TWO MULTIALLELIC MATING COMPATIBILITY LOCI SEPARATELY REGULATE ZYGOTE FORMATION AND ZYGOTE DIFFERENTIATION IN THE MYXOMYCETE PHYSARUM POLYCEPHALUM

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ABSTRACT

The mating of Physarum polycephalum amoebae, the ultimate consequence of which is a "plasmodium," was recently shown to be governed by two compatibility loci, matA (or mt) and matB (DEE 1978; YOUNGMAN et al. 1979). We present evidence that matA and matB separately regulate two discrete stages of mating: in the first stage, amoebae (which are normally haploid) fuse in pairs, with a specificity determined by matB genotype, to form diploid zygotes; subsequent differentiation of the zygotes into plasmodia is regulated by matA and is unaffected by matB. Mixtures of amoebae carrying unlike matA and matB alleles formed diploids to the extent of 10 to 15% of the cells present, and the diploids differentiated into plasmodia. When only the matB alleles differed, diploid cells still formed to a comparable (5 to 10%) extent, but rather than differentiating, these diploids remained amoebae. When strains carried the same alleles of matB, formation of diploid cells was greatly reduced: in like-matB, like-matA mixtures, none of 320 cells examined was diploid; in like-matB, unlike mat-A mixtures, differentiating diploids could be detected, but at only $10^{-3}$ to $10^{-2}$ the frequency of unlike-matB, unlike-matA mixtures. The nondifferentiating diploid amoebae recovered from unlike-matB, like-matA mixtures were genetically stable through extensive growth, even though they grew more slowly than haploids (10-hr vs. 8-hr doubling period), and could be crossed with both haploids and diploids. The results of such higher ploidy and mixed ploidy crosses indicate that karyogamy does not invariably accompany zygote formation and differentiation.

THE sexual cycle of Myxomycetes (plasmodial slime molds) includes a haploid amoebal form and a diploid plasmodial form. Amoebae differ substantially from plasmodia in several aspects of cell structure and physiology, and differentiation of amoebae into plasmodia has recently attracted interest as a model eucaryotic developmental system amenable to both genetic and biochemical analysis (ADLER and HOLT 1975; COOKE and DEE 1975; YOUNGMAN et al. 1977; HONEY, POULTER and TEALE 1979; GORMAN, DOVE and SHAIBE 1979; AN-

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In the amoebal form, the cells are microscopic and uninucleate. Amoebae typically feed on bacteria, and proliferate by ordinary mitotic cell division. When two amoebae of different “mating type” encounter one another under appropriate conditions, they may fuse to form a binucleate cell, and the nuclei within such binucleate cells soon fuse to yield diploid zygotes. These diploid zygotes then differentiate into plasmodial cells. This differentiation process radically alters the pattern of mitosis; the nuclei continue to divide (without, in contrast to amoebal cell division, dispersion of the nuclear envelope), but no further division of the cell mass occurs. The multinucleate, developing zygotes fuse with one another as they grow, soon creating a very large, syncytial plasmodial cell with diploid nuclei. Like amoebae, plasmodia are capable of extensive vegetative growth, if their nutritional requirements are met; however, under nutritional stress and in the presence of light, they form spores. Meiosis occurs during spore formation, and the spores germinate to yield haploid amoebae of different mating types, completing the cycle.

More detailed accounts of the events of the Myxomycete sexual cycle are given in the reviews of Collins (1979), Rusch (1970) and Hüttermann (1973), in monographs by Gray and Alexopoulos (1968) and Ashworth and Dee (1975) and in a recent collection of articles edited by Rusch and Dove (1980).

The present work concerns the genetic regulation of mating (zygote formation and zygote differentiation) in the species Physarum polycephalum, the most widely studied plasmodial slime mold. The genetics of mating compatibility in *P. polycephalum* was first investigated in the “Wisconsin” isolate by Dee (1960), who found that a single locus, subsequently called *mt*, was a principal determinant of mating specificity, and that only mixtures of amoebal strains carrying different *mt* alleles produced plasmodia. There were unexplained aspects to Dee’s studies, however: the extent of mating was generally poor, there was considerable variation in extent from one cross to another and, in several cases, combinations of “*mt*-compatible” amoebae failed entirely to produce plasmodia. Dee (1966) later examined a second isolate of *P. polycephalum* and showed that *mt* was multiallelic. Additional isolates from diverse geographic regions have since been studied by other workers (Collins and Tang 1977), bringing the total number of known alleles to 14. Each isolate carries distinct *mt* alleles, suggesting that a very large number of such alleles may exist in nature.

It was at first thought probable that *mt* alleles would be found to govern the specificity with which amoebal cells fuse during mating (Dee 1966). Later, Adler and Holt (1975) found that certain diploid *mt*-heterozygous amoebae were able to differentiate rapidly into plasmodia and interpreted this finding as suggesting that *mt* might regulate the ability of zygotes to differentiate into plasmodia.

We have recently reported the discovery of an additional multiallelic genetic locus that regulates mating specificity (Youngman et al. 1979). Some explanation for discrepancies in the earlier studies may be found in the fact that plasmodium formation is 10²- to 10⁴-fold more extensive when mating strains carry different alleles at both *mt* and the newly discovered locus than when mated
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strains differ only in mt genotype. To make our mating type terminology appropriate to the two-locus ("tetrapolar") regulation of mating specificity in *P. polycephalum*, we adopted new notation: "matA" was introduced in place of mt, and the newly discovered locus was called "matB". Three alleles of matB were reported in the original work, and KIROVAC-BRUNET, MASSON and PALLOTTA (1980) have since discovered several more in plasmodial samples from diverse regions, suggesting that matB, like matA, may possess a large number of natural alleles (the two alleles of the locus "rac," identified by Dee (1978), are probably matB alleles identical to two of the three alleles reported in our original studies). Alleles of matA and matB segregate independently.

