Linkage of mating-type loci distinguishes bipolar from tetrapolar mating in basidiomycetous smut fungi
(basidiomycetes/ustilaginiales/Ustilago/a locus/b locus)

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ABSTRACT Sexual compatibility requires self vs. non-self recognition. Genetically, two compatibility or mating-type systems govern recognition in heterothallic basidiomycete fungi such as the edible and woodrotting mushrooms and the economically important rust and smut phytopathogens. A bipolar system is defined by a single genetic locus (MAT) that can have two or multiple alleles. A tetrapolar system has two loci, each with two or more specificities. We have employed two species from the genus Ustilago (smut fungi) to discover a molecular explanation for the genetic difference in mating systems. Ustilago maydis, a tetrapolar species, has two genetically unlinked loci that encode the distinct mating functions of cell fusion (a locus) and subsequent sexual development and pathogenicity (b locus). We have recently described a b locus in a bipolar species, Ustilago hordei, wherein the existence of an a locus has been suspected, but not demonstrated. We report here the cloning of an allele of the a locus (al) from U. hordei and the discovery that physical linkage of the a and b loci in this bipolar fungus accounts for the distinct mating system. Linkage establishes a complete MAT locus in U. hordei; this locus appears to be in a region suppressed for recombination.

A hallmark of the heterothallic basidiomycete fungi is the fusion of cells of different compatibility to form a dikaryotic mycelium in which each cell contains a pair of nuclei. The interaction of two non-self nuclei within one cell triggers a program of sexual development (1–5). Among the homobasidiomycetes, which include the mushrooms such as Schizophyllum commune and Coprinus cinereus (6, 7), ~65% of the species regulate sexual compatibility genetically via a tetrapolar (bifactorial) mating system. In these fungi, two genetic loci, each with two or more allelic specificities, control sexual development. Approximately 25% of the species have a bipolar (unifactorial) mating system in which compatibility is governed by a single genetic locus. In the bipolar species, this locus can have two or multiple alleles, the latter being common in the homobasidiomycetes and the non-parasitic heterobasidiomycetes. The remaining 10% consist of homothallic species (6, 8). Among the parasitic heterobasidio-
mycetes, such as the phytopathogenic rust, bunt, and smut fungi, the diallelic bipolar mating system is predominant with certain exceptions (9, 10). These exceptions include a multiallelic bipolar system for the bunt fungus Tilletia controversa (11) and the occurrence of a tetrapolar mating system in several smut species including Ustilago maydis and Ustilago longissima (10, 12). To our knowledge, the molecular mechanisms underlying the genetic differences between these mating systems have heretofore been unknown.

Mating and dikaryon formation are intricately connected with pathogenicity in the smut fungi, and the mating-type loci in the tetrapolar smut pathogen U. maydis have recently been

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Isolated and characterized (10, 13–19). The a locus has two specificities (a/ and a2) and consists of a gene complex encoding pheromones and pheromone receptors (14, 15); this locus controls fusion of mating partners (20). The b locus has at least 25 naturally occurring allelic specificities and consists of a gene complex encoding homoeodomain polypeptides, bW and bE (12, 17–19). Independent assortment of the a and b loci of U. maydis promotes outbreeding because only 25% of the mating interactions in an F1 population of meiotic products will be successful. In contrast, 50% of the interactions in diallelic bipolar smut fungi, such as the barley pathogen Ustilago hordei, will be successful because a single locus (MAT) controls mating (8, 21, 22).

The sequences at the MAT locus in bipolar smut fungi (MAT-1 or MAT-2; refs. 23 and 24) presumably control fusion events by a mechanism similar to that of the a locus of U. maydis. In fact, sequences that cross-hybridize with the U. maydis a locus and factors that may be diffusible pheromones have been detected in U. hordei (25, 26). In addition, bipolar smut fungi do have homologs of the U. maydis bE and bW genes, and the b gene complexes from U. hordei are functional when transferred to U. maydis (25, 27). These results raise questions such as (i) why hasn’t a b function been identified genetically in bipolar smut fungi and (ii) what is the organization of the b locus relative to the classically defined MAT locus in U. hordei? In this report, we present evidence that the a and b loci are genetically and physically linked in the bipolar species U. hordei to establish the MAT locus and describe the cloning of an allele (al) of the a locus.

