Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 4912–4916, July 1990
Genetics

Neurospora crassa A mating-type region

(sexual reproduction/filamentous fungus/vegetative incompatibility)

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Communicated by David D. Perkins, April 16, 1990

ABSTRACT **The mating-type locus of the haploid filamentous fungus *Neurospora crassa* is a regulatory region that controls entry into the sexual cycle and prevents formation of mixed mating-type heterokaryons in the vegetative phase. The locus consists of alternative sequences called *A* and *a*. The *A* mating-type DNA sequence of *Neurospora crassa* is composed of a region of 5301 base pairs that has little similarity to the sequence present at the mating-type locus in an *a* mating-type strain. However, the sequences flanking the mating-type locus in the *A* haploid and *a* haploid genome are essentially identical. The region of the *A* mating-type sequence required for expression of the heterokaryon incompatibility and sexual functions has been localized to a single open reading frame (ORF) encoding a polypeptide of 288 amino acids. Sequence analysis of sterile, heterokaryon-compatible mutants reveals frameshift mutations in this same ORF. The putative 288-amino acid product has a region of similarity to the MATα1 polypeptide of *Saccharomyces cerevisiae*.**

The filamentous fungus *Neurospora crassa* requires fusion of cells of opposite mating types for initiation of events associated with the sexual cycle; its two mating types are designated *A* and *a*. The sexual cycle is initiated when a female structure (protoperithecium with its associated trichogyne) contacts the male donor cell (conidium or hypha) and fuses with it. The attraction of the trichogyne to the male cell is mediated by mating-specific pheromones (1, 2). The fusion of cells of opposite mating types initiates a series of developmental events leading to the formation of a fruiting body (perithecium) containing many asci, each with ascospores that are the haploid products of meiosis. Heteroallelism at the mating-type locus is necessary and sufficient for entry into the sexual cycle; that is, otherwise isogenic strains will mate provided that they are of opposite mating type.

In addition to its role in sexual reproduction, the mating-type locus determines heterokaryon incompatibility; if hyphae of opposite mating type fuse during vegetative growth, the resulting heterokaryotic cells are inhibited in their growth (3, 4). Except for a unique fertile, compatible mutant (a^{m33}), a series of *A* and *a* mating-type mutants selected for the loss of heterokaryon incompatibility were sterile and revertants to fertility simultaneously reacquired heterokaryon incompatibility (5, 6).

The *A* and *a* mating-type alleles of *N. crassa* were originally defined functionally as entities that must be heteroallelic if the sexual cycle is to occur. The *A* and *a* alleles have recently been redefined on a structural basis as regions of DNA that have little or no sequence similarity as judged by DNA hybridization studies (7). We introduced the term "idiomorph" to denote sequences, like those of mating types *A* and *a*, that occupy the same locus in different strains but are not related in sequence or (probably) common descent (8, 9). Henceforth in this report, we use this term instead of the familiar but inappropriate term "allele."

The introduction of either the *A* or *a* cloned idiomorphs by transformation into sterile mutants restores fertility partially (if integration is ectopic) or fully (if integration is homologous), the mating specificity being that of the transforming DNA (7). The cloned *A* and *a* idiomorphs were also shown to contain the heterokaryon-incompatibility function of the mating-type locus; viable transformants that were partial diploids for *A* and *a* sequences displayed the heterokaryon-incompatibility phenotype.

This study describes the physical characterization of the *A* mating-type idiomorph of *N. crassa* and its comparison to the *a* mating-type idiomorph.† The fertility and heterokaryon incompatibility functions of the *A* idiomorph were localized by sequence analysis of mating-type mutants and by functional assays to a 288-amino acid open reading frame (ORF) consisting of two exons (*mt A-1*). Transcriptional analyses, cDNA isolation, and sequencing were performed to confirm the presence and exact position of the intron. A comparison of the *mt A-1* ORF with known polypeptide sequences revealed a region of similarity between the *N. crassa mt A-1* ORF and the *MATα1* polypeptide of *Saccharomyces cerevisiae*. In the accompanying paper (10) on the *N. crassa a* mating-type idiomorph, Staben and Yanofsky report a region of the *mt a-1* ORF with amino acid sequence similarity to the product of *Schizosaccharomyces pombe matM$_c$*.

