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Deletion of the *Schizophyllum commune* Aα Locus: The Roles of Aα *Y* and *Z* Mating-Type Genes

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ABSTRACT

The Aα locus is one of four master regulatory loci that determine mating type and regulate sexual development in *Schizophyllum commune*. We have made a plasmid containing a *URA1* gene disruption of the Aα *Y1* gene. *Y1* is the sole Aα gene in Aα1 strains. We used the plasmid construction to produce an Aα null (*i.e.*, AαΔ) strain by replacing the genomic *Y1* gene with *URA1* in an Aα1 strain. To characterize the role of the Aα genes in the regulation of sexual development, we transformed various Aα *Y* and *Z* alleles into AαΔ strains and examined the acquired mating types and mating abilities of the transformants. These experiments demonstrate that the Aα *Y* gene is not essential for fungal viability and growth, that a solitary *Z* Aα mating-type gene does not itself activate development, that Aβ proteins are sufficient to activate the A developmental pathway in the absence of Aα proteins and confirm that *Y* and *Z* genes are the sole determinants of Aα mating type. The data from these experiments support and refine our model of the regulation of A-pathway events by Y and Z proteins.

SEXUAL development, *i.e.*, the formation of the dikaryon, follows fusion of homokaryotic mycelia with different mating types in the basidiomycete fungus, *Schizophyllum commune*. Classical genetic studies (Kniep 1920; Papazian 1951; Raper 1966) have shown that mating events comprise two independently regulated developmental pathways, A and B, which in concert comprise the transition from homokaryon to dikaryon. The A pathway is controlled by two loci, Aα and Aβ. There are 9 Aα mating types and 32 Aβ mating types in the worldwide population of *S. commune*. The A pathway is activated by mates that differ for Aα and/or Aβ mating types. Thus, Aα and Aβ are considered redundant in the sense that either is sufficient to activate A-regulated development. The B pathway is controlled in a similar fashion by two loci, Bα and Bβ, also considered redundant to one another.

We have shown that the Aα locus contains two divergently transcribed genes (*Y* and *Z*), which encode mating type proteins, Y and Z (Figure 1, Stankis *et al.* 1992). Each Aα locus contains a unique pair of *Y* and *Z* alleles (*e.g.*, Aα3 contains alleles *Y3* and *Z3*), except for Aα1, which encodes only the Y allele, *Y1*. The Y and Z proteins contain homeodomains and other motifs that suggest that they may be DNA-binding regulatory proteins. Transformation experiments suggest that Aα activation of the A pathway occurs when Y and Z proteins from different mating types (non-self pairs) of Aα are brought together in the fusion cell formed by two mates (Y_i+Z_j where $A\alpha_i \neq A\alpha_j$) (Specht *et al.* 1992). Mutational analysis shows that the homeodomain of the Y protein is necessary for activation of the A pathway, but the homeodomain of Z protein is not (Luo *et al.* 1994). Assays using the yeast two hybrid system (Magae *et al.* 1995) have shown that protein interactions occur between *non-self* (*e.g.*, Y4 Z5), but not *self* (*e.g.*, Y4 Z4), pairs of Y and Z proteins.

The Aα locus is flanked by genes *SMIP* and *X* with function apparently unrelated to sexual development (Figure 1). The *SMIP* gene is located to the left of the Aα locus. The *SMIP* gene encodes mitochondrial intermediate peptidase, an enzyme involved in processing nuclear encoded peptides as they are being imported to the mitochondrion (Isaya *et al.* 1995). Gene *X* is located to the right of the Aα locus. Three alleles of *X*: *X1*, *X3* and *X4* have been sequenced (Marion *et al.* 1995). Neither the DNA sequences nor the protein sequences derived from the DNA sequences match known sequences of protein or DNA databases. Gene disruptions of the *X* gene cause no alteration of prototropy, mating or homokaryotic or dikaryotic phenotypes under the conditions used. Therefore we consider the *X* gene to have an undefined function which is likely to be unrelated to sexual development.

Other basidiomycete fungi have similar mating-type systems. In *Coprinus cinereus* and *Ustilago maydis*, development is initiated when non-self pairs of homeodomain proteins are brought together by fusion of haploid cells (Kües *et al.* 1994; Gillissen *et al.* 1992, respectively). Protein affinity assays of the *C. cinereus* A mating-

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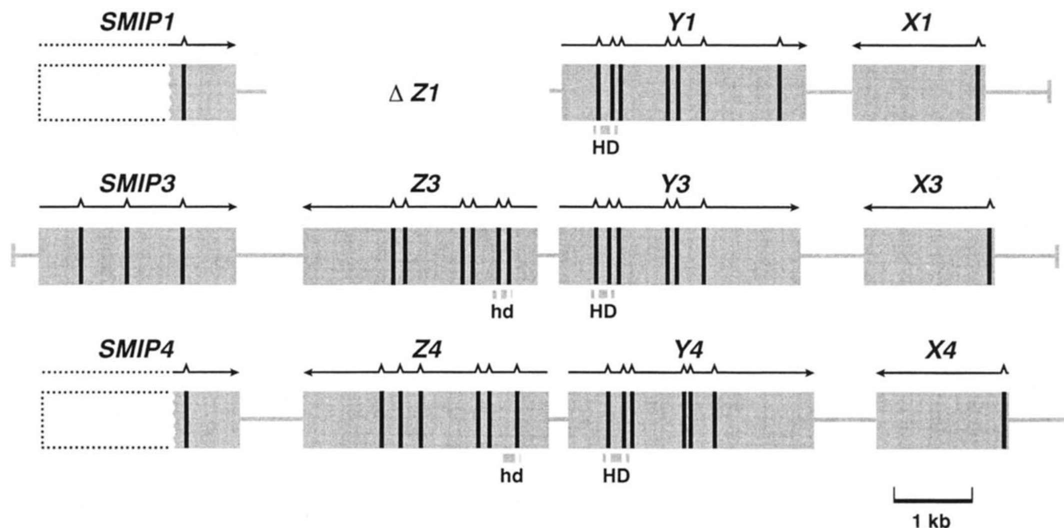

