

# The *pro1*<sup>+</sup> Gene From *Sordaria macrospora* Encodes a C<sub>6</sub> Zinc Finger Transcription Factor Required for Fruiting Body Development

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## ABSTRACT

During sexual morphogenesis, the filamentous ascomycete *Sordaria macrospora* differentiates into multicellular fruiting bodies called perithecia. Previously it has been shown that this developmental process is under polygenic control. To further understand the molecular mechanisms involved in fruiting body formation, we generated the protoperithecia forming mutant *pro1*, in which the normal development of protoperithecia into perithecia has been disrupted. We succeeded in isolating a cosmid clone from an indexed cosmid library, which was able to complement the *pro1*<sup>-</sup> mutation. Deletion analysis, followed by DNA sequencing, subsequently demonstrated that fertility was restored to the *pro1* mutant by an open reading frame encoding a 689-amino-acid polypeptide, which we named PRO1. A region from this polypeptide shares significant homology with the DNA-binding domains found in fungal C<sub>6</sub> zinc finger transcription factors, such as the GAL4 protein from yeast. However, other typical regions of C<sub>6</sub> zinc finger proteins, such as dimerization elements, are absent in PRO1. The involvement of the *pro1*<sup>+</sup> gene in fruiting body development was further confirmed by trying to complement the mutant phenotype with *in vitro* mutagenized and truncated versions of the *pro1* open reading frame. Southern hybridization experiments also indicated that *pro1*<sup>+</sup> homologues are present in other sexually propagating filamentous ascomycetes.

**S**EXUAL development in filamentous fungi is usually characterized by the formation of complex fruiting bodies that contain meiosporangia with either four or eight asco- or basidiospores. In basidiomycetes as well as in ascomycetes, fruiting body formation requires the temporal and spatial control of cell differentiation, a process that is under polygenic control (Dyer *et al.* 1992). Currently, the molecular mechanisms underlying fruiting body morphogenesis in fungi are poorly understood. What is known has been obtained largely from studies in which developmentally regulated genes were identified by their preferential expression during fruiting body morphogenesis. For example, in *Neurospora tetrasperma* and *Sordaria brevicollis*, a number of perithecium-specific proteins were identified in phase-specific protein extracts (Nasrallah and Srb 1973, 1977; Broxholme *et al.* 1991). In the case of *N. tetrasperma*, one of these proteins was shown to be localized in the perithecium's extracellular matrix, in which the asci and paraphyses are embedded (Nasrallah and Srb 1978). In a molecular genetic study that used subtractive hybridization, Nelson and Metzberg (1992) were able to subclone 14 *N. crassa* sexual development genes. One of these genes, *asd-1*, has been shown to encode a puta-

tive rhamnogalacturonase necessary for ascus development (Nelson *et al.* 1997a). In large-scale analyses, fruiting body-specific expressed sequence tags (ESTs) from *Aspergillus nidulans* and *N. crassa* were detected and sequenced for further molecular characterization (Lee *et al.* 1996; Nelson *et al.* 1997b). In such experimental approaches, large efforts have to be made to obtain detailed information about mutant phenotypes and their corresponding functional genes, genes which are usually highly transcriptionally expressed. However, genes encoding components of the regulatory network of signal transduction pathways and transcription factors, which are transcribed mostly at lower rates, are more difficult to isolate. We therefore set out to generate morphological mutants of the ascomycete *S. macrospora* as a means of identifying genes controlling fungal multicellular development. In this homothallic fungus, fruiting body development is an apandrous process, lacking the cooperative interaction of two opposite mating-type strains, as is the case in the closely related heterothallic species *N. crassa* and *Podospora anserina*. Because *S. macrospora* provides such a favorable genetic system in which to generate developmental mutants (Esser and Straub 1958), we have chosen to use it in our studies to identify regulatory genes involved in fruiting body formation.

During vegetative growth, chemical and physical stimuli such as biotin or light induce branching of hyphal tips, which is followed by adhesion of several hyphae to each other. This entry into the sexual phase results in the formation of a three-dimensional network of inter-

This article is dedicated to Professor Karl Esser on the occasion of his 75th birthday.

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connecting hyphae, leading to the first of two morphological stages: first, the development of fruiting body primordia (protoperithecia); and second, the transition of protoperithecia into mature fruiting bodies, called perithecia. In the homothallic fungus *S. macrospora*, transition between these two stages takes ~72 hr. In heterothallic fungi, this transition requires crossing two opposite mating-type cultures.

In this article we describe the generation of the developmental mutant *pro1*, a mutant that is unable to undergo transition from the protoperithecial to the perithecial stage. To characterize this mutant at the molecular level, we used molecular tools recently developed for use in *S. macrospora* (Walz and Kück 1995; Pöggeler *et al.* 1997a). This included the construction of an indexed cosmid library, which enabled us to isolate and characterize the mating-type locus from this homothallic fungus, as well as various genes with metabolic functions (Pöggeler *et al.* 1997a,b; Nowrousian and Kück 1998). A further development, the use of complementation transformation, demonstrated that the ATP citrate lyase gene (*ac11*) is required for fruiting body maturation (Nowrousian *et al.* 1999). Using a similar experimental approach, we report here the identification of a gene encoding a putative C<sub>6</sub> zinc finger protein that restores fertility to the sterile mutant *pro1*. To our knowledge, this is the first description of a fungal transcription factor that is required for the transition of fruiting body primordia into mature fruiting bodies during sexual reproduction.

## MATERIALS AND METHODS

**Strains and culture conditions:** The *S. macrospora* strains K (isolates L 3346 and S 1957) from our laboratory collection and D (DSM 997) display wild-type phenotypes. For morphological studies, wild-type and mutant strain *pro1* were cultivated on cornmeal medium (Esser 1982). For the isolation of genomic DNA and the preparation and regeneration of protoplasts, cultivation was performed as described by Pöggeler *et al.* (1997a). For DNA isolation, the following fungal strains were used: *N. crassa* (FGSC 4317); *N. dodgei* (FGSC 1692); *N. pannonica* (FGSC 7221); *N. terricola* (FGSC 1889); *Podospira anserina* (Esser 1974); *S. brevicollis* (FGSC 1903); *S. fimicola* (FGSC 2918); and *S. sclerogenia* (FGSC 2740). Conventional genetic analysis was undertaken as previously described by Esser (1982, 1996).

**Scanning electron microscopy:** After growth in liquid cornmeal medium for 7 days without agitation, mycelial samples for scanning electron microscopy (SEM) investigation were fixed directly in petri dishes for 1 hr, using Carnoy's fixative (60% ethanol, 30% chlorophorm, 10% acetic acid; Romeis 1968). Small pieces of mycelium (~0.5 cm<sup>2</sup>) were dried using the critical point method (Gersterberger and Leins 1978) and a Bal-Tec (Middlebury, CT) critical point dryer CDP 030. Samples were fixed on metal stubs and sputter-coated with gold before being viewed with a Zeiss (Thornwood, NY) DSM 950 scanning electron microscope.

