Functional Analyses of the *Neurospora crassa* MT a-1 Mating Type Polypeptide

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ABSTRACT

The *Neurospora crassa* *mt a-1* gene, encoding the MT a-1 polypeptide, determines a mating type properties: sexual compatibility and vegetative incompatibility with A mating type. We characterized *in vivo* and *in vitro* functions of the MT a-1 polypeptide and specific mutant derivatives. MT a-1 polypeptide produced in *Escherichia coli* bound to specific DNA sequences whose core was 5'-CTTTG-3'. DNA binding was a function of the MT a-1 HMG box domain (a DNA binding motif found in high mobility group proteins and a diverse set of regulatory proteins). Mutation within the HMG box eliminated DNA binding *in vitro* and eliminated mating *in vivo*, but did not interfere with vegetative incompatibility function *in vivo*. Conversely, deletion of amino acids 216–220 of MT a-1 eliminated vegetative incompatibility, but did not affect mating or DNA binding. Deletion of the carboxyl terminal half of MT a-1 eliminated both mating and vegetative incompatibility *in vivo*, but not DNA binding *in vitro*. These results suggest that mating depends upon the ability of MT a-1 polypeptide to bind to, and presumably to regulate the activity of, specific DNA sequences. However, the separation of vegetative incompatibility from both mating and DNA binding indicates that vegetative incompatibility functions by a biochemically distinct mechanism.

THE mating type locus of *Neurospora crassa*, like that of yeast (HERSKOWITZ *et al.* 1992), is a master regulatory locus that controls the sexual life cycle of the fungus (GLASS and STABEN 1990). This locus determines the alternate mating types,¹ *a* and *A*. The *a* and *A* mating types contain completely different DNA sequences, called idiomorphs, at the mating type locus (GLASS *et al.* 1990; METZENBERG and GLASS 1990; STABEN and YANOFSKY 1990). The *a*-specific DNA segment is 3.2 kbp; the *A*-specific segment is 5.3 kbp. These idiomorphs control certain vegetative functions and also regulate the morphologically complex sexual development of *N. crassa*.

The primary vegetative form of *N. crassa* is a haploid, multinucleate mycelium. *N. crassa*, like many fungi, including *Saccharomyces cerevisiae*, produces mating type-specific pheromones (BISTIS 1981, 1983). Production of pheromone by *a* cultures depends upon the *mt a-1* gene encoded by one half of the *a* idiomorph (BISTIS 1981; STABEN and YANOFSKY 1990). However, the *N. crassa* mating type locus has an additional role in vegetative growth unlike any associated with *S. cerevisiae* mating type. The *mt a-1* and *mt A-1* genes encoded by the mating type idiomorphs prohibit the formation of stable *a/A* heterokaryons (GLASS *et al.* 1990; STABEN and YANOFSKY 1990). Such barriers to heterokaryosis are called vegetative incompatibility systems (GLASS and KULDAU 1992).

¹ The nomenclature for *N. crassa* mating type, the genes that specify mating type, and their encoded polypeptides present problems with respect to uniform naming. After discussion with other workers in this field and with editors of *Genetics*, we have adopted the following conventions. The mating type locus is the *mt* locus. The two idiomorphs, *mt a* and *mt A*, may each contain multiple genes. We designate these genes *mt a-1*, *mt A-1*, etc. The polypeptides encoded by these genes will be designated MT a-1, MT A-1, etc., respectively.

The mating type locus has multiple roles during the sexual cycle of *N. crassa* (GLASS and STABEN 1990; RAJU 1992). Differentiated female structures respond to mating pheromones in a mating-type specific manner (BISTIS 1981). Fertilized females develop morphologically complex fruiting bodies and produce zygotic asci. Genetic evidence suggests that *a* plays roles in post-fertilization events (RAJU 1992). A single gene, *mt a-1*, appears to be both necessary and sufficient for all *a* mating type functions. Replacement of a resident *A* idiomorph with a DNA segment containing only the *mt a-1* gene was sufficient to specify all functions of the *a* mating type, including fertility (S. CHANG and C. STABEN, in preparation). Sequence and functional analyses of *mt a-1* genes from a wild type strain and from mating mutants (STABEN and YANOFSKY 1990) indicate that the MT a-1 polypeptide encoded by this region is the essential determinant for both vegetative incompatibility and mating. We infer that MT a-1 is the *a* product that is necessary for mating and vegetative incompatibility and sufficient for ascospore formation. However, the biochemical mechanisms by which the MT a-1 polypeptide acts are not known.