FIGURE 1.—Structure of the Aα1, Aα3, and Aα4 mating-type loci (STANKIS *et al.* 1992). *SMIP*, *Y*, *Z*, and *X* genes thought to encode proteins are boxed; shaded regions have been sequenced. Proposed introns are indicated by solid boxes. Arrows indicate the direction of transcription. *SMIP* encodes mitochondrial intermediate peptidase (ISAYA *et al.* 1995). *Y* and *Z* genes encode Aα mating proteins Y and Z. *X* is a gene of unknown function (MARION *et al.* 1995). HD, homeodomain motif; hd, similarity to a homeodomain motif.

type proteins (BANHAM *et al.* 1995) and both protein affinity assays and yeast two-hybrid assays of the *U. maydis* b proteins (KÄMPER *et al.* 1995), show that non-self pairs of homeodomain proteins interact, self pairs do not.

The most complete functional analysis of mating-type proteins has been done in the biallelic mating-type system of the ascomycete, *Saccharomyces cerevisiae*, where the regulatory properties of **a**1, α1 and α2 mating-type proteins have been defined (for a review, see HERSKOWITZ 1989). By comparison to the work presented here, it is noteworthy that the **a**1/α2 protein complex has been shown to function as a negative regulator (GOUTTE and JOHNSON 1988; DRANGINIS 1990).

Here we describe experiments in which we replaced the *Y* gene of the Aα1 mating type with a functional *URA1* gene. Because the Aα1 locus contains *Y1* but no *Z1* allele, this replacement has created an Aα-null (A$\alpha\Delta$) strain of *S. commune* devoid of Aα mating-type genes. The A$\alpha\Delta$ strain was used as a recipient in transformation studies of Aα regulation of the A developmental pathway. These experiments provide insights to the function of the *Y* and *Z* genes and the relation of Aα *Y* and *Z* genes to Aβ genes. The results confirm and extend our model of the means by which Y and Z proteins regulate development (LUO *et al.* 1994).

MATERIALS AND METHODS

Strains: Homokaryotic strains of *S. commune* used or created in this work are shown in Table 1. *Escherichia coli* strain DH5αF'IQ was used for gene cloning.

Construction of the Aα *Y1* deletion vector, pIR5: Plasmid pIR5, which contains a *URA1* gene disruption of the Aα *Y1* gene, is shown in Figure 2. Genomic sequences flanking the *Y1* gene were subcloned from cosmid pLAB08 (GIASSON *et al.* 1989). A 2.2-kb *Bam*HI fragment containing sequence from the right flank of the *Y1* gene was subcloned to plasmid vector pUC-18 (YANISCH-PERRON *et al.* 1985). This fragment contains the 3′-most 116 nucleotides of the *Y1* gene and the 1.7-kb *X* gene lacking the 5′-most 342 nucleotides. A 3.2-kb *Bam*HI-*Bgl*II fragment containing sequence from the left flank of the *Y1* gene was subcloned into plasmid vector pUC18. This fragment contains the 2.5-kb *SMIP* gene lacking 399 nucleotides from the 3′ end. These flanking sequences were then sequentially ligated into plasmid pEF3 (FROELIGER *et al.* 1989), which consists of plasmid pIBI30 (International Biotechnologies Inc.) containing *S.commune URA1*. The 2.2-kb *Bam*HI fragment containing the right flank of the *Y1* gene was ligated into a *Bam*HI site on the right flank of the *URA1* gene. Proper orientation of the insert was shown by *Mlu*I and *Pst*I digests. The 3.2-kb *Bam*HI-*Bgl*II fragment containing the left flank of the *Y1* gene was made blunt-ended with the Klenow fragment of DNA Polymerase I and ligated into a *Xho*I site on the left flank of the *URA1* gene. Proper orientation of the insert was shown by *Kpn*I and *Pst*I digests.

Construction of plasmid, pIR6, containing the full length *SMIP* gene: Plasmid, pIR6, containing the *SMIP* and *TRP1* genes was constructed for use in transformations. A 7-kb *Bam*HI fragment containing the *SMIP* gene and all but the 3′-most 291 nucleotides of the *Y1* gene was subcloned from cosmid pLAB08 to plasmid vector pUC18. This plasmid was digested with *Dra*III and made blunt ended with T4 DNA polymerase. Digestion with *Dra*III removed nucleotides 1300–3436 of the Aα1 locus containing the promoter and the 5′-most 1991 nucleotides of the *Y1* gene. A 4.5-kb *Eco*RI-*Hind*III fragment containing a functional *TRP1* gene was made blunt ended with T4 polymerase and ligated into the *Dra*III cut, blunt ended plasmid. The construction retains 740 nucleotides from the midregion of the *Y1* gene. Confirmation of the construction and orientation of the insert were shown by digestion with *Bgl*II.

