A Mushroom-Inducing DNA Sequence Isolated From the Basidiomycete, *Schizophyllum commune*

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ABSTRACT

A DNA sequence capable of inducing the *de novo* development of fruiting bodies (mushrooms) when integrated into the genome of unmated, nonfruiting strains of the Basidiomycete *Schizophyllum commune* has been isolated and partially characterized. This sequence, designated *FRT1*, overrides the normal requirement of a mating interaction for fruiting in this organism. It has been shown to integrate stably in different chromosome locations and appears to be *trans*-acting. It also enhances the normal process of fruiting that occurs after mating. Additional DNA sequences with similarity to *FRT1* were detected within the genome of the strain of origin by hybridization of labeled *FRT1* DNA to blots of digested genomic DNAs. *FRT1* and the genomic sequences similar to it were shown to be genetically linked. Southern hybridization experiments suggested sequence divergence at the *FRT1* locus between different strains of *S. commune*. A testable model for how *FRT1* may act as a key element in the pathway for the differentiation of fruiting bodies is presented as a working hypothesis for further investigation.

FRUITING bodies in the homobasidiomycete *Schizophyllum commune* normally develop from dikaryotic cells, the dikaryon being formed by the mating of two haploid homokaryons heteroallelic for the mating-type genes *A* and *B* (reviewed in RAPER 1988; STANKIS, SPECHT and GIASSON 1990). The *A* and *B* genes are unlinked to each other and are each comprised of two linked loci α and β. Two homokaryons are fully compatible if they are different at $A\alpha$ and/or $A\beta$, and different at $B\alpha$ and/or $B\beta$. Each of the four loci is multiallelic (RAPER, BAXTER and ELLINGBOE 1960; KOLTIN, RAPER and SIMCHEN 1967; STAMBERG and KOLTIN 1972), with the number of possible mating types in the worldwide population exceeding 20,000. Sexual reproduction takes on special significance in light of the fact that *S. commune* has no specialized structure for asexual reproduction in its life cycle. In addition to the requirements for heteroallelism at the mating-type loci, fruiting has a dependence upon other genetic loci of undetermined location (RAPER and KRONGELB 1958). Appropriate environmental conditions such as light, low CO_2 tension and temperature are necessary for the induction of fruiting (NIEDERPRUEM 1963; PERKINS and GORDON 1969; RAUDASKOSKI and VIITANEN 1982). There exists, however, the relatively rare phenomenon of homokaryotic fruiting, which in contrast to dikaryotic fruiting, does not usually culminate in mature fruiting bodies; homokaryotic fruits seldom sporulate. The activity of the mating-type genes seems to be required for sporulation to occur. Homokaryotic strains containing mutations for constitutive function in the $A\beta$ and $B\beta$ mating-type loci (called *Acon Bcon*) are self-fertile and are able to both fruit and sporulate (RAPER, BOYD and RAPER 1965). Fruiting in other homokaryons with no mutations in the mating-type loci may occur either spontaneously (RAPER and KRONGELB 1958; ESSER, SALEH and MEINHARDT 1979; LESLIE and LEONARD 1980), in response to mechanical injury (LEONARD and DICK 1973), or in response to the addition of the biochemical fruit-inducing substance, FIS (LEONARD and DICK 1968). The response to these three stimuli are thought to be related but genetically separable (LESLIE and LEONARD 1979a). A minimum of six genes have been hypothesized to be required for control of the initiation of homokaryotic fruiting of the three types listed above (LESLIE and LEONARD 1979b). Certain cerebrosides have been identified, including those from *S. commune*, that induce the formation of fruiting bodies (KAWAI 1987; KAWAI and IKEDA 1982).

MULDER and WESSELS (1986) isolated a number of cDNA clones that correspond to mRNAs that increase in abundance during dikaryotic fruiting. Expression of these genes (called *Sc* genes) is controlled by environmental factors that are necessary for fruiting, such as CO_2 and light (WESSELS, MULDER and SPRINGER 1987; YLI-MATTILA, RUITERS and WESSELS 1989) and is also probably regulated at least indirectly by the mating-type genes (RUITERS, SIETSMA and WESSELS 1988). Some of these fruiting-specific genes are also expressed during homokaryotic fruiting (RUITERS, SIETSMA and WESSELS 1988; YLI-MATTILA *et al.* 1989). A mutation that prevents homokaryotic fruit-

ing in an *Acon Bcon* strain also blocks the expression of a number of the *Sc* genes (SPRINGER and WESSELS 1989). The concept of a common developmental pathway shared by homo- and dikaryotic fruiting has been strengthened by recent genetic studies in which it was demonstrated that certain homokaryotic fruiting alleles also affected dikaryotic fruiting (YLI-MATTILA *et al.* 1989).

In the present study we describe the isolation and preliminary characterization of a DNA sequence from *S. commune* that induces the production of fruiting bodies in recipient transformants upon integration into the genome of nonfruiting homokaryons. This sequence, designated *FRT1*, appears to override the usual controls on fruiting imposed by the mating-type genes, and induces fruiting at a premature stage in the life cycle of *S. commune*.

MATERIALS AND METHODS

Strains, growth conditions and genetic techniques: *S. commune* strain V 57-34 (*Aα3 Aβ5, Bα2 Bβ2, dom2, ura1, trp1*) was derived by a series of two crosses in which H 9-4 (*Aα3 Aβ5, Bα2 Bβ2, dom2*) was first crossed with 12-44 (*Ax Bx, ura1*; FROELIGER *et al.* 1987) to combine *Aα3 Aβ5, Bα2 Bβ2, dom2* with *ura1* to generate the strain V 55-21. This strain was then crossed with 72-4 (*Aα5 Aβ7, Bα? Bβ?, trp1*; MUNOZ-RIVAS *et al.* 1986) to incorporate the *trp1* auxotrophic marker into strain V 57-34 . Both V 57-34 and 72-4 were used as transformation recipients for detecting the biological activity of the cloned *FRT1* sequence. H 4-6 (*Aα4 Aβ6, Bα1 Bβ1*) and H 1-40 (*Aα1 Aβ1, Bα3 Bβ2*) were used for testing the effect of *FRT1* in transformants on dikaryotic fruiting. Unless mentioned otherwise, standard techniques for culturing and genetic analysis of *S. commune* were employed (RAPER and HOFFMAN 1974). Trp^+ *S. commune* strains were cultured on CYM medium, while Trp^- strains were cultured on CYM supplemented with 0.8 g/liter L-tryptophan (Sigma).

