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Complementation of *npf* mutations in diploid amoebae of *Physarum polycephalum*: the basis for a general method of complementation analysis at the amoebal stage

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SUMMARY

Haploid amoebae of *Physarum polycephalum* may form plasmodia by crossing, a sexual process that involves cellular and nuclear fusions, or by selfing, an asexual process in which the development of a single amoeba into a plasmodium may involve neither cellular nor nuclear fusion. Mutant strains (*npf*) in which selfing is suppressed were previously assigned to several functional groups on the basis of their ability to cross with one another in certain combinations. In the present study hybrid, diploid amoebae were isolated from both crossing-compatible and incompatible mixtures of *npf* mutants. The diploid amoebae from mixtures of compatible strains readily formed plasmodia by selfing, but selfing was suppressed in the diploids from incompatible mixtures. Thus the crossing tests between *npf* mutants may be viewed as complementation tests: their results reflect the differing selfing abilities of the hybrid, diploid amoebae that formed in each mixture. Genetical and environmental factors affecting the efficiency of formation of diploid amoebae were studied, and the diploids were shown to be stable during repeated subcultures. Although diploid amoebae carrying complementing *npf* mutations readily formed plasmodia by selfing at 26 °C, they could be cultured without plasmodium formation at 30 °C, a temperature that also inhibited selfing of the haploid npf^+ strains. Ways are discussed of exploiting this combination of properties in a general procedure for isolating and testing diploids for dominance and complementation of amoebal mutations in *P. polycephalum*.

1. INTRODUCTION

A focal point of studies on the Myxomycete *Physarum polycephalum* is the differentiation of uninucleate amoebae into giant, multinucleate cells called plasmodia. In the past, a problem with genetical studies on plasmodium formation, and also with studies on some other phenomena in *P. polycephalum*, was that

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diploid or heterokaryotic cells suitable for dominance and complementation testing were available only in the plasmodial phase of the life cycle, whereas the mutations of interest were expressed only in amoebae. This problem was largely overcome when we developed a method for the isolation of diploid amoebae of known genotype (Youngman, Anderson & Holt, 1981). We describe here the first use of such diploids to examine complementation in amoebae, the mutations under test being ones that affect the differentiation of amoebae into plasmodia.

The formation of plasmodia (reviewed by Dee, 1982) normally occurs as part of the sexual cycle. Haploid amoebae fuse in pairs to form diploid amoebae that function as zygotes; the zygotes develop into macroscopic, diploid plasmodia by undergoing successive mitotic cycles in the absence of cell division. This sexual type of plasmodium formation, termed crossing, occurs only in mixtures of unlike amoebae and is regulated by two unlinked, multiallelic mating factors, *matA* and *matB* (Dee, 1966; Youngman *et al*, 1979). We recently presented evidence that *matB* affects the amoebal fusion step of crossing and that *matA* affects the differentiation of the fusion products into plasmodia (Youngman *et al*. 1981). Plasmodia may be cultured indefinitely or may be induced to sporulate. Meiosis occurs during sporulation, and the spores release haploid amoebae upon germination.

Plasmodium formation may also occur in clonal cultures of haploid or diploid amoebae. In this case the process, termed selfing, appears to involve fusion neither of amoebae nor of nuclei. Instead, individual amoebae behave like the zygotes that form during a cross; they develop into multinucleate plasmodia by undergoing successive mitoses in the absence of cell divisions (Anderson, Cooke & Dee, 1976; Collett, Holt & Huttermann, 1983). Consequently the plasmodia that result from selfing are of the same ploidy as the amoebae from which they are derived. Clonal cultures of most haploid strains give rise to plasmodia only rarely, the frequency of selfing being typically less than one plasmodium formed per 10^8 amoebae, but strains of certain genotypes may undergo selfing much more often. For example, favourable conditions will permit amoebae carrying an exceptional allele of *matA*, known as *matAh*, to self at a frequency of about 10% (Youngman *et al*. 1977), and mutants of the class *gad* (= greater asexual differentiation) show selfing phenotypes similar to that of *matAh* strains (Adler & Holt, 1977). Most of the *gad* mutations map close to *matA*, and there is some evidence that the *matAh* allele may have arisen as a result of a *gad* mutation occurring in a *matA2* strain, less than 0·13 cM from the genetic region defining *matA* specificity (Anderson, 1979; Shinnick, Anderson & Holt, 1983).

Haploid amoebae lacking the ability to self at high frequency are readily obtained as mutants of *matAh* or *gad* strains. Several studies have been reported in which such *npf* (= no plasmodium formation) mutants were assigned to different functional groups on the basis of tests of their ability to form plasmodia when mixed with one another (Wheals, 1973; Anderson & Dee, 1977; Davidow & Holt, 1977; Anderson, 1979; Honey, Poulter & Teale, 1979; Anderson & Holt, 1981; Honey, Poulter & Aston, 1982). For example, *npf* mutants of *matAh* strains formed four groups, designated *npfA*, *npfB*, *npfC* and *npfF* (formerly *aptA*). Crossing occurred to give diploid, hybrid plasmodia when representatives of any two groups

were mixed together, but no hybrid plasmodia were formed in mixtures containing amoebae from only one group. Similar *npf* groups have been found among *gad*-derived mutants.