MATERIALS AND METHODS

Strains, Media, and Mating Tests. U. maydis strains, 521 (alb1) and 518 (a2b2) (16), and U. hordei strains, Uh112 (MAT-1, ade) and Uh100 (MAT-2, ade) (27), have been described. The bipolar smut strains (25) and all other U. hordei strains were obtained from P. Thomas (Agriculture Canada, Winnipeg, MB, Canada). Strains were grown on potato dextrose broth (PDB) or agar (PDA) (Difco) at 22°C. Mating tests were performed with liquid cultures grown in PDB and spotted onto plates containing complete medium with activated charcoal (28). Mixtures of compatible strains give mycelial colonies and incompatible strains form yeast-like colonies (12, 28).

Isolation of the U. hordei al Locus. A cosm id library containing Uh112 (MAT-1) genomic DNA in pJW42 (27) was screened by hybridization with a 3.3-kb Xho I-BamHI fragment containing the U. maydis panl gene (14) as described (27). Subclones were constructed in an integrative bleomycin-resistance vector, pUb35 (27). All probes were labeled

Abbreviations: RFLP, restriction fragment length polymorphism; Mb, megabase pair(s); Uhprof, U. hordei prof.

†The sequence reported in this paper has been deposited in the GenBank data base (accession no. U079399).
RESULTS AND DISCUSSION

A b Locus Restriction Fragment Length Polymorphism (RFLP) Is Genetically Linked to MAT in U. hordei. Upon analysis of the b gene complex cloned previously from U. hordei (27), we discovered a RFLP, detected by a probe specific for the b locus (bE1 probe, Fig. 1) that cosegregated with mating type (MAT-1 or MAT-2) in 86 meiotic progeny from two telospore populations. The results for 25 of those progeny are shown in Fig. 2A. This result indicated that the classically defined MAT locus in U. hordei might embody a complex region consisting of loci for both the a and b functions found in U. maydis.

Cloning of the U. hordei aLocus. To prove the existence of an a locus in U. hordei, we drew on the knowledge that in U. maydis the a1 locus is 2.5 centimorgans (2–6 kb) from a pantothenic acid biosynthesis gene (panl; ref. 14). Similarly, a pan gene is linked to the MAT locus in U. hordei (24), and hybridization of the U. maydis panic probe to DNA from 86 progeny of U. hordei detected a RFLP that cosegregated (100%) with the MAT locus (data not shown). This probe was used to identify a cosmids, panMAT-1, from a genomic library of DNA from a U. hordei MAT-1 strain. Subsequently, the cosmids panMAT-1 and several subclones (Fig. 1) were tested for mating-type-specific activity by transformation into a U. hordei MAT-2 strain. Introduction of DNA fragments carrying the a1 locus should result in transformants that exhibit dual

**FIG. 1.** Map of the U. hordei MAT-1 mating-type loci. The upper line represents the genomic region. The open boxes represent the position of the bl locus consisting of two open reading frames, bW1 and bE1, isolated on cosmids panMAT-1 (27). Cosmid panMAT-1 carries the a1 locus (hatched boxes and enlarged map). Note that the orientation relative to the bl locus has not been determined. The stippled arrow within the hatched box indicates the location and direction of transcription of the U. hordei phenomone receptor gene, Uhp01 (Fig. 4). Subclones (lower four lines) were tested for the presence of the a1 locus (mating activity) as shown in Fig. 3B. An, Apl1; B, BamH1; Bg, BglII; Bw, BssHII; K, Kpn1; N, Not1; R, EcoRI; Sa3, SaA3; SI, SacI; Sl, SacII; Sp, Sp1; Xb, Xba1. Solid bars denote the hybridization probes used in this study; the region of hybridization to the U. maydis panic probe is indicated on panMAT-1.

