Patterns of Inheritance, Development and the Mitotic Cycle in the Protist *Physarum polycephalum*

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I. Introduction

Physarum polycephalum has commonly been cited in the GenBank sequence database as a plant, and in the Medline bibliographic database as a fungus; other descriptors such as "protostelid" have also been proposed (for a review, see Alexopoulous, 1982). Recent taxonomy places *P. polycephalum* convincingly in the protist kingdom (Margulis and Schwartz, 1982), but the unpretentious vernacular description "plasmodial slime mould" remains unchanged. The variety of cell types observed during the life cycle (Fig. 1) reveals how one might be led astray in trying to classify the organism: the amoeba and the plasmodium appear so different that they could easily be mistaken for two organisms from different kingdoms.

The wealth of biological variation in this organism provides a broad array of opportunities for experimental analysis. The different cell types and developmental pathways of *P. polycephalum* provide abundant opportunities for analysis of problems in cellular and developmental biology; the natural mitotic synchrony of the plasmodium provides unique opportunities for experimental analysis of the unperturbed mitotic cycle; and genetic analysis is made possible by the sexual and meiotic alternations between amoebal and plasmodial stages of the life cycle.

Beneath this veneer of variation lies a remarkably conserved array of fundamental biological structures and processes. The amoebal microtubular cytoskeleton is built from conserved tubulins, orchestrated by organizing centres like the counterparts in the cells of animals. And amoebal mitosis follows the same basic pattern as mitosis in the animal cell. In the plasmodium, mitosis occurs inside the intact nuclear membrane, as it does in fungi, while the classical eukaryotic cycle of chromatin condensation and decondensation is preserved during the naturally synchronous mitotic cycle, based on a complement of histones and modifying enzymes similar to those of most other eukaryotes. Many other fundamental eukaryotic processes are conserved in this protean protist.

In this review we summarize the biology of *P. polycephalum*, and give examples of how the organism has been utilized for analysis of patterns of inheritance, development and the mitotic cycle. We introduce recent

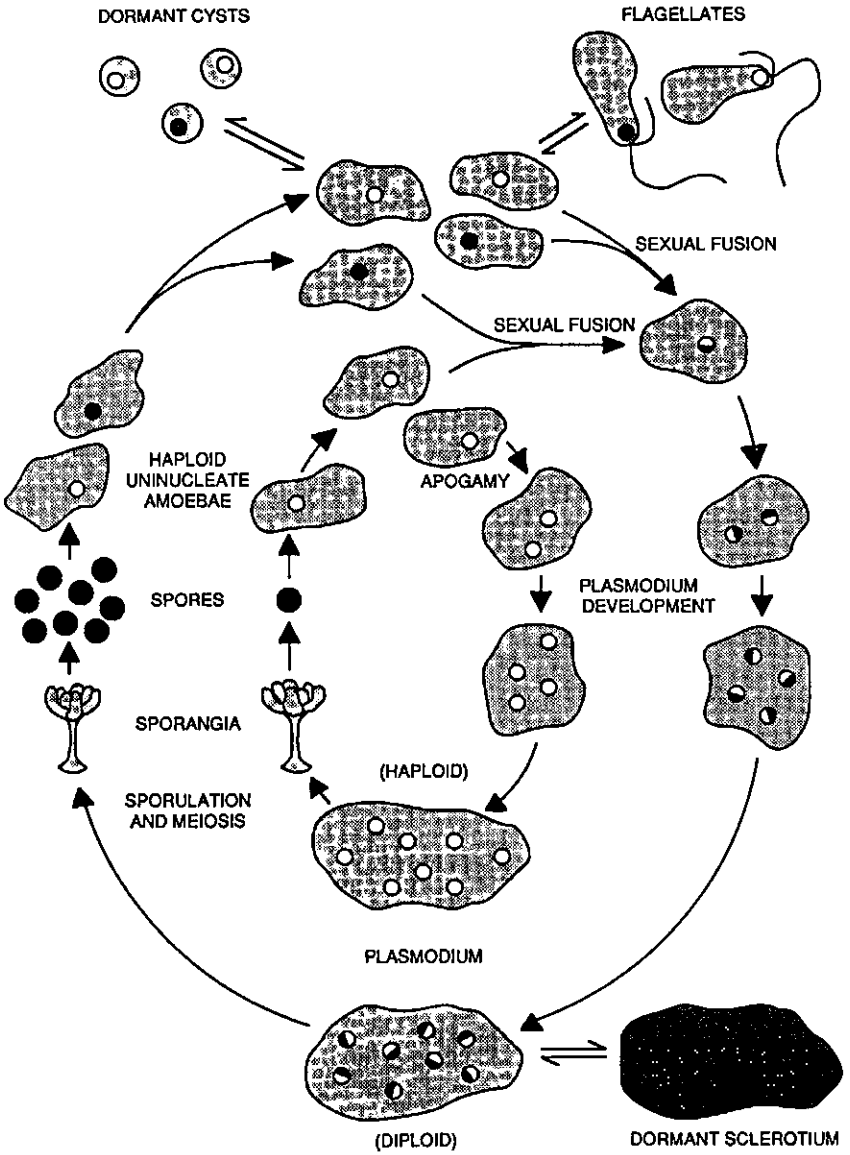


FIG. 1. Life cycle of *Physarum polycephalum*. The outer circuit summarizes the life cycle of heterothallic strains, typical of the species isolated from nature. The inner circuit summarizes the life cycle of apogamic strains, such as the *matA2 gadAh* mutant CL. Apogamic strains retain the ability to cross with heterothallic strains of appropriate mating types. Redrawn from Burland (1978).

advances in DNA transformation and gene targeting in the organism, and point to definitive experiments now possible using the new technology with the inveterate biology of this plasmodial slime mould.

II. Life Cycle

A. AMOEBAL PHASE

Among the distinct cell types of *P. polycephalum*, only the amoeba and plasmodium are capable of proliferation. The uninucleate amoebae are usually haploid, though some isolates of the close relative *Didymium iridis* are diploid (Collins and Betterley, 1982). Amoebae live primarily in the soil, feeding phagocytically on bacteria and other microbes (Olive, 1975). In the laboratory, amoebae are grown on lawns of *Escherichia coli* but, in order to grow amoebae of *P. polycephalum* axenically, mutant strains had to be selected (Dee *et al.*, 1989).

On transfer to water, amoebae develop reversibly into flagellates (Fig. 1), changing their mode of locomotion from amoeboid crawling to swimming. Upon return to a solid substrate or nutrient medium, flagellates revert to the amoebal cell type. Under adverse conditions, amoebae develop reversibly into cysts which, unlike amoebae and flagellates, possess a cell wall (Raub and Aldrich, 1982). Cysts germinate to form amoebae or flagellates upon exposure to favourable conditions. The third developmental option for amoebae is irreversible transition to the plasmodium (Fig. 1).

B. PLASMODIAL PHASE

The plasmodium of *P. polycephalum* is most commonly found in the litter of the forest floor. In this yellow-pigmented, multinucleate syncytium, growth and synchronous nuclear division continue in the absence of cytokinesis; the plasmodium increases in mass as long as nutrients are available. As far as we know, there is no limit to the size of a single plasmodial cell, although we have not grown a synchronous plasmodium larger than a 30 cm or so in diameter in the laboratory. The plasmodium can be grown axenically on a surface or in submerged, shaken liquid culture. In the latter situation, a large plasmodium breaks into pieces a millimetre or less in diameter; such "microplasmodia" grow to culture densities of over 100 mg wet weight ml^{-1}, and without agitation readily settle to the bottom of the container at unit gravity. With these characteristics, in combination with emerging molecular technologies and absence of a cell

wall, the plasmodium could serve as an efficient biofactory for research and commercial products.

When two plasmodia carrying identical alleles of the *fusA*, *fusB* and *fusC* loci come into contact, they fuse together (Poulter and Dee, 1968). Nuclei and cytoplasm soon mix, although the nuclei do not fuse, so that a heterokaryon is formed. Plasmodial heterokaryons are useful for analysis of mitotic regulation, somatic compatibility and genetic complementation testing.

The plasmodium has two developmental options, each requiring starvation for induction. In the dark, the starving plasmodium encysts, forming cell-walled structures known as sclerotia or spherules; these revert to active plasmodia when conditions become favourable. If starving plasmodia are illuminated, irreversible meiotic sporulation occurs. Each haploid spore cell is encased in a wall and, upon exposure to favourable conditions, spores "hatch" to release amoebae or, in moist conditions, flagellates, thereby completing the life cycle (Fig. 1).

C. THE SEXUAL CYCLE AND INHERITANCE

Mating and meiotic recombination in *P. polycephalum* were first demonstrated by Dee (1960, 1966, 1982). Spores of wild-type diploid plasmodia hatch to yield haploid progeny amoebae, which carry alternate alleles, such as *matA1* or *matA2*, of the primary mating-type locus. Plasmodium development does not normally occur from amoebal clones of either mating type, but does occur in mixtures of *matA1* and *matA2* amoebae, as a result of sexual fusion between haploid amoebae of different mating type, yielding diploid heterozygous plasmodia. Mating in *P. polycephalum* is thus heterothallic. Pairs of alleles of two other mating-type loci, *matB* and *matC*, also segregate among the meiotic progeny of most plasmodial isolates. Allelic difference at *matA* is required for fusion of nuclei of different mating types and subsequent plasmodium development (Dee, 1982), while allelic difference at *matB* is required for efficient cell fusion (Youngman *et al.*, 1981), and allelic difference at *matC* improves the efficiency of crossing at certain pH values (Kawano *et al.*, 1987b). These requirements for genetic difference in amoebal mating contrast with the genetic similarity required for somatic fusion of plasmodia.

From analysis of distinct natural isolates of *P. polycephalum*, at least 13 *matA* alleles (Collins and Tang, 1977) and 13 *matB* alleles (Kirouac-Brunet *et al.*, 1981) have been identified, and it seems likely that outbreeding is rampant in natural populations. The multiplicity of mating-type alleles is reminiscent of mating types in basidiomycetes (e.g. Metzenberg, 1990), although cellular changes that occur under control of the mating loci appear completely different in the two groups of organisms.

To simplify genetic and cell-biological analyses, apogamic strains of *P. polycephalum* have been derived in which a haploid amoeba can develop into a haploid plasmodium without cell fusion and without change in ploidy (Fig. 1; Cooke and Dee, 1974). Such apogamy, or "selfing", usually arises in the laboratory through mutation at or very close to *matA*, further implicating this locus as a major regulator of the development of amoebae into plasmodia. Apogamic strains retain the ability to cross with heterothallic strains (Fig. 1), facilitating both isolation and genetic analysis of mutants. Although viability of spores from haploid apogamic plasmodia is generally low, it is adequate for laboratory analysis, and correlates well with the frequency of rare diploid nuclei found in the otherwise haploid plasmodia (Laffler and Dove, 1977).

Somatic inheritance of specific markers appears to be very stable; for example, there is no known example of an amoeba changing mating specificity after extended subculture, nor have nuclear genome rearrangements been observed between different cell types (Sweeney *et al.*, 1987). Nevertheless, with continued extensive subculture of plasmodia in the laboratory, various sublines of the same original isolate have inherited distinct characteristics (Mohberg and Babcock, 1982). Of more concern, after extended subculture plasmodia can become heteroploid (Kubbies and Pierron, 1983; Kubbies *et al.*, 1986), and this heteroploidy is associated with poor synchrony of DNA replication, which is potentially a problem for mitotic-cycle analysis. Therefore, the preferred strategy for maintaining strains in the laboratory is to generate plasmodia periodically from fresh crosses of various well-characterized pairs of amoebal stocks. There are no difficulties with heteroploidy in such plasmodia (Cunningham, 1992). Appropriate amoebal stocks can be stored frozen, recloned from time to time, and are publicly available; thus, to maintain precise, homogeneous ploidy, isogenicity and experimental consistency with *P. polycephalum* is a matter of elementary microbiological technique.

III. Genome Organization

A. NUCLEAR CHROMOSOMAL GENOME

The nuclear genome is distributed among approximately 40 chromosomes (Mohberg, 1982b), which appear small and of similar size under the light microscope; a karyotype has not been determined. The haploid unreplicated DNA content of the nucleus in *P. polycephalum* is 0.3 pg (Mohberg, 1982b), corresponding to $2.7 \cdot 10^8$ bp in each genome—about the same as in *Drosophila melanogaster*. Although *P. polycephalum* has a

variety of cell types in its life cycle, *Dr. melanogaster* has far more, and it would seem unlikely that all of the DNA in *P. polycephalum* encodes protein or RNA products. Gross analysis of nuclear DNA by reassociation kinetics suggests that no more than two-thirds of the genome is single copy. The repetitive sequences include both inverted-repeat and direct-repeat structures, the most abundant of which appear to be methylated (for a review, see Hardman, 1986).

The predominant repeat structure, Tp1 (transposon *Physarum* 1), is related to classical retrotransposon-like sequences such as *copia* in *Dr. melanogaster* (Rothnie *et al.*, 1991), and the next most abundant repeat structure, Tp2, appears to be a member of the same group (McCurrach *et al.*, 1990). Organization of Tp1 repeats into scrambled tracts of up to 50 kb suggests that, over evolutionary time, the elements have retrotransposed into already integrated transposons, although transposition of Tp elements in *P. polycephalum* has not been demonstrated in the laboratory. A single Tp1 element consists of an 8343 bp sequence flanked by 277 bp LTRs (long terminal repeats) that are terminated by short inverted repeats (Rothnie *et al.*, 1991). The LTRs contain putative transcriptional signals as well as sites analogous to initiation sites for DNA synthesis found in both retrotransposons and retroviruses. Tp1 elements include open-reading frames (ORFs) corresponding to homologues of the protease, endonuclease, reverse transcriptase and nucleic acid-binding products of *copia* (Rothnie *et al.*, 1991). However, the transposon-like ORFs found so far appear incomplete, which is not surprising if indeed Tp1 tracts derive from multiple integrations of one transposon into another; only a minority of transposons might retain a complete structure.

If any of the Tp elements are functional retrotransposons, they may have practical value for recombinant DNA applications in *P. polycephalum*, notably for integrative DNA transformation. And whether or not functional retrotransposons are present, elements like Tp1 are of interest in elucidating the evolutionary origins of retrotransposons. Sequence homologies between Tp1 and other retrotransposons, such as *copia* from an insect, Ty1 and Ty3 from a yeast, and Tal and Tnt1 from plants, indicate that this group of transposons originated from a common ancestor. An interesting question is whether these related transposons populated genomes of the diverse group of hosts prior to the hosts' divergence, or afterwards by horizontal transmission. Comparisons of sequences of transposons in closely related species with those of distantly related species are beginning to address this issue (Rothnie *et al.*, 1991).

Cloning of structural genes from *P. polycephalum* has sometimes been difficult, due to instability in bacteria of some of the repeated DNA structures and other unusual sequences (e.g. Nader *et al.*, 1986). However,

sequences that present cloning problems tend to be regularly dispersed throughout the genome, typically with 5–10 kb of single-copy sequence interspersed. Structural genes of interest can be obtained by cloning DNA fragments of the order of 5–7 kb or less (e.g. Monteiro and Cox, 1987a; Gonzalez-y-Merchand and Cox, 1988; Adam *et al.*, 1991). Structural genes examined so far typically contain several introns (e.g. Adam *et al.*, 1991), but these are small relative to the size of introns in mammalian genes. Where detailed genomic structure is not needed, cDNA libraries have proved to be an efficient means of elucidating the coding potential of specific genes (e.g. Pallotta *et al.*, 1986; Paul *et al.*, 1992).

B. NUCLEOLAR DNA GENOME

The genes for 26S, 19S and 5.8S rRNAs in *P. polycephalum* are encoded on 60 kb, linear extrachromosomal DNA molecules present at about 150 copies in each haploid nucleus. These rDNA molecules are located in the nucleolus, which greatly simplifies their purification from chromosomal and mitochondrial DNA populations. Each rDNA molecule is a palindrome containing two sets of rRNA genes, each set being transcribed away from the centre (for a review, see Hardman, 1986). The two 13.3 kb transcription units are separated by a 23 kb central non-transcribed spacer, which contains a variety of repetitive elements and can vary in size even within one strain.

In possessing multicopy extrachromosomal rDNA molecules, *P. polycephalum* is typical of many protists. A linear, palindromic structure with two transcription units is found in *Dictyostelium discoideum* (Welker *et al.*, 1985) and *Tetrahymena thermophila* (Engberg and Nielsen, 1990), though *D. iridis* has only a single transcription unit (Johansen, 1991). In other protists, circular molecules with one or two transcription units have been found, and the presence of multiple copies of rDNA sequences is typical of genomes in a wide variety of organisms (Long and Dawid, 1980).

The rDNA molecules in *P. polycephalum* have a repeated $(T_2AG_3)_n$ telomere structure (Forney *et al.*, 1987), similar to telomeres in chromosomes of *Trypanosoma* spp., *Neurospora* spp., humans and other organisms (Zakian, 1989; Coren *et al.*, 1991). Such sequences facilitate recognition of telomeres by the telomerase needed to complete replication (Blackburn, 1991) and probably also facilitate binding of other proteins to prevent exonuclease degradation of the ends of linear chromosomes, thereby stabilizing the linear topology. A protein, PPT, has been identified in *P. polycephalum* that specifically binds to the T_2AG_3 repeats of the rDNA molecule (Coren *et al.*, 1991). Binding of the protein is resistant to ribonuclease, and thus PPT seems not to be a telomerase, which recognizes telomere sequences through its RNA moiety (Blackburn, 1991). It may

rather be a structural protein that protects replicated telomeres. Further study should yield information relevant not only for rDNA from *P. polycephalum*, but also for the general nature of linear chromosome maintenance in eukaryotes.

Unlike chromosomal genes, rDNA molecules are inherited in a non-Mendelian fashion. Using restriction fragment-length polymorphism (RFLP) markers for rDNA in crosses, Ferris *et al.* (1983) found that amoebal-progeny clones carry either one or other parental rDNA species, but not both. However, the ratio of the two rDNAs among the progeny is biased in favour of one species; the older the plasmodium before sporulation and meiosis, the more biased is the distribution among the progeny. These observations suggest that replication efficiencies of different rDNA molecules are dissimilar, leading to an unequal proportion of the two rDNA molecules, and that a single master copy of rDNA is chosen randomly at meiosis to be passed on to the progeny (Ferris *et al.*, 1983). One implication of this hypothesis is that no nuclear chromosomal master copy of rDNA need exist.

Molecules of rDNA in *P. polycephalum* contain two or occasionally three so-called group-I introns (Muscarella and Vogt, 1989) in the gene encoding 26S rRNA. The third intron, intron-3, has so far been found in only one isolate, namely Carolina. Intron-3 occurs at the same position as another group-I intron in the highly conserved rRNA gene in *Tetrahymena* spp. (Muscarella and Vogt, 1989), and the 3′ portion of intron-3 is remarkably similar to the intron found in *Tetrahymena* spp., including conserved sequences involved in self-splicing.

One powerful feature of the biology of *P. polycephalum* is the simplicity with which functions in the plasmodium can be compared between diploid heterozygotes, formed by crossing together amoebae of different mating types, and haploid heterokaryons, formed by fusing together plasmodia of identical fusion type. This feature was used to elucidate intron-3 action. When a strain of amoebae from the mould carrying intron-3 is crossed with a strain lacking intron-3, and the crossed plasmodium is analysed, the intron transposes site specifically into all of the rDNA molecules in the diploid heterozygous nuclei of the plasmodium (Muscarella and Vogt, 1989). In plasmodial heterokaryons formed by fusing together plasmodia carrying intron-3^+ and intron-3^- nuclei, breakage of rDNA in some of the intron-3^- nuclei is detectable, consistent with synthesis of a specific, intron-3-encoded endonuclease in the cytoplasm and subsequent entry into the nuclei. However, transposition is not observed in the heterokaryon, indicating as expected that intron-3 is nucleus limited. It is now clear that intron-3 encodes a site-specific endonuclease, called I-*Ppo*I (Muscarella *et al.*, 1990). It appears that I-*Ppo*I is encoded from a transcript synthesized by RNA polymerase I (Muscarella and Vogt, 1989).

