VEGETATIVE INCOMPATIBILITY AND THE MATING-TYPE LOCUS IN THE CELLULAR SLIME MOLD *DICTYOSTELIUM DISCOIDEUM*

GILLIAN E. ROBSON AND KEITH L. WILLIAMS

Genetics Department, Research School of Biological Sciences, The Australian National University, P. O. Box 475, Canberra City, A.C.T., 2601, Australia

Manuscript received February 13, 1979
Revised copy received May 21, 1979

ABSTRACT

The genetic basis of vegetative incompatibility in the cellular slime mold, *Dictyostelium discoideum*, is elucidated. Vegetatively compatible haploid strains form parasexual diploids at a frequency of between 10^{-6} and 10^{-5}, whereas "escaped" diploids are formed between vegetatively incompatible strains at a frequency of $\sim 10^{-8}$. There is probably only a single vegetative incompatibility site, which appears to be located at, or closely linked to, the mating-type locus. The nature of the vegetative incompatibility is deduced from parasexual diploid formation between wild isolates and tester strains of each mating type, examination of the frequency of formation of "escaped" diploids formed between vegetatively incompatible strains, and examination of the mating type and vegetative incompatibility of haploid segregants obtained from "escaped" diploids.

IN eukaryotes, there are two classical genetic systems, the sexual and parasexual cycles. Since the sexual cycle (meiosis) has associated with it a high frequency of recombination, it can be used to determine the complexity of genetic loci and to map the relative distances between and the order of linked markers. On the other hand, the parasexual cycle, if it involves a relatively stable diploid phase, allows complementation tests to be performed easily. In addition, since the frequency of mitotic recombination is low, whole chromosomes segregate independently during the haploidization phase of the parasexual cycle, making linkage assignment of genetic markers relatively easy (PONTECORVO and KAFER 1958). This is important in simple eukaryotes where an individual chromosome often comprises several meiotic linkage groups *e.g.*, *Aspergillus nidulans* (KAFER 1977) and *Saccharomyces cerevisiae* (SHERMAN and LAWRENCE 1974). Clearly, there is merit for the investigator in having both genetic systems working in the same organism. However, a complete parasexual cycle has been described only in the fungi (*e.g.*, *A. nidulans*, PONTECORVO and KAFER 1958) and a few other simple eukaryotes (*e.g.*, the cellular slime mold *Dictyostelium discoideum*, JACOBSEN and LODISH 1975). Only in *A. nidulans* have both the sexual and parasexual genetic systems been extensively exploited (KAFER 1977).

We are attempting to set up both sexual and parasexual genetic analysis in *D. discoideum*. Although the parasexual cycle is well understood and is used extensively for genetic analysis, the sexual cycle has not yet been exploited (WALLACE and RAPER 1979; NEWELL 1978). There are still major unsolved problems, both practical (*e.g.*, macrocyst dormancy and germination) and theoretical, (*e.g.*, the fate of the meiotic products) concerning the sexual cycle of *D. discoideum* (WALLACE and RAPER 1979). Before tackling these problems, we plan to construct strains appropriate for use in both sexual and parasexual genetic analysis. We consider it important to use isogenic strains to avoid problems of genetic background. The parasexual system of *D. discoideum* has been established using the type strain, NC4 (RAPER 1935). Since this strain is now well-marked genetically, it would be advantageous to construct the sexual genetic system around it. *D. discoideum* is primarily a heterothallic organism, with mating most probably being controlled by a one locus two allele mating system (ERDOS, RAPER and VOGEN 1973; CLARKE, FRANCIS and EISENBERG 1973; ROBSON 1978). In order to obtain genetically marked strains of opposite mating type in predominantly isogenic strains, we planned to transfer the mating-type locus from the V12 isolate (*mata2*) into the NC4 (*matA1*) genetic background. Since such an approach is not feasible using the sexual cycle at this stage, attempts were made to do this using the parasexual system. However, preliminary experiments indicated that parasexual diploid formation between haploid *D. discoideum* strains of opposite mating type is rare (WILLIAMS, unpublished); there is only one report of parasexual diploid formation between such strains (MOSSES, WILLIAMS and NEWELL 1975).

In organisms that have different mating types, vegetative fusion between strains of unlike mating type is often restricted. Such self, not-self recognition systems have been observed within many species from fungi (GRINDLE 1963a and 1963b; KWON and RAPER 1967; MYLYK 1975) to Ascidians (OKA 1970). It has been suggested that even the major histocompatibility complex, which is a complex group of genes controlling allograft reactions and the immune response in vertebrates, may have its origin in primitive self, not-self systems (KLEIN 1975). In heterothallic Ascomycetes (*e.g.*, *Neurospora crassa*), there exist vegetative incompatibility (self, not-self) systems controlled by several loci, including the mating-type locus (MYLYK 1975). In such systems, normal heterokaryons fail to form in strains carrying different alleles at any of the vegetative incompatibility loci (including the mating-type locus). It seems likely that the failure of parasexual diploid formation in *D. discoideum* between strains of opposite mating type may result from a vegetative incompatibility system involving at least the mating-type locus. In this report, we elucidate the vegetative incompatibility system of *D. discoideum*. We show that there is probably only a single site of vegetative incompatibility, which resides at, or closely linked to, the mating-type locus.

MATERIALS AND METHODS

Media

SM agar (SUSSMAN 1966) and inhibitor-containing SM agar were prepared exactly as described previously (WILLIAMS 1978). Sodium arsenate (1.5 mg per ml) SM agar was pre-

pared by adding an appropriate amount of a sterile Na_2AsO_4 solution (100 mg per ml) to melted SM agar.

Strains

D. discoideum was grown on a cobalt-resistant strain of *Klebsiella aerogenes* at 21 ± 1° (WILLIAMS and NEWELL 1976). This bacterial strain grew on all of the inhibitor-containing SM agar media used in this study. During experimental periods, strains of *D. discoideum* were maintained by clonal passage on SM agar plates (21 ± 1°) at weekly intervals. Stocks of all strains were maintained at 4° desiccated on silica gel (WILLIAMS and NEWELL 1976). Most genetically marked strains of *D. discoideum* are derived from the type strain, NC4 (RAPER 1935) or V12 (isolated by K. B. RAPER in 1937) and are described in Table 1. Other strains of *D. discoideum*, which were all obtained from K. B. RAPER (University of Wisconsin), are named in Table 2 and details of them have been published previously (ERDOS, RAPER and VOGEN 1973; WALLACE 1977).

