MUTATIONS OF THE *a* MATING-TYPE GENE IN *NEUROSPORA CRASSA*

A. J. F. GRIFFITHS AND A. M. DELANGE

Department of Botany, The University of British Columbia, Vancouver V6T 1W5, Canada

Manuscript received September 12, 1977
Revised copy received November 7, 1977

ABSTRACT

In Neurospora, the mating-type locus controls both mating ($A + a$ is fertile) and heterokaryosis ($A + a$ is incompatible). The two alleles appear stable: no novel fertility reactions have ever been reported, and attempts to separate fertility and heterokaryon incompatibility functions by recombination have been unsuccessful. In the present approach the locus was studied through a mutational analysis of heterokaryon incompatibility function. A selection system was used that detects vigorous ($A + a$) heterokaryotic colonies against a background of inhibited growth. Twenty-five mutants of an *a* strain were produced following mutagenic treatment with UV and NG: 15 were viable as homokaryons and 10 were not. All but one were infertile, but most showed an abortive mating reaction involving the production of barren, well-developed perithecia with *A* and (surprisingly) *a* testers. None of the mutants complement each other to restore fertility. Seven mutants have been mapped to the mating-type locus region of chromosome *1*. Restoration of fertility was used to detect revertants, and these were found in five out of the eight mutants tested. (A dose response was observed). In four cases incompatibility was fully restored and in one case it was not.—The results suggest two positive actions of the locus when in heterozygous (A/a) combination (the stimulation of some stage of ascus production and the inhibition of vegetative heterokaryosis), and one positive action in homozygous combination (the production of a perithecial inhibitor).

MATING is the union of individuals for the purpose of establishing a diploid zygote during sexual reproduction, and successful mating involves plasmogamy and karyogamy. After mating, the other stages of reproduction, meiosis and the production of fertile meiotic products, are the normal consequences. In heterothallic fungi, mating is achieved only if the individuals paired are of different mating type. Mating type is determined by mating-type genes. In bipolar forms such as *Neurospora crassa*, there are two mating types, *A* and *a*, each determined by a codominant allele of the single mating-type locus, located on the left arm of linkage group I. The success of the mating is detected not through the observation of plasmogamy and karyogamy *per se*, but by the sexual morphogenesis associated with sexual reproduction. Usually, this involves the observation of macroscopic perithecia and later the "shooting" of large numbers of black ascospores. Although successful mating requires heterozygosity for the mating-

type alleles, the extent of their involvement in the mating process and subsequent events is not known: do these genes simply generate a signal for the initiation of mating, or is there continuing involvement at later stages of mating and even meiosis? The present study was initiated to pursue these questions through a mutational analysis of the mating-type locus.

In Neurospora, the mating-type alleles appear to be stable: no novel fertility reactions have ever been reported that might suggest a third allele, nor has mutational interconversion been reported. (If such changes were common, they should have been detected by the regular appearance of rare fertile perithecia in supposedly pure cultures). Mutations to null function, on the other hand, would probably result in male and female sterility. Female-sterile mutants are commonly encountered, but they are not usually simultaneously male sterile, and none have been associated with the mating-type locus (MYLYK and THRELKELD 1974; JOHNSON 1978). Thus, the mating functions of the locus did not seem amenable to mutational analysis.

However, the Neurospora mating-type locus is known to be bifunctional, controlling not only mating ($A + a$ is fertile) but also vegetative heterokaryosis ($A + a$ is incompatible) (BEADLE and COONRADT 1944). In the latter function, the locus acts just like the other known incompatibility loci in Neurospora (GARNJOBST and WILSON 1956; MYLYK 1975). The two functions have been found to be inseparable by recombination analysis (NEWMEYER, HOWE and GALEAZZI 1973). The present study utilizes the incompatibility function in a selection system for mutations affecting the mating-type locus. In this system, ($A + a$) heterokaryons were selected after mutagenic treatment of the *a* component, and the mutants, which were effectively null for the incompatibility function, were then studied further with respect to their mating functions and, as far as possible, to their genetic locus. In such a system, several possible types of mutational changes are possible *a priori*. First, mutator-like mutants of the kind shown to cause "homothallism" in yeast could be responsible for the change in *a* function by mutating *a* to *A* (see HARASHIMA, NOGI and OSHIMA 1974 for the HO system; HOPPER and HALL 1975b for the *cmt* system; and BLAMIRE and MELNICK 1975 for the *dmt* system). Second, the mating-type locus functions could be modified by unlinked mutations, such as *CSP* (HOPPER and HALL 1975a) and some *ste* mutations (MACKAY and MANNEY 1974) in yeast, or *tol* and *tol*-like mutations in Neurospora (NEWMEYER 1970, METZENBERG and AHLGREN 1973). Third, deletion of one mating type could occur. This is found to occur in spontaneous escape from mating-type generated heterokaryon incompatibility in Neurospora cultures growing in long growth tubes (DELANGE and GRIFFITHS 1975). Fourth, stable changes at the mating locus itself could be responsible: mutations at mating-type loci have been reported in *Saccharomyces cerevisiae* (see MACKAY and MANNEY 1974 for some *ste* mutations; and KASSIR and SIMCHEN 1976 for a^* mutations), *Schizosaccharomyces pombe* (see MEADE and GUTZ 1976 for mutations of the *h* locus), and the tetrapolar *Schizophyllum commune* (see RAPER and RAPER 1973 for mutations of the Bβ locus). From the

evidence to be presented, we conclude that the mutations induced in our system are mutations at the mating locus itself.

MATERIALS AND METHODS

Media and routine procedures

The media and routine manipulative procedures used were those described in DAVIS and DE SERRES 1970.

Strains

The following mutant alleles were used: *ad-3A* (2–17–814), *ad-3B* (2–17–128, 2–17–114, complementing) IL, *cyh-1* (KH52) IR, *fl* (fl^{p}) IIR, *his-3* (74A-Y152-M9) IR, *nic-2* (43002) IR, and *un-3* (55701-t) IL.