The *npf* mutants were isolated as a consequence of their inability to self but were classified, somewhat paradoxically, according to their ability to cross with one another. If it could be shown that amoebal fusions occurred to generate diploid

Table 1. *Alternative gene symbols*

Symbols used in this paper		Alternative symbols	
matA	(Youngman *et al.* 1979)	*mt*	(Dee, 1966)
matB	(Youngman *et al.* 1979)	*rac*	(Dee, 1978)
gad	(Adler & Holt, 1977)	*cat*	(Gorman, Dove & Shaibe, 1979)
		het	(Honey, Poulter & Winter, 1981)
npfB	(Anderson & Dee, 1977)	*aptB*	(Davidow & Holt, 1977)
		difB	(Honey *et al.* 1979)
npfC	(Anderson & Dee, 1977)	*aptC*	(Davidow & Holt, 1977)
		difA	(Honey *et al.* 1979)
npfF	(Shinnick *et al.* 1983)	*apt-1*	(Wheals, 1973)
		aptA	(Davidow & Holt, 1977)
fusA	(Adler & Holt, 1974)	*f*	(Poulter & Dee, 1968)

amoebae (zygotes) in all the mixtures of *npf* mutants, it would be possible to view the crossing tests simply as tests of diploid amoebae for complementation between mutations affecting selfing. Nevertheless, in the absence of any evidence that hybrid amoebae had been formed in the mixtures that failed to produce hybrid plasmodia, the early studies could not exclude the possibility that some or all of the *npf* mutations might also affect cellular or nuclear fusions between amoebae, and the interpretation of the results of the crossing tests was thus problematic (Anderson & Dee, 1977). To clarify the significance of the groups defined in the crossing tests, we now present evidence concerning the formation and behaviour of diploid amoebae carrying various combinations of *npf* mutations.

2. MATERIALS AND METHODS

(i) *Loci*

Several loci in *P. polycephalum* have been given more than one name. We suggest that the gene symbols shown below should be used in preference to the alternative designations listed in Table 1.

matA: mating-type locus affecting zygote differentiation (Dee, 1966; Youngman *et al.* 1979, 1981). There are at least thirteen *matA* alleles (Collins & Tang, 1977).

matB: mating-type locus affecting zygote formation (Dee, 1978; Youngman *et al.* 1979; 1981). There are at least thirteen *matB* alleles (Kirouac-Brunet, Masson & Pallotta, 1981).

gad: greater asexual differentiation (Adler & Holt, 1977). Mutant alleles promote selfing, resulting in the formation of haploid plasmodia. The spores of such plasmodia germinate to yield *gad* amoebal progeny. Several loci are known but most mutations, including *gad-5*, map at or near *matA* (Shinnick *et al.* 1983).

npf: no plasmodium formation (Anderson & Dee, 1977). Functional groups are *npfA*, *npfB*, *npfC* (Anderson & Dee, 1977), *npfD*, *npfE* (Anderson & Holt, 1981) and *npfF* (Wheals, 1973; Shinnick *et al.* 1983). Mutations of the *npfB* and *npfC* groups are always associated with *matAh* or *matA2*, while *npfD* and *npfE* are associated with *matA3* (Anderson & Dee, 1977; Anderson & Holt, 1981).

fusA, *fusC:* plasmodial fusion loci (Poulter & Dee, 1968; Adler & Holt, 1974). Alleles *fusA1* and *fusA2* are codominant; *fusC2* is dominant to *fusC1*. Plasmodia of identical fusion phenotype fuse on contact but non-identical plasmodia do not fuse.

imz: ionic modulation of zygote formation (Shinnick *et al.* 1978). Alleles *imz-1* and *imz-2*. The efficiency of crossing between amoebal strains tends to be lower in *imz-1* × *imz-1* mixtures than in *imz-1* × *imz-2* or *imz-2* × *imz-2* mixtures, particularly under conditions of high pH or low ionic strength.

(ii) *Strains and culture methods*

All strains were largely isogenic with the Colonia isolate (Adler & Holt, 1974; Cooke & Dee, 1975). Principal strains are listed in Tables 2 and 3. Amoebae and plasmodia were cultured as described by Anderson (1979).

