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Map and function of *gad* mutations in *Physarum polycephalum**

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SUMMARY

Amoebae and plasmodia are alternate vegetative forms in the life cycle of the acellular slime mould *Physarum polycephalum*. Haploid amoebae carrying heterothallic alleles of the *matA* (or *mt*) locus ordinarily form plasmodia only by crossing, but occasionally give rise to mutants that form plasmodia by selfing as well as by crossing. Twelve independently isolated mutants of this type have been studied. Eight carry mutations (termed *gad* or greater asexual differentiation mutations) within approximately 0·2 map units of *matA*. Another mutation (*gad-12*) is linked neither to *matA* nor to any of 9 other markers tested. The remaining three mutations are linked to *matA* and map as follows: *matA*–0·5 units – *gad-4*–4 units – *gad-6* – 8 units – *gad-11*. One mutation, *gad-11*, has been tested in strains carrying each of the five *matA* alleles (*matA1*, *2*, *3*, *4*, and *h*) available in a common genetic background; the mutation is expressed with all five alleles. The mutation *npfF1* (formerly *aptA1*), which was isolated as a suppressor of selfing in Colonia (*matAh*) amoebae, suppresses the action of each of the 12 *gad* mutations. The similarly isolated mutation *npfA1* is also epistatic to eight of the mutations, but permits selfing with *gad-5*, *6*, *12* and *13*. For double mutant strains containing *gad-12* and *gad-1*, *2*, *4*, *6* or *11*, the selfing behaviour of each double mutant differs from that of either single mutant. Mixtures of gad^- *npfF1* with gad^+ npf^+ amoebae readily form plasmodia, a result suggesting that *gad* mutations are dominant or semi-dominant. We conclude that the commitment of a cell to differentiate into a plasmodium is under the control of a complex group of genes linked to *matA*.

1. INTRODUCTION

The life cycle of the acellular slime mould *Physarum polycephalum* includes two distinct vegetative forms, the uninucleate amoeba and the multinucleate plasmodium (Gray & Alexopoulos, 1968). Amoebae are ordinarily haploid. They form plasmodia by crossing, which involves pairwise cell fusion and doubling of ploidy (Mohberg & Rusch, 1971), or by selfing, which involves neither (Cooke &

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Dee, 1974; Anderson, Cooke & Dee, 1976; Mohberg, 1977). Amoebae carrying heterothallic alleles (*matA1*, *matA2*, *matA3*,...) of the *matA* (or *mt*) locus usually form plasmodia only by crossing (Dee, 1962, 1966). Amoebae carrying the allele *matAh* can both cross and self (Wheals, 1970). The life cycle is completed by sporulation of the plasmodium and germination of the resulting spores to yield amoebae. Sporulation is a meiotic process, apparently even in the predominantly haploid plasmodia that arise by selfing. Although such plasmodia contain mostly haploid nuclei, they also contain a few diploid nuclei that may undergo meiosis to yield haploid, viable amoebae (Laffler & Dove, 1977; Shinnick & Holt, unpublished observations).

The specificity of crossing is determined by *matA* and a more recently discovered locus, *rac* or *matB* (Dee, 1978; Youngman *et al.* 1979). Optimal crossing occurs between strains differing at both *matA* and *matB*. Strains differing only at *matA* cross, but inefficiently; strains carrying the same allele of *matA* ordinarily do not cross at all. Diploid amoebae heterozygous only for *matB* do not self (Youngman, Anderson & Holt, 1981), whereas those heterozygous only for *matA* do self readily (Adler & Holt, 1975). Cultures containing two *matB* types form diploid cells at a much higher frequency than cultures containing only a single *matB* type, regardless of *matA* type (Youngman *et al.* 1981). Thus, it appears that *matB* affects the cell fusion step in crossing and *matA* affects the conversion of cells from the amoebal form to the plasmodial form.

Although selfing normally occurs in heterothallic strains only very rarely, certain mutations can bring about selfing phenotypes similar to that of *matAh* amoebae (Adler & Holt, 1977; Gorman, Dove & Shaibe, 1979; Honey, Poulter & Winter, 1981). We term such mutations *gad*, for greater asexual differentiation. Of an initial group of eight *gad* mutations that were mapped, only one (*gad-12*) was found to recombine freely with *matA* (Adler & Holt, 1977). Subsequently, one of the *matA*-linked mutations (*gad-11*) was shown to lie approximately 12 map units from *matA* (Shinnick & Holt, 1977).

Mutations of another type have the effect of reducing selfing by *matAh* or *gad* amoebae to levels typical of heterothallic strains (Wheals, 1973; Anderson & Dee, 1977; Davidow & Holt, 1977; Honey, Poulter & Teale, 1979; Anderson & Holt, 1981). The mutations that reduce selfing appear to fall into six complementation groups (Anderson, 1979; Anderson & Holt, 1981). Four of the groups (*npfB*, *npfC*, *npfD* and *npfE*) contain all but two of approximately 100 mutations and are tightly linked to *matA*. The other two groups, *npfA* and *npfF*, are linked neither to *matA* nor to one another (Wheals, 1973; Anderson & Dee, 1977). The *npfF* group was previously named *aptA* but we have now adopted a uniform terminology.

In this paper we describe analyses of twelve *gad* mutants, including the eight mutants originally studied by Adler & Holt (1977). The *matAh* strain was included in most of the tests too, since the strain behaves genetically and phenotypically as if it were a (*matA2*-derived) *gad* mutant (Anderson, 1979). Our objectives were to map the *gad* mutations, and to obtain clues to the function of *gad* genes by means of gene interaction experiments.