**Formation of protoplasts:** Protoplasts of *S. macrospora* were prepared according to the method of Walz and Kück (1995) with the following modifications. The mycelium from three

Fernbach flasks was harvested and treated with 20 ml Novozym 234 (Novo Nordisk, Denmark) solution (10 mg/ml in protoplast buffer) as described by Le Chevanton and Leblon (1989). Protoplasts were separated from the mycelium using a no. 1 filter funnel (Schott, Germany).

**UV-mutagenesis to generate developmental mutants:** For UV-mutagenesis, protoplast suspensions from the wild-type strain D were exposed to UV-light (254 nm) for 15 min with a survival rate of 0.1% and plated on CCM medium (Walz and Kück 1995) supplemented with 10.8% sucrose for regeneration. After 24 hr individual clones were transferred to cornmeal medium to observe clones with phenotypic variations in fruiting body development. One of these clones led to the isolation of mutant *pro1*, which was tested for mitotic stability, before a single-spore isolate was generated for further molecular analysis.

**Transformation of *S. macrospora*:** Transformation of *S. macrospora* was carried out as described by Walz and Kück (1995) with slight modifications. A total of  $2 \times 10^7$  protoplasts derived from mutant *pro1* were incubated with either 10–20 µg of plasmid DNA or 20 µl of pooled cosmid DNA from the *S. macrospora* cosmid library (Pöggeler *et al.* 1997a). After 4–6 hr of protoplast regeneration, agar plates were overlaid with top-agar (0.8 M NaCl, 0.8% agar) containing hygromycin B at a final concentration of 110 units/ml. For morphological analysis, individual transformants were inoculated on cornmeal medium containing 110 units/ml hygromycin B and were then transferred to a nonselective medium after 24 hr. After genomic complementation with DNA from cosmid pools, transformants were transferred onto selective and nonselective media as replicas of the original transformation plate.

**Nucleic acids analysis:** Nucleic acids were isolated from fungal strains as previously described (Hoge *et al.* 1982; Lecellier and Silar 1994). The isolation of poly(A)<sup>+</sup> RNA was performed with the PolyATtract mRNA Isolation System (Promega, Madison, WI). Digested DNA and mRNA were separated by gel electrophoresis, transferred onto nylon membranes, and hybridized with radioactively labeled dsDNA probes, according to conventional methods (Southern 1975; Sambrook *et al.* 1989).

**Electrophoretic karyotyping:** Protoplasts were treated as described previously to isolate intact chromosomes (Walz and Kück 1991). The CHEF Mapper system (Bio-Rad, Richmond, CA), which uses the separation conditions reported by Walz and Kück (1995), was used to perform separation of chromosomal DNA.

**Plasmid constructions:** Cosmid clone D6 (Figure 2A), isolated from pool VI 1298-1345 of the indexed cosmid library (Pöggeler *et al.* 1997a), carries the *pro1*<sup>+</sup> gene described in this article. Restriction of D6 with *Xba*I, followed by religation, resulted in the construction of cosmid clone D6ΔX. A second overlapping cosmid clone, C10, was isolated from pool III 171-213 of the cosmid library. This clone contains further sequences upstream of the *pro1*<sup>+</sup> gene and is given in Figure 2A. Transformation vectors pNP2-1 (Osiewacz and Nuber 1996) and pBChygro (Silar 1995) were used to construct several derivatives of this cosmid clone, which resulted in the generation of the following recombinant plasmids: pPro31, pPro31.1, pPro31.2, pPro41, pPro41.1, pPro41.2, pPro41.3, pPro41.3.1, pPro41.4, and pPro42. The inserts carried by all of these recombinant plasmids are given in Figure 3C. Plasmid pPro41.3.1 contains a frameshift mutation and was generated by restriction at the unique *Hind*III digestion site, followed by Klenow polymerase treatment and religation. Plasmid pPro41.4 contains an internal deletion of 246 bp (amino acid codon no. 60 to no. 142, leaving an overhang of three nucleotides, which encodes a threonine) and was constructed by exonuclease III/nuclease S1 treatment of the *Hind*III-digested

*pro1*<sup>+</sup> gene. The deletion of 83 codons does not lead to a truncation of the open reading frame.

**PCR amplification and cDNA cloning:** cDNA clones of the *pro1* transcript were generated by reverse transcription PCR (RT-PCR) following a few modifications to a previously published procedure (Kempken and Kück 1996). After 5 µg of total RNA was treated with DNase I, reverse transcription was performed with AMV reverse transcriptase (Boehringer, Mannheim, Germany), with a hexamer-oligonucleotide mix (Boehringer) used as primer. Aliquots of the cDNA were used for PCR amplification with specific primers in accordance with Kempken and Kück (1996). PCR products were separated from surplus primers and nucleotides with a QIA Quick Spin PCR-purification kit (QIAGEN, Hilden, Germany) and cloned into the *Sma*I restriction site of the pBluescript/KS<sup>+</sup> vector (Stratagene, La Jolla, CA). The following sets of oligonucleotides were used as primers for the generation of three overlapping cDNA-clones, as given in parentheses (Figure 3B): oligonucleotides 851 [5'-AGCAAGCCGGACTTGCTCAGACCC] and 840 [5'-GCATTGAAGCCCAGATGCTTGCA] (pCP1); oligonucleotides 841 [5'-CCAAGACCAAGGCGACCGCAAA GGC] and 842 [5'-CCGCTTGAAATGTTGGTGCCAGGG] (pCP2); and oligonucleotides 850 [5'-ACGACATCATGCCG CACCGCTATGC] and 848 [5'-TCCCTCGGTCTGCATGACA TCCCGC] (pCP3). Homologous fragments of the *pro1*<sup>+</sup> gene from other filamentous fungi were generated using oligonucleotides 841 (sequences already given) and 865 (5'-CCGA GATGATACATGACG).

**DNA sequencing and sequence analysis:** A T7-polymerase sequencing kit (Pharmacia, Germany) was used to perform sequencing of double-stranded DNA templates with the dideoxy chain-terminating method (Sanger *et al.* 1977). To facilitate sequencing of the *pro1*<sup>+</sup> gene, a nested deletion kit (Pharmacia) was used to generate nested DNA fragments. DNA fragments of *pro1*<sup>+</sup> homologues from *S. brevicollis* and *N. crassa* were sequenced by the QIAGEN customer sequencing service (Hilden, Germany). The programs from the Husar/genius server (Heidelberg, Germany) and FASTA (Pearson 1990) were used to compare nucleotide and amino acid sequences. The methods of Lupas *et al.* (1991) and Berger *et al.* (1995) were used to predict putative coiled-coil secondary structures.