(iii) *Isolation of diploid amoebae*

Diploids were isolated essentially as described by Youngman *et al* (1981). Equal numbers of amoebae of two strains were mixed in a concentrated suspension of *Escherichia coli*. The mixed amoebal suspension was used to inoculate 0·1 ml 'mating spot' cultures on non-nutrient agar plates containing 3 mM sodium citrate pH 5 and 10 mM-$MgSO_4$. The cultures were incubated at 30 °C and harvested when the amoebal density reached approximately 10^6 per culture, after 65–72 h. The harvested amoebae were diluted in water and replated at 26 °C with bacteria to give 30–50 well-separated colonies per plate. For selfing diploids, replating was on 1·5% agar containing 3 mM sodium citrate pH 5 and 1 g/l Oxoid liver infusion powder (LIA5). The plates were screened for selfing colonies after 6–7 days. For non-selfing diploids, replating was carried out on agar containing only liver infusion (LIA).

(iv) *Progeny analyses*

OD103: The presence of the *npfA1* and *matAh* alleles in all amoebal progeny of the putative diploid OD103 was confirmed by showing that all the progeny were able to cross with npf^+ *matA2* testers at 26 °C, but not with *npfA1 matAh* testers (Anderson & Dee, 1977; Anderson, 1979). Scoring of *matB* genotypes was carried out by visually assessing the rate and extent of plasmodium formation in mixtures with tester strains of the two *matB* types concerned (Youngman *et al.* 1979). Plasmodia formed in these crosses were tested for their ability to fuse with plasmodia of known *fusA* genotypes; since the *fusA* genotypes of the tester amoebae were known, the genotypes of the progeny could be deduced (Poulter & Dee, 1968; Cooke & Dee, 1975). Progeny were classified for *imz* alleles by testing their ability to cross with a *matA1 matB3 imz-1* tester strain at elevated pH (Shinnick *et al.* 1978).

Table 2. *Haploid strains*

Strains	Relevant genotypes							Reference or origin
APT1	*npfF1*	*matAh*	*matB1*	*imz-1*	*fusA2*	*fusC1*		Wheals, 1973
CH207	*matA4*	*matB2*	*imz-1*	*fusA2*	*fusC2*			Anderson & Dee, 1977
CH242	*matA3*	*matB3*	*imz-2*	*fusA2*	*fusC2*			Adler, 1975
CH361	*npfB361*	*matAh*	*matB1*	*imz-1*	*fusA2*	*fusC1*		Davidow & Holt, 1977
CH495	*gad-11*	*matA3*	*matB3*	*imz-2*	*fusA2*	*fusC2*		Shinnick & Holt, 1977
CH508	*matA2*	*matB3*	*imz-2*	*fusA2*	*fusC2*			Youngman *et al.* 1979
CH771	*npfA1*	*matAh*	*matB2*	*fusA1*	*fusC2*			CH207 × LU906
CH810	*matA2*	*matB2*	*imz-1*	*fusA1*	*fusC1*			Anderson, 1979
CH818	*npfF1*	*matAh*	*matB2*	*imz-1*	*fusA1*	*fusC1*		Anderson, 1979
CH821	*npfA1*	*matAh*	*matB2*	*imz-1*	*fusA2*	*fusC1*		Anderson & Holt, 1981
CH822	*npfB4*	*matAh*	*matB2*	*imz-1*	*fusA1*	*fusC1*		Anderson, 1979
CH825	*npfC3*	*matAh*	*matB2*	*imz-1*	*fusA2*	*fusC1*		Anderson, 1979
CH894	*npfE894*	*gad-5*	*matA3*	*matB3*	*imz-1*	*fusA2*	*fusC2*	Anderson & Holt, 1981
CH925	*matA1*	*matB2*	*imz-1*	*fusA1*	*fusC1*			CH810 × LU897
CH944	*npfE894*	*gad-5*	*matA3*	*matB1*	*imz-1*	*fusA1*	*fusC1*	CH894 × LU913
CH954	*npfC3*	*matAh*	*matB1*	*imz-2*	*fusA2*	*fusC1*		CH508 × LU881
CH955	*npfC3*	*matAh*	*matB3*	*imz-2*	*fusA1*	*fusC1*		CH508 × LU881
CH958	*npfB4*	*matAh*	*matB3*	*imz-2*	*fusA1*	*fusC1*		CH242 × CH822
CH966	*npfF1*	*matAh*	*matB3*	*fusA2*				APT1 × CH495
CL6129	*npfB4*	*matAh*	*matB1*	*imz-1*	*fusA2*	*fusC1*		Anderson & Dee, 1977
CL6143	*npfC3*	*matAh*	*matB1*	*imz-1*	*fusA2*	*fusC1*		Anderson & Dee, 1977
LU867	*npfA1*	*matAh*	*matB1*	*imz-1*	*fusA1*	*fusC1*		Anderson & Dee, 1977
LU874	*npfB4*	*matAh*	*matB1*	*imz-1*	*fusA1*	*fusC1*		Anderson, 1976
LU881	*npfC3*	*matAh*	*matB1*	*imz-1*	*fusA1*	*fusC1*		Anderson, 1976
LU896	*matA1*	*matB1*	*imz-1*	*fusA2*	*fusC1*			Anderson, 1977
LU897	*matA1*	*matB1*	*imz-1*	*fusA2*	*fusC1*			Anderson & Truitt, 1983
LU906	*npfA1*	*matAh*	*matB1*	*fusA1*	*fusC1*			LU867 × LU896
LU913	*matA3*	*matB1*	*fusA1*	*fusC1*				Anderson & Holt, 1981
OX104	*npfA1*	*matAh*	*matB1*	*imz-2*	*fusA1*	*fusC1*		CH508 × LU867
OX109	*npfF1*	*matAh*	*matB3*	*imz-2*	*fusA1*	*fusC1*		CH508 × CH818