2. MATERIALS AND METHODS

(*i*) *Culture methods*

Amoebal strains were maintained in two-member cultures with *E. coli* on LIA (liver infusion agar; Cooke & Dee, 1974). Crosses were performed by inoculating 10^4 amoebae of each strain on to a spot of bacteria on a dPRM agar plate (Adler & Holt, 1974*a*) and incubating at 30 °C. Newly formed plasmodia were cultured once on PRM-agar (Adler & Holt, 1974*a*) containing 250 μg/ml streptomycin

Table 1. *gad mutants*

Strain	Genotype	Plaque diameter (mm) when plasmodia form at 26°	Reference
CH405	*matA2 matB1 fusA1 fusC1 gad-1*	3–6	Adler & Holt 1977
CH403	*matA2 matB2 fusA2 fusC1 gad-2*	3–5	Adler & Holt 1977
CH404	*matA2 matB1 fusA1 fusC1 gad-3*	4–6	Adler & Holt 1977
CH480	*matA2 matB1 fusA1 fusC1 gad-4*	3–5	Adler & Holt 1977
CH479	*matA3 matB1 fusA2 fusC2 gad-5*	1–3	Adler & Holt 1977
CH484	*matA4 matB3 fusA2 fusC2 gad-6*	5–7	Adler & Holt 1977
CH495	*matA3 matB3 fusA2 fusC2 gad-11*	4–6	Shinnick & Holt, 1977
CH478	*matA1 matB1 fusA1 fusC1 gad-12*	> 20*	Adler & Holt 1977
CH526	*matA4 matB1 fusA2 fusC2 gad-13*	2–4	Adler & Holt 1977
LU884	*matA2 matB1 fusA1 fusC1 gad-14*	5–7	Anderson, 1976
CH806	*matA2 matB1 fusA1 fusC1 gad-15*	3–5	This study
CH807	*matA2 matB2 fusA2 fusC1 gad-16*	4–6	This study
CH1†	*matAh matB1 fusA1 fusC1*	3–5	Adler & Holt 1977

* This strain forms plasmodia at 30 °C only; see Table 8.

† The strain is similar to CL (Cooke & Dee, 1975), but forms plasmodia somewhat more slowly.

sulphate and subsequently on PRM-agar or PYE-agar (Youngman *et al.* 1981). Sporulation of fully grown cultures was induced by placing them on a windowsill (northern exposure). Spores were germinated by placing a clump of spores in a test tube containing 0·2 ml H_2O, crushing with a glass rod to disperse the spores, adding 1 ml H_2O and mixing vigorously on a vortex mixer. The spore suspension was left at room temperature for at least 1 h prior to dilution and plating. For strain construction, progeny strains were established by picking well-isolated clones and subjecting them to two rounds of single-colony purification prior to characterization.

(ii) *Strains*

Strains with a Colonia genetic background (Adler & Holt, 1974*a*; Cooke & Dee, 1975) were used throughout this study.

Information on the *gad* mutants is given in Table 1. The two *fus* loci control fusion between plasmodia and do not affect the formation of plasmodia by amoebae (Poulter & Dee, 1968; Adler & Holt, 1974*a*). The numbers assigned to various *gad* mutations (*gad-1*, *gad-2*, etc.) are serial numbers indicating independently isolated mutations. Mutant strains CH806 and CH807 were isolated as described earlier (Adler & Holt, 1977) from LU648 (Cooke & Dee, 1975) and CH396 (Adler, 1975), respectively.

Strain CH538 was constructed by Adler (1975), LU910 and CH871 by Anderson (unpublished), and CH834 as part of this study. The genotypes of these strains are given in Table 6. The locus *kilA* affects the viability of heterokaryons formed between *fus*-compatible plasmodia (Adler & Holt, 1974*a*). Plasmodia are normally yellow; the recessive mutation *whi-1* causes white coloration (Anderson, 1977). The recessive mutation *leu-1* confers a leucine requirement on plasmodia (Cooke & Dee, 1975). The alleles eme^{s} and eme^{R}-*4* confer emetine sensitivity and resistance, respectively, on amoebae (Adler & Holt, 1974*a*). The mutations *npfF1* and *npfA1* interfere with plasmodium formation (Wheals, 1973; Anderson & Dee, 1977).

Strains carrying a given *gad* mutation and either the *npfF1* or *npfA1* mutation were constructed by crossing the original *gad* mutant (e.g. the *matA2 gad-1* strain) to a heterothallic strain carrying the *npf* mutation (e.g. *matA1 npfF1*) and screening the progeny as follows. Progeny carrying the *npfF1* mutation were identified by their inability to cross with a strain carrying a different *matA* allele and *npfF1*. Strains with the genotype *matAh npfA1* were identified by their behaviour in complementation tests with other *matAh* npf^{-} strains (Anderson, 1979). The presence of the *npfA1* mutation in a strain with a *matA* allele other than *matAh* was determined by crossing the strain to a *matAh* npf^{+} strain and analysing 50 of the progeny of this cross for *matAh npfA1* progeny as above. The presence of the *gad* mutation was determined by crossing the strain to a heterothallic strain (e.g. *matA1*) and analysing the progeny for the selfing phenotype associated with the *gad* mutation.

Strains carrying a *matA*-linked *gad* mutation and the *gad-12* mutation were constructed by crossing the *matA1 gad-12* strain to another *gad* mutant. Progeny carrying both *gad* mutations were identified as follows. The *matA* specificity of the progeny was determined, and the progeny displaying the *matA* specificity of the *matA*-linked *gad* mutation were crossed to a *matA1* strain. The progeny of this cross were analysed as above to show whether or not both gad^{-} alleles were present. Further details regarding strain construction are available in a thesis (Shinnick, 1978).