Macrocyst formation

The standard method for making macrocysts of *D. discoideum* is to grow together, under appropriate conditions, the two strains to be tested; macrocysts are formed directly on the growth plates (ERDOS, RAPER and VOGEN 1973). We wanted to develop a method for making macrocysts by growing the amoebae separately before combining them for macrocyst formation. To this end, we modified techniques for making macrocysts in liquid (FILOSA and DENGLER 1972; CLARKE, FRANCIS and EISENBERG 1973) and used multiwell plates for incubation of the combined amoebae for macrocyst formation. This allowed quantitative analysis of macrocyst formation, using an inverted microscope. As a result of a series of unpublished experiments (POWELL and WILLIAMS), 20 mM $CaCl_2$ was found to be a satisfactory liquid medium for macrocyst production in the dark. For the experiments reported here, a rapid and reliable mating-type test, the "toothpick test," was used: One ml of sterile 20 mM $CaCl_2$ was dispensed into each of 24 flat-bottomed clear plastic wells (capacity ~3.2 ml) of a sterile Linbro FB-16-24TC multiwell dish. Amoebae of two strains, one of known mating type, were picked into each well from clonal SM-agar plates, using a toothpick. For good macrocyst production, large picks of cells were required (~10^6 to 2×10^6 amoebae per pick). The multiwell dish was swirled briefly before covering with foil for dark incubation. The dish was stored at 21 ± 1° for seven days and was not shaken during this period. Macrocysts were scored at 40 × magnification with an Olympus CK inverted microscope with a mechanical stage that held the Linbro tissue culture dish. For reasons not understood, macrocyst production was poor if the smaller 96-well Falcon 3040 microtest plates (containing 0.1 ml 20 mM $CaCl_2$) were used.

Parasexual diploid formation

Co-aggregation of haploid amoebae: In all experiments, except those described in Figure 1, amoebae were co-aggregated in 20 mM $CaCl_2$ with shaking for 17 to 18 hr at 150 cycles per min on an orbital shaker at 21 ± 1°, in either a 96-well Falcon 3040 plate with 3041 lid (0.1 ml of $CaCl_2$ per well) or a 24-well Linbro FB-16-24TC plate (1 ml of $CaCl_2$ per well). The Falcon 3040 plate was used for rapid crosses in which amoebae of strains to be crossed were toothpicked into the wells (WILLIAMS and NEWELL 1976), while the Linbro FB-16-24TC plates were used for accurate quantitive crosses where cells were washed and counted (WILLIAMS, KESSIN and NEWELL 1974a).

Selective conditions for obtaining the diploids: 10^5 to 10^6 amoebae from crosses between two haploid strains carrying different growth temperature-sensitive mutations were usually plated on SM agar at 26.8 ± 0.2°, which kills the haploids but allows the growth of diploids with complementing growth temperature-sensitive mutations (LOOMIS 1969; KATZ and SUSSMAN 1972). 10^5 to 10^6 amoebae from crosses between wild-type (not growth temperature-sensitive) isolates and a cobalt-resistant, growth temperature-sensitive strain were plated on 300 μg per ml or 350 μg per ml $CoCl_2 \cdot 6H_20$-SM agar (WILLIAMS 1978). Diploids were purified by clonal passage on SM agar at 21 ± 1°, their genotype was examined, and they were stored on silica gel at 4°.

TABLE 1

Haploid strains of D. discoideum

Strain†	Parent‡	Genotype*											
		whi	*bwn*	*tsg*	*cyc*	*acr*	*cob*	*axe*	*spr*	*ebr*	*ars*	*mat*§	*man*
NP84	NP73	+	+	-350	+	+	+	-352	B2	+	+	a2	+
NP86	NP73	+	+	-351	+	+	+	-352	B2	+	+	a2	+
NP158	NP84	+	+	-350	+	+	+	-352	B2	-350	+	a2	+
HU1	DP1	A1	A1	D12,E13	+	A351	+	+	A1	+	+	A1	+
HU77	DU220	A1	+	B3	A1	A2	+	NS	+	+	+	A1	+
HU89	NP158/HU1	+	A1	-350	+	+	+	+	B2	+	+	a2	+
HU156	HU89	+	A1	-350	+	+	-364	+	B2	+	+	a2	+
HU184	HU156	+	A1	-350	+	A356	-364	+	B2	+	+	a2	+
HU227	DU405	A1	+	A1,D12	A1	A1,D369	-353	B1	H351	+	A351	A1	A1
HU235	DU429	A1	+	A1,D12	A1	A1,D369	-353	B1	H351	+	A351	A1	A1
HM3	V12M2	-900	+	-901	-900	-900	+	+	+	+	+	a2	+

* The symbols used to describe the mutant loci have been defined previously (WILLIAMS and NEWELL 1976; WRIGHT, WILLIAMS and NEWELL 1977; FREE, SHIMKE and LOOMIS 1976) except for *mat*, which denotes the mating-type locus, and *ars*, which denotes genes whose phenotype is resistance or sensitivity to sodium arsenate. Two such loci, alleles at which exhibit arsenate resistance, have been discovered (WILLIAMS, in preparation).

† All strains derived in this laboratory, except HM3, which was obtained from J. H. TRENT and R. R. KAY (ICRF. Mill Hill, London, U.K.).

‡ Parent strains: NP73 (WILLIAMS 1976); DP1 is a diploid formed between M28 and TS12; HU1 is a methanol-resistant (*acrA351*) strain of a haploid segregant from DP1; DU220 is a methanol resistant diploid (*acrA2/acrA2*) obtained by mitotic crossing over from DP32 (TS12 × NP12, WILLIAMS, KESSIN and NEWELL 1974b). HU77 is a haploid segregant from DU220, which is recombined in linkage group II. HU77 has a region proximal to *acrA*, including *whiA1*, derived from strain TS12 and the region distal to (and including) *acrA2* from the AX3-derived strain NP12; hence *tsgD*12 is crossed out. This is useful as HU77 is a strain which has the *whiA1* allele, but does not also have a *tsg* mutation linked to it; NP158 and HU1 are the parents in the cross from which HU89 was isolated; the diploid was lost; HU227 and HU235 are NC4-derived strains containing markers on six linkage groups. Since their origin is complex and they carry some undescribed mutations, they will be published in detail elsewhere.