The sequences of *mt a-1* from wild type and from *a^m* mating type mutants (STABEN and YANOFSKY 1990) suggested that MT a-1 had at least two functional regions. First the MT a-1 amino terminus contains an HMG box motif found in DNA binding proteins (GRIESS *et al.* 1993). The HMG box region is strongly conserved in the mating type factors homologous to MT a-1 from *Schizosaccharomyces pombe* (*mat-Mc*) (KELLY *et al.* 1988), *Podospora anserina* (*FPR1*) (DEBUCHY and COPPIN 1992), and *Cochliobolus heterostrophus* (*MAT-2*)

(TURGEON *et al.*, 1993). Therefore, we suspected that the HMG box region was a DNA binding domain essential for mating. The carboxyl terminus of MT a-1 is rich in acidic and proline residues, similar to regions found in transcriptional transactivator proteins (HAHN 1993). Mutations in this portion of MT a-1 eliminate either vegetative incompatibility or both mating and vegetative incompatibility (STABEN and YANOFSKY 1990). Also, the other fungal mating factor homologs differ greatly from MT a-1 in regions other than the HMG box. However, one homolog, *P. anserina* *FPR1*, confers mating, but not vegetative incompatibility in *N. crassa* (ARNAISE *et al.* 1993). These observations suggested that the carboxyl terminus contained some essential determinants for mating, and that determinants of mating and of vegetative incompatibility differ.

In this study, we determined that MT a-1 binds to specific DNA sequences within and surrounding the *a* and *A* idiomorphs. We compared the *in vitro* DNA binding properties of MT a-1 mutants to the mating and vegetative incompatibility functions of these gene products *in vivo*. These observations suggest a model for the actions of the MT a-1 polypeptide relating the structure of the MT a-1 polypeptide to its functions in mating and in vegetative incompatibility.

MATERIALS AND METHODS

Chemicals, media and oligonucleotides: Chemicals, restriction enzymes and media were from standard suppliers. Hygromycin B was purchased from Calbiochem. Oligonucleotides were prepared by the MSAF facility at the University of Kentucky.

Construction of MT a-1 expression vectors: A DNA sequence encoding the presumed MT a-1 polypeptide was constructed by oligonucleotide-directed mutagenesis (KUNKEL *et al.* 1987) to remove intron DNA from cloned *mt a-1* genomic DNA. An *NdeI* site was introduced at the initiation codon to facilitate insertion into T7-promoter based expression vectors (STUDIER *et al.* 1990). Base changes were confirmed by sequence analysis; the modified plasmid also conferred *a* activity in *N. crassa* transformation assays.

Additional DNA sequence changes were introduced by oligonucleotide-directed mutagenesis (KUNKEL *et al.* 1987) into DNA templates bearing the open reading frame construct and a hygromycin-resistance marker for selection in *Neurospora* transformations (pCSK31). These altered coding regions were also introduced into T7-promoter based expression vectors pET11a or pET22b (Novagen).

Purification of MT a-1 polypeptides expressed in *Escherichia coli*: BL21(DE3)pLysS cells bearing pET expression plasmids were cultured at 37° in ZBM9-broth with ampicillin (100 µg/ml) to an optical density of 0.5–0.7 (600 nm). Isopropyl β-D-thiogalactopyranoside was added to a concentration of 0.4 mM. After 30 min, rifampicin was added to a final concentration of 200 µg/ml and cells were incubated for an additional 4 hr. Addition of rifampicin, which inhibits *E. coli* RNA polymerase, was essential to visualize induced product. Cells were harvested by centrifugation and frozen at –80° or processed immediately.

In a typical preparation of purified MT a-1 full length polypeptide, 4 g of *E. coli* cells were resuspended in 12 ml buffer A (50 mM Tris-Cl, pH = 8.0, 1 mM EDTA). Phenylmethylsulfonyl fluoride was added to 1 mM as cells thawed. The extract was frozen and thawed three times. Endogenous T7 lysozyme lysed the cells and 80 µl DNase I were added to re-

duce viscosity of the extract. After incubation on ice for 30 min, the extract was centrifuged at 16,000 × *g* for 5 min. The pellet was resuspended in the original volume of buffer A and repelleted. This pellet was washed twice with buffer A/0.5% Triton X-100. The final pellet was solubilized in buffer A/8 M urea. Solubilized proteins were chromatographed on a 2.5 × 10-cm column of P11 phosphocellulose (WHATMAN) equilibrated in buffer A/8 M urea. The column was washed with buffer A/8 M urea until protein no longer appeared in the eluent. Then, the column was eluted with a 10 column volume linear gradient of 0–250 mM KCl in buffer A/8 M urea. Polypeptide used in these experiments eluted at approximately 150 mM KCl. A second, separable peak of MT a-1 eluted prior to this peak. Although the properties of fractions eluting early and late appeared to be identical, early fractions were contaminated with other host proteins and were not used. We presumed that the fractions differed in a minor modification, such as the presence or modification state of the amino-terminal methionine because they had similar amino acid compositions. Concentrations of protein were measured by dye-binding with bovine serum albumin as a standard (Bio-Rad Protein Assay). Purification was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Amino acid compositions were determined by acid hydrolysis and high performance liquid chromatography by MIKE CLARK, Schering Corporation. Amino acid composition of the purified MT a-1 polypeptide was consistent with that expected from the gene sequence (data not shown). Several renaturation protocols were compared. All protocols yielded soluble, active protein. The simplest protocol, dialysis *vs.* buffer A at 4° with two changes, was used in all experiments.