(iii) *Measurement of selfing ability*

Gad mutants display obvious differences in asexual plasmodium-forming ability. Although a quantitative kinetics assay is available (Youngman *et al.* 1977; Adler & Holt, 1977) for measuring such differences, it is not convenient for the analysis of large numbers of strains. We use instead a 'plaque diameter assay' (Davidow & Holt, 1977). The amoebae to be tested were plated on a lawn of live bacteria on duplicate LIA pH 5·0 (Anderson & Holt, 1981) or dPRM agar plates; either a suspension dilute enough to give separate clones was spread on the plate, or amoebae were picked up on the end of a toothpick and the toothpick was stabbed into the agar. The plates were examined daily from 5 to 15 days of incubation. When nascent plasmodia were first visible in each plaque, the diameters of the plaques were measured and recorded. The plaque diameter when plasmodia form is the same whether the plaques begin from single amoebae or from stabs. If one ranks *gad* mutants according to plasmodium-forming ability with this method

(Table 1), one obtains essentially the same rank as with the earlier 'T(50)' method (Adler & Holt, 1977).

(iv) *Crosses between gad mutants*

Crosses involving *gad* mutants were performed at 30 °C except as noted in the text. Crossed plasmodia were obtained within 3 trials when the *gad* mutants carried different *matB* alleles; up to 10 trials were necessary when strains carried the same *matB* allele. A preliminary indication of crossing was the formation of a plasmodium with a hybrid fusion type. Confirmation of crossing was obtained by measuring the recombination between unlinked loci (*matA* and *fusC*, *matA* and *fusA*, or *matA* and *matB*) except for the cross CH479 × CH526, where the presence of equal numbers of *matA3* and *matA4* progeny was used as the criterion for crossing.

For each pair of *gad* mutants, progeny analysis was performed on at least two independently isolated, crossed plasmodia and the data pooled. In no case did the data for one plasmodium differ significantly from those for another that involved the same mutants.

Classification of progeny by asexual plasmodium-forming capacity was accomplished as follows. Well-separated amoebal plaques on spore germination plates were sampled with toothpicks and stabbed on to pre-spread lawns of *E. coli* on duplicate dPRM-agar or LIA pH 5·0 plates. The plates were incubated at 26 °C or 30 °C. The 'plaque diameter' was determined as above. Since 21 stabs were placed on each plate, a fraction (typically 1–2 %) of all stabs had to be retested because the plaques were unable to grow to a diameter of greater than 10 mm before plasmodia, formed in a neighbouring plaque, overran them.

(v) *Dominance tests*

The experiments on dominance of *gad* mutations involve mixing amoebae of two strains and monitoring plasmodium formation by the mixture. The mixtures contained equal numbers of the two types of amoebae and were placed as 'spots' on dPRM-agar plates with formalin-killed bacteria (Haugli, 1971) or on non-nutrient plates at pH 5 with 'concentrated' live bacteria (Youngman *et al.* 1981). The two strains carried different alleles of *matB*, *gad* and *npfF* or *npfA* (see Results). In addition, one of the two strains carried *fusA1* and the other strain carried *fusA2*. To see if plasmodia that arose in the mixtures were crosses, they were subjected to fusion testing and progeny analysis. Crossed plasmodia have the fusion type *fusA1/fusA2* and yield recombinants between unlinked markers. Nearly all the plasmodia were crosses.

3. RESULTS

(i) *Mapping studies*

(a) *Crosses between gad mutants.* We crossed *gad* mutants with one another in all *matA*-compatible combinations as well as, where possible, with a *matAh* strain. Progeny of the crossed plasmodia were scored for their selfing ability. Two classes

were distinguished, the CPF (clonal plasmodium-forming) progeny, which form plasmodia in plaques before the plaques have reached 15 mm diameter, and the non-CPF progeny, which do not form plasmodia by the time plaques have grown to 15 mm diameter. Selfing ability is scored easily, which permits large numbers of progeny to be examined. The results of the 36 crosses analysed in this fashion are presented in Table 2; the interpretation of the results is presented in subsequent sections.

Table 2. *Proportion of non-CPF progeny of* gad × gad *crosses**

Genotype of strain 1	Genotype of strain 2			
	matA3 gad-5	*matA4 gad-6*	*matA3 gad-11*	*matA4 gad-13*
	(number of non-CPF progeny/total number of progeny)			
matAh	0/6500	66/3125	296/4460	0/6237
matA2 gad-1	0/1260	26/1132	82/1554	0/1050
matA2 gad-2	1/1092	24/1330	115/1827	0/920
matA2 gad-3	0/1320	28/1260	65/1200	0/1050
matA2 gad-4	4/1260	26/1320	151/2416	5/2436
matA2 gad-14	0/3150	36/1912	48/900	0/2100
matA2 gad-15	0/1580	24/1260	67/1285	0/1460
matA2 gad-16	0/2135	43/2150	98/1652	1/1236
matA3 gad-5	—†	28/1472	—	0/2605
matA4 gad-6	—	—	55/1540	—
matA3 gad-11	—	—	—	127/2100

* Progeny of strain 1 × strain 2 crosses were analysed for selfing ability. Progeny scored as non-CPF were those failing to form plasmodia in plaques up to or beyond 15 mm diameter. The strains are those given in Table 1.

† The cross is shown elsewhere in the table or the cross was not possible because both strains carry the same *matA* allele.

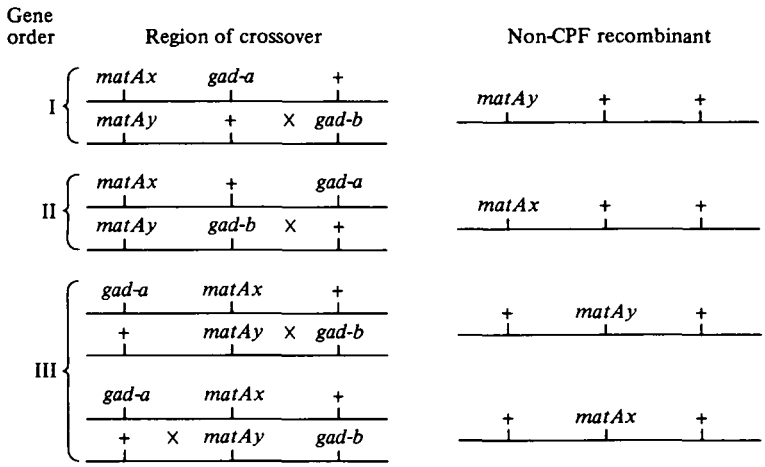
(b) *gad mutations tightly linked to one another.* Many of the crosses between *gad* mutants yielded no non-CPF progeny (Table 2). To interpret this observation, we view such crosses as three-factor crosses, and write the three possible orders of the genes (Table 3). As shown in the table, a single recombination event between the two *gad* mutations will always produce a chromosome lacking *gad* mutations, regardless of the gene order. Thus, the absence of non-CPF recombinants from a particular progeny set shows that the two *gad* mutations involved in the cross are tightly linked.

