COMMENTARY

Cell division in Aspergillus

JOHN H. DOONAN

Department of Cell Biology, John Innes Institute, Colney Lane, Norwich NR4 7UH, UK

Summary

Amenable to sophisticated genetic and molecular analysis, the simple filamentous fungus Aspergillus nidulans has provided some novel insights into the mechanisms and regulation of cell division. Mutational analysis has identified over fifty genes necessary for nuclear division, nuclear movement and cytokinesis. Molecular and cellular analysis of these mutants has led to the discovery of novel components of the cytoskeleton as well as to clarifying the role of established cytoskeletal proteins.

Mutations leading to defects in the kinases (i.e. p34^cdc2) and phosphatases (i.e. cdc25 and PP1), which are known to regulate mitosis in other eukaryotes, have been identified in Aspergillus. Additional, as yet novel, mitotic regulatory molecules, encoded by the nimA and bim^E genes, have also been discovered in Aspergillus.

Key words: Aspergillus, cell division, mutations.

Introduction

Progress in solving the riddle of how the cell replicates and divides has come from two different approaches: one biochemical, the other genetical. Biochemists have exploited the special characteristics of animal oocytes (large cell size and predictable oscillation between interphase and mitosis) to isolate and characterize a factor which can regulate entry into and exit from mitosis. This factor, mitosis or maturation promoting factor (MPF), has the properties of a protein kinase and is itself regulated by phosphorylation/dephosphorylation (reviewed by Maller, 1991). Genetic analysis of eukaryotic microbes provides an alternative route to the identification of molecules involved in the cell division cycle. Such analysis affords a powerful, yet elegant, means to dissect these complex biological processes and has allowed at least some of the participating genes to be identified. In practice, genetic approaches have identified many regulatory factors, some of which are very similar to those found by biochemical analysis. Regulatory factors are often present in very low abundance, and therefore are more difficult to purify by biochemical means. Over the past few years genetic-based approaches have allowed rapid progress to be made in unravelling the complexities of cell division in several organisms including the budding and fission yeasts, the filamentous fungus, Aspergillus nidulans and the fly, Drosophila.

This commentary will introduce Aspergillus as an experimental organism and its uses in the study of nuclear division, for some of the most informative insights into eukaryotic cell division have come from studies on nuclear division-defective mutants (Morris and Enos, 1992; Oakley, 1992). An additional attraction of Aspergillus for such studies is that this fungus undergoes a simple, well-defined morphogenesis, which is also amenable to genetic dissection (see Timberlake, 1990, for review). This species therefore offers the possibility to study how the cell cycle is regulated during development. The first part of this review will deal with basic cell cycle controls in Aspergillus. The second part will introduce the idea that cell cycle controls may interface with those regulating development.

Genetic and molecular tools available for Aspergillus

A long history of classical genetic analysis has produced a deep understanding of both the physiology of Aspergillus and the organisation of its genome. The organism is normally haploid, with a complement of eight chromosomes. These correspond to the eight genetically defined linkage groups which contain hundreds of mapped genes affecting diverse metabolic and developmental characters (Clutterbuck, 1990). In addition to the haploid state, the fungus will grow as a vegetative diploid. The diploid is generally stable but can be induced to return to the haploid state by growth on substances which encourage chromosome loss. The production and breakdown of diploids is known as the parasexual cycle and allows rapid mapping of new mutations to chromosome. Complementation between different mutations can be undertaken in either diploids or in heterokaryons (strains containing two or more different haploid nuclei in a common cytoplasm). Different strains can be crossed simply by mixing the parental mycelia on suit-
able media and sealing to reduce oxygen supply. This induces sexual development and within 10 days to three weeks the fruiting bodies are formed. There are no mating types and therefore no restrictions on which strains may be crossed to which.