Mutant polypeptides were induced and purified by differential centrifugation of insoluble protein fractions as above. These partially purified preparations (~30% pure MT a-1 polypeptide) were solubilized in buffer A/8 M urea and renatured by dialysis against buffer A. Full-length polypeptide in such fractions behaved identically to purified MT a-1 in gel retardation assays. Protein extracts prepared from cells containing vector had no DNA binding activity, nor did such extracts interfere with binding by purified polypeptide.

Electrophoretic assays of DNA binding: DNA binding was assayed by alteration of electrophoretic mobility. The standard DNA fragment for assay was a 133-base *TaqI* fragment from base 3106 to 3239 of the published *mt a-1* sequence (STABEN and YANOFSKY 1990). Additional fragments were derived from bases 2750–3250 of the *A* mating type sequence (GLASS *et al.* 1990) or bases 5489–6022 of DNA flanking the *a* idiomorph (numbers relative to (STABEN and YANOFSKY 1990). Fragments were labeled with Klenow fragment and dNTPs to yield labeled, blunt ended, molecules which were purified by agarose gel electrophoresis. Typically, 9 fmol of DNA fragments were incubated with indicated amounts of polypeptide in the absence of magnesium at room temperature (TRAVIS *et al.* 1991). Autoclaved, deproteinized salmon sperm DNA or dl-dC appeared to be equally effective as nonspecific competitors; 1 µg salmon sperm DNA was used in these experiments. After incubation, samples were loaded onto a low-salt Tris-acetate, 4% polyacrylamide gel and electrophoresed 2–3 h at 100 V with buffer recirculation. Dried gels were autoradiographed.

DNaseI footprinting of DNA-protein complexes: Specific protein complexes were footprinted essentially as described (TRAVIS *et al.* 1991). The concentration of KCl in the assays was increased to 250 mM to increase binding specificity. At this KCl concentration, the mobility shift assays showed the clearest retained specific bands and the least nonspecific binding.

***In vivo* assay of mating type activities:** Both mating and vegetative incompatibility properties of wild-type and mutant forms of MT a-1 were determined by transformation

into an a^{m30} , $ade3B$ strain of *N. crassa* (GLASS *et al.* 1988; STABEN and YANOFSKY 1990). This mutant has a 212-bp insertion in the HMG box region of *mt a-1* that causes loss of both mating and vegetative incompatibility functions. This strain was transformed with the wild type *mt a-1* gene or mutant derivatives carried on the plasmid pCSK31, which also contains the hygromycin B resistance gene from pCSN43 (STABEN *et al.* 1988). At least ten individual hygromycin resistant transformants were tested for mating or vegetative incompatibility.

Mating function was assayed as the production of enlarged, darkened perithecia when recipients were mated to a *flA* tester strain. These strains did not mate with a *flA* tester strain. Untransformed a^{m30} cultures formed no perithecia when crossed to *A*; transformants bearing functional mating type genes formed perithecia. Such transformants were never fertile, so ascosporeogenesis functions could not be assayed.

Vegetative incompatibility was assayed in two ways, with similar results. First, *mt a-1* DNAs were transformed into *A* recipients. Only DNA segments lacking vegetative incompatibility function transformed a wild type *A* recipient at high efficiency. Second, a^{m30} mutants were transformed with *mt a-1* DNA constructs. Transformants bearing DNA segments lacking the vegetative incompatibility function were able to form a vigorous prototrophic heterokaryon with a *A, his-3* strain (FGSC6103); when *mt a-1* function was restored, heterokaryons did not form. Several independent transformants, representing unique nonhomologous integration events (typical for *N. crassa*), were assayed when appropriate.

RESULTS

Expression of functional MT a-1 polypeptides: All MT a-1 polypeptides expressed in *E. coli* accumulated in an insoluble fraction. The migration on SDS-PAGE and amino acid composition (data not shown) of pure MT a-1 matched that expected from its DNA sequence. The altered forms of MT a-1 were expressed in similar amounts and matched their expected migrations in SDS-PAGE. The insoluble MT a-1 polypeptides were re-natured by dialysis and used for experiments described in Figures 1–3.

