Escape From *het-6* Incompatibility in *Neurospora crassa* Partial Diploids Involves Preferential Deletion Within the Ectopic Segment

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ABSTRACT

Self-incompatible *het-6*^{OR}/*het-6*^{PA} partial diploids of *Neurospora crassa* were selected from a cross involving the translocation strain, *T(IIL → IIIR)AR18,* and a normal sequence strain. About 25% of the partial diploids exhibited a marked increase in growth rate after 2 weeks, indicating that "escape" from *het-6* incompatibility had occurred. Near isogenic tester strains with different alleles (*het-6*^{OR} and *het-6*^{PA}) were constructed and used to determine that 80 of 96 escape strains tested were *het-6*^{PA}, retaining the *het-6* allele found in the normal-sequence LGII position; 16 were *het-6*^{OR}, retaining the allele in the translocated position. Restriction fragment length polymorphisms in 45 escape strains were examined with probes made from cosmids that spanned the translocated region. Along with electrophoretic analysis of chromosomes from three escape strains, RFLPs showed that escape is associated with deletion of part of one or the other of the duplicated DNA segments. Deletions ranged in size from ~70 kbp up to putatively the entire 270-kbp translocated region but always included a 35-kbp region wherein we hypothesize *het-6* is located. The deletion spectrum at *het-6* thus resembles other cases where mitotic deletions occur such as of tumor suppressor genes and of the hprt gene (coding for hypoxanthine-guanine phosphoribosyltransferase) in humans.

VEGETATIVE incompatibility is responsible for intraspecific self/nonself recognition during the assimilative phase of the life cycle in filamentous fungi. Vegetative incompatibility in *Neurospora crassa* can be caused by heteroallelism for any of at least ten heterokaryon incompatibility (*het*) loci, or for the mating-type locus, *mt* (PERKINS 1988). *N. crassa het* loci have been identified in two ways. Heterokaryon incompatibility function at *mt* and *het-c, -d, -e,* and *het-i* were first established by heterokaryon testing (BEADLE and COONRADT 1944; GARNJOBST 1955; WILSON and GARNJOBST 1966). Strains with complementary auxotrophic markers and different alleles at any one of these loci are unable to establish a stable, rapidly growing heterokaryotic mycelium on minimal medium. In contrast, *het-5, -6, -7, -8, -9* and *het-10,* were identified by analysis of partial diploids (MYLYK 1975). One-third of the viable progeny from a cross between an insertional or quasi-terminal translocation-bearing strain and a normal karyotype strain will have the translocated region duplicated (Figure 1). Homokaryotic progeny carrying this duplication will be visibly self-incompatible if the translocated region contains a *het* locus and different alleles occur at this locus in the two parental strains. This method circumvents the laborious preparation of near-isogenic strains that are required in conventional heterokaryon tests.

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A characteristic of both incompatible heterokaryons and self-incompatible partial diploids is that they can exhibit "escape" from the incompatible growth phenotype (PITTENGER 1964; NEWMEYER and TAYLOR 1967; DELANGE and GRIFFITHS 1975; VELLANI *et al.* 1994). Within 1–2 weeks, slow-growing incompatible colonies can suddenly exhibit an increase in growth rate. Based on analyses of genetic markers linked to *het* loci (NEWMEYER and GALEAZZI 1977; DELANGE and GRIFFITHS 1975), it is apparent that escape from heterokaryon incompatibility is due to the alteration of one of the incompatibility alleles. The physical basis of escape remained to be investigated, however, and several means of escape could be hypothesized. First, mitotic recombination or conversion in the duplicated region would result in escape through a loss of heterozygosity. Escape could also proceed following chemical modifications or point mutations within the duplicated DNA segments that silence genes in and around the incompatibility locus. Transient inactivation of genes in *N. crassa* through quelling (ROMANO and MACINO 1992), for example, could account for some characteristics of escape. Finally, self-incompatibility could be overcome by a mitotic deletion event within one of the duplicated regions. Possible mechanisms for inactivation of duplicated genes in plants, fungi and animals have been reviewed by FLAVELL (1995) and JUDELSON and WHITTAKER (1995). The objective of this study was to examine the physical basis of escape from self-incompatibility in

het-6 heterozygous duplications involving the insertional translocation in *T(IIL → IIIR)AR18.* We hypothesized that modifications, if detectable, would be those that selectively silence the *het-6* incompatibility function. Through molecular analyses, we found that escape is associated with large DNA deletions at the *het-6* locus. Similar methods would be useful for cloning other genes that restrict mitotic growth when duplicated.

MATERIALS AND METHODS

Strains and culture conditions: The genotypes and origin of the *N. crassa* strains used in this study are listed in Table 1. Throughout this paper "OR" in parentheses signifies that all unspecified *het* loci are those present in the Oak Ridge wild-type strains. The strain *T(IIL → IIIR)AR18,* subsequently referred to as *T(AR18),* has an interstitial segment of the left arm of LGII inserted into the right arm of LGIII (PERKINS and BARRY 1977). The translocated segment contains *het-6.* RFLP mapping strains (METZENBERG and GROTELUESCHEN 1992) were obtained from the Fungal Genetics Stock Center (FGSC, Department of Microbiology, University of Kansas Medical School, Kansas City, Kansas). Crossing and maintenance of Neurospora strains were as described in DAVIS and DE SERRES (1970). The Orbach/Sachs genomic library of *N. crassa* strain 74OR23-IV*A* in the cosmid vector pMOcosX (ORBACH 1994) was obtained from the FGSC and handled as described in VOLLMER and YANOFSKY (1986).

Growth rates were determined in one of two ways. Strains were inoculated at one end of 40-cm long race tubes containing 30 ml of Vogel's medium (VOGEL 1964) and appropriate nutritional supplements. Incubation was at 22° and the mycelial front was measured daily. Alternatively, petri plates containing Vogel's medium plus supplements were inoculated with strains at the center of the plate. Maximum colony radius was measured daily or at 2-day intervals, as appropriate.