Transformation of *S. commune* and analysis of transformants: Transformation was done as previously described (SPECHT *et al.* 1988; FROELIGER *et al.* 1989). Transformation of protoplasts derived from strain T54 with plasmid pIR5 to produce an A$\alpha\Delta$ transformant used 2.5 μg of plasmid pIR5

TABLE 1

S. commune homokaryotic strains and genotypes used

Strains	Mating type Aα	Aβ	Bα	Bβ	Auxotrophic markers
T54	1	1	2	2	*ura1*[a]
18-1	Δ	1	2	2	
18-2[b]	Δ	5	4	2	
18-5	Δ	1	2	2	*trp1*[a]
18-11[c]	Δ	1	3	2	
18-17	Δ	1	2	2	
18-18	Δ	1	2	2	
20-1	Δ *Y1*[d]	1	2	2	
20-2	Δ *Y1*[d]*Z4*[d]	1	2	2	
20-3	Δ *Z3*[d]	1	2	2	
20-4	Δ *Z4*[d]	1	2	2	
20-5	Δ *Z3*[d]	1	3	2	
T2	4	1	3	2	*ura1 trp1*
T11	4	1	1	6	*ura1 trp1*
T13	4	5	3	2	*ura1 trp1*
T14	4	5	2	2	*ura1*
T17	3	1	3	2	
T29	2	1	3	2	*ura1 trp1*
T30	6	1	3	2	*ura1*
T31	8	1	3	2	*ura1 trp1*
T32	9	1	3	2	*ura1 trp1*
T33	3	1	3	2	*ura1 trp1*
T34	5	1	3	2	*ura1 trp1*
T35	7	1	3	2	*ura1 trp1*
T52	3	5	4	2	*ura1*
8-7	3	5	3	2	*arg7*[a]
12-43	3	5	2	2	*ura1*
19-2	1	1	1	1	

[a] *ura1* mutant for orotidine-5′-phosphate decarboxylase (DIRUSSO *et al.* 1983); *trp1* mutant for indole-3-glycerol phosphate synthetase (MUÑOZ-RIVAS *et al.* 1986); and *arg7* mutant for arginine biosynthesis.

[b] Derived from strains 18-1 × T52.

[c] Derived from strains 18-1 × T13.

[d] Genes inserted by transformation.

and selection for Ura$^+$ phenotype on minimal medium (SPECHT *et al.* 1988). Cotransformations of AαΔ strains with Aα genes, *Y* or *Z*, used 10 μg of plasmid containing either *Y* or *Z* and 2.5 μg of plasmid pAM1 containing the *TRP1* gene, (MUÑOZ-RIVAS *et al.* 1986). Selection was for Trp$^+$. The DNA fragments containing the *Y* and *Z* genes (specified below) were cloned in plasmid vector pUC18.

The mating types of transformants were determined by mating tests. Each transformant was paired with a tester strain of appropriate mating type, incubated for 3 days at 30° and scored for the mating reaction (see below).

Cells of strain T54 transformed with plasmid pIR5 were screened for gene replacements (*i.e.*, AαΔ) by pairing Ura$^+$ transformants with tester strain T31. Strains T54 and T31 are identical for Aβ1 mating-type, but differ for the Aα mating-type, (*i.e.*, Aα1 and Aα8, see Table 1). Therefore, in a pairing of strain T54 with strain T31, activation of the A pathway depends on the presence of the *Y1* gene in strain T54; replacements yielding AαΔ would be incapable of activating A-regulated development because only the Aα8 locus of strain T31 would be present. Matings between strains identical at the Aα and Aβ loci, but different at either the Bα and/or the Bβ loci, produce mycelia with a distinctive morphology (*i.e.*, sparse hyphae with short, perpendicular branches and mycelium appressed to the agar with few aerial hyphae). This morphology is termed the "flat" phenotype. In contrast, matings between strains differing in both the A and the B mating types activate both the A and B pathways and produce dikaryotic cells with copious aerial mycelium and lateral appendages of the hyphae at every septum. These appendages are termed "clamp connections"; they are readily visualized with a light microscope at ×200 magnification.

Transformations of AαΔ strains used plasmids, (specified below) containing Aα DNA (*i.e.*, *Y* and/or *Z*), and plasmid pAM1 (MUÑOZ-RIVAS *et al* 1986) containing the *TRP1* gene. Trp$^+$ transformants were selected on CYM medium (RAPER and HOFFMAN 1974). Trp$^+$ transformants were crossed with appropriate tester strains containing a different Aα, an identical Aβ, and different Bα and/or Bβ mating types. Pairings were made on CYM and examined microscopically after incubation at 30° for 3 days in order to distinguish between dikaryotic and flat mycelia. Introduction of Aα DNA by transformation would produce a difference at the Aα locus between the mates. Thus both the A and B pathways would be activated leading to the production of clamp connections. If *TRP1*, but not Aα, DNA were introduced the B pathway would be active but the A pathway would not; in this case the matings result in the flat phenotype. The morphologies described above were used to assay for activation of the A and B pathways.

Extraction of DNA from Schizophyllum: Genomic DNA for use in Southern analysis and PCR was extracted from *S. commune* as previously described (SPECHT *et al.* 1982).