Endonuclease I-*Ppo*I cuts rDNA at the site of integration of intron-3, both *in vitro* and in *E. coli*, indicating that intron-3 catalyses its own transposition. The recognition sequences for two other intron-encoded endonucleases, I-*Sce*I and I-*Sce*II, found in the mitochondrial rDNA of *Sacch. cerevisiae* are large compared with the more familiar bacterial restriction endonucleases (Delahodde *et al.*, 1989). Likewise, the I-*Ppo*I recognition sequence is 13–15 nucleotide pairs long, encompassing the sequence 5′ CTCTCTTAA ↓ GGTAGC 3′, where the arrow indicates the site of cleavage (E. L. Ellison and V. Vogt, personal communication; Lowery *et al.*, 1992). Endonucleases of this type are expected to be useful for mapping large genomes, since the rare cleavage-site frequency allows a chromosome to be subdivided into large, discrete units for physical analysis. Endonuclease I-*Ppo*I appears to have the catalytic efficiency and stability needed for *in vitro* analyses, and the enzyme is now commercially available (Lowery *et al.*, 1992).

In view of the efficiency with which transposition occurs in a cross between intron-3$^+$ and intron-3$^-$ strains, it is interesting that this intron has been found only in one strain of *P. polycephalum*. The Carolina isolate which carries intron-3 may be geographically isolated. Alternatively, the presence of intron-3 may be deleterious and selected against. Perhaps it is a recent invader of the Carolina isolate soon to spread to other strains.

The nucleotide sequences of rRNA genes are among the most conserved through evolution, and sequences for both 19S rRNA (Johansen *et al.*, 1988) and 26S rRNA (Hasegawa *et al.*, 1985) have been used to estimate phylogenetic relationships between *P. polycephalum* and other eukaryotes (e.g. Baroin *et al.*, 1988; Lenaers *et al.*, 1988). These comparisons suggest that *P. polycephalum* represents a line of descent that was one of the earliest to diverge from other eukaryotic lines (Johansen *et al.*, 1988). In this respect, it is remarkable that fundamental cellular processes like mitosis and the chromatin condensation–decondensation cycle in the slime mould are more similar to vertebrates than to those of some apparently later-diverging fungi. This may reflect a decreased spectrum of motility functions in fungi compared with slime moulds and vertebrate cells, leading to loss of unnecessary functions in fungi. Other functions may also be more similar between slime moulds (and other protists) and vertebrates, since motility embraces so many aspects of cell structure and function.

C. MITOCHONDRIAL GENOME

Mitrochondrial DNA (mtDNA) comprises 5–10% of cellular DNA in *P. polycephalum*. Estimates of size for the mitochondrial genome from a variety of strains range from 56–86 kb (Kawano *et al.*, 1982, 1987a; Jones

et al., 1990; Takano *et al.*, 1990). As with nuclear gene markers, different isolates display abundant mtDNA RFLP, and complete restriction maps are available for several mtDNAs. The 86 kb size estimate includes a map showing near-terminal duplications of 19.6 kb stretches (Takano *et al.*, 1990), which could explain why many estimates are around 60 kb; either other mtDNAs lack this duplication or it was overlooked. However, mtDNAs can vary in size within several organisms, and it is possible that some of the size variation reported for mtDNA from *P. polycephalum* is due to natural variation (e.g. Kawano *et al.*, 1987a) rather than different experimental interpretations. Both linear and circular topologies have been proposed for mtDNA from this slime mould (Jones *et al.*, 1990; Takano *et al.*, 1990). The observation that a specific mtDNA restriction fragment that maps close to the proposed linear terminus is preferentially sensitive to exonuclease digestion strongly favours a linear rather than a circular topology, at least for the mtDNA from the Colonia genetic background (Takano *et al.*, 1990). It remains possible, however, that both linear and circular mtDNAs could exist, perhaps related by recombination.

Restriction fragment-length polymorphism markers were used to establish that mtDNA inheritance in *P. polycephalum* is uniparental (Kawano *et al.*, 1987a), as is so in a broad array of eukaryotes. However, uniparental inheritance in isogamous organisms such as *P. polycephalum* is remarkable in that both gametes contribute mitochondria at sexual fusion. Therefore, a mechanism must exist to select or eliminate one genome. Meland *et al.* (1991) showed that one of the mtDNA genomes from this slime mould is specifically eliminated within two cell cycles after sexual fusion of amoebae. Interestingly, which mtDNA is eliminated depends upon the *matA* alleles carried by the parents; different *matA* alleles can be ranked in linear hierarchical order of dominance for determining loss of mtDNA (Meland *et al.*, 1991). For example, in a *matA7*×*matA2* cross, the mtDNA carried by the *matA7* parent is inherited while the mtDNA from the *matA2* parent is lost; in a *matA2*×*matA11* cross, the mtDNA of the *matA2* parent is inherited; and from this pair of relationships, it can be deduced that, in a *matA7*×*matA11* cross, the *matA7*-associated mtDNA will be inherited (Meland *et al.*, 1991). This is the first evidence for active degradation of mitochondrial genomes in sexual crosses. The phenomenon bears a remarkable resemblance to degradation of chloroplast genomes in crosses in *Chlamydomonas reinhardtii* (Kuroiwa, 1985). Thus, although rDNA and mtDNA are both inherited in a non-Mendelian fashion in *P. polycephalum*, as they are in *D. iridis* (Silliker and Collins, 1988), the mechanisms that distort segregation of rDNA and mtDNA genomes are distinct and act at different stages of development.

Mitochondria in *P. polycephalum* typically possess a single spherical mass

of material that stains with diamidinophenylindole, which is known as the mt nucleus and presumably represents the mitochondrial genome. Fusion of mitochondria in zygotes and during sporulation occurs in the Ng isolate of *P. polycephalum* and most of its derivative strains, leading to larger, knotted, multinucleate mitochondria (Kawano *et al.*, 1991). Other strains tested do not exhibit this mitochondrial fusion. Following mitochondrial fusion, fusion of mt nuclei occurs. Remarkably, at spore germination, fused mitochondria and their mt nuclei divide to yield the original spherical mitochondrial-nuclear morphology (Kawano *et al.*, 1991). Genetic evidence supports these morphological phenomena: rather than the usual uniparental inheritance of mtDNA, progeny of such crosses exhibit recombination of mtDNA RFLP markers. Kawano *et al.* (1991) suggest these observations reflect a mitochondrial meiotic cycle.

Occurrence of this mitochondrial cycle correlates with the presence of a 16 kb linear Mif (mitochondrial fusion) plasmid in the mitochondrion (Kawano *et al.*, 1991; Takano *et al.*, 1991). Most progeny of the Ng isolate contain the plasmid and, when they are mated with another strain, whether it contains the plasmid or not, mitochondrial fusions ensue. The plasmid is inherited by nearly all of the progeny of such crosses, essentially displaying uniparental inheritance, and these progeny in turn transmit the Mif phenotype to their progeny. The Mif plasmid is thus acting like a selfish gene. However, one of the mtDNA species in each Mif cross benefits in that the mitochondrial fusions preserve the mtDNA that otherwise would have been eliminated according to the *matA* hierarchy (Meland *et al.*, 1991). Hurst (1991) points out that this mechanism fits well with proposals as to how sex may have initially evolved. The DNA transformation system for *P. polycephalum* (see Section VI.C) has been developed for integration into the nucleus but, if it can be extended to the mitochondrion, the specific detailed functions of the Mif plasmid would be open to investigation.

Another remarkable characteristic of the mitochondrial genome in the slime mould is the editing of RNA for the α-subunit of ATP synthase (Mahendran *et al.*, 1991). Insertion of cytosine residues at 54 sites is required to generate a functional reading frame for the mRNA from the gene. This is the first example of RNA editing by insertion of cytosine residues, contrasting, for example, with insertion of uridine residues in the RNA of *Trypanosoma brucei* (Feagin *et al.*, 1988). This type of insertional editing observed in organelles of these and other protists appears to be distinct from substitutional editing found in plant mitochondria and in vertebrates (Scott, 1989; Benne, 1990). Given the apparent susceptibility of the nuclear and nucleolar genomes in *P. polycephalum* to transposition, usually with no obvious benefit to the host, it is curious that an editing requirement for mtDNA genes has been maintained during evolution. If a transposon

could enter and function in the mitochondrion, even if only rarely over evolutionary time, one might predict that it would be more efficient and therefore advantageous if the editing system were replaced by retro-integration of an edited transcript.

IV. Cytoskeletal Organization

The cytoskeleton of *P. polycephalum* has been a major focus of research, covering the roles of tubulin, actin, myosin, titin, profilin and other cytoskeletal proteins (Dove *et al.*, 1986). The plasmodium is a particularly useful source of non-muscle actomyosin and related proteins, since it contains substantial quantities of cytoplasmic actin and myosin, and is easy to culture to the large mass needed for protein biochemistry. As Hatano (1986) adroitly phrased it, "Ten plastic buckets of 10 L each are used for cultivation of surface plasmodia in order to collect 100–200 g of material every two days". Such prolific growth has spawned far more actomyosin biology than we can reasonably review here. Hence, we review principally the microtubular cytoskeleton, with only passing mention of other cytoskeletal elements.

A. MICROTUBULE ORGANIZATION

Microtubules are fibres 25 nm in diameter that are constructed principally from heterodimers of α-tubulin and β-tubulin polypeptides. They are major components of several eukaryotic organelles, including mitotic and meiotic spindles, centrioles, axonemes and the cytoskeleton. Each of these structures is found in one or more cell types in *P. polycephalum*.

Microtubule organelles are usually organized by distinct organizing centres (MTOCs). The amoebal cytoskeletal microtubules in *P. polycephalum* radiate from a single MTOC juxtaposed to a centriole pair beside the nucleus. The nucleus–centriole complex can be isolated structurally intact, and retains the capacity to nucleate microtubule assembly *in vitro* (Roobol *et al.*, 1982). During mitosis in amoebae, cytoskeletal microtubules disappear, the nuclear membrane breaks down, the centriole pair separates and duplicates, and daughter centriole pairs migrate to opposite spindle poles, while the mitotic spindle and associated astral microtubule arrays assemble (Aldrich, 1969; Wright *et al.*, 1980). Mitosis is accompanied by the usual cycle of chromatin condensation and decondensation. Following mitosis and cytokinesis, spindle microtubules disassemble and cytoskeletal microtubules reappear, remaining throughout the interphase. The pattern of amoebal mitosis is reminiscent of mitosis in animal cells. However,

replication of centrioles during amoebal mitosis contrasts with that in animal cells, where centriole replication occurs throughout the cell cycle (Kochanski and Borisy, 1990). The fact that centriole duplication can be limited to mitosis in the amoebal cell cycle raises doubts about the hypothesis that the centrosome is a cog that helps to couple cell growth with cell division (Bailly and Bornens, 1992).

Upon development of flagellates, the cytoskeleton reorganizes dramatically. The nucleus moves to the anterior of the cell, with the associated centriole pair acting as basal bodies for the axonemes. Two flagella (one long and one short) assemble, while cytoskeletal microtubules form a cone around the nucleus and basal bodies (Havercroft and Gull, 1983). Flagellar and cone microtubules are highly organized, with five distinct MTOCs recognized, of which that designated MTOC1 is considered to be the same structure which organizes the mitotic spindle (Wright *et al.*, 1988). While this reorganization of microtubules is occurring, the microfilament system is also substantially reorganized (Pagh and Adelman, 1988; Uyeda and Furuya, 1985).

In the plasmodium, the most prominent microtubule structure is the mitotic spindle, present only during the synchronous mitoses. In contrast to extranuclear mitosis in amoebae, the plasmodial mitotic spindle is organized by an intranuclear MTOC, and has no astral microtubules (Aldrich, 1969; Tanaka, 1973; Havercroft and Gull, 1983). Plasmodial mitosis thus resembles mitosis in fungi, but the intranuclear MTOC in the plasmodium appears distinct from the spindle-plaque type of MTOC observed in fungi (Aldrich, 1969). Salles-Passador *et al.* (1991) observed cytoskeletal microtubules in the mature plasmodium, contradicting previous failures to detect cytoplasmic microtubules in this syncytium. So far it is not known whether these microtubules are nucleated by classic MTOCs. The function of plasmodial cytoplasmic microtubules is obscure, as the structure of the plasmodium is thought to be determined principally by the microfilament cytoskeleton, while vigorous protoplasmic streaming is thought to facilitate intracellular transport.

B. TUBULIN GENES AND POLYPEPTIDES

Given the multifunctional role of microtubules, detection of multiple forms of α-tubulin or β-tubulin polypeptides within a single cell type has raised interest in the multitubulin hypothesis, namely the question of whether different tubulins provide distinct functions for microtubules (Fulton and Simpson, 1976). *Physarum polycephalum* was the first microbe from which assembly-competent tubulin was purified (Roobol *et al.*, 1980, 1984), and multiple α-tubulin and β-tubulin polypeptides are found in different cell

TABLE 1. Summary of expression and utilization patterns of tubulins in *Physarum polycephalum*

Gene	Tubulin	Expression pattern[a]			Utilization[b]			
		Amoeba	Flagellate	Plasmodium	csk	msp (am)	msp (pla)	fla
altA	α1A	+++	+++	+	+	+	+	+
	α3	+	+++	−	−	−	−	+
altB(N)	α1B	−	−	+++			+	
altB(E)	α2B	−	−	+			+	
betA	β1A	+/−	+++	−	+			
betB	β1B	++	++	+	+		+	
betC	β2	−	−	++	+	+	+	+

csk indicates cytoskeleton; msp, mitotic spindle (am, amoebal; pla, plasmodial); fla, flagellar axoneme and cone.

[a] Expression patterns are deduced from RNA levels or for polypeptide levels or for both.

[b] Utilization means detection in the specified structure; it does not mean that the isotype is usually found there. For example, β2 tubulin has been detected in the flagellum on rare occasions, but it is not normally expressed when flagella are present. Where a matrix element is left blank, utilization of the tubulin has not been tested and cannot be deduced from present data.

types (Burland *et al.*, 1983). These advances increased the attraction of the organism to examine the multitubulin hypothesis. Evidence that α- and β-tubulins are encoded by multiple genes in eukaryotes (for a review, see Sullivan, 1988) also raised interest in the function of different tubulin gene products.

Genetic mapping using RFLPs as markers and heterologous tubulin genes as probes reveals four loci in *P. polycephalum* for α-tubulin, namely *altA*, *altB*, *altC* and *altD*, and three loci for β-tubulin, namely *betA*, *betB* and *betC* (Burland, 1986). The *altB* locus comprises two tightly linked α-tubulin genes (Schedl *et al.*, 1984c; Green *et al.*, 1987), but there is no evidence for multiple sequences at any of the other tubulin loci. Monteiro and Cox (1987a) termed the two linked *altB* genes Eα-Tu and Nα-Tu; we propose combining the original nomenclature of Schedl *et al.* (1984c), which follows published rules of genetic nomenclature for *P. polycephalum* (Anderson *et al.*, 1986), with Monteiro and Cox's refinement so that the two *altB* genes be referred to as *altB(E)* (Eα-Tu) and *altB(N)* (Nα-Tu).

The polypeptide products of *altA*, *altB(E)*, *altB(N)*, *betA*, *betB* and *betC* have all been identified, and complete or partial sequences deduced either from direct protein sequencing or from sequencing DNA clones. These genes show distinct patterns of expression in different cell types (Table 1). This has prompted various searches for clues as to the reasons for differential expression. Sequence differences among α1A, α1B and α2B

TABLE 2. Percentage identities of α-tubulin polypeptides in various organisms

	Pp1B	Pp2B	Ng1	Cr1	S11	Mm1	Dm1	At1	Sp2	Sc1	Sc3	Sp1
Pp1A	91.5	91.5	91.3	90.6	89.5	86.6	85.8	85.8	71.6	71.9	71.2	70.3
Pp1B		96.4	87.3	86.6	85.3	82.1	81.7	84.4	71	70.8	69.8	69.5
Pp2B			87.5	87.2	86	82.3	81.7	84.4	70.8	69.8	68.9	69.4
Ng1				93.1	92.2	85.7	85.5	86.6	71.7	69.3	69.3	70.4
Cr1					92.2	86.3	85.8	86.9	71.9	69.7	70	70.5
S11						85.3	84.5	85.7	69.6	68.6	68.2	69.4
Mm1							96.9	79.4	76.5	74.7	73.4	76.3
Dm1								78.9	76.3	74.9	74.3	75.8
At1									68.2	66.8	67.1	67.9
Sp2										75.5	73.9	85.7
Sc1											90.8	76.3
Sc3												74.5

Pp1A indicates *Physarum polycephalum* α1A tubulin; Pp1B, *Physarum polycephalum* α1B tubulin; Pp2B, *Physarum polycephalum* α2B tubulin; Ng1, *Naeglaeria gruberi* α1 tubulin; Cr1, *Chlamydomonas reinhardtii* α1 tubulin; S11, *Stylonichia lemmae* α1 tubulin; Mm1, *Mus musculus* α1 tubulin; Dm1, *Drosophila melanogaster* α1 tubulin; At1, *Arabidopsis thaliana* α1 tubulin; Sp2, *Schizosaccharomyces pombe* α2 tubulin; Sc1, *Saccharomyces cerevisiae* α1 tubulin; Sc2, *Saccharomyces cerevisiae* α2 tubulin. The sequences were obtained from the GenBank database.

tubulins (Table 2) fall well within the typical range of α-tubulin sequence differences found within a single eukaryote (Singhofer-Wowra *et al.*, 1986b; Cunningham *et al.*, 1993). The α1B and α2B polypeptides, whose genes are closely linked, show greater sequence identity to one another than to α1A. Comparing across species, α-tubulins from *P. polycephalum* are more similar to α-tubulins from other protists, plants, vertebrates and insects than to those from known fungi (Table 2). The α1B- and α2B-tubulin polypeptides are distinct from other known α-tubulins in having a methionine residue at their *C*-termini, instead of the more usual glycine residue. In other organisms a terminal glycine residue is thought to be necessary for tyrosination (Gunderson *et al.*, 1987), but neither tubulin tyrosine ligase nor Tyr-tubulin carboxypeptidase has been detected in *P. polycephalum*.

In the β-tubulin gene family in *P. polycephalum*, the *betA* and *betB* genes encode almost identical β1-tubulin polypeptides even though the two genes differ in 15% of their nucleotide residues (Werenskiold *et al.*, 1988; Paul *et al.*, 1992). By contrast, the β2-tubulin polypeptide encoded by *betC* differs from the β1 tubulins in 17% of its residues. Comparing across species, β1 tubulins in *P. polycephalum*, like its α-tubulins, are more similar to those from protists, insects and vertebrates than to those from fungi (Fig. 2), but β2 tubulin stands out as being highly divergent in its sequence, showing no particular similarity to any other β-tubulin (Burland *et al.*, 1988).