MATERIALS AND METHODS

Strains. *Escherichia coli* NM522 (11) was used as the recipient for bacterial transformation experiments. The following *Neurospora* strains were used: 74-OR23-IV *A* (FGSC 2489). *qa-2; aro-9 A* (RLM 52-22). *qa-2; aro-9 a* (RLM 52-21). *ad-3B cyh-1 a*^{m1} (FGSC 4564). *un-3 ad-3A nic-2 cyh-1 A*^{m42} (FGSC 4569). *un-3 ad-3A nic-2 cyh-1 A*^{m44} (FGSC 4570). *un-3 ad-3A nic-2 cyh-1 A*^{m54} (FGSC 4571). *un-3 ad-3A nic-2 cyh-1 A*^{m56} (FGSC 4572). *un-3 ad-3A A*^{m64} (FGSC 4573). *fl a* (FGSC 4347).

Materials. Restriction enzymes were from Promega. Moloney murine leukemia virus (M-MLV) reverse transcriptase was obtained from Bethesda Research Laboratories. DNA oligonucleotides were synthesized at the University of Wisconsin Biotechnology Center. [^{32}P]dCTP and [^{35}S]dATP were obtained from Amersham or DuPont. Oligo(dT)-cellulose was obtained from Pharmacia. *Thermus aquaticus* DNA polymerase (*Taq* I polymerase) was from Perkin–Elmer/Cetus and was used according to the manufacturer's suggestions with a Perkin–Elmer DNA thermal cycler.

Transformation. Competent spheroplasts of *N. crassa* strains were prepared according to the method of Schweizer

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Abbreviation: ORF, open reading frame.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M33876).

et al. (12) using the modification of Akins and Lambowitz (13). Transformation procedures into *N. crassa* competent spheroplasts to screen for mating-type activity and heterokaryon incompatibility were according to Glass *et al.* (7).

RNA Analysis. RNA from *N. crassa* cultures grown in synthetic crossing medium or in Vogel's minimal medium (14) was isolated according to Lucas *et al.* (15). Poly(A)$^+$ RNA isolation was according to Aviv and Leder (16). Northern blot procedures were according to Fourney *et al.* (17).

Amplification of cDNA. A cDNA specific for *mt A-1* was synthesized by using 1 μg of poly(A)$^+$ RNA (isolated from FGSC 2489 grown in crossing medium), a primer that spanned the putative stop codon (nucleotides 4843–4867), M-MLV reverse transcriptase, and reverse transcriptase buffer (Bethesda Research Laboratories). The *mt A-1* cDNA was amplified by polymerase chain reaction with a primer specific for the 5′ end of the *mt A-1* (nucleotides 4032–4048) plus additional 3′ end primer. The procedure for amplification of the cDNA was according to Frohman *et al.* (18).

DNA Sequencing. A series of overlapping deletions was constructed by using the Erase-a-Base kit (Promega). DNA sequence was determined primarily by the dideoxynucleotide chain-termination method of Sanger *et al.* (19). Single-stranded template DNA was obtained by using pGEM-3Zf vectors (Promega) according to the manufacturer's suggestions. The region surrounding and including the polypyrimidine tract was sequenced according to Maxam and Gilbert (20).

DNA Sequence and Protein Analyses. Computer sequence analyses and DNA (European Molecular Biology Laboratory) sequence bank and protein (National Biomedical Research Foundation) data bank searches were performed by programs available from the Wisconsin Genetics Computer Group (21).

