Genet. Res., Camb. (1977), **29**, *pp.* 21–34
With 1 *text-figure*
Printed in Great Britain

Isolation and Analysis of Amoebal–Plasmodial Transition Mutants in the Myxomycete *Physarum polycephalum*

By R. W. ANDERSON and JENNIFER DEE
Department of Genetics, University of Leicester, Leicester LE1 7RH, England

(*Received* 21 *June*, 1976)

SUMMARY

Plasmodium formation in the Myxomycete *Physarum polycephalum* normally involves fusion of haploid amoebae, carrying different alleles at the mating type (*mt*) locus, to give diploid plasmodia. Strains carrying the mt_h allele are capable of undergoing the amoebal–plasmodial transition with high efficiency within amoebal clones, resulting in the formation of haploid plasmodia. NMG mutagenesis of mt_h amoebae, followed by an enrichment procedure, was used to isolate mutants in which such clonal plasmodium formation was either delayed or absent. Thirteen mutants of the second type were analysed. Three of these were temperature-sensitive for plasmodium formation. All thirteen mutants were able to form diploid crossed plasmodia when mixed with a mt_1 strain. Three new genes were identified and designated *npf*A, *npf*B and *npf*C. A mutant allele of *npf*A rendered *clonal* plasmodium formation temperature-sensitive, but did not prevent crossing at the non-permissive temperature with derived strains carrying the same mutant allele. No recombination was detected between *npf*B or *npf*C and *mt*, but *npf*A was unlinked to *mt* and a locus (*apt*-1) shown in a previous study to be involved in plasmodium formation. The genes *npf*B and *npf*C were distinguished by complementation analysis. Strains of the genotype *npf*B$^-$; *npf*C$^+$ behaved in the same way as strains carrying the mt_2 allele. The nature of the mutants and the role of the mating-type locus in the initiation of plasmodium formation are discussed.

1. INTRODUCTION

The true slime mould (Myxomycete) *Physarum polycephalum* can be cultured as either of two morphologically distinct vegetative phases: microscopic, uninucleate amoebae or macroscopic, multinucleate, syncytial plasmodia. Under appropriate conditions plasmodia give rise to spores which hatch to yield amoebae. The life-cycle is completed when amoebae undergo a developmental transition to become plasmodia. This 'amoebal–plasmodial transition' is of particular interest as a developmental process since it is potentially suitable for the combined application of genetical, cytological and biochemical analyses (Wheals, 1973; Dee, 1975). The aim of this work was to isolate mutants defective in the amoebal–plasmodial transition, since the analysis of such mutants should yield information on the number and functions of genes involved.

Plasmodium formation in *P. polycephalum* normally involves cell and nuclear fusion of pairs of amoebae carrying different alleles at the mating-type (*mt*) locus (Ross, 1957; Dee, 1960; Dee, 1966; Wheals, 1970). Measurements of nuclear DNA content and chromosome number show that plasmodia formed in this way from haploid amoebae are diploid (Mohberg & Rusch, 1971; Mohberg *et al.* 1973). Plasmodia may also be formed within single clones of amoebae of at least some mating types and the plasmodia so formed are haploid (Cooke & Dee, 1974; Adler & Holt, 1975). These clonal plasmodia arise in only a small proportion of cultures (Adler & Holt, 1975) except in the case of mt_h strains, all cultures of which are normally capable of forming plasmodia (Wheals, 1970, 1973; Cooke & Dee, 1975).

The efficiency of plasmodium formation within clones of haploid mt_h amoebae makes them suitable for the isolation of mutants defective in plasmodium formation. Amoebae plated on a suitable agar medium in a lawn of *Escherichia coli* proliferate to form separate colonies or plaques. After a period of incubation, the length of which is dependent on environmental factors, each plaque of mt_h amoebae gives rise to a number of plasmodia (Wheals, 1973; Cooke & Dee, 1975). Amoebae of other mating types do not form plasmodia under these conditions. Wheals (1973) screened 5×10^5 amoebal clones derived from mutagenized cultures of mt_h amoebae and isolated four which lacked the ability to form plasmodia within clones. All four strains formed plasmodia when mixed with one another, though the formation of hybrid plasmodia was not demonstrated. Wheals analysed one strain (APT1) extensively and showed that it carried a mutation of a nuclear gene (*apt*-1) unlinked to *mt*. In the course of other work, Cooke & Dee (1975) used an enrichment technique without mutagenesis to isolate two strains which consistently formed plasmodia several days later than the strains from which they were derived and thus from much larger plaques than normal. These strains may carry mutations at or closely linked to the mating-type locus, though they have not been fully characterized (Cooke, 1974).

2. MATERIALS AND METHODS

(i) *Loci*. *mt*, amoebal mating type. Alleles mt_1, mt_2, mt_3, mt_4 (Dee, 1966). mt_h (Wheals, 1970, 1973) confers the ability to form plasmodia within amoebal plaques.