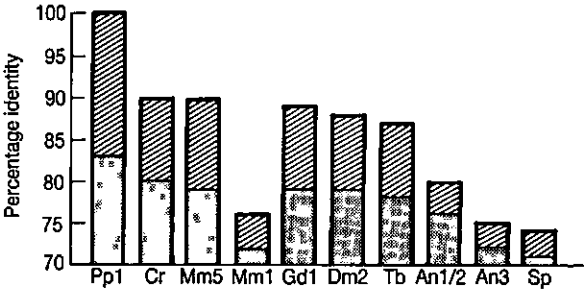


FIG. 2. Comparison of β-tubulin polypeptides. The shaded portion of each bar indicates the percentage identity between β2 tubulin from *Physarum polycephalum* and other β-tubulins. The lighter hatched of each bar indicates the percentage identity from *Physarum polycephalum* β1 tubulin and other β-tubulins. The β2 polypeptide is more diverged in sequence than β1 tubulin for all pairwise comparisons. Pp1 indicates *Physarum polycephalum* β1 tubulin; Cr, *Chlamydomonas reinhardtii* β-tubulin; Mm5, *Mus musculus* β5 tubulin; Mm1, *Mus musculus* divergent β1 tubulin; Gd1, *Gallus domesticus* β1-tubulin; Dm2, *Drosophila melanogaster* β2 tubulin; Tb, *Trypanosoma brucei* β-tubulin; An1/2, *Aspergillus nidulans* β1/2 tubulin; An3, *Aspergillus nidulans* divergent β3 tubulin; Sp, *Schizosaccharomyces pombe* β-tubulin. The sequences were obtained from the GenBank database.

C. TUBULIN UTILIZATION

If different tubulin polypeptides possess distinct functional properties, discovering the location of tubulin isotypes in various microtubular organelles may elucidate their functional differences. In *P. polycephalum*, it has been possible to distinguish several specific tubulins using a variety of methods.

1. α1A and α3 Tubulins

Although the α1A tubulin from *P. polycephalum* shows remarkable similarity to α-tubulins from organisms as evolutionarily distant as mammals, it appears to have at least one distinctive sequence characteristic, namely the presence of a lysine residue at position 40 in combination with a tyrosine residue at position 44. Birkett *et al.* (1985a,b) raised a monoclonal antibody to α-tubulin from *P. polycephalum*, namely KMP-1, specific for α1A tubulin from the slime mould. The KMP-1 epitope encompasses residues Lys_{40} and Tyr_{44} in α1A tubulin (Walden *et al.*, 1989a). Western and Northern blotting indicates that α1A tubulin is abundant in amoebae and flagellates, but only small amounts relative to the other α-tubulins are present in the plasmodium (Birkett *et al.*, 1985a,b; Cunningham *et al.*, 1993).

So far as we know, *altA* is the only α-tubulin gene expressed in amoebae and flagellates, and it seems likely that the α3 tubulin found in amoebae and flagellates is an acetylated form of the *altA* product (Table 1; Green and Dove, 1984; Cunningham *et al.*, 1993). The monoclonal antibody KMP-1 does not recognize amoebal α-tubulin when residue Lys_{40} is acetylated (Walden *et al.*, 1989a), and can therefore detect non-acetylated α1A tubulin in microtubules also populated with acetylated α3 tubulin. Using immunofluorescence microscopy, α1A tubulin has been found in microtubules in the amoebal and flagellate cytoskeleton, the flagellar axoneme, and amoebal and plasmodial mitotic spindles (Diggins and Dove, 1987; Sasse *et al.*, 1987). Given that α1A tubulin is expressed in the plasmodium, and that expressed tubulin gene products are usually not excluded from any microtubular organelles, it is likely that α1A tubulin is also utilized in plasmodial microtubules.

The α3 polypeptide can be distinguished by its unique electrophoretic mobility on two-dimensional gels (Burland *et al.*, 1983). The antibody 6–11B–1 (Piperno and Fuller, 1985), which specifically recognizes an α-tubulin epitope only when residue Lys_{40} is acetylated, facilitates specific detection of acetylated α-tubulins such as α3 tubulin in microtubular organelles in individual fixed cells. The α3 tubulin in *P. polycephalum* is present principally in microtubules of the flagellate (Diggins and Dove, 1987; Sasse *et al.*, 1987), both in cytoplasmic microtubules of the flagellar cone and in axonemal microtubules of flagella. In amoebae, α3 tubulin has been detected only in centriole-associated MTOCs, and not in the microtubules of the cytoskeleton or mitotic spindle (Diggins and Dove, 1987; Sasse *et al.*, 1987). Thus, α3 tubulin appears to be located in the more stable classes of microtubules found in *P. polycephalum*. When amoebae develop into plasmodia, α3 tubulin is present at early stages in the MTOCs; at later stages of development and in mature plasmodia, α3 tubulin is no longer detectable (Solnica-Krezel *et al.*, 1990) either by Western blotting of whole cell lysates or by immunofluorescence microscopy. Since the α1A-tubulin substrate for acetylation, containing residue Lys_{40}, is present in the plasmodium (Cunningham *et al.*, 1993), it would seem either that acetylase is absent or that de-acetylation is highly efficient in the plasmodium.

2. *α1B and α2B Tubulins*

The α1B- and α2B-tubulin polypeptides, the products of *altB(N)* and *altB(E)*, respectively, each have a glycine residue at position 44 which precludes KMP-1 reactivity (Walden *et al.*, 1989a). The presence of α1B tubulin, which virtually co-electrophoreses with α1A tubulin, is conveniently inferred by the presence of an α-tubulin that does not react with the

KMP-1 antibody (Birkett *et al.*, 1985a,b), although this criterion runs the risk of mistaking for α1B tubulin the products of other genes, such as *altC* or *altD*, whose products are not known. The α2B polypeptide is readily detected by its unique electrophoretic mobility (Burland *et al.*, 1983). Expression of α1B and α2B tubulins appears to be co-ordinate, and has so far been detected only in the plasmodium (Green *et al.*, 1987; Monteiro and Cox, 1987b; Walden *et al.*, 1989b). Isotype-specific polyclonal antibodies reveal the presence of α1B and α2B tubulins in plasmodia (Walden *et al.*, 1989b), and analysis of the tubulins contained within plasmodial spindles confirms the presence of α2B tubulin as well as an α1 tubulin (Paul *et al.*, 1987).

3. *β1A and β1B Tubulins*

For wild-type amoebae, β1A and β1B tubulins co-migrate on two-dimensional gels (Burland *et al.*, 1984). However, the *benD210* mutation, which confers resistance to antitubulin benzimidazole drugs, causes a structural alteration in the β1B-tubulin polypeptide, giving it a unique, altered electrophoretic mobility (Burland *et al.*, 1984). Using this distinction, and isolating cytoskeletons from amoebae and mitotic spindles from synchronous plasmodia, Paul *et al.* (1989) showed that β1B tubulin is utilized both in cytoskeletal microtubules of amoebae and intranuclear mitotic-spindle microtubules of plasmodia. Thus, the *betB* gene product is found in two classes of microtubular organelles, and in two distinct cellular compartments. Hence, there is no evidence for a specific function for the *betB* gene product in one class of microtubules. The β1A-tubulin isotype is also found in amoebal cytoskeletons (Paul *et al.*, 1989) in a lower abundance than β1B, consistent with the lower expression of *betA* relative to *betB* in amoebae (Table 1). It is not known whether β1A or β1B tubulin is present in the flagellum, although both genes are expressed in flagellates so that both β1-tubulins probably have an opportunity to participate in this structure.

When tubulin from amoebae from *P. polycephalum* is assembled *in vitro*, β1A and β1B tubulins assemble into microtubules with a stoichiometry similar to that in cells whence they were purified (Foster *et al.*, 1987). Moreover, tubulin from amoebae carrying the *benD210* mutation assembles into microtubules *in vitro* in the presence of antitubulin benzimidazoles to which the mutant is resistant (Foster *et al.*, 1987). Remarkably, the stoichiometry of the mutant β1B-210 and non-mutant β1A tubulins is similar when assembly *in vitro* occurs in the presence or absence of benzimidazoles (Foster *et al.*, 1987). Thus, even under artificial strong selection in the laboratory, there seems to be no preferential association of one of these two β-tubulins with assembled microtubules.

4. β2 Tubulin

The β2-tubulin isotype is distinct in electrophoretic mobility and immunogenicity, allowing analysis of its distribution by both biochemical and immunological techniques. Expression of β2 tubulin, principally in plasmodia and not in amoebae or flagellates (Solnica-Krezel *et al.*, 1990), normally restricts the spectrum of microtubular organelles in which this protein has the opportunity to function. Originally, we believed that this expression pattern restricted β2 tubulin mainly to mitotic spindles in plasmodia (Burland *et al.*, 1988), but detection of β2 tubulin in cytoplasmic microtubules in developing plasmodia (Solnica-Krezel *et al.*, 1990) leads us to question this view. Further, it is likely that β2 tubulin would be used in the cytoplasmic microtubules recently detected in mature plasmodia (Salles-Passador *et al.*, 1991). Thus, there are opportunities for β2 tubulin to assemble into several classes of microtubule.

Using the distinct electrophoretic mobility of β2 tubulin, Paul *et al.* (1987, 1989) demonstrated its presence in mitotic spindles isolated from plasmodia. A polyclonal antibody specific for β2 tubulin (Diggins-Gilicinski *et al.*, 1989) revealed the distribution of the antigen using immunofluorescence microscopy and Western-blotting experiments. It is now clear that β2 tubulin can assemble *in vivo* into the microtubules of the cytoskeleton, the astral mitotic spindle, the MTOCs and even flagella (Diggins-Gilicinski *et al.*, 1989; Solnica-Krezel *et al.*, 1990, 1991), as well as into the intranuclear anastral mitotic spindle. Thus, there is no direct evidence that β2 tubulin has a specific role in a particular microtubular function.

D. FUNCTION OF MULTIPLE TUBULINS

Acetylated α3 tubulin is the only tubulin isotype in *P. polycephalum* for which there is evidence of preferential utilization in particular organelles. Its association with more stable microtubules is dramatically preferential in amoebae, where α3 tubulin appears exclusively in the MTOC, and not in cytoskeletal microtubules. In contrast, this isotype is present in cytoskeletal microtubules of the flagellate, as well as in the flagellar axoneme. This distribution of acetylated α-tubulin in different microtubules of the slime mould is analogous to the distribution of acetylated α-tubulin in a wide variety of organisms, where this protein is found in more stable classes of microtubules (Ledizet and Piperno, 1991). It seems that acetylation of the Lys_{40} residue is a characteristic that evolved before divergence of major eukaryotic groups, having been observed in members of both the kingdoms Protista and Animalia. The absence from fungi may reflect the restricted mobility functions of these organisms, notably absence of flagella.

The lack of an obvious functional specificity for different tubulin gene products in *P. polycephalum* is typical of a wide variety of organisms (Sullivan, 1988). And, as might be expected from the conserved nature of α1A and β1 tubulins from the slime mould, they can assemble *in vivo* into microtubules in mammalian cells (Prescott *et al.*, 1989). However, experiments which examine only assembly of proteins into microtubules do not elucidate whether different microtubular organelles function normally; it is conceivable, for example, that β2 tubulin is functionally deleterious on the rare occasions when it is incorporated into the flagellum or the astral mitotic spindle during plasmodium development (cf. Hoyle and Raff, 1990).

Regarding the function of multiple tubulin gene products in a single organism, it is possible to observe certain correlations for the gene family in *P. polycephalum*. The *betC* gene exhibits a distinct sequence that places its product, β2 tubulin, into the class known as divergent β-tubulins; two others in this class are the murine β1 tubulin and the β3 tubulin from *Aspergillus nidulans* (see Fig. 2). These three (and other) divergent β-tubulins are restricted in their pattern of expression to specific cell types, and thereby they are utilized in only a subset of microtubular organelles found in the respective organisms (Burland *et al.*, 1988). The murine β1 tubulin is expressed principally in erythrocytes, which limits its utilization to the marginal band of microtubules in these cells (Wang *et al.*, 1986); the β3 tubulin from *A. nidulans* is expressed largely in conidiating tissues, where it is utilized mainly in mitotic spindles (Weatherbee *et al.*, 1985); and the β2 tubulin from *P. polycephalum* is expressed principally in the plasmodium, where it is limited to the mitotic spindle and presumably whatever cytoskeletal microtubules are present. Thus, fewer functional constraints may be placed on these divergent β-tubulins, allowing some degree of neutral drift (Burland *et al.*, 1988). In *Dr. melanogaster*, divergent β3 tubulin is normally expressed at only a low level in a restricted set of cell types (Kimble *et al.*, 1989). When expressed beyond a 20% threshold level in the testis, assembly of microtubules in axonemes is disrupted, indicating that divergent β3 tubulin does not function properly in axonemal microtubules (Hoyle and Raff, 1990). For *P. polycephalum*, the expression pattern for β2 tubulin leaves this isotype normally absent from the flagellar axoneme, highlighting the view that utilization of a particular tubulin in the flagellar axoneme may be a powerful force for conservation of primary sequence (Little *et al.*, 1986; Singhofer-Wowra *et al.*, 1986a); this may reflect the greater number of specific protein–protein interactions in the flagellum than in other microtubular organelles. Thus, it may be specifically the absence from the flagellum that permits more neutral drift in β2 tubulin sequence than in tubulins used in the flagellum. Conservation of the β1 tubulins in *P. polycephalum* is consistent with this view. Despite their

distinct expression patterns, both β1A and β1B tubulins are probably utilized in the flagellar axoneme, and the sequences of these two β-tubulins show much more similarity to tubulins in other organisms that possess flagella (or cilia) than they do to β2 tubulin in the slime mould (or to other divergent β-tubulins). In fact, the α-tubulin gene family in *P. polycephalum* mirrors the β-tubulin family, although in a less dramatic way. The more diverged α1B and α2B tubulins are expressed only in the plasmodium, where flagella have not been found, while the more conserved α1A tubulin is the principal, and possibly only, α-tubulin gene product in amoebae and flagellates (Cunningham *et al.*, 1993). Conservation of primary sequence among tubulin gene families is notable among other protists that possess flagella (Silflow, 1991).

Whether or not distinct tubulin gene products are used for specific functions, the fact remains that multiple α- and β-tubulin genes are the norm among eukaryotes. It is clear from studies of the tubulin gene family in *P. polycephalum*, as well as from tubulin gene families from other eukaryotes, that multiple tubulin genes are expressed in distinct patterns in different cell types. The flexibility this arrangement offers for differential gene expression may be a characteristic that was selected for during evolution, resulting in multiple tubulin genes for most eukaryotes (Raff, 1984; Paul *et al.*, 1992).

E. MICROTUBULE-ASSOCIATED PROTEINS

The lack of evidence for distinct tubulin gene products determining specific microtubular functions points to other potential molecules for this role, notably microtubule-associated proteins (MAPs; Olmsted, 1986). Microtubule-associated proteins were originally defined as those that copurify when mammalian brain tubulins are purified by their capacity for self-assembly *in vitro* (Borisy *et al.*, 1975). When tubulins from *P. polycephalum* were first purified in this way, the high molecular-weight MAPs observed in brain-tubulin preparations were absent, although smaller proteins with molecular weights of 49,000, 57,000 and 59,000 were observed (Roobol *et al.*, 1980). Phosphocellulose chromatography of the assembly-purified tubulin from *P. polycephalum* eliminates its self-assembly capacity, which can be restored by addition of brain MAPs, suggesting that those from the slime mould are present in the assembly-purified material and are removed, like brain MAPs would be, by chromatography (Roobol *et al.*, 1980).

Albertini *et al.* (1990) found six candidates for higher molecular-weight MAPs after adding the taxol analogue baccatine to the concentrated amoebal extracts used for tubulin purification. One such MAP resembles

kinesin, while another, a 125 kDa polypeptide, promotes microtubule assembly *in vitro* from mammalian brain tubulin and protects microtubules from disassembly upon dilution. The 125 kDa MAP is, like certain mammalian brain MAPs, heat-soluble, suggesting conservation of more than simply microtubule-binding capacity. However, this 125 kDa polypeptide does not cross-react with antibodies to the well-characterized mammalian brain MAP2 and τ proteins (Albertini *et al.*, 1990). Nevertheless, now that MAPs have been clearly identified in *P. polycephalum*, more detailed analyses of their structures and functions will be possible, and elucidation of the evolutionary origins of these important proteins should follow.

F. THE CYTOSKELETON IN DEVELOPMENT

The microtubule cytoskeleton is remarkably different in amoebae, flagellates and plasmodia. It is of interest to determine how cytoskeletal changes occur during developmental transitions between these cell types, not only for an understanding of cytoskeletal dynamics, but also to gain insights into fundamental developmental processes. Protists like *P. polycephalum* are amenable to these studies because individual transitions generally occur by conversion of an entire population from one cell type to another; dissections or cell-separation schemes are not needed to determine specific developmental changes. With *P. polycephalum*, simple manipulations of culture conditions can be used to induce either the amoeba–flagellate transition or the amoeba–plasmodium transition.

1. The Amoeba–Flagellate Transition

The amoeba–flagellate transition is induced in the laboratory by suspending amoebae in non-nutrient solutions, and can be reversed by returning cells to a nutrient medium or on to a solid substrate. The distinction between amoeba and flagellate in *P. polycephalum* is well defined in terms of cytoskeletal architecture, as seen by both immunofluorescence and electron microscopy (e.g. Aldrich, 1968, 1969; Pagh and Adelman, 1988; Havercroft and Gull, 1983; Wright *et al.*, 1988). Uyeda and Furuya (1985) made a detailed immunofluorescence study of cytoskeletal changes during this transition using antitubulin antibody to detect microtubules, and nitrobenzdiazole–phallacidin to detect microfilaments, in the same cells, simultaneously. They recognized seven distinct stages in the developmental sequence amoeba → flagellate → amoeba, compiling the sequence of events from analysis of individual cells fixed for microscopy over the time-course of development (Fig. 3). In most cells, the first major events are migration

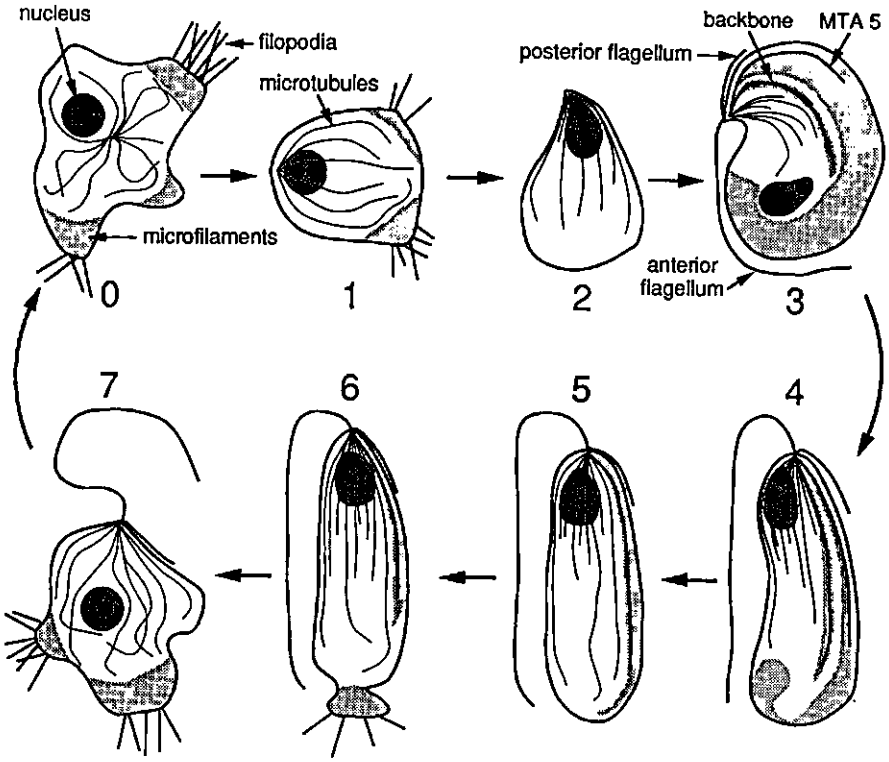


FIG. 3. The amoeba–flagellate and flagellate–amoeba transitions. Shading in the cytoplasm indicates regions showing immunofluorescence staining with nitrobenzdiazole–phallacidin (microfilaments) and curved lines indicate antitubulin antibody fluroescence (microtubules). MTA 5 indicates microtubular array 5. Redrawn from Uyeda and Furuya (1985).

of the nucleus to the anterior end of the cell, concomitant with development of the anterior cone of microtubules; this is usually followed by extrusion of flagella (Fig. 3). The nuclear migration is sensitive to microfilament poisons but not to microtubule poisons, suggesting that migration is mediated by an actin-generated force (Ohta *et al.*, 1991). This contrasts with nuclear migration in fungi, which is sensitive to microtubule poisons, and requires functional tubulin (Oakley and Morris, 1980). Analysis of *P. polycephalum* strains with abnormal flagellar organization suggests that the force for nuclear migration acts on the centrosome, which is connected to the nucleus, rather than on the nucleus itself (Ohta *et al.*, 1991). Around the time of flagellar extrusion, a prominent ridge of microfilaments develops

around the dorsal surface of the cell; the function of the ridge is not clear, and it disappears by stage 5, which Uyeda and Furuya (1985) consider the mature flagellate (Fig. 3). Another interesting structure observed during the amoeba–flagellate transition is the "backbone", which on light-microscope resolution appears to be made up of coincident bundles of microtubules and microfilaments (Fig. 3). In contrast to the microfilament ridge, this backbone persists in the mature flagellate. Pharmacological and electron-microscopic evidence suggests that movement of the backbone structure involves interaction between microfilaments and microtubules, and Uyeda and Furuya (1989) propose that ATP induces reciprocal sliding between microtubules and microfilaments in the backbone. The precise function of the backbone structure, however, remains to be determined.

