Multiple Genes Encoding Pheromones and a Pheromone Receptor Define the Bβ1 Mating-Type Specificity in *Schizophyllum commune*

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ABSTRACT

The genes defining multiple B mating types in the wood-rotting mushroom *Schizophyllum commune* are predicted to encode multiple pheromones and pheromone receptors. These genes are clustered in each of two recombinable and independently functioning loci, Bα and Bβ. A difference in specificity at either locus between a mated pair of individuals initiates an identical series of events in sexual morphogenesis. The Bα1 locus was recently found to contain genes predicted to encode three lipopeptide pheromones and a pheromone receptor with a seven-transmembrane domain. These gene products interact in heterospecific pairs, the pheromone of one Bα specificity with the receptor of any one of the other eight Bα specificities, and are likely to activate a signaling cascade similar to that known for mating in *Saccharomyces cerevisiae*. We report here that the Bβ1 locus also contains at least three pheromone genes and one pheromone receptor gene, which function similarly to the genes in the Bα1 locus, but only within the series of Bβ specificities. A comparison of the DNA sequences of the Bα1 and Bβ1 loci suggests that each arose from a common ancestral sequence, allowing us to speculate about the evolution of this unique series of regulatory genes.

FRUITING and sporulation in the wood-rotting Homobasidiomycete *Schizophyllum commune* occur only after a mating of two sexually compatible individuals. Compatibility is determined by multiple, multiallelic genes that define an estimated 20,000 or more mating types in the worldwide population of *S. commune* (RAPER 1966). How this fungus distinguishes self from such a multitude of nonselves has been a subject of interest and research for many years. It is only recently, since the molecular characterization of mating-type genes in this and other fungi, that we have come to recognize a commonality in the kinds of genes that regulate compatibility in a variety of fungi, from those with only two mating-types to those like *S. commune* with multitudes (WENDLAND *et al.* 1995; PARDO *et al.* 1996; BAKKEREN and KRONSTAD 1996; also see BÖLKER and KAHMANN 1993; KOTHE 1996; VAILLANCOURT and RAPER 1996 for reviews).

The life cycle of *S. commune* involves an alternation of two phases, the unmated homokaryon and the mated dikaryon. Only the dikaryon is competent to fruit and sporulate. Spores of *S. commune* are produced by specialized cells called basidia that line the gills of the fruiting bodies (mushrooms). The spores germinate and develop into multicellular, filamentous homokaryons. Each cell in the homokaryotic mycelium contains a single haploid nucleus. All the nuclei in a homokaryon are genetically identical. Two homokaryons will undergo plasmogamy freely, but sexual development is initiated only if they are of compatible mating types. The first step in sexual morphogenesis is reciprocal migration of nuclei from one mate into and throughout the other. Septa between cells are rapidly broken down to allow passage of fertilizing nuclei. Nuclear migration continues until the fertilizing nuclei reach growing tip cells to initiate a dikaryon. Propagation of the dikaryotic tip cells proceeds through a highly organized process of hook-cell formation, conjugate nuclear division, and septal formation, resulting in the establishment and proliferation of a dikaryotic mycelium. Each cell of the dikaryon contains two haploid nuclei, one derived from each of the parent strains. The dikaryon can grow indefinitely, and when environmental conditions are right it will produce fruiting bodies. Karyogamy, immediately followed by meiosis, occurs in the basidial cells of the fruiting body. The four postmeoitic nuclei are separately included in four haploid spores, each of which gives rise to a homokaryotic mycelium and begins the life cycle again.

Mating type in *S. commune* is determined jointly by two unlinked genetic complexes named A and B, each consisting of two linked loci called α and β. These loci each contain two or more master regulatory genes that control a cascade of events in sexual morphogenesis. The two complexes regulate separate but complemen-

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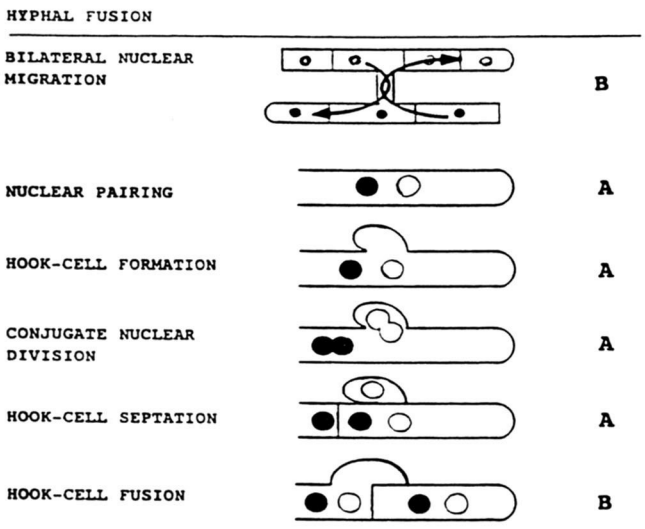

FIGURE 1.—Regulation of different steps in dikaryon formation and maintenance by the A and B mating-type loci of *S. commune*. Initial hyphal fusion is not regulated by either A or B. Reciprocal migration of fertilizing nuclei is induced by a difference at either of the two B loci. Pairing of nuclei in the terminal cells, hook-cell formation, conjugate nuclear division, and hook-cell formation are all controlled by a difference at either of the two A loci. Hook-cell fusion to form the mature clamp connection is regulated by a difference at the B loci.

tary aspects of mating and dikaryon formation (Figure 1; also see RAPER 1966; RAPER 1983 for reviews). There are estimated to be nine different specificities of Aα, 32 of Aβ, and nine each of Bα and Bβ in the worldwide population. The minimal requirement for a fully compatible mating is a difference at either of the two A loci and at either of the two B loci. Thus α and β of each complex are functionally redundant. The two loci also appear to operate independently of one another in that recombination between α and β of either complex never results in an alteration of activity or specificity of the relevant loci.

Different developmental fates result from hemi-compatible matings between two homokaryons differing only at the A locus (A-on/B-off) or only at the B locus (A-off/B-on). Hemi-compatible matings yield heterokaryons that are infertile and that have distinct morphologies. The A-off/B-on heterokaryon displays a characteristic phenotype called "flat," in which the mycelium grows submerged in the substrate. Microscopically, the individual hyphae appear irregular and highly branched when compared with normal homokaryotic hyphae (Figure 2). Activation of the B mating-type pathway in the absence of the A pathway allows continuous septal breakdown and cell to cell nuclear migration.

