Scooter, a New Active Transposon in *Schizophyllum commune*, Has Disrupted Two Genes Regulating Signal Transduction

Thomas J. Fowler and Michael F. Mitton

Microbiology and Molecular Genetics Department, University of Vermont, Burlington, Vermont 05405

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ABSTRACT

Two copies of *scooter*, a DNA-mediated transposon in the basidiomycetous fungus *Schizophyllum commune*, were characterized. *Scooter* is the first transposon isolated from *S. commune*. *Scooter* creates 8-bp target site duplications, comparable to members of the *hAT* superfamily, and has 32-bp terminal inverted repeats. Both copies of *scooter* are nonautonomous elements capable of movement. Southern blot hybridizations show that *scooter*-related sequences are present in all *S. commune* strains tested. *Scooter-1* was identified initially as an insertion in the Bβ2 pheromone receptor gene, *bbr2*, leading to a partial defect in mating. *Scooter-2* spontaneously disrupted a gene to produce the frequently occurring morphological mutant phenotype known as thin. The *scooter-2* insert permitted cloning of the disrupted gene, *thn1*, which encodes a putative regulator of G protein signaling (RGS) protein. Spontaneous insertion of *scooter* into genes with identifiable mutant phenotypes constitutes the first evidence of active transposition of a DNA-mediated transposon in a basidiomycete.

DNA-MEDIATED transposons from the *hAT*, *Fot1/pogo*, and Tc1/*mariner* superfamilies have been identified in the filamentous fungi (for review, see Kempken and Kück 1998). Members of the *hAT* (*hobo/Ac/Tam3*) superfamily have characteristic 8-bp target site duplications (TSD) and similarities in amino acid sequence among their putative transposases (Calvi *et al.* 1991). Until now, the sole *hAT*-like transposon identified in the basidiomycetous fungi was *Abr1* from the button mushroom, *Agaricus bisporus* (Sonnenberg *et al.* 1999). *Abr1* does not encode its own transposase and is atypical among the *hAT* transposons with its 7-bp TSD and the possibility of a preferred sequence motif into which it integrates. *Abr1* has not been observed to move and appears stably inherited in cultivated *A. bisporus*.

Active transposition of *hAT* family transposons in filamentous fungi has been demonstrated in several ways. Transposon trapping, a technique by which a known gene is disrupted via insertion of an active transposon, was used to identify *Folyt1* from *Fusarium oxysporum* f.sp. *lysopersici* (Gómez-Gómez *et al.* 1999). In the hyphomycete *Tolypocladium inflatum*, the transposon *restless* was shown to be active by its spontaneous insertion into *tnir*, a previously uncharacterized gene involved in nitrogen metabolism (Kempken and Kück 2000). Mutant phenotypes that revert to wild type with high frequency can also be indicative of transposon activity. Excision of a transposon from an insertionally inactivated gene can lead to restoration of gene function, resulting in an unstable, or mutable, phenotype (McClintock 1950). The *hAT* transposon *Ascot-1* of *Ascobolus immersus* was isolated from a mutable allele of *b2*, a spore color gene (Colot *et al.* 1998). *Folyt1*, in addition to being captured by a transposon trap, was shown to cause an unstable mutant phenotype due to occasional excision from the *nit1* gene (Gómez-Gómez *et al.* 1999).

We describe in this article an insertional mutation that was identified through a mating defect in the homobasidiomycetous fungus *Schizophyllum commune* (Raper and Raper 1973). An early step during mating in *S. commune* is the regulated exchange of nuclei between partners, a process controlled by two linked mating-type loci, Bα and Bβ (Raper 1966; Koltin and Flexer 1969). Each of these loci has nine versions, or specificities, defined by a combination of genes encoding a single, unique G protein-coupled pheromone receptor and several unique lipopeptide pheromones (Koltin *et al.* 1967; Stamberg and Koltin 1972; Wendland *et al.* 1995; Vaillancourt *et al.* 1997). Pheromones control donation of fertilizing nuclei from one mate to the other while pheromone receptors control acceptance of nuclei from a mating partner during the process of nuclear migration (Wendland *et al.* 1995; Vaillancourt *et al.* 1997; Fowler *et al.* 1998). The pheromones and pheromone receptor encoded within a single B specificity are normally incapable of interacting to trigger nuclear migration.

We applied molecular techniques to the study of several mutations that alter mate recognition during Bβ-dependent matings. These mutations were generated in previous studies and had been mapped in or near specificity 2 of the Bβ locus, Bβ2 (Parag 1962; Raper

Corresponding author: T. J. Fowler, Department of Microbiology and Molecular Genetics, 208 Stafford Hall, University of Vermont, Burlington, VT 05405. E-mail: tfowler@zoo.uvm.edu

et al. 1965; Raper and Raudaskoski 1968; Raper and Raper 1973). Among the mutants was one of a group historically called primary B-on mutants in which the B-regulated pathway of sexual development is constitutively functioning. The B-on phenotype is characterized by septal breakdown, continuous intercellular nuclear migration, and a morphological phenotype with few aerial hyphae and short irregular hyphal branches (Parag 1962; Koltin and Flexer 1969). The mutation eliciting this B-on phenotype is in a pheromone gene (Fowler *et al.* 1998). The encoded mutant pheromone is capable of activating the resident Bβ2 pheromone receptor to transduce a signal for B-regulated development within the unmated homokaryon and also promotes donation of migrant nuclei in a mating with the progenitor Bβ2 strain. In subsequent experiments, this B-on mutant was treated with X rays and a variety of secondary mutants were selected for normal homokaryotic morphology, indicating suppression of the constitutively active B-regulated pathway (Raper and Raper 1973). However, some of these secondary mutants retained the ability to donate migrant nuclei to the grandprogenitor Bβ2 strain and also showed additional mating defects that mapped to the Bβ2 locus. One secondary mutant contained a lesion first called *f* and later called *def* (Raper and Raper 1973; Raper 1988). The *def* mutant mates unilaterally: it can donate fertilizing nuclei to compatible mates, including the grandprogenitor Bβ2 strain, but is incapable of accepting fertilizing nuclei in any mating dependent upon Bβ compatibility. The *def* phenotype would be logically interpreted to reflect a continued presence of the mutant pheromone but a complete loss of Bβ2 receptor function. In the original analysis, however, this secondary mutation appeared to map to a position just outside the Bβ locus rather than to the receptor gene (Raper and Raper 1973).

