

Analysis of cellular events during plasmodium

development in *Physarum polycephalum*

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ABSTRACT

In *Physarum polycephalum*, uninucleate, haploid amoebae develop into multinucleate syncytial plasmodia. Plasmodium development is controlled by the mating-type locus *matA*. Sexual development involves the fusion of pairs of amoebae carrying different alleles of *matA*; fusion between amoebae carrying the same allele of *matA* does not lead to plasmodium formation. Apogamic development is caused by mutations at this locus.

Time-lapse cinematographic analysis of *matA*-heteroallelic and *matA*-homoallelic cultures indicated that amoebae were able to fuse at any age. In *matA*-heteroallelic cultures, amoebal fusion was followed by nuclear fusion, in interphase, to give a diploid zygote. The zygote underwent an extended period of growth before forming a binucleate plasmodium by mitosis unaccompanied by cytokinesis. During this cell cycle, the cells lost the ability to transform into flagellates and became irreversibly committed to development. Immunofluorescence microscopy showed that the change from amoebal to plasmodial microtubule organisation began during this cell cycle. In *matA*-homoallelic fusion cells, the cell cycle was not extended and there were no alterations in microtubule organisation.

In apogamic strains, single haploid amoebae could develop into haploid plasmodia; developing amoebae entered an extended cell cycle ending in the formation of a binucleate plasmodium. As in sexual development, growth continued during this cell cycle, ability to undergo the amoeba-flagellate transformation was lost, the developing cell became committed to development and microtubule organisation began to alter.

Development was analysed in two apogamic strains carrying additional mutations blocking plasmodium development. In both strains, development began with an extended cell cycle, leading to the formation of a binucleate plasmodium; development became abnormal shortly after this time. In one strain, the mutation had apparently affected the cytoskeleton or the cell membrane. In the other strain, nuclear structure appeared to be affected by the mutation.

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FOREWORD

Ode to a Slime Mould

Physarum is

the singular aroma of a cool damp forest
the vibrant yellow of a saucy lemon
the relentless rhythms of nature's multiple cycles
the storehouse of countless answers
the tantalizing challenge that lures us on

kram namleda
(Mark Adelman)

1985

CHAPTER 1

General Introduction

1.1 The Myxomycetes

Myxomycetes have an unusual life cycle in which two prolonged vegetative phases are linked by two irreversible transitions. One irreversible transition is that of uninucleate, microscopic, vegetative amoebae (or myxamoebae; Fig. 1.1a) into multinucleate, syncytial, vegetative plasmodia (Fig. 1.1b). It is this multinucleate, syncytial plasmodium that separates the Myxomycetes from the Acrasiales or cellular slime moulds (e.g. *Dictyostelium discoideum*); in the Acrasiales, the pseudoplasmodium, or "slug", is a multicellular structure. The other irreversible transition of the Myxomycete life cycle is the differentiation of plasmodia into spores during sporulation; the spores hatch to yield amoebae and, thus, complete the life cycle.

1.1.1 Evolutionary relationships

The origin of the Myxomycetes and their relationship to other organisms is obscure. It has been argued that Myxomycetes are related to fungi since they sporulate (see Alexopoulos, 1982); however, as has been pointed out by several authors (see Alexopoulos, 1982), the Myxomycetes could just as easily be related to protozoa since neither group possesses a cell wall in the motile stages and all Myxomycetes and many protozoa feed by phagocytosis. Studies of the spore wall showed that the Myxomycete spore wall was of very different composition to that of both fungi and

protozoa (reviewed by Alexopoulos, 1982). Recent comparisons between the sequences of the small subunit of ribosomal RNA from *Physarum polycephalum* and those from other lower eukaryotes (e.g. *Dictyostelium*, *Tetrahymena* and *Saccharomyces*) indicated that *P. polycephalum* diverged from other eukaryotes about 1.5 - 2.0 billion years ago; that was about 200 - 300 million years before the appearance of the cellular slime mould *Dictyostelium*, which on this sequence evidence, is its closest relative outside the true, acellular, slime moulds (Johansen et al., 1988).

1.1.2 The diversity of the Myxomycetes

The Myxomycetes have been known to biologists for some 300 years and there are at least 450 known species (Gray and Alexopoulos, 1968; Madelin, 1984). Since, the plasmodia often sporulate in exposed places, such as on bark or soil, they are easily found and much of the early work, therefore, involved descriptions of the gross morphology of the fruiting bodies (or spores). The morphology of the spores has been used as a major character in the classification of Myxomycete species. With the exception of three exosporous species, the spores of all the Myxomycetes species are contained within spore cases. Spores are generally about 10 μ m in diameter but may be as small as 4 μ m or as large as 20 μ m (Gray and Alexopoulos, 1968). Each species has a range of spore sizes, however, and so this characteristic alone is not sufficient to distinguish between species.

The pattern of wall ornamentation of the spore cases has also been used to help classify Myxomycete species. The walls

of the spores may be smooth, or ornamented with spines or warts; however, the pattern of wall ornamentation may vary with the conditions under which the spores formed and so care must be taken when using this characteristic for classification (Alexopoulos, 1963; Gray and Alexopoulos, 1968). Another characteristic of spores that has been used in separating the Myxomycetes into Orders, is colour. Spores of the orders Physarales and Stemonitales are usually black while those of the Trichiales and Echinostales are usually brown or yellow (Gray and Alexopoulos, 1968).

Alexopoulos (1960) used the size and morphology of the syncytial plasmodium to divide the Myxomycetes into three types. Phaneroplasmodia, (e.g. the plasmodia of *Physarum polycephalum*) the most common, were defined as being macroscopic with thick veins at the rear which merged into a fan at the front; reversible cytoplasmic streaming occurred in the veins. Many of these plasmodia were pigmented with colours ranging from white to yellow, red, olive-green and blue (Alexopoulos, 1960, 1982). The aphanoplasmodia, (e.g. the plasmodia of *Stemonitis herbatica*), the second type described by Alexopoulos (1960), were also macroscopic and had a delicate, flat, branched structure with no pigmentation, although some became pigmented on sporulation. This type also showed reversible cytoplasmic streaming in the veins. Some intermediates have been found between these two types (e.g. the plasmodia of *Arcyria cinerea*); in nature there may be a whole range of species with plasmodia of all intermediate types. The last type of plasmodia described by Alexopoulos (1960), the protoplasmodia, (e.g. the plasmodia

of *Echinostelium minutum*) were normally microscopic plasmodia with tiny fruiting bodies. These plasmodia also showed streaming but had no distinct veins. Unlike the other two types where fusion occurred between plasmodia, protoplasmodia split apart when large and did not fuse when they met. Plasmodia of all types were covered in a thick slime sheath and all were actively mobile.

The variation in the life cycle has been examined in several species of Myxomycetes and it has been found that the transition from amoeba to plasmodium can be a heterothallic or non-heterothallic (selfing) process. Different isolates of the same species may differ with some isolates being heterothallic and others non-heterothallic (Collins and Betterley, 1982). In *Physarum polycephalum*, all natural isolates are heterothallic but selfing strains have arisen during culture in the laboratory; in *Didymium iridis* both heterothallic and non-heterothallic natural isolates are known. In both these species, heterothallic plasmodium development is governed by allelic differences in a mating-type gene (Dee, 1960; Collins and Betterley, 1982); in *P. polycephalum*, however, other genes are also involved in the control of plasmodium formation. Heterothallic development involves the fusion of amoebae, followed by nuclear fusion to give a zygote; the zygote then develops into a plasmodium. In selfing isolates, plasmodium development occurs within clones of amoebae but it is not known whether clonal development is apogamic (i.e. a single amoeba can develop into a plasmodium) or homothallic (i.e. involves fusion of amoebae carrying the same allele of the

mating-type gene).

1.1.3 Geographic distribution

Myxomycete plasmodia have been found in most parts of the world. At least one-third of the species are worldwide in their distribution (e.g. *Fulgio septica*; Gray and Alexopoulos, 1968) but some species have been found only in tropical (e.g. *Perichaena microspora*) or temperate regions (e.g. *Hemitrichia clavata*) or in the Far East (e.g. *Arcyria glauca*; data from Gray and Alexopoulos, 1968). However, more detailed analysis of the ecology and abundance of the Myxomycetes may alter these distribution patterns. The time of year at which sporulation occurs varies with latitude; fruiting bodies have been found all the year round in Jamaica but only in the summer months in the northern United States (Gray and Alexopoulos, 1968).

Little was known about the numbers of myxamoebae present in soil, where they may have considerable ecological importance, until the study of Feest and Madelin (1985) who isolated myxomycetes from a variety of soils including those of evergreen forests, beech woods, grasslands (e.g. meadows and lawns) and deserts. In their study, Myxomycete numbers, defined as "plasmodium forming units" (pfu) per gram of soil, ranged between a minimum of 2 pfu / gram of soil (Death Valley, U.S.A.) and a maximum of 9000 pfu / gram of soil (from a mangold field in Germany); the normal level was between 10 and 1000 per gram. In general, more Myxomycetes were present in grassland and agricultural soils than in woodland (Feest and Madelin, 1985). In common with the

situation for fruiting plasmodia, the number of plasmodium forming units was subject to seasonal variation in temperate climates (Feest and Madelin, 1988a; 1988b). A plasmodium forming unit was probably a microcyst or amoeba (Madelin, 1984; Feest and Madelin, 1985).

1.1.4 Ecology

Spores germinate to release one or several amoebae or flagellates. Amoebae have been successfully hatched from spores over 60 years old (Madelin 1984) but spore viability declines with age. Amoebae transform into biflagellated cells in moist conditions and revert to amoebae in dry conditions. Amoebae feed on bacteria and fungal spores in the soil, but in many species the flagellates do not appear to be able to feed. In adverse conditions, for example nutrient deprivation, the amoebae reversibly transform into resistant microcysts with tough, smooth walls. The encystment process takes several hours, and the cysts have been shown to remain viable for about a year. On excystment, a single amoeba emerges from the cyst via a small pore in the cyst wall.

Plasmodia ingest chiefly microbial prey; including bacteria, fungal spores, hyphae and fruiting bodies, algae and Myxomycete amoebae, microcysts, flagellates and spores. Many plasmodia have been shown to secrete a wide range of extracellular enzymes (Kilpatrick and Stirling, 1977; see Madelin, 1984 for a more complete list) which are presumably used for breaking down materials such as wood or fungal hyphae; the products of extracellular digestion are taken into the cell by direct absorption and by pinocytosis. Both

amoebae (Konijn and Koevenig, 1971; Jacobson, 1980) and plasmodia (Knowles and Carlile, 1978a; 1978b) show chemotactic responses towards likely food sources such as carbohydrates and bacterial extracts. Plasmodia reversibly transform into resistant sclerotia in adverse conditions. Sclerotia can remain viable for several years and will germinate to yield plasmodia in good conditions. In certain environmental conditions, the plasmodia sporulate and nuclei are encapsulated in the spores. In most of the species examined (Laane and Haugli, 1976; Mohberg, 1982) meiosis occurs within the spores. In some species (e.g. *P. polycephalum*), 3 of the 4 meiotic products normally disintegrate following meiosis (Laane and Haugli, 1976).

In the laboratory, both amoebae (Fig. 1.1a) and plasmodia can be grown on bacterial lawns. Colonies of amoebae, which appear as clear round patches on the surface of agar plates, arise by repeated cell cycles. In some strains, the amoebae can also grow in liquid axenic culture. Amoebae divide by mitosis with cytokinesis but in syncytial plasmodia, the nuclei enter mitosis synchronously and mitosis is not accompanied by cytokinesis. A single plasmodium about 9cm in diameter can contain 10^9 nuclei which divide with almost perfect synchrony; plasmodia have been grown which cover an area of several square metres (Achenbach, 1989). In the laboratory, plasmodia of some species can be cultured axenically as veined macroplasmodia on solid surfaces (Fig. 1.1b) or under liquid, and also in liquid, axenic, shaken culture as veinless microplasmodia, each containing several hundred nuclei. Unlike the situation in many amoebae (e.g.

those of *P. polycephalum*; McCullough et al., 1978 and Dee et al., 1989), axenic growth of plasmodia does not appear to be the result of a mutation.

1.2 *Physarum polycephalum*

Of all the Myxomycetes, *Physarum polycephalum* has been the most intensively studied and it is the species of choice for this work. Both amoebae and plasmodia of *P. polycephalum* have proved easy to culture in the laboratory and the plasmodia can be induced to sporulate with little difficulty. The genetics of plasmodium formation have been well characterised in this species. There are great structural differences between amoebae and plasmodia which are related to substantial alterations in cellular organisation and gene expression that occur during the transition from amoeba to plasmodium. At the start of this work, however, very little was known about the timing and sequence of these alterations.

1.2.1 Genetic analysis of development in *P. polycephalum*

Early experiments by Dee (1960) indicated that plasmodia developed in *P. polycephalum*, only when mating occurred between amoebae carrying different alleles of the mating-type gene *mt*; this gene has since been renamed *matA* (Youngman et al., 1979). Fourteen different alleles of *matA* have now been characterised in *P. polycephalum* (Collins and Tang, 1977). Almost every isolate of *P. polycephalum* has been found to carry two new alleles of *matA*, suggesting that the number in nature might be much larger; the alleles of this gene are

intercompatible. In *Didymium iridis*, 18 different alleles of the mating-type gene have been characterised, so a multiallelic system may be a feature of plasmodium formation in Myxomycetes (Collins, 1981; Madelin, 1984).

Selfing strains of *P. polycephalum*

Dr. H. A. von Stosch (reviewed by Dee, 1982) was the first to describe a strain of *P. polycephalum* in which plasmodia developed within clones of amoebae (i.e. the amoebae could self); this first "Colonia" isolate arose during culture in the laboratory rather than in the wild. The selfing ability of Colonia strains was stably inherited by many generations of clonal progeny (Wheals, 1970). Unlike sexual development, selfing is temperature sensitive; by using this characteristic, it was possible to mate amoebae from Colonia strains with sexually compatible, heterothallic amoebae and thus carry out genetic analysis. During crosses of Colonia strains with heterothallic strains, Wheals (1970) obtained a segregation of 1:1 between selfing ability and the heterothallic allele of the *matA* gene; he, therefore, postulated that Colonia strains arose by mutation at *matA* and carried a new *matA* allele, *matAh*.

Selfing strains have now been isolated from several different *matA* backgrounds (Adler & Holt 1977; Gorman *et al.*, 1979, Honey *et al.*, 1981) and further genetic analysis has shown that selfing strains retain the mating specificity of the heterothallic strain from which they were isolated (Adler and Holt, 1977). The Colonia strain CL (Colonia Leicester) was shown by Anderson (1979), to cross with all heterothallic strains tested except those carrying *matA2*; Anderson (1979)

concluded that CL had arisen as a result of a mutation in a *matA2* strain. The mutation causing selfing thus appeared to be affecting a function other than *matA* specificity but mapping at the same locus. Selfing strains were designated as carrying a *gad* mutation (greater asexual differentiation; Adler and Holt, 1977) and their genotypes are written in the form *matAx gady* where *matAx* is the heterothallic allele from which the selfing strain was isolated. CL can thus be said to have the genotype *matA2 gadh* but *matAh* is often used. Two selfing strains have been utilised in the present study; details are given in Section 2.2.

Heterothallic development in *P. polycephalum*

Dee (1978) and Youngman *et al.* (1979) both demonstrated that heterothallic plasmodium formation was influenced, not only by *matA* but also by another mating-type gene, *matB*; *matB*, like *matA*, was multiallelic and 13 alleles have so far been identified (Kirouac-Brunet *et al.*, 1981). Youngman *et al.* (1981) suggested, using evidence from experiments assaying the kinetics of mating and from microscopic analysis of mating cultures, that *matB* influenced the frequency of amoebal fusion but had no influence on the subsequent development of the fusion cell. This hypothesis was later confirmed by Shipley and Holt (1982) who showed that, when two strains of amoebae carrying different alleles of the *matB* gene were mixed, the level of cell fusions achieved was approximately 1000 times higher than when the mixed strains carried the same allele of *matB*. Youngman *et al.* (1981) further showed that, in mixtures of strains heteroallelic for *matB* but homoallelic for *matA*, cell fusion occurred but

plasmodia did not develop. It therefore appeared that amoebal fusion was controlled by *matB* and development of the fusion cell was under the control of *matA*; *matA* apparently had no effect on amoebal fusion.

A third mating-type gene, which also influences the frequency of amoebal fusion, has been identified. This gene was originally named *imz* (ionic modulation of zygote formation) because it acted in a pH-related way. Between pH5.4 and pH6.0, strains carrying different *imz* alleles mated faster than strains carrying identical *imz* alleles; *imz* did not affect the frequency of amoebal fusion at pH values outside this range (Shinnick et al., 1978, Kawano et al., 1987). Initially two alleles were found, *imz-1* and *imz-2*, with *imz-2* being dominant to *imz-1* (Shinnick et al., 1978). A later study (Kawano et al., 1987) identified another allele, *imz-3*, and showed that all the alleles were, in fact, codominant; *imz* has been renamed *matC* (Kawano et al., 1987).

Mutations blocking plasmodium formation

Strains which carry a mutation blocking plasmodium formation have been isolated from selfing strains. The developmental mutants isolated by the different research groups have been given different names; those of Anderson and Dee (1977) were called *npf* (no plasmodium formation) mutants, while those of Wheals (1973) and Davidow and Holt (1977) were *apt* (amoebal plasmodial transition) mutants and those of Honey et al. (1979) were *dif* (differentiation) mutants. The mutations carried by these blocked strains have been analysed genetically and fall into two classes: 1) Those which map at *matA*. 2) Those which map to other loci.

The loci *difA* (Honey *et al.*, 1979), *npfB* and *npfC* (Anderson and Dee, 1977), *npfD* and *npfE* (Anderson and Holt, 1981) have all been demonstrated to be tightly linked to *matA*. Further genetic analysis (Anderson *et al.*, 1989) suggested that mutations at the *npfB* and *npfC* loci affected gene functions necessary for plasmodium development but different from the function controlled by *matA*. The phenotypes of all the strains carrying the *matA*-linked, *npf* and *dif* mutations were very similar; development did not appear to be initiated in any of them and only amoebae were present during culture at any temperature. The location of these mutations close to *matA* indicated that this complex genetic region played a vital role in the initiation of development.

Wheals (1973) isolated, from a *Colonia* strain, 4 strains blocked in apogamic development; he named these strains APT1 to APT4. Genetic analysis indicated that all 4 mutations had occurred at different loci, and at least one mutation (*apt1*) was in a gene unlinked to *matA*; unfortunately no strains carrying the other *apt* mutations still exist. Other workers subsequently isolated more strains carrying mutations affecting plasmodium formation that were unlinked to *matA*. These mutations did not affect initiation of development, but apparently affected processes essential for the formation of a normal plasmodium. In most of these strains, development began but halted at different stages with a wide variety of terminal phenotypes. Two strains carrying *matA*-unlinked mutations affecting plasmodium development have been utilised during part of the work described in this thesis; both

strains were isolated by Dr. R.W. Anderson at the University of Sheffield. Details of these two strains are given in Section 2.2.

1.2.2 DNA content in *P. polycephalum*

In *P. polycephalum*, the nuclei of amoebae have been shown to contain about 0.6pg of DNA, distributed between about 40 very small chromosomes (Mohberg, 1982). *Didymium iridis* also has many small chromosomes (Collins and Betterley, 1982), so this may be a feature of Myxomycetes in general. Plasmodia formed by heterothallic mating had twice as much DNA per nucleus as amoebae; i.e. amoebae were haploid and plasmodia were diploid (Mohberg, 1982; Collins and Betterley, 1982). Initial studies on the DNA content of two selfing *Colonia* strains suggested that these strains were aneuploid (Mohberg et al., 1973) and did not support the suggestion of Wheals (1970) that *Colonia* was homothallic (i.e. diploid plasmodia were formed by fusion of haploid amoebae of the same mating-type). Subsequent analysis of the DNA content of amoebae and plasmodia from the *Colonia* strain CL (Cooke and Dee, 1974) indicated that both vegetative phases had the same DNA content.

1.2.3 Events during development in *P. polycephalum*

Commitment, the inducer and mating competence

Plasmodium formation in most selfing strains was found to be temperature sensitive; e.g. in the strain CL, development occurred only at temperatures below about 29°C and CL amoebae

could be cultured at 30°C without development. Youngman *et al.* (1977) set up cultures of CL amoebae on bacterial lawns and incubated them at a temperature where development would occur. At various times after inoculation, they resuspended the cells, diluted them and replated them on assay plates. At the early timepoints, all the cells formed amoebal colonies when replated but at later timepoints, some of the cells gave rise to plasmodia when replated. Such cells were termed "plateable plasmodia" and were said to be irreversibly committed to plasmodium formation at the time of replating (Youngman *et al.*, 1977). The time at which the first committed cells appeared depended upon the medium used, the incubation temperature, the initial cell density, and the numbers of bacteria present. The results of Youngman *et al.* (1977) did not indicate whether committed cells were uninucleate or multinucleate. Using a similar method to Youngman *et al.* (1977), Burland *et al.* (1981) determined the number of committed cells present in a CL culture at various times after inoculation. They also counted, by microscopic analysis, the number of multinucleate cells present at each timepoint. At each timepoint where committed cells were detected, there were fewer multinucleate cells than committed cells. Burland *et al.* (1981) concluded, therefore, that commitment occurred in uninucleate cells; this result was later confirmed by Collett *et al.* (1983).

Youngman *et al.* (1977) also concluded that a diffusible inducer was involved in the initiation of development. They placed sparse cultures of CL amoebae on Nuclepore filters with 0.2µm pores through which cell processes could not

reach. These filters were placed over dense cultures of vegetative amoebae and the sparse CL cultures were assayed for committed cells at various times after inoculation; the underlying dense amoebal cultures accelerated development in the sparse CL cultures, suggesting that one or more extracellular factors were influencing development. The inducer produced by *Didymium iridis* amoebae has also been shown to influence development in *P. polycephalum* (Shipley & Holt, 1982). Partial purification of the active substance from cultures of *D. iridis* indicated that it had a molecular weight of about 120,000 Daltons and was possibly a glycoprotein (Nader *et al.*, 1984).

Pallotta *et al.* (1979) used experimental techniques similar to those of Youngman *et al.* (1977) to show that heterothallic plasmodium development commenced at the same cell density no matter how many bacteria were present; thus mating was not influenced by food supply. In another experiment, Pallotta *et al.* (1979) placed a sparse culture containing two mating compatible strains of amoebae on a Nuclepore filter, over a dense culture of amoebae; the dense culture accelerated mating in the sparse culture, suggesting that, as in apogamic development, one or more extracellular factors were influencing mating. The idea that amoebae produced substances that influenced mating had been suggested by Ross (1957). Shipley and Holt (1982) showed that amoebae had to become "competent" to mate before they were able to undergo amoebal fusion; competence was acquired during exponential growth in clonal cultures. In addition, Shipley and Holt (1982) noted that competent cells induced competence

in a low density culture when the two cultures were separated by the same type of Nuclepore filters as were used by Pallotta *et al.* (1979) and Youngman *et al.* (1977). The results suggested that the same substance might induce mating competence and influence plasmodium formation. Only partial purification of the inducer has been achieved (Nader *et al.*, 1984) and it is not known whether the same molecule is involved in all types of induction.

Amoeba-flagellate transformation

In moist conditions, amoebae can reversibly transform into biflagellates which swim with the flagella foremost. Flagellates possess one long (anterior) flagellum and one short (posterior) flagellum which is usually pressed against the side of the cell and is difficult to observe. Wright *et al.* (1979) demonstrated that the two centrioles acted as the basal bodies for the flagella. The centrioles usually duplicated and divided shortly before mitosis (Havercroft and Gull, 1983) but this can not happen when the centrioles are acting as basal bodies for the flagella. Flagellates, therefore, do not undergo mitosis. Plasmodia cannot transform into flagellates. By comparing the proportion of committed cells in a developing culture with proportion of cells unable to flagellate, Blindt *et al.* (1986) showed that ability to undergo the amoeba-flagellate transformation was lost by uninucleate cells several hours before commitment.

Analysis of plasmodium formation by time-lapse cinematography

The observation that amoebae and plasmodia of the *Colonia*

strain CL were both haploid suggested that plasmodium development was not homothallic (Cooke and Dee, 1974). It was suggested that plasmodium development might be apogamic (i.e. a single amoeba developing into a plasmodium) or might involve coalescence (i.e. amoebal fusion without nuclear fusion). In order to investigate the origin of plasmodia formed within colonies of CL amoebae, Anderson *et al.* (1976) undertook a time-lapse cinematographic study of development in CL. They observed the formation of binucleate plasmodia by mitosis without cytokinesis in large uninucleate cells. Larger plasmodia arose from the binucleate cells by rounds of synchronous mitoses and by fusions between multinucleate plasmodia (Fig. 1.2b). Unfortunately, Anderson *et al.* (1976) were unable to trace the developing uninucleate cells back to their origin as amoebae. They concluded that development was probably apogamic but they could not rule out the possibility that amoebal fusion was involved in clonal plasmodium formation.

Ross (1957) had observed that, in heterothallic plasmodium formation, cell fusion was followed by nuclear fusion during interphase, giving rise to a diploid zygote. Holt and Huttermann (1979) analysed time-lapse films of heterothallic plasmodium development. They confirmed the observations of Ross (1957) and additionally showed that, some time after nuclear fusion, the diploid zygote underwent mitosis without cytokinesis to become a binucleate diploid plasmodium (Fig. 1.2a). Larger plasmodia arose by rounds of synchronous mitoses and by fusions between multinucleate plasmodia. Holt and Huttermann (1979) also analysed time-lapse films of the

events in mixtures of cells homoallelic for *matA* and showed that although amoebal fusions occurred, nuclear fusion did not occur during interphase. In *matA*-homoallelic fusion cells, the nuclei were sometimes observed to enter mitosis synchronously some hours after cell fusion (Holt and Huttermann, 1979; Holt *et al.*, 1979). Nuclear fusion occurred at mitosis and two diploid amoebae were formed following cytokinesis. Unfortunately the films of Holt and Huttermann (1979) cannot be used to give a complete picture of the events that occurred in mixtures of strains either heteroallelic or homoallelic for *matA* because individual cells were only filmed for short periods of time, thus making it impossible to trace the origins or subsequent fates of the cells observed. Diploid amoebae produced in mixtures of strains homoallelic for *matA* were first isolated by Youngman *et al.* (1981); such amoebae have proved useful in genetic analysis by allowing dominance and complementation tests on mutants isolated in amoebae (e.g. Anderson and Youngman, 1985; Dee *et al.*, 1989).

1.2.4 Differences between amoebae and plasmodia

Differences in protein synthesis and gene expression

Using 2-dimensional gel-electrophoresis, Turnock *et al.* (1981) showed that 26% of 306 abundant proteins, synthesised by amoebae and plasmodia of strain CL under identical growth conditions, were phase specific; 36 were amoeba-specific and 43 were plasmodium-specific. In addition to this, 54 (18%) of the proteins visualised showed much higher levels of

expression in one of the two vegetative phases, although they were present in both phases; many of these differences were thought to be due to post-transcriptional and post-translational modifications. Pallotta et al. (1984) found differences in the pattern of proteins following two-dimensional gel-electrophoresis of the proteins present in plasmamembranes isolated from amoebae and plasmodia.

Following on from this work, several groups have looked for differences in the mRNAs synthesised in amoebae and plasmodia. Cox (1986) reported finding several differentially expressed mRNAs but no mRNAs that were only present in either of the two vegetative phases. Pallotta et al. (1986) and Sweeney et al. (1987) identified cDNA clones that detected mRNAs in either amoebae or plasmodia but not both; these cDNA probes were assumed to be detecting phase-specific genes. The experimental techniques followed by Pallotta et al. (1986) and Sweeney et al. (1987) was very similar; poly (A)⁺ RNA was isolated from amoebae and plasmodia and cDNA libraries were made from this RNA. The libraries were screened against RNA from amoebae and plasmodia to detect any differences in gene expression. Pallotta et al. (1986) found that 16% of cDNAs were plasmodium specific and 2.3% were amoeba specific. The results of Sweeney et al. (1987) were slightly different; 5% of clones were plasmodium specific and 5.7% were amoeba specific. One of the main drawbacks of these studies was that they could only detect messages which represented more than 0.1% of the total mRNA in the cells; for example, Pallottta et al. (1986) identified a cDNA clone that hybridised to a message that represented 4.8% of the mRNA in plasmodia. Many

of the cDNA clones identified by both groups were sequenced (Sweeney, 1987; D. Pallotta, personal communication) but the sequences found did not share significant homology with any known protein sequences. No comparison has been made of the sequences found by the two groups, so it is not clear how many were common to both investigations. It seems likely that, since a similar experimental approach was used by both groups, at least some genes were identified by both groups.

Blindt *et al.* (1986), enriched for cells unable to transform into flagellates by passing a population of developing CL cells which had been induced to flagellate, down a glass bead column; the non-flagellates remained on the column and could be harvested. Since ability to flagellate was lost shortly before commitment, a population enriched for cells unable to flagellate was also enriched for committed cells. Using the method of Blindt *et al.* (1986), Sweeney *et al.* (1987) carried their investigation further. They obtained populations of developing CL cells which contained predominantly uninucleate committed cells, or binucleate plasmodia, or quadrinucleate plasmodia and attempted to identify the developmental phase at which cDNA clones first (or last) detected a transcript. Three of 4 amoeba-specific clones did not detect mRNAs in uninucleate committed cells or in any later stages of development; the genes coding for these mRNAs were presumably switched off early in development. The fourth amoeba-specific clone detected an mRNA that was present at very much lower levels in uninucleate committed cells than in amoebae and was not detectable after the quadrinucleate plasmodium stage. Of the

5 plasmodium-specific cDNA clones examined in this way, two detected mRNAs only in mature plasmodia while the other three detected mRNAs in all developing populations from the uninucleate committed cell onwards and were presumably activated early in development.

Differences in microtubule organisation and tubulin gene expression

Interphase amoebae have been shown to possess an extensive network of cytoplasmic microtubules that radiate from a microtubule organising centre (MTOC; Havercroft & Gull, 1983). Wright *et al.* (1979) demonstrated that a pair of centrioles was associated with the MTOC. Havercroft *et al.* (1981) demonstrated that the MTOC and its associated centrioles were linked to the nucleus, although the exact nature of the link was unclear. At mitosis, the MTOC and centrioles duplicated and divided so that an MTOC and a pair of centrioles were present at each pole of the mitotic spindle; the nuclear membrane did not remain intact during amoebal mitosis (Havercroft and Gull, 1983). Cleavage gave rise to two daughter cells each of which possessed an MTOC, a pair of centrioles and associated cytoplasmic microtubules (Havercroft & Gull, 1983). A study of microtubule organisation in interphase *D. discoideum* amoebae by Roos *et al.* (1984), found a very similar pattern to that previously found for *P. polycephalum* (Havercroft and Gull, 1983). However, some *D. discoideum* amoebae possessed a second MTOC which was not attached to the nucleus (Roos *et al.*, 1984). At mitosis, *D. discoideum* amoebae underwent intranuclear

mitosis, rather than open mitosis as occurred in *Physarum* amoebae; the *Dictyostelium* mitotic spindle appeared to consist of a short rod of microtubules rather than an open network as observed for the *Physarum* amoebal spindle (Roos *et al.*, 1984; Havercroft and Gull, 1983).

In plasmodia, microtubules were present in the mitotic spindle but were absent during interphase (Havercroft & Gull 1983); there were no centrioles present. The nuclear membrane remained intact during plasmodial mitosis and the spindle, which was nucleated by an intranuclear MTOC, formed inside the nuclear membrane (Howard, 1932; Tanaka, 1973; Heath, 1980). Laffler *et al.* (1981) found that, in plasmodia, the majority of the microtubular proteins required for mitosis were synthesised in the 3 hours prior to mitosis.

In the apogamic strain CL, the alteration from the amoebal type of microtubule organisation to that characteristic of plasmodia occurred gradually over several cell cycles. The first change in organisation was observed at the mitosis by which the uninucleate cell became binucleate; this mitosis appeared to be of the intranuclear plasmodial type (Blindt, 1987; Gull *et al.*, 1985). Some mitotic large uninucleate, presumably committed, cells possessed a cytoplasmic MTOC and / or centrioles which did not appear to be nucleating the intranuclear mitotic spindle. It has been suggested that these cytoplasmic MTOCs were the remnants of the amoebal MTOC (Blindt, 1987; Gull *et al.*, 1985). Most binucleate plasmodia were observed to have microtubules radiating from either one or two MTOCs. The remaining interphase MTOCs and microtubules were lost over the next few cell cycles (Blindt, 1987; Gull

et al., 1985).

In addition to the differences in microtubule organisation between amoebae and plasmodia, differences in the tubulin isotypes utilised by the two vegetative cell types have been demonstrated. *Physarum*, in common with many other organisms including man, chicken, *Drosophila*, *Trypanosomes* and *Chlamydomonas* (Cleveland, 1983; Sullivan, 1988), has multiple tubulin genes for both α -tubulin and β -tubulin. Schedl et al. (1984) demonstrated, by restriction fragment length polymorphisms, that there were at least 4 unlinked α -tubulin loci (*altA* to *altD*) and 3 unlinked β -tubulin loci (*betA* to *betC*) in the *Physarum* genome. Burland et al. (1983) identified, by 2-dimensional gel electrophoresis, only two tubulin isotypes in amoebae, α_1 and β_1 , but four in plasmodia. Flagellates were shown, however, to express a novel α -tubulin isotype, α_3 ; this isotype was shown to arise by post-translational modification of the α_1 -isotype (Burland et al., 1983; Green and Dove, 1984). The flagella of both *Polytomella* and *Chlamydomonas* have also been shown to contain an α_3 -isotype which arose by post-translational modification of an α_1 -isotype (McKeithan et al., 1983). The α_1 -isotype in both *Chlamydomonas* and *Physarum* was subsequently shown to be modified to α_3 by post-translational acetylation (L'Hernault and Rosenbaum, 1985; Sasse et al., 1987; Diggins and Dove, 1987). Further analysis in *Physarum* indicated that, although α_3 occurred mainly in flagellates, small amounts of α_3 were present in amoebal centrioles (Sasse et al., 1987; Diggins and Dove, 1987). Piperno et al. (1987) showed that the centrioles of some mammalian cells also contained acetylated

α -tubulin. Microtubules containing acetylated α -tubulin have been observed to be more stable and more resistant to antimicrotubular drugs than their non-acetylated counterparts (LeDizet and Piperno, 1986; Piperno *et al.*, 1987).

Plasmodia, unlike amoebae, utilised tubulin only in the mitotic and meiotic spindles, but expressed two additional isotypes; α_2 and β_2 (α_3 was not present in plasmodia; Burland *et al.*, 1983, Roobol *et al.*, 1984, Diggins & Dove, 1987). Thus, on evidence from 2-dimensional gel electrophoresis, four tubulin isotypes were produced by 7 genes. Subsequent work has indicated that the amoebal α_1 -isotype consists of a single protein species whereas the plasmodial α_1 -isotype consists of at least two proteins (Birkett *et al.*, 1985; Singhofer-Wowra *et al.*, 1986a). In addition, the β_1 -isotype consists of two proteins in amoebae but only one in plasmodia (Burland *et al.*, 1984; Singhofer-Wowra *et al.*, 1986b). More recently, Solnica-Krezel *et al.* (1988) demonstrated that, in CL, the alterations in the pattern of tubulin isotypes present began in the uninucleate committed cell; the plasmodium specific β_2 -isotype (product of the *betC* locus) was present in many of these cells and was apparently utilised in the mitosis by which the uninucleate cell became binucleate (Diggins-Gillicinski *et al.*, 1989).

1.3 Aims of this work

The aim of this work was to examine the sequence and timing of events during the amoebal-plasmodial transition; in order to do this several techniques were used.

Time-lapse cinematography was used to determine the

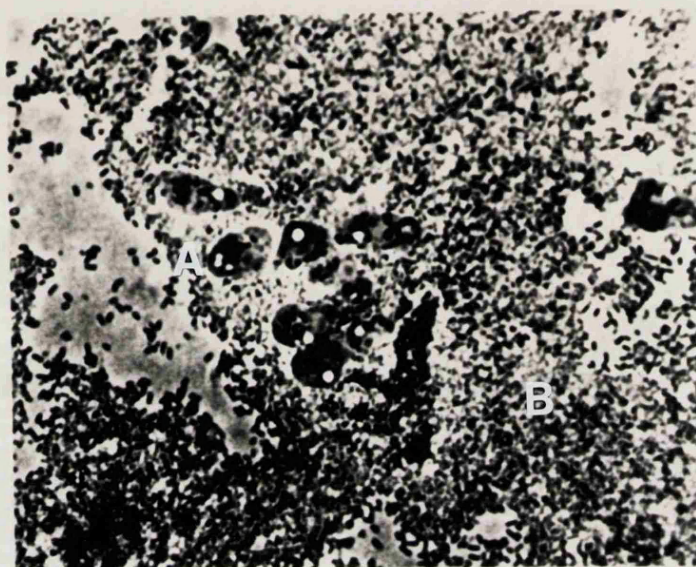
origins and fates of developing cells in both clonal and heterothallic plasmodium formation; neither Anderson *et al.* (1976), nor Holt and Huttermann (1979), had been able to follow individual cells through the whole amoebal-plasmodial transition and thus left unanswered vital questions. For example, is development in selfing strains apogamic? What is the relationship between the events of heterothallic plasmodium formation and the events of clonal plasmodium formation? Is there a relationship between the events of the amoebal-plasmodial transition and the cell cycle? Blindt (1987; Gull *et al.*, 1985) showed that, in CL, the alteration from amoebal to plasmodial microtubule organisation occurred gradually over several cell cycles. Is there a similar gradual change during heterothallic plasmodium development? What effect does amoebal fusion have on microtubule organisation during mating? These and other questions are addressed in Chapters 3 and 4.

Strains carrying mutations which block plasmodium formation have been analysed genetically (e.g. Anderson *et al.*, 1989) but the effects of the lesions on plasmodium formation are not known and many questions remain to be answered. For example, when does development first appear abnormal? Is the developmental lesion related to alterations in the pattern of gene expression and cellular organisation? Chapters 5 and 6 deal with two mutants which are blocked in apogamic development; both were analysed by several different techniques.

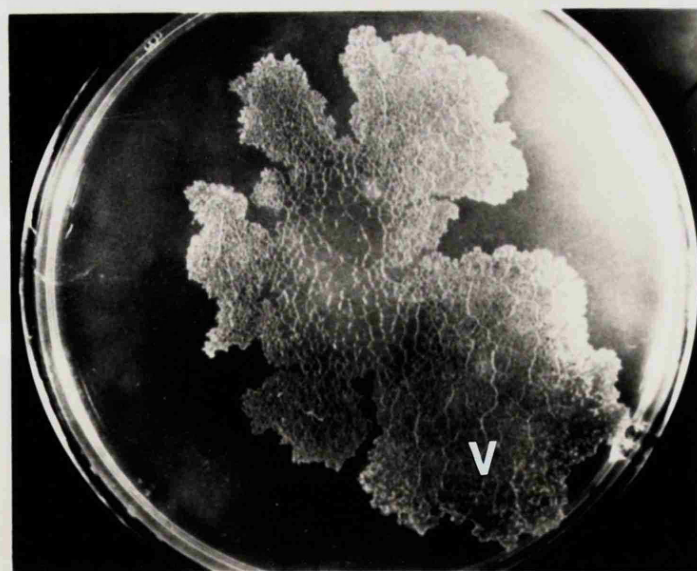
Figure 1.1 The two vegetative forms of the Myxomycete
Physarum polycephalum.

a) Haploid, uninucleate amoebae (A) growing on a bacterial
lawn (B) Bar = 10 μ m

b) Macroscopic pigmented (yellow) plasmodium growing on an
agar plate. The veins (V) are clearly visible. Bar = 1cm



a



b



Figure 1.2 The two life cycles of *Physarum polycephalum*.

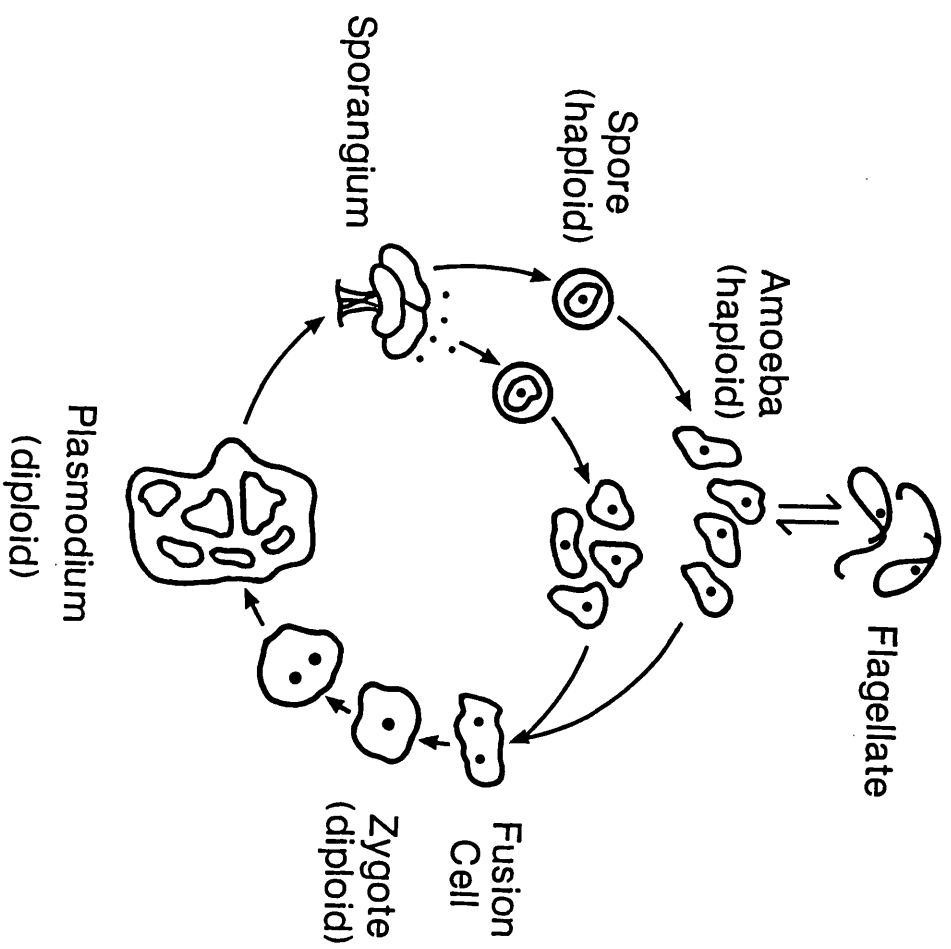
a) Heterothallic Life Cycle.

In moist conditions amoebae are able to reversibly transform into flagellates. Compatible haploid amoebae undergo cell and nuclear fusion to give a diploid zygote which develops into a plasmodium by rounds of synchronous mitosis unaccompanied by cytokinesis. When plasmodia starve in the light they sporulate. Meiosis occurs in the spores which hatch to yield haploid amoebae in suitable conditions.

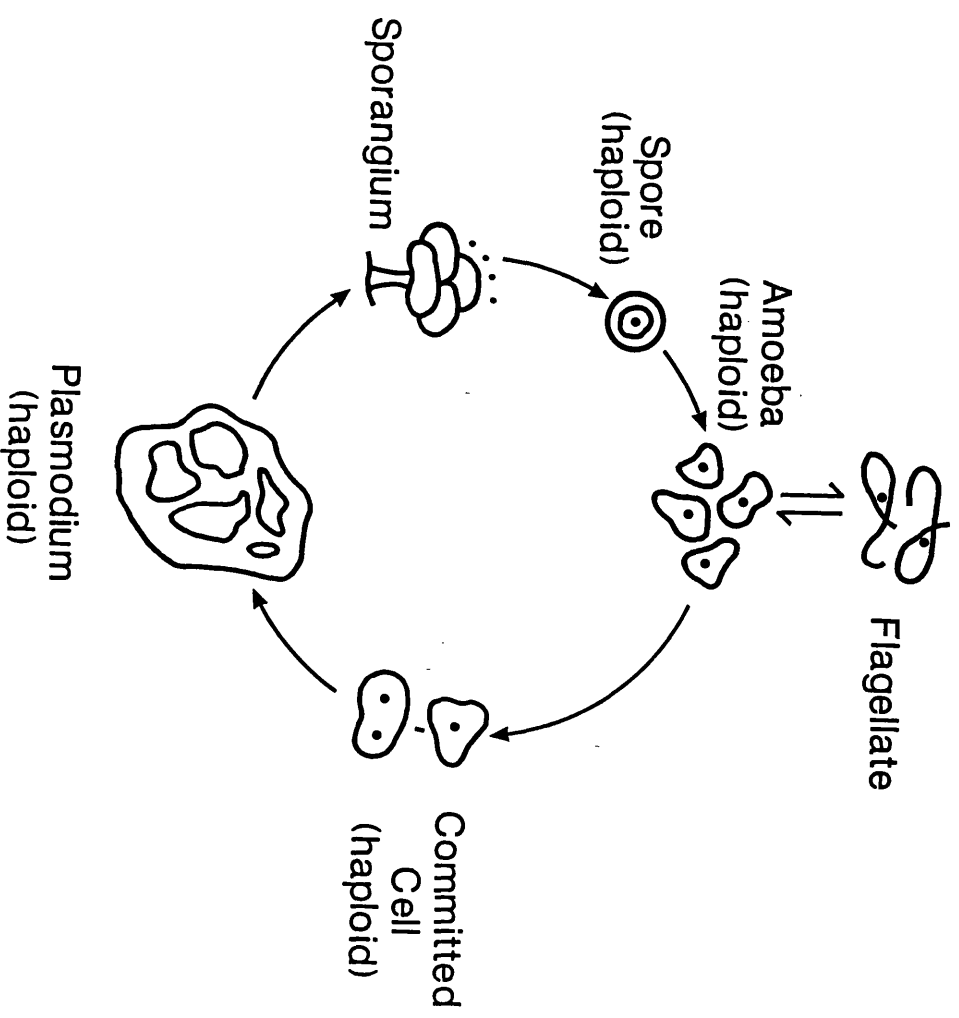
b) Apogamic Life Cycle.

A single apogamic amoeba is thought to develop into a haploid plasmodium. Apogamic amoebae become committed to develop into plasmodia while still uninucleate; plasmodia form by rounds of synchronous mitoses unaccompanied by cytokinesis. The plasmodia sporulate in the light and the viable spores are thought to result from meiosis in the small number of diploid nuclei.

Both figures are based on those of Dee (1987).



(a)



(b)

CHAPTER 2

MATERIALS AND METHODS

2.1 Genetic loci relevant to this work

See Section 1.2.1 for details about the action of these genes.

matA Gene involved in the control of plasmodium formation after cell fusion. At least 14 alleles (*matA1*, *matA2*, *matA3* etc.) exist and all are intercompatible.

matB Gene influencing amoebal fusion. At least 13 alleles (*matB1*, *matB2*, *matB3* etc.) exist and all are intercompatible.

matC Gene influencing amoebal fusion. Three alleles of this gene have been identified (*matC1*, *matC2* and *matC3*) and these are intercompatible.

gad (greater asexual differentiation) Mutations at these loci allow apogamic development. Most *gad* mutations are closely linked to *matA*.

npf (no plasmodium formation) Mutations at these loci block apogamic development; many *npf* loci are known, both linked and unlinked to *matA*.

2.2 Amoebal strains used in this work and their origins

Underlined genes are closely linked.

Heterothallic strains

LU648	<i>matA1</i> , <i>matB1</i> , <i>matC1</i> (Cooke and Dee, 1975)
LU859	<i>matA2</i> , <i>matB1</i> , <i>matC1</i> (Cooke, 1974)
CH508	<i>matA2</i> , <i>matB3</i> , <i>matC2</i> (Youngman et al., 1979)

Selfing strains

Two selfing strains were used in this study, CL and RA376. Two strains blocked in plasmodium formation were also used.

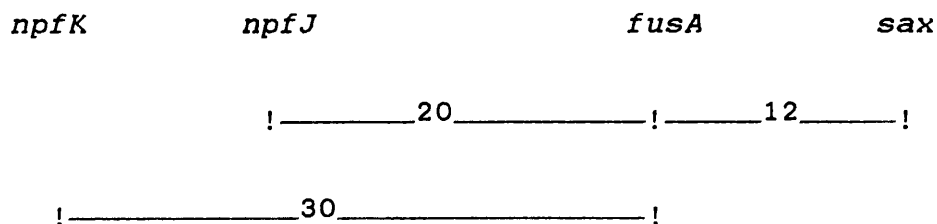
Both these strains were isolated by Dr. R. W. Anderson (RA612 from CL and RA614 from RA376) using a published method (Anderson *et al.*, 1989). Briefly, amoebae were grown for 48 hours in the presence of caffeine before being UV irradiated for 10 sec, and regrown for another 48 h still in the presence of caffeine. The amoebae were then replated at a dilution that allowed the formation of separate plaques on the assay plates. Colonies which failed to form plasmodia, or which formed abnormal plasmodia were selected and recloned.

CL matA2 gadh, matB1, matC1

Plasmodia, in CL, form at temperatures below about 29°C. CL is thought to have arisen by mutation in a *matA2* strain and carries the mutation *gadh*, closely linked to *matA*. This strain has been intensively studied; the relevant previous work on CL has been summarised in Section 1.2.

RA612 matA2 gadh, matB1, matC1, npfK1

RA612, was isolated following mutagenesis of amoebae of the strain CL. The *npfK1* mutation carried by RA612 was shown by genetic analysis to be unlinked to *matA* and *npfL*; it was linked, however, to the following loci: *fusA* (a gene governing fusion between plasmodia); *sax* (sensitivity to axenic medium; a plasmodium-specific gene); *npfJ* (mutation at this locus also blocks plasmodium formation). A map showing the distance between these genes is shown below; the numbers indicate the estimated distances between the genes in centiMorgans (cM; Dr. R. W. Anderson, personal communication).



Microscopic analysis of this strain indicated that it formed microplasmodia but they were of abnormal fissured morphology (see Chapter 5). The mating-type specificity and temperature range for development were the same as for CL.

RA376 matA3 gad111, matB3, matC2

From the heterothallic strain CH21 (Adler and Holt, 1974), Adler and Holt (1977) isolated a selfing strain, CH496, which gave rise to many plasmodia at 26°C but none at all at 30°C. The *gad* mutation carried by CH496 was named *gad11*; the strain had retained *matA3* mating specificity (Adler and Holt, 1977). Shinnick and Holt (1977) carried out genetic analysis on CH495 which was a sibling strain of CH496 and carried the same *matA* (*matA3*) and *gad* (*gad11*) alleles as CH496; both strains had the genotype *matA3 gad11*. Shinnick and Holt (1977) demonstrated that *gad11* was linked to *matA* but would recombine with *matA*; the estimated map distance between *matA* and *gad11* was 12.3 cM. Later work by Shinnick et al. (1983) confirmed the separation of *matA* and *gad11*. When Dr. R. W. Anderson moved to the University of Sheffield from the laboratory of Dr. C. E. Holt, he took a sample of strain CH495. In crosses involving this strain in his laboratory, no recombination could be detected between *matA* and *gad11* (Anderson and Hutchins, 1986). Since it was not possible to repeat under identical conditions, the earlier experiments which had established the recombination frequency between these two functions, Anderson and Hutchins (1986) re-designated the gene causing apogamic development in this strain, *gad111* and the strain which carried this mutation was named RA376; the genotype of RA376 was *matA3 gad111*.