In considering the ways in which matB might regulate matings, YOUNGMAN *et al.* (1979) raised the possibility that the role of matB was to govern, independently of matA, the frequency of zygote formation; matA would control only zygote differentiation. A crucial untested prediction of the model is that non-differentiating "zygotes" (diploid amoebae) should form in mixtures of strains with the same matA alleles, but different matB alleles, to approximately the same extent that differentiating zygotes form in mixtures of strains with different matA and matB alleles. The model also predicts that such diploid cells should form only rarely in mixtures of strains with the same matA and matB alleles. The major aim of the present work was to test these predictions.

MATERIALS AND METHODS

Strains and culture media: The strains CH242 (matA3 matB3 imz-2 whi+ fusA2 fusC2) (ADLER 1975), CH351 (matA3 matB1 imz-1) (ADLER 1975) CH508 (matA2 matB2 imz-2 whi+ fusA2 fusC2) (ADLER 1975), CH792 (matA2 matB3 imz-2) (YOUNGMAN *et al.* 1979), CH813 (matA3 matB2 imz-1 whi+ fusA2 fusC1) (ANDERSON, unpublished), CH924 (matA1 matB1 imz-1 fusA1 fusC1 whi-1) (ANDERSON and TRUJT, unpublished), CH929 (matA1 matB3 imz-1 whi-1 fusA1 fusC1) (ANDERSON and TRUJT, unpublished), CH930 (matA1 matB3 imz-1 whi-1 fusA1 fusC1) (ANDERSON 1976) and LU897 (matA1 matB1 imz-1 whi-1 fusA2 fusC1) (ANDERSON 1977) were constructed from strains made partially isogenic to Colonia by repeated backcrossing. Mating testers and fusion testers were constructed for this work, also from strains inbred to the Colonia background.

Liver infusion agar (LIA) contains 1 g Oxoid liver infusion per liter of 1.5% agar. Peptone-yeast extract medium (PYE) is the "simplified medium" of BREWER and PRIOR (1976), but with N-Z-Case instead of Tryptone, and 4.2 g citric acid per liter instead of 3.6 g; dilute PYE-agar (dPYE agar) is a 20-fold dilution of PYE into 1.5% agar, and PYE-agar is a mixture of equal volumes of PYE and 3% agar with 1/100 volume 0.5% hematin in 1% NaOH.

Mating tests and phenotypic scoring: To score for matA and matB, an amoebal strain was tested for plasmodium formation with a set of amoebal mating testers that usually included all possible parental and nonparental combinations of matA and matB genotypes (YOUNGMAN *et al.* 1979). In the present work, standard mating test conditions involved the use of nonnutrient plates buffered at pH 5.0 with 3 mm sodium citrate, and the use of "concentrated" live *Escherichia coli* bacteria as the source of food for the mating amoebae. The concentrated bacteria were prepared as follows: *E. coli* were grown to late log or early stationary phase in "LB broth" (LEVINE 1957) at 37°; the bacterial suspensions were then washed twice by centrifugation and resuspension in sterile H2O, and the final suspension of washed bacteria, which was typically concentrated approximately 6-fold with respect to the growth broth, was stored at 4° until use. Such suspensions were sometimes stored longer than 2 months in this condition; most of the bacteria do not remain alive in storage, but this does not seem to alter their nutritive value to the amoebae. When the
bacteria were used to start mating cultures, they were diluted 2- to 3-fold when combined with amoebae. This produced a "mating spot" (see Youngman et al. 1979) with a bacterial density approximately equivalent to that achieved when the bacteria are grown in situ, in mating spots, on dPYE agar plates. Under these standard conditions, plasmodium formation was very extensive in instances when the tester and tested strains carried different matA and matB alleles; fusion of numerous individual plasmodia yielded a single, large, syncytial cell that engulfed the entire mating spot after 3 days of incubation at 26°. When the tester and tested strains carried different matA alleles, but the same matB alleles, plasmodium formation of limited extent was visible at isolated foci after 4 days of incubation. When neither strain carried the imz-2 allele (Shinnick et al. 1978), 5-10 foci of plasmodium formation could be seen in typical mating spots; whereas, 50-100 foci were visible in mixtures when the imz-2 allele was carried by one or both strains. Plasmodium formation was never observed after 4 days when tester and tested strains carried the same matA alleles, irrespective of matB genotypes.

The segregation of imz-1 and imz-2 alleles was determined from the numbers of plasmodial foci formed in mating spots when the tester and tested strains carried the same matB alleles, but different matA alleles, and when the tester strains carried the imz-1 allele (see above). To score segregation of the two alleles at the whi locus (Anderson 1977), which controls plasmodial color, the strains to be tested were mated with tester amoebae carrying the recessive mutant allele whi-I, which confers white color upon plasmodia; formation of yellow plasmodia in such tests indicated that the tested amoebae carried the whi+ allele. To score segregation of alleles at the fusA and fusC loci, plasmodia subcultured on PYE-agar were paired with plasmodial fusion testers of "opposite" color, as described by Anderson (1976, 1977). The properties of fusA alleles (Cooke and Dee 1975) and fusC alleles (Adler and Holt 1974) have been previously described.

**RESULTS**

**Increasing the yield of zygotes:** In order to facilitate a search for the non-differentiating "zygotes" predicted by the hypothesis that matB governs amoebal cell fusions during mating, we sought conditions under which mating would occur with high efficiency. Recent work in our laboratory (Shinnick et al. 1979) showed that when the tester and tested strains carried different matB alleles, in situ mating in a "mating spot" produced a single, large, syncytial cell that engulfed the entire mating spot. This method was modified by diluting the bacteria 2- to 3-fold when combined with amoebae. This produced a "mating spot" with a bacterial density approximately equivalent to that achieved when the bacteria are grown in situ, in mating spots, on dPYE agar plates. Under these standard conditions, plasmodium formation was very extensive in instances when the tester and tested strains carried different matA and matB alleles; fusion of numerous individual plasmodia yielded a single, large, syncytial cell that engulfed the entire mating spot after 3 days of incubation at 26°. When the tester and tested strains carried different matA alleles, but the same matB alleles, plasmodium formation of limited extent was visible at isolated foci after 4 days of incubation. When neither strain carried the imz-2 allele (Shinnick et al. 1978), 5-10 foci of plasmodium formation could be seen in typical mating spots; whereas, 50-100 foci were visible in mixtures when the imz-2 allele was carried by one or both strains. Plasmodium formation was never observed after 4 days when tester and tested strains carried the same matA alleles, irrespective of matB genotypes.