Representative specificities of all four of the mating-type loci of *S. commune* have now been cloned. The two A loci each contain sets of genes encoding homeodomain proteins that interact in heterologous pairs. The paired proteins are thought to transcriptionally regulate A-specific genes that in turn activate the A mating-type pathway (NOVOTNY *et al.* 1991; MAGAE *et al.* 1995; SHEN *et al.* 1996). The Bα1 locus contains pheromone and pheromone receptor genes similar to those found in other fungi (WENDLAND *et al.* 1995). We have now characterized the Bβ1 locus and in this paper we will show that its organization is similar to that of the Bα1

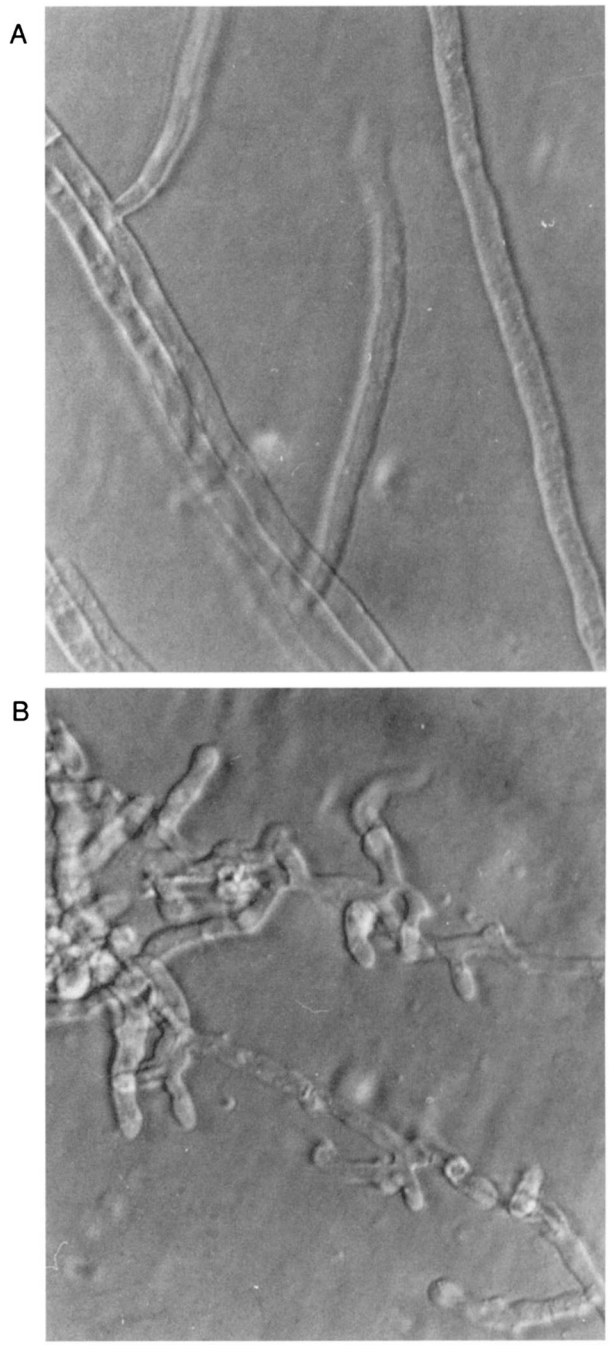

FIGURE 2.—Photomicrographs depicting normal homokaryotic mycelia (A) and "flat" mycelia (B). The flat phenotype is elicited when the B mating-type pathway is activated in the absence of the A pathway. In flat mycelia, the hyphae are irregular with disrupted septa. They grow submerged in the substrate and frequently branch at right angles to the main hyphae.

TABLE 1

Strains of *Schizophyllum commune*

Strain	A mat	B mat	Comments, other markers
4-40	α4-β6	α1-β1	Source of Bβ1 clone
4-39	α1-β1	α3-β2	Used for RNA
4-8	α4-β6	α3-β2	Used for RNA
T12[a]	α4-β6	α2-β1	*ura1, trp1* (T, M)[c]
HMS-8[a]	α5-β7	α2-β1	*ura1, trp1* (M)
T26[a]	α7-β1	α2-β2	*ura1, trp1* (T, M)
12-43	α3-β5	α2-β2	*ura1* (M)
V113-9	α3-β20	α3-β3	*trp1* (T, M)
V113-14	α5-β7	α3-β3	*trp1* (M)
V142-3	α4-β7	α8-β4	*trp1* (T)
V114-17	α5-β7	α3-β5	*trp1* (T)
T11[a]	α4-β1	α1-β6	*ura1, trp1* (T, M)
HMS-6[a]	α5-β7	α1-β6	*ura1, trp1* (M)
V118-4	α2-β2	α9-β6	*trp1* (T, M)
V118-7	α5-β7	α9-β6	*trp1* (M)
V112-3	α5-β7	α5-β7	*trp1* (T, M)
V112-17	A3[b]	α5-β7	*trp1* (M)
V115-4	A15[b]	α1-β8	*trp1* (T)
V119-19	α5-β7	α7-β9	*trp1* (T)

[a] Obtained from Dr. CHARLES NOVOTNY, University of Vermont.

[b] α and β comprising this specificity are unknown.

[c] T, used as a transformation recipient; M, used in matings.

locus. We will provide evidence that the Bβ1 locus contains a set of Bβ-specific pheromone and pheromone receptor genes, the products of which interact in hetero-specific pairs to activate the B-regulated pathway of sexual morphogenesis.

MATERIALS AND METHODS

Fungal and bacterial strains: Strains of *S. commune* used in this study and their relevant genotypes are given in Table 1. All strains were derived from the original collection of J. R. RAPER and associates at Harvard University (RAPER *et al.* 1958; PARAG and KOLTIN 1971). The fungal strains were cultured as described previously (RAPER and HOFFMAN 1974). *Escherichia coli* strains DH5α and XL1-Blue MRA were used for routine maintenance of plasmid and cosmid clones.

Plasmids: The *S. commune trp1* gene (MUNOZ-RIVAS *et al.* 1986) was used as a selectable marker in transformation and cotransformation experiments with *S. commune.* Plasmids pUC18 and pBluescript SK+ were used for routine subcloning and as transformation shuttle vectors. PCR products were amplified with *taq* polymerase (Perkin-Elmer), gel-purified, and cloned into the pT7-Blue T-vector (Novagen).

Screening of a cosmid library: The Bα1-β1 cosmid library used in this study was constructed previously (GIASSON *et al.* 1989). We ordered the library in 96-well microtiter plates. A total of 4800 clones were screened, which should represent the complete genome of strain 4-40 with a probability of ~95%.