*fus*A and *fus*B, plasmodial fusion loci (Poulter & Dee, 1968; Poulter, 1969; Cooke & Dee, 1975). Alleles *fus*A1 and *fus*A2 are co-dominant, *fus*B2 is dominant to *fus*B1. Plasmodia fuse only if they have the same fusion types.

apt-1, a locus affecting the amoebal–plasmodial transition (Wheals, 1973). mt_h;*apt*-1$^-$ strains fail to form plasmodia within amoebal clones. They cross with heterothallic *apt*-1$^+$ strains but not with *apt*-1$^-$ strains.

leu, a locus affecting plasmodial requirement for leucine (Cooke & Dee, 1975).

(ii) *Strains*. See Table 1. All strains except i are of Colonia genetic background. LU862 and LU863 are progeny of plasmodia formed by crossing CH188 (mt_3) and CH207 (mt_4), strains supplied to this laboratory by Dr P. N. Adler and Dr C. E.

Holt, with LU640, a mt_h;*fus*A1:*fus*B1 strain derived by Cooke (1974); LU866 is a progeny clone of the plasmodium (APT1 × LU648)3 (Anderson, unpublished).

(iii) *Cultural conditions.* Amoebae were routinely maintained in culture at 26 °C with *E. coli* on liver infusion agar, LIA (1 g Oxoid liver infusion powder per litre 2 % agar). To subculture amoebal strains cells were picked up on the tips of toothpicks, stirred into 0·2 ml puddles of bacterial suspension on new plates and spread with a glass spreader. Separate amoebal plaques were visible after 3–4 days' incubation. One nutrient agar plate of *E. coli*, streaked out and grown overnight at 37 °C, was sufficient for about 10 ml of bacterial suspension. Plasmodia were routinely maintained at 26 °C on semi-defined medium agar (SDM agar; Dee & Poulter, 1970). Spore formation, spore plating and the isolation of clones were carried out as described by Wheals (1970), except that spore counts were not made and toothpicks were used for cloning. Amoebal strains were routinely stored at 4 °C.

Table 1. *Amoebal strains*

Strains	Genotypes
CL (Cooke & Dee, 1975)	mt_h; *fus*A2; *fus*B1
CL5001 (Cooke & Dee, 1975)	mt_h: *fus*A2; *fus*B1; *leu*$^-$
LU648 (Cooke & Dee, 1975)	mt_1; *fus*A1; *fus*B1
LU688 (Cooke & Dee, 1975)	mt_2; *fus*A1; *fus*B1
LU861 (Cooke, 1974)	mt_2; *fus*A2; *fus*B1
LU862	mt_3; *fus*A1; *fus*B1
LU863	mt_4; *fus*A1; *fus*B1
LU866	mt_1; *fus*A2; *fus*B1; *apt*-1$^-$
i (Dee, 1966)	mt_2; *fus*A2; *fus*B2

(iv) *Microscopic observation of growing amoebae.* Filming slide cultures were set up as described by Anderson *et al.* (1976) and daily observations were made with phase-contrast optics and a magnification of × 400.

(v) *Plasmodium formation.* Amoebal clones were tested for the formation of plasmodia in plaques by plating with *E. coli* on dilute semi-defined medium agar (DSDM agar; 10 ml liquid SDM per 100 ml 2 % agar). mt_h strains formed plasmodia in every plaque on a plate within 3–4 days. Crosses were set up by co-inoculating two amoebal strains into 0·2 ml bacterial puddles on DSDM agar. Plates were incubated for up to 6 weeks, though mixtures of compatible strains generally gave plasmodia within 1–2 weeks. Scoring was complicated by the occasional formation of 'selfed' plasmodia by one of the strains in incompatible matings. To overcome this problem crosses were usually set up in duplicate and formation of plasmodia in both puddles was required for a positive result, unless other markers (e.g. plasmodial fusion type) demonstrated the production of hybrid plasmodia.

(vi) *Plasmodial fusion tests.* The fusion behaviour of a plasmodium was classified by testing for fusion with plasmodia of known fusion genotypes. Two blocks, each approximately 30 mm × 4 mm × 4 mm, were cut from plates of growing plasmodia and placed on fresh SDM agar plates. The blocks were placed about 1 cm apart,

with the surfaces carrying plasmodial growth facing one another. The edges of the plasmodia met after 24–48 h growth and fusion or non-fusion could be scored within a few hours. Limited fusion, followed by clear non-fusion, was sometimes observed between plasmodia of the genotypes *fus*A1/*fus*A2 and *fus*A2. This was easily recognized and scored as non-fusion. Fusion, followed by a lethal interaction (Carlile & Dee, 1967) was observed in a few cases.

(vii) *Mutagenesis.* The method described by Cooke & Dee (1975) for the mutagenesis of exponentially growing amoebae on membrane filters was employed, with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NMG) as mutagen. An inoculum size of about 5×10^4 cells was used and mutagenesis was carried out after 48 h incubation. No postmutagenic incubation period was allowed before enrichment.