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**Determination of the time course of plasmodium formation:** The assay of Youngman et al. (1977) was applied to mating mixtures essentially as described previously (Youngman et al. 1979; Pallotta et al. 1979), except that nonnutrient agar plates buffered at pH 5 with 3 mM sodium citrate and supplemented with 10 mM MgSO4 were used as "mating plates." LIA plates were used for assay, as in the previous work.

**Recovery of rare, asexually formed plasmodia from heterothallic amoebae:** Strains were plated as whole-plate cultures with concentrated E. coli food bacteria on nonnutrient plates buffered at pH 5.0 with 3 mM sodium citrate. Both in yield of rare plasmodia and in the health of the plasmodia recovered, this method proved superior to the procedure described previously by Adler and Holt (1977).

**Preparation of slides for microscopic observation:** Drops of 2% agar, buffered at pH 5.0 with 3 mM sodium citrate and supplemented with 10 mM MgSO4, were deposited with Pasteur pipettes on 75 x 25 mm microscope slides heated for a few seconds on a hot plate. Cover glasses (22 x 50 mm) were then immediately placed over the agar drops in such a way that the agar spread evenly over the slide and no air bubbles formed between the slide and the cover glass. These agar-covered slides were stored in a covered container at 4° until use. A few minutes before use, a slide was placed at room temperature, and the cover slip was raised and removed from the agar by lifting the corner with the blade of a scalpel. A drop of water was then placed on the agar-slide surface, and culture material transferred to it with a flat toothpick. The toothpick was used to stir the culture material until it appeared to be evenly suspended in the drop. Excess moisture was evaporated under an air jet and the preparation was covered with a fresh cover glass for observation with phase contrast optics at 500X.

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1978) showed that the efficiency of mating between strains carrying different matA and matB alleles is enhanced in the presence of MgSO₄, and that plasmodium formation is most extensive when at least one of the mated strains carries the imz-2 allele at the imz locus (not a specificity locus). This work also confirmed an earlier finding that mating is inhibited when the pH of the culture rises above pH 5.0 (Collins and Tang 1973) and showed that the use of live E. coli bacteria, as food source for the mating amoebae, can inhibit the mating process by elevating the pH of the medium. We therefore measured the time course and extent of (differentiating) zygote formation in a mating involving an imz-2 strain on pH 5.0 agar plates containing 10 mM MgSO₄ but lacking nutrients that would permit bacterial growth; concentrated, live bacteria were used as food for the mating amoebae. Figure 1 shows the results of such a measurement. The maximum extent of plasmodium formation seen in this experiment and other similar experiments was 3- to 10-fold greater than in previous studies (Pallotta et al. 1979; Youngman 1979). In other matings under these conditions that involved strains with different matA but the same matB alleles, we found 10²- to 10³-fold less plasmodium formation (Youngman 1979).

In experiments of the type reported in Figure 1, the titer of plasmodia is obtained by sampling replicate mating mixtures, at different times, to determine the number of cells "committed" to plasmodium formation (Youngman et al. 1977). Cells in mating mixtures are washed from plates in a known volume of sterile water or buffer, diluted and plated for assay under conditions that strongly inhibit zygote formation, but that readily permit the development of zygotes into plasmodial cells (Pallotta et al. 1979; Youngman 1979). Thus, the titer of plasmodia in Figure 1 actually reflects the number of zygotes formed in the mixtures, only some (if any) of which would have been "recognizable" plasmodia when sampled. It is possible to estimate from the data in Figure 1, therefore, that differentiating zygotes had represented 10 to 15% of the total mixture population by the 65th hour.

Recovery of nondifferentiating "zygotes" from mixtures of strains carrying the same matA alleles: To search for nondifferentiating diploids, the matA2 strains CH508 (matA2 matB3 imz-2 fusA2 fusC2 whi+) and LU887 (matA2 matBl imz-1 fusAl fusCl whi-) were mixed and inoculated onto plates under the same culture conditions as those employed in the experiments reported in Figure 1. The strains were marked such that any diploid amoebae formed by cell fusions between them would be heterozygous at several genetic loci. Although we could not predict, a priori, what the phenotype of diploid amoebae would be, we hoped that diploids would be genetically stable, that they would proliferate essentially as do ordinary haploid amoebal cells and that they might be clearly distinguishable from haploid CH508 or LU887 amoebae by virtue of being heterozygous at matB and fusA. Growth was monitored in these matA-homoallelic "mating mixtures" by taking periodic hemacytometer counts of suspensions made by washing the cultures from plates in known volumes of water, and the cultures were harvested at a time between 65 and 70 hours when the cell density
Figure 1.—The time course of plasmodium formation in a mating performed under improved conditions. The strains involved were CH351 (matA3 matB1 imz-1) and CH792 (matA2 matB3 imz-2), and the mating inocula contained equal numbers of amoebae of the two strains. The amoebal titer (Δ) was determined by making hemacytometer counts, and the plasmodial titer (▲) was determined with an assay plating, as described in MATERIALS AND METHODS and RESULTS.

had reached a level of approximately 10⁶ cells/culture. Harvesting consisted of washing the mixtures from the “mating plates” and replating the washes at a dilution expected to produce well-isolated amoebal colonies (30–40 per plate).