RA614 matA3 gad111, matB3, matC2, npfL

Strain RA614 was isolated following mutagenesis of amoebae of the strain RA376; RA614 carries the mutation *npfL1*. Genetic analysis showed that *npfL* was not linked to any of the other genes tested, including *matA* (Dr. R. W. Anderson; personal communication). Microscopic analysis of this strain revealed that development was initiated but no macroplasmodia were formed; instead many large, rounded cells were found in which no nuclei could be observed (see Chapter 6). The temperature range for development and the mating-type specificity were the same as in RA376.

2.3 *Escherichia coli* suspensions used as a food source for amoebae

SBS - Standard Bacterial Suspension

E. coli, strain B145, were streaked onto Luria agar plates (see Section 2.4) and the plates were incubated overnight at 37°C. The following day the bacteria were washed off the agar plate in 5ml of sterile distilled water and pipetted into a sterile test-tube. The suspension was vortexed for at least 5 sec before use.

FKB - Formalin Killed Bacterial Suspension

1ml of *E. coli* suspension, prepared as described above, was placed into a 2l flask containing 1l of sterile nutrient broth (see Section 2.4) which was then incubated overnight on a rotatory shaker at 37°C. Next day, the culture was spun down and resuspended (in a smaller sterile flask) in 50ml of buffer (see Section 2.4); 4ml of 38% formaldehyde was added to the buffer before the flask was stood overnight at 4°C. The next day, the cells were spun down and resuspended in 50ml of buffer; 5ml of 1M glycine were added to the flask which was then shaken at 37°C for at least 30 min. After shaking the cells were spun down and washed twice in 50ml of buffer. Finally the cells were resuspended in 20ml of buffer before being dispersed into 1ml aliquots in sterile screw-cap bottles and stored at 4°C. A sample of the FKB was streaked onto a luria agar plate and incubated at 37°C overnight to check for the presence of live bacteria.

2.4 Media and Materials

Nutrient broth

25g of Oxoid No. 2 nutrient broth powder was added to 1l of distilled water which was then sterilised in an autoclave at 15 psi for 15 min.

Buffer used in preparation of FKB

The following were added to 1l of distilled water.

Na_2HPO_4	7.0g
KH_2PO_4	3.0g
NaCl	4.0g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.1g

The solution was sterilised in an autoclave at 15 psi for 15 min.

1M citrate buffer

The following were added to 1l of distilled water and the pH was adjusted to pH5.0.

73.5g Citric acid monohydrate

191.0g trisodium citrate dihydrate

The solution was sterilised in an autoclave at 15 psi for 15 min.

0.5M sodium phosphate buffer

The following were added to 1l of distilled water and the pH was adjusted to pH6.8.

78.0g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$

70.98g Na_2HPO_4

The solution was sterilised in an autoclave at 15 psi for 15 min.

Luria agar

The following were added to 1l of distilled water and the pH was adjusted to pH7.0.

10.0g Tryptone

5.0g yeast extract

5.0g NaCl

15g (1.5%) agar

The solution was sterilised in an autoclave at 15 psi for 15 min.

SDM - Semi-defined liquid medium

The following were added to 1l of distilled water and the pH was adjusted to pH4.6 with 20% NaOH.

Glucose	10.0g	
Soytone	10.0g	(Difco-bacto)
KH ₂ PO ₄	2.0g	
CaCl ₂ .6H ₂ O	1.35g	
MgSO ₄ .7H ₂ O	0.6g	
FeCl ₂ .4H ₂ O	0.039g	
ZnSO ₄ .7H ₂ O	0.034g	
Citric acid	3.54g	
EDTA	0.224g	(Disodium salt)
Biotin	0.005g	
Thiamine	0.04g	

The medium was sterilised in an autoclave at 15 psi for 15 min. 1ml of 0.05% Hematin (in 1% NaOH) was added per 100ml immediately before use.

SDM Agar

Equal amounts of molten 3% agar and SDM were mixed just before use.

DSDM agar (Dilute SDM agar)

62.5ml of SDM were added to 1l of 1.5% agar just before use.

Liver infusion stock solution

100g of Oxoid dessicated liver (product number L26) in 1l distilled water was sterilised in an autoclave at 15 psi for 15 min.

LIA (Liver infusion agar)

40ml of liver infusion stock solution and 10ml sodium phosphate buffer were added to 1l of molten 1.5% agar before use.

LIA+CIT

LIA buffered with 3ml of sodium citrate buffer per litre of 1.5% agar.

LIA+DSDM

62.5ml SDM and 40ml liver stock solution were added to 1l molten 1.5% agar.

Stock phosphate buffered saline (PBS) used during preparation of cells for immunofluorescence

NaH₂PO₄ 2.56g

Na₂HPO₄ 22.4g

The above were added to 500ml of distilled water and the pH adjusted to pH7.3. NaCl (87.66g) was added and the volume made up to 1l. The solution was sterilised in an autoclave for 15 min at 15 psi. This stock was diluted 1 in 10 with sterile distilled water before use.

Poly-L-lysine coated coverslips

5mg of poly-L-lysine powder (Sigma No. P1399) was dissolved in 100ml of 10mM Tris buffer (pH8.0) and stored at -20°C in 20ml aliquots. Coverslips (Chance no. 1.5) were washed in detergent, rinsed thoroughly in distilled water, and drained of excess water. The damp coverslips were rinsed in alcohol for about 30 mins before being allowed to air dry. The washed and dried coverslips were soaked in poly-L-lysine solution for 10min at room temperature and then allowed to air dry. The coated coverslips were stored in a rack in an airtight container at room temperature; coverslips prepared this way can be stored for a few days before use.

DAPI/anti-fade mountant

100mg of p-phenylenediamine (Sigma No. P6001) was added to 10ml of 1M tris HCl (pH7.5) and then the volume was made to 100ml with glycerol. 2.5mg of the DNA specific stain, DAPI (4,6-diamidine-2-phenylindole, Sigma No. D1388), was added to the glycerol solution which was subsequently stored at -20°C.

Agarose overlays for immunofluorescence microscopy

This method was based on that of Yumura *et al.* (1985). A microscope slide was placed on a flat surface and a coverslip was placed on each end so that about half of the coverslip rested on the slide. About 1ml of molten 2% agarose, in PBS,

was pipetted onto the slide and a second slide was placed on top of the first. Gentle pressure on the top slide spread the agarose into an even layer about 1mm thick. After the agarose had set, the slides were separated using a razor blade. The ends of the agarose sheet were trimmed and it was cut in two using a razor blade. Then the agarose sheets were lifted off the microscope slide and placed in distilled water at room temperature, where they could be kept overnight; sheets containing holes were discarded.

Lugol's iodine for staining flagellates

KI 0.6g

I₂ 0.4g

H₂O 10ml

(Wenrick & Diller 1950)

2 or 3 drops were added to a 1ml suspension of flagellates in order to fix the cells and stain the flagella.

Mithramycin stock solution

137.5µg/ml Sigma mithramycin complex (M6766) in 0.1M Tris-HCl (pH7.4), 0.1M NaCl and 30mM MgCl₂

2.5 Culture of amoebae and plasmodia

Frozen stocks of amoebae

Stocks of amoebae were routinely kept frozen at -80°C and were prepared in the following way. Amoebae growing on LIA (see Section 2.4) were allowed to grow to confluence and encyst. The cells were resuspended in 4ml ice-cold sterile distilled water and then 0.8ml of cell suspension was placed in a 1.5ml screw-cap plastic tube and 0.2ml of 50% glycerol was added. The contents of the tube were mixed well and then stored in the freezer at -80°C; cells frozen this way will remain viable for several years.

Maintenance of amoebal stocks

Amoebal cultures were started from frozen stocks by spreading 0.1ml of stock suspension and 0.1ml SBS (see Section 2.3) on LIA (see Section 2.4) and incubating at a

temperature that allows amoebal growth but not plasmodium formation; incubation temperature varied with the strain used.

Subculturing was carried out at weekly intervals by spreading 0.1ml SBS over a LIA plate and transferring cells from old plates to new using a sterile wooden toothpick. The amoebal strains were incubated at the following temperatures; 26°C for heterothallic strains, 28.5°C for RA376 and RA614, and 24 hours at 26°C prior to shifting to 30°C for CL and RA612. Stocks cultured this way encysted at a density of $1-2 \times 10^7$ cells / plate. Encysted amoebae on agar plates were stored at 4°C for a few months. Every 3 months, a new culture was inoculated from the frozen culture.

Maintenance of plasmodia

Macroplasmodia were subcultured by cutting a block of agar 10mm by 15mm, with a whole plasmodium or part of a plasmodium growing on it, from an agar plate. This block of agar was placed on its side on the surface of an SDM agar plate. The plasmodium quickly moved onto the surface of the SDM agar plate and in 4-5 days had grown to cover the whole plate.

Induction of sporulation and harvesting of spores

To induce sporulation, plasmodia which covered the whole of an SDM plate, were placed in a specially built box containing a light which was on for 12 h and then off for 12 h (Dee *et al.*, 1989). After sporulation, the spores were scraped off the plate and could be stored in small screw-cap tubes for several years. In order to induce spores to hatch, the spores were crushed in water and allowed to soak for about an hour before being plated out on LIA. Amoebal colonies formed after a few days.

2.6 Assaying cultures for different cell types

At time zero, replicate cultures on LIA+CIT or DSDM plates (see Section 2.4) were prepared from a suspension of cysts. 0.1ml FKB (diluted 1:2 with water) and 0.1ml cyst suspension were spread over a 5cm by 5cm square on each plate. The number of cysts inoculated depended upon the experiment; the

plates were incubated at 22°C where development occurred.

Assay for committed cells

At various times after inoculation, plates were harvested and analysed as follows. The cells were washed off 2 plates in 5 ml of water /plate; the suspensions from the 2 plates were assayed separately. The suspensions were serially diluted as follows: a = washoff, b = 1/5a, c = 1/5b, d = 1/5c, e = 1/5d, f = 1/5e. Two LIA+DSDM (see Section 2.4) plates were inoculated with 0.1ml SBS and 0.1ml of cell suspension, giving a total of 12 plates, 2 for each dilution. These assay plates were incubated at 22°C. After 2 - 3 days plates were scored for plateable plasmodia and after 4 - 7 days for amoebal colonies (Youngman et al., 1977). Both replicate assay plates were scored for the dilution that gave 30 - 100 colonies per plate and the scores from the two assay plates were averaged; multiplying this average by the dilution factor gave the number of cells per culture.

Assay for cells able to undergo the amoeba-flagellate transformation

In order to assay developing cultures for the percentage of cells able to transform into flagellates, the density of the wash-off prepared above, was adjusted to 1×10^6 cells / ml and 1ml of the suspension was placed in a tube on a reciprocating shaker at 26°C. After 3 hours, the tube was removed and the contents stained with Lugol's Iodine (Section 2.4). A drop of the stained suspension was placed on a slide and then a cover slip was placed on top. 100 - 500 cells were scored for presence or absence of flagella using phase contrast microscopy at a magnification of x400; it was not possible to see nuclei within the stained cells.

Assay for cells with different numbers of nuclei

To determine the numbers of nuclei within cells, a drop of the cell suspension was pipetted onto a thin agar slide made as described in Section 2.7. The drop was allowed to dry into the agar before a coverslip was placed on top. 100 - 500 cells were scored for the number of nuclei present, using

phase contrast microscopy at a magnification of x400.

2.7 Slide cultures

Thin agar slide cultures

Four or 5 drops of molten 2% water agar were dropped onto a slide and allowed to cool for a few seconds, but not to solidify and a coverslip was placed on top. After the agar had set the coverslip carefully was removed to leave a flat smooth surface and a drop of cell suspension was placed on the agar. Once the liquid had dried, the coverslip was replaced and the cells were examined using phase contrast microscopy. Cultures prepared this way will only live for a few hours.

Filming slide cultures

The method is based on that of Anderson et al. (1976). Slides were obtained which had a glass cavity (internal diameter approximately 10mm) mounted onto a glass microscope slide (Griffin & George No. YSH-190-G). A coverslip was placed partly over the cavity and molten 2% LIA+CIT agar was injected into the cavity, while the coverslip was held steady, to form a 6 - 8mm diameter block which touched the top and bottom of the chamber. It was important that the agar block did not completely fill the cavity or the cells would die from lack of oxygen. In addition, the block must not touch the sides of the cavity or the liquid and cells run off the block onto the surrounding glass. Once the agar had set, the coverslip was removed and cell suspension was placed on the block. The liquid was allowed to dry before the coverslip was dropped on top and sealed in place with molten wax; good contact was essential between the agar block and the coverslip. Cells will survive in slide cultures of this type for about 10 days.

To make the cell suspension for inoculating onto the agar block, 0.4ml of sterile distilled water was added to 0.1ml FKB; 0.2ml of this suspension was placed in a test-tube. A loopful of the bacterial suspension was picked up using a tungsten loop and rubbed across a plate of encysted amoebae

to collect cells. The loop was returned to the bacterial suspension and agitated to remove the cells. Three loops of cells were collected for selfing strains; this gave a cell density of about 4×10^5 cells / ml. Three loops of each strain were used for *matA*-heteroallelic and *matA*-homoallelic mixtures. The suspension was vortexed vigorously before use to break up clumps of cells. The same tungsten loop was used to transfer cells and bacteria from the suspension to the agar block in the slide. One loopful of cell suspension was used per slide culture; this resulted in a monolayer of bacteria on the agar block. FKB was used in preference to live bacteria because the continued growth and movement of live bacteria in the culture slides made accurate observation of the *Physarum* cells difficult.

2.8 Making and analysis of films

Time-lapse films were made using Ilford PanF 50 ASA film (100ft reels of 16mm black and white cine film) which was developed and printed by Studio Film and Video Labs. Ltd., London. The equipment consisted of a Nikon CFMA cine-autotimer and Bolex H16-reflex cine camera attached to a Wild microscope fitted with Wild Fluotar phase contrast lenses. Two magnifications were used: x20 objective with x6 photo eyepiece (CL film) and x10 objective with x10 photo eyepiece (all other films). One frame of film was exposed every 15 or 30 sec with an exposure time of 0.2 sec; each reel of film was run for 4000 frames. The equipment was placed in a 22°C constant temperature room on a marble bench to dampen vibrations.

Films to be analysed were projected onto a screen divided into 88 squares, each 5cm by 5cm. These squares were labelled A - K along the horizontal axis and 1 - 8 along the vertical. The analysing projector used (Lafayette AAP-305-A; Lafayette Instrument Company, Lafayette, Indiana) was fitted with a frame counter and a facility for still frame projection as well as forward and reverse projection at various speeds (1 - 24 frames / sec). These facilities allowed events to be accurately measured by use of a frame number in conjunction with a code designating the position on the screen (e.g.

frame 2134, square B6). Since it was not possible, during filming, to identify in advance the cells that would be of interest, analysis had to be done retrospectively. During analysis of the films, cells were traced back to their origins at a previous mitosis. All times are given as mean in hours $\pm$ standard deviation; n is the number of observations.

Cell area was measured placing a sheet of paper on the screen over the cell of interest and then drawing round the cell. These drawings were converted to areas by placing a grid drawn on a transparent acetate sheet over the cells, and counting the number of squares that the cell covered. The grid consisted of 5mm by 5mm squares, 100 of which were equal to one large square on the screen; the area each small square represented on the slide culture could be calculated since the magnification factor was known. This method proved to be a valid method of measuring cell growth during a film (see Chapter 3); however, the degree of cell flattening varied between slide cultures and so values obtained from different cultures could not be combined. In the text, all area measurements are given in arbitrary units as mean $\pm$ standard deviation, n is the number of observations.

All statistical tests carried out were t-tests unless stated otherwise and only significance levels are given in the text. During film analysis, each cell was arbitrarily given a number. The progeny of a cell were given number / letter codes which began with the number given to the parent cell. These numbers and number / letter codes are shown on some of the cell pedigrees.

2.9 Flow cytometry

At time zero, 5 DSDM plates were inoculated with 5×10^5 cysts / plate and 0.1ml FKB (diluted 1:2) as food source. Mixtures of strains were set up at an initial density of 2.5×10^5 cells of each strain / plate. These cultures were grown at 22°C so that development could take place. Five LIA plates were inoculated with the same number of cysts from a single strain as an amoebal control. These control plates were incubated at temperatures where development would not occur, i.e. 22°C (LU648, LU859, CH508) or 28.5°C (RA376, RA614) or

30°C (CL, RA612). In all experiments, the cells were spread over the whole plate. After 3 days (unless otherwise stated), the cells were harvested and suspended in SDM (without hematin; see Section 2.4) at 10^7 / ml. To permeabilise the cell membranes, 50 μ l of 0.2% Triton in 0.1M NaCl was added to 100 μ l of cell suspension; the cells were then incubated at room temperature for 1 min. To this suspension was added 400 μ l of mithramycin stock solution (see section 2.4) and the mixture was incubated at room temperature for 5 min and then kept on ice for 1 - 2 hours until analysis. Stained cell suspensions were analysed on a FACS 420 flow cytometer linked to a Consort-30 accessory computer (Becton-Dickinson Immunocytometry Systems). The 4W argon laser was tuned for an excitation wavelength of 457.9nm at 120mW output and fluorescence emission was measured through a 530nm bandpass filter with a 30nm band width. The intensity of fluorescence emitted by each cell was regarded as directly proportional to its DNA content (Crissman & Tobey 1974; Taylor & Milthorpe 1980). In all flow cytometry experiments described in this work, a haploid amoebal strain was examined first as a control. The same settings on the FACS machine were used for every experiment. In order to avoid counting the cell and bacterial debris present in the lower channels, the threshold value was set at about Channel 25.

2.10 Preparation, fixation and staining of cells for immunofluorescence microscopy

Cell cultures

All cultures were set up as detailed in Section 2.9. Cells were harvested, fixed and stained after 3 days. Three different methods of fixation were used but only one staining technique.

Fixation on a glass surface

At various times after inoculation, the cells were washed off each of 5 plates with 2ml water and the suspensions were combined. The cells were spun down and resuspended in water at 5×10^6 / ml. Drops of suspension (25 μ l) were placed on

poly-L-lysine coated coverslips (see Section 2.4) and the cells were allowed to settle for 40 min before being fixed by immersion in -20°C methanol for 10 sec. The cells were rehydrated in PBS (see Section 2.4) and stained as described below.

Fixation in suspension

A different preparation method was required for flagellates because flagella are quickly resorbed when the cells are placed on a surface. Cells were washed off the culture plates and resuspended at 5×10^6 cells / ml in water. Flagellate formation was induced as detailed in Section 2.6. The cells were fixed, in suspension, by adding 0.1 volumes of formaldehyde (38% stock solution). After one hour, the cells were thoroughly washed in PBS (3 x 5 min), placed in -20°C methanol for 10 sec, and finally rinsed in acetone for 5 min before being thoroughly washed in PBS (3 x 5 min). The cells were resuspended in PBS at $1 - 5 \times 10^6$ cells / ml. Drops of suspension ($25\mu\text{l}$) were placed on poly-L-lysine coated coverslips and allowed to dry; the cells were then stained as described below.

Fixation on an agar plate

In order to preserve the natural shapes of the cells, they were fixed in situ on an agar plate using the following method. The cells were fixed by pipetting 3ml of 3.8% formaldehyde, in PBS, onto the agar plates. After 10 min, the cells were removed from the agar plates and thoroughly rinsed in PBS (3 x 5 min). The cells were resuspended in PBS at a density of about 10^7 cells /ml. Poly-L-lysine coated coverslips were placed on top of slides. Drops of suspension ($50\mu\text{l}$) were placed on the coverslips and an agarose overlay (see Section 2.4; Yumura et al., 1985) was placed on each coverslip. The excess liquid was sucked from under the overlay using a tissue; as the liquid was removed, the cells were pulled onto the poly-L-Lysine coverslips and stuck there. The coverslip was removed from the slide and the gel overlay was removed from the coverslip by gentle washing in PBS. The cells were stained by placing the coverslips, cell

side down, onto Nescofilm on which had been placed drops of antibody. The coverslips were removed to trays of PBS for washing. All timings and washes were as described below.

Staining of fixed cells

After fixing by any method, 50 μ l of the primary anti- β -tubulin antibody (Amersham International PLC No. N357 diluted 1:1000 in PBS before use; Blose et al., 1982) was added for one hour. Following this, the cells were washed for 3 x 5 min in PBS (see Section 2.4), before 50 μ l of the FITC tagged secondary antibody was added for one hour (FITC conjugated goat-anti-mouse IgG from one of two sources: Tago Inc (No. 4350) or Amersham International PLC (No. N1031). Both were diluted 1:50 in PBS before use). The coverslips were then re-washed in PBS (3 x 5 min). After staining was complete, the coverslips were mounted onto glass microscope slides using the DAPI/anti-fade mountant (see Section 2.4) and sealed in place with clear nail varnish.

Slides were examined using a Zeiss axiophot microscope fitted with a x100 Plan neo-fluar objective, a mercury light source and excitation filters for FITC and DAPI. Unfortunately, the microscope was not fitted with a x100 phase-contrast objective so it was not possible to examine the cells by this method as well.

2.11 Abbreviations

cm - centiMorgan
 PBS - Phosphate buffered saline
 FITC - Fluorescein isothiocyanate
 DAPI - 4,6-diamidine-2-phenylindole
 IMT - Intermitotic time
 MTOC - Microtubule organising centre
 SDM - Semi-defined medium
 LIA - Liver infusion agar
 FKB- Formalin killed bacteria
 SBS - Standard bacterial suspension

CHAPTER 3

ANALYSIS OF DEVELOPMENT IN THE APOGAMIC STRAINS CL AND RA376

3.1 INTRODUCTION

Time-lapse cinematographic analysis by Anderson *et al.* (1976) showed that development in CL involved the formation of binucleate plasmodia by mitosis without cytokinesis in large uninucleate cells with large nuclei (see Section 1.2.3). Anderson *et al.* (1976) were unable to trace the origin of the developing uninucleate cells but suggested that development was apogamic. The aim of the study described in Section 3.2 was to confirm, by time-lapse cinematography, that development in CL was apogamic and to investigate cell growth and the lengths of the cell cycles during the amoebal-plasmodial transition. Once the lengths of the cell cycles were established, other techniques could be used to place the times of commitment and loss of ability to flagellate within these cell cycles and to investigate whether the increased nuclear area in cells at the time of binucleate cell formation reflected an increase in DNA content.

The apogamic strain RA376 arose by mutation in a *matA3* strain, unlike CL which apparently arose by mutation in *matA2* (see Section 2.2). The aim of Section 3.3 was to compare the sequence of events in apogamic development of RA376 with that found for CL. It was thought that the sequence of events during development might differ in these two strains because

of their different *matA* origins. CL and RA376 have both been used as parental strains in the isolation of *npf* mutants and it was necessary to establish the sequence events during apogamic development to determine which aspects of the phenotype of the *npf* strains are a result of the *npf* mutation.

The work on CL described in this Chapter has been published (Bailey et al., 1987). Some of the initial studies on RA376 were undertaken in conjunction with Miss. Sarah Parkinson, a 3rd year undergraduate at the University of Leicester.

RESULTS

3.2 Development in CL

3.2.1 Analysis of growth and development by time-lapse cinematography

Cell pedigrees

Filming slide cultures were set up as detailed in Section 2.7. Filming was started when the cells had excysted and divided to give colonies of 8-16 cells (about 50 hours after inoculation) and filming was continued for 66 hours (8000 frames at one every 30 sec). During the analysed film of one culture, 15 cells became binucleate by mitosis without cytokinesis and many subsequently underwent a second mitosis to become quadrinucleate or fused with other multinucleate cells. By the end of the film the field of view was occupied by one large plasmodium.

The origin of 5 of the cells that became binucleate could be traced; each came from an apparently normal amoebal division. No fusions were seen between uninucleate cells. These observations confirmed the suggestion of Anderson *et al.* (1976) that development in CL was apogamic. Fig. 3.1 shows 2 examples of cell pedigrees involving binucleate cell formation that were traced from the film. One amoebal division was analysed from which both daughter cells became binucleate (Fig. 3.1b) and three amoebal divisions were seen where only one daughter cell became binucleate (Fig. 3.1a); these observations showed that the origin of binucleate cells did not involve a fixed cell lineage.

In amoebal mitoses, nucleolar disappearance was quickly followed by disappearance of the nucleus and the appearance of a cleavage furrow. This suggested that CL amoebae underwent normal open amoebal mitosis (i.e. the nuclear membrane broke down during mitosis; Havercroft and Gull, 1983). In many uninucleate developing cells, however, nucleolar disappearance was followed by the appearance, inside the nucleus, of the metaphase plate and subsequently by chromosome separation. Thus most of the mitoses by which uninucleate cells became binucleate were probably of the plasmodial type where the nuclear membrane remains intact throughout mitosis; this agrees with the observations of Blindt (1987). In many binucleate cells becoming quadrinucleate, mitosis appeared to be of the plasmodial type. Incomplete cleavage furrows were seen in some uninucleate cells undergoing mitosis to become binucleate and also in some binucleate cells undergoing mitosis to become

quadrinucleate.

Measurement of intermitotic time

Intermitotic times (IMT) were measured from nucleolar disappearance in one cell cycle to nucleolar disappearance in the next. From the pedigrees shown in Fig. 3.1 it can be seen that the cell cycle ending in binucleate cell formation was about 2.4 times as long as an average amoebal cell cycle (28.7 ± 1.85 h, $n=5$ compared with 11.8 ± 2.2 h, $n=18$; $p > 0.001$; Fig. 3.8). Unexpectedly, the cell cycles of binucleate cells that became quadrinucleate (7.6 ± 1.03 h, $n=11$) were significantly shorter than those of amoebae (11.8 ± 2.2 h, $n=18$; $p > 0.001$; Fig. 3.8).

Cell growth and nuclear area during development

In the film analysed, one arbitrary unit of area was equal to $4.86 \mu\text{m}^2$ on the slide culture. Cells that underwent a normal amoebal cell cycle approximately doubled in area during the cell cycle growing from 9.7 ± 1.6 units ($n=18$) to 25.9 ± 3.9 units ($n=18$; Fig. 3.8); this doubling indicated that cell area could provide a reliable means of investigating cell growth. At their birth at the previous amoebal division, cells that became binucleate were not significantly different in area from cells that underwent a normal amoebal division (9.8 ± 1.2 units, $n=5$ compared with 9.7 ± 1.6 units, $n=18$; $p < 0.05$; Fig. 3.8). Where only one daughter cell from an amoebal division became binucleate, it was the same size as its sister cell when that cell divided (23.0 ± 6.6 units, $n=3$ compared with 25.9 ± 3.9 units, $n=18$; $p < 0.05$; Fig. 3.8); the

two were morphologically indistinguishable until this time. At binucleate cell formation, the large uninucleate cell was approximately twice as big as an amoeba at mitosis. For cells whose origin could be traced, the mean area at mitosis was 43.3 ± 10.7 units, $n=5$ (Fig. 3.8) which was not significantly different from the mean for all cells which became binucleate (46.1 ± 8.44 units, $n=15$; $p < 0.05$).

Binucleate cells that became quadrinucleate ($n=11$) did not show a large increase in cell area during the cell cycle, growing from 47.7 ± 8.9 units to 57.0 ± 7.1 units. During this cell cycle, cell thickness apparently started to increase and the cells became much more rounded; thus area measurements may underestimate growth at this stage.

By measuring cell areas at different times during growth of individual cells, comparison was made of the patterns of growth of amoebae and uninucleate committed cells. IMTs were normalized to 100 units for amoebal cell cycles ($n=9$) and to 240 units for cell cycles ending in binucleate cell formation ($n=5$). Fig. 3.2 shows mean cell area plotted against normalized IMT. The two cell types showed virtually identical growth curves until the amoebae divided at 100 units. Cells which became binucleate continued to increase in area until approximately 210 units; there was little increase in area during the remaining 10% of the cell cycle before division followed at 240 units.

In amoebae, nuclear area approximately doubled during a cell cycle, increasing from 2.3 ± 0.4 units ($n=18$) to 3.8 ± 0.7 units ($n=18$). When the uninucleate committed cell entered mitosis at the end of the extended cell cycle, nuclear area

had increased to 4.9 ± 1.0 units ($n=5$). After mitosis, nuclear area in binucleate cells had decreased to 2.8 ± 0.4 units ($n=8$) and during the following cell cycle did not double but increased to only 3.1 ± 0.8 units ($n=21$). In the newly formed quadrinucleate cell, the nuclei were not significantly different in area from those of amoebae at birth (2.46 ± 0.6 units, $n=23$ compared with 2.30 ± 0.4 units, $n=18$; $p < 0.05$). These observations are in agreement with those of Anderson *et al.* (1976).

Plasmodial fusion

In developing cultures of CL, fusions between cells with 2 or more nuclei have frequently been observed (Anderson *et al.*, 1976; Collett *et al.*, 1983) but no fusions involving uninucleate cells have been recorded. During the development of the cells described above, 29 fusions occurred between multinucleate cells (e.g. Fig. 3.3) but no fusions involving uninucleate cells were observed. To increase the probability of contacts involving large uninucleate cells, films were made of dense populations of cells which had been enriched for committed cells by the method of Blindt *et al.* (1986). In one film, 11 fusions were recorded one of which involved the fusion of a large uninucleate cell with a plasmodium containing at least 16 nuclei. The nucleus of the large uninucleate cell was considerably larger than the plasmodial nuclei and was visible within the cell until filming ended 2.5 h later; none of the nuclei entered mitosis during this time.

3.2.2 Timing of commitment in the amoebal-plasmodial transition

Burland *et al.* (1981) showed that cells became committed to plasmodium formation while they were still uninucleate and microscopically indistinguishable from uncommitted cells. In order to relate the sequence of events observed in the films to the time of commitment, replating experiments were carried out using culture conditions very similar to those used for filming. DSDM plates were inoculated with 1×10^4 cells / plate and at intervals after inoculation, the cells were assayed for numbers of amoebae and committed cells (see Section 2.6).

The results (Fig. 3.4 and Table 3.1) gave an amoebal doubling time of about 10 hours, close to that measured from the film. There was an initial lag period of about 24 h where amoebal numbers did not increase; this was the time required for amoebae to hatch from cysts and begin growth. After a period of exponential growth, the amoebal growth rate declined and finally the number of amoebae present in the culture started to decrease; the maximum number of amoebae present during the experiment was 3.3×10^5 (114 h). The decrease in amoebal growth rate occurred for the following 2 reasons: 1) Each cell that became committed resulted in the loss of an amoeba from the population of dividing cells. 2) During the later stages of the experiment the amoebae began to encyst due to lack of food and were no longer dividing. The decline in amoebal numbers was a consequence of the ingestion of amoebae and cysts by developing plasmodia.

The first committed cells were present at about 50 hours

after inoculation (Fig. 3.4); after this time there was a rapid increase in plasmodial numbers as more amoebae became committed to development. The number of plasmodia present reached a maximum of 1.3×10^5 at 114 h, and then decreased. Fusions between plasmodia contributed to the decrease in the number of plasmodia present.

Binucleate cell formation in relation to commitment

Slide cultures were inoculated with cells from a developing culture so that the development of committed cells could be followed (see Section 1.2.3). A dilute cell suspension was used so that the inducer concentration would be below that required to initiate development and thus only cells that were already committed would be able to continue development. The use of a dilute suspension also minimised the number of fusions between multinucleate cells. Cells that possessed 2 or more nuclei at the time of inoculation continued to develop. Some uninucleate cells also continued to grow and became binucleate; these cells must have been committed at the time of inoculation. The remaining uninucleate cells proliferated as amoebae and were therefore not committed at the time of inoculation. Cells with 2 or more nuclei were counted every 2 hours until a plateau was reached (Fig. 3.5 and Table 3.2).

The results were interpreted by assuming that, at the time of inoculation, the uninucleate committed cells were randomly distributed at various stages between commitment and binucleate cell formation. The uninucleate cells that had most recently become committed would be the last to become

binucleate, by which time some of the first uninucleate cells to become binucleate would have become quadrinucleate. Thus the results are plotted in terms of the increase in multinucleate cells rather than the increase in binucleate cells alone. As shown in Fig. 3.5, the number of multinucleate cells reached a maximum about 13.5 hours after inoculation; this was taken as the maximum interval between commitment and binucleate cell formation.

Timing of loss of ability to undergo the amoeba-flagellate transformation in relation to commitment

By determining both the proportion of cells unable to flagellate and the proportion of committed cells in differentiating cultures, Blindt et al. (1986) estimated that ability to undergo the amoeba-flagellate transformation was lost about 5 hours before commitment. I repeated their experiment (see Section 2.6), using the culture conditions used for filming, and obtained the data shown in Table 3.3 and Fig. 3.6. The number of committed cells and the number of cells unable to transform into flagellates were scored using the methods described in Section 2.6.

In the early samples, before the level of committed cells reached 1% (0 - 60 h), about 92% of the cells transformed into flagellates. The number of cells which did not transform into flagellates rose before the number of committed cells started to increase and was greater than the number of committed cells for the remainder of the experiment. This agrees with the conclusion of Blindt et al. (1986) that ability to transform into a flagellate is lost before

commitment. From the distance between the curves (T in Fig. 3.6) it was possible to estimate that, under the culture conditions used in this study, ability to transform into a flagellate was lost 3 - 5 hours before commitment.

3.2.3 DNA content of developing cells

It had been found, by filming analysis, that the nuclei of uninucleate committed cells were larger than those of amoebae and binucleate cells (Section 3.2.1), but the nuclei of quadrinucleate cells were similar in size to those of vegetative amoebae. To determine whether this transient increase in nuclear area reflected a transient increase in DNA content, CL cells were stained and the DNA content of the cells was analysed by flow cytometry (see Section 2.9).

By carrying out flow cytometry on isolated amoebal nuclei, Turner *et al.* (1981) demonstrated that *Physarum* amoebae, which are haploid, spend the bulk of the cell cycle in G2 phase, with little or no G1 phase and a short S phase. This conclusion was confirmed by Fry and Matthews (1987), who carried out flow cytometry on intact amoebae.

The results from flow cytometry are shown in Fig. 3.7. The control amoebal population (Fig. 3.7a) had a main peak with mode in Channel 50 which contained roughly 80% of the cells; the results of Turner *et al.* (1981) indicated that this peak corresponded to haploid G2 phase. (Haploid G2 phase amoebae contain about 0.6pg of DNA which is distributed between about 40 small chromosomes [Mohberg, 1982]). A second peak with mode around Channel 100 contained roughly 10% of the cells; the results of Turner *et al.* (1981) indicated that this peak

corresponded to diploid uninucleate or haploid binucleate cells in G2 phase. The higher fluorescence values were assumed to correspond to polyploid and clumped cells.

The distribution of fluorescence values for a developing culture is shown in Fig. 3.7b. Microscopic analysis showed that only 3% of the cells contained more than one nucleus but replating assays demonstrated that 56% of the cells were committed; thus the population contained 53% uninucleate committed cells. The distribution of fluorescence values in Fig. 3.7b is similar to that in Fig. 3.7a with a main peak of mode 56 which contained about 80% of the cells. A secondary peak with mode at Channel 112 contained about 8% of the cells. Therefore, the uninucleate committed cells did not show an increase in DNA content proportional to the increase in nuclear area revealed by filming. However, the peaks in the developing culture are shifted slightly upwards relative to those of the non-differentiating culture; similar increases were found in three further experiments (data not shown).

The data described in Section 3.2 are summarised in Fig. 3.8

3.3 Development in RA376

3.3.1 Introduction

Initial filming analysis and replating studies on RA376 (Parkinson, 1988) indicated that the sequence of events in this strain was substantially similar to that observed for CL, but there were also some differences. Due to the small size of the sample obtained from this study, it was not possible to determine whether the differences in the sequence of events during development between CL and RA376 were statistically significant. Further filming analysis was carried out on two sublines of RA376. The first subline (RA376.1) was a stock stored on silica gel and was obtained from Dr. R. W. Anderson. The second subline (RA376.2) originally came from Dr. R. W. Anderson as cysts on an agar plate; the cells were subcultured for some time before a frozen stock was made. The initial studies had been carried out using RA376.2 by an undergraduate student at Leicester University (Parkinson, 1988).

3.3.2 Assays for committed cells and for cells unable to transform into flagellates in RA376.2

In order to determine the numbers of committed cells at various times after inoculation and the rate at which they formed, assays were carried out as described in Section 2.6. RA376.2 was set up at a starting density of 1×10^4 cells / LIA+CIT plate. The results of the assays are shown in Fig. 3.9 and Table 3.4. The RA376 amoebal doubling time was approximately 12 hours (slightly longer than that of CL

amoebae; see Section 3.2.2) and the first committed cells were present at about 40 hours (slightly earlier than found for CL). The number of amoebae peaked at 2×10^5 /plate (100 h) and the maximum number of plasmodia (8×10^4 /plate) was reached at the same time.

The timings of loss of ability to flagellate and commitment were estimated using replating experiments, similar to those described for CL (see Section 3.2.2). The results indicated that commitment in RA376.2 occurred in uninucleate cells at a maximum of 14.3 h before binucleate cell formation. The time of loss of ability to flagellate could not be separated from the time of commitment (Parkinson 1988).

3.3.3 Time-lapse cinematographic analysis of development in RA376.1 and RA376.2

Cell pedigrees

Time-lapse films were made of both RA376 sublines using the same initial cell density as was used for CL; filming was begun at the same time after inoculation as for CL. The magnification used was different from that used for the film of CL; in the RA376 films, one arbitrary unit was equal to $7.30 \mu\text{m}^2$. Examples of pedigrees traced from the film of RA376.1 are shown in Fig. 3.10; those for RA376.2 are essentially similar. The data for both sublines are summarised in Table 3.5.

In common with the situation in CL, apogamic development in RA376 involves an extended cell cycle ending in the

formation of a binucleate cell (Cells 6A, 3A, 3B, 1A in Fig. 3.10). This extended cell cycle was followed by a short cell cycle ending in the formation of a quadrinucleate cell (Cell 3A, Fig. 3.10b). The IMTs measured from the films of RA376.1 and RA376.2 are shown in Table 3.5. There was a significant difference (at the 5% level) between the amoebal IMTs of the two sublines; the IMT of RA376.2 was almost the same as that measured from the kinetics experiment (see Section 3.3.2).

During the film of RA376.1, one cell divided at the end of the extended cell cycle; during the film of CL no cells divided at this time. Ten other RA376.1 cells became binucleate at the end of the extended cell cycle (Table 3.5); one of these subsequently split into two uninucleates several hours after mitosis (Cell 1A, Fig. 3.10c). One cell was seen to divide at the end of the long cell cycle in the film of RA376.2; four cells became binucleate at the end of the extended cell cycle. The sample obtained from the two films was too small to determine whether the difference between the RA376 sublines, in the number of cells which divided or developed at the end of the long cell cycle, was statistically significant. Neither was it possible to determine whether there was a statistically significant difference between CL and RA376 in the number of cells which divided at the end of the long cell cycle. Of the four uninucleate cells formed following mitosis and cytokinesis in the two large uninucleate cells at the end of the extended cell cycle, only one could be traced for more than a few hours. This cell, from RA376.1, became binucleate 10.43 h after the previous division; this IMT was shorter than the

average amoebal IMT for this subline, and equal to the time between binucleate cell formation and quadrinucleate cell formation for this subline (Table 3.5). The subsequent fate of this cell could not be determined.

As in CL, all divisions at the end of an amoebal cell cycle appeared to be of the open amoebal type. Some of the mitoses by which large uninucleate cells became binucleate appeared to be of the plasmodial type in which the nuclear membrane remained intact. As in CL, incomplete cleavage furrows were seen in some uninucleate cells undergoing mitosis to become binucleate and also in some binucleate cells undergoing mitosis to become quadrinucleate. Two cells failed to complete cytokinesis at the end of a normal amoebal IMT and formed binucleate cells; the subsequent fate of these binucleate cells could not be traced.

Cell growth

In the films of both RA376 sublines, amoebal cell area approximately doubled during each cell cycle (Table 3.5). Growth continued throughout the extended cell cycle, so that at the mitosis at which a binucleate cell was formed, the developing cell was about twice as big as an amoeba at mitosis (Table 3.5). Cell area did not double during the short cell cycle between binucleate and quadrinucleate cell formation. In the film of RA376.1, the amoebae which carried on to develop were significantly larger at birth than amoebae which completed a normal amoebal cell cycle (Table 3.5).

Plasmodial fusions

In the two films of RA376, a total of 6 fusions were seen which involved the fusion of a large, uninucleate cell with a multinucleate cell. All the uninucleate cells which fused with a multinucleate cell and whose origins could be traced arose from apparently normal amoebal divisions and were at least 20 h old at the time of fusion (0.75 of the extended cell cycle). Ten fusions were seen which involved two multinucleate cells. Two fusions were observed between two large, uninucleate cells. In one fusion both the cells were more than 20 h old at fusion; the subsequent fate of the binucleate cell formed in this fusion could not be traced. The other fusion between uninucleate cells gave rise to an abnormal cell and is described below.

Abnormal cells

During the films of RA376, 3 multinucleate cells were seen to become abnormal. Two of these had become binucleate at the end of an extended cell cycle, but during the following interphase the nuclei disappeared; the cells became thick and rounded and showed vigorous cytoplasmic movement but did not move about the field of view. The other abnormal cell became binucleate as a result of fusion between two large, uninucleate cells, one of which was 27.4 h old at fusion, while the origin of the other could not be traced. The two nuclei in the cell entered mitosis synchronously and a quadrinucleate cell was formed; nuclear fusion followed in interphase to give a binucleate (presumably diploid) cell. This binucleate cell subsequently became thick and rounded,

and exhibited vigorous cytoplasmic movement so that the nuclei were no longer visible.

3.3.4 DNA content during development in RA376.2

Analysis of DNA content in developing cells of CL had shown that the increased nuclear area in large uninucleate cells was not due to increased DNA content. A similar flow cytometry study was carried out using RA376.2; DNA content was analysed as detailed in Section 2.9 and the results are shown in Fig. 3.11.

Fig. 3.11a shows the control amoebal population; microscopic analysis showed that the population contained 76.3% uninucleate cells and 23.3% amoebal cysts. The main, haploid G2 peak (see Section 3.2.3) had its mode at about Channel 50 and contained 89.2% of the cells. The secondary diploid G2 peak with mode at about Channel 100 contained 3.4% of cells. The cells at higher fluorescence values were assumed to correspond to polyploid and clumped cells.

The developing population from 22°C is shown in Fig. 3.11b. Microscopic analysis showed that the population contained only 2.7% binucleate cells; all the rest were uninucleate cells. Replating assays demonstrated that 47.4% of the population were committed; thus the population contained 44.7% uninucleate committed cells. The main fluorescence peak in Fig. 3.11b had its mode at about Channel 50 and contained 84.6% of the population; a secondary peak with mode at about channel 100 contained 6.1% of the cells. The remaining cells had higher DNA contents and were assumed to be polyploid and clumped cells. Therefore, as for CL, the

large increase in nuclear area in developing uninucleate cells did not reflect a similar large increase in DNA content.

3.4 DISCUSSION

3.4.1 Results from filming analysis

The cell pedigrees analysed in the film of CL development confirmed that plasmodium formation in CL was apogamic; the uninucleate cells that became binucleate arose from apparently normal amoebal divisions. Since either or both daughter cells from an amoebal division could become binucleate, the significant events leading to binucleate cell formation were presumably not initiated until after this division. The cell cycle ending in binucleate cell formation was approximately twice as long as an amoebal cell cycle, and the cells continued to grow throughout the cycle, so that at mitosis they were about twice the size of an amoeba at mitosis. Analysis of development in the two sublines of RA376 showed that development was also apogamic in this strain. Although there were some differences between the two RA376 sublines in the mean lengths of the cell cycles, the relative lengths were constant. Both sublines of RA376 followed a similar pattern of growth and development to CL.

The divergence of developing cells from vegetative cells could not be detected until the former failed to undergo mitosis and cytokinesis at the expected time. It seems likely that failure to enter mitosis at the end of an amoebal cell cycle resulted from changes initiated earlier in the cell

cycle. The differences in cell fate (divide or develop) observed between sister cells from an amoebal division did not appear to be due to a difference in cell size at birth, except in the film of RA376.1. It is not clear why there should be this difference between RA376.1, and CL and RA376.2.

3.4.2 Timing of events relative to commitment

The experiments in which cells were removed from the presence of inducer and observed during their continued development allowed an estimate of the time in the extended cell cycle at which development becomes irreversible. The maximum estimated time interval between commitment and binucleate cell formation was 13.5 hours for CL and 14.3 hours for RA376.2; this maximum interval corresponded to about 50% of the extended cell cycle for both strains. Since the cells were not developing synchronously, however, it was not possible to estimate how much the timing of commitment varied between individual cells nor whether commitment occurred at a particular cell size. Using the same experimental approach but different culture conditions, Blindt et al. (1986) estimated the maximum time between commitment and binucleate cell formation in CL as 5-10 hours. This shorter interval may have been related to the conditions used (higher temperature and live bacteria as food) which gave an amoebal doubling time approximately half as long as that measured for CL under our experimental conditions.

Blindt et al. (1986) estimated that cells of strain CL undergoing the amoebal-plasmodial transition lost the ability to transform into flagellates about 5 hours before

commitment. In the present study, using a similar method, but different culture conditions leading to slower growth, the time between these two events, for CL, was estimated to be 3-5 hours. For RA376.2, however, commitment and loss of ability to a flagellate could not be separated.

To find the time at which ability to undergo the amoeba-flagellate transformation was lost, relative to the extended cell cycle in the developing CL uninucleate cell, the average time between commitment and loss of ability to flagellate (4 h) was added to the time between commitment and binucleate cell formation ($13.5 + 4 = 17.5$ h); this total was subtracted from the mean length of the extended cell cycle (28.7 h; Fig. 3.8). This led to the conclusion that, for CL, ability to flagellate was lost at about the time ($28.7 - 17.5 = 11.2$ h) that the cell would have undergone mitosis and cytokinesis had it continued to cycle vegetatively (11.8 h). A similar calculation led to the same conclusion for RA376.2, even though the time of loss of ability to flagellate could not be clearly separated from commitment. This correlation between loss of ability to flagellate and failure to undergo mitosis may be due to a change in centriole activity, since it is known that both flagella formation and amoebal mitosis require centrioles, whereas the mitosis by which a committed cell becomes binucleate is thought to be of the intranuclear plasmodial type in which centrioles are not required (Gull *et al.*, 1985; Blindt 1987).

3.4.3 Results from flow cytometry

The flow cytometry results using a developing CL culture

(Fig. 3.7b) showed that the major peak was shifted up relative to the control culture (Fig. 3.7a). Since 44% of the cells in the major peak were uncommitted and no similar increase in DNA content was found in developing cultures of RA376.2 (Fig. 3.11), it seemed unlikely that the increases in nuclear area observed in uninucleate developing cells reflected an increase in nuclear DNA content associated with commitment. It was possible that the slightly lower DNA content of CL vegetative amoebae relative to CL developing cells was a consequence of growing the amoebae at 30°C.

The flow cytometry results using non-differentiating amoebae from both apogamic strains (Figs. 3.7a & 3.11a) indicated that there were some amoebae which possessed double the normal amount of the DNA. The two RA376 amoebae which became binucleate as a result of incomplete cleavage at mitosis (see Section 3.2.3) would have a doubled DNA content. If nuclear fusion occurred at mitosis in binucleate amoebae (as observed in *matA*-homoallelic cultures by Holt and Huttermann, 1979), diploid amoebae would arise following cytokinesis; these cells would also have a doubled DNA content. Both mechanisms might be expected to contribute to the small secondary peak present in all clonal cultures of amoebae.

The results from flow cytometry indicated that there was no increase in nuclear DNA content during apogamic development in CL or RA376; thus, the cytoplasm:DNA ratio is greater in large uninucleate cells at mitosis than it is in amoebae. The ratio is apparently reduced again during the short cell cycle ending in the formation of a quadrinucleate

cell. Laffler and Tyson (1986) suggested that cell cycle regulation in *Physarum* operates by a size control mechanism; attainment of a critical protein:DNA ratio triggers mitosis. If this is correct, the critical ratio required to trigger mitosis appeared to be the same in amoebae and plasmodia but was altered in the uninucleate cell committed to plasmodium development. Sweeney (1987) showed that large uninucleate cells contain substantially elevated levels of RNA; such an increase in transcription might be associated with the transient increase in nuclear size observed during development.

3.4.4 Plasmodial fusions

In the films of CL, 39 fusions were observed between multinucleate cells but only one was seen between a uninucleate cell and a multinucleate cell; this suggested that the ability of developing plasmodia to fuse with one another was acquired at about the time of binucleate cell formation. In the films of RA376, however, almost as many fusions were observed between a uninucleate cell and a multinucleate cell (6), as there were fusions between multinucleate cells (10). Thus, in RA376, ability to undergo plasmodial fusions was apparently acquired earlier than in CL. In the films of RA376, it was possible to trace the origins of two of the uninucleate cells that fused with plasmodia; both were more than 20 h old and so were about three quarters of the way through the extended cell cycle and, presumably, some hours past the point of commitment. Two examples of fusion between two uninucleate cells were

observed during the films of RA376. In one of these pairs of uninucleate cells, both cells had been in the field of view for more than 20 h which was close to the length of the extended cell cycle (about 28 h). Thus, in RA376 at least, fusion might occur between two normally developing, large, uninucleate cells which were both at a late stage of the extended cell cycle.

3.4.5 Cleavage and mitosis

It was suggested by Blindt (1987) that, in CL, mitosis had altered from the open amoebal type to the closed plasmodial type by the time of the mitosis at which the developing uninucleate cell becomes binucleate. However, the plasmodium-specific $\beta 2$ -tubulin isotype, which first appeared at about the time of commitment, has been detected in mitoses of the amoebal type in large uninucleate cells (Solnica-Krezel *et al.*, 1988; Diggins-Gillicinski *et al.*, 1989). This suggested that not all mitoses that lead to the formation of a binucleate cell were necessarily of the intranuclear plasmodial type.

Partial cleavage furrows were observed in large uninucleate and binucleate cells at mitosis, suggesting that cleavage was incompletely suppressed for some time after development had begun. In two RA376 uninucleate cells, cleavage was completed at the end of the long cell cycle, giving rise to two uninucleate daughter cells rather than a single binucleate cell. Completion of cleavage at mitosis might depend partly upon the type of mitosis being used. In uninucleate cells undergoing plasmodial mitosis, cleavage

might be initiated at mitosis but not completed. Cleavage might be completed, however, in large uninucleate cells undergoing amoebal mitosis at the end of the long cell cycle.