In this study we show that the *def* secondary mutation was caused by disruption of the Bβ2 pheromone receptor gene, *bbr2*, through insertion of a previously uncharacterized transposon called *scooter-1*. We propose that the conflict this finding creates with previous mapping data can be resolved by considering that the disrupted receptor gene, *bbr2-1*, can occasionally be restored to function by excision of the transposon. A second copy of *scooter* spontaneously tagged *thn1*, a gene encoding a putative regulator of G protein signaling (RGS) protein. Disruption of *thn1* has pleiotropic effects on vegetative growth and sexual development in *S. commune*. *Scooter* is the first active transposon identified in a basidiomycete.

MATERIALS AND METHODS

***S. commune* strains, growth conditions, transfection, and mating:** The following *S. commune* strains were used for these studies and are part of a collection maintained at the University of Vermont. The primary B-on mutant has the Bβ designation *Bβ2(1)*. Gene symbols shown following the designated Bβ mating-type specificity indicate mutations in the strain: 4-8, *Bβ2*; 4-40, *Bβ1*; 8-7, *Bβ2 arg7*; V11-20, *Bβ2(1) def trp1*; V15-34, *Bβ2(1) pab1 bug*; V112-3, *Bβ7 trp1*; V113-9, *Bβ3 trp1*; V119-19, *Bβ9 trp1*; V145-2, *Bβ2 def trp1*; V168-3, *Bβ2 trp1 thn1*; and V168-5, *Bβ2 trp1 thn1*. Plate cultures of *S. commune* were grown at 30° on complete yeast extract medium (CYM)-1.2% agar medium (Raper and Hoffman 1974), supplemented with 2.5 mm tryptophan as necessary. Liquid cultures of *S. commune* were grown as described in Fowler *et al.* (1999). Protoplast preparation and transfections were done according to Specht *et al.* (1988), using Novozyme 234 (InterSpex Products, Foster City, CA) as the wall digesting enzyme, with modifications described by Horton and Raper (1991). Mating of strain 8-7 *thn1* with V113-9, sporulation, and isolation of germlings were according to standard methods (Raper and Hoffman 1974).

DNA isolation, digestion, and cloning: *S. commune* total DNA was isolated from cultures grown in liquid medium by a DNA extraction method using hexadecyltrimethylammonium bromide (CTAB; Saghai-Maroof *et al.* 1984) with the following change in the protocol. Mycelial pellets were vacuum filtered from the medium, then frozen in liquid nitrogen and powdered with a mortar and pestle, rather than lyophilized. Digestion of DNA with restriction endonucleases was according to the manufacturer's recommendations for the particular enzymes (Life Technologies, Grand Island, NY). Cloned DNA was prepared from bacterial or phage cultures by standard methods (Maniatis *et al.* 1982) with the exception that plasmid DNA used for sequencing or transfection of *S. commune* was prepared using affinity chromatography kits (QIAGEN, Valencia, CA). DNA subcloning was by standard procedures (Maniatis *et al.* 1982) into phagemid pBluescript KS+ (Stratagene, LaJolla, CA). Plasmids were electroporated into *Escherichia coli* strain DH5α using the Gene Pulser system according to manufacturer's instructions (Bio-Rad, Hercules, CA).

Library construction and screening: Two subgenomic libraries were constructed in bacteriophage vector λgt10 (Life Technologies). *Eco*RI restriction fragments from *S. commune* genomic DNA of the appropriate sizes, 6 kb for the library of *def* mutant strain V145-2 and 4 kb for the library of the *thn1* mutant of 8-7, were isolated from a 0.8% agarose gel with the GeneClean II kit (Bio101, Vista, CA). Approximately 25 ng of insert DNA was included with 500 ng vector in the ligation reactions. Ligated DNA was packaged using the Gigapack II XL system (Stratagene) and the packaged phage were used to infect *E. coli* strain C600*hfl*. For each subgenomic library, ~5000 plaques were screened by plaque lift assay (Maniatis *et al.* 1982) using ^{32}P-labeled DNA. The V145-2 library was screened with the insert of plasmid pTF2076-9, which is 2.1 kb and contains a 3′-truncated, functional copy of the wild-type *bbr2* gene. A PCR product containing all of *scooter-1*, as well as 152 bp of *bbr2* located adjacent to *scooter-1*, was amplified from pTF4061-3 with primers 97723-3 (5′-GGCATGGAAC TCGGGCAC) and 971021-1 (5′-CAGCTAAACAAAGGCCAC) and used as a probe for the 8-7 *thn1* library. Conditions for the hybridizations and washes during the screening of all libraries were as described for Southern analyses (below), except that final washes were in 0.1× saline sodium citrate (SSC) and 0.1% sodium dodecylsulfate (SDS). Positive plaques were reisolated two times to identify single plaque clones and the *Eco*RI inserts of the clones were subcloned into plasmid vectors.