Protoplast preparation and transformation of *S. commune*: Preparation of protoplasts from homokaryotic mycelia of *S. commune* and PEG-mediated transformation were performed as described by MUNOZ-RIVAS *et al.* (1986) and SPECHT *et al.* (1991), as modified by HORTON and RAPER (1991), except that Mureinase (USB) was used in place of Novozyme for protoplast preparation.

Assay of transformants; test matings: Transformants selected for tryptophan prototrophy were screened for evidence of cotransformation with B mating-type genes. Introduction of a B mating-type gene of a different specificity into a homokaryotic strain resulted in activation of the B-regulated pathway. By the method used, transforming DNA was integrated randomly into the genome of *S. commune.* Activation of the B pathway in the transformant could be readily detected by development of a typical "flat" phenotype after 3–5 days of growth on regeneration medium (see Figure 2). If the flat transformant was mated to a tester strain that had a different A mating type but the same B mating type as the transformation recipient, a normal dikaryon was the eventual result. If the transformant was able to donate migrant fertilizing nuclei, the outer colony margins of the test-mate were rapidly converted to dikaryotic hyphae. If the transformant was not able to donate migrant nuclei, the test-mate remained homokaryotic, and a dikaryon eventually resulted only after proliferation of fused cells at the intersection of the two colonies. All transformation experiments involved examination of samples ranging from ~30 to 200 trp^+ transformants, and the transformation recipients in each case were derived from one or two different strains of each Bβ specificity. Cotransformation by B mating-type genes ranged from 12 to 50% and averaged ~35%.

Nucleotide sequencing and analysis: Sequencing of plasmid and PCR-amplified DNAs was done with the dsDNA Cycle Sequencing System kit (Life Technologies, Gaithersburg). Sequence data were analyzed and sequence alignments were made using the MacVector software package (International Biotechnologies, Inc. New Haven, CT). Sequence similarity and motif searches were performed using the BLAST and BLOX alignment tools at the National Center for Biotechnology Information (NCBI).

Amplification and sequencing of pheromone-receptor cDNA: Total RNA was purified from mycelium of the A-off/B-on heterokaryon with an RNA Isolation Kit (Stratagene). The mycelium was prepared by grinding a flat colony (the result of a hemi-compatible mating between strains 4-8 and 4-40) in a Waring blender, and then spreading the mycelial fragments onto dialysis membranes laid on nutrient agar plates. The mycelium was harvested from the membranes after 48 hr of growth at 30°, and immediately frozen at −80°. Messenger RNA was prepared from total RNA with a mRNA separator kit (Clontech). The mRNA was reverse-transcribed, and fragments of the cDNA of the pheromone receptor gene *bbr1* were amplified, using specific primers and the Superscript Preamplification System (Life Technologies). The cDNA products were gel-purified and sequenced directly.

Northern and Southern blotting: Genomic DNA was isolated from homokaryons of *S. commune* as described previously (SPECHT *et al.* 1982). Southern blotting was performed using standard methods (SAMBROOK *et al.* 1989).

Total RNA for Northern blotting was extracted from mycelia grown as follows: the compatible homokaryons 4-40 and 4-39 were cultured separately for 24 hr on semipermeable cellophane membranes placed on complete nutrient medium. One membrane was then "sandwiched," with its adherent mycelium, upside-down on top of another membrane containing the intended mate on complete nutrient medium. The mycelia were harvested after 6, 12, 24, and 44 hr of growth at 30°, frozen immediately in liquid nitrogen, and then stored at −80° until the RNA extraction. RNA extraction and Northern blotting were performed as described (RUSSO *et al.* 1992). All hybridizations were done at high stringency.

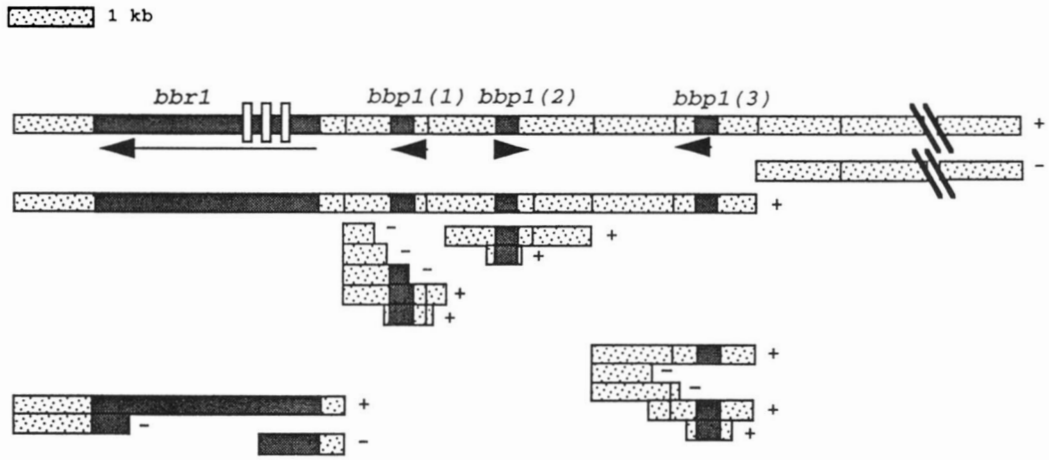

FIGURE 3.—Summary of Bβ1 subcloning experiments. Patterned bars represent DNA subclones. Black bars within the patterned bars represent ORFs predicted to encode one pheromone receptor, Bbr1 (at left), and three pheromones designated Bbp1(1), Bbp1(2), and Bbp1(3), respectively (at right). These were identified by sequencing and functional testing in DNA-mediated transformation experiments. A + to the right of the clone signifies activation of the B mating-type pathway in one or more nonself transformation recipients; a − signifies that the clone did not activate the B mating-type pathway in any of the nonself recipients tested. Introns are indicated within the *bbr1* ORF by vertical white bars. Direction of transcription is indicated by the arrowheads.

RESULTS

A 3-kb genomic fragment conferring Bβ1 mating-type activity was initially cloned by functional complementation and plasmid rescue (SPECHT 1996). This fragment was used to probe a cosmid library of strain 4-40, the same strain used to make the original plasmid library. A cosmid with a 25-kb genomic insert that hybridized to the 3-kb probe was isolated and subjected to further analysis.

The Bβ1 locus is complex, consisting of at least four active elements embedded within an 8.5-kb region of unique DNA: The cosmid was tested for B mating-type activity in transformation assays. It induced B-regulated development in strains representing all Bβ mating types, with the exception of Bβ1.