Along with increases in acetylation of α1A tubulin to make α3 tubulin, substantial increases in levels of tubulin RNA occur during the amoeba–flagellate transition in *P. polycephalum* (Green and Dove, 1984; Paul *et al.*, 1992; Cunningham *et al.*, 1993), as occurs in algae after loss of flagella (Lefebvre and Rosenbaum, 1986). For α- and β-tubulin, a 5–7 fold increase in message occurs (Green and Dove, 1984); for α-tubulin, the increase is mostly or entirely in *altA* transcript, since *altA* is the principal or only α-tubulin gene expressed (Cunningham *et al.*, 1993). But for β-tubulin, two genes are expressed, namely *betA* and *betB* (see Table 1), with the level of *betB* transcript remaining approximately constant while that of *betA* transcript increases 100-fold from an initial low level (Paul *et al.*, 1992).

The burst of tubulin expression during the amoeba–flagellate transition is a pulse rather than a sustained increase. Before all of the cells in the population possess mature flagella, the level of *altA* transcript is already on the decline (Paul *et al.*, 1992). The level of actin transcript declines only slightly during flagellate development, contrasting with flagellate development in the protist *Naeglaeria gruberi*, where a dramatic fall in the level of actin transcript occurs (Lai *et al.*, 1984).

The amoeba–flagellate transition is reversible, in that flagellates revert to amoebae, although the sequence of events in the flagellate–amoeba transition is not a simple reversal of amoeba–flagellate transition. Rather, distinct morphologies are detectable with respect to organization of microtubules and microfilaments (Fig. 3; Uyeda and Furuya, 1985). The flagellar axoneme retracts into the cytoplasm, a process that takes only a few seconds (Glyn and Gull, 1990), and appears to persist there for some time. Judging from immunofluorescence, de-acetylation of microtubules takes perhaps 45 minutes (Glyn and Gull, 1990).

The amoeba–flagellate transition depicted in Fig. 3 shows a discrete pattern of development by progressive morphological change. However, Uyeda and Furuya (1985) noticed that, even after extended incubation in

a non-nutrient solution, a few cells in populations undergoing the transition were in stages 3 and 4, rather than the expected stage 5 (Fig. 3). They suggested that, while this could mean that some cells stay in these intermediate stages for longer times than others, it could instead reflect reversibility of the developmental sequence: stage 3 → stage 4 → stage 5. Further, in analysing individual cells for their microtubule and microfilament structures during the transition, Uyeda and Furuya (1985) noted that some cells did not show the expected characteristics for the developmental sequence they described. Pagh and Adelman (1988) similarly detected apparent plasticity in changes in the cytoskeletal pattern during the transition. For example, under optimum conditions for flagellate development, occasional cells can be found from which flagella are extruded but the microtubule cone around the nucleus is not formed, inconsistent with the sequence of events shown in Fig. 3 in which the cone is formed before flagellar extrusion (Uyeda and Furuya, 1985). One interpretation is artefactual loss of the cone during fixation. However, another explanation is that, although development progresses by the same sequence of events in most cells, it follows alternative sequences of events in a minority of cells. Where such alternatives are not substantially detrimental, variability in development may be a characteristic of natural populations.

In the future, it would be interesting to ascertain expression patterns for other cytoskeletal-protein genes during the transition; probes for both myosin (Kobayashi *et al.*, 1988) and profilin (Binette *et al.*, 1990) are available and could be used to determine expression of these genes. Further elucidation of functional relationships between cytoskeletal changes and flagellar motility should result from analysis of conditional mutants defective for the transition (Mir *et al.*, 1979), and from disruption of individual members of cytoskeletal gene families, a technique that has recently become feasible in *P. polycephalum* (see Section VI.C).

2. *The Amoeba–Plasmodium Transition*

The development of amoebae into plasmodia, the amoeba–plasmodium transition, is a dramatic transition in cell type in the life cycle of *P. polycephalum*. The developmental changes in the amoeba–plasmodium transition are most readily observed in apogamic strains, where cinematographic analysis indicates that a single haploid amoeba can develop into a plasmodium without cell or nuclear fusion (Anderson *et al.*, 1976; Bailey *et al.*, 1987).

The amoeba–plasmodium transition is induced in the laboratory by growing apogamic amoebae to a high cell density on lawns of bacteria.

Developing cells can be fractionated on glass-bead columns to obtain a population of largely uninucleate cells committed to development (Blindt *et al.*, 1986) but, under optimum culture conditions, synchrony of development is satisfactory for most applications (Solnica-Krezel *et al.*, 1988). A diffusible inducer of plasmodium development is released at a high cell density (Youngman *et al.*, 1977; Nader *et al.*, 1984), but it has not yet been characterized.

The sequence of changes related to the cytoskeleton during the amoeba–

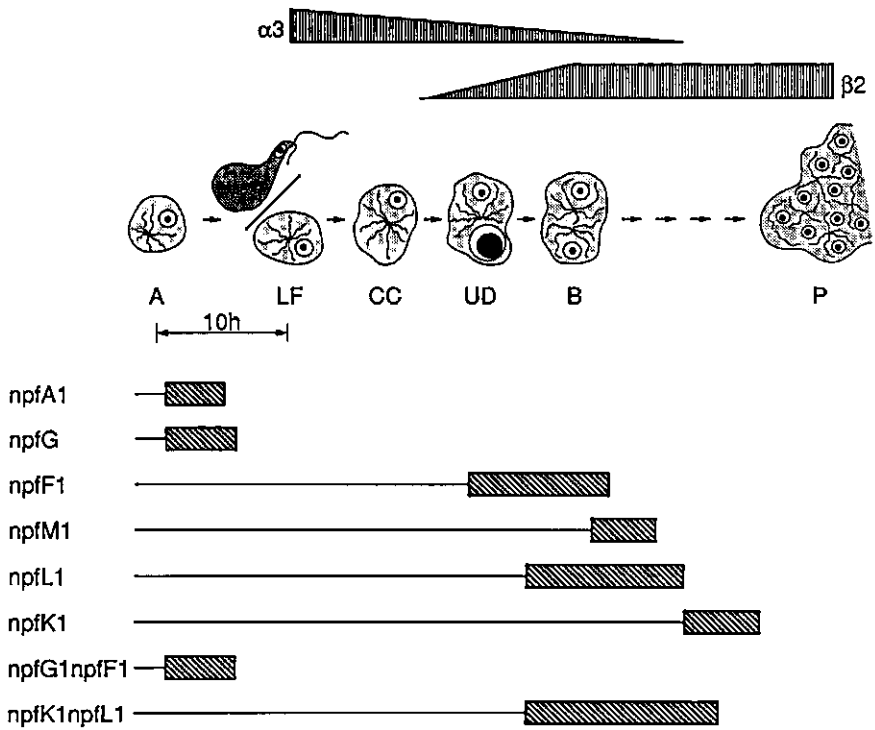


FIG. 4. The amoeba–plasmodium transition. The shaded boxes at the top indicate levels of α3 and β2 tubulins in microtubules as detected by immunofluorescence. Below are shown cellular events in the transition: A, amoeba, with nucleus and microtubules; LF, stage at which cells lose the ability to undergo the amoeba–flagellate transition; CC, stage of cell commitment to plasmodium development; UD, uninucleate developing cell that has gained the ability to ingest amoebae and cysts; B, binucleate cell; P, young plasmodium. The double-headed arrow defines a time interval of 10 hours for development of strain CL, though timing of events in other strains may vary. The shaded boxes at the bottom indicate stages of development at which single and double mutants arrest development.

plasmodium transition in the apogamic strain CL is summarized in Fig. 4. Initiation of apogamic development is thermosensitive, but, once initiated, development can proceed at any growth temperature. The first detectable change after the thermosensitive period is loss of the ability to undergo the amoeba–flagellate transition (Blindt *et al.*, 1986), which occurs about one amoebal intermitotic time after the previous cell division. The next key event detected is commitment to development, the stage beyond which cells develop into plasmodia even when removed from the high-density conditions and plated individually (Youngman *et al.*, 1977). Commitment occurs in uninucleate cells of strain CL (Burland *et al.*, 1981) about 3–5 hours after loss of the ability to flagellate (Bailey *et al.*, 1987), but before major changes in the cytoskeleton are evident (Solnica-Krezel *et al.*, 1990, 1991). The timing of the commitment event places it on average around the middle of the extended cell cycle that distinguishes the first developmental cell cycle from the typical amoebal cell cycle (Bailey *et al.*, 1987; Fig. 4). The time of commitment corresponds to the stage at which wild-type cells acquire the ability to grow axenically (Blindt *et al.*, 1986), one of the first clear physiological characteristics of the plasmodium. Around the same time, uninucleate developing cells acquire other plasmodial characteristics, including ability to ingest amoebae (Solnica-Krezel *et al.*, 1990).

In heterothallic myxomycete development, cell fusion appears to define the point of commitment (Shipley and Holt, 1982), and flagellates as well as amoebae of compatible mating types are capable of cell fusion (Ross, 1957; Bailey *et al.*, 1990). Thus, in heterothallic development, loss of ability to flagellate occurs no sooner than the time of commitment, rather than some hours before as in apogamic development of strain CL. Heterothallic amoebal cell fusion can occur at any stage in the cell cycle, and the two cells that fuse need not be at the same stage (Bailey *et al.*, 1990). Nevertheless, an extended cell cycle is observed after cell fusion, with nuclei fusing about two hours after the amoebae fuse; subsequently, events are remarkably similar to apogamic development (Bailey *et al.*, 1990).

The extended developmental cell cycle is over twice as long as an amoebal cell cycle, for both apogamic and heterothallic development (Bailey *et al.*, 1987, 1990). At the end of this cell cycle, the uninucleate developing cell, substantially larger than an amoeba, is ready for mitosis. A key question is when during the amoeba–plasmodium transition the pattern of mitosis switches from the amoebal to the plasmodial type, since this is an essential change for sustaining the multinucleate state. One difficulty in analysing this problem is that it is impossible to determine whether a cell is committed simply by looking at it under the microscope, since commitment is defined operationally as the point beyond which cells will develop into plasmodia,

rather than amoebal colonies, when plated at a low density (Youngman *et al.*, 1977). However, by correlative studies of strain CL, it is clear that β2 tubulin can, with minor exceptions, first be detected in microtubules just after the time of the commitment event (Fig. 4; Solnica-Krezel *et al.*, 1990), so that the presence of β2 tubulin in microtubules can be used with reasonable reliability to determine whether a cell is committed to development. Thus, mitotic spindles in most committed cells should contain β2 tubulin. Furthermore, the extranuclear MTOC that nucleates the amoebal mitotic spindle contains α3 tubulin but the MTOC that nucleates the intranuclear mitotic spindle of the mature plasmodium does not (Diggins and Dove, 1987; Sasse *et al.*, 1987). Thus, α3 and β2 tubulins can be used as developmental markers to investigate changes in mitosis during development (Fig. 4). Using these criteria, four types of mitosis can be observed in committed cells (Solnica-Krezel *et al.*, 1991). These are:

(a) Amoebal mitosis, which in developing cells is similar to mitosis in normal amoebae, except that β2 tubulin can be present. The spindle is organized by extranuclear, α3 tubulin-positive MTOCs, the spindle poles radiate astral microtubules, and cytokinesis usually follows mitosis.

(b) Plasmodial mitosis occurs within the nuclear membrane, is organized by intranuclear MTOCs that lack α3 tubulin, and is anastral. However, in developing cells, extranuclear MTOCs may also be present, nucleating cytoplasmic microtubules, while some of these MTOCs divide in the usual manner during mitosis, even though they take no part in the spindle. Cytokinesis does not occur.

(c) Chimeric mitosis is characterized by an astral mitotic spindle nucleated by MTOCs that apparently lack α3 tubulin. Microtubule-organizing centres that are α3 tubulin-positive are usually present outside the spindle, nucleating cytoplasmic microtubules; these additional MTOCs often appear to block cytoplasmic furrow development, leading to a cell with only a partial furrow, probably impairing cytokinesis. Chimeric mitosis probably results from initiation of intranuclear mitosis followed by nuclear-membrane breakdown during mitosis (Solnica-Krezel *et al.*, 1991).

(d) Star microtubule structures, which may be defective spindles, consist of a condensed mass of chromatin at the centre of a radial array of microtubules, and usually contain two α3 tubulin-positive MTOC-like structures near the middle of the array.

As a population of cells develops, the frequencies of the four different patterns of mitosis change (Solnica Krezel *et al.*, 1991). Early in development, only amoebal mitoses are observed but, when committed cells first

appear in the population, plasmodial and star mitoses also become apparent. Chimeric mitoses have not been detected in amoebae, and they remain rare throughout development. The frequency of plasmodial and star mitoses continues to increase, while the frequency of amoebal mitoses decreases as the proportion of committed cells increases in the developing population. Eventually, when the binucleate stage of development is reached, all subsequent mitoses appear to be of the plasmodial type (Solnica-Krezel *et al.*, 1991).

It seems likely that both the plasmodial and amoebal types of mitosis, and probably also chimeric mitosis, produce viable somatic progeny, but the fate of cells undergoing star mitoses is unclear. Star mitoses comprise up to 20% of mitoses at one stage of the amoeba–plasmodium transition (Solnica-Krezel *et al.*, 1991) so, if this is a non-viable event, it is a significant cost of development. Such loss may be analogous to elimination of abnormal nuclei during development of embryos of *Dr. melanogaster* (Sullivan *et al.*, 1990).

From the binucleate-cell stage onwards, the young plasmodia of apogamic strain CL increase in mass not only by growth but also by fusion with other multinucleate (and, rarely, large uninucleate) cells. Such cell fusions are a clear plasmodial characteristic, under control of three *fus* loci (Poulter and Dee, 1968). In contrast to the action of the *matB* gene, where allelic difference promotes cell fusion, similarity at the *fus* loci is required for plasmodial fusions. Although cytoplasmic microtubules are readily detectable in young plasmodia, the MTOCs that nucleate them no longer contain α3 tubulin and, as the cells increase in size, the MTOCs are barely apparent, while cytoplasmic microtubules become more diffuse.

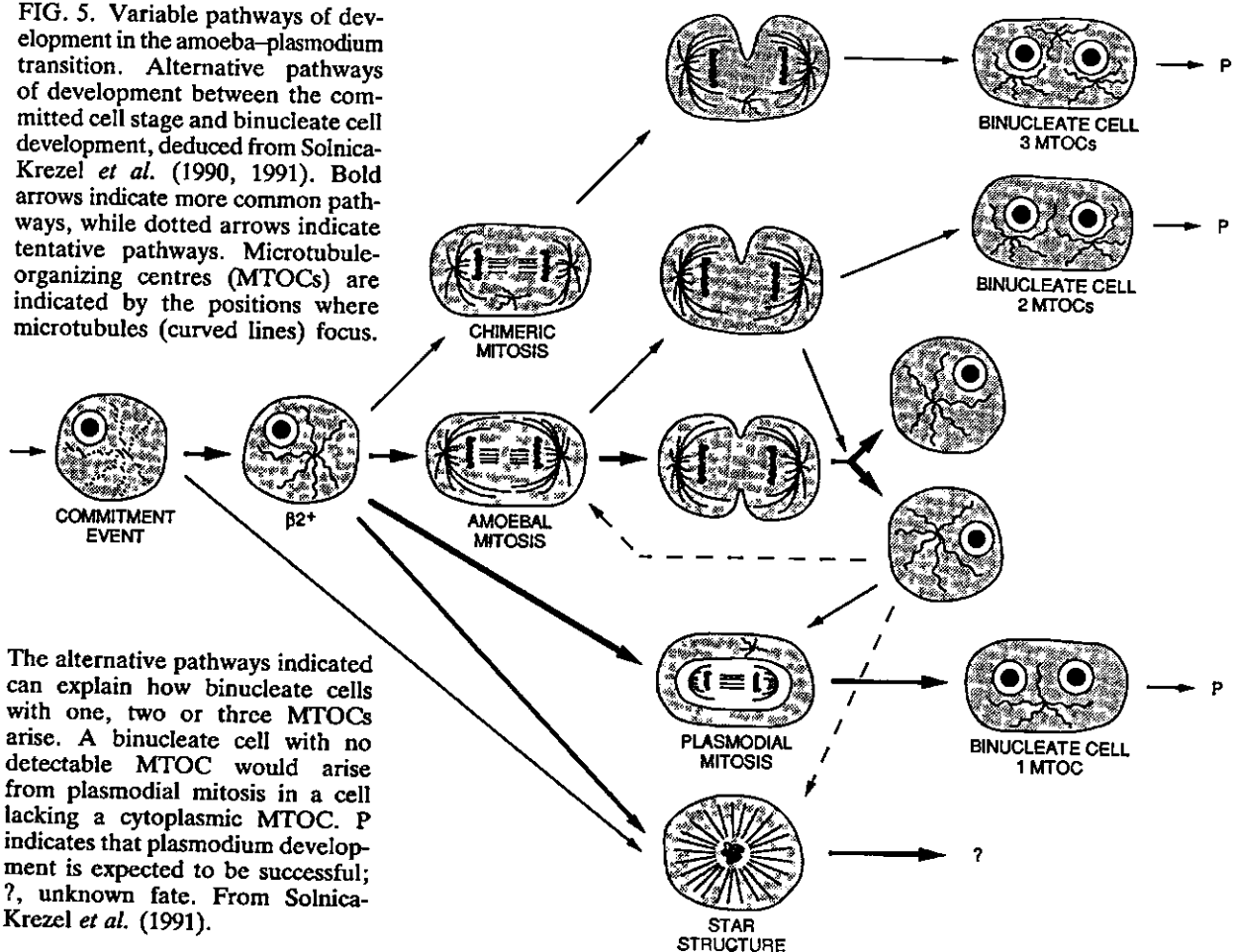
The frequency changes and nature of the different classes of mitosis in developing populations of strain CL suggest that changes in organization of the mitotic spindle occur gradually during the amoeba–plasmodium transition, probably reflecting gradual loss of factors required for amoebal mitosis and gradual accumulation of factors required for plasmodial mitosis. The fact that different types of mitosis are found at the same time in a population of developing cells in part may reflect asynchrony of the transition, but biological variability also probably contributes to these differences. For example, if commitment does not always occur at the same point in the cell cycle, then commitment-dependent accumulation of plasmodial factors would vary between different cells by the time mitosis occurs.