Other strains used in this paper whose genotypes have already been published are: M28, TS12, X9 (KATZ and SUSSMAN 1972; WILLIAMS, KESSIN and NEWELL 1974b); HU32 (WILLIAMS 1978).

§ The two mating-type alleles are distinguished as *matA* (formerly Type A_1, ERDOS, NICKERSON and RAPER 1973) and *mata* (formerly Type A_2, ERDOS, NICKERSON and RAPER 1973). The NC4 isolate is referred to as *matA1* and the V12 isolate *mata2*. Other wild isolates (Table 2) are given different isolation numbers, but are coded as *matA* or *mata*, depending on mating type, *e.g.*, strain WS472 is *matA1659* and strain WS582 is *mata355* (see Table 2). NS: not scored.

TABLE 2

Macrocyst formation (sexual cycle) and parasexual diploid formation in "sexy" wild isolates of D. discoideum

Haploid‡ strain	Mating§ type	Macrocyst formation with‖ Self	*matA* tester	*mata* tester	Parasexual diploid† formation with *matA* tester	*mata* tester
WS472	*matA1659*	—	—	++	+	—
WS1956	*matA1654*	—	—	++	+	—
WS2054	*matA1655*	—	—	++	+	—
WS583	*matA351*	—	—	++	+	—
WS51 (WS10)	*matA352*	—	—	++	+	—
HU182* (WS585)	*matA1651*	—	—	(++)	+	—
HU188**	*matA353*	—	—	++	+	—
WS655	*matA354*	—	—	++	+	—
WS582	*mata355*	—	++	—	—	+
WS656	*mata356*	—	++	—	—	+
WS7	*mata1650*	—	++	—	—	+

† Parasexual diploid formation was tested in two experiments involving at least 2×10^6 amoebae using rapid crosses involving toothpicks of amoebae (see MATERIALS AND METHODS) and selecting diploids at 26.8° on SM-$CoCl_2$ agar plates. Cobalt-resistant tester strains were HU32 (*matA1*) and HU156 or HU184 (*mata2*). +: At least one diploid obtained; —: no diploids obtained in either experiment.

‡ These strains are the partially and fully sexually competent strains described in ERDOS, RAPER and VOGEN (1973); WS655 and WS656 are two other sexually competent strains (WALLACE 1977). All of these strains were obtained from K. B. RAPER (University of Wisconsin). Strains WS10 and WS585 were diploids. HU182* and HU188** are haploid strains obtained in this laboratory from diploid strains WS10 and WS585, respectively, by haploidizing on ben late (20 μg per ml)-SM agar (WILLIAMS and BARRAND 1978).

§ See legend to Table 1.

‖ Macrocyst formation was assayed using the "toothpick test" (see MATERIALS and METHODS) using strain HU1, TS12, HU235 or WS583 as the *matA* tester and strain HU89 or WS582 as the *mata* tester. —: no macrocysts; ++: macrocysts formed; (++): poorly developed macrocysts.

Haploidization of diploids

Haploids were segregated by plating about 2×10^4 diploid amoebae, heterozygous for a recessive resistance marker, on an appropriate inhibitor-containing SM agar plate; *e.g.*, 2% (v/v) methanol-SM agar for *acrA*/+ diploids (WILLIAMS, KESSIN and NEWELL 1974b), or 35 μg per ml ethidium bromide-SM agar for *ebrA*/+ diploids (WRIGHT, WILLIAMS and NEWELL 1977). Some diploids were haploidized by clonal platings on SM agar containing 20 μg per ml or 50 μg per ml ben late (WILLIAMS and BARRAND 1978). The genotype of haploid segregants was determined by examining spore shape and fruiting body pigmentation on SM agar plates at 21 ± 1°, and by picking amoebae with toothpicks on to SM agar at 26.8 ± 0.2° and appropriate inhibitor-containing SM agar plates at 21 ± 1°.

RESULTS

Mating type of the strains used

In this first report on vegetative incompatibility, only results with strains showing strong mating reactions for one of the two well-established mating types (*matA*, formerly Type A_1, or *mata*, formerly Type A_2, see legend to Table 1)