Restriction fragments from the 25-kb cosmid insert were subcloned, and each subclone was tested for activity in two or more different Bβ transformation recipients (Figure 3). Approximately 8.5 kb at one end of the cosmid was found to contain four separate regions, each of which was capable of independently activating B-regulated development. A single subclone that contained the entire contiguous 8.5-kb region induced B-regulated development in all Bβ mating types, with the exception of Bβ1.

The active subclones of the 25-kb cosmid insert occupied a region of at least 8.5 kb, present in different Bβ1 strains but not in strains of the Bβ2 specificity, as determined by Southern blots of genomic DNA (data not shown). This mating-type specific region was flanked, at least on one end, by DNA of high sequence similarity to that bordering the Bβ2 locus; these flanking regions cross-hybridized in Southern blot experiments performed at high stringency (data not shown). Sequence dissimilarity flanked by sequence similarity is typical of other fungal mating-type loci (reviewed in BÖLKER and KAHMANN 1993; VAILLANCOURT and RAPER 1996).

The Bβ1 locus contains one pheromone receptor gene and three pheromone genes: Nucleotide sequence data were generated and analyzed for the entire cloned, active region of the Bβ1 locus (GenBank accession number U74495). There were four open reading frames (ORFs) of particular interest, corresponding to each of the four fragments conferring Bβ1 activity (Figure 3). The predicted amino acid sequence encoded by the largest of these ORFs is similar to known fungal pheromone-receptor genes (Figure 4). We named this gene *bbr1*.

The other three ORFs are shorter, and each of the proteins they are predicted to encode terminates in a CaaX motif (C-aliphatic-aliphatic-X), a characteristic of known fungal pheromone-precursor genes. We named these genes *bbp1(1)*, *bbp1(2)*, and *bbp1(3)* (Figure 5).

Suspected introns within the ORFs were identified by the presence of conserved sequences that signal cleavage sites in fungi (BALLANCE 1990). Three potential introns were detected in the pheromone receptor gene *bbr1*, and these were subsequently confirmed by sequencing several contiguous cDNA fragments spanning 1.9 kb of the region containing the putative introns. These introns, ranging in size from 52 to 55 bp, are shown in Figure 3.

No introns were detected by the presence of conserved cleavage sites in the putative pheromone genes. These genes were not analyzed for cDNA sequence. Minimal DNA fragments containing each of the three putative pheromone genes were amplified, cloned into

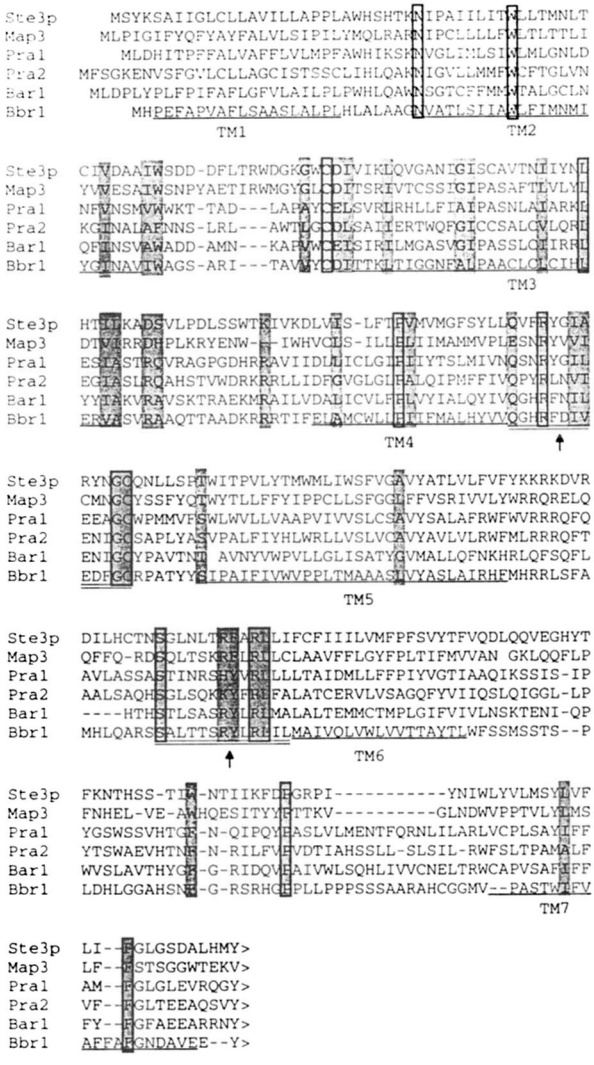

FIGURE 4.—Alignment of the predicted amino acid sequence of the Bbr1 receptor with other proteins in the Ste3p receptor family. Residues identical in all of the six sequences are boxed and shaded. Residues conserved in all six sequences are shaded. The seven predicted hydrophobic transmembrane regions are underlined and numbered. Two regions that are highly conserved among the six sequences are indicated by arrows and double underlines. The proteins are truncated because the C-termini are not similar and cannot be aligned. Ste3p is from *S. cerevisiae* (NAKAYAMA *et al.* 1985; HAGEN *et al.* 1986). Map3 is from *S. pombe* (TANAKA *et al.* 1993). Pra1 and Pra2 are from *U. maydis* (BÖLKER *et al.* 1992), and Bar1 is from the Bα1 locus of *S. commune* (WENDLAND *et al.* 1995).

a dT vector and sequenced to ensure that no mutations had been introduced. Each subclone contained between 250 and 350 bp of DNA upstream of the coding region, and not more than 300 bp downstream. These subclones were tested for activity by introducing them into Bβ2 and Bβ6 recipient strains *via* transformation. A 630-bp fragment containing *bbp1(1),* an 800-bp fragment containing *bbp1(2),* and a 560-bp fragment containing *bbp1(3)* each had full activity in these transformation assays.

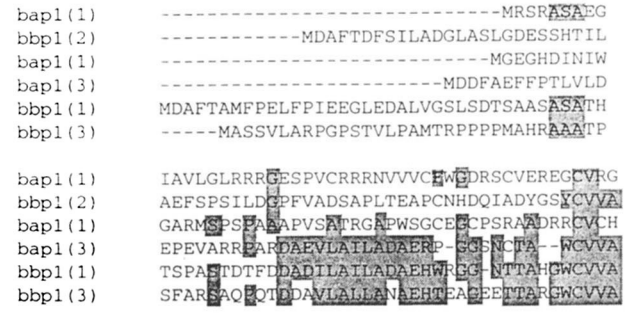

FIGURE 5.—Alignment of predicted amino acid sequences of the six pheromone precursors from *S. commune.* Amino acid residues that are similar or identical in at least three of the six are shaded.