## RESULTS

**Generation of the developmental mutant *pro1* with defects in fruiting body formation:** In our effort to elucidate the molecular basis of fruiting body formation in the filamentous ascomycete *S. macrospora*, we chose a forward genetic approach. Using UV-mutagenesis, we generated several nonallelic developmental mutants with defects in sexual morphogenesis. One mutant, named *pro1* (*protoperithecia*), shows defects in early fruiting body development and was chosen for detailed analysis. Figure 1 displays scanning electron micrographs of a mutant protoperithecium and mature perithecia from the wild-type strain. In mutant *pro1* sexual differentiation leads only to the formation of protoperithecia. However, no ascus primordia were detected inside *pro1* protoperithecia, which indicates that transition from protoperithecia to perithecia is arrested in *pro1* and thus leads to the lack of any mature perithecia. To investigate whether a single gene is responsible for the mutant phenotype, *pro1* was crossed with the wild

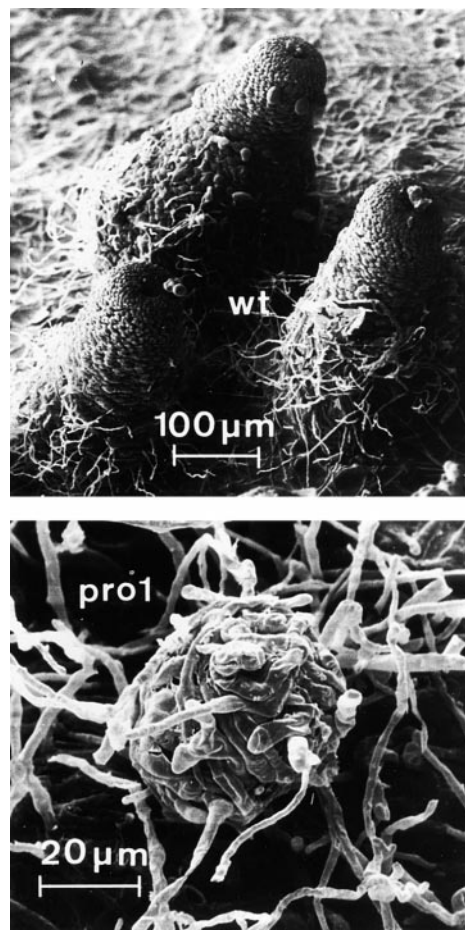


Figure 1.—Scanning electron micrograph, *Sordaria macrospora* wild-type and mutant *pro1* strain. Wild-type (wt) perithecia and a protoperithecium from mutant *pro1* were prepared for SEM as described in materials and methods. Note the different magnification scales.

type as well as various mutant strains. The 437 tetrads analyzed showed a Mendelian segregation (4:4) of the mutant phenotype. These data indicate the involvement of a single locus in the mutant phenotype and lead to a calculated distance (postreduction frequency) between the *pro1*<sup>+</sup> locus and the centromere of 142.3 cM.

**Complementation analysis of mutant *pro1* using an indexed cosmid library:** To isolate the developmental gene responsible for the *pro1* phenotype, we undertook a genomic complementation strategy, using a recently described genomic cosmid library (Pöggeler *et al.* 1997a). This library consists of 4608 cosmid clones, which are arranged in 96 cosmid pools, each bearing 48 individual clones. After transformation of DNA from 34 pools, transformants were selected on medium containing 110 units hygromycin B/ml. In a second screening of nearly 10,000 transformants, a single cosmid pool was identified that yielded fertile transformants that showed perithecia and ascospore formation. Further subdivision of this pool led to the identification of a single clone, designated D6, which was suitable for geno-