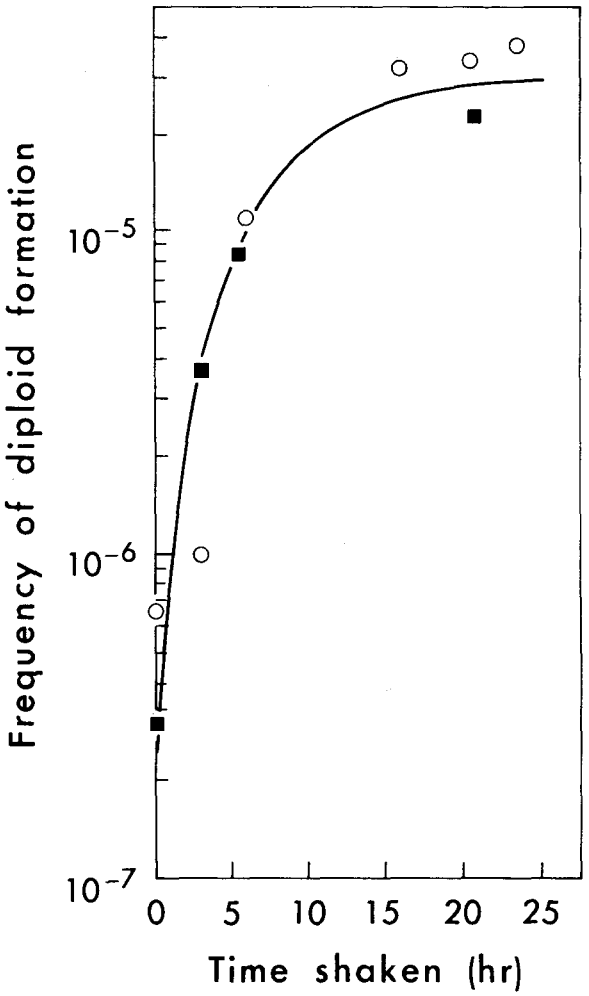

FIGURE 1.—Time course of diploid formation between growth temperature-sensitive haploid strains M28 (*matA1*) and TS12 (*matA1*) co-aggregated in Linbro FB-16-24TC plates. 5×10^6 washed amoebae of each strain were shaken at 150 cycles per min on an orbital shaker at 21 ± 1° in 1 ml H_2O (O), or 1 ml Bonner's Salt solution: NaCl, 0.6g; KCl, 0.75g; $CaCl_2$ 0.3g dissolved in 1 l of water. (■). The amoebae were plated on SM agar at the restrictive temperature and diploids obtained as described in the MATERIALS AND METHODS. The frequency of diploid formation is the number of diploids formed divided by the total number of amoebae plated at the restrictive temperature, as estimated by haemacytometer counts (WILLIAMS, KESSIN and NEWELL 1974a). This frequency is higher than that ($\sim 7 \times 10^{-6}$) obtained on the basis of diploids per 10^6 amoebae plated, which assumes 100% recovery of amoebae. This is probably due to large clumps of amoebae being excluded from the haemacytometer chamber.

were used. Studies on asexual, bisexual and homothallic strains will be reported elsewhere. Strains NC4 (*matA1*) and V12 (*mata2* were used as reference strains. The nine sexually competent isolates of ERDOS, RAPER and VOGEN (1973) plus two other isolates, WS655 and WS656, were tested for macrocyst

production, using the "toothpick test." All showed good mating competence, which confirmed the results of ERDOS, RAPER and VOGEN (1973) and, in the case of strains WS655 and WS656, the results of WALLACE (1977) (Table 2). The strains WS10 and WS585 were diploids; they were haploidized on ben late-SM agar (WILLIAMS and BARRAND 1978); the haploids, named HU182 and HU188, respectively, were used in this work instead of strains WS10 and WS585.

Parasexual diploid formation

Vegetative incompatibility is defined here as the failure of two strains to form stable parasexual diploids at the usual frequency of between 10^{-5} and 10^{-6}. To study this phenomenon, many crosses were performed, and hence a simple, yet reliable, method for making diploids was needed. Over the past few years, we have examined in some detail conditions required for parasexual diploid formation. Vegetative haploid amoebae mixed together briefly form diploids at a frequency of about 10^{-7}. A major increase in the frequency of diploid formation (to about 10^{-5}) is obtained by co-aggregating haploid amoebae to be fused on filters (LOOMIS 1969) or in liquid salt solution for at least 17 hours (Figure 1).

Varying the pH, temperature, molarity and composition of the incubation solution (*e.g.*, NaCl, KCl, $CaCl_2$, LiCl, $MgSO_4$ and K_3PO_4 have been tested), while showing some differences, resulted in no significant and reproducible increase in diploid formation above about 10^{-5}. These experiments and all previously published crosses in *D. discoideum* parasexual genetics (except for the cross involving diploid DP72, MOSSES, WILLIAMS and NEWELL 1975) involved crosses between strains derived from the NC4 isolate. To establish that the frequency of diploid formation observed previously was not isolate-specific, a quantitative experiment (see MATERIALS AND METHODS) was conducted using strains derived from the NC4 (*matA1*) and V12 (*mata2*) isolates of *D. discoideum*. The frequency of diploid formation, calculated from the number of diploids formed per 10^6 amoebae plated (see legend to Figure 1) was 7.4×10^{-6} for the cross HM3 (*mata2*) × HU89 (*mata2*) and 7.2×10^{-6} for the cross M28 (*matA1*) × TS12 (*matA1*). No diploids were obtained in the cross between strains X9 (*matA1*) and NP158 (*mata2*) of opposite mating type, which was conducted at the same time, although between 35 and 40 diploids would have been expected had the frequency been the same as that found for within mating-type crosses. For the rapid crosses, amoebae were toothpicked from clonal plates and the bacteria were not removed. Results obtained from these crosses were almost identical to those obtained for the quantitative technique, except that incubation in water alone was more variable (see WILLIAMS and NEWELL 1976). Incubation in 20 mM $CaCl_2$ resulted in reliable diploid formation; hence it has been used as the incubation solution for co-aggregation of amoebae in almost all experiments.

Demonstration of vegetative incompatibility: parasexual diploid formation between wild isolates and tester strains of either matA *or* mata *mating type*

In order to test whether there is a series of vegetative incompatibility loci in *D. discoideum* as there is in *N. crassa* (MYLYK 1975), or whether vegetative

incompatibility is associated only with the mating-type locus, crosses were performed between wild isolates of *D. discoideum* and tester strains of each mating type. Since all of the strains (detailed in Table 2) were wild type (not growth temperature sensitive), the conventional method for constructing parasexual diploids in *D. discoideum*, involving complementation between different growth temperature-sensitive mutants (LOOMIS 1969; KATZ and SUSSMAN 1972), was not possible. However, recently we have shown that diploids can be formed between wild-type strains and strains carrying a growth temperature-sensitive mutation plus a dominant mutation whose phenotype is growth in the presence of toxic levels of $CoCl_2$ (WILLIAMS 1978). Temperature-sensitive, $CoCl_2$-resistant strains of both mating types (HU32, *matA1* and HU156 or HU184, *mata2*) were constructed (Table 1). The isolation of HU32 has been described previously (WILLIAMS 1978). HU156 (*cob-364*) is one of three spontaneous $CoCl_2$-resistant strains (HU156, HU157, HU158) isolated at a frequency of 10^{-6} by plating the growth temperature-sensitive *mata2* strain HU89 on SM agar containing 300 μg per ml or 350 μg per ml $CoCl_2 \cdot 6H_2O$. The dominance of the three *cob* mutations was tested by constructing diploids between each cobalt-resistant mutant and strain HM3. The two strains HU157 (*cob-365*) and HU158 (*cob-366*) had *cob* mutations that were recessive to wild type, while *cob-364* (in strain HU156) showed a high degree of dominance to wild type on SM agar containing 300 μg per ml $CoCl_2$. Strain HU184 was isolated as a methanol-resistant (*acrA*) mutant of HU156.