A complete genomic library of wild-type *S. commune* (4-8) was constructed by ligating *Sau*3AI fragments into λDASH II predigested with *Bam*HI (Stratagene). The library was amplified in *E. coli* strain XL1-Blue (Maniatis *et al.* 1982). A total of 15,000 plaques from the amplified library were screened

with ^{32}P-labeled insert DNA from pTF6099-1. Plasmid pTF-6099-1 contains an insert of 2.7 kb derived from DNA that flanks the *scooter-2* insertion in *thn1* of *S. commune* mutant 8-7 *thn1*. A hybridizing plaque containing a 15-kb insert was identified and the insert was subcloned into plasmid vectors.

A wild-type *S. commune* cDNA library (4-40, courtesy of M. Raudaskoski) was screened as described above for the complete genomic library, except that only 10,000 plaques were used. The phage vector for this library was λZAP II (Stratagene) and plasmid clones containing the *S. commune* insert were released from the phage genome according to the manufacturer's instructions.

Southern analyses and probes: Digested DNAs were size-fractionated on 0.8% agarose gels and transferred to nylon membranes (Hybond-N; Amersham Pharmacia, Piscataway, NJ) by capillary blotting. DNA was crosslinked to the filters with UV light and then prehybridized a minimum of 10 min in standard hybridization buffer (Maniatis *et al.* 1982) at 65° prior to addition of the probe. Hybridizations were incubated at least 16 hr at 65° and the filters were subsequently washed twice in a solution of 2× SSC and 0.5% SDS for 30 min at 65°. Probes were stripped from filters with a boiling solution of 0.1% SDS and checked by autoradiography.

Probes for all hybridizations were made from plasmid insert DNA isolated with the GeneClean II kit and labeled with ^{32}P by the random primer method (Feinberg and Vogelstein 1983). Southern hybridization data with *Pst*I-*Eco*RI double-digested *S. commune* genomic DNA showed that *bbr2* probes hybridize to a single band, *bbr2*, for strains having the Bβ2 mating-type specificity and that the same probes hybridize to either one band or show no hybridization for strains of other Bβ specificities (data not shown). Therefore, at most, one band displayed on the Southern hybridization shown in Figure 3 may be due to hybridization with *bbr2*-related sequences rather than *scooter-1* when probed with DNA containing *scooter-1* and 152 bp of *bbr2*.

DNA sequencing and sequence comparisons: DNA sequencing reactions were done at the Vermont Cancer Center DNA sequencing facility at the University of Vermont using a dideoxynucleotide method and fluorescent labeling system (ABI Prism kit; Perkin Elmer-Cetus, Norwalk, CT). Oligonucleotide primers for sequencing and PCR were purchased from Genosys (The Woodlands, TX). DNA sequences have been deposited as GenBank accessions as follows: *scooter-1*, AF267871; *scooter-2*, AF267872; and *thn1*, AF267870. DNA and protein sequence comparisons were made using BLAST and BLAST 2 Sequences programs located at www.ncbi.nlm.nih.gov (Altschul *et al.* 1997; Tatusova and Madden 1999) and the Pileup program (Henikoff and Henikoff 1992) from the University of Wisconsin Package (Genetics Computer Group).

Microscopy and photographs: *S. commune* cultures used for microscopy were grown on thin layers of CYM medium on glass slides. Photomicrographs were taken on a Nikon Eclipse 400 microscope at 40× magnification using pseudo-Nomarski optics (*i.e.*, the phase ring was partially disengaged). Photographs of *S. commune* colonies were taken at the medical photography studio at the University of Vermont.

RESULTS

The Bβ2 receptor gene *bbr2* complements the *def* mutant phenotype: Inability of the *def* secondary mutant strain to accept fertilizing nuclei in a Bβ-dependent mating resembles the phenotype predicted for loss of Bβ pheromone receptor function. Absence of Bβ2 receptor function would also explain a lack of self-activation of the B-regulated pathway by the B-on mutant pheromone in this secondary mutant (Fowler *et al.* 1998). We wondered whether addition of the wild-type Bβ2 receptor gene, *bbr2*, to a *def* strain might overcome the missing receptor function to restore the B-on mutant phenotype. A *def* mutant strain auxotrophic for tryptophan (V11-20) was cotransfected with pTF2076-9 containing the wild-type *bbr2* and pRHV1 (Horton and Raper 1995) containing wild-type *trp1*. In two separate experiments, 5 of 11 and 10 of 21 prototrophic colonies were restored to the B-on phenotype, indicative of constitutive activity of the B-regulated pathway. A control transformation of the *def* mutant strain with *trp1* alone resulted in 22 prototrophs, all with normal homokaryotic morphology. Restoration of the B-on mutant phenotype by *bbr2* led us to investigate the Bβ2 receptor gene of the *def* mutant.

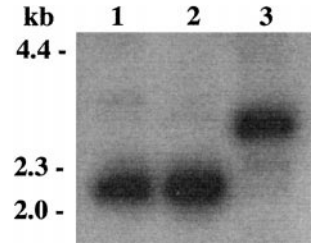

Figure 1.—The *bbr2* gene has a restriction fragment length polymorphism between wild-type, B-on mutant, and *def* secondary mutant strains. Genomic DNA was double digested with *Eco*RI and *Pst*I and analyzed by Southern hybridization. The Bβ2 *S. commune* strains represented are wild type (strain 4-8, lane 1), B-on mutant (strain V15-34, lane 2), and *def* secondary mutant (strain V11-20, lane 3). The probe was a 2.1-kb *Eco*RI-*Pst*I cloned restriction fragment (pTF2076-9) containing only wild-type *bbr2* DNA. Approximately 1 μg of DNA was loaded in each lane.