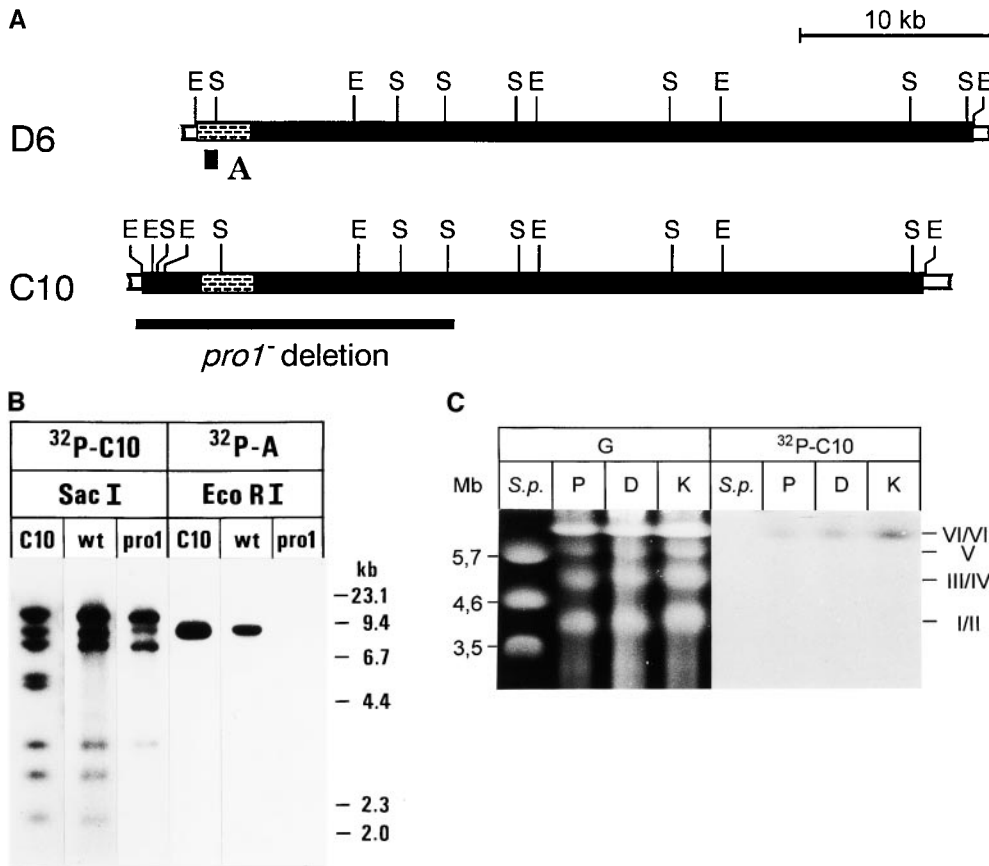


Figure 2.—Genomic organization and chromosomal localization of the *pro1*<sup>+</sup> locus. (A) Restriction maps of cosmid clones D6 and C10. The stippled region indicates a fragment, which is able to restore fertility in mutant *pro1*. The bar below the map shows the location of the mapped deletion present in mutant *pro1*. E, *EcoRI*; S, *SacI*. (B) Autoradiograph from a Southern hybridization. Genomic DNA isolated from *S. macrospora* wild-type and mutant strains, as well as DNA from the cosmid clone C10, restricted with either *SacI* or *EcoRI*. Cosmid C10 or fragment A in A were used as labeled probes. The sizes of the molecular weight standards are given in kilobases. (C) Localization of the *pro1*<sup>+</sup> locus. Ethidium bromide-stained CHEF gel of the *S. macrospora* mutant strain *pro1* (P) and two wild-type strains D and K (G). Southern hybridization was undertaken using the radiolabeled cosmid C10. Chromosomes of *Schizosaccharomyces pombe* (*S.p.*) were used as size

markers; sizes of the marker chromosomes are indicated on the left. The relative positions and the numbers of the *S. macrospora* chromosomes (which have a calculated size of 38 Mb) are given on the right.

mic complementation. A subfragment of D6, stippled in Figure 2A, served as a probe for the isolation from the indexed library of a second independent cosmid clone (C10) with homology to D6. The restriction maps of the two overlapping cosmid clones, with insert sizes of 36.5 kb (D6) and 37 kb (C10), are given in Figure 2A. In subsequent complementation experiments, we succeeded in isolating a 2.6-kb subfragment of cosmid D6 (stippled region in Figure 2A) that was able to complement mutant *pro1*.

To demonstrate that restored fertility results from genomic complementation and is not a consequence of reversion or suppressor mutations, a total of 15 tetrads from five complemented strains were isolated and characterized. Usually, transformed protoplasts carry transformed as well as untransformed nuclei. Therefore tetrads from these heterokaryons show a segregation of the hygromycin B-resistant fertile phenotype and the hygromycin B-sensitive *pro1* phenotype. All ascospore lines derived from homokaryotic hygromycin B-resistant mycelia showed a stable fertile phenotype. From these results, together with Southern hybridization data (not shown), we concluded that restoration of fertility in *pro1* transformants results from integration of cosmid DNA into the fungal genome.

For a more detailed characterization of the mutant

locus, Southern hybridizations were performed. Following restriction and electrophoretic separation, genomic DNA, isolated from both *pro1* and wild-type strain, was probed with cosmid DNA as well as cosmid-derived subfragments. The sample autoradiograph shown in Figure 2B indicates that, in mutant *pro1*, DNA has been deleted from the wild-type genome. Analysis of *SacI*-restricted DNA clearly shows that at least three fragments are missing in the mutant strain. Further analysis of all our hybridization data revealed a deletion of ~11.5 kb in *pro1*, including the 2.6-kb fragment that complements the sterile *pro1* phenotype.

To map the *pro1*<sup>+</sup> locus on an electrophoretic karyotype, chromosomal DNA from *pro1* and two isolates of the wild-type strain were used to perform pulsed-field gel electrophoresis. Because of similar size, only four bands, three of them consisting of comigrating chromosomes, can be resolved under our electrophoretic conditions as can be seen from Figure 2C. Southern hybridization, with cosmid clone C10 as a probe, showed that the largest chromosomal band, which corresponds to chromosomes VI/VII, carries the *pro1*<sup>+</sup> locus.

**Sequence analysis of the *pro1*<sup>+</sup> gene region and corresponding cDNA clones:** A 5.5-kb *EcoRI*/*EcoRV* DNA fragment, covering a genomic region sufficient to restore fertility to the *pro1* mutant and its flanking regions,

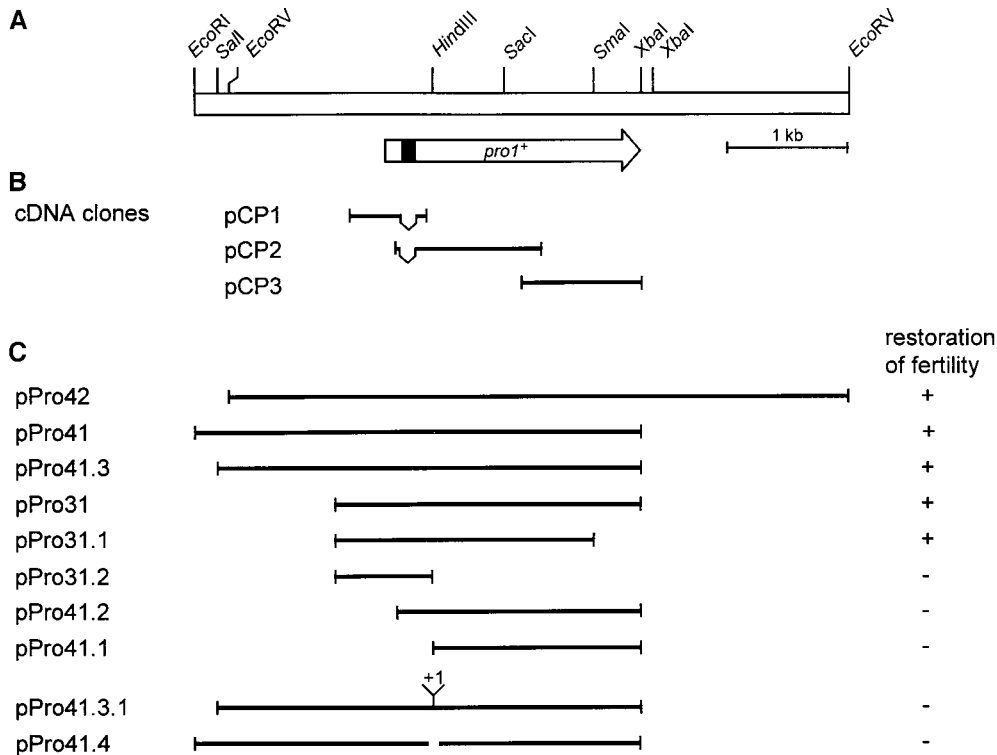


Figure 3.—Molecular organization of the *proI*<sup>+</sup> gene. (A) Restriction map of the sequenced 5.5-kb *EcoRI/EcoRV* fragment carrying the *proI*<sup>+</sup> gene. The location of the open reading frame is indicated by an arrow, whereas the single intron is marked by a black box. (B) Extension of three overlapping cDNA clones, which were generated by RT-PCR. (C) Recombinant plasmids carrying inserts of the *proI*<sup>+</sup> gene, which were used in complementation experiments. For each construct the ability to restore fertility in mutant *pro1* is indicated. A fertile transformant phenotype is indicated by a “+”, while no complementation is shown by a “-”.

was completely sequenced on both strands following standard procedures. We were able to identify an open reading frame encoding a polypeptide of 689 amino acids. To map intronic sequences, we generated three cDNA fragments using RT-PCR. As shown in Figures 3B and 4, the open reading frame is interrupted by a 92-bp intron, which is located 157 bp downstream of the ATG start codon. The 5'- and 3'-sequences, as well as the intron branch site, correspond with the consensus sequences proposed for *S. macrospora* introns (Pöggeler 1997). Starting with the first ATG codon at position 1603, the open reading frame has a length of 2159 bp and terminates with TAG at position 3762 (Figure 4). The amino acid sequence of the predicted polypeptide and the corresponding DNA sequence with the exact intron position are shown in Figure 4.