The study is based on two main types of strains that are the potential components of a heterokaryon. The genotype of one of these (to be called the reference strain) is *un-3*, *A*, *ad-3A*, *nic-2*. The genotype of the other (the treatment strain) is usually *a*, *ad-3B*$_{114}$, *cyh-1*, or in a preliminary experiment *a*, *ad-3B*$_{128}$, *cyh-1*+. The approximate linkage arrangement of markers in these strains is shown in Figure 1. Wild types used were standard Oak Ridge strains 74-OR8-1a and 74-OR23–1A. Heterokaryon tests showed that all the strains used had the same *het* locus constitution except for mating type, and that there was no *tol* (NEWMEYER 1970) associated compatibility.

Experimental design

Two cultures of compatible *het* locus constitution, but of opposite mating type, do not normally form a vigorous heterokaryon (see DELANGE and GRIFFITHS 1975 for discussion). If one component strain is treated with a mutagen, then change in the mating-type locus associated incompatibility function can be selected by observing heterokaryotic growth on minimal medium. In this basic system, the markers used had the following functions: the complementing *ad-3* alleles, and the *nic-2* allele were heterokaryon forcing markers, and also assisted in identifying the nuclear components. The *cyh-1* allele confers resistance to cycloheximide. Initial simulation experiments revealed that when heterokaryons heterozygous for this marker were plated on supplemented medium containing 10 mg/l of cycloheximide, two colony types were seen: large dense colonies that proved to be mainly *cyh-1*, or small wispy colonies which proved to be *cyh-1*+ or heterokaryotic. Although a minority of large colonies prove to be heterokaryotic, the production of appreciable numbers of large colonies provides a good indication of the presence of a viable *cyh-1* component in the heterokaryon, and also assists in the isolation and purification of this component. Cycloheximide resistance is evidently recessive in heterokaryons unless there is a high proportion of *cyh-1* nuclei. (In the preliminary experiment using a *cyh-1*+ strain, the recoverability of the mutated component could be checked only by the identification of *ad-3B*, *nic-2*+ isolates). *fl* (*fluffy*) is an allele that renders cultures aconidial. The *fluffy* strains are also highly fertile, so that these are very useful as protoperithecial parents in mating-type reaction tests. *un-3* is a nonsupplementable, heat-sensitive allele very closely linked

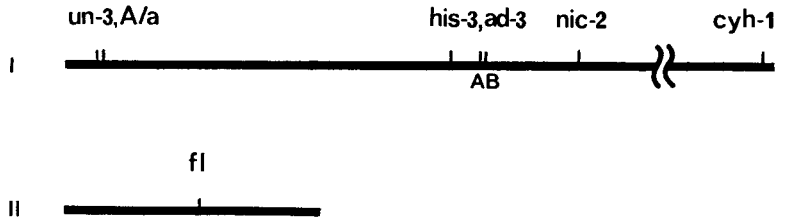


FIGURE 1.—Approximate linkage in genetically marked strains.

(< 0.1 mu) to the mating-type locus. Thus, any mutational event that deletes the mating-type locus might be expected to delete an adjacent *un-3*+ allele, rendering an *un-3*+/*un-3* heterokaryon temperature sensitive. *his-3* was used as a heterokaryon-forcing marker and as a nuclear marker in a specialized procedure to be described below.

Mutant induction and isolation

The treatment strain was mutagenized either with UV at 5×10^3 ergs/cm^2 for 0, 15, 30, 45, 70 or 100 sec in a stirred 20 ml conidial suspension, or with nitrosoguanidine (pH7) at 1.125 mM for 0 or 3 hours. Approximately 10^4 treated conidia were mixed with approximately 10^6 conidia from the reference strain and plated together on a plate of sorbose/fructose/glucose medium made with washed agar and supplemented with 50 mg/l nicotinic acid plus 1 mg/l adenine sulphate using an overlayer technique (NEWMEYER 1954). The latter supplements are limiting to both strains, but simulation experiments had proved that the supplements are necessary in order to permit sufficient growth for heterokaryotic contact to be made. For each treatment, a total of 10^6 conidia of the treatment strain were mutagenized. Two UV-induction experiments were run, the preliminary one using *ad-3B*$_{128}$, the main one using *ad-3B*$_{114}$, *cyh-1*; and one experiment was run with nitrosoguanidine using *ad-3B*$_{114}$, *cyh-1*. These will be referred to as experiments I, II and III, respectively.

After seven days, the plates were observed for growth. At this time the plates were covered with a background "lawn" of weak growth. Superimposed on this lawn, rare dense colonies could be seen that were then isolated. These colonies represent heterokaryons containing the presumptive mutations affecting incompatibility function, probably in close physical association, or even cytoplasmic contact, with the background genotypes. The colonies were grown on slants of minimal medium, then plated on minimal medium at low densities and reisolated. This procedure was repeated once more. By this stage, the heterokaryons presumably contained only reference strain nuclei and mutated treatment strain nuclei, and this was in fact borne out by later findings. In the treatments involving *cyh-1* components, conidia from each heterokaryon were next plated onto medium supplemented with adenine sulfate and cycloheximide for the purpose of recovering the mutated nuclear type as a homokaryon. The desired homokaryons were manifest as large colonies, distinct from the co-present wispy colonies. Some heterokaryons did not produce any large colonies when plated on this medium. The mutants in these cases were classified as "nonrecoverable," and were maintained as heterokaryotic stocks. Cultures from recoverable mutant nuclei were tested to confirm their *ad-3B*, *nic-2*+, *cyh-1* genotype. In the experiment using the *cyh-1*+ treatment strain, the heterokaryons were plated on supplemented medium, and approximately 200 colonies were isolated and tested for the *ad-3B*, *nic-2*+ genotype, which would indicate that the treated component was viable.