The Bβ2 receptor gene of the *def* strains has been altered: A comparison was made between Bβ2 wild-type, primary B-on mutant and *def* secondary mutant strains to determine whether any differences existed in their *bbr2* genes. DNA extracted from these *S. commune* strains was analyzed by Southern blot hybridization. Figure 1 shows the *bbr2* probe hybridized strongly to a band in each strain and that the hybridizing band in the *def* mutant strain was ~600 bp larger than the hybridizing bands in the wild-type and B-on mutant strains. The probe for *bbr2* used in this hybridization was the cloned equivalent of the 2.1-kb hybridizing band from the wild-type strain and contained only *bbr2*. Therefore, the observed difference in size between the hybridizing restriction fragments of the two mutants indicated that a change in *bbr2* occurred at the time of secondary mutagenesis.

The insert in the *bbr2* gene of the *def* mutant has characteristics of a DNA-mediated transposon: We set out to identify the nature of the change in the *bbr2* allele of the *def* mutant, now termed *bbr2-1*. Additional

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ggggcaacAG GACCCCGGAA TATACGCACA AAGGCCGTCC TGGAAAATCC GGCTCTGGCG   52
CCCATTTATC CACTTTTTTC TTGGGTCTCC GACCCCCAGA TTTATCCGTT TTCTGGGACG 112
GCCTCTCGGG ACCCCCCGGA TTTATCCGTT TTCTCGGGAC GGCCTTCAAA AATGGCAATA 172
AAATCGGGAA AATCTGATTT TTTTCTCCCA GGGTCCCTAC GCGCTCTGCC CGTAGCGTCT 232
GCGACGAAAG AAGGTTGAGA AGTACGAATG AGAGAGCTAC TTGCCTCCCA TACCCATCTG 292
AGGTGCTACC ACGCACCGCT GTCCCTCGGA ATATCGAGCC TGCCAATGGT AGAAGGTGCC 352
AATGGGCACA GGGAGCTGAA CTAGGGTGCT ACTGCCCGCT GTAACAAATG GTCAATTAAC 412
TTATCGGTAT CGCGTGCGTC TAGTTTGAGG GTAGGTCGGC GGTTCGAATC GACCGCTCGA 472
TATCCACTTT TTCGGAACGG CCTCCCCGTC TCCCCTGTAT ATATCCGCGT TTTTGAACCG 532
GCCTTCGAGC CCTCAGGTCC CCGATTCATC CATTTTTTGG ACCGGCCTAC CGGCCGGGAC 592
CCCCACGATT TATCCGCACC GCTGGACGGC CTTTGTGCGT ATATTCCGGG GTCCTggggc 647
aac
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FIGURE 2.—Nucleotide sequence of *S. commune* transposon *scooter-1*. The 647-bp *scooter-1* element is shown in uppercase letters and is numbered on the right. The 32-bp terminal inverted repeat sequences are shown in boldface and subterminal repeats are underlined. The 8-bp target site duplication of the *bbr2* gene is shown in lowercase and corresponds to base pairs 234–241 of *bbr2* (GenBank accession no. AF148501).

Southern hybridizations indicated that *bbr2-1* resided within an *Eco*RI restriction fragment of ~6 kb (data not shown). A subgenomic phage library was constructed from a *def* mutant strain using *Eco*RI restriction fragments of ~6 kb. Plaques that hybridized to the *bbr2* probe were shown to contain a 2.7-kb *Pst*I-*Eco*RI restriction fragment within the insert (data not shown), as expected from the original hybridization results (Figure 1). A comparison of sequences between the 2.7-kb restriction fragment containing *bbr2-1* and the wild-type *bbr2* allele (FOWLER *et al.* 1999) showed that *bbr2-1* contained a single change, a 655-bp insertion interrupting the first exon of *bbr2* between codons 62 and 63. The inserted sequence introduces stop codons in all reading frames of *bbr2* such that a receptor produced from this allele would include only the N-terminal 10% of wild-type receptor unless alternative splicing of the primary transcript occurred.

The inserted DNA contained hallmarks of a DNA-mediated, or class II, transposable element (Figure 2; FINNEGAN 1989). One characteristic of DNA-mediated transposition is creation of a short duplication of the interrupted DNA at the site of insertion. This "target site duplication" flanks each end of the inserted transposon. In *bbr2-1*, 8 bp of the *bbr2* coding sequence (GGGGCAAC) located adjacent to the 5′ end of the 655-bp insertion are duplicated directly following the 3′ end of the novel sequence of the insertion. Short terminal inverted repeats and subterminal repeats are also typical of class II transposons. The ends of the novel insert in *bbr2-1* (*i.e.*, excluding the 8-bp target site duplication) are inverted 32-bp perfect repeats and several subterminal repeated sequences are also present (Figure 2). Although a transposon may maintain autonomy by encoding its own transposase, this insert does not contain a significant open reading frame or any similarity to sequences in public databases. We conclude that this segment of DNA transposed into *bbr2* at the time of secondary mutagenesis and is a previously unknown nonautonomous transposon. We have designated this sequence *scooter-1*.

The transposon *scooter-1* is part of a small group of related sequences: It is common for related versions of a transposon to exist in a single genome and for the different versions to share significant regions of DNA sequence identity (STRECK *et al.* 1986; FEDOROFF 1989). To determine whether sequences with strong similarity to *scooter-1* exist in the *S. commune* genome, a Southern blot analysis was done. Total DNA from seven *S. commune* strains was digested with *Pst*I and *Eco*RI, restriction enzymes that have no recognition sites within *scooter-1*. Southern hybridization of these seven strains with the *scooter-1* probe showed from 8 to ~20 discrete hybridizing restriction fragments (Figure 3). Analyses using additional strains and other restriction endonucleases with 6-bp recognition sites produced similar results. The minimum number of hybridizing restriction fragments in any strain was 3 (data not shown). *Scooter-1* or *scooter*-related sequences may be ubiquitous in *S. commune*, inasmuch as we have analyzed 14 strains collected from five continents and have failed to identify any strain that is devoid of *scooter*-related sequences.