OD106 × *CH925*: All progeny forming large, clearly-defined colonies were shown by the following behaviour (see Anderson, 1979) to carry the *npfF1* and *matAh* alleles: all crossed with *npfF*$^+$ *matA1* testers at 30 °C, and with *npfF*$^+$ *matA2* testers at 26 °C, but did not cross with *npfF*$^+$ *matA2* testers at 30 °C or with *npfF1 matAh* testers at 26 °C. Alleles of *matB* and *fusA* were scored as described for OD103 progeny.

Table 3. *Diploid strains*

Strains	Origin
CH963	CH771/CH966
CH965	CH361/CH966
OD103	CH821/OX104
OD104	CH821/OX104
OD106	APT1/OX109

Selfing diploids: Progeny selfing at high frequency were classified as recombinants carrying only wild-type *npf* alleles (see Anderson, 1979). Non-selfing progeny were scored for *npf* alleles by testing their ability to cross at 26 °C with testers carrying the relevant *npf* mutations (Anderson, 1979; Anderson & Holt, 1981). Alleles of *matB* were scored as described for OD103.

3. RESULTS

(i) *Isolation of diploid amoebae from crossing-incompatible mixtures of* npf *mutants*

If the tests of crossing between *npf* mutants are to be viewed simply as complementation tests of selfing mutations, it is necessary to know that amoebal fusions generated diploid amoebae even in those mixtures that did not give rise to plasmodia. We therefore investigated the formation of diploid amoebae in mixed cultures in which both strains belonged to the same *npf* class. The classes tested were: *npfA*, *npfB*, *npfC*, *npfE* and *npfF*. In each mixture the haploid amoebal strains both carried the same *npf* mutation, but differed in their alleles at other loci. Since heteroallelism for *matB* was known to favour amoebal fusions (Youngman *et al.* 1981), all the mixtures were made heteroallelic for *matB*. The *matB* locus was also exploited during the identification of diploids (see below). One strain of each pair usually carried the *imz-2* allele, which is known to promote efficient crossing under sub-optimal conditions of pH and ionic strength (Shinnick *et al.* 1978). As an aid to the subsequent genetic analysis of diploids (see below), all the mixtures were made heteroallelic for *fusA*, a locus that affects fusion of plasmodia.

The haploid strains were mixed, incubated at 30 °C to permit fusions to occur, and then harvested when the cultures reached approximately 10^6 cells. Harvested amoebae were replated at low density and allowed to proliferate to give well-separated colonies, which were recloned once. In order to identify those clones which were diploid, cells from each were mixed with two *matA*-compatible tester strains, each of which carried one of the parental *matB* alleles. Our earlier study (Youngman *et al.* 1981) had led us to expect that *matB*-heterozygous diploids would yield only a few plasmodia in crosses with either tester, while haploids would give many plasmodia in the *matB*-heteroallelic cross, and few in the homoallelic one.

The results of all five diploid searches are summarized in Table 4. Clones showing the crossing behaviour expected of diploids were identified in all the searches, and all the putative diploids shared a non-selfing phenotype. It therefore appeared that crossing in these incompatible mixtures was blocked during the development of diploid amoebae into plasmodia, rather than during the amoebal fusion step. The

Table 4. *Isolation of non-selfing, putative diploid amoebae*

Genotype	Strains A	Strains B	Deduced origin of screened clones* Strain A	Strain B	Hybrid	% putative diploids
npfA1 matAh	CH821	OX104	78	20	2	2
npfB4 matAh	CH958	CL6129	16	97	7	6
npfC3 matAh	CH955	CL6143	79	30	11	9
npfE894 gad-5 matA3	CH894	CH944	21	13	3	8
npfF1 matAh	APT1	OX109	93	6	1	1

* Deduced from *matB* phenotypes.

frequency of recovery of diploid clones was quite variable, in the range 1–9 % of clones tested. Since the experiments were performed over a period of months, in two separate institutions, differences in the rate of recovery of diploids from different mixtures in Table 4 are not regarded as significant. In earlier work (Youngman *et al.* 1981; Youngman, 1979), also using mixtures heteroallelic for *matB* and *imz*, we found 5–10 % of tested clones to be diploid.