(viii) *Enrichment.* A modification of the method employed by Cooke & Dee (1975) in the isolation of CLd was used. Each mutagenized culture was harvested by flooding with 2 ml water and scraping with a glass spreader. This was done immediately after mutagenesis. The harvested culture was either all inoculated on to a single DSDM agar plate or divided between two plates. Each plate represented the beginning of a single enrichment line. It was incubated at $28{\cdot}5 \pm 0{\cdot}5$ °C (the maximum temperature for the formation of plasmodia in plaques by CL and CL5001) or at 22 °C until plasmodia became visible (5–7 days), then held at 4 °C for 24 h to reduce plasmodial viability. Harvesting in 2 ml water by gentle agitation yielded an amoebal suspension which was enriched for any mutants able to proliferate but unable to form plasmodia. This suspension, with *E. coli*, was inoculated on to a further plate. Only amoebae grew for the first few days of incubation, showing that viable plasmodia were not replated. The enrichment procedure was repeated up to five times. At each replating dilutions of the suspension were plated on DSDM agar, incubated at 28·5 °C or 22 °C, as appropriate, and examined for plaques which did not form plasmodia at the same time as CL or CL5001. These appeared in most lines after 3–5 réplatings. They were subcultured to LIA plates and recloned.

(ix) *Estimation of nuclear DNA contents.* Amoebal and plasmodial nuclei were isolated essentially as described by Mohberg & Rusch (1971); underlaying with 1 M sucrose was omitted in plasmodial nuclear isolation and 0·1 % Triton was used in the amoebal procedure. Phase-contrast microscopic observation of glycerol/ethanol fixed smears (Mittermayer *et al.* 1965) was used to ensure that plasmodia were harvested in G_2 phase. Amoebal nuclei were isolated from exponentially growing cultures and were thus mainly in G_2 phase (Mohberg & Rusch, 1971). Methods described by Cooke & Dee (1974) were used for the storage, Feulgen staining and measurement of stain intensity of nuclei. Chicken erythrocyte nuclei were isolated (Mohberg, 1976) and included on all slides to give a reference standard taken to be 2·45 pg DNA/nucleus (Rasch *et al.* 1971). Stain intensities recorded for 50 *Physarum* nuclei on each slide were compared with the mean value of ten erythrocyte nuclei from the same slide.

3. RESULTS

(i) *Isolation of mutants*

Exponentially growing cultures of CL and CL5001 amoebae were mutagenized and enriched for mutants with delayed plasmodium formation. Out of 130 independent enrichment lines 96 yielded some amoebal plaques which did not form plasmodia at the same time as those of CL and CL5001. These amoebae were cloned, plated on DSDM agar and incubated for 2 weeks at 22 °C and 28·5 °C. Ten clones isolated from separate enrichment lines failed to form plasmodia at either temperature and three formed a few plasmodia per plate at 22 °C (and 26 °C) but none at 28·5 °C. These 13 strains (Table 2) were selected for further analysis. The remaining strains all formed plasmodia at both temperatures, one or more days later than CL or CL5001.

Table 2. *Mutant strains selected for analysis*

		Plated on DSDM agar	
Amoebal strains	Derived from	Plasmodia at 22 °C	Plasmodia at 28·5 °C
CL6049	CL	–	–
CL6082	CL	–	–
CL6089	CL	–	–
CL6099	CL	–	–
CL6100	CL	few	–
CL6111	CL	few	–
CL6115	CL	few	–
CL6129	CL	–	–
CL6130	CL	–	–
CL6134	CL	–	–
CL6136	CL	–	–
CL6143	CL	–	–
CL5001/8	CL5001	–	–
CL		+	+
CL5001		+	+

+ = plasmodia in plaques. – = no plasmodia.

Plasmodia formed at 22 °C from each of the temperature-sensitive mutants were tested for growth at 32 °C, the maximum temperature for growth of CL plasmodia. All grew normally, showing that they were not temperature-sensitive. Spores were obtained from the plasmodia and 20 amoebal progeny clones of each were plated on DSDM agar at 28·5 °C. All grew normally as amoebae but did not form plasmodia, showing that the plasmodia which were tested for temperature-sensitivity were not revertants. Thus the temperature-sensitive mutants were temperature-sensitive only for plasmodium formation, not for amoebal or plasmodial growth.

Amoebae of all the mutant strains grew at similar rates to CL and CL5001. Microscopic examination of growing cultures of the mutants showed only uninucleate cells.

To determine whether plasmodium formation was possible in the mutant strains, each was incubated in 50 bacterial puddles for 6 weeks at 26 °C or 28·5 °C, as appropriate. All strains gave rise to at least one plasmodium. LU648 (mt_1), LU688 (mt_2) and LU861 (mt_2) were tested in the same way and also gave rise to some plasmodia. Investigation of progeny of plasmodia derived from mutant strains showed that some were revertants, while some retained the mutant phenotype. The origin of the rare clonal plasmodia is being investigated further.

(ii) *Crosses of the mutants with LU648*

To determine whether each mutant carried a single nuclear gene mutation, it was crossed with a mt_1 strain. Crosses were set up at 26 °C or 28·5 °C of all the mutants (which carried the *fus*A2 plasmodial fusion allele) with LU648 (mt_1;*fus*A1). In each case plasmodia which fused with heterozygous *fus*A1/*fus*A2 tester plasmodia were recovered, indicating the formation of hybrids in the crosses. The mean nuclear DNA content was estimated for one hybrid plasmodium derived from each cross. Values of 1·1–1·5 pg DNA/nucleus were obtained. These were approximately twice the values obtained for the mutant strains, CL and LU648 (0·5–0·8 pg DNA/nucleus). Thus all the hybrid plasmodia appeared to be diploid. Histograms showing distributions of nuclear DNA contents for one mutant strain (CL6129) and the corresponding crossed plasmodium (CL6129 × LU648) are shown in Fig. 1.

