Functional analysis of the C₆ zinc finger gene pro1 involved in fungal sexual development

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Abstract

The pro1 gene, controlling fruiting body development in the homothallic ascomycete Sordaria macrospora, encodes a C₆ zinc finger protein with a typical DNA binding domain of GAL4-like C₆ zinc finger proteins as well as a putative nuclear targeting signal. In the corresponding mutant pro1, the pro1 gene is deleted, and the transition of primordia into mature fruiting bodies is prevented. To further characterize the PRO1 polypeptide, the yeast system was used for identifying a transactivation domain in the N-terminal half of PRO1, which probably also functions in S. macrospora. The functional analysis was extended by using truncated versions of the pro1 gene in complementation transformations of a Δpro1 mutant. Interestingly, the 5' part of the pro1 gene encoding the DNA binding and transactivation domain as well as putative nuclear targeting signals was sufficient to restore fertility in the sterile pro1 mutant. In vitro mutagenesis verified that the DNA binding domain is essential for normal fruiting body development. This was concluded from transformation experiments with eight pro1 derivatives containing triplet substitutions in conserved codons of the DNA binding domain; some, but not all, failed in restoring the wild-type phenotype in mutant pro1. Using a PCR-based cloning strategy, pro1 homologs from the two related heterothallic species Neurospora crassa and Sordaria brevicollis were isolated, showing similarities in the predicted amino acid sequences of 91 and 90%, respectively. When a N. crassa pro1 cDNA clone was used in complementation transformations, we succeeded in restoring the wild-type phenotype to the S. macrospora pro1 mutant. These data suggest that pro1 homologs from heterothallic species can provide the pro1 function in homothallic ascomycetes. Based on the published sequence of the N. crassa genome, we identified hpro1A, another transcriptionally expressed gene, with a similarity of 40% to the pro1 genes, which is present as a single copy gene in N. crassa as well as in S. macrospora.

Keywords: C₆ zinc finger transcription factor; Sexual development; Sordaria macrospora

1. Introduction

Fruiting body formation is a major developmental step during the sexual reproduction of most filamentous fungi. In basidiomycetes as well as in ascomycetes, genetic loci have been identified that control basidiocarp and ascocarp development, respectively (for review see Kües, 2000; Moore, 1998). Genetic work with Neurospora crassa or Sordaria macrospora has provided clear evidence for a multigenic control of fruiting body formation (Esser and Straub, 1958; Raju, 1992). In recent years, components that play a role in signal transduction pathways have been identified through molecular genetic approaches. However, molecular events leading to complex sexual structures containing several tissue layers are poorly understood.

In recent years, it was successfully demonstrated that S. macrospora serves as a suitable experimental system for exploring this multicellular differentiation process in more detail (Masloff et al., 1999; Nowrousian et al., 1999; Walz and Kück, 1995). In contrast to heterothallic ascomycetes, such as N. crassa or Podospora anserina, S. macrospora shows the formation of single mating-type fruiting bodies (perithecia). The life cycle can be completed without the presence of other crossing partners.
carrying opposite mating-type loci. Recently, we have described the mutant pro1, which was isolated from a screen of UV-induced mutants showing a defect in fruiting body development. Mutant pro1 forms protoperithecia only and is unable to perform the transition into mature perithecia (Masloff et al., 1999). The pro1 gene encodes a polypeptide with significant homology to the DNA binding domain of fungal C6 zinc finger proteins, such as the Gal4p protein from yeast. The Zn(II)$_2$Cys$_6$ binuclear cluster DNA binding domain contains six cysteine residues, which complex two zinc ions. In PRO1, the C6 zinc finger motif is located in the amino terminus, as in most C6 zinc finger transcription factors. However, further amino acid sequence comparisons did not reveal other domains of C6 zinc finger factors, which typically include a coiled-coil region for dimerization and a less conserved middle homology region (Schjerling and Holmberg, 1996). Consequently, questions arise as to whether the conserved DNA binding domain of the PRO1 transcription factor is actually essential for the control of fruiting body formation and also whether the developmental function of PRO1 is exclusive to homothallic ascomycetes. The yeast system was used to identify an activation domain that, together with the adjacent DNA binding domain, readily restores fertility in mutant pro1. In vitro mutagenesis demonstrated that the Zn(II)$_2$Cys$_6$ binuclear cluster is a prerequisite for the developmental function of the PRO1 protein. In addition, isolated pro1 homologs from two heterothallic ascomycetes showed their ability to complement the developmental defect in a homothallic species.