Isolation of *het-6*^{PA} strains free of other incompatibilities with Oak Ridge strains: The *het-6*^{PA} allele used in this work originated in a strain of *N. crassa* from Panama, FGSC 2190, that also carried the linked *het-c*^{PA} and a number of other *het* alleles incompatible with their Oak Ridge counterparts (Table 1). It was necessary to introgress the *het-6*^{PA} allele into an otherwise Oak Ridge-compatible background. In brief, the strategy used was to cross FGSC 2190 to Oak Ridge-compatible RLM57-25 *pyr-4; inl; pan-2; nic-3 A. pyr-4* is fairly closely linked to *het-6* and even more closely linked to *het-C* (Figure 2). For the second step of introgression, a single *pyr-4*$^{+}$ *a* progeny with the above vitamin markers was crossed to an Oak Ridge-compatible *pyr-4 A* strain. Next, an *inl* mating type *A* derivative of a *pyr-4*$^{+}$ progeny was crossed to Oak Ridge-compatible RLM57-26 *pyr-4 arg-5; inl; pan-2 a.* Progeny were chosen that were *pyr-4*$^{+}$ *arg-5, i.e.,* they included Oak Ridge alleles to the right of *pyr-4.* For the fourth step of introgression, an isolate from the third cross, provisionally identified as *het-6*^{PA} *het-c*^{PA} *arg-5; inl; nic-3 a* was crossed to an Oak Ridge-compatible *pyr-4 A* strain, and progeny were scored for compatibility with Oak Ridge testers. Among *pyr-4*$^{+}$ isolates, 14/15 were incompatible, and among the *pyr-4* progeny, 4/16 were incompatible, suggesting that all remaining incompatibilities were linked to *pyr-4.* Of the four incompatible *pyr-4* progeny, putative crossovers between the *het-6*^{PA}–*het-c*^{PA} region and *pyr-4,* one was carried forward as RLM58-18. The presence of *het-6*^{PA} in RLM58-18 was confirmed by outcrossing it to two Oak Ridge-compatible *het-6* tester strains, *T(II → VI)P2869* and *T(II → V)NM149,* both of which are known to produce partial diploids for the *het-6* region (MYLYK 1975; PERKINS 1975). Unlike *T(NM149),* duplications from *T(2869)* include *het-6* but not *het-C* (Figure 2). These crosses gave severely inhibited partial diploid progeny characteristic of *het-6* heteroallelism.

Southern analysis with cosmid probes from the translocated region showed that RLM58-18 *het-6*^{PA} *het-C*$^{PA?/OR?}$ *pyr-4; inl a* (OR) had multiple RFLPs when compared to *T(AR18),* whereas DJJ987-51 [*ro-7 un-24 het-6*^{PA} *thr-2 A* (OR), constructed by D. J. JACOBSON, and kindly provided to us at this stage of the study] did not (data not shown). To construct testers carrying *het-6*^{PA} of RLM58-18 and the *thr-2* marker, we selected *ro-7*$^{+}$ *un-24*$^{+}$ colonies that required threonine (*thr-2*) but not uridine (*pyr-4*$^{+}$) from a cross between RLM58-18 and DJJ987-51. Two of these strains, C2(2)-1 and C2(2)-9, were carried forward as *het-6* tester strains (Table 1). A third, C2(3)-41, was found to have RLM58-18-derived RFLPs throughout the *T(AR18)* translocation region and was therefore used as the parental strain to generate escape strains in this study (Table 1).

The presence of *het-c*^{PA} in RLM58-18 is inferred from the cross between strains C2(2)-5 *het-6*^{PA} *het-c*^{PA} *thr-2 a* (which was compatible with RLM58-18) and RLM57-30 *pyr-4; cyh-1 A* (OR). This cross gave a class of *pyr-4*$^{+}$ *thr-2* progeny that as severely incompatible with *het-6*^{PA} *het-c*^{PA} and *het-6*^{PA} *het-C*^{OR} testers. In addition, these *pyr-4*$^{+}$ *thr-2* progeny produced the characteristic slow growing, spidery colony that is typical of *het-C*-incompatible reactions (PERKINS 1975) when forced with *het-6*^{OR} *het-C*^{OR} testers. Further, when conidia of C2(2)-9 *het-6*^{PA} *het-C*^{OR} *thr-2 a* were placed together on minimal Vogel's medium with conidia of RLM58-18, the resultant heterokaryons grew slowly, with a spidery *het-C*-incompatible phenotype. The tester strains 6-19 *arg-1 ad-3B; het-6*^{PA}; *inl a* (OR) and 6-28 *arg-1 ad-3B; het-6*^{PA}; *inl A* (OR) were selected from the cross, I-9-26 *arg-1 ad-3B A* (OR) × RLM58-18 *het-6*^{PA} *het-c*^{PA} *pyr-4; inl a* (OR), as *pyr-4*$^{+}$ progeny that proved to be heterokaryon compatible with C2(2)-9 and C2(2)-1, respectively.

Heterokaryon testing: Heterokaryon compatibility tests were performed by overlaying aqueous conidial suspensions of escape strains and *het-6* tester strains (Table 1) on a nutrient-limited medium that would not support growth of either strain alone. The presence of compatible (*i.e.,* identical) *het-6* alleles was evident after 3 days at 30° as abundant aerial growth and conidiation, whereas *het-6*-incompatible combinations exhibited no hyphal growth. Multiple heterokaryon tests were also performed on single petri plates containing sorbose medium (DAVIS and DE SERRES 1970).

DNA isolation and electrophoresis conditions: Genomic DNA was extracted from *N. crassa* dried mycelium as described previously (OAKLEY *et al.* 1987). Cosmid DNA was isolated from 1.5 ml overnight *Escherichia coli* cultures by the alkaline lysis procedure of BIRNBOIM and DOLY as described in SAMBROOK *et al.* (1989). Restriction enzyme digests were performed according to the enzyme manufacturers' directions (Boehringer-Mannheim, Laval, Québec; Promega Corp., Madison, WI). Agarose gel electrophoresis and DNA hybridization conditions were as described previously (GLASS and SMITH 1994). Densitometry was performed on autoradiographs of restriction fragments from escape strains 1–20 (XRS 3cx scanner, X-Ray Scanner Corp., Torrance, CA). Chromosome sized DNA for Orthogonal Field Agarose Gel Electrophoresis (OFAGE) was prepared as in Orbach *et al.* (1988). Electrophoresis conditions for OFAGE were as described previously (GLASS and SMITH 1994).