Mating and heterokaryon incompatibility tests on mutants

The mating reactions of the mutants were tested in several ways. First, the mutants were tested in heterokaryons (the stage just prior to final homokaryon isolation). The heterokaryons were inoculated into large test tubes containing supplemented liquid crossing medium and a strip of filter paper (see NEWCOMBE and GRIFFITHS 1972). The tubes were later scored for the presence or absence of perithecia and ascospores. Second, the mutant cultures, as homokaryons when recoverable or as heterokaryons when nonrecoverable, were jointly inoculated into liquid crossing medium with a wild-type strain of *A* or *a* mating type. Third, the mutant cultures were used both as conidial and as protoperithecial parents in standard crosses with wild types of both mating types in tubes of solidified crossing medium. Fourth, mutant cultures were used as conidial parents to fertilize fluffy protoperithecial parents of both mating types, grown on 100 mm diameter Petri plates. In this approach, the mating reaction was photographed. Interfertility between mutants was tested by inoculating all possible pair combinations of mutant cultures (including nonrecoverables) into liquid crossing medium. This was done to see if any new mating types had resulted from the mutations. Heterokaryon compatibility of mutants with *A* testers was confirmed by combining conidial suspensions of the mutant cultures with a suspension of the complementing reference strain of opposite mating type in unslanted tubes of

minimal medium. Also, the resulting heterokaryons were subcultured and placed at 37° to test for expression of the *un-3* allele.

Inter-complementarity of mutants

Since experiment I and experiments II and III utilized two different but complementing *ad-3B* alleles, recoverable mutant strains from each type could be combined to form heterokaryons on minimal medium. The resulting heterokaryons could then be used as protoperithecial parents in crosses on solid medium, with standard *A* and *a* wild-type conidial parents to determine whether or not the mutated components could complement each other and allow a fertile cross with wild type.

Carrying the mutants through a sexual cycle using heterokaryons

MYLYK and THRELKELD (1974) showed that female-sterile mutants could be carried through a sexual cycle by combining them with a normal nuclear component in a heterokaryon and using this heterokaryon as protoperithecial parent. We used this approach to try to carry the *a* mutants through a sexual cycle. The mutants were combined with a carrier strain of genotype *a, his-3, nic-2* to form a heterokaryon on minimal medium. This was crossed to an *A* wild-type conidial strain and the ascospores plated onto cycloheximide-containing medium. The production of large colonies would be an indication that the *a, ad-3, cyh-1* mutant component is present and has been carried through the cross. Such large colonies may be sampled and tested for the presence of the appropriate marker genes.

Reversion studies

Reversion to the incompatibility function is difficult to detect. However, since most mutants proved to be infertile, reversion to fertility was used instead. Homokaryons of recoverable mutants, or heterokaryons of nonrecoverable ones were irradiated with UV at 5×10^3 ergs/cm^2 for 0, 50 or 100 seconds in the case of *ad-3B, cyh-1* experiments and for 0, 30, 60 or 90 seconds in the case of *ad-3B, cyh-1+* experiments, in stirred 20 ml conidial suspensions. A total of about 10^8 conidia were irradiated at each dose. The irradiated suspensions were then used to fertilize *fl A* protoperithecial parents grown on Petri dishes. 100 plates were used for each dose. Reversion was detected by the appearance of rare perithecia and ascospores in restricted areas of the plates. Ascospores were isolated and heat shocked, and the resulting cultures were checked for appropriate marker segregations, as well as for heterokaryon incompatibility function.

RESULTS

No presumptive mutant-containing heterokaryons were produced spontaneously. In the three induction experiments, the following numbers of colonies were obtained: Experiment I (UV) 0 colonies at 0 seconds, 1 at 15, 5 at 30, 4 at 45, and 9 at 70. Experiment II (UV) 0 colonies at 0 seconds and 16 at 100. Experiment III (NG) 0 colonies at 0 hr and 14 at 3 hr. Thus, there was a total of 49 presumptive mutants. Of these, 13 failed to grow or grew very poorly upon transfer to tubes of minimal medium; these were rejected. The remainder produced vigorous orange heterokaryons at the initial isolation stage and throughout the purification platings. When subcultures of the final heterokaryons were grown at 37°, all grew, indicating that none expressed the *un-3* phenotype. Hence, the mutants probably did not contain deletions of the mating-type and adjacent regions of the type shown by DELANGE and GRIFFITHS (1975) to be responsible for some cases of escape from mating-type incompatibility in growth tubes.

The final heterokaryons were plated on cycloheximide-containing medium or other media as described in MATERIALS AND METHODS to determine whether or not the mutated components were recoverable as viable homokaryons. In Experiment I, five were recovered and 12 were not; in Experiment II, nine were recovered and three were not, and in Experiment III one was recovered and six were not. The UV treatment produced a higher proportion of recoverable mutants than did the NG treatment.

The recoverable mutants were then rigorously tested for the complete set of markers borne by that nuclear component. All bred true to the type expected, that is, *un-3*$^+$, *ad-3B*, *nic-2*$^+$ in Experiment I and *un-3*$^+$, *ad-3B*, *nic-2*$^+$, *cyh-1* in Experiments II and III. They were then combined with the reference strain to confirm their *het* compatibility: all gave vigorous orange heterokaryons, whereas the unmutagenized parental treatment strain gave the standard ($A + a$) heterokaryon reaction, consisting of small amounts of low wispy aconidial mycelium, with an associated brown pigment on the surface of the medium. This set of tests was repeated twice with identical results. Such heterokaryon tests confirm that the strains are genuine mutants.

Next the mating behavior of the mutants was tested. First, the final heterokaryons were allowed to try to "self" in liquid crossing medium. Only one mutant, 33, produced perithecia with ascospores; all the others produced neither in this test. In tests using homokaryons (or heterokaryons in the case of nonrecoverable mutants) as protoperithecial or conidial parents with Oak Ridge wild-type strains of either mating type, or in double inoculation crosses with the same Oak Ridge wild types, no perithecia or ascospores were produced, except for the case of mutant 33. When the mutants were used as conidial parents onto highly fertile fluffy protoperithecial parents on plates, however, a heterogeneous set of reactions was obtained, probably due to the highly protoperithecial nature of the fluffy strains and to the fact that large, uniform surface areas were now under examination instead of the rather restrictive conditions in test tubes. These fluffy crosses were attempted with all mutants from Experiments II and III, and representative ones from Experiment I. The results are summarized in Table 1 and photographic examples are shown in Figure 2. Several features of these data are listed below:

1. The unmutagenized control treatment strain does show a very weak reaction with the *fl a* strain, which is not seen in the water blank.