Apart from the crossing behaviour that initially permitted their identification, the putative diploids showed other phenotypic similarities to the confirmed diploids which had been isolated previously. For example, colonies of all strains enlarged more slowly than colonies of the haploids from which they were formed, and microscopic observation of amoebae on agar-coated slides showed that all the putative diploids were uninucleate and noticeably larger than haploid cells. For the *npfA* and *npfF* mixtures, we measured the sizes of haploid and putative diploid cells. The amoebae were first induced by starvation to become resistant cysts, in which form they were roughly spherical. They were then mounted in water, and their diameters measured at a magnification of 400 ×, using a phase-contrast microscope equipped with a micrometer eyepiece. The three putative diploids were found to have volumes roughly twice those of the corresponding haploids (see Fig. 1).

Confirmation that the putative diploid clones had been correctly classified was obtained from genetic analyses. In crosses with tester amoebae of known *fusA* genotype, all the putative diploid clones gave rise to plasmodia of hybrid, *fusA1/fusA2* behaviour, whether the crosses were with *fusA1* or *fusA2* testers. This result demonstrates the presence of both *fusA* alleles in all the diploid strains (Youngman *et al.* 1981). Two representative clones were subjected to more detailed analysis. OD103, a putative *npfA1/npfA1* diploid, was allowed to self at 21 °C, a temperature at which *npfA1* haploid clones self readily. The OD103 plasmodium

was induced to sporulate and, following germination of the spores, 30 amoebal progeny clones were obtained. Colonies of all the progeny strains enlarged more rapidly than colonies of the parental OD103 amoebae, at a rate typical of haploids. Each clone was tested (see Materials and Methods), and all were found to carry the alleles *npfA1* and *matAh*. Segregation of alleles of *matB*, *fusA* and *imz* did not

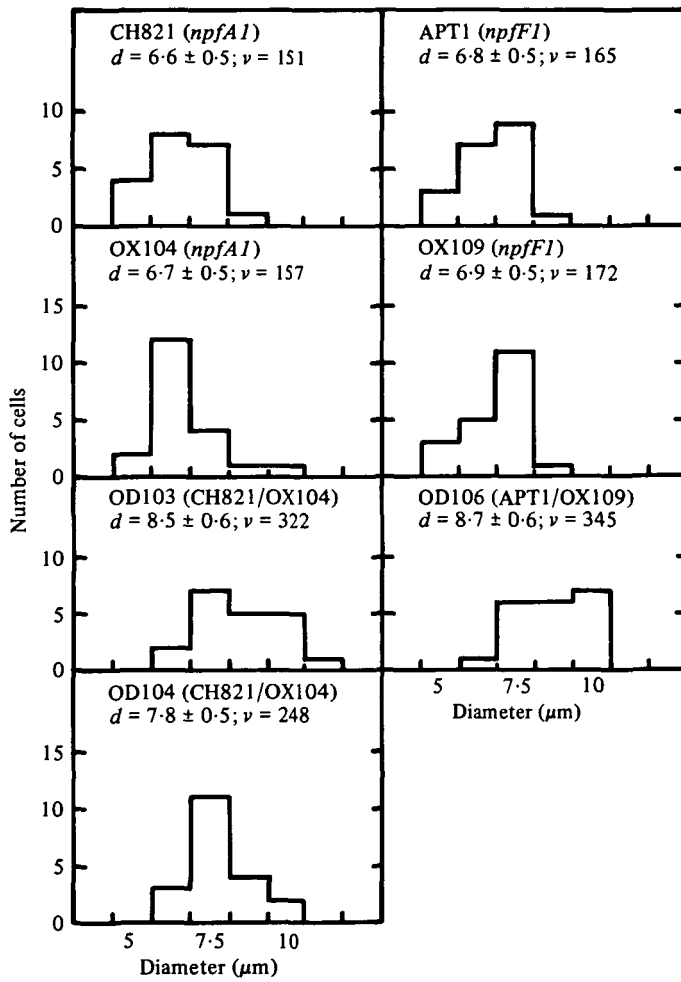

Fig. 1. Diameters of haploid and putative diploid, encysted amoebae. d = mean diameter (μm) $\pm$ 95% confidence limits; v = volume (μm^3) = $\pi d^3/6$.

differ significantly from 1 : 1 ($P > 0{\cdot}05$) and there was no significant deviation from free recombination between these three loci ($P > 0{\cdot}05$). Thus the results (see Table 5*a*) confirm the expected genotype of OD103.