Since cell division is essential for normal development, mutants defective in cell division must be conditional. This means that such mutants can grow under so-called permissive conditions but exhibit their phenotype under other conditions (restrictive conditions). Filamentous fungi are rather adaptable organisms: *Aspergillus* will grow at reasonable rates between 15°C and 44°C, providing an exceptionally wide range of temperatures in which to look for temperature- and cold-sensitive mutations. Conditional mutations provide useful insights into the function of the temperature-sensitive gene products because they often allow rapid inactivation following a shift to restrictive conditions. Such mutations also effectively “tag” the gene and facilitate the isolation of a wild-type copy by DNA-mediated transformation. One of the primary attractions of *Aspergillus* is a large collection of such mutations defective in various aspects of cell division.

The relatively small size of the *Aspergillus* genome, coupled to efficient DNA-mediated transformation, permits functional complementation of interesting mutations with genomic DNA libraries. Subsequent marker rescue in *Escherichia coli* allows recovery of the wild type version of the gene (Osmani et al. 1987). If the library is constructed in a cosmids vector, the DNA can be packaged into bacteriophage λ heads directly from the genome and delivered into bacteria (Yelton et al. 1985). Chromosome-specific cosmid collections (Brody et al. 1991) are available, greatly facilitating complementation and gene isolation (Ehinger et al. 1990).

Stable transformation involves integration of exogenous DNA into the genome. Genes can be targeted to specific sites within the genome, allowing site-directed mutagenesis of target genes. Both gene replacement and molecular disruption are routine. Even essential genes can be disrupted and the progeny analysed using heterokaryon rescue (Osmani et al. 1988a). Primary transformants usually contain a mixture of nuclei, some contain a disrupted copy of the gene of interest while others contain the complete gene. Because the hyphae are multinucleate, the heterokaryon is usually viable and, through sporulation, the two types of nuclei are separated into uninucleate conidiospores. Germination of these spores under suitable conditions allows clear determination of the phenotype resulting from the lack of particular gene product.

Conversely, the effects of excess gene product can also be easily ascertained. Well characterised regulatable promoters are available in expression vectors for manipulating the level of gene expression. For instance, expression from the alcohol dehydrogenase (*alcA*) promoter can be either induced or repressed by choice of suitable carbon source (Pateman et al. 1983). This promoter has been used for modulating expression of *Aspergillus* cytoskeletal genes (Waring et al. 1989); of cell cycle control genes (Osmani et al. 1988b) and of developmental control genes (Adams et al. 1988). The *alcA* promoter is also useful for functional complementation experiments with genes from other organisms (Doonan et al. 1991).

In addition to the traditional genetic map, the eight chromosomes can be physically separated and identified (Brody and Carbon, 1989). Pulsed-field gel electrophoresis followed by probing with chromosome-specific markers provides the physical location of any cloned gene. The electrophoretic karyotype is particularly useful if no convenient mutation exists in a newly cloned gene (Ehinger et al. 1990).

### Nuclear division in filamentous fungi

Filamentous fungi form a multicellular colony consisting of branched networks of tubular cells that constitute the hyphae. Depending on the species and the developmental stage, these cells may be uninucleate, binucleate or, in the case of vegetative phase of *Aspergillus*, multinucleate. In *Aspergillus* the colony is established from a uninucleate haploid asexual spore. These spores are dispersed as dormant cells arrested in G1 - each has an unreplicated complement of DNA. The first hyphal nuclear division cycle takes about 75-120 minutes, depending on growth conditions. Mitosis is estimated to last 5 minutes at 37°C; G2, 30 minutes; S-phase, 25 minutes; and G1, 15 minutes. Under different conditions the duration of S and M-phase remain similar but the G1 and G2 phases vary (Bergen and Morris, 1983).

Mitosis, the process by which the cell’s nucleus is divided into two daughter nuclei, involves the coordinated rearrangement and separation of nuclear components. Some aspects of nuclear division are common to all eukaryotes. The cell’s genetic material, the chromosomes, is partitioned on a highly specialised and intricate spindle of microtubules. The spindle is assembled at mitosis, and its purpose may be to increase the fidelity with which the chromosomes are divided into the daughter nuclei. Other nuclear components (including some proteins, parts of the nucleolus) are segregated by a spindle-independent mechanism, whereby they are dispersed through the cytoplasm early in mitosis and are subsequently taken up into the daughter nuclei at the end of mitosis. To permit such dispersal and to allow spindle-dependent partition without damage, the structure of the nucleus undergoes profound rearrangements at mitosis. Mitotic alteration of nuclear structure is most extreme in animal and higher plant cells where large parts of the nucleus and the nuclear envelope are disassembled, but all eukaryotes (with the possible exception of budding yeast) undergo some modification of their nucleus in preparation for, and during, mitosis.