**FIG. 2.** Segregation of RFLPs within the U. hordei a and b loci with mating type (MAT). Lane labels 1 or 2 refer to mating types MAT-1 or MAT-2, respectively (for a subset of 25 random progeny). The specificity at the MAT locus was determined by a mating assay on culture medium. The same DNA blot of total genomic BamHI-digested DNA was hybridized with a U. hordei b locus-specific probe, bE1 (A), a U. hordei a1 locus-specific probe (panMAT-1, B, 8.5-kb Sp1 fragment) (B), or a U. hordei MAT-1-specific probe (panMAT-1, 2.1-kb EcoRI-SacI fragment) (C) (see Fig. 1 for locations of probes). The MAT-1-specific probe (2.1-kb EcoRI-SacI fragment; Fig. 1) hybridizes specifically to genomic DNA from MAT-1 strains and not to DNA from MAT-2 strains.
Fig. 3. Conjugation tube formation and cell–cell interactions in *U. hordei*. The conjugation assay (30 hr) was performed as described (30). (A) Conjugation tube formation in a compatible mating reaction between a MAT-I strain (Uh112) and a MAT-2 strain (Uh100). (Inset) Budding wild-type cell of strain Uh100 (MAT-2). (B) "Self-mating" behavior (conjugation tube formation and cell aggregation) after introduction of the 8.5-kb SphI fragment carrying the *U. hordei* al locus (Fig. 1) in a *U. hordei* MAT-2 strain. (Uh100). (Bars = 10 µm.)

mating behavior, i.e., formation of conjugation tubes and cell aggregation in the absence or presence of cells of opposite mating type. Normally, *U. hordei* responds to mating partners by forming conjugation tubes that mediate fusion (26). A typical mating structure from a mixture of wild-type cells of opposite mating type is shown in Fig. 3A. Similar observations have been made for *U. maydis* (30). When tested for mating activity, the episomal cosmids pmAT-1 and 8.5-kb SphI and 5.5-kb BamHI fragments, but not adjacent 4.8-kb and 4.5-kb BamHI fragments (Fig. 1), induced the formation of conjugation tubes and cell aggregation upon transformation into a MAT-2 strain (Fig. 3B). Moreover, when introduced into a *U. maydis* strain, the *U. hordei* 8.5-kb SphI fragment allowed the transformants to interact specifically with a *U. hordei* MAT-2 strain but not with a MAT-1 strain (G.B., unpublished results). Haploid strains of *U. hordei* and *U. maydis* do not normally interact, regardless of mating type.

DNA sequence analysis confirmed the existence of a pheromone receptor gene, *U. hordei* (Uh) *pral*, having 62% DNA sequence identity with the *pral* gene of *U. maydis* (15); its predicted amino acid sequence is 64% identical (and 82% similar) to the predicted *pral* gene product of *U. maydis* (Fig. 4). The *Uhpral* gene spans the BamHI restriction site between the 4.8- and 5.5-kb BamHI fragments (Fig. 1). The fact that the 5.5-kb BamHI fragment has mating-type activity might indicate that it harbors a gene coding for a *U. hordei* mating factor (Uhmsf1). We conclude that the 8.5-kb SphI fragment from pmAT-1 contains the *U. hordei* al locus. These results allowed us to sequence this fragment from the *U. hordei* al-specific hybridization probes for subsequent analysis of the linkage between *a* and *b* loci.

The *a* and *b* Loci of *U. hordei* Are Genetically Linked and Together Define the Classical MAT Locus. Hybridization with