The genomic library of *S. commune* was constructed by GIASSON *et al.* (1989) in the cosmid vector pTC20 using DNA from strain H 9-1 (*Aα1 Aβ1(1), Bα3 Bβ2(1), pab1, bug*) containing constitutive mutations for the mating-type loci *Aβ* and *Bβ* (*Acon Bcon*). This homokaryotic strain is self-fertile and both fruits and sporulates, unlike the other strains used in this study. *Bug* (*Bug's ear*) is a fruiting mutation that results in (but does not induce *de novo*) a great number of small fruiting bodies that sporulate profusely (RAPER and KRONGELB 1958). Genomic DNAs from strains H 4-40 (*Aα4 Aβ6, Bα1 Bβ1*), V 46-14 (*Aα4 Aβ1(1), Bα1 Bβ2(1), def1, trp1*), along with strains H 9-1, 72-4, and V 57-34 were isolated for use in DNA-DNA hybridization experiments. DNA fragments of cosmid clone pSF1 were subcloned in pGEM-7Zf(+) (Promega Corp., Madison, Wisconsin). Plasmids and cosmids were propagated in *Escherichia coli* strains DH5α and DH1, respectively. Subclones containing unidirectional deletions from both ends of the *FRT1* subclone pSF3 were produced using the Erase-a-Base system (Promega), based on a procedure developed by HENIKOFF (1984). Molecular sizes of DNA fragments separated by gel electrophoresis were estimated by using the 1-kb ladder DNA (BRL Life Technologies, Gaithersburg, Maryland) as a size standard.

DNA isolation, transfer and hybridization conditions: *S. commune* DNA was isolated by the mini-prep method of ZOLAN and PUKKILA (1986). Cosmid and plasmid DNAs were isolated and manipulated utilizing standard techniques (AUSUBEL *et al.* 1987; MANIATIS, FRITSCH and SAMBROOK 1982). DNA was electrophoretically separated in agarose gels and transferred to nylon membranes (Hybond-N, Amersham Corp., Arlington Heights, Illinois). Labeling of DNA probes was by the random hexamer primer method of FEINBERG and VOGELSTEIN (1983) using [α-^{32}P]dCTP (>3000 Ci/mmol, Amersham) to a specific activity of at least 2×10^8 cpm/μg. High stringency hybridization (6 × SSC, 65°) of labeled probes to nylon filters and subsequent washes (0.1 × SSC, 65°) were performed according to the manufacturer's procedures (Hybond-N, Amersham).

Pulsed-field gel electrophoresis of chromosomal DNAs: Chromosomal-sized DNA of *S. commune* was prepared and separated by pulsed-field gel electrophoresis as described previously (HORTON and RAPER 1991). Methods of chromosomal DNA transfer and hybridization conditions were as described for other DNAs except that gels were soaked in two changes of 0.25 M HCl for 15 min each in order to nick the DNA before transfer to the membranes.

Screening of a *S. commune* cosmid library: A cosmid library made by GIASSON *et al.* (1989) of genomic DNA from *S. commune* strain H 9-1 (*Acon Bcon*) and containing the *S. commune TRP1* gene was screened with a labeled probe made from DNA of the chromosome containing the *B* mating-type loci (linkage group (LG) II; RAPER 1990). The chromosomal DNA was eluted from a preparative pulsed-field gel of separated *S. commune* (strain 72-4) chromosomes (band 2, HORTON and RAPER 1991). For efficient recovery of the chromosomal DNA, gel slices were first equilibrated with the appropriate restriction enzyme buffer and the DNA was digested overnight with *Eco*RI. DNA fragments were then eluted from the gel slices by the Gene Clean II procedure (BIO 101, La Jolla, California), as described by the manufacturer. Colony blots of the plated cosmid library were hybridized with a probe of α-^{32}P-labeled *B* chromosomal DNA under high stringency conditions according to the manufacturer's procedures (Hybond-N, Amersham). Both strongly and weakly hybridizing colonies were picked as putative *B* chromosome clones. DNA from cosmids and plasmids was isolated using standard CsCl-EtBr ultracentrifugation techniques (AUSUBEL *et al.* 1987; MANIATIS, FRITSCH and SAMBROOK 1982), and used to transform protoplasts from Trp^- *S. commune* strains to prototrophy. Plasmid clone pAM1 (MUNOZ-RIVAS *et al.* 1986) containing the *S. commune TRP1* gene was used in transformation experiments as a control. Plasmid clone pEF3 (FROELIGER *et al.* 1987) containing the *S. commune URA1* gene (LGII) was used as a check to see if this gene was present on a clone among the putative *B* chromosome clones selected.

***S. commune* transformation and screening of transformants:** Protoplasts from Trp^- strains were prepared for transformation experiments by a modification of the methods of SPECHT *et al.* (1988), as described by HORTON and RAPER (1991). Transformation of *S. commune* was performed as described by SPECHT *et al.* (1988), except that β-mercaptoethanol (final concentration 100 μM) was added to the protoplast-DNA mixture and protoplasts were regenerated in CYM + 0.6 M sucrose. In screening of Trp^+ transformants for fruiting body development, transformation plates were placed inverted in the light at room temperature (24°) after 3 days of incubation in the dark at 30°.

Matings to test the effect of *FRT1* on dikaryotic fruiting: Twelve Frt^+ Trp^+ transformants (using pSF1 cosmid DNA containing *FRT1* and *TRP1*) and 12 Trp^+ transformants (using *TRP1*-containing pAM1 DNA) were selected to be

tested in matings for fruiting competence. Half of each transformant type (six Frt^+ Trp^+ and six Trp^+) were derived from the Trp^- strain V 57-34, while the other half were derived from the Trp^- strain 72-4. Each of the 24 transformants was mated with two wild-type tester strains, H 4-6 and H 1-40. A mating of a Frt^+ Trp^+ transformant (the experimental mating) was always performed on the same plate with the same tester as a control mating of a Trp^+ transformant derived from the identical recipient strain. The same pair of transformants were mated on a separate plate with the other tester strain. Forty-eight matings were performed in all.

Inocula for matings were cut to identical size from the growing edge of 3-day-old subcultures growing on semisolid CYM agar plates at 30° in the dark. Paired inocula were placed 5 mm apart and 15 mm from the edge of the Petri plate. The pairs of inocula for each of the two matings (four separate inocula in all) were on opposite sides of the plate, 5.3 cm apart. The plates containing the matings were incubated inverted for 53 hr in the dark at 30°, then transferred, also inverted, to constant light (13 μmol s^{-1} m^{-2} quantum, cool white fluorescent light) for 175 hr at room temperature (24°). During this period the matings were examined for dikaryon formation (as signified by clamp connections), development of fruiting bodies and sporulation.