Activities of the pheromone genes and pheromone-receptor genes differ quantitatively and qualitatively: Subclones containing each of the four Bβ1 genes were integrated by transformation into one or more different strains representing each of the nine Bβ mating types (Table 2). The number of transformants analyzed in each case ranged from 30 to 200. Each gene had a specific and unique range of activity. Some had higher levels of activity in certain specificities than others, as evidenced by a faster developing flat reaction that involved more of the colony and exhibited a more intense phenotype (see Figure 2). There was no clear evidence of a position effect on the expression of any of these clones as indicated by the consistency in phenotype of B mating-type transformants within each experiment. Interestingly, a single clone containing both pheromone genes *bbp1(1)* and *bbp1(2)* elicited a much stronger reaction in the Bβ2, Bβ4, Bβ5 and Bβ6 strains than did each of two smaller clones that were derived by subcloning the two halves of the larger clone and that contained either *bbp1(1)* or *bbp1(2)* alone.

Transformants of the Bβ2, Bβ5, and Bβ6 specificities containing the ectopically inserted Bβ1 receptor gene by itself and transformants containing each of the three

TABLE 2

Relative ability of clones containing Bβ1 genes to activate B-regulated development in strains of all other Bβ specificities

Bβ of recipient strain	*bbr1*	*bbp 1(1)*	*bbp 1(2)*	*bbp 1(3)*
β1	−	−	−	−
β2	++	++	+	+
β3	++	−	−	−
β4	−	+	+	++
β5	++	++	++	++
β6	++	++	++	++
β7	++	++	−	++
β8	++	−	−	−
β9	++	−	−	−

−, no visible activity; ++, moderately to highly active; +, slightly active.

TABLE 3

Ratios of B-on transformants resulting from cotransformation of two different recipients with *trp1* and complete or truncated Bβ1 pheromone genes

	Transformation recipients[a]	
Cloned gene	T26 (β2)	T11 (β6)
bbp1(1)	55/157 (35)	14/43 (32)
*bbp1(1)*Δ	0/174	1/55 (2)
bbp1(3)	11/91 (12)	9/46 (20)
*bbp1(3)*Δ	0/154	0/16

[a] Values are number of B-on cotransformants/total number of trp+ transformants examined, with percentage in parentheses.

Bβ1 pheromone genes were tested for their ability to donate fertilizing nuclei to test mates. In each case the tester strain was of the same B specificity as the original transformation recipient before transformation. Even though all the transformants had been activated and exhibited the flat phenotype, those containing the introduced nonself receptor gene were not able to donate migrating nuclei to test mates, while transformants containing any of the three pheromone genes could donate migrating nuclei to the test mates.

The CaaX motif is important for pheromone gene function: Truncated pheromone genes were constructed by using the PCR to incorporate a downstream primer that included a stop codon in place of the cysteine codon four nucleotides from the end of the coding sequence. The upstream primers were those used previously for amplification of minimal active subclones for each of the pheromones. The truncated products were cloned into a dT vector, and the clones were sequenced to ensure that no other mutations had been introduced.

Truncated *bbp1(1)* and *bbp1(3)* pheromone constructs were tested in Bβ2 and Bβ6 transformation recipients using the method of cotransformation in which the *trp1* gene of *S. commune* was incorporated in a separate clone as a selectable marker. In combined results of the two experiments, 89 of the 337 tryptophan-competent cotransformants expressed the B-on (flat) phenotype when cotransformed with the wild-type, full-length versions of either gene, while only one of 399 transformants expressed the B-on phenotype when cotransformed with the truncated constructs of these genes (Table 3). The one exception in the latter case could be an artifact, possibly due to position effect or contamination.

Pheromone genes appear to be upregulated between 12 and 24 hr after mating is initiated, during the period of nuclear migration: Minimal, active subclones of the pheromone genes were used as hybridization probes against total RNA isolated from "self/nonself" and "self/self" confrontations at various times after the tester strains were brought into contact. All three of the pheromone transcripts appeared to have increased in abundance during heterokaryon formation in the mating of the two compatible strains (designated 1-2 in Figure 6). These strains differed in both A specificity and B specificity. Strain 1 contained Bα1-β1 and strain 2 contained Bα3-β2. The transcripts appeared to have increased between the 6- and 12-hr time points in these self/nonself confrontations. Transcripts of two of the pheromone genes, *bbp1(1)* and *bbp1(2),* continued to be abundant at 24 hr, while the transcript of *bbp1(3)* had begun to diminish by this time point. The B-regulated process of nuclear migration coincided with the 12- to 24-hr time period after a compatible mating under the test conditions used. All three genes appeared to have been downregulated in the compatible confrontation at 44 hr, the time at which nuclear migration was nearing an end and the A-regulated dikaryon was beginning to form. The *bbp1(2)* probe hybridized to two differently sized transcripts in these Northern blot analyses. This result was repeatable and we cannot explain it at the present time. It could be due to the presence of all or part of another gene within the clone, although sequence analysis of both DNA strands of the clone and flanking regions produced no evidence of this. On the

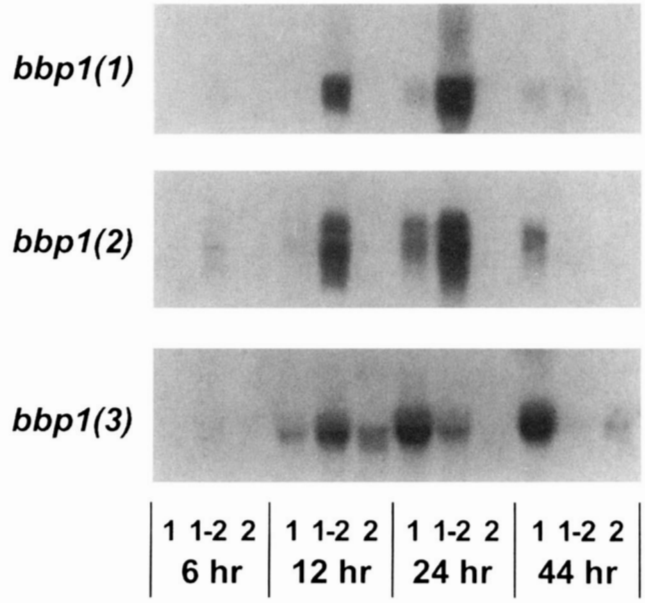

FIGURE 6.—Northern blot illustrating relative levels of transcripts of the three pheromone genes, *bbp1(1), bbp1(2),* and *bbp1(3),* over time in self/self *vs.* self/nonself confrontations. Lanes are labeled as follows: 1, self/self confrontation of the homokaryon 4-40 (Aα4-β6, Bα1-β1 × Aα4-β6, Bα1-β1); 1-2, self/nonself confrontation of the two homokaryons 4-40 × 4-39 (Aα4-β6, Bα1-β1 × Aα1-β1, Bα3-β2); and 2, self/self confrontation of the homokaryon 4-39 (Aα1-β1, Bα3-β2 × Aα1-β1, Bα3-β2). Total RNA was collected at 6, 12, 24, and 44 hr after confrontations. RNA loading varied no more than a factor of two as judged by ethidium bromide staining and by probing the blots with the constitutively expressed *S. commune* gene encoding glyceraldehyde-3-phosphate dehydrogenase (not shown).