Sequence-specific DNA binding by MT a-1 polypeptide: Although no genetic evidence suggested a direct effect of MT a-1 upon the mating type locus, we tested the binding of MT a-1 to fragments from both the *a* and *A* mating type idiomorphs. Somewhat to our surprise, certain fragments within and surrounding the cloned *N. crassa* mating type idiomorph DNAs (both *A* and *a*) were bound by MT a-1 (Figure 1). Addition of antibody specific for the MT a-1 polypeptide to binding reactions altered the mobilities of the complexes, verifying that the complexes contained MT a-1 polypeptide (data not shown). Several other DNA fragments, including specific DNA fragments from a plasmid vector, pBSSK⁺, were not bound (Figure 1). All of the bound fragments contained at least one copy of the DNA sequence that we determined was the core of the specific MT a-1 binding sequence (see below). Unbound fragments lacked this sequence.

To examine the specificity of DNA binding by MT a-1, we characterized in greater detail the interaction between MT a-1 and the DNA 5' to its own coding region (the Taq133 fragment). MT a-1 polypeptide protected

FIGURE 1.—Electrophoretic mobility assay of DNA binding by MT a-1 polypeptides. Each set of lanes shows DNA binding assays with different specific fragments of DNA and various preparations of MT a-1 polypeptide. The DNA fragments indicated at the top are: Taq133 fragment (bases 2872–3005 of the *a* idiomorph), *A* (bases 2750–3250 of *A* idiomorph), *a* (bases 5489–6022 of *a* idiomorph), SK⁺ (bases 699–792 of BSSK⁺). These fragments were incubated with 0 (–), 2, 5, or 5 μg boiled (5Δ) of purified MT a-1 polypeptide.

two regions of the Taq133 fragment from DNaseI digestion when bound to the DNA (Figure 2). One protected region includes the sequence 5'-CTTTG-3', an element found in the binding sites of other HMG box polypeptides (see DISCUSSION). The CTTTG sequence is found in each fragment bound by MT a-1 and is absent from unbound DNA fragments. The other protected region contains the related sequence 5'-CTTCG-3'. Approximately 5 residues flanking the core site on either side were also protected from digestion.

In vitro DNA binding properties of mutant MT a-1 polypeptides: Truncated or altered forms of the MT a-1 polypeptide were analyzed to determine which regions were critical for DNA binding *in vitro*. All forms that contained an intact HMG box domain bound DNA (Figure 3). Removal of the carboxyl terminal 188 amino acids (Δ194–382), or deletion of a short segment within the carboxyl terminus (Δ216–220) did not prevent DNA binding. A radical mutation that removes 102 amino acids from the N terminus and 187 amino acids from the C terminus also did not prevent *in vitro* DNA binding (Δ1–3:107–195). The latter deletion removes from MT a-1 essentially all amino acids except the HMG box, which still bound to DNA. In contrast, deletion of residues 117–120 within the HMG box eliminated DNA binding activity (Δ117–120). These results are compared to *in vivo* properties in Figure 6. We conclude that the HMG box is necessary and sufficient for binding to DNA *in vitro*.

In vivo properties of *mt a-1* genes encoding altered MT a-1 polypeptides: To assay the mating functions associated with MT a-1, an a^{m30} mutant strain was transformed with plasmids encoding normal or mutant *mt a-1* genes. The a^{m30} mutation is a large insertion within the HMG box coding region (STABEN and YANOFSKY 1990) that causes a null phenotype (no mating and no vegetative incompatibility). We transformed a^{m30}

FIGURE 2.—DNase I footprint of MT a-1 bound to bases 2872–3005 of *mt a-1* DNA. DNase I digestion pattern of end-labeled Taq133 fragment incubated with 0, 1, 3, 9 μ g purified MT a-1 polypeptide prior to digestion with DNase I. Blocks of protected DNA sequence are indicated at right with the cores of the protected sequences capitalized.

spheroplasts with pCSK31 or altered derivatives. Plasmid pCSK31 contains the full length *mt a-1* gene, in which expression of the MT a-1 polypeptide is driven by its normal promoter, and a selectable hygromycin resistance marker. Transformants selected for hygromycin resistance (STABEN *et al.* 1988) were tested for the ability to mate with a *A* tester strain. Individual transformants, even those transformed by the wild type *mt a-1* gene, varied in their ability to mate with the *A* tester. Such variation is normally observed in transformation assays. (In *Neurospora*, transforming DNA integrates at random chromosomal locations that may vary in transcription activity and integration may also disrupt portions of the plasmid during integration.) Therefore, at least 10 transformants with each construct were tested in mating type assays (Figure 4). Negative mating results were confirmed by testing at least 10 additional transformants. Ability to mate was scored as the induction of large, black fruiting bodies on the *A* tester lawn. As expected, the full-length *mt a-1* gene conferred mating ability on the *a^{m30}* recipient. The $\Delta 216$ –220 construct also conferred normal mating. In contrast, neither the $\Delta 194$ –382 construct, which encodes a polypeptide similar in length to the *mat-Mc* polypeptide from *Schizosaccharomyces*