Cosegregation of a *scooter*-related sequence and the thin mutant phenotype: A spontaneously occurring mutant phenotype called thin, commonly seen in some *S. commune* strains, is caused by mutation of the *thn1* gene (RAPER and MILES 1958; RAPER *et al.* 1958). The thin phenotype appears as a fast-growing sector during colonial growth. While the thin sector may look fluffier than the wild-type growth from which it arose (Figure 4A), it grows in a relatively submerged fashion on semisolid medium once subcultured away from wild type (Figure 4, B and C). A similar observation had been previously made by SCHUREN (1999). Thin hyphae are distinguished microscopically by their characteristic corkscrew morphology (SCHWALB and MILES 1967) as contrasted to the relatively smooth morphology of wild-type hyphae (Figure 4, D and E). The high frequency of sectoring to the thin phenotype in certain strains led us to hypothesize that mutagenesis by a transposon might be responsible for the change. To test whether a *scooter*-related sequence is involved, a spontaneous sector of thin mutant hyphae was identified and subcultured from a colony with otherwise normal homokaryotic hyphal morphology. The original strain (8-7) and its thin mutant derivative were compared by Southern blot analysis. Hybridization of a *scooter-1* probe to genomic DNA digested with *Eco*RI showed that the mutant had three distinct hybridizing restriction fragments not present in the wild type (Figure 5A).

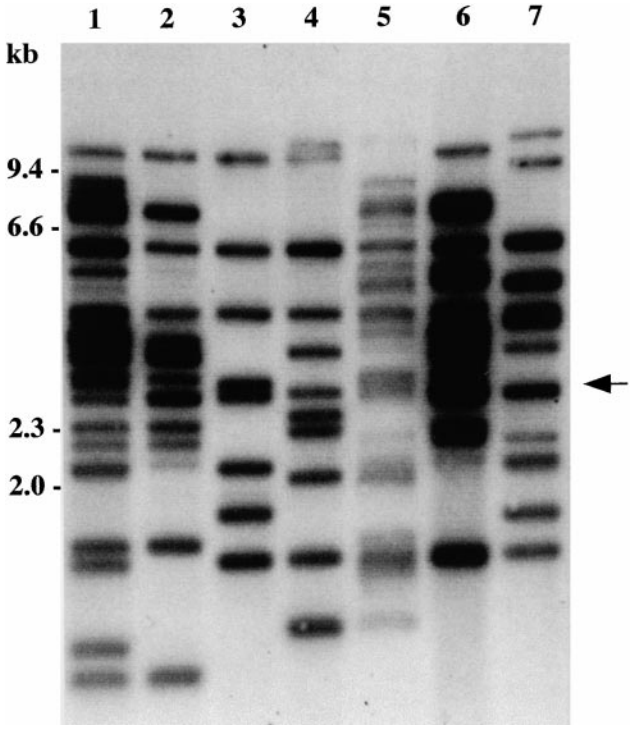

FIGURE 3.—The *S. commune* genome has a family of sequences related to *scooter-1*. Southern analysis of seven *S. commune* strains shows hybridization to a small family of sequences. The probe consisted of the entire *scooter-1* element as well as 152 bp of *bbr2*. Under the hybridization conditions used here, probes containing only *bbr2* either would hybridize to a single band or would not hybridize at all. Therefore, at most, one band of hybridization in each lane might be due to *bbr2* and not *scooter-1*. Lane 7 contains the *def* secondary mutant strain from which *scooter-1* was originally isolated; the arrow indicates the restriction fragment corresponding to the *scooter-1* insertion into *bbr2*. Strains in lanes 3–7 are derived from crosses that have one common parental strain. Loading order (lane, strain): 1, 4-40; 2, 4-8; 3, V113-9; 4, V119-19; 5, V112–3; 6, V11-20; 7, V145-2. Approximately 1 μg of genomic DNA, double-digested with *Eco*RI and *Pst*I, was loaded for each lane.

This mutant was mated with a compatible wild-type strain (V113-9) and the progeny of this mating were tested for segregation of the thin mutant phenotype with one or more of the restriction fragment polymorphisms. The progeny showed a 1:1 segregation ratio for wild type *vs.* thin (13:13) as would be expected for a trait controlled by a single gene. Southern hybridization analysis of nine offspring, four wild type and five thin, using a *scooter-1* probe showed a perfect correlation between a hybridizing *Eco*RI restriction fragment of ~4 kb and the thin phenotype (Figure 5B). None of the wild-type progeny exhibited this particular band. A comparable analysis of six more individuals (four thin and two wild type, data not shown) exhibited the same correlation.

Isolation of a second *scooter* element and flanking DNA: Cosegregation of the thin phenotype and a restriction fragment identified by a *scooter-1* probe indicated that a *scooter-1*-like sequence was either in the *thn1* gene or closely linked to it. We proceeded to construct a subgenomic phage library of 4-kb *Eco*RI restriction fragments from one of the thin mutant offspring of the mating. Screening of the library with a *scooter-1* probe yielded several positive clones. *Eco*RI inserts of two slightly different sizes were represented among the clones, corresponding to two bands of ~4 kb seen in genomic Southern analysis (Figure 5B, last lane). A clone with the larger insert size, which corresponded to the cosegregating band, was selected for restriction analysis, subcloning, and sequencing. The clone contained a *scooter* transposon that is 91% identical in nucleotide sequence to *scooter-1*. This second copy of *scooter*, named *scooter-2*, created an 8-bp target site duplication (AGGCGATG) at the point of insertion and has 32-bp imperfect terminal inverted repeats that are identical at 31 positions. The 3′ terminal inverted repeat of *scooter-2* differs from the 3′ end of *scooter-1* by a single base pair, a T to A difference (Figure 2, base pair 625). Subterminal repeats in *scooter-2* are also nearly identical to those identified in *scooter-1*, having a single difference in one of the repeat units (Figure 2, base pair 131, G to A).