3.4.6 Conclusions

The results detailed above, and those of many other authors (e.g. Blindt 1987; Sweeney 1987), suggest that the changes leading to the formation of a plasmodium are initiated during the extended cell cycle, and that amoebal characteristics are gradually lost and plasmodial ones acquired over several cell cycles. Although the sequence of events was substantially similar in the two apogamic strains, there appeared to be differences in the relative timings of loss of ability to transform into a flagellate and gaining of ability to undergo plasmodial fusions. Since apogamic strains arise as a result of mutation at the *matA* locus, these differences could be related to the different *matA* alleles from which the strains arose. *MatA* is the major locus controlling heterothallic plasmodium formation and it is, therefore, important to compare the sequence of events in heterothallic and apogamic development.

TABLE 3.1
Kinetics of growth and development in CL

Hours after inoculation	Numbers of colonies per differentiation plate			
	Amoebae	Average	Plasmodia	Average
0	5×10^3		0	
	4.8×10^3	4.9×10^3	0	0
24	3.3×10^3		0	
	5.7×10^3	4.5×10^3	0	0
30	6.6×10^3		0	
	6.4×10^3	6.5×10^3	0	0
36	9.8×10^3		0	
	9.3×10^3	9.6×10^3	0	0
42	5.7×10^3		0	
	1.0×10^4	7.8×10^3	0	0
48	9.8×10^3		0	
	1.4×10^4	1.2×10^4	0	0
54	1.0×10^4		0	
	1.7×10^4	1.4×10^4	0	0
60	1.8×10^4		0	
	1.6×10^4	1.7×10^4	0	0
66	3.4×10^4		1.5×10^2	
	3.0×10^4	3.2×10^4	1.0×10^2	1.3×10^2
72	7.0×10^4		3.5×10^2	
	5.9×10^4	6.5×10^4	1.8×10^2	2.7×10^2
78	1.4×10^5		1.5×10^3	
	7.0×10^4	1.1×10^5	5.3×10^2	1.0×10^3
84	1.7×10^5		2.2×10^3	
	1.1×10^5	1.4×10^5	9.3×10^2	1.6×10^3
90	1.4×10^5		2.8×10^3	
	1.5×10^5	1.5×10^5	3.2×10^3	3.0×10^3
96	1.5×10^5		6.6×10^3	
	2.8×10^5	2.2×10^5	1.8×10^4	1.2×10^4
102	2.4×10^5		5.8×10^4	
	2.1×10^5	2.3×10^5	1.8×10^4	3.8×10^4
108	2.8×10^5		5.9×10^4	
	3.3×10^5	3.1×10^5	8.9×10^4	7.4×10^4
114	4.0×10^5		1.7×10^5	
	2.6×10^5	3.3×10^5	9.0×10^4	1.3×10^5
120	3.0×10^5		1.2×10^5	
	2.0×10^5	2.5×10^5	9.6×10^4	1.1×10^5
136	1.5×10^5		7.4×10^4	
	1.3×10^5	1.4×10^5	7.1×10^4	7.3×10^4

The average numbers of amoebae and plasmodia per plate at each timepoint are plotted on Fig. 3.4.

TABLE 3.2

Time between commitment and binucleate cell formation in CL

Slide	Hours after start	Numbers of 2 nuclei	of multinucleate 4 nuclei	cells with more nuclei	total cells	% of maximum no. of multinucs.
1 ▲	0	37	9	1	47	23
	4	59	13	5	77	38
	6	61	18	5	84	42
	8	89	28	8	125	62
	11	136	46	8	190	95
	13	148	43	4	195	97
	15	135	58	7	200	100
2 Δ	0	19	7	3	29	23
	4	35	7	1	43	34
	6	41	9	1	51	40
	8	59	15	5	79	62
	12	82	30	4	116	91
	14	86	36	5	127	100
	15.5	78	43	5	126	99
	17.5	72	39	9	120	95

For Slide 1 the maximum number of multinucleate cells was 200 (15h).

For Slide 2 the maximum number of multinucleate cells was 127 (14h).

These data are plotted in Fig. 3.5

TABLE 3.3

Timing of loss of ability to undergo the amoeba-flagellate transformation in CL

Hours after start	% Committed cells	% Flagellates
24	0	93.2
42	0.01	91.0
50	0.1	92.0
55	0.2	91.6
60	0.3	93.2
64	0.3	89.2
68	15.6	75.2
76	42.0	62.4
84	36.7	49.2
96	67.4	16.4

These data are plotted in Fig. 3.6.

Table 3.4
Kinetics of growth and development in RA376.2

Hours after inoculation	Numbers of colonies per differentiation plate			
	Amoebae	Average	Plasmodia	Average
0	5.5×10^3		0	
	5.6×10^3	5.6×10^3	0	0
24	6.4×10^3		0	
	7.9×10^3	7.2×10^3	0	0
31	8.5×10^3		0	
	7.1×10^3	7.8×10^3	0	0
48	2.0×10^4		2.5×10^1	
	1.9×10^4	2.0×10^4	2.5×10^1	2.5×10^1
55	6.3×10^4		1.0×10^2	
	4.8×10^4	5.6×10^4	1.3×10^2	1.2×10^2
72	7.6×10^4		4.7×10^3	
	1.2×10^5	9.6×10^4	3.3×10^3	4.0×10^3
80	1.3×10^5		1.0×10^4	
	1.0×10^4	1.2×10^5	2.0×10^4	1.5×10^4
96	1.6×10^5		6.4×10^4	
	3.1×10^5	2.4×10^5	8.4×10^4	7.4×10^4
103	2.2×10^5		6.4×10^4	
	2.3×10^5	2.3×10^5	1.1×10^5	7.7×10^4
120	1.7×10^5		—	
	1.3×10^5	1.5×10^5	3.8×10^4	3.8×10^4

The average numbers of amoebae and plasmodia per plate at each timepoint are plotted on Fig. 3.9.

Table 3.5

Summary of data from time-lapse cinematography of RA376.1 and RA376.2

Measurement	RA376.1	RA376.2
Amoebal IMT	13.4 ± 3.0 h (24)	11.4 ± 2.7 h (13)
Length of extended IMT	29.7 ± 4.2 h (10)	27.1 ± 1.3 h (5)
Binucleate to quadrinucleate IMT	10.6 ± 3.3 h (2)	-
Amoebal area at birth	10.6 ± 1.63 (23)	9.7 ± 1.1 (13)
Amoebal area at mitosis	23.4 ± 5.6 (23)	22.3 ± 5.2 (13)
Area at birth of cells which become binucleate	15.6 ± 5.0 (10)	10.0 ± 1.2 (5)
Area at binucleate cell formation	58.0 ± 21.6 (10)	40.8 ± 10.0 (5)
Area at quadrinucleate cell formation	104.0 ± 12.7 (2)	-
Number of cells which divide at the end of the extended cell cycle	1/11	1/5

Measurements are given as mean $\pm$ standard deviation and the number of observations is given in parentheses. Times are given in hours (h) and areas are given in arbitrary units.

Figure 3.1

Representative cell pedigrees for CL.

↪ Time of nucleolar disappearance preceding mitosis

⊙ Uninucleate cell

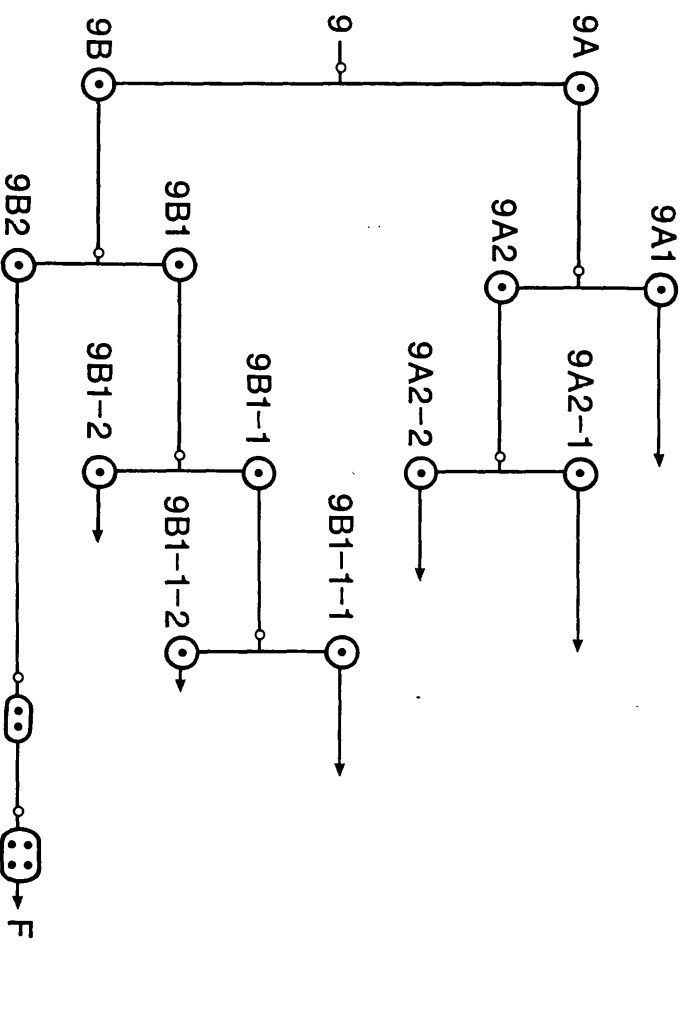
⊖ Binucleate cell

⊖ Quadrinucleate cell

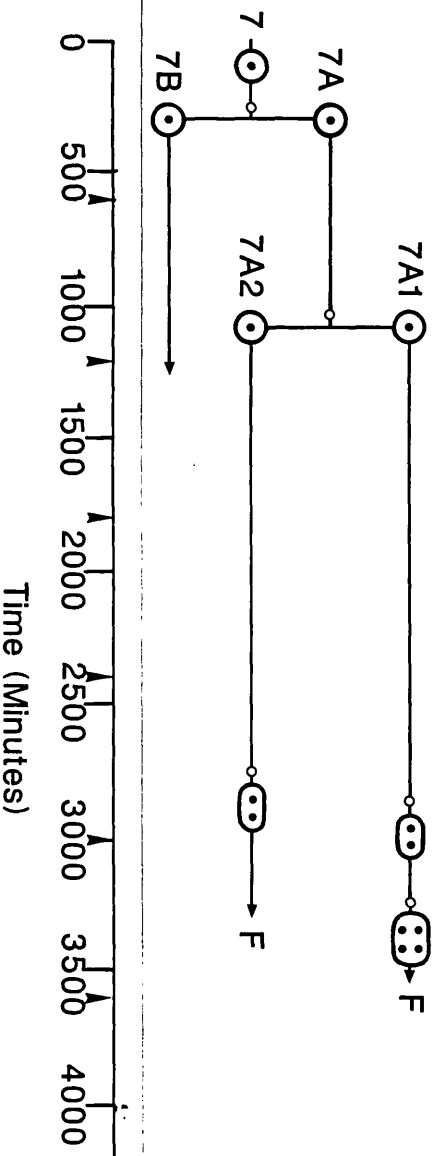
F - Cell fused with another multinucleate cell

→ Cell left field

Filming began at 0 hours. The arrows (➤) on the time axis indicate 10 hour intervals.



a



b

Figure 3.2

Cell area in relation to position in the cell cycle for CL. Amoebal intermitotic time (IMT) was normalized to 100 units. $\circ$ - shows mean for 9 cells. IMT for cell cycles ending in binucleate cell formation was normalized to 240 units. $\square$ - shows mean for 5 cells. Bars indicate range of measured cell areas for the 5 cells which became binucleate. 1 arbitrary unit = $4.86\mu\text{m}^2$ (see Section 2.8).

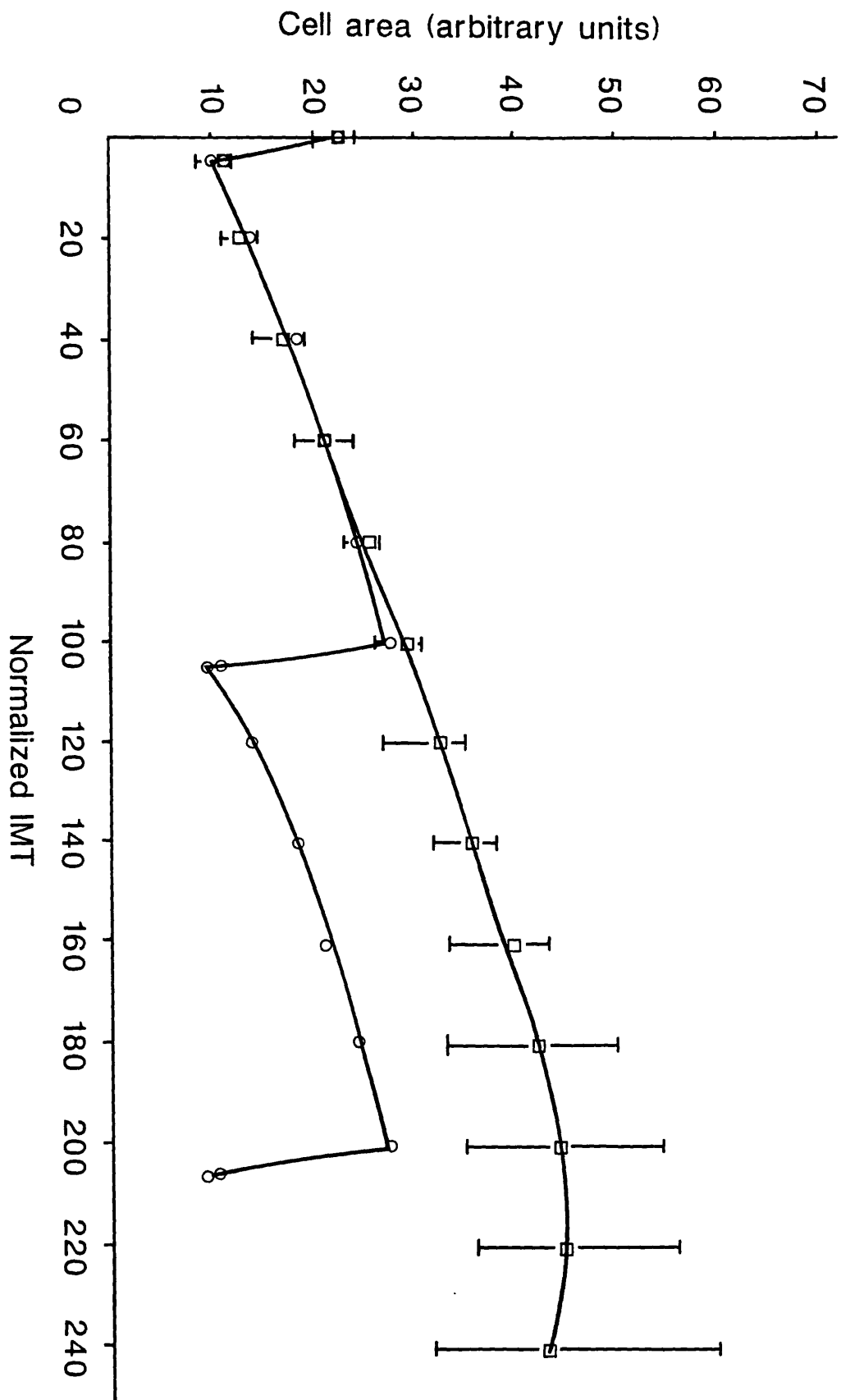


Figure 3.3

Fusion of CL multinucleate cells. P1,P2 & P3 multinucleate cells. B bacterial lawn. Bar = 10 μ m. The time between a and d is about 50 minutes. a) P1 and P2 are in close contact, P3 lies between two other unlabelled multinucleate cells. b) P1 and P2 have now fused. c) The product of fusion (P1&2) has moved across the field towards P3. P3 has moved out from between the other two multinucleate cells. d) P3 has fused with P1&2.

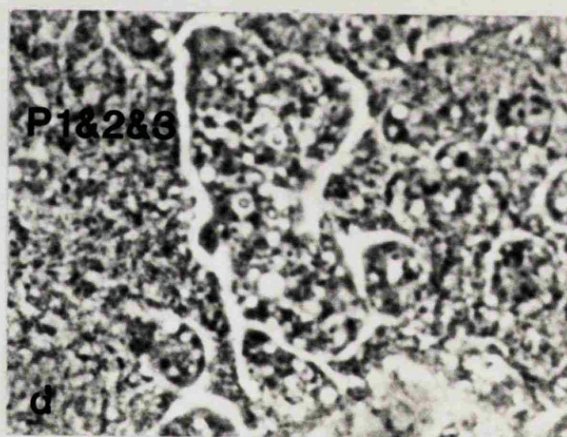
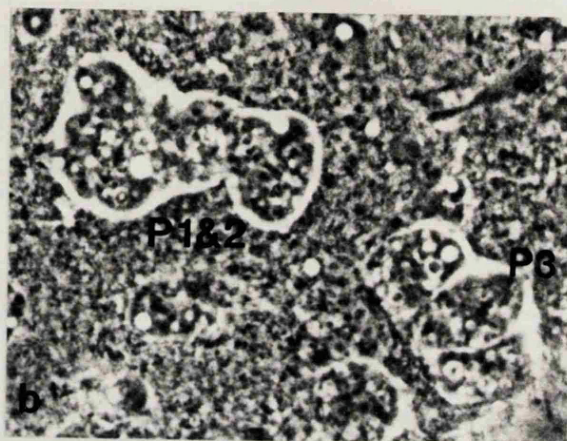


Figure 3.4

Kinetics of amoebal growth and plasmodium formation in CL cultures inoculated with cysts at time zero. Two plates were assayed, every 6 hours, for amoebae (O) and plateable plasmodia (●). Extrapolation of the plasmodial curve indicates that the first cell became committed to plasmodium formation at about 50 hours.

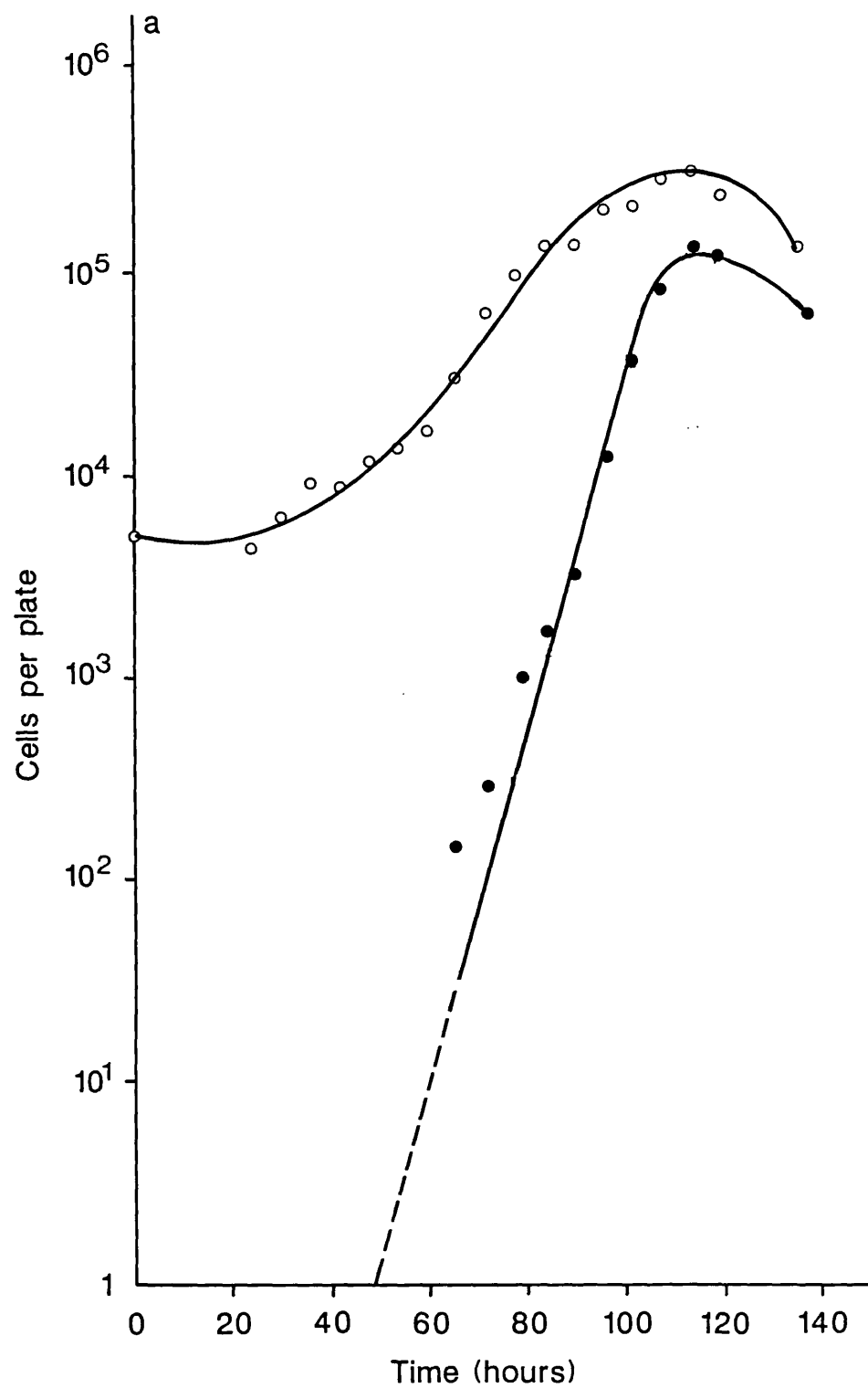


Figure 3.5

Kinetics of multinucleate cell formation in slide cultures inoculated with CL committed cells (see Text). The number of multinucleate cell was counted at each time point and is expressed as a percentage of the maximum number of multinucleate cells present during the experiment. ▲ = slide culture 1. Δ = slide culture 2.

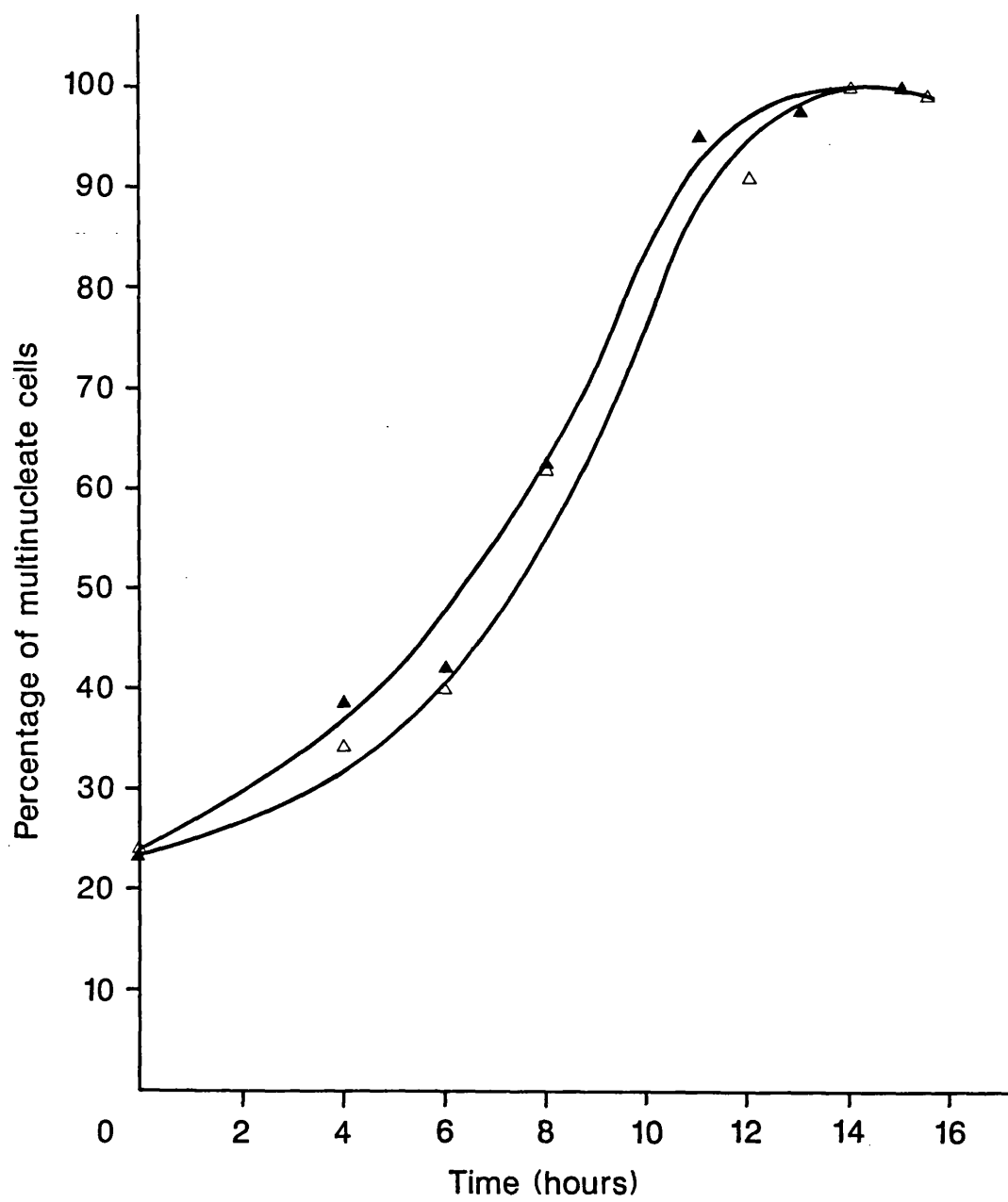


Figure 3.6

Percentage of CL committed cells and percentage of cells unable to transform into flagellates over a time period of 100 hours. Percentages of committed cells (X) were calculated from a graph similar to that shown in Fig. 3.4. Percentages of cells unable to transform into flagellates (O) were calculated by counting the number of flagellates in 200 stained cells at each time point. F - loss of ability to flagellate; C - commitment; T - mean time between C and F.

% committed cells (o) and % non flagellates (x)

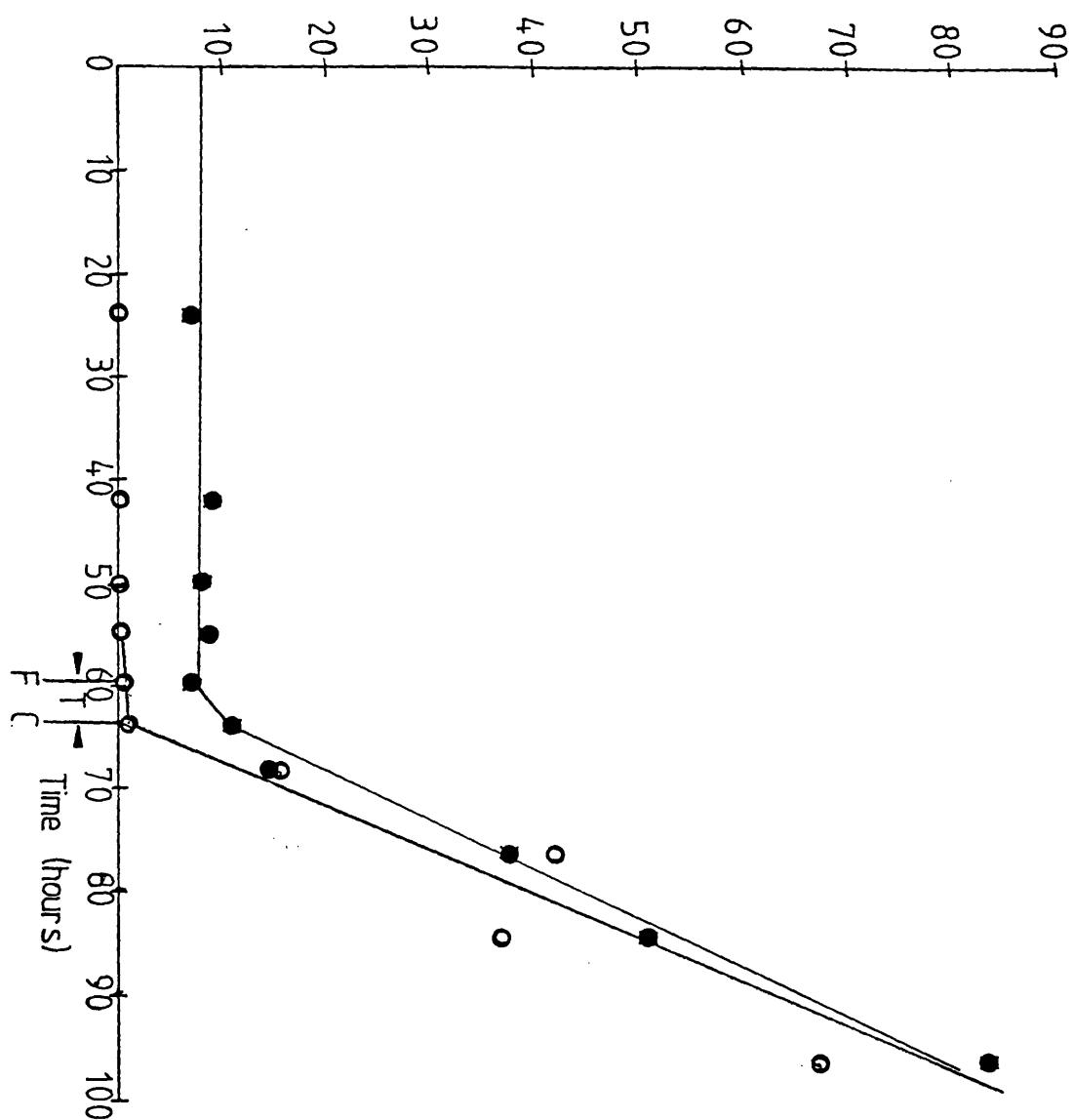


Figure 3.7

Distribution of cellular DNA contents in strain CL estimated by flow cytometry. a) Vegetative amoebae at 30°C
b) Developing population at 22°C. Channel number is proportional to DNA content (see Section 2.9). 10^4 cells were counted for each sample. On each panel the percentages of cells (i) in the main peak with the mode at or near channel 50, and (ii) in the secondary peak with the mode at or near channel 100, are indicated. The remaining cells had higher DNA contents.

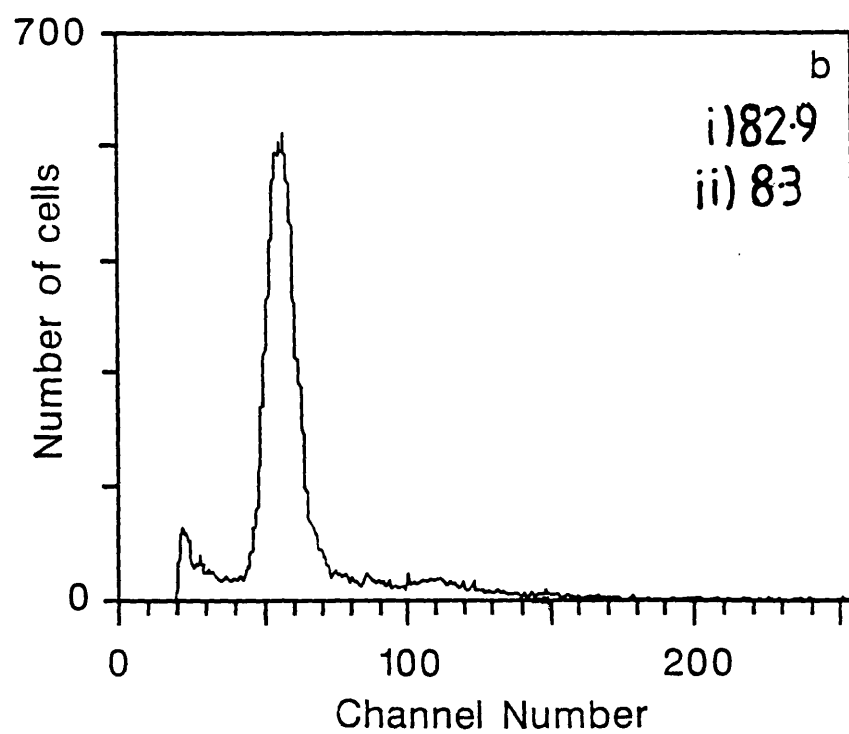
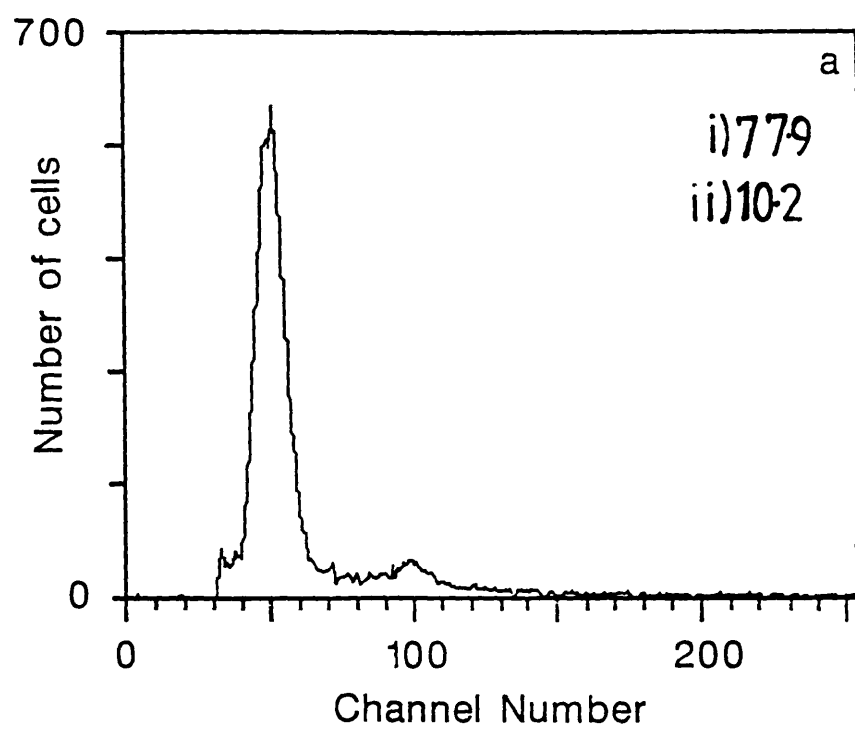
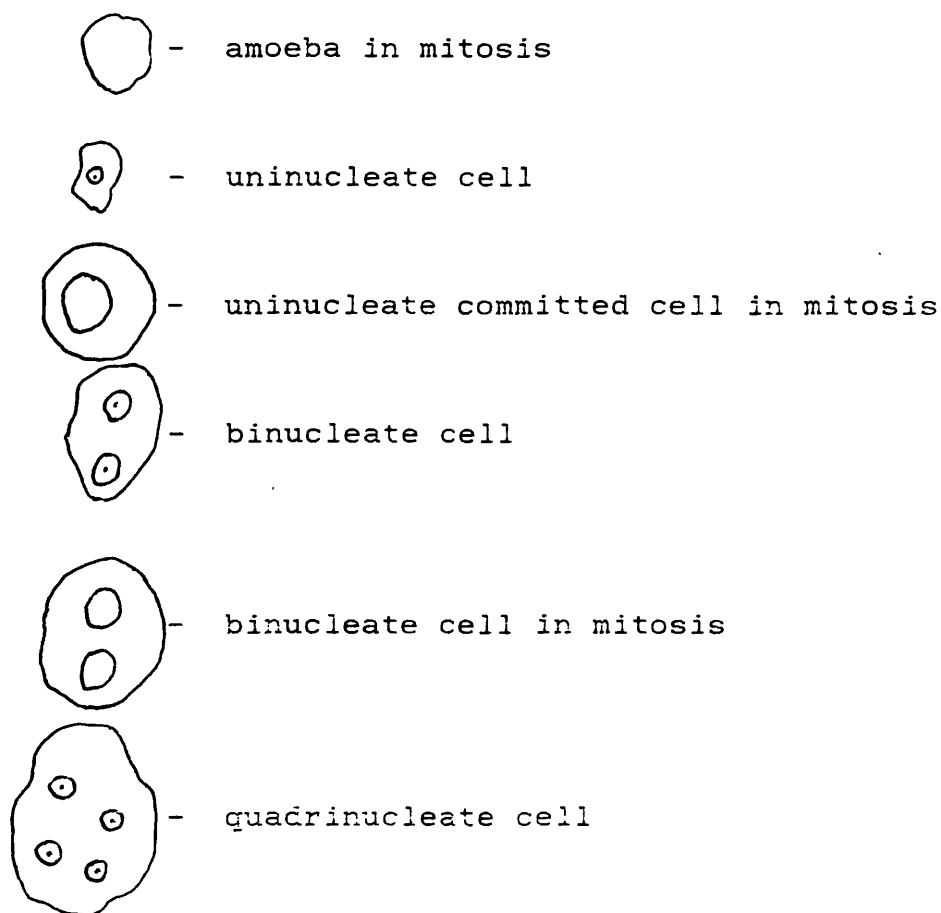


Figure 3.8

Sequence of events and some timings in the amoebal plasmodial transition of CL.



h - hours

M - mitosis

a - area in arbitrary units.

All values given are mean values (see Text). Area is given to the nearest whole arbitrary unit.

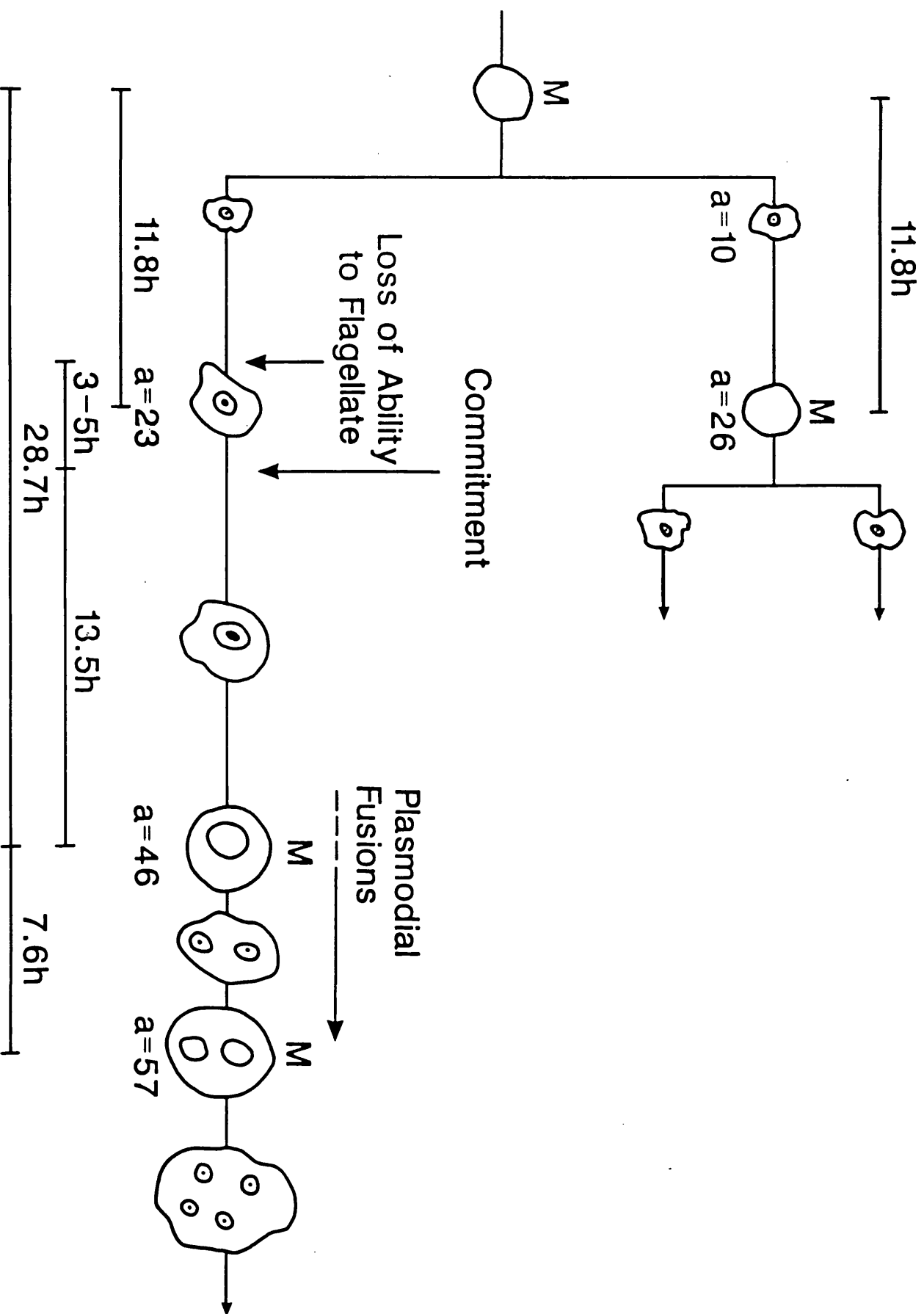


Figure 3.9

Kinetics of amoebal growth and plasmodium formation in RA376.2 cultures inoculated with cysts at time zero. Two plates were assayed, every 6 hours, for amoebae (●) and plateable plasmodia (○). Extrapolation of the plasmodial curve indicates that the first cell became committed to plasmodium formation at about 40 hours.

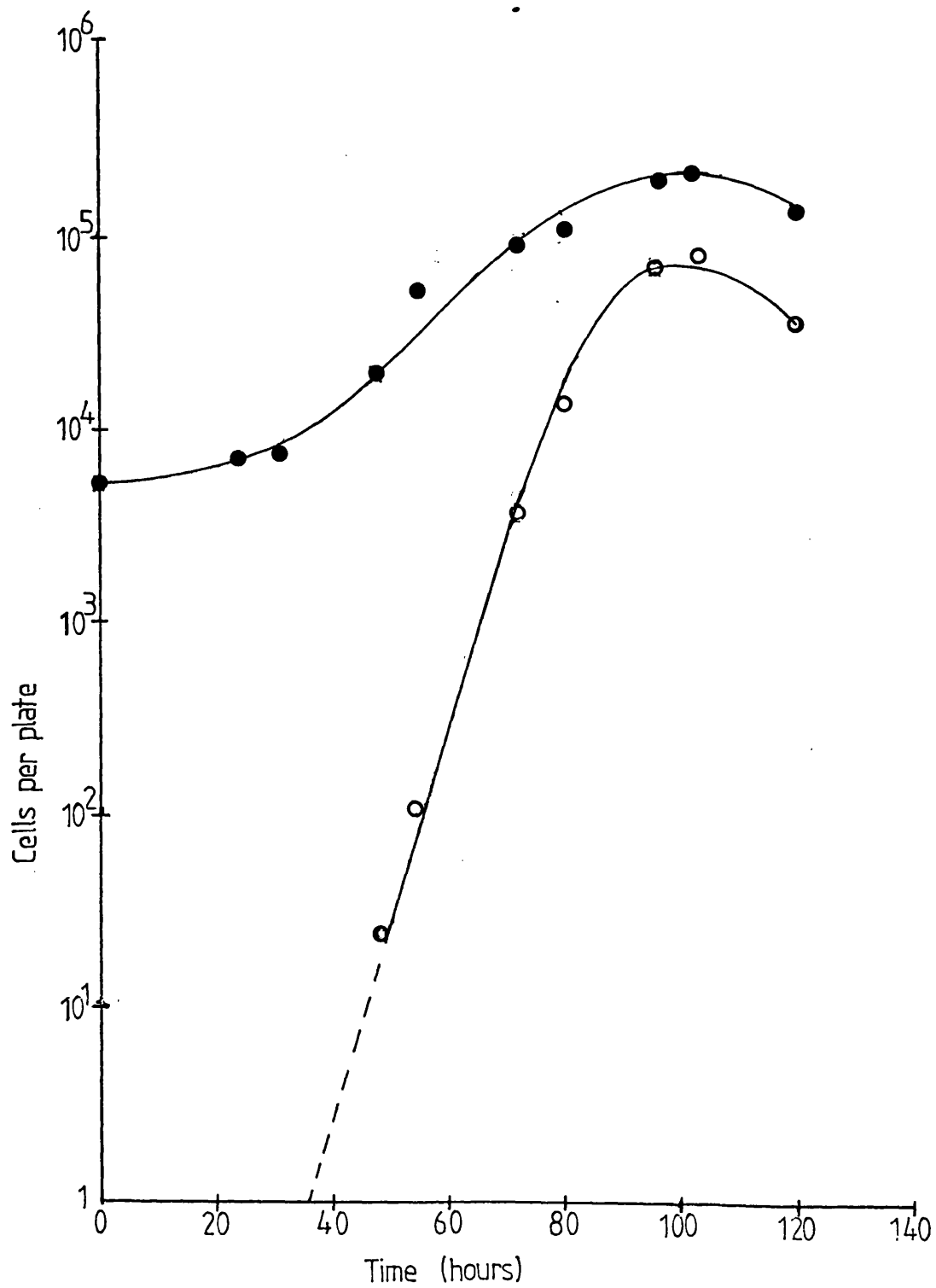


Figure 3.10

Representative cell pedigrees for RA376.

→ Time of nucleolar disappearance preceding mitosis

⊙ Uninucleate cell

⊞ Binucleate cell

⊞⊞ Quadrinucleate cell

F - Cell fused with another multinucleate cell

→ Cell left field

S - Cell split into two

Filming began at 0 minutes and finished at END after 66.7 hours.

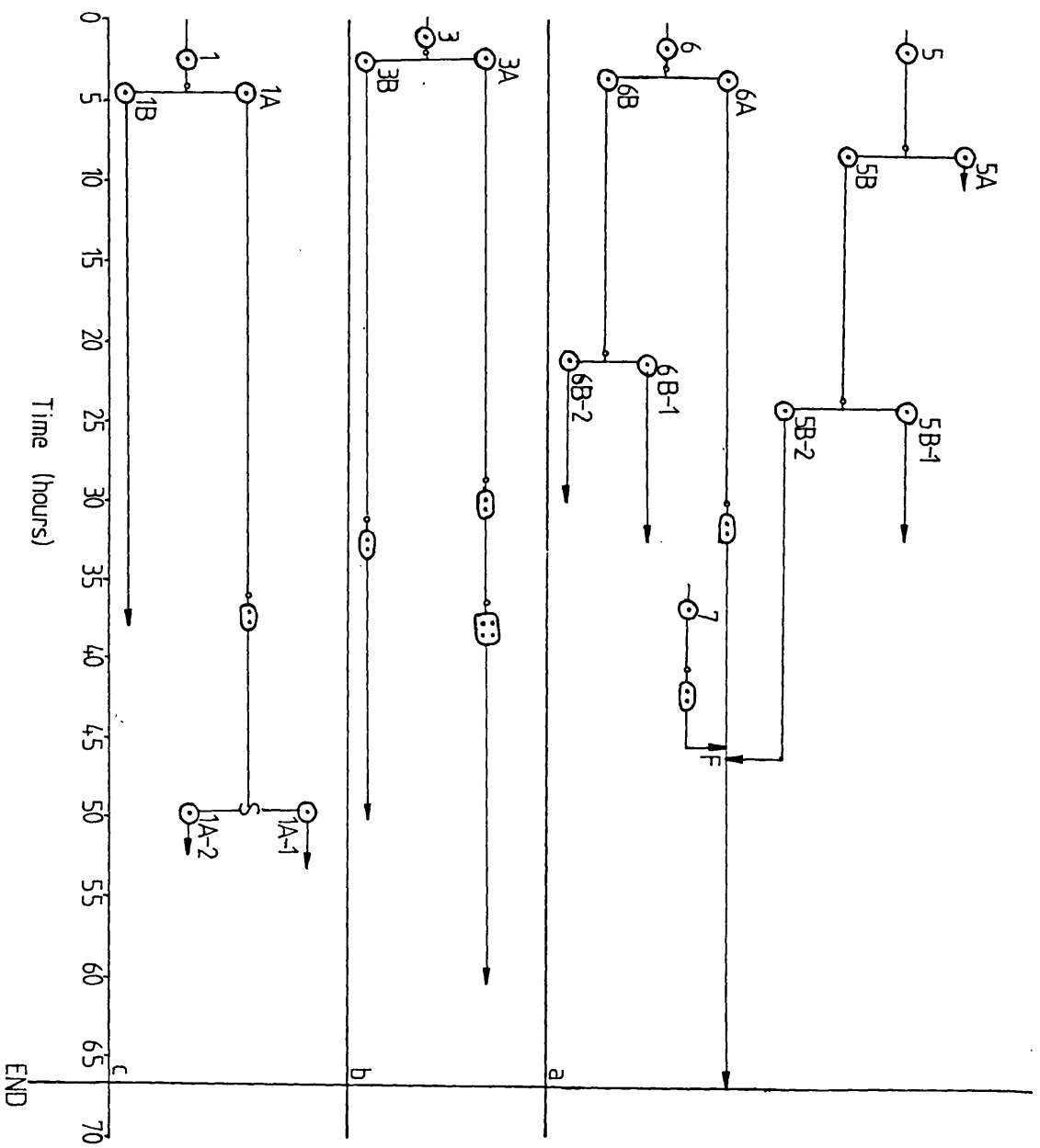


Figure 3.11

Distribution of cellular DNA contents in strain RA376.2

estimated by flow cytometry. a) Vegetative amoebae at 28.5°C

b) Developing population at 22°C. Channel number is

proportional to DNA content (see Section 2.9). 10^4 cells were

counted for each sample. On each panel the percentages of

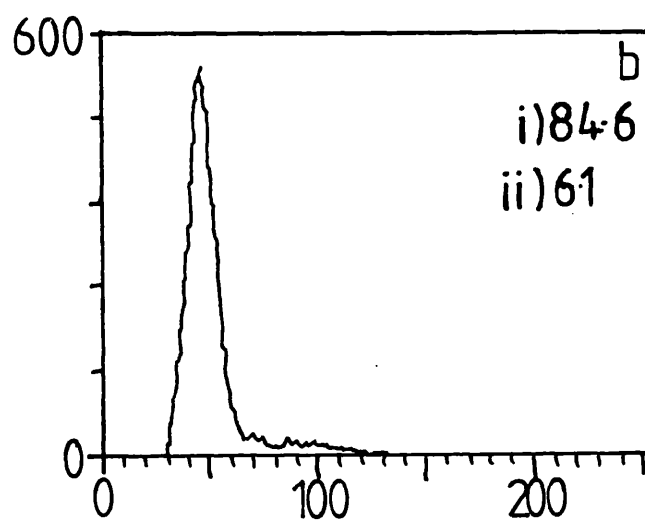
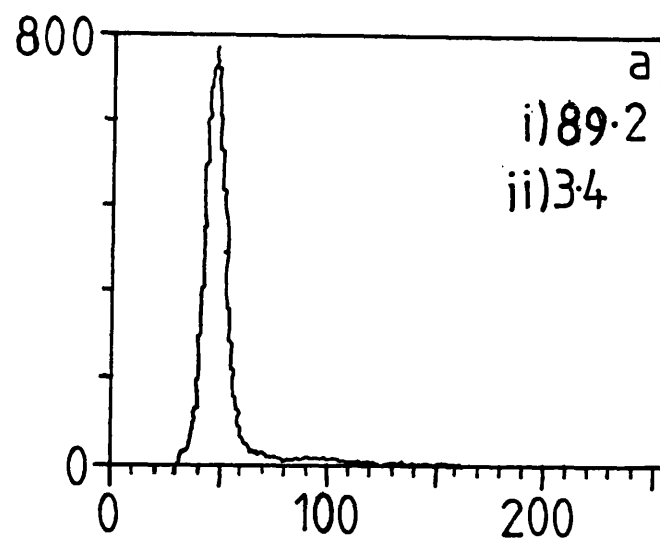
cells (i) in the main peak with the mode at or near channel

50, and (ii) in the secondary peak with the mode at or near

channel 100, are indicated. The remaining cells had higher DNA

contents.