Amoebae from 100 clonal colonies produced from the diluted harvest washes of CH508/LU887 mixtures were crossed with tester strains that would permit the scoring of matB, fusA, imz and whi genotypes. This, we reasoned, would probably detect at once any diploids among the harvest colonies; unless one of the matB alleles showed complete dominance over the other, matB1/B3 amoebae could be expected to mate readily with amoebal tester strains of both or neither
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*matB*-type, depending upon the assumptions one makes concerning *matB* functions. On the basis of the mating-test results (Table 1), the clones recovered from the CH508/LU887 mixture were judged to comprise 3 classes: Class 1 clones, which displayed *matB1*, *imz-1*, *whi-1* behavior (the same as LU887); Class 2 clones, which displayed *matB3*, *imz-2*, *whi*<sup>+</sup> behavior (the same as CH508); and Class 3 clones, which displayed poor mating with both *matB1* and *matB3* testers, and thus represented a novel class unlike either haploid component of the CH508-LU887 mixture. Subsequent tests with Class 3 amoebeae revealed that they mate as poorly with *matB2* testers as they do with *matB1* and *matB3* testers. Furthermore, Class 3 amoebeae were found to grow with a doubling period of 10 hours, as compared with the period of 8 hours typical of haploid strains, and to be both uninucleate and clearly larger than haploid cells. These various properties and characteristics of Class 3 amoebeae suggested that they were diploid cells that had resulted from CH508-LU887 fusions. This was also supported by the fact that plasmodia arising in mating test crosses between Class 3 amoebeae and the four testers were all yellow (Table 1), a characteristic expected of plasmodia with a *whi-1*/*whi-1*/*whi*<sup>+</sup> triploid genotype, since the *whi*<sup>+</sup> allele is dominant even when present in the minority (Anderson 1977).

The somatic fusion phenotypes of plasmodia that arose in the mating tests of Table 1 were also examined (Table 2). The known alleles of *fusA* (*fusA1* and *fusA2*) are co-dominant and govern the somatic fusion of plasmodial cells by the rule that only cells with the same *fusA* genotype are able to fuse (*fusA* has no effect on amoebal fusions). Thus, for instance, a diploid plasmodium with the genotype *fusA1*/*A2* will not fuse with plasmodia with the genotypes *fusA1*/*A1* or *fusA2*/*A2*. Any amoebal diploids formed by CH508-LU887 fusions would have the genotype *fusA1*/*A2*, and these diploids would be expected to generate plasmodia with triploid genotypes when mated with haploid amoebal tester

<table>
<thead>
<tr>
<th>Phenotypic class of recovered clone</th>
<th>Extent of plasmodium formation and color of resulting plasmodium when clone was combined with</th>
<th>Number of clones in class</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CH924</td>
<td>LU887</td>
</tr>
<tr>
<td></td>
<td>matB1</td>
<td>matB1</td>
</tr>
<tr>
<td>1 (LU887-like)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>2 (CH508-like)</td>
<td>(n)</td>
<td>(n)</td>
</tr>
<tr>
<td>3 (&quot;diploid type&quot;)</td>
<td>--</td>
<td>(y)</td>
</tr>
</tbody>
</table>

Amoebal strains CH924, CH929, CH930 and LU897 are all *matA1* *whi-1* *imz-1*. "++" indicates extensive plasmodium formation by the 3rd or 4th day; "+-" indicates that 50 to 100 isolated plasmodial foci were visible on the 4th day (typical of homoallelic combinations at *matB* where at least one of the combined strains carries the *imz-2* allele); "-" indicates that fewer than 10 foci were visible on the 4th day (typical of homoallelic combinations at *matB* where both strains carry the *imz-1* allele). Color was scored after plasmodia in a test culture fused to form a single large plasmodium: "y", yellow; "w", white; "n", either plasmodia were not formed or were not cultured long enough to score for color.
TABLE 2
Fusion tests of plasmodia formed in mating tests

<table>
<thead>
<tr>
<th>Class of amoebal clone from CH508/LU887 mixture</th>
<th>Strain the clone was combined with</th>
<th>Number fusing with fusA1/A2 fusA1/A2 fusC1 tester fusC2 tester</th>
<th>Genotype of class</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 CH929 (fusA1)</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 CH930 (fusA2)</td>
<td></td>
<td>70</td>
<td>0</td>
</tr>
<tr>
<td>2 CH929 (fusA1)</td>
<td></td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>2 LU897 (fusA2)</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3 CH924 (fusA1)</td>
<td></td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>3 LU897 (fusA2)</td>
<td></td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>3 CH929 (fusA1)</td>
<td></td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>3 CH930 (fusA2)</td>
<td></td>
<td>0</td>
<td>6</td>
</tr>
</tbody>
</table>

* See Table 1.
† These strains carry the recessive allele fusCl.
‡ Presumably fusC1/fusC2, which is phenotypically indistinguishable from fusC2.

strains: fusA1/A1/A2 when mated with a fusA1 tester, and fusA1/A2/A2 when mated with a fusA2 tester. Such triploid plasmodia might still be expected to display a “fusA1/A2 phenotype,” despite the disproportionate representation of alleles. (By similar reasoning, such triploid plasmodia would be expected to display the phenotype associated with the dominant fusC2 allele.)

As shown by the data in Table 2, the three classes recovered from the CH508/LU887 mixtures were, in fact, also distinguishable on the basis of their inferred somatic fusion genotypes: Class 1 clones, like LU887, were fusA1; Class 2 clones, like CH508, were fusA2; and Class 3 clones showed the novel behavior of forming plasmodia with fusA1/A2 phenotypes when mated with either fusA1 or fusA2 testers, the behavior suggested for amoebal diploids with the heterozygous genotype fusA1/fusA2. Thus, on both the basis of matB behavior and apparent fusA genotype, nondifferentiating, matA-homozygous, diploid amoebal “zygotes” appeared to have formed in the CH508/LU887 mixtures to an extent of about 6% of the total number of cells in the mixtures.

To date, we have performed six diploid clone-recovery experiments similar to the one described above, involving different pairs of strains. In each of these, the results were similar to those of the CH508/LU887 mixtures: from mixtures of amoeboae carrying the same matA alleles, but different matB alleles, we consistently obtained 5 to 10% “diploid-type” cells. Moreover, the “diploid-type” cells display a consistent phenotype: they show the anomalous matB specificity of CH508/LU887 diploids, grow more slowly than ordinary haploid amoebae, are uninucleate and appear distinctly larger under microscopic examination than haploid cells.

