The Specificity Determinant of the Y Mating-Type Proteins of *Schizophyllum commune* Is Also Essential for Y-Z Protein Binding

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ABSTRACT

This paper concerns the manner in which combinatorial mating proteins of the fungus, *Schizophyllum commune*, recognize one another to form complexes that regulate target gene expression. In Schizophyllum, tightly linked *Y* and *Z* mating-type genes do not promote development in the combinations present in haploid strains (*i.e.*, self combinations). When the *Y* and *Z* genes from two different mating types are brought together by the fusion of two haploid cells, the Y and Z proteins from different mating types recognize one another as *nonself*, form a complex and activate development. Several *Y* and *Z* alleles are present in the population and all *nonself* combinations of *Y* and *Z* alleles are equally functional. We have made chimeric genes among *Y1*, *Y3*, *Y4* and *Y5* and examined their mating-type specificities by transformation and mating tests. These studies show that the specificity of Y protein recognized by Z protein is encoded within a short region of N-terminal amino acids. The critical region is not precisely the same in each Y protein and in each Y-Z protein interaction. For Y3 protein compared with Y4 protein, the critical residues are in an N-terminal region of 56 amino acids (residues 17–72), with 40% identity and 65% similarity. Two-hybrid studies show that: the first 144 amino acids of Y4 protein are sufficient to bind Z3 and Z5 proteins, but not Z4 protein, and proteins deleted of the Y4 specificity region do not bind Z3, Z4 or Z5 protein. Thus the specificity determinant of the Y protein is essential for protein-protein recognition, Y-Z protein binding and mating activity.

THE developmental fate of eukaryotes is determined in many cases by interactions of combinatorial regulators. Features inherent in the proteins determine which partners may pair and, thereby, the sets of target genes regulated. In basidiomycetes, sexual development is regulated by the genetic constitution of the mating-type loci of interacting cells; the gene products of these loci distinguish between *self* and *nonself* combinations, and thereby, determine developmental fate. The multiallelic mating-type loci of *Schizophyllum commune* (GIASSON *et al.* 1989; SPECHT *et al.* 1992; STANKIS *et al.* 1992), *Ustilago maydis* (KRONSTAD and LEONG 1990; SCHULZ *et al.* 1990; BANUETT 1992; GILLISSEN *et al.* 1992; KÄMPER *et al.* 1995) and *Coprinus cinereus* (MUTASA *et al.* 1990; KÜES *et al.* 1992) and the biallelic mating-type locus of *U. hordei* (BAKKEREN and KRONSTAD 1993) are examples of developmental systems that exhibit these protein-protein interactions.

Our work concerns mating type in *S. commune*, where mating type is determined by four complex loci: Aα, Aβ, Bα and Bβ. Multiple alternatives of these loci are found in the natural population: nine Aα, 32 Aβ, nine Bα and nine Bβ (RAPER *et al.* 1960; KOLTIN *et al.* 1967). Aα and Aβ are redundant regulators in the sense that a difference between the mates at either locus is sufficient to activate A-regulated development. Similarly, the redundant B loci control the B-regulated pathway. Activation of both the A and B pathways allows the two sterile, haploid, homokaryotic mates to develop into a fertile dikaryon.

The Aα locus of *S. commune* contains two genes, *Y* and *Z* (STANKIS *et al.* 1992). Each Aα mating type has its unique pair of *Y* and *Z* alleles (*e.g.*, Aα3: *Y3* and *Z3*) except Aα1, which contains only one allele, *Y1*. The deduced Y and Z proteins encode homeodomains and other motifs; this suggests that the proteins function as transcription factors. Previously we showed, by molecular genetic analysis, that the combination of Y protein from one Aα mating type and Z protein from a different Aα mating type activates A-regulated development (SPECHT *et al.* 1992). We proposed that Y and Z proteins from different Aα mating types form heteromultimers, whereas Y and Z proteins from a haploid strain do not (LUO *et al.* 1994). Subsequently we obtained evidence in support of this hypothesis with two-hybrid assays in yeast (MAGAE *et al.* 1995). The results reported here describe the region in Y proteins that encodes allelic specificity and confers allele specific Y-Z protein interactions. Our studies show that the specificity region is essential to Y-Z protein-protein interactions.

MATERIALS AND METHODS

Strains: Table 1 shows the genotypes of *S. commune* strains used in this study. Yeast strain, SFY526, from the Clontech Matchmaker Kit was used for two-hybrid studies.