RESULTS

Functional and Physical Characterization of the *A* Idiomorph. Previously, we estimated the length of the *A* idiomorph as 4.4 kilobase pairs (kbp) based on the failure of hybridization of restriction fragments to *a* genomic DNA (7). Sequence analysis of the *A* idiomorph reveals a region of 5301 bp that has little or no sequence similarity with the *a* idiomorph (Fig. 1). The *a* idiomorph is 3235 bp long by the criterion of dissimilarity between the *A* and *a* genomes [for physical characterization of the *a* idiomorph, see the accompanying paper (10)]. Surrounding the mating-type locus are sequences that are highly conserved between the two mating-type genomes. The transition from near identity to dissimilarity between the two idiomorphs is very abrupt (Fig. 1*B*). A comparison of the right and left border regions of the *A* idiomorph to each other and to border regions of the *a* idiomorph failed to reveal repeated structures or regions of dyad symmetry.

Introduction of the *A* idiomorph into spheroplasts of sterile mutants confers the ability to induce perithecium formation when the transformants are crossed to an *a* mating-type tester strain. Although perithecia develop in crosses with these transformants, ascospores are rarely produced. The Barren phenotype of the transformants resembles that of *N. crassa* strains bearing chromosomal duplications (22, 23). Microscopic examination of perithecial contents shows that sexual development stops at, or prior to, the formation of ascogenous hyphae. The phenotype of transformants is similar whether a^{m1} or A^m (A^{m42}, A^{m44}, A^{m54}, A^{m56}, and A^{m64}) mutants are used as recipients. Deletion analysis of the 5.3-kbp *A* idiomorph localized the mating-type activity to a 1-kbp fragment (*mt A-1*) toward the centromere-distal end (Fig. 1*A*). The introduction of fragments of the *A* idiomorph other than *mt A-1* into sterile mutants (a^{m1} and A^{m64}) did not confer