Two crosses, *matA2 gad-2* × *matA3 gad-5* and *matA2 gad-16* × *matA4 gad-13* each gave a single non-CPF progeny. Both these progeny displayed fuzzy plaque morphology, slow growth, and inefficient mating with a compatible tester strain. Spores from crosses between these progeny strains and a *matA1* strain displayed poor germination (i.e. less than 0·1 % of the spores were viable as compared to about 10 % in most crosses). Amoebae from the spores were grossly heterogeneous in plaque morphology and growth rate. Furthermore, the *fusA* and *whi* loci displayed a 2 : 1 segregation of alleles in the crosses. These data suggest that these two non-CPF progeny are aneuploids rather than haploid recombinants (Adler & Holt, 1974*b*). Thus, for our present purposes, we regard the *gad-2* × *gad-5* and *gad-16* × *gad-13* crosses as ones that gave no non-CPF progeny.

In toto, the data define a set of 8 *gad* mutations that are very closely linked to

one another. The set is composed of *gad-1*, *gad-2*, *gad-3*, *gad-5*, *gad-13*, *gad-14*, *gad-15* and *gad-16*. At least 1000 progeny were examined for each of the crosses giving no non-CPF recombinant. The detection of one recombinant out of 1000 would have reflected a recombination frequency of 2/1000 or 0·2 map units, which is therefore a rough measure of the maximum size of the set.

Table 3. *Formation of non-CPF recombinants*



(c) *Crosses with matAh*. Four crosses between *gad* mutants and a *matAh* strain were analysed (Table 2). Earlier we regarded such crosses as two-factor crosses, assuming implicitly that *matAh* is allelic to *matA3* and *matA4* (Shinnick & Holt, 1977). This assumption is called into question by the recent suggestion (Anderson, 1979) that *matAh* may actually have the structure *matA2 gad-h*, where *gad-h* would represent a mutation outside the genetic region defining *matA* but nevertheless linked to it. Consistent with the suggestion are the facts that *matAh* strains mate relatively poorly with *matA2* strains (Youngman *et al.* 1979), that *matA2* strains mutate to strains with *matAh* characteristics in one step (Adler & Holt, 1977), and that *matAh* can mutate to *matA2* in one step (Davidow & Holt, 1977; Anderson & Dee, 1977).

Adler & Holt (1975) examined 666 progeny of *matA3/matAh* and *matA4/matAh* plasmodia. None of the 330 non-CPF progeny had a *matA2* specificity, showing that if *gad-h* is indeed separate from *matA*, it is not readily separable. We repeated these measurements, looking this time at 910 progeny from the cross CL (*matAh*) × CH21(*matA3*) and testing *matA* types with the improved efficiency possible since the discovery of *matB*. All 448 non-CPF progeny were *matA3*, showing that no recombination between *matA* and the putative *gad-h* mutation had occurred. (The 462 CPF progeny were uniformly *matA2*, which is compatible with the hypothesis that *gad-h* is very closely linked to *matA*, but does not further support it, since one does not know *a priori* whether or not *gad-h* would be expressed with *matA3*.) Using the same rough measure of maximum distance as above, and summing the earlier and present sets of data, we conclude that *gad-h* is less than 0·13 map units from *matA*.

The crosses *matAh* × *matA3 gad-5* and *matAh* × *matA4 gad-13* yielded no non-CPF progeny in over 6000 progeny each. Thus *gad-5* and *gad-13* are closely linked to *matA*, the maximum distance being set by the resolution with which we have mapped the postulated allele *gad-h* to *matA*. Since *gad-5* and *gad-13* belong to the set of closely linked mutations already described, we may also conclude that the set itself is closely linked to *matA*.

Table 4. matA *types of non-CPF progeny**

Cross(es)	Non-CPF progeny: number and *matA* type
matA3 gad-11 × *matA2 gad-4*	150 *matA3*, 1 *matA2*
matA3 gad-11 × *matA4 gad-6*	53 *matA3*, 2 *matA4*
matA2 gad-4 × *matA4 gad-6*	26 *matA4*
The 8 other crosses with *gad-11*	898 *matA3*
The 8 other crosses with *gad-6*	275 *matA4*
The 2 other crosses with *gad-4*	9 *matA2*

* The non-CPF progeny described in Table 2 were analysed for *matA*-type as described under Materials and Methods. Two exceptional progeny classified non-CPF are described in the text and are not included in this table.

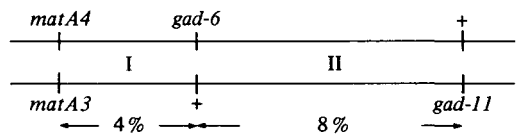
(d) *Map position of gad-11.* All 10 crosses involving the *matA3 gad-11* strain produced non-CPF recombinants (Table 2). Seven of these crosses were with strains carrying *gad* mutations from the group so far inseparable from *matA*, and an eighth was with the *matAh* strain. The non-CPF recombinants must have arisen, in these 8 cases, from recombination between *gad-11* and *matA* or its closely associated *gad* sites. The non-CPF class includes at least the genotype *matA3 gad*$^+$, and might also include the novel reciprocal recombinant, *matAx gad*$^-$ *gad-11*, where *matAx* and *gad*$^-$ come from the strain crossed with *matA3 gad-11*. However, all the non-CPF recombinants displayed the *matA3* specificity (Table 4), which implies that the reciprocal recombinants are not included in the non-CPF class. Thus the frequency of non-CPF progeny from these crosses is one-half the recombination frequency between the *matA* group and *gad-11*. The resultant *matA*, *gad-11* map distance is 10·4–13·2 map units, in accord with the earlier measurement of 12·3 map units (Shinnick & Holt, 1977).