Chromosome walk in the *T(AR18)*-translocated region: Cosmids were identified from the *T(AR18)*-translocated region through sequential hybridizations of the cosmid library with OFAGE-separated LGII DNA of strain 74-OR23-IV*A* and LGIII DNA of *T(AR18)* (SMITH and GLASS 1996). Overlapping cosmids within the translocated region were subsequently selected by one of two procedures. The cosmid library was probed with end-specific (α^{32}P)-UTP (Amersham, Oakville, Ontario)-labeled probes, prepared from the T3 or T7 bacte-

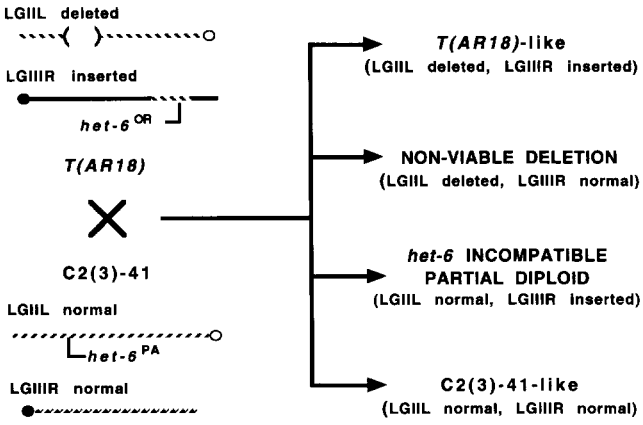

FIGURE 1.—Diagram of LGs II and III in parental strains and production of *het-6*-incompatible progeny. *T(AR18)*-like and C2(3)-41-like progeny grow at wild-type rates. Before escaping, partial diploids from this cross are extremely slow growing, due to heterozygosity at *het-6*.

riophage promoters in pMOcosX (ORBACH 1994). Alternatively, probes were prepared [(α^{32}P)-dCTP, Amersham; T7 Quick Prime, Pharmacia, Baie d' Urfe, Québec] from *Bam*HI fragments that did not overlap with previous cosmids. The location of the overlapping cosmids within the left arm of LGII was then verified by RFLP mapping (METZENBERG *et al.* 1985; METZENBERG and GROTELUESCHEN 1992), and within the *T(AR18)*-translocated region by hybridization to OFAGE blots of *T(AR18)* and 74-OR23-IV*A* chromosomes.

RESULTS

Escape from *het-6* self-incompatibility: Self-incompatible partial duplication strains were generated by crossing the normal sequence strain, C2(3)-41 *het-6*^{PA} *thr-2 A*, with *T(AR18) het-6*^{OR} *a* (Figure 1). After 16 hr approximately one-third of the germinated ascospores were observed to give rise to extremely slow growing colonies, which appeared as hyphal buds. The remaining germlings grew at rates comparable to the two parental strains and exhibited wild-type colony morphology. Four hundred slow-growing, self-incompatible progeny were transferred to individual test tubes containing liquid medium with nutritional supplements and incubated at 30°.

Escape from *het-6* incompatibility was recognized by a sudden increase in growth rate at which point the colonies were transferred to solid medium plus supplements. No cultures exhibited escape until after 12 days. Tubes were monitored for a period of up to 8 weeks, at which time a total of 96 colonies had escaped. Most escape events occurred at, or shortly after, day 20. Before escape, growth rates of all self-incompatible colonies were 0.1 to 0.3 mm/day at 30°. The first 20 escape strains recovered had a mean growth rate (±SD) of 5.67 ± 1.47 mm/day at 30°. However, none of the escape strains achieved wild-type growth rates (70 ± 10 mm/day), nor the mean growth rate of 64 ± 8.4 mm/day exhibited by C6-3, a *het-6*$^{OR/OR}$ partial diploid. In addition to a marked increase in growth rate, escape from self-incompatibility was evident by the formation of aerial hyphae and, in most cases, conidiation. Conidia from 34 escape strains were used to fertilize protoperithecia of *flA* and *fla* strains. In each case, developing perithecia were observed after 5 days with either *flA* or *fla*, but not both. However, even after 1 month, ascospores were not observed in any of these crosses.

***het-6* self-incompatibility and escape characteristics:** We constructed near isogenic tester strains that differed at *het-6* and did not contain modifiers (JACOBSON *et al.* 1995) of the incompatibility growth phenotype. As was communicated by D. JACOBSON (PERKINS *et al.* 1993), we found that heteroallelism at *het-6* prevented the formation of a heterokaryotic mycelium under forcing conditions. The phenotype of *het-6*-incompatible heterokaryons is characterized by the absence of appreciable growth, in contrast to the limited growth that is characteristic of *het-C*- and *mt*-incompatible heterokaryons. The characteristics of heteroallelism at *het-6* in both heterokaryons and self-incompatible partial diploids were similar and are among the most severe incompatibility reactions described for *N. crassa*, perhaps most similar to those of *het-e* (PERKINS 1975).

Heterokaryon tests were performed with each of 96 escape strains on minimal medium with the *het-6*^{PA} tester strains, 6-19 and 6-28, and OR background tester strain, 4571 (Table 1). Strain 4571 has the mutant A^{m54} allele and is therefore heterokaryon compatible with both *A* and *a* mating types (GRIFFITHS 1982). Where escape strains carried no auxotrophic markers, as a result of a crossover between the right breakpoint of

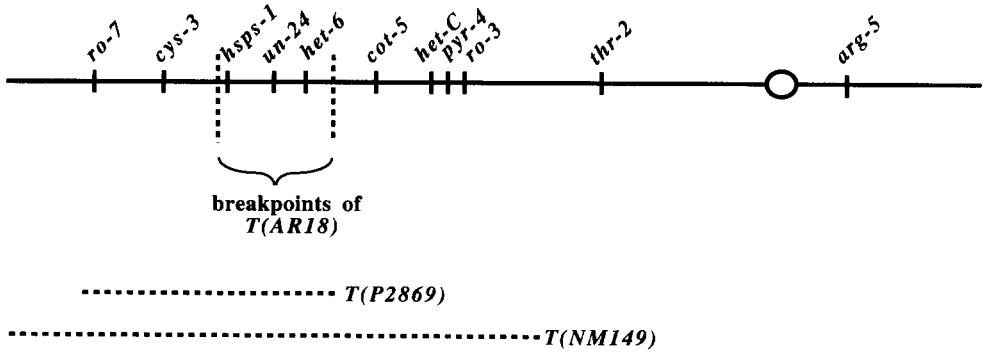

FIGURE 2.—Map of relevant part of LGII. Adapted from PERKINS *et al.* (1982, 1993) and SMITH and GLASS (1996).

