The pro1+ Gene From Sordaria macrospora Encodes a C6 Zinc Finger Transcription Factor Required for Fruiting Body Development

Sandra Masloff, Stefanie Pöggeler and Ulrich Kück
Lehrstuhl für Allgemeine Botanik, Ruhr-Universität, 44780 Bochum, Germany
Manuscript received September 25, 1998
Accepted for publication January 22, 1999

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− 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 C6 zinc finger transcription factors, such as the GAL4 protein from yeast. However, other typical regions of C6 zinc finger proteins, such as dimerization elements, are absent in PRO1. The involvement of the pro1+ 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+ homologues are present in other sexually propagating filamentous ascomycetes.

SEXUAL development in filamentous fungi is usually characterized by the formation of complex fruiting bodies that contain meiosporangia with either four or eight ascospores 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 peritheciun-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 peritecium’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 Metzenberg (1992) were able to subclone 14 N. crassa sexual development genes. One of these genes, asd-1, has been shown to encode a putative 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 andStraub 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-
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 (acl1) 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 C6 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 5675) 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. dodeg (FGSC 1692); N. pannonica (FGSC 7221); N. terricola (FGSC 1889); Podospora anserina (Esser 1974); S. brevicaulis (FGSC 1903); S. fimicola (FGSC 2918); and S. sclerotiorum (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% chloroform, 10% acetic acid; Romeis 1968). Small pieces of mycelium (~0.5 cm²) 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 Chevallier 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 × 10⁷ 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 cosmids, 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)⁺ 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⁺ gene described in this article. Restriction of D6 with XbaI, 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⁺ 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 pPro41.4 contains an internal deletion of 246 bp (amino acid residues 950 scanning electron microscope.
pro1 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 SmaI restriction site of the pBluescript/KS+ 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'-AGCAAGCCGACCTTGCTCACGACCC] and 840 [5'-GCATTGAAGCCCGAGATGCTTGCA] (pCP1); oligonucleotides 841 [5'-CCAGAGCCAAGGGCGACCGCAA GCC] and 842 [5'-CCCGTGGAAATGTTGGTGCCAGGG] (pCP2); and oligonucleotides 850 [5'-ACGACATCATGCCACCGCTATGC] and 848 [5'-TCCCTCGGTCTGCATGACATTCCCG] (pCP3). Homologous fragments of the pro1 gene from other filamentous fungi were generated using oligonucleotides 841 (sequences already given) and 865 [5'-CGGAGATGAGATACATGACG].

**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 gene, a nested deletion kit (Pharmacia) was used to generate nested DNA fragments. DNA fragments of pro1 homologues from S. brevicolis and N. crassa were sequenced by the QIAGEN customer sequencing service (Hilden, Germany). The programs from the Husar/rospora 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.

### 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 protoperithecium. However, no ascus primordia were detected inside pro1 protoperithecia, which indicates that transition from protoperithecium 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 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 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 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 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.

omic 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 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 locus.

Sequence analysis of the pro1 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,
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 pro1+ 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 pro1+ 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)$_2$Cys$_6$ 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$_z$ 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)$_2$Cys$_6$ zinc binuclear cluster domain is missing in PRO1, as is a central inhibitory domain (Schjerling and Holmberg 1996; Poch 1997). Thus, the Zn(II)$_2$Cys$_6$ zinc binuclear cluster domain is the only characteristic feature of PRO1 with obvious similarity to members of the GAL4 family.

**Functional analysis of the pro1+ gene:** To further understand the regulatory role that the PRO1 polypeptide plays during sexual development, truncated versions of the pro1+ gene were generated. Constructs, as shown in Figure 3C, were used to transform pro1, which carries a complete deletion of the pro1+ gene region as described above. Best results were obtained with plasmid pPro41, which carries the complete pro1+ coding region.
and 1.6 kb of the sequence 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 and pPro41.2), which included codons for the N-terminal part of the polypeptide failed to produce a functional PRO1 polypeptide and, hence, were unable to restore fertility in the pro1 mutant. Similarly, no fertile(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.

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...
Fruiting Body Development

Figure 5.—Transcript analysis of the pro1+ gene. Poly(A)+ 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 32P-labeled pro1+ specific probe, which carries the amino acid coding region only. For calibration, the same membrane was hybridized with a 32P-labeled tubA α-tubulin specific probe (Doshi et al. 1991).

from fruiting body-forming ascomycetes gave strong signals with the pro1+ 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+ 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+ 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-}

**Figure 6.**—Amino acid sequence comparison of the DNA binding domain of C6 zinc finger proteins. The consensus sequence of Zn(II)2Cys6 domains from C6 zinc finger transcription factors: The pro1+ gene from S. macrospora encodes a polypeptide that shares significant homology with the Zn(II)2Cys6 DNA-binding domain of fungal reg-

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

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 matingtype 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+ gene encodes a C6 zinc finger protein that is structurally different from other C6 zinc finger transcription factors: The pro1+ gene from S. macrospora encodes a polypeptide that shares significant homology with the Zn(II)2Cys6 DNA-binding domain of fungal reg-
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 Cys6/His2 type or the Cys6/Cys4 type. In the Zn(II)2Cys6 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)2Cys6 domain of GAL4 family members. All of the conserved cysteine residues are also present in PRO1. Previous mutation analyses of different C6 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 C6 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 C6 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)2Cys6 DNA-binding domain, a large number of C6 zinc finger proteins carry adjacent 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 C6 zinc finger proteins, such as ARGRII from S. cerevisiae (De Rijk 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)2Cys6 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 C6 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 Ebbel 1998). In another example, sexual development in the basidiomycete Lentinus etodes, the prB gene transcript, which encodes a C6 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 C6 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; Osiuszcz and Nuber 1996; Ito et al. 1997; Wu and Miller 1997).

Our finding that the pro1+ gene controls cell differentiation in S. macrospora enables us to study the role of C6 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+ homologues in the related heterothallic species N. crassa and S. brevicollis.

We thank Ms. S. Schlewinski and S. Giessmann for excellent technical assistance and Mr. H.-J. Rathke for preparing figures. We thank Prof. Stützel (Bochum) for his advice during the SEM studies. S. Masloff received a stipend according to the Graduiertenförderung des Landes Nordrhein-Westfalen, Germany. This work was funded by grants from the Deutsche Forschungsgemeinschaft (Ku 517/9 and SFB 480; Bonn Bad-Godesberg, Germany).

**LITERATURE CITED**


Fruiting Body Development


Lenovuel, F., I. Nikolae b and B. Felenbok, 1997 In vitro recognition of specific DNA targets by AlcR, a zinc binuclear cluster activator different from the other proteins of this class. J. Biol. Chem. 272: 15521–15526.


Panozzo, C., V. Capuano, S. Fillinger and B. Felenbok, 1997 The zinc binuclear cluster activator AlcR is able to bind to single sites but requires multiple repeated sites for synergistic activation of the alc gene in Aspergillus nidulans. J. Biol. Chem. 272: 22859–22865.