Number of cells



Channel number

CHAPTER 4

THE EFFECT OF *MATA* ON CELLULAR EVENTS DURING DEVELOPMENT

4.1 INTRODUCTION

The work described in the previous Chapter showed that apogamic development involved an extended cell cycle during which growth continued, cells became committed to development, and ability to undergo the amoeba-flagellate transformation was lost. Apogamic strains arise as a result of mutation at *matA* in heterothallic strains, and sexual plasmodium formation occurs only by fusion of amoebae that carry different alleles of *matA* (see Section 1.2.2). In mixtures of strains that are homoallelic for *matA*, amoebae fuse but plasmodium development does not occur. By comparing the sequence of cellular events during sexual development with those that occur in *matA*-homoallelic fusion cells, it should be possible to determine the role of *matA* more precisely. Comparison of the sequence of events in sexual development with those already determined for apogamic development should identify those events that are common to both types of development.

In the work described in the present Chapter, time-lapse microcinematography was used to examine mixtures of cells either heteroallelic or homoallelic for *matA*, to determine the timing of cell fusion and subsequent events in relation to the amoebal cell cycle. DNA content was examined by flow cytometry. Immunofluorescence microscopy was used to investigate the changes in microtubule organisation that follow amoebal fusion in *matA*-heteroallelic and

matA-homoallelic cultures, and to compare these with the extensive changes that have been observed during apogamic development (Havercroft and Gull, 1983; Gull et al., 1985; Blindt, 1987).

This work has been described in a paper accepted for publication (Bailey et al., 1990).

RESULTS

4.2 Time-lapse Cinematography of *matA*-heteroallelic and *matA*-homoallelic cultures.

4.2.1 Events preceding amoebal fusion

Filming slides were set up as described in Section 2.7. Three films were made of mixed cultures inoculated with a pair of amoebal strains carrying different alleles of *matA*, *matB*, and *matC* (CH508 x LU648), and two films of mixed cultures in which the two strains carried the same *matA* allele, but different alleles of *matB* and *matC* (CH508 x LU859). Amoebal divisions continued throughout all 5 films and fusions between amoebae were observed during the course of all 5 films. Sexual development occurred in the *matA*-heteroallelic cultures and by the end of each film the field of view was occupied by large plasmodia. In the *matA*-homoallelic cultures, plasmodium development did not occur. Films were also made of each amoebal strain growing in clonal culture.

Amoebal intermitotic times.

It was not possible to differentiate visually between the amoebae of the two strains present in a mixed culture. Since amoebal fusions did not occur during the films of clonally cultured amoebae, it was assumed that amoebae observed to fuse in the films were of different strains. Intermitotic times were measured for amoebae throughout the 3 films of sexual development (CH508 x LU648) and no indication was found of a bimodal distribution; the mean IMTs were: Film 1: 9.38 ± 1.56 h, $n=20$; Film 2: 9.11 ± 1.24 h, $n=26$; Film 3: 8.94 ± 1.56 h, $n=23$. Analysis of variance of the mean amoebal IMTs for the 3 films of sexual development showed no significant differences between the films ($F=0.51$; $p>0.05$) and the data were therefore pooled for further analysis (Table 4.1A). The distributions of the amoebal IMTs measured from the 2 films of *matA*-homoallelic cultures (CH508 x LU859) also appeared unimodal and were not significantly different from each other ($p>0.05$; Film 1: 9.93 ± 1.50 h, $n=26$; Film 2: 10.26 ± 1.37 h, $n=19$); these data were pooled for further analysis (Table 4.1A). The apparently unimodal distribution of the amoebal IMTs from each of the 5 films suggested that the two strains in each mixture did not show characteristically different IMTs. This conclusion was supported by the analysis of films of each amoebal strain cultured alone under the same conditions used for the mixed cultures; no significant differences were found between the IMTs in these clonal cultures ($p>0.05$; CH508: 10.76 ± 1.52 h, $n=36$; LU648: 10.20 ± 2.02 h, $n=76$; LU859: 10.34 ± 1.63 h, $n=29$). There was, however, a significant difference ($p<0.001$)

between amoebal IMTs in *matA*-heteroallelic cultures and the amoebal IMTs for all other cultures (Table 4.1A; see Discussion).

Origins of fusing cells.

An example of a pedigree traced from a film of sexual development is shown in Fig. 4.1. Out of 68 amoebal fusions observed in the films of sexual development, 66 involved pairs of uninucleate amoebae and 2 were multiple fusions, each of which involved 3 amoebae. Most of the amoebae participating in fusions (101/138) could be traced back to a previous amoebal division, and from 45 of these divisions it was possible to determine the fates of both daughter cells. There were 21 divisions from which both daughter cells mated (e.g. daughters of Cell A in Fig. 4.1) and 24 divisions from which one sister mated and the other completed an apparently normal amoebal mitosis (e.g. daughters of Cell B in Fig. 4.1). The mean IMT of the sister cells that completed an apparently normal amoebal cell cycle was 8.92 ± 1.60 h ($n=24$), not significantly different from the mean for all amoebal cell cycles ($p>0.05$; Table 4.1A). In 24 cases it was possible to measure the length of the cell cycle preceding the one in which fusion occurred; the mean length of this cycle (8.72 ± 1.18 h, $n=24$) was not significantly different from the mean for all amoebal cell cycles ($p>0.05$; Table 4.1A).

Fig. 4.2 shows examples of pedigrees traced from the two films of *matA*-homoallelic cultures. The events preceding amoebal fusion in these cultures were very similar to those seen in the *matA*-heteroallelic cultures. Forty-nine fusions

were observed, of which 2 were multiple fusions involving 3 amoebae. Seventy of the amoebae that were seen to fuse were traced back to their origin at the previous amoebal division. Among 24 divisions from which the fates of both daughter cells could be determined, there were 17 divisions from which both daughter cells underwent cell fusion (e.g. daughters of Cell E in Fig. 4.2c) and 7 divisions from which one daughter cell divided and the other fused (e.g. daughters of Cell D in Fig. 4.2a). The mean IMT of sister cells that completed an apparently normal amoebal cell cycle (9.85 ± 1.48 h, $n=7$) was not significantly different from the mean for all amoebal cell cycles ($p>0.05$; Table 4.1A). The mean length of the cell cycle preceding the one in which cell fusion occurred (10.02 ± 1.45 h, $n=23$) also showed no significant difference from the mean for all amoebal cell cycles ($p>0.05$; Table 4.1A).

Age of amoebae at cell fusion.

The films were analysed to determine whether the fusion of amoebae occurred at a constant time after mitosis. Figure 4.3a shows the time from the start of mitosis (defined by nucleolar disappearance in prophase) to cell fusion in the *matA*-heteroallelic cultures; Fig. 4.3b shows the same information for the *matA*-homoallelic cultures. No fusions occurred for about 20 min following the start of mitosis; this period corresponds to the time taken to complete mitosis and cell division (M and D in Figs. 4.3a,b). Thereafter, amoebal fusions were distributed throughout a period roughly equal to the mean length of an amoebal cell cycle (marked as

IMT on Figs. 4.3a,b; Table 4.1A,B). The data were further analysed to determine whether there was a consistent relationship between the ages of a fusing pair (Fig. 4.4). For example, if an amoeba could fuse only with another of similar age, the points in Fig. 4.4 would lie along Line A. Alternatively, if the combined age of each pair of amoebae was constant, the points in Fig. 4.4 would lie along Line B. The distribution of points in Fig. 4.4 suggests that fusions between amoebae occur irrespective of cell age.

Multiple amoebal fusions.

In 3 of the 4 multiple fusions observed during filming, the origins of the fusing amoebae could be traced. In one fusion, in a culture heteroallelic for *matA*, a cell 4.9 h old fused simultaneously with a pair of sister cells 1.9 h old. In another fusion, in a *matA*-homoallelic culture, a cell 4.7 h old fused simultaneously with sister cells 1.7 h old. The final fusion in which the origin of the fusing cells could be traced did not involve the fusion of sister cells; this fusion, also in a *matA*-homoallelic culture, began with the fusion of 2 cells 4.0 and 4.7 h old and was followed 2 minutes later by fusion with a third cell 4.3 h old.

4.2.2 Events following amoebal fusion

In *matA*-heteroallelic cultures, nuclear fusion occurred in interphase about two hours after cell fusion (2.05 ± 0.67 h, $n=59$). In *matA*-homoallelic cultures, no nuclear fusions were observed to occur during interphase. Fourteen *matA*-homoallelic fusion cells remained binucleate until both

nuclei entered mitosis synchronously some hours later (Fig. 4.2b,c); the remaining 31 fusion cells split without mitosis to generate pairs of uninucleate cells (Fig. 4.2a) and 7 of these uninucleate cells later fused with different partners. In *matA*-homoallelic cultures there was no consistent relationship between the ages of amoebae at cell fusion and the fates of fusion cells (Fig. 4.4).

The films showed that sexual development in *matA*-heteroallelic cultures included an extended cell cycle preceding binucleate plasmodium formation. The length of this cell cycle was calculated by adding the average age of the pair of fusing amoebae to the time between cell fusion and the following mitosis at which the zygote became binucleate (Table 4.1E). The extended cell cycle was approximately 2.3 times the length of the mean amoebal cell cycle; the following cell cycle, leading to formation of a quadrinucleate cell, was shorter than the mean amoebal cell cycle (Table 4.1F).

In *matA*-homoallelic cultures, no extended cell cycle was observed; in fusion cells that remained binucleate until mitosis, the mean time between the mitosis preceding cell fusion and the synchronous mitoses in the fusion cell was not significantly different from the mean amoebal IMT ($p > 0.05$; Table 4.1A,E). For fusion cells that split to yield 2 uninucleates, the mean time between the mitosis preceding and the mitosis following amoebal fusion was significantly shorter than the mean length of an amoebal cell cycle ($p < 0.001$; Table 4.1A,E).

When *matA*-homoallelic fusion cells entered mitosis, both

nuclei proceeded synchronously (Fig. 4.5). The nucleolus moved to the side of the nucleus before disappearing and shortly afterwards the prophase nuclei vanished. This sequence of events was different from that observed in zygotes, in which the nucleus remained visible throughout mitosis. Metaphase plate formation and chromosome separation were clearly visible in zygotes, as they were in multinucleate plasmodia (Fig. 4.6). In some *matA*-homoallelic fusion cells, nuclear fusion occurred during prophase and 2 uninucleate, presumably diploid, daughter cells were formed after cytokinesis (Table 4.2, Class a); two such diploid daughter cells could be followed until the next mitosis at which time both divided into three. The mean IMT for these putative diploid amoebae (8.95 ± 0.27 h) was significantly shorter than the mean IMT for haploid amoebae ($p < 0.001$). In other fusion cells, nuclear fusion was not observed during mitosis and the two daughter cells were both binucleate (Table 4.2, Class b; Fig. 4.2b). The binucleate daughter cells showed the same range of fates as their binucleate parent cells, either splitting into 2 uninucleates (3 cases) or entering mitosis as a binucleate cell (1 case; Fig. 4.2b). Two other *matA*-homoallelic fusion cells observed in mitosis divided to give three daughters (Table 4.2, Classes c,d; Fig. 4.2c).

Multiple amoebal fusions.

In *matA*-heteroallelic cultures, it was possible to determine the fates of the 3 nuclei in each of the fusion cells formed by multiple amoebal fusions. Two of the nuclei

in each cell fused together about 2 h after cell fusion, followed by fusion with the third nucleus 30-40 min later. The resulting (presumably triploid) zygotes became binucleate 16-17 h after cell fusion and one was observed to become quadrinucleate 5.2 h after becoming binucleate. Both cells eventually fused with larger plasmodia and the subsequent fates of their nuclei could not be determined.

In both of the multiple fusions involving amoebae homoallelic for *matA*, the resulting trinucleate cells split to form a binucleate and a uninucleate. One binucleate cell subsequently separated into two uninucleates whose final fates could not be determined. The other binucleate cell entered mitosis and gave rise to 2 binucleate daughters whose subsequent fates could not be determined.

4.2.3 Cell growth

In *matA*-heteroallelic cultures, amoebae approximately doubled in area in one cell cycle, growing from 7.6 ± 1.3 units to 17.9 ± 1.9 units ($n=19$; Fig. 4.7). Amoebae that participated in mating were not significantly different in area, at birth, from amoebae that divided to yield daughter cells ($p>0.05$; Fig. 4.7). At the time of mating, amoebal areas ranged from 7.5 to 21.0 arbitrary units; this large variation in area was consistent with the variation in cell age at fusion (Fig. 4.3a). During the extended cell cycle, zygotes continued to grow until, at the first plasmodial mitosis, they were approximately 3 times the area of amoebae in mitosis (Fig. 4.7). Cell area failed to double in the short cell cycle that ended with the formation of a quadrinucleate plasmodium,

increasing by a factor of only 1.6 (Fig. 4.7); this failure to double cell area may be related to an increase in cell thickness.

Increase in plasmodial size occurred both by growth of individual plasmodia and by fusions between plasmodia. Many fusions were observed between plasmodia with two or more nuclei, but no fusions were seen between uninucleate zygotes. Two zygotes fused with multinucleate plasmodia; both zygotes were undergoing mitosis at the time of fusion and completed mitosis after fusion.

Following nuclear fusion, nuclear area increased in zygotes during the remainder of the extended cell cycle; at mitosis, nuclear area was substantially greater than twice the nuclear area of a haploid amoeba entering mitosis (compare nuclear areas in Fig. 4.8a,b,c). Nevertheless, in quadrinucleate cells, the area of each diploid nucleus was approximately twice the area of a haploid amoebal nucleus (not illustrated).

During amoebal cell cycles in *matA*-homoallelic cultures, cells approximately doubled in area, growing from 7.8 ± 1.0 ($n=26$) units to 19.0 ± 2.9 units ($n=13$). *MatA*-homoallelic fusion cells in mitosis were approximately 1.5 times the area of amoebae in mitosis (29.5 ± 4.8 units, $n=8$ compared with 19.0 ± 2.9 units, $n=13$). The 2 (putative) diploid amoebae that arose as a result of nuclear fusion at mitosis in *matA*-homoallelic fusion cells, were larger, at mitosis, than amoebae at mitosis, (25.0 ± 0.0 units, $n=2$ compared with 19.0 ± 2.9 units, $n=13$). Each of these putative diploid cells divided to give three daughters of almost equal size (8.3 ± 1.0

units, $n=6$). In *matA*-homoallelic cultures, the observed changes in nuclear area were consistent with the changes in ploidy assumed to have occurred at mitosis.

4.3 Cellular DNA Content in *matA*-heteroallelic and *matA*-homoallelic cultures.

The cellular DNA contents of amoebae in clonal cultures of the 3 strains used in this Chapter were analysed by flow cytometry. The cellular DNA contents of *matA*-heteroallelic and *matA*-homoallelic cultures were also examined. The cells were set up and harvested as described in Section 2.9 and the results are shown in Fig. 4.9.

Microscopic analysis of the clonal cultures of amoebae showed that all of the amoebae that were not encysted (about 80%) were uninucleate. Flow cytometry indicated that more than 78% of the cells had DNA contents in the main, haploid G2 peak with mode at about channel 50 (Fig. 4.9a,b,c; see Section 3.2.3). The second, diploid G2 peak had a mode at about channel 100 and contained 7 - 10% of the cells (see Section 3.3). The remaining cells, with DNA contents at higher fluorescence values, were assumed to correspond to polyploid and clumped cells.

Microscopic analysis of a *matA*-heteroallelic culture indicated that it contained 98.8% uninucleate cells, 0.5% binucleate fusion cells and 0.7% binucleate plasmodia. Replating analysis indicated that this culture contained 12.3% committed cells of which 11.6% ($12.3 - 0.7$) would have been uninucleate zygotes or binucleate fusion cells. Flow

cytometry indicated that the main, haploid G2 peak (Fig. 4.9d), with mode at about channel 50, contained 73.9% of the cells. The secondary, diploid G2 peak at about 100 units contained 16.2% of the cells. The remaining cells with DNA contents at higher fluorescence values were assumed to correspond to multinucleate and clumped cells. The simplest explanation for the distributions observed was that zygotes and fusion cells were in diploid G2 phase.

Microscopic analysis of the *matA*-homoallelic culture indicated that 98% of the cells were uninucleate, 1.6% were binucleate and 0.4% were trinucleate. Flow cytometry indicated that the main, haploid G2 peak, with mode at about channel 50, contained 70.8% of the cells. There was a second group of 20.4% of the cells whose DNA contents lay in a region consisting of the shoulder to the right of the main peak and a secondary peak at about 100 units (Fig. 4.9e); these cells were assumed to correspond to diploid cells in G2 phase and aneuploid cells which arose as a result of tripolar divisions in diploid amoebae (see Section 4.2.2). The remaining cells with higher DNA contents were assumed to correspond to polyploid and clumped cells.

4.4 Microtubule organisation in *matA*-heteroallelic and *matA*-homoallelic cultures.

Alterations in microtubule organisation following cell fusion in *matA*-heteroallelic and *matA*-homoallelic cultures were studied using immunofluorescence microscopy.

Mata-heteroallelic cells were fixed on glass but *matA*-homoallelic cells were fixed on agar plates. Both types of fixed cells were double-stained with an anti- β -tubulin monoclonal antibody and the DNA-specific fluorochrome DAPI using the method described in Section 2.10.

4.4.1 Microtubule organisation in *matA*-heteroallelic cultures during plasmodium development

Counts were made of the number of MTOCs present in a sample of interphase fusion cells, zygotes and binucleate plasmodia (Table 4.3). Binucleate fusion cells were recognised by the presence of 2 nuclei similar in size to those of vegetative amoebae (Fig. 4.8a); the majority of these cells contained 2 MTOCs although some possessed 0 or 1 MTOC (Table 4.3; Fig. 4.10a,b). Zygotes were recognised as large cells containing a single large nucleus (Fig. 4.8a,b); only 3% of these possessed 2 MTOCs, the majority possessing one (Table 4.3; Fig. 4.10c,d). Binucleate plasmodia were identified by the presence of 2 large nuclei within a large cell (Fig. 4.8c); most of these did not possess an MTOC (Table 4.3). Microtubules were present in all binucleate plasmodia, but in cells lacking an MTOC, the microtubules did not radiate from a single focus (not illustrated). Plasmodia with more than two nuclei were excluded from consideration because they could have been the result of fusion between smaller plasmodia.

In amoebae stained with the anti- β -tubulin antibody, a pair of small dots was seen closely associated with the MTOC; similar structures were present at the spindle poles of

mitotic amoebae (Fig. 4.13a,b), suggesting that they were the centrioles. A stained cell population was used to determine the number of pairs of centrioles present in cells at different stages of sexual development. Out of 98 zygotes in which centrioles could be scored, 77 possessed two pairs of centrioles; these were either present as a group of four (62 cells; Fig. 4.10e,f) or as two separate pairs (15 cells; Fig. 4.10g,h). The remaining 21 zygotes each possessed only one pair of centrioles. Centrioles could not be observed in 71 of the zygotes examined.

Of zygotes in mitosis, 18 (35%) possessed an extranuclear structure labelled by the anti- β -tubulin antibody; it was not possible to determine whether this structure was an MTOC or one or more pairs of centrioles. This extranuclear structure lay either on the opposite side of the cell to the dividing nucleus (Fig. 4.11e,f) or at one pole of the spindle (Fig. 4.11c,d) and thus was evidently not nucleating the spindle. No extranuclear MTOC or centrioles were visible in the remaining 34 (65%) zygotes examined (Fig. 4.11a,b).

Ten out of 29 binucleate plasmodia possessed centrioles; 3 had one pair, 6 had 2 pairs and one had 4 pairs. No binucleate plasmodia in mitosis were observed to possess extranuclear centrioles or an extranuclear MTOC (Fig. 4.11g,h).

4.4.2 Microtubule organisation in cultures homoallelic for *matA*

In *matA*-homoallelic cultures uninucleate, putative diploid amoebae were distinguished from uninucleate, haploid amoebae

by the larger size of the cells and nuclei. A count of the number of MTOCs in interphase cells homoallelic for *matA*, showed that all putative haploid amoebae possessed one MTOC (Fig. 4.12a,b,c,d). Of the binucleate cells, 67% (46) possessed 2 MTOCs (Fig. 4.12e,f,g,h) and only 4.3% (9) of putative diploid, uninucleate amoebae possessed two MTOCs.

Filming analysis indicated that there were several possible outcomes for mitosis in a binucleate cell homoallelic for *matA* (Fig. 4.2; Table 4.2). Immunofluorescence microscopy of five binucleate cells in mitosis showed that the mitotic spindles lay in various orientations. In one cell, the spindles lay end to end (Fig. 4.13g,h) and in another cell only three spindles poles were observed (Fig. 4.14a,b). Since a cleavage furrow usually forms at the equator of a spindle (Rappaport, 1971), the mitosis shown in Fig. 4.14a,b would be expected to produce three daughter cells (Fig. 4.2c) of which one would contain either two haploid nuclei (Table 4.2, class c) or one diploid nucleus (Table 4.2, class d). Two binucleate cells were observed in which the mitotic spindles were parallel (Fig. 4.14c,d); cytokinesis in such cells would be expected to produce two daughter cells, which might be uninucleate or binucleate (Fig. 4.2b,c; Table 4.2, Classes a,b). In the fifth cell the spindles were approximately perpendicular (Fig. 4.13e,f).

Examples of apparently normal amoebal mitoses in uninucleate, haploid amoebae are shown in Fig. 4.13 (a,b,c,d). The mitotic spindle was also examined in putative diploid amoebae, of which 37 contained bipolar spindles and

12 contained tripolar spindles (Fig. 4.14e,f). The presence of tripolar spindles was consistent with the conclusion from filming analysis that diploid amoebae could divide to give three daughter cells.

4.4.3 Flagellate formation

To determine the stage of sexual development at which ability to undergo the amoeba-flagellate transformation was lost, flagellate formation was induced in a *matA*-heteroallelic population and a *matA*-homoallelic population. A sample of each cell type, as defined in Sections 4.4.1 and 4.4.2, was scored for the presence or absence of the long flagellum. Of the 500 small uninucleate cells scored, which were presumably derived from amoebae, 87% were flagellates and all of these possessed one long flagellum (Fig. 4.15a,b). All 5 fusion cells observed possessed flagella; 4 possessed one long flagellum and the other possessed two. Only 46 flagellates were observed amongst 420 zygotes; 41 possessed one long flagellum (Fig. 4.15c,d) and 5 possessed two. No flagella were observed on the 50 binucleate and multinucleate plasmodia scored.

In a *matA*-homoallelic culture only 4% of cells failed to transform into flagellates. Of the uninucleate cells which transformed into flagellates, 95.1% (312/328) had one long flagellum (Fig. 4.16a,b) while the remainder possessed two long flagella (Fig. 4.16c,d). Of the 17 binucleate cells that transformed into flagellates, 6 possessed two long flagella (Fig. 4.16e,f) and 6 cells possessed one long flagellum; the number of flagella on the remaining binucleate cells could

not be determined.

4.5 DISCUSSION

4.5.1 The effect of *matA* on cell fusion

Using a combination of genetic analysis of diploid amoebae from *matA*-homoallelic mixtures, and visual examination of cell mixtures homoallelic for *matA*, Youngman et al. (1981) suggested that *matA* had no effect on cell fusion. Filming analysis (see Section 4.2) showed that amoebal fusions occurred frequently in cultures either heteroallelic or homoallelic for *matA*, and that the distributions of cell ages at the time of cell fusion were almost identical in both types of culture (Fig. 4.3); both these observations supported the suggestion of Youngman et al. (1981). In cultures either heteroallelic or homoallelic for *matA*, the maximum age at cell fusion was approximately equal to the mean length of an amoebal cell cycle. The mean amoebal IMT was shorter in *matA*-heteroallelic cultures than in all other cultures, suggesting that amoebal growth may be influenced by the presence of multiple alleles of *matA* and implying that an amoeba might be able to detect the *matA* allele carried by other amoebae before cell fusion. If this is so it would not agree with the suggestions of Youngman et al. (1977). The observed shortening of the amoebal IMT might, however, be an indirect effect of the presence of plasmodia in the culture; this would not disagree with the suggestion of Youngman et al. (1977). This relationship between shortening of amoebal

IMT and the presence of two different *matA* alleles in a culture, could be examined further by measuring amoebal IMT in a variety of cultures containing two amoebal strains which each carry different *matA* alleles.

Youngman *et al.* (1981) also suggested that, in *matA*-homoallelic cultures, the binucleate fusion cells were likely to split apart into two uninucleates unless the nuclei were in similar stages of the cell cycle at the time of cell fusion. Flow cytometric analysis of *matA*-heteroallelic mixtures and *matA*-homoallelic mixtures (see Section 4.3) suggested that the amoebal cell cycle was normal in fusing cells. Evidence from Dee *et al.* (1989) and Turner *et al.* (1981) suggested that S phase occupied about 7% of the cell cycle in axenically grown amoebae with an IMT of 25 - 30 hours; i.e. S was about 1.5 - 2 hours. Braun *et al.* (1965) estimated that S phase occupied about 1.5 hours in plasmodia with an 8 - 10 hour IMT. Since the mean amoebal IMT in the films was 9 - 10 hours, it seems reasonable to estimate that S phase was largely completed about 2 hours after mitosis. In the films of *matA*-homoallelic cultures, cell fusions between cells of somewhat less than 2 hours old (presumably in S phase) were observed to end in either cell splitting or mitosis. Cell fusions between cells of substantially more than 2 hours old (presumably in G2 phase; Fig. 4.4) also ended in either cell splitting or mitosis; cell age at the time of cell fusion, thus, did not appear to influence the fate of the fusion cell. Therefore, the suggestion of Youngman *et al.* (1981), which related the fate of fusion cells to cell age, was not supported by the filming data.

In *matA*-heteroallelic fusion cells, also, the stage of the cell cycle of the nuclei at cell fusion apparently did not affect the outcome of cell fusion; no *matA*-heteroallelic fusion cells were observed to split apart following cell fusion. The time between cell fusion and nuclear fusion was fairly constant at about 2 h. Heteroallelism at *matA* may act to overcome any differences in the stage of the cell cycle by interposing the 2 hour gap between cell fusion and nuclear fusion, in this time an S phase nucleus could complete DNA replication, so that all fusing nuclei would presumably be in G2 phase.

4.5.2 Mating competence and the cell cycle

In many unicellular organisms there is visible evidence of mating competence, such as alteration in cell shape (shmooing) in yeast, or the production of small gametes in Dinoflagellates (Bhaud *et al.*, 1988) and *Dictyostelium* (O'Day *et al.*, 1987). In *Physarum*, the situation appears to be different. The amoebae which underwent cell fusion were presumably mating competent (see Section 1.2.2); however, the films of both *matA*-heteroallelic and *matA*-homoallelic mixtures indicated that there was no visible difference between vegetative and mating competent amoebae.

Physarum amoebae, like plasmodia, apparently have no G1 phase in their vegetative cell cycle (Turner *et al.*, 1981; Nygaard *et al.*, 1960). A cell cycle that lacks a G1 phase occurs in several unicellular organisms, including *Acanthamoeba*, *Amoeba*, *Naegleria* (reviewed by Byers, 1979, 1986), *Dictyostelium discoideum* (Weijer *et al.*, 1984a but see

also Sharpe and Watts, 1985), *Schizosaccharomyces pombe* (reviewed by Prescott, 1987), and the micronuclei of *Tetrahymena* (reviewed by Brunk, 1986), as well as a few multicellular organisms (e.g. *Hydra attenuata*; Campbell and David, 1974). In the sexual development of many unicellular organisms, cell fusion occurs in the G1 phase of the cell cycle (e.g. *Chlamydomonas*, *Oxytricia* and *Saccharomyces*; reviewed by Crandall, 1977 and Cross et al., 1988). The possibility that mating occurred only during a G1 phase in *Physarum* could not be excluded, but if this was so, the mean length of time for which an amoeba must have remained in G1 prior to mating was 3.5 hr, about one third of the length of an amoebal cell cycle (Fig. 4.3; Table 4.1A). Such an extended G1 period seems unlikely since measurements of DNA content by flow cytometry (Fig. 4.9; Fry and Matthews, 1987; Dee et al., 1989) indicated that there was no G1 peak in exponentially growing, clonal cultures, which should contain a significant proportion of mating competent amoebae (Shipley and Holt, 1982). Flow cytometric measurements of DNA content in developing populations of cells (Fig. 4.9d) suggested that both fusion cells and zygotes were in G2 phase.

Amongst organisms with no G1 phase, development and differentiation can be initiated in G2 phase; for example, there is evidence that *Tetrahymena* micronuclei are in G2 phase at the time of cell fusion (Doerder and De Bault, 1975). During the starvation and aggregation phases of *Dictyostelium* slug formation, G2 phase cells differentiate into prespore cells, while S phase cells become prestalk cells (Weijer et al., 1984b). In *Acanthamoeba*, encystment is

initiated from a particular point in G2 phase (Stohr *et al.*, 1987), while in *Hydra attenuata*, differentiation of interstitial cells is initiated from G2 phase (Campbell and David, 1974). Thus *Physarum* would not be unique if development was initiated during G2 phase; it would, however, be unusual if a G1 phase were added to a cell cycle that did not normally contain one.

4.5.3 Nuclear fusion during sexual development

In agreement with the observations of Holt and Huttermann (1979), the first evidence that plasmodium development had been initiated in the *matA*-heteroallelic mixtures was the occurrence of nuclear fusion in interphase about two hours after amoebal fusion; interphase nuclear fusion did not occur in *matA*-homoallelic mixtures. Therefore nuclear fusion appeared to be under the control of *matA*. Szabo and O'Day (1983) classified fusion of sexual nuclei into two types; direct fusion, where the nuclear membranes remain intact during fusion, and envelope vesiculation, where the nuclear membranes break down and the two sets of condensed chromosomes mix. Envelope vesiculation is usually followed immediately by mitosis, whereas direct fusion may precede mitosis by some time. The films suggest that *Physarum* undergoes direct nuclear fusion since the nuclei appear to remain intact during fusion, and the zygote nucleus remains in interphase for about 16 h after nuclear fusion.

There is evidence that microtubules have an essential function in nuclear movement and fusion in many organisms including *Chlamydomonas* (Dutcher, 1988), *Aspergillus nidulans*

(Oakley and Morris, 1980), *Tetrahymena* (Hamilton and Suhr-Jessen, 1980), and *Saccharomyces cerevisiae* (Hasek et al., 1987). During zygote formation in *S. cerevisiae*, cell fusion led to contact, in the neck of the zygote, between the bundled cytoplasmic microtubules from the two, haploid, fusing cells. Shortening of the microtubule bundles led to association of the haploid spindle pole bodies; this association was followed by nuclear fusion (Hasek et al., 1987). The immunofluorescence studies suggested that microtubules may have a similar function in karyogamy in *Physarum*, since changes in microtubule organisation apparently began before nuclear fusion. Although each member of a fusing pair of amoebae presumably contributed one MTOC to the fusion cell, two MTOCs were found in only 52% of *matA*-heteroallelic fusion cells and 3% of zygotes. One possibility was that the reduction in the number of MTOCs that accompanied nuclear fusion was caused by association of the amoebal MTOCs as a result of shortening of microtubule bundles that linked the MTOCs; in some fusion cells, the two MTOCs did appear to be linked by a microtubule bundle. Many zygotes possessed two pairs of centrioles but only one MTOC. Since the MTOC and centrioles are linked, two pairs of centrioles would be seen near a single MTOC if the amoebal MTOCs moved together (and possibly fused) to facilitate nuclear fusion. It was not possible to ascertain whether the zygote MTOC consisted of a single fused MTOC, or two closely apposed amoebal MTOCs. In contrast, most binucleate cells homoallelic for *matA* retained two clearly separate amoebal MTOCs and associated microtubules. It would be possible to

elucidate the role of microtubules in nuclear fusion by examining nuclear fusion in the presence of drugs which affect microtubules (e.g. benomyl); benomyl resistant strains of amoebae have been isolated (Burland *et al.*, 1984). Such studies have confirmed that microtubules are involved in nuclear fusion in a variety of organisms including *Chlamydomonas* (Dutcher, 1988) and yeast (Cross *et al.*, 1988).

4.5.4 Nuclear fusion in binucleate cells from *matA*-homoallelic cultures

Holt and Huttermann (1979) observed that nuclear fusion in binucleate fusion cells homoallelic for *matA* occurred at mitosis, and that cytokinesis gave rise to two uninucleate, presumably diploid daughters. Youngman *et al.* (1981) found, by a combination of genetic analysis and microscopic examination of developing cultures, that the frequency of diploid amoebae formed in *matA*-homoallelic mixtures (5-10%) was lower than the frequency of zygotes formed in *matA*-heteroallelic cultures (10-15%); to account for this difference, they assumed that *matA* had no effect on cell fusion and suggested that a minority of the fusion cells homoallelic for *matA* might split apart before mitosis. In the films of *matA*-homoallelic cultures, the majority (75%) of fusion cells were observed to split apart before mitosis and some cells underwent 2 or even 3 rounds of successive fusion and splitting.

The filming studies also showed that even when a *matA*-homoallelic fusion cell reached mitosis, mitosis did not always result in the formation of two diploid amoebae (Fig.

4.2; Table 4.2). The immunofluorescence studies showed that synchronous mitosis in *matA*-homoallelic binucleate cells did not always result in spindle fusion, and that, when spindle fusion did occur, it sometimes involved only one pole from each spindle. Assuming that cytokinesis occurred at the equator of the spindle in each case (Rappaport, 1971), the different spindle orientations observed by immunofluorescence accounted for all the different types of products observed in the films (Figs. 4.2; 4.13; 4.14). The occurrence of spindle fusion suggests that mitosis was of the amoebal type with breakdown of the nuclear envelope. No mitoses were observed which were not accompanied by cytokinesis, indicating that the cells homoallelic for *matA* had retained the amoebal characteristics of open mitosis accompanied by cytokinesis.

Some diploid amoebae were observed to divide into three, consistent with the observation of tripolar spindles in uninucleate cells during the immunofluorescence studies. The daughter cells would presumably be aneuploid and would not be expected to survive; this may account for the reports that diploid amoebae grow more slowly than haploid amoebae (Youngman *et al.*, 1981).

4.5.5 Events during plasmodium development

Comparison of the sequences of events during sexual and apogamic plasmodium formation allows identification of events that may be essential for development. Comparison of the events in *matA*-heteroallelic cultures with those in *matA*-homoallelic cultures, indicated which events are associated with *matA*-heteroallelism, and thus, presumably,

directly or indirectly under the control of *matA*. Some of these events are discussed below.

Nuclear fusion cannot be essential for plasmodium formation since it does not occur in apogamic strains.

The extended cell cycle

A striking similarity between sexual and apogamic development is the existence of an extended cell cycle, approximately 2.3 times the length of an amoebal cell cycle, ending with the formation of a binucleate cell by mitosis without cytokinesis (Chapter 3; Bailey et al., 1987). This extended period may be essential for development and may be required for the completion of a series of developmentally regulated events leading to the onset of the plasmodial type of nuclear division cycle and the suppression of cytokinesis. Even though there was variation in the size of amoebae at cell fusion, there was still a minimum interval of about 1.5 amoebal cell cycles between amoebal fusion and binucleate plasmodium formation. There may also be a minimum size required to trigger the mitosis by which the zygote becomes binucleate; if a zygote had completed the sequence of developmentally regulated events, but was under a certain size, mitosis might be delayed until that size was attained. Conversely, a zygote that reached the minimum size for mitosis before completing the sequence of developmentally regulated events, would continue to grow until the required events were completed and would then enter mitosis. The fact that apogamic strains arose as a result of mutation at *matA*,

and that there was no extension to the cell cycle in *matA*-homoallelic fusion cells indicated that extension of the cell cycle was under the control of *matA*.

In both sexual and apogamic plasmodium formation, the extended cell cycle was accompanied by continuing growth, leading to an increased cytoplasm:DNA ratio in the cell entering mitosis; a large uninucleate apogamically developing cell was, on average, twice as big at mitosis as an amoeba at mitosis and a zygote was, on average, three times as big, at mitosis, as an amoeba at mitosis. Thus, if mitosis was triggered by attainment of a critical DNA:cytoplasm ratio, that ratio was altered from that of vegetative cells, in sexual development as well as in apogamic development (see Section 3.4). Following the extended cell cycle in both types of development, there was a short cell cycle, about 0.7 times the length of an amoebal cell cycle, ending in the formation of a quadrinucleate cell. This short cell cycle may be essential for plasmodium formation or it may be present as a consequence of the extended cell cycle, restoring the normal DNA:cytoplasm ratio. At the end of this short cell cycle, a sexually-formed, diploid, quadrinucleate plasmodium was, on average, 11 times the mean area of an amoeba at birth. In CL, a haploid, quadrinucleate plasmodium was approximately 6 times the mean area of an amoeba at birth. These figures suggest that the cytoplasm:DNA ratio, at this stage of development, was similar in both sexual and apogamic development. In *matA*-homoallelic fusion cells, there was no increase in the cytoplasm:DNA ratio; mitosis with cytokinesis

occurred at the end of a period equal to the length of a normal amoebal cell cycle (Table 4.1A,E).

An increase and subsequent decrease in nuclear area occurred during the extended and shortened cell cycles in both types of development and may be related to increased levels of RNA synthesis (see Section 3.4). This alteration in nuclear area was common to plasmodium formation in all strains examined and may, therefore, be essential for plasmodium formation. Since, there was no increase or decrease in nuclear area in *matA*-homoallelic cells, the alteration in nuclear area was presumably under the control of *matA*.

Commitment to plasmodium development

Analysis of the timing of events in apogamic strains (see Chapter 3 and Bailey et al., 1987) showed that cells became committed to development during the extended cell cycle. After commitment, a time slightly greater than one amoebal cell cycle elapsed before the mitosis at which a binucleate plasmodium was formed; this interval estimates, however, the maximum interval between commitment and binucleate cell formation and the interval may vary in different cells (see Section 3.4). From kinetic studies, Shipley and Holt (1982) suggested that, in sexual development, commitment to plasmodium formation coincided closely with the time of amoebal fusion. The length of time between amoebal fusion and binucleate plasmodium formation, measured from the films of sexual development, varied from 13.6 to 20.2 h. Thus, if commitment coincides with amoebal fusion, binucleate plasmodium formation does not occur at a fixed time after

commitment. This variable interval between commitment and binucleate plasmodium formation suggests that the timing of events during the extended cell cycle may not be fixed, nor occur at a fixed size, but may vary between individual cells. There may also be a difference in the meaning of commitment in sexual and apogamic development; commitment is defined operationally, as the ability of a developing uninucleate cell to survive replating. The stage at which this ability is acquired may be different in zygotes and apogamically developing uninucleate cells.

Loss of ability to undergo the amoeba-flagellate transformation

In the apogamic strain CL, ability to undergo the amoeba-flagellate transformation was lost during the extended cell cycle, 3-5 hours before commitment, at about the time that the cell would have divided had it continued to cycle vegetatively (Blindt *et al.*, 1986; Chapter 3; Bailey *et al.*, 1987); in the apogamic strain RA376, however, ability to transform into a flagellate was apparently lost closer to the time of commitment (Chapter 3). The immunofluorescence studies of sexual development showed that all fusion cells and some zygotes could transform into flagellates when suspended in water. Thus in sexual development, unlike apogamic development in CL, cells are still able to transform into flagellates at about the time of, or even after, commitment; this is consistent with previous studies in which mating was observed between flagellates (Ross, 1957). However, no zygotes in mitosis, nor binucleate plasmodia, were observed with flagella, indicating that ability to

undergo the amoeba-flagellate transformation was lost by the time of binucleate cell formation. In contrast, in *matA*-homoallelic cultures the majority of cells transformed into flagellates when suspended in water. The differences, between sexual development and development in RA376 and CL, in the timing of loss of ability to flagellate, relative to commitment, suggested that the order of events during the extended cell cycle might not be inflexible.

Alterations in microtubular organisation

Even though many zygotes had two pairs of centrioles, very few of them possessed two long flagella. This is consistent with observations on microtubule organization during apogamic development in CL and suggested that a change in centriolar function rather than a loss of centrioles was responsible for the loss of ability to undergo the amoeba-flagellate transformation and the failure to undergo amoebal mitosis (Chapter 3; Blindt, 1987; Bailey *et al.*, 1987). In some zygotes, the two pairs of centrioles did not show the close association with the MTOC that is usual in amoebae (Fig. 4.10); this is not inconsistent with the suggestion that a change in centriolar function occurred during the extended cell cycle.

During apogamic development in CL, the first change in microtubule organisation detected by immunofluorescence studies, occurred at the mitosis by which the developing uninucleate cell became binucleate; this mitosis appeared to be of the intranuclear plasmodial type (Blindt, 1987). The films of heterothallic development suggested that the mitosis by which the zygote became binucleate might be intranuclear

since it resembled the mitosis observed in multinucleate plasmodia rather than mitosis in amoebae. The immunofluorescence studies of sexual development showed that an extranuclear MTOC, presumably the remains of the amoebal MTOC, was present in some zygotes at mitosis. In the following interphase, more binucleate plasmodia possessed two MTOCs than there were zygotes with two MTOCs, suggesting that the amoebal MTOC had duplicated even though it was not utilised during the first intranuclear plasmodial mitosis. Similar observations were made on apogamic development in CL (Blindt, 1987). In both sexual and apogamic development, the amoebal microtubule organisation was lost only gradually over several cell cycles (Gull et al., 1985; Blindt, 1987).

Alterations in fusion behaviour

In *matA*-heteroallelic cultures, no fusions were observed between amoebae and fusion cells, or between zygotes and amoebae, suggesting that the ability of amoebae to fuse was lost almost immediately after cell fusion. The loss of ability to fuse could be due to the presence of two different *matB* products on the surface of the fusion cells; such a hybrid cell might not be able to fuse with amoebae of either of the *matB* types it carries. Alternatively, ability to undergo amoebal fusion may be actively switched off in the fusion cell.

During the extended cell cycle, in both apogamic and sexual development, ability to undergo plasmodial fusion appeared to be acquired at about the same time as, or slightly after, ability to ingest amoebae; plasmodia were never observed to ingest developing cells. Ability to ingest

amoebae may simply be related to the large size of the developing cells, since amoebae were sometimes observed apparently attempting to ingest one another. Such alterations in fusion behaviour during plasmodium development, which were first described by Ross (1957), could be caused by alterations in the cell surface during development; such changes would influence the recognition of other cells as either fusion partners or food. Since the changes in cell fusion pattern began during the extended cell cycle, they may be under the influence of *matA*. Plasmodial fusion is under the control of at least 3 fusion (*fus*) genes and, unlike the situation in amoebae, genetically identical plasmodia are able to fuse. The function of the *fus* gene products is unknown but it is possible that the *fus* genes code for cell surface receptors. Some of the observed alterations in cell fusion pattern may be related to the appearance of the products of these plasmodial fusion genes on the surface of the developing plasmodium. Pallotta *et al.* (1984) showed that some of the proteins present in the cell membranes of amoebae were absent from those of plasmodia, and vice versa. Ingestion of amoebae has also been observed during macrocyst development in *Dictyostelium discoideum*, where the developing zygote attracts and ingests many amoebae (reviewed by O'Day and Lewis 1981).

4.5.6 Conclusions

The comparisons made between the cellular events that occurred in sexual development, in apogamic development and in *matA*-homoallelic cultures indicated that many events

during plasmodium development appeared to be under the control of *matA*. These events included nuclear fusion, extension of the cell cycle, acquisition of the ability to ingest amoebae and to undergo plasmodial fusions, cessation of cytokinesis, loss of the ability to transform into a flagellate, and extensive changes in microtubule organisation. Sweeney *et al* (1987) showed that, during the extended cell cycle, some of the amoebal-specific genes are switched off, and some of the plasmodium-specific genes are switched on. It is unlikely that *matA* controls all these events directly; the product of the *matA* gene is more likely to act as an activator or repressor for a whole series of genes, resulting in a cascade of gene action.

Dr. R. W. Anderson, at the University of Sheffield, has isolated mutant strains that show normal amoebal growth, but are defective in the function of a gene essential for the formation of a mature plasmodium; the mutant genes are candidates for genes activated by *matA*. Analysis of plasmodium development in the mutant strains, and of the defects that they show, will help to elucidate the mechanisms of plasmodium formation and the action of *matA*. The analysis of two such mutant strains forms the basis for the next two chapters.

Table 4.1 Timings of events in *matA*-heteroallelic and *matA*-homoallelic cultures

EVENT	STRAINS	
	CH508 x LU648 (Heteroallelic for <i>matA</i>)	CH508 x LU859 (Homoallelic for <i>matA</i>)
A: amoebal IMT	9.13±1.46 (69)	10.06±1.44 (45)
B: mitosis (M_1) to cell fusion	3.46±1.77 (98)	#1) 3.30±2.21 (72) 2) 3.94±2.46 (19) 3) 3.12±2.11 (48)
C: cell fusion to mitosis (M_2)	16.90±1.44 (26)	2) 4.89±2.00 (14)
*D: cell fusion to split		3) 2.38±1.23 (31)
E: M_1 to M_2	20.58±1.73 (37)	2) 9.35±2.32 (20) 3) 8.15±1.15 (12)
F: binucleate to quadrinucleate	6.13±0.62 (12)	

Times are given as means in hours ± standard deviation;
numbers of observations are given in parentheses.

*D - see text.

#1) All cells

2) Fusions where mitosis occurred in the fusion cell.

3) Fusions in which cell fusion was followed by splitting
into 2 uninucleates.

Table 4.2

Products of mitosis in *matA*-homoallelic fusion cells

*Class	No. of cells undergoing mitosis	<u>Daughter Cells</u>		Daughter nuclei
		uninucleate	binucleate	
a	7	2	0	2
b	3	0	2	4
c	1	2	1	4
d	1	3	0	3

* Class a: 2, uninucleate daughter cells; Class b, 2 binucleate daughter cells; Class c, 1 binucleate and 2 uninucleate daughter cells; Class d, 3 uninucleate daughter cells.

Table 4.3.

Numbers of cells with 0,1 or 2 MTOCs at different stages of sexual development in *matA*-heteroallelic cultures.

STAGE OF DEVELOPMENT	<u>NUMBER OF INTERPHASE CELLS WITH</u>			TOTAL SCORED
	0 MTOC	1 MTOC	2 MTOCs	
Fusion cell	4 (16%)	8 (32%)	13 (52%)	25
Zygote	110 (33%)	212 (64%)	11 (3%)	333
Binucleate plasmodium	26 (58%)	10 (22%)	9 (20%)	45

Figure 4.1. Representative cell pedigree from a *matA*-heteroallelic culture.

—○— time of nucleolar disappearance preceding mitosis

⊙ uninucleate cell

⊖ binucleate cell

⊖ quadrinucleate cell

CF - cell fusion

NF - nuclear fusion

F - fusion of multinucleate cells

E - amoeba ingested by plasmodium

→ cell left field.

A,B,C - see text. Filming began at 0 h.

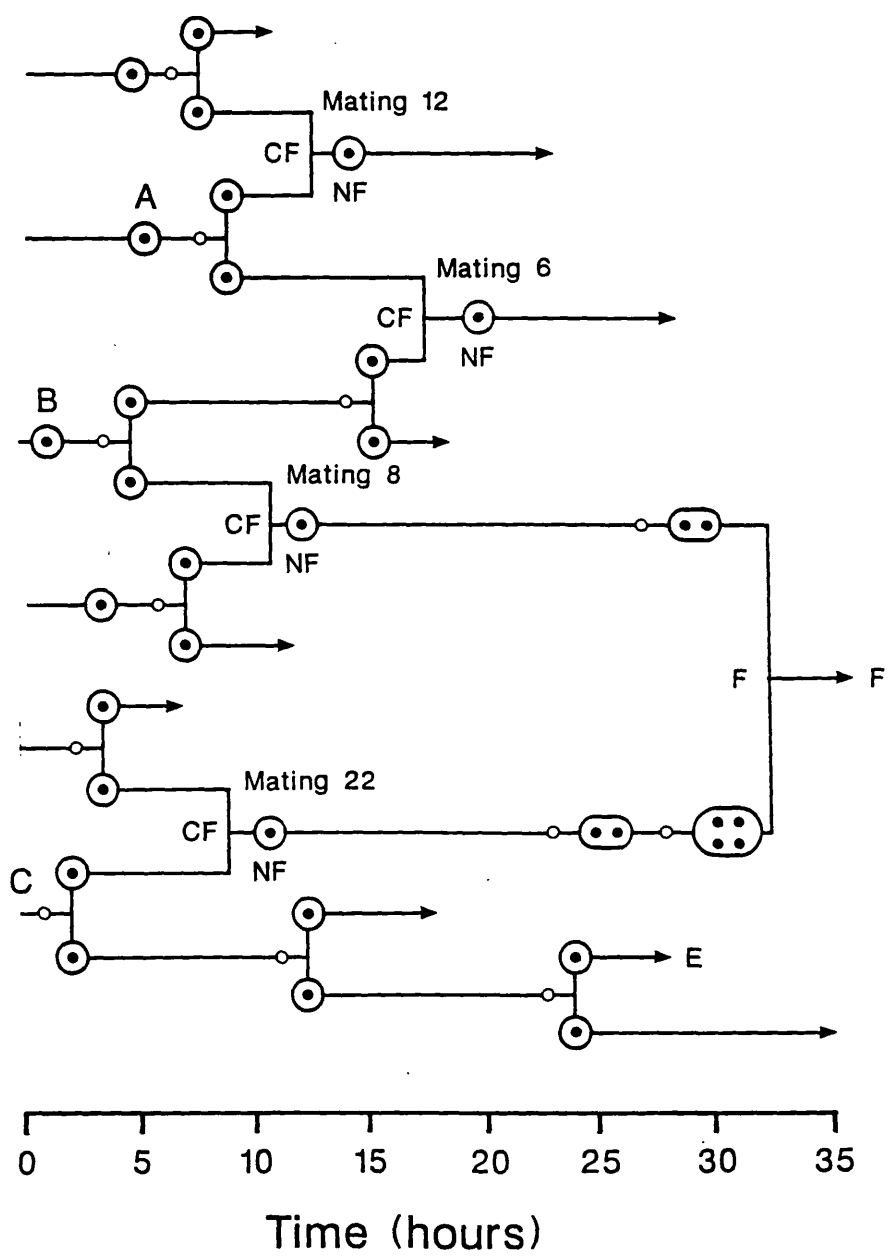


Figure 4.2. Representative cell pedigrees from a *matA*-homoallelic culture.