TABLE 1

***N. crassa* strains used in this study**

Strain designation	Origin
Escape strains 1–96	C2(3)-41 × *T(IIL → IIIR)AR18*
T(IIL → IIIR)AR18 a (OR)[a]	FGSC 1562
T(II → VI)P2869	FGSC 1828
T(II → V)NM149	FGSC 3879
C2(3)-41 *het-6*^{PA} *thr-2 A* (OR)	RLM58-18 × DJJ987-51
74-OR23-IVA (OR) standard wild-type	FGSC 2489
C6-3 *het-6*$^{OR/OR}$ *a* (OR) partial diploid[b]	74-OR23-IVA X *T(AR18) a*
RLM57-25 *pyr-4; inl; pan-2; nic-3 A* (OR)	R. L. METZENBERG
RLM57-26 *pyr-4 arg-5; inl; pan-2 a* (OR)	R. L. METZENBERG
DJJ987-51 *ro-7 un-24 Het-6*^{PA} *thr-2 A* (OR)	D. J. JACOBSON
2190 *a* (f_1 of QM#4838, PA at *het-C, -5, -6, -8, -9*)	FGSC 2190
I-20-26 *arg-1 ad-3B A* (OR)	A. J. F. GRIFFITHS
153 *un-3 ad-3A nic-2 cyh-1 A* (OR)	A. J. F. GRIFFITHS
flA fluffy tester (OR)	FGSC 4317
fla fluffy tester (OR)	FGSC 4347
het-6 tester strains	
RLM58-18 *het-6*^{PA} *het-c*^{PA} *pyr-4; inl a* (OR)	R. L. METZENBERG
6-13 *arg-1 ad-3B; het-6*^{PA} *het-c*^{PA} *pyr-4 A* (OR)	I-20-26 × RLM58-18
6-19 *arg-1 ad-3B; het-6*^{PA}*; inl a* (OR)	I-20-26 × RLM58-18
6-28 *arg-1 ad-3B; het-6*^{PA}*; inl A* (OR)	I-20-26 × RLM58-18
4571 *un-3 ad-3A nic-2 cyh-1 A*^{m54} (OR)	FGSC 4571
C2(2)-1 *het-6*^{PA} *thr-2 A* (OR) (=FGSC 8236)	RLM58-18 × DJJ987-51
C2(2)-9 *het-6*^{PA} *thr-2 a* (OR) (=FGSC 8237)	RLM58-18 × DJJ987-51

[a] (OR) indicates Oak Ridge alleles at unspecified *het* loci.

[b] Crosses between C6-3 and fluffy tester strains were barren, indicating C6-3 contains the translocated segment as a duplication.

T(AR18) and *thr-2*, compatible heterokaryons were easily recognized by increased conidiation and a wild-type growth rate compared to that of the escape strains alone. Of the 96 escape strains tested, 80 carried the PA allele and 16 carried the OR allele at *het-6*. No escape strains were observed to be compatible with both *het-6*^{PA} and *het-6*^{OR} testers.

Chromosome walk across the *T(AR18)*-translocated region: Six *T(AR18)* translocation-specific cosmids were isolated by sequentially probing the cosmid library with LGIII DNA of *T(AR18)* and LGII DNA of wild-type strain 74-OR23-IV*A* (OR). In this way, cosmids were identified that were specific to the *T(AR18)* translocation sequences (SMITH and GLASS 1996). Cosmids X19:F:1, G25:F:11, X18:H:11 and G8:F:7 were used to initiate a chromosome walk. The 10 cosmids shown in Figure 3 represent a nearly unbroken contiguous segment across the *T(AR18)*-translocated region. The segment extends from X7:G:5, which covers the right breakpoint of the *T(AR18)* translocation (SMITH and GLASS 1996), for ~200 kbp to cosmid X18:H:11. To the left of X18:H:11 there is a gap followed by an additional 60 kbp covered by the overlapping cosmids G25:F:11 and X19:F:1. A second gap in the segment occurs between X19:F:1 and the left breakpoint. Attempts to walk through these two gaps were unsuccessful. Neither gap appears to be large, however, since the 10 cosmids shown in Figure 3 cover ~260 kbp and, based on chromosome size estimates for *T(AR18)* (SMITH and GLASS 1996), this represents nearly the entire 270 ± 90 kbp translocated region.

Physical characteristics of duplicated regions following escape: The RFLP patterns (Figure 4) across the *T(AR18)*-duplicated region were examined in 33 *het-6*^{PA} and 12 *het-6*^{OR} escape strains and the two parental strains. Southern transfers of genomic DNAs of escape and parental strain DNAs digested with the restriction enzyme *Hin*dIII were probed separately with cosmids, X19:F:1, G8:F:7, G12:E:3, G8:G:1, X14:C:1, X5:F:11 and X7:G:5 (Table 2). From this, it was inferred that 20 escape strains had only PA RFLPs in the duplicated region, and three escape strains had only OR RFLPs. Analysis of the remaining 22 escape strains indicated the presence of heterozygous duplicated sequences in regions covered by one or more of the cosmid probes. The heterozygous regions were evidenced by the presence of all restriction enzyme fragments of both *het-6*^{OR} and *het-6*^{PA} parents, or by all restriction enzyme fragments of one parent and some, but not all, fragments of the other parent. With some cosmid probes, escape strains exhibited a restriction fragment that was not found in either parent (Table 2).

Figure 3 includes the 22 escape strains where heterozygous duplications were evident from RFLP data. For each escape isolate, one parental pattern is complete across the entire translocated region as indicated at the left of Figure 3. The solid (OR) and hatched (PA) lines represent the extent of heterozygous DNA. The blank