Sampling of basidiospores and quantitation of sporulation: Basisiospores were collected from the lid of the Petri plate onto which the spores had been released from the fruiting bodies. The spore prints that emanated from the two matings on a plate were distinct and spores from each mating were collected separately in 1 ml of sterile distilled water. A standard curve was constructed which showed a positive linear relationship between the absorbance of the spore suspensions at 550 μm and spore density as determined by quantitation with a haemocytometer. This linear relationship was valid between optical density readings of 0.05 to 0.75, corresponding to a spore density of 0.4×10^7/ml to 5.5×10^7/ml. Optical density measurements were converted to number of spores/ml by reading from the standard curve. Samples were read either immediately, or stored at −20° for later measurement. Frozen spore suspensions were thawed on ice and thoroughly mixed before quantitation. Measurements of the spore samples made before and after freezing were identical.

RESULTS

Isolation of a cosmid clone that induces fruiting bodies: Our initial goal was to isolate a genomic clone containing either a *Bα* or *Bβ* mating-type gene, or a clone that complemented a closely linked morphological mutant, *dom2*, which could be used to isolate the *B* genes by chromosome walking. These genes are present on LGII, as is the auxotrophic mutation *ura1* (RAPER 1990). A cosmid library made by GIASSON *et al.* (1989) from *S. commune* genomic DNA isolated from an *Acon Bcon* strain H 9-1 , and containing the selectable marker *TRP1* was screened with a probe made from *B* chromosome DNA (derived from strain 72-4) that was eluted from a preparative pulsed-field gel (band 2, HORTON and RAPER 1991). One hundred and fifty-three clones that hybridized to the probe were selected. A probe made from *URA1* DNA eluted from the plasmid clone pEF3 (FROELIGER *et al.* 1987) hybridized to two distinct clones in the pool (not shown), indicating there was some enrichment for *B* chromosomal sequences. DNA from sets of five cosmid clones each was used to transform the recipient strain V 57-34 (*Aα3 Aβ5, Bα2 Bβ2, dom2, ura1, trp1*) to prototrophy for tryptophan. During the course of screening for *DOM2* and the *B* mating-type genes, we noticed that a significant proportion (17%) of the Trp^+ transformants from one particular set of five cosmid clones were fruiting. Fruiting bodies appeared a few days after the transformation plates had been brought out of the incubator (30°, dark) into the laboratory (24°, light) for observation. The DNAs from each of the five cosmid clones from this set were tested individually, and one clone (pSF1), was found to induce fruiting bodies in approximately 75% of the over one thousand Trp^+ transformants examined (Figure 1a). GIASSON *et al.* (1989) found that a similar proportion (46 out of 61) of Trp^+ transformants expressed *Aα* mating-type activity when a cosmid containing the *Aα4* and *TRP1* genes was used to transform a *Aα1 trp1* recipient of *S. commune*. No homokaryotic fruiting was observed among thousands of Trp^+ transformants when either a plasmid clone containing the *S. commune TRP1* gene (pAM1, MUNOZ-RIVAS *et al.* 1986) or cosmid clones other than pSF1 were used as the source of donor DNA. The fruit-inducing ability of clone pSF1 was not restricted to one recipient strain; a similar percentage of Trp^+ transformants also produced fruiting bodies when another homokaryotic Trp^- strain 72-4 was used. We have called this fruit-inducing DNA sequence *FRT1*.

It was possible that the transforming DNA was simply complementing a fruiting mutation present in both recipients used in these experiments. This possibility was unlikely, however, for several reasons. There was no morphological or genetic evidence of altered mating-type activity in Frt^+ transformants. The two strains used as transformation recipients were wild type except for *trp1* and *dom2*, and were therefore unlikely to contain a fruiting mutation that could be complemented by the cloned *FRT1* sequence. Furthermore, each strain was capable of normal fruiting when mated with each other and other strains to form a dikaryon. The degree of fruiting body development and the number of fruiting bodies produced varied somewhat depending upon the individual transformant and the strain used as a recipient for transformation (Figure 1, b and c). None of the fruiting bodies induced to form in the recipient homokaryons were observed to sporulate. Interestingly, some of the preformed homokaryotic fruiting bodies did sporulate after dikaryotization through mating with a compatible homokaryon, and produced postmeiotic haploid spores that when germinated, were shown to segregate in a ratio of 1:1 for the Frt^+ *vs.* Frt^- phenotype.

