Isolation of *Neurospora crassa A* Mating Type Mutants by Repeat Induced Point (RIP) Mutation

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ABSTRACT

In the filamentous fungus, *Neurospora crassa*, mating type is regulated by a single locus with alternate alleles, termed *A* and *a*. The mating type alleles control entry into the sexual cycle, but during vegetative growth they function to elicit heterokaryon incompatibility, such that fusion of *A* and *a* hypha results in death of cells along the fusion point. Previous studies have shown that the *A* allele consists of 5301 bp and has no similarity to the *a* allele; it is found as a single copy and only within the *A* genome. The *a* allele is 3235 bp in length and it, too, is found as a single copy within the *a* genome. Within the *A* sequence, a single open reading frame (ORF) of 288 amino acids (*mt A-1*) is thought to confer fertility and heterokaryon incompatibility. In this study, we have used repeat induced point (RIP) mutation to identify functional regions of the *A* idiomorph. RIP mutations in *mt A-1* resulted in the isolation of sterile, heterokaryon-compatible mutants, while RIP mutations generated in a region outside of *mt A-1* resulted in the isolation of mutants capable of mating, but deficient in ascospore formation.

THE filamentous fungus, *Neurospora crassa*, exists as a population containing two mating types, each of which is self-sterile. Mating type is controlled by a single locus with alternate alleles, termed *A* and *a* (see PERKINS, TURNER and BARRY 1976; see PERKINS and TURNER 1988). The DNA sequences specifying *A* and *a* function have been cloned and sequenced (VOLLMER and YANOFSKY 1986; GLASS *et al.* 1988; GLASS, GROTELUESCHEN and METZENBERG 1990; STABEN and YANOFSKY 1990). The *A* allele consists of 5301 bp and has no sequence similarity to the *a* allele; it is found as a single copy and only within the *A* genome. The *a* sequence is 3235 bp in length, and it, too, is found as a single copy, and only within the *a* genome. The term "idiomorph" has been introduced in place of "allele" to denote sequences (such as *A* and *a*) at the same locus in different strains that have no sequence similarity and probably do not share a common evolutionary relationship (METZENBERG and GLASS 1990; GLASS and LORIMER 1991). The extent of the two idiomorphs is defined in terms of the regions of dissimilarity.

Although the mating type locus controls entry into the sexual cycle, during vegetative growth it functions as a heterokaryon-incompatibility locus such that forced heterokaryons between *A* and *a* mating types are inhibited in their growth (BEADLE and COONRADT 1944; SANSOME 1946). Selecting for mutants (A^m and a^m) that are heterokaryon-compatible with the opposite mating type selects simultaneously for sterility, and selection of revertants to fertility simultaneously selects for the regaining of heterokaryon incompatibility (GRIFFITHS and DELANGE 1978; GRIFFITHS 1982).

Functional studies and sequence analysis of the *A* and *a* idiomorphs revealed ORFs (*mt A-1* and *mt a-1*, respectively) that confer both fertility and heterokaryon incompatibility (GLASS *et al.* 1988; GLASS, GROTELUESCHEN and METZENBERG 1990; STABEN and YANOFSKY 1990). The *mt A-1* and *mt a-1* ORFs are thought to encode polypeptides involved in transcriptional activation of genes that confer both recognition of the opposite mating type and recognition *by* the opposite mating type. In support of this hypothesis, the *mt A-1* ORF has a region of amino acid similarity to the *Saccharomyces cerevisiae* α cell-type regulator, *MAT*α1 (GLASS, GROTELUESCHEN and METZENBERG 1990). The *N. crassa mt a-1* ORF has a region of amino acid similarity to the M_c ORF of *Schizosaccharomyces pombe* (STABEN and YANOFSKY 1990). The M_c polypeptide is thought to be a transcriptional activator of genes that confer *M* mating type to *S. pombe* cells (KELLY *et al.* 1988; EGEL 1989).

Although the *A* idiomorph is 5.3 kbp long, only a 1-kbp region containing the *mt A-1* ORF has a defined function. To further analyze *A* function and to define any additional regions that might be required for sexual development and meiosis, we took advantage of a genetic process termed repeat induced point (RIP) mutation to generate new *A* mating type mutants (SELKER *et al.* 1987; SELKER and GARRETT 1988; GRAYBURN and SELKER 1989; for review, see SELKER

TABLE 1

Neurospora crassa strains

Strain genotype[a]	Mating phenotype	Reference[b]
74 ORS *A*	Fertile	Gift from R. L. METZENBERG
qa-2; aro-9 A (326M237; Y325M6)	Fertile	Gift from R. L. METZENBERG
qa-2; aro-9 a (326M237; Y325M6)	Fertile	Gift from R. L. METZENBERG
un-3 a (55701(t))	Fertile	FGSC 4529
inl; fl A (37401; P)	Fertile	FGSC 3631
inl; fl a (37401; P)	Fertile	FGSC 3632
un-3, ad-3A, nic-2, cyh-1 A^{m64} (55701(t) 2-17-814 43002 KH52(r) 64)	Nonmating	FGSC 4573
$A^{I\ RIP}$	Nonmating	This study
arg-1 $A^{I\ RIP}$ (36703)	Nonmating	This study/R. L. METZENBERG
$A^{II\ RIP}$	Ascospore deficient	This study
fl $A^{II\ RIP}$ (P)	Ascospore deficient	This study

[a] Alleles of each locus are identified parenthetically.
[b] FGSC = Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas City, Kansas.