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Fig. 4. Molecular analysis of the potential pheromone receptor gene, *Uhpral*, at the *U. hordei* al locus. (A) DNA sequence analysis of the *Uhpral* gene. Sequence comparison with the *U. maydis* *pral* gene (15) indicated three potential introns (lowercase type). The following features are depicted in order in boldface type: start codon; *Kpn I*, EcoRI, Bgl II, and BamHI restriction sites (compare Fig. 1); and stop codon. (B) Predicted product of 359 amino acids translated from the DNA sequence depicted in A, excluding the three potential introns to maintain a proper open reading frame. (Uh100, Uh.) This polypeptide has been compared with the 359-amino acid predicted product of the *U. maydis* *pral* gene (*U. maydis*, Um). Only amino acids that differ are shown; dots indicate a gap. (Sequence alignment was generated with the GCG sequence analysis software package.)
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the U. hordei al probe detected a RFLP, among the 86 progeny of U. hordei, that showed cosegregation (100%) with mating type (MAT-1 or MAT-2; Fig. 2B) and with the RFLPs revealed by the beI probe (Fig. 2A) and the U. maydis pan1 probe (data not shown). This result confirmed genetic linkage of the a and b loci. In addition, a 2.1-kb EcoRI-SacI fragment from paMAT-1 (Fig. 1) was found to be a MAT-1-specific probe; i.e., it hybridized only to DNA from MAT-1 strains (Fig. 2C). This suggests that the a sequences of U. hordei are idiomorphs, i.e., nonhomologous sequences that occupy the same chromosomal locus, as described for the a sequences of U. maydis (14, 15, 31).

The a and b Loci Are Located on the Same Chromosome in U. hordei. To test whether the cosegregation of the a and b loci in U. hordei indicated physical linkage, we hybridized the a and b sequences to electrophoretically separated chromosomes (molecular karyotypes) of U. hordei (29) and U. maydis (ref. 32; Fig. 5 A and E). In U. maydis, the pan1 probe detected the a locus on a 1.5-Mbp linkage (Mbp) chromosome in both aI and a2 strains (Fig. 5B; compare bands indicated by the arrow in Fig. 5F). The b locus was found on a 2-Mbp chromosome (Fig. 5 D and G; see arrow). The U. maydis pan1 probe and the U. hordei al and beI probes hybridized to a 2- to 3-Mbp chromosomal DNA band in U. hordei.

Fig. 6. Variation among alleles of the b genes from bipolar smut fungi. The geographic origins of the collections are given in parentheses. Specificity at the MAT locus was determined by a mating assay on culture medium; all the bipolar species used are interfertile. Open boxes with arrowheads represent the U. hordei open reading frames, Uh6W1 and -2 and Uh6E1 and -2 (see Fig. 1). Hatched boxes represent introns, asterisks denote homeodomains (27), and V indicates the variable N-terminal regions. In the latter regions, Uh6W1 differs by 275 bp of 511 bp from Uh6W2 and Uh6E1 differs by 193 bp of 365 bp from Uh6E2 (27). Solid squares indicate the positions of the oligonucleotide primers, p1 and p2 (including XbaI cloning sites [Xbh], used to amplify the indicated region by the PCR. For example, PCR amplification in the MAT-1 strain resulted in a product of 1082 bp consisting of, respectively, the N-terminal 511 bp (170 amino acids) of Uh6W1, a 206-bp intergenic region, and the N-terminal 555 bp (211 amino acids) of Uh6E1. Subsequently, the sequence of the PCR products was determined. For example, 0.511 means that no base pair change was found in the 511-bp N-terminal region of Uh6W1. ND, not determined; U., Ustilago.

aStrains and DNA sequences as described (27).

bTwo independent sets of basidiospores were analyzed.

cConservative change.

dSingle base-pair change found at the same position in Ustilago avenae and Ustilago kolleri.

Loss of a BamHI restriction site with consequent loss of a RFLP (see figure 3A, lane K, in ref. 25).

hordei (Fig. 5 B–D). The use of electrophoretic conditions to achieve resolution of the high molecular weight chromosomes revealed that the a and b loci were present on the largest chromosome in U. hordei (=3 Mbp; Fig. 5 E–G). These results provide physical evidence for linkage and confirm the genetic evidence for cosegregation of the a and b loci in U. hordei (Fig. 2).