—○— time of nucleolar disappearance preceding mitosis

⊙ uninucleate cell

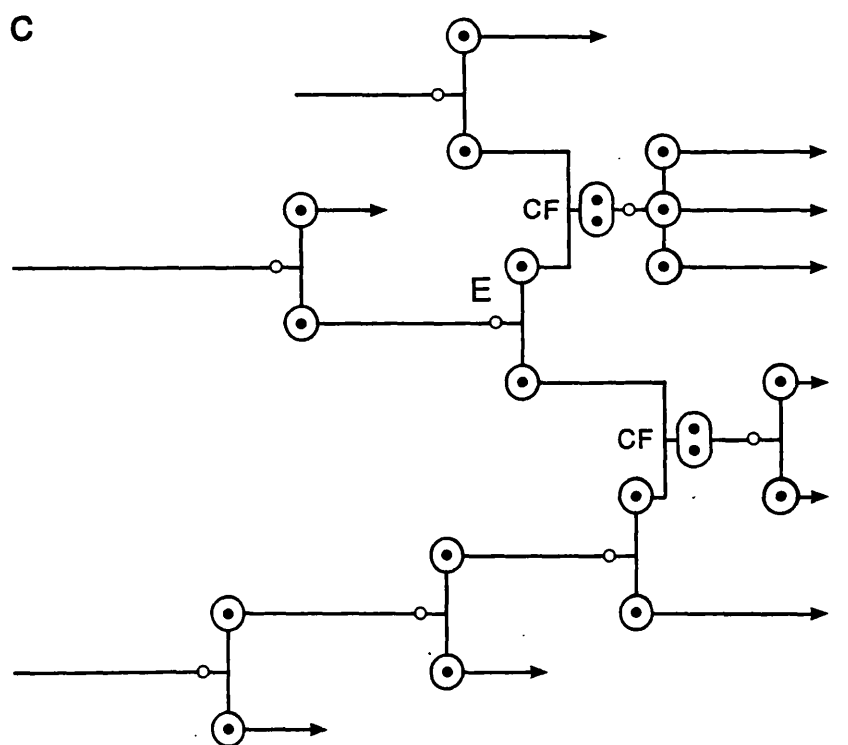
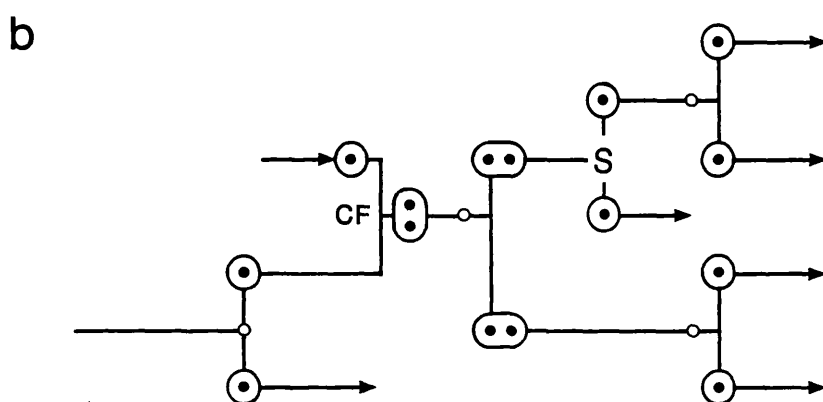
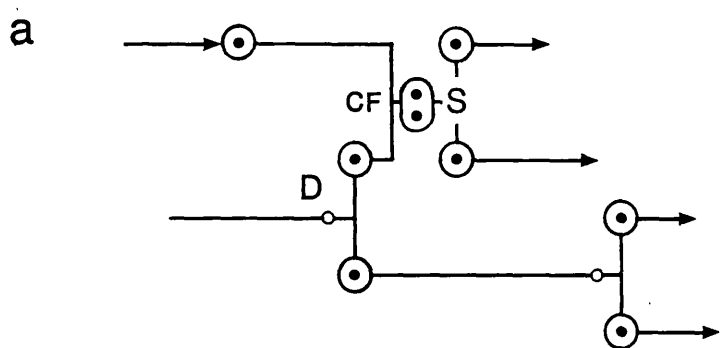
⊖ binucleate cell

CF - cell fusion

S - cell splits apart

→ - cell left field.

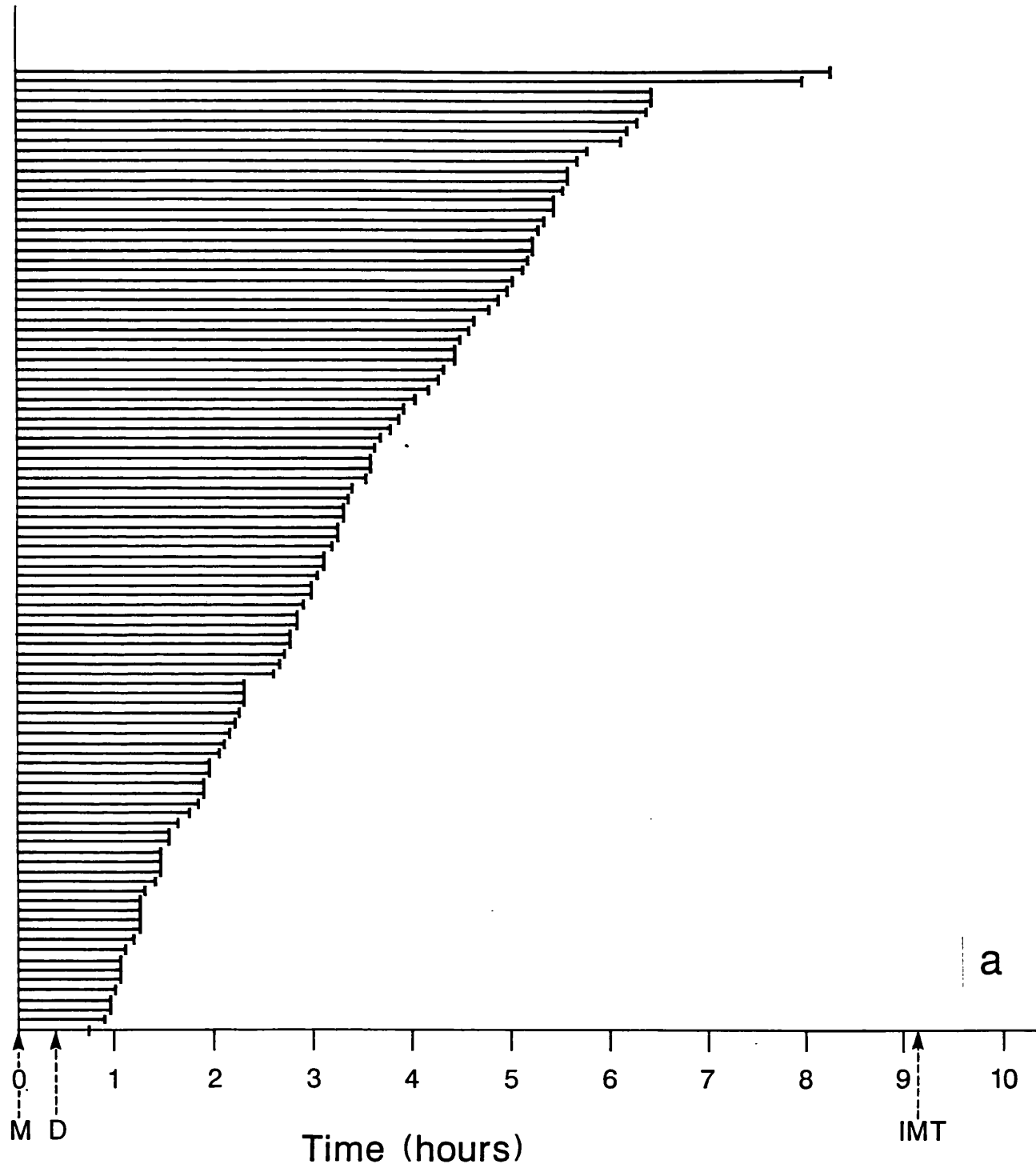
D,E - see text. Filming began at 0 h.



0 5 10 15 20 25 30 35

Time (hours)

Figure 4.3. Ages of amoebae at cell fusion of (a) *matA*-heteroallelic cells, (b) *matA*-homoallelic cells. Each bar (—) represents the age of one amoeba at the time of cell fusion measured from nucleolar disappearance at mitosis (M). The interval between M and D represents the mean time between mitosis and cell division. IMT represents the mean amoebal IMT. Fig. 4.3b represents the data on first fusions only (see Text).



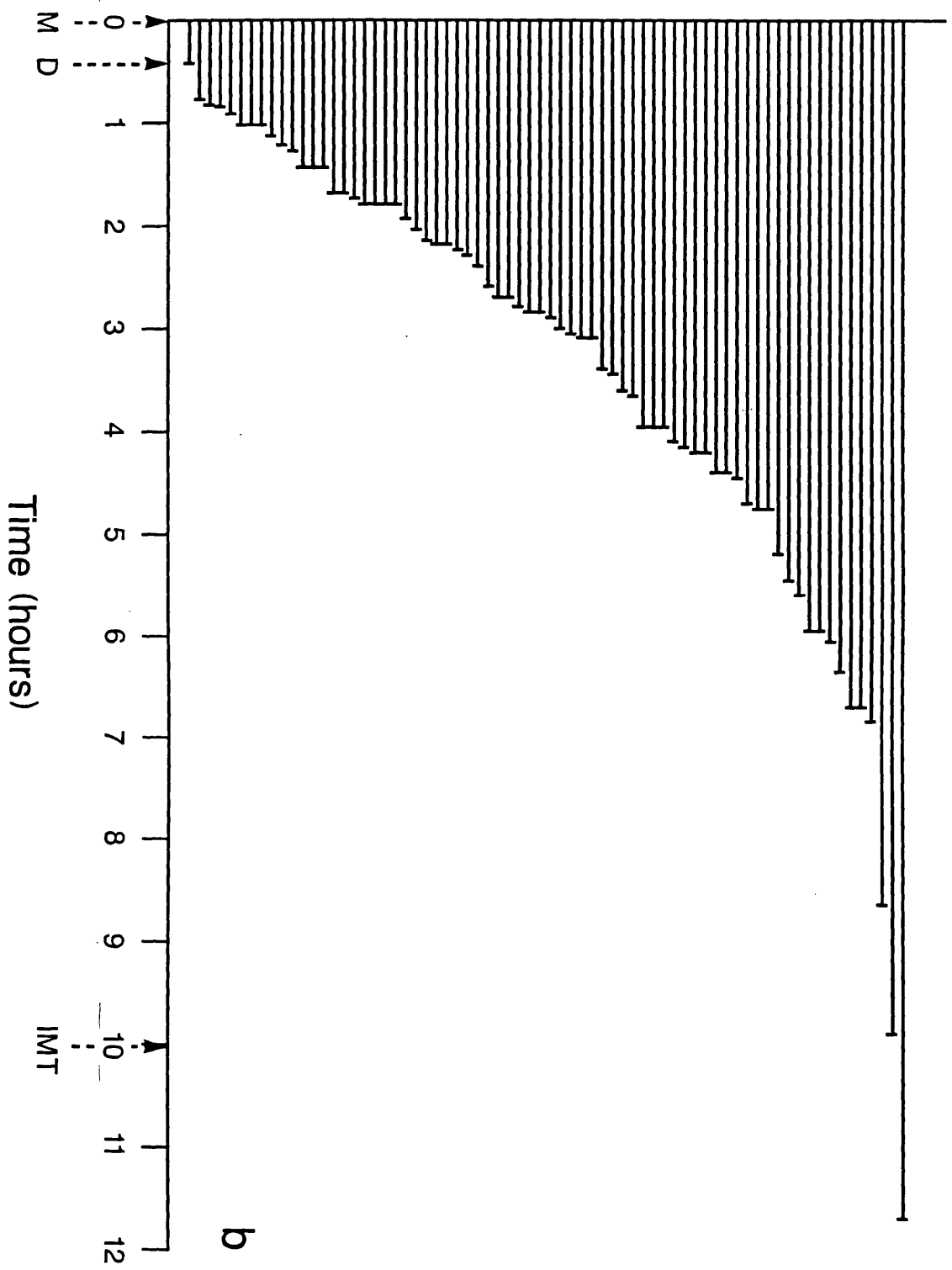


Figure 4.4. Ages of pairs of fusing amoebae. x - fusions in *matA*-heteroallelic cultures ● - fusions in *matA*-homoallelic cultures where fusion ends with mitosis, o - fusions in *matA*-homoallelic cultures where fusion ends in cell splitting. Line A - expected distribution if only amoebae of similar age are able to fuse. Line B - expected distribution if the combined age for each pair of mating amoebae was similar. See text for further explanation.

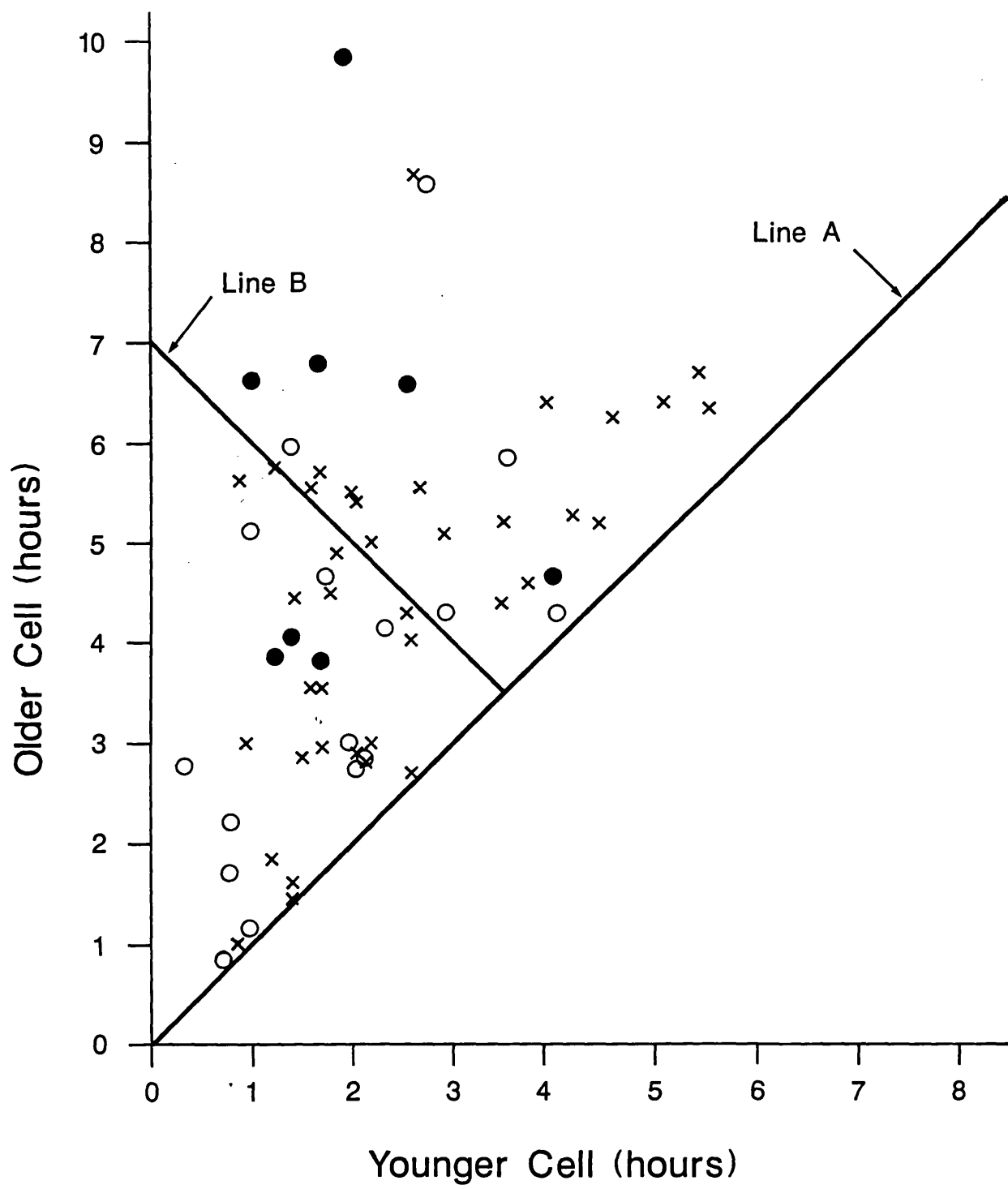
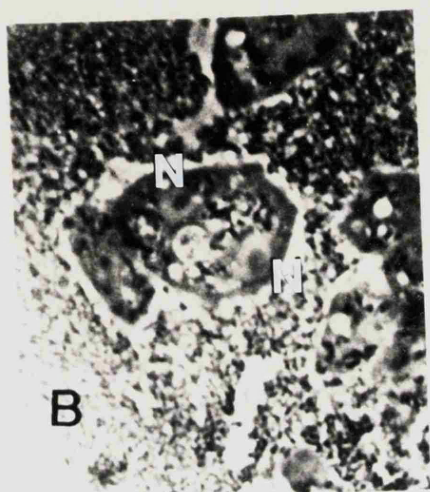
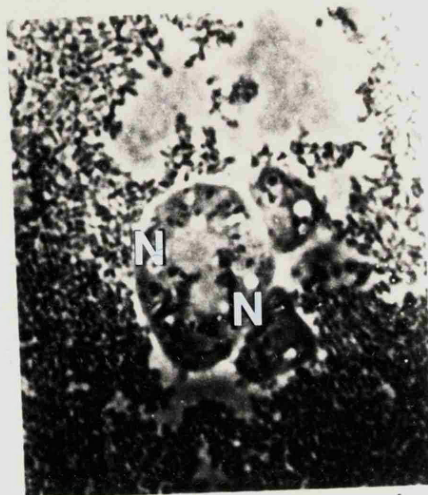


Figure 4.5. Synchronous mitosis in a binucleate cell from a *matA*-homoallelic culture; the cells are growing on a bacterial lawn (B). N - nucleus. Bar = 5 μ m. (a) interphase binucleate cell. (b) 0 min: A binucleate cell entering mitosis; the nucleoli have vanished. (c) +15 min: cleavage furrow (>) in the same cell. (d) +30 min: daughter cells (D) after mitosis in the cell in (b).



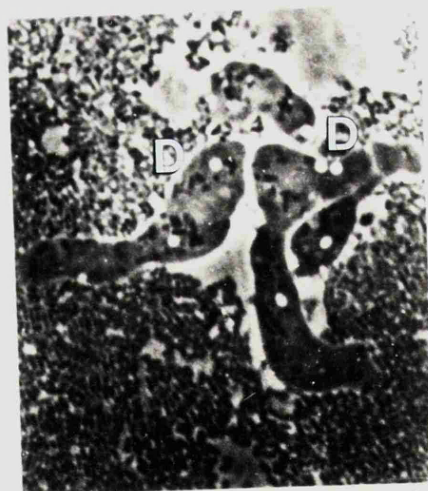
a



b

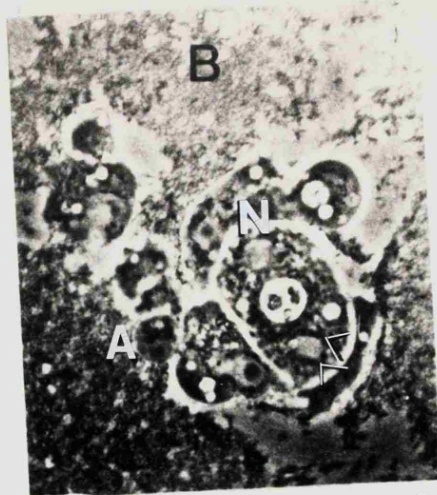


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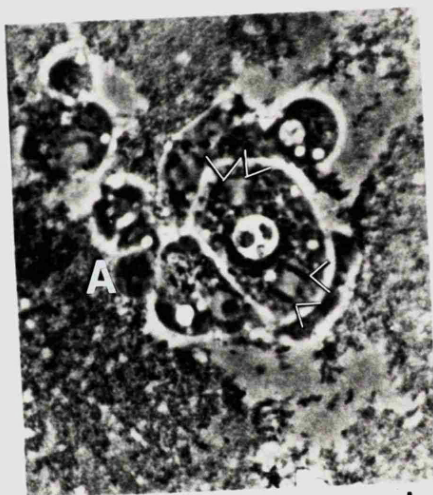


d

Figure 4.6. Mitosis in a binucleate plasmodium from a *matA*-heteroallelic culture; the cells are growing on a bacterial lawn (B). A - amoeba, N - nucleus, > - chromosomes. Bar = 5 μ m. (a) 0 min: the nucleolus has disappeared in one nucleus, while in the other two sets of chromosomes are visible. (b) +30 sec: two sets of chromosomes are visible in each nucleus. (c) +1 h: nuclear division and nucleolar reconstruction are completed; the cell is now quadrinucleate.



a



b



I

c

Figure 4.7. Areas of cells during sexual development in *matA*-heteroallelic cultures. Mean area is given in arbitrary units ($\pm$ standard deviation) for a sample of cells at each stage indicated on the pedigree. All measurements are taken from one film; one arbitrary unit = $7.30\mu\text{m}^2$ (see Materials and Methods). n = number of cells measured

M - mitosis

CF - cell fusion

NF - nuclear fusion

 - amoeba in mitosis

 - amoeba

 - fusion cell

 - zygote

 - zygote in mitosis

 - binucleate plasmodium

 - binucleate plasmodium in mitosis

 - quadrinucleate plasmodium.

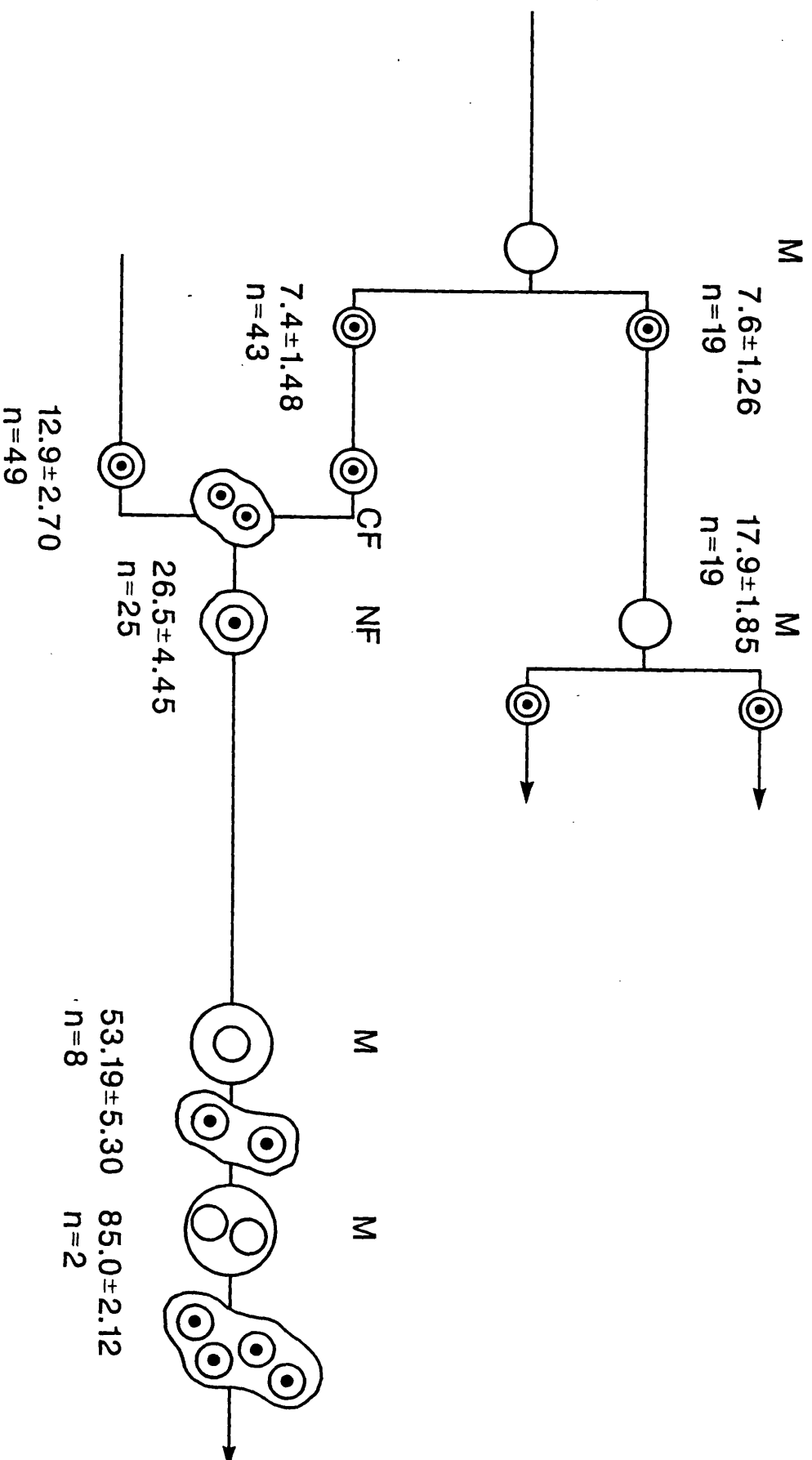


Figure 4.8. Developing cells, from a *matA*-heteroallelic culture, growing on a bacterial lawn. > - nucleus. Bar = 10 μ m. (a) A - amoeba, FC - fusion cell with two small nuclei, Z - zygote just after nuclear fusion. (b) Z - zygote in mitosis; note large size of cell and nucleus relative to those in (a). (c) binucleate plasmodium; the two nuclei (>) are smaller than the nucleus of the zygote illustrated in (b).

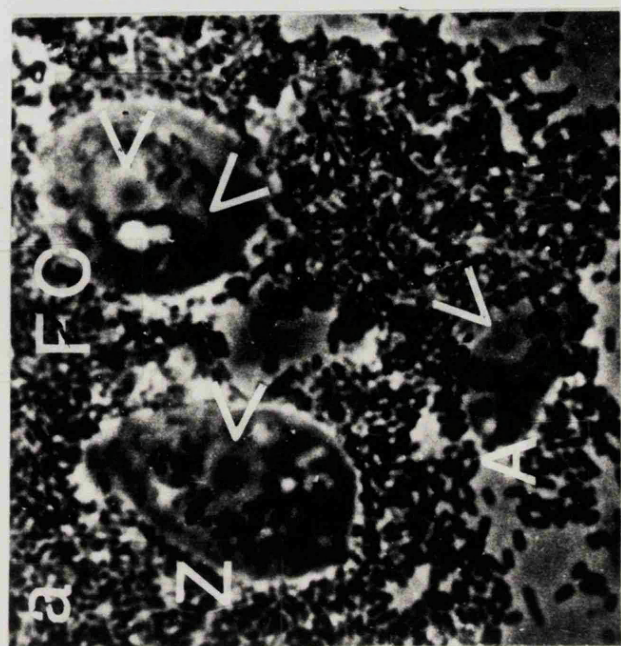
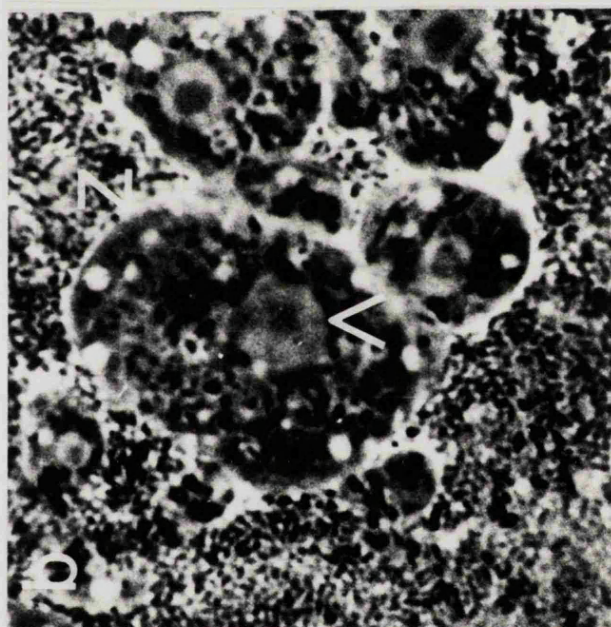
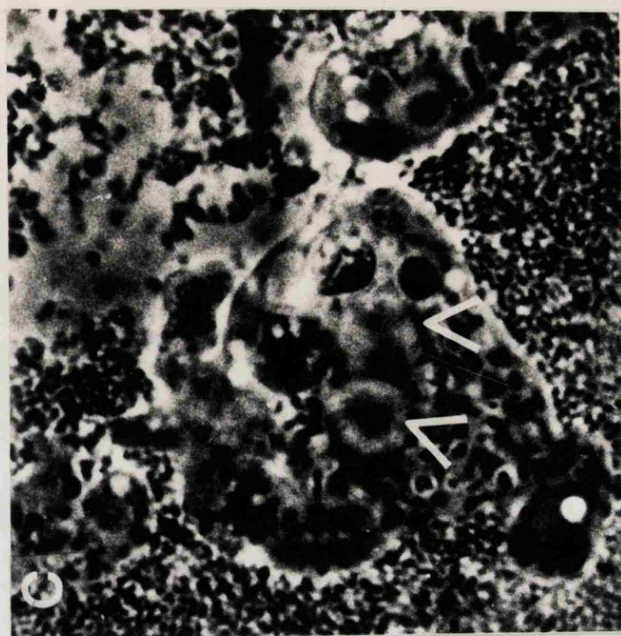
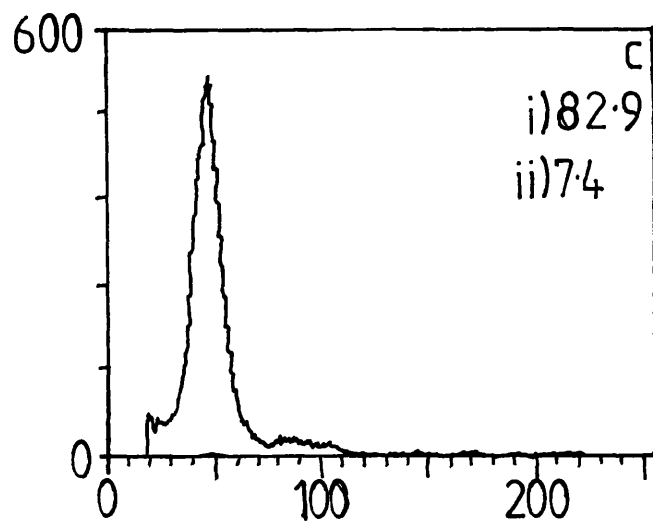
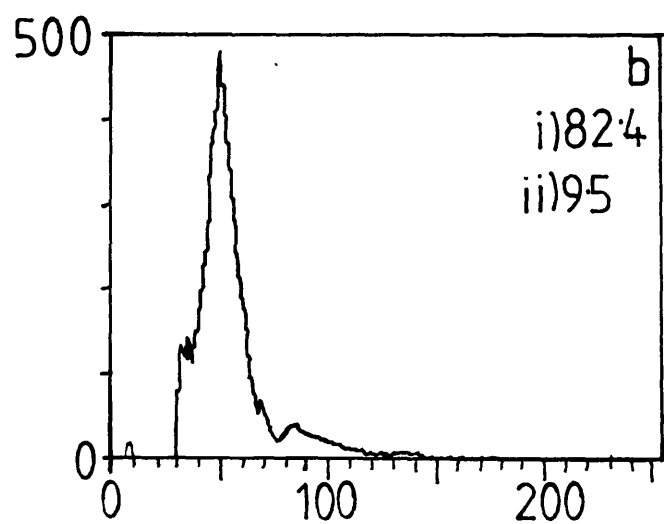
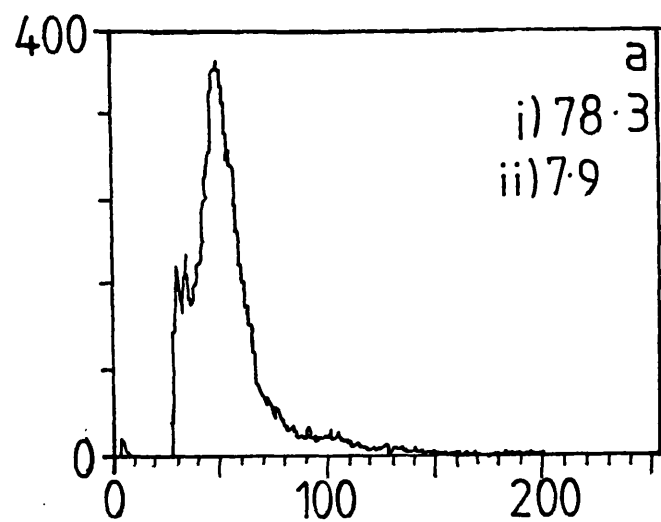


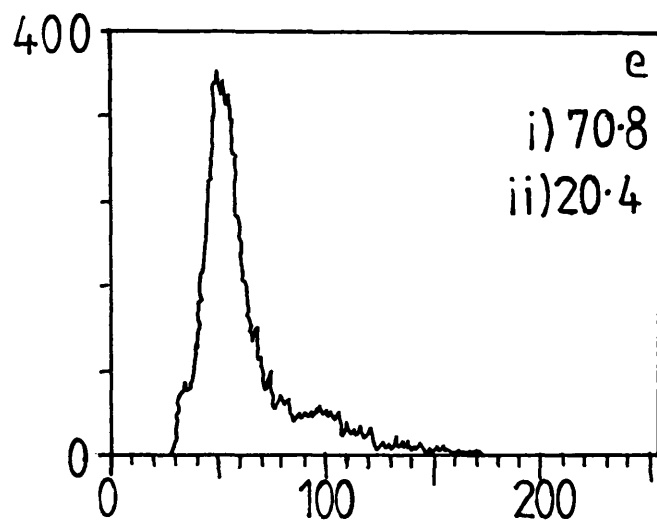
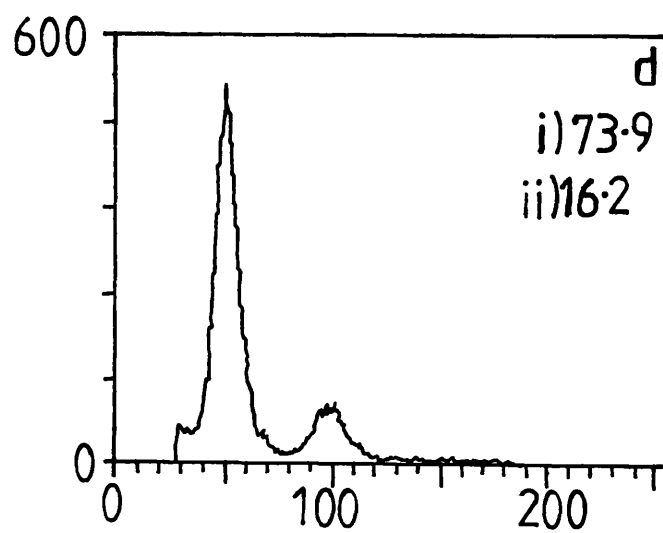
Figure 4.9. Distribution of cellular DNA contents estimated by flow cytometry. (a) CH508 (b) LU859 (c) LU648 (d) CH508 x LU648 (*matA*-heteroallelic) at 22°C. (e) CH508 x LU859 (*matA*-homoallelic) at 22°C. Channel number is proportional to DNA content (see Section 2.9). 10^4 cells were counted for each sample. On each panel the percentage of cells (i) in the main peak with the mode at or near channel 50, and (ii) in the secondary peak with the mode at or near channel 100, is indicated. The remaining cells had DNA contents above the second peak. In (e) the second value (ii) contains cells with DNA contents between 70 and 130 units.

Number of cells



Channel number

Number of cells



Channel number

Figure 4.10. Interphase cells from a *matA*-heteroallelic culture, double-stained with β -tubulin antibody to show microtubules (a,c,e,g) and with DAPI to show DNA (b,d,f,h). The dots in the cytoplasm in b,d,f,h are a mixture of mitochondria and ingested bacteria. Bar=5 μ m. (a,b) fusion cell with two MTOCs (>) and two small nuclei. (c,d) zygote with one MTOC (>) and a single large nucleus. (e,f) zygote with two pairs of centrioles in a cluster (>) and a single large nucleus. (g,h) zygote with two separate pairs of centrioles (>) and a single large nucleus.

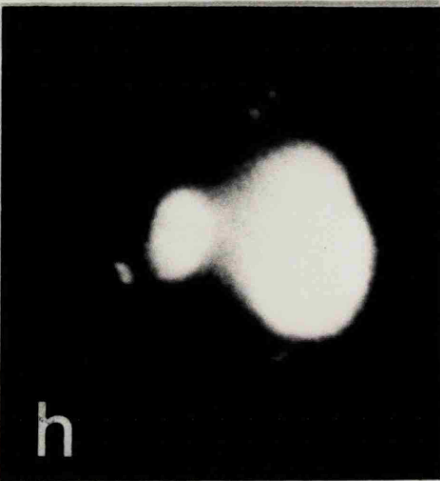
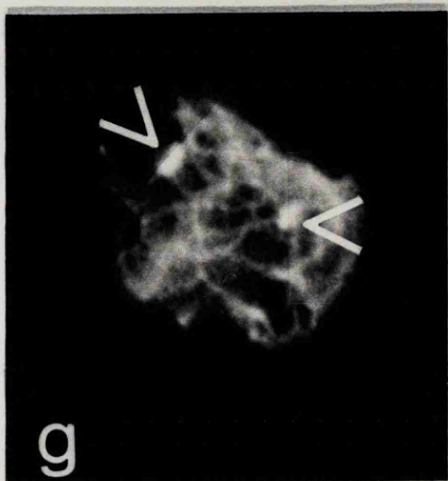
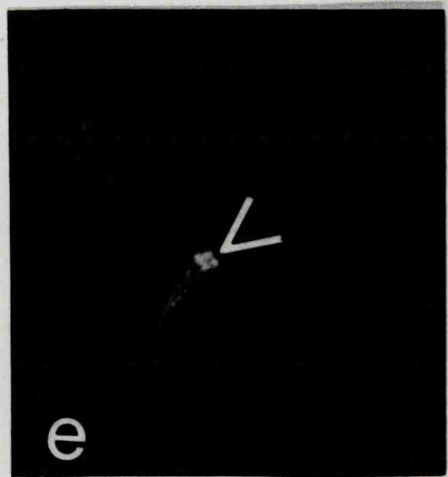
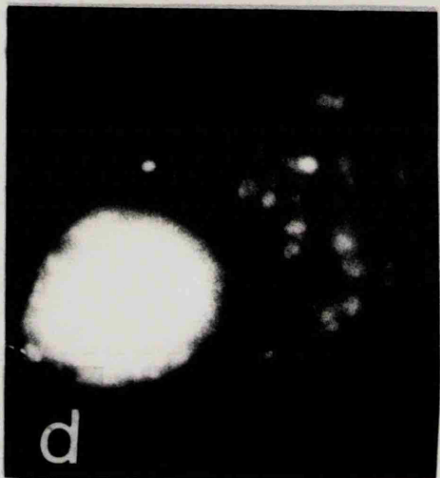
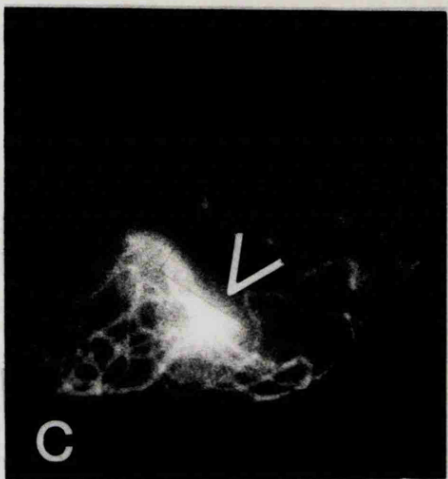
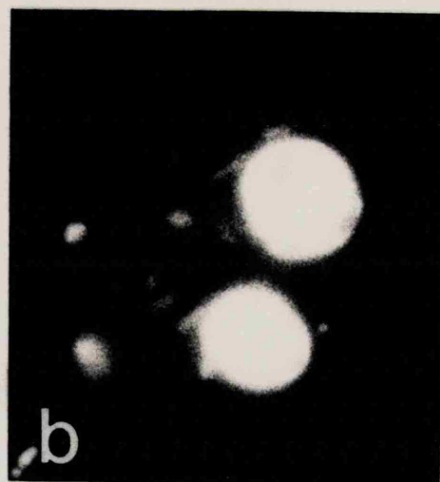
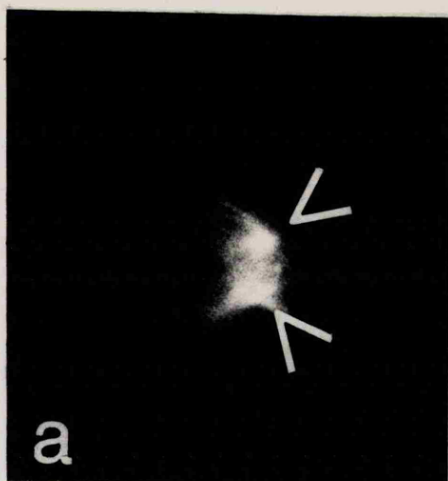


Figure 4.11. Mitotic cells from a *matA*-heteroallelic culture, double-stained with β -tubulin antibody to show microtubules (a,c,e,g) and with DAPI to show DNA (b,d,f,h). The dots in the cytoplasm in b,d,f,h are a mixture of mitochondria and ingested bacteria. Bar=5 μ m. (a,b) zygote in mitosis with no extranuclear MTOC; the small object to the right of the mitotic nucleus is the remains of an ingested amoeba with nucleus and microtubules still visible. (c,d) zygote in mitosis with centrioles present at one pole of the spindle (>). (e,f) zygote in mitosis with an extranuclear MTOC (>); no ingested amoebae are present. (g,h) binucleate plasmodium in mitosis; no extranuclear MTOC is present.

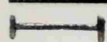
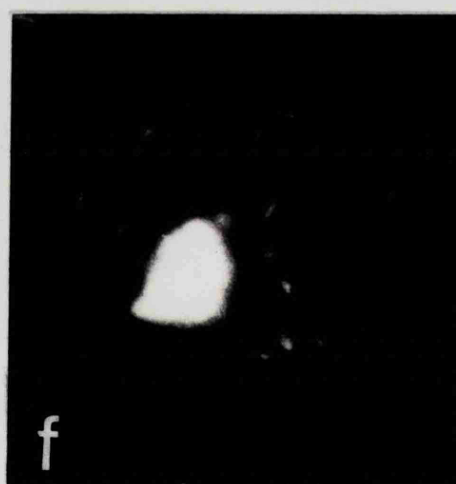
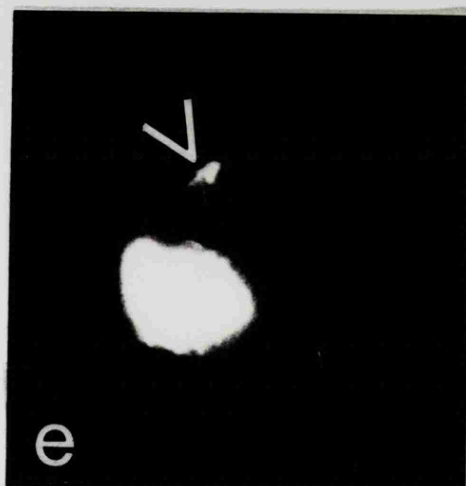
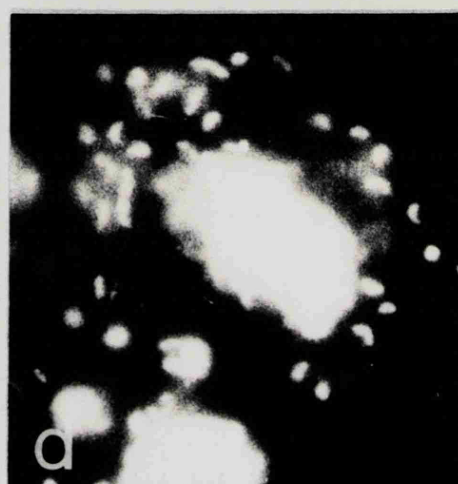
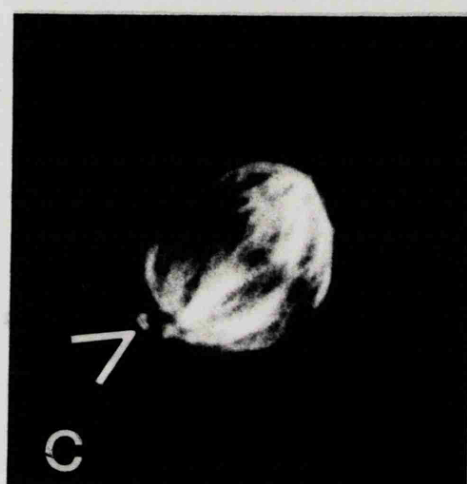
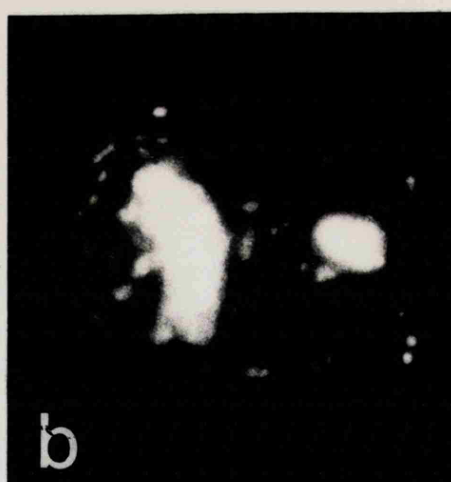
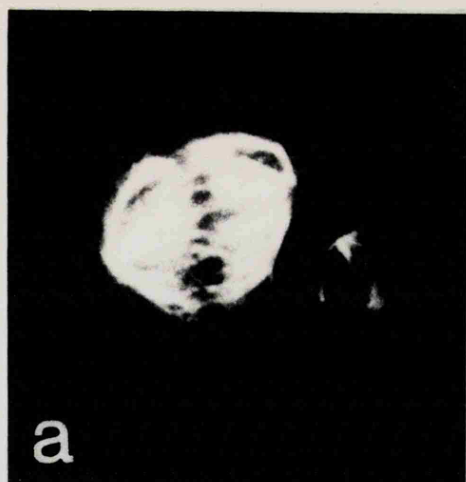
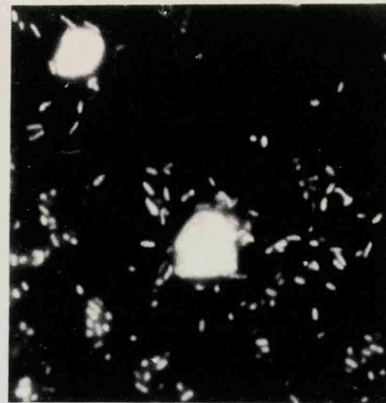


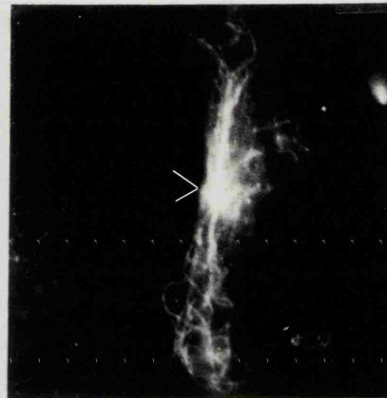
Figure 4.12. Interphase cells from a *matA*-homoallelic culture double-stained with β -tubulin antibody to show microtubules (a,c,e,g) and with DAPI to show DNA (b,d,f,h). The dots in the cytoplasm in b,d,f,h are a mixture of mitochondria and ingested bacteria. Bar = 5 μ m. > - MTOC (a,b, c,d) uninucleate cells with a single MTOC. (e,f, g,h) binucleate cells with two MTOCs.



a



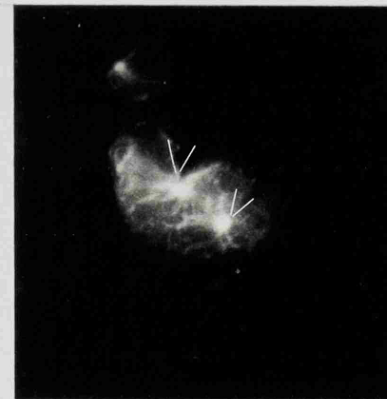
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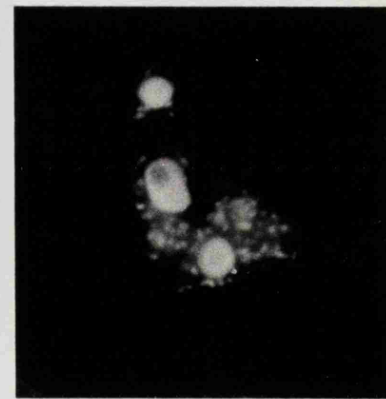
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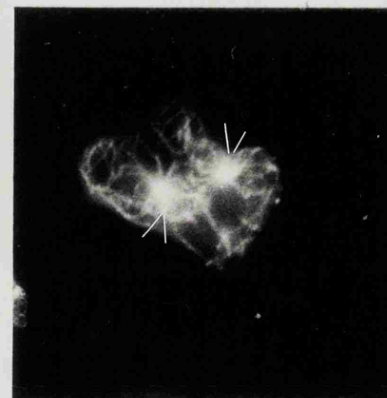
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e



f

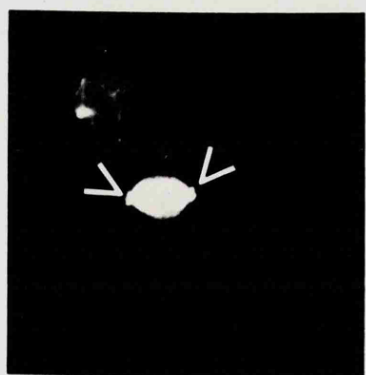


g

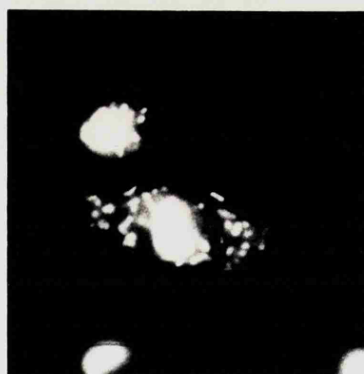


h

Figure 4.13. Mitotic cells from *matA*-homoallelic cultures double-stained with β -tubulin antibody to show microtubules (a,c,e,g) and with DAPI to show DNA (b,d,f,h). The dots in the cytoplasm in b,d,f,h are a mixture of mitochondria and ingested bacteria. Bar = 5 μ m. (a,b) haploid amoeba in metaphase; centrioles appear to be present at the spindle poles (>). (c,d) haploid amoeba in anaphase; two sets of chromosomes are visible. (e,f) binucleate cell at metaphase in synchronous mitosis; the spindles are approximately perpendicular. Two sets of chromosomes are visible. (g,h) binucleate cell at metaphase in synchronous mitosis; the spindles lie almost end to end. Two sets of chromosomes are visible (>).