other hand, the two transcripts might be explained by alternate start sites for the *bbp1(2)* message. Other fungal genes have been shown to have alternate start sites for transcription (MILLER *et al.* 1992; PRADA and TIMBERLAKE 1993). Even if one of these two transcripts was derived from a different gene, both transcripts appeared to be coregulated with respect to the time period monitored. We conclude that the *bbp1(2)* gene, as well as the *bbp1(1)* and *bbp1(3)* genes, are all upregulated during the period of nuclear migration.

For an unknown reason, pheromone transcripts also accumulated over time in the Bα1-β1, self/self confrontations (designated 1 in Figure 6). The signal was especially strong for the *bbp1(3)* gene. In contrast to the transcript pattern in the self/nonself confrontations however, transcript levels in these self/self confrontations did not decrease but remained elevated between the 24- to 44-hr time points. It is possible that under our test conditions, B gene transcripts start at a low constitutive level and then accumulate to high levels due to absence of downregulation by an activated B pathway, an activated A pathway, or a combination of the two. The *bbp1(3)* gene also appeared to hybridize to total RNA from the Bα3-β2 self/self confrontations (designated 2 in Figure 6) at the 12-hr point. This was unexpected, although it could be explained by the presence of a transcript of a similar pheromone gene in either the Bβ2 or the Bα3 locus of homokaryotic strain number 2.

Surprisingly, levels of the pheromone receptor *bbr1* transcript were extremely low, even in Bβ1 tissue where the B mating-type pathway was activated: transcripts could not be detected in any case using a *bbr1* cDNA probe against total RNA extracted from B-on cells. Furthermore, cDNA could not be amplified from total RNA using *bbr1*-specific primers, but could be amplified from a preparation that had been enriched for mRNA, suggesting that the transcript was present, but only in very low amounts. Because we could not detect the transcript on our total RNA blots, we were unable to determine if the *bbr1* gene is also upregulated in B-on tissue.

The sequences of the Bα1 and Bβ1 loci are related, perhaps suggesting a common origin: It is possible to align the nucleotide sequences of the left and right halves of the 8.5-kb Bβ1 locus (Genbank accession number U74495) in an inverse orientation using the Pustell DNA matrix analysis function of the MacVector sequence analysis software package. It is also possible to align the left or the right halves of the Bβ1 locus to the left or the right halves of the Bα1 locus, and to align the left and right halves of the Bα1 locus to one another in a tandem orientation. The sequence of each half is ~50% identical overall to the other three. Attempts to similarly align other published DNA sequences from *S. commune,* or several nucleotide sequences selected at random from the Genbank database, failed. Our interpretation of this is that the Bβ1 locus is comprised of two imperfect inverse duplications, and the Bα1 locus contains the same sequence imperfectly duplicated twice in a tandem orientation. This suggests to us that the Bα1 and Bβ1 loci arose from a common ancestral sequence by a series of duplications and translocations.

DISCUSSION

Until the discovery of putative pheromone and pheromone receptor genes in the Bα1 locus of *S. commune* (WENDLAND *et al.* 1995), it was not suspected that these proteins might regulate mating in the Homobasidiomycetes. This is because Homobasidiomycetes display no obvious evidence of extracellular signaling, as do the Hemiascomycete yeasts and certain Hemibasidiomycetes (see KURJAN and TAYLOR 1993; KOTHE 1996; VAILLANCOURT and RAPER 1996 for reviews). It was a surprise also because Homobasidiomycetes have so many mating types, far more than the two common to all fungi previously known to utilize pheromone-receptor recognition systems. In the previously known systems, heterospecific pheromone/receptor interactions are required for cell fusion. In the Homobasidiomycetes, cell fusion occurs regardless of mating type, and there is no clear evidence that the mating-type genes play a role before cell fusion. In addition to *S. commune,* it is now known that the B mating-type locus of *Coprinus cinereus,* a related Homobasidiomycete with multiple mating types, also contains multiple pheromone and pheromone-receptor genes (L. CASSELTON, personal communication). By analogy to known functions of the pheromone-receptor systems in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe,* it is likely that the pheromones and receptors encoded by the B mating-type genes in the Homobasidiomycetes interact in heterologous pairs to initiate a signal transduction cascade leading to sexual development.

In this study, we have found evidence that self/nonself recognition among the nine different Bβ mating-type specificities of *S. commune* is based upon positive interactions between pheromones encoded within one specificity of the Bβ locus and receptors encoded within another. A similar mechanism of activation by heterospecific pheromone/receptor pairing was suggested by results from comparable studies within the series of nine specificities of the Bα mating-type locus (WENDLAND *et al.* 1995). The numerous different pheromone and receptor genes within each series apparently regulate the identical pathway of sexual morphogenesis, yet there appears to be no interaction between the gene products of Bα and Bβ to activate the pathway. Possibly none of the Bα pheromones is capable of binding productively to any of the Bβ receptors, and *vice versa.* This may be due to differences in the two types of molecules within each series.