Table 2 shows the results of mating-type tests and success or failure of parasexual diploid formation between the wild isolates and *matA* or *mata* tester strains. Parasexual dipolids were formed at the normal frequency (between 10^{-5} and 10^{-6}) in all crosses between strains of the same mating type, but no diploids were formed between strains of opposite mating type. The 11 isolates shown in Table 2 were isolated in different geographical regions; yet, all were vegetatively compatible with a tester strain of their own mating type. This shows that we have encountered no vegetative-incompatibility locus unrelated to the mating type (see DISCUSSION). All parasexual diploids formed were shown to be heterozygous for several markers; *e.g.*, diploids formed with HU32 were segregated on ethidium bromide (35 μg per ml) SM agar and shown to be heterozygous for *ebrA1* and *bwnA1* (WRIGHT, WILLIAMS and NEWELL 1977). Diploids formed with HU184 were segregated on methanol (2% v/v) SM agar to show heterozygosity for *acrA356*, while *bwnA1* segregants were also detected; diploids formed with HU156 carry no selective markers; they were segregated on benlate (20 μg per ml) SM agar to show heterozygosity for *bwnA1*. It was important to segregate the diploids, as on a few occasions poorly growing isogenic diploids, caused by chromosome doubling or fusion of alike amoebae, were encountered. Why these diploids tolerate the selective conditions better than haploids is not known, but this phenomenon has also been observed in ts/ts crosses within NC4 strains (WILLIAMS unpublished).

Quantitative data on parasexual diploid formation between strains of like and unlike mating type

Most of the experiments described so far involved crosses between wild isolates and derivatives of strains NC4 (*matA1*) and V12 (*mata2*). In no case was a diploid formed in parasexual crosses between strains of opposite mating type. We investigated whether the vegetative incompatibility between strains of opposite mating type could be overcome, and if so at what frequency. Since NC4 and V12 are perhaps the best known isolates of *D. discoideum*, NC4 in particular being the strain in which almost all parasexual genetic analysis has been conducted, semi-quantitative results were collected for the frequency of parasexual diploid formation within and between derivatives of strains NC4 and V12. A series of such experiments, plus the wild-isolate crosses (Table 2), are summarized in Table 3. While some of the crosses in Table 3 were performed quantitatively (see MATERIALS AND METHODS), most of the crosses were done using the rapid technique in which cells were "toothpicked" and therefore not counted (see MATERIALS AND METHODS). For this reason only approximate frequencies of

TABLE 3

Quantitative data on parasexual diploid formation between D. discoideum *strains of like and unlike mating type*

Mating types of strains crossed	Origin of strains crossed*	Total number of amoebae crossed†	Number of diploids isolated	Approximate frequency of parasexual diploid formation¶
matA/matA	NC4/NC4	1.0×10^8	464	4.6×10^{-6}
	NC4–V12/NC4	1.5×10^8	384	2.5×10^{-6}
	Other isolates/NC4	2.0×10^7	41	2.0×10^{-6}
matA/mata	NC4/V12	2.5×10^8	3‡	1.2×10^{-8}
	NC4–V12/V12	1.1×10^8	1§	9.1×10^{-9}
	Other isolates/V12	2.0×10^7	0	$<5 \times 10^{-8}$
mata/matA	NC4–V12/NC4	1.7×10^8	1‖	6.0×10^{-9}
	Other isolates/NC4	6.0×10^6	0	$<10^{-7}$
mata/mata	V12/V12	2.4×10^7	52	2.2×10^{-6}
	NC4–V12/V12	8.0×10^7	186	2.3×10^{-6}
	Other isolates/V12	6.0×10^6	5	8.0×10^{-7}

* All strains used in these crosses were derived from the NC4 (*matA1*) or V12 (*mata2*) isolates, or from the wild isolates (Table 2) referred to as "other isolates". NC4–V12: haploid strains with a mixture of NC4-derived and V12-derived chromosomes.

† Most of the experiments reported here were from rapid crosses in which amoebae were picked up on toothpicks, so that the number of amoebae per cross is only approximate. An average of 5×10^5 amoebae per toothpick was assumed, except in some difficult crosses where more amoebae ($\sim 10^6$ per toothpick) were used.

‡ These are the diploids DP72, DU260 and the haploid HU89, whose parent diploid was lost.

§ Inconclusive result as neither the diploid, nor a haploid segregant of it, was purified.

‖ Diploid DU454.

¶ Number of diploids per 10^6 amoebae plated. The frequency would have been between two and three times greater had haemacytometer counts been used. See legend to Figure 1.

diploid formation can be given. Since Table 3 is complex, the results will be discussed in detail.

Parasexual crosses between haploid strains of the same parental origin and therefore of the same mating type (NC4/NC4 or V12/V12): These experiments represent "standard" crosses in parasexual genetic analysis. The NC4/NC4 experiments reported in Table 3 are the most recent 100 crosses conducted in this laboratory, using a variety of NC4-derived haploids. The reliability of the method is illustrated by the fact that the average number of diploids formed per cross ($\sim 10^6$ amoebae) was 4.6. To examine the frequency of diploid formation in V12/V12 experiments, 12 crosses were performed between the V12-derived strains HM3 and NP84, and a further 12 crosses between the V12-derived strains HM3 and NP86. The frequency of diploid formation, $\sim 2.2 \times 10^{-6}$, was similar to that found for NC4/NC4 crosses, $\sim 4.6 \times 10^{-6}$.