1990). RIP operates during mitotic divisions of haploid, opposite mating type nuclei in ascogenous hyphae and efficiently alters both copies of duplicated sequences, whether genetically linked or not. All of the changes are G-C to A-T transition mutations (CAMBARERI *et al.* 1989). In this study, we used RIP to generate mutants in *mt A-1* that are sterile and heterokaryon compatible. RIP mutations generated elsewhere in the *A* idiomorph result in strains capable of being induced for perithecial development, but deficient in ascospore formation.

MATERIALS AND METHODS

Strains: The *Neurospora crassa* strains used in this study are described in Table 1. *Escherichia coli* NM522 (GOUGH and MURRAY 1983) was used as the transformation recipient for plasmid constructions.

Materials: Restriction enzymes were from Bio-Rad and Promega. [α-^{32}P]dCTP was obtained from Amersham Corp. Nylon membranes were obtained from AMF Cuno, Inc., Meriden, Connecticut, and chemicals were obtained from Sigma Chemical Co.

Cloning and hybridizations: The A^{m64} gene was cloned from the *un-3 ad-3A nic-2 cyh-1* A^{m64} mutant by digesting genomic DNA with *Pst*I and *Sal*I and ligating fragments into a pQa2 vector which was constructed by inserting a *Bam*HI fragment containing the *qa-2* gene of *N. crassa* (SCHWEIZER *et al.* 1981) into pBluescript$^+$ (Stratagene, Inc.). The pSV50 vector (VOLLMER and YANOFSKY 1986), which contains a benomyl-resistance gene, was obtained from the C. YANOFSKY laboratory.

Transformation methods: Competent spheroplasts of *N. crassa* were prepared according to the method of SCHWEIZER *et al.* (1981) using the modification of AKINS and LAMBOWITZ (1985). Transformation of competent *N. crassa* spheroplasts was according to GLASS *et al.* (1988).

Crosses and microscopic examination of mutants: Crosses were performed on synthetic crossing medium (WESTERGAARD and MITCHELL 1947). Following fertilization, developing perithecia were dissected and the perithecial contents were examined under the light microscope. Some microscopic preparations were stained with acriflavin (Sigma Chemical Co.), or with iron-hematoxylin (RAJU and NEWMEYER 1977; RAJU 1978).

DNA isolation and hybridization: Genomic DNA was isolated by a modification of the method of STEVENS and METZENBERG (1982). DNA was cut with the appropriate enzyme, subjected to agarose gel electrophoresis, and then blotted to Zetabind membrane (AMF Cuno, Inc.). Probes were labeled with [α-^{32}P]dCTP by the random priming method (FEINBERG and VOGELSTEIN 1983). Prehybridizations and hybridizations conditions used were as described by MANIATIS, FRITSCH and SAMBROOK (1982).

Scanning electron microscopy: Wild-type strain ORS 74 *A* and the $A^{II\ RIP}$ mutant were grown in separate Petri dishes of mating medium to induce differentiation of female reproductive structures. After 4 days, conidia from the male parent (*un-3 a*, Table 1) was seeded onto the plates. At intervals during an 18-day time course, 0.5 cm × 0.5-cm agar blocks were cut from the plates; excess agar was sliced away. Each block was vapor-fixed for 12 h with 2% v/w OsO_4 in a sealed container. The samples were then dehydrated by passing them through a graded ethanol series: 50%-70%-90%-100%-100%, each for 15 min. The samples were then frozen in liquid nitrogen and fractured. After warming to 25° in 100% ethanol, the samples were critical-point dried (Balzers CPD 020), mounted on stubs and sputter-coated with gold (Nanotech Semprep II Sputter Coater). The samples were examined with a Cambridge 250 scanning electron microscope and photographs were taken with Polaroid 55 instant 4 × 5-inch negative and positive film.

RESULTS

Isolation of fragment I RIP mutants: The region of the *A* idiomorph that encompasses the *mt A-1* ORF and functions in controlling mating type and heterokaryon incompatibility is contained within fragment I (Figure 1). Frameshift mutations identified within the *mt A-1* ORF (GLASS, GROTELUESCHEN and METZENBERG 1990) are thought to cause both sterility and heterokaryon compatibility in A^m mutants (GRIFFITHS 1982). The strategy for RIPing of the *mt A-1* ORF is outlined in Figure 2. A 1.7-kbp fragment of the *A* sequence (Fig. 1, fragment I) from a sterile mutant, A^{m64} (GRIFFITHS 1982) (Table 1) was isolated from a

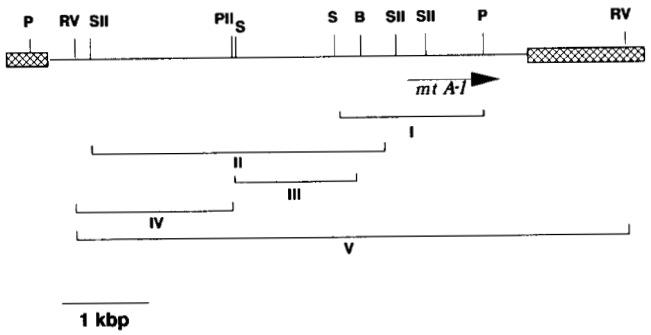

FIGURE 1.—Restriction map of the 5301-bp *A* idiomorph of *N. crassa*. Sequences surrounding the mating type locus in *A* and *a* strains that are homologous between the two are shown as hatched boxes. The position and direction of transcription of *mt A-1* is shown by an arrow. Fragments I–V are cloned fragments that were used in this study to delineate functions of the *A* idiomorph. The functional portion of the *mt A-1* ORF is contained within fragment I. B, *Bcl*I; *P*, *Pst*I; PII, *Pvu*II; RV, *Eco*RV; S, *Sal*I; SII, *Sst*II.