The transcript analysis shown in Figure 5 revealed that two *proI*<sup>+</sup>-specific transcripts of 2.8 and 3.1 kb are detectable using wild-type mRNA. As expected from our deletion analysis, no *pro1* transcripts were detected in mutant *pro1*. At present, we do not know whether the smaller transcript is a processing derivative of a 3.1-kb precursor molecule. Because the intron has a size of 92 bp, the 2.8-kb transcript is not merely the spliced precursor RNA.

Comparison of the *proI*<sup>+</sup>-encoded polypeptide with sequences in the EMBL, SWISS-Prot, and GenBank databases revealed considerable homology to fungal transcription factors. In the deduced PRO1 amino acid sequence, a region sharing significant homology with Zn(II)<sub>2</sub>Cys<sub>6</sub> zinc binuclear cluster domains of eukaryotic

transcription factors of GAL4 family members has been identified (Schjerling and Holmberg 1996; Poch 1997). This putative DNA-binding domain is located in the amino terminus, a characteristic feature of C<sub>6</sub> zinc finger transcription factors. An alignment of typical zinc binuclear cluster domains of GAL4 family members with the putative zinc finger-binding domain of the PRO1 polypeptide is shown in Figure 6. In PRO1, highly conserved amino acid residues are found, including six cysteines, which are essential for the coordination of two zinc ions. In the loop region, which connects two substructures of the domain, a structurally important proline sequence is conserved. The zinc cluster's C-terminal region is characterized by a high content of basic residues with putative functions in nuclear localization. In contrast to most members of the GAL4 family, a characteristic coiled-coil dimerization domain C-terminal to the Zn(II)<sub>2</sub>Cys<sub>6</sub> zinc binuclear cluster domain is missing in PRO1, as is a central inhibitory domain (Schjerling and Holmberg 1996; Poch 1997). Thus, the Zn(II)<sub>2</sub>Cys<sub>6</sub> zinc binuclear cluster domain is the only characteristic feature of PRO1 with obvious similarity to members of the GAL4 family.

**Functional analysis of the *proI*<sup>+</sup> gene:** To further understand the regulatory role that the PRO1 polypeptide plays during sexual development, truncated versions of the *proI*<sup>+</sup> gene were generated. Constructs, as shown in Figure 3C, were used to transform *pro1*, which contains a complete deletion of the *proI*<sup>+</sup> gene region as described above. Best results were obtained with plasmid pPro41, which carries the complete *proI*<sup>+</sup> coding region

964 GCACCAACTAACGCAGCTAGGGGGTCAAATCGGGAAAAGCAGTTAATAGCCCGCTTTTCAAATAAGAACAGGTGGCCAATCACCGATTCCACCACACGCTGGCCCATCGACCGGGCC

1084 GATATAAGAATTTGCCACTTCTCTCCCAAGTTTCCGTTTGGTCTTCTCATCTGCAGGCCAAAATGAAGAGAAGGATAGTGAGATCTAGGTGTCGTCAGTCCAGTCCAGTCAACAAA

1204 CCTTTTCCCTCTTTTAAATACACTCAACCTCACGCTACCCAGTCAAAACGCTTTTCGACTCGACGAGTTCATCATCGATCCAACCTCCGACCCAGTCTACTCAGCAAGCCGGACTTG

1324 CTCACGACCAGTCATTTCATTCGACGATACAATAATCTAATAATCTGATACAAGAGAGTGTTTTGGGTATAAGCCCCCTAGCGATTGGCCCTGGGACCTTCTGGACCGACGACGGA

1444 TAGAGAGCCCGACGCCATTTCCCTCGGCTAGCTGAAGTTACCACCCCTGGTCCCGACCCCTCTCCGCTAGCACCCCCACCACCCCTGGTCCCGGTGTGTGTCTCCATAAC

1564 TCACCTAAAAGAGTACTTCCATACAAGGGTACAATGTCAACACGGCCCCCAACCACCGGAGACATCAGGAACACATCTCAGTCAACATGACGACCACAACCAACCAAG

1 1 M S T R P P N H H G D I T N T S S V N M T T T T T T K

1684 ACCAAGCGACCGCAAAGCCCGCACCAATGCCCGCCCAAGCAAAGACACAGATGCATCGCCGCTCAAGGACAG gtttgtttgcctcttttactaccgctatcttatcacttacaca

28 T K A T A K A G T N A A P K Q K T Q M H R R S R T

1803 cttctcttcgacaacgttggaccgggaacgctgactttacagcgtgaagGATGTTACACATGCAGGCTGAGGAGGAAGTGCACGAGGGTTCCTCCATGTGCACAGCTTCAAGCAT