In three independent control experiments, we harvested cells from mixtures of two strains that carried the same matA and matB alleles. The strains mixed carried different fusA alleles to permit the identification of “diploid types” on
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the basis of fusA-heterozygosity. Among 320 clones harvested from such mixtures, we found no diploid types. Thus, the formation of stable, diploid cells is at least 20-fold more frequent when mixed strains carry different matB alleles.

Verification of the genotype of a presumptive CH508/LU887 diploid strain: As Adler and Holt (1975) demonstrated, even strains that normally form plasmodia only by the heterothallic sexual process may spontaneously "self," though at an extremely low frequency (usually in the range of $10^{-19}$). This mode of asexual plasmodium formation occurs without change in ploidy and usually without mutational alteration (Adler 1975). Thus, it was possible to verify the genetic constitution of our presumptive CH508/LU887 diploids by analyzing the progeny of a rare asexually formed plasmodium from a clonal culture of the "diploid-type" Class 3 amoebae. The same was done for a "haploid-type" Class 1 and a "haploid-type" Class 2 clone, as controls.

The results of the analysis of 86 progeny of a rare plasmodium obtained from a culture of Class 3 amoebae are displayed in Table 3. These results demonstrate free recombination among the five loci that would be heterozygous in a CH508/LU887 diploid. Twenty progeny of a plasmodium from a Class 1 amoebal culture were analyzed, and all were found to have the genotype matA2 matB1 imz-1 whi-1 fusA1 fusC1, as does LU887. Twenty progeny of a plasmodium from a Class 2 amoebal culture were analyzed, and all were found to have the genotype matA2 matB3 imz-2 whi+ fusA2 fusC2, as does CH508.

Microscopic studies of cultures in which diploids form: The recovery of diploid cells from matA-homoallelic mixtures only when the mixed strains have different matB genotypes clearly indicates that the self-sterile specificity function of matB is to regulate the zygote formation process. It may be suspected, then, that matB alleles govern the specificity with which cells fuse during mating.

---

TABLE 3

Analysis of progeny of a presumptive diploid

<table>
<thead>
<tr>
<th>Marker pair</th>
<th>Number of progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parental</td>
</tr>
<tr>
<td>matB, imz</td>
<td>39</td>
</tr>
<tr>
<td>matB, whi</td>
<td>41</td>
</tr>
<tr>
<td>matB, fusA</td>
<td>37</td>
</tr>
<tr>
<td>matB, fusC</td>
<td>37</td>
</tr>
<tr>
<td>imz, whi</td>
<td>42</td>
</tr>
<tr>
<td>imz, fusA</td>
<td>42</td>
</tr>
<tr>
<td>imz, fusC</td>
<td>46</td>
</tr>
<tr>
<td>whi, fusA</td>
<td>48</td>
</tr>
<tr>
<td>whi, fusC</td>
<td>40</td>
</tr>
<tr>
<td>fusA, fusC</td>
<td>44</td>
</tr>
</tbody>
</table>

Parents: CH508 (matB3 imz-2 whi+ fusA2 fusC2), LU887 (matB1 imz-1 whi-1 fusA1 fusC1).

* A total of 86 progeny were scored for the 5 markers. Four additional progeny displayed "matB heterozygote specificity" (they mated poorly but equally well with all matB types) and were presumably diploids. The four were not scored for the other genotypes and are not included in the data shown.
theless, this need not be the case; the \textit{matB} locus might, for instance, regulate nuclear fusion or some process that affects the stability of binucleate cells that are the immediate products of random cell fusions. In an attempt to distinguish among these possibilities, we made microscopic observations of cells sampled from mixtures at times expected to correspond to the periods of most extensive diploid formation. In these experiments, cultures were established as for diploid isolation experiments, but, instead of harvesting cultures for dilution and plating, we scraped material from the cultures with flat toothpicks and transferred it to moistened agar slides (see Materials and Methods). Cultures were sampled in the 60th hour of incubation when they had attained a density of $7.3 \times 10^5$ cells/culture, a time and cell density that is presumed just to precede the period of maximum zygote formation in matings of amoebae with different \textit{matA} and \textit{matB} genotypes (see Figure 1).

Two types of cultures were examined: mixtures in which strains possessed the same \textit{matA} and \textit{matB} genotypes and mixtures in which the strains possessed the same \textit{matA} genotype, but different \textit{matB} genotypes. Cells were scored at 500× magnification with phase-contrast optics, and the numbers of cells with one, two or more nuclei were recorded. The results, summarized in Table 4, show the presence of binucleate cells at a level of approximately 3% of the total cell population only in mixtures heteroallelic for \textit{matB}. Although it would seem very likely that the binucleate cells seen in these experiments were formed from the fusion of uninucleate amoebae, no amoebal cell fusions were actually observed on the slides.

In addition to scanning slides to make cell counts, slides were made for the purpose of intensive observation of particular cell groups (cells remained motile and healthy in appearance for 30 to 60 minutes under these conditions). Not only did we fail to observe the fusion of uninucleate amoebae in these groups, but

\begin{table}[h]
\centering
\caption{Frequencies of binucleate cells in \textit{matA} homoallelic mixtures sampled in the 60th hour of growth}
\begin{tabular}{|l|c|c|c|}
\hline
Composition of mixtures & Numbers of cells containing the following numbers of nuclei: & Frequency of cells with more than one nucleus \\
& & 1 & 2 & other \\
\hline
CH242 (mat\textit{A}3 mat\textit{B}3) & 250 & 9 & 1\dagger & 3.3\% \\
CH813 (mat\textit{A}3 mat\textit{B}2) & & & & \\
CH242 (mat\textit{A}3 mat\textit{B}3) & 250 & 0 & 0 & 0.0\% \\
CH763 (mat\textit{A}3 mat\textit{B}3) & & & & \\
CH508 (mat\textit{A}2 mat\textit{B}3) & 250 & 7 & 0 & 2.7\% \\
A (mat\textit{A}2 mat\textit{B}1)* & & & & \\
CH508 (mat\textit{A}2 mat\textit{B}3) & 250 & 0 & 0 & 0.0\% \\
B (mat\textit{A}2 mat\textit{B}3)* & & & & \\
\hline
\end{tabular}
\end{table}

* Strains “A” and “B” are progeny clones from the cross CH508 × LU881.
\dagger Quadrinucleate cell.
we also failed to observe nuclear fusions in binucleate amoebae studied for extended periods. Explanations for this will be considered in the discussion.