2. Only 33 is truly fertile, producing many black ascospores. Thus 33 has lost only its incompatibility function.

3. Since none of the other mutants produce ascospores, their incompatibility and fertility functions have been simultaneosuly inactivated by the mutation event(s). (The infertility of the nonrecoverable strains with the *fl A* tester is a good indication that these heterokaryons no longer contain a fertile *a* component).

4. Although complete fertility has been lost in the majority of cases, most still retain a vestige of *a* mating reaction, involving considerable advancement

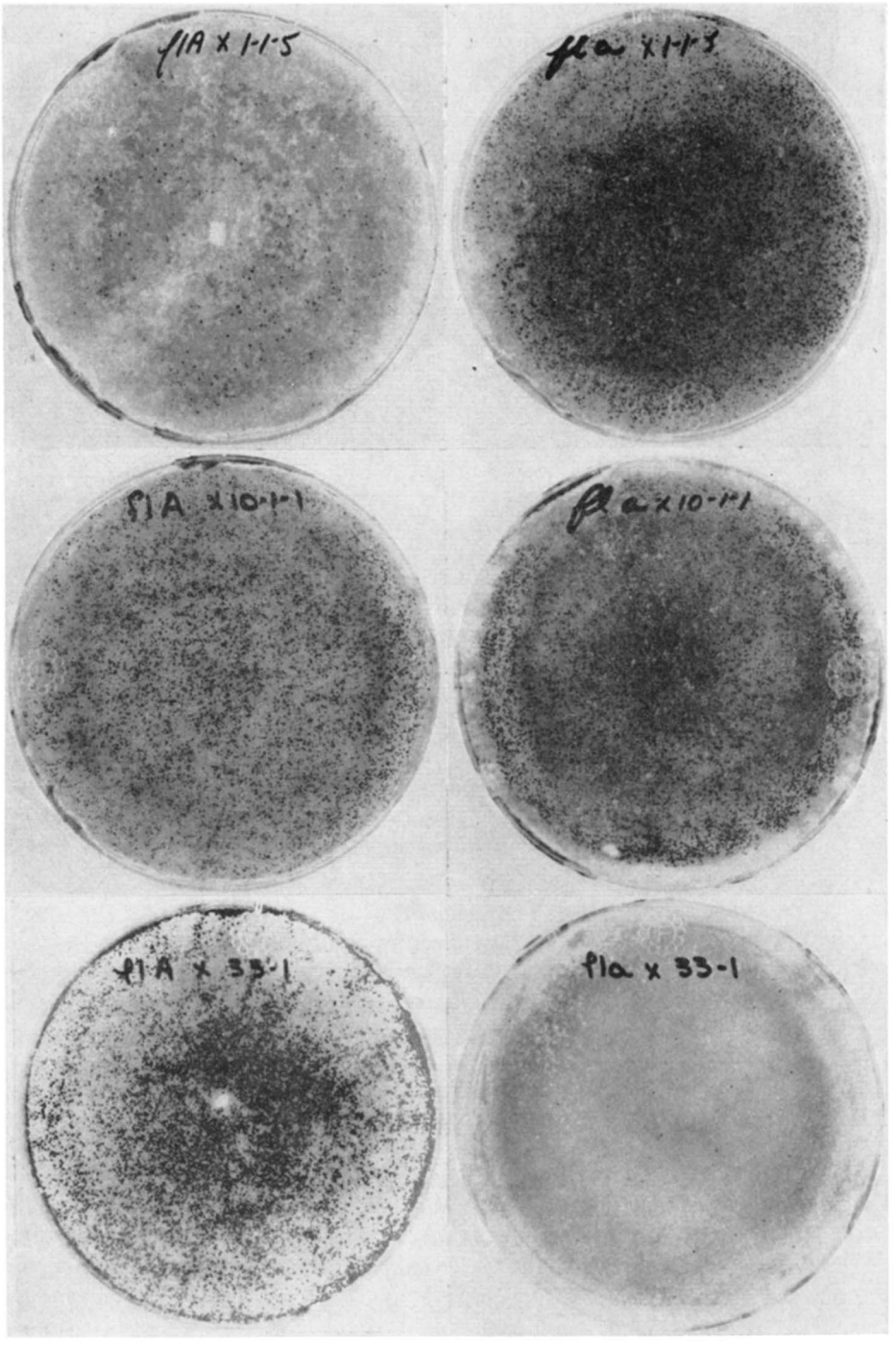
TABLE 1

Mating reactions of mutants using fluffy females

Mutant number	Induced by UV or NG in experiment I, II, III	× *fl A* (*a* mating reaction)		× *fl a* (*A* mating reaction)	
		No. perithecia per cm²*	Presence of ascospores**	No. perithecia per cm²	Presence of ascospores
Recoverable					
1	UV II	9	—	205	—
2	UV II	3	—	113	—
5	UV II	26	—	207	—
7	UV II	7	—	122	—
10	NG III	180	—	138	—
12	UV II	57	—	125	—
16	UV II	25	—	110	—
18	UV II	0	—	2	—
24	UV II	104	—	203	—
26	UV II	105	—	163	—
29	UV I	21	—	104	—
30	UV I	9	—	67	—
31	UV I	159	—	112	—
32	UV I	40	—	102	—
33	UV I	223	+	1	—
Nonrecoverable					
4	UV II	6	—	140	+
6	UV II	164	—	199	+
9	NG III	15	—	169	+
11	UV I	0	—	151	+
13	UV II	198	—	182	+
17	NG III	1	—	222	+
19	NG III	6	—	186	+
21	NG III	5	—	189	+
27	NG III	0	—	183	+
28	NG III	1	—	199	+
Control	—	151	+	11	—
Water blank	—	0	—	0	—

UV = ultraviolet radiation.
NG = nitrosoguanidine.
* Mean of three counts.
** Only two states observed; abundant ascospores, or none.