*Pst*I/*Bam*HI genomic digest and cloned into a vector containing the *qa-2* gene of *N. crassa* (SCHWEIZER *et al.* 1981). This construct was introduced into *qa-2; aro-9 A* (Table 1) spheroplasts by transformation, thus creating a duplication of both the *qa-2* and the *mt A-1* sequences. (The A^{m64} fragment, rather than wild-type *A* fragment, was used to make partial diploids for *mt A-1* because the presence of the mutant sequence would (presumably) not affect the mating process.) Transformants with either a single copy insertion (A/A^{m64}) or a multicopy insertion ($A/(A^{m64})_n$) were crossed with an opposite mating type strain, *un-3 a* (Table 1). During the premeiotic proliferation of nuclei, a certain percentage of the A/A^{m64} duplicated sequences will have undergone RIP. After 2 weeks, ascospores were isolated, heat-shocked and transferred to 33° for vegetative growth. This procedure selects against the *a* mating type because of the close linkage of the temperature-sensitive mutation, *un-3*.

To detect phenotypic alterations in mating function, single ascospore isolates from the A/A^{m64} × *un-3 a* and $A/(A^{m64})_n$ × *un-3 a* crosses were spotted onto *A* and *a* mating type tester strains, *inl; fl A* and *inl; fl a* (Table 1). Fourteen of 72 progeny from the A/A^{m64} × *un-3 a* cross failed to undergo any of the steps associated with mating when crossed with either mating type. A single ascospore isolate (out of 72 assayed) from the $A/(A^{m64})_n$ × *un-3* cross was also sterile.

Phenotypic characterization of fragment I RIP mutants: Sterility in Neurospora can occur by mutations either in genes controlling the formation of the male or female reproductive structures (WEIJER and VIGFUSSON 1972; VIGFUSSON and WEIJER 1972; MYLYK and THRELKELD 1974) or in the *A* and *a* idiomorphs (GRIFFITHS 1982; GLASS, GROTELUESCHEN and METZENBERG 1990; STABEN and YANOFSKY 1990). To determine the basis of the sterility of the fragment I mutants ($A^{1\ RIP}$), we examined each of them for protoperithecial and male conidial development.

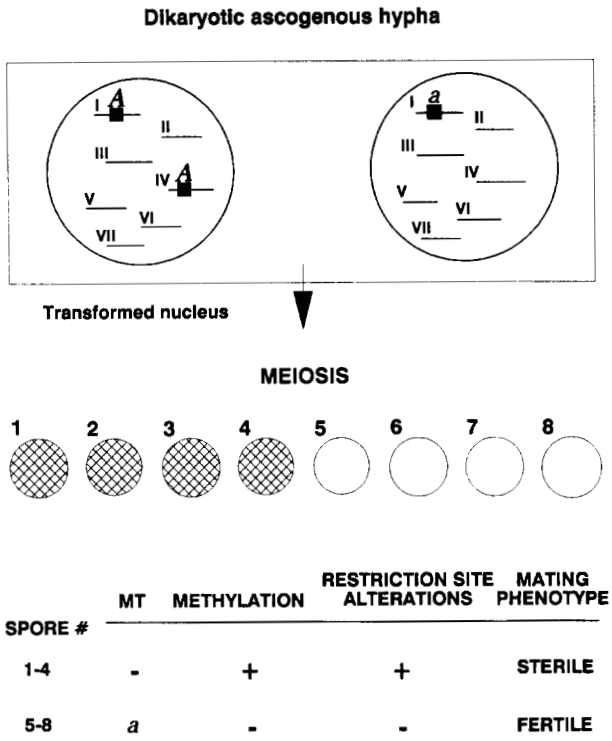

FIGURE 2.—Illustration of RIP mutation. A pQa2 vector containing the 1.7-kbp A^{m64} fragment (fragment I, Figure 1) is introduced into an *A* mating type isolate by DNA-mediated transformation to create a strain with a duplication for fragment I. The transformant containing the duplication is then crossed to an *a* mating type strain. Prior to premeiotic DNA replication, cytosines within the duplicated fragment I *A* sequences suffer methylation and C to T transition mutations. Following karyogamy, meiosis and a single mitotic division, eight ascospores are delimited within the ascus. Four ascospore isolates display *a* mating type behavior and four are sterile and heterokaryon compatible. In this study, approximately 20% of random progeny from the above cross were sterile and heterokaryon compatible.

In all cases, the mutants were indistinguishable from wild type in the formation of the sexual structures preliminary to mating. Although the $A^{1\ RIP}$ mutants were capable of forming both male and female reproductive structures, they could not function as either a male or female parent in a cross.

The ability of the $A^{1\ RIP}$ mutants to form compatible heterokaryons was tested by selecting for the fusion of strains with complementing auxotrophic mutations. As expected, vegetative growth in a control heterokaryon between *un-3 a* and *qa-2; aro-9 A* on minimal medium at 34° was very sparse and conidia were not formed. In contrast, heterokaryons formed between an *arg-1* $A^{1\ RIP}$ mutant and *qa-2; aro-9 a* (Table 1) grew as vigorously on minimal medium as did either strain alone on supplemented medium. The growth phenotype of an *arg-1* $A^{1\ RIP}$ and *qa-2; aro-9 a* heterokaryon was similar to that of a compatible heterokaryon between *un-3 ad-3A nic-2 cyh-1* A^{m64} and *qa-2; aro-9 a*.