(e) *Map position of gad-6.* The 10 crosses involving *matA4 gad-6* also produced non-CPF progeny (Table 2). The seven crosses with *gad* mutations in the *matA* group plus the cross with *matAh* gave frequencies of non-CPF progeny in the range from 1·8 to 2·3 %. The non-CPF progeny from these 8 crosses were all *matA4*, which by the reasoning in the preceding paragraph implies that only one-half the *matA*, *gad-6* recombinants are included. The distance between *gad-6* and *matA* is, therefore, about 4 map units.

The order of the three sites *matA*, *gad-6* and *gad-11* may be deduced from data on the cross *matA4 gad-6* × *matA3 gad-11*. The cross produced 3·6 % non-CPF recombinants (Table 2) in the ratio 53 *matA3* to 2 *matA4* (Table 4). Referring to Table 3, and letting *matA4 gad-6* × *matA3 gad-11* be represented by *matAx gad-a* × *matAy gad-b*, respectively, we note first that order III predicts *matA4* (*matAx*) progeny at a frequency of about 2 % or higher. (The frequency would be reduced

by one-half the frequency of double recombinants, which should be negligible, and increased, if classes other than *matA4 gad*$^+$ were non-CPF strains with *matA4* specificity.) Since the observed frequency of *matA4* non-CPF progeny was only 0·14%, order III is incorrect. Order II contradicts the observed *matA*, *gad-6* spacing relative to the *matA*, *gad-11* spacing, as well as predicting a much larger number of *matA4* non-CPF progeny than observed. The surviving order I, which places *gad-6* between *matA* and *gad-11*, is compatible with the data.

Table 5. *Predicted recombinant frequencies*



Genotype of progeny	Crossover(s)	Frequency (per cent)
matA4 gad-6 + / *matA3* + *gad-11*	None	—
matA3 + +	Region II	4
matA4 gad-6 gad-11		4
matA3 gad-6 +	Region I	2
matA4 + *gad-11*		2
matA4 + +	Regions I and II	0·16*
matA3 gad-6 gad-11		0·16*

* Assuming that the coefficient of coincidence = 1.

An analysis of the frequencies of various types of progeny expected for order I is given in Table 5. The single recombinant class *matA3 gad*$^+$ is expected to appear at a frequency of about 4%, which agrees adequately with the observed frequency of 3·4%. The double mutant *matA4 gad-6 gad-11* is also expected at 4%, and since there were so few *matA4* non-CPF progeny, we assume that the double mutant is CPF. The two single recombinants *matA3 gad-6* and *matA4 gad-11* are expected at about 2% each. We know from earlier work (Shinnick & Holt, 1977) that *gad-11* is expressed with *matA4*, which explains the absence of the former recombinant from the non-CPF class. To see if *gad-6* is expressed with *matA3*, we tested the *matA* type of 149 CPF progeny from the cross *matA4 gad-6* × *matA3 gad*$^+$. Five of the CPF progeny had *matA3* specificity (the remainder were *matA4*), which shows that *gad-6* is expressed with *matA3*. Thus, the expected 2% *matA3 gad-6* recombinants would not be found in the *matA3* non-CPF class, as is compatible with our interpretation of the data. The double recombinant *matA4 gad*$^+$ is expected at a frequency of 0·16%, which is also in agreement with the observation of only two *matA4* non-CPF progeny. The reciprocal of this, the double mutant *matA3 gad-6 gad-11*, is also expected at 0·16%; we have no information on the phenotype of this recombinant. The recombinant could, in any case, be in any of the progeny classes without upsetting the good agreement between the observations and the model. In conclusion, the map *matA* (4%) *gad-6* (8%) *gad-11* accords well with a detailed analysis of the data.

(f) *Mapping of gad-4.* The crosses *matA2 gad-4* × *matA3 gad-5* and *matA2 gad-4* × *matA4 gad-13* each gave a few non-CPF progeny (Table 2). There were a total of nine such progeny, and all were *matA2* (Table 4). The *matA* to *gad-4* distance is therefore 18/3696 or about 0·5 %.

Like *gad-6*, *gad-4* lies on the same side of *matA* as *gad-11*. Results from two crosses support this conclusion. First, in the cross *matA2 gad-4* × *matA3 gad-11*,

Table 6. *Linkage studies with* gad-12

Parents: CH538 (*matA4 matB1 fusA2 fusC2 kilA2 whi*$^+$ *leu*$^+$ *eme*^{S} *gad-12*)
LU910 (*matA3 matB3 fusA1 fusC1 kilA1 whi-1 leu-1 eme*^{r}*-4 gad*$^+$)

Gene pair	Number of progeny: Parental	Recombinant
matA, gad-12	48	50
fusA, gad-12	17	24
fusC, gad-12	19	22
kilA, gad-12	21	20
whi-1, gad-12	69	57
leu-1, gad-12	21	18
eme-4, gad-12	24	26
matB, gad-12	80	68

Parents: CH538 (*matA4 gad-12*) × CH834 (*matA1 npfF1*)

Type and number of progeny:		
	npf$^+$ *gad*$^+$	15
	npf$^+$ *gad-12*	17
	npfF1 gad$^+$	13
	npfF1 gad-12	15

Parents: CH478 (*matA1 gad-12*) × CH871 (*matA4 npfA1*)

Type and number of progeny:		
	npf$^+$ *gad*$^+$	17
	npf$^+$ *gad-12*	14
	npfA1 gad$^+$	15
	npfA1 gad-12	14

only one of 151 non-CPF progeny was *matA2* (Table 4). If *gad-4* were on the other side of *matA* (corresponding to order III in Table 3), *matA2* and *matA3* progeny would have appeared in the same ratio as the ratio of distances of *gad-4* and *gad-11* to *matA*; that is, one would have expected (0·5/12·5)151 = 6 rather than one *matA2* progeny to have been found. Second, in the cross *matA2 gad-4* × *matA4 gad-6*, all 26 non-CPF progeny were *matA4* (Table 4). If *gad-4* were on the other side of *matA*, then one would have expected (0·5/4·5)26 = 3 rather than no *matA2* progeny to have been found. The dearth of *matA2* non-CPF progeny is readily accounted for, in both cases, by the assumption that *gad-4* lies to the same side of *matA* as *gad-6* and *gad-11*, for then *matA2* non-CPF progeny arise only as double recombinants.