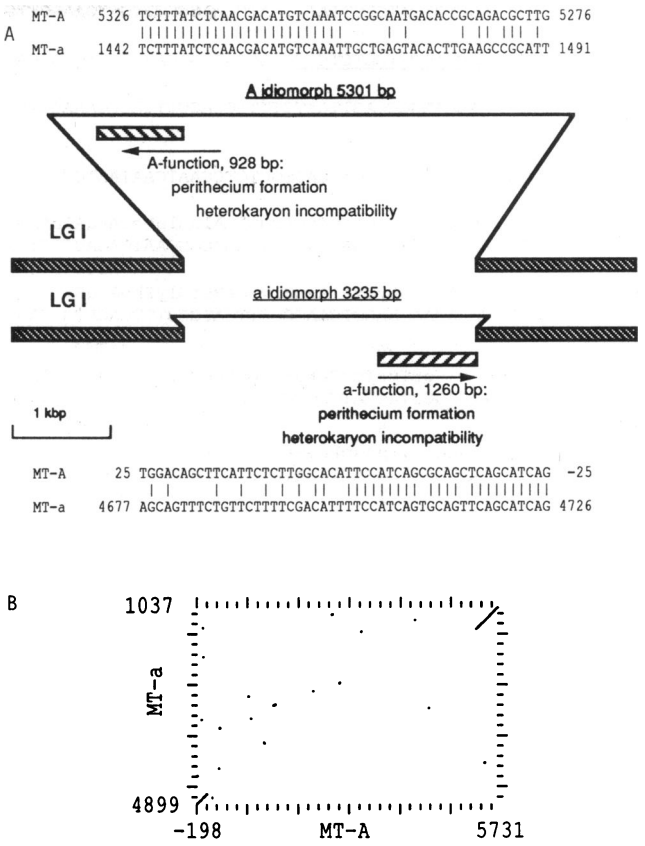

FIG. 1. (*A*) Comparison between the *A* idiomorph and the *a* idiomorph at the mating-type locus. LG1 hatched area is essentially identical between the *A* and *a* haploid genomes. The *A* idiomorph and the *a* idiomorph are dissimilar in sequence. The sequence of the left (centromere distal) boundary region of similarity/dissimilarity is shown above; the sequence of the right (centromere proximal) boundary region is shown below. The transcript-like strand of the *a* region and the non-transcript-like strand of the *A* region are shown. The regions of the *A* and *a* idiomorph that confer fertility and heterokaryon incompatibility are hatched boxes within the idiomorphs. (*B*) Dot plot (21) comparison between the *A* and *a* idiomorphs including sequences surrounding the mating-type locus. The *A* and *a* idiomorphs are numbered from the centromere proximal side and centromere distal side, respectively. The regions of dissimilarity run from +1 to +5301 for the *A* and from +1467 to +4701 for *a*.

fertility or any obvious sexual or vegetative phenotype upon the transformants.

In addition to its function during the sexual cycle, the cloned *A* idiomorph was shown to contain the heterokaryon incompatibility function of the mating-type locus (7). The region of the *A* idiomorph conferring heterokaryon incompatibility is contained within *mt A-1*. The introduction of *mt A-1* into *a* spheroplasts by DNA transformation to form heterozygous partial diploids for mating type (*mt A-1/a*) reduced the recovery of transformants 100-fold compared with transformation into *A* mating-type spheroplasts. Surviving transformants either contained disrupted copies of *mt A-1* or they continued to exhibit the phenotype associated with the heterokaryon incompatibility response. Both the fertility and heterokaryon-incompatibility functions were lost concurrently when deletions were made from either the 5′ or 3′ end of the 1-kbp fragment; the smallest fragment that retained the two activities was 750 bp.

Sequence Analysis of the *A* Idiomorph. The only plausible ORF in the *A* idiomorph was in the region already identified as *mt A-1*. This ORF consisted of two exons separated by a consensus sequence for an intron 163 bp downstream of the first ORF ATG start site (Fig. 2). If the putative 59-bp intron