2. Material and methods

2.1. Strains and growth conditions

The S. macrospora wild-type strain K (S 17.736) and the sterile mutant pro1 (M 8871) (Masloff et al., 1999) were derived from our laboratory collection. Sordaria brevicollis mating type A (FGSC 1903) and N. crassa mating types A (FGSC 4317) and a (FGSC 4347) originate from the Fungal Genetic Stock Center (FGSC, Kansas City, KS)$^{3}$ For morphological analysis, strains were grown on cornmeal medium with 0.8% malt extract (Esser, 1982). For protoplast preparation and isolation of genomic DNA, strains were cultivated on liquid CM medium (Nowrousian et al., 1999) as described by Pöggeler et al. (1997a). Transformants with derivatives of the zinc finger DNA binding domain were regrown on minimal medium (Le Chevanton and Leblon, 1989) with or without Zn$^{2+}$ ions (0.7 µM). Saccharomyces cerevisiae strain PJ69-4A (James et al., 1996) was used for the transactivation assay. The strain contains three reporter genes under the control of different GAL4-inducible promoters (GAL2-ADE2, GAL1-his3, and GAL7-lacZ). PJ69-4A was propagated on YEPD medium, while transformants were selected on SD minimal medium lacking uracil (Sherman et al., 1979).

2.2. Nucleic acid analysis

Nucleic acids were isolated from fungal strains by previously described methods (Lecellier and Silar, 1994). Digested DNA was separated by gel electrophoresis, and used in hybridization experiments, according to conventional methods (Sambrook et al., 1989).

2.3. Isolation of genomic and cDNA clones

pro1 homologs from S. brevicollis and N. crassa were amplified by a PCR amplification strategy using two sets of oligonucleotide primers (Nos. 1119 × 866 and 850 × 1208), as shown in Table 1. Amplicons of 0.7 and 1.0 kb, respectively, have been cloned and sequenced. A third fragment, overlapping with the 0.7- and 1.0-kb fragments, was amplified from both species using primers 841 and 865 (Table 1). In a separate experiment, the cDNA clone pIG111 from N. crassa was isolated by plaque filter hybridization of a N. crassa perithecial cDNA library P-1 (Nelson et al., 1997) obtained from the FGSC. A 1.5-kb BgIII/SacI fragment from plasmid pPRO-21 (Masloff et al., 1999) served as a pro1-specific probe. The S. macrospora pro1A gene was amplified by using different sets of primers (1591 × 1595 and 1592 × 1594) given in Table 1.

2.4. DNA sequencing and sequence analysis

DNA sequencing was performed by custom sequencing services (QIAGEN, Hilden, Germany, and MWG Biotech, Ebersberg, Germany). FASTA ( Pearson, 1990) was used for comparisons of nucleotide and amino acid sequences and sequence alignments were made using the CLUSTAL W program (Thompson et al., 1994).