in the path of sexual morphogenesis. Strain 13 is the best example of this, producing large numbers of barren perithecia. Mutant 6 also produced large numbers of barren perithecia. Mutants 11, 17, 18, 27 and 28, at the opposite extreme, show complete absence of *a* mating reactions. In mutants showing a vestige of the *a* mating reaction, the perithecia were large, of a size comparable to normal perithecia, had no pronounced ostioles or necks, and contained no ascospores, asci or ascus initials. The stage of advancement through meiosis is not known. In no cases were ascospores shot onto the petri dish lid.


flA x 1-1-5
fla x 1-1-3
flA x 10-1-1
fla x 10-1-1
flA x 33-1
fla x 33-1

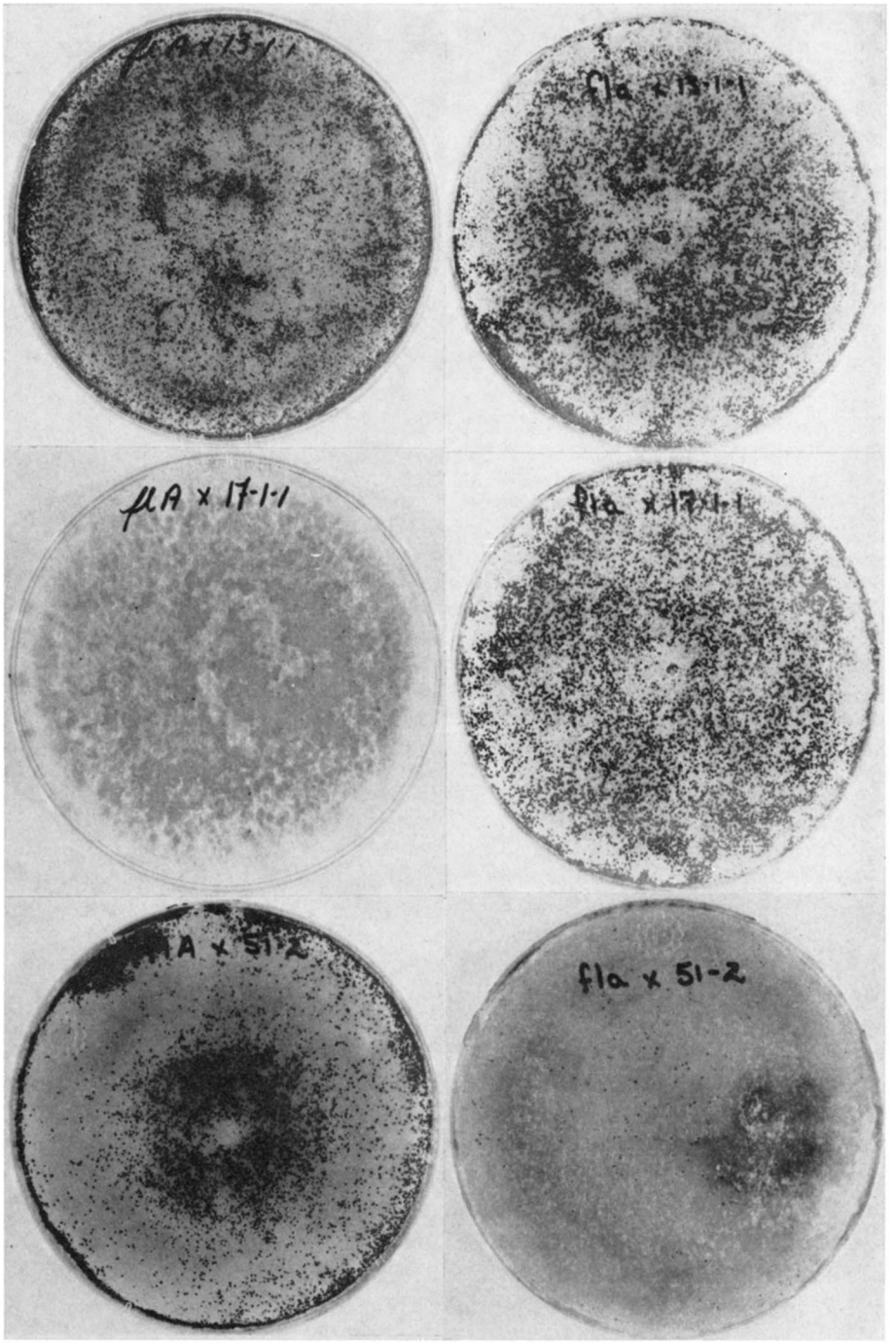


FIGURE 2.—Photographs of representative mating reactions of mating-type mutants. Mutants 1 and 10 are nonfertile recoverable, 33 is fertile recoverable, 13 and 17 are nonfertile non-recoverable, and 51 is an untreated control strain. In each of the pairs, the fluffy *A* female parent is to the left, and the fluffy *a* female parent is to the right.

5. The *A* mating reaction exhibited by all the nonrecoverable mutants is expected and attributable to the *A* reference component of these heterokaryons.

6. Very surprising are the *A* mating reactions shown by the homokaryotic recoverable mutants. Although none produced ascospores, the majority of such strains showed a stronger *A* than *a* mating reaction in terms of total numbers of perithecia. In such reactions the perithecia-like bodies were apparently identical to their counterparts in the *a* mating reactions, although no size quantification was attempted.

These test crosses were repeated with essentially similar results.

The mutant strains were inoculated in all possible pair combinations into crossing medium, but no perithecia were produced except, as expected, when 33 was paired with the heterokaryons of nonrecoverable mutants.

The homokaryotic recoverable mutants from Experiment I, bearing the *ad-3B*$_{128}$ allele and those from Experiments II or III, bearing the complementary *ad-3B*$_{114}$ allele have been paired in all possible combinations on minimal medium. All resulting heterokaryons grew vigorously, indicating successful complementation of *ad-3B* alleles. These heterokaryons were then crossed with Oak Ridge *A* wild type, doubly inoculated into liquid medium, and as male and female parents on solid medium in test tubes. No perithecia or ascospores resulted. Thus, the mutations did not complement to allow sexual reproduction. From the cross of $33 \times fl\ A$, ascospores were isolated, 20 of which proved to be *ad-3B, a*. These were each combined with the original reference strain to check their heterokaryon compatibility reaction. All 20 produced vigorous heterokaryons, indicating that the altered compatibility function in mutant 33 is linked to the *ad-3B–a* region, and, by inference, represents a mutation of the *a* locus itself.