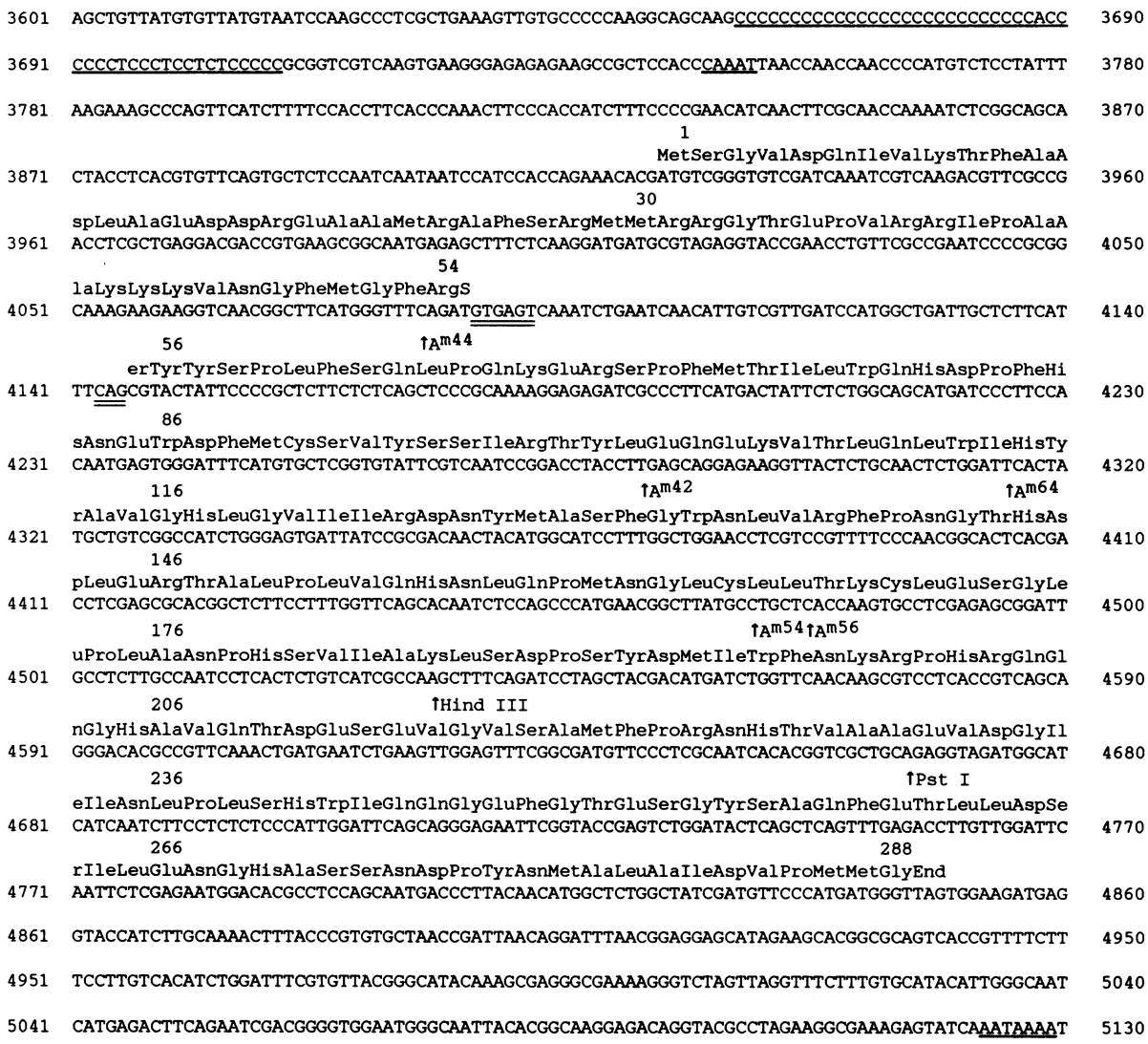

FIG. 2. Sequence of the functional region conferring fertility and heterokaryon incompatibility of the *A* idiomorph. The nucleotide sequence is numbered as described in the legend to Fig. 1. Deduced amino acid sequence of *mt A-1* is shown above the DNA sequence. The 5′ and 3′ splice junctions of the 59-nucleotide intervening sequence are double underlined. Locations of A^m mutations are indicated by arrows. The 49-nucleotide polypyrimidine tract is underlined as are the CAAT and polyadenylylation sequences.

were spliced out, the first and second exon would code for a translational product encoding a 288-amino acid polypeptide with a predicted molecular weight of 32,732 and a pI of 6.51. The *mt A-1* ORF was analyzed for *Neurospora* codon usage (21); it is likely to be protein coding.

The initial constructs of the *A* idiomorph used for complementation of the sterile mutants all terminated at the *Pst* I site (Fig. 2). However, the first in-frame stop codon, which is presumably the physiologically important one, is 182 bp 3′ to the *Pst* I site. No differences in complementation of sterile mutants could be detected between recipients containing the *Pst* I-terminated clones versus recipients containing the entire sequence of *mt A-1*. However, constructs in which an additional 124 bp were deleted from the 3′ end (*Hin*dIII site; Fig. 2) fail to complement sterile recipients.

The most striking structural feature of the *mt A-1* sequence is the presence of a 49-bp polypyrimidine tract interrupted by only 1 purine base (Fig. 2). This polypyrimidine tract is 215 bp 5′ to the ATG of *mt A-1* ORF. However, deletion constructs missing this region complement sterile mutants. A CAAT consensus sequence is present 172 bp 5′ to the translation start site, but a TATA consensus sequence could not be identified. A polyadenylylation site (AATAAAA) consensus sequence is located 274 bp 3′ to the stop codon of *mt A-1* ORF.