PCR: Ura$^+$ transformants unable to activate the A developmental pathway in Aα-dependent pairings were screened by PCR for gene replacement of Aα1. Two primers were used. Primer 1: 5′CAAGAGCACGCTCACGCACG was specific to the *URA1* gene sequence and primer 2: 5′CCGTTCTCTACGAGGCAGCT was specific to the *X* gene sequence (Figure 2). Reactions were run for 35 cycles (45 sec at 95°, 1 min at 65° and 1 min at 72°). PCR was concluded with a general extension step (10 min at 72°). Each reaction used 100 ng of template DNA, 200 nM concentration of each primer, 200 μM dNTPs, 3 mM MgCl and 5 units of *Taq* polymerase in 1× buffer supplied by Perkin Elmer. PCR products were electrophoresed on a 1% agarose gel, stained with ethidium bromide, visualized with UV light. The size of the PCR products was estimated against a 1-kb ladder (GIBCO).

Southern analysis: DNA was digested with *Eco*RI or *Bam*HI, separated on an agarose gel, denatured and transferred to Zetaprobe membrane (BioRad, USA) according to the manufacturer's specifications. The transfer was made in a downward direction according to the protocol of KOETSIER *et al.* (1993). [α-^{32}P]dCTP was used to label probes to specific activities of ~1 × 10^8 cpm/μg using a random primer DNA labeling kit (U.S. Biochemical). Prehybridizations and hybridizations were done in 1 mM EDTA, 0.5 M $NaHPO_4$, pH 7.2, 7% SDS. Hybridizations were done overnight at 70°. Membranes were washed twice in 1 mM EDTA, 40 mM $NaHPO_4$, pH 7.2, 5% SDS at 70° for 30 min. and once in 1 mM EDTA, 40 mM $NaHPO_4$, pH 7.2, 1% SDS at 70° for 45 min. Following these washes the membranes were exposed to Kodak X-OMAT film for periods of 1–14 days.

RESULTS

Deletion of the *Y1* gene to produce an AαΔ strain: Structural analysis suggests that the Aα1 mating-type differs from the other eight Aα mating-types in that it lacks a *Z* gene and contains only a *Y* gene, allele *Y1* (STANKIS *et al* 1992; SPECHT *et al.* 1994). Thus, deletion

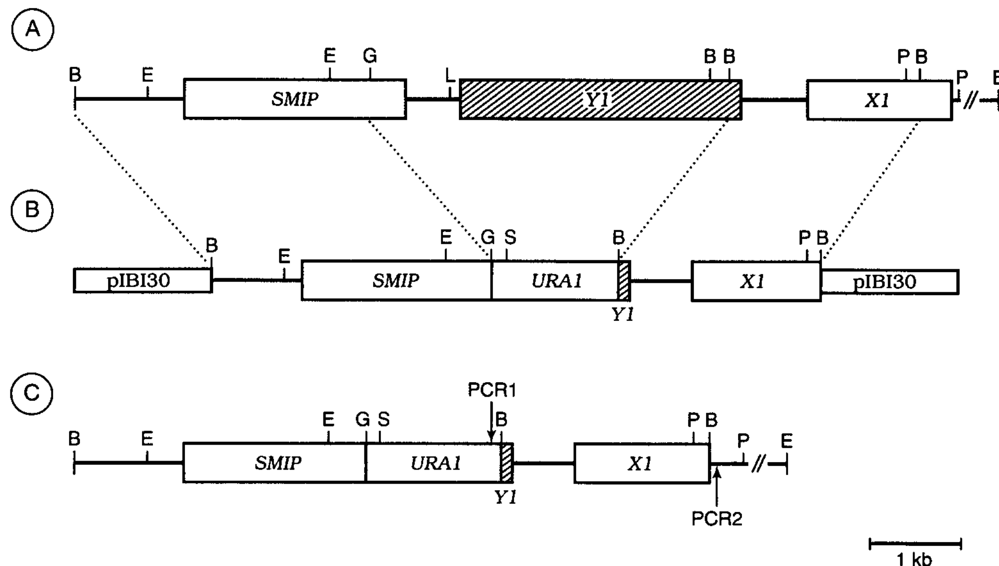

FIGURE 2.—Structure of the Aα1 locus, the *URA1* disruption of the *Y1* gene in pIR5 and the A$\alpha\Delta$ locus. B, *Bam*HI; G, *Bgl*II; E, *Eco*RI; P, *Pst*I; L, *Bal*I; S, *Sal*I. (A) Genomic structure of the Aα1 locus and neighboring *SMIP* and *X* genes. (B) *Y1* disruption construct, pIR5, used to make the *Y1* gene replacement. The construction process is described in MATERIALS AND METHODS. Hatched box shows 3′ 116 nucleotides of the *Y1* gene remaining in pIR5. (C) Structure of the genomic gene replacement of the *Y1* gene generating the A$\alpha\Delta$ mutation. Primers used for PCR (PCR1 and PCR2) are complementary to sequences specified by arrows. The *X* gene primer binds outside and to the right of the flanking sequence included in pIR5. Homologous recombination of pIR5 places the *URA1* gene in the Aα locus linking *URA1* to the *X* gene. A 2.3-kb PCR product is anticipated using primers 1 and 2 described above and substrate DNA from strain 18-1 as a template.

of the *Y1* gene should yield a strain with no Aα mating-type genes, *i.e.*, an A$\alpha\Delta$ mutant. Strain T54 with Aα1 mating type was transformed with pIR5 circular plasmid DNA. Ura$^+$ transformants could contain sequence from plasmid pIR5 by ectopic integration and/or gene replacement. A homologous event would replace all but the 3′-most 116 nucleotides of the *Y1* gene with the *URA1* gene.