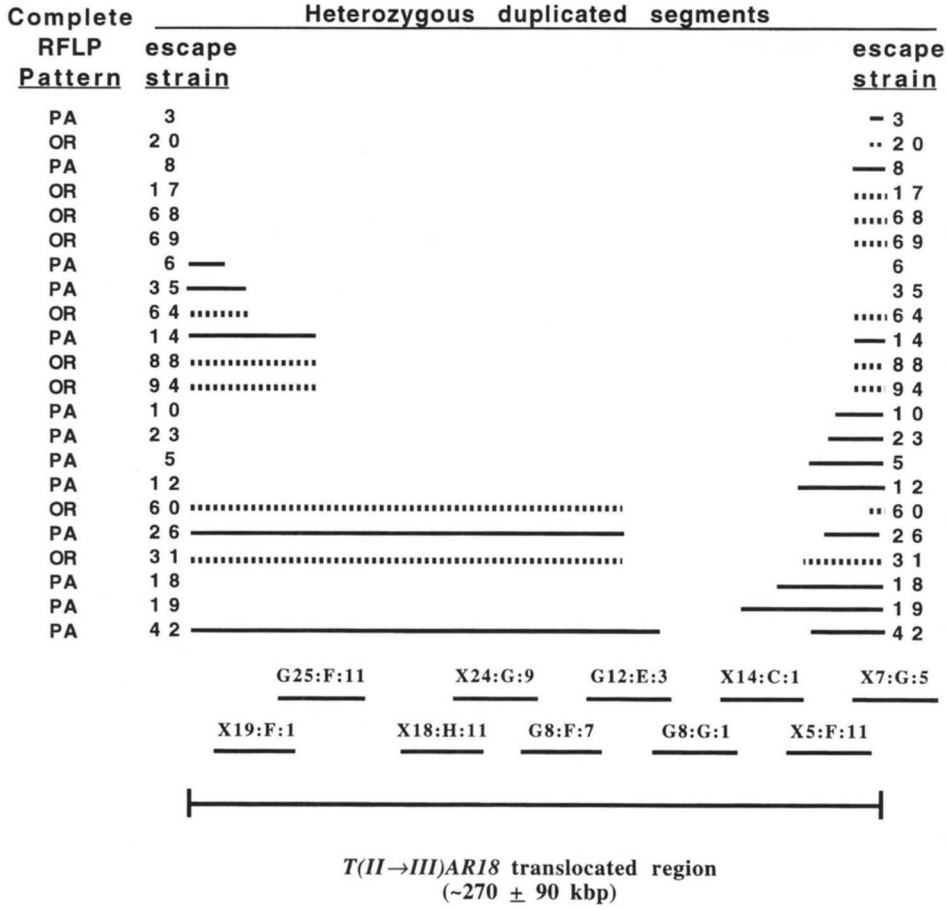

FIGURE 3.—Cosmid walk through *T(AR18)*-translocated region and extent of heterozygous DNA detected in escape strains. The identity and relative position of cosmids across the *T(AR18)* translocation are presented at bottom. One of the duplicated parental components in escape strains was found to be complete (indicated at left); part of the other component was deleted in 22 escape strains. The remaining, undeleted OR DNA (solid lines) or PA DNA (hatched lines) is represented for each escape strain within the heterozygous duplicated region.

areas represent regions wherein only one parental DNA was evident from RFLP analysis. The escape isolates are arranged with respect to the approximate amount of heterozygous duplicated DNA detected. It is evident from this arrangement that heterozygous duplications were detected in one or more escape strains in all but the region covered by cosmid G8:G:1. In fact, the RFLP pattern present at cosmid G8:G:1 was positively correlated to the *het-6* phenotype for all 45 escape strains examined (Table 2). In every case, escape strains determined to be *het-6*^{PA} by heterokaryon tests also exhibited the PA RFLP pattern of parent C2(3)-41 at cosmid G8:G:1; the 12 phenotypically *het-6*^{OR} escape strains all had the OR pattern of parent *T(AR18)*.

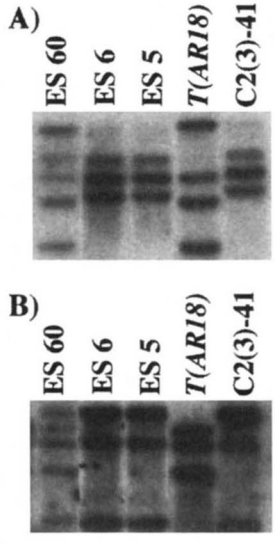

FIGURE 4.—Autoradiograms of parental and escape strains (ES) 5, 6 and 60 genomic DNAs probed with DNA of cosmid G12:E:3 (A) and cosmid G8:F:7 (B). Escape strains 5 and 6 show only PA parental fragments with both cosmids. Escape strain 60 has all OR and PA parental fragments at G8:F:7. At G12:E:3, escape strain 60 has all OR and some PA parental fragments, identifying the left margin of the deleted segment of PA DNA.

The areas not covered by either a solid or a hatched line in Figure 3 represent continuous regions for which RFLPs of one parent were not observed. The absence of one of the parental patterns in these regions suggests that deletion of one copy occurred to give hemizygosis, or that the missing parental type was altered by gene conversion, to give homozygosis. To determine whether one or both of these mechanisms was involved in escape from *het-6* incompatibility, densitometry was performed on autoradiographic images of restriction fragments from escape strains 1–20. If the areas represented homozygous duplications, we would expect the autoradiographic intensity of fragments from these regions to be approximately twice that of comparably sized fragments outside of the homozygous region. Densitometry should reveal diploidy in regions of transition from two to one parental pattern as occurs, for example, at cos-

TABLE 2

RFLPs in escape strain duplication region

Escape strain	*het-6* phenotype[a]	RFLP pattern[b] for cosmid probe						
		X19:F:1	G8:F:7	G12:E:3	G8:G:1	X14:C:1	X5:F:11	X7:G:5
1	PA	PA	PA	PA	PA	PA	PA	PA
2	PA	PA	PA	PA	PA	PA	PA	PA
3	PA	PA	PA	PA	PA	PA	PA	H(PA)
4	PA	PA	PA	PA	PA	PA	PA	PA
5	PA	PA	PA	PA	PA	PA	H(PA)	PA/OR
6	PA	H(PA)	PA	PA	PA	PA	PA	PA
7	PA	PA	PA	PA	PA	PA	PA	PA
8	PA	PA	PA	PA	PA	PA	PA	PA/OR
9	PA	PA	PA	PA	PA	PA	PA	PA
10	PA	PA	PA	PA	PA	PA	H(PA)	H(PA)
11	OR	OR	OR	OR	OR	OR	OR	OR
12	PA	PA	PA	PA	PA	D	D	H(PA)
13	PA	PA	PA	PA	PA	PA	PA	PA
14	PA	PA/OR	PA	PA	PA	PA	PA	PA/OR
15	PA	PA	PA	PA	PA	PA	PA	PA
16	OR	OR	OR	OR	OR	OR	OR	OR
17	OR	OR	OR	OR	OR	OR	OR	PA/OR
18	PA	PA	PA	PA	PA	H(PA)	H(PA)	PA/OR
19	PA	PA	PA	PA	PA	D	PA/OR	PA/OR
20	OR	OR	OR	OR	OR	OR	OR	H(OR)
21	PA	PA	PA	PA	PA	PA	PA	PA
22	PA	PA	PA	PA	PA	PA	PA	PA
23	PA	PA	PA	PA	PA	PA	N(PA)	H(PA)
24	PA	PA	PA	PA	PA	PA	PA	PA
26	PA	PA/OR	PA/OR	H(PA)	PA	PA	H(PA)	H(PA)
27	OR	OR	OR	OR	OR	OR	OR	OR
31	OR	PA/OR	PA/OR	H(OR)	OR	OR	H(OR)	H(OR)
32	PA	PA	PA	PA	—	PA	—	PA
35	PA	PA/OR	PA	PA	—	PA	PA	PA
36	PA	PA	PA	PA	PA	PA	PA	PA
37	PA	PA	PA	PA	PA	PA	PA	PA
42	PA	PA/OR	PA/OR	PA/OR	PA	PA	N(PA)	PA/OR
50	PA	PA	PA	PA	PA	PA	PA	PA
52	PA	PA	PA	PA	—	PA	PA	PA
55	PA	PA	PA	PA	—	PA	PA	PA
59	PA	PA	PA	PA	—	PA	PA	PA
60	OR	PA/OR	PA/OR	H(OR)	OR	OR	OR	H(OR)
64	OR	PA/OR	OR	OR	OR	OR	OR	PA/OR
68	OR	OR	—	—	OR	OR	—	PA/OR
69	OR	OR	—	—	OR	OR	OR	PA/OR
71	PA	PA	PA	PA	PA	PA	PA	PA
76	PA	PA	PA	PA	PA	PA	PA	PA
79	PA	PA	PA	PA	PA	PA	PA	PA
88	OR	PA/OR	OR	OR	OR	OR	OR	PA/OR
94	OR	PA/OR	OR	OR	OR	OR	OR	PA/OR