2.5. Transformation of S. macrospora

Transformation of S. macrospora was carried out as described by Masloff et al. (1999) with slight modifications. With a glass pipette, the solution containing transformed protoplasts was plated on CM plates (Nowrousian et al., 1999) which were supplemented with 10.8% sucrose (CMS medium). Agar plates were directly overlaid with top agar containing hygromycin B (CMS medium, 0.8% agar).
2.6. Plasmid constructions

2.6.1. Site-directed mutagenesis

PCR-based mutagenesis (Ho et al., 1989) was performed to introduce amino acid substitutions in the PRO1 DNA binding domain. This method consists of two PCR rounds using three pairs of oligonucleotide primers. The 5\(^\prime\) primer overlaps with the unique Bgl\(^{II}\) site in the pro1 promoter at position 1160, while the 3\(^\prime\) primer is located at the Hind\(^{III}\) recognition site at position 2029. F (forward) primers contain the desired nucleotide substitutions and R (reverse) primers are reverse complement to the corresponding F primer. In a first round PCR, 5\(^\prime\) and R primers or F and 3\(^\prime\) primers were used separately, producing two overlapping PCR products, when plasmid pPRO41 (Masloff et al., 1999) was used as template. After purification of both PCR fragments, they served together as template for the second round PCR with primers 5\(^\prime\) and 3\(^\prime\). The resulting PCR fragment was subcloned in the Sma\(^{I}\)-digested vector pT3T7. The wild-type 0.9-kb Bgl\(^{II}\)/Hind\(^{III}\) fragment of pPRO41.4 was then replaced by the mutated Bgl\(^{II}\)/Hind\(^{III}\) fragment. Vector pPRO41.4 is a derivative of pPRO41, in which the Hind\(^{III}\) recognition site of the polylinker is destroyed by Klenow filling.

With the method described above, the following pro1 mutant alleles have been generated:

- pro1C54S (primers F1 and R1, see Table 1);
- pro1R61P (primers F2 and R2);
- pro1R61A (primers F3 and R3);
- pro1D65L (primers F4 and R4);
- pro1P69R (primers F5 and R5), and
- pro1P69G (primers F6 and R6). Primer 5\(^\prime\)B was used as 5\(^\prime\) primer for the generation of allele pro1C74P (primers F7 and R7). In this case the subcloned PCR fragment was ligated as a Hind\(^{III}\) fragment in plasmid pPRO41.4.

### Table 1

Sequences of oligonucleotides used in this study

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<th>Oligonucleotide</th>
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<td>R1 primer</td>
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<td>PCR primers for the isolation of the pro1A gene</td>
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<td>Oligonucleotide used for the construction of GAL4-PRO1 fusion plasmids</td>
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2.6.2. Fusion of *S. macrospora* PRO1 with the GAL4 DNA binding domain

Plasmid pPRO21.4 carries a 1.7-kb *HindIII/XbaI*-fragment carrying part of the *pro1* coding region (Lys-110–Ile-689). By insertion of a linker in the *XbaI* recognition site, a *BglII* site was generated, resulting in plasmid pPRO21.5. Using pPRO21.5, five subfragments of the *pro1* coding region were cloned in vectors pGBDU-C1 and pGBDU-C3 (James et al., 1996), as shown in Fig. 1. The resulting plasmid pPBD1 contains the complete 1.7-kb insert of pPRO21.5 cloned as a *Clal/BglII* fragment (Lys-110–Ile-689). pPBD3 carries a 0.6-kb *XhoI/BglII* (Glu-493–Lys-689) and pPBD4 a 1.1-kb *Clal/XhoI* (Lys-110–Lys-492) fragment. In pPBD5, a 0.4-kb *HindIII/HindII* fragment (Lys-110–Ser-249) is inserted and pPBD6 carries a 0.6-kb *HindII* fragment (Ser-249–Ala-455).