FIGURE 3.—DNA binding assays of altered forms of MT a-1. Each set of three lanes shows DNA binding assays conducted with the Taq133 fragment and 1, 3, or 9 μ g of partially purified extract from *E. coli* cells expressing the indicated MT a-1 polypeptides. The first three assays represent extracts prepared from control cells bearing only vector (–). The next sets represent: MT a-1 (full-length, amino acids 1–382), $\Delta 117$ –120 mutant, $\Delta 194$ –382 mutant, $\Delta 216$ –220 mutant, and $\Delta 1$ –3:107–195 mutant.

FIGURE 4.—Assay of mating function. Individual transformants bearing plasmids with the indicated normal or mutant *mt a-1* genes were spotted onto a plate of *flA* mycelia. The presence of black fruiting bodies indicates *a* mating type function in transformants bearing the *mt a-1* gene or the $\Delta 216$ –220 mutant gene.

pombe, nor the $\Delta 1$ –3:107–195 construct had mating activity. Also, the $\Delta 117$ –120 deletion, which disabled *in vitro* DNA binding, eliminated mating *in vivo*. We did not notice any other effects of the mutations upon mating properties. None of the transformed strains acquired the ability to mate with *a*. In addition, none of the

transformants that formed fruiting bodies produced spores, which is characteristic of ectopic mating type genes in *N. crassa*. Based on these observations, we conclude that both the DNA binding domain and a portion of the carboxyl terminal region of MT a-1 were required for mating functions.

The vegetative incompatibility properties of *mt a-1* derivatives were determined by two different transformation assays. The first assay depended upon the observation that plasmids encoding a vegetative incompatibility do not transform wild type *A* strains efficiently (GLASS *et al.* 1988). Plasmids containing the $\Delta 216-220$, $\Delta 194-382$, and 1-3:107-195 mutant *mt a-1* genes transformed the *A* recipient as efficiently as the control vector containing only the hygromycin resistance gene (Figure 5). The $\Delta 216-220$ transformants also gained the ability to mate as *a*, which clearly indicated that these transformants expressed *mt a-1* activity, but that the mutant construct did not encode vegetative incompatibility function. In contrast, the normal *mt a-1* gene on pCSK31, as expected, prevented efficient transformation. In addition, the $\Delta 117-120$ mutation plasmid, though unable to confer mating activity and encoding a polypeptide unable to bind DNA *in vitro*, prevented efficient transformation. All of these plasmids transformed *a* spheroplasts with equal efficiencies. Therefore, we concluded that an intact DNA binding domain was not essential for vegetative incompatibility functions, but that specific residues within the C-terminal region were essential to this function.

To confirm the results of the *A* transformation assays, we attempted to form heterokaryons of the *a^{m30}* transformants described in the mating assays with a *A,his-3⁻* strain. Transformed *a^{m30},ade-3b* strains bearing a hygromycin resistance vector (pCSN43) or the $\Delta 216-220\Delta$, $\Delta 194-382$, or 1-3:107-195 mutants formed heterokaryons with the *A,his-3* strain that grew on minimal agar (not shown). Neither parent strain alone could grow on the minimal medium. Transformed *a^{m30},ade-3B* strains bearing pCSK31 or the $\Delta 117-120$ mutant *mt a-1*, did not form a heterokaryon with the *A,his-3* strain, and did not make vigorous mycelia on minimal agar. These results confirmed those of the transformation assays (Figure 5), which indicated that only the normal *mt a-1* and the $\Delta 117-120$ mutant retained the vegetative incompatibility activity.

DISCUSSION

Sequence-specific DNA binding by *N. crassa* MT a-1 polypeptide: The MT a-1 polypeptide bound to specific DNA sequences. DNaseI footprinting (Figure 2) revealed that the core of one bound site was the sequence 5'-CTTTG-3' (or 5'-CAAAG-3' on the complementary strand). Approximately 5 bp on either side of this core sequence were also protected from DNaseI digestion. The CTTTG sequence appears in all of the tested DNA fragments that bound the polypeptide, and does not appear in fragments that were not bound. We concluded

FIGURE 5.—Assay of vegetative incompatibility function. Spheroplasts (*A*) transformed with the indicated constructs were plated on hygromycin media. The presence of large numbers of transformants indicates the lack of vegetative incompatibility function. The small number of transformants from $\Delta 117-120$ presumably result from rare integration events that inactivated the *mt a-1* gene.

from the spectrum of fragments bound and from the DNaseI footprinting experiments that MT a-1 binds specific DNA sequences *in vitro*, whose core is 5'-CTTTG-3'.