In an attempt to carry infertile homokaryotic recoverable mutants through a sexual cycle, heterokaryons were formed by pairing six mutants with an *a*, *his-3*, *nic-2* strain. These heterokaryons were then crossed with an *A* male parent. Five out of the six crosses were performed in duplicate. Ascospores from these six crosses, when plated on cycloheximide-containing medium, produced appreciable numbers of distinct, large, colonies, and these colonies were sampled. In each sample, about half of the resulting cultures proved to be *ad-3B*, *cyh-1*, demonstrating that these individuals were derived from mutant nuclei and must have been carried through the cross. These ascospore isolate cultures were tested for their mating reactions. Thirty-seven cultures were tested from mutant 1, thirty-six from 2, thirty-eight from 5, seventeen from 7, thirty-four from 12 and sixty from 24. All gave the standard mutant *a* mating reaction on *fl* testers, that is a reaction with both *A* and *a* strains, and a stronger reaction with *fl a*. Thus, the mutation event in these cases is linked to the *ad-3* locus and by inference involves the mating-type locus itself. It is noteworthy that none of the cultures tested were recombinant for *ad-3B* and mating type. Based on recombinant frequencies in other crosses of comparable genetic background, about nine recombinants would have been expected in this total sample. The reason for the failure to recover recombinants is not known at present.

An attempt was made to revert a representative sample of eight mutants (no more attempts have been made because of the large number of plates needed). Three mutants, 1, 31, and 32 produced no revertants (perithecia and associated ascospores—see MATERIALS AND METHODS) at any UV dose. Strain 31 was used in a further attempt and still no revertants were observed. Strains, 5, 11, 13, 18 and 26, however, produced rare revertants and these results are plotted in Figure 3. In some cases, the experiment was repeated with results as indicated, A response to dose is evident in most cases. The ascospores discharged in the cases of 5, 11 and 26 were mostly (> 90%) black. Mutants 13 and 18, however, produced ascospores that showed a high proportion of white and intermediate shades in each perithecium produced by reversion: 20% to 31% for 13 and 64% to 68% for 18.

One hundred to 200 black ascospores were isolated from each reversion experiment and heat shocked. These were combined from three perithecia in the case of mutant 1, three in mutant 5, three in mutant 13, four in mutant 18, and four in mutant 26. Germination was low in the cases of 13 (30%) and 18 (12%). The *fl*+ ascospores were tested against *fl A* and *fl a* testers to determine mating-

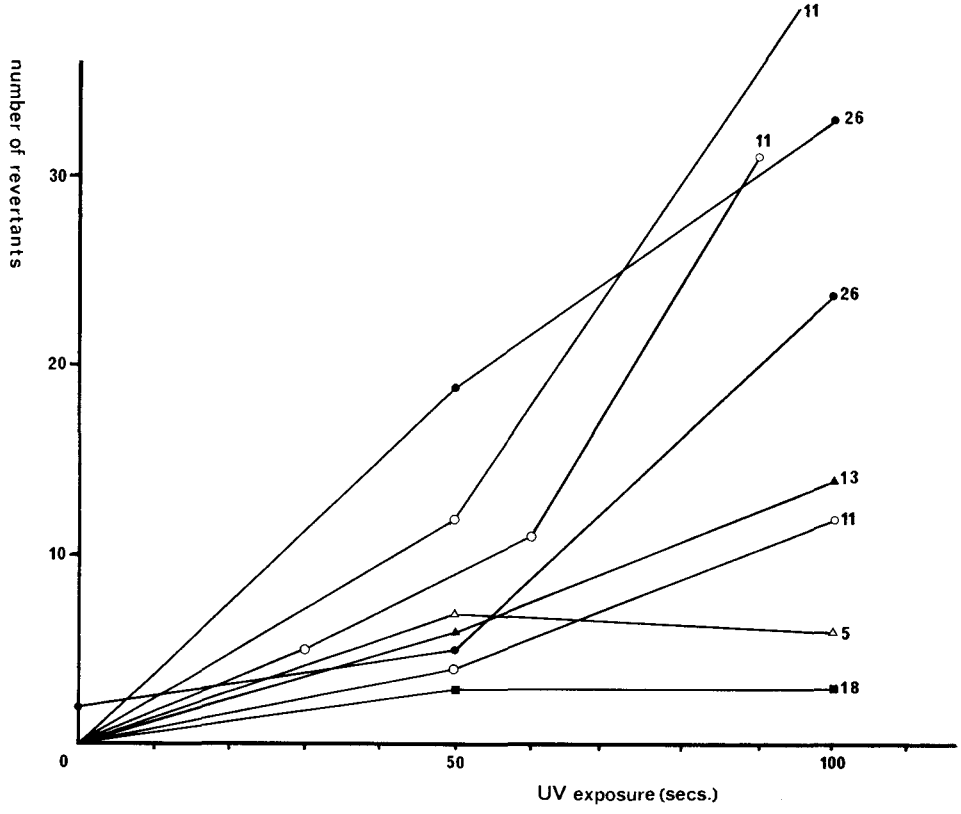


FIGURE 3.—Revertants obtained at various UV doses. (Each revertant is manifested as a fertile perithecium).