2.7. Yeast transformation and β-galactosidase reporter assays

GAL4-PRO1 fusion constructs were transformed into *S. cerevisiae* strain PJ69-4A (MATa trp1-901 leu2-3, 112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ) (James et al., 1996) by the electroporation method of Becker and Guarante (1991). Selection of transformants was performed on synthetic dextrose (SD) medium supplemented with the required amino acids, but lacking uracil (SD/ura-). In addition, selection on SD medium minus adenine (SD/ade-) or failure to grow on selective media. The 689-amino-acid sequence of PRO1 displays characteristic features of a transcriptional regulatory protein with a Zn(II)$_2$Cys$_6$ binuclear cluster DNA binding motif at the N-terminus and a putative nuclear localization signal (NLS). However, the 598-amino-acid C-terminal sequence is of indeterminate function (Masloff et al., 1999). A large number of Zn(II)$_2$Cys$_6$ factors possess a coiled-coil domain, C-terminal to the zinc cluster, which is involved in dimerization (Todd and Andrianopoulos, 1997). The presence of a coiled-coil domain was investigated by the programs COILS (Lupas, 1996) and PAIRCOIL (Berger et al., 1995). Neither program predicted a coiled-coil domain in the PRO1 protein.

As most C$_6$ zinc finger proteins are transcriptional activators, the same function can be assumed for the PRO1 polypeptide. To identify putative activation domains, we examined fragments of the PRO1 polypeptide, which are capable of activating transcription in *S. cerevisiae*. Appropriate constructs were designed, which fuse various regions of PRO1 to the yeast GAL4-DNA binding domain (Fig. 1). The ability of the various fusion proteins to activate the expression of three reporter genes controlled by different GAL promoters (GAL1-HIS3, GAL2-ADE2, and GAL7-lacZ) was utilized to determine the transactivation potential of each PRO1 region.

The carboxy terminus (pPBD3) does not show any transactivation activity, suggesting that the activation domain is located separately from these regions. In contrast, one construct carrying the carboxy terminal region of the DNA binding domain encompassing amino acid residues 110–689 (pPBD1) displays transcriptional activation function, which results in a high level of β-galactosidase activity in the yeast system (Fig. 1). Similar activities were obtained with clone pPBD5, which contains the region, which is limited by amino acid residues 110 and 249. Protein extracts of transformants carrying pPBD4 can grow on media by the method of Bradford (1976) and activity was normalized to protein concentration in the samples.

2.8. Nucleotide sequence accession numbers

The nucleotide sequences are deposited in the EMBL database under the following accession numbers: *S. brevicollis* hpro1 gene, AJ238536; *N. crassa* hpro1 gene, AJ238440; *N. crassa* hpro1 cDNA, AJ238557; *N. crassa* hpro1A, AJ318498.

3. Results

3.1. Identification of regions of PRO1 associated with transcription activation in *S. cerevisiae*

The 689-amino-acid sequence of PRO1 displays characteristic features of a transcriptional regulatory protein with a Zn(II)$_2$Cys$_6$ binuclear cluster DNA binding motif at the N-terminus and a putative nuclear localization signal (NLS). However, the 598-amino-acid C-terminal sequence is of indeterminate function (Masloff et al., 1999). A large number of Zn(II)$_2$Cys$_6$ factors possess a coiled-coil domain, C-terminal to the zinc cluster, which is involved in dimerization (Todd and Andrianopoulos, 1997). The presence of a coiled-coil domain was investigated by the programs COILS (Lupas, 1996) and PAIRCOIL (Berger et al., 1995). Neither program predicted a coiled-coil domain in the PRO1 protein.

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lacking adenine or histidine and display β-galactosidase activity. However, in yeast transformants containing constructs pPBD3 or pPBD6, the β-galactosidase activity was similar to that of the negative control, which carries only the GAL4 DNA binding domain (pGBDU-C1, 1–147), with activities of 8.8 ± 4.2 U/mg of protein. These data indicate that the activation domain is located between amino acid residues 110 and 249.

3.2. Deletion derivatives of the pro1 ORF restore fertility in the sterile mutant pro1

Previously we have shown that 3′ truncated versions of the pro1 gene are able to restore fertility in mutant pro1 (Masloff et al., 1999). This study was expanded by using pro1 derivatives, which had additional truncations of the 3′ end. As shown in Fig. 2, deletion derivative pPro52 (encoding amino acids 1–312 of the PRO1 polypeptide) carrying the DNA binding domain as well as the putative nuclear targeting signals is sufficient to restore the fertile phenotype in the sterile recipient. This fragment also encodes the putative transactivation domain, which is described above. Clone pPro31.2 (Masloff et al., 1999) carrying the DNA binding domain but lacking the C-terminal region including the putative activation domain is unable to complement the sterile mutant pro1.