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TABLE 1

Strains of *S. commune*

Strain	Genotype	Use[a]
UVM T4a	Aα1 Aβ1 Bα2 Bβ2 *trp1*	r
UVM T5	Aα1 Aβ1 Bα7 Bβ4	t
UVM T33	Aα3 Aβ1 Bα4 Bβ1′ *trp1 ura1*	r
UVM T22	Aα3 Aβ1 Bα2 Bβ2 *trp1 ura1*	t
UVM T11	Aα4 Aβ1 Bα1 Bβ6 *trp1 ura1*	r
UVM 4-24	Aα4 Aβ1 Bα3 Bβ1	t
UVM T2	Aα4 Aβ1 Bα3 Bβ2 *trp1 ura1*	r
UVM T8	Aα4 Aβ1 Bα7 Bβ4 *trp1 ura1*	t
UVM T41	Aα4 Aβ7 Bα1 Bβ4 *ura1*	r
UVM T46	Aα4 Aβ7 Bα2 Bβ2	t
UVM T24	Aα5 Aβ1 Bα2 Bβ2 *trp1 ura1*	r
UVM T34	Aα5 Aβ1 Bα1 Bβ1 *trp1 ura1*	t
UVM 4-40	Aα4 Aβ6 Bα1 Bβ1	RNA

[a] r, Recipient in transformation; t, strain used for test matings; RNA, strain used to extract polyA+RNA.

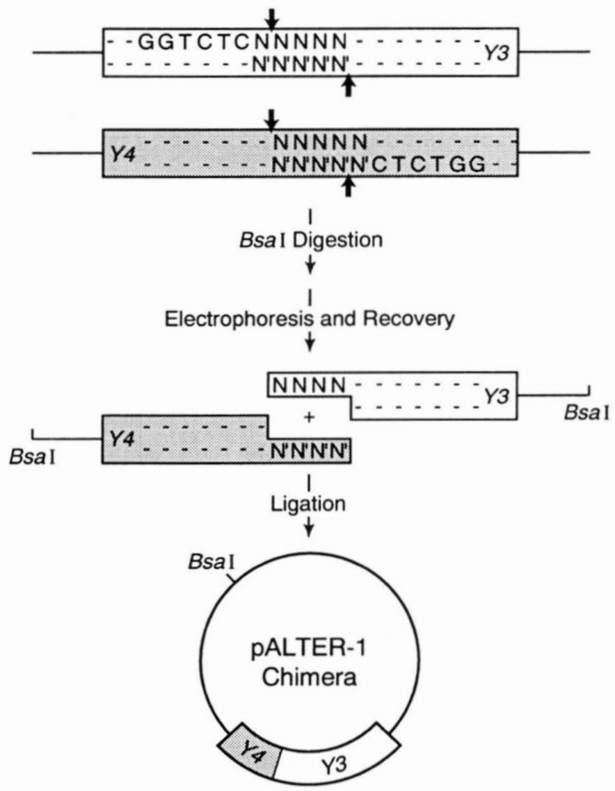

FIGURE 1.—Strategy for *in vitro* construction of chimeric genes using *Y3* and *Y4* cloned in pALTER-1. Open box, *Y3*; shaded box, *Y4*; single line, pALTER-1 cloning vector. N and N′ designate complementary bases of any sequence common to *Y3* and *Y4*. Site-directed mutagenesis (Altered Sites Mutagenesis System, Promega) was employed to introduce a *Bsa*I recognition sequence (GGTCTC) into *Y3* and *Y4* at corresponding sites. The pALTER-1 vector contains one *Bsa*I site; therefore, cleavage of the mutated *Y3* or *Y4* DNA with *Bsa*I yields two fragments. Fragments possessing the wild-type *Y3* and *Y4* partial coding sequences were recovered after electrophoresis and ligated together to form the chimeric *Y4/Y3* allele. This strategy requires only a short sequence in common at corresponding positions in the two *Y* alleles.

Construction of chimeric genes: Restriction fragments containing either *Y3* or *Y4* DNA (*Y3*, 3.4-kb fragment c and *Y4*, 3.1-kb fragment c; SPECHT *et al.* 1992) were ligated into plasmid vector pALTER-1 (Altered Sites Mutagenesis System, Promega). Each fragment is fully functional as demonstrated by activating A-regulated development when transformed into a strain containing a *Z* gene from a different Aα mating type. The strategy for making chimeric genes that contain coding sequences from both *Y3* and *Y4* alleles is described in Figure 1. Using this strategy, *Y4/Y3* (5′/3′) chimeric genes consisting of wild-type *Y4* and *Y3* sequences were constructed with junctions occurring at codons 1, 16, 26, 41, 62, 73, 118 and 224, respectively (Figure 2). The mutated *Y4* and *Y3* sequences created in the course of synthesizing the *Y4/Y3* chimeras were also tested for mating-type specificities as shown in Table 3.