Eighty-five Ura$^+$ transformants of strain T54 (*Aα1 Aβ1 Bα2 Bβ2*), were selected on minimal medium. These transformants were tested for Aα-dependent mating competence by pairing them with strain T31 (*Aα6 Aβ1 Bα3 Bβ2*). One transformant produced a "flat" mycelium in this mating assay. The "flat" reaction, diagnostic of A pathway off, B pathway on, is anticipated of an A$\alpha\Delta$ strain derived from T54 in a mating with tester strain T31 (Table 1). The transformant was tested further in mating assays using tester strains T11, T29, T32 and T35 containing Aα4, Aα2, Aα9 and Aα7 mating types respectively (Table 1). Each of these pairings is dependent on the presence of the *Y1* gene to activate the A pathway because each tester contains the *Aβ1* mating type, as does the putative A$\alpha\Delta$ strain. Each of these pairings gave a flat reaction. This putative A$\alpha\Delta$ transformant was named strain 18-1, and examined further by PCR.

PCR primers were chosen complementary to sequence in *URA1* and in *X1* sequence located outside of the flanking sequence used in the construction of pIR5 (Figure 2). Because *URA1* and *X* are normally unlinked, a PCR product resulting from the use of these primers would indicate that *URA1* had integrated contiguous to the *X1* flanking sequence. A PCR product of the anticipated length, 2.3 kb, was obtained when DNA of transformant strain 18–1 was used as a PCR template (data not included). The 2.3-kb product was not observed when these primers were used with template DNA from the progenitor strain, T54. This result is consistent with transformant 18-1 being an A$\alpha\Delta$ produced by gene replacement.

Further analysis of the replacement strain was conducted by Southern analysis (Figure 3). The *Y1* probe (2.8-kb *Bal*I-*Bam*HI fragment, Figure 2) lacks the 3′ most 291 nucleotides of the *Y1* allele and hybridizes to the anticipated 9.4-kb *Eco*RI fragment in DNA from the progenitor, strain T54, but not to DNA of strain 18-1. The 1.2-kb *Bam*HI-*Sal*I *URA1* probe hybridizes to the resident *URA1* locus contained on an *Eco*RI fragment >24 kb in length in DNA from strains T54, 18-1, and progeny of 18-1: strains 18-2 and 20-1. A 7.1-kb *Eco*RI fragment unique to the DNA of transformant 18-1, and its progeny, strains 18-2 and 20-1, is identified by the *URA1* probe. Finally a 0.7-kb *Pst*I fragment containing *X1* DNA reveals the 9.4-kb *Eco*RI fragment of T54 and the 7.1-kb *Eco*RI fragment of transformant 18-1 to contain *X1* sequence. The results confirm the deletion of *Y1* to produce A$\alpha\Delta$ strain, 18-1, by homologous recombination.

The results of mating tests with the A$\alpha\Delta$ strain 18-1 confirm earlier analyses (STANKIS *et al.* 1992; SPECHT *et al.*. 1994) that Aα1 strains contain only a single Aα mating-type determinant.

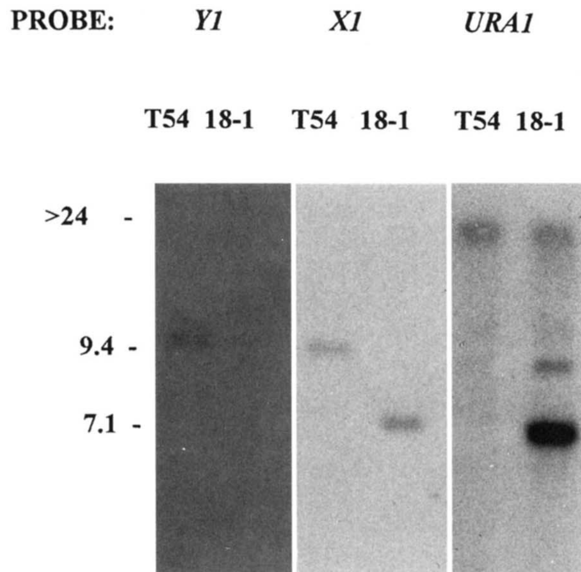

FIGURE 3.—Southern analysis of DNA from progenitor strain T54 and AαΔ strain 18-1 to show deletion of the *Y1* gene. Probes: *Y1*, 2.8-kb *Bal*I-*Bam*HI fragment; *X1*, 0.7-kb *Pst*I fragment; *URA1*, 1.2-kb *Sal*I-*Bam*HI fragment; each fragment used as a probe is identifiable in Figure 2.

Phenotype of AαΔ: Strain 18-1 was crossed with other strains to associate the AαΔ with other Aβ, Bα and Bβ mating types and auxotrophic markers. AαΔ strains have normal homokaryotic phenotypes (including simple septa) in all but two respects: they cannot activate the A pathway by virtue of the Aα locus and they have a slightly reduced growth rate (radial extension ~85% of the progenitor, strain T54). When mated with strains containing different Aβ and B mating types, AαΔ strains demonstrate normal A-pathway development, produce typical dikaryons and have normal ability to fruit, sporulate and make viable progeny.