3.3. In vitro mutagenesis of the C6 zinc finger region

Fig. 2 clearly demonstrates that only those derivatives carrying the DNA binding domain as well as the putative activation domain (and nuclear signals) are able to restore the fertile phenotype to mutant pro1. These findings strongly suggest that the Zn(II)2Cys6 DNA binding domain is essential for the developmental function of PRO1. To test this hypothesis, a PCR-based protocol (Ho et al., 1989) for in vitro mutagenesis was used to substitute conserved residues of the DNA binding domain. In particular, as shown in Fig. 3, three of the six conserved cysteines, which can be found in all C6 zinc finger transcription factors, were altered (Schjerling and Holmberg, 1996). Cys-54 was substituted by serine (pro1C54S) and Cys-71 by glycine (pro1C71G). These changes should prevent a correct coordination of the zinc ions, but should still allow for normal conformation of the domain, as the substituted amino acids carry small residues. A substitution of Cys-74 by proline was expected to destroy the overall conformation of the DNA binding domain. The activity of the three mutated polypeptides was examined by transformation of the sterile mutant pro1 with mutated copies of pro1. None of the resulting transformants displayed restoration of fertility.

In an extended approach further conserved amino acid residues of the PRO1 Zn(II)2Cys6 DNA binding domain were substituted (Fig. 3). In vitro mutagenesis, of codons for Arg-61, Asp-65, and Pro-69, which have been shown to be conserved in fungal C6 zinc finger transcription factors, resulted in the generation of the following five derivatives of the pro1 wild-type gene: pro1R61P, pro1R61A, pro1D65L, pro1P69R, and pro1P69G. While pro1R61A was able to restore fertility of the pro1 mutant strain, the pro1R61P construct was unable to restore the mutant phenotype, indicating that the conformation of the domain may have been destroyed. The pro1D65L mutation did not diminish the in vivo function of the PRO1 polypeptide. Although the conserved proline (Pro-69) has structural functions in Gal4p of S. cerevisiae (Marmorstein et al., 1992), the two substitution derivatives of Pro-69 retained their ability to restore fertility in mutant pro1. Similarly, substitution of Asp-65, which is conserved in 51 of 76 C6 zinc finger proteins (Schjerling and Holmberg, 1996), did not result in a loss of function.

The Pro-69 residue is highly conserved in C6 transcription factors and substitution should therefore result in a loss of function. Some of the transformants, carrying the pro1P69G derivative, were reexamined to verify the rather unexpected results. DNA from single
ascospore isolates was extracted, followed by amplification of the gene fragment encoding the DNA binding domain. Sequencing confirmed that all transformants carried the expected codon substitution. Johnston (1987) has previously shown that a loss of function mutant (Pro → Leu) of the Gal4 protein of S. cerevisiae can be corrected in vivo by high concentrations of Zn ions. The corresponding residue in PRO1 is Pro-69. We therefore tested growth of the transformants on solid minimal media containing either 0.7 μM ZnSO₄ or no ZnSO₄. In neither case was the function of the PRO1 derivative with the Pro-69 Gly substitution abolished. As a control, we used the pro1C71G derivative. Transformants, carrying pro1C71G, were unable to restore the developmental defect on medium containing a surplus of Zn.

3.4. Characterization of the S. brevicollis and N. crassa pro1 homologs

Previous hybridization experiments have already shown that homologs of the S. macrospora pro1 gene are present in related homothallic or heterothallic species (Masloff et al., 1999). Using a PCR-based cloning strategy, we succeeded in isolating genomic copies of pro1 homologs from N. crassa and S. brevicollis. In a first set of experiments, PCR amplifications were performed with two pairs of primers as described under Materials and methods. Using genomic DNA as a template, two fragments of 0.7 and 1.0 kb in size were amplified, which cover the 5′ or the 3′ end of the N. crassa and S. brevicollis homologs. DNA sequence determination allowed the synthesis of a third pair of primers (841 bp insert and encodes a protein of 696 amino acids.