Plasmodia which fused with *fus*A2 testers were also recovered from some mixtures of the mutants with LU648, and in some strains these 'selfed' plasmodia arose at frequencies higher than those at which plasmodia were formed in puddles of the mutants alone. This stimulation of selfing is being investigated and will be fully reported elsewhere.

Spores were obtained from the hybrid plasmodia whose nuclear DNA contents had been estimated. About 100 amoebal progeny clones were isolated from each spore batch. Each clone was plated separately on DSDM agar and incubated for 2 weeks at 26 °C or 28·5 °C to test for the formation of plasmodia in plaques. It was expected that, if the mutant phenotype was due to mutation in a gene (*npf*) unlinked to the mating type locus, one-quarter of the progeny clones would be mt_h;npf^+ recombinants, and would therefore give plasmodia in plaques. The results indicated that only CL6111 carried a mutant allele (designated *npf*A1$^-$) at a locus unlinked to *mt* (see Table 3). Four other crosses each gave rise to one amoebal progeny clone which formed plasmodia in plaques (see Table 3). These four clones were shown not to be normal haploid products of meiosis; those derived from CL6130 × LU648 and CL6136 × LU648 both yielded plasmodia of hybrid *fus*A1/*fus*A2 fusion behaviour, while plasmodia formed in plaques from amoebal progeny of CL6049 × LU648 and CL5001/3 × LU648 were not of hybrid fusion type but gave rise to amoebal clones which did not form plasmodia in plaques and which included both mt_h and mt_1 types. These results were consistent with all four clones being heterozygous at the mating-type locus (Adler & Holt, 1975).

Progeny clones of all the crosses, except CL6111 × LU648, were classified for

mating type alleles by testing for plasmodium formation in puddles with LU648 (mt_1) and LU688 (mt_2). Clones which gave plasmodia with LU648 were classified as mt_h, while those which gave plasmodia with LU688 but not with LU648 were classified as mt_1. The plasmodia were classified for fusion type and recombination

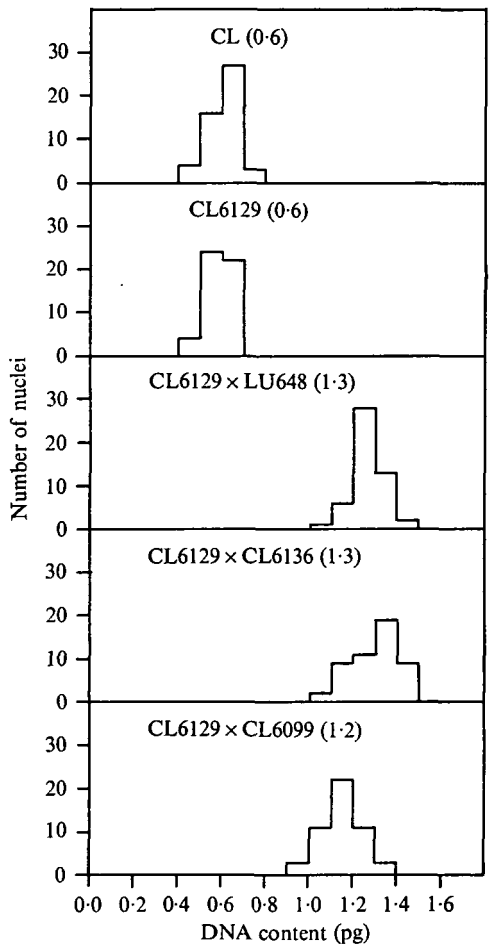

Fig. 1. Microdensitometric estimation of nuclear DNA content. CL and CL6129 were amoebal strains, the remainder plasmodial. Mean values are shown in parentheses.

of *mt* and *fus*A alleles was detected in every case (see Table 4). Where sufficient numbers were classified allele ratios were not significantly different from 1:1 ($P > 0{\cdot}05$ except in one case where $P > 0{\cdot}01$) and there was no significant deviation from free recombination of *mt* and *fus*A ($P > 0{\cdot}05$).

The analysis of crosses with LU648 suggested that every mutant except CL6111 carried a *npf*$^-$ mutation closely linked to *mt*, since mt_h;*npf*$^+$ recombinants were apparently recovered only in the cross CL6111 × LU648. However, it was also possible that the mutants carried extranuclear mutations inherited by all progeny

https://doi.org/10.1017/S0016672300017080 Published online by Cambridge University Press

of the crosses with LU648. To test whether the mutants carried extranuclear mutations, two non-revertant plasmodia formed clonally from CL6089 and CL6099 were fused with CL5001 plasmodia (mt_h;*leu*$^-$) to form heterokaryons. Fifty amoebal