[a] *het-6* phenotype determined by heterokaryon tests.

[b] PA, Panama RFLP pattern only; OR, Oak Ridge RFLP pattern only; PA/OR, both Panama and Oak Ridge RFLP patterns; H, one complete pattern, either OR or PA as indicated in parentheses, with some restriction fragments from other pattern; N, one complete pattern (indicated in parentheses) with additional, nonparental-sized restriction fragment; D, nonparental stoichiometry of some restriction fragments; —, not determined.

mid X5:F:11 with escape strain 5 (Table 2). In the several cases where transition from two patterns to one was evident, densitometry readings in excess of two times the expected values were detected only at cosmids X14:C:1 and X5:F:11 in escape strain 12, and at cosmid X14:C:1 in escape strain 19. In both of these strains the anomalous readings were restricted to one or two fragments within the transition zone and did not extend into the region where only a single parental pattern was evident.

In addition to differences in band stoichiometry based on RFLP analysis, it should be possible to differentiate homozygosis and deletion by analysis of electrophoretic karyotypes. Provided that the tracts are large enough, escape by deletion, but not by homozygosis, should result in increased electrophoretic mobility of

TABLE 3

Size of parental LGs II and III and mean size estimates for altered LGs in three escape strains

Strain	*het-6* phenotype	LGII (Mb)	LGIII (Mb)	Event
C2(3)-41	PA	4.60	5.10	—
T(AR18)	OR	4.33	5.37	—
ES 5	PA	4.60	5.05 ± 0.03[a]	~320-kbp deletion of LGIII
ES 6	PA	4.60	5.04 ± 0.05[a]	~330-kbp deletion of LGIII
ES 60	OR	4.50 ± 0.07[a]	5.37	~100-kbp deletion of LGII

[a] Values are ± SD.

the chromosome. Size estimates of LGs II and III for escape strains 5, 6, and 60 were compared in four electrophoresis experiments to those of C2(3)-41 and *T(AR18)* following OFAGE (Table 3). The size standards used were LGs II and III from wild type (ORBACH 1992) and *T(AR18)* (SMITH and GLASS 1996). Escape strains 5 and 6 both lack OR RFLPs over some portion of the translocated region (Figure 4) and both were scored as *het-6*^{PA} in heterokaryon tests (Table 2). LGIII, which carries *het-6*^{OR}, was reduced in size by ~320 and 330 kbp for escape strains 5 and 6, respectively, compared to that of *T(AR18)*. The deleted region in both of these strains is equivalent to the 270 ± 90 kbp size estimate of the *T(AR18)* translocation. When OFAGE blots of these two strains were probed with the X7:G:5 cosmid, which is known to span the centromere proximal breakpoint of the *T(AR18)* region (SMITH and GLASS 1996), hybridization to LGII was most evident (Figure 5). On the original autoradiographs some hybridization signal at LGIII is evident in escape strain 5, but not in escape strain 6. This is consistent with RFLP data that indicate that the deleted region of escape strain 6 extends at least to the right translocation breakpoint and perhaps beyond it into unique DNA of LG III, whereas escape strain 5 retains some duplicated sequences adjacent to the *T(AR18)* translocation insertion point (Table 2). LGII of escape strain 60 consistently migrated to an intermediate position between LGII of *T(AR18)* and that of C2(3)-41 (Figure 5). Based on RFLP analysis, we expected that ~90 kbp of LGII should be deleted in escape strain 60 and that LGIII in escape strain 60 should be unaltered from that of LGIII in parental strain *T(AR18)*. Results from the OFAGE analysis of escape strain 60 were consistent with these expectations. From OFAGE, the estimated size of the stretch of DNA deleted from LGII in escape strain 60 is ~100 kbp.

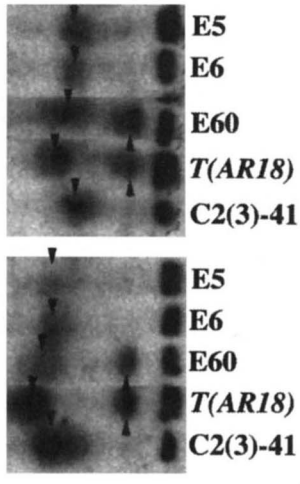

FIGURE 5.—OFAGE blots from two separate electrophoresis experiments (top and bottom) of parental strains and escape strains (ES) 5, 6 and 60 probed with DNA from cosmid X7:G:5 that covers the right breakpoint of the *T(AR18)* translocation. Arrows from top, LGII, from bottom, LGIII, indicate the middle of major hybridization signals, from which LG size estimates were taken (Table 3).

DISCUSSION

This is the first study of the molecular basis of escape from self-incompatibility in fungi. The data indicate that the primary means of escape is through deletion of tracts of DNA in which genes responsible for self-incompatibility are located. First, hybridization signals with cosmids from the translocated region were consistent with deletion rather than chemical modifications or homozygosis. The few cases where double intensity fragments were evident (Table 2) could have resulted from overlap of a parental fragment and a new, nonparental fragment. Second, OFAGE analysis of chromosome sizes in three escape strains clearly showed that escape in these cases was due to deletion of part of one of the duplicated components. The size and identity of the deletions apparent through OFAGE were consistent with the RFLP data for these three escape strains.