Fully Aβ-dependent mating: Wild-type strains that differ at either the Aα or Aβ loci activate the A pathway when paired. We wished to determine if the Aα and Aβ loci function entirely independently of each other, *i.e.*, does activation of development by Aβ require participation by Aα protein? To answer this question, a fully Aβ-dependent mating was made between two AαΔ strains in the following manner. Strain 18-1 was mated with strain T52 and a recombinant *AαΔ Aβ5* strain, 18-2, was recovered. Strain 18-1 (*AαΔ Aβ1 Bα2 Bβ2*) was crossed with 18-2 (*AαΔ Aβ5 Bα4 Bβ2*). In the mating of strains 18-1 × 18-2 development was normal; both the A and B pathways were activated, clamp connections were produced and both fruiting bodies and spores were formed. The spores had typical viability with >90% germination. Because activation of the A pathway was dependent solely on the Aβ locus we conclude that the A-developmental pathway may be activated by Aβ in the complete absence of Aα proteins.

Cotransformations with *TRP1* and *Y1*: Strain 18-5 (*AαΔ Aβ1 Bα2 Bβ2 trp1*) was cotransformed with plasmids containing the *Y1* and *TRP1* genes. For the *Y1* gene, fragment b of SPECHT *et al.* (1992) was cloned into plasmid vector pUC18. Twenty-five Trp^+ transformants were examined for the presence of the *Y1* gene by mating tests using strain T30 (*Aα6 Aβ1 Bα3 Bβ2*) as a tester. Clamp connections were produced by 12 of 25 Trp^+ transformants in these matings (12 of 25 is within the expected frequency of cotransformation under the conditions used, SPECHT *et al.* 1988). *Y1* transformants crossed with strain T30 formed dikaryons with clamp connections, produced fruiting bodies and sporulated. The spores had typical viability. *Y1* transformants paired with strain 19-2 (*Aα1 Aβ1 Bα1 Bβ1*) gave flat mating reactions anticipated of strains with identical Aα and Aβ loci. Transformation with *Y1* did not restore a wild-type rate of growth. Genetic analysis showed that the *Y1* gene integrated ectopically (*i.e.*, unlinked to either the A or B loci, data not included). We conclude that the *Y1* gene restores Aα1 mating-type specificity to the AαΔ strain 18-5 and that the *Y1* gene functions normally when ectopically integrated.

Cotransformation with *TRP1* and *Z4*: Strain 18-5 (*AαΔ Aβ1 Bα2 Bβ2 trp1*) was cotransformed with plasmids containing the *Z4* and *TRP1* genes. For the *Z4* gene, fragment g of SPECHT *et al.* (1992) was cloned in plasmid vector pUC18. Twenty-five Trp^+ transformants were examined for the acquisition of the *Z4* gene by mating with tester strain 19-2 (*Aα1 Aβ1 Bα1 Bβ1*). Eighteen of 25 Trp^+ transformants contained the *Z4* gene as shown by formation of clamp connections in these test matings. We tested if *Z4* transformants would activate the A pathway with eight tester strains, each possessing one of the other eight Aα mating types. Four *Z4* transformants were chosen at random and paired in Aα dependent matings (*i.e.*, Aβ the same), with strains 19-2, T29, T30, T31, T32, T33, T34 and T35 (Table 1). Each pairing activated the A pathway as evidenced by the production of clamp connections. The matings were normal with respect to the ability to fruit, sporulate and produce progeny. The *Z4* transformants did not acquire the wild-type rate of growth. We also tested each of the 18 *Z4* transformants in pairings with tester strain T2 (*Aα4 Aβ1 Bα3 Bβ2*). Each of these pairings produced "flat" mating reactions. These results show that *Z4* transformants of the AαΔ strain behave as Aα4 strains in mating. Analysis of one of the transformants showed the *Z4* gene to be integrated ectopically and unlinked to either the A or B loci, (data not included). We conclude that Z protein does not activate the A pathway in the absence of Y protein, that ectopically integrated *Z4* confers an Aα mating-type upon the cell and that this cell has Aα4 mating-type specificity.

Cotransformations with *TRP1*, *Y4* and *Z4*: Strain 18-5 (*AαΔ Aβ1 Bα2 Bβ2 trp1*) was cotransformed with three plasmids, each containing one of the following genes: *TRP1*, *Y4* and *Z4*. Fragment c of SPECHT *et al.* (1992) containing the *Y4* gene was cloned in plasmid

vector pUC18; the other plasmids are described above. Twenty-five Trp^+ transformants were tested for the presence of the *Z4* and/or *Y4* genes in mating tests. Tester strain 19-2 (*Aα1 Aβ1 Bα1 Bβ1*) was used to detect the *Z4* gene; this exploits the fact that Aα1 strains possess the *Y1* gene but no *Z* allele. The test to detect the *Y4* gene in the Trp^+ transformants of strain 18-5 relied upon the presence of the ectopically integrated *Z3* gene in tester strain 20-5 (*AαΔ Aβ1 Bα3 Bβ2 Z3*). Strain 20-5 was created from a *Z3* transformant of strain 18-5 by crossing the transformant with T32. Mating tests to detect the *Y4* and *Z4* genes show that 11 of the 25 Trp^+ transformants received *Z4*, 11 received *Y4* and 8 received both *Y4* and *Z4*. The reduced growth rate persisted in all of the transformants.