The switch from amoebal to plasmodial mitosis raises a key question as to the organization of the two types of spindles: are the spindle organizers modified forms of the same basic structure or are they independent structures? Wild-type haploid amoebae carry one major MTOC, termed

MTOC1, which nucleates cytoskeletal microtubules during interphase and microtubules of the spindle during amoebal mitosis. Some diploid amoebal strains, constructed by abortive crossing between, for example, *matA1–matB1* and *matA1–matB3* strains, carry two or three of these MTOCs (Wright *et al.*, 1988). The presence of the extra MTOCs correlates with an increased frequency of normally rare multipolar mitoses, and the ranges of the frequencies are distinct for strains carrying one, two or three MTOCs. Akhavan-Niaki *et al.* (1991) crossed diploid amoebae carrying different numbers of MTOCs and examined mitoses in the resulting tetraploid plasmodia for multipolar frequency, using this frequency as a proxy for the number of spindle-organizing centres. These indirect data are consistent with the hypothesis that crossed plasmodia contain the same number of spindle-organizing centres present in one or other of the amoebal parents, and neither the sum nor the average of the two. Selfed, haploid wild-type amoebae with one MTOC also give rise to plasmodia that contain a single spindle-organizing centre, but Akhavan-Niaki *et al.* (1991) observed that one strain of amoeba with three MTOCs, when selfed, gave rise to plasmodia with frequencies of multipolar mitoses typical of strains with three MTOCs. These results are consistent with structural continuity of MTOCs throughout development. Alternatively, a similar number of MTOCs in amoebae and plasmodia could simply indicate that a mechanism that regulates their number is conserved in amoebae and plasmodia.

This issue has also been addressed by light-microscope observation of MTOCs during the amoeba–plasmodium transition. For heterothallic development, fusion of two amoebae results in a cell with two nuclei and two major MTOCs (Bailey *et al.*, 1990). Subsequently, the number of major cytoplasmic MTOCs observed in each nucleus falls, with most zygotes containing one or none. Among binucleate developing plasmodia, 20% contain two MTOCs (i.e. one per nucleus), 22% contain one MTOC and 58% contain none (Bailey *et al.*, 1990). These data suggest that, at the light-microscope level, the major cytoplasmic MTOCs, which in amoebae reorganize to nucleate the spindle at mitosis, are lost gradually during development, and indicate that, in many cells, all of them are lost. Thus, structural continuity of amoebal and plasmodial spindle-organizing centres cannot be demonstrated from these data. Obviously, an MTOC is needed in these cells that apparently lack one, to organize the spindle at mitosis, but, as in the mature plasmodium, this would appear to be a structure which has not been detected during the interphase (Paul *et al.*, 1987). Is it possible that the organizer for the intranuclear plasmodial spindle is synthesized *de novo* for each mitosis? Now that γ-tubulin has been established as a molecular marker for MTOCs, it would be worth seeking the plasmodial spindle-organizing centre with anti-γ-tubulin antibodies.

FIG. 5. Variable pathways of development in the amoeba–plasmodium transition. Alternative pathways of development between the committed cell stage and binucleate cell development, deduced from Solnica-Krezel *et al.* (1990, 1991). Bold arrows indicate more common pathways, while dotted arrows indicate tentative pathways. Microtubule-organizing centres (MTOCs) are indicated by the positions where microtubules (curved lines) focus.



The alternative pathways indicated can explain how binucleate cells with one, two or three MTOCs arise. A binucleate cell with no detectable MTOC would arise from plasmodial mitosis in a cell lacking a cytoplasmic MTOC. P indicates that plasmodium development is expected to be successful; ?, unknown fate. From Solnica-Krezel *et al.* (1991).

However, in a variety of cells, anti-γ-tubulin immuno-staining of MTOCs is most intense at mitosis (Zheng *et al.*, 1991), so the disappearing plasmodial organizing centre may yet remain cryptic.

For apogamic strains, there appears to be only one major MTOC in each uninucleate developing cell but, in binucleate cells, there can be as many as three MTOCs, depending upon the type of mitosis that gives rise to the binucleate cell (Fig. 5; Solnica-Krezel *et al.*, 1990, 1991). Clearly, control over the number of organizing centres is not tightly regulated during the amoeba–plasmodium transition. However, it is possible to detect α3 tubulin-containing, extranuclear MTOCs of the amoebal type in the same cell that contains intranuclear, α3 tubulin-negative spindle-organizing centres of the plasmodial type (Solnica-Krezel *et al.*, 1991). This is difficult to reconcile with a proposal (Akhavan-Niaki *et al.*, 1991) that the plasmodial spindle-organizing centre arises from the major amoebal organizing centre MTOC1, unless only a subset of amoebal MTOCs act as progenitors for the plasmodial one.

It is clear that development of amoebae into plasmodia can occur via different pathways (Fig. 5). The pathway shown in Fig. 4 appears to be the most common, but other pathways are possible. For example, cytoplasmic MTOCs may or may not contain α3 tubulin by the binucleate cell stage of development. Perhaps more remarkable is the option for a developing cell to undergo both karyokinesis and cytokinesis before binucleate cell development occurs, rather than completing the extended cell cycle by forming a binucleate cell (Solnica-Krezel *et al.*, 1991; Bailey *et al.*, 1992).

Whether the mitosis that gives rise to a binucleate cell is of the amoebal or plasmodial type appears to depend on the status of numerous developmental variables at the time mitosis is initiated. Variation in the length of the cell cycle itself may account for much of the variability observed in the pathway for cytoskeletal changes. Such variability is reminiscent of occasional variations seen during the amoeba–flagellate transition, implying that the asignment of a strict deterministic sequence of events to execute a specific developmental process may be inappropriately rigid.

G. THE CYTOSKELETON IN DEVELOPMENTAL MUTANTS

1. Mutations Linked to matA

Isolation of haploid mutant apogamic (selfing) strains from heterothallic strains facilitates selection for mutants defective in plasmodium development (Wheals, 1973; Cooke and Dee, 1974). The most commonly used apogamic mutation, obtained from a *matA2* strain, is *gadAh* (*gad*, greater asexual differentiation). The *gadAh* mutation has not been separated from

matA by recombination (Anderson *et al.*, 1989). Names of loci identified through mutants of *gadA* strains that no longer self are usually referred to as *npf* (no plasmodium formation).

In early genetic screens, the most common developmental block detected resulted from mutation in one of two complementation groups, namely *npfB* or *npfC*, tightly linked to, or perhaps integral units of, *matA* (Anderson *et al.*, 1989). The mating specificities in most *npf* mutants appear unchanged, despite the fact they were isolated through the two-step mutational pathway heterothallic → apogamic → Npf. This suggests that *npfB* and *npfC* encode functions separate from the mating specificity function of *matA* (Anderson *et al.*, 1989). Similar barriers to mutational changes in mating specificity are apparent in basidiomycetes (Metzenberg, 1990). Mutations identified in *npfB* and *npfC* appear to prevent initiation of development; cells of these strains cultured under conditions favourable to development remain indistinguishable from amoebae.

2. *Mutations Unlinked to* matA

The first developmental mutation isolated from a *gadAh* strain was *apt-1* (Wheals, 1973), now called *npfF1*, which is unlinked to *matA*. Like most other developmental mutations, *npfF1* is defective for development under all culture conditions. Since *gadAh npf*$^+$ development is thermosensitive, failing to occur at 30°C or above, it is difficult to isolate mutants thermosensitive for the amoeba–plasmodium transition. However, Anderson and Dee (1977) were able to isolate one mutation, *npfA1*, which is thermosensitive for the apogamic transition. This mutation is unlinked to *matA* and is the only mutant allele known for the *npfA* gene. Using modified screening methods to eliminate the original bias towards selecting mutants blocked early in development, several more genes essential for apogamic development have recently been detected, denoted *npfD* through *npfM*, all of which are unlinked to *matA* (R. W. Anderson, personal communication; Solnica-Krezel *et al.*, 1992; Bailey *et al.*, 1992).

The changes that occur in the microtubule cytoskeleton during the amoeba–plasmodium transition are useful cellular markers for the stage of development at which mutants arrest or go wrong. Solnica-Krezel *et al.* (1992) used phase-contrast and immunofluorescence microscopy to analyse the effects of mutations in the *npfA*, *npfF*, *npfG*, *npfK*, *npfL* and *npfM* genes on microtubule organization during the amoeba–plasmodium transition. The results are summarized in Fig. 4.

a. The *npfA1* Mutant

This mutant arrests development at the uninucleate stage and does not lose the ability to form flagellates. Many cells encyst, while mitotic spindles

among active cells all appear to be of the amoebal type. Moreover, β2 tubulin is only sporadically detected in microtubules, and its appearance probably reflects incomplete penetrance of the mutation (Solnica-Krezel *et al.*, 1992). Clearly, *npfA1* is blocked at a very early stage of development.

b. The *npfG1*, *npfG2* and *npfG3* Mutants

These mutants also arrest development at the uninucleate stage, and retain the ability to flagellate. Star-microtubule arrays are occasionally observed in *npfG* cells during abortive development, although mitotic spindles formed are mainly of the amoebal type and rarely if ever contain detectable levels of β2 tubulin. Thus, *npfG* mutants appear to be blocked early in development.

c. The *npfF1* Mutant

Many *npfF1* cells encyst during the abortive development, but some enlarge and a few become binucleate. Some cells lose the ability to form flagellates, some express β2 tubulin, and some lose detectable α3 tubulin from MTOCs. A few of the mitoses observed are of the plasmodial type, indicating that *npfF1* cells arrest later in development than cells with *npfA* and *npfG* mutations. Curiously, occasional *npfF1* cells can be observed, under immunofluorescence optics, to contain thick, α3 tubulin-positive microtubules, a structure generally observed neither in amoebae nor in plasmodia.

d. The *npfM1* Mutant

Development in the *npfM1* mutant progresses as far as small multinucleate cells, many of which have lost α3-tubulin staining and express β2 tubulin. Mitotic spindles of the plasmodial type are readily detected in both uninucleate and binucleate cells, suggesting that development progresses to an advanced stage before arrest. Cinematographic analysis shows that developing *npfM1* cells pass through an extended cell cycle, as do npf^+ apogamic strains, although cell fusions have not been observed in *npfM1* cells, as are observed in development of npf^+ cells by the stage at which the *npfM1* mutation arrests (Bailey *et al.*, 1987).

e. The *npfL1* Mutant

This mutant also develops to the stage where α3 tubulin is lost and β2 tubulin is present in microtubules, although abnormalities become evident

at the uninucleate stage. Spindles either of the amoebal or plasmodial type can be detected, but many of the mitoses are abnormal, including not only the star microtubule arrays seen in the wild type but also some morphologies peculiar to the *npfL1* mutation (Bailey *et al.*, 1992). A remarkable nuclear abnormality involves the tight condensation of chromatin, which cinematography suggests occurs after *npfL1* cells pass through the extended cell cycle. Although this abnormality is seen occasionally in development of *npf* $^{+}$ cells, it is much more common during development of *npfL1* cells. The abnormal cells can grow quite large, but nuclear organization is clearly defective (Bailey *et al.*, 1992). Many *npfL1* cells round up and begin to pulsate, a characteristic that appears as a "boiling" phenotype when viewed cinematographically at high speed (Bailey *et al.*, 1992). Like some *npfF1* cells, microtubules in *npfL1* developing cells often appear under immunofluorescence to thicken dramatically. These abnormal processes appear to result in cell death. This phenomenon may represent a protistan version of apoptosis, an active process long recognized in animal cells, where cell death proceeds through a distinct pathway of morphological changes, including the "boiling" phenotype and disintegration of the nucleus (Bailey *et al.*, 1992).

f. The *npfK1* Mutant

Development in cells with the *npfK1* mutation proceeds to the binucleate cell stage and beyond, with loss of ability to flagellate, loss of α3 tubulin, and appearance of β2 tubulin. Mitosis of the plasmodial type is found in both uninucleate and binucleate cells, and cells with many more nuclei can be detected. However, the cells are morphologically abnormal, and larger cells take on a stringy appearance. Thus, these cells progress farther in development than other mutants.

In addition to focusing on the effects of lesions in specific genes, this work provides information on mechanisms of the transition from the amoebal to the plasmodial type of mitosis. Three aspects of cytoskeletal organization, namely activation of β2-tubulin expression, loss of α3 tubulin, and formation of plasmodial mitotic spindles, were not separated by any of the mutations studied. It is therefore possible that these three events are regulated co-ordinately.

Mutants such as *npfK1* and *npfL1*, which appear to execute several developmental functions successfully, indicate that development occurs on parallel pathways, some of which can continue when others fail. Analysis of double mutants is consistent with this interpretation. For example, cells of the *npfF1 npfG1* double mutant arrest development at a stage characteristic of *npfG* cells (Fig. 4), suggesting that *npfG* function is

executed on a pathway on which *npfF* function is dependent (Solnica-Krezel *et al.*, 1992). By contrast, the *npfK1 npfL1* double mutant arrests with characteristics of both *npfK1* and *npfL1* single mutants, suggesting that the two genes function on parallel rather than dependent developmental pathways. Much of the variation observed in the staging of events in the amoeba–plasmodium transition may reflect flexible timing of events that occur on parallel pathways, while events occurring on the same dependent pathway presumably occur in a stricter sequence.

Control of development by both dependent and parallel pathways of events is analogous to mitotic-cycle control (Hartwell, 1991). It is thought that parallel pathways in the mitotic cycle must eventually be integrated at key check-points (e.g. Nurse, 1991). Variations on pathways of development during the amoeba–plasmodium transition appear to originate during the extended developmental cell cycle. This may reflect a paucity of developmental check-points during most of the cycle, with key check-point(s) being located later in development.

H. OTHER GENES DIFFERENTIALLY EXPRESSED IN DEVELOPMENT

Amoebae and plasmodia show distinct patterns of expression of many genes. It has been suggested that amoebae and plasmodia largely express "different sets of genes" (Gingold *et al.*, 1976; Wheals *et al.*, 1976), based on the observation that plasmodia derived from thermosensitive mutant amoebae did not appear to be thermosensitive and, likewise, thermosensitive mutant plasmodia arose from amoebae that did not express a thermosensitive phenotype. More careful analyses indicate that, in fact, most thermosensitive mutations express a mutant phenotype in both amoebal and plasmodial cell types (DelCastillo *et al.*, 1978; Burland and Dee, 1979, 1980), suggesting that only a minority of genes are expressed in a cell type-specific manner. Confirmation that only a minority of genes show distinct expression patterns between amoebal and plasmodial phases of the life cycle comes from analysis of cDNA libraries representing the more abundant amoebal or plasmodial mRNAs. Only 5% of the cDNAs from either cell type represent mRNAs specifically expressed in only one of the cell types (Pallotta *et al.*, 1986; Sweeney *et al.*, 1987). Switches to turn off amoeba-specific gene expression and to turn on plasmodium-specific gene expression patterns occur during the first few cell cycles during the amoeba–plasmodium transition (Sweeney *et al.*, 1987).

Although there are five actin genes at four unlinked loci in *P. polycephalum* (Schedl and Dove, 1982), the *ardA*, *ardB* and *ardC* loci are isocoding, and most of the actin transcripts in amoebae and plasmodia derive from the *ardB* and *ardC* genes (Hamelin *et al.*, 1988). However, a

fourth actin gene, *ardD*, encodes an actin that is only 84% identical to the *ardB* and *ardC* actin products, which is a low identity given that the isocoding *ardB* and *ardC* products are 95% identical to human cytoplasmic actin (Adam *et al.*, 1991). Expression of the *ardD* gene has been detected only at a very low level in plasmodia, at a higher level in encysting plasmodia, and not at all in amoebae.

In addition to genes for actin and tubulin, other cytoskeletal genes expressed in a cell type-specific pattern include profilins (Binette *et al.*, 1990) and myosins (Uyeda and Kohama, 1987). The sequences of the two profilin genes, *proA* (amoebal) and *proP* (plasmodial), are so distinct that it would be difficult to detect one with the other by nucleic acid-hybridization techniques. This may explain why this was the first occasion on which cell type-specific differences in profilin genes were detected in any organism (Binette *et al.*, 1990). The prevalence of cell type-specific cytoskeletal elements contrasts with the general interpretation of analyses of thermosensitive mutants, cDNA expression studies and two-dimensional electrophoresis of proteins (Turnock *et al.*, 1981) taken to indicate that most genes expressed in amoebae are also expressed in plasmodia, and vice versa. Distinct expression patterns for cytoskeletal genes may be related to the dramatically different cellular organization and developmental potentials of amoebae and plasmodia.

The approach of screening cDNA libraries made from a particular cell type of *P. polycephalum* has also been successful in identifying cell type-specific genes for sporulating plasmodia (Martel *et al.*, 1988) and for encysting plasmodia (Bernier *et al.*, 1986, 1987; Savard *et al.*, 1989).