On the DNA level, the N. crassa hpro1 gene shows 88% and the S. brevicollis hpro1 gene 87% identity to the S. macrospora hpro1 gene. Based on consensus sequences, it may be concluded that both homologs carry a single intron at the same position as the S. macrospora pro1 gene. This was substantiated when the cDNA sequence from N. crassa was compared to the corresponding genomic sequence. The intervening sequences of the two homologs display the regions with the highest sequence variability. While the intron of the S. macrospora pro1 gene has a size of 92 bp, the introns of hpro1 from N. crassa and S. brevicollis have lengths of 98 and 97 bp, respectively. Despite this variability, all intron consensus sequences, including the 5′ and 3′ as well as the intron branch sites, are completely identical in the three homologs and correspond closely to the consensus sequences defined for N. crassa and S. macrospora (Bruchez et al., 1993; Pöggeler, 1997). On the amino acid level the degree of homology of the pro1 homologs is even higher. The HPRO1 polypeptides from N. crassa and S. brevicollis show 91 and 90% identity to the corresponding S. macrospora PRO1 polypeptide. Using the recently released sequence of the N. crassa genome of the Neurospora Sequencing Project (Whitehead Institute/MIT Center for Genome Research; www.genome.wi.mit.edu), we were able to identify other similar N. crassa genes. Database search analysis with the BLAST program revealed a similarity between the HPRO1 protein and 12 putative ORFs of the N. crassa genome. All ORFs share a significant sequence similarity in a region containing the C₆ zinc finger DNA binding domain, but one ORF shows a 40% similarity in a 574-amino-acid overlap. The encoded protein was named HPRO1A and has been compared with the translation products of the three PRO1 homologs, as shown in Fig. 4.

From the alignment it becomes obvious that the open reading frames show a high level of homology, including the DNA Zn(II)₂Cys₆ binding domain. This domain is completely identical in S. brevicollis HPRO1 polypeptide, whereas the N. crassa HPRO1 DNA binding domain shows a single deviating cysteine residue at position 81 in a less conserved position of the C₆ zinc finger domain. As can be seen in Fig. 4, the only remarkable sequence differences between the three pro1 homologs are detectable in the carboxy terminal regions of the PRO1 polypeptides. With respect to amino acid position 671 of the S. macrospora PRO1 polypeptide, HPRO1 from N. crassa and S. brevicollis contain six or seven additional amino acids, respectively. It remains to be resolved whether this amino acid extension is a typical feature of pro1 homologs in heterothallic species. The N. crassa HPRO1A sequence shows significant homology not only to the three PRO1 homologs in the DNA binding domain, but also with the C-terminal end of the predicted polypeptide.

Using PCR primers as given in Table 1, which were originally designed in consideration of the N. crassa nucleotide sequence, we were able to amplify 2.4- and 1.2-kb fragments from the hpro1A gene using the genomic DNA from N. crassa or S. macrospora as a template. These amplicons served as probes in Southern hybridizations under stringent conditions, which detected a single 1.7-kb EcoRI and a 3.2-kb PsI fragment with genomic DNA from S. macrospora. Similarly, a single 12-kb EcoRI and a 3.2-kb SalI fragment were found with N. crassa genomic DNA, suggesting that hpro1A is a single copy gene in both ascomycetes. Finally, Northern hybridizations with RNA from vegetative mycelia, grown for 3 d, verified that both genes are
transcriptionally expressed, since a 2.6-kb mRNA could be detected (data not shown).