One transformant that acquired both the *Y4* and *Z4* genes was paired with Aα4 strain T2 (*Aα4 Aβ1 Bα3 Bβ2*). This mating produced the flat mating reaction expected of an Aα4 strain and shows that *Y4 Z4* transformants behave as Aα4 strains. Furthermore, the matings with compatible strains were entirely normal with respect to dikaryotic phenotype and the ability to fruit, sporulate and produce viable progeny. We conclude that the integrated *Y4* and *Z4* genes confer Aα4 specificity on the AαΔ strain.

Cotransformations with *TRP1, Y1* and *Z4*: Strain 18-5 (*AαΔ Aβ1 Bα2 Bβ2 trp1*) was cotransformed with the *TRP1*, *Y1* and *Z4* genes (plasmids described above), each on a separate plasmid. Twenty-five Trp^+ transformants were tested for the presence of the *Y1* and *Z4* genes by mating tests using tester strain 18-11 (*AαΔ Aβ1 Bα3 Bβ2*). Transformants that receive both the *Y1* and *Z4* genes should be constitutive for A-regulated development, due to the presence of a non-self *Y-Z* pair in the cell. Three of 25 Trp^+ transformants produced clamp connections in pairings with strain 18-11, showing that they had received genes *Y1* and *Z4*. These three unmated cotransformants have the reduced rate of growth of their progenitor and an abnormal homokaryotic phenotype; they make pseudoclamps, *i.e.*, clamp connections that are not fused to the subterminal hyphal cell. Pseudoclamps are normally found in cells activated for the A-pathway but not for the B pathway (*e.g.*, in A≠B= heterokaryons and constitutive developmental mutants of the A loci, RAPER 1966). Thus pseudoclamps on the unmated *Y1 Z4* transformants are anticipated and indicative of activation of the A pathway by genes *Y1* and *Z4*. When the *Y1 Z4* transformants were mated the matings were normal with respect to the A pathway phenotype, dikaryotic phenotype and the ability to fruit, sporulate and produce progeny. These experiments show that a cell constitutive for the A pathway of development can be created by cotransformation with non-self pairs of *Y* and *Z* genes.

Transformation with the *SMIP* gene alleviates the reduced growth rate of AαΔ strains: AαΔ strains grow more slowly than strain T54 from which they were derived. One possible explanation of the reduced growth rate is that truncation of the *SMIP* gene may produce a form of the endopeptidase that is functional but less efficient than the wild-type protein. Construction of the *Y1* gene replacement plasmid, pIR5, removed 399 nucleotides from the 3′ end of the *SMIP* gene (Figure 2). Gene replacement of the *Y1* gene by pIR5 would thus truncate the *SMIP* gene and the resultant Schizophyllum mitochondrial intermediate peptidase would lack amino acids 693–727 (however, it would retain the *SMIP* active site, amino acids 549–553). Mitochondrial intermediate peptidases cleave amino-terminal octapeptides from imported mitochondrial proteins (HENDRICK *et al.* 1989). AαΔ strains transformed with plasmids containing the *Y1*, *Z4*, *Y4* and *Z4*, or *Y1* and *Z4* genes retain the slow growth phenotype.

To confirm that the slow growth of the AαΔ strains was due to a mutant *SMIP*, we transformed strain 18-5 with plasmid pIR6, which contains the *TRP1* gene and the complete *SMIP* gene. Two Trp^+ transformants, strains 18-17 and 18-18, were isolated for Southern analysis. Strains 18-17 and 18-18 have normal growth rate equal to that of strain T54, the AαΔ progenitor. Southern analysis of these transformants shows that a probe consisting of the 2-kb *Eco*RI fragment encoding most of *SMIP* hybridizes to a single *Bam*HI fragment in DNA from either strain T54 or 18-1. These fragments contain the endogenous wild-type *SMIP* gene in strain T54 and the truncated *SMIP* gene in strain 18-1. The *SMIP* probe hybridizes to two *Bam*HI fragments in DNA isolated from strains 18-17 and 18-18. One of these *Bam*HI fragments contains the truncated endogenous *SMIP* gene and the other fragment contains the full-length *SMIP* gene ectopically integrated (data not included). Transformation of the *SMIP* gene into strain 18-5 restores the wild-type growth rate, and we conclude that the reduced rate of growth of AαΔ strains is most likely due to the truncation of the *SMIP* gene.

DISCUSSION

By creating an AαΔ strain and transforming it with *Y* and *Z* alleles we have studied the roles of Y and Z proteins in Aα-regulated activation of the A developmental pathway. The studies also refine our understanding of the relationship between genes of the Aα and Aβ loci in A-regulated development. Our results permit conclusions about the mechanisms by which the Aα proteins regulate the A pathway.

Z protein alone does not activate the A pathway: No Aα mating types are known to contain only a *Z* gene. Therefore, it was unknown if a Z protein in the absence of Y protein would activate the A pathway. Because *Z4* did not activate the A pathway when introduced into AαΔ strain 18-5, we conclude that Z protein is not sufficient to activate A-pathway events in a homokaryon. Nevertheless, in Aα-dependent pairings the ectopically

integrated *Z4* behaves as expected of an Aα4 specificity. *Z4* activates the A-pathway in matings that provide a non-Aα4 *Y* gene and does not activate the pathway in matings with Aα4 strains.