I. INFERENCES

Different analyses of the amoeba–plasmodium transition have given several insights into protist development, some of which may also be relevant for mechanisms of development in species from other kingdoms. First, the maintenance of different cell types involves differential expression of a substantial number of genes, albeit a small fraction of the total number. Second, a key regulator of the developmental transition between amoeba and plasmodium is the *matA* gene, including the tightly linked complementation groups *gadA*, *npfB* and *npfC*. Third, the *mat* and *npf* genes usually act in a consistent sequential order: *matB* and *matC* appear to function before *matA*, while the *npf* genes execute their developmental functions after *matA*. Fourth, the fact that amoebae and plasmodia of the *npfA*, *npfB* and *npfC* mutants appear perfectly normal for all functions except selfing suggests that some key genes may function specifically during development. This suggestion will be directly testable when the *npf* genes

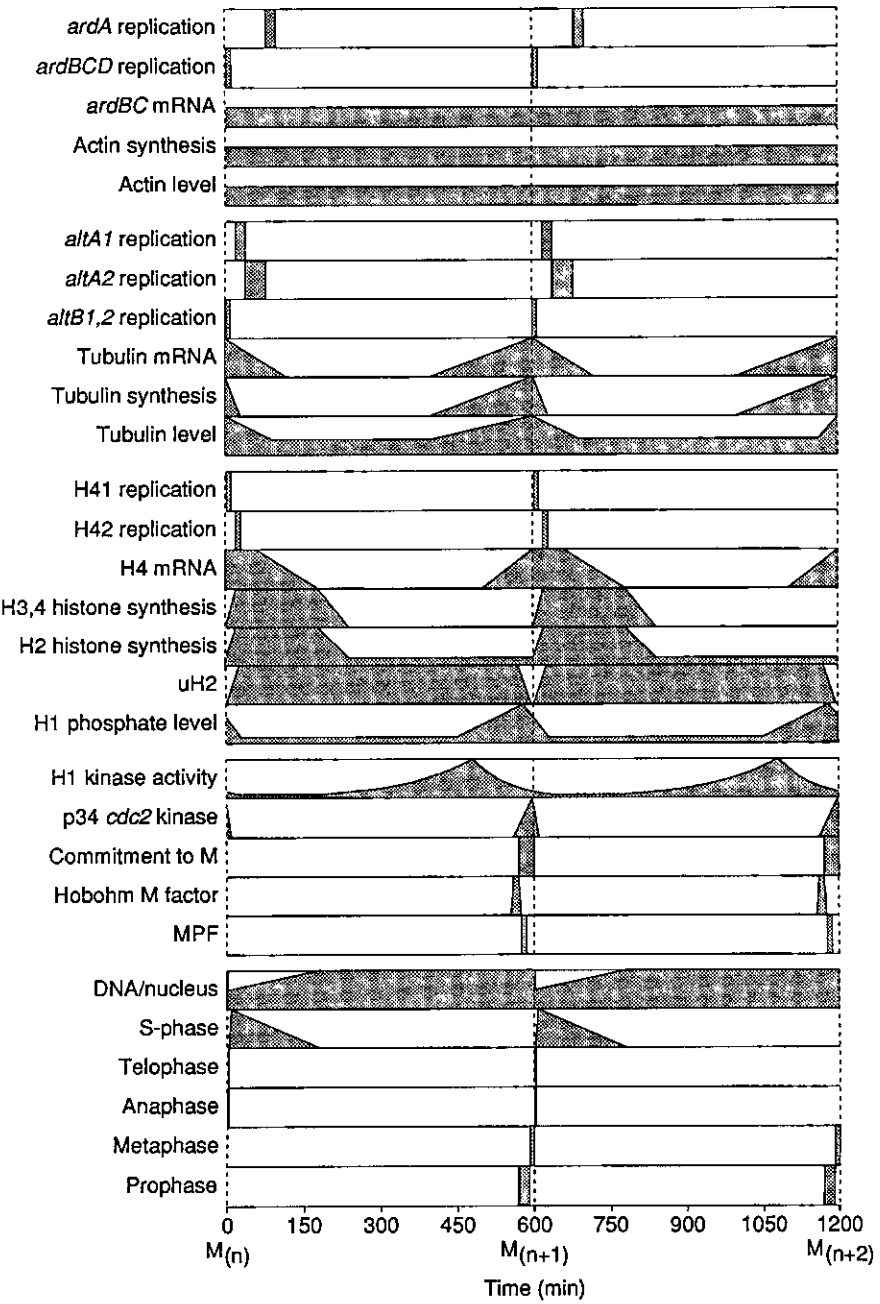
in question are cloned. Fifth, the abilty to analyse many individual cells undergoing the same developmental transition, from one cell type to another, reveals that the order of events in development is not absolutely fixed. Some events in the amoeba–plasmodium transition, such as loss of ability to develop flagella or activation of a specific tubulin gene, may be executed out of the usual turn; some unusual sequences of events may be lethal but, for others, a viable plasmodium may be formed. This principle of variability on a developmental pathway may apply to other organisms, although it would be easy to overlook. On developmental pathways that are known principally from analysis of populations of cells, occasional variations could be undetectable. In other organisms, the number of cells analysed individually may not be large enough to detect the rarer, although legitimate, alternative paths, and there is a tendency to discount occasional experimental variation as artefacts. There may be more variation on other developmental pathways than we presently know. Even the mitotic cell cycle is not always the invariate sequence of events described in textbooks.

V. The Mitotic Cycle

A. THE PLASMODIAL MITOTIC CYCLE

Mitotic cycle studies with *P. polycephalum* are typically carried out on the plasmodium to exploit its natural synchrony and large cellular mass. A key property of the plasmodium is the ability to remove multiple pieces, large or small, for analysis without disturbing the synchronous progression of the mitotic cycle (e.g. Schedl *et al.*, 1984a,b). In addition, progression of the mitotic cycle can simply and immediately be monitored by phase-contrast microscopy of tiny biopsies of the plasmodium (Mohberg, 1982a). Together, these features allow accurate, simultaneous high-resolution analysis of multiple events at many stages of consecutive, unperturbed mitotic cycles.

The plasmodial mitotic cycle differs from the amoebal cycle in its absence of cytokinesis, and occurrence of mitosis within the nuclear membrane, orchestrated by intranuclear MTOCs. This arrangement presumably prevents nuclear fusion during mitosis, which occurs in multinucleate amoebae (Burland *et al.*, 1981). In addition, there are no astral microtubules radiating from the plasmodial mitotic spindle poles (Aldrich, 1969; Tanaka, 1973). The occurrence of plasmodial mitosis without dissolution of the nuclear membrane is reminiscent of fungi (Heath, 1982) and some animal embryos (Harel *et al.*, 1989). However, unlike some yeasts, the usual cycles of chromatin condensation–decondensation and mitotic-spindle assembly–


ardA replication
ardBCD replication
ardBC mRNA
Actin synthesis
Actin level
altA1 replication
altA2 replication
altB1,2 replication
Tubulin mRNA
Tubulin synthesis
Tubulin level
H41 replication
H42 replication
H4 mRNA
H3,4 histone synthesis
H2 histone synthesis
uH2
H1 phosphate level
H1 kinase activity
p34 *cdc2* kinase
Commitment to M
Hobohm M factor
MPF
DNA/nucleus
S-phase
Telophase
Anaphase
Metaphase
Prophase
0 150 300 450 600 750 900 1050 1200
M(n) M(n+1) M(n+2)
Time (min)

disassembly do occur at mitosis. Synchrony in the plasmodium includes not only mitosis but also DNA replication, facilitating analysis of replication timing. As in amoebae, there appears to be no G_1 phase following mitosis in growing cells (Fry and Matthews, 1987; Dee *et al.*, 1989), although a G_1 phase has been reported for pre-encystment amoebae (Fry and Matthews, 1987). The S-phase occupies 2–3 hours of plasmodial interphase, the balance of the 8–10 hour cell cycle consisting of a long G_2 phase and 20–30 minute mitosis (Evans *et al.*, 1982). Salles-Passador *et al.* (1991) report that cytoplasmic microtubules can be detected in the plasmodium (Salles-Passador *et al.*, 1992) during interphase. These cytoplasmic microtubules were observed to disassemble during intranuclear mitosis of the plasmodium (Salles-Passador *et al.*, 1992), as occurs during extranuclear mitosis in amoebae. This contrasts with some observations made on cells undergoing the amoeba–plasmodium transition, where assembled cytoplasmic microtubules were observed in occasional cells fixed during intranuclear mitosis (see Section IV.F.2; Solnica-Krezel *et al.*, 1991). It will be interesting to learn what structures organize cytoplasmic microtubules in plasmodia.

Some have questioned the use of the plasmodium to analyse the eukaryotic mitotic cycle, viewing the cycle as abnormal. In fact, the multinucleate state, intranuclear mitosis and absence of a G_1 phase between mitosis and the S-phase are characteristics shared with organisms beyond the kingdom Protista. Early embryos of many animals are multinucleate syncytia and, in the case of embryos of *Dr. melanogaster*, for example, mitosis is intranuclear for the first several divisions (Harel *et al.*, 1989). The G_1 phase, defined by Howard and Pelc (1953) as a "gap", has long been known as a dispensable time period in eukaryotes, including cultured

FIG. 6. The plasmodial mitotic cycle schedule. The time-scale at the bottom spans two consecutive mitotic cycles, with the vertical dashed lines indicating the end of metaphase (M). The occurrence of an event, or presence of an activity or molecular species, is indicated by shading. MPF indicates maturation promotion factor or M-phase factor; Hobohm M factor, mitotic advancing activity detected by Hobohm *et al.* (1990), which occurs at the same time as the mitotic activity detected by Grobner and Loidl (1985); commitment to M, commitment to mitosis; H1, 2, 3, 4, histones 1, 2, 3, 4; uH2, ubiquitinated histone H2; *altA1*, *altA2*, *altB1*, *altB2*, alleles of the *AltA* and *AltB* α-tubulin genes; *ardA*, *ardB*, *ardC*, *ardD*, actin genes. Data are taken from the references indicated in the text, and normalized to a 10 hour mitotic cycle. Times of occurrence are plotted as accurately as possible, but the changes in amplitude are not intended to represent absolute values of the data; rather, they indicate the general nature of the changes. For example: the actin protein synthesis rate, protein level and mRNA level remain approximately constant as functions of cell mass throughout the mitotic cycle; $p34^{cdc2}$ kinase activity shows a sharp peak; replication of individual genes and alleles occurs in defined segments of the S-phase.

mammalian cells (Prescott, 1976). The fact that key events occur in the G_1 phase in many organisms does not mean that those events must occur between mitosis and the S-phase. And, as discussed later, it is now clear that fundamental aspects of mitotic regulation are common to most if not all eukaryotes. Summarizing, the plasmodium is far from abnormal and, as experimental evidence indicates, continues to be a useful system for mitotic cycle studies. Since Pierron (1986) and Laffler and Tyson (1986) reviewed the status of mitotic-cycle studies in *P. polycephalum*, significant progress has been made in this area.

B. PERIODIC VARIATIONS

The most important criterion for establishing mitotic-cycle periodicity of specific events is to detect the same variations at the identical stage in consecutive mitotic cycles. One plasmodium can be grown to sufficient mass so that samples for biochemical analysis can be taken at many consecutive times, over consecutive cycles, without decay of synchrony, making this key criterion unusually straightforward to establish. Variations relating to tubulins and histones (Fig. 6) illustrate the practical value of the plasmodium.

1. Tubulins

Tubulin expression varies dramatically over the cell cycle of the plasmodium, for both protein synthesis and transcript level (Fig. 6; Laffler *et al.*, 1981; Schedl *et al.*, 1984b). This expression is low in the late S-phase and peaks 40–80-fold higher at metaphase. The rise in the protein-synthesis rate is co-ordinate for all expressed tubulins, namely α1A, α1B, α2B, β1B and β2 (Schedl *et al.*, 1984b; Cunningham *et al.*, 1993), although, after metaphase, synthesis of α2B and β2 tubulins decreases more rapidly than synthesis of α1 and β1B tubulins (Schedl *et al.*, 1984b). The high level of expression of tubulin transcripts in the late G_2 phase can be suppressed by fusing a late G_2-phase plasmodium with a plasmodium in the S-phase (Laffler, 1987), indicating that tubulin expression is negatively regulated during the S-phase.

The stability of tubulin protein also varies over the cell cycle, with the tubulin protein level falling 50% or more during the S-phase (Ducommun and Wright, 1989), when the tubulin synthesis rate is falling (Fig. 6). Inhibition of DNA synthesis with hydroxyurea prevents tubulin degradation, suggesting some coupling between these events (Ducommun and Wright, 1989). The function of tubulin degradation is not clear. Inability to detect cytoplasmic microtubules in the plasmodium leads us to believe

that tubulin would not be utilized outside the mitotic phase of the cell cycle, but detection of cytoplasmic microtubules (Salles-Passador *et al.*, 1991, 1992) suggests that tubulin is indeed utilized. It would be useful to know what fraction of available tubulin in the plasmodium is utilized in cytoplasmic microtubules.

Despite some degradation of tubulin during the mitotic cycle, not all of the tubulin is lost. First, all tubulin isotypes are readily detectable in the S-phase, as well as in the G_2 phase, by silver staining of proteins resolved on two-dimensional gels (Schedl *et al.*, 1984a). Second, analysis of tubulin radiolabelled *in vivo* indicates that some of the tubulin protein synthesized in one mitotic cycle is used in the mitotic-spindle microtubules at the end of the following cycle (Paul *et al.*, 1987). Although these experiments do not establish whether a tubulin molecule used in one mitotic spindle is reutilized in the spindle in the following mitosis, they clearly indicate that a significant fraction of the tubulin pool escapes degradation and remains available for use in more than one mitotic cycle.

For tubulin transcripts, the variation in level appears to be regulated mainly by changes in the transcript-degradation rate, and to a lesser extent by the rate of synthesis (Green and Dove, 1988). Tubulin transcripts are substantially less stable after metaphase than before metaphase, and the lower stability correlates with a decrease in the length of the poly(A) tail on the mRNA (Green and Dove, 1988). The length of poly(A) tails correlates with mRNA stability for transcripts of several eukaryotic genes, both *in vivo* and *in vitro* (Bernstein and Ross, 1989).

Just as tubulin expression increases in the flagellate shortly before a new microtubule structure, the axoneme, is assembled, tubulin expression increases on in the mitotic cycle of the plasmodium shortly before the mitotic spindle is assembled. It is nevertheless interesting that tubulin expression is already substantially rising over three hours before the metaphase. Given the apparent stability of tubulin proteins at this time, it seems unlikely that the upturn in tubulin expression is based on a negative-feedback loop wherein depletion of the tubulin pool by assembly into microtubules leads to increased tubulin-gene expression (Yen *et al.*, 1988). The first visible sign of the mitotic spindle does not occur until 60 minutes before the metaphase, when a dot of tubulin-containing material can be detected in the nucleus by immunofluorescence microscopy (Paul *et al.*, 1987). This structure is presumably related to the organizing centre for the mitotic spindle, and it increases in size for the next 30 minutes until the classical prophase can be discerned (Paul *et al.*, 1987). Simultaneous measurements of tubulin-transcript levels and tubulin-synthesis rates, together with light- and electron-microscope structure determinations confirm that tubulin expression peaks at the metaphase in the plasmodium (Schedl *et al.*, 1984b).

One might wonder why the dramatic variation in tubulin expression over the plasmodial mitotic cycle has not been observed in mitotic cycles in other organisms. However, evidence has been obtained for a 2–3-fold increase in tubulin expression late in the mitotic cycle for HeLa cells (Bravo and Celis, 1980). Since these experiments were performed on a population of imperfectly synchronous cells, it seems likely that the change in expression is greater for each individual cell. In a fascinating theoretical reconstruction experiment, Matthews and Bradbury (1982) compared the amplitude of changes in levels of H1-histone phosphorylation observed during the mitotic cycle in a plasmodium with changes in phosphorylation levels that would be observed if the nuclei in the plasmodium were as asynchronous as a typical "synchronous" culture of mammalian cells used for the same experiment. They found that the imaginary imperfectly synchronous plasmodium showed a remarkably similar pattern of change in H1-phosphorylation level to that observed for mammalian cells, with a smaller amplitude of change than observed in a synchronous plasmodium. Thus, it would be unwise to conclude that because the plasmodium in the slime mould shows greater amplitudes for mitotic cycle changes than observed for other organisms, there is something odd about *P. polycephalum*. The analysis by Matthews and Bradbury highlights the power of the natural synchrony for revealing changes of small amplitude or of short period that may be overlooked when imperfectly synchronous populations of cells are analysed.

Histones

Chromatin in *P. polycephalum* contains a complement of histones similar to those of most other eukaryotes, namely H1, H2A, H2B, H3 and H4 (Matthews and Bradbury, 1982; Hardman, 1986), as well as the more recently discovered $H1^0$ (Yasuda *et al.*, 1986). In contrast to the co-ordinately expressed tubulins, different histones display distinct expression patterns during the mitotic cycle of the plasmodium.

Estimating synthesis rates of pulse-labelled nuclear proteins resolved on gels, Loidl and Grobner (1987a) reported that: (a) H3 and H4 histones are synthesized primarily during the S-phase; (b) H2A and H2B histones are synthesized at a high rate during the S-phase and at a moderate rate in the G_2 phase; and (c) H1-histone synthesis peaks towards the end of the S-phase (Fig. 6). When DNA replication is inhibited by hydroxyurea, synthesis of H3 and H4 histones is also inhibited, but synthesis of H2A and H2B histones is unaffected, indicating that the two types of histones are regulated by distinct mechanisms (Loidl and Grobner, 1987a).

The two isocoding H4-histone genes in *P. polycephalum* show co-ordinate

expression through the mitotic cycle (Wilhelm and Wilhelm, 1989). The transcript level for histone H4 rises in the late G_2 phase, remains high when the S-phase initiates, then gradually falls during the S-phase (Fig. 6; Carrino *et al.*, 1987). The lack of H4-histone synthesis in the G_2 phase, when transcript is present, appears to be due to sequestration of the message in ribonucleoprotein particles in the cytoplasm (Wilhelm *et al.*, 1988).

When DNA replication is blocked by hydroxyurea, the H4-transcript level falls with a half-life of 15 minutes, in parallel with the hydroxyurea-induced fall in the S-phase synthesis of the H4 protein. But the increased level of H4 transcript in the late G_2 phase is unaffected by hydroxyurea (Carrino *et al.*, 1987). The effects of hydroxyurea are confirmed when transcription of H4 genes is assayed by nuclear run-on *in vitro* (Carrino *et al.*, 1987), suggesting that changes in transcription rates as well as stability are important in regulating H4-transcript levels. The 15 minute half-life of H4 transcript in the S-phase is comparable to the estimate of less than 19 minutes for the half-life of tubulin transcripts in the S-phase (Green and Dove, 1988), and H4 transcripts appear more stable in the late G_2 phase, just as tubulin transcripts do. It has been postulated (Carrino and Laffler, 1986; Laffler and Carrino, 1986) that this reflects co-ordinate control of histone and tubulin expression during the mitotic cycle. However, this model does not account for the distinct patterns of regulation of the different histones.

Histone modifications, such as phosphorylation, ubiquitination and acetylation, may be important for the transcriptional state of the chromatin, for the chromatin condensation–decondensation cycle or for chromosome replication. Of interest in the mitotic cycle of the plasmodium of *P. polycephalum* is the parallel pattern of changes in the phosphate content of H1 histone, and activity of H1 kinase. Peak kinase activity is reached ahead of metaphase, falling to early interphase levels by the time division occurs (Matthews and Bradbury, 1982). Activity of H1 kinase thus peaks somewhat ahead of chromosome condensation at mitosis (Fig. 6), although the precise timing and any causal connection between H1 phosphorylation and chromosome condensation are the subject of debate. Nevertheless, phosphorylation of H1 histone in *P. polycephalum* has demonstrable structural effects *in vitro* (Jerzmanowski and Krezel, 1986); it seems likely that such profound changes would have biological significance.

Jerzmanowski and Malaszewski (1985) detected individual H1-histone variants that differed stepwise from 0 to as many as 28 phosphate residues per polypeptide. During mitosis, H1 histone is present in variants with 14–28 phosphate residues per molecule, compared with 8–20 residues in the late S-phase, reaching a minimum in the mid-G_2 phase of 6–14 phosphates residues per molecule. Thus, although H1-histone molecules with the

highest level of phosphorylation occur at mitosis, when chromatin is condensed, dephosphorylation does not minimize the phosphate content in H1 histone until well after decondensation of chromatin. Since the number of phosphate residues per H1 molecule is variable at any one time, Jerzmanowski and Malaszewski (1985) suggested that several phosphorylations may be needed to generate a quantum change in H1 conformation.

All of the core histones in *P. polycephalum* appear to be susceptible to acetylation, at multiple sites on each molecule. Waterborg and Matthews (1984) observed acetate turnover on H2A and H2B histones only in the S-phase, but H3 and H4 acetylation turned over in both the S- and G_2 phases. However, the pattern of turnover of acetate on H3 and H4 histones differed in the S- and G_2 phases, and Waterborg and Matthews (1984) proposed a correlation between mono-acetylated H4 histone and inactive chromatin. Meanwhile, H4 deacetylase activity changes only two-fold during the mitotic cycle (Waterborg and Matthews, 1982).

Loidl and Grobner (1987b) found a correlation between the ability of protamine to displace histone H4 from chromatin *in vitro* and the stage of the mitotic cycle whence the chromatin was isolated. Core histones were more readily released from S-phase chromatin than from G_2-phase chromatin, and the more highly acetylated H4 histones were more readily released by protamine than less highly acetylated molecules. Acetylation of core histones may serve to release them more readily from chromatin for the purposes of both DNA replication and transcription (Loidl, 1988).