Table 3. *Formation of plasmodia in plaques by progeny of hybrid plasmodia*

Hybrid plasmodia	Progeny plated on DSDM agar: Plasmodia in plaques	Progeny plated on DSDM agar: Amoebae only
(CL6049 × LU648)1	1	104
(CL6082 × LU648)3	0	99
(CL6089 × LU648)9	0	96
(CL6099 × LU648)3	0	89
(CL6100 × LU648)10	0	100
(CL6111 × LU648)3	21	79
(CL6115 × LU648)4	0	100
(CL6129 × LU648)3	0	105
(CL6130 × LU648)3	1	99
(CL6134 × LU648)2	0	98
(CL6136 × LU648)5	1	98
(CL6143 × LU648)11	0	100
(CL5001/8 × LU648)12	1	99

Table 4. *Recombination of* mt *and* fus*A alleles in the progeny of hybrid plasmodia*

Hybrid plasmodia	Recombinants: mt_h; *fus*A1	Recombinants: mt_1; *fus*A2	Parentals: mt_h; *fus*A2	Parentals: mt_1; *fus*A1
(CL6049 × LU648)1	24	34	16	30
(CL6082 × LU648)3	20	26	29	24
(CL6089 × LU648)9	30	19	20	27
(CL6099 × LU648)3	22	25	17	25
(CL6100 × LU648)10	4	3	6	7
(CL6115 × LU648)4	2	4	2	2
(CL6129 × LU648)3	27	25	22	31
(CL6130 × LU648)3	4	4	1	1
(CL6134 × LU648)2	3	1	3	3
(CL6136 × LU648)5	2	2	2	4
(CL6143 × LU648)11	4	3	5	8
(CL5001/8 × LU648)12	7	7	2	4
Total	149	153	125	166

progeny of each heterokaryon were isolated and plated on DSDM agar. Some progeny formed plasmodia in plaques and were shown to be *leu*$^-$. The remainder failed to form plasmodia in plaques and were shown to be *leu*$^+$. Thus none of the *leu*$^-$ progeny acquired the mutant phenotype and this was strong evidence that CL6089 and CL6099 did not carry extranuclear mutations.

It was concluded that all the mutant strains, except CL6111, probably carried nuclear mutations at or closely linked to the mating type locus.

Table 5. *Complementation analysis*

CL6049	CL6082	CL6089	CL6129	CL6130	CL6134	CL6099	CL6136	CL6143	CL5001/8		Assigned genotype
−	−	−	−	−	−	+	+	+	+	CL6049	*npf*B1⁻; *npf*C⁺
	−	−	−	−	−	+	+	+	+	CL6082	*npf*B2⁻; *npf*C⁺
		−	−	−	−	+	+	+	+	CL6089	*npf*B3⁻; *npf*C⁺
			−	−	−	+	+	+	+	CL6129	*npf*B4⁻; *npf*C⁺
				−	−	+	+	+	+	CL6130	*npf*B5⁻; *npf*C⁺
					−	+	+	+	+	CL6134	*npf*B6⁻; *npf*C⁺
						−	−	−	−	CL6099	*npf*B⁺; *npf*C1⁻
							−	−	−	CL6136	*npf*B⁺; *npf*C2⁻
								−	−	CL6143	*npf*B⁺; *npf*C3⁻
									−	CL5001/8	*npf*B⁺; *npf*C4⁻

\+ = hybrid plasmodium formation. − = no hybrid plasmodia.

Table 6. *Crosses of mutants with strains of various mating types*

Strains		mt_1	mt_2	mt_3	mt_4
CL6049	*npf*B1⁻	+	−	+	+
CL6082	*npf*B2⁻	+	−	+	+
CL6089	*npf*B3⁻	+	−	+	+
CL6129	*npf*B4⁻	+	−	+	+
CL6130	*npf*B5⁻	+	−	+	+
CL6134	*npf*B6⁻	+	−	+	+
CL6100	*npf*B7⁻	+	−	+	+
CL6115	*npf*B8⁻	+	−	+	+
CL6099	*npf*C1⁻	+	+	+	+
CL6136	*npf*C2⁻	+	+	+	+
CL6143	*npf*C3⁻	+	+	+	+
CL5001/8	*npf*C4⁻	+	+	+	+

\+ = hybrid plasmodium formation. − = no hybrid plasmodia.

(iii) *Complementation analysis*

The complementation of the mutants which apparently carried mutations at or closely linked to the mating-type locus was tested. A single amoebal clone of the class designated mt_h;*fus*A1 in Table 4 was selected from the progeny of each plasmodium formed by crossing the mutants with LU648. All possible mixtures of these derivative strains with the original mutants were set up in bacterial puddles. Each mixture was inoculated into five puddles which were incubated for 6 weeks at 26 °C, or 28·5° C if temperature-sensitive strains were involved. Plasmodia which arose were tested for fusion with known *fus*A1/*fus*A2 plasmodia and a number of combinations of strains was found to give rise to hybrid plasmodia. The results obtained for non-temperature-sensitive strains (Table 5) were consistent with the interpretation that the mutants each contained a mutation affecting one or other of two genes, which were designated *npf*B and *npf*C. All *npf*B$^-$ × *npf*C$^-$ combinations gave hybrid plasmodia but no *npf*B$^-$ × *npf*B$^-$ or *npf*C$^-$ × *npf*C$^-$ combinations did so. While mixtures of strains carrying different mating-type alleles routinely give hybrid plasmodia in nearly 100% of puddles, only about 40% of puddles of the complementing combinations shown in Table 5 did so. The efficiency of complementation was even lower with puddles involving temperature-sensitive strains at 28·5 °C; only one puddle of each of two combinations gave hybrid plasmodia. These were CL6100 × CL6099 and CL6115 × CL5001/8, allowing the mutant alleles *npf*B7$^-$ and *npf*B8$^-$ to be tentatively assigned to the temperature-sensitive strains CL6100 and CL6115, respectively.