Molecular characterization of fragment I RIP mutants: When taken through the sexual cycle, duplica-

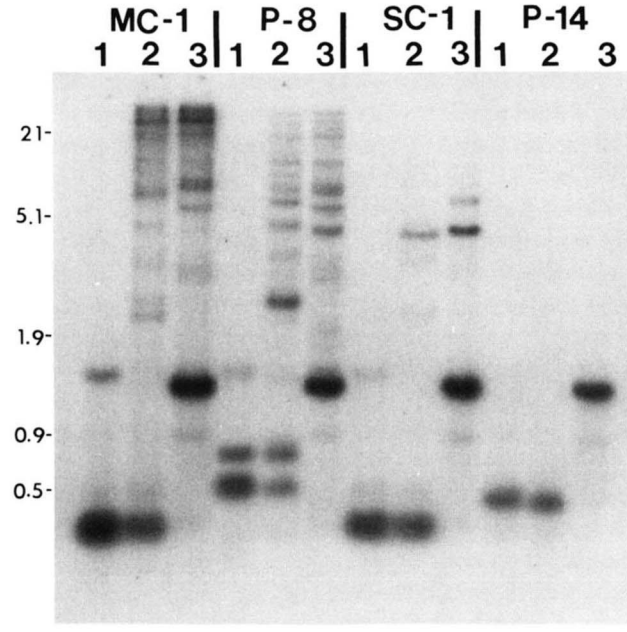

FIGURE 3.—Autoradiogram of restriction analysis of parental A/A^{m64} transformants and their sterile progeny. MC-1 panel: transformant containing multiple copies of the A^{m64} sequence ($A/(A^{m64})_n$). P-8 panel: sterile and heterokaryon-compatible strain from an $A/(A^{m64})_n \times$ *un-3 a* cross. SC-1 panel: transformant bearing a single copy of the transformed A^{m64} sequence (A/A^{m64}). P-14 panel: sterile and heterokaryon-compatible strain from an $A/A^{m64} \times$ *un-3 a* cross. Lanes 1, DNA digested with *Sau*3A; lanes 2, DNA digested with *Mbo*I; lanes 3, DNA digested with *Hpa*II. Fragment I (Figure 1) was used as the probe for the autoradiogram.

tions within the genome of *N. crassa* have been shown to undergo cytosine methylation and G-C to A-T mutations that result in altered restriction patterns (SELKER *et al.* 1987; SELKER and GARRETT 1988; CAMBARERI *et al.* 1989). The occurrence of RIP can be detected by use of restriction enzymes that have a 4-base recognition sequence containing C residues. The enzymes *Mbo*I and *Sau*3A are particularly useful for detecting RIP because they are isoschizomers, but differ in their capacity to digest methylated DNA. We isolated DNA from the initial transformants (A/A^{m64} and $A/(A^{m64})_n$) and their sterile progeny. The DNA samples were digested with *Mbo*I, *Sau*3A and *Hpa*II, transferred to Zetabind membrane after electrophoresis, and probed with fragment I (Figure 1). Nine *Sau*3A (*Mbo*I) fragments were present, the majority of which are less than 125 bp in length. Three *Sau*3A fragments of approximately 350 bp appeared as a single hybridization band in both the A/A^{m64} (SC-1) and $A/(A^{m64})_n$ (MC-1) transformants (Figure 3). Methylation of fragment I could be detected in both the A/A^{m64} and $A/(A^{m64})_n$ transformants, although the level of methylation was greater in the $A/(A^{m64})_n$ transformant. Alterations in restriction patterns of the 350-bp *Sau*3A fragments could be detected in sterile progeny of the $A/(A^{m64})_n \times$ *un-3 a* cross (P-8) and progeny of the $A/A^{m64} \times$ *un-3 a* cross (P-14). The remaining 13 $A^{I\ RIP}$ mutants generally contained detectable alterations in *Sau*3A restriction sites in addition to methylation (data not shown). These data are consistent with the sterile and heterokaryon-compatible phenotype of $A^{I\ RIP}$ mutants having been generated by RIP.

Complementation of fragment I RIP mutants: The normal sequence (NS) fragment I (Figure 1) was introduced into $A^{I\ RIP}$ spheroplasts by cotransformation with a vector conferring benomyl resistance (pSV50) (VOLLMER and YANOFSKY 1986). Approximately 50% of the transformants were capable of supporting perithecial development when crossed as a male or a female to an *a* mating type strain, although ascospores were not produced. The NS fragment I could be detected in restriction digests of DNA from $A^{I\ RIP}$ transformants capable of being induced for perithecial differentiation (data not shown).

Isolation of fragment II RIP mutants: Because of the success in generating mutants in fragment I using RIP, we chose this technique to search for mating-related functions in the remaining 4.3 kbp of the *A* idiomorph. We used a 3.0-kbp *Sst*II fragment of the *A* idiomorph 250 bp 5′ to *mt A-1* (Figure 1, fragment II). *qa-2*; *aro-9 A* spheroplasts were cotransformed with Fragment II and pQa2 to make duplications for the *A Sst*II fragment. A single-copy transformant, A/A^{sII}, and a multicopy transformant, $A/(A^{sII})_n$, were crossed to *un-3 a*. After 2 weeks, ascospores were isolated, heat-shocked and grown at 33° to select against the *un-3* and hence against the closely linked *a* idiomorph. All of the 157 ascospore progeny underwent full perithecial development when crossed to an *a* mating type tester strain, and all but one formed abundant ascospores. This strain, called $A^{II\ RIP}$, was isolated from the A/A^{sII} transformant $\times$ *un-3 a* cross. The $A^{II\ RIP}$ mutant was morphologically indistinguishable from the wild type in early perithecial development, but was drastically reduced in its capacity to produce ascospores.