Perhaps the most dramatic change in histone modification during the cell cycle is ubiquitination of H2A and H2B histones (Fig. 6; Mueller *et al.*, 1985). Both histones are ubiquitinated during most of the mitotic cycle, but are de-ubiquitinated at prophase and metaphase, then re-ubiquitinated at anaphase. This modification pattern may be a general feature of the eukaryotic mitotic cycle, as H2A histone in metaphase chromosomes of several eukaryotes lacks ubiquitin (Bradbury, 1992).

Histidine kinase activity in extracts of nuclei from *P. polycephalum* phosphorylates the His_{75} residue of free histone H4 *in vitro* (Huebner and Matthews, 1985), although not H4 in nucleosomes (Wei *et al.*, 1989). Phosphohistidine is found in histone-like proteins in nuclei from *P. polycephalum* (Pesis *et al.*, 1988), but the precise substrate(s) of the histidine kinase and the biological significance of phosphorylation of histidine in nuclear proteins remain to be determined. Purification of a protein histidine kinase from yeast that phosphorylates specifically His_{75} in H4 histone *in vitro* (Huang *et al.*, 1991) suggests that this activity may have broad biological relevance.

Considering the variety of covalent modifications that can be made to

various histones, the possible combinations of modifications that might occur sometimes seem discouragingly high. Nevertheless, correlations between modifications and functional and structural changes are too remarkable, and occur in too broad an array of eukaryotes (e.g. Bradbury, 1992) to ignore. It would help greatly if chromatin structure could be analysed for a single gene over the course of consecutive synchronous plasmodial mitotic cycles, where both the replication time and expression pattern are accurately known. Such a test system may be feasible using plasmids carrying specific replication origins linked to various cloned genes. Given the progress being made in mapping replication origins (see Section V.C) and in DNA transformation (see Section VI.C) in *P. polycephalum*, this feasibility may also soon be at hand. Finally, conditional mutants defective in mitotic-cycle progression would help to provide the functional analysis needed to complement the biochemical progress. This last resource will be simple to achieve (Burland, 1978, 1986; Burland and Dee, 1980; Burland *et al.*, 1981).

3. *Significance of Periodicity*

Universally periodic events in the mitotic cycle, such as DNA synthesis and mitosis are presumably regulated by some underlying periodic changes, be they transcriptional, translational or post-translational (for a review, see Tyson, 1982). But some events, such as changes in thymidine kinase activity over the mitotic cycle (Sachsenmaier *et al.*, 1970), can occur as a peak or "step" change, depending upon culture conditions (Wright and Tollon, 1979). Thus, observation of periodicity does not prove that the periodicity is necessary for orderly mitotic progression. An equally disconcerting problem is that events which show mitotic periodicity may not need to occur at the particular stage at which they are observed. This is another issue that mitotic mutants can help us understand better.

Intermitotic time, which remains at a constant mean value for a given cell type under specific conditions, shows substantial variations between individual cells (e.g. Bailey *et al.*, 1987). These variations in part reflect asymmetry of division, whereby daughter cells of unequal size, growing at a constant rate, take different times to reach the same DNA-to-mass ratio for the next division. However, mass at division also varies, further contributing to intermitotic time variation. In the plasmodium, natural mitotic synchrony occurs in billions of nuclei in the same cell, without cytokinesis. Thus, variation in cycle time due to variation in mass at division will tend to be averaged out as an experimental variable. This may contribute to the ready detection of periodicity of events in the plasmodial mitotic cycle compared with mitotic cycles in other organisms, particularly

for events such as increased tubulin expression that occur later in the cycle.

Analysis of cell-cycle mutants in a yeast shows that certain functions need to be carried out in a particular sequence for the orderly progression of the mitotic cycle (Hartwell, 1991), but the absolute stage of execution of certain functions often seems less critical than their sequence. However, precise timing of the execution of key functions is probably vital for events that occur during mitosis, and this is one area where the large-scale mitotic synchrony of the plasmodium offers powerful opportunities for experimental analysis.

C. CHROMOSOME REPLICATION

In the plasmodium, DNA synthesis begins immediately after mitosis (Nygaard *et al.*, 1960). This feature provides a clear and simple cytological marker for the beginning of the S-phase. A fundamental principle of the temporal organization of chromosome replication, demonstrated in the plasmodium, is that DNA sequences replicated during a specific stage of the S-phase in one mitotic cycle are replicated at the same stage in the subsequent mitotic cycle (Braun *et al.*, 1965). Thus, at the gross genome level, DNA replication occurs in a defined sequential order in each cycle. The size distribution of newly replicated DNA (i.e. pulse-labelled DNA, denatured and size fractionated) indicates that Okazaki fragments are synthesized throughout the S-phase. During the first hour of the S-phase, when replication is at its maximum rate, the size of the newly synthesized DNA increases to $1 \cdot 10^7$–$2 \cdot 10^7$ Da, the proposed size of a replicon (Funderud *et al.*, 1978a). Subsequently, further replication and joining of replicons leads to discontinuous increases in the size of newly replicated DNA around 90 minutes and again at 120 minutes into the S-phase (Funderud *et al.*, 1978a). These observations indicate that specific replicons are synchronous between all of the nuclei in the plasmodium, and that different replicons proceed with DNA replication at various times during the S-phase; isochronous replicons tend to lie in clusters. Funderud *et al.* (1978b) also obtained evidence that most if not all replicons initiate at the beginning of the S-phase. Thus, the initiation of replication and the elongation of replication forks are distinct events that can be separated by substantial intervals of time. It is remarkable that replication remains rather synchronous later in the S-phase, since many replicons proceed with DNA replication long after initiation has occurred. Flow cytometry of nuclei isolated from a plasmodium progressing through the S-phase confirms that replication begins very soon after mitosis and that individual nuclei within

a single plasmodium increase their DNA content at approximately the same rate (Kubbies and Pierron, 1983; Kubbies *et al.*, 1986).

1. Replication Timing of Individual Genes

Given that the genome in *P. polycephalum* replicates synchronously in all of the nuclei in a plasmodium, that specific portions of the genome replicate in a defined temporal sequence, and that replicons are substantially larger than structural genes, it should be possible to investigate the replication timing of individual genes during the S-phase in a plasmodium. If replication is allowed to progress in the presence of bromodeoxyuridine, newly replicated DNA can then be separated by isopycnic centrifugation from unreplicated DNA, on the basis of its higher density. If the separated DNA species are then analysed by Southern blotting, replicated DNA fragments will be detected in the heavier DNA while unreplicated fragments will be detected in the lighter DNA fraction. Using this technique to analyse replication of actin genes in the plasmodium of *P. polycephalum*, Pierron *et al.* (1984) found that three actin loci, *ardB*, *ardC* and *ardD*, are replicated early in the S-phase while the fourth, *ardA*, is replicated late in the S-phase (Fig. 6). The bromodeoxyuridine heavy labelling strategy has also been used to estimate the time of replication of the H4-histone genes, H41 and H42, in the plasmodial mitotic cycle. Jalouzot *et al.* (1985) found that H41 replicates in the first 10 minutes of the S-phase while H42 replicates between 20 and 30 minutes into the S-phase (Fig. 6).

One limitation to this technique is that bromodeoxyuridine labelling must be carried out for extended periods, usually beginning before the S-phase, to obtain a sufficient density increase for resolution of newly replicated and unreplicated DNA. To circumvent this problem, and to increase temporal resolution, Southern blots can be run for unlabelled DNA extracted at different stages of the S-phase, and the intensity of bands representing specific restriction fragments can be quantified; band intensity should double upon replication. To eliminate errors caused by inaccurate DNA loads in different lanes of a Southern blot, band intensities need to be normalized to other bands in the same lane; therefore, the time of replication can only be determined by this method if two or more fragments detected replicate at different times, since only then will the relative band intensities change. With appropriate care, relative band intensities can be estimated to within ±10%, well within the accuracy needed for this purpose (Cunningham, 1992). Using this strategy, Pierron *et al.* (1984) established that the actin genes *ardB*, *ardC* and *ardD* replicate in the first 10 minutes of the S-phase, when 10% of the genome is replicated; the *ardA* gene replicates much later, around 80–100 minutes into the S-phase, when 75%

of the genome is replicated (Fig. 6). Since the plasmodium used is heterozygous for restriction-fragment length at *ardC* and *ardA*, Pierron *et al.* (1984) were able to demonstrate for the first time contemporaneous replication of different alleles of the same gene. The use of the parameter "percentage of genome replicated" (% G) may prove more reliable as a measure of the stage of the S-phase than clock time into the S-phase. This value can be obtained accurately by flow cytometry of a sample of the nuclei used for replication analysis.

While contemporaneous replication of a pair of alleles may be the norm, the exquisite synchrony of the S-phase in the plasmodium provides an opportunity to observe significant differences in replication timing should any pair of alleles behave this way. Two alleles tested for the *altB* α-tubulin locus in *P. polycephalum* show contemporaneous replication in a diploid plasmodium heterozygous for restriction-fragment length. By contrast, two alleles of *altA* replicate at distinct times (Cunningham and Dove, 1993; Fig. 6). This is the first compelling example of distinct times of replication for alleles of the same locus. Thus, to obtain a clear picture of DNA-replication timing in any diploid cell, it is essential to recognize that the time of replication may differ for each allele.

2. *Timing of Replication Relative to Timing of Transcription*

The idea that transcription and replication of a gene are coupled in eukaryotes has gained wide currency (e.g. Goldman, 1988), bolstered by some observations that expressed genes replicate early in the S-phase while silent genes replicate late. In *P. polycephalum*, electron-microscope examination of DNA samples from the S-phase plasmodium reveals that transcription units are often associated with replication "bubbles", consistent with a coupling between replication and transcription (Pierron *et al.*, 1983).

The establishment of reliable, high-resolution methods to determine the time of replication of individual genes in the plasmodium, together with isolation of cDNA clones of genes that show distinct patterns of expression (Pallotta *et al.*, 1986), facilitated a rigorous test of the temporal patterns of replication and transcription of several genes. Ten cDNAs were chosen, representing single-copy genes; six are expressed in the plasmodium, while the other four are not. Of the four not expressed in the plasmodium, two are expressed in the amoeba, a third during spherulation, and the fourth during sporulation (Pallotta *et al.*, 1986; Pierron *et al.*, 1989). One of the six genes expressed in the plasmodium, LAV3-2, is also expressed in the amoeba, but the transcripts are of different size in the two cell types. Four of the six genes expressed in the plasmodium replicate early in the S-phase,

but the other two genes, LAV3-2 and the plasmodium-specific gene LAV1-4, replicate late (Pierron *et al.*, 1989). This clearly contradicts the general rule that expressed genes are replicated early. Among the genes not expressed in the plasmodium, the spherulation-specific gene replicates late, the two amoeba-specific genes replicate in the mid-S-phase and the sporulation-specific gene replicates early. Again, the results argue against a general rule of coupling between replication and transcription.

3. Identifying Replication Origins

The natural synchrony of DNA replication in the plasmodium renders detection of short-lived replication intermediates much simpler than in other organisms (e.g. Hamlin *et al.*, 1991), circumventing the need for artificial synchronization or gene amplification to detect replication intermediates. Funderud *et al.* (1978a) showed that newly replicated DNA from the plasmodium can be detected initially as low molecular-weight single-stranded fragments, which increase in length per minute by about 1.5 kb, approximately the size of a typical gene. Benard and Pierron (1990) reasoned that synchronous replication of a specific gene in the plasmodium would generate nascent ssDNA fragments containing the gene which would increase in size during the S-phase. Very high molecular-weight parental ssDNA fragments bearing the gene would always be present. Therefore, if DNA is isolated from the plasmodium at consecutive stages of the S-phase, denatured, then resolved on agarose gels and blotted to filters, replication intermediates of a specific gene will be detectable by hybridizing the filters with a nucleic-acid probe for the gene. The smallest size of the replication intermediate first detected in the S-phase for a particular gene would depend upon the distance of the gene from the origin of replication; a gene containing a fixed origin of replication would show a replication intermediate at the lowest detectable size, while a gene 10 kb from a replicon would show a minimum replication intermediate of 20 kb, assuming bidirectional replication from a fixed origin with the two forks displaying the same replication rate. For the LAV1-2 gene (Pallotta *et al.*, 1986), Benard and Pierron (1990) found the first replication intermediate, only 5 minutes (less than 5% G) into the S-phase, with a minimum size of around 6 kb, indicating very early replication of this gene, and a replicon within 3 kb of the 1.2 kb gene, i.e. very close to the gene itself. This technique reveals the increasing size of the replication intermediates in samples taken from consecutive stages of the S-phase, and can thus reveal the rate of elongation of the replication fork. The replication intermediate for LAV1-2 increases in size at about 1 kb each minute, in remarkably close agreement with the data of Funderud *et al.* (1978a) for average replicon growth rates in *P. polycephalum*.

Benard and Pierron (1990) also noted that the replication intermediates for LAV1-2 showed some size heterogeneity. One explanation for this heterogeneity is asynchrony. In fact, differences in timing of initiation of the LAV1-2 replicon in different nuclei could be at most 3–4 minutes for asynchrony alone to account for the heterogeneity, not bad synchrony for a 10 hour mitotic cycle. However, other factors may contribute to the size heterogeneity of ssDNA, including degradation during DNA isolation, variation in the rate of fork movement, and possibly initiation of replication from close but separate sites. Even with the size heterogeneity observed, the small, discrete size of the first replication intermediates detected provides a compelling case for a limited region of replication initiation in eukaryotes (cf. Hamlin *et al.*, 1991). Further application and refinement of this strategy should lead to physical location of replication origins with respect to specific DNA sequences with greater accuracy, perhaps down to the 150–200-nucleotide size of Okazaki fragments. In parallel with this type of analysis, developments in DNA transformation in *P. polycephalum* provide a basis for functional assays for replication-origin activity of specific DNA fragments, both *in vitro* and *in vivo*.

D. RIBOSOMAL DNA REPLICATION

In the 60 kb palindromic extranuclear multicopy rDNA molecule from *P. polycephalum*, two pairs of symmetrically arranged replication origins can be observed by electron microscopy, although only one replicon appears to be active on any one molecule (Vogt and Braun, 1977). As for chromosomal genomes, replication of rDNA is semi-conservative and bidirectional, although most replication occurs in the late S-phase and in the G_2 phase (Braun and Evans, 1969; Zellweger *et al.*, 1972; Newlon *et al.*, 1973; Vogt and Braun, 1977). Regions of rDNA molecules around the four potential replication origins are heavily methylated (5-methyldeoxycytidine), but the replicon that is active on any one molecule is hypomethylated (Cooney *et al.*, 1988).

Extracts of the plasmodium of *P. polycephalum* can selectively initiate replication of rDNA *in vitro*, at the same sites that replication initiates *in vivo* (Daniel and Johnson, 1989). Plasmid pPHR21 contains a segment of the rDNA that includes one of the replication origins (Ferris, 1985). Extracts taken from synchronous plasmodia catalyse replication of this plasmid *in vitro*, with highest catalytic activity coming from prophase plasmodia, when the nucleolus disperses. Moderate activity is found in the early S-phase but, by the time the nucleolus is fully reorganized later in the S-phase, little activity can be detected (Daniel and Johnson, 1989).

Electron microscopy pinpoints the origin of rDNA replication *in vitro*

at the same position as estimated *in vivo*, corresponding to a region of 78 tandem repeats of an element with a 31 bp consensus sequence (Daniel and Johnson, 1989). It will be interesting to compare the rDNA origins with chromosomal replicons, which will soon be identified (Benard and Pierron, 1990), and to determine whether replication is controlled by the same or distinct factors for chromosomal and rDNA. This is particularly interesting given that chromosomal DNA replicates only once in each mitotic cycle, while some rDNA molecules can replicate more than once in a cycle in which others do not replicate at all (Vogt and Braun, 1977).

E. MITOTIC REGULATION

1. Heteroploidic Fusion

Plasmodia of *P. polycephalum* which carry identical alleles of the *fusA*, *fusB* and *fusC* loci fuse naturally when they come into contact. Fusion occurs regardless of the ploidy of the two plasmodia. Dee and Anderson (1984) fused together haploid and diploid plasmodia that carried distinct genetic markers. They observed that the phenotypes associated with the diploid nuclei were always lost from these heteroploid plasmodia, and by careful reciprocal experiments eliminated the possibility that residual genetic differences could account for directional loss of the nuclei observed (Dee and Anderson, 1984). It seems likely that this phenomenon is related to some fundamental aspect of mitotic-cycle regulation. Analysis of replication timing for haploid and diploid nuclei would be illuminating, and could be achieved by analysing replication times for two alleles of the same gene that normally replicate at the same time. For example, the heteroploidic heterokaryon could be made with haploid *altB1* nuclei and diploid *altB2/altB2* nuclei (see Section V.C).

2. Heterophasic Fusions

The natural capacity for genetically similar plasmodia to fuse permits unusually straightforward analysis of mitotic averaging. For example, if plasmodium A is scheduled to divide at 2 p.m. and plasmodium B at 6 p.m., a plasmodium created by the fusion of A and B around noon will initiate mitosis between 2 p.m. and 6 p.m.; the greater the mass that plasmodium B contributes to the mixed plasmodium, the closer to 6 p.m. will be the initiation of mitosis (for a review, see Tyson, 1982). Such results implicate cytoplasmic, rather than nucleus-limited, factor(s) controlling the timing of mitosis.

Plasmodial fusion experiments have been used to elucidate the time of

commitment to mitosis, defined as the stage beyond which fusion of plasmodium A, early in the mitotic cycle, to plasmodium B, later in the cycle, can no longer retard initiation of mitosis in nuclei from plasmodium B. Commitment occurs typically about an hour (0.1 of a cycle time) before metaphase, although the time of commitment *in vivo* may correspond to the onset of prophase (15–30 minutes before metaphase), depending on the time correction made for plasmodial fusion and cytoplasmic mixing to occur (Loidl and Sachsenmaier, 1982).

Beyond timing events, the technique of plasmodial fusion permits analysis of specific molecular events. For example, when plasmodial nuclei in the S-phase are introduced by brief fusion into a plasmodium in the G_2 phase, the S-phase nuclei continue to replicate, but do not induce G_2 nuclei to replicate (Guttes and Guttes, 1968). Nevertheless, after the G_2 nuclei ultimately pass through nuclear division, they can then replicate normally, highlighting the now common knowledge that replicated nuclei cannot normally replicate again before nuclear division is initiated (Guttes and Guttes, 1968). Use of fluorouracil to inhibit DNA replication in plasmodia had already shown that mitosis is dependent on prior DNA synthesis (Sachsenmaier and Rusch, 1964). Thus, heterophasic fusion experiments completed the picture of the now-familiar cycle of mutual dependency between DNA synthesis and mitosis in eukaryotes. These experiments paved the way for similar experiments with mammalian cells, which confirmed that this fundamental regulatory mechanism is a general feature of eukaryotic mitotic-cycle control (Rao and Johnson, 1970). The DNA synthesis–mitosis dependency cycle is perturbed only in rare cases in nature, for example to develop polytene chromosomes, although it can be interrupted in the laboratory not only by inhibitors but also by mutation (e.g. Broek *et al.*, 1991).

3. Mitotic Factors

A logical next step from fusing whole plasmodia at different phases of the mitotic cycle is to make extracts of plasmodia at specific points in the mitotic cycle and add these extracts to plasmodia at other stages in the cycle. Two features of the plasmodium facilitate this experimental approach. First, since one plasmodium can be cut into two or more pieces without detriment to its mitotic progression, one piece can be used as a robust control sample for other experimental pieces. Secondly, substances placed on top of a plasmodium are usually taken up, obviating the need for injection. Since Oppenheim and Katzir (1971) showed that extracts from plasmodia in the late G_2 phase could advance mitosis in plasmodia in the early G_2 phase by at least an hour, many other studies have confirmed the presence of mitotic

factors in plasmodia about to undergo mitosis. Application of partially purified mammalian histone kinase to a plasmodium advances the time of mitosis by up to 40 minutes (Bradbury *et al.*, 1974), strongly suggesting a role in mitotic regulation for histone kinase or a copurifying substance.