The genetic basis of escape has been best characterized for the Neurospora mating-type locus, which has numerous closely linked genetic markers. DELANGE and GRIFFITHS (1975) observed an apparent loss of genetic markers linked to the *mt* locus following escape of forced incompatible heterokaryons. In a series of genetic experiments, NEWMEYER and GALEAZZI (1977) showed that heterozygosity in partial diploids of the mating-type locus results in somatic events that eliminate the incompatibility function. Two classes of escape strains were detected in their experiments. The first class of escape strains were fully fertile, producing ample ascospores in test crosses. The second class re-

sembled the escape strains recovered in the present study. These strains were barren in that they produced perithecia, but few ascospores in test crosses, suggesting that the euploid condition was not restored. From these previous studies, several mechanisms can be proposed to account for the genetic characteristics of escape, including mitotic crossing over with segregation, homozygosis through conversion of one of the duplicated regions, and inactivation of one *het* gene by chemical modification or mutation. Based on the present study, the most plausible means of escape from self-incompatibility at *het-6* is by chromosomal deletion.

In contrast to studies on the suppression of mating type-associated incompatibility (NEWMEYER 1970; VELLANI *et al.* 1994), no suppressors of *het-6* incompatibility were identified in our study. The recessive suppressor mutation of mating-type incompatibility, *tol*, was originally recovered as an escape in partial diploids of *A/a* (NEWMEYER 1970). In the present study, a dominant suppressor would have been evident from escape strains that were compatible with both *het-6*^{OR} and *het-6*^{PA} tester strains. An escape strain with a recessive suppressor would have been incompatible with both *het-6*^{OR} and *het-6*^{PA} testers.

Escape from *het-6* self-incompatibility in our experiments was both slow (most escapes occurred after 20 days) and infrequent (only ~24% of 400 original partial diploids had escaped after 2 months). This contrasts with heterozygous partial diploids of *mt*, *het-c* and *het-e*, where escape begins to occur after 3–10 days (PERKINS 1975) and, in the case of *mt*, nearly all are observed to escape (VELLANI *et al.* 1994). The low frequency and the extended time for escape in our experiments is likely a result of the type of translocation in *T(AR18)*. The *T(AR18)* translocation involves an interstitial portion of LGII inserted into the right arm of LGIII (PERKINS *et al.* 1982). Deletions within this translocation would involve a complex mechanism, whereas escape from *het* incompatibility had previously been examined only in duplications derived from terminal or quasiterminal rearrangements (NEWMEYER and TAYLOR 1967; DELANGE and GRIFFITHS 1975; NEWMEYER and GALEAZZI 1977; TURNER 1977), and these would require only a single break to escape, plus restoration or preservation of the telomere.

We observed that the translocated *het-6*^{OR} allele was nearly five times more likely to be deleted than was the normal position *het-6*^{PA} allele. Bias toward deletion of the translocation copy of a heterokaryon incompatibility locus is interesting and apparently characteristic of escape in partial diploids (METZENBERG *et al.* 1973; PERKINS and BARRY 1977). There are at least two general models that would explain this bias. First, the translocated component of the partial diploid may be preferentially recognized, perhaps because of an unusual chromosome structure due to the insertion of the translocated segment. Second, the bias could be at the level of selection for growth of equally frequent deletion mutants, rather than differential frequency of deletion.

Loss of heterozygosity through mitotic deletions are associated with many human diseases (reviewed in MÜLLER and SCOTT 1992). The series of deletions observed in this study is strikingly similar to deletion spectra of the *hprt* gene (LIPPERT *et al.* 1995) and of putative tumor suppressor genes (LEISTER *et al.* 1990; ZENKLUSEN *et al.* 1995). In all cases, the deletions are variable in size but include a specific region of DNA that apparently contains a gene involved in growth suppression. In the case of escape from *het-6* incompatibility, the region that is always deleted is covered by cosmid G8:G:1. From this, we can hypothesize that *het-6* is located within G8:G:1, and that removal of one copy permits hyphal growth. Independent support for this hypothesis comes from other experiments (SMITH and GLASS 1995, and unpublished results) that identify crossover points proximal and distal to *het-6*, which also closely flank G8:G:1. Another common feature to large deletion spectra is the occurrence of what may be breakpoint hotspots (LEISTER *et al.* 1990; LIPPERT *et al.* 1995; ZENKLUSEN *et al.* 1995). In Figure 3, breakpoints are especially common in regions covered by cosmids G25:F:11, G12:E:3 and X7:G:5, the last of which also straddles the centromere proximal breakpoint of the *T(AR18)* translocation. The common features to these deletion spectra suggest a general DNA deletion mechanism that results in a release from growth suppression. Such deletions could be used to pinpoint the location of any gene that has a recessive allele that allows growth under some stress condition (*e.g.*, various genes for antimetabolite resistance) but that does not allow growth under the same condition when the resistance allele is in heterozygous combination with its wild-type allele.

While the biochemical mechanism(s) of large mitotic deletions in Neurospora and the analogous human systems has not been determined, several models have been proposed. These involve intrachromosomal rearrangement, excision of chromatidal loops or DNA replication errors (CARON *et al.* 1994; GORDON and HALLIDAY 1995; LEGUERN *et al.* 1996). Neurospora should be useful for testing these models of eukaryotic mitotic deletion mechanism since large deletions are predictably recovered following escape from self-incompatibility.

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LITERATURE CITED

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

CARON, H., P. VAN SLUIS, N. VAN ROY, J. DE KRAKER, F. SPELEMAN, *et al.*, 1994 Recurrent 1;17 translocations in human neuroblastoma

reveal nonhomologous mitotic recombination during the S/G2 phase as a novel mechanism for loss of heterozygosity. Am. J. Hum. Genet. **55:** 341–347.

DAVIS, R. H., and F. J. DE SERRES, 1970 Genetic and microbiological techniques for *Neurospora crassa.* Methods Enzymol. **17A:** 79–143.

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.

FLAVELL, R. B., 1995 Inactivation of gene expression in plants as a consequence of specific sequence duplication. Proc. Natl. Acad. Sci. USA **91:** 3490–3496.

GARNJOBST, L., 1955 Further analysis of genetic control of heterokaryosis in *Neurospora crassa.* Am. J. Bot. **42:** 444–448.

GLASS, N. L., and M. L. SMITH, 1994 Structure and function of a mating-type gene from the homothallic species *Neurospora africana.* Mol. Gen. Genet. **244:** 401–409.

GORDON, A. J. E., and J. A. HALLIDAY, 1995 Inversions with deletions and duplications. Genetics **140:** 411–414.