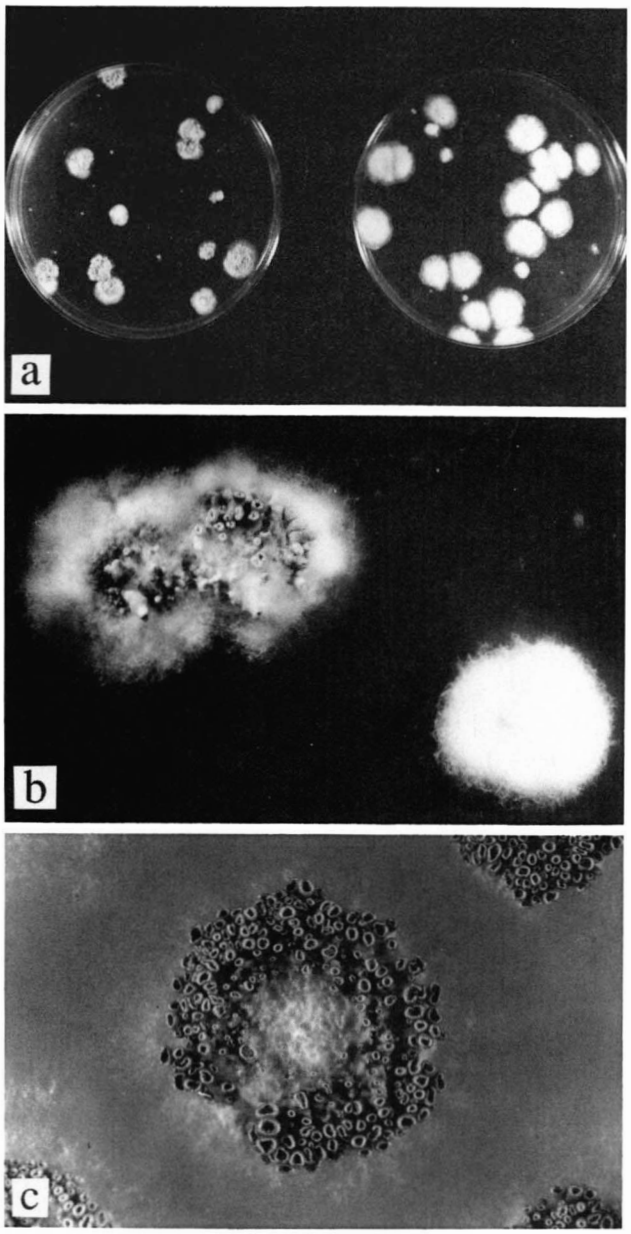

FIGURE 1.—*FRT1* induces fruiting in homokaryotic recipients in transformation. (a) On the plate (87-mm diameter) at left are Trp$^+$ transformants of a *dom2* strain (V 57-34) of *S. commune*, most of which are producing fruiting bodies induced by *FRT1* (contained in the pSF1 cosmid clone). On the plate at right are nonfruiting transformants of the same strain transformed with the *TRP1* gene only. (b) Close-up of two fruiting and one nonfruiting Trp$^+$ transformants. (c) Subcultures of fruiting Trp$^+$ Frt$^+$ transformants of strain 72-4. Note the differences in morphology of the induced fruiting bodies in this strain *vs.* V 57-34.

The fruiting phenotype is stable through meiosis and is expressed when *FRT1* is integrated at various genomic locations: Matings between a Trp$^-$, nonfruiting homokaryotic strain (72-4) and four different Frt$^+$ Trp$^+$ transformants of strain V 57-34 (using pSF1 as donor DNA) produced progeny of which roughly half were Trp$^+$. In a total of four crosses, 87 out of 107 of the Trp$^+$ progeny expressed the fruiting phenotype (Table 1); none of the Trp$^-$ progeny from these crosses were observed to fruit. This result was indicative of a relatively stable integration of pSF1 cosmid clone DNA (*TRP1* ectopically linked to *FRT1*) into the genome of each of the transformants tested. In cross 4 (transformant 4 × 72-4), there were more Frt$^-$ Trp$^+$ progeny observed (11 out of 30 Trp$^+$, Table 1) than expected. We suspect that in transformant 4 there had been two separate integration events during transformation: one involving *TRP1* linked to *FRT1* (on pSF1), the other involving only *TRP1*, which might have been separated from *FRT1* prior to integration. To test whether or not the *FRT1* sequence had integrated in the same genomic location in the four different transformants, representative Frt$^+$ Trp$^+$ progeny from these crosses were mated in various combinations. Progeny of the transformants were mated, rather than the transformants themselves, because the latter were all of the same mating-type and were therefore incompatible. The progeny were scored for Trp$^+$ because *TRP1* was present on the cosmid pSF1, and therefore should be closely linked to *FRT1* when integrated into the recipient's genome, except perhaps for one of the integrated *TRP1* sequences of transformant 4. Almost all of the progeny in this second series of crosses would be expected to be Trp$^+$ if the transforming DNA had integrated in similar genomic locations in the original, individual transformants. A 3:1 segregation ratio for Trp$^+$:Trp$^-$ would be expected if integration of the transforming DNA was in different (unlinked) genomic locations. By this reasoning, it appears that *FRT1* was integrated in a similar genomic location in those progeny derived from transformants 1, 2 and 3; 93–95% of the second generation progeny were Trp$^+$ (crosses 5 and 6, Table 2). Crosses between Trp$^+$ Frt$^+$ progeny derived from transformant 4 and progeny derived from transformants 2 and 3 produced progeny of which only 54 and 39% were Trp$^+$, respectively (crosses 7 and 8, Table 2). The reason for an observed ratio of less than 3:1

TABLE 1

Analysis of first generation progeny from outcrosses of four Frt$^+$ Trp$^+$ transformants mated with the Trp$^-$ strain 72-4 to test for cosegregation of *TRP1* and *FRT1*

Cross	Mating of Trp$^+$ Frt$^+$ transformant × Trp$^-$ tester	Trp$^+$ Progeny	
		Sample size	% Frt$^+$
1	Transformant 1 × 72-4	25	84
2	Transformant 2 × 72-4	26	85
3	Transformant 3 × 72-4	26	96
4	Transformant 4 × 72-4	30	63
Total		107	87

The cosmid clone pSF1 containing the *S. commune TRP1* gene and 38 kb of genomic DNA from strain H 9-1 including *FRT1* was used to transform the Trp$^-$ strain V 57-34. None of the Trp$^-$ progeny from any of the crosses were observed to be Frt$^+$.

TABLE 2

Analysis of second generation progeny obtained by crossing selected, compatible Frt$^+$ Trp$^+$ progeny of crosses 1–4 listed in Table 1

Cross	Mated pair of Trp$^+$ Frt$^+$ progeny	Sample size	% Trp$^+$
5	Progeny cross 1 × progeny cross 2	110	93
6	Progeny cross 2 × progeny cross 3	44	95
7	Progeny cross 2 × progeny cross 4	80	54
8	Progeny cross 3 × progeny cross 4	78	39

Progeny were screened for their ability to grow in the absence versus presence of L-tryptophan in the medium, in order to test whether or not cosmid pSF1 had integrated in similar genomic locations in the original transformants. Two different progeny from transformant 4 (cross 4 of Table 1) were tested in crosses 7 and 8. Fruiting of the progeny was not scored.

for Trp$^+$:Trp$^-$ is unclear; it is certainly not because of the possible presence of a second integrated copy of *TRP1* in the isolates derived from transformant 4. If an additional copy of *TRP1* had been present, a ratio of greater than 3:1 would have been expected for Trp$^+$:Trp$^-$. The proposed second integration event involving *TRP1* was probably at a sufficient distance from the first integration that the *TRP1* gene was segregated away from *FRT1-TRP1* in the Frt$^+$ Trp$^+$ progeny of transformant 4 that were tested. The data are best explained by the integration of *FRT1* at a different genomic location in transformant 4 as compared to that of the other three transformants analyzed. In all four cases, spore germination on tryptophan-supplemented medium exceeded 85% and the Frt$^+$ phenotype segregated independently of the *B*-linked *dom2* mutation present in strain V 57-34.

The presence of *FRT1* enhances dikaryotic fruiting and sporulation: To determine whether or not *FRT1* has an effect in dikaryons as well as in homokaryons, the rate and quantity of fruiting body development and basidiospore production were compared in dikaryons containing an integrated *FRT1* sequence (experimentals) *vs.* isogenic dikaryons without integrated *FRT1* (controls). Twenty-four Trp$^+$ transformants, half of which had been transformed with the *FRT1, TRP1*-containing cosmid pSF1, the other half with the *TRP1*-containing control plasmid pAM1, were tested in matings for fruiting competence. Half of all transformants were derived from the Trp$^-$ strain V 57-34, while the other half were derived from another Trp$^-$ strain (72-4) of a different mating type. All 24 transformed isolates were mated with the two compatible, wild-type tester strains, H 4-6 and H 1-40, which were selected because they were known to be capable of good fruiting in most dikaryotic combinations.