Similarly, a 2.8-kb *Ball-Bam*HI fragment of allele *Y1* (Fragment b; SPECHT *et al.* 1992) and a 1.8-kb *Sal*I fragment of allele *Y5* were used to produce *Y1/Y3* and *Y5/Y3* chimeras each fused at codon 73.

Each chimeric construction was checked by restriction analysis and/or sequencing across the fusion point. DNA sequencing was done with a Sequenase kit (U.S. Biochemical Corp.).

Transformation of chimeric constructions into *S. commune*: Chimeric constructions were integratively cotransformed with plasmid DNA containing either the *TRP1* or *URA1* gene and transformants selected on media lacking either tryptophan or uracil using the methods of SPECHT *et al.* (1988). Because only 20–80% of the selected transformants are cotransformants, for each chimeric construction ≥25 transformants were subjected to mating tests to determine the Aα mating type of each chimeric *Y* gene.

Mating type analysis of transformants: When A-regulated development is active (*i.e.*, Aα and/or Aβ of the two mates differ) lateral appendages called clamp connections form near the hyphal septa. The absence of clamp connections indicates that A-regulated development has not been activated and that the two mates have identical mating type for Aα and Aβ. This distinction forms the basis of the mating test for allelic specificity of transformants. The test is most easily applied when the two mates differ for Bα and/or Bβ mating type; therefore, the recipient strains for transformation and their respective testers differ for either Bα and/or Bβ, but contain the same Aα and Aβ specificities (Table 1). When a recipient (that has been transformed with a chimeric *Y* gene) is mated with its tester strain, clamp connections will be observed only if the chimeric gene differs in Aα mating type from that of the recipient and the tester. Each chimeric gene was transformed into at least two strains, each carrying one of the two parental *Y* alleles, *Y3* and *Y4*. Test matings were incubated for 3 days at 30° before microscopic examination for clamp connections. Transformants with wild-type *Y* genes were included as positive and negative controls in each set of experiments. The results of test matings identify the allelic specificity of the transformed chimeric *Y* gene.

cDNA synthesis, plasmid construction and two-hybrid assay: To synthesize the full-length *Y4* cDNA, polyA+RNA was isolated from Aα4 homokaryotic strain UVM 4–40 as previously described (YANG *et al.* 1995). The polyA+RNA was used as template for first-strand cDNA synthesis in the presence of random hexamers (First-Strand cDNA Synthesis Kit, Pharmacia) and the products of the first strand synthesis were PCR amplified with specific primers corresponding to the 5′ and 3′ ends of the *Y4* gene. The *Y4* cDNA (encoding all 928 amino acids) is active in mating tests when ligated to its natural promoter and transformed into Schizophyllum.

Plasmids pGBT9 and pGAD424, containing the Gal4 DNA-binding domain (Gbd) and Gal4 activation domain (Gad)

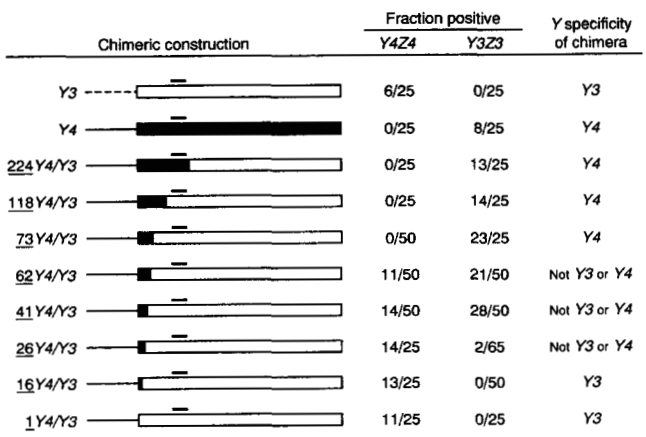

FIGURE 2.—*Y4/Y3* chimeric constructions and their *Y* mating-type specificities as determined from transformation and mating tests. Open box, *Y3* coding region; filled box, *Y4* coding region. – – –, *Y3* promoter region; ——, *Y4* promoter region. Bar above each box indicates position of the homeodomain. Underlined number specifies the most N-terminal amino acid of *Y3* in the derived fusion protein. For example, 224*Y4/Y3* has *Y4* sequence through codon 223, and *Y3* sequence from codon 224 to the C-terminal end of the fusion protein. Fraction positive, fraction of transformants forming clamp connections. *Y4Z4*, Aα4 recipient was strain T11, Aα4 tester was strain 4–24; *Y3Z3*, Aα3 recipient was strain T33, Aα3 tester was strain T22 (See Table 1 for genotypes).