Molecular characterization of fragment II RIP mutant: Genomic DNA was isolated from the A/A^{sII} transformant, from the $A^{II\ RIP}$ mutant, and from fertile *A* and *a* progeny from the $A/A^{sII} \times$ *un-3 a* cross. The DNA was digested with *Sau*3A and *Mbo*I, subjected to agarose gel electrophoresis and then transferred to nylon membranes. The membranes were then probed with fragment II (Figure 1); four fragments ranging in size from 910, 530, 335 and 240 bp can be seen on the autoradiogram (Figure 4). Methylation of fragment II sequences and restriction site alterations of the 530 bp fragment could be detected in the $A^{II\ RIP}$ mutant, but were not apparent in either the A/A^{sII} transformant or fertile *A* and *a* progeny from the $A/A^{sII} \times$ *un-3 a* cross (Figure 4).

Molecular characterization of fragment II second generation RIP mutants: The $A^{II\ RIP}$ mutant produces

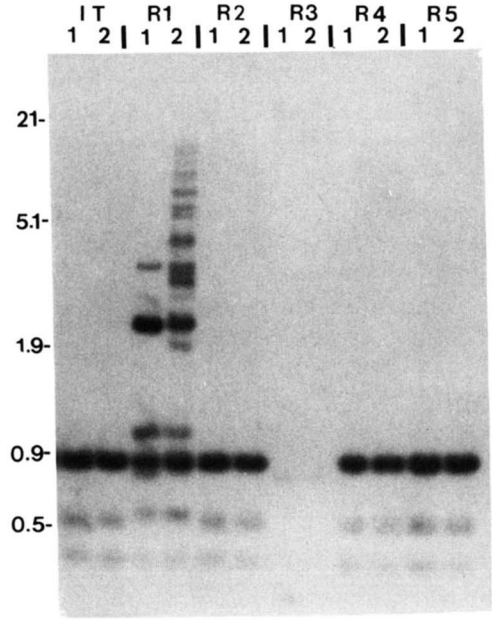

FIGURE 4.—Autoradiogram of restriction analysis of $A^{II\ RIP}$ mutant compared to fertile *A* and *a* progeny from $A/A^{sII} \times$ *un-3 a* cross. IT panel: initial A/A^{sII} transformant. R1 panel: $A^{II\ RIP}$ mutant from $A/A^{sII} \times$ *un-3 a* cross. R2, R4, R5 panels: fertile *A* progeny from $A/A^{sII} \times$ *un-3 a* cross. R3 panel: fertile *a* strain from $A/A^{sII} \times$ *un-3 a* cross. Lanes 1, DNA digested with *Sau*3A; lanes 2, DNA digested with *Mbo*I. Probe for the autoradiogram is fragment II (Figure 1).

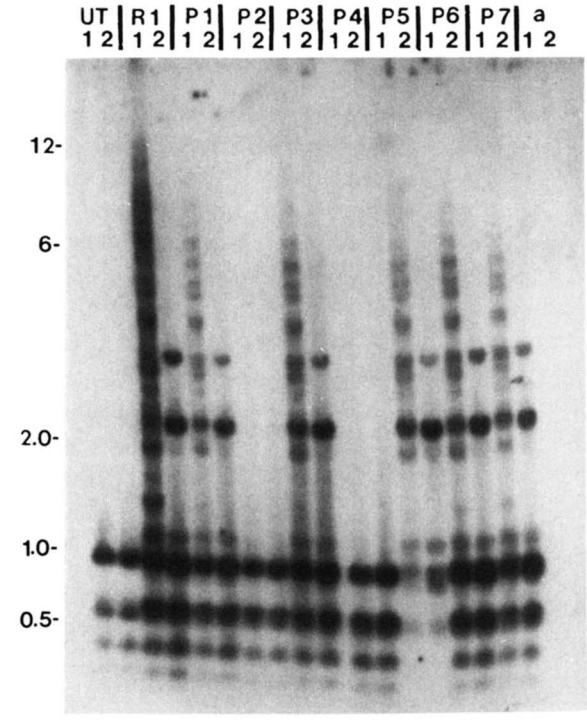

FIGURE 5.—Autoradiogram of restriction analysis of representative progeny from $A^{II\ RIP} \times$ *inl*; *fl a* cross. UT panel: *qa-2*; *aro-9*; *A*. R1 panel: $A^{II\ RIP}$ mutant from $A/A^{sII} \times$ *un-3 a* cross. P1–P7 panels: progeny from $A^{II\ RIP} \times$ *inl*; *fl a* cross. Panels numbered P1, P2, P3, P4, P6 and P7 are *A* mutants that either completely lack the ability to produce ascospores or produce very few. Panel numbered P5 is a normal, fertile *a* strain. *a* panel: *qa-2*; *aro-9 a*. Lanes 1, DNA digested with *Mbo*I; lanes 2, DNA digested with *Sau*3A. Probe for the autoradiogram is fragment II (Figure 1).

normal numbers of perithecia when crossed to an *a* mating type strain but ascospore formation is drastically reduced. Thirty-two ascospores were recovered from a $A^{II\ RIP} \times$ *inl*; *fl a* cross (Table 1); in contrast, thousands of ascospores are produced from a comparable cross between *A* and *a* wild-type strains. The 32 ascospores were analyzed for mating phenotype. Eighteen were found to be mating type *a* and produced abundant perithecia and ascospores when crossed to a mating type *A* strain. The remaining 14 ascospore isolates were mating type *A* and produced abundant perithecia when crossed to *a* mating type strain, but produced few or no ascospores when used as either a female or male parent.