The interphase nucleus in *Aspergillus*, as observed by EM, contains a darkly stained nucleolus, surrounded by more lightly stained nucleoplasm, and the entire organelle is delimited by a typical double membrane nuclear envelope (Fig. 1A). Embedded within the nuclear envelope is a multi-layered structure, variously referred to as the nuclear plaque, spindle plaque or spindle pole body (the latter term will be used here). One of the first events in the nuclear duplication cycle (as observed by EM) is duplication of the spindle pole body. This occurs in interphase, probably
Cell division in Aspergillus during S-phase. The duplicated spindle pole bodies remain together until the cell traverses the G2/M boundary. During interphase, the spindle pole bodies nucleate few, if any, microtubules but as the cell enters mitosis microtubules are nucleated on the nuclear face of the spindle pole bodies.

As these intranuclear microtubules grow, the spindle pole bodies move apart and a short spindle of interdigitating microtubules is established between them. By mid-mitosis ("metaphase") the spindle pole bodies lie at opposite ends of the nucleus (Fig. 1B). Coincident with establishment of

Fig. 1. Nuclear structure in Aspergillus nidulans during interphase (A) and mitosis (B). A nimA5 strain was grown at restrictive temperature to produce a population of cells in interphase (A), and then shifted to permissive temperature to allow synchronous entry into mitosis (B). The interphase nuclei have intact nuclear envelopes (ne), discrete nucleoli (no), and spindle pole bodies (spb). Mitotic nuclei contain an intranuclear spindle of microtubules (s) and lack a nucleolus. Chromosomes (ch) are visible as stained regions within the nucleoplasm. (Modified from Osmani et al. (1987) with permission.)
the spindle the nucleolar region becomes less distinct and disappears. Dark-stained regions of condensed chromatin are apparent in metaphase nuclei and are connected to the poles by bundles of microtubules. The pole-to-pole microtubule bundle elongates during anaphase and increasing numbers of astral microtubules associate with the cytoplasmic face of the spindle pole body. At the end of mitosis, the daughter nuclei return to an interphase configuration with a discrete nucleolus and no intranuclear microtubules.

Mitosis can also be observed by phase-contrast microscopy (Robinow and Caten, 1969; Clutterbuck, 1970): the apical-most nucleus normally enters mitosis first and a wave of mitotic activity passes down the hyphae. The nucleus visibly changes; first, the nucleolus disappears and then the interface between nucleus and cytoplasm subsequently becomes less distinct. EM studies show that although the nuclear envelope does not disassemble completely during mitosis the membranes become less continuous. Because Aspergillus cells are so small, various cytological aids have been used to aid interpretation of mitotic events.

Monitoring the cell cycle

The isolation and analysis of cell cycle mutants in Aspergillus has relied heavily on microscopic examination of temperature-sensitive strains. The advent of improved fluorescent probes has provided new insights into the molecular changes which occur during the nuclear division cycle. The DNA-specific dye, DAPI, provides a convenient tool for monitoring number, distribution and conformation of nuclei within cells (Figs 2 and 3). In fixed cells, stained with DAPI, the interphase nucleus has three discernable regions: the chromatin stains as a bright blue crescent surrounding a single dark nucleolus and a single small densely stained very bright “DAPI-philic spot” is observed, usually at the edge of the chromatin. This spot may be heterochromatin underlying the spindle plaque - an area of osmiophilic material is observed in this region in EM micrographs (Fig. 1A). In mitosis, these separate regions are no longer discrete: the chromatin region condenses and the nucleolar region disappears.