respectively, were used as the expression vectors in yeast two-hybrid assays (Matchmaker Two-Hybrid System, Clontech). The full-length *Y4* cDNA and a set of 3′ truncations (see Table 4) were cloned in the pGAD424 vector to generate in-frame Gad-Y4 fusions. The deletion of *Y4* sequence encoding amino acids 10–80 was made in the following manner. Site-directed mutagenesis was used to produce a *Pst*I site at nt 240 in the full-length *Y4* cDNA. Digestion at the native *Pst*I site at nt 30 and the introduced *Pst*I site at nt 240 released a 210-nt *Pst*I fragment (encoding amino acids 10–80). Ligation of the remaining fragments of *Y4* DNA led to in-frame deletion of amino acids 10–80. The *Y4* deletion construct was subsequently cloned in pGAD424 to yield pGadY4(1–9, 81–928); numbers in parentheses specify amino acids encoded. Truncations of this construct at the 3′ end by restriction and ligation led to the production of pGadY4(1–9, 81–228) and pGadY4(1–9, 81–144). Plasmid construction pGbdZ5(1–101) was made by ligating the *Bam*HI-*Sal*I fragment of *Z5* into the respective sites of the vector pGBT9. GbdZ3(1–110) was made by PCR with primers specific to the region; the primers were designed to introduce terminal *Bam*HI and *Eco*RI sites to clone the fragment into pGBT9. Plasmid pGbdZ4(1–267) was made by ligating the *Eco*RI fragment that encodes the first 267 amino acids of Z4 into pGBT9. All the constructs were sequenced across the ligation junction using a cycle sequencing kit (Gibco BRL); no deviations from the expected sequences were found.

Each pairwise combination of *Y* and *Z* fusions in the yeast two-hybrid vectors was transformed into cells of yeast strain SFY526. The filter assays for β-galactosidase activities of selected transformants were performed according to manufacturer's directions (Clontech).

RESULTS

Identification of a specificity region within the *Y3* and *Y4* alleles: A total of eight chimeric genes containing