Since the recessive *npfF1* allele blocks plasmodium formation at all temperatures, it was not possible to self OD106, the putative *npfF1/npfF1* diploid. Instead, a plasmodium was formed by crossing OD106 with an *npfF*$^{+}$ *matA1* haploid strain, CH925. We previously found (Youngman *et al.* 1981) that viable progeny of a

diploid × haploid cross formed colonies of two types. Many of the colonies were small and diffuse and were presumably composed of aneuploid cells derived from meiosis in triploid nuclei. Other colonies were large and clearly defined; these proved to be mainly the products of meiosis in unfused nuclei of the diploid parent. Thus, to obtain meiotic products of the putative diploid OD106, we searched for

Table 5. *Analysis of progeny of representative diploids*

Deduced genotypes*	Numbers
(*a*) Progeny of OD103 (*npfA1 matAh matB1 imz-2 fusA1/npfA1 matAh matB2 imz-1 fusA2*):	
npfA1 matAh matB1 imz-1 fusA1	2
npfA1 matAh matB1 imz-1 fusA2	4
npfA1 matAh matB1 imz-2 fusA1	4
npfA1 matAh matB1 imz-2 fusA2	3
npfA1 matAh matB2 imz-1 fusA1	5
npfA1 matAh matB2 imz-1 fusA2	4
npfA1 matAh matB2 imz-2 fusA1	5
npfA1 matAh matB2 imz-2 fusA2	3
Total	30

Deduced genotypes*	Numbers
(*b*) Progeny of OD106 × CH925 (*npfF1 matAh matB1 fusA2/npfF1 matAh matB3 fusA1* × *npf*$^{+}$ *matA1*):	
npfF1 matAh matB1 fusA1	6
npfF1 matAh matB1 fusA2	3
npfF1 matAh matB3 fusA1	4
npfF1 matAh matB3 fusA2	3
npf^{+} *matAh*	0
matA1	0
Total	16

Deduced genotypes*	Numbers
(*c*) Progeny of CH963 (*npfA1 npfF*$^{+}$ *matAh matB2/npfA*$^{+}$ *npfF1 matAh matB3*):	
npfA^{+} *npfF*$^{+}$ *matAh*	10
npfA^{+} *npfF1 matAh matB2*	6
npfA^{+} *npfF1 matAh matB3*	3
npfA1 npfF^{+} *matAh matB2*	4
npfA1 npfF^{+} *matAh matB3*	7
npfA1 npfF1 matAh	7
Total	37

* See Materials and Methods.

large, clearly-defined colonies amongst the progeny of the cross OD106 × CH925. Sixteen suitable clones were identified and tested, as shown in Table 5*b*. All sixteen were of the genotype *npfF1 matAh*, indicating that they were indeed derived from nuclei of the putative diploid. Alleles of *matB* and *fusA* segregated 1:1 and independently ($P > 0{\cdot}05$), behaviour that is consistent with the expected diploid genotype of OD106.

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(ii) *Isolation of diploid amoebae from crossing-compatible mixtures of* npf *amoebae*

If crossing between a pair of *matAh*-derived *npf* mutants simply reflects complementation between their selfing defects, the behaviour of the zygotes formed in such a cross should be predictable: their selfing phenotype should resemble that of *npf*$^+$ *matAh* haploid amoebae. The known effect of high temperature (30 °C) on these crosses lends some support to the prediction, since unlike crossing in *matA*-heteroallelic mixtures but like selfing of *npf*$^+$ *matAh* haploids, crossing between *npf matAh* strains is strongly inhibited at high temperature (Anderson, 1979). In order to permit more detailed study of the zygotes formed in crosses between *npf* mutants, we isolated diploid amoebae from mixtures representing all six inter-group combinations of *npfA*, *npfB*, *npfC* and *npfF* in *matAh* strains. We also isolated diploids from an *npfD*/*npfE* mixture containing mutants derived from a *gad-5* strain. Crossing in this mixture, like selfing in *npf*$^+$ *gad-5* strains, is inhibited at 30 °C, although the inhibition is less than in *matAh* mixtures.

The strains were incubated at 30 °C in mixtures heteroallelic for *matB* and, in some cases, *imz*. Since amoebal fusions are not blocked at 30 °C, it was expected that zygotes would form in the mixtures but that the high temperature would inhibit development of the zygotes into plasmodia. The cultures were harvested as before and replated at 26 °C to give well-separated colonies on LIA5 plates. These conditions favour selfing of *npf*$^+$ matAh and *npf*$^+$ *gad-5* amoebae, and putative diploid colonies were therefore identified as those in which plasmodia formed. Putative diploid clones were identified from all the crossing-compatible mixtures of *npf* mutants, at frequencies in the same range as found for crossing-incompatible mixtures (data not shown).

When plasmodia first became visible in each selfing colony, a large number of undifferentiated amoebae were also still present, and samples of these putative diploid amoebae were easily removed with toothpicks. The amoebae showed little tendency to form plasmodia when cultured on LIA at 30 °C, but consistently gave rise to plasmodia on LIA5 at 26 °C. Colonies of all the putative diploid amoebae enlarged more slowly than haploid colonies. Genetic analysis was carried out on putative diploids representing all the *npf* combinations by permitting the amoebae to self at 26 °C and then testing amoebal progeny of the plasmodia for *matB* and *npf* alleles (see Table 5*c* for example). In every case the progeny analysis confirmed the expected, heterozygous constitution of the parental clone.