Consistent with this idea is the fact that the predicted protein product encoded by the Bβ1 receptor gene

bbr1 has relatively little primary sequence identity (only ~15%) with the predicted proteins encoded by the three Bα pheromone receptor genes that have been sequenced to date. In sharp contrast, the three putative Bα receptor proteins are remarkably similar to one another, sharing at least 90% identity (WENDLAND *et al.* 1995; L. VAILLANCOURT, unpublished results). Nevertheless, the predicted receptor proteins of both series, for example Bbr1 and Bar1, are similar in secondary structure to other receptor proteins with seven transmembrane segments, and each shares similarity in amino acid sequence at the N-terminus with the Ste3p family of fungal lipopeptide pheromone receptors, *i.e.*, Ste3p of *S. cerevisiae*, Map3 of *S. pombe* and Pra1 and Pra2 of *U. maydis*. As indicated by the arrows in Figure 4, two regions in particular are highly conserved among all members of this latter group. One is in the predicted third intracellular loop, an area thought to be important for interaction with the Gα subunit of a heterotrimeric G protein (see SAVARESE and FRASER 1992 for review). This suggests that the Bβ and Bα receptors of *S. commune* also interact with a heterotrimeric G protein. The other conserved area is in the second extracellular loop. This region is found only in the Ste3p lipopeptide-pheromone receptor family. It has not yet been associated with any important activity, but one interesting possibility is that it plays a role in a nonspecific interaction with the lipid portion of the pheromones. A study of the effects of site-directed mutagenesis in these conserved regions should help to determine whether or not they are functionally significant.

A comparison of the six putative pheromone-gene products of the α and β series did not reveal any obvious differences between the two groups. As shown in Figure 5, all six end in CaaX or CaaX-like motifs, and one of the three Bα pheromones is in fact quite similar to two of the Bβ pheromones at its C-terminus. In many proteins, a CaaX box motif serves as a signal to attach an isoprenyl modifying group to the C-terminus of the mature peptide. For fungal pheromones that have been studied, the isoprenyl modification is always a farnesyl group. Removal of the farnesyl group results in a decrease in activity (see DUNTZE *et al.* 1993 for review). In our experiments, deletion of the CaaX box motif from the gene products of *bbp1(1)* and *bbp1(3)* resulted in loss of activity (Table 3). We postulate that this was due to loss of a farnesylation signal, resulting in failure of the protein to be modified. Lack of modification may interfere with binding to the receptor and/or transport of the pheromone from cell to cell. Although replacement of the cysteine codon with a stop codon would not be expected to destabilize the protein significantly, we cannot discount the possibility that alteration of the gene in this way resulted in a nonspecific decrease in levels of the protein due to instability, or for some other reason. Other fungal pheromone precursors are generally posttranslationally processed at the N-terminus to yield an active form that is secreted from the cell. In our comparisons of the six *S. commune* pheromone genes sequenced to date, no obvious sequence similarities were found in any of the predicted protein products that might indicate a cleavage site for a processing enzyme. It is therefore difficult to predict where, and even if, the *S. commune* pheromones might be processed.

The three pheromone genes of Bβ1, like the three pheromone genes of Bα1, are unique in sequence, and each has a different spectrum of activity with respect to all other specificities of the relevant series. The Ascomycetes and some of the Hemibasidiomycetes also have more than one pheromone gene contained within the locus of a single specificity. These, however, are relatively similar, encoding identical or nearly identical pheromones, with apparent identical function as in *S. cerevisiae*, *S. pombe*, and *Rhodosporidium toruloides* (reviewed in KURJAN and TAYLOR 1993; VAILLANCOURT and RAPER 1996), or are pseudogenes, as in *U. maydis* (URBAN *et al.* 1996). The Homobasidiomycete *S. commune* differs not only in its multitude of B mating types, but also in the multiplicity of different pheromone genes of distinguishable phenotypes within a given locus. Furthermore, each specificity of each locus, Bα1 and Bβ1, seems to have built-in redundancy with respect to function within its own series. Our evidence is consistent with the idea that each pheromone gene encodes a distinct peptide capable of activating different receptors of several other specificities; for example, the Bbp1(1) pheromone can activate receptors in all other specificities except Bβ's 3, 8, and 9. Our evidence also suggests that a single receptor may be activated by several different pheromones; for example, Bbr1 is activated by pheromones encoded by all other specificities except Bβ4. The ability of single pheromones to interact with more than one different receptor, and of single receptors to be activated by more than one different pheromone, appears to be a unique feature of the Homobasidiomycete system when compared with other fungi.

The quantitative differences in phenotype elicited by different pheromones when introduced via transformation into each of the other mating types of *S. commune* may be a reflection of the relative strength of their physical interactions with various receptors. It is interesting that a single clone containing both the pheromone genes *bbp1(1)* and *bbp1(2)* produced a much stronger reaction in the Bβ2 strain than did two smaller clones, consisting of each half of the larger clone, and containing only one of these genes each. One possibility is that the two pheromones together had a synergistic effect because each bound to different activating sites in the Bβ2 specificity, either on the same receptor, or on two different receptors. Another possibility is that in the process of cutting the larger clone in half, important upstream regulatory elements for each gene were destroyed.