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    17          30          40          50          60          72
Y3  DLASFALSRGASPIPQPVGLTDVTFDPLPLPDLNALHRRLKDAGLPPKTTKSAIKA
    :. . : :::::.   :. :  :: :  :.:. .. ::..: :::::. :... :
Y4  DMMALARSRGATGSR-PTPTTLPHFDELLPPNLDFVRTRLQEARLPPKAIKGTLSA
```

FIGURE 3.—Comparison of Y3 and Y4 amino acid sequences determining specificity in tests with Aα3 and Aα4 cells. Alignment by MACAW plus visual inspection. Numbers refer to residues. –, gap giving best alignment between Y3 and Y4; :, identity; ., conservative change (FENG *et al.* 1985; SCHWARTZ and DAYHOFF 1978).

5′ *Y4* sequence and 3′ *Y3* sequence (*Y4/Y3*) were constructed. Each chimera was cotransformed with *TRP1* DNA into Aα3 and Aα4 recipient strains and the Trp^+ transformants tested for *Y* allelic specificity. The results are shown in Figure 2. Chimeras yielding Y4/Y3 proteins with the fusion occurring at amino acids 224, 118 or 73 possess the *Y4* mating-type specificity. The chimera fused at amino acid 16 has the mating-type specificity of the *Y3* allele. Therefore, in Aα3 and Aα4 interactions the critical region determining *Y3* and *Y4* specificity is between amino acids 17 and 72 (Figure 3).

Y4/Y3 chimeras yielding hybrid proteins with junctions at amino acids 62, 41 or 26 activate both Aα3 and Aα4 strains and thus have neither the *Y3* nor *Y4* allelic specificity. Two possible mechanisms may explain this phenotype. The fusions may create mutant proteins active with any Z protein, or they may create a self-active (*i.e.*, constitutive) Y protein that requires no Z protein to activate Aα-regulated development. To test these possibilities, each of these chimeras was transformed into an Aα1 strain that naturally lacks the *Z* gene. The three chimeras did not activate Aα-regulated events in the Aα1 recipient (data not included). Therefore, activation by these chimeras requires Z protein and the fusions are not constitutive for development, as such they may represent novel Aα mating-type specificities.

Chimera 1*Y4/Y3* containing the *Y4* promoter region and the entire *Y3* coding sequence was constructed to determine if Y protein may affect development by altering transcription from the Y promoter in an allele-dependent manner (Figure 2). Transformation and mating tests using Aα3 and Aα4 cells demonstrate that this chimera displays the specificity of the *Y3* allele. The chimera was also transformed into an Aα5 strain (T24), then mated with an appropriate Aα5 tester strain (T34). Aα-regulated development was activated in an entirely normal fashion (eight of 25 transformants tested developed clamp connections) in the absence of Y4 protein; therefore, there are no promoter-specific effects associated with *Y* specificity. A construction (not shown) that contains the *Y3* promoter and the *Y4(8)* mutant structural gene (see Table 3) also confirms that allele specific promoter effects are not involved in determining specificity or controlling Aα-regulated development.

Further definition of the region encoding specificity: The experiments with *Y4/Y3* chimeras above revealed the specificity of the *Y4* gene product to be encoded

TABLE 2

***Y1/Y3* and *Y5/Y3* chimeric constructions and their *Y* mating-type specificities as determined from transformation and mating tests**

Chimeric construction[a]	Fraction positive			*Y* specificity of chimera
	Y1[b]	*Y3Z3*[b]	*Y5Z5*[b]	
73*Y1/Y3*	0/40	0/40	17/25	*Y3*
73*Y5/Y3*	0/25	18/40	8/25	Not *Y5* or *Y3*

[a] Sequence of *Y1* or *Y5* to codon 72, *Y3* sequence thereafter.
[b] *Y1*, Aα1 recipient T4a, tester T5; *Y3Z3*, Aα3 recipient T33, tester T22; *Y5Z5*, Aα5 recipient T24, tester T34 (see Table 1).

N-terminal of amino acid 73 and that of the *Y3* gene product to be encoded C-terminal of amino acid 16. Chimeras with junctions between amino acid 17 and 72 were neither *Y3* nor *Y4* in specificity (Figure 2). Thus the determinants of *Y4* compared with *Y3* specificity reside between amino acids 17 and 72. We decided to examine if the same region determines specificity in other Y proteins. To accomplish this, we constructed *Y1/Y3* and *Y5/Y3* chimeras each fused at amino acid 73. The results of the specificity assays are shown in Table 2. Aα1 cells have no *Z* gene; therefore, the *Y1/Y3* and *Y5/Y3* chimeras do not activate Aα-regulated development in Aα1 cells. The *Y1/Y3* chimera activates development in Aα5 cells, but not in Aα3 cells; this shows that the *Y1/Y3* chimera encodes a critical element of *Y3* specificity C-terminal of amino acid 73. In contrast, the *Y5/Y3* chimera activates both Aα3 and Aα5 strains and thus reveals *Y3* specificity C-terminal of amino acid 73 and *Y5* specificity N-terminal of amino acid 73. This chimera also may represent a novel Aα specificity. These experiments demonstrate that the region of Y protein defining specificity is dependent upon the particular Z protein with whom complex formation occurs.