The diameter of amoebal colonies at the time when plasmodia first appear is a roughly reproducible measure of the selfing capacity of a selfing strain (Shinnick & Holt, 1977). In spite of their slower growth rates, the selfing diploids all gave rise to plasmodia at approximately the same diameters as the corresponding *npf*$^+$ haploids, within the range 2–4 mm at 26 °C. More detailed information on selfing behaviour was obtained by applying the method of 'kinetics analysis' (Youngman *et al.* 1977) to CH965, an *npfB matAh*/*npfF matAh* diploid strain. Replicate cultures were inoculated at 0 h and harvested at intervals. The harvested cultures were replated under conditions in which cells committed to plasmodium formation would form 'assay plasmodia'. Fig. 2 shows that the doubling time of CH965 amoebae was about 10 h, compared with roughly 8 h for haploid strains. Despite

the slow growth shown by CH965, the 'assay plasmodia' curve in Fig. 2 is of the characteristic form shown by haploid *matAh* and *gad* strains (see Youngman *et al.* 1977).

(iii) *Factors affecting the frequency of recovery of diploids*

A number of genetic and environmental factors are known to affect the frequency of plasmodium formation in 'normal' crosses, that is crosses between amoebae that differ in their *matA* alleles. We tested the effects of three of these

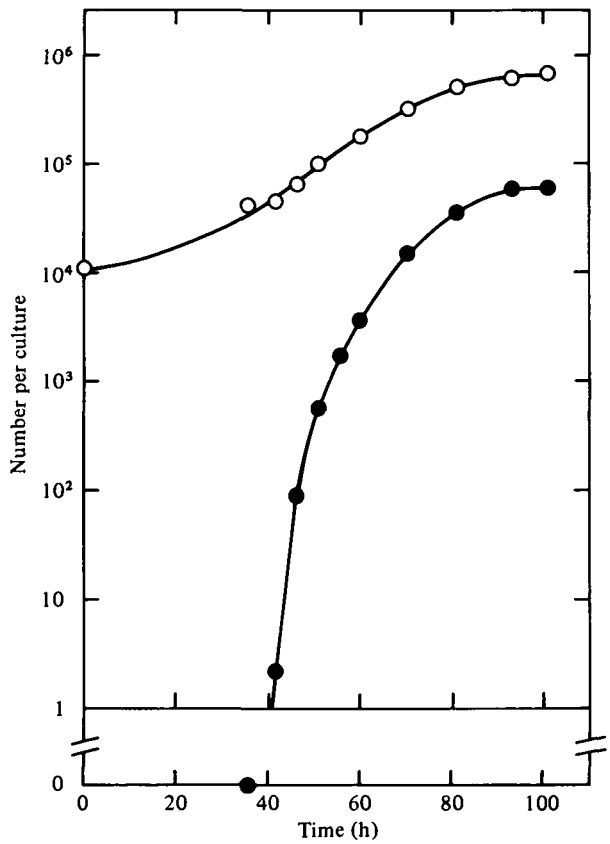

Fig. 2. Time course of plasmodium formation in CH965 (*npfB361 npfF*$^+$ *matAh*/*npfB*$^+$ *npfF1 matAh*). ○, amoebae; ●, cells committed to plasmodium formation.

factors upon the frequency of recovery of diploid amoebae from mixtures of *npf* mutants. The diploids were ones in which the *npf* mutations complement, since the ability of these diploids to self makes them extremely easy to recognise on the screening plates. Table 6 shows the results of three experiments which were carried out to test the effects of *matB* and *imz* under the conditions used for previous diploid isolations. The experiments were all carried out at the same time, in the same laboratory, and with the same pair of complementing *npf* mutations (*npfB4* and *npfC3*). Comparison of the results for mixtures A and B in Table 6 reveals a

marked effect of *matB*: the frequency of recovery of selfing colonies was 100× higher in the heteroallelic mixture than in the homoallelic one. In contrast, the results for A and C suggest that the presence of the *imz-2* allele had little or no effect under these cultural conditions, which are close to the optimum pH and ionic strength for 'normal' crosses (Shinnick *et al.* 1978). In other experiments, not

Table 6. *Effects of* matB *and* imz *alleles on diploid recovery*

Mixtures	Genotypes	Strains	Numbers of clones screened	Numbers of putative diploids	% putative diploids
A	*matB1 imz-1* / *matB3 imz-2*	CL6129 / CH955	720	36	5·0
B	*matB1 imz-1* / *matB1 imz-2*	LU874 / CH954	27000	17	0·06
C	*matB1 imz-1* / *matB2 imz-1*	LU874 / CH825	2000	41	2·1

shown, mixtures similar to A and C in Table 6 were incubated in the absence of $MgSO_4$, to test the effect of *imz* under non-optimal cultural conditions. In these experiments, *imz-1* × *imz-2* mixtures still yielded approximately 5 % selfing colonies, but the frequency of selfing colonies in *imz-1* × *imz-1* mixtures was reduced to 0·1–0·3 %. The *matB* and *imz* genotypes of the diploids appeared to have no effect upon their selfing phenotypes.