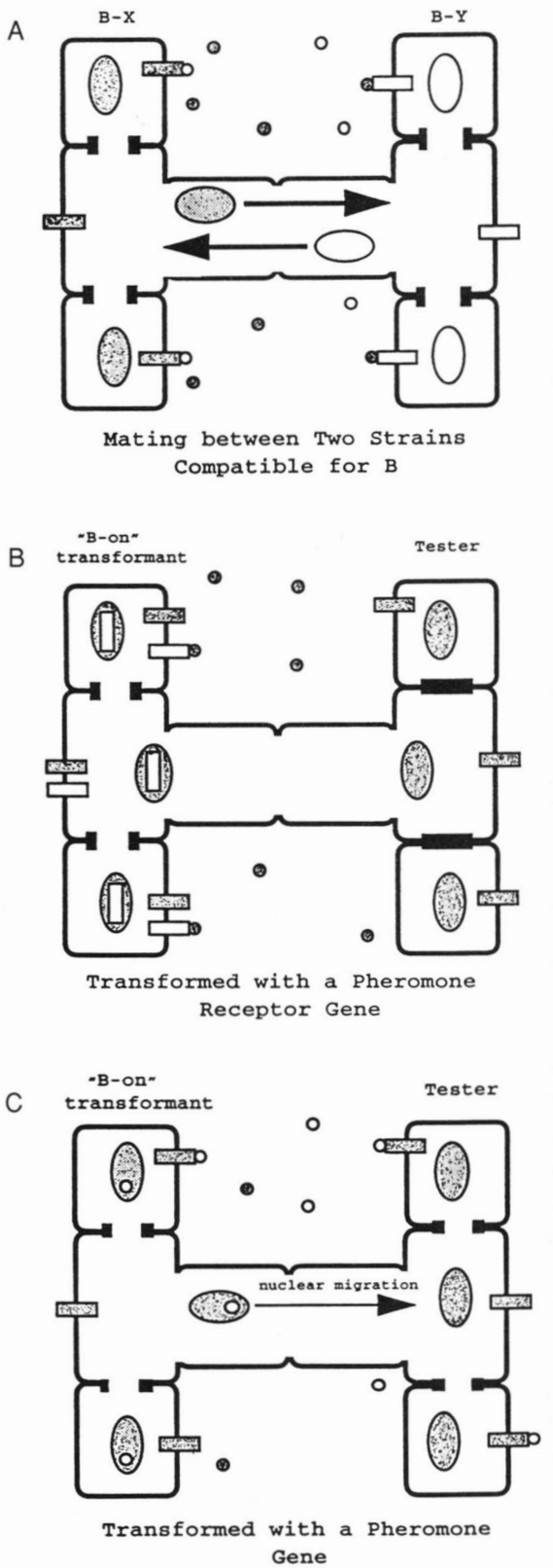

FIGURE 7.—A depiction of how pheromones and pheromone receptors might regulate bilateral nuclear migration. In A, a mating compatible for the B mating-type genes is shown. The bars represent membrane-bound pheromone receptors, and the circles represent diffusible pheromones. Each mate produces pheromones that activate the receptors of the opposite mate, inducing a signaling pathway that culminates in activation of the cellular machinery involved in nuclear migration. This includes the factors responsible for septal dissolution, a requirement for passage of nuclei from one cell to the next. Septal dissolution is illustrated by open gates between cells. In B and C, a bar or circle shown inside the nucleus represents a transgene encoding a nonself pheromone receptor or pheromone, respectively. B illustrates a test mating involving a strain transformed with a nonself pheromone-receptor gene. The presence of a compatible receptor activates the B pathway within the transformant, but migration of nuclei from the transformant into the tester does not occur because the compatible, membrane-bound receptor molecules cannot diffuse to nearby cells of the tester strain. C represents a test mating involving a strain transformed with a nonself pheromone gene. The presence of a compatible pheromone gene activates the B pathway not only within the transformant but within the tester as well. This is because the compatible pheromone can diffuse to nearby tester cells and activate receptors in advance of the fusion cell, thus preparing the way for acceptance of fertilizing nuclei.

We have shown that the Bβ1 genes of *S. commune* occupy a large piece of DNA that is unique to the β1 specificity. This is true also of the Bα1 genes. In fact, the disimilarity in sequence of mating-type loci is a feature common to all Basidiomycetes so far studied (see VAILLANCOURT and RAPER 1996 for a review). This may be an adaptation to preclude recombination between the genes of different specificities, an occurrence that might result in self-activation. One can see why constitutive activation of the B-regulated pathway in *S. commune* would have a selective disadvantage: the flat phenotype is a rather sickly one with relatively poor growth characteristics. A selective disadvantage is not so evident in other fungi, however, where pheromone/receptor activation of the relevant pathway does not lead to any clear difference in growth potential.

The qualitatively different activities of the pheromone and pheromone receptor genes of both Bα and Bβ are consistent with a hypothesis, which has been described previously (WENDLAND *et al.* 1995; KOTHE 1996) for how diffusible pheromones and membrane-bound receptors might operate to control nuclear migration (see Figure 7). Our Northern data indicating that the period of maximal levels of pheromone gene transcripts correspond to the period of nuclear migration in compatible matings are also consistent with this hypothesis. We propose that during this early time period the mated mycelia produce large amounts of pheromones to induce and sustain migration of nuclei into the opposite mate. After this migration has been completed, the pheromone genes are downregulated because less pheromone is required at that time. If this hypothesis is correct, we may not have identified all the genes relevant to the Bβ1 specificity. There could be another, as yet unidentified, receptor in the Bβ1 locus. Evidence in support of this is that the receptor encoded by the *bbr1* gene fails to activate the Bβ4 strain in transformation assays. If we assume that each interaction involves reciprocal binding of pheromone to receptor,

then there must be a second Bβ1 receptor that is responsive to the pheromones of the Bβ4 specificity. This second receptor might be encoded by the DNA adjacent to the original 25-kb cosmid clone investigated in the present work and shown in Figure 3. We tried to isolate the relevant overlapping clone by walking in the cosmid library, but unfortunately that DNA was not represented in our clone bank. There is also reason to believe that the Bβ1 locus might contain at least one other pheromone gene, since the β3, β8, and β9 strains were not responsive to the three β1 pheromone genes we have cloned (Table 2). Theoretically, Bβ-dependent mating could occur between Bβ1 strains and strains containing Bβ3, or 8, or 9, or 4, even if the three pheromone and one receptor genes do represent the full complement of Bβ1, but migration of fertilizing nuclei would be expected to be unilateral in such matings. According to our observations on the qualitative difference in pheromone *vs.* receptor activity, each mate must produce at least one pheromone capable of interacting with one receptor of the other mate to activate reciprocal nuclear migration. In actuality nuclear migration was shown to be bilateral in all of the above pairings, indicating that at least one additional pheromone and one additional receptor might exist in the Bβ1 strain.

It has often been suggested that the α and β loci of the B mating-type complex of *S. commune* had a common origin, given their functional redundancy and the similarity in phenotype of mutations within these loci (RAPER 1966; RAUDASKOSKI *et al.* 1976). Comparison of the overall nucleotide sequences of the Bα1 and Bβ1 loci provides some evidence in favor of a common evolutionary origin for these two loci. Studies of additional Bα and Bβ loci could further substantiate this connection and might allow a parsimony analysis to be performed that would indicate what specificities are most related and thus may have diverged most recently. Although this may give us some clues about the order in which these mating-type specificity loci could have evolved, it would tell us relatively little about why they are so diverse in nature.

The pheromone and receptor genes have been multiplied dramatically in *S. commune.* Our Southern hybridizations indicated that no two specificities contain identical B genes, either at the α locus or at the β locus (data not shown). By extrapolation from the results obtained so far, we might logically assume that each of the nine Bβ specificities produce at least one pheromone receptor and three pheromones and that each of the pheromones is capable of activating about half of the receptors of the other eight specificities. This would produce a possible 108 different heterospecific pairs of activating pheromones and pheromone receptors within the Bβ series alone. The addition of comparable figures for the nine specificities of the Bα series produces a grand total of 216 different pheromone/receptor pairs that are capable of activating the B-regulated pathway of sexual morphogenesis. The astonishing multiplicity of pheromone and pheromone receptor genes in *S. commune* not only demonstrates the flexibility of the two types of molecules these genes encode but also reflects a significant difference in the evolutionary development of Homobasidiomycetes as compared to the Ascomycetes and the Hemibasidiomycetes.

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