A genomic Southern blot analysis was used to confirm

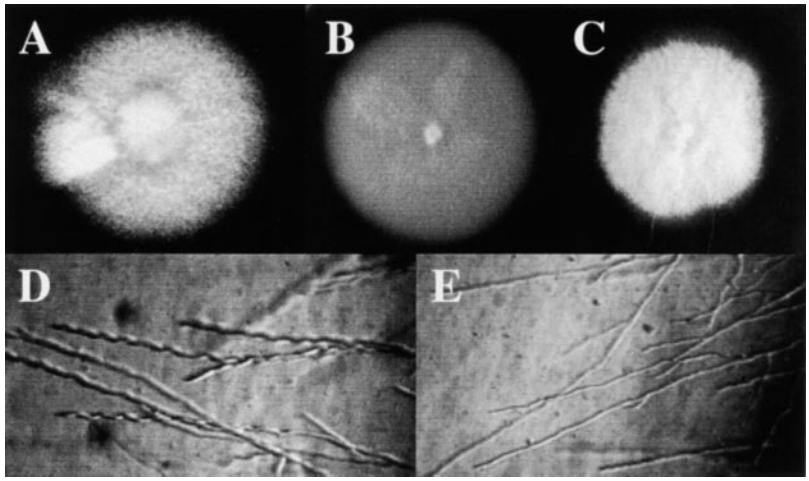

FIGURE 4.—Phenotype of a *S. commune thn1* mutant. (A) Wild-type colony with a wedge-shaped spontaneous *thn1* mutant sector on the left; (B) *thn1* mutant colony grown in isolation; and (C) wild-type colony, progenitor of colony shown in B. Colonies shown in B and C were inoculated and grown simultaneously under identical conditions and are shown at the same scale. (D) *thn1* mutant hyphae; and (E) wild-type hyphae. Strains shown in D and E were grown under identical conditions.

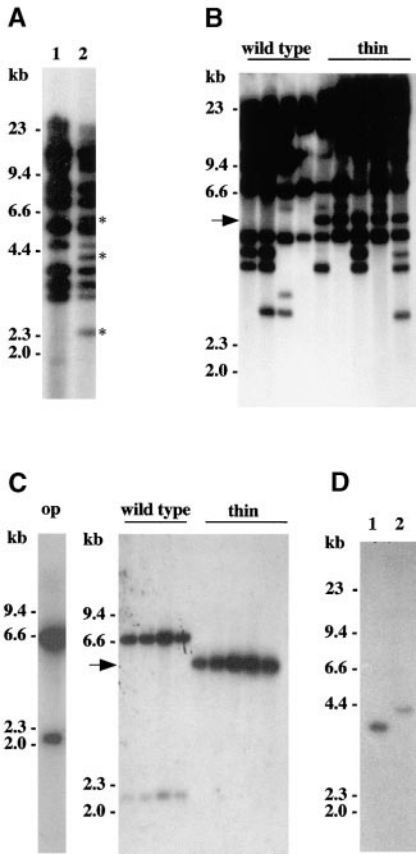

FIGURE 5.—Cosegregation analysis of *scooter-2* and the thin mutant phenotype. (A) Genomic Southern analysis of a wild-type strain (8-7, lane 1) and its thin mutant derivative (lane 2) with a *scooter-1* probe. Asterisks indicate polymorphisms. (B) Southern hybridization with a *scooter-1* probe was used to compare nine progeny from a cross between the thin mutant (A, lane 2) and an unrelated wild-type strain (V113-9). The phenotype of each individual is indicated above the lane, and the arrow indicates a band that segregates with the thin mutant phenotype. (C) Autoradiograms of two filters probed with 2.7 kb of DNA that flanks the *scooter-2* insertion in the thin mutant (pTF6099-1). The single lane on the left shows the hybridization pattern of the wild-type outcross parent (op). A filter with samples from the segregating progeny shows two distinct patterns of hybridization for wild-type *vs.* thin mutants (right, identically loaded as in B). The arrow indicates the same band as identified in B. (D) An autoradiogram of the filter used in A, reprobed with 2.7 kb of DNA that flanks *scooter-2*. As in A, the wild-type progenitor (lane 1) is compared to its thin mutant derivative (lane 2). All DNA samples were digested with *Eco*RI and loaded at ~1–2 μg per lane.

that the DNA flanking the cloned *scooter-2* also segregated with the thin mutant phenotype among the progeny of a cross of an unrelated wild-type strain (V113-9) and the thin mutant, suggesting this adjacent DNA was also linked to the *thn1* gene. The probe was 2.7 kb of DNA flanking the *scooter-2* insertion derived from the original 4-kb subclone of thin mutant DNA. *Eco*RI restriction fragments of 6.6 and 2.2 kb were identified in the wild-type parent and in the wild-type progeny from the cross by this probe made from "flanking DNA" (Figure 5C). The same probe hybridized to DNA isolated from thin mutant progeny only at the 4-kb *Eco*RI restriction fragment (Figure 5C) originally identified by the *scooter-1* probe (Figure 5B). There are no *Eco*RI sites in the 653-bp *scooter-2* sequence; we therefore predicted that the probe made from DNA flanking *scooter-2* would hybridize to a 3.4-kb *Eco*RI band in the wild-type strain (8-7) from which the original thin sector arose. This expectation was confirmed by Southern blot analysis (Figure 5D).