Limited Variability Exists Between b Alleles of Bipolar Smut Species. Physical linkage of the a and b loci in bipolar smut fungi would ensure that every fusion event mediated by the a locus of MAT-1 and MAT-2 strains would bring together b loci of opposite specificity and result in formation of the infectious dikaryon. This scenario suggests that the bipolar smut fungi require only two b specificities, one linked to each a specificity. To test this hypothesis, we analyzed the variability in the predicted amino acid sequences of the N-terminal regions of the bW and bE genes from a world-wide collection of U. hordei strains. The N-terminal region is variable among different alleles of the bW and bE genes in U. maydis (17–19) and U. hordei (27) and harbors specificity determinants (33). The sequence analysis (Fig. 6) revealed that there are only two classes of b gene complexes in U. hordei and that there is very little variability within each class. As expected, one class of bW and bE genes is associated with the MAT-1 mating type and the other is associated with MAT-2. The bW and bE variable regions from MAT-1 and MAT-2 isolates of five additional Ustilago species with bipolar mating systems also showed similar classes when compared with U. hordei (Fig. 6). Overall, the finding of only two classes of b loci supports the hypothesis that most bipolar smut fungi have only two allelic specificities.

Recombination Between the a and b Loci Is Suppressed. Given the linkage of the a and b loci in U. hordei, meiotic recombination could occur between the loci to generate progeny with genotypes a1 b2 or a2 b1 that are unable to mate with either parental strain (a1 b1 or a2 b2). However, a search among 2182 random progeny, obtained from three collections of teliospores, failed to yield candidate recombinant strains with altered mating specificity. Although genetic and physical distances have not been correlated in U. hordei, it is known that the pan1 gene and the a1 locus in U. maydis are separated by 2.5 centimorgans (2–6 kb; ref. 14). Recombination must be suppressed in the MAT region containing the a and b mating-type loci in U. hordei because preliminary linkage mapping with rare-cutting restriction enzymes and pulse field gel analysis suggested that the a and b loci are >150 kb apart (data not shown). Recombination in this region might be suppressed due to genetic control or because of differences in the genomic organization of the MAT region in the two mating types. In Chlamydomonas reinhardtii, the mating-type region contains many genes, is recombinational suppressed, and displays intrachromosomal translocations, inversions, and large deletions (spanning at least 190 kb) between the two mating types (34).

The linkage of sex determinants (i.e., a and b sequences) to establish the bipolar mating system may reflect the need to keep functionally related genes together. Such a situation would make biological sense given the requirement for two distinct mating types to establish the infectious dikaryon. Certainly, the clustering of genes for sex determination has been proposed as the reason for mosaic sex chromosomes in higher eukaryotes (35). In this context, it is conceivable that additional genes for sexual development (and pathogenicity) are also clustered within the MAT region of U. hordei.

The finding that the genome organization of conserved mating-type loci in smut fungi accounts for apparent major differences in mating systems suggests that the mechanisms for establishing the infectious dikaryon are also conserved in this group of fungi. Because smut fungi are obligately depen-
dent on infection to produce the diploid teliospores necessary for meiosis, sexual development is dependent not only on compatibility but also on the host species or cultivar preferences of the mating partners. In this regard, it is possible that host range differences contributed to the evolution of the two mating systems in smut fungi. Although it is not known whether one type of mating system evolved from the other (for opposing viewpoints, see refs. 5 and 6), it is likely that tetrapolary mating evolved after the divergence of basidiomycete ascomycete fungi because tetrapolary mating systems have not been found in the latter group (1). Based on relative complexity and on the suggestion that basidiomycetes evolved from ascomycetes (5), one might imagine that the tetrapolary system (two loci) evolved from the bipolar system (one locus). Initially, translocation events in a diadic bipolar species could have separated the a and b loci. Subsequent mutation of b genes would be then required to generate multiple allelic specificities. It is also possible that the loci might have been separated after multiallelic factors evolved. Generally, it is argued that tetrapolary mating systems give the selective advantage of increased outbreeding capacity and, therefore, increased adaptability (8). However, even though 65% of the basidiomycetes are tetrapolary, increased outbreeding capacity is not necessarily the driving force in evolution. For example, an inbreeding or nonbreeding fungal species may be highly specialized and very successful in its claimed niche. Certainly, bipolar smut fungi are quite specialized and successful in their ability to parasitize grass and cereal hosts. Ustilago nuda is an example of one such bipolar smut species. This species is an embryo-infecting smut that can establish a dikaryon by mating between the four meiotic progeny immediately after germination of a teliospore on floral tissue of barley and wheat plants. In this species, the opportunity for outbreeding is minimal because of the short haploid stage in the life cycle.

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