The stage of fruiting was observed to be more advanced at a given time point, and enhanced overall, in those dikaryons which were derived from a *FRT1*-containing transformant, as compared to dikaryons of the control matings. The degree of enhancement varied with strain differences but was consistently apparent between isogenic pairs. The overall enhancing effect of *FRT1* on dikaryotic fruiting is illustrated in Figure 2, which shows the relative degree of fruiting in dikaryons derived from two isogenic isolates of *S. commune strain* V 57-34, one a Frt$^+$, Trp$^+$ transformant, the other a Trp$^+$ transformant, each mated with the tester strain H 4-6. The *FRT1*-containing dikaryon (left, Figure 2) consistently displayed more advanced fruiting as compared to the control (right, Figure 2)

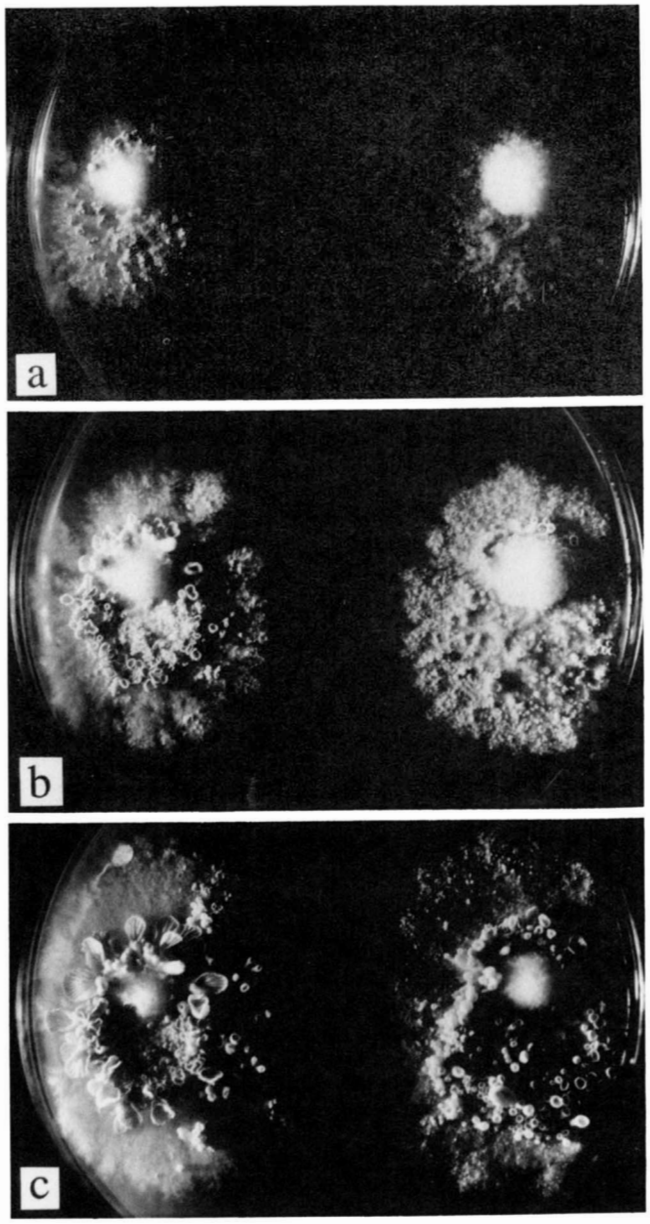

FIGURE 2.—The effect of *FRT1* on dikaryotic fruiting. On the left is a mating between a Frt$^+$, Trp$^+$ transformant of *S. commune* strain V 57-34 (top) and tester strain H 4-6 (bottom). On the right of the same plate (87-mm diameter) is a control mating between a Trp$^+$ transformant of strain V 57-34 and the same tester. Note the earlier and more extensive production of fruiting bodies in the mating with the Frt$^+$ transformant at time points (a) 92 hr, (b) 132 hr, (c) 156 hr after coinoculation of the plates.

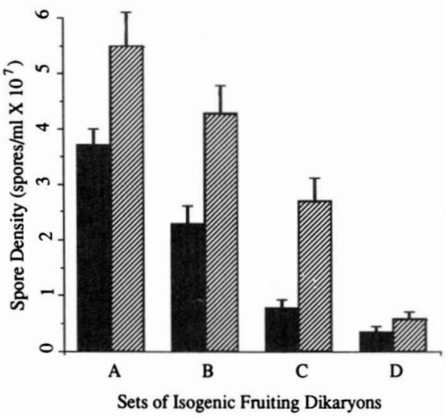

FIGURE 3.—The effect of *FRT1* on spore production by dikaryotic fruiting bodies. Comparative sporulation in non-*FRT1* containing (solid bars) *vs.* *FRT1*-containing (hatched bars) sets of isogenic, fruiting dikaryons. The lines above the bars represent standard error of the mean number of spores released by six dikaryons. The mating sets were: (A) V 57-34 transformants × H 4-6 ; (B) 72-4 transformants × H 4-6; (C) V 57-34 transformants × H 1-40; and (D) 72-4 transformants × H 1-40. The matings in set C fruited and sporulated so much more poorly than those in the other three sets that the spores were collected at a later time (228 hr after inoculation) as compared to the others (156 hr after inoculation).

at each recorded time point. By 156 hr after coinoculation of the cultures (Figure 2c), the experimental mating (containing *FRT1*) had produced well developed and sporulating fruiting bodies, while the control had not. The observed differences in the rate of fruiting did not result from different rates of dikaryon formation between the isogenic pairs; dikaryons were formed in both experimentals and controls at about the same time, within a period of 53–68 hr after mating.