Parasexual crosses between haploid strains derived from NC4 and V12 and therefore of opposite mating type (NC4/V12): In crosses between NC4(*matA1*)-derived and V12(*mata2*)-derived haploids, two diploid strains and one recombinant haploid strain were recovered from $\sim 2.5 \times 10^8$ amoebae crossed. One diploid, DP72, was formed in a cross between NP84 (*mata2*) and X23 (*matA1*) (MOSSES, WILLIAMS and NEWELL 1975); the second diploid, DU260, was formed in a cross between NP158 (*mata2*) and HU1 (*matA1*). In the third case, a diploid from a cross between NP158 (*mata2*) and HU1 (*matA1*) was lost, but a haploid, HU89, containing chromosomes from both parents and the *mata2* mating type (Table 1) was isolated. Diploids DP72 and DU260 were haploidized and attempts were made to recover haploids with all combinations of chromosomes. This could not be done with certainty in these diploids, since DP72 is marked on linkage groups II and IV only, while DU260 has linkage groups I, II, IV and possibly III, marked. However, haploids were segregated that carried both parental and recombinant combinations of marked chromosomes. DU260 was examined in the greatest detail and 100 independently derived haploid segregants were obtained. The 100 segregants fell into eight phenotypic classes on the basis of spore shape, spore color and colony morphology (ROBSON 1978). All haploids in six of these classes gave a strong *matA* mating reaction, while haploids in the two remaining classes exhibited a weak *matA* mating reaction. None of the 100 haploid segregants from DU260 formed macrocysts with a *matA* tester strain; therefore, there was no evidence for the presence of the *mata* mating type. These results suggest that DU260 does not carry both mating-type alleles, but that it is homozygous at the mating-type locus (*matA1/matA1*). Similar analysis of DP72 suggests that this diploid is also homozygous at the mating-type locus, being *mata2/mata2*.

Parasexual crosses involving wild isolates (other isolates/NC4 and other isolates/V12): These are the crosses described earlier (Table 2) in which diploids were obtained between wild isolates and growth temperature-sensitive, cobalt-resistant NC4- or V12-derived haploids at 27° on SM agar containing $CoCl_2$. The frequency of diploid formation in crosses between strains of the same mating type ($\sim 2 \times 10^{-6}$) was similar to that found in the normal crosses described

above. While exhaustive crosses were not done, no diploids were formed in any of the crosses between wild isolates and strains of opposite mating type.

Parasexual crosses between haploid NC4- or V12-derived strains and haploid strains that carry various combinations of NC4 and V12 chromosomes: The crosses between strains of opposite mating type described so far involve those in which the participating strains were of different geographic origin. To examine the effect of genetic background on parasexual diploid formation, haploids with a mixed complement of NC4 and V12 chromosomes were segregated from DP72 and DU260; the haploid HU89 was also used. These mixed haploids were backcrossed with pure NC4- or V12-derived haploids.

Parasexual crosses between strains of the same mating type (NC4-V12 (matA1)*/NC4 or NC4-V12*(mata2)*/V12)*: We made 150 crosses between NC4-derived strains (*matA1*) and 23 independently obtained haploid segregants of DU260 (all *matA1*), which comprised representatives of all eight phenotypic groups. Diploids were obtained at a frequency of $\sim 2.5 \times 10^{-6}$, which is similar to that found in normal NC4/NC4 crosses (Table 3). All 23 haploid segregants of DU260 showed a similar frequency of diploid formation when crossed with the NC4-derived *matA1* strain, HU77. Eighty crosses between V12-derived strains and haploid segregants of DP72 (all *mata2*) or strain HU89(*mata2*) resulted in diploid formation at a frequency of $\sim 2.3 \times 10^{-6}$, which is the same as that found in V12/V12 crosses.

Parasexual crosses between strains of different mating type (NC4-V12 (matA1)*/V12 or NC4-V12*(mata2)*/NC4)*: Haploid strains containing various combinations of NC4 and V12 chromosomes, but all of *matA1* mating type, were segregated from DU260. The 23 independently obtained haploid segregants from DU260 used in the previous section were crossed several times with the V12-derived strain HM3. From $\sim 1.1 \times 10^8$ amoebae crossed, only one diploid was formed; this result is inconclusive since the diploid was lost before its genotype was established. Strain HU89 (*mata2*) and three independently obtained haploid segregants of DP72 (all of *mata2* mating type) were crossed repeatedly to NC4-derived strains. The crosses involved $\sim 1.7 \times 10^8$ amoebae, yet only one diploid, DU454, which was formed in a HU89 × HU227 cross, was obtained. Analysis of DU454 and haploid segregants from this diploid (ROBSON 1978) show that it is heterozygous for all loci examined, involving markers on six linkage groups, except for the mating-type locus which appears to be homozygous *matA1/matA1*.

To summarize the results in Table 3, all parasexual crosses between strains of the same mating type, regardless of the genetic background of the strains, resulted in diploid formation at a frequency of between 10^{-6} and 10^{-5}. Conversely, no diploids containing both mating type alleles were found in parasexual crosses between strains of opposite mating type involving $\sim 5.6 \times 10^8$ amoebae. However, three diploids apparently homozygous at the mating type locus and one recombinant haploid strain were isolated, showing that diploids are obtained from such crosses at a frequency of approximately 10^{-8}, or 10^{-2} to 10^{-3} times the frequency found between strains of the same mating type.

DISCUSSION

Two types of diploid are formed between haploid amoebae of heterothallic strains of *D. discoideum*: sexual diploids and vegetative diploids. Successful sexual fusion occurs only between haploid amoebae of opposite mating type during macrocyst formation, the sexual stage of the life cycle; there is no strong evidence for selfing in heterothallic strains (WALLACE 1977). In *D. discoideum* it is likely, although not definitively shown, that the diploid so formed does not divide mitotically. Instead, the diploid probably proceeds into meiosis (ERDOS, NICKERSON and RAPER 1972). We will show elsewhere (ROBSON and WILLIAMS in preparation) that under appropriate conditions this sexual fusion occurs at high frequency (greater than 1%).

The results presented here show that successful vegetative fusion, leading to relatively stable mitotically dividing diploid amoebae, occurs at a frequency of 10^{-6} to 10^{-5} between all tested strains that are of the same mating type. Since the stability of diploid strains of *D. discoideum* has been a controversial issue in the past, the discussion section of the paper by BRODY and WILLIAMS (1974) should be consulted for our views. A system of vegetative incompatibility has been elucidated here; the phenotype of this effect is failure to form stable, mitotically dividing diploids between strains of opposite mating type. While the mechanism of the vegetative incompatibility in *D. discoideum* will be discussed elsewhere, we have shown here that it has a simple genetic basis. There are three lines of evidence consistent with there being only a single vegetative incompatibility locus, and indicating that this locus is almost certainly closely linked to, or coincident with, the mating-type locus (*mat*).