Anti-tubulin antibodies provide an alternative means of monitoring the cell cycle (Gambino et al. 1984; and Fig. 4). Microtubule distribution cycles between an interphase organisation, where longitudinally orientated cytoplasmic microtubules run down the length of the hyphae, and a mitotic organisation where cytoplasmic microtubules disassemble as the intranuclear spindle is set up. Few cytoplasmic microtubules are present at metaphase, but as the cell enters anaphase, spindle pole associated (or astral) microtubules increase in length and abundance.

Fig. 2. Genetic analysis of cell growth and division in Aspergillus nidulans. Germination of uninucleate conidiospore produces an elongate multinucleate, multicellular hyphae. Several classes of mutation conditionally prevent normal hyphal morphogenesis at restrictive temperature, producing the phenotypes shown here. Mutations which affect the cell division cycle include nim (never in mitosis); bim (blocked in mitosis); nud (nuclear distribution); and sept (septation). Some genes may have multiple roles in cell division and morphogenesis (e.g. bimG).
A third cytological marker for cell cycle studies is the MPM-2 antibody. MPM-2, a monoclonal antibody raised against mammalian mitotic cells, recognises mitosis-associated phosphoproteins in a wide range of eukaryotes (Vandré et al. 1986). MPM-2 reacts with the spindle pole body from the middle of G₂ phase until the end of mitosis, implying that this structure is phosphorylated during this phase of the cycle (Engle et al. 1988). Accordingly, MPM-2 has provided a useful preliminary screen for mutations defective in regulation of protein phosphorylation (Doonan and Morris, 1989). In addition, other antibodies are now available which recognise specific cellular components. One notable example is anti-γ-tubulin antibodies which recognise the spindle poles.

Genetic analysis of the nuclear division cycle

By mutational analysis, over thirty genes have been implicated in DNA replication and nuclear division. These have been isolated either as temperature-sensitive lethals by Morris (1976) and Orr and Rosenberger (1976), as aneuploid generating mutants (Upshall and Mortimore, 1984) or indirectly as a result of screens for fungicide resistance (Van Tuyl, 1977). Several mutations which are sensitive to DNA damage have also been isolated, but only one of these has been cloned (Oza and Kafer, 1990).

Classification of nuclear division mutants

Morris (1976) isolated mutants conditionally defective in nuclear division but not in cell elongation. At restrictive temperature, these strains could produce hyphae. However, such hyphae contained fewer nuclei per unit length, suggesting that nuclear division was impaired. They were then classified on the basis of chromatin configuration and presence or absence of spindles at restrictive temperature. Those mutants with interphase-like nuclei were given the acronym nim, never in mitosis; those with condensed nuclei bim, blocked in mitosis.

To establish where the interphase mutations had their
point of action, 22 nim mutants were tested using a reciprocal shift method similar to that used by Hartwell (1976), but instead of using completion of cell division as the end point of the cell cycle, Morris used completion of nuclear division. The logic of this method relies on being able to place the temperature-sensitive arrest point of a cell cycle mutant relative to the arrest in S-phase caused by hydroxyurea (HU), an inhibitor of DNA synthesis (Bergen et al. 1984). By imposing a HU block before or after imposition of the temperature-sensitive block and scoring for ability to complete nuclear division under these two conditions one can infer whether the mutation has its effect before, at the same time, or after the HU block. Firstly, the uninucleate spores are germinated in HU at permissive temperature or in the absence of HU but at restrictive temperature. The cells are allowed to arrest at the respective HU-sensitive and ts block point, respectively. The spores grown on HU at permissive temperature are then shifted to HU-free medium at restrictive temperature; those grown on HU-free medium at restrictive temperature are moved to HU medium at permissive temperature. The ability of germlings to complete nuclear division is then scored. For cell cycle mutants which arrest in interphase there are four possible results: (1) G1 arrest: only restrictive temperature followed by HU will prevent completion of the cell cycle; (2) S-phase: both types of shift will prevent completion of the cell cycle; (3) G2: only HU followed by restrictive temperature will prevent completion of the cell cycle; (4) if neither type of shift prevents completion of the cell cycle, then the mutation probably affects a HU-independant pathway.