Examination of mutant alleles: To make the eight *Y4/Y3* chimeras containing wild-type *Y4* and *Y3* sequences (Figure 2), eight *Y3* and eight *Y4* mutant alleles were created (see MATERIALS AND METHODS). The 16 mutant *Y3* and *Y4* alleles were each tested for their Aα specificities. The predicted amino acid substitutions, the results of transformation and mating tests, as well as the *Y* specificities, are shown in Table 3. Each mutant *Y* allele has the same *Y* specificity as its wild-type progenitor. None of the amino acid substitutions alter the *Y* mating-type specificities or functions of the mutant alleles. Thus the *Y3* and *Y4* mutant alleles either do not have their mutations in the specificity region or have a change that is not critical to specificity. These mutants prove that not every one of the 56 amino acids in this region is critical to specificity.

One other mutant was contrived for inclusion in our study. This construct, Y4(1–9, 81–928), deletes amino acids 10–80 of the Y4 protein. Our results above suggest that this region contains amino acids essential to defining the specificity of Y4 protein in interaction with Z3 protein. When tested in transformations and mating assays with Aα3 cells, this construct was unable to activate Aα-regulated development. This strongly suggests the essential nature of the putative specificity region for mating activity.

Two-hybrid analyses: a role for the specificity region in Y-Z protein interactions: Previous two-hybrid studies showed that *nonself* pairs of Y and Z proteins (*e.g.*, Y4 and Z5) interact in yeast cells, while *self* pairs (*e.g.*, Y4 and Z4) do not (MAGAE *et al.* 1995). We used the yeast two-hybrid system to determine whether the specificity region of the Y proteins is essential for interactions with Z proteins. Various *Y4* cDNAs encoding C-terminal deletions of Y4 protein were fused in-frame to the Gal4 activation domain (Gad). Truncations of various *Z* cDNAs were ligated in-frame to the Gal4 DNA-binding domain (Gbd) to encode Gbd fusion proteins [Z3(1–110), Z4(1–267) and Z5(1–101)]. The truncated Z proteins were used because we find they give stronger signals than full-length Z proteins in the two-hybrid analyses. The results of the two-hybrid assays are shown in Table 4. Each fusion protein construction is inactive in combination with yeast plasmid encoding the alternative domain (*i.e.*, Gad or Gbd), but lacking a fusion to Schizophyllum protein. The constructions, either full length or truncated, retain their mating-type specificity in the two-hybrid assay, *i.e.*, Y4 proteins bind Z3 or Z5 proteins, but not Z4 proteins. Full-length or truncated Y4 proteins with the Y3 *vs.* Y4 specificity region deleted (amino acids 10–80) lose the ability to interact in the two-hybrid assays. This indicates that the region containing the Y specificity determinant also provides a function essential to Y-Z protein interactions. Further study shows that Y4(1–144) supports two-hybrid interaction, but Y4(1–80) does not. Therefore elements extending C-terminal of the Y3 *vs.* Y4 specificity region are also essential to Y-Z protein interactions.

DISCUSSION

Identification of a specificity region within the *Y3* and *Y4* genes: Using *Y4/Y3* chimeras fused at various sites, we have defined the region of critical amino acids (residues 17–72) encoding allelic specificity of *Y3* compared with *Y4* (Figures 2 and 3). When the junction of the hybrid protein is outside this region, the chimera displays the specificity of the *Y* allele encoding amino acids 17–72. The chimeras joined within this region activate both parental strains, *i.e.*, the chimeras encode the mating-type specificity of neither progenitor. These chimeras do not activate Aα-regulated development in Aα1 cells (which naturally lack a *Z* gene); therefore, they are not constitutive creations, and they continue to require a *Z* gene to activate the Aα-regulated develop-

TABLE 3

Mutant *Y3* and *Y4* alleles and their *Y* mating-type specificities as determined from transformations and mating tests

Mutant	Predicted amino acid substitutions	Fraction positive		*Y* specificity of mutant
		Y4Z4	*Y3Z3*	
Y3(1)	I215M, P216V, M217S	5/25	0/25	*Y3*
Y3(2)	Q116V, K117S	8/25	0/25	*Y3*
Y3(3)	I70M, K71V, A72S	9/25	0/25	*Y3*
Y3(4)	No substitution	5/25	0/25	*Y3*
Y3(5)	V39G, T40L	8/25	0/25	*Y3*
Y3(6)	L23R	9/25	0/25	*Y3*
Y3(7)	T13G, S14L	9/25	0/25	*Y3*
Y3(8)	No substitution[a]	13/25	0/25	*Y3*
Y4(1)	F225R, E226P	0/25	6/25	*Y4*
Y4(2)	Y119R, E120P	0/25	11/25	*Y4*
Y4(3)	S74R, A75P	0/25	16/25	*Y4*
Y4(4)	K63E, A64T	0/25	20/25	*Y4*
Y4(5)	L43T	0/25	10/25	*Y4*
Y4(6)	A27G, T28D, G29R	0/25	22/25	*Y4*
Y4(7)	D17E, M18T	0/25	14/25	*Y4*
Y4(8)	E3R, L4P	0/25	21/25	*Y4*

[a] Nucleotides mutated in promoter region. Recipients and testers as in Figure 2.