(iv) *Stability of diploid amoebae*

Many of the diploid amoebae were subcultured several times in the course of their analysis, in some cases over a period of months, during which time their growth and selfing phenotypes remained stable. It seemed possible, however, that some degree of mitotic instability might exist, and we sought evidence of such instability by looking closely for non-selfing segregants among diploid, selfing amoebae. Non-selfing segregants, if they arose, might be the results of mitotic crossovers or of chromosome loss: mitotic crossing over between an *npf* gene and its centromere might generate diploid segregants that were homozygous for an *npf* mutation; and haploid or aneuploid, non-selfing segregants might be generated following the loss of a chromosome carrying an npf^+ allele. We searched for rare, non-selfing segregants by subjecting selfing diploids to an enrichment procedure that was originally developed for the isolation of *npf* mutant amoebae from mixtures containing a large excess of npf^+ *matAh* amoebae (Davidow & Holt, 1977). The enrichment is achieved by permitting extensive plasmodium formation to occur in the mixtures, so that conversion of the npf^+ amoebae into plasmodia effectively removes them from the cultures. Undifferentiated amoebae that remain are replated to give well-separated colonies, which are readily scored for the presence or absence of selfing. In the original *npf* mutant isolations, the frequency of *npf* amoebae was increased by a factor of approximately 10^3 during the course of enrichment. We applied the enrichment procedure to two selfing diploids. These were CH963 (*npfA1* $npfF^+$ *matAh*/$npfA^+$ *npfF1 matAh*) and CH965 (*npfB361*

npfF$^+$ *matAh*/*npfB*$^+$ *npfF1 matAh*). Following enrichment, we failed to detect any non-selfing segregants among approximately 1500 colonies of each diploid. This result must be interpreted with some caution, since we cannot satisfactorily estimate the enrichment factors that might apply to non-selfing, mitotic segregants of the various possible types. Nevertheless, we conclude that diploid amoebae are sufficiently stable to permit their use in routine genetic analyses, such as dominance and complementation testing.

4. DISCUSSION

In this paper we have presented strong evidence that the results of crossing tests between *npf* mutants simply reflect the ability or inability of particular selfing defects to complement one another in diploid amoebae. We have shown not only that amoebal fusions occur readily in the crossing-incompatible mixtures we have tested, but also that the selfing behaviour of diploid amoebae from the compatible mixtures closely resembles that of haploid *npf*$^+$ *matAh* or *npf*$^+$ *gad-5* amoebae. It remains possible, of course, that *npf* mutations which block amoebal fusions will be found to exist, and it will therefore be necessary to test each new *npf* group as it is discovered.

Although it now emerges that *npf* crosses depend upon complementation between selfing defects, the nature of the intracellular interactions remains obscure. An intriguing puzzle is posed by the clustering of *matA* and most of the *gad* and *npf* mutations in a single locus. Perhaps these mutations define a group of genes with related functions. It has also been suggested, however, that the clustered mutations might bring about a variety of changes in the structure and function of a single, *matA* gene product. For more detailed information and discussion of these ideas, see Anderson & Holt (1981), Dee (1982) and Sauer (1982).

Apart from its contribution to our understanding of plasmodium formation, this work will be of immediate interest to all those geneticists who wish to carry out dominance and complementation tests of almost any type of mutation that is expressed in *P. polycephalum* amoebae. Although such tests have been possible, in principle, since we first constructed diploid amoebae of known genotype (Youngman *et al.* 1981), the screening method employed in the original procedure was both difficult and laborious, being the same as that used here to isolate non-selfing diploids. We suggest that selfing diploids containing complementary *npf* mutations represent a much more realistic choice of material for dominance and complementation tests. The ability of these strains to self at 26 °C makes their identification extremely simple, and raises the possibility of testing complementation in quite large sets of mutants. Maintenance and phenotypic testing of the diploids will normally be carried out at 30 °C, since incubation at this temperature effectively blocks selfing. Nevertheless, testing at lower temperatures will often be possible, because plasmodium formation in the selfing amoebae is always preceded by a period of amoebal proliferation. Some types of mutation will remain difficult to test in diploid amoebae. For example, mutations that prevent amoebal growth at 30 °C may not be readily incorporated into selfing diploids. Probably the most difficult challenge, however, would be presented by the discovery of mutations that

interfered with fusion itself. Selective techniques now being investigated might prove powerful enough to recover diploids even from mixtures in which fusion was an extremely rare event.

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