The mating of a haploid strain with a diploid strain: Since mating of haploid amoebae ordinarily involves both cell and nuclear fusion with subsequent differentiation of a diploid zygote, it is to be expected that the mating of a haploid with a diploid strain would yield triploid amoebal zygotes that would differentiate to form plasmodia with triploid nuclei. Since the chromosome number for haploid amoebae appears to exceed 40 (Mohberg 1977), the successful meiotic processing of such triploid nuclei is problematical; one should expect serious aneuploidy among the progeny.

We examined a mating of this type, involving the diploid strain CH508/LU887 (matA2/A2 matB1/B3 imz-2/1 whi+/−1 fusA1/A2 fusC1/C2) and the haploid strain CH813 (matA3 matB2 imz-1 whi+ fusA2 fusC1). As expected, the germinating spores from this cross produced a heterogeneous assortment of progeny clones. The great majority of amoebal colonies (approximately 95 to 98%) were aberrant, presumably because the cells were aneuploid; the colonies were small, many hardly developing at all. Most had fuzzy edges, some were very diffuse and many showed rapid clonal differentiation to form plasmodia (a property expected of amoebae heterozygous for matA alleles). Thus, triploid nuclei appear to have been formed in the haploid/diploid mating.

The remaining 2–5% of the spore clones from the haploid/diploid mating grew in the fashion characteristic of ordinary haploid strains; their colonies were uniformly large, had sharp edges and showed no clonal plasmodium formation. Amoebae from 92 of the large sharp colonies were recloned and then tested for matA, matB, imz, whi, fusA, fusC genotypes. They behaved in these tests as normal haploid strains, and the results revealed that they comprised both matA2 and matA3 progeny, in the ratio of 64:28. The matA2 progeny showed segregation of alleles at all five loci for which the CH508/LU887 parent was heterozygous. Moreover, only those alleles carried by the CH508/LU887 diploid were represented among the matA2 progeny: no matA2 progeny carried the matB2 allele. Analysis of the 28 matA3 progeny revealed them all to have the CH813 genotype; i.e., matB2 imz-1 whi+ fusA2 fusC1. Thus, it appears that progeny with normal colony morphology did not arise from the meiotic processing of triploid plasmodial nuclei.

A mating between diploid strains: The CH508/LU887 diploid recovery experiment described above produced clones with the genotype matA2/A2 matB1/B3 imz-1/2 whi+/−1 fusA1/A2 fusC1/C2. Using the same approach as in that experiment, we combined approximately equal numbers of the matA3 strains CH242 and CH813 to generate diploid clones with the genotype matA3/ A3 matB2/B3 imz-1/2 whi+/+ fusA2/A2 fusC1/C2. In total, 120 clones were recovered from CH242/CH813 mixtures and scored for matB and imz phenotypes; 29% (35/120) were of the CH242 “parental type,” 63% (75/120) were of the CH813 “parental type” and 8% (10/120) displayed the anomalous matB specificity and slower growth characteristic of diploid strains (these we presumed to be diploids with the genotype indicated above).
Some of the matA3/A3, CH242/CH813 presumptive diploids recovered in this experiment were combined in mating mixtures with matA2/A2, CH508/LU887 diploids. Plasmodia formed in these mixtures, and several of independent origin were cultured to produce spores for genetic analysis. It was expected that the spores would germinate to yield diploid progeny generated by the meiotic processing of the presumably tetraploid nuclei produced in these diploid × diploid matings.

Among the diploid spore progeny expected as products of the meiotic processing of matA2/A2/A3/A3 nuclei would be diploids heterozygous for matA alleles. With free re-association of chromosomes, these matA-heterozygotes should represent 56–67% of the diploid progeny, depending upon the degree of linkage between matA and its centromere. Since matA-heterozygous diploids possess the distinctive property of rapid clonal differentiation (Adler and Holt 1975), the matA heterozygous diploid spore progeny could be readily identified directly on the spore germination plates.

Five independent sets of spores from diploid/diploid matings were analyzed. Unexpectedly, only 1 to 4% of the clones displayed the characteristic matA heterozygote differentiation behavior; moreover, nearly all of the spore clones exhibited the rapid growth typical of haploids. Amoebae from 233 of the clones (not chosen with a bias related to colony size or morphology) were crossed with tester strains appropriate for scoring matA, matB, whi, fusA and fusC genotypes, and nearly all (225) behaved in the tests as typical haploid strains. The progeny of each of the five plasmodia were either all matA2, or included both matA2 and matA3 types with matA2 progeny predominating (27:0, 30:0, 19:10, 26:1 and 98:14). Each of the progeny genotypes identified could be derived entirely from only one or other of the diploid parents, indicating that there had been no recombination between the two parental genomes: no matA2 progeny were matB2; no matA3 progeny were either matB1 or whi-1; and no matA3 progeny were fusA1, even though fusC alleles segregated 1:1. Among the 233 progeny examined, eight crossed poorly, but equally well with testers of all matB-types, a behavior we have observed to be typical of diploid amoebae that are heterozygous for matB alleles; these 8 presumptive diploids were all matA2.

**DISCUSSION**

We have shown that diploid amoebal “zygotes” form in mixtures of strains that carry the same matA alleles, but different matB alleles. These diploids form to an extent (approximately 5 to 10% of the total mixture population) comparable to the extent to which plasmodial zygotes form (10 to 15% of the total mating population) in mating mixtures in which amoebae carry different alleles at both matA and matB. Diploid amoebae are easily distinguishable from the haploids whose fusion formed them by their mating specificity, slower growth, larger size and (if they were formed from the fusion of haploids carrying different fusA alleles) apparent fusA genotype.

A rare asexually formed plasmodium was obtained from a culture of a presumably diploid amoebal strain, and an analysis of its progeny revealed the 1:1
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This both confirms the diploid genotype and suggests that the diploid amoebae maintain a stable chromosome complement. The tendency of amoebal pairs to form diploid amoebal zygotes appears to be regulated by the matB locus, since combinations of strains carrying the same alleles at both matA and matB do not generate diploids at a measurable frequency.

The hypothesis that matB controls only the zygote formation stage of mating and that matA controls only the zygote differentiation stage (Youngman et al. 1979) arose largely from the study of rare diploid spore progeny heterozygous for matA alleles. Most of the progeny of a sexually formed plasmodium are, of course, haploid. However, a small, variable percentage are diploid and heterozygous at matA, and these show a remarkable behavior: they differentiate very rapidly into plasmodia. It has been proposed that matA heterozygotes are the meiotic products of tetraploid nuclei, which are sometimes observed at low frequency in the cytoplasm of vegetative plasmodia (Adler and Holt 1975; Laffler and Dove 1977; Shinnick 1978). Adler and Holt (1975) associated the differentiation behavior of these unusual spore clones with the presence, within the same cell, of two different matA alleles, and they argued that the amoebae in the clones may differentiate into plasmodia as a direct consequence of matA heterozygosity. More recent studies involving the analysis of mutations that affect differentiation have further implicated matA as a regulator of differentiation to the plasmodial state. Nearly all such mutations map very close to matA, and most have been inseparable from matA by recombination (Shinnick and Holt 1977; Anderson and Dee 1977; Adler and Holt 1977; Davidow and Holt 1977; Honey, Poulter and Teale 1979; Gorman, Dove and Shaibe 1979; Shinnick, Anderson and Holt, unpublished). In contrast, no available information suggests a connection between matB genotype and the tendency of a cell to differentiate. No mutations affecting differentiation are linked to matB (Shinnick, Anderson and Holt, unpublished), and matA heterozygotes from spores differentiate rapidly in clones, regardless of whether they are homozygous or heterozygous at matB (Davidow and Youngman, unpublished).

To summarize our present knowledge concerning the functions of matA and matB: diploids form in mixtures of strains carrying different matB alleles, whether or not the strains carry different matA alleles; diploids form at a much lower frequency in mixtures of strains carrying the same matB alleles, whether or not the strains carry different matA alleles; when diploids form to produce a cell heterozygous at the matA locus, the diploids differentiate rapidly to yield plasmodia; matB genotype has no apparent influence upon the tendency of a matA-heterozygote to differentiate; diploids homozygous at matA and heterozygous at matB exhibit the same very limited tendency as haploids to differentiate clonally; and finally, mutations associated with the tendency to differentiate are generally linked to matA and are never, among those analyzed thus far, linked to matB. On the basis of these considerations, we conclude that matA imposes specificity on the mating process only by governing the tendency of zygotes to segregate of alleles at all loci for which a true amoebal diploid would have been heterozygous.
differentiate into plasmodia, and that \( \text{matB} \) imposes specificity only by governing the tendency of haploid amoebae to form diploid zygotes.

It is tempting to draw a more specific conclusion concerning the role of \( \text{matB} \): that it governs the specificity of cell fusion during mating. The microscopic studies reported here tend to support this suggestion, but fall short of proof. Although it is probably justified to regard the binucleate cells that we observed in \( \text{matB} \) heteroallelic mixtures as evidence of cell fusion, the absence of binucleates in the \( \text{matB} \) homoallelic mixtures does not necessarily imply the absence of cell fusion in those mixtures; binucleate cells may have formed and then become uninucleate by either separation or nuclear fusion.

Confirmation that \( \text{matB} \) affects the cell fusion stage has come from another quarter, however. Microcinematographic studies of amoebal mixtures have recently been performed in Göttingen, at the Institute für den Wissenschaftlichen Film, in collaboration with our laboratory (Holt et al. 1979). The films show clearly that cell fusions are extensive in \( \text{matB} \) heteroallelic mixtures, regardless of \( \text{matA} \) genotypes, and very rare in \( \text{matB} \) homoallelic mixtures.

In addition, the film studies reveal an interesting and unexpected difference between like-\( \text{matA} \) and unlike-\( \text{matA} \) cell fusions. In the binucleate cells formed from cell fusions in mixtures of strains with the same \( \text{matA} \) alleles, but different \( \text{matB} \) alleles, no fusion of interphase nuclei was observed; nuclear coalescence occurred only when the nuclear membranes had dispersed during mitosis. By contrast, the interphase nuclei of binucleates formed from cell fusions in mixtures of strains with different \( \text{matA} \) and \( \text{matB} \) alleles fused before mitosis within approximately 60 min after cell fusion.

The effect that the \( \text{matA} \) genotype apparently has upon the probability of a nuclear fusion within a binucleate cell may help to explain why the extent of \( \text{matA} \) homozygous diploid formation (5 to 10%) always seems to be slightly less than the extent of \( \text{matA} \) heterozygous plasmodial zygote formation in matings: binucleates may tend to separate if nuclear fusion does not occur. Moreover, in the case of cell fusions involving amoebae carrying the same \( \text{matA} \) alleles, where nuclear fusion occurs only at mitosis, there may be a requirement that the two nuclei involved be in similar stages of the cell cycle; otherwise, binucleate separation is the likely outcome.

The analysis of progeny from haploid \( \times \) diploid and diploid \( \times \) diploid crosses produced results that were both interesting and difficult to explain. The haploid \( \times \) diploid cross involved a \( \text{matA3} \) haploid strain and a \( \text{matA2/A2} \) diploid. Such a cross might be expected to produce a plasmodium with triploid nuclei and, thus, to produce progeny that were virtually all aneuploids. Nearly all of the progeny did, in fact, appear to be aneuploid, as inferred from their poor growth and abnormal colony morphology; some, however, resembled normal haploid strains in growth, colony morphology and mating characteristics. These included \( \text{matA3} \) and \( \text{matA2} \) progeny strains in a ratio of 28:64. Among these presumably haploid progeny, there was no evidence of recombinational exchange between the haploid and diploid parents, suggesting that these progeny were the meiotic products of unfused nuclei. The \( \text{matA2} \) progeny were probably the meiotic products of the few diploid \( \text{matA2/A2} \) nuclei that remained unfused after the fusion of amoebae to
form zygotes in the diploid × haploid cross. Such an explanation cannot, however, account for the presence of matA3 progeny, since unfused haploid matA3 nuclei could not successfully be processed during ordinary meiosis. It becomes necessary to suggest either an unusual meiotic processing of these haploid nuclei in which a reductional division did not occur, or that some unfused matA3 nuclei became diploid (see Lafler and Dove 1977).

The diploid × diploid cross involved matA2/A2 and matA3/A3 diploid amoebae. Such a cross might be expected to produce a plasmodium with tetraploid nuclei, and thus to produce only diploid progeny, 56 to 67% of which would be matA heterozygous. Instead, a very small number of matA heterozygotes was evident on the spore plates, and the great majority of the progeny appeared to be haploid. Moreover, the haploid progeny showed no evidence of recombination with respect to matA, suggesting that they arose from the meiotic processing of unfused nuclei. Thus, instead of producing a plasmodial homokaryon containing tetraploid nuclei, the diploid × diploid cross appears to have produced a plasmodial heterokaryon containing mostly unfused diploid nuclei. This suggests, among other things, an interesting feature of the regulation, by matA, of differentiation to the plasmodial state: heterologous matA alleles need not be within the same nucleus to promote differentiation, but only within the same cell.

Notwithstanding the obvious complexities and unexplained aspects of the diploid × diploid and haploid × diploid crosses, a useful result stands out: the haploid progeny from both types of crosses appear to be derived from the meiotic processing of unfused nuclei. Thus, in the case of the two specific crosses presented here, for instance, analysis of only the matA2 progeny is equivalent to (and far easier than) analyzing the progeny of a rare asexually formed plasmodium obtained from a clonal culture of the matA2/A2 diploid. This suggests a simple, general method for directly verifying the genotype of a diploid strain.

The distinct and independent functions of matA and matB demonstrated here have no direct analogy in other taxons in which heterotypic-compatible, tetrapolar-breeding systems are known. Such systems have been described only among the Basidiomycetes (to our knowledge), where approximately 55% of the characterized species are tetrapolar (Fincham and Day 1971). In all cases of Basidiomycete tetrapolarity, the A and B factors regulate only developmental processes (nuclear migration, clamp connection formation, septum morphology and fruiting body production) and have no apparent effect on the frequency of hyphal fusions to form a heterokaryon (Raper 1966; Koltin, Stanberg and Lenks 1972; Carlile and Gooday 1978).

The one possible exception to this concerns the breeding system that operates in Ustilago maydis. In this smut fungus, sexual fusions occur in the unicellular, yeast-like stage, producing a dikaryon that must infect a suitable host plant to permit normal mycelial growth and, ultimately, to produce a fruiting body (Fincham and Day 1971; Holliday 1975). There are two "mating loci" in U. maydis, called a and b; the a locus has two alleles, and the b locus is multiallelic (Rowell 1955). The a locus governs the specificity of sexual fusions in the yeast-like state of the organism, and the b locus governs the parasitic development of the dikaryon mycelium in the host plant (Holliday 1961). Thus, the a locus of
U. maydis is roughly analogous to the MatB locus of P. polycephalum, and the b locus of U. maydis is roughly analogous to the matA locus in P. polycephalum. No other Ustilago species is known to employ such tetrapolar mechanisms (Whitehouse 1951; Holliday 1961; Fincham and Day 1971).

In most of the lower fungi (reviewed by Carlile and Gooday 1978), the ciliate protoza (reviewed by Miyake 1978) and the unicellular algae (reviewed by Crandall 1977; Goodenough 1977), cell fusions (other than sexual fusions) do not occur in the natural course of vegetative growth (as is frequently the case in the higher fungi and in the plasmodial form of Myxomycetes). In these systems, sexual cell fusions occur only between specialized complementary structures or between complementary “gametic forms”, where complementarity is usually a property specified by both genetic and epigenetic mating-type factors (Crandall 1977). The ability of complementary structures to fuse is generally not constitutive; “agglutination competence” or “fusion competence” is induced by chemical gamones, cell contact, environmental factors or by some combination of these. We have previously shown that differentiation of amoebae into plasmodia by both the asexual (Youngman et al. 1977) and the sexual process (Youngman 1979; Pallotta et al. 1979) is activated by a diffusible substance (or substances) secreted by growing amoebae. Thus, by analogy with the regulation of sexual activity in other unicellular organisms, we suspect that the competence for matB-governed sexual fusions of amoebae may be induced by this substance. This is under active investigation.

Thus far, our studies of mating compatibility in the Myxomycetes have been almost exclusively restricted to P. polycephalum, and we readily concede that there is little basis, at the present time, for generalizations that apply to other Myxomycete species. We (Youngman, unpublished) have, however, made limited studies with the Myxomycete Didymium iridis, and have already demonstrated the involvement in D. iridis matings of a secreted substance similar to the one involved in P. polycephalum matings. Preliminary studies also suggest the existence among some D. iridis strains of factors with functions similar to that of matB (Youngman, unpublished). There is some reason to believe, therefore, that P. polycephalum is not atypical in its mating regulation.

In addition to clarifying our understanding of the mating process in P. polycephalum (and, perhaps, in Myxomycetes in general), knowledge and use of the properties of the matB locus should have an important impact upon the practice of genetics in this field. Because of the ease with which crosses may be performed in P. polycephalum, it has long been regarded as a useful “model system”, amenable to both biochemical and genetic study. Yet, before the work described here, one of the most powerful genetic tools was not at our disposal: the capability for creating amoebal diploids of any desired genetic constitution for the purpose of testing the dominance and complementation of amoebal mutations. By employing a strategy similar to the one we have described for the isolation of CH508/LU887 diploids, these fundamental genetic tests should now become routinely possible. In fact, we have already completed the first such studies, which will be published elsewhere (Anderson and Youngman, unpublished).
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LITERATURE CITED


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