ment. These chimeras contain determinants of specificity from both *Y* alleles and consequently activate both Aα3 and Aα4 strains. In this respect, they represent a new mating-type specificity. Similar findings were reported by YEE and KRONSTAD (1993) for *U. maydis* where the b locus consists of two multiallelic genes, *bE* and *bW*. Chimeras made between *bE1* and *bE2* identified an N-terminal region 49 amino acids in length (amino acids 39–87) that contains the determinants of *bE1* specificity compared to *bE2* specificity. As in *S. commune*, chimeras fused within this region had a specificity different from both parental alleles. DAHL *et al.* (1991) used convenient restriction sites to make chimeric constructions and determined the distinction in specificity of bE2 protein compared with bE3 protein to be encoded between residues 56–115. Similarly, KÜES *et al.* (1994) made two chimeric constructions from alleles of the A loci of *C. cinereus*; in both cases sequence N-terminal of the homeodomain (>160 amino acids) determines specificity. A study of the allelic specificity of the *Z* genes of *S. commune* reveals that a 42 amino acid, N-terminal region between residues 18 and 60 includes the specificity determinants of *Z4* compared with *Z5* (WU *et al.* 1996). The results gathered from eight pairs of mating-type genes encoded in four loci from three basidiomycetes are strikingly similar. They suggest that a relatively small region in the N-terminal end of these mating-type proteins determines specificity. The mechanism of protein-protein recognition encoded by each may be similar.

TABLE 4

Two-hybrid assays of Y and Z protein interactions

	Gbd	GbdZ5(1–101)	GbdZ3(1–110)	GbdZ4(1–267)
Gad	–	–	–	–
GadY5FL[a]	–	–	NT	++
GadY4(1–928)	–	+++	+	–
GadY4(1–438)	–	+	+	–
GadY4(1–228)	–	+++	++	–
GadY4(1–144)	–	+++	+++	–
GadY4(1–80)	–	–	–	–
GadY4(1–9, 81–928)	–	–	–	–
GadY4(1–9, 81–228)	–	–	–	–
GadY4(1–9, 81–144)	–	–	–	–

Gad, fusions to Gal4 activation domain; Gbd, fusions to Gal4 DNA-binding domain. NT, not tested. +++, blue color detected within 2 hr; ++, blue color detected between 2 and 8 hr; +, blue color detected between 8 and 16 hr; –, no blue color detected within 16 hr. Numbers in parentheses indicate amino acids present.

[a] Y5 full-length protein.

Further definition of the region encoding specificity: The region of critical importance in determining *Y* mating-type specificity depends upon the allelic pairs being tested. The experiments with chimeras *Y4/Y3*, *Y5/Y3* and *Y1/Y3* were designed to test this point (Table 2). *Y4/Y3* chimeras tested against Aα3 and Aα4 strains demonstrated that the region from amino acids 17–72 contains the critical determinants for discriminating between Z3 and Z4 proteins (Figure 2). However, the *Y5/Y3* and *Y1/Y3* chimeras (each fused at amino acid 73) showed different results (Table 2). Chimera 73*Y5/Y3* activates both Aα3 and Aα5 strains, as though it has a specificity different from either parent; this indicates that residues determining specificity are located on each side of amino acid 73. Similarly, chimera 73*Y1/Y3* showed that elements determining specificity of the *Y3* allele also extend C-terminal of amino acid 73. In other words, the critical elements defining specificity of Y3 protein compared with Y4 protein reside within amino acids 17–72, but those of Y3 compared with Y5 and Y3 compared to Y1 extend further C-terminal. Therefore, different regions of a given Y protein may discriminate between the Z proteins of different mating types. This is also true of the *U. maydis bE* alleles. DAHL *et al.* (1991) found the specificity region of *bE2* compared with *bE3* to be within the region of amino acids 56 to 115, whereas YEE and KRONSTAD (1993) found that the specificity region of *bE1* compared with *bE2* resided between amino acids 39–87.

Figure 3 compares the Y3 and Y4 amino acid sequences that determine specificity in tests with Aα3 and Aα4 cells. Y3 and Y4 have 40% identity and 65% similarity within this region; this is not significantly different than the overall identity of the Y3 and Y4 proteins. Specificity may be determined by a few critical amino acids or scattered subregions, while other amino acids may be less critical or not critical. Several of the mutant amino acids (Table 3) are within the Y3/Y4 specificity region. These mutations have no impact on specificity and prove that not every amino acid of the region is critical to specificity. The situation in *U. maydis* appears to be similar where KÄMPER *et al.* (1995) have shown that only some scattered residues in *bE2* impact specificity.

Two-hybrid studies of the Y-Z interaction domain: Our previous two-hybrid studies (MAGAE *et al.* 1995) demonstrated interactions between Y and Z proteins encoded from different Aαs (*e.g.*, Y5 protein and Z4 protein), but not between proteins encoded from the same Aα (*e.g.*, Y4 and Z4). The results reported in Table 4 extend our previous two-hybrid results by defining a region essential to *nonself* protein interactions. The N-terminal regions of Z3 and Z5 are sufficient to bind the N-terminal region of Y4 [*i.e.*, Gbd Z5(1–101) and GbdZ3(1–110) interact with GadY4(1–144)]. The negative results of Y4(1–80) suggest that the 80 N-terminal amino acids of Y4 protein are insufficient to bind Z5 or Z3 protein, but we are presently unable to discount the possibility that this result is due to instability of the truncated Y4 protein in yeast.