53 G Y T R L R R R K K C D E G S P M C T A C K H

1923 CTCGGGCTCAATCGAATAACAAGAGGCCAATGTGGTGGAGCAACACGATTGGCGGGAGCAGCAGGATGAGATCAAGATGATCATCAGGAGGAGGCTTCGSGAGAAGTCTGCG

77 L G L Q E Y K R P M W W S N N D L R R R K H K D E I K M I I K R K K L S E K S S

2043 CATACCATCCAGACTCAATCAACACACCGCCCGGCTCTCCGACTCGCTCCGACGCTCAGCGACCTTTCCGGTCCGCTTGATCGCAACAGGTCGCTTCGATCGATTTGGC

117 H T I Q T S I N T P P G L S H S L P T S A T F S G P L D R N R S A S I D S H F G

2163 TTCACCTTCAACCCCTCACAATGGCCCAAGATTTCACACCTTTGCGACGCCCAATAAACAATCAATGGAGAGTTCATCTCCACCCTTCTCTCCATCAGGAGATCGACATGAAGCC

157 F N F N S P H N G Q D F T P F A T P Q I N I N G E F I F P F S P Y E I D M K T

2283 GAGCGGAAATATTCATCAACGATGCCCGACCTTCGTGAATCAACAGTATCAACCTTCAGCACATATCAGACCCGCTCTCCGGGTACAATTCCTCCGCTATCCCTCTGGAAGT

197 E R Q I F I N D V P T L R E S T V S T F S T Y Q T P P P P G T I L P S F P L E G

2403 GAATGGACCGAGCAGGTGTTCTCGGAGCGGAGAGTCTGTGACGGAGGAGACTTTAATGCCAACTTCTCGACTTGTCTGCGATCCGGCTTGGCTTCTCGCAAGTGGCAGTTGAG

237 E W T E Q V F S E R R E S L T E E T F N A N F F D F A C D P A L A S S Q V A V E

2523 TTGGATGAGGTGACCAGAAGCTTCGATCACTTTGTGCAACAGCTTTCPCGACATCTTCCCAATTCGGAATCCAACCAGCATGGCTCAATCAGTCTGAGCTCATCTCCCATCA

277 L D E E G D Q K L F D H F V Q H V L P T I F P I L E S N Q H G S I S S E L I L P S

2643 CTGGCCAAACAAGGGTTACCTGCACTGCTGTGAGCATGGCGCGCAACACTCAAGTCTACAACGGGCATTGAGCGGAGAGATCGACAACGACATCATGCCACCCTGATGCC

317 L A N N K G Y L H C C L S I A A Q H F K S T T G I Q S E E I D N D I M R H R Y A

2763 ACCATCACCTGGCTGCGGAGGCACTCAATCGCGATGAGAACCACCGCAATTCGATGCCACCTTGGCTTGATTTCTTTCAGTGCATCGTTGGCGCTCCGAGGATACCTCTCT

357 T I T W L C E A L N R D E N H Q P I I L D A T L G L I F F Q C I V G R P E D T L P

2883 GATATTCCTGGCACCACATTTCCAAGCGGTGGTATCGCTTGTGCAAGCTCGATCTTGTGACTTGTATCGGATCTCGCCAGCCTCTCGCCACACCCCTTTCACATGACACTC

397 D I P W H Q H F Q A V V S L V Q K L D L A G L V S D L A K P L A H T P F N M T L

3003 ACGTCTGGATTGACATTTGGCGCCACCATGCTGGGTAGTTCCCCGATGTCGGGCACACTTCCGACCAAGCATCTTCGGTCAACAACCATCGCTCGGCTCGGTGAGTTGATG

437 T S W I D I L G A T M L G S S P M F A H T Y R H K H L S V N N H S L G L R E L M

3123 GGCTGCAAGACCGGCTCATGTATCTCATCTCGGAGATTGCCTGCCTCGATCGCTCAAGAACCGGGCATGGACGACATTACCCCTTTCGACGACGCTCCGTGGCTTGGCGACGATC

477 G C E D R V M Y L I S E I A C L E S L K N Q G M D D I T L C Q H V R G L G D E I

3243 AGCAATACCGAGATCAACGAGGGCAGCTCGTCGAGCCTTACAACGCCACCGCACCTTGGCCCTAAACAACCTGAGCAAGAATCAACCGCGCTTCCGCTTGTGCTCGCATCTAC

517 S N T E I N E G T L V E P Y N A N G T L S P K Q L S K N I T A F R L A A R I Y

3363 CTTCGACGCTGGTCCCGGGCTTCCACCTGCCAGCCAGCTGCATGGGTCTCGTCGAGAGCTCACCGGGTCCCTCAGCTGATCCCTCGGGGTCAACGGGATGACCGCTCACTC

557 L C S L V P G F H P A Q P S C M G L V E K L T A V L Q L I P S G V N G Y D R S L

3483 ACCTGGGTCTACCTGATCGGTGGATCCGTCAGCGTGGACGGCTCTCCCTCCGGACTTTTCGAGACCCGCTCGCCAGCTCGGGGACCGGCGAGCTCGGGGAGCTTTGGACGACG

597 T W V T Y L I G G S V S V D G S S F R D P F E T R I A Q L G D A A S S G A S S F G R T

3603 ACCGCTCCTCTCGCGAGACCTGGGTGCAATATCGCCCGCGTGGCGGGTACGAGCGCTCAAGCGGCGAGCGGAAATTCATCTCTCAGCCACAAGGCCTTACGTCAGGTGGCG

637 T V L L R E T W V Q Y A A A V A Y E A S K A A G A G N S S P Q P E P Y V R W R

3723 GATGTCATCGACCGAGGGATGGGACTACTTGTCTATCTAGACAACGACACCGTTCCTTCCAGGCTGGTTACTGGAACACAATTTCTTCTTCTTATACCACCTTTTTTATACAC

677 D V M Q T E G W D Y L L I \*

Figure 4.—Nucleotide and deduced amino acid sequences of the *S. macrospora pro1+* gene and its flanking regions. The intron sequence is indicated in lower case. Conserved intron sequences are underlined, while putative TATA and CAAT boxes are double underlined. Cysteine residues of the putative Zn(II)<sub>2</sub>Cys<sub>6</sub> DNA-binding domain are indicated by circles. The putative nuclear localization signal is boxed according to Dingwall and Laskey (1991). The nucleotide and deduced polypeptide sequences are numbered on the left, starting with nucleotide 964 according to the numbering of the complete sequence of 5479 bp deposited in the EMBL database under accession no. AJ010151.

and 1.6 kb of the *pro1* gene located upstream. With this construct we received complete restoration of fertility in ~40% of all *pro1* transformants. Ectopic integration of the transformed plasmid DNA into the recipient's genomic DNA may avoid efficient *pro1+* gene expression in many transformants. Similar effects have already been observed in many recombinant strains of other fungi (Fincham 1989). Truncated promoter regions in recombinant plasmids (pPro31, pPro41.3, pPro42), however, often led to the formation of fertile but stunted perithecia. A reduced ability to restore fertility in mutant *pro1* was observed when 127 amino acids from the carboxy terminus were deleted (plasmid pPro31.1). In this case, 4% of the transformants developed only a few stunted perithecia. This demonstrates that with respect to the *pro1+* gene activity, the carboxy terminus is functional but may not be essential for partial function of PRO1. Major truncations from the carboxy terminus were also tested for their ability to restore fertility. In plasmid pPro31.2, nearly 84% of the polypeptide was deleted, while in plasmid pPro41.3.1, a frame-shift muta-

tion at the *Hind*III recognition site of the open reading frame at amino acid 111. Neither construct was able to complement the *pro1-* mutation. Constructs with deletions in the *pro1+* gene's 5' region (plasmids pPro41.1 and pPro41.2), which included codons for the N-terminal part of the polypeptide failed to produce a functional PRO1 polypeptide and, therefore, were unable to restore fertility in the *pro1* mutant. Similarly, no fertile transformants were observed when an *in vitro*-mutagenized *pro1+*-coding region with an internal deletion of 83 amino acids (pPro41.4) was used. This deletion included half of the Zn(II)<sub>2</sub>Cys<sub>6</sub> zinc binuclear cluster domain and its flanking region. This confirmed the necessity of the putative DNA-binding domain for *pro1+* gene function.

