 80-21; Figure 7D).

**DISCUSSION**

The molecular structure, pattern of expression, and cellular localization of Asm-1+, the *N. crassa* homologue of the *A. nidulans* *stuA* gene, has been determined. Analogous to loss-of-function of *stuA* in *A. nidulans*, deletion of *Asm-1*+ in *N. crassa* abolishes protoperithecial formation and affects the development of aerial hyphae, suggesting that these genes have similar regulatory functions during early sexual development (CUTTERBUCK 1969; MILLER 1990; MILLER et al. 1991).

ASM-1 and StuA polypeptides have extensive regions of homology outside the APSES domain (see Figure 2), and have an overall identity of 50.9% (Table 2) (DEVEREUX et al. 1984). The *S. cerevisiae* Phd1 and Sok2, and the *Candida albicans* EF1 proteins also have regions of identity with ASM-1 outside the APSES domain, though to a lesser degree (Table 2, DEVEREUX et al. 1984). The high overall identity between the conserved domains of these proteins and ASM-1 marks them as belonging to the same subfamily of related proteins (APSES domain proteins, Table 2).

In contrast, Blast searches of databases identified only Swi4, Mbp1 (*S. cerevisiae*), Res1'/Sac1', Res2'/Pct1', and Cdc10' (S. pombe), and Mbp1+ (K. lactis) when the conserved domain of ASM-1 (amino acid residues 110 to 226) was used as query (ALTSCHUL et al. 1990). The overall identity of the conserved domain of ASM-1 with that of the conserved domain of Swi4, Mbp1 (+S. cerevisiae), Res1/Sac1, Res2/Pct1, Cdc10 (S. pombe), and Mbp1+ (K. lactis) ranges from 26.3% (ASM-1 × Sp-Cdc10, Table 2) to 22.1% (ASM-1 × Sc-Mbp1, Table 2).

This group of proteins belongs to the Swi4/Mbp1/Res family of transcription factors regulating START, the period preceding a new round of DNA replication (S phase, reviewed in ANDREWS and MASON 1993; KOCH and NASMYTH 1994). Deletion of any one of these genes alone is not lethal to the cell but destroys the regulation of its target genes. This contrasts with the phenotype of the double mutants [for example *swi4 mbp1* (+S. cerevisiae) or *res1 res2* (S. pombe)], which are not viable (KOCH et al. 1993; MIYAMOTO et al. 1994). Similarly to the Swi4/
Mbp1/Res subfamily of transcription factors, the subfamily of ASM-1-like proteins may have more than one member in the genomes of typical filamentous fungi. In *S. cerevisiae*, there are two ASM-1-like proteins, Phd1 and Sok2, with different but interrelated functions. Overexpression of the *PHD1* gene allows pseudohyphal growth in rich medium. Paradoxically, however, even *PHD1* loss-of-function mutants can undergo pseudohyphal development in nitrogen-starvation medium, suggesting the existence of additional genes that affect the process. One such candidate is *SOK2*, which is involved in the activation of genes that control pseudohyphal development and cell proliferation. Surprisingly, deletion of *SOK2* induces an exaggerated degree of pseudohyphal development in a Phd1-dependent manner, suggesting that these genes together serve to either activate (Phd1) or repress (Sok2) the expression of the genes required for pseudohyphal development (*WARD et al. 1995*). Sok2 protein is also thought to be regulated by cAMP levels, perhaps through a direct interaction with the camp-dependent protein kinase A (*WARD et al. 1995*). *LIU et al.* (1993) demonstrated that elements of the mating signal transduction pathway are also required for filamentous growth. However, the relationship between pseudohyphal growth and the mating-kinase cascade with genes like *PHD1* and *SOK2* is poorly understood, and it is likely that the identification of new members of this protein family will help explain some observations. For example, in contrast to the lethality of *swi4 mbp1*, and *res1 res2* double mutants, *phd1 sok2* double mutants are viable (*WARD et al. 1995*). This suggests either that the APSES-domain proteins as a group are not essential for life, or that *PHD1* and *SOK2* functions can be fulfilled by other genes. Functional redundancy may explain why deletion of *Asm-1*+, though very detrimental is not lethal. Conceivably ASM-1 makes part of a transcription factor involved in sensing or responding to internal or external signals (e.g., cAMP levels or nitrogen deprivation) or in inducing the transcription of genes necessary for premeiotic G1 arrest (perhaps an essential step in protoperithelial development). In *S. pombe*, the interaction of START genes (like *res1*+ and *res2*+) with a B-type cyclin (*cyc17*) controls sexual development in a crucial but poorly understood manner (*Obara-Ishihara and Okayama 1994*). The cloning, disruption, and characterization of all members of the ASM-1-like subfamily in several organisms will be needed to reveal the roles of the various gene products and their connections to the rest of the cell machinery.

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