DNA was isolated from the 14 mutant *A* progeny from the $A^{II\ RIP} \times$ *inl*; *fl a* cross, digested with *Sau*3A or *Mbo*I and probed with fragment II (Figure 1). Ten out the 14 of the $A^{II\ RIP}$ progeny showed extensive methylation of fragment II, while in the remaining four progeny, the DNA was unmethylated, or the amount of methylation was reduced. All 14 progeny inherited an altered *A* restriction pattern from the parental $A^{II\ RIP}$ mutant, presumably reflecting a combination of the effects of methylation and C-G to A-T transition mutations. Six representative mutant *A* (P1, P2, P3, P4, P6 and P7) and a single *a* (P5) progeny are shown in Figure 5. The P5 *a* progeny has inherited the ectopic copy of fragment II from the $A^{II\ RIP}$ parent, but mates normally as an *a* strain and produces abundant perithecia and ascospores.

Phenotypic and developmental characterization of the fragment II RIP mutants: Although a functional fragment II is required for a normal yield of ascospores, it is apparently not required for heterokaryon incompatibility. Previous results showed that heterokaryon incompatibility can be assayed by a reduction in transformation efficiencies; when *mt A-1* (fragment I) is introduced into *a* spheroplasts, the few recoverable transformants either displayed the incompatibility phenotype or received a *mt A-1* sequence which was disrupted during the transformation event (GLASS *et al.* 1988). In contrast to these results, transformants were recovered in equal numbers when fragment II (Figure 1) was introduced into *a* spheroplasts as compared to controls. In addition, compatible heterokaryons between $A^{II\ RIP}$ mutants and *un-3*, *a* (Table 1) could not be demonstrated.

To determine when the block in sexual development occurs in the $A^{II\ RIP}$ mutants, wild-type and mutant perithecia were dissected at various times during development and examined with both the light microscope and scanning electron microscope. Under starvation conditions, a single mating type culture (either

A or *a*) produces numerous female mating structures, termed protoperithecia (DODGE 1935; BACKUS 1939). Development of protoperithecia was normal in the $A^{II\ RIP}$ mutant and similar numbers of female structures were produced as compared to wild-type *A* (Figure 6, A and B). Following fertilization by *un-3 a*, the $A^{II\ RIP}$ and *A* protoperithecia enlarged and became blackened due the deposition of melanin. Three days post-fertilization, paraphysoidal hyphae within the central cavity of the perithecium (centrum) can be seen in both $A^{II\ RIP}$ and *A* (Figure 6, C and D). Paraphyses are thought to be composed of female tissue and are involved in the expansion of the centrum (LUTTRELL 1951). The development of an ostiole (a round opening in the top of the perithecium) and development of periphyses (hyphae that line the perithecial beak) in the $A^{II\ RIP}$ mutant were indistinguishable from wild type.

Four to five days post-fertilization, the development of the ascogenous hyphae with its characteristic croziers can be detected within the wild-type perithecium. Karyogamy and meiosis occur in the penultimate cell of croziers, and asci that develop from these cells can be seen among the paraphyses of the wild-type cross (Figure 6E). In the $A^{II\ RIP}$ mutant, development of the centrum is arrested at this point (Figure 6F). Formation of ascogenous hyphae is infrequent, although occasional croziers can be observed among the paraphyses. The nuclei ($A^{II\ RIP} \times a$) in these few croziers undergo normal karyogamy and meiosis and form mature asci and eight viable ascospores. The paraphyses persist in the $A^{II\ RIP}$ mutant and can still be observed within the centrum 12 days post-fertilization (Figure 6H). In the wild type, the paraphyses dehisce as the asci continue to develop until the centrum is filled with asci, each containing eight ascospores (Figure 6G). The perithecia continue to enlarge during this process and form prominent beaks. The $A^{II\ RIP}$ perithecia are comparable in size to those of the wild type, although the perithecial beak does not enlarge to the same extent (Figure 6H).

Complementation of fragment II RIP mutants: Restoration of ascospore formation in the $A^{II\ RIP}$ mutants was not obtained by the ectopic introduction of fragment II, by smaller fragments, or by the entire *A* idiomorph (Figure 1). No significant difference in mating phenotype could be seen between the original $A^{II\ RIP}$ mutant and the $A^{II\ RIP}$ mutant transformed with fragments II, III, IV or V (Figure 1).

DISCUSSION

The aim of the this study was to delineate mating functions of the *A* idiomorph by RIP mutation. Two functional regions have been identified by mutational analyses. The first region is the *mt A-1* ORF; RIP mutations within this region result in sterile and heterokaryon-compatible mutants. A second region of the *A* idiomorph (contained within a 3.0-kbp *Sst*II fragment) is apparently required after mating has taken place but before meiosis. In the fragment II RIP mutants, mating occurs and perithecia are formed (indicating that *mt A-1* function is intact), but further development is arrested. These mutants thus represent a novel class of *A* mating mutants that has not previously been described.

This study confirms that mutations generated by RIP in *mt A-1* result in the simultaneous loss of fertility and of heterokaryon incompatibility. Thus, a single gene product is necessary for conferring both of these functions on *N. crassa* strains. It is likely that *mt A-1* is a transcriptional activator that regulates *A* mating type-specific genes during haploid growth. Mating type-specific pheromones that mediate the attraction between the trichogyne and a male cell in *N. crassa* have been detected (BISTIS 1981, 1983), and these could conceivably be target genes for *mt A-1*. In support of this hypothesis, no discernable mating type reactions are observed when the *mt A-1* mutants are crossed with the opposite mating type, although male and female mating structures are present.