Sequence and Functional Analyses of A^m Mutants. Single frameshift mutations in *mt A-1* ORF are responsible for both the sterility and heterokaryon compatibility of the A^m mutants. Spheroplasts from A^m mutants (A^{m42}, A^{m44}, A^{m54}, A^{m56}, and Am^{64}) were transformed with the wild-type sequence of *mt A-1*; the ability to form perithecia was restored in all five mutants, although few ascospores were produced. Sequence analysis of the five A^m mutants revealed the presence of either small deletions or insertions within *mt A-1*. These mutations, which correspond to frameshift mutations in the ORF, were all located in the first 550 bp of *mt A-1* (Fig. 2); none occurred in the intron of *mt A-1*. In addition, the introduction of a cloned mutant *A* sequence (A^{m64}) did not restore fertility when transformed into sterile recipients.

Characterization of the *A* Idiomorph Transcripts. Northern blots of poly(A)$^+$ RNA isolated from *A* cultures grown under nutrient conditions either conducive or nonconducive to mating were probed with labeled DNA from *mt A-1*. A 1.2-kb mRNA that included all of *mt A-1* was detected at a low level under both mating and nonmating nutrient conditions. The interpretation that the *A* mRNA is the product of splicing of two exons was confirmed by cDNA isolation and sequencing.