The degree of sporulation was enhanced in the dikaryons of each mated pair that contained a Frt^+ transformant. Within each set of matings, the experimentals always produced more spores at a given time point than did the controls; however, there was considerable variation in sporulation between sets of matings involving different strains. Overall, matings in set A (V 57-34 transformants × the H 4-6 tester) sporulated most prolifically, while sets B (72-4 transformants × H 4-6) and D (72-4 transformants × H 1-40) showed an intermediate level (Figure 3). Matings in set C (V 57-34 transformants × H 1-40) had not sporulated to any extent at 156 hr after inoculation, the time at which spores from the other sets of matings (A, B, D) were collected. Spores were collected from these matings at 228 hr after inoculation, by which time enough spores had been released to be measurable. The spore density at this time was actually greater than that determined for set D at 156 hr (Figure 3). We also tested the effect of *FRT1* when present in both mates. Frt^+ Trp^+ transformants of two compatible strains, V 57-34 and 72-4 were mated and compared to matings of Frt^+ Trp^+ transformants

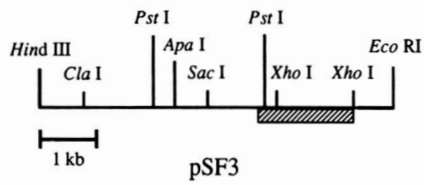

FIGURE 4.—Map of selected restriction enzyme recognition sites of the 6.3-kb insert of pSF3, which contains *FRT1*. The terminal *Eco*RI site is contained within a short segment of DNA derived from the cosmid vector pTC20. The smallest sized fragment (1.7 kb) that has so far been determined to be active in transformation is indicated by the shading.

× Trp^+ transformants, each plate having its own control (Trp^+ × Trp^+). No greater enhancement of fruiting or sporulation was seen when both partners contained *FRT1* as compared to when only one partner was transformed with *FRT1* (data not shown).

Subcloning of *FRT1* and defining its minimum active sequence: Eight *Hin*dIII fragments of the cosmid clone pSF1 were subcloned into the plasmid vector pGEM-7Zf(+) and tested for biological activity. Because the plasmid vector did not contain a selectable marker for transformation of *S. commune*, the subcloned fragments were introduced into protoplasts from the Trp^- strain 72-4 in cotransformation experiments with *TRP1* DNA from pAM1. Only one of the subcloned fragments tested (pSF2), containing 6.3 kb of *S. commune* DNA and 3 kb of cosmid DNA, was active in inducing fruiting in transformation recipients. The inclusion of a 3-kb sequence derived from the cosmid vector was verified by localizing the unique *Pst*I site of the ampicillin resistance gene of pTC20 [the *Pst*I site is not present in the same gene found in pGEM-7Zf(+)], as well as by DNA-DNA hybridization experiments using vector DNA as a probe (data not shown). The 6.3-kb *Eco*RI-*Hin*dIII fragment was then subcloned, and the resulting recombinant plasmid, pSF3, was also able to induce fruiting upon transformation into recipient strains. Although a direct comparison of the percentage of Trp^+ transformants induced to fruit by pSF1 and pSF3 was not possible, we routinely observed that in experiments using pSF3 and pAM1, 25–35% of the Trp^+ transformants obtained were also Frt^+ (and therefore cotransformed with pSF3). A map of recognition sites for selected restriction enzymes was constructed for pSF3 (Figure 4). Subclones of pSF3 containing unidirectional deletions from either side of the clone were generated by the procedure of HENIKOFF (1984) using exonuclease III digestion. These deletion subclones were tested for biological activity by cotransformation of strain 72-4 with pAM1. The minimum region of pSF3 determined to be necessary for fruiting has so far been defined to be 1.7 kb (shown as the shaded portion of the restriction map, Figure 4), a segment of the clone that is almost completely overlapped by a 1.4-kb *Xho*I fragment of pSF3. It is possible that the minimum-

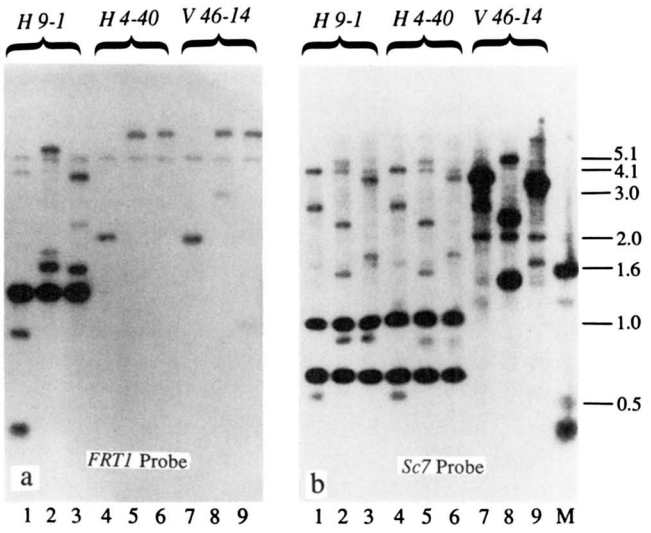

FIGURE 5.—Southern blot of digested *S. commune* genomic DNAs hybridized with *FRT1* and *Sc7* probes. In each grouping of three lanes (*e.g.*, 1–3, 4–6, 7–9) the first, second and third lanes correspond to genomic DNAs digested with *Xho*I and *Pst*I, *Xho*I and *Cla*I, *Xho*I and *Hin*dIII, respectively. Genomic DNAs were isolated from the three *S. commune* strains indicated at the top. The *FRT1* probe was made from the 1.4-kb *Xho*I fragment of pSF3. Note the hybridization to different-sized bands obtained with the *FRT1* (a) and *Sc7* cDNA (b) probes. The *FRT1* probe hybridizes much more intensely with the genomic DNA of the strain from which it was isolated (H 9-1) than with DNAs from the other two strains examined. Lane M contained kb ladder DNA which was used as a molecular size marker. Hybridization of the *Sc7* probe to DNA in this lane is to fragments derived from pBR322.