(1) All 11 independent, sexually competent isolates (Table 2) formed vegetative diploids with tester strains of their own mating type at the expected frequency. While exhaustive crosses were not done, the results are consistent with all isolates being vegetatively incompatible (no diploids formed) with tester strains of opposite mating type.

(2) The relatively high frequency at which vegetative incompatibility can be overcome (10^{-2} to 10^{-3}) suggests a simple genetic basis for the phenomenon. "Escape" from vegetative incompatibility, (*i.e.*, stable diploid formation between strains of opposite mating type) can perhaps be explained by a mitotic recombination event (which occurs at $\sim 10^{-3}$, MOSSES, WILLIAMS and NEWELL 1975) leading to homozygosis for vegetative incompatibility. The mating-type locus is strongly implicated in this phenomenon since the mating-type alleles also became homozygous in three independently isolated "escaped" diploids (DP72, DU260, DU454) despite the fact that these experiments selected only for overcoming vegetative incompatibility.

(3) Segregation of haploids from "escaped" diploids allowed examination of the effect of genetic background on vegetative incompatibility. In every haploid examined, the presence or absence of vegetative incompatibility could be related to the mating-type allele, rather than to the rest of the genotype. For example, haploid strains carrying several chromosomes from a *mata2* strain, but the *matA1* mating-type allele, exhibited vegetative incompatibility with *mata2*

strains, but were compatible (formed vegetative diploids at 10^{-6} to 10^{-5}) with *matA1* strains.

Vegetative incompatibility systems in fungi, plants and animals have been reviewed by ESSER and BLAICH (1973). Vegetative incompatibility has also been observed in the acellular slime molds. This is fundamentally different from the process described here since it involves incompatibility between diploid plasmodia (CARLILE 1973). More relevant are the studies on vegetative incompatibility involving failure of stable heterokaryon formation in the fungi, particularly the Ascomycetes. Among the best studied organisms are Aspergillus species (GRINDLE 1963a,b; KWON and RAPER 1967; CATEN 1971) and *Neurospora crassa* (GARNJOBST and WILSON 1956; MYLYK 1975, 1976). Vegetative incompatibility in *N. crassa* is controlled by at least ten *het* loci (MYLYK 1976). Strains are vegetatively incompatible unless they are homozygous for alleles at all *het* loci. One of these *het* loci is the mating-type locus (GARNJOBST and WILSON 1956; NEWMEYER 1970; GRIFFITHS and DELANGE 1978). The multiple *het* loci in *N. crassa* make analysis of vegetative incompatibility considerably more complex than that described here in *D. discoideum*. For example, whereas all 11 wild isolates of *D. discoideum* were vegetatively compatible with tester strains of their own mating type, only two strains were found to be vegetatively compatible at all five *het* loci studied from tests on 64 pairs of wild *N. crassa* strains of the same mating type (MYLYK 1976). Similar studies on wild isolates of Aspergillus species (GRINDLE 1963a,b; KWON and RAPER 1967; CATEN 1971) show that most wild isolates are vegetatively (heterokaryon) incompatible, because there is a low probability that any two strains will have the same alleles at all of several *het* loci. Why is the vegetative incompatibility system less complex in *D. discoideum* than in the Ascomycetes? In fungal mycelia (*e.g.*, *N. crassa*), extensive hyphal fusion occurs, so that a network of hyphae is formed. This would occur between genetically unlike hyphae in the absence of vegetative incompatibility. It has been suggested that vegetative incompatibility may represent a defense mechanism against cytoplasmic invasion (*e.g.*, by viruses) or exploitation of heterokaryons by nuclei that are nonadaptive in homokaryons (CATEN 1971; HARTL, DEMPSTER and BROWN 1975). Since *D. discoideum* is primarily a uninucleate amoeba, and amoebal fusion is a rare event, elaborate isolating mechanisms seem unnecessary.

Our studies have revealed a barrier to sexual and parasexual genetic analysis in the same strains of *D. discoideum*, since parasexual analysis is feasible only between strains of the same mating type, and sexual analysis is feasible only between strains of opposite mating type. We are currently exploring the possibility of inactivating the vegetative incompatibility function, yet maintaining the mating function. Such a mutation has been found at the mating-type locus in *Neurospora crassa* recently (GRIFFITHS and DELANGE 1978). If this approach is successful, it would be possible to conduct sexual and parasexual genetic analysis in the same strains of *D. discoideum*.

We thank K. B. RAPER and M. WALLACE for the wild isolates of *D. discoideum*, and J. H. TRENT and R. R. KAY for *D. discoideum* strain HM3. PAUL WHITINGTON, DENNIS WELKER,

K. B. RAPER, F. ROTHMAN and R. R. KAY made helpful comments on the manuscript. M. WALLACE kindly sent a pre-print of his manuscript.

LITERATURE CITED

BRODY, T. and K. L. WILLLIAMS, 1974 Cytological analysis of the parasexual cycle in *Dictyostelium discoideum*. J. Gen. Microbiol. 82: 371–383.

CARLILE, M. J., 1973 Cell fusion and somatic incompatibility in Myxomycetes. Ber. Deutsch. Bot. Ges. Bd. **86**: 123–139.

CATEN, C. E., 1971 Heterokaryon incompatibility in imperfect species of *Aspergillus*. Heredity **26**: 299–312.

CLARKE, M. A., D. FRANCIS and R. EISENBERG, 1973 Mating-types in cellular slime molds. Biochem. Biophys. Res. Commun. **52**: 672–678.

ERDOS, G. W., A. W. NICKERSON and K. B. RAPER, 1972 Fine structure of macrocysts in *Polysphondylium violaceum*. Cytobiologie **6**: 351–366.

ERDOS, G. W., K. B. RAPER and L. K. VOGEN, 1973 Mating types and macrocyst formation in *Dictyostelium discoideum*. Proc. Natl. Acad. Sci. U.S. **70**: 1828–1830.

ESSER, K. and R. BLAICH, 1973 Heterogenic incompatibility in plants and animals. Advan. Genet. **17**: 107–152.

FILOSA, M. F. and R. E. DENGLER, 1972 Ultrastructure of macrocyst formation in the cellular slime mold, *Dictyostelium mucoroides*: extensive phagocytosis of amoebae by a specialized cell. Develop. Biol. **29**: 1–16.