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30 MRRGTEPVRRIPAAKKKVNGFMGFRSYYSPLFSQLPQKERSPF  72 mt A-1
          |   || :|:||:|| ||| : | : |   | :
76 TQFRQFNKTSIKSSKKYLNSFMAFRAYYSQFGSGVKQNVLSSL 118 MAT α1
```

FIG. 3. Region of amino acid sequence alignment of *mt A-1* ORF and MATα1. Identities are indicated by vertical lines. :, Conservative changes.

The *mt A-1* transcripts could be detected in *A tol* + *a tol* heterokaryons [the *tol* mutation allows growth of mixed mating-type heterokaryons by partly suppressing the heterokaryon incompatibility function of the mating-type locus (24); suppression is only partial when defined medium is used (R.L.M., unpublished results)]. This indicates that, at least under these conditions, transcription of *mt A-1* is not markedly decreased when *A* nuclei are in a common cytoplasm with *a* nuclei.

The *A* idiomorph is 5301 bp long, yet only 1 kbp of the *A* region has any defined function based on mutational analyses and functional assays. When poly(A)$^+$ RNA from *N. crassa* grown on crossing or vegetative medium was probed with fragments from the *A* idiomorph other than *mt A-1*, no mRNAs encoded by this region were detected. However, initial mutational analysis of this region has resulted in the isolation of mutants with unusual phenotypes with respect to fertility. The exact nature and location of the mutation(s) within the *A* idiomorph that confers the mutant phenotype have not yet been determined.

Similarity Between *mt A-1* ORF and *MATα1* Polypeptide. The 926-bp *mt A-1* DNA sequence and predicted protein sequence were compared with the European Molecular Biology Laboratory data bank, the National Biomedical Research Foundation nucleic acid sequence data bank, and the National Biomedical Research Foundation protein data bank to search for similarities to any recorded DNA or protein sequence. Of the polypeptide sequences in the data banks, the *MATα1* polypeptide (amino acids 90–104) of *S. cerevisiae* had the greatest similarity to the *mt A-1* ORF (amino acids 45–59) (Fig. 3). Of a region of 15 consecutive amino acids, 10 are identical between mt A-1 ORF and MATα1, and 3 can be interpreted as conservative substitutions. A comparison of the remainder of the polypeptide sequences did not reveal additional significant regions of amino acid sequence similarity between mt A-1 polypeptide and MATα1.

DISCUSSION

The *N. crassa A* and *a* mating-type idiomorphs are defined by regions of sequence dissimilarity between the *A* and *a* haploid mating-type genomes, which include sequences necessary for the initiation of the sexual cycle. By the criterion of dissimilarity, the *A* idiomorph is 5301 bp and the *a* idiomorph is 3235 bp long. The sharpness of the border between dissimilarity and identity or near-identity needs comment. Ordinarily, two examples of a particular sequence may be kept similar or invariant between individuals either because they code for a strongly selected macromolecular species or because the sequences are fairly recent descendants of a particular molecule. The former is exemplified by the yeast mating-type alleles, *MAT***a** and *MATα*, and their flanking sequences. *Mat***a** and *Matα* are composed of dissimilar DNA sequences of 642 bp (Y**a**) and 747 bp (Yα), respectively (25). However, the DNA sequences flanking the Y**a** and Yα regions at the *HML*, *HMR*, and *MAT* loci are essentially identical. The sharp boundary between identity and dissimilarity at the *MAT* locus is maintained because mRNAs encoding MATα2, MATα1, and MAT**a**2 originate in the Yα and Y**a** regions but terminate in the flanking region of identity (26, 27).

Unlike the yeast mating-type mRNAs, the mRNA for the functional region of the *A* idiomorph, *mt A-1*, is completely coded by the region of sequence dissimilarity. We might expect that sequences flanking the mating-type locus, but not essential to it, would diverge mutationally between strains of the two mating types because they would very rarely be separated from mating type by crossing-over or gene conversion. According to this idea, the transition between dissimilarity and similarity should be gradual, reflecting the recentness of historical cross-over or conversion events. The fact that the border is sharp, not gradual, suggests that some other mechanism preserves sequence identity immediately flanking the idiomorphs—perhaps strong selection for maintenance of some unknown function.

Although the *N. crassa A* idiomorph is 5.3 kbp, only 1 kbp of it is required for events associated with the initiation of the sexual cycle. Entry into the sexual cycle requires the fusion of cells of opposite mating types, apparently mediated by the production of mating-specific pheromones and their receptors (1, 2). The A^m mutants are incapable of initiating the sexual cycle; A^m trichogynes are not attracted to, nor do they fuse with, an opposite mating-type male cell, nor are A^m male cells able to attract *a* trichogynes (C. Myers and A. J. Griffiths, personal communication). However, the A^m mutants are otherwise indistinguishable from wild type in the development and morphology of their female and male reproductive structures. The implication from these results is that *mt A-1* regulates the production of mating-specific *A* pheromone(s) and receptor(s) for the *a*-specific mating pheromone(s).