No strains were identified as blocking in G1. Several mutations produced nuclei which could not be scored due to abnormal nuclei. In addition, three mutations (in the nimL, nimM and nimN genes) were irreversible conditional lethals whose position of blockage could not therefore be determined. However, the latter three are all super-sensitive to low doses of HU, implying that they may be involved in DNA metabolism (Doonan, unpublished data). Five mutations, sodB1, nimC3, nimG10, nimK14 and nimQ20, conditionally block in S-phase. Mutations in seven genes conditionally prevent the transition from G2 to M. These include nimA, nimB, nimE, nimT, nimU, HfaB and HfaF, of which the first five have been cloned. The proposed block points of several nim mutations are shown in Fig. 5.

Regulation of the G2/M transition in Aspergillus

The reorganisation of cellular structure which occurs during mitosis is dramatic and extensive: cytoplasmic microtubules disappear as the spindle is established, the spindle pole is activated and extensive changes in nuclear structure occur. However, before any of these changes can occur, certain interphase events must be completed. These include the completion of DNA synthesis and in some organisms the attainment of a minimum cell size. Preventing mitosis from occurring until everything is ready ensures that a full com-

Fig. 4. The microtubule cycle in hyphae: antitubulin staining of hyphae shows the long cytoplasmic microtubules in interphase cells (Cyt) and the various stages of spindle development: early (S1), mid (S2) and late M-phase spindles (S3). (Reproduced from Osmani et al. 1988a.) ×2000.
Fig. 5. Summary of genes involved in the nuclear division cycle in *Aspergillus*. As in other organisms, different aspects of the duplication cycle are temporally separated. DNA is replicated in S-phase (S) and the nucleus is divided during mitosis (M). Genetic and cellular analysis of cell cycle mutations have allowed various interferences to be made with regard to the function of various genes: arrowheads denote gene products which are required for a particular phase of the division cycle; reversed arrowheads denote gene products which inhibit a particular phase. The small inner circle represents consecutive rounds of DNA synthesis without intervening mitosis.

Supplement of genetic information and sufficient cellular material are available to make two new, viable daughter cells. The integrity of the parental DNA is also important for the health of the daughter cells: monitoring mechanisms exist to ensure that DNA damage is repaired before division ensues. Hartwell and Weinert (1989) have postulated that the completion of events in interphase are monitored by "checkpoints". Such checks may represent molecular mechanisms whereby completion of particular events is monitored (Enoch and Nurse, 1991). If these checks are not satisfied, then subsequent events are not permitted. In this way temporal order could be imposed on what would otherwise be independent processes.

The decision to begin the division process is one of the critical transitions in the cell cycle; because if taken at the wrong time this would be effective suicide. The interphase to mitosis transition is dependent on a protein phosphorylation cascade, a central component of which is the p34<sup>cdc2</sup> protein kinase. Activation of this mitosis-specific histone H1 kinase is required for entry into mitosis in eukaryotes and is a major component of the G2/M checkpoint.

Transition into M requires at least two kinase activities in *Aspergillus*. One of these resembles the p34<sup>cdc2</sup> kinase and reaches a maximum as cells enter mitosis and correlates with its dephosphorylation (Osmani et al. 1991b). Genes encoding two of the regulatory subunits of this kinase have been identified from the analysis of mutations which block the cell cycle in G<sub>2</sub> (O’Connell et al. 1992). *nimT* encodes a phosphotyrosine phosphatase, similar to the fission yeast cdc25 phosphatase. Mutant *nimT*23 strains block in G<sub>2</sub> with phosphorylated p34<sup>cdc2</sup> kinase. Both of these proteins are involved in the mitotic activation of the cdc2 kinase in fission yeast and the evidence in *Aspergillus* indicates a similar situation here. *nimE* encodes a cyclinB subunit, similar to that of cdc13 of *Schizosaccharomyces pombe*. CyclinB is a regulatory subunit of p34<sup>cdc2</sup> kinase which is essential for its mitotic activation. An extra copy of the *nimE* gene can partially compensate for mutation of the *nimT* gene. O’Connell et al. (1992) suggest that the extra *nimE* may increase the amount of pre-MPF (tyrosine phosphorylated cyclinB/cdc2) for *nimT*23-mediated activation and, eventually, the mutant phosphatase activates enough kinase to allow entry into mitosis.