Mean nuclear DNA contents of 14 different hybrid plasmodia were estimated. Values of 1·2–1·4 pg DNA/nucleus were obtained, indicating that the plasmodia were diploid (see Fig. 1).

(iv) *Effects of* npf*B and* npf*C on mating specificity*

To determine whether the *npf*B$^-$ or *npf*C$^-$ mutants had altered mating specificities they were each inoculated into bacterial puddles with strains carrying various mating-type alleles (Table 6). All the mutants formed heterozygous diploid plasmodia when crossed with LU648 (mt_1), as already shown in section (ii). Plasmodia of appropriate hybrid fusion type were also obtained from all puddles with LU862 (mt_3) and LU863 (mt_4), but preliminary tests indicated that LU688 (mt_2) formed plasmodia very inefficiently with the mutants. Wheals (1970) had also found that the efficiency of certain mt_h × mt_2 crosses was very low. Since Dee (1966) had noted that crosses between closely related strains were particularly prone to failure, a mt_2 strain which was not of Colonia genetic background was used to obtain the results shown in Table 6. This strain, i, was mixed with mt_h;*fus*A1 progeny of the mutant × LU648 crosses. Five puddles of each test were set up. All puddles involving *npf*B$^+$;*npf*C$^-$ strains gave hybrid plasmodia but no hybrids were produced with *npf*B$^-$;*npf*C$^+$ strains.

(v) *Analysis of progeny of the cross CL6111 × LU648*

Amoebal progeny clones of the plasmodium (CL6111 × LU648)3 were classified for *mt*, *fus*A and a proposed gene, *npf*A (Table 7). Clones which formed plasmodia in plaques when plated at 28·5 °C were designated mt_h;*npf*$^+$. The remaining clones were tested for the formation of plasmodia in bacterial puddles at 26 °C, and those which gave plasmodia were classified as mt_h;*npf*A1$^-$. All other clones were classified as mt_1. The segregation mt_h:mt_1 did not differ significantly from 1:1 ($P > 0{\cdot}05$), and the segregation *npf*A$^+$:*npf*A1$^-$ in mt_h clones was also not significantly different from 1:1 ($P > 0{\cdot}05$).

Table 7. *Analysis of progeny of the cross CL6111 × LU648*

Parental genotypes: CL6111 mt_h; *fus*A2; *npf*A1$^-$
LU648 mt_1: *fus*A1; *npf*A$^+$

		*npf*A$^+$	*npf*A1$^-$	totals			
mt_h	*fus*A1	9	18	27	47 mt_h	51 *fus*A1	47 *fus*A2
	*fus*A2	12	8	20			
mt_1	*fus*A1	(2)*	(2)	24	51 mt_1		
	*fus*A2	(2)	(2)	27			
		25	30	98			

* Figures in parentheses refer to a sample of only eight mt_1 clones classified for *npf*A.

All progeny were classified for their *fus*A alleles (mt_1 progeny were crossed with an appropriate mt_2 strain to give plasmodia to be tested). The segregation *fus*A1:*fus*A2 did not differ significantly from 1:1 ($P > 0{\cdot}05$).

To detect the segregation of *npf*A$^+$:*npf*A1$^-$ in the mt_1 progeny, attempts were made to cross them (at 28·5 °C) with mt_h;*npf*A1$^-$ clones. It was expected that *npf*A1$^-$ strains would be unable to cross with one another and that half the mt_1 progeny (*npf*A1$^-$) would therefore fail to form plasmodia of hybrid fusion type with mt_h;*npf*A1$^-$ strains carrying different *fus*A alleles. However, all the mt_1 clones gave rise to hybrid *fus*A1/*fus*A2 plasmodia in these crosses. To determine whether any of the mt_1 clones carried the *npf*A1$^-$ allele, it was necessary to analyse mt_h progeny of these hybrid plasmodia. Twenty amoebal clones derived from each of eight hybrid plasmodia were isolated; mt_h clones from four plasmodia showed segregation of *npf*A$^+$ and *npf*A1$^-$, but all mt_h progeny of the remaining four plasmodia carried the *npf*A1$^-$ allele. Segregation of *fus*A1 and *fus*A2 was detected in the mt_h progeny of all eight plasmodia. It was concluded that four hybrid plasmodia had arisen from mt_1;*npf*A$^+$ clones and four from mt_1;*npf*A1$^-$ clones. Thus, although the *npf*A1$^-$ allele prevented clonal plasmodium formation, it did not prevent plasmodium formation in crosses between *npf*A1$^-$ strains.