The precise nature of the MT a-1-DNA complex was not clear. We frequently observed multiple bands in electrophoretic mobility shift assays. Bands of greatly decreased mobility predominated at high protein concentrations. Two factors may account for this observation. First, two regions of the Taq133 fragment were protected, so multiple MT a-1 polypeptides may bind this DNA. Since the protected regions are 46 bp apart, they would be on opposite sides of the DNA helix, approximately 15 nm apart, and are probably bound by at least two different MT a-1 molecules. Second, we suspect from migration on molecular sieve columns (not shown) that the renatured polypeptide is present primarily as large soluble aggregates, whose binding properties are likely to depend on protein concentration.

Structure/function analysis of the MT a-1 polypeptide *in vitro*: The MT a-1 HMG box domain was essential to and sufficient for DNA binding. The $\Delta 1-3:107-195$, which contained little but the HMG box still bound to DNA. However, the MT a-1 DNA binding domain was disrupted by deletion of the PRPP residues ($\Delta 117-120$) that are conserved in *N. crassa* MT a-1, *P. anserina* FPR1, *C. heterostrophus* MAT-2, and *S. pombe* mat-Mc. Protein sequence similarities and DNA binding specificity suggest that there may be several distinct types of HMG box domains. HMG boxes from polypeptides that bind CTTTG sequences are most closely related to MT a-1 (GRIESS *et al.* 1993). These proteins include *S. pombe* STE11 (SUGIMOTO *et al.* 1991), LEF-1(TCF-1) (TRAVIS

		DNA Binding	Mating	Incompat.
<i>mt a-1</i>		+	+	+
$\Delta 117-120$		-	-	+
$\Delta 194-382$		+	-	-
$\Delta 216-220$		+	+	-
$\Delta 1-3:107-195$		+	-	-

1	MDGNSTHPAP	NLKTTHMAWS	ISNQLGHWND	RKVIAIPLSD	FLNTHPDQIS	GIIEAFKKAT
61	GEEGMFARDP	ESLGIMLLGP	VKLFKPDSVV	VDGNLFWDPK	GIHASAPKEQ	QKKAKIPRPP
121	NAYILYRKDH	HREIREQNP	LHNNEISVIV	GNMWRDEQPH	IREKYFNMSN	EIKTRLLLEN
181	PDYRNPRRS	QDIRRRVSPY	LKIKLLNYDV	NGNLLWGTVN	AEDAALIRTH	FHGVVRVEEM
241	DDGCRIVCRP	VAGSRKLRAA	VVDTWMPRYT	VDTPVTEDD	DAQAFNFNDP	LGGAYFPLNE
301	HLWITVNQNP	PFNAPPPNPN	PHLDFVHPDG	MEAVVHNQVN	MIAQVQEANE	AAALTLPPPP
361	PLRLLSLRLW	LMPIPLQLSF	PL			

FIGURE 6.—Summary of MT a-1 Properties *in vitro* and *in vivo*. The portions of the MT a-1 polypeptide used in each test are indicated by the open boxes, deletions within the polypeptide by dark vertical lines. The locations of the HMG box and the proline-rich, acidic region are indicated.

et al. 1991; WATERMAN *et al.* 1991; VAN DE WETERING and CLEVERS 1992), hSRY protein (ALEXANDER-BRIDGES *et al.* 1992; HARLEY *et al.* 1992) and the IRE-ABP protein (NASRIN *et al.* 1991; ALEXANDER-BRIDGES *et al.* 1992). Some less closely related HMG box polypeptides, such as hUBF (JANTZEN *et al.* 1990) or the testis-specific HMG (BOISSONNEAULT and LAU 1993) bind different, but specific, DNA sequences. Finally, many HMG box polypeptides bind to DNA in a sequence-independent fashion (BIANCHI *et al.* 1989, 1992; DIFFLEY and STILLMAN 1992; FISHER *et al.* 1992). Because the HMG box domain of MT a-1 and other proteins appears to be sufficient for DNA binding, this domain must be the primary determinant of DNA binding specificity. Although certain residues are conserved in all HMG boxes we suspect that residues conserved in CTTTG-binding polypeptides may be essential to both the HMG box DNA binding activity and to specificity for CTTTG sequences. Point mutations in the HMG boxes of hSRY (HARLEY *et al.* 1992) and LEF-1 (GIESE *et al.* 1991) do interfere with DNA binding. However, their wild type amino acid sequences differ from MT a-1 in these regions. Although even subtle changes like substitution of a leucine for valine cripples hSRY DNA binding (HARLEY *et al.* 1992), the MT a-1 polypeptide functions with a proline in the homologous position. Sequence constraints within HMG box polypeptides that bind specific DNA sequences are not yet clear, but they appear to be very loose.