3.5. Functional complementation of the pro1 mutant phenotype using heterologous Nchpro1 cDNA

To further analyze the function of the *N. crassa* gene, the derived cDNA clone was used for complementation studies in *S. macrospora*. Since no fungal selection marker is present on plasmid pIG111 containing the *N. crassa* cDNA, pIG111 was cotransformed with plasmid pRP81-1 (Ridder and Osiewacz, 1992) into the recipient strain *pro1*. A third of about 350 hygromycin-B-resistant *S. macrospora* transformants showed restoration of fertility. This is most probably due to the fact that not all transformants carry both recombinant plasmids. Moreover, expression of the promoterless *N. crassa* cDNA gene copy is dependent upon ectopic integration into the genomic DNA adjacent to expression sequences; consequently, not all transformants show full fertility complementation. Fertile transformants were able to generate mature perithecia containing asci with eight viable spores, thus demonstrating that the heterologous *N. crassa* gene can functionally restore the defect in the *S. macrospora pro1* recipient strain. These data confirm that PRO1 function is not restricted to homothallic fungi.

4. Discussion

*S. macrospora* carries a single mating-type locus with four open reading frames showing homology to both idiomorphs of the heterothallic ascomycetes *N. crassa* and *P. anserina* (Pöggeler et al., 1997b; Pöggeler and Kück, 2000). Additionally, homokaryons from *S. macrospora*, but not from *N. crassa* or *P. anserina*, are able to form fertile fruiting bodies during their sexual pathway. A candidate gene regulating fruiting body development downstream of the mating-type genes is *pro1*, which controls the transition of protoperithecia into perithecia. The encoded gene product PRO1 shares considerable homology to C6 transcription factors, which so far have been identified in ascomycetes and basidiomycetes only. For example, 56 C6 transcription factors have been identified in yeast (Schjerling and Holmberg, 1996) on the basis of genomic sequences. The detection of *hpro1A* in *N. crassa* as well as in *S. macrospora* indicates that structural similarities between PRO1 and other C6 transcription factors are not restricted to the DNA binding domains. However, mutant analysis shows that functional complementation does not exist between PRO1 and PRO1A in *S. macrospora*.

PRO1 shares the presence of a Zn(II)$_2$Cys$_6$ zinc binuclear cluster with other C6 transcription factors but lacks other features, such as a middle homology region or
a coiled-coil domain. Fungal transcription factors containing a Zn(II)$_6$Cys$_6$ binuclear cluster usually bind to specific DNA sequences as homodimers. Coil-coiled elements have been found to be involved in dimerization (Marmorstein and Harrison, 1994; Marmorstein et al., 1992). Analysis of the three investigated PRO1 sequences showed no significant coiled-coil domain following the Zn(II)$_6$Cys$_6$ binuclear cluster motif. These findings suggest that _pro1 _homologs do not form homodimers, or they might interact with themselves through different amino acid motifs. Recently, Tsuji et al. (2000) identified a novel type of fungal transcriptional activators containing Cys-2–His-2 zinc finger motifs in addition to a zinc Zn(II)$_2$Cys$_6$ DNA binding motif. They suggested that the transcriptional activators Cmr1p and Pig1p of _Colletotrichum lagenarium_ and _Magnaporthe grisea_, which also lack coiled-coil elements, might interact via their Cys-2–His-2 zinc fingers. Similarly, an activation domain, which is characteristic of all analyzed C$_6$ transcription factors, could not be predicted from the primary amino acid sequence. We used fusions of the GAL4 DNA binding domain with parts of the PRO1 coding region to identify domains that are able to activate transcription of reporter genes under the control of GAL4-inducible promoters (James et al., 1996). This approach has already been successfully applied to NIT-4, a C$_6$ transcription factor, which mediates nitrate induction in _N. crassa_. In vivo analysis in yeast identified three separate activation domains in the 1090-amino-acid polypeptide (Feng and Marzluf, 1996). Similarly, a transactivation domain was identified in transcription factor GRISEA from _Podospora amerina_, which activates reporter genes in yeast in a copper-modulated manner (Borghouts and Osiewacz, 1998).