(g) *Mapping of gad-12.* The *gad-12* mutation is not linked to *matA* (Adler & Holt, 1977) nor to *matB*, *fusA*, *fusC*, *kilA*, *whi*, *leu*, *eme-4*, *npfA* or *npfF* (Table 6). (See Materials and Methods for a description of the loci and scoring procedures.) A summary of the mapping information on all twelve *gad* mutations is given in Fig. 1.

(h) *Selfing ability of non-CPF recombinants.* The non-CPF progeny described in Tables 2 and 4 were further tested to detect even minimal selfing ability. This was

accomplished by allowing plaques to grow up to 50 mm diameter and observing at what plaque diameter, if at all, plasmodia arose. There were 21 crosses that gave non-CPF progeny. For 19 of these crosses, the non-CPF progeny did produce selfed plasmodia when plaques were from 17 to 35 mm diameter. The number of foci of plasmodium production was fewer than 20 per plaque. For example, the non-CPF

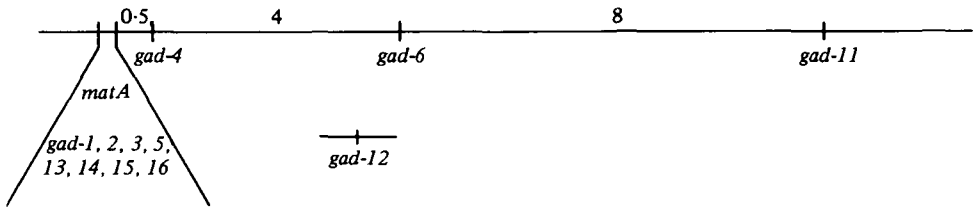


Fig. 1. Map of *matA* and *gad* mutations.

progeny of the cross CH403 (*matA2 gad-2*) × CH484 (*matA4 gad-6*) gave 1–10 plasmodia per plaque when plaques reached diameters in the range of 20–30 mm. In contrast, CH396 (*matA2 gad*$^+$), the parent of CH403, and CH329 (*matA4 gad*$^+$), the parent of CH484, almost never form plasmodia in plaques up to 50 mm diameter. Thus, even though the non-CPF progeny show only very infrequent, late plasmodium production, the progeny are not identical to the parents of the GAD strains. The results suggest that the *gad*$^+$ alleles may differ slightly from strain to strain (Shinnick & Holt, 1977).

Two of the 21 crosses, CH480 (*matA2 gad-4*) × CH479 (*matA3 gad-5*) and CH480 × CH526 (*matA4 gad-13*), behaved differently. The nine non-CPF progeny from these crosses gave no plasmodia in plaques up to 50 mm diameter. These progeny are recombinants that arise from crossing over in the small region between *gad-4* and *matA*, so that the *matA2* allele in the recombinant lies adjacent to an intact region from *gad-4* through *gad-11*.

(ii) *Gene interaction studies*

(a) *Expression of gad-11 with different matA alleles.* The mutation *gad-11* is expressed in strains carrying any one of the alleles *matA3*, *matA4* and *matAh* (Shinnick & Holt, 1977). Amoebae with the genotype *matA4 gad-11* self with approximately the same efficiency and temperature sensitivity as *matA3 gad-11* amoebae. Amoebae with the genotype *matAh gad-11* self even more rapidly than *matAh* amoebae. The plaque diameters at plasmodium appearance for *matAh* amoebae are 3–5 mm at 26° and > 30 mm at 30°; for *matAh gad-11* amoebae, the figures are 1–3 mm at 26° and 7–14 mm at 30°. (The figure 7–14 mm at 26° given in Shinnick & Holt (1977) is an error.) To find out if *gad-11* is expressed with the two other *matA* alleles available in the Colonia genetic background, we crossed CH495 (*matA3 gad-11*) with LU648 (*matA1*) and with CH394 (*matA2*) and analysed their progeny. Of 148 CPF progeny amoebae from the former cross, 14 were *matA1* and the remainder were *matA3*. Of 106 CPF progeny amoebae from the latter cross, 13 were *matA2* and the remainder were *matA3*. Thus, in both cases,

recombinant CPF amoebae appeared at the frequency expected for *matA1 gad-11* or *matA2 gad-11* progeny. We conclude that *gad-11* stimulates selfing with all 5 *matA* alleles tested.