The analysis of the cross CL6111 × LU648 showed no significant deviation from free recombination of *npf*A, *mt* and *fus*A ($P > 0{\cdot}05$).

https://doi.org/10.1017/S0016672300017080 Published online by Cambridge University Press

(vi) *Test for allelism of* npf*A1*$^-$ *and* apt-*1*$^-$

In order to test for allelism of *npf*A1$^-$ and *apt*-1$^-$, amoebae of strain LU867 (*mt*$_h$;*fus*A1;*npf*A1$^-$) were crossed with LU866 amoebae (*mt*$_1$;*fus*A2;*apt*-1$^-$) and plasmodia which fused with heterozygous *fus*A1/*fus*A2 tester plasmodia were recovered. Spores were obtained from one of these crossed plasmodia (LU866 × LU867)1 and germinated. Two hundred amoebal progeny clones were isolated, plated on DSDM agar and incubated at 26 °C for 2 weeks. It was expected that, if *npf*A1$^-$ and *apt*-1$^-$ were mutant alleles at two unlinked loci, approximately one-eighth of progeny clones would form plasmodia in plaques (*mt*$_h$;*npf*A$^+$;*apt*-1$^+$ recombinants), one-eighth would form a few plasmodia per plate (*mt*$_h$;*npf*A1$^-$; *apt*-1$^+$) and the remainder would fail to form plasmodia. The absence of clones able to form plasmodia in plaques would indicate that *npf*A1$^-$ and *apt*-1$^-$ were alleles of the same locus, or of closely linked loci. Twenty-three clones formed plasmodia in plaques (11 *fus*A1: 12 *fus*A2) and 33 formed a few plasmodia per plate. Neither of these numbers differed significantly ($P > 0{\cdot}05$) from the expectation for two unlinked loci.

4. DISCUSSION

The amoebal and plasmodial phases of the life-cycle of *P. polycephalum* show differences of morphology, behaviour and biochemistry. Thus it might be expected that mutation of any one of many genes could result in the failure of plasmodium formation. This expectation was reinforced when Wheals (1973) isolated, from a *mt*$_h$ strain, four mutants which failed to form plasmodia within clones, and which apparently all complemented with one another. One mutant (APT1) was extensively analysed and shown to carry a mutation unlinked to the mating-type locus.

The current work has identified mutations in only three genes. The use of an enrichment step in the isolation procedure restricted the mutant classes recoverable to those in which amoebae, or small multinucleate cells, retained the ability to proliferate at nearly the same rate as wild-type amoebae. (The fact that all the mutants isolated in this work grew as uninucleate amoebae may indicate that multinucleate cells can no longer proliferate.) The enrichment procedure probably limited the mutants recovered to those defective in the *initiation* of plasmodium formation, and it follows that there are probably very few loci involved in this process. It may be necessary to dispense with the enrichment in order to isolate mutants with defects of later stages in plasmodium formation. However, mutants isolated in this study may not be entirely different from those isolated by Wheals, since reconstruction experiments indicate that enrichment for APT1 amoebae is readily achieved in mixtures with CL amoebae.

The analysis of the temperature-sensitive mutant CL6111 indicated that a mutation at a locus (*npf*A) unlinked to *mt* affected the ability of *mt*$_h$ amoebae to form plasmodia in plaques, but did not affect crossing, even when both strains involved in a cross carried the same mutant allele. This may suggest that the formation of plasmodia in plaques requires the function of some product not

necessary in the formation of crossed plasmodia, though it would seem likely that most functions involved in plasmodium formation are the same for both selfing and crossing. CL6111 and other strains carrying the *npf*A1$^-$ allele provide an alternative to the use of APT1 in the analysis of certain recessive mutations expressed only in the plasmodial phase (Dee *et al.* 1973), and an alternative to the use of CLd in the isolation of plasmodial mutants (Cooke & Dee, 1975).

Twelve of the thirteen strains analysed in this work carried mutations at or closely linked to the mating type locus, and the crosses between these mutants defined two complementation groups. However, more than two genes could have been involved, since cell fusion was not demonstrated in those combinations of strains which did not complement; if cells failed to fuse there would be no possibility of intracellular complementation.

The complementation of strains designated *npf*B$^-$ and *npf*C$^-$ gave rise to diploid plasmodia in every case tested, rather than haploid heterokaryons. Since this is the normal behaviour of strains carrying different mating type alleles, the behaviour of these strains was consistent with their carrying mutations at the mating-type locus.

Early studies on *P. polycephalum* suggested that the role of the mating-type locus in the regulation of plasmodium formation was the control of cell and nuclear fusion; plasmodia were formed only by cell and nuclear fusion of amoebae carrying different mating-type alleles (Dee, 1966). An alternative possibility was that amoebae of the same mating type were able to fuse but unable to develop into plasmodia (Dee, 1966; Adler & Holt, 1975). However, no conclusive evidence of amoebal fusions within clones has been reported. Studies on nuclear DNA content showed that nuclear fusion was not involved in plasmodium formation in mt_h clones but left open the question of whether cell fusion occurred (Cooke & Dee, 1974). Adler & Holt (1975) showed that amoebae containing apparently diploid nuclei heterozygous for mating type formed diploid plasmodia within plaques; thus in these clones also there was no nuclear fusion. Adler & Holt concluded that the presence of two different mating type alleles within the same cell promoted plasmodium formation, and suggested that mt_h might consist of two different mating-type alleles, closely linked on the same chromosome. A cinematographic analysis of plasmodium formation in clones of mt_h amoebae demonstrated plasmodium formation without cell or nuclear fusion (Anderson *et al.* 1976). Amoebal fusion is thus ruled out as an absolute requirement in plasmodium formation, but the involvement of the mating-type locus in the control of amoebal fusions is not excluded; plasmodium formation might normally be triggered by cell fusion, but the presence of products of two mating types within the same cell might bypass the requirement for fusion.