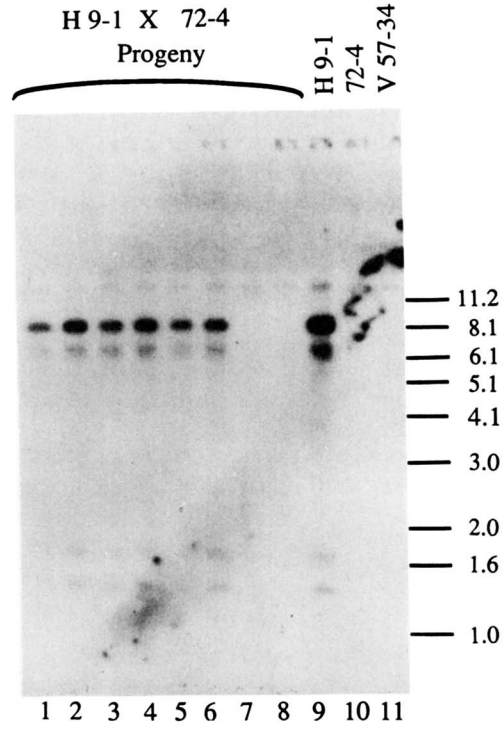

FIGURE 6.—*FRT1* and similar genomic sequences cosegregate through meiosis. A Southern blot of digested genomic DNAs from progeny (lanes 1–8) of a cross between strains H 9-1 and 72-4 was probed with the 1.4-kb *Xho*I fragment (containing *FRT1*) from pSF3. Lanes 9 and 10 correspond to digested genomic DNAs of the parental strains H 9-1 and 72-4, respectively. Lane 11 is genomic DNA from strain V 57-34. All DNAs were digested with *Eco*RI and *Hin*dIII. DNAs from six out of eight progeny shown in the figure had a hybridization pattern like that of the H 9-1 parent, while DNAs from two of the progeny hybridized in a manner similar to strain 72-4. In all, 18 out of 24 progeny analyzed had the H 9-1 pattern; six showed faint hybridization to the *FRT1* probe characteristic of the 72-4 parent. A background smear is evident across the top of lanes 10 and 11, and on the bottom of lanes 3 and 4.

sized piece of DNA active in transformation could be still smaller, as we have not yet analyzed deletions within the 1.7-kb region.

Evidence of sequence divergence at the *FRT1* locus: The genomic organization of *FRT1* was examined by probing digested genomic DNAs of three *S. commune* strains with the 1.4-kb *Xho*I fragment that is overlapped by the active region of *FRT1*. It is apparent from an examination of Figure 5a that the *FRT1* probe hybridizes much more intensely to the genomic DNA of the strain from which it was derived (H 9-1), than to the DNAs of two other homokaryotic strains, H 4-40 and V 46-14. This differential hybridization intensity between strains was not observed when the same blot was reprobed with *Sc7* (Figure 5b), a cDNA clone corresponding to an mRNA expressed differentially in fruiting dikaryons (MULDER and WESSELS 1986). THE *Sc7* cDNA probe was also observed to hybridize to some fragments of the 1-kb ladder DNA used as a molecular size standard. The probable cause for this was the presence of contaminating vector sequences (pGEM) in the hybridization probe made from gel-purified *Sc7* cDNA. The pGEM sequences would then hybridize to those bands in the 1-kb ladder DNA which were derived from the vector pBR322. The genomic DNAs from the two strains used as transformation recipients for testing *FRT1* activity, 72-4 and V 57-34, also did not hybridize very intensely to the same probe (Figure 6). The weak hybridization was more detectable after overexposure of the autoradiogram. A probe made from the 6.3-kb insert of pSF3 also showed differential hybridization between H 9-1 genomic DNA and that of strains H 4-40 and V 46-14 (data not shown). A possible explanation for these results is that there exists some sequence divergence at the *FRT1* locus between different *S. commune* genomes. Restriction fragment length polymorphisms were also evident between the DNAs of the strains examined. Enough sequence conservation must be present at this locus to account for the observed binding of the *FRT1* probe to the other genomic DNAs analyzed in these hybridization experiments.

***FRT1* has similarity to other sequences in the *S. commune* genome:** Since there are no recognition sites for *Pst*I, *Cla*I, or *Hin*dIII within the 1.4-kb *Xho*I fragment from pSF3 (Figure 4), a single copy sequence would be expected to hybridize to a single fragment of this size in probings of genomic DNA digested with

both *Xho*I and any of the enzymes listed above. Fragments of H 9-1 genomic DNA of the predicted size (1.4 kb) did bind this probe, but the presence of additional hybridization signals of varying intensity to other bands (Figure 5a) suggests that other sequences with similarity to *FRT1* exist in this genome.

The 1.4-kb *Xho*I fragment containing *FRT1* was also used as a probe in hybridizations of blots of genomic DNAs digested with enzymes that cut only outside of this fragment (see restriction map of pSF3, Figure 4). Four *Eco*RI-*Hin*dIII fragments of H 9-1 genomic DNA of sizes 8.7, 6.7, 1.75 and 1.4 kb were observed to hybridize to this probe, the largest sized band hybridizing most intensely (Figure 6). Four fragments of 22, 12.5, 8.2 and 6.2 kb were observed to bind to the same probe in H 9-1 genomic DNA digested with *Eco*RI only (data not shown). Taken together, these data strongly suggest the presence in the genome of other distinct sequences with similarity to *FRT1*.

***FRT1* and similar genomic sequences cosegregate through meiosis:** The *Acon Bcon* strain (H 9-1) containing *FRT1* was crossed with one of the strains used as a transformation recipient (72-4) and a sample of 24 Trp^- homokaryotic progeny (not *Acon Bcon*) were analyzed for segregation of *FRT1* sequences. Genomic DNA isolated from these progeny was digested with *Eco*RI and *Hin*dIII and the blots probed with the 1.4-kb *Xho*I fragment containing *FRT1*. The hybridization pattern of eighteen of these progeny was the same as that of the H 9-1 parent. The digested genomic DNA from the other six progeny bound to the same probe only faintly, similar to the 72-4 parent. Hybridization of genomic DNA from eight progeny is shown in Figure 6, along with DNAs from the two parents (H 9-1 and 72-4), and the other strain used as a recipient in transformation studies with *FRT1*, V 57-34. Three quarters of the progeny had the H 9-1 pattern of hybridization to the *FRT1* probe, while one quarter had the 72-4 *FRT1* genotype. *FRT1* and the genomic sequences similar to it were inherited together, indicating that they are linked. Restriction mapping and hybridization analysis of the pSF1 cosmid (not shown) indicated that in addition to the *FRT1* sequence found on pSF3, another sequence with strong similarity to *FRT1* lies within the cosmid insert, and is no more than 25 kb away. This finding supports the concept of close linkage of *FRT1* and its related sequences.

Wild-type homokaryons of the H 9-1 *FRT1* "type" do not express the fruiting phenotype and cannot be induced to fruit when transformed with cloned *FRT1*: None of the 24 Trp^- progeny (wild type for the mating-type genes) that were analyzed from the cross of H 9-1 and 72-4 were observed to fruit spontaneously. This included the 18 progeny whose genomic DNA hybridized intensely to the *FRT1* probe, and therefore have the H 9-1 type of *FRT1* genomic organization. This indicates that in the absence of both the *A* and *B* developmental pathways being activated (through *Acon Bcon* or mating), that the presence of the linked *FRT1* sequences of the H 9-1 type is not sufficient to allow fruiting to occur. When five Trp^- progeny of the H 9-1 *FRT1* type were cotransformed with pSF3 and pAM1, none of the over 200 Trp^+ transformants obtained were observed to fruit. As expected, the controls transformed with pAM1 only did not fruit either. Fruiting was observed in approximately 30% of the Trp+ transformants derived from each of the four Trp^- progeny of the 72-4 *FRT1* type that were cotransformed with pSF3 and pAM1. These results suggest that the presence of *FRT1* sequences derived from H 9-1 does not allow the expression of the fruiting phenotype when transformed with cloned *FRT1* (pSF3).