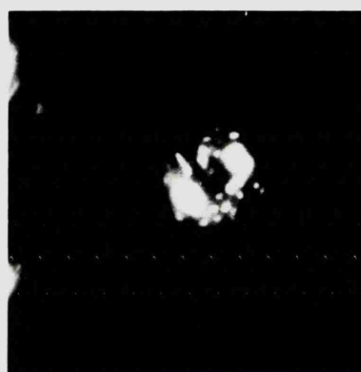
a



b



c



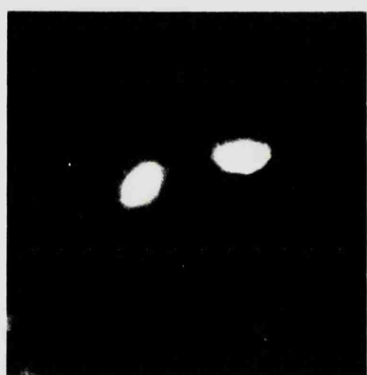
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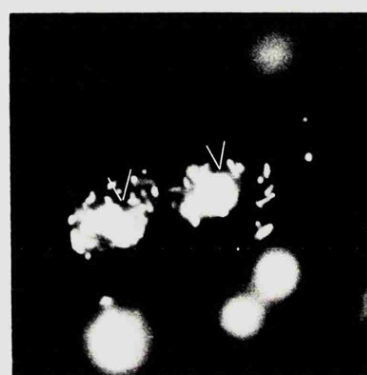
e



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g



h

Figure 4.14. Mitotic cells from *matA*-homoallelic cultures double-stained with β -tubulin antibody to show microtubules (a,c,e) and with DAPI to show DNA (b,d,f). The dots in the cytoplasm in b,d,f are a mixture of mitochondria and ingested bacteria. Bar = 5 μ m. > - spindle poles. (a,b) binucleate cell in synchronous mitosis; the spindles have fused at one pole. (c,d) binucleate cell in synchronous mitosis; four sets of chromosomes and four spindle poles are visible. (e,f) two uninucleate cells in mitosis; one spindle is bipolar while the other is tripolar.

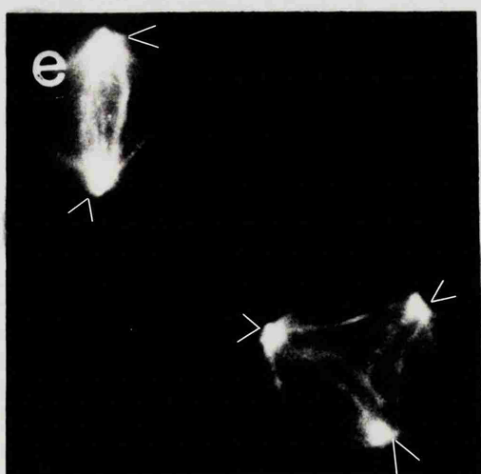
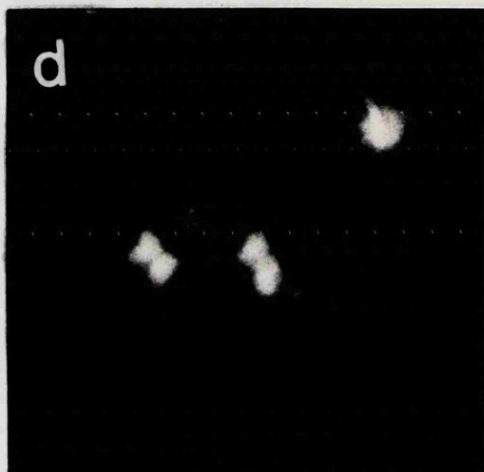
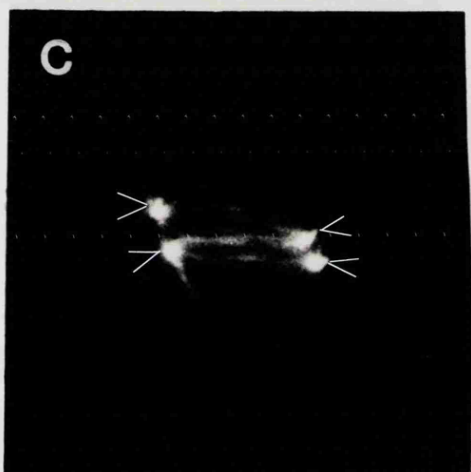
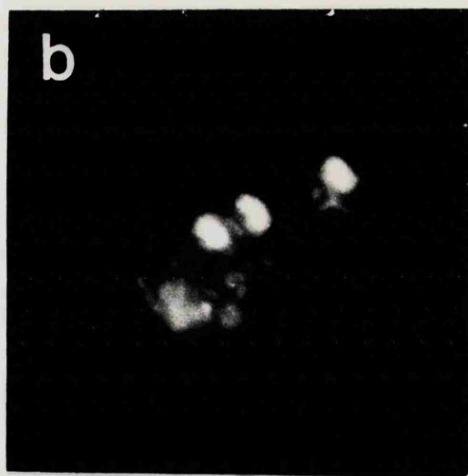
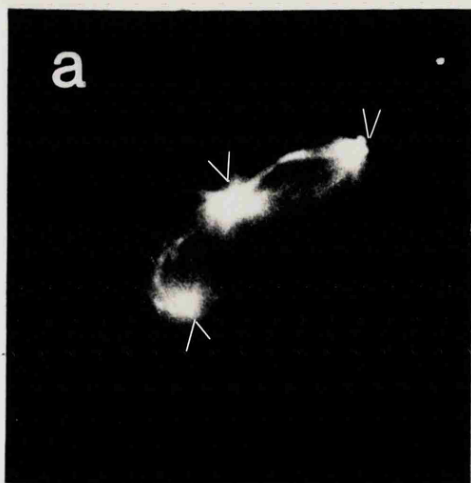


Figure 4.15. Flagellates from a *matA*-heteroallelic culture, double-stained with β -tubulin antibody to show microtubules (a,c) and with DAPI to show DNA (b,d) The dots in the cytoplasm in b,d are a mixture of mitochondria and ingested bacteria. Bar=5 μ m. (a,b) flagellate presumably derived from an amoeba. (c,d) flagellate presumably derived from a zygote.

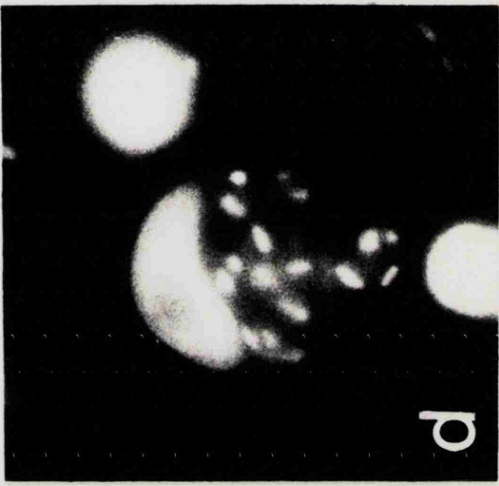
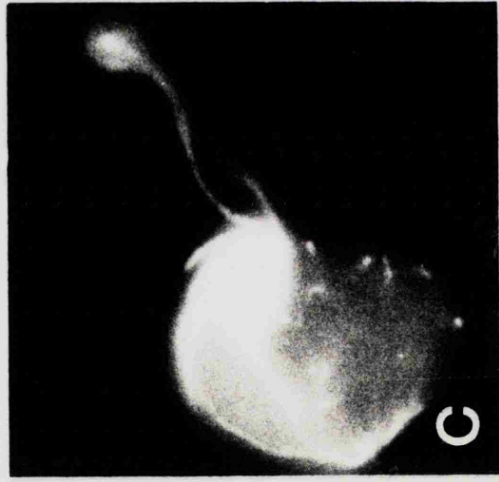
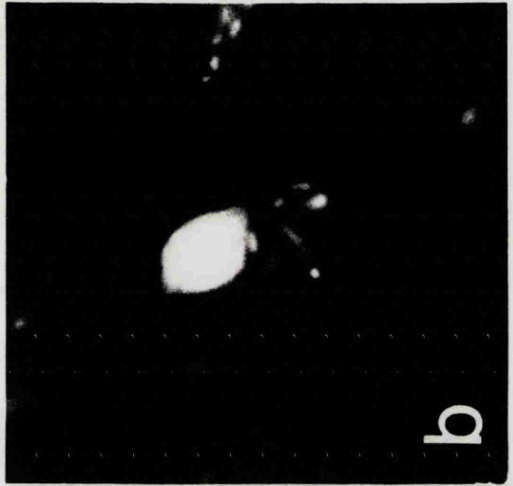
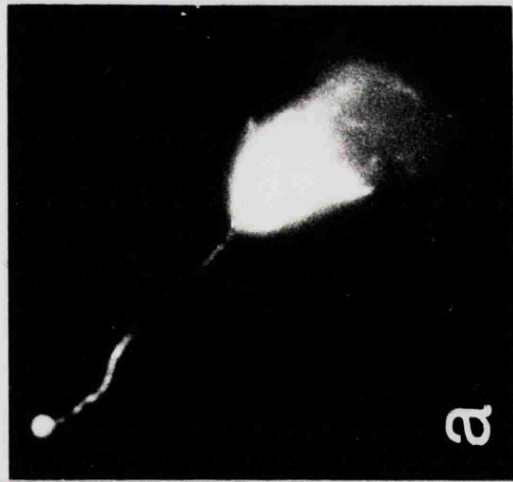
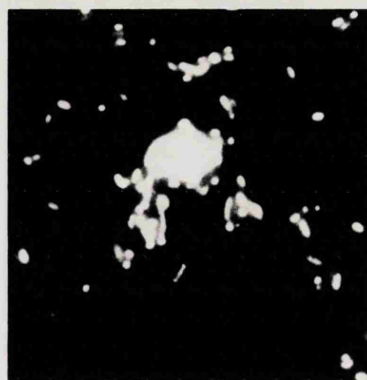


Figure 4.16. Flagellates from a *matA*-homoallelic culture, double-stained with β -tubulin antibody to show microtubules (a,c,e) and with DAPI to show DNA (b,d,f). The dots in the cytoplasm in b,d,f are a mixture of mitochondria and ingested bacteria. Bar=5 μ m. > - flagella. (a,b) Flagellate with a single long flagellum. (c,d) Flagellate with two long flagella. (e,f) Binucleate cell, presumably derived from a *matA*-homoallelic fusion cell, with two long flagella.



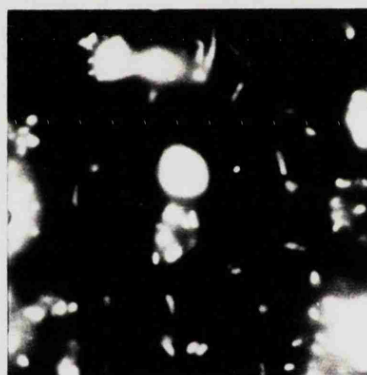
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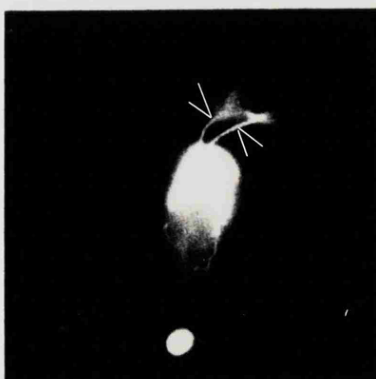
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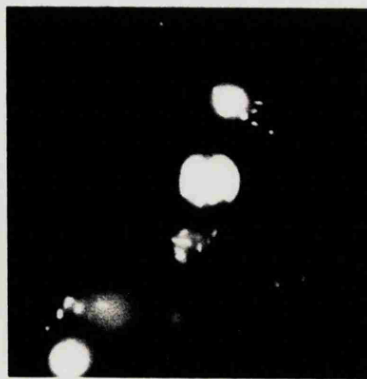
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f

CHAPTER 5

ANALYSIS OF DEVELOPMENT IN THE MUTANT STRAIN RA612

5.1 INTRODUCTION

The mutant strain described in this Chapter, RA612, was isolated from CL and carries a mutation in the gene *npfK*. The mutation was unlinked to *matA*; see Section 2.2 for more details of the genetic analysis. At their largest size, RA612 plasmodia, growing on agar plates, were just visible to the naked eye and under the microscope appeared structureless with no veins (Fig. 5.1). Filming analysis was used to determine the sequence of events during development of the abnormal plasmodia, and to examine the effect of the mutant *npfK* allele on development. Analysis of phase-specific mRNAs was undertaken in an attempt to detect alterations in the pattern of gene expression during the amoebal-plasmodial transition in this mutant strain. DNA content during the amoebal-plasmodial transition was examined using flow cytometry.

RESULTS

5.2 Kinetics of multinucleate cell formation

Before time-lapse cinematographic analysis of plasmodium development in RA612 could be carried out, it was necessary to estimate the numbers of plasmodia that formed and the rate

at which they appeared under the experimental conditions used; therefore the kinetics of multinucleate cell formation were examined as described in Section 2.6 using an initial density of 5×10^4 cells / plate on LIA+CIT plates. The differentiation cultures had to be grown at 23°C instead of 22°C because the 22°C constant temperature room was out of action for an extended period of time (about 9 months). In developing cultures of RA612, the first plasmodia were detected at a density of about 2.0×10^5 cells / plate (52 h; Table 5.1); extrapolation of the plasmodial curve in Fig. 5.2 indicated that the first cell became committed at about 25 h. The number of plasmodia reached a maximum at 94 h (7.4×10^4 plasmodia / plate). Even though the initial and maximum numbers of amoebae in the differentiating RA612 cultures (5×10^4 and 8.7×10^5 respectively) were greater than those in CL cultures (1×10^4 and 3.3×10^5 respectively; Table 3.1), fewer plasmodia were formed; the maximum number of plasmodia was 7.4×10^4 for RA612, whereas it was 1.3×10^5 for CL. Since RA612 plasmodia, unlike those of CL, were almost immobile and left very short trails on the assay plates, it was very difficult to distinguish plasmodia from pieces of agar. A plateable plasmodium was only scored as such when a definite identification could be made; thus, the number of plasmodia scored was probably an underestimate.

At each time-point during the kinetics experiment, microscopic analysis was carried out on the cell suspensions washed off the differentiation plates; about 300 cells from each time-point were scored for the number of nuclei each contained. The results are shown in Table 5.2. Committed

cells were present before multinucleate cells appeared in the population, and there were always more committed cells in the population than there were multinucleate cells (Table 5.2); this suggested that, as in CL and RA376, commitment occurred in uninucleate cells. During these cell counts many binucleate cells and some quadrinucleate cells were observed; however, no cells with more than 4 nuclei were observed, even in cultures where plasmodia were visible on the differentiation plates; no cells were visible on the plates after washing off. This suggested that many of the multinucleate cells had burst when they were washed off, presumably because they were very fragile; therefore the number of multinucleate cells scored at each time-point was probably an underestimate. Thus, the number of plasmodia formed in developing cultures of CL and RA612 may not, in fact, be very different.

5.3 Cinematographic analysis of development

5.3.1 Introduction

Two films were made of development in RA612; one lasted 66 h and the other 33 h. Unfortunately, the 66 h film was damaged during processing, leaving a large part of the first third of the film blank; this film did not yield any useable data on IMTs. For the 66 h film, cultures were set up at the same initial cell density as for CL and RA376; a density one third higher than this was used for the 33 h film. The magnification used gave an arbitrary unit of area equal to $7.30\mu\text{m}^2$. Filming was started, in both films, at the same time

after inoculation as for CL and one frame was exposed every 30 sec. All the data on IMTs and areas were obtained from the 33 h film and are shown in Table 5.3; representative pedigrees are shown in Fig. 5.3. Observations from the later part of the 66 h film (after the blank section), and from all the 33 h film were used in compiling the description of the phenotype of this mutant.

5.3.2 Events preceding binucleate plasmodium formation

The mean amoebal IMT was 9.74 ± 1.6 h ($n=21$) which was slightly shorter than the IMT for CL amoebae (Fig. 3.8). Amoebal area approximately doubled during an amoebal cell cycle (Table 5.3) as in CL and RA376 amoebal cell cycles. These measurements suggested that the mutation in the *npfK* gene carried by RA612 was not affecting amoebal growth, thus confirming the results of the initial experiments carried out by Dr. R. W. Anderson. As in CL and RA376, development involved an extended cell cycle, 2.3 times the length of an amoebal cell cycle, ending in the formation of a binucleate cell (Fig. 5.3a,b,c). As in the two apogamic strains, amoebae which entered the extended cell cycle and became binucleate arose from apparently normal amoebal divisions (Fig. 5.3). The mean area of an amoeba which went on to become binucleate was no different, at birth, from the mean area of an amoeba which divided at the end of an amoebal cell cycle (Table 5.3; $p < 0.005$). Growth continued during the extended cell cycle, so that at mitosis the developing uninucleate cell was, on average, twice the area of an amoeba at mitosis (Table 5.3); this ratio was the same as that observed for CL and RA376.

Amoebal mitoses appeared normal in RA612 (see Section 3.2.1). In many developing uninucleate cells, mitosis appeared to be of the closed plasmodial type. Partial cleavage furrows were observed in some uninucleate cells undergoing mitosis to become binucleate.

5.3.3 Events following binucleate plasmodium formation

As in CL and RA376, the cell cycle between binucleate cell formation and the next mitosis, at which a quadrinucleate cell was formed, was, on average, only about 0.6 of an amoebal cell cycle in length and cell area did not double during this short cell cycle; a binucleate cell at mitosis was, on average, only 1.6 times the area of a developing uninucleate cell at mitosis (Fig. 5.3b; Table 5.3). Nuclear area increased during the extended cell cycle (compare the nuclei of the amoeba and the large uninucleate cell in mitosis in Fig. 5.4a) and later decreased again (compare the nuclei of the plasmodium and the amoeba in Fig. 5.5b); a similar increase and decrease was observed in both CL and RA376. The mitoses observed in multinucleate cells appeared to be of the closed plasmodial type rather than the open amoebal type. As CL and RA376, partial cleavage furrows were observed in some binucleate cells undergoing mitosis to become quadrinucleate.

Of 11 cells which completed the extended cell cycle, 10 formed a binucleate cell by mitosis without cytokinesis. The other cell underwent mitosis and cytokinesis at the end of the long cell cycle, but the subsequent fate of the two uninucleate daughter cells could not be determined (Fig.

5.3a).

5.3.4 Plasmodial fusions

Plasmodial fusions occurred at a very low rate between RA612 plasmodia; only 6 fusions were seen throughout the 33 h film, compared with 29 fusions in a single film of CL. RA612 plasmodia were often observed to remain in contact for some time without fusing, whereas in CL contact between plasmodia was usually rapidly followed by fusion. Four of the six fusions took place between multinucleate cells (e.g. Fig. 5.3b); the other two involved the fusion of a uninucleate cell with a multinucleate cell; in neither case could the origin of the uninucleate cell be traced. Two binucleate cells were observed to split into two uninucleates some time after mitosis; the subsequent fates of the uninucleate cells could not be determined. At the end of both films of RA612 many small plasmodia were present, whereas at the end of the film of CL a few large plasmodia occupied the field of view.

5.3.5 Abnormal behaviour and morphology of RA612 plasmodia

Although the relative lengths of the cell cycles, the timing of commitment and the mitoses appeared normal, the morphology of the developing RA612 plasmodia began to appear abnormal shortly after binucleate cell formation (compare the abnormal binucleate cell in Fig. 5.4b with the normal CL cells in Fig. 3.3). As development proceeded, the phenotype became more pronounced. RA612 plasmodia were often observed to extend pseudopodia towards an amoeba without fusing around it, only 3 amoebae were observed to be ingested by plasmodia;

a food vacuole containing the remains of an amoeba can be seen in the plasmodium in Fig. 5.5a. In contrast, in CL cultures, multinucleate cells often contained several ingested amoebae (not illustrated).

The RA612 plasmodia continuously changed in shape as cells extended and retracted pseudopodia. This led to irregularly-shaped, fissured plasmodia, an example is shown in Fig. 5.5b, where the multilobed plasmodium has extended pseudopodia round a cell but has not fused with or ingested the cell. Often a plasmodium would extend several pseudopodia in different directions at once, rather than in a single direction as observed for CL; these pseudopodia were often narrow and multilobed and altered shape continuously. The extension of several pseudopodia at once in different directions often led to the development of long, narrow plasmodia where two sections were attached together by a thin bridge of cytoplasm; this is in great contrast to CL plasmodia which were thick and rounded at the same stage of development (Fig. 3.3). Fusions sometimes occurred between one of these long, narrow sections and another plasmodium; in this event the narrow part sometimes split across the thin cytoplasmic bridge, thus giving rise to two plasmodia again.

The developing uninucleate cells appeared normal in morphology and movement, but it was noticeable that, after about the binucleate to quadrinucleate stage, very little net movement of the plasmodium occurred although the RA612 plasmodia were continually extending pseudopodia. In contrast to CL, RA612 plasmodia with more than about 4 nuclei did not show the pulsation normally observed. The relative flatness

of the RA612 plasmodia, compared with CL plasmodia, can be seen by comparing Fig. 5.5b (where at least 12 nuclei can be seen) with Fig. 3.3, which shows CL plasmodia of similar size but very different morphology; in the CL plasmodia no nuclei can be seen and the slime layer is prominent.

5.4 DNA content during development

The results of measurement of DNA by flow cytometry are shown in Fig. 5.6. Microscopic analysis of the control amoebal culture indicated that it contained only encysted and uninucleate cells. The main haploid G2 peak for this culture, with mode at about channel 50, contained 85.0% of the cells (see Section 3.2.3). The second, diploid G2 peak, with mode at about channel 100, contained about 7% of the cells. The remainder of the cells had higher DNA contents and were assumed to correspond to polyploid and clumped cells.

In the developing culture, large plasmodia were visible on the differentiation plate, but microscopic analysis of the cell suspension showed that it contained 2.3% binucleate cells and 0.4% quadrinucleate cells; the majority of the cells (97.3%) were uninucleate. This suggested that the multinucleate plasmodia had burst when washed off the differentiation plates. Flow cytometry (Fig. 5.6b) indicated that the major haploid G2 peak, with mode at about channel 50, contained 78.5% of the cells. The second peak, corresponding to uninucleate diploid or binucleate haploid G2, had a mode at about channel 100 and contained 14.4% of the cells. The remaining cells had higher DNA contents, and

were presumably polyploid cells or clumped cells. Replating assays indicated that 18.3% of the cells were committed; thus, 15.6% ($18.3 - 2.3 - 0.4$) of the total cells were uninucleate committed cells. This value for the number of committed cells was probably an underestimate for the reasons stated in Section 5.2. The secondary peak contains 14.4% of the cells, some of these cells were binucleate plasmodia (2.3%) and some were probably diploid amoebae. It was possible that some of the developing uninucleate cells could have had DNA contents at about channel 100, but the number of cells in this secondary peak was too small to account for all the developing uninucleate cells scored (15.6%). It, therefore, seems likely that in RA612, as in CL, most of the uninucleate committed cells had the same nuclear DNA content as amoebae.

5.5 Expression of phase-specific mRNAs

It has been shown that some of the genes expressed in *P. polycephalum* are specific to a single stage of the life cycle (see Section 1.2.4). Sweeney (1987; Sweeney *et al.*, 1987) isolated RNA from amoebae and plasmodia of CL and constructed two cDNA libraries from the RNA; differential screening allowed identification of cDNAs that hybridised with phase-specific mRNAs. By examining the pattern of gene expression during the amoebal-plasmodial transition in mutants blocked in apogamic development, it was hoped to build up an activation sequence for some phase-specific genes and possibly to identify genes that were not activated

correctly in specific mutant strains. The work described here was carried out in collaboration with Glen Sweeney, while he was a postgraduate student in the Biochemistry Department at Leicester University. The results have been reported in Sweeney (1987) and Anderson *et al.* (1987). The methods used are described in Sweeney (1987) and Sweeney *et al.* (1987).

RNA was isolated from three populations of RA612 cells: amoebae, plasmodia and a developing culture enriched for cells unable to flagellate, which were prepared as follows. The amoebae were set up on LIA plates at a density of 1×10^5 cells / plate and grown for 5 days at 30°C before being harvested for RNA extraction. Plasmodia were obtained by growing amoebae at 26°C for 10 days before they were harvested and RNA was extracted.

A developing culture was obtained by culturing amoebae at 22°C for three days; the amoebae were set up at an initial cell density of 1×10^5 cells / plate. After three days the developing cultures were harvested and microscopic analysis showed that the culture contained 83.5% uninucleate cells, 15.5% binucleate cells and 1.0% quadrinucleate cells; no large plasmodia were visible on the differentiation plates. This developing culture was enriched for cells unable to transform into flagellates by the method of Blindt *et al.* (1986; see Section 1.2.4). This resulted in an population of cells enriched for cells unable to transform into flagellates; the population contained 75% uninucleate cells, 22% binucleate cells and 3% quadrinucleate cells as indicated by microscopic analysis. Since ability to undergo the amoeba-flagellate transformation was lost some hours before

commitment (Blindt *et al.*, 1986; Bailey *et al.*, 1987), a population of cells enriched for cells unable to flagellate was expected to contain a high proportion of developing cells. RNA was extracted from this enriched population.

The RNA from the three populations of RA612 cells, together with CL plasmodial RNA as a control, were Northern blotted and then probed with a cDNA clone which had previously been shown to be phase-specific in expression (Sweeney, 1987; Sweeney *et al.*, 1987). It must be remembered when considering the results of Sweeney *et al.* (1987) and those of this work, that the developing cultures did not contain only developing cells. For example, the populations of uninucleate committed cells used by Sweeney *et al.* (1987; Sweeney, 1987) contained 30% - 50% amoebae.

The results of the Northern blots are shown in Fig. 5.7. The clone P210 was expressed in amoebae and plasmodia from both CL (Sweeney *et al.*, 1987) and RA612. Clones P325, P57 and P46 were not present in the amoebae of either CL (Sweeney *et al.*, 1987) or RA612. In CL, P325 and P46 were expressed in uninucleate committed cells and all later stages of development; both clones were expressed in the RA612 enriched developing culture (C in Fig. 5.7) and in RA612 plasmodia (M in Fig. 5.7), but P46 was at a very low level compared with that observed in CL (Sweeney *et al.*, 1987). Clone P57 was expressed only in large plasmodia in CL (Sweeney *et al.*, 1987) and could not be detected at all in RA612. The two amoeba-specific clones (A195 and A273) were both strongly expressed in RA612 amoebae (A in Fig. 5.7), and much more weakly expressed in the enriched developing culture and the

plasmodia; this was the same pattern as had been observed for CL by Sweeney *et al.* (1987). The only detected difference in gene expression between RA612 and CL was the absence of the P57 transcript from RA612 microplasmodia.

5.6 Other aspects of development in RA612

In CL, ability to grow in liquid medium was acquired while committed cells were still uninucleate (Blindt *et al.*, 1986). In order to see whether RA612 had also acquired this ability, small (5mm x 10mm) blocks of agar were cut out of a DSDM agar plate on which developing RA612 cells were growing. Two blocks of agar were placed in the bottom of each of two 250ml baffled flasks. Enough SDM (containing penicillin and streptomycin both at 250 units / litre) was added to just cover the blocks of agar. After 2 days stationary culture at 26°C, the microplasmodia had moved off the blocks into the liquid; the amoebae, which cannot grow in SDM, had died. The cultures were transferred to a shaker, and enough SDM was added to give a final volume of about 50ml. After a week in shaken culture the flasks contained many yellow microplasmodia. The microplasmodia were subsequently subcultured weekly by inoculating 0.1ml of cell suspension into 100ml of SDM in a 500ml flask; the cultures were kept on a shaker at 26°C. The growth rate of RA612 microplasmodia appeared to be similar to that of CL microplasmodia inoculated in the same manner but the RA612 culture fluid appeared much yellower than that of CL. The morphology of both strains of microplasmodia looked similar; many of the

microplasmodia of both strains were round or dumb-bell shaped (not illustrated). In many of the dumb-bell shaped plasmodia, from both strains, cytoplasmic streaming could be observed across the neck of the cell; no veins with distinct walls were present however.

At intervals, a drop of RA612 microplasmodial suspension was placed on SDM agar and allowed to grow. Within 3 days the plasmodia had fused to give a small macroplasmodium which rapidly grew to cover the whole plate; the morphology of the plasmodium looked normal (compare Fig. 5.5c with Fig. 1.1b). Other workers (Lilianna Solnica-Krezel, University of Wisconsin and Duncan Gruer, Sheffield University; personal communication) have also obtained RA612 macroplasmodia, often after a few weeks of culturing amoebae on agar plates.

There were several possible explanations for the appearance of RA612 macroplasmodia. Firstly, revertants may have been selected during culture in liquid medium (and during extended growth on plates). Secondly, the mutation may not completely block the functioning of the *npfK* gene product. Lastly, the product of the mutant gene may only be required transiently during development; if so, growing the cells in liquid medium may have allowed them to pass the point at which the gene product was required.

In order to distinguish between the first two of these possibilities, five RA612 macroplasmodia were allowed to sporulate; spores were obtained from each one. Spores from 4 of the plasmodia were plated on LIA and the resultant amoebal colonies were examined for the presence of plasmodia; of 1000 colonies scored after one week at 26°C, all contained

plasmodia with the morphology typical of RA612; no CL-like plasmodia were detected. Similar results were obtained by Duncan Gruer (personal communication). It appeared, therefore, that reversion of the mutation had not occurred, at least not at a detectable level. The other two possibilities will be discussed further in Section 5.7.2.

5.7 DISCUSSION

5.7.1 Events common to development in CL and RA612

The results from all experiments suggested that the early stages of development in RA612 were very similar to those of CL, from which RA612 had been isolated. The kinetics analysis indicated that commitment occurred while the cells were still uninucleate, as in CL and RA376. Filming analysis identified an extended cell cycle, during which growth continued; this was followed, as in CL, by a short cell cycle during which cell area did not double. As with all other strains, nuclear area increased in uninucleate committed cells and subsequently decreased. The increase and subsequent decrease in nuclear area did not appear to be correlated with fluctuations in nuclear DNA content in the majority of committed cells. Large uninucleate cells were observed to fuse with multinucleate plasmodia suggesting that, as in CL, ability to undergo plasmodial fusion was acquired while the cells were still uninucleate. Ability to grow in axenic culture was also acquired by the developing RA612 plasmodia.

The pattern of gene expression detected by the phase-specific cDNA clones indicated that, for the small

number of clones examined, RA612 and CL had very similar patterns of gene expression during the amoebal-plasmodial transition (Anderson *et al.*, 1987; Sweeney 1987; Sweeney *et al.*, 1987). All the clones, except P57, hybridised to RNA that appeared (or disappeared) at the same developmental stage in both CL and RA612. The absence of hybridisation between clone P57 and RNA from RA612 was not altogether surprising because the gene identified by this clone was apparently not active during the early stages of CL development. Since RA612 rarely forms mature plasmodia it may not have reached the developmental stage at which the product of P57 was expressed. The final pattern of gene expression seen in RA612 microplasmodia (M in Fig. 5.7) fitted well with the terminal phenotype of the strain; it expressed amoeba-specific, and early plasmodium-specific genes, but not late plasmodium-specific genes. The defective *npfK* message did not appear to be represented in the small number of clones screened but this result was not surprising since the cDNA library contained mainly RNA species comprising more than 0.1% of the total mRNA (Sweeney, 1987). If the *npfK* gene product was present at a lower level than this, it would probably not be represented in the library. If the *npfK* gene product was active only during the amoebal-plasmodial transition, it would not feature in the library.

Filming analysis indicated that there was a switch from open mitosis in amoebae to closed mitosis in plasmodia, as in CL (Blindt, 1987). Other evidence (Lilianna Solnica-Krezel, personal communication) suggested that the pattern of tubulin gene expression observed in the developing RA612 cells was

developmentally regulated in the same way as in CL; the β 2-tubulin isotype was detected in some uninucleate cells and the acetylated- α isotype (α 3) was absent in some uninucleate cells (Solnica-Krezel *et al.*, 1988; Diggins-Gilicinski *et al.*, 1989).

5.7.2 Explanations for the formation of macroplasmodia

Three suggestions were put forward in Section 5.6 to explain the appearance of macroplasmodia in cultures of RA612.

The first possibility was reversion of the mutation. Results from the analysis of spores obtained from the macroplasmodia indicated that no revertants were present. Other laboratories have also shown that macroplasmodia that arose on plates of RA612 amoebae were not revertants (Duncan Gruer, personal communication).

Secondly, the mutation might not completely destroy the function of the *npfK* gene product. It is possible that the defective gene product can function with a low efficiency, or that most of the transcripts from the gene are short but a few full length messages are produced and these allow very slow growth of the RA612 plasmodia. However, if the *npfK* gene was partially functional, it might be expected that more macroplasmodia would arise from cultures on agar plates than were observed.

The third possibility was that the defective gene product was important during part of the amoebal-plasmodial transition but not later. Growth in liquid axenic culture does not require phagocytosis; nutrients are absorbed directly and by pinocytosis. Plasmodial fusions do not occur

in this medium, in fact liquid grown plasmodia are kept small by fragmentation. Since, RA612 was defective in both phagocytosis and plasmodial fusion, liquid axenic culture might provide the ideal environment for growth of this strain. Placing RA612 microplasmodia in liquid culture may allow the cells to continue growth thus passing the size at which the defective gene product was important.

Although it was possible to rule out reversion as a cause of the appearance of RA612 macroplasmodia, it was not possible to conclusively rule out either of the other two possibilities.

5.7.3 Possible explanations for the abnormal morphology of RA612 plasmodia

The time-lapse films indicated that the developing RA612 plasmodia began to appear abnormal at about the time of binucleate cell formation, suggesting that the product of the *npfK* gene was first required early in plasmodium formation. The low levels of plasmodial fusion and amoebal ingestion both contributed to the slow growth of the plasmodia; both are important for growth in normal plasmodia. The complex phenotype observed from the films suggested that the mutation might be affecting the membrane and / or cytoskeleton in the developing RA612 plasmodium. There are several ways in which these could be affected.

A defect in the delivery of newly synthesised membrane components to the required place might lead to the observed slow locomotion and also to the abnormal shape of the developing plasmodia and to the presence of multiple, lobed

pseudopodia. In such a defect, alterations in the cytoskeleton could be implicated, as could a defect in the intracellular signalling system. It is known that microtubules are important components of the intracellular transport system in many organisms (Allen, 1987). In developing plasmodia, however, the number of microtubules gradually declined (Blindt, 1987; see Section 4.4.1 and Section 5.7.1) and their function would, presumably, be taken over by the developing veins. The macroplasmodia of RA612 possessed distinct, walled veins but the microplasmodia on plates or in liquid did not, although streaming was observed; the development of walled veins might, however, be a function of the size of a plasmodium since CL microplasmodia grown in liquid did not possess walled veins either. Plasmodia express more than one isotype of both α - and β -tubulin (Burland et al., 1983; Roobol et al., 1984); it is unlikely, therefore, that a defect in a single tubulin gene would halt development in the way observed in this mutant. It is possible, however, that one of the more minor components of the cytoskeleton is defective in this strain. With reference to this suggestion, results from experiments using *Dictyostelium discoideum* indicated that, in this species at least, the absence of α -actinin (Wallraff et al., 1986) did not affect cell growth, aggregation or development; the disruption of myosin, however, reduced (but did not halt) cell growth and locomotion, delayed aggregation and halted development at the mound stage (Wessels et al., 1988; Knecht and Loomis, 1988).

Other possible explanations for the defect observed in this strain lie in the actual mechanism of membrane fusion;

membrane fusion is a complex process during which several barriers to fusion need to be overcome. These barriers include recognition of fusion sites, movement of the membranes into apposition, alterations in membrane packing, fusion itself and reestablishment of membrane composition after fusion (Blumental 1987). A defect in any aspect of membrane fusion would explain the observed defects in phagocytosis and plasmodial fusion.

Recognition of the fusion sites in plasmodia may be under the control of the *fus* genes. Plasmodia express three genetically well characterised *fus* genes which control fusion of plasmodia; other fusion genes may exist (reviewed by Dee, 1982). The mechanism by which these genes mediate fusion is unknown. The alleles of the *fus* genes carried by a plasmodium govern its fusion partners, suggesting that the *fus* genes are involved in intercellular recognition. Unlike the situation in amoebae, only genetically identical plasmodia can fuse. Failure to recognise fusion compatible membranes, which would include parts of the same plasmodium, would lead to the observed failure to fuse around amoebae or with other plasmodia. However, genetic analysis has shown that the mutation carried by RA612 does not map to the position of any of the 3 well characterised plasmodial fusion genes although it is loosely linked to one of them (*fusA*; see Section 2.2); thus, if the mutation affects intercellular recognition, the mutation must have occurred in another, as yet undetected, gene governing plasmodial fusion.

Unlike the situation in CL, developing RA612 plasmodia often spent long periods in very close contact before fusion.

One possibility, therefore, is that the RA612 plasmodia cannot overcome the steric and electrostatic repulsion between the two membranes so that fusion can occur.

It has been shown that the distribution of membrane components is altered in the area in which membrane fusion occurs (reviewed by Blumental, 1987). Presumably, membrane fusion requires newly synthesised membrane components in the areas where fusion or phagocytosis are occurring. A defect in intracellular signalling could lead to incorrect alterations in the distribution of membrane components, or no alterations at all, and to the observed alterations in fusion. The fusions which did occur appeared normal but the magnification used in filming did not allow detection of any defects in the actual process of fusion at the microscopic level.

5.7.4 Conclusions and further experiments

No one simple explanation appears to explain the complex phenotype observed in RA612. Further investigations are needed to learn more about the action and distribution of the components of the membrane and cytoskeleton. It might be possible to look for the defective proteins by isolating plasmodial cell membranes from both CL and RA612 at a similar stage of development, and then separating them on 2-dimensional gels. Comparison of the pattern of spots given by the two strains, should reveal any differences in proteins present but would not detect a defective protein unless the electrophoretic mobility or size was altered. An immunofluorescence study of the cytoskeleton using antibodies specific to various cytoskeletal components might indicate if

any of the components of this system were absent, or present in the wrong place. There is always the possibility, with both these approaches, that any differences detected might be a secondary consequence of the mutation at the *npfK* gene and not the primary defect.

Another approach, which might result in isolation of the defective gene, would be to make a more detailed analysis of gene expression in different cell types using the cDNA libraries prepared by Sweeney (1987). However, enrichment is not very efficient and messages that are present at low levels would not be detected. In addition, unless hybridisation conditions were very stringent, the mutation might not be detected if it involved only minor changes in the DNA (and hence RNA) sequence of the gene. Advances in cell culture (Solnica-Krezel *et al.*, 1988) have made it possible to obtain cultures containing up to 60% uninucleate cells without using the enrichment method of Blindt *et al.* (1986). Recent advances in cloning techniques have made it possible to remove, by cDNA subtraction, many of the abundant messages from an mRNA population and leave only the low abundance messages (Davis, 1986). Using such an approach might allow detection of differences in expression of many low abundance mRNAs.

There are, therefore, several possible avenues to explore when continuing the search for the molecular defect underlying the complex phenotype of RA612.

Table 5.1
Kinetics of growth and development in RA612

Hours after inoculation	Numbers of colonies per differentiation plate			
	Amoebae	Average	Plasmodia	Average
0	2.9×10^4		0	
	3.7×10^4	3.3×10^4	0	0
22	3.8×10^4		0	
	5.5×10^4	4.7×10^4	0	0
28	2.6×10^4		0	
	3.8×10^4	3.2×10^4	0	0
34	7.3×10^4		0	
	7.3×10^4	7.3×10^4	0	0
46	1.2×10^5		0	
	1.0×10^5	1.1×10^5	0	0
52	3.1×10^5		4.5×10^2	
	2.5×10^5	2.8×10^5	1.2×10^3	8.3×10^2
58	3.8×10^5		1.2×10^3	
	2.8×10^5	3.3×10^5	4.5×10^2	8.3×10^2
70	7.1×10^5		3.8×10^4	
	5.3×10^5	6.2×10^5	3.8×10^4	3.8×10^4
76	5.3×10^5		5.3×10^4	
	7.1×10^5	6.2×10^5	1.5×10^4	3.4×10^4
82	1.0×10^6		3.0×10^4	
	7.3×10^5	8.7×10^5	4.1×10^4	3.6×10^4
94	8.4×10^5		6.4×10^4	
	8.1×10^5	8.3×10^5	8.3×10^4	7.4×10^4
100	4.1×10^5		6.4×10^4	
	3.4×10^5	3.8×10^5	9.8×10^3	1.5×10^4

The average numbers of amoebae and plasmodia are plotted on Fig. 5.2.

Table 5.2

Percentage of different cell types during development in RA612

Hours after inoculation	% of cell type in population			% committed cells
	uninucleate	binucleate	quadrinucleate	
0	all cysts	0	0	0
22	100	0	0	0
28	100	0	0	0
34	100	0	0	0
46	100	0	0	0
52	100	0	0	0.3
58	100	0	0	0.3
70	99.5	0.5	0	5.8
76	97.4	2.3	0.3	5.2
82	96.3	3.7	0	4.0
94	92.1	7.2	0.7	8.2
100	-	-	-	3.8

- not counted

Table 5.3**Summary of data from time-lapse cinematography of RA612**

<u>Measurement</u>	
Amoebal	9.74+1.6 h
IMT	(21)
Uninucleate to	22.56+1.7 h
binucleate IMT	(11)
Binucleate to	5.96+1.8 h
quadrinucleate IMT	(3)

Amoebal area	8.6+1.1
at birth	(21)
Amoebal area	18.1+1.7
at mitosis	(21)
Area at birth	8.7+0.7
of cells which	(11)
become binucleate	
Area of cells	37.3+4.7*
at binucleate	(11)
cell formation	
Area of binucleate	59.7+10.1
cells at mitosis	(3)
to quadrinucleate	

Number of cells which divided at the end of the extended cell cycle: 1/11

*One of these cells divided at the end of the extended cell cycle.

Measurements are given as mean $\pm$ standard deviation and the number of observations is given in parentheses. Times are given in hours (h) and areas are given in arbitrary units.

Figure 5.1

RA612 plasmodia (>) developing at the junction of two amoebal colonies. A - amoebae. Bar = 0.3mm



A

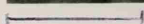


Figure 5.2

Kinetics of amoebal growth and plasmodium formation in cultures inoculated with RA612 cysts at time zero. Two cultures were assayed at intervals, for amoebae (x) and plateable plasmodia (o). Extrapolation of the plasmodial curve indicates that the first cell became committed to plasmodium formation at about 25 hours.

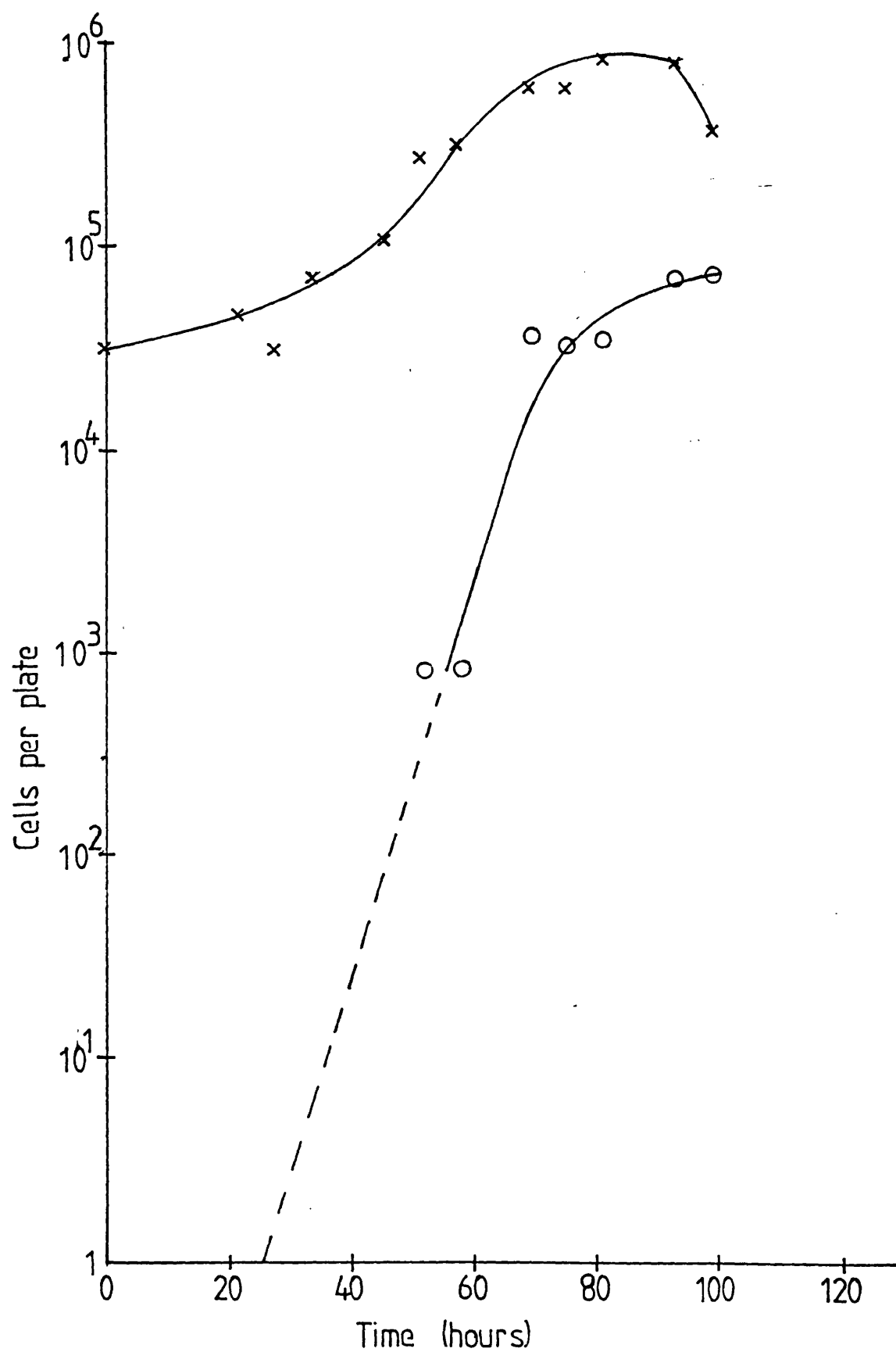


Figure 5.3

Representative RA612 cell pedigrees.

—● Time of nucleolar disappearance preceding mitosis

⊙ uninucleate cell

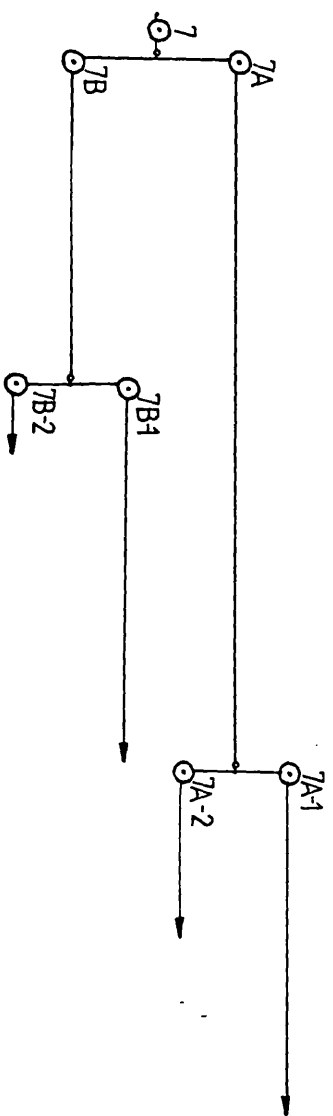
⊖ binucleate cell

⊖ quadrinucleate cell

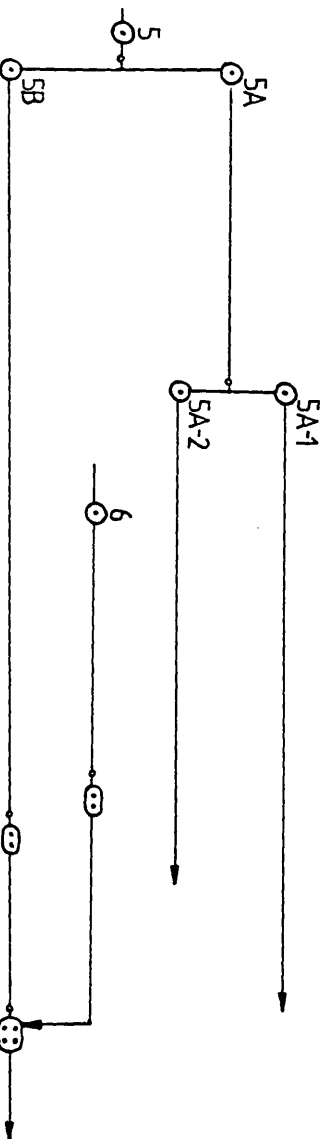
F - cell fused with another multinucleate cell

→ cell left field.