TABLE 2

Genotypes of ascospores resulting from reversion of mating-type mutants

Mutant number	Genotype of *fl*+ revertant ascospores				Total
	ad a	*ad A*	+ *a*	+ *A*	
1	23	0	1	25	49
5	24	2	3	26	55
13	14	0	0	13	27
18	8	0	0	6	14
26	15	0	1	20	36

type. In each case a normal mating-type reaction with only one tester was observed. The results, shown in Table 2, indicate that a functional *a* locus has been restored, appropriately linked to the *ad-3B* allele; and, since all *ad-3B* strains are fertile, argue against the notion that the phenotypic reversion is by a suppressor mutation. The proportions of white spores in crosses of *a*-revertant ascospore isolates to wild type were quite homogeneous within the mutant strains and averaged 17% for strain 1 (25 isolates), 15% for strain 5 (26 isolates), 16% for strain 13 (13 isolates), 41% for strain 18 (4 isolates), and 14% for strain 26 (16 isolates). Similar percentages were obtained in crosses of *A* ascospores to wild type, except that in the case of mutant 18, three out of the four isolates tested showed low percentages of white spores (about 15%) and in only one isolate was a high percentage of 66% white spores obtained. This suggests that the white spores and low germination percentages in the cases of mutants 18 and 13 are produced by some factor (possibly a rearrangement) that may or may not be lost by recombination. However, it is evidence that most *a* revertant ascospore isolates show normal crossing behavior.

Revertant ascospores of genotype *ad-3B, a,* (the numbers are seen in Table 2) were combined in heterokaryons with the original *A* reference strain on minimal medium. Revertants from mutants 1, 13, 18 and 26 produced reactions of the typical (*A* + *a*) incompatible type, indicating that the incompatibility function had been simultaneously regenerated upon reversion of the fertility function. In the case of mutant 5 revertants, however, 21 of the heterokaryons showed vigorous growth and three showed incompatibility. The simplest interpretation of this is that the reversion event has occurred at the *a* locus to restore fertility independent of the incompatibility function, making the revertants of mutant 5 similar to the forward mutant 33. The three strains showing incompatibility probably originated from a separate, independent reversion event.

DISCUSSION

We have described the isolation of mutants affecting the function of the mating-type locus. Furthermore, we believe the mutants to be of the *a* mating-type allele itself, for the following reasons.

1. Heterokaryon incompatibility and fertility, both known functions of the

mating-type locus, are, in the great majority of cases, mutated and backmutated simultaneously.

2. Linkage to the *a–ad-3B* region was demonstrated in all seven mutants tested. These were mutant 33, which maintained normal fertility function, and six mutants, which, in the absence of fertility function, were carried through a cross by a normal *a* heterokaryon component.

3. The inability of the mutant strains to complement each other in their fertility function indicates that the mutations are allelic or pseudoallelic, and by inference alleles of the mating-type locus. This point is strengthened by the demonstration that mutants may be carried through a sexual cycle by a normal nuclear component in a heterokaryon. Since the normal component succeeds in supplementing the inadequacies of a mutant nucleus, it is evident that internuclear transmission of gene products relevant to these mutants is effected, either in the vegetative mycelium or when the mutant nucleus has become isolated in a perithecium.

We propose the symbol a^m to designate the mutant *a* mating-type alleles.

Three broad classes of mutants were produced: recoverable infertile (a total of 14), nonrecoverable infertile (a total of 10) and recoverable fertile (1 only). The demonstration of recoverable mating-type mutants suggests that the known functions of this locus are not necessary for normal vegetative growth. Furthermore, these mutants grow at rates within the range normally encountered in Neurospora. The identification of the single recoverable fertile mutant and the fertile revertant of mutant 5, both of which have lost the vegetative incompatibility function, shows that while in most cases fertility function is lost concomitant with incompatibility function, the two functions are distinct and separable. This separation had already been demonstrated through the specific action of *tol* on the incompatibility function (NEWMEYER 1970). Nonrecoverable mutants could be attributed to lethal damage at the mating-type locus itself or to lethal damage elsewhere in the genome. The prevalence of these types of mutants in the nitrosoguanidine-treated set indicates that the second explanation is more likely; however, some mating type mutants could be lethal. The production of perithecia-like organs (called perithecia henceforth) in most ($a^m + a$) reactions was unexpected. Since this function mutates and backmutates with the other two functions, a third function of the mating-type locus is suggested.

Thus, we may distinguish and analyze three functions of the mating type locus on the basis of this study: vegetative incompatibility (manifested as unsuccessful heterokaryosis), the stimulation of some stage of ascus production (manifested ultimately by the production of normal ascospores), and the production of perithecia. The locus appears to exert its effects in some cases through heterozygosity and in others through homozygosity, as shown below.

In general, two main types of schemes can be imagined to account for differences in function between heterozygosity and homozygosity at one genetic locus, and these will be called type I and type II. In type I, heterozygosity provides the active state, and homozygosity of either allele provides the inactive state.

Either each allele provides a separate component essential for function, or both complement to give one functional component. If we assume that mutation generally acts in the direction of specificity to nonspecificity, then mutation of either allele may be expected to inactivate the heterozygous interaction. Thus, $(A + a^m)$, for example, would be functionally equivalent to $(A + A)$ or $(a + a)$. Since the selection system used in the present study was based on the detection of mutation in the direction from incompatibility to compatibility, it is likely that heterokaryon incompatibility represents the active state in Neurospora (rather than compatibility), and that upon mutation this function is lost. Thus, the incompatibility function shows a type I reaction. Ascus production, too, shows a type I reaction in that all the mutants but one (33) failed to produce ascospores.

In type II, homozygosity of either allele provides the active state, and heterozygosity the inactive. Here, mutation, to a^m for example, would generate the combinations $(a + a^m)$ and $(A + a^m)$ which would be functionally inactive like $(A + a)$, in contrast to the active combinations $(a + a)$ and $(A + A)$. Perithecial production appears to show a type II reaction because the majority of mutants produce $(a + a^m)$ combinations that resemble $(A + a)$ rather than $(a + a)$ or $(A + A)$ in this regard. According to this reasoning, the active state produced by $(A + A)$ or $(a + a)$ must involve active inhibition of perithecial development since the homozygous combinations show no perithecia. Such a perithecial inhibitor might be produced when a trichogyne and conidum of the same mating type come into contact, and would provide a positive block to selfing.