(b) *Effect of npfA1 and npfF1 on selfing induced by gad mutations.* The mutations *npfA1* and *npfF1* suppress plasmodium formation in *matAh* strains but are unlinked to *matA*. We constructed amoebal strains with the generalized genotypes

Table 7. *Plaque size tests of* gad⁻ npfA1 *strains*

Genotype	Plaque diameter (mm)*	
	26 °C	30 °C
matA3 gad-5	2–4	5–8
matA3 gad-5 npfA1	7–14	> 50
matA4 gad-6	5–7	10–15
matA4 gad-6 npfA1	8–10	20–30
matA4 gad-13	2–3	5–7
matA4 gad-13 npfA1	4–6	> 50
matA1 gad-12	> 20	14–17
matA1 gad-12 npfA1	> 20	14–17

* Plaque diameter at the time when plasmodia first became visible.

matAx gad⁻ npfA1 and *matAx gad⁻ npfF1* and determined the extent of selfing in each of the strains. All the *gad* mutations listed in Table 1, as well as *gad-8* (Adler & Holt, 1977) were tested. The amoebae carrying *npfF1* almost never selfed, that is, *npfF1* suppressed selfing to the level seen in amoebae lacking a *gad* mutation. Amoebae carrying most of the *gad* mutations and *npfA1* also formed plasmodia at very low frequency. The *gad* mutations in this category are those numbered *1*, *2*, *3*, *4*, *11*, *14*, *15* and *16*. Strains carrying one of these *gad* mutations and *npfA1* formed no plasmodium at all in spots incubated up to 10 days at 26° and 30°. Further incubation at 26° occasionally led to the production of a few foci of plasmodium formation, something that did not occur in amoebae carrying *npfF1*. Slight leakiness and temperature sensitivity of *npfA1* has been noted previously (Anderson & Dee, 1977). Thus *npfF1* is epistatic to all tested *gad*s, and *npfA1* is epistatic to most of them.

Amoebae with the genotypes *matA3 gad-5 npfA1*, *matA4 gad-6 npfA1*, *matA4 gad-12 npfA1* and *matA4 gad*-13 *npfA1* readily formed plasmodia at either 26° or 30°. In the case of the *gad*-12 *npfA1* amoebae, plasmodium formation was greater at 30°, which is the same behaviour as that of *gad-12* amoebae lacking the *npfA1* mutation (Adler & Holt, 1977). In the other three cases, plasmodium formation was greater at 26°, which is also the same as the behaviour of *gad-5*, *gad-6* and *gad-13* amoebae lacking an *npfA1* mutation (Adler & Holt, 1977). A more direct, quantitative comparison of strains with and without *npfA1* reveals that *npfA1* does reduce selfing induced by *gad-5*, *gad-6* and *gad-13*, particularly at 30° with *gad-5* and *gad-13* (Table 7). None the less, *npfA1* has much less effect overall when combined with *gad-5*, *gad-6*, *gad-12* or *gad-13* than with the other *gad* mutations tested.

Another functional difference between *npfF1* and *npfA1* is known from studies

on crossing: crossing between *npfF1* amoebae is blocked (Wheals, 1973), but crossing between *matA*-compatible *npfA1* amoebae is not (Anderson & Dee, 1977).

(c) *Effect of gad-12 on other gad mutations.* The interaction of *gad-12* with certain *matA*-linked *gad* mutations and with *matAh* was studied by constructing the appropriate doubly mutant, haploid amoebal strains and determining their plasmodium-forming capabilities. The plaque diameters at which each of the

Table 8. *Selfing with and without* gad-12

Genotype	Plaque diameter (mm)*	
	26°	30°
matA1 gad-12	> 20	13–16
matA4 gad-12	> 20	13–17
matAh	3–5	> 20
matAh gad-12	3–5	13–17
matA2 gad-1	3–6	> 20
matA2 gad-1 gad-12	3–6	13–17
matA2 gad-2	3–5	> 20
matA2 gad-2 gad-12	3–6	13–17
matA2 gad-4	3–5	> 20
matA2 gad-4 gad-12	3–5	13–16
matA4 gad-6	5–7	12–15
matA4 gad-6 gad-12	3–5	9–12
matA3 gad-11	4–6	> 20
matA3 gad-11 gad-12	4–6	13–16

* Plaque diameter at the time when plasmodia first became visible.

strains first produced visible plasmodia are listed in Table 8. For all strains carrying two *gad* mutations except the *matA4 gad-6 gad-12* strain, plasmodium formation at both 26° and 30 °C appeared to be determined by the *gad* mutation promoting the greatest plasmodium formation at the particular temperature. For example, at 26°, *matA3 gad-11 gad-12* amoebae selfed at the same plaque diameter as *matA3 gad-11* amoebae, and at 30°, *matA3 gad-11 gad-12* selfed at the same plaque diameter as *matA1 gad-12* (Table 8). On the other hand, the plaque diameter at which *matA4 gad-6 gad-12* amoebae selfed was significantly less at both 26° and 30 °C than the plaque diameters characteristic of *gad-6* amoebae and *gad-12* amoebae.

(d) *Dominance of gad mutations.* In a series of experiments designed to study the complementation of *gad* mutations, we prepared (as controls) mixtures of *matAx matBy gad*$^-$ *npfF1* and *matAx matBz gad*$^+$ *npf*$^+$ amoebae and exposed the mixtures to crossing conditions at 26°. (The symbols *matBy* and *matBz* represent different alleles of *matB*, and *gad*$^-$ represents a *gad* mutation.) Unexpectedly, the mixtures all produced numerous plasmodia. Selfed plasmodia were not expected, since the first strain carried *npfF1* and the second carried neither *matAh* nor a *gad* mutation. Crossing of the usual sort was also not expected, since both strains carried the same allele of *matA*. Analysis of the plasmodia revealed that essentially all were the result of crossing between the two types of amoebae in the mixtures. Such crossing can be accounted for by the hypothesis that *gad* mutations are

dominant or semi-dominant, so that the diploid products of the fusions in each mixture expressed the *gad* mutation and developed into diploid plasmodia. (The *npfF1* mutation is already known to be recessive to npf^+ in crosses between heterothallic strains.) Support for this hypothesis is provided by the failure of plasmodia to form in mixtures identical to the above but with gad^+ replacing gad^- or with *matB* homoallelic rather than heteroallelic.

The *gad* mutations studied as described above are those numbered *1*, *2*, *3*, *4*, *5*, *6*, *8*, *11*, *13*, *14*, *15* and *16*. The numbers of crossed plasmodia produced by the various gad^-/gad^+ mixtures were not measured accurately but, for all except two of the *gad* mutations, exceeded 50 plasmodia per mating spot. The mixtures involving *gad-11* and *gad-14* were the exceptions, producing only 5–50 plasmodia per spot. All the mutations may be described as dominant or semi-dominant, but the degree of expression of the mutations in the presence of the wild-type alleles may vary.