RIP is characterized by numerous G-C to A-T transition mutations and occurs during divisions of nuclei of opposite mating type in the ascogenous hyphae. Numerous $A^{I\ RIP}$ mutants were obtained when a strain containing a duplication of fragment I was taken through a cross. These $A^{I\ RIP}$ mutants could not mate, although they were initially obtained from a cross. Therefore, the accumulation of *mt A-1* mRNA must already have been sufficient for the completion of karyogamy and meiosis at the time RIP occurred. In support of this hypothesis, *mt A-1* transcripts could be detected in *A* cultures grown in vegetative or mating media (GLASS, GROTELUESCHEN and METZENBERG 1990), but the level of *mt A-1* mRNA is reduced to absent in perithecia 5 days post-fertilization (T. RANDALL and N. L. GLASS, unpublished results).

The mating type idiomorphs were recently cloned from the filamentous fungus, *Podospora anserina*, a close relative of *N. crassa*. This was accomplished by DNA hybridization of the *A* idiomorph of *N. crassa* to a genomic library from a *P. anserina* mat^- strain (PICARD, DEBUCHY and COPPIN 1991). The mat^+ sequences were cloned by DNA hybridization of sequences surrounding the mat^- idiomorph that are highly homologous between the mat^+ and mat^- genomes. As in the *N. crassa A* idiomorph, functional analyses of the 4.7-kbp *P. anserina* mat^- idiomorph confirmed the presence of two regions; one required for fertilization (*FMR1*) and a second region required for sporulation. The *FMR1* ORF in the mat^- fertilization region of *P. anserina* has a high degree of similarity to *mt A-1* ORF of *N. crassa*; in the amino

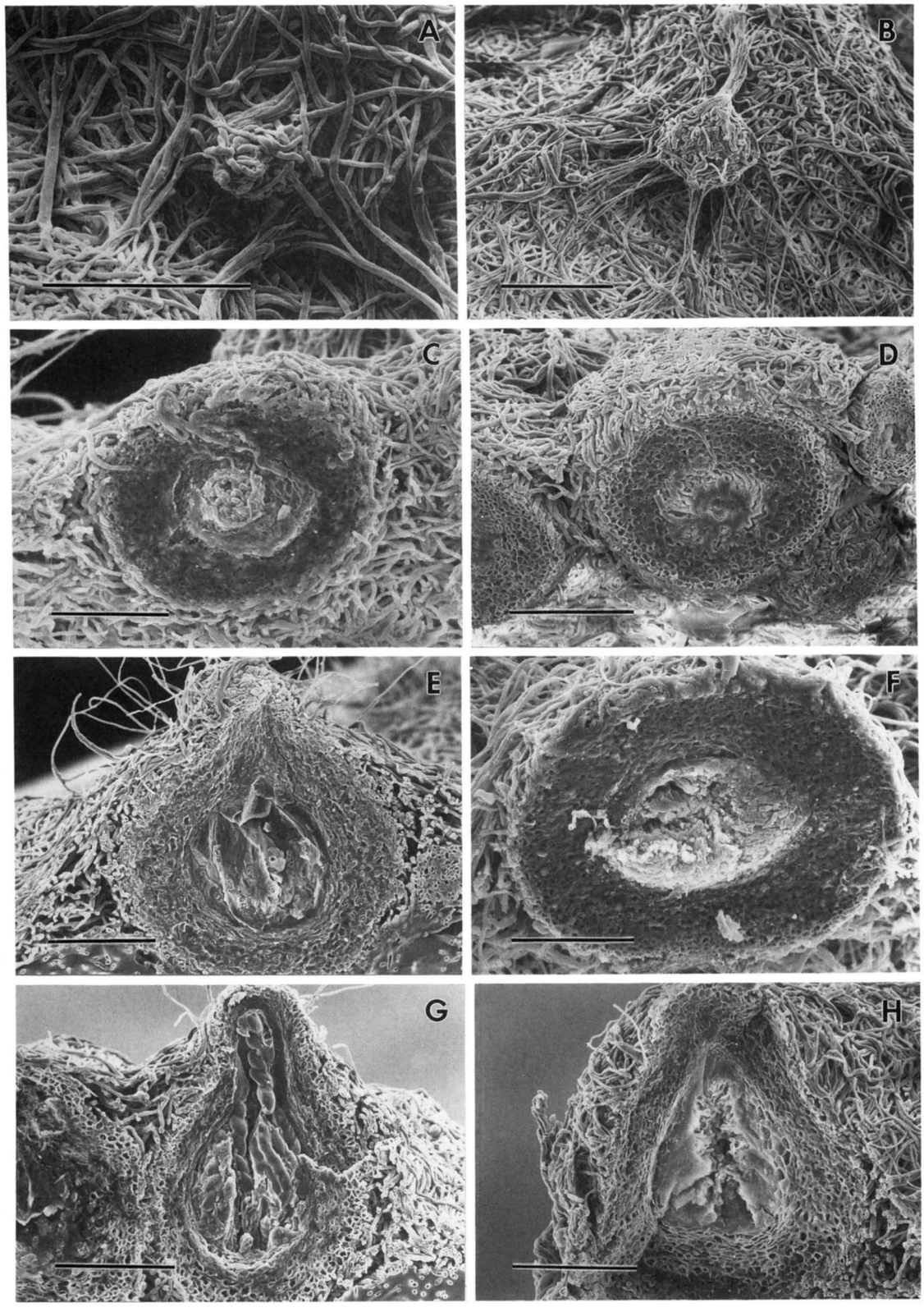

FIGURE 6.—Scanning electron micrographs of perithecial development during a 12-day period. Wild-type 74-ORS *A* (Table 1) (A, C, E, G) and $A^{II\ RIP}$ mutant (B, D, F, H). A and B: unfertilized protoperithecia. C and D: 3 days post-fertilization with *un-3 a*. E and F: 5 days post-fertilization. G and H: 12 days post-fertilization. The bar in each photograph represents 100 μm.

terminal portion of the two ORFs, 106 amino acids are identical out of 196 (DEBUCHY and COPPIN 1992). The introduction of *FMR1* DNA into *N. crassa* sterile A_m spheroplasts results in transformants capable of crossing with a *N. crassa a* mating type strain (N. L. GLASS, unpublished results).