DISCUSSION

We have isolated a DNA sequence, *FRT1*, that can induce the development of fruiting bodies upon integration into the genomes of vegetatively growing homokaryons that otherwise will not fruit. This induction of homokaryotic fruiting has been demonstrated to occur in a number of different Trp^- strains tested so far, and is stable both mitotically and meiotically. Preliminary genetic analysis of Frt^+ transformants indicated that *FRT1* can induce its developmental effect when integrated in at least two genomic locations, suggesting that it acts in *trans*. Although homologous integration of large DNA fragments via transformation is the rule rather than the exception in *S. commune* (C. A. SPECHT, personal communication), we have found evidence of nonhomologous integration of the 48-kb pSF1 cosmid DNA (counting vector) in at least one of four Frt^+ Trp^+ transformants examined. Small DNA fragments (<11 kb) integrate homologously only rarely into the genome of this organism, no more than 2% of the time. The relatively high incidence of Trp^+ Frt^+ transformants when cotransformed with pAM1 (*TRP1*) and small subclones of *FRT1* DNA (6.3 kb or less) supports the idea that expression of *FRT1* is not dependent upon homologous integration.

The positive effect of integrated, cloned *FRT1* is not confined to the induction of fruiting in homokaryons, but appears to influence dikaryotic fruiting as well. An enhancing effect on the early development of normal dikaryotic fruiting bodies was evident from a comparison of the time of fruiting and spore production in dikaryons derived from matings involving Frt^+ Trp^+ *vs.* Frt^- Trp^+ transformants with compatible tester strains. Further evidence that *FRT1* is a key element in the pathway for differentiation of dikaryotic fruiting bodies came from the observation that

preformed homokaryotic fruiting bodies, induced by cloned *FRT1*, could be dikaryotized by migrating nuclei from a compatible mate and, under the influence of the now activated mating-type genes, could produce postmeiotic spores.

The homokaryotic strain from which *FRT1* was isolated (H 9-1) is capable of fruiting and sporulating on its own. It differs genotypically in two main respects from the nonfruiting homokaryotic strains that can be induced to fruit by integration of cloned *FRT1*: (1) H 9-1 has constitutive mutations in the mating-type genes which "turn on" the dikaryotic pathway, and (2) H 9-1 has other genomic sequences with similarity to *FRT1*. By contrast, strains 72-4 and V 57-34 have wild-type genes for mating and their genomes contain sequences that only faintly hybridize to *FRT1*. An analysis of the progeny from a cross between H 9-1 and 72-4 showed not only that *FRT1* and its related sequences from strain H 9–1 were inherited together and independently of the mating-type factors, but that the endogenous *FRT1* sequence was incapable of inducing homokaryotic fruiting in progeny wild type for the mating-type genes. These results suggest that the mating-type genes must be activated in order for endogenous *FRT1* to be expressed. Furthermore, these nonfruiting progeny that contained endogenous *FRT1* and related sequences of the H 9-1 "type" did not express the fruiting phenotype when transformed with cloned *FRT1*, whereas transformants of wild-type progeny with *FRT1* of the 72-4 "type" did fruit. In summary, fruiting occurs in strains that contain *FRT1* of the H 9-1 type only when the mating-type genes are activated; cloned *FRT1* is able to induce fruiting when the mating-type genes are inactive, but only when it is integrated into a genome that does not contain endogenous *FRT1* of the H 9-1 (self) type.

How can these seemingly paradoxical phenomena be explained? The data presented here support neither a position effect nor alteration in copy number as an explanation for the fruit-inducing activity of cloned *FRT1* when introduced into recipient homokaryons. Rather, *FRT1* appears to be functioning in a *trans*-acting and nonadditive manner. Cloned *FRT1* might be a mutated version of the endogenous sequence, or perhaps the clone contains an active region of the gene that has been separated from a linked regulatory element that exists in the genome of origin. If it is a mutated sequence (not the result of cloning), it would have to be dominant and functionally constitutive. It therefore should be expressed when residing in homokaryons in which the mating-type genes are not activated (wild type); this was not observed in any of the progeny of this type derived from a cross of strains H 9-1 and 72-4. If the sequence had somehow become mutated in the process of cloning, then it would be expected to induce fruiting when transformed into "self"; this did not occur either. More likely, endogenous *FRT1* is a nonmutated sequence which is normally under the control of a regulatory element (probably a repressor) and which was separated from this element in the process of cloning. The active region of cloned *FRT1* lies within 0.7 kb of the end of the cloned sequence. The putative repressor could be fairly closely linked. An obvious test of this idea would be the isolation of clones overlapping the genomic region we have cloned already, and the testing of these clones in transformation experiments. An overlapping clone that cannot induce fruiting would be highly suggestive of a linked controlling element not present in pSF1 that normally represses *FRT1* activity. A controlling element some distance away (over 35 kb) from *FRT1* would not be detected by this kind of analysis.

We propose a testable model for *FRT1* action that can serve as a working hypothesis for further investigation. Although other models are possible, we feel that this one fits best all of the observations to date. In a normal wild-type homokaryon, fruiting does not occur; therefore *FRT1* activity must be repressed. When *A* and *B* mating-type loci are activated, either by mating or in an *Acon Bcon* homokaryon, *FRT1* and the fruiting pathway are "turned on." In this model, *FRT1* has different allelic forms, and linked to this locus is an allele-specific repressor. When *A* and *B* are expressed, there is an inactivation of the repressor's function; *FRT1* is "turned on" (not repressed). Presumably there is a common element in every allele-specific repressor of *FRT1* which interacts with a factor produced either directly or indirectly as a result of the activity of the mating-type genes. If our cloned version of *FRT1* is lacking its allele-specific repressor, then it would not be repressible when integrated into a transformation recipient with a different *FRT1* allele linked to a different repressor. *FRT1* would then be expressed despite the absence of mating-type activity. Endogenous repressor specific to the cloned *FRT1* allele is present in transformation recipients with the H 9-1 *FRT1* type, and is able to repress the activity of both endogenous and cloned copies of *FRT1*. According to this model, the rare phenomenom of spontaneous homokaryotic fruiting observed in wild-type strains [see STAHL and ESSER (1976) for a review] could be explained by "leakyness" of the repressor system.

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