GRIFFITHS, A. J. F., 1982 Null mutants of the *A* and *a* mating type of *Neurospora crassa.* Can. J. Genet. Cytol. **24:** 167–176.

JACOBSON, D. J., J. OHRNBERGER and R. A. AKINS, 1995 The Wilson-Garnjobst heterokaryon incompatibility tester strains of *Neurospora crassa* contain modifiers which influence growth rate of heterokaryons and distort segregation ratios. Fungal Genet. Newsl. **42:** 34–40.

JUDELSON, H. S., and S. L. WHITTAKER, 1995 Inactivation of transgenes in *Phytophthora infestans* is not associated with their deletion, methylation, or mutation. Curr. Genet. **28:** 571–579.

LEGUERN, E., R. GOUIDER, N. RAVISÉ, J. LOPES, S. TARDIEU *et al.,* 1996 A *de novo* case of hereditary neuropathy with liability to pressure palsies (HNPP) of maternal origin: a new mechanism for deletion in 17p11.2? Hum. Mol. Genet. **5:** 103–106.

LEISTER, I., A. WEITH, S. BRÜDERLEIN, C. CZIEPLUCH, D. KANGWANPONG *et al.,* 1990 Human colorectal cancer: high frequency of deletions at chromosome 1p35. Cancer Res. **50:** 7232–7235.

LIPPERT, M. J., J. A. NICKLAS, T. C. HUNTER and R. J. ALBERTINI, 1995 Pulsed field analysis of *hprt* T-cell large deletions: telomeric region breakpoint spectrum. Mutat. Res. **326:** 51–64.

METZENBERG, R. L., and J. GROTELUESCHEN, 1992 Restriction polymorphism maps of *Neurospora crassa:* update. Fungal Genet. Newsl. **39:** 50–56.

METZENBERG, R. L., M. K. GLEASON and B. S. LITTLEWOOD, 1973 Genetic control of alkaline phosphatase synthesis in Neurospora: the use of partial diploids in dominance studies. Genetics **77:** 25–43.

METZENBERG, R. L., J. N. STEVENS, E. U. SELKER and E. MORZYCKA-WROBLEWSKA, 1985 Identification and chromosomal distribution of 5S RNA genes in *Neurospora crassa.* Proc. Natl. Acad. Sci. USA **82:** 2067–2071.

MÜLLER, H., and R. SCOTT, 1992 Hereditary conditions in which the loss of heterozygosity may be important. Mutat. Res. **284:** 15–24.

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

NEWMEYER, D., 1970 A suppressor of the heterokaryon-incompatibility associated with mating type in *Neurospora crassa.* Can. J. Genet. Cytol. **12:** 914–926.

NEWMEYER, D., and D. R. GALEAZZI, 1977 The instability of Neurospora duplication *Dp(IL → IR)H4250,* and its genetic control. Genetics **85:** 461–487.

NEWMEYER, D., and C. W. TAYLOR, 1967 A pericentric inversion in Neurospora, with unstable duplication progeny. Genetics **56:** 771–791.

OAKLEY, C. E., C. F. WEIL, P. L. KRETZ and B. R. OAKLEY, 1987 Cloning of the *ribo B* locus of *Aspergillus nidulans.* Gene **53:** 293–298.

ORBACH, M. J., 1992 Fungal Genet. Newsl. **39:** 92.

ORBACH, M. J., 1994 A cosmid with a Hy^R marker for fungal library construction and screening. Gene **150:** 159–162.

ORBACH, M. J., D. VOLLRATH, R. W. DAVIS and C. YANOFSKY, 1988 An electrophoretic karyotype of *Neurospora crassa.* Mol. Cell. Biol. **8:** 1469–1473.

PERKINS, D. D., 1975 The use of duplication-generating rearrangements for studying heterokaryon incompatibility genes in Neurospora. Genetics **80:** 87–105.

PERKINS, D. D., 1988 Main features of vegetative incompatibility in Neurospora. Fungal Genet. Newsl. **35:** 44–46.

PERKINS, D. D., and E. G. BARRY, 1977 The cytogenetics of *Neurospora.* Adv. Genet. **19:** 133–285.

PERKINS, D. D., A. RADFORD, D. NEWMEYER and M. BJÖRKMAN, 1982 Chromosomal loci of *Neurospora crassa.* Microbiol. Rev. **46:** 426–570.

PERKINS, D. D., J. F. LESLIE and D. J. JACOBSON, 1993 Strains for identifying and studying individual vegetative (heterokaryon) incompatibility loci in *Neurospora crassa.* Fungal Genet. Newsl. **40:** 69–73.

PITTENGER, T. H., 1964 Spontaneous alterations of heterokaryon compatibility factors in Neurospora. Genetics **50:** 471–484.

ROMANO, N., and G. MACINO 1992 Quelling: transient inactivation of gene expression in *Neurospora crassa* by transformation with homologous sequences. Mol. Microbiol. **6:** 3343–3353.

SAMBROOK, J. E., F. FRITSCH and T. MANIATIS, 1989 *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

SMITH, M. L., and N. L. GLASS 1995 Characterization of two incompatibility genes at *het-6* in *Neurospora crassa.* Fungal Genet. Newsl. **42A:** 74.

SMITH, M. L., and N. L. GLASS 1996 Mapping translocation breakpoints by orthogonal field agarose gel electrophoresis. Curr. Genet. **29:** 301–305.

TURNER, B. C., 1977 Euploid derivatives of duplications from a translocation in Neurospora. Genetics **85:** 439–460.

VELLANI, T. S., A. J. F. GRIFFITHS and N. L. GLASS, 1994 New mutations that suppress mating-type vegetative incompatibility in *Neurospora crassa.* Genome **37:** 249–255.

VOGEL, H. J., 1964 Distribution of lysine pathways among fungi: evolutionary implications. Am. Nat. **98:** 435–446.

VOLLMER, S. J., and C. YANOFSKY, 1986 Efficient cloning of the genes in *Neurospora crassa.* Proc. Natl. Acad. Sci. USA **83:** 4869–4873.

WILSON, J. F., and L. GARNJOBST, 1966 A new incompatibility locus in *Neurospora crassa.* Genetics **53:** 621–631.

ZENKLUSEN, J. C., J. C. THOMPSON, A. J. P. KLEIN-SZANTO and C. J. CONTI, 1995 Frequent loss of heterozygosity in human primary squamous cell and colon carcinomas at 7q31.1: evidence for a broad range tumor suppressor gene. Cancer Res. **55:** 1347–1350.

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