The activation domain of PRO1 together with the DNA binding domain is sufficient to complement the mutant phenotype of the _pro1_ strain. Other regions, lacking the zinc finger motif, but carrying the carboxy terminus, failed to restore the wild-type phenotype (Masloff et al., 1999). Most of the analyzed C$_6$, zinc finger proteins contain regulatory functions as activators in primary and secondary metabolism as well as in drug resistance (Todd and Andrianopoulos, 1997). So far, only a few cases have been reported in which morphogenetic functions are attributed to C$_6$ zinc finger proteins. One example is fluffy (fl) of _N. crassa_, which activates conidiation-specific genes (Bailey and Ebbole, 1998).

In vitro mutagenesis of the N-terminal Zn(II)$_6$Cys$_6$ binuclear cluster DNA binding domain clearly demonstrates that this region is required for sexual morphogenesis in _S. macrospora_. The alteration of any one of three out of six key cysteines impairs restoration of fertility in mutant _pro1_ and indicates that _pro1_ function in vivo requires an intact Zn(II)$_2$Cys$_6$ motif. In analogy with other C$_6$ transcription factors, it may be suggested that sequence-specific DNA binding is prevented in the mutants. For example, mutations of the first or the fourth cysteine of the DNA binding domain from the yeast activator HAP1, or from _Kluyveromyces lactis_ LAC9, eliminated or reduced substantially the binding to transcription factor target sites (Pfeifer et al., 1989; Witte and Dickson, 1988). Another example is that of the _facB_ gene, a major regulator gene involved in acetamide and acetate utilization in _Aspergillus nidulans_. Mutation in the fourth cysteine (C → S) completely abolishes in vivo activity (Todd et al., 1997). The results with derivatives of the conserved proline residue at position 69 are most surprising. Substitution by arginine or glycine did not result in a loss of function. This is in contrast with data obtained from the NIT4, MAL63p, PRNA, or GAL4p transcription factors. Mutants with a substituted proline residue show a significantly reduced activity or even the loss of function (Cazelle et al., 1998; Hu et al., 1999; Johnston and Dover, 1987; Yuan et al., 1991). In the case of GAL4p, however, the loss of function can be suppressed when high concentrations of ZnCl$_2$ are present in the medium (Johnston, 1987). We observed no loss of function when the corresponding transformant was grown on medium containing non-detectable amounts of Zn$_2$SO$_4$.

The high amino acid sequence homology of PRO1 to the corresponding polypeptides from _N. crassa_ and _S. brevicollis_ suggests that the developmental function of _pro1_ is not restricted to homothallic fungi. This was subsequently confirmed in complementation studies with the _pro1_ recipient strain. To date, few cases have been reported in which genes involved in the sexual cycle were functionally exchanged between homothallic and heterothallic fungi. For example, the mating-type locus from the homothallic fungus _Neurospora africana_ functions as a mating activator in _N. crassa_ (Glass and Smith, 1994). Similarly, the mating-type locus from _S. macrospora_ is able to complement the single mating-type genes (_mat$^-$ or _mat$^+$_) in the heterothallic fungus _P. anserina_ (Pöggeler et al., 1997b). In this study the _pro1_ homolog from _N. crassa_ is able to restore fertility in the homothallic _S. macrospora_ _pro1_ mutant. Although _pro1_ is not sufficient to trigger the transition of protoperithecia to perithecia in _N. crassa_, it may be speculated that the products of both mating-type loci from _N. crassa_ _mat A_ and _mat a_ strains are a prerequisite for the morphogenetic function of _pro1_.

Currently, PRO1 is the only identified gene product controlling the transition from protoperithecia to perithecia in _S. macrospora_. However, further factors are most likely involved in fruiting body differentiation. Recently, we have isolated over 40 nonallelic mutants which show a developmental block after protoperithecia formation (Masloff and Kück, unpublished data). Consequently, it must be assumed that numerous factors downstream of the mating-type genes contribute to this developmental process.
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