The position of the heterogeneous set of $(a^m + a^m)$ combinations in this scheme is not clear. They might be expected to represent heterozygosity and hence show perithecia, yet few peritheria were observed in the testtube crosses attempted. However, these reactions involve no fluffy component: fluffy could well provide

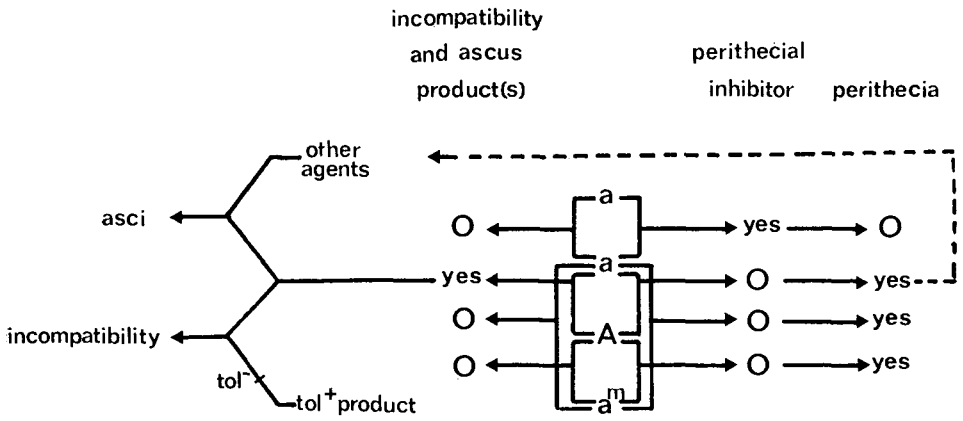


FIGURE 4.—Summary of the actions of the mating-type alleles, (the $A + A$ reaction is omitted).

an optimal background in which to observe the reactions of the mating-type locus. The precise role of fluffy in these reactions is presently under study.

The above conclusions are based on the behavior of the majority of the mutants at the phenotypic level. No inference about the gene products involved is implicit. The only assumption made is that mutation generally proceeds from an active to an inactive conformation. Undoubtedly the mutants represent a diverse set, and to attempt to explain all the various minority reactions would be premature.

It is apparent that the mating-type locus in Neurospora has at least three functions as revealed by this study. All functions generally mutate and back-mutate together, indicating that they are closely located at a single genetic locus, rather than at separate loci prevented from recombining (NEWMEYER, HOWE and GALEAZZI 1973).

The actions of the mating type locus as revealed by the present experiments are summarized in Figure 4.

Technical assistance was provided by SHERRY CHEW. Research was supported by Grant A6599 from the National Research Council of Canada.

LITERATURE CITED

BEADLE, G. W. and V. L. COONRADT, 1944 Heterocaryosis in *Neurospora crassa*. Genetics **29**: 291–308.

BLAMIRE, J. and L. M. MELNICK, 1975 The mating reaction in yeast. I. A new mutation involved in the determination of mating type. Molec. Gen. Genet. **140**: 243–252.

DAVIS, R. H. and F. J. DE SERRES, 1970 Genetic and microbiological research techniques for *Neurospora crassa*. In: *Methods in Enzymology*, Vol. XVIIA. Edited by H. TABOR and C. TABOR. Academic Press, New York.

DELANGE, A. M. and A. J. F. GRIFFITHS, 1975 Escape from mating-type incompatibility in bisexual ($A + a$) *Neurospora* heterokaryons. Can. J. Genet. Cytol. **17**: 441–449.

GARNJOBST, L. and J. F. WILSON, 1956 Heterocaryosis and protoplasmic incompatibility in *Neurospora crassa*. Proc. Nat. Acad. Sci. U.S. **42**: 613–618.

HARASHIMA, S., Y. NOGI and Y. OSHIMA, 1974 The genetic system controlling homothallism in Saccharomyces yeasts. Genetics **77**: 639–650.

HOPPER, A. K. and B. D. HALL, 1975a Mating type and sporulation in yeast. I. Mutations which alter mating-type control over sporulation. Genetics **80**: 41–59. ——, 1975b Mutation of a heterothallic strain to homothallism. Genetics **80**: 77–85.

JOHNSON, T. E., 1978 Isolation and characterization of perithecial development mutants in Neurospora. Genetics **88**: 27–47.

KASSIR, Y., and G. SIMCHEN, 1976 Regulation of mating and meiosis in yeast by the mating-type region. Genetics **82**: 187–206.

MACKAY, V. and T. R. MANNEY, 1974 Mutations affecting sexual conjugation and related processes in *Saccharomyces cerevisiae*. II. Genetic analysis of nonmating mutants. Genetics **76**: 273–288.

MEADE, J. H. and H. GUTZ, 1976 Mating-type mutations in *Schizosaccharomyces pombe*: Isolation of mutants and analysis of strains with an h^- or $h+$ phenotype. Genetics **83**: 259–273.

METZENBERG, R. L. and S. K. AHLGREN, 1973 Behaviour of *Neurospora tetrasperma* mating-type genes introgressed into *N. crassa*. Can. J. Genet. Cytol. **15**: 571–576.

MYLYK, O. M., 1975 Heterokaryon incompatibility genes in *Neurospora crassa* detected using duplication-producing chromosome rearrangements. Genetics **80**: 107–124.

MYLYK, O. M., and S. F. H. THRELKELD, 1974 A genetic study of female sterility in *Neurospora crassa*. Genet. Res. **24**: 91–102.

NEWCOMBE, K. D. and A. J. F. GRIFFITHS, 1972 Adjustable platforms for collecting shot asci. Neurospora Newsl. **20**: 32.

NEWMEYER, D., 1954 A plating method for genetic analysis in Neurospora. Genetics **39**: 604–618. ——, 1970 A suppressor of the heterokaryon-incompatibility associated with mating-type in *Neurospora crassa*. Can. J. Genet. Cytol. **12**: 914–926.

NEWMEYER, D., H. B. HOWE, JR. and D. R. GALEAZZI, 1973 A search for complexity at the mating-type locus of *Neurospora crassa*. Can. J. Genet. Cytol. **15**: 577–585.

RAPER, C. A., and J. R. RAPER, 1973 Mutational analysis of a regulatory gene for morphogenesis in *Schizophyllum*. Proc. Nat. Acad. Sci. U.S. **70**: 1427–1431.

Corresponding editor: P. DAY