**Presence of *pro1+* gene homologues in other filamentous fungi:** To identify *pro1+* homologues in other fungi, the genomic DNA isolated from various fungi and a labeled probe that carries the *S. macrospora pro1+* coding region (pPro41.2) were used to perform Southern hybridizations. As can be seen in Figure 7, genomic DNA

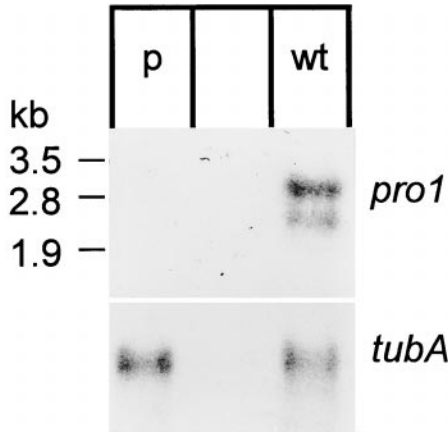


Figure 5.—Transcript analysis of the *pro1*<sup>+</sup> gene. Poly(A)<sup>+</sup> RNA from 3-day-old mycelium, from both wild-type strain (wt) and mutant *pro1* (p), has been separated on a denaturing formaldehyde gel and transferred to a nylon membrane. The upper part shows the autoradiograph from hybridization with a <sup>32</sup>P-labeled *pro1*<sup>+</sup>-specific probe, which carries the amino acid coding region only. For calibration, the same membrane was hybridized with a <sup>32</sup>P-labeled *tubA* α-tubulin-specific probe (Doshi *et al.* 1991).

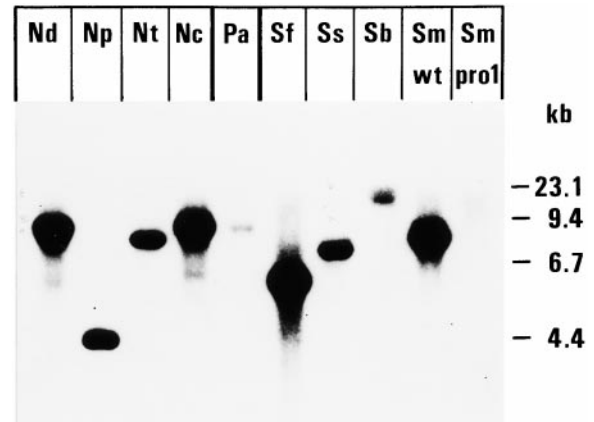


Figure 7.—Autoradiograph of a heterologous hybridization of fungal genomic DNAs with the *pro1*<sup>+</sup> gene-labeled probe. The *EcoRI*-restricted DNA was isolated from strains as indicated. Nc, *Neurospora crassa*; Nd, *N. dodgei*; Np, *N. pannonica*; Nt, *N. tericola*; Pa, *Podospora anserina*; Sb, *Sordaria brevicollis*; Sf, *S. fimicola*; Sm, *S. macrospora*; Ss, *S. sclerotigena*.

from fruiting body-forming ascomycetes gave strong signals with the *pro1*<sup>+</sup> probe. This includes homo- as well as heterothallic species. Remarkably, the hybridization signal with genomic DNA from *P. anserina* is less distinct. An even weaker signal was obtained with DNA from *A. nidulans* (data not shown). Further characterization was undertaken by amplifying fragments of the *pro1*<sup>+</sup> homologues from the heterothallic species *N. crassa* and *S. brevicollis* (as described in materials and methods). Subsequent sequencing of a fragment of about 1.5 kb, which carries the open reading frame from amino acid position 35 to 481, revealed a high degree of homology of 89% on the nucleotide level and 94% on the amino acid level between the *S. macrospora pro1*<sup>+</sup> gene with its homologues from these two heterothallic species (data not shown). Interestingly, even the position of the single intron is conserved within these three species. More-

over, the same homology is found when the two heterothallic species are compared with each other.

DISCUSSION

The homothallic nature of *S. macrospora* enables the generation of morphological mutants with defects in the transition from protoperithecia to perithecia, without the need to cross two strains of opposite mating-type during mutant screening. Our data also demonstrate that *S. macrospora* is a suitable experimental system for isolating genes that control cell differentiation during sexual development. The identification of transcription factor PRO1 suggests that a signal transduction pathway is involved in the control of sexual reproduction.

**The *pro1*<sup>+</sup> gene encodes a C<sub>6</sub> zinc finger protein that is structurally different from other C<sub>6</sub> zinc finger transcription factors:** The *pro1*<sup>+</sup> gene from *S. macrospora* encodes a polypeptide that shares significant homology with the Zn(II)<sub>2</sub>Cys<sub>6</sub> DNA-binding domain of fungal reg-

Cons > 33%		KR R	ACD	CR RK KCD	P C	C K	C Y																																
Cons > 15%		KK KRKVS	KS	KRRVR	GKK	SR IRRNLP	TFS PPKKR																																
Cons > 15%		K R		R I	R R	N G	V RRK																																
PRO1	<i>Sm</i>	44	TQMHR	RSRTG	CYTCRLR	KKKCD	EGSPMCTACKHLGLQ	CEYKRP	PMWWS																														
Gal4p	<i>Sc</i>	1	MKLLSSIE	QACDI	CRLK	KKLKS	KEKPKCAKCLKNNWE	CRYSPKT	KRS																														
Ppr1p	<i>Sc</i>	24	NIGISK	SR	TACKR	CRLK	KKIKCDQEF	PSCKRCAKLEVP	CVSLDP	PATGK																													
Lys14p	<i>Sc</i>	149	TVK	RRKYS	RNGCSE	EKKRR	RMKCD	ETKPTC	WQCARLNRQ	CVYV	LNPKNK																												
LAC9	<i>Kl</i>	85	KKSS	EVMHQ	ACD	ACR	KKKWKCSKT	VPTCTNCL	KYNLD	CVYS	PQVVRT																												
AFLR	<i>Af</i>	19	TRR	ARKLR	DS	CTSC	ASSKVR	CTKEK	PACARCI	ERGLA	COYMVS	KRMG																											
FACB	<i>An</i>	14	NNT	QSR	IAQ	ACDR	CRSK	KLRC	DGIR	PCCTQ	CANVGF	ECKT	SDKLS	RR																									
UAY	<i>An</i>	57	SSSS	ENVS	A	CNRC	CR	RK	NRCD	QRL	PRCQ	ACEK	AGVR	CVGY	DPIT	KR																							
NIT4	<i>Nc</i>	43	NQ	RRRC	V	STAC	IA	CR	RR	RSK	CD	GAL	PS	CA	ACAS	VY	GTE	CI	YDPNS	DHR																			
FL	<i>Nc</i>	1	M	PR	QHL	T	PN	ACL	V	CR	KK	R	T	K	C	D	G	Q	M	P	CR	R	C	R	S	R	G	E	E	C	A	Y	E	D	K	K	W	R	T