GbdZ5(1–101) reacts more rapidly with GadY4 (1–928) than does GbdZ3(1–110) (Table 4). GbdZ5(1–120) reacts at the rate of GbdZ3(1–110) (data not included). The altered rate of reactions may be related to the additional amino acids, rather than the difference in mating type.

The constructions which produce Y4 proteins containing a deletion of amino acids 10–80 demonstrate that the Y3 *vs.* Y4 specificity determinant is essential to Y-Z interaction. The concern that these deletion proteins may be unstable in yeast is assuaged by direct protein interaction assays that show that GST-Y4(1–9, 81–485) is unable to bind Z5(1–101) and Z5(1–120) whereas undeleted GST-Y4(1–485) does bind (Y. ASADA, pers. commun.).

A similar relationship of specificity to binding may pertain to *C. cinereus* A mating-type proteins. In *C. cinereus*, the A specificity determinants have been localized to a >160 amino acids N-terminal region (BANHAM *et al.* 1995). BANHAM *et al.* (1995) used protein interaction assays to show that the N-termini interact. The 163 and 96 amino acids of HD1 proteins (b1-1 and b1-3, respectively) interact with HD2 GST-fusion proteins (b2-1 and b2-3) containing 257 or 224 N-terminal amino acids, respectively. Thus in *C. cinereus* N-terminal polypeptides thought to contain the specificity determinants will also form complexes provided the proteins are derived from different mating types.

In *U. maydis*, the b mating-type genes are constituted of variable and constant regions (KRONSTAD and LEONG 1990). As would be anticipated, the specificity determinant that encodes the uniqueness of the protein is found in the variable region. KÄMPER *et al.* (1995) have shown by two-hybrid assays and protein interaction assays that the N-terminal variable regions of *U. maydis* bE and bW polypeptides contain the protein binding domains.

In *S. commune*, we have defined the Y3 *vs.* Y4 specificity region (amino acids 16–72) and then demonstrated that this region plays an important role in Y-Z protein binding. Similar studies with *S. commune* Z protein show that the regions required for specificity (amino acids 19–60) and protein interaction (amino acids 40–101) also overlap (WU *et al.* 1996). These studies extend our understanding of the binding activities and protein-complex formation for proteins derived from the multiallelic, homeobox, mating-type genes of basidiomycetes.

Structural analysis: HO *et al.* (1994) identified 3,4-hydrophobic heptad repeats within the N-termini of the yeast, *Saccharomyces cerevisiae*, ***a1*** and α2 mating-type proteins. They believe these motifs mediate dimerization by two leucine zipper-like coiled coils. Other studies suggest that the C-terminal 22 amino acids of α2

form an α-helical interaction with **a1** (STARK and JOHNSON 1994). Our two-hybrid results clearly show that the specificity regions of Y and Z proteins function both in Y-Z protein-protein recognition and complex formation. We applied the algorithm of LUPAS *et al.* (1991, as ported by L. HARVIE) to assay the deduced protein sequences of Y and Z for potential coiled-coil elements. The algorithm predicts essentially 100% probability for a pair of sequences (14–28 amino acids long) capable of forming coiled-coils in each Y and Z protein, but none are in the specificity regions and those in Z fall within regions not essential to activity (SPECHT *et al.* 1992; WU *et al.* 1996). The algorithm shows low probability for several short elements in the specificity regions of both the Y and Z proteins (multiple short elements have also been noted in *U. maydis* bE by KÄMPER *et al.* 1995). Although these short elements are unlikely to form coiled-coils in the accepted sense (O'SHEA *et al.* 1992), we can not exclude the possibility that they may participate in complex formation between Y and Z proteins.

There is reasonable likelihood that a common theme exists as the basis for *nonself* protein recognition and binding in the multi-isoform, homeodomain, mating-type proteins in different species of basidiomycetes. Nevertheless, the structural basis of that theme has not yet been made lucid. In this paper, we make a case for two functions of the N-terminal region of the Y proteins of *S. commune*. These functions are essential to the activation of Aα-regulated development. In one role, the region confers uniqueness or specificity to the proteins for the purpose of recognition. A second function in which this region has been implicated is the binding of Y protein to Z protein. It is not yet clear if the Y protein specificity domain itself seizes the Z protein, or if touching by the specificity domain propagates a conformational shift to an additional domain actually responsible for complex formation. It is conceivable that the latter possibility is reflected by the lack of activity in two-hybrid assays of the Y4(1–80) construct, which contains little more than the specificity determinant.

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