We also conducted a series of experiments of the same design as those described, but with *npfA1* replacing *npfF1*. The gad^- *npfA1*/gad^+ npf^+ mixtures with *gad-1*, *2*, *3*, *4*, *15* and *16* formed crossed plasmodia, but more slowly and in smaller numbers than with the corresponding gad^- *npfF1*/gad^+ npf^+ mixtures. The *npfA1*/npf^+ mixtures with *gad-11* and *gad-14* formed no crossed plasmodium at all, which correlates with the small number of crossed plasmodia produced in the corresponding *npfF1*/npf^+ mixtures. The only other *gad* mutation studied in *npfA1*/npf^+ mixtures was *gad-5*. In this case, crossed plasmodia formed as readily as in the corresponding *npfF1*/npf^+ mixture. Nevertheless, this may still be regarded as consistent with the general pattern of reduced plasmodium formation with *npfA1*/npf^+ mixtures, as *gad-5* has the greatest stimulatory action of any of the *gad* mutants tested (Table 1), and this may put any *npfA1*, *npfF1* difference out of the range of the tests. The results suggest that *npfA1*, unlike *npfF1*, is not completely recessive to its wild-type allele.

4. DISCUSSION

The existence of four *npf* complementation groups closely linked to *matA* has already indicated that the *matA* region is functionally complex. Further indications of functional complexity are provided by our data on the interactions of the *matA*-linked *gad* mutations with *npfA1* and *gad-12* (see text and Tables 7 and 8). The mapping studies reported here also reveal structural complexity. Although most of the *matA*-linked *gad* mutations were, like most *npf* mutations, inseparable from *matA*, three of them – *gad-4*, *gad-6*, and *gad-11* – have proved to be separable both from *matA* and from one another. We cannot state categorically that these three mutations are in separate genes, but the map distances between them are great enough that it would be surprising if the recombination were in fact intragenic.

The linkage of several genes of related function in *P. polycephalum* cannot be viewed as fortuitous. Not only is the haploid chromosome number approximately 40 (Mohberg, 1977), but of over 50 other gene pairs previously tested for recombination, only three pairs showed linkage: *fusA* and *sax* (Cooke & Dee, 1975),

fusC and *npfF* (Pallotta *et al.* 1979), and *imz* and *eme* (Shinnick *et al.* 1978). Thus, our finding that there are apparently at least four *gad* loci closely linked to each other, to the *matA* locus, and to the four *npf* complementation groups is clearly of some significance. One possibility is that efficient functioning of these clustered genes, which are all involved in plasmodium formation, may be somehow facilitated by their proximity. A second possibility is that each *matA* allele may be associated with a unique '*gad* region' that is maintained as a unit by selection. Our observation that the non-CPF recombinants from crosses between *gad* mutants were generally not as fully suppressed for plasmodium formation as wild-type heterothallic strains is consistent with this possibility, since such non-CPF strains would contain recombinant *gad* regions unlike those in wild isolates.

The clustering of *gad* and *npf* mutations at and near *matA* is somewhat reminiscent of the situation found in *Schizophyllum commune*, a tetrapolar Basidiomycete that, like *P. polycephalum*, possesses multiallelic incompatibility loci. The B mating-type factor of *S. commune* has been the subject of intense investigation and it has been found that this factor forms part of an extended genetic region, within which map a number of mutations that switch on or switch off morphogenetic sequences normally initiated by interaction between unlike B alleles (Raper & Hoffman, 1974). In *Schizophyllum*, as in *Physarum*, the reason for the clustering of mutant sites is unknown.

The *matA* locus was originally defined in terms of its effect on mating specificity (Dee, 1962, 1966). Subsequently, it was found that the gene responsible for the selfing behaviour of Colonia strains is allelic to *matA*, and we now know that most mutations that increase or decrease selfing map to the same genetic region. Thus, the *matA* region affects not only mating specificity, but selfing as well. The recent work on the control of zygote formation by *matB* has also focused attention on *matA* as the gene controlling conversion of the zygote to a plasmodium. Since this conversion is an event that can follow diploid cell formation by many generations (Adler & Holt, 1975; Youngman *et al.* 1981), we see that the apparently dual functions of *matA* – control of mating specificity (i.e. control of differentiation of the diploid zygote to a plasmodium) and of selfing – are probably in fact different reflections of a single function.

It seems most likely that *matA* genes make active products in amoebae. This conclusion comes from a consideration of the fact that the two different *matA* genes in a zygote must interact with one another, and it would be surprising if this occurred directly at the DNA level. One could of course propose that only some *matA* genes make products, but the large number of *matA* alleles and the symmetry of their interactions make this unlikely. The large number of alleles also limits the types of interactions that seem plausible. One attractive possibility (Anderson & Holt, 1981) is that the product of *matA* is a polymer, and is fully active only in the homopolymeric form. The function of the polymer would be as an inhibitor of plasmodium formation or as a stimulator of amoebal function. In a zygote, subunits from different *matA* alleles would 'poison' one another by making inactive heteropolymers. We have presented detailed arguments elsewhere to show that the observed behaviour of the *matA*-inseparable *gad* and *npf* mutations can be accounted for by alterations affecting the structure of the hypothetical *matA*

product (Anderson & Holt, 1981). The existence of *gad* mutations separable from *matA* can be accommodated within this model. For example, if the separable *gad* mutations actually lie in different genes, we would suggest the *matA* polymer interacts with these genes or their products to control plasmodium formation. Another possibility is that the separable *gad* mutations are the products of duplication of the *matA* gene and subsequent mutation of one copy to gad^-; plasmodium formation then would occur by subunit poisoning, as proposed for gad^+/gad^- heterozygous diploids.

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