Structure/function analyses of MT a-1 polypeptide functions *in vivo*: The properties of our *mt a-1* mutants are compared in Figure 6. The wild-type *mt a-1* gene complemented both mating and vegetative incompatibility defects in an a^{m30} mutant. However, the $\Delta 117-120$ mutant polypeptide, which was unable to bind DNA *in vitro*, had vegetative incompatibility function but no mating function *in vivo*. In contrast, the $\Delta 216-220$ mutant polypeptide bound DNA *in vitro*, had mating function *in vivo*, but lacked vegetative incompatibility function. However, both mating and vegetative incompatibility relied upon sequences in the carboxyl terminal

region. The $\Delta 194-382$ truncation polypeptide bound DNA *in vitro*, but the corresponding gene did not confer either mating or vegetative incompatibility upon transformation.

Other variants of the mating type polypeptides are also relevant to the structure-function analysis. The a^{m1} mutation is a frameshift at amino acid 247 (STABEN and YANOFKY 1990) that eliminates both mating and vegetative incompatibility. The a^{m1} MT a-1 polypeptide should have an intact HMG box, but lack the acidic C-terminal tail of wild type MT a-1, suggesting again that the carboxyl terminus contains essential determinants for both functions. However, the sequences within the terminal 180 amino acids required for mating do not appear to be very specific; the *P. anserina* *FPRI* gene confers mating in *N. crassa* (ARNAISE *et al.* 1993), although its polypeptide sequence is quite different from that of MT a-1. The MT a-1 mutants that retain mating activity (a^{m33} and $\Delta 216-220$), the *FPRI* polypeptide, and the related *C. heterostrophus* *MAT-2* polypeptide, all have acidic, proline-rich C-terminal tails. Mutant polypeptides lacking acidic tails did not confer mating activity. We infer that these acidic tails have a function required for mating, perhaps transcription activation, that can be satisfied by unrelated polypeptide segments of similar, acidic, composition.

Although an acidic transactivation domain may be necessary for mating and many different sequences can fulfill this requirement, the vegetative incompatibility depends upon other specific determinants. Either the a^{m33} mutation, a change of R258S (STABEN and YANOFKY 1990), or the $\Delta 216-220$ mutation inactivate only the vegetative incompatibility function. In addition, although the *P. anserina* *FPRI* gene has the mating function of *mt a-1* and its polypeptide has an acidic tail, *FPRI* does not have the vegetative incompatibility function (ARNAISE *et al.* 1993). Although other portions of *mt a-1* that we have not probed by mutagenesis may be important for the vegetative incompatibility function, we propose that specific residues in the carboxyl terminal MT a-1 segment, probably within segment 210–260, are essential for the vegetative incompatibility function of *mt a-1*, but not for mating.

An alternative interpretation of these phenotypes is

that mutated residues might be essential for proper production and localization of MT a-1 rather than for function. Although we have not determined production or localization for MT a-1, both the $\Delta 117-120$ and $\Delta 216-220$ mutant genes conferred a mating phenotype on the null recipient. This suggested that stable, active polypeptide was present in at least these cases.

Unfortunately, our mating type tests do not assay all functions essential for fertility. Mating type genes transformed into ectopic sites do not confer fertility upon recipients (GLASS *et al.* 1990; STABEN and YANOFKY 1990). The reasons for this infertility are not clear. None of the mutations that we tested conferred unexpected fertility upon the transformants.

We conclude that two *in vivo* activities of *mt a-1*, mating and vegetative incompatibility, rely on biochemically distinct mechanisms. Mating requires DNA binding activity by MT a-1 as well as functions encoded by the C terminus. The C-terminal acidic domain may have a role in transcription activation that can be filled by many different sequences. In contrast, vegetative incompatibility does not require the DNA binding activity of MT a-1, but may require specific residues within the C terminus that are not essential for mating. Postfertilization activities are likely to involve some of the same biochemical mechanisms, though these experiments have not tested these requirements.

Roles of MT a-1 DNA binding sequences in *N. crassa* idiomorphic DNA: The ability of MT a-1 to bind to specific DNA sequences suggests a mechanism by which it could control the many target genes we presume are required to determine *a* mating type properties and to regulate sexual reproduction. However, the relevance of MT a-1 DNA binding to mating depends upon the identification of genes regulated by MT a-1. The interaction of MT a-1 with DNA immediately upstream of its own coding region suggested the possibility of autogenous regulation of MT a-1 synthesis. However, initial data suggest that the CTTTG site in this region may not be relevant to mating (M. L. PHILLEY and C. STABEN, unpublished).