Independence of Aα and Aβ proteins in regulation of the A pathway: Matings that pair AαΔ strains containing different Aβ mating types are fertile. This shows that Aα proteins are not necessary for Aβ proteins to activate the A pathway. We do not currently know enough about the structure of the Aβ locus to make an Aβ knockout mutation. Without this mutant we cannot test whether Aα proteins can activate the A pathway in the absence of Aβ proteins.

***Y* is not an essential gene:** The existence of AαΔ homokaryons demonstrates that the *Y* gene is not essential for growth. However, we cannot rule out the possibility that an Aβ gene (*e.g.*, Aβ *V*, SHEN *et al.* 1996) may be analogous to the *Y* gene and provide an essential function in the absence of the Aα *Y* gene.

Further, it may be anticipated that Aα and Aβ proteins provide a function beyond mating-type specificity to homokaryotic cells. For example, note the role of *S. cerevisiae* α1 protein in activating α-specific genes (HERSKOWITZ 1989). However, we detect no change in phenotype for the homokaryotic AαΔ strains as a consequence of deletion of the *Y1* gene. The slightly reduced rate of growth in the AαΔ strains is alleviated by transformation with full length *SMIP*. Nevertheless, it is possible that an Aβ gene analogous to *Y1* provides a function that masks a phenotypic change, which would otherwise appear in the absence of the *Y1* gene in the AαΔ strains.

Normal regulation by ectopically integrated Aα genes: The linkage analysis of an AαΔ strain transformed with the *Y1* gene and another transformed with the *Z4* gene show that the integrated genes assort independently of the Aα and Aβ loci. These transformants mate normally as Aα1 and Aα4 strains, respectively. We conclude that the *Y* and *Z* genes can function normally when integrated ectopically and the *Y* and *Z* genes need not be located in the Aα locus to provide normal function.

***Y* and *Z* genes are the sole determinants of Aα mating type:** AαΔ strains were derived from strain T54, an Aα1 strain. Transformation of the *Z4* and *Y4* genes into an AαΔ strain (derived from a progenitor whose genetic background is Aα1), produced a strain with homokaryotic phenotype and Aα4 mating type specificity. The gene deletion and reconstruction experiments demonstrate that the *Y* and *Z* genes are the sole determinants of Aα mating type as suggested previously, (STANKIS *et al.* 1992).

Gene replacement: This study employed the standard methods of ROTHSTEIN (1983) to make gene replacements in *S. commune*. One of the 85 URA$^+$ transformants selected had the expected deletion of gene *Y1* (*i.e.*, 1.2%). The percentage of gene replacements may have been higher if the plasmid had been linearized as was the case for other *S. commune* gene replacements: *TRP1*, 4% (unpublished results) and *X*, 33% (MARION *et al.* 1995).

Y protein is not a simple negative regulator of the A pathway: Because the A pathway is not activated in AαΔ homokaryons, it can be surmised that Y protein is not a simple negative regulator of the A pathway. Simple negative regulation of sexual development by homeodomain proteins is not unprecedented in fungi. α2 protein is a simple negative regulator of **a**-specific genes in *S. cerevisiae*. α2 protein binds to the operator of **a**-specific genes as a multimer in combination with MCM1 protein (KELEHER *et al.* 1989). In **a1**/α2 diploid cells, α2 also acts in combination with yeast protein **a1**, binding tightly to the operators of haploid specific genes and marking them for repression (GOUTTE and JOHNSON 1994). Conservation of an intron within the region that encodes the conserved WF.N.R motif of the homeodomain in the *Y* genes of *S. commune*, *HD2* genes of *C. cinereus*, *bW* genes in *U. maydis* and the **a1** gene of *S. cerevisiae* led KÜES *et al.* (1994) to suggest a shared lineage among these genes. It is therefore appropriate to ask whether these proteins act in a manner similar to **a1**. Deletion of both *bE* and *bW* genes from *U. maydis* causes an inability to activate development in pairings (GILLISSEN *et al.* 1992). Thus bE and bW proteins are not simple negative regulators. Similarly, our results in *S. commune* show that Y protein is not a simple negative regulator and strongly suggest that, unlike **a1**, Y protein is more likely a positive regulator of sexual development. Also the redundant, but independent, function of Aα and Aβ argue against Y protein acting as a simple negative regulator.

Support of model for Aα activation of the A pathway: The results of experiments reported here support our model of the functions of Aα proteins in activation of A-pathway events. We proposed (LUO *et al.* 1994) that Y and Z proteins from different Aα mating types form multimeric complexes (possibly with other proteins) and bind to the recognition sites of developmental target genes to be controlled in A-regulated development. The results reported here demonstrate that the Aβ proteins function independently of Aα proteins. We speculate that the converse is likely to be true, and therefore Aβ proteins are likely not part of the Y/Z protein complex. Regulation of transcription could be by activation or, as in the case of **a1** and α2 proteins in yeast, repression. In light of the findings that Y protein is not a simple negative regulator and Z protein is not by itself an activator, we propose that the Y_i+Z_j $i \neq j$ protein complex is most likely an activator of A-pathway genes. There remains a remote possibility that Y is a negative regulator of a negative regulator.

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