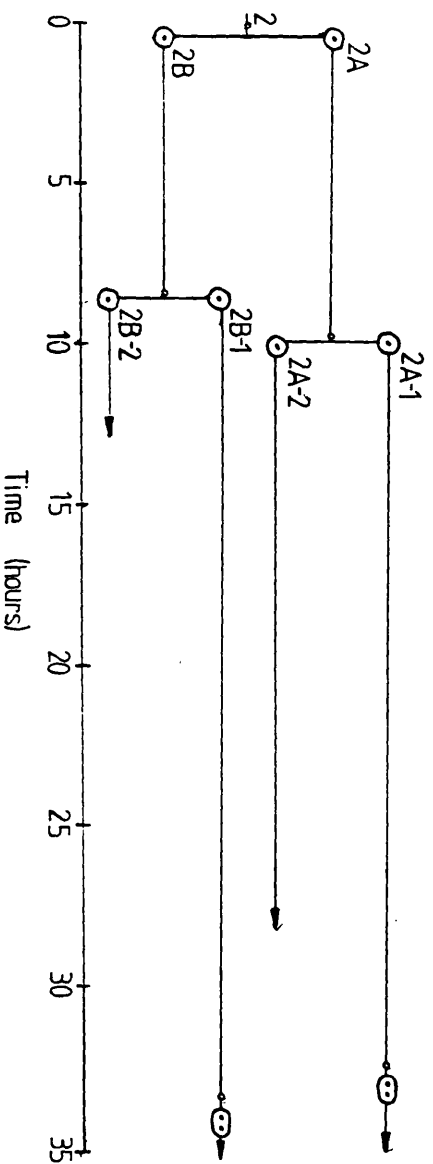
Filming began at 0 minutes.



a



b



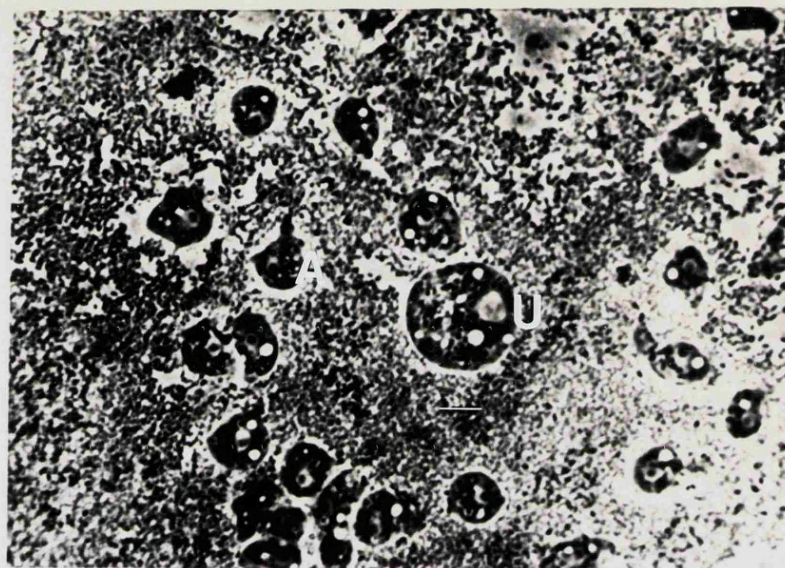
c

Time (hours)



Figure 5.4

Developing RA612 cells on a bacterial lawn. A - amoeba, U - large uninucleate cell. Bar = 10 μ m. a) A large uninucleate cell in mitosis; the nucleolus has disappeared and the cell is very rounded. b) abnormal stretched cell (B).



a



b

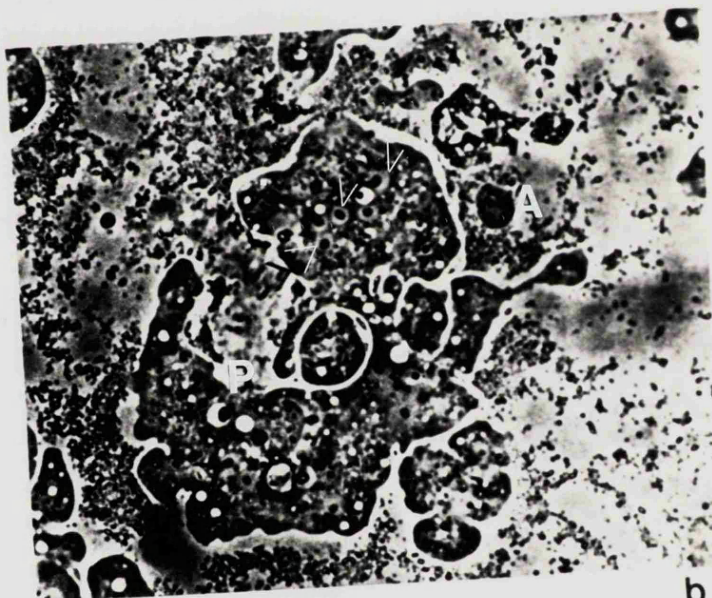


Figure 5.5

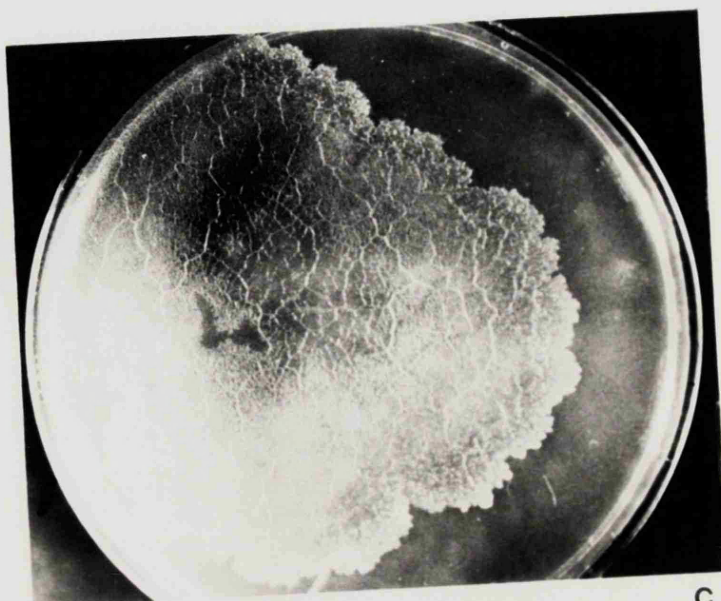
Developing RA612 plasmodia. a&b) Abnormal microplasmodia (P) showing the fissured shape. An amoeba (A) is indicated for size comparison. Bar = 10 μ m. a) abnormal plasmodium which has ingested an amoeba into a vacuole (V). b) An abnormal plasmodium that has extended pseudopodia around two multinucleate cells but has not fused with them. Several nuclei are visible (>). c) Multinucleate plasmodia grown by placing drops of RA612 liquid culture on SDM agar. The vein structure appears normal as does the shape. Bar = 1cm.



a



b



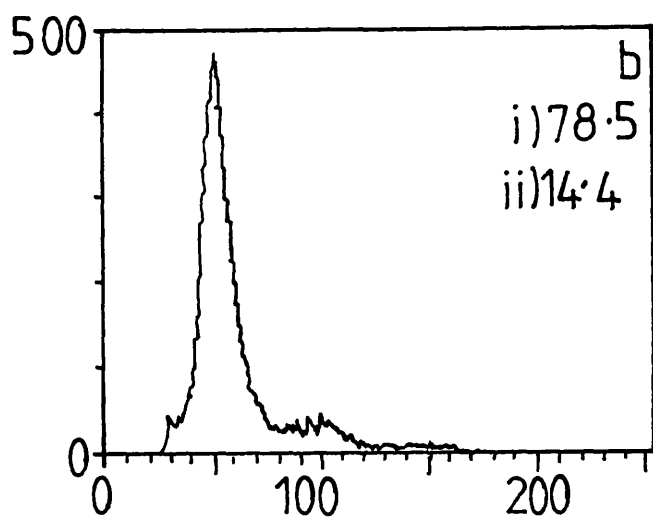
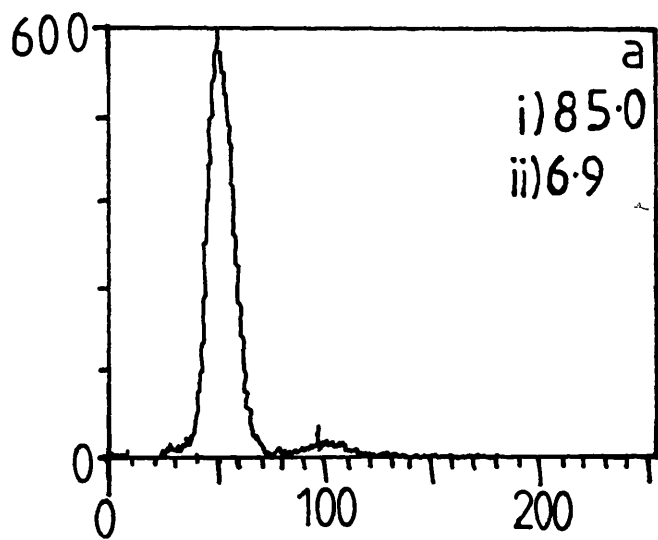
c

I

Figure 5.6

Distribution of cellular DNA contents in RA612 estimated by flow cytometry. a) vegetative amoebae at 30°C b) developing population at 22°C. Channel number is proportional to DNA content (see Section 2.9). 10^4 cells were counted for each sample. On each panel the percentages of cells (i) in the main peak with the mode at or near channel 50, and (ii) in the secondary peak with the mode at or near channel 100, are indicated.

Number of cells



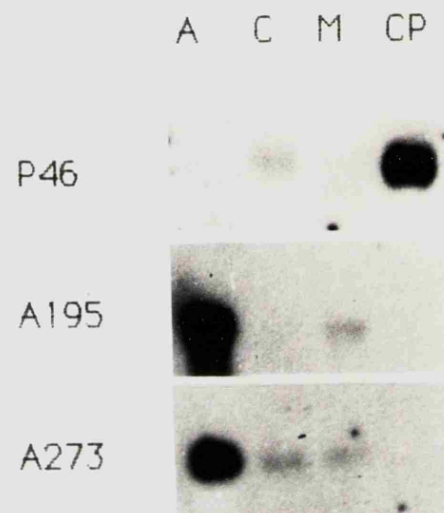
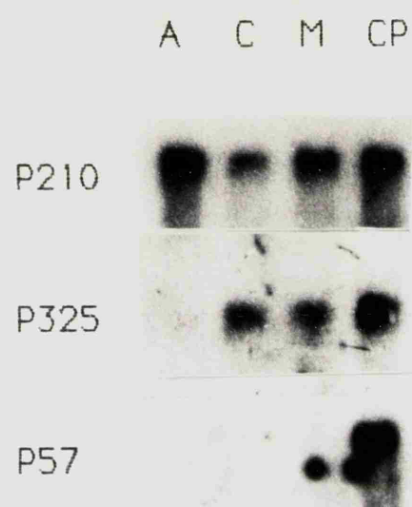
Channel number

Figure 5.7

Expression of phase-specific mRNAs in RA612

RNA from amoebae (A), from an enriched developing culture (C), from plasmodia (M) were extracted and Northern blotted. RNA from CL plasmodia (CP) was included as a control. The Northern blots were then probed with the indicated clones. See Sweeney (1987) and Sweeney et al. (1987) for experimental details.

RA612



CHAPTER 6

ANALYSIS OF DEVELOPMENT IN THE MUTANT STRAIN RA614

6.1 INTRODUCTION

The mutant strain described in this Chapter, RA614, was isolated by Dr. R. W. Anderson following mutagenesis of RA376 amoebae. RA614 carries a mutation in a gene (*npfL*) unlinked to *matA*; see Section 2.2 for details of the genetic analysis. Phase contrast microscopic analysis of development in this strain, by Dr. R. W. Anderson, showed that although some binucleate cells formed during development very few quadrinucleate cells were observed, suggesting that development halted at an early stage. The terminal stage of development was a cell which lacked visible nuclei under phase contrast microscopy. Unlike the situation in RA612 (see Chapter 5), macroplasmodia never formed. The events leading to the formation of apparently anucleate cells were examined using time-lapse microcinematography. Analysis of phase-specific mRNAs was undertaken in an attempt to detect alterations from the normal pattern of gene expression during the amoebal-plasmodial transition. The DNA content of the cells that lacked visible nuclei was examined using flow cytometry. The involvement of microtubules in the development of the apparently anucleate cells was studied using immunofluorescence microscopy. In this section of the results, comparison is made with the alterations in microtubule organisation observed during development in RA376.

RESULTS

6.2 Kinetics of anucleate cell formation

Although the terminal phenotype of RA614 had been identified, the relationship between the number of anucleate cells in a culture and time after inoculation, was unknown. Before time-lapse cinematography could be used to investigate the origins of the anucleate cells, it was necessary to examine the above relationship under the experimental conditions used for filming. The experiment was carried out using the method outlined in Section 2.6. LIA+CIT plates were inoculated with 5×10^4 cells / plate; at each timepoint the cells were washed off two plates and the suspensions were combined. Using thin agar slides, prepared as described in Section 2.7, the number of nuclei in each of about 300 cells was counted; the results from these counts are shown in Fig. 6.1 and Table 6.1.

From 0 - 60 hours, all the cells were uninucleate; the first binucleate cells were detected at 60 hours. The number of binucleate cells increased to a maximum of 5.4% (96 hours) and then remained almost constant, at around 5%, until 168 hours (7 days) when it dropped to 2.9% (Fig. 6.1; Table 6.1). The number of cells without any visible nuclei increased rapidly after 78 hours, reaching a maximum of 36% after 168 hours (Fig. 6.1; Table 6.1). No quadrinucleate or octanucleate cells were observed.

6.3 Cinematographic analysis of development

6.3.1 Introduction

Two films were made of development in RA614; both lasted 66 hours and the interval between frames was 30 sec. The same magnification was used for both films. The slide culture used for Film 1 was inoculated with cysts at the same density used for the films of RA376 and CL; filming began at the same time after inoculation as in the RA376 and CL films (see Section 3.2.1) at a time when all the cells were uninucleate. The slide culture used for Film 2 was inoculated with cells from a culture in which development had begun; some binucleate cells were present at the start of this film. By the end of Film 1, the cells had exhausted the available bacterial food supply but few binucleate and anucleate cells had formed. The slide used for Film 2 was therefore inoculated with more bacteria; the food supply did not run out during Film 2. Data on IMTs (Table 6.2) and cell fates were obtained from both films; IMT is given in hours and is measured from nucleolar disappearance in one cell cycle to nucleolar disappearance in the next. All the data on cell areas during development came from Film 1 (Table 6.2); cell areas are given in arbitrary units (one unit equals $7.30\mu\text{m}^2$ on the slide culture).

6.3.2 Growth and division in amoebae

There was a significant difference between the mean amoebal IMTs measured from the films of the two slides (Table 6.2); the mean amoebal IMT from Film 2 was significantly shorter than the mean amoebal IMT in Film 1 ($p > 0.005$). This difference may, however, be related to the culture conditions

of the amoebae; Film 2 was inoculated with growing amoebae which had an abundant food supply, whereas Film 1 was inoculated with cysts and fewer bacteria as a food source. Amoebal area approximately doubled during one cell cycle; the mean at birth was 14.9 ± 3.0 and the mean size at mitosis was 34.0 ± 6.0 units (Table 6.2). Amoebal mitoses appeared normal (see Section 3.2.1).

6.3.3 The terminal phenotype of RA614 cells

In all RA614 cells which began the amoebal-plasmodial transition, the films indicated that nuclei eventually vanished giving rise to an apparently anucleate cell. Nuclear disappearance occurred at several different stages of the amoebal-plasmodial transition but was most common in binucleate cells (see Section 6.3.4). A characteristic phenotype was observed in terminally developed RA614 cells. After nuclear disappearance, the developing cells began to accumulate contractile vacuoles (Fig. 6.2) and the cytoplasm began to churn around. Speeding up this protoplasmic movement by projection of the time-lapse films made the cells appear as if the contents were boiling. The "boiling" cells gradually became smaller as time went on and some eventually burst or ceased movement. Once the cells had begun to "boil", locomotion ceased. Developing cells with normal movement sometimes fused with the cells that had begun "boiling". Amoebae were ingested by the "boiling" cells if they came close enough.

6.3.4 Development of cells lacking visible nuclei

From the two films, it was possible to trace a total of 14

cells from their origin as amoebae to the mitosis at the end of the extended cell cycle. As in RA376, the extended cell cycle was 2.3 times the length of an amoebal cell cycle (Table 6.2) and the cells which entered the extended cell cycle arose from apparently normal amoebal divisions and were no different in area at birth to amoebae which completed an apparently normal amoebal cell cycle (Table 6.2; $p < 0.005$). As in RA376, growth continued during the extended cell cycle so that, at mitosis, the developing cell was, on average, twice the area of an amoeba at mitosis (Table 6.2; Fig. 6.3a). As in RA376, nuclear area increased during the extended cell cycle. Chromosomes were visible inside the nucleus in some uninucleate cells during the mitosis at the end of the extended cell cycle, but in other uninucleate cells, the nucleus disappeared following nucleolar disappearance at this mitosis.

At the end of the extended cell cycle, 10 of the 14 cells traced from their origin as amoebae, became binucleate by mitosis without cytokinesis (Fig. 6.4, cells 13B and 13A-1-1); the remaining 4 cells divided to yield 2 uninucleate daughter cells each (a total of 8 daughter cells; Fig. 6.4, cell 14B-1). Of the 10 cells which were observed to become binucleate, 7 could be traced further; in all of these, the nuclei vanished during interphase before the next mitosis and the cells began to exhibit the cytoplasmic movement characteristic of this strain. After nuclear disappearance, 2 of the 7 cells fused with large uninucleate cells; both the large uninucleate cells were more than 15 h old at fusion (Fig. 6.4, cells 3B, 14B-2). In one of these fusion cells, the large nucleus from the uninucleate

cell (Fig. 6.4, cell 14B-2) was observed to enter mitosis some hours after cell fusion; it was not possible to distinguish any other nuclei in the cell.

Of the 8 daughter cells that had been formed by mitosis and cytokinesis in large uninucleate cells, at the end of the extended cell cycle, 6 could be followed further. One of these 6 cells (Fig. 6.5, cell 2A-1-1) fused with a "boiling" cell. The other 5 daughter cells entered mitosis after a cell cycle which was slightly shorter than (Film 1; Table 6.2) or approximately equal to (Film 2; Table 6.2) the mean length of an amoebal cell cycle. In one of the 5 cells that entered mitosis, no nuclei reappeared and the cell began to exhibit vigorous cytoplasmic movement. The other 4 cells became binucleate by mitosis without cytokinesis (e.g. Fig. 6.4, cell 14B-1-1) and a variety of fates were observed. In one of the binucleate cells, the nuclei vanished in interphase (Fig. 6.5, cell 2A-1-2) and the cell began to "boil". In another cell, the two nuclei entered mitosis some hours after the cell had become binucleate but no nuclei reappeared (Fig. 6.5, cell 2A-2-2) and vigorous cytoplasmic movement was observed. The nuclei in the remaining 2 binucleate cells fused in interphase to produce 2 uninucleate, presumably diploid cells (Fig. 6.4, cell 14B-1-1; Fig. 6.5, cell 2A-2-1). These uninucleate diploid cells underwent mitosis without cytokinesis and gave rise to binucleate, presumably diploid cells before the nuclei vanished and the cells began to show the cytoplasmic movement characteristic of this strain.

Three cells, which had entered the field of view as binucleate cells, underwent mitosis without cytokinesis and

which the quadrinucleate cells formed appeared to be of the intranuclear plasmodial type (Fig. 6.3b). In one of the 3 quadrinucleate cells, the nuclei vanished and the cell began to "boil". In the other 2 quadrinucleate cells, the 4 nuclei entered mitosis synchronously a few hours after the cells had become quadrinucleate. In both cells, instead of 8 nuclei being formed at mitosis, only two giant nuclei were observed (Fig. 6.5, cell 3); these nuclei eventually disappeared and the cells began to show vigorous cytoplasmic movement.

Filming analysis elucidated the sequence of events leading to the formation of cells lacking visible nuclei but it could not reveal whether the nuclei in the apparently anucleate cells were intact nor whether they had fragmented or condensed. Filming analysis could not reveal how much DNA was present in the apparently anucleate cells. These questions were examined using flow cytometry and immunofluorescence microscopy.

6.4 DNA content of anucleate RA614 cells

Flow cytometry was used to determine whether the terminally-differentiated apparently anucleate cells contained any DNA. The amoebae were grown as normal but the developing cultures were grown for 4 or 8 days before being harvested and the cells stained as described in Section 2.9. The results are shown in Fig. 6.6. Phase contrast microscopy of the control amoebal culture showed that it contained only uninucleate cells and cysts. Flow cytometry (Fig. 6.6a) indicated that, for this control culture, the main haploid G2

peak (see Section 3.2.3) had a mode at about Channel 50 and contained about 90% of the cells. The secondary diploid G2 peak had its mode about Channel 100 and contained about 4% of the cells. The remainder of the cells had higher DNA contents and were assumed to correspond to polyploid and clumped cells.

Microscopic analysis, by phase contrast microscopy, of the cultures that had been developing for 4 days indicated that they contained 87.0% uninucleate cells and 6.5% binucleate cells; no nuclei were visible in the remaining 6.5% of cells. Flow cytometry of cells from the same culture (Fig. 6.6b) indicated that the major haploid G2 peak, with mode about Channel 50, contained about 50% of the cells. The second peak, uninucleate diploid G2 or binucleate haploid G2, had a mode at about Channel 100 and contained about 23% of the cells. The remaining cells had higher DNA contents and were presumably polyploid or clumped cells. The second peak contained 23% of the cells but only 6.5% binucleate cells were scored. This difference, together with the discrepancy between uninucleate cell numbers (87.0%) and the number of cells with haploid G2 DNA content (49.8%), suggested that some of the uninucleate cells were diploid, a suggestion which was supported by the filming analysis (see Section 6.3.4).

The developing culture grown for 8 days was shown, by microscopic analysis, to contain 65.0% uninucleate cells and 0.8% binucleate cells; no nuclei were visible in the remaining 33.2% of cells. Flow cytometry (Fig. 6.6c) indicated that the major haploid G2 peak in this culture had a mode at about Channel 50 and contained 53% of the cells. There were however, more uninucleate cells (65%) than there

were haploid cells (53%); suggesting that, as in the 4 day culture, some of the uninucleate cells were diploid. The second peak, with mode at about Channel 100 contained 27% of the cells; this peak was assumed to contain diploid uninucleate and haploid, binucleate cells in G2 phase. The remainder of the cells had higher DNA contents and were assumed to correspond to polyploid and clumped cells. Only 0.8% binucleate cells were scored by microscopic analysis but 27% of the cells had DNA content in the secondary peak suggesting, that some anucleate cells had DNA content equivalent to binucleate haploid cells. In addition there were cells with DNA contents equivalent to that of quadrinucleate and higher multinucleate cells, although no cells with more than 2 nuclei had been observed by microscopic analysis. This suggested that some anucleate cells had the DNA content of quadrinucleate cells, which agreed with the developmental stages at which the nuclei disappeared during filming.

Although flow cytometry indicated that the apparently anucleate cells did contain DNA, it was not possible to tell whether this was contained within nuclei or not.

6.5 Expression of phase-specific mRNAs

The method used to examine the expression of phase specific genes was the same as that used for RA612 (see Section 5.5); a summary of this work has been published (Anderson *et al.*, 1987). RNA was isolated from RA614 amoebae, set up at 1×10^5 cells / plate on LIA and incubated at 28.5°C for 3 days. Other cultures were set up at the same

density but on DSDM agar and were grown at 22°C for 3 days, to allow development to begin, before being harvested. The developing cultures contained some cells with more than one nucleus or with no visible nuclei. The RNA from these two populations was Northern blotted, together with CL plasmodial RNA as a control, and then probed with phase-specific cDNA clones. The results of these experiments are shown in Fig. 6.7.

Sweeney *et al.* (1987) showed that, in CL, the cDNA clone P210 hybridised to RNA from all cell types; this pattern of expression was also found in RA614 (Fig. 6.7). Clone P325 hybridised to RNA from the RA614 culture which had contained developing cells (Fig. 6.7, E) but not to RNA from RA614 amoebae (Fig. 6.7, A); this expression pattern was the same as that for CL (Sweeney *et al.*, 1987). The expression patterns of P46 and A195 in RA614 differed from those found in CL (Sweeney *et al.*, 1987). In CL (Sweeney *et al.*, 1987), P46 showed the same expression pattern as P325 but in RA614, this clone did not hybridise to RNA from either amoebae or developing cells. The cDNA clone A195 hybridised to RNA from both RA614 amoebae and RA614 developing cells (see Discussion). This is in contrast to the situation in CL where A195 only hybridised to RNA from amoebae and not to RNA from uninucleate developing cells or any later developmental stages. It therefore appears that the expression of some genes is developmentally regulated as normal in RA614 but the expression of other genes may not be correctly regulated.

6.6 Nuclear sizes and numbers during development in RA376 and RA614

The flow cytometry results had suggested that many of the RA614 cells which appeared anucleate by phase contrast microscopy did contain DNA but it was not possible to determine whether the DNA was contained in intact or fragmented nuclei. To investigate this question, cells of both RA376 and RA614 were set up for fluorescence microscopy as indicated in Section 2.10. RA376 was included in this analysis because it was the strain from which RA614 was derived and, thus, any differences between them may be related to the mutation carried by RA614. After 3 days growth, a sample of the unfixed cells of each strain was assayed for the number of nuclei within each cell, using thin agar slides set up as described in Section 2.7. The remaining cells were fixed as described in Section 2.10 and the number of nuclei in a sample of cells was counted using the fluorescence microscope to look at the DNA-specific stain DAPI. The results are shown in Table 6.3.

Phase contrast microscopic analysis of RA376 (Table 6.3, column 1) indicated that most of the cells were uninucleate, although there were a few binucleate cells and cells with more than 4 nuclei; only 1.7% of the cells appeared to be anucleate. When the nuclei of the stained cells were examined, using fluorescence microscopy, (Table 6.3, column 2), the distribution of cell types did not differ greatly from that counted by phase contrast microscopy; however, no anucleate cells were observed (a few anucleate cells were observed in later experiments; see Section 6.7). Some of the

cells (3%) had condensed nuclei; condensed nuclei were defined as being less than half the size of normal nuclei and without nucleoli (Fig. 6.8).

Phase-contrast microscopic analysis of the RA614 sample (Table 6.3, column 3) indicated that 78% of the cells were uninucleate. The second largest class (15.6%) contained the anucleate cells. Examination of the nuclei of the stained RA614 sample using fluorescence microscopy (Table 6.3, column 4; Fig. 6.8) showed that the number of uninucleate, binucleate, quadrinucleate and cells with more than 4 nuclei was similar in the two samples but only 0.6% of the cells were anucleate; 9.6% of the cells had condensed nuclei (Fig. 6.8). The presence, in both strains, of cells with condensed nuclei and the absence of large numbers of anucleate cells, suggested that the cells which appeared anucleate by phase-contrast microscopy contained condensed nuclei.

During the fluorescence microscopy studies of RA614, two cells were observed in which there were no nuclei; both were about the same size as an octanucleate cell. Although these cells did not contain intact nuclei, both contained large numbers of what appeared to be condensed metaphase chromosomes; one example of such a cell is shown in Fig. 6.9c. Possible ways in which these cells might develop are discussed in Section 6.8.2.

6.7 Microtubule organisation in interphase cells during development in RA376 and RA614

Extensive studies of RA614 (Lilianna Solnica-Krezel, personal communication) suggested that many (up to 70%) of

the mitotic cells in developing cultures of RA614 contained abnormal spindles; very few abnormal mitotic spindles were observed in cultures of RA614 vegetative amoebae. However, interphase microtubule organisation was not examined nor was microtubule organisation in RA614 compared with that in RA376. Therefore, microtubule organisation in RA614 and RA376 was examined and compared. Cells from both strains were stained for anti-tubulin immunofluorescence as described in Section 2.10 using the method for fixing cells on agar plates. Interphase cells of each strain were scored for number of MTOCs. Cells with more than 4 nuclei were excluded from the count because of the large number of fusions between multinucleate cells in both strains. Mitotic cells from both strains were also examined (see Section 6.7.2).

6.7.1 Interphase RA376 cells with normal nuclei

The results of this study are shown in Table 6.4a. Most RA376 uninucleate cells possessed one MTOC (Fig. 6.10c,d) although a few cells had either 2 MTOCs or no MTOC (Table 6.4a). There were more binucleate cells with 2 MTOCs (Fig. 6.10i,j) than there were uninucleate cells with 2 MTOCs, suggesting that some uninucleate cells had duplicated their MTOC at the mitosis by which they became binucleate. There were more binucleate cells with no MTOC than there were uninucleate cells with no MTOC (Table 6.4). The RA376 quadrinucleate cells scored had between 0 and 4 MTOCs; the cells were almost evenly distributed between these classes (Table 6.4a). RA376 microplasmodia with more than 4 nuclei showed a few scattered microtubules and MTOCs rather than the ordered array seen in cells with fewer nuclei (Fig. 6.9a,b).

All uninucleate, binucleate and quadrinucleate cells examined possessed microtubules although these were unorganised in the absence of a MTOC (not illustrated).

6.7.2 Interphase cells of RA614 with normal nuclei

The results of this study are shown in Table 6.4b. Most RA614 uninucleate cells possessed 1 MTOC (Fig. 6.10 c,d) although some possessed either 0 or 2 MTOCs. There were more RA614 uninucleate cells with no MTOC (Table 6.4b) than there were RA376 uninucleate cells with no MTOC (Table 6.4a). Unlike the situation in RA376, there were almost equal numbers of RA614 uninucleate cells with no MTOC and RA614 binucleate cells with no MTOC (Table 6.4). These differences may be related to the conclusion, from both filming analysis and flow cytometry, that some of the RA614 uninucleate cells are diploid.

There were more RA614 binucleate cells with 2 MTOCs (Fig. 6.10i,j) than there were uninucleate cells with 2 MTOCs. As in RA376, the quadrinucleate cells were almost equally distributed between cells with 0,1,2 or more MTOCs. All RA614 cells with 4 or fewer nuclei possessed microtubules although these were unorganised in the absence of a MTOC. Very few RA614 cells with more than 4 nuclei were observed; the microtubule organisation in these cells was similar to that of RA376 microplasmodia. It therefore appeared that the alterations in microtubule organisation that occurred during development in RA614 were very similar to those observed in RA376.

6.7.3 RA376 and RA614 interphase cells with no nuclei or with condensed nuclei

The results of these studies are given in Tables 6.4a and 6.4b. In both RA376 and RA614, anucleate cells (as defined by DAPI) were only present in very small numbers although more anucleate cells (as defined by phase contrast microscopy) were present in RA614 than in RA376 (Table 6.3). The anucleate RA376 and RA614 cells all contained microtubules which, in some cells, radiated from an MTOC; no anucleate cells (defined by DAPI) were observed to have more than one MTOC (Tables 6.4a,b). In many of the anucleate cells from both strains, the microtubules were 2 - 3 times as thick as normal microtubules and fewer in number (Fig. 6.10a,b) although in other cells the microtubules appeared normal in number and thickness.

Most of the cells with a single condensed nucleus, from both strains, had a single MTOC but there were more cells with no MTOC and fewer cells with 2 MTOCs than were observed for normal uninucleate cells of the same strain (Tables 6.4a,b). Where 2 condensed nuclei were present, the percentages of cells with 0,1 or 2 MTOCs were very similar to those observed for cells with one condensed nucleus; most of the cells had one MTOC but a significant proportion possessed no MTOC (Tables 6.4a,b). Not enough cells with 4 condensed nuclei were observed, from either strain, to give a significant count; these cells are not included in Table 6.4. As in cells with normal nuclei, all cells contained microtubules although these were unorganised in the absence of a MTOC (not illustrated). Many of the cells with condensed nuclei, from both strains, contained thick cable like

microtubules (Fig. 6.10 e,f and g,h) similar to those observed in the anucleate cells (defined by DAPI); other cells with condensed nuclei contained an apparently normal microtubule array (not illustrated).

6.8 Microtubule organisation at mitosis in RA376 and RA614.

Immunofluorescence microscopy of mitosis in RA614 suggested that many of the mitotic spindles were abnormal (Lilianna Solnica-Krezel, personal communication). In order to confirm this observation, the mitotic spindle was examined in cells of RA614 and RA376.

The various types of spindle were classified as normal or abnormal according to their morphology. The following 3 subgroups were classified as normal: 1) "Normal amoebal" mitotic spindles had distinct astral microtubules. 2) "Normal plasmodial" mitotic spindles did not possess astral microtubules (Fig. 6.11a,b). 3) Spindles which appeared normal but which could not be fitted into either of the above groups, with certainty, were classed as "normal undefined". There were 2 subgroups of abnormal mitotic spindle: 1) "Star spindles" in which all the microtubules radiated from a single point (Fig. 6.11e,f). 2) All other mitotic spindles which were not normal were placed in the remaining class of abnormal spindles; there were a wide variety of types in this subgroup.

Filming analysis had indicated that uninucleate cells entering mitosis at the end of the extended cell cycle were twice the size of amoebae at mitosis and had larger nuclei (Table 6.2). By estimating the size of mitotic uninucleate

cells which had been stained for immunofluorescence, it was possible to separate them into 2 groups; small mitotic cells, which were assumed to be amoebae, and large mitotic cells, with large nuclei which were assumed to be cells which had completed the extended cell cycle. These two classes of mitotic uninucleate cell have been treated separately in the following analysis. It should be noted that some mitotic uninucleate cells may have been wrongly classified by the size criterion. Mitosis was also examined in multinucleate cells. In addition to scoring the type of mitosis, the number of extranuclear MTOCs present was also scored.

6.8.1 Mitosis in amoebae

Examination of mitotic amoebae, of both RA614 and RA376, indicated that all normal mitoses were of the amoebal type with asters at the poles. There were between 0% and 10% abnormal mitoses, most of which were of the star type; the number of abnormal mitoses varied between experiments. These data were confirmed by Lilianna Solnica-Krezel (personal communication).

6.8.2 Mitosis in developing cells of RA376

The results of this study are shown in Table 6.5. In RA376, most of the mitoses in large uninucleate cells were normal and many of these appeared to be of the intranuclear plasmodial type (Table 6.5a). Many of the mitotic uninucleate cells undergoing plasmodial and undefined normal mitoses possessed 1 or 2 extranuclear MTOCs; very few of the uninucleate cells undergoing what appeared to be open amoebal mitosis possessed extranuclear MTOCs. The proportion of large

uninucleate cells undergoing amoebal mitosis (23.7%) was approximately equal to the increase in the number of cells with 2 MTOCs that was observed between interphase uninucleate (0.4%) and interphase binucleate cells (21.2%; Table 6.4a). There was a surprisingly high number of abnormal mitoses in the RA376 large uninucleate cells (30%); most of these were of the star type. Some of the uninucleate cells undergoing abnormal mitosis possessed extranuclear MTOCs (Fig. 6.11g,h).

Almost all the mitotic binucleate cells of RA376 appeared to be undergoing closed plasmodial mitosis (Table 6.5b and Fig. 6.12a,b); very few abnormal mitoses were observed in binucleate cells. Few mitotic binucleate cells possessed extranuclear MTOCs. Eight mitotic RA376 cells with more than 2 nuclei were observed; all were undergoing what appeared to be plasmodial mitosis and none possessed any extranuclear MTOCs. An example of a microplasmodium in synchronous plasmodial mitosis is shown in Fig. 6.12g,h.

6.8.3 Mitosis in developing cells of RA614

The results of this study are shown in Table 6.6. RA614 (Table 6.6a) showed a higher level of mitotic abnormalities in large uninucleate cells (50%) than was observed in RA376 (30%); most of the abnormal mitoses in uninucleate cells were of the star type (Fig. 6.11e,f) but other types of abnormality were also observed (Fig. 6.11i,j and k,l). Amongst the normal mitoses in uninucleate cells, there were roughly equal proportions of the three types (amoebal, plasmodial and undefined; Table 6.6a). As in RA376, the proportion of large uninucleate cells undergoing amoebal mitosis (11.8%) was approximately equal to the increase in

the number of cells with 2 MTOCs that was observed between interphase uninucleate (3.6%) and interphase binucleate cells (15.9%; Table 6.4b). Very few uninucleate cells undergoing amoebal mitosis possessed any extranuclear MTOCs (Table 6.6a).

Few mitotic binucleate cells were observed in the RA614 cultures; this fits well with the observations from filming that in most cells the nuclei disappeared at the binucleate stage. There were more RA614 binucleate cells in amoebal mitosis than there were RA376 binucleate cells in amoebal mitosis (Table 6.6b), although this difference may not be statistically significant since the samples were small. As in RA376, few mitotic binucleate cells possessed extranuclear MTOCs. In RA614, there were 3 mitotic binucleate cells in which the two nuclei were not of the same mitotic type (Unclassified in Table 6.6b); in all 3 cells, one nucleus was undergoing what appeared to be intranuclear plasmodial mitosis while the other nucleus was dividing abnormally. Only 5 mitotic cells with more than 2 nuclei were observed in RA614 cultures; no cells had more than 8 nuclei. Four of these 5 cells were undergoing what appeared to be plasmodial mitosis (Fig. 6.12c,d) while the other multinucleate cell appeared to be in amoebal mitosis.

6.9 DISCUSSION

6.9.1 Development of RA614

During the kinetics of anucleate cell formation in RA614 (see Section 6.2), binucleate cells appeared but no quadrinucleate cells were observed. Cells which lacked

visible nuclei under phase contrast microscopy appeared instead; these apparently anucleate cells represented the terminal stage of development in RA614. In analyses of this type (e.g. RA612; Table 5.2), quadrinucleate cells are detected shortly after the first binucleate cells were detected. The absence of quadrinucleate cells from the analysis of anucleate cell formation in RA614 suggested that development halted at an early stage in this mutant strain.

Filming analysis suggested that amoebal growth and the early stages of the amoebal-plasmodial transition were normal. An extended cell cycle, during which growth continued and nuclear area increased, was an integral part of development in RA614, as it had been in all other strains studied. The filming analysis indicated that development started to become abnormal at about the binucleate stage; this agreed with the results from the kinetic analysis. Although most developing RA614 cells became binucleate (10 cells) at the end of the long cell cycle, 4 cells divided into two uninucleate daughter cells instead. Some of these daughter cells became binucleate by mitosis without cytokinesis after a short cell cycle. The proportion of cells which divided at the end of the extended cell cycle was approximately twice the level observed for RA376 (see Table 3.5); however, given the small sample size, this difference may not be significant. During filming analysis, the nuclei in developing RA614 cells disappeared (as defined by phase contrast); at the time of nuclear disappearance all the affected cells had 2 or more nuclei. Nuclear disappearance occurred with or without nuclear fusion, and either at mitosis or in interphase. Once the nuclei had vanished, the cell

began to accumulate contractile vacuoles and the cytoplasm began to move vigorously. Plasmodial fusions and ingestion of amoebae both occurred during development in RA614, suggesting that the *npfL* mutation carried by this strain was not affecting these processes.

6.9.2 Anucleate cells and cells with condensed nuclei

The results from flow cytometry of RA614 suggested that the apparently anucleate cells formed during development did contain DNA. Examination of the nuclei in cells from developing cultures of RA614, using fluorescence microscopy, suggested that most of the cells which appeared anucleate under phase-contrast microscopy contained one or two condensed nuclei. However, flow cytometry indicated that cells which appeared anucleate by phase contrast microscopy, contained DNA equivalent to that in binucleate haploid or quadrinucleate haploid cells. The apparent contradiction between the fluorescence microscopy and flow cytometry results can be resolved if it is assumed that each of the condensed nuclei contained the diploid amount of DNA. One way in which condensed nuclei might have arisen from normal nuclei was for a cell with normal nuclei to undergo chromosome condensation at mitosis but to fail to undergo chromosome separation; if the chromosomes remained in a condensed form (giving the small, anucleolate nuclei) but the DNA duplicated as if mitosis had been completed normally, the observed nuclear appearance would arise.

Two cells were observed which contained many metaphase chromosomes; these chromosomes were not contained within nuclei but were scattered in the cytoplasm, suggesting that

the nuclear structure had broken down in these cells. If the nuclear structure in anucleate cells broke down, releasing DNA into the cytoplasm as intact chromosomes, then the cells containing chromosomes might represent anucleate cells which had reached mitosis and undergone chromosome condensation. Spindle formation would presumably not occur if the chromosomes were not contained within nuclei. Examination of the microtubules in anucleate cells and in cells with condensed nuclei showed that there appeared to be two different types; normal and cable-like. The data do not suggest an explanation for the presence of cable-like microtubules but they presumably arose from normal microtubules and might consist of bundles of normal microtubules.

6.9.3 Alterations in gene expression

The expression pattern of some of the phase-specific genes tested differed from that observed for CL (Sweeney, 1987; Sweeney *et al.*, 1987). Clones P210 and P325 (see Section 6.5) showed the same expression pattern in CL and RA614; clones P46 and A195 did not. Clone A195 detected transcripts in CL amoebae but not in CL developing cells. In contrast, A195 detected a transcript in both RNA samples from RA614; the detection of this gene product in developing cells of RA614 may, however, be due to contamination of the sample of developing cells with amoebae. Clones P46 and P315 detected transcripts in all developing CL cells but not in CL amoebae. Neither P46 nor P325 detected transcripts in RA614 amoebae. The detection of a transcript that hybridised to P325, in RA614 cells, indicated that developing cells of this strain

did express some plasmodium-specific genes. Clone P46 did not detect a transcript in RA614 developing cells suggesting that the gene product to which clone P46 hybridised was not present in RA614 cells. Further experiments are necessary to determine whether the absence of hybridisation between P46 and RNA from RA614 developing cells was an experimental artefact or a real alteration in the pattern of gene expression in RA614.

6.9.4 Microtubule organisation during development

The pattern of loss of MTOCs during plasmodium development in RA376 and RA614 cells with normal (not condensed) nuclei, was similar to that observed for apogamic development in CL (Blindt, 1987) and for sexual development (Chapter 4; Bailey *et al.*, 1989). In both strains, most uninucleate cells had one MTOC but as development proceeded more and more cells with no MTOC were present. In both RA376 and RA614, there were more binucleate cells with 2 MTOCs than there were uninucleate cells with 2 MTOCs suggesting that some uninucleate cells which became binucleate had apparently not lost the amoebal characteristic of duplicating the MTOC at mitosis. This suggestion was supported by the observation that some large uninucleate cells appeared to be undergoing amoebal mitosis. Very few large uninucleate cells undergoing amoebal mitosis possessed extranuclear MTOCs (6.4%, RA376; 8.3%, RA614; see Section 6.8), consistent with the idea that, in these cells, the amoebal MTOCs were forming the poles of the spindles. Most of the developing uninucleate cells in plasmodial mitosis possessed extranuclear MTOCs, consistent with the suggestion of Blindt (1987) that these were the

remains of the amoebal MTOC.

Even though no binucleate cells with 4 MTOCs had been observed in either strain, some quadrinucleate cells possessed 4 MTOCs; this suggested that some of the binucleate cells might have duplicated their MTOCs at the mitosis by which they became quadrinucleate. This suggestion was supported by the observation that some binucleate cells, of both strains, appeared to be undergoing amoebal mitosis. However, the possibility that these quadrinucleate cells were the result of fusion cannot be ruled out.

The pattern of alterations in microtubule organisation, in RA614 cells with normal nuclei, was very similar to that observed for RA376, suggesting that the *npfL* mutation carried by RA614 was not directly affecting microtubule organisation. Work in progress (Lilianna Solnica-Krezel, personal communication) suggests that, in RA614 developing uninucleate cells as in those of CL, the α 3-tubulin isotype, which is found in amoebae and flagellates only, is not present and the plasmodium-specific β 2-tubulin isotype is present (Solnica-Krezel *et al.*, 1988; Diggins-Gillicinski *et al.*, 1989).

6.9.5 Abnormal mitotic figures

About 50% of the mitotic figures observed by immunofluorescence microscopy in RA614 developing uninucleate cells, and 30% of those in RA376 developing uninucleate cells were abnormal; however, very few mitotic cells became abnormal during the films of either strain. This discrepancy can be explained by assuming that mitosis could be completed in some cells with abnormal spindles.

Most of the abnormal spindles were of the star type which appeared to be unipolar with the microtubules radiating outwards. How could the star type of abnormal mitotic spindle arise? At amoebal mitosis, the MTOC duplicates and divides to form the spindle poles; the nuclear membrane also breaks down (Havercroft and Gull, 1983). If the MTOC failed to duplicate at amoebal mitosis, a unipolar spindle from which microtubules radiated in all directions would be formed; this would be the star mitosis. Could a star mitosis arise if mitosis was of the intranuclear plasmodial type? If the intranuclear MTOC failed to duplicate at plasmodial mitosis, a unipolar spindle would be formed. However, the spindle microtubules would not be able to radiate outwards because they would be confined within the nuclear membrane. It therefore seems more likely that the star mitoses were abnormal amoebal mitoses. This suggestion was supported by the observation that very few star mitoses had extranuclear MTOCs (8.4%, RA376; 18.2%, RA614). It would be interesting to know whether cells undergoing star mitosis could complete mitosis. It is possible that the star mitoses are a previously unrecognised stage in normal mitosis but there is no evidence to support this idea.

6.9.6 Conclusions and further experiments

Nuclear fusion, condensation of nuclei and abnormal mitoses all suggested that the *npfL* mutation was affecting the nuclei of the developing cells in RA614. The observation that some RA614 cells were anucleate suggested that the nuclear membrane was defective. The characteristic phenotype (i.e. "boiling") observed in the developing cells, may be a

consequence of the defect in the nuclei rather than a cause. The fact that nuclear fusion and nuclear disappearance occurred in interphase as well as at mitosis suggested that the mutation was not affecting mitosis directly; the abnormal mitoses observed may be a secondary consequence of the nuclear defect rather than the cause. There were 3 times as many cells with condensed nuclei in RA614 as in RA376, so this feature may be due to the mutation carried by RA614. It must be noted, however, that all the phenotypes of RA614 were observed, to a lesser extent in RA376 suggesting that, perhaps, the mutation present in RA614 is also found in RA376 but in a less extreme form. Alternatively, RA376 might have a defect in mitosis; RA614 would possess this lesion but would also have an additional unique mutation affecting (e.g.) nuclear structure.

It is known that the type of mitosis changes from open amoebal to closed plasmodial during plasmodium development. In most cells, this change probably occurs at the mitosis which gives rise to a binucleate cell (Blindt, 1987 and this work). One of the main differences between amoebal and plasmodial mitosis is whether or not the nuclear membrane remains intact during mitosis (Howard, 1932; Havercroft and Gull, 1983). The switch from breakdown of the nuclear membrane to non-breakdown could be caused by alterations in the nuclear membrane itself or by alterations in the enzymes required to disassemble the nuclear membrane at amoebal mitosis. An alteration in the structure of the nuclear membrane, caused by the *npfL* mutation, would give rise to most of the aspects of the RA614 phenotype, including nuclear fusion in interphase and the formation of condensed nuclei.

If the nuclear membrane alters as amoebae develop into plasmodia, the *npfL* mutation, which does not cause abnormalities in amoebae, may be affecting the switch from amoebal to plasmodial nuclear membrane organisation giving rise to cells with unstable nuclear membranes.

An alteration in the enzymes which cause nuclear envelope breakdown might result in the enzymes being switched on at mitosis or being switched on throughout the cell cycle. The presence of the enzymes at mitosis, might result in developing plasmodia which retained a tendency to break down the nuclear membrane at mitosis. In such cells, amoebal mitosis might continue, leading to spindle fusion. In RA614 although abnormal mitotic spindles were observed, abnormal events, such as nuclear fusion and disappearance, occurred in interphase as well as at mitosis. This idea, therefore, does not explain all the observed events.

If the enzymes which acted on the nuclear envelope were present at all stages of the cell cycle, rather than only at mitosis, the nuclear membranes would be unstable (at best) or absent (at worst). This scenario might give rise to the observed abnormalities in RA376 if the nuclear envelope was present but unstable.

The observations made during this work suggest that the mutation in RA614 is affecting the nuclear membrane and causing it to be unstable. The observed phenotype of RA614 does not exclude the possibility that the *npfL* mutation affects the amoebal nuclear membrane as well as the plasmodial one; if this is correct, RA614 is not a true developmental mutant. Even if the nuclear membrane was defective, since amoebae are uninucleate, nuclear fusion

would not occur and amoebal growth would appear normal.

Further experiments are needed in order to decide which, if any, of these possible explanations is correct.

Examination of nuclear structure in RA614 cells using electron microscopy and immunofluorescence microscopy, might reveal more about nuclear structure and the effects of the *npfL* mutation on developmentally regulated alterations in its structure. More detailed analysis of the pattern of gene expression during development, using phase-specific cDNA clones, might identify gene transcripts whose presence or absence is associated with the phenotype of RA614.

Table 6.1

% of different cell types during development of RA614

Hours after inoculation	% of cell type in population		
	uninucleate	binucleate	anucleate
0	100		
24	100		
29	100		
36	100		
48	100		
54	100		
60	99.1	0.9	
72	100		
78	95.9	3.1	0.9
84	83.1	3.9	12.9
96	74.8	5.4	19.7
102	71.7	4.8	23.5
108	66.9	5.3	27.8
121	68.0	4.1	27.9
168	60.8	2.9	36.3

These numbers are plotted on Fig. 6.1.

Table 6.2

Summary of data from time-lapse cinematography of RA614

Measurement	Film 1	Film 2
Amoebal IMT	13.1 $\pm$ 1.8 h (38)	10.3 $\pm$ 3.3 h (20)
Uninucleate to binucleate IMT	32.8 $\pm$ 1.4 h (3)	24.5 $\pm$ 2.7 h (7)
IMT for cells that divided after long cell cycle	29.0 h (1)	28.6 $\pm$ 5.4 h (3)
Time to next M for daughters of cells that divided after long cell cycle	11.1 h (1)	10.7 $\pm$ 1.3 h (4)

Amoebal area at birth	14.9 $\pm$ 3.0 (38)	-
Amoebal area at mitosis	34.0 $\pm$ 6.0 (38)	-
Area at birth of cells which entered long cell cycle	16.8 $\pm$ 2.7 (4)	-
Cell area at end of long cell cycle	71.3 $\pm$ 20.3 (4)	-

Film 1: The slide was inoculated with cysts.

Film 2: The slide was inoculated with cells from a culture which contained developing cells.

Area measurements are given in arbitrary units and times are given in hours.

Table 6.3

Number and relative sizes of nuclei during development in RA376 and RA614

Cell type	RA376			RA614	
	% of cell type in population scored by phase	DAPI		% of cell type in population scored by phase	DAPI
Uninucleate	88.1	85.6	!	78.2	77.8
Binucleate	7.8	4.0	!	5.5	6.1
Quadrinucleate	0.9	2.3	!	0.0	0.9
More than 4 nuclei	0.5	1.0	!	0.0	0.9
Mitotic	0.9	4.0	!	0.8	3.0
1 condensed nucleus	0.0	1.7	!	0.0	3.3
2 condensed nuclei	0.0	1.3	!	0.0	6.1
4 condensed nuclei	0.0	0.0	!	0.0	1.2
no nuclei	1.7	0.0	!	15.6	0.6
Total cells counted	632	299	!	385	329

Two samples of cells from each strain were scored. One sample, of living cells, was scored using phase contrast microscopy (columns 1 & 3). The other sample, of fixed cells, was scored using fluorescence microscopy (columns 2 & 4). Since the % are rounded to the nearest 0.1%, the total for each experiment may not add up to 100%.

Table 6.4

a) Number of MTOCs in interphase RA376 cells

No. of nuclei	0 MTOC	1 MTOC	% of cells with			Total cells
			2 MTOC	3 MTOC	4 MTOC	
1	3.1	96.5	0.4	0.0	0.0	260
2	12.8	63.5	21.2	2.6	0.0	156
4	14.3	35.7	21.4	10.7	17.9	28
0	57.9	42.1	0.0	0.0	0.0	19
1 cond	36.4	60.6	3.0	0.0	0.0	33
2 cond	34.4	65.6	0.0	0.0	0.0	32

b) Number of MTOCs in interphase RA614 cells

No. of nuclei	0 MTOC	1 MTOC	% of cells with		Total cells
			2 MTOC	more MTOCs	
1	10.6	85.8	3.6	0.0	282
2	13.6	70.5	15.9	0.0	44
4	28.6	14.2	28.6	28.6	14
0	93.8	6.2	0.0	0.0	16
1 cond	44.7	54.1	1.2	0.0	85
2 cond	32.7	64.3	3.0	0.0	101

Since all % are rounded to the nearest 0.1%, the total for each class may not add up to 100%. Cells with condensed nuclei (cond) are separated from cells with the same number of noncondensed nuclei. The number of cells in each class does not represent the proportion of that cell type in the population. The number of nuclei was defined by examining DAPI staining. The number of MTOCs was defined by immunofluorescence microscopy of the β -tubulin antibody.