The results obtained in the present work are consistent with a model similar to that described by Adler & Holt. If the mt_h strains from which the mutants were derived each contained two mating-type alleles, mutation causing loss of expression of one allele would result in a strain expressing only the other mating type. Two complementation groups would be predicted, as was found for the mutants carrying mutations associated with the mating-type locus. The failure of *npf*B$^-$;

*npf*C$^+$ strains to cross with a mt_2 tester strain would suggest that the *npf*C$^+$ allele might be identical to the mt_2 allele.

We wish to thank Mrs Jennifer Foxon for excellent technical assistance and Dr Dave Cooke for much useful discussion. R. W. A. was in receipt of a Science Research Council Studentship, which is acknowledged with thanks.

Footnote added in proof. Mutants which may correspond to the strains designated *npf*B$^-$ and *npf*C$^-$ have also been isolated from mt_h strains by L. S. Davidow and C. E. Holt (personal communication).

REFERENCES

ADLER, P. N. & HOLT, C. E. (1975). Mating type and the differentiated state in *Physarum polycephalum*. *Developmental Biology* **43**, 240–253.

ANDERSON, R. W., COOKE, D. J. & DEE, J. (1976). Apogamic development of plasmodia in the Myxomycete *Physarum polycephalum*: A cinematographic analysis. *Protoplasma* **89**, 29–40.

CARLILE, M. J. & DEE, J. (1967). Plasmodial fusion and lethal interaction between strains in a Myxomycete. *Nature* **215**, 832–834.

COOKE, D. J. (1974). Studies on the Colonia isolate of *Physarum polycephalum*. Ph.D. thesis, University of Leicester.

COOKE, D. J. & DEE, J. (1974). Plasmodium formation without change in nuclear DNA content in *Physarum polycephalum*. *Genetical Research* **23**, 307–317.

COOKE, D. J. & DEE, J. (1975). Methods for the isolation and analysis of plasmodial mutants in *Physarum polycephalum*. *Genetical Research* **24**, 175–187.

DEE, J. (1960). A mating type system in an acellular slime mould. *Nature* **185**, 780–781.

DEE, J. (1966). Multiple alleles and other factors affecting plasmodium formation in the true slime mould *Physarum polycephalum* Schw. *Journal of Protozoology* **13**, 610–616.

DEE, J. (1975). Slime moulds in biological research. *Science Progress* **62**, 523–542.

DEE, J. & POULTER, R. T. M. (1970). A gene conferring actidione resistance and abnormal morphology on *Physarum polycephalum* plasmodia. *Genetical Research* **15**, 35–41.

DEE, J., WHEALS, A. E. & HOLT, C. E. (1973). Inheritance of plasmodial valine requirement in *Physarum polycephalum*. *Genetical Research* **21**, 87–101.

MITTERMAYER, C., BRAUN, R. & RUSCH, H. P. (1965). The effect of actinomycin D on the timing of mitosis in *Physarum polycephalum*. *Experimental Cell Research* **38**, 33–41.

MOHBERG, J. (1976). Nuclear DNA content and chromosome number throughout the life cycle of the Colonia strain of the Myxomycete *Physarum polycephalum*. (Submitted for publication.)

MOHBERG, J., BABCOCK, K. L., HAUGLI, F. B. & RUSCH, H. P. (1973). Nuclear DNA content and chromosome numbers in the Myxomycete *Physarum polycephalum*. *Developmental Biology* **34**, 228–245.

MOHBERG, J. & RUSCH, H. P. (1971). Isolation and DNA content of nuclei of *Physarum polycephalum*. *Experimental Cell Research* **66**, 305–316.

POULTER, R. T. M. (1969). Senescence in the Myxomycete *Physarum polycephalum*. Ph.D. thesis, University of Leicester.

POULTER, R. T. M. & DEE, J. (1968). Segregation of factors controlling fusion between plasmodia of the true slime mould *Physarum polycephalum*. *Genetical Research* **12**, 71–79.

RASCH, E. M., BARR, H. J. & RASCH, R. W. (1971). The DNA content of sperm of *Drosophila melanogaster*. *Chromosoma* **33**, 1–18.

ROSS, I. K. (1957). Syngamy and plasmodium formation in the Myxogastres. *American Journal of Botany* **44**, 843–850.

WHEALS, A. E. (1970). A homothallic strain of the Myxomycete *Physarum polycephalum*. *Genetics* **66**, 623–633.

WHEALS, A. E. (1973). Developmental mutants in a homothallic strain of *Physarum polycephalum*. *Genetical Research* **21**, 79–86.