Wild-type genomic and cDNA clones of *thn1* are identified using DNA flanking the *scooter-2* insert: Preliminary sequence analysis of the thin mutant clone suggested the *scooter-2* element had interrupted the coding region of a gene. To characterize this putative gene, genomic and cDNA libraries constructed from wild-type *S. commune* strains were screened using the probe made from DNA flanking *scooter-2*. A phage clone isolated from the genomic library yielded an ~15-kb insert that was subsequently subcloned into plasmid vectors. Comparison of sequences derived from the wild-type and mutant strains confirmed the target site duplication created by *scooter-2*.

Screening of the cDNA library yielded six positive clones. All six cDNA clones, including the one containing the largest insert of 2.5 kb, were partially or completely sequenced and were found to represent the same gene. The cDNA sequence was identical to the regions of the wild-type genomic clone that had been sequenced, except for the presence of one intron in the genomic clone. This comparison of sequences, in conjunction with a genomic Southern blot that showed only a single band of hybridization when the full-length cDNA was used as a probe (data not shown), confirmed that these clones represent a single gene, *thn1*. This gene was interrupted by *scooter-2* between codons 104 and 105 in the *thn1* mutant (Figure 6).

Thin mutants can be complemented by addition of the wild-type *thn1* gene: To be assured that we had identified the gene responsible for the thin phenotype, we attempted to complement a thin mutant with a genomic copy of the putative wild-type *thn1* gene. A plasmid containing this 3.1 kb of wild-type genomic DNA was co-

FIGURE 6.—Comparison of deduced amino acid sequences of RGS domain proteins Thn1 (*S. commune*), FlbA (*A. nidulans*), and Sst2p (*S. cerevisiae*). Identical amino acids at each position are shown in a black background, conservative substitutions are shown in a gray background, and gaps are indicated by dots. Regions of Thn1 that fit the RGS domain consensus are underlined. Numbering is according to SwissProt Accessions P38093 for FlbA and P11972 for Sst2p. Alignments were done using the Pileup program (Genetics Computer Group).

transfected with a *trp1* plasmid into protoplasts isolated from an auxotrophic *thn1* mutant strain. The plasmid insert containing *thn1* included a genomic region that corresponded with the longest cDNA, as well as ~600 bp of DNA upstream of the transcribed region. In two separate transfection experiments, 5 of 16 and 6 of 37 prototrophic transformants were restored to wild-type hyphal and colonial morphology. A control transfection with only the *trp1* plasmid yielded only thin mutant colonies. These experiments confirm that we have isolated *thn1*, a gene responsible for maintaining normal hyphal morphology and colonial growth.

Deduced amino acid sequence indicates that Thn1 is an RGS protein related to FlbA and Sst2p: A search of protein databases using the amino acid sequence predicted from the longest *thn1* cDNA revealed a strong similarity to a large number of protein sequences featuring the RGS domain. Proteins of the RGS domain family function as signal regulators by acting as GTPase-activating proteins (GAPs) for Gα subunits of heterotrimeric G proteins (Berman *et al.* 1996a,b) or by directly interfering with activation of effector molecules by the Gα subunits (Hepler *et al.* 1997). Two RGS proteins from the fungi are well known, FlbA of *Aspergillus nidulans* (Lee and Adams 1994a) and Sst2p of *Saccharomyces cerevisiae* (Dietzel and Kurjan 1987). Their sequences are compared to the predicted amino acid sequence of the *thn1* protein, Thn1, in Figure 6. Pairwise comparison of the deduced amino acid sequences of Thn1 and FlbA revealed 36% identity and 54% similarity over the length of Thn1, while a similar comparison of Thn1 with Sst2p showed 22% identity and 39% similarity.

DISCUSSION

Scooter-1 and *scooter-2* of *S. commune* are closely related copies of the first transposon shown to be active in a basidiomycete. *Scooter* appears to belong to the *hAT* (*hobo/Ac/Tam3*) superfamily of class II transposons, having an 8-bp target site duplication, short terminal inverted repeats, and subterminal repetitive sequences like other *hAT* superfamily members (Calvi *et al.* 1991). Two other class II transposon-like sequences have been identified in basidiomycetes: *Abr1* in *A. bisporus*, which has been tentatively placed in the *hAT* superfamily based on a 7-bp target site duplication and subterminal repeats (Sonnenberg *et al.* 1999), and *Pce1* of *Phanerochaete chrysosporium* (Gaskell *et al.* 1995). There is no DNA sequence identity between the currently identified members of these three transposon groups.

Scooter-1 and *scooter-2* appear to be nonautonomous elements that were mobilized by a transposase encoded elsewhere in the *S. commune* genome. Each of these *scooter* elements has terminal inverted repeats and subterminal repeats that may constitute *cis*-acting sequences for transposase binding as is the case in the *Ac/Ds* transposons (Kunze and Starlinger 1989). Each also has a relatively short length and no long open reading frame capable of encoding a transposase. Identification of a transposase gene for the *scooter* transposon would clarify whether *scooter* is truly part of the *hAT* superfamily. One *S. commune* strain that must produce transposase, indicated by the recent movement of *scooter-2* into *thn1*, was shown to have several *scooter*-related sequences in its genome (Figure 5A). One of the restriction fragments to which *scooter-1* hybridizes may contain an autonomous element, since DNA sequence identities are commonly found between autonomous and nonautonomous members of transposon groups (Döring *et al.* 1984; Pohlman *et al.* 1984; Streck *et al.* 1986).