Table 6.5**Types of mitosis and numbers of extranuclear MTOCs in RA376****a) Mitotic large uninucleate cells**

Type of mitosis	% cells with extranuclear MTOCs			Total cells	% of total mitoses
	0 MTOC	1 MTOC	2 MTOCs		
Amoebal	93.5	3.2	3.2	31	23.7
Plasmodial	39.6	56.3	4.2	48	36.6
Unidentified	58.3	41.7	0.0	12	9.2
Star	91.7	5.6	2.8	36	27.5
Other abnormality	100.0	0.0	0.0	4	3.0
				131	

b) Mitotic binucleate cells

Type of mitosis	% cells with extranuclear MTOCs			Total cells
	0 MTOC	1 MTOC	2 MTOCs	
Amoebal	100.0	0.0	0.0	1
Plasmodial	68.8	31.2	0.0	16
Unidentified	100.0	0.0	0.0	4
Star	100.0	0.0	0.0	1
Other abnormality	100.0	0.0	0.0	1
				23

Since the % are rounded to the nearest 0.1%, the totals may not add up to 100%.

Table 6.6

Types of mitosis and numbers of extranuclear MTOCs in RA614**a) Mitotic large uninucleate cells**

Type of mitosis	% cells with extranuclear MTOCs			Total cells	% of total mitoses
	0 MTOC	1 MTOC	2 MTOCs		
Amoebal	91.7	8.3	0.0	12	11.8
Plasmodial	55.0	40.0	5.0	20	19.6
Unidentified	78.9	21.1	0.0	19	18.6
Star	84.1	18.2	0.0	44	43.1
Other abnormality	100.0	0.0	0.0	7	6.9
				102	

b) Mitotic binucleate cells

Type of mitosis	% cells with extranuclear MTOCs			Total cells
	0 MTOC	1 MTOC	2 MTOCs	
Amoebal	100.0	0.0	0.0	2
Plasmodial	100.0	0.0	0.0	3
Unidentified	0.0	100.0	0.0	2
Unclassified	100.0	0.0	0.0	3
				10

Since the % are rounded to the nearest 0.1%, the totals may not add up to 100%.

Figure 6.1

Kinetics of binucleate and anucleate cell formation in cultures inoculated with RA614 cysts at time zero. Two cultures were assayed at intervals for amoebae (x), binucleate cells (o) and anucleate cells (●). The graph is plotted on log paper so only the counts that are over 1% appear on the graph.

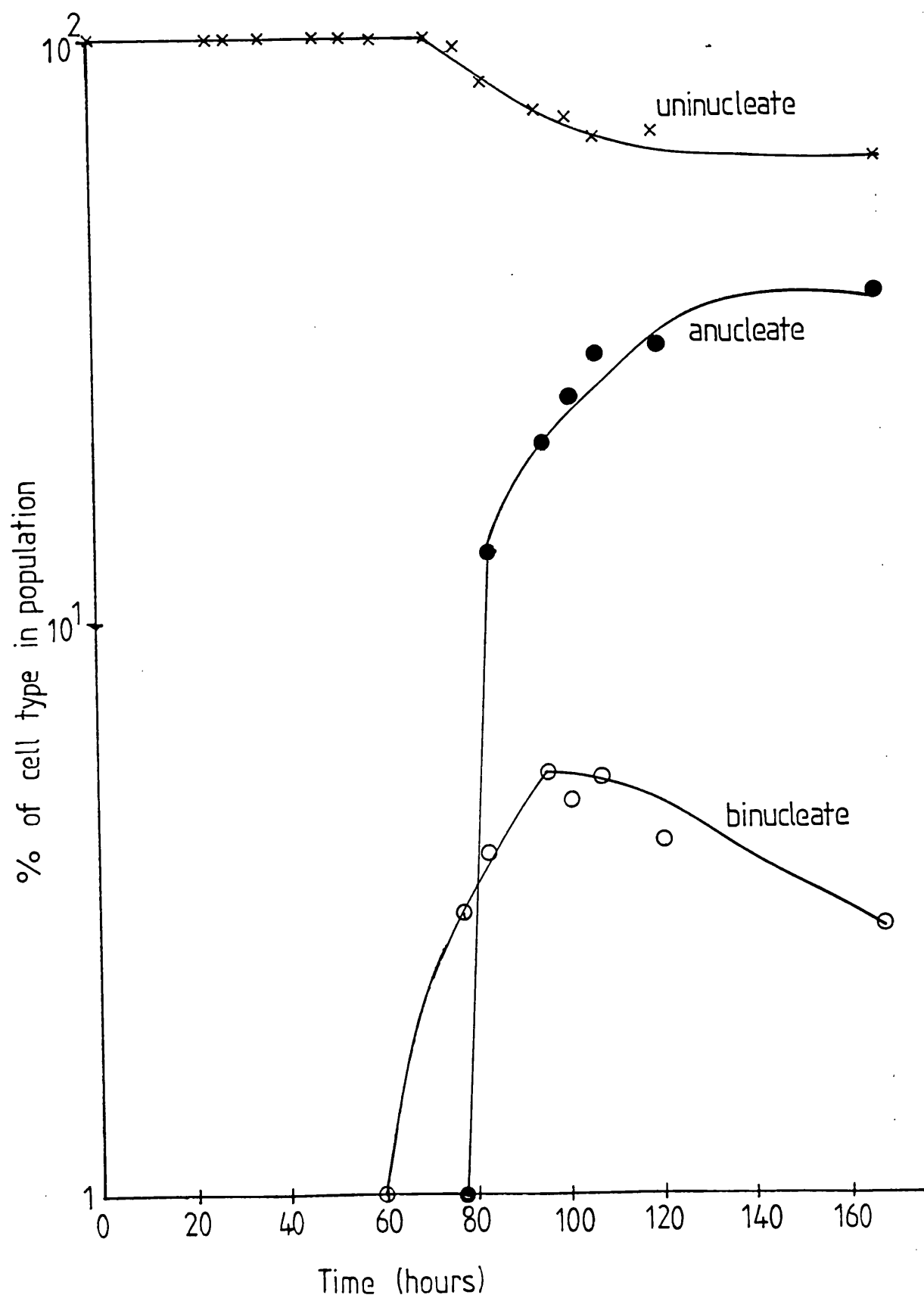
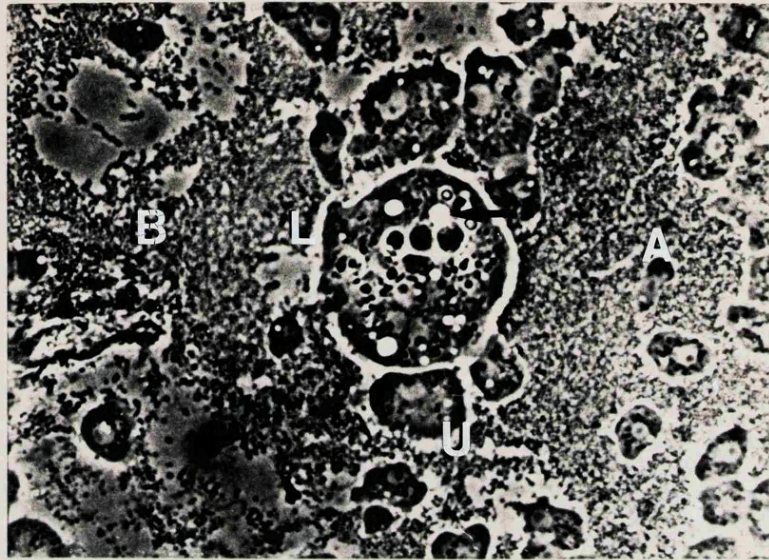


Figure 6.2

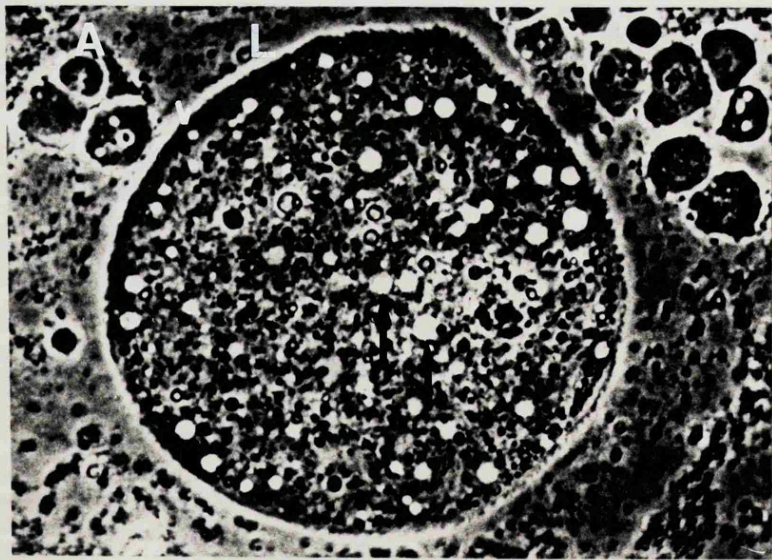
Terminally differentiated RA614 cells on a bacterial lawn.

Bar = 10 μ m. $\longrightarrow$ Contractile vacuole.

a) The large uninucleate cell (U) is much smaller than the apparently anucleate cell (L). Contractile vacuoles (bright spots) and ingested amoebae in food vacuoles are visible in the large cell (L). b) A large cell (L) in which no nuclei are visible. There are many contractile vacuoles; compare the size of L with the amoeba (A) in the same photograph.



a



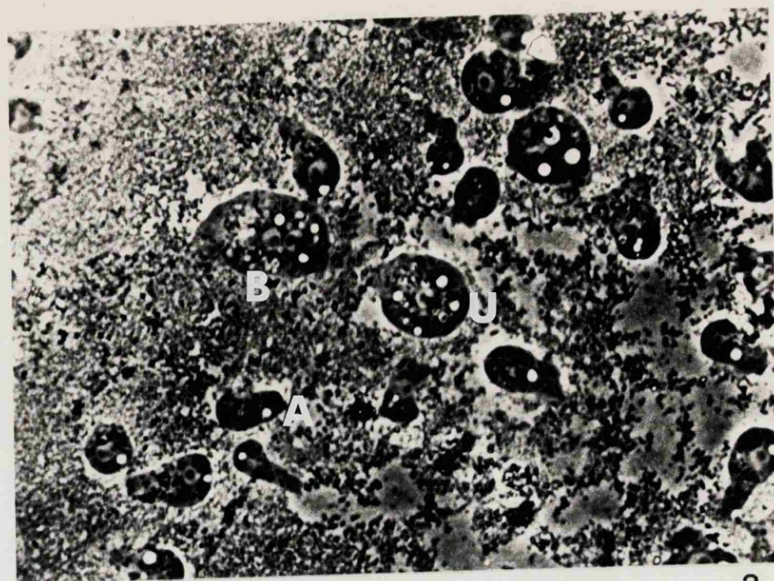
b

I

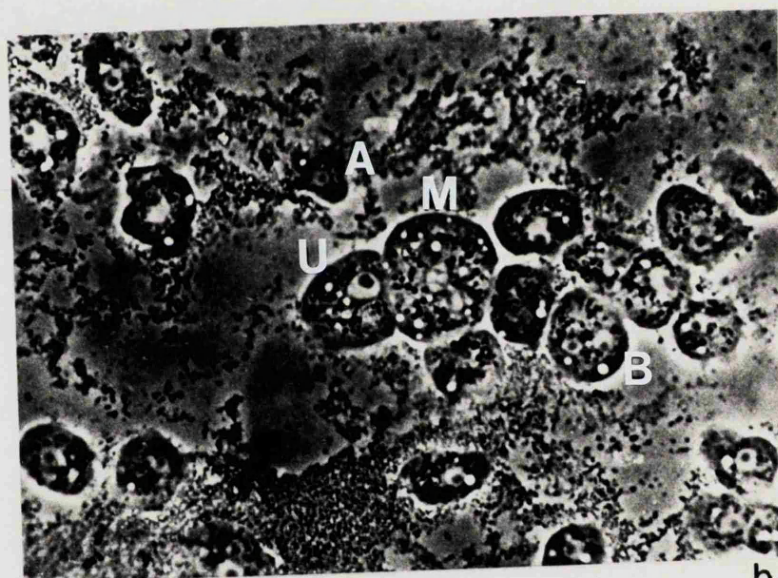
Figure 6.3

Developing RA614 cells on a bacterial lawn. Bar = 10 μ m.

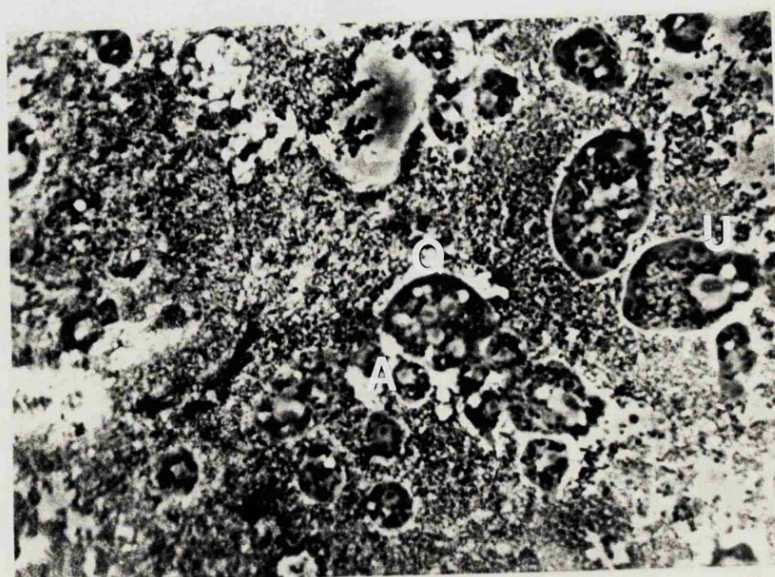
a) Amoeba (A), large uninucleate cell (U) and binucleate cell (B) to show relative areas. b) As in (a) plus a binucleate cell (M) in what appears to be closed mitosis; two sets of chromosomes are visible inside each nucleus of this cell. c) Amoeba (A) and quadrinucleate cell (Q); the nuclei of the quadrinucleate cell are the same size as the nucleus of the amoeba. The single nucleus in the large cell (U) is much larger than those of the quadrinucleate cell.



a



b



c

Figure 6.4

Representative RA614 cell pedigrees.

—o— Time of nucleolar disappearance preceding mitosis

⊙ uninucleate cell

⊙ binucleate cell

○ anucleate cell

F - cell fused with another multinucleate cell

→ cell left field

NF - nuclear fusion

B - cell showed vigorous cytoplasmic movement

Filming began at 0 minutes.

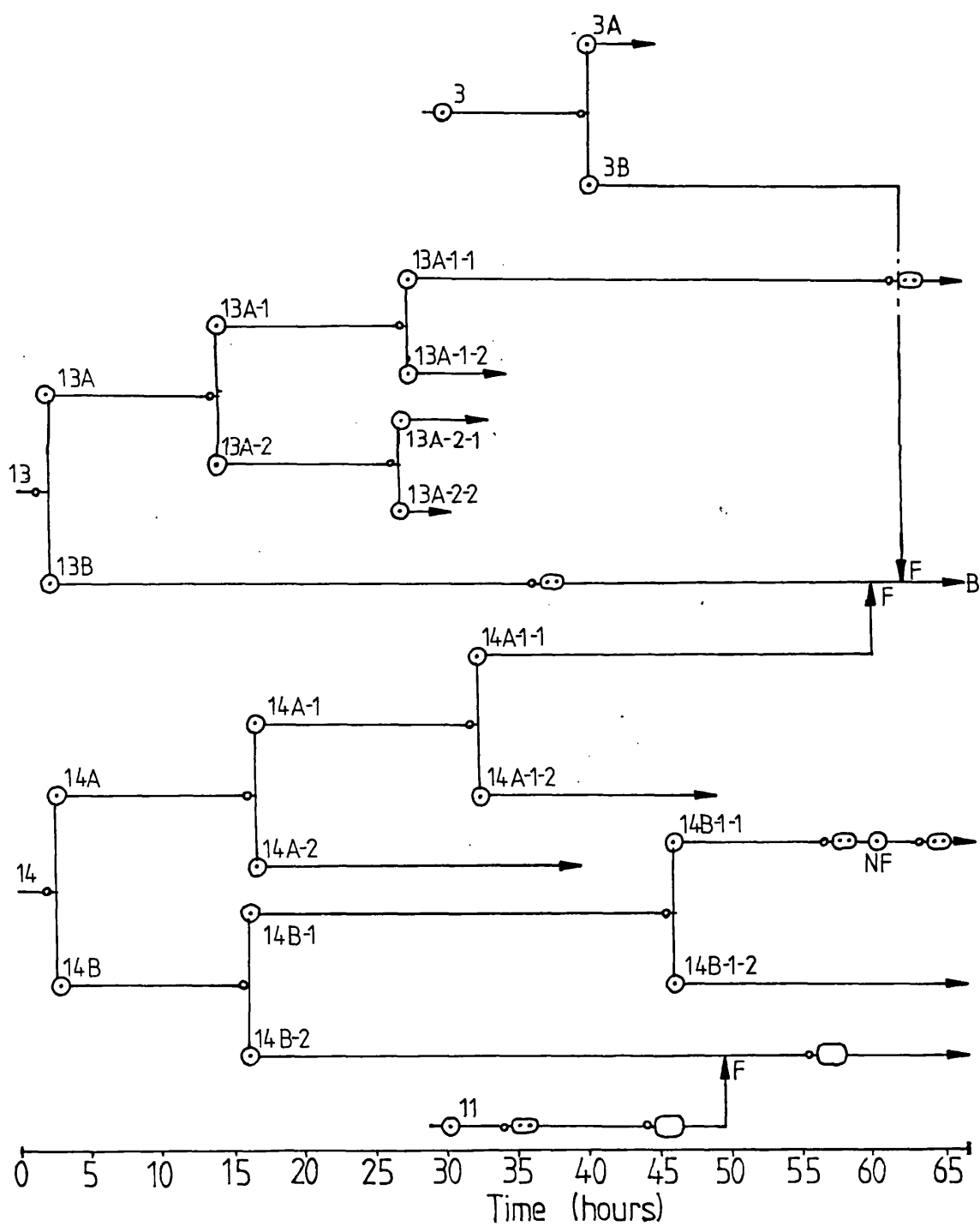


Figure 6.5

Another representative RA614 pedigree.

 - quadrinucleate cell

NV - nuclei vanish

All other symbols are as shown for Fig. 6.4. Filming began at
0 minutes.

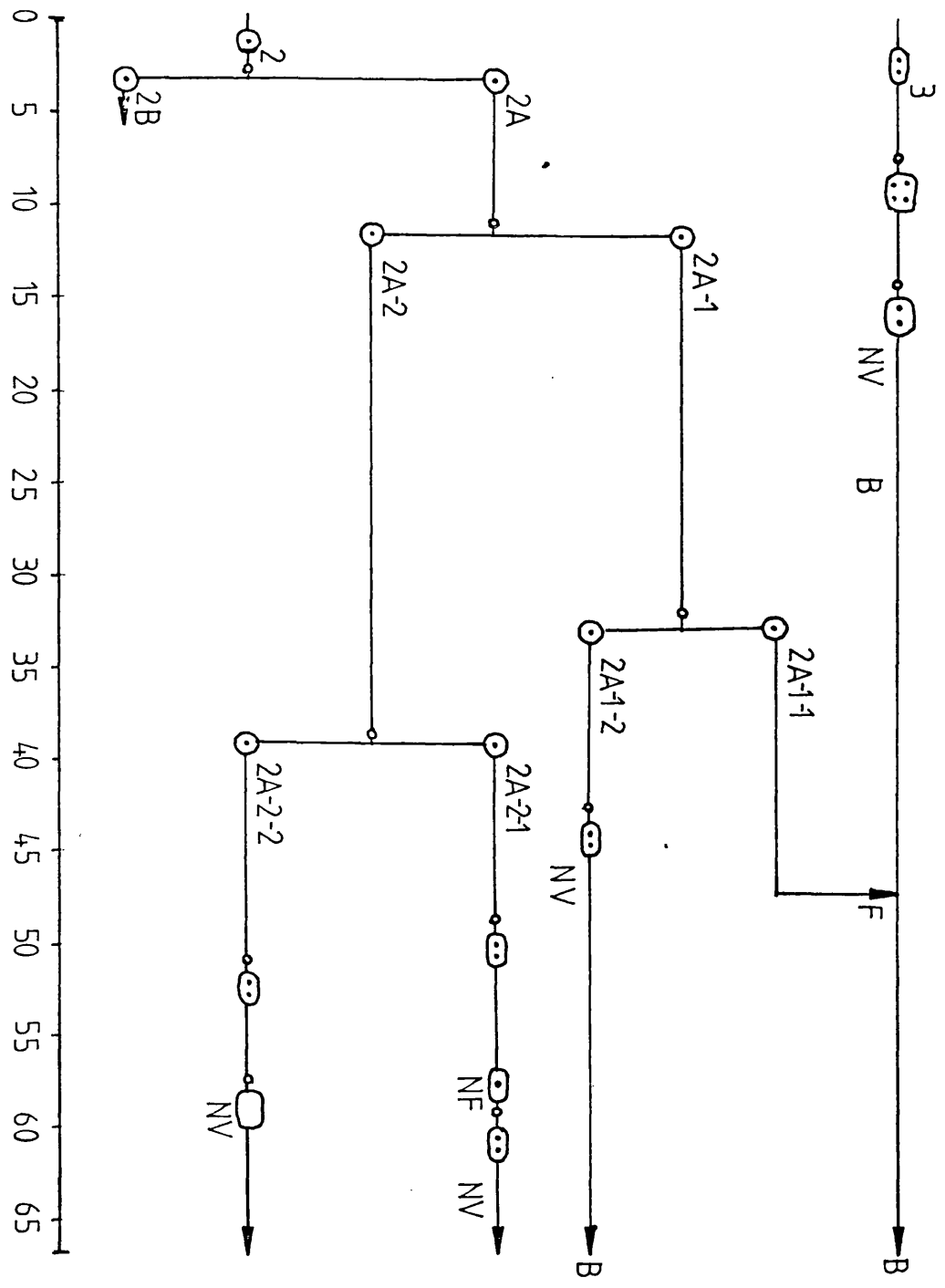
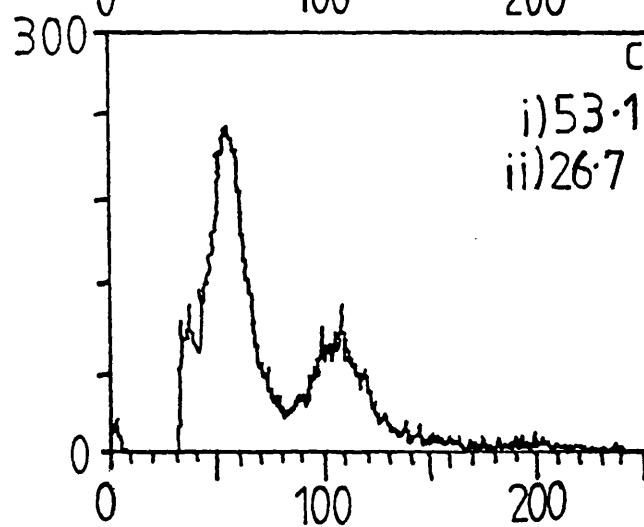
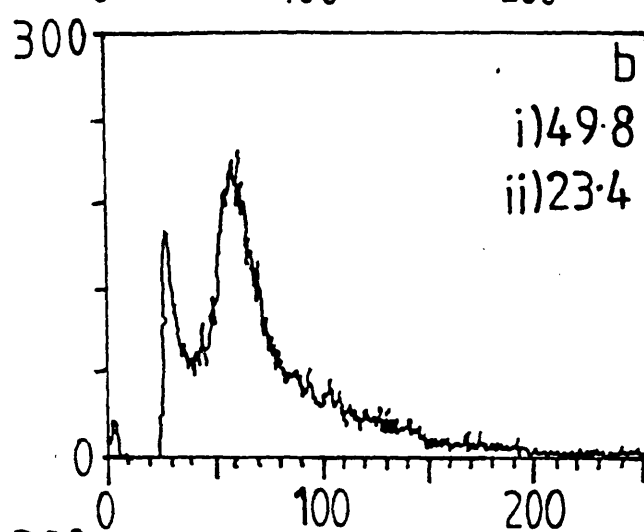
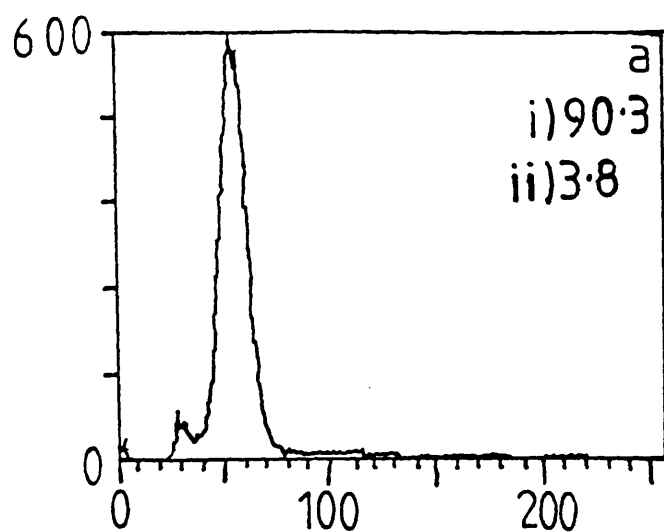


Figure 6.6

Distribution of cellular DNA contents in RA614 estimated by flow cytometry.

a) Vegetative amoebae at 28.5°C. b) Four day developing culture at 22°C. c) Eight day developing culture at 22°C. Channel number is proportional to DNA content (see Section 2.9). 10^4 cells were counted for each sample. On each panel the percentages of cells (i) in the main peak with mode at or near Channel 50, and (ii) the secondary peak with mode at or near Channel 100, are indicated.



Channel number

Number of cells

Figure 6.7

Expression of phase-specific mRNAs in RA614

RNA from amoebae (A) and from a developing culture (E) were extracted and Northern blotted. RNA from from CL plasmodia (CP) was included as a control. The northern blots were then probed with the indicated clones. See Sweeney (1987) and Sweeney *et al.* (1987) for experimental details.

RA614

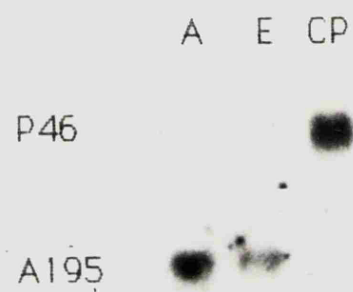
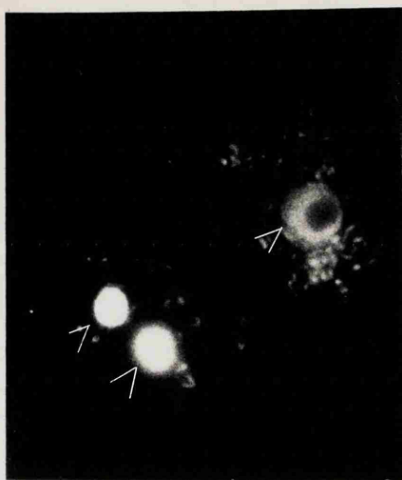


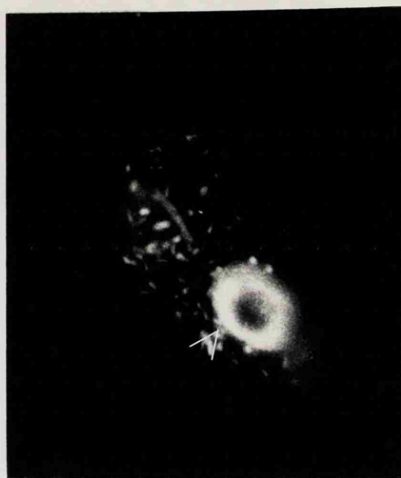
Figure 6.8

Nuclear size in RA614 cells stained with DAPI to show DNA.

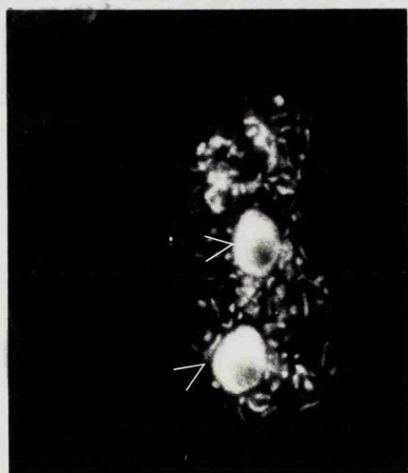
The dots in the cytoplasm are a mixture of mitochondria and ingested bacteria. Bar = $3\mu\text{m}$. > - Nucleus. (a) Amoeba with a normal nucleus and a binucleate cell with condensed nuclei. (b) A large uninucleate cell with a normal nucleus. (c) A binucleate cell with normal nuclei. (d) A quadrinucleate cell with condensed nuclei.



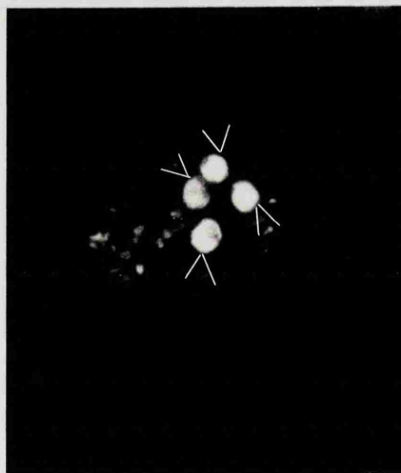
a



b



c



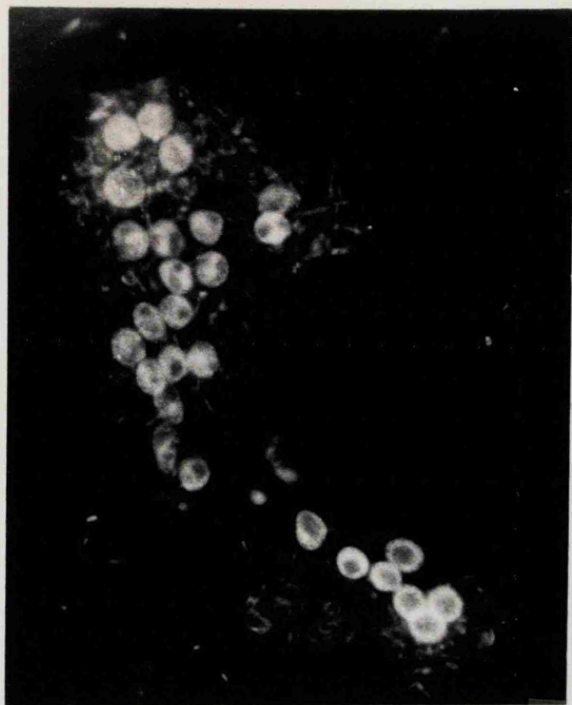
d



Figure 6.9

a & b) Interphase RA376 microplasmodium double-stained with β -tubulin antibody to show microtubules (b, >) and with DAPI to show DNA (a). The dots in the cytoplasm (a) are a mixture of mitochondria and ingested bacteria. Bar = 5 μ m.

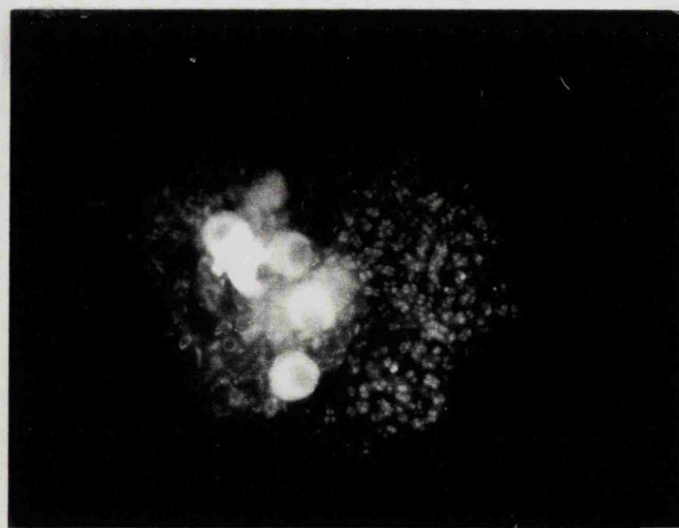
(c) Two RA614 cells stained with DAPI to show DNA. The dots in the cytoplasm are a mixture of mitochondria and ingested bacteria. One cell, on the left, has 6 visible nuclei while the cell in the right, which is almost the same size as the cell on the left, does not contain intact nuclei but possesses many chromosomes. Bar = 3 μ m



a



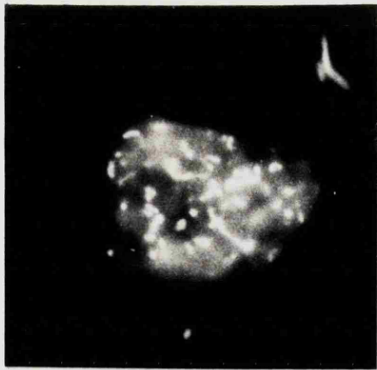
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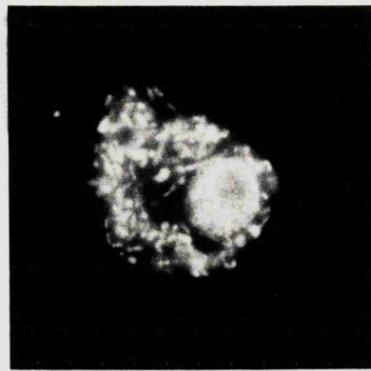
c

Figure 6.10

Interphase cells of RA376 and RA614 double-stained with β -tubulin antibody to show microtubules (b,d,f,h,j)) and with DAPI to show DNA (a,c,e,g,i). The dots in the cytoplasm (a,c,e,g,i) are a mixture of mitochondria and ingested bacteria. Bar = $3\mu\text{m}$. > - MTOC. (a & b) RA376 anucleate cell with cable-like microtubules and no MTOC. (c & d) RA376 uninucleate cell with a normal nucleus and normal microtubules radiating from a single MTOC. (e & f) RA614 binucleate cell with condensed nuclei and cable-like microtubules radiating from a single MTOC. (g & h) RA614 cell with 4 condensed nuclei and cable-like microtubules radiating from 2 MTOCs. (i & j) RA614 cells. The uninucleate cell has microtubules radiating from a single MTOC while the binucleate cell has microtubules radiating from 2 MTOCs.



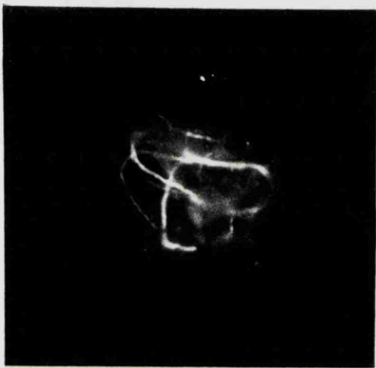
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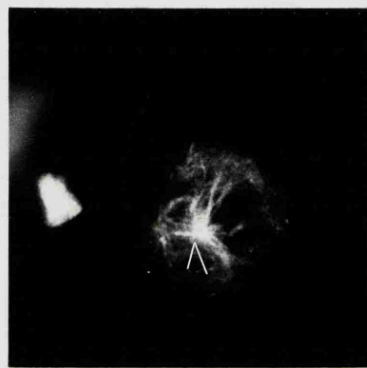
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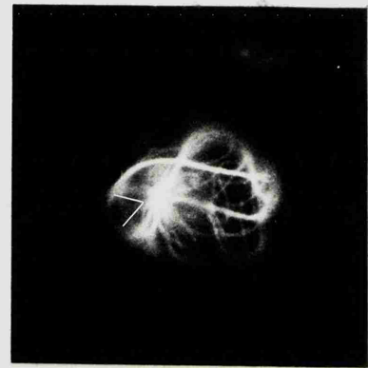
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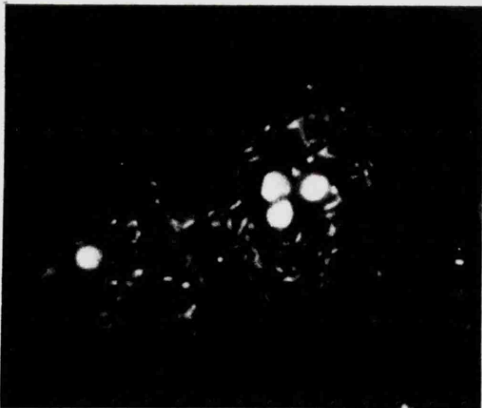
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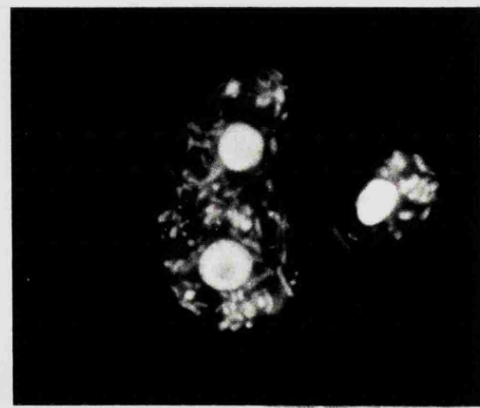
d



f



g



i



h



j



Figure 6.11

Mitotic RA376 and RA614 large uninucleate cells double-stained with β -tubulin antibody to show microtubules (b,d,f,h,j,l) and with DAPI to show DNA (a,c,e,g,i,k). The dots in the cytoplasm (a,c,e,g,i,k) are a mixture of mitochondria and ingested bacteria. Bar = 3 μ m. (a & b) RA376 large uninucleate cell in plasmodial mitosis; one extranuclear MTOC (>) is present. (c & d) RA376 large uninucleate cell in amoebal mitosis. (e & f) RA614 large uninucleate cell in star mitosis. (g & h) RA376 large uninucleate cell in star mitosis with two extranuclear MTOCs (>). (i & j; k & l) RA614 large uninucleate cells with abnormal spindles.



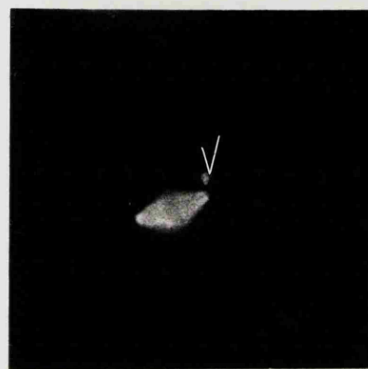
a



c



e



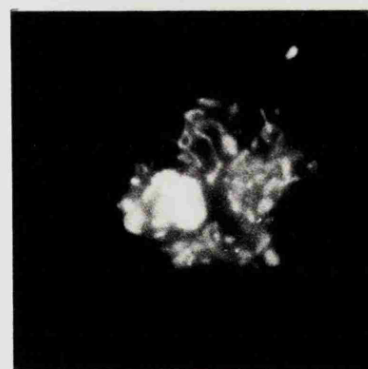
b



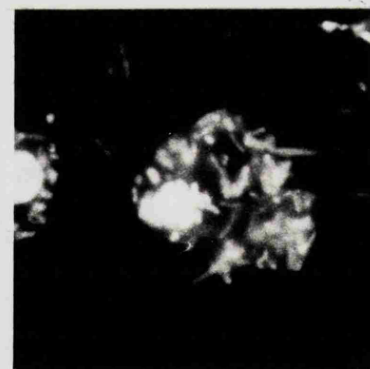
d



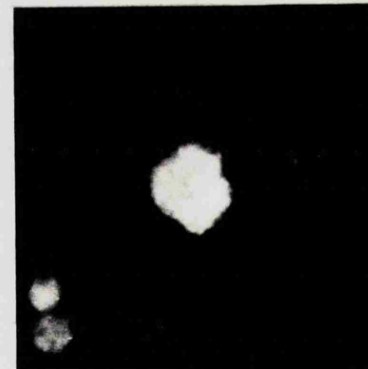
f



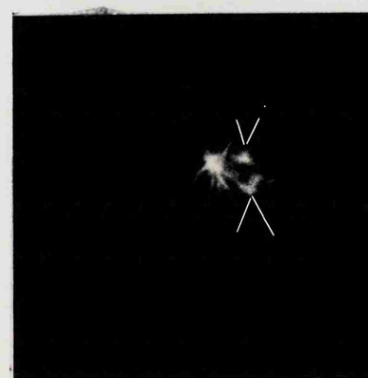
g



i



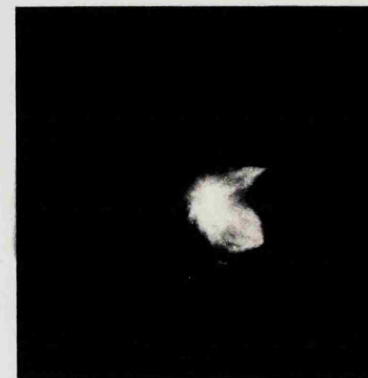
k



h



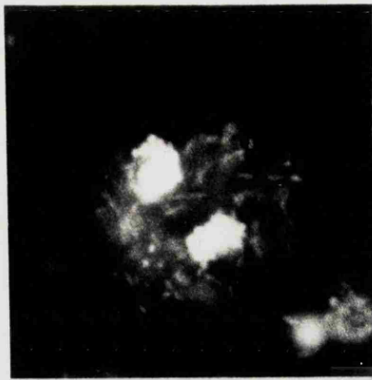
j



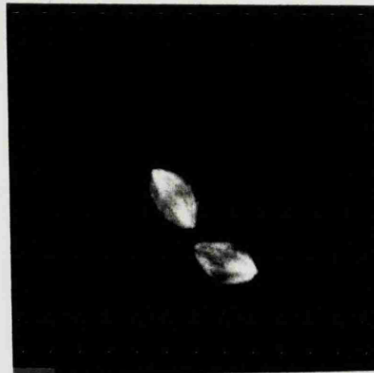
l

Figure 6.12

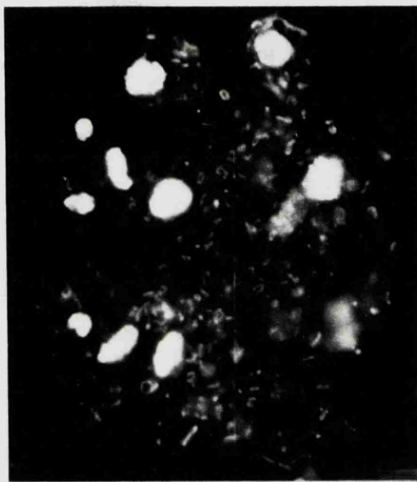
Mitotic RA376 and RA614 cells in synchronous plasmodial mitosis double-stained with β -tubulin antibody to show microtubules (b,d,f) and with DAPI to show DNA (a,c,e). The dots in the cytoplasm (a,c,e) are a mixture of mitochondria and ingested bacteria. Bar = $3\mu\text{m}$. (a & b) RA614 binucleate cell (c & d) RA614 octanucleate cell. There are 8 spindles (>) but 10 nuclei. The extra nuclei are the remains of ingested amoebae. (e & f) Section of a RA376 microplasmodium in mitosis.



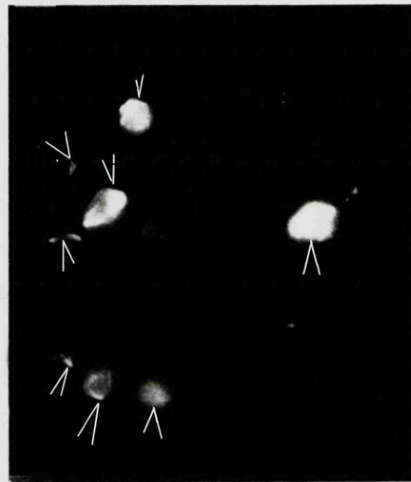
a



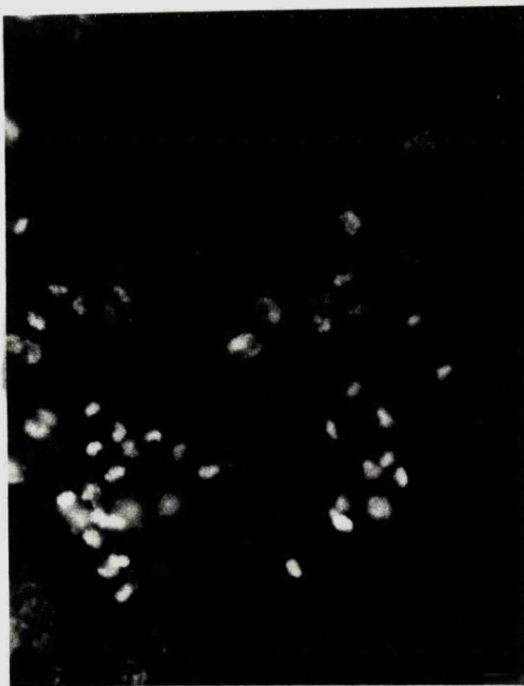
b



c



d



e



f

CHAPTER 7

General Discussion

The long-term aim of studying the amoebal-plasmodial transition in *Physarum* is to be able to relate changes in gene expression to alterations in cell organisation and, ultimately, to changes in cell behaviour. In order to achieve this aim, it is necessary to use a variety of techniques to examine different aspects of the amoebal-plasmodial transition. During this work, a number of experimental approaches were utilised. Some of the techniques permit analysis of individual cells, either over an extended period of time or at an instant in time, whereas other techniques only allow analysis of cell populations.

By using time-lapse cinematography, it is possible to follow individual cells over an extended period of time and to trace their origins and fates. The events in individual cells can be examined and used to build up a picture of the sequence and timing of events during the amoebal-plasmodial transition in relation to mitosis. Time-lapse cinematography can not, however, reveal the changes in cellular organisation that occur during development nor the timing of changes in gene expression. In contrast, by using immunofluorescence microscopy, it is possible to examine microtubule organisation in individual cells at the time of fixation and to built up a picture of the alterations in microtubule organisation that accompany plasmodium formation. It is not possible, however, using immunofluorescence microscopy, to

determine the ages or the origins of the cells nor the stage of the cell cycle at which the alterations in microtubule organisation occur. Coupling flow cytometry with microscopic analysis of cell populations allows estimates to be made of the amount of DNA in cells of different developmental types but this technique can not reveal the DNA content of individual cells at a particular stage of development. The experiments which examine the kinetics of development and the pattern of gene expression during development are carried out on populations of cells which are not developing synchronously. These studies, therefore, can not reveal the timing of commitment or of gene activation in individual cell pedigrees.

Combining the results from all these techniques gives a picture of the timings and sequence of events that accompany plasmodium formation. An extended cell cycle, ending in the formation of a binucleate cell by mitosis without cytokinesis, is an essential part of plasmodium development. Many of the changes leading to the formation of a plasmodium appear to be initiated during this extended cell cycle. These changes include commitment to plasmodium development, alterations in microtubule organisation and gene expression, and alterations in cell behaviour. Cell and nuclear area increase during the long cell cycle and ability to undergo the amoeba-flagellate transformation is lost. Ability to undergo plasmodial fusions is gained. The extended cell cycle occurs during development in all the apogamic strains examined. Extension of the cell cycle occurs in *matA*-heteroallelic cultures but not in *matA*-homoallelic

cultures. Extension of the cell cycle, therefore, appears to be under the control of *matA*. Although many of the changes associated with plasmodium formation are initiated during the extended cell cycle, it is not possible to identify whether these events occur at a particular stage of the cell cycle (e.g. G1-phase or G2-phase).

During cell and nuclear fusion in mating cells of most organisms, the nuclei are synchronised so that they are in the same stage of the cell cycle. This stage is often G1-phase but may also be G2-phase (see Section 4.5.3). In *Saccharomyces cerevisiae*, cells which are able to mate become arrested in G1-phase and cell and nuclear fusion occur at this stage. G1-phase arrest is caused by mating pheromones which also affect gene expression (Cross et al., 1988; McCaffrey et al., 1987). *Physarum amoebae*, like cells of *S. cerevisiae* and *Dictyostelium*, produce a chemical substance, the inducer (Shipley and Holt, 1982; O'Day and Lewis, 1981). It is possible that the inducer produced by *Physarum amoebae* acts as a sex pheromone. If so, how and when does the inducer act on amoebae? The available evidence suggests that the inducer causes *Physarum amoebae* to become mating competent, but does not arrest them in G1-phase or any other phase of the cell cycle (see Section 4.5.2; Shipley and Holt, 1982). Although the inducer does not appear to cause cell cycle arrest, it may act at only one point in the cell cycle. It is possible that *Physarum amoebae* can become competent to mate only when acted on by the inducer immediately after mitosis, before the cells start S-phase. If this is so, *Physarum amoebae* would become competent to mate at a particular point

in the cell cycle but might be able to fuse at any stage in the cell cycle. If *Physarum* amoebae can fuse at any stage in the cell cycle, fusion would sometimes occur between an S-phase cell and a G2-phase cell. Such a fusion would give rise to a fusion cell with nuclei at different stages in the cell cycle, which might cause difficulties at the next mitosis. Normally, the nuclei in a common cytoplasm are at the same stage of the cell cycle. In plasmodia of *Physarum*, the G1-phase lasts a maximum of a few minutes (see Section 4.5.2). If the same is true in amoebae, and the evidence suggests that it is (see Section 4.5.2), then amoebal S-phase might begin before cell division was completed. The nucleus of an S-phase amoeba would presumably continue DNA replication after cell fusion. The gap between cell and nuclear fusion would then serve to allow both the nuclei in the fusion cell to reach G2-phase by the time of nuclear fusion.

In order to examine the cell cycle during development and the effect of the inducer on amoebae, synchronous cultures of developing cells are required. It has proved possible with *S. cerevisiae* to produce synchronised cell cultures by blocking cells in G1-phase with hydroxyurea and then releasing the block by washing the cells (Slater, 1973). If synchronised cultures of *Physarum* amoebae could be produced, it might be possible to induce development in these cultures by addition of partially-purified inducer (Nader *et al.*, 1984). The effect of adding the inducer at different stages of the cell cycle can then be investigated. Possible methods of producing synchronised amoebae are currently being studied

in a number of laboratories.

In apogamic strains, a single amoeba can develop into a plasmodium without change in nuclear DNA content (see Section 3.4.3). Apogamic development, like sexual development, involves an extended cell cycle (see Section 3.4.1). The fact that apogamic strains arise as a result of mutation at *matA* reinforces the conclusion drawn from comparisons of *matA*-heteroallelic and *matA*-homoallelic cultures, that *matA* is the main gene controlling plasmodium formation in *Physarum*. The isolation of mutants which are blocked in apogamic development (see Chapters 5 & 6) demonstrates that many genes are required for plasmodium development, although it is not possible at present to estimate the total number of genes that affect plasmodium formation. Analysis of development in these mutants suggests that plasmodium development involves a series of parallel processes rather than a single developmental sequence. These processes appear to be partly independent and if one is blocked, others may continue for some cell cycles. For example, filming analysis indicates that developing RA614 cells continue to ingest amoebae and undergo plasmodial fusions even though the nuclei have apparently vanished.

It has been possible to investigate the effect of mutation on apogamic development at a phenotypic level (see Chapters 5 & 6). However, in order to reveal more about the underlying processes which control development, it is necessary to study the mutant genes at a molecular level. A DNA transformation system in which mutant rescue experiments could be performed might lead to the isolation of genes which are necessary for

plasmodium formation. Such a DNA transformation system would also make it possible to carry out gene disruption experiments where the action of specific genes can be examined. Possible methods of achieving DNA transformation in *Physarum* are currently being investigated in several laboratories.

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