Fusion of cells of opposite mating types in the sexual cycle results in meiosis; fusion in the vegetative phase results in heterokaryon incompatibility. Both the heterokaryon incompatibility function as well as the fertility function have been localized to *mt A-1* of the *A* idiomorph. In the accompanying paper by Staben and Yanofsky (10), the fertility and heterokaryon incompatibility function of the *a* idiomorph was localized on a single ORF termed *mt a-1*. In the sexual phase, the heterokaryon incompatibility function of *mt A-1* and *mt a-1* must be nullified or counterbalanced by some other activity to allow the conjugate divisions of opposite mating-type nuclei in the ascogenous hyphae. Thus, the physiological status of the organism (either vegetative or mating competent) is important in determining the end result of fusion of opposite mating types. This suggests that the *mt A-1* and *mt a-1* products interact with a factor or factors reflective of the physiological status of the organism.

Introduction of the entire *mt A-1* sequence is not required for initiation of the sexual cycle in A^m and a^m sterile mutants. Deletion constructs lacking 200–250 bp of sequence at the 3′ end of *mt A-1* are able to confer the capacity for perithecium formation upon transformants when they are crossed to an *a* mating-type strain. This indicates that the carboxyl-terminal portion of the mt A-1 polypeptide is dispensable for this function. Since all of the A^m mutants are frameshift mutants, it is not known whether the fertility and heterokaryon incompatibility functions have separate domains within *mt A-1*. In contrast, a point mutation in *mt a-1* [a^{m33} (5)] results in an amino acid substitution, which makes the strain heterokaryon compatible without abolishing fertility [see accompanying paper (10)].

Transformants containing copies of the *A* idiomorph at random sites in the genome do not form ascospores. Occasionally, however, transformation of A^m and a^m sterile mutants with the *A* idiomorph gives full complementation when the transformant is paired with an *a* strain and ascospores are formed. When DNA samples prepared from fertile transformants were examined, it was clear that all of the insertion events that resulted in full fertility were gene replacements (7). This suggests that cis-acting sequences are important for proper expression of *mt A-1*.

The RNA for *mt A-1* is transcribed under mating and nonmating conditions; however, little is known about the sequences or factors that control its expression. A long polypyrimidine tract is present in the *A* idiomorph 215 bp 5′ to the *mt A-1* translational start site. Poly(dG)-poly(dC) tracts have been associated with putative regulatory regions of various genes and have been correlated with gene expression in fungi as well as cell-specific gene expression in higher eukaryotes (28, 29). However, constructs of *mt A-1* lacking the polypyrimidine tract complement sterile mutants as efficiently as constructs containing it.

Of the 5301-bp *A* idiomorph, only the *mt A-1* fragment has a clearly defined function. However, DNA hybridization studies show that the entire region is conserved in all species of *Neurospora* examined (7) as well as in related genera (N.L.G., N. Raju, and R.L.M., unpublished data), indicating that the entire region may be important for *A* function. Initial mutational analyses of the *A* idiomorph indicate that regions in addition to *mt A-1* are required for the completion of the sexual cycle. It is possible that structural sequences involving regions other than *mt A-1* are required after the fusion of opposite mating types and may be involved in the formation of ascogenous hyphae or during the process of meiosis itself.

A computer data bank comparison between the *mt A-1* ORF and protein sequences present in the National Biomedical Research Foundation protein data bank revealed a region of significant similarity to MATα1 of *S. cerevisiae*. Within a 15-amino acid region, both basic and hydrophobic residues are conserved. In *S. cerevisiae*, the initial mating recognition events have been shown to be mediated by mating-type-specific pheromones and their receptors. The gene for pheromone production and the *a*-factor pheromone receptor are under transcriptional control by MATα1 (30, 31). MATα1 interacts with a non-mating-type-specific transcriptional factor, MCM1 (32, 33), which binds to the promoter regions of *MAT*α-specific haploid genes, such as the genes for α factor (*MF*α*1*, *MF*α*2*) and the receptor for a factor (*STE3*) (34). It is possible that in *N. crassa* the product of *mt A-1* ORF interacts with an analogous MCM1-like factor to control genes that mark a strain as an *A* mating type. Abolition of the *A* function would then result in a strain that is both female and male sterile: female sterile because it lacks the *a* receptor and is unable to recognize an *a* male, and male sterile because it fails to produce the *A* pheromone and cannot attract an *a* trichogyne.

In contrast to the putative product of *mt A-1*, the *a* idiomorph mating-type product has a region of similarity to *Schizosaccharomyces pombe mat-M*$_c$ product [see accompanying paper (10)]. Evolutionary studies indicate that *S. cerevisiae* and *Sc. pombe* are not at all closely related. Nevertheless, a distant common ancestor of both these species and of *N. crassa* may have contained DNA sequences that were ancestral to *MAT*α*1*/*mt A-1* and to *mat-M*$_c$/*mt a-1*. The proteins encoded by these sequences comprise a family of cell type-controlling regulatory factors that have been sufficiently preserved to be recognizable today.

The authors are grateful to Drs. David Perkins, Chuck Staben, MaryAnne Nelson, and Charles Yanofsky for their suggestions, and to Chuck Staben and Charles Yanofsky for sharing their data with us in advance of publication. This work was supported by U.S. Public Health Service Grant GM 08995 (to R.L.M.). N.L.G. was a Postdoctoral Fellow of the American Cancer Society during most of the course of this work.

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