The *N. crassa* A idiomorphic DNA and its immediate flanks contain 21 CTTTG or CAAAG sequences in 5928 bp (1 per 300 bp, similar to the distribution expected from chance). Seven of these CTTTG sequences cluster between nucleotides 2750 and 3250. This segment of DNA is bound by MT a-1 (Figure 1). This binding site is upstream of the *mt A-1* gene (GLASS *et al.* 1990), but within a segment essential for ascospore development (GLASS and LEE 1992). Although no transcript from the region has been identified, one postfertilization function of MT a-1 could be to regulate expression of a gene in this region after mating brings *A* and *a* nuclei together.

Another cluster of 4 CTTTG sequences in the centromere distal flank (bases 5500–6000) of the *mt a-1* gene (S. CHANG and C. STABEN, in preparation, and GenBank M54787) is bound by MT a-1 *in vitro*. Although

this region is adjacent to a long open reading frame, we have not identified a transcript from the region (S. CHANG and C. STABEN, unpublished), and mutants in which this region has been deleted are viable and mate (BADGETT and C. STABEN, unpublished).

Analysis of CTTTG clusters associated with the mating type idiomorphs has not confirmed that the interaction of MT a-1 with DNA sequences *in vitro* is relevant to mating *in vivo*. However, the correlation that we observe between *in vitro* DNA binding and *in vivo* mating ability strongly indicates that DNA binding must be important to mating type action. Identification of a gene regulated by MT a-1 is necessary to directly test this hypothesis.

CTTTG clusters in mating-related DNA sequences from other fungi: We have noted clustered CTTTG sequences in a number of DNA sequences involved in the regulation of mating in fungi. Although any 5-bp sequence or its complement are expected every 500 bp in most DNAs, the frequency and arrangement of CTTTG sequences in mating related genes is unusual. The *Ustilago maydis mfa1* gene contains 11 repeats of the sequence CTTTG (or CAAAG) immediately 5' to the presumed pheromone open reading frame. The opposite, *mfa2* allele, contains five repeats (BOLKER *et al.* 1992). The *S. pombe MFm1* and *MFm2* (pheromone genes) contain 3 and 4 CTTTG repeats, respectively (DAVEY 1992). These genes are transcribed *only* in *mat-M* cells and *only* when STE11 polypeptide is produced. Because both STE11 (SUGIMOTO *et al.* 1991) and, (by homology to MT a-1), *mat-Mc* bind CTTTG sequences, these observations suggest that the complex regulation of the *MFm* pheromone genes may be mediated by both STE11 and *mat-Mc* binding, though the role of the CTTTG sequence has not been directly determined.

A model for MT a-1 action: Although DNA binding has not been demonstrated *in vivo*, and many components of mating type control remain to be identified, our results suggest a simple working model for mating type control in *N. crassa*, analogous to those used to describe mating type control in *S. cerevisiae*. We suggest that MT a-1 binds to and activates specific target genes essential to specify *a* properties, such as the *a* pheromone gene. MT a-1 may control gene expression by altering the conformation of the DNA to which it binds (a function of many HMG box proteins) (BIANCHI *et al.* 1989; DIFFLEY and STILLMAN 1992; FISHER *et al.* 1992; GIESE *et al.* 1992), or by interacting with other protein factors. Interactions with other protein factors may affect either the specificity of DNA binding or its consequences during different phases of the life cycle. For example, MT a-1 could associate with an unidentified *A*-specific factor to assume a new function after mating much as the $\alpha 2-a1$ complex confers novel properties upon *S. cerevisiae* diploids (HERSKOWITZ *et al.* 1992). Interactions of MT a-1 with other polypeptides may be very relevant to interaction of *a* and *A* during fruiting body development and

ascosporogenesis. We wish to note in particular that the A-2 region essential for fruiting body maturation can encode an HMG box polypeptide called R3i (GLASS and LEE 1992), and that HMG1 polypeptides dimerize via the HMG box (BIANCHI *et al.* 1992).

Any model for mating type action must also explain the vegetative incompatibility function of the locus. This function does not appear to depend upon MT a-1 DNA binding activity, so we propose that a protein-protein interaction between MT a-1 and an A-specific gene product triggers the incompatibility response in a fashion independent of interaction with DNA. The A-specific gene product may either be MT A-1 or a gene product controlled by *mt A-1*, which is the only region of the A idiomorph required for vegetative incompatibility (GLASS *et al.* 1988; GLASS *et al.* 1990).

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