Loidl and Grobner (1982) improved methods for uptake of extracts into plasmodia, and found that mitosis can be advanced by up to two hours by addition of extracts from a plasmodium in the late G_2-phase. The protease-sensitive factor they assayed was not detected in extracts from other stages of the mitotic cycle (Loidl and Grobner, 1982).

Once the presence of mitotic factors is established for a particular extract (which need not be from *P. polycephalum*), enrichment and purification are necessary. Grobner and Loidl (1985) reasoned that, if the late G_2-phase mitotic factor were absent from other stages of the mitotic cycle, it should be possible to enrich the factor by making antibodies to S-phase plasmodial extracts and, using the antibodies, to subtract S-phase proteins from late G_2-phase extracts. They succeeded in decreasing the number of abundant proteins in a late G_2-phase extract by a factor of 35, and increasing the specific activity of the mitotic stimulating factor 10-fold in the immuno-depleted extract (Grobner and Loidl, 1985). However, they did not detect a novel protein in the G_2-phase extract, although there are several reasons why the factor may be difficult to detect: the mitotic factor may be in low abundance; it may have properties that preclude its identification on the two-dimensional gels used; the active factor may be a complex mixture like M-phase factor (see below); and assay for the factor may not be linear.

Hobohm *et al.* (1990) used direct injection of 3–5 μl volumes of plasmodial extracts to evaluate their capacity to advance the timing of the next mitosis. They found that only extracts from a 20–30 minute period just before prophase advanced the timing of mitosis in plasmodia, and then only when injected into plasmodia early in the mitotic cycle. The timing of the presence of this mitotic factor correlates rather well with the time of commitment of plasmodial nuclei to mitosis (Fig. 6; Loidl and Sachsenmaier, 1982). Hobohm *et al.* (1990) then sought to purify the mitotic factor from late G_2-phase extracts. They estimated that the factor has a mass of around 2500 Da, a very small protein. Such a protein would not have been detected on the two-dimensional gels used by Grobner and Loidl (1985), so it could be the same factor. Following purification, the low molecular-weight factor retained its capacity to advance mitosis. Characterization of this protein is keenly awaited.

4. MPF / p34^{cdc2}

The temptation to compare mitotic factors detected in plasmodia from *P. polycephalum* with the universal mitotic regulators MPF and p34^{cdc2}

(Lewin, 1990; Nurse, 1990) is irresistable. Oocytes from *Xenopus laevis* are naturally blocked in progression just before the prophase of meiosis I. Maturation of oocytes, which begins with germinal vesicle breakdown and chromosome condensation, can be induced by extracts of cytoplasm from cells that have matured to the second meiotic metaphase (Masui and Markert, 1971). This activity is called MPF (M-phase factor, or maturation-promoting factor), and is now recognized as a complex protein involving the $p34^{cdc2}$ protein kinase and cyclin (Gautier *et al.*, 1988; Dunphy *et al.*, 1988). MPF can be detected in extracts of plasmodia in the late G_2 phase, but not in extracts from other mitotic-cycle stages, and its activity peaks 10–20 minutes before metaphase in plasmodia (Adlakha *et al.*, 1988). This timing is similar to the timing of the peak in mitotic-factor activity found by Hobohm *et al.* (1990) (Fig. 6). Clearly, the 2500 Da mitotic factor detected by Hobohm and his colleagues is too small to be MPF, but it will be interesting to determine whether it is a completely separate entity, a previously unknown component or a key activator. The MPF activity detected by Adlakha *et al.* (1988) shares several biochemical characteristics with similar factors from other sources, suggesting a strong evolutionary conservation of the structure and activity of this mitotic regulator.

Inhibitors of MPF have been detected in the G_1 phase in various cell types (e.g. Adlakha *et al.*, 1983) while, in CHO V79-8 cells, which like plasmodia do not exhibit a G_1 phase, the inhibitors are found in the S-phase. Adlakha *et al.* (1988) found MPF-inhibitor activity in plasmodia in the S-phase but not in plasmodia in the G_2 phase. The activity inhibited MPF from both plasmodia and from HeLa cells, again pointing to evolutionary conservation of mitotic regulators.

An intriguing comparison is between $p34^{cdc2}$, a known kinase that can phosphorylate H1 histone *in vitro*, and the H1-histone kinase that can advance timing of mitosis in plasmodia (Bradbury *et al.*, 1974). Shipley and Sauer (1989) detected a homologue of $p34^{cdc2}$ in a plasmodium, and found constant levels of the protein throughout the mitotic cycle, as found in yeasts. Ducommun *et al.* (1990) assayed activity of the $p34^{cdc2}$ kinase over the mitotic cycle in a plasmodium, using the $p13^{suc1}$ protein of *Schizosaccharomyces pombe* to purify the kinase, and histone H1 or SV40 large T-antigen as the substrate. They found that the kinase activity of the protein from *P. polycephalum* is low during most of the mitotic cycle, but that activity rises abruptly in the late G_2 phase, peaking in mitosis at early metaphase (Fig. 6). Thereafter, $p34^{cdc2}$ kinase activity plunges dramatically. This experiment marks the most accurate estimate of the profile of $p34^{cdc2}$ kinase activity in an unperturbed, ongoing mitotic cycle. Delaying mitosis with microtubule poisons or a DNA-synthesis inhibitor causes the peak in kinase activity to be delayed until mitosis occurs, while blocking a

plasmodium in mitosis by treating with cycloheximide late in the G_2 phase leads to a sustained increase in kinase activity; these results confirm that kinase activation and mitosis are tightly coupled (Ducommun *et al.*, 1990).

The peak in $p34^{cdc2}$ kinase activity occurs later in the mitotic cycle than the peak in H1-histone kinase activity described earlier for plasmodia (for a review, see Matthews and Bradbury, 1982). It therefore seems unlikely that the two activities are the same. Nevertheless, the H1 kinase that peaks earlier remains a candidate as a regulator of mitosis. The early H1 kinase could represent an alternative activity of $p34^{cdc2}$, or the two activities could be distinct kinases acting either independently or in a dependent series.

The timing and sharpness of the peak in $p34^{cdc2}$ kinase activity at early metaphase in a plasmodium suggests that mitotic factors detected slightly earlier in the cycle by Loidl and Sachsenmaier (1982) and Hobohm *et al.* (1990) are distinct from $p34^{cdc2}$ kinase, although they may act on the same pathway, or in combination with $p34^{cdc2}$ at earlier times. With respect to MPF, it would be useful to re-examine timing of its peak in activity with timing of the $p34^{cdc2}$ kinase peak, with both activities determined from the same plasmodium. This would clarify whether the $p34^{cdc2}$ kinase peak coincides with or follows the MPF peak, a matter of some concern for evaluating cause-and-effect relationships in this conserved mitotic regulatory pathway. And now that the structure and activity of MPF and its components are better understood, it should be possible to evaluate the other mitotic factors from *P. polycephalum* to determine whether they are part of active MPF or contribute to its activation.

One clear message from the studies of MPF, $p34^{cdc2}$ and other mitotic factors is the remarkable evolutionary conservation of key regulators. Homologous gene products regulate mitotic and meiotic cycles as diverse as the fast embryonic cycle of oocytes of *X. laevis*, the budding mitotic cycle of *Sacch. cerevisiae* with its mitotic spindle assembly long before the classical M-phase, the textbook M-G1-S-G2 mitotic cycle of mammalian cells cultured *in vitro*, and the G_1-less mitotic cycle of the syncytial plasmodium of *P. polycephalum* (Nurse, 1990; Ducommun *et al.*, 1990), a remarkable variety of mitotic cycle strategies. From this perspective, it is logical to consider the natural mitotic-cycle synchrony of the plasmodium as a valid tool to elucidate key events in the unperturbed eukaryotic mitotic cycle. Now that utility of the plasmodium for analysing periodic molecular events has been amply demonstrated, and DNA-transformation and gene-targeting systems have been developed, use of plasmodia from *P. polycephalum* can be productive and informative.

One resource still needed for research on *P. polycephalum* is a set of mitotic mutants. Such mutants can be isolated by elementary microbial-genetic techniques (Burland, 1986) and, in addition to clarifying some of

the cause–effect relationships for known molecular events, should also identify new mitotic regulators. This strategy has worked admirably in research on *A. nidulans*, where analysis of mitotic mutants identified gene products that were not known from the collection of yeast cell-cycle mutants (Morris and Enos, 1992). For example, mitosis in *A. nidulans* requires not only $p34^{cdc2}$ kinase activity, but also the activity of another protein kinase, NimA, which acts independently of $p34^{cdc2}$ (Osmani *et al.*, 1991). Other mitotic regulators probably await discovery (Hartwell, 1991), and *P. polycephalum* is an obvious organism in which to seek them.

VI. Expression of Introduced Molecules

A. DIFFUSION UPTAKE

One of the most exciting possibilities for molecular analysis in the plasmodium of *P. polycephalum* is the introduction of exogenous molecules. Growth of the plasmodium on a surface, such as a filter paper laid on top of a nutrient medium, allows easy access to the biomass. Radiolabelled precursors are readily taken up by the plasmodium from the medium underneath, or, very efficiently, from droplets containing radiolabelled solutes placed on top. Passive uptake of exogenous molecules even occurs when substances are added to submerged liquid "microplasmodial" cultures. Perhaps the most remarkable demonstration of this phenomenon is the uptake into microplasmodia of derivatized H3 histone, which becomes incorporated into nucleosomes (Prior *et al.*, 1980). This powerful capability is one of the most underutilized features of the plasmodium. Nevertheless, for molecules that are difficult to obtain in large quantities, more direct methods may be needed for uptake into this receptive giant cell.

B. MACROINJECTION

A mammalian cell has a volume of about 1 pl. The oocyte of *X. laevis* is useful for analysing biological activity of specific molecules in part because upwards of 50 nl of liquid can be injected into one cell, 50,000 times the volume of a mammalian cell. By contrast, several microlitres of liquid can be injected into a small plasmodium at each of several sites (Kazarinoff and Ruth, 1986), that is, about a million times the volume of one mammalian cell. This is enough material for analysis not only of biological activity but also for recovering the injected material to evaluate molecular changes or specific associations. Rapid mixing of injected liquid is aided by rapid protoplasmic streaming in the plasmodium. This technique is useful for

reintroducing mitotic activators into the plasmodium after purification to verify biological activity, and may also be useful to text extracts or specific gene products for ability to complement mitotic and other mutations. Given the large volumes that can be introduced, it should eventually be possible to macro-inject not only purified proteins in quantities large enough for easy detection, but perhaps also multiprotein complexes and structural assemblies such as DNA–histone complexes. This seems to be another technique worthy of more intense exploitation. For example, MPF or one of its components could be isolated from a plasmodium at one stage of the mitotic cycle, labelled or derivatized in some way, and injected into plasmodia at other stages of the mitotic cycle. One could then analyse not just its biological activity, but also its stability, cellular localization and association with other proteins.

C. DNA TRANSFORMATION

Were Jane Austen a late 20th-century molecular biologist, she might well write a novel, as she did nearly two centuries ago, entitled *Pride and Prejudice*, modifying the first sentence only slightly, to read

> It is a truth universally acknowledged that a single-celled organism in possession of good cell biology must be in want of DNA transformation.

Such is the scientific value of introducing heterologous or modified genes into a cell that no organism is considered worthy of the attention of researchers today unless DNA transformation can be applied routinely. Fortunately, a DNA-transformation system is a technique no longer absent from the toolkit of research into *P. polycephalum*.

1. Transient Expression

The *hph* (hygromycin phosphotransferase) gene confers selectable resistance to the antibiotic hygromycin in both prokaryotes and eukaryotes (Gritz and Davies, 1983). The sequence immediately 5′ to the *ardC* actin gene from *P. polycephalum* (*PardC*) drives expression of *hph* in both fission yeast and budding yeast, conferring hygromycin resistance on these ascomycetes (Burland *et al.*, 1991). Thus, *PardC* can act as a functional promoter in a broad range of eukaryotes.

To develop transformation systems for *P. polycephalum*, *PardC* was tested in transient expression experiments. The promoter was fused to the *cat* (chloramphenicol acetyltransferase) gene and amoebae were electroporated in the presence of plasmids carrying the *PardC–cat* fusion. Under precise buffer and electrical conditions, achievable using the Bio-Rad Gene

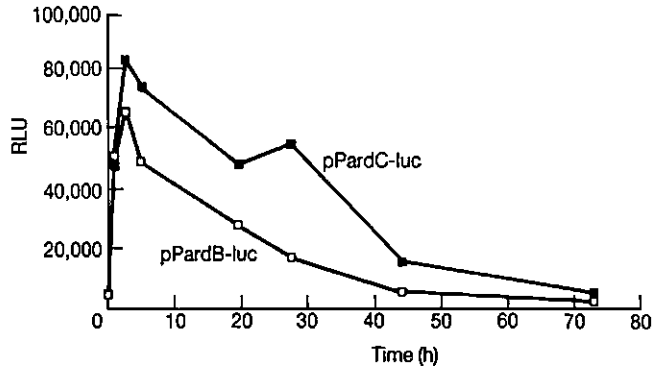


FIG. 7. Transient expression of luciferase in *Physarum amoebae*. Amoebae were electroporated by standard procedures (Burland *et al.*, 1992a) in the presence of vectors carrying either *PardB–luc*, the promoter region of the *ardB* actin gene translationally fused to the firefly luciferase (*luc*) gene, or *PardC–luc*, the promoter region of the *ardC* actin gene translationally fused to *luc*. RLU indicates relative light units (background emission without vector addition is 500 RLU). Following electroporation (time zero), equal numbers of amoebae were harvested and lysed after incubation in the growth medium, and luciferase activity in the lysates was measured with a luminometer, using the assay kit provided by Promega Inc. (Madison, Wisconsin, USA).

Pulser, expression of the *cat* gene can be detected (Burland *et al.*, 1992a). The promoter region for the *ardB* actin gene (*PardB*) from *P. polycephalum* also drives *cat* expression in amoebae, although at a lower level than for *PardC*, reflecting the relative *in vivo* activities of the actin genes (Burland *et al.*, 1992a). This is not the first report of transient expression of *cat* in amoebae, but the plasmid originally described for expressing *cat* in amoebae, pE1ori-CAT (McCurrah *et al.*, 1988), does not express *cat* in our tests. The development of reproducible methods for transient expression now allows further optimization of transformation conditions.

Expression peaks only a few hours after transformation with plasmid DNA, then falls steadily (Burland *et al.*, 1992a). The same is true whether the *PardC* or *PardB* promoter is driving *cat*, and expression of the *luc* (firefly luciferase) gene under the control of *PardB* or *PardC* shows similar kinetics (Fig. 7; J. Bailey, W. F. Dove and T. G. Burland, unpublished findings). The sequence *TardC* is the transcriptional terminator located downstream of the *ardC* gene, and includes the putative polyadenylation site. Therefore, *TardC* was expected to make mRNA expressed from plasmids more stable, but it has no measurable effect on level or duration of plasmid-borne gene expression in amoebae (Burland *et al.*, 1992a). It remains to be determined how expression of introduced genes can be

sustained at high levels over time. However, the availability of luciferase expression vectors (J. Bailey, W. F. Dove and T. G. Burland, unpublished findings) greatly accelerates progress in refining transformation techniques, as the assays are simple, non-radioactive, quantitative and rapid (deWet *et al.*, 1987).

2. *Stable Expression*

Once conditions were refined for transient expression, rational attempts could be made to transform amoebae of *P. polycephalum* to stable, selectable hygromycin resistance using vectors carrying the *PardC* element translationally fused to *hph*. Following electroporation of amoebae with vectors carrying *PardC–hph*, several hygromycin-resistant transformants have been isolated, and all of those analysed carry the *PardC–hph* fusion integrated into the genome at one copy in each nucleus (Burland *et al.*, 1992b). Hygromycin resistance is heritable through the life cycle in crosses, a necessary characteristic for many gene-disruption experiments, since disruptions will need to be carried out on diploid amoebae for genes that might be essential in the amoebal phase of the life cycle. The frequency of stable hygromycin-resistant transformants in these first experiments was less than 10^{-7} per treated cell, but further refinement of transformation conditions and efficiency are in progress. Identification of the protistan equivalent of yeast *ars* elements should increase transformation efficiency sharply. The high-resolution physical mapping of replicons of *P. polycephalum* made possible by the synchrony of the plasmodium (Benard and Pierron, 1990) may aid in this goal.

3. *Gene Targeting*

The stable transformation system developed allows addition of specific genes to the nuclear genome of *P. polycephalum*. However, geneticists prefer also to subtract information from the genome and, with integrative DNA transformation available, this can be achieved by gene disruption. As a test system, we sought to disrupt the *ardD* actin gene, which encodes an actin protein of divergent sequence and unknown function (Adam *et al.*, 1991). The *ardD* gene encodes a product that appears not to be expressed in amoebae (Adam *et al.*, 1991), and is not expected to be essential for amoebal viability. Therefore, in trying to disrupt *ardD*, we used the same haploid strain of amoebae, LU352 (Dee *et al.*, 1989), that was used in all of the other transformation experiments.

A genomic clone for *ardD* already contains a deletion around the intron-5/exon-6 junction (Adam *et al.*, 1991). We made a further 5′ deletion of

the first 0.75 kb of the coding region, leaving a sequence of approximately 1.6 kb of unbroken homology with the wild-type *ardD* allele. We then cloned this doubly deleted allele into a vector containing *PardC–hph* alongside the *ardD* allele, and transformed amoebae to hygromycin resistance. Southern blotting indicated that, in a set of five transformants, the wild-type allele of *ardD* was replaced by the deleted allele in three, indicating that gene disruption will be readily feasible for any gene for which a DNA clone exists (Burland *et al.*, 1992b). Considering that integrative DNA transformation was developed for *P. polycephalum* less than a year before the *ardD* targeting experiments, we can hardly be disappointed at this progress. The principal capability now sought is increasing transformation efficiency to a level that will permit DNA-mediated complementation of mutants.

VII. Concluding Remarks

The protists have been overlooked as experimental organisms among much of the research community. Some protists have been difficult to work with, partly due to their prodigious wealth of cell-biological features. Also, the genetic acrobatics possible with fungi has rightly attracted cell biologists to ascomycetes and basidiomycetes. Yet, despite the heavy weighting of research towards non-protists, key breakthroughs have been made with protists. Self-splicing RNA was discovered in the protist *Tetrahymena thermophila* (see Gold, 1990); acetylation of α-tubulin, now known to be widespread among eukaryotes, was discovered in the protist *Chlamydomonas reinhardtii* (L'Hernault and Rosenbaum, 1983; Piperno and Fuller, 1985); a defined temporal order of replication for individual genes and pairs of alleles was established in the protist *P. polycephalum* (Pierron *et al.*, 1984); and the exquisite mitotic synchrony of the plasmodium in *P. polycephalum* has now revealed that, for some genes, two alleles at a locus can replicate at distinct times in the S-phase (Cunningham and Dove, 1993).

It is ironic that protists, named from the Greek *protos*, meaning "first", should be last in line as research organisms. Now that sophisticated molecular approaches to cell-biological problems are possible in *P. polycephalum* and in other protists, further studies will inevitably reveal more of the knowledge that has been awaiting discovery in these organisms for so many millenia.

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