The other kinase activity, NIMA, is a distinct kinase which appears to act alongside the p34 kinase as a positive regulator of mitosis. At restrictive temperature, mutations in the *nimA* gene block in G<sub>2</sub> (Bergen et al. 1984) but the block is readily reversible - return of cells to the permissive temperature results in a synchronous entry into mitosis within a few minutes (Oakley and Morris, 1983). The level of *nimA* mRNA is under tight cell cycle regulation (Osmani et al. 1987), increasing to a maximum at mitosis and falling precipitously as the cells return to interphase. If cells are blocked in M (with antimicrotubule drugs or by mutations such as *bimE7*) *nimA* mRNA levels remain high. This suggests that the *nimA* mRNA is destroyed as the cells enter interphase. Inappropriate production of *nimA* mRNA from an inducible promoter can drive cells into a premature mitosis, even in the presence of a HU block (Osmani et al. 1988b). When multiple copies of *nimA* are overexpressed, cells persist in mitosis with elongated spindles and condensed fragmented chromatin. This suggests that exit from mitosis requires low levels of *nimA*. Antibodies raised against peptides based on the *nimA* amino acid sequence immunoprecipitate a casein kinase activity from *Aspergillus* extracts which reaches a maximum at G<sub>2</sub>/M. *nimA*, therefore, encodes a cell cycle-regulated β-casein kinase (Osmani et al. 1991a) that reaches a peak in mitosis.

When p34<sup>cdc2</sup> kinase activity was examined in a *nimA*5 mutant background, a surprising result was obtained. Despite the fact that the *nimA*5 mutant at restrictive temperature is blocked in interphase, it has a fully activated p34 H1 kinase (Osmani et al. 1991b). The p34 kinase is dephosphorylated on tyrosine; this is thought to be one of the final steps in its activation. Therefore, an active p34 kinase is insufficient to allow *Aspergillus* cells to enter mitosis.

A second surprise was in store. Other G<sub>2</sub> *nim* mutations block with p34 phosphorylated on tyrosine and low H1 kinase activity. One of these, *nimT*, is a homolog of the tyrosine protein phosphatase encoded by the *S. pombe* cdc25 gene (O’Connell et al. 1992). NIMA kinase is fully activated in a *nimT*23 strain at restrictive temperature despite the lack of active cdc2 kinase, suggesting that NIMA activation is independent of p34 kinase activation. These results demonstrate that active NIMA kinase and active p34 kinase are both required before an *Aspergillus* cell can enter mitosis (Osmani et al. 1991b).
bimE inhibits premature mitotic events during interphase

The NIMA protein kinase also interacts in an interesting way with another cell cycle control gene, bimE. The bimE7 mutation causes cells to block in metaphase at restrictive temperature, but the wild-type bimE gene product may act in interphase to prevent premature mitosis. The bimE gene product encodes a protein of 2073 amino acids. It has several potential membrane spanning domains (Engle et al. 1990) and may be a component of a G2 checkpoint which monitors, amongst other things, NIMA kinase (Osmani et al. 1991a). Loss of bimE function over-rides a variety of interphase blocks, driving cells into premature mitosis (Osmani et al. 1988a). These blocks include hydroxyurea and several mutations which arrest in S-phase. bimE/nimK14, bimE/nimC3, bimE/HU double blocks enter M with similar kinetics to a single bimE7. Spindles and chromatin condensation in these double-block experiments are typical of metaphase. The interaction between bimE7 and mutations in nimA are quite different. Although the bimE7 mutation will eventually drive a nimA mutant cell into M, the entry is much delayed compared to the bimE7 in a nimA+ background. Another difference is that the tubulin immunofluorescence reveals that spindles in the double mutant strain, bimE7/nimA5, are disorganised whereas in the single bimE7 mutant spindles appear in typical metaphase conformation. When the mitotic phenotype of strains carrying both a ts nimA5 and a ts bimE7