By taking advantage of RIP, we were able to detect a second functional region of the *A* idiomorph that is required for the production of a normal yield of ascospores. The $A^{II\ RIP}$ mutant phenotype segregates with the *A* idiomorph and is correlated with altered restriction patterns in fragment II. The $A^{II\ RIP}$ mutant is capable of mating with an *a* strain and perithecial differentiation is induced, but ascospores are formed only in small numbers. Thus, the mutant phenotype is specific for *A* mating type and can not be complemented by the presence of wild-type *a* nuclei in ascogenous hyphae.

Microscopic and scanning electron microscopic examination of developing $A^{II\ RIP} \times a$ perithecia suggests that the block in sexual development occurs during formation of ascogenous hyphae. *A* and *a* nuclei must recognize each other within the syncytium of nuclei in the perithecium for formation of ascogenous hyphae, conjugate division, and karyogamy. Mutations that block recognition and/or compartmentation of opposite mating type nuclei would presumably give a phenotype similar to that observed in the $A^{II\ RIP}$ mutants. The few croziers observed displayed normal meiosis, indicating that if opposite mating type nuclei are capable of pairing and undergoing fusion, meiosis proceeds normally.

In this study, many more $A^{I\ RIP}$ mutants were isolated than $A^{II\ RIP}$ mutants. The fact that so few fragment II mutants were obtained suggests that the functional part of this region is required for the completion of karyogamy and meiosis. Mutations within this region would therefore greatly decrease or abolish ascospore production. Alternatively, fragment II may be less susceptible to RIP than fragment I of the *A* idiomorph, so that the majority of spores coming through a cross are unaltered. From the results presented in Figures 3 and 4, it is apparent that methylation of introduced DNA is quite variable in *N. crassa* and is apparently dependent upon the sequence and location of insertion. Methylation of duplicated sequences is thought to be a requirement for the RIP process (SELKER and GARRETT 1988; SELKER 1990).

As in fragment II of *N. crassa*, the *mat*$^-$ sporulation region of *P. anserina* does not play a role in fertilization, but is required for ascospore formation. A number of small ORFs are conserved between the *N. crassa* fragment II and the sporulation region from *P. anserina*. Sequence analyses of the *N. crassa* fragment II and protein database searches (DEVEREUX, HAEBERLI and SMITHIES 1984) indicate that a conserved ORF (R3i) shows similarity to the *S. pombe* mating type polypeptide, M_c (KELLY *et al.* 1988). The R3i ORF is 3.0 kbp from the *mt A-1* ORF, approximately 200 bp from the distal *Sst*II site (Figure 1) and would be transcribed in the opposite orientation. The R3i ORF is 30% identical and 50% similar to the 181-amino acid M_c ORF. Perhaps more significantly, the region of similarity is within the high mobility group (HMG) box motif (WRIGHT and DIXON 1988; JANTZEN *et al.* 1990) of the M_c ORF (data not shown).

Two mRNAs specific for fragment II of the *A* idiomorph have been detected in low levels in poly(A$^+$) RNA isolated from a *fl A* strain grown in mating media and from perithecia 5 days post-fertilization (N. L. GLASS, unpublished results). One of the mRNAs spans the *A* idiomorph junction and continues into a region for which DNA sequence information is not available. A report of mRNA transcripts and cDNA analyses of the *A* and *a* idiomorphs during vegetative and perithecial development will be presented elsewhere.

The introduction of the *A* idiomorph into the A^m or $A^{I\ RIP}$ mutants by transformation and ectopic integration restores heterokaryon incompatibility and the capacity for perithecial development, although ascospores are rarely produced. Similarly, the introduction of the *A* idiomorph into $A^{II\ RIP}$ mutants does not result in a detectable increase in ascospore formation. In a certain percentage of transformants, RIP could be expected to inactivate the introduced *A* idiomorph and thus reduce ascospore formation. However, from what we know of the RIP process, some of the transformants should escape RIP and thus produce abundant ascospores. This suggests that introduced copies of *A* idiomorph that are ectopic may be incorrectly expressed, or must integrate at the mating type locus for meiosis to proceed normally. Restoration of ascospore formation in A^m and a^m mutants has been correlated with homologous integration of an introduced copy of *A* or *a* idiomorphs (GLASS *et al.* 1988). It is possible that *cis*-acting features surrounding the mating type locus play a role in mating type function.

This study indicates the usefulness of RIP to create mutants that otherwise would have been difficult to isolate and analyze. RIP has also been useful in analyzing the function of *A* mating type-specific cDNAs that were isolated by subtractive cDNA hybridization (NELSON and METZENBERG 1992). Thus, RIP is a general tool for the initial characterization of cloned DNA of unknown function, such as fragment II of the *A* idiomorph of *N. crassa*. In addition, it has allowed isolation of a variety of mating mutants that will be useful in the further genetic, biochemical and molecular analyses of *A* mating type function.

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