Our current discovery that the *def* secondary mutation in Bβ2 was caused by insertion of *scooter-1* into the Bβ2 receptor gene provides evidence of recent mobility of the *scooter* element. Previous mapping data indicated recombination between the B-on mutation in the Bβ2 locus and the *def* mutation, now called *bbr2-1* (Raper and Raper 1973). Reinterpretation of these data following discovery of the transposon strongly supports the idea that *scooter-1* occasionally excised from its position in *bbr2-1*. Two outcrosses between the *def* mutant and wild-type strains appeared to yield 13 recombinants among the progeny analyzed, suggesting that the *def* mutation was located 0.8 cM outside the Bβ locus. The number of recombinants in the two expected classes were extremely skewed, however, with 12 of the apparent recombinants fitting into one class and only 1 into the second class. Members of the majority class had the phenotype of the B-on mutant. In retrospect, the preponderance of individuals with the B-on phenotype can best be explained as the result of excision of *scooter-1* from *bbr2-1* in a manner that restored Bβ2 receptor function while in the presence of the mutant pheromone that activates this receptor. The one member of the minority recombinant class, and perhaps a small number of the majority class as well, may represent true recombinations between the pheromone gene and the receptor gene, which are now known to lie ~1.2 kb apart within the Bβ2 locus. Direct molecular evidence to support this interpretation is not possible to obtain since these strains no longer exist.

Scooter-2 acted as a molecular tag for identification of *thn1*. Mutations in *thn1* give rise to a frequently encountered morphological mutant of *S. commune*. The predicted gene product of *thn1* is a RGS protein, one of a few RGS proteins currently identified in the fungi. More than 40 genes encoding putative RGS proteins have been identified in animals as well. The common feature of all RGS proteins is a consensus domain of ~120 amino acids (De Vries *et al.* 1995) that is thought to interact directly with the Gα subunit of heterotrimeric G protein. This interaction modulates the rate of conversion of GTP-bound Gα protein to GDP-bound Gα protein to regulate signals passed from an activated seven-transmembrane-domain receptor to downstream effector molecules via the heterotrimeric G protein. The

founding member of the RGS protein family, Sst2p of *S. cerevisiae*, contributes to the process of desensitization and recovery from pheromone stimulation during mating (Chan and Otte 1982; Dietzel and Kurjan 1987). The other well-characterized fungal RGS protein is FlbA of *A. nidulans* (Lee and Adams 1994a). FlbA belongs to a signaling pathway that leads to inhibition of vegetative hyphal proliferation and to promotion of asexual sporulation. It is thought to act as a GTPase-activating protein for the Gα protein, FadA (Yu *et al.* 1996; Adams *et al.* 1998). A knock-out mutation of *flbA* results in *A. nidulans* colonies with increased hyphal proliferation, reduced sporulation, and eventual autolysis of the older hyphae (Lee and Adams 1994a). Like FlbA, disruption of *thn1* results in pronounced changes in vegetative hyphal morphology. The effects on asexual sporulation in *A. nidulans* have no parallel in *S. commune* because *S. commune* does not produce asexual spores. One characteristic of *thn1* mutants, however, is the inability to produce fruiting bodies from a compatible mating homoallelic for the *thn1* mutation (Schwalb and Miles 1967).

The three deduced fungal RGS protein sequences contain conserved regions outside of the RGS domain, suggesting that these proteins may contain regions important for other common functions. There is strong sequence identity (36%) between Thn1 and FlbA throughout the entire Thn1 sequence. Thn1, like Sst2p, does not have a region similar to the N-terminal 204-amino-acids region of FlbA, which has no defined function as yet. Thn1 and Sst2p share identical start positions relative to FlbA, suggesting that the N-terminal extension of FlbA is an acquisition more recent than the divergence of ascomycetes and basidiomycetes. In all other respects, the primary sequences of Thn1 and FlbA are much more similar to each other than either of these proteins is to Sst2p. Of great interest is a region of Thn1 between amino acid residues 51 and 110 that shares a very high level of similarity with FlbA, but not with Sst2p. Particularly striking is a string of seven consecutive threonine residues (amino acids 87–93).

The strong sequence similarity of Thn1 to other RGS proteins, such as FlbA and Sst2p, suggests a similarity in biochemical functions. One consequence of the loss of Thn1 function is a reduction or loss of mRNAs encoding hydrophobins (Wessels *et al.* 1991). These hydrophobins include one normally expressed in both homokaryons and dikaryons (Sc3) and two that are dikaryon specific (Sc1 and Sc4). We hypothesize that Thn1 is regulating a heterotrimeric G protein-signaling pathway that, in turn, regulates hydrophobin expression. Our observation that the *thn1* mutant has an altered macroscopic phenotype when isolated from its sector within the wild-type colony corroborates earlier observations of Schuren (1999). He noted a similar phenotypic switch of *thn1* mutants from the production of abundant aerial hyphae in the presence of wild-type hyphae to a relatively submerged habit of growth in isolation. He also produced some evidence that a diffusible molecule <8 kD may be responsible for this effect. This finding has similarity to the response of *A. nidulans fluG* mutants to a small molecule produced by *A. nidulans* wild-type hyphae, resulting in a shift from fluffy vegetative growth to conidiation (Lee and Adams 1994b). The small molecule proposed by Schuren is obviously not Thn1, but it could be part of a pathway in which Thn1 operates. Since some, but not all, of the pleiotropic effects of *thn1* mutations were overcome by incubation with wild-type hyphae (Schuren 1999), Thn1 may be part of a complex network of signaling, as is true for FlbA in *A. nidulans* (Hicks *et al.* 1997; Adams *et al.* 1998; Yu *et al.* 1999).

Identification of *thn1* provides a starting point for elucidating the components of what is likely to be a G protein-mediated signaling system that regulates pathways for vegetative and fruit body development in *S. commune*. It remains to be determined whether Thn1 exerts any effect on the G protein-regulated pathway of pheromone response that controls sexual development, a pathway in which there is a longstanding interest. As more is learned about *scooter*, we hope this transposon will become useful as a tool for gene tagging in the quest to understand these signaling pathways.

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