Figure 6.—Amino acid sequence comparison of the DNA binding domain of C<sub>6</sub> zinc finger proteins. The consensus sequence of Zn(II)<sub>2</sub>Cys<sub>6</sub> domains from C<sub>6</sub> zinc finger proteins determined by Schjerling and Holmberg (1996) is shown. A distinction was made for amino acids conserved in >33% (black) or 15% (gray) of 79 analyzed fungal regulatory proteins (Schjerling and Holmberg 1996).

ling and Holmberg 1996). All sequences are referenced in Todd *et al.* (1997) and Schjerling and Holmberg (1996). *Af*, *Aspergillus flavus*; *An*, *Aspergillus nidulans*; *Kl*, *Kluyveromyces lactis*; *Nc*, *Neurospora crassa*; *Sc*, *Saccharomyces cerevisiae*; *Sm*, *Sordaria macrospora*.



ulatory proteins. The zinc binuclear cluster domain has previously been identified in >80 fungal regulatory proteins, many of which have been shown to act positively as regulators of specific biosynthetic pathways, such as the well-studied GAL4 protein from *Saccharomyces cerevisiae* (for review see Schjerling and Holmberg 1996; Poch 1997; Todd and Andrianopoulos 1997). The zinc binuclear cluster domain differs clearly in its structure from typical zinc finger motifs of the Cys<sub>2</sub>/His<sub>2</sub> type or the Cys<sub>2</sub>/Cys<sub>2</sub> type. In the Zn(II)<sub>2</sub>Cys<sub>6</sub> DNA-binding domain, six cysteines participate in complexing two zinc ions. Each zinc ion is bound by four cysteine residues, with cysteine 1 and cysteine 4 acting as bridging ligands that contribute to the binding of both ions.

As indicated in the sequence comparison in Figure 6, the putative DNA-binding domain of the PRO1 polypeptide shares clear homology with the Zn(II)<sub>2</sub>Cys<sub>6</sub> domain of GAL4 family members. All of the conserved cysteine residues are also present in PRO1. Previous mutation analyses of different C<sub>6</sub> zinc finger proteins have demonstrated the importance of cysteine residues for proper functioning of these polypeptides (*e.g.*, Witte and Dickson 1988; Yuan *et al.* 1991; Todd *et al.* 1997). In all these cases, either substitution or deletion of one of the cysteines resulted in a complete loss of function. Preliminary data from *in vitro* mutagenesis indicate that a substitution of cysteine 5 by a proline residue in the PRO1 polypeptide prevents any complementation of the developmental defect in *pro1* (S. Masloff, unpublished data). Furthermore, the zinc binuclear cluster of PRO1 contains a conserved proline, which is located in a loop region connecting two substructures, each containing three of the cysteines. This proline is important to the avoidance of strain in the loop region. A further characteristic feature of this type of DNA-binding domain is a basic region between the second and third cysteine, while the fourth amino acid following cysteine 2 plays a crucial role in specific DNA binding. Although 61 of 79 identified C<sub>6</sub> zinc finger proteins have a lysine in this position, there are 13 examples, such as PRO1, where an arginine is carried instead of a lysine (*e.g.*, Lys14p in Figure 6; Feller *et al.* 1994).

Comparative analyses of C<sub>6</sub> zinc finger proteins have demonstrated that most members of this family of transcription factors share several structural features. Besides the amino-terminal location of the Zn(II)<sub>2</sub>Cys<sub>6</sub> DNA-binding domain, a large number of C<sub>6</sub> zinc finger proteins carry adjacently a coiled-coil motif that serves as a dimerization element. The region between the DNA-binding domain and the coiled-coil motif, the so called "linker," is of variable length and determines specificity of DNA binding. In PRO1 no coiled-coil motif was detected, indicating that PRO1 may bind as monomer to single asymmetric DNA-binding sites. A similar mechanism has been postulated for a number of C<sub>6</sub> zinc finger proteins, such as ARGRII from *S. cerevisiae* (De Rijcke *et al.* 1992) and AlcR from *A. nidulans* (Lenouvel *et al.*

1997; Panozzo *et al.* 1997). Interestingly, 19 out of 79 proteins studied, with a Zn(II)<sub>2</sub>Cys<sub>6</sub> DNA-binding domain, lack a coiled-coil motif (Schjerling and Holmberg 1996) and may also bind as monomers to DNA.

**Fungal developmental genes that control sexual reproduction:** The role of C<sub>6</sub> zinc finger transcription factors in cell differentiation is poorly understood. In *N. crassa*, for example, the FL protein participates in the activation of the previously characterized conidiation-specific (*con*) genes, *con-6* and *con-10*. It has been suggested that FL acts as a developmentally regulated transcription factor required for asexual morphogenesis (Bailey and Ebbolle 1998). In another example, sexual development in the basidiomycete *Lentinus edodes*, the *priB* gene transcript, which encodes a C<sub>6</sub> transcription factor, is abundant in fruiting body primordia. However, significantly lower transcript levels have been observed in mycelia and mature fruiting bodies, which suggests that the PRIB protein plays a regulatory role during morphogenesis (Endo *et al.* 1994). To date, the participation of C<sub>6</sub> zinc finger transcription factors in signal transduction during sexual morphogenesis is unresolved. However, other components involved in fruiting body formation have been determined, some of which often have additional functions in the vegetative phase or asexual reproduction (*e.g.*, Aramayo *et al.* 1996; Osiewacz and Nuber 1996; Ito *et al.* 1997; Wu and Miller 1997).

Our finding that the *pro1*<sup>+</sup> gene controls cell differentiation in *S. macrospora* enables us to study the role of C<sub>6</sub> zinc finger transcription factors in the controlled differentiation of fungal cell types. Further studies will be aimed at revealing other interacting factors that direct cell differentiation in this multicellular eukaryote. Especially, it is of importance to study the expression pattern of the *pro1*<sup>+</sup> homologues in the related heterothallic species *N. crassa* and *S. brevicollis*.

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