FREE, S. J., R. T. SCHIMKE and W. F. LOOMIS, 1976 The structural gene for α-mannosidase-1 in *Dictyostelium discoideum*. Genetics **84**: 159–174.

GARNJOBST, L. and J. F. WILSON, 1956 Heterokaryosis and protoplasmic incompatibility in *Neurospora crassa*. Proc. Natl. Acad. Sci. U.S. **42**: 613–618.

GRINDLE, M., 1963a Heterokaryon compatibility of unrelated strains in the *Aspergillus nidulans* group. Heredity **18**: 191–204. ——, 1963b Heterokaryon compatibility of closely related wild isolates of *Aspergillus nidulans*. Heredity **18**: 397–405.

GRIFFITHS, A. J. F. and A. M. DELANGE, 1978 Mutations of the *a* mating-type gene in *Neurospora crassa*. Genetics **88**: 239–254.

HARTL, D. L., E. R. DEMPSTER and S. W. BROWN, 1975 Adaptive significance of vegetative incompatibility in *Neurospora crassa*. Genetics **81**: 553–569.

JACOBSON, A. and H. F. LODISH, 1975 Genetic control of development of the cellular slime mold *Dictyostelium discoideum*. Ann. Rev. Genet. **9**: 145–185.

KAFER, E., 1977 Meiotic and mitotic recombination in *Aspergillus* and its chromosomal aberrations. Advan. Genet. **19**: 33–131.

KATZ, E. R. and M. SUSSMAN, 1972 Parasexual recombination in *Dictyostelium discoideum*. Selection of stable heterozygotes and stable haploid segregants. Proc. Natl. Acad. Sci. U.S. **69**: 495–498.

KLEIN, J., 1975 Many questions (and almost no answers) about the phylogenetic origin of the major histo-compatibility complex. Advan. Exptl. Med. Biol. **64**: 467–478.

KWON, K. J. and K. B. RAPER, 1967 Heterokaryon formation and genetic analysis of color mutants of *Aspergillus heterothallicus*. Am. J. Botany **54**: 49–60.

LOOMIS, W. F., 1969 Temperature-sensitive mutants of *Dictyostelium discoideum*. J. Bacteriol. **99**: 65–69.

MOSSES, D., K. L. WILLIAMS and P. C. NEWELL, 1975 The use of mitotic crossing-over for genetic analysis in *Dictyostelium discoideum*: mapping of linkage group II. J. Gen. Microbiol. **90**: 247–259.

MYLYK, O. M., 1975 Heterokaryon incompatibility genes in *Neurospora crassa* detected using duplication-producing chromosome rearrangements. Genetics **80**: 107–124. ——, 1976 Heteromorphism for heterokaryon incompatibility genes in natural populations of *Neurospora crassa*. Genetics **83**: 275–284.

NEWELL, P. C., 1978 Genetics of the cellular slime molds. Ann. Rev. Genet. **12**: 69–93.

NEWMEYER, D., 1970 A suppressor of the heterokaryon-incompatibility associated with mating type in *Neurospora crassa*. Can. J. Genet. Cytol. **12**: 914–926.

OKA, H., 1970 Colony specificity in compound Ascidians. The genetic control of fusibility. pp. 196–206. In: *Profiles of Japanese Science and Scientists*. Edited by H. YUKAWA, Kodansha, Tokyo.

PONTECORVO, G. and E. KAFER, 1958 Genetic analysis based on mitotic recombination. Advan. Genet. **9**: 71–104.

RAPER, K. B., 1935 *Dictyostelium discoideum*, a new species of slime mold from decaying forest leaves. J. Agr. Res. **50**: 135–147.

ROBSON, G. E., 1978 *Mating and vegetative incompatibility in the cellular slime mould Dictyostelium discoideum*. M.Sc. thesis, Australian National University, Canberra.

SHERMAN, F. and C. W. LAWRENCE, 1974 Saccharomyces. pp. 359–394. In: *Handbook of Genetics*. Vol. 1, Ch. 24. Edited by R. C. KING. Plenum Press, New York.

SUSSMAN, M., 1966 Biochemical and genetic methods in the study of cellular slime mold development. pp. 397–410. In: *Methods in Cell Physiology*. Vol. 2. Edited by D. PRESCOTT. Academic Press, New York.

WALLACE, M. A., 1977 *Cultural and genetic studies of the macrocysts of Dictyostelium discoideum*. Ph. D. thesis, University of Wisconsin, Madison.

WALLACE, M. A. and K. B. RAPER, 1979 Genetic exchanges in the macrocysts of *Dictyostelium discoideum*. J. Gen. Microbiol. **113**: 327–337.

WILLIAMS, K. L., 1976 Isolation of strains of the cellular slime mold *Dictyostelium discoideum* capable of growing after a single passage in axenic medium. Appl. Envir. Microbiol. **32**: 635–637. ——, 1978 Characterization of dominant resistance to cobalt chloride in *Dictyostelium discoideum* and its use in parasexual genetic analysis. Genetics **90**: 37–47.

WILLIAMS, K. L. and P. BARRAND, 1978 Parasexual genetics in the cellular slime mould *Dictyostelium discoideum*: haploidization of diploid strains using ben late. FEMS Microbiol. Letters **4**: 155–159.

WILLIAMS, K. L., R. H. KESSIN and P. C. NEWELL, 1974a Genetics of growth in axenic medium of the cellular slime mould *Dictyostelium discoideum*. Nature **247**: 142–143. ——, 1974b Parasexual genetics in *Dictyostelium discoideum*: mitotic analysis of acriflavin resistance and growth in axenic medium. J. Gen. Microbiol. **84**: 59–69.

WILLIAMS, K. L. and P. C. NEWELL, 1976 A genetic study of aggregation in the cellular slime mould *Dictyostelium discoideum* using complementation analysis. Genetics **82**: 287–307.

WRIGHT, M. D., K. L. WILLIAMS and P. C. NEWELL, 1977 Ethidium bromide resistance, a selective marker located on linkage group IV of *Dictyostelium discoideum*. J. Gen. Microbiol. **102**: 423–426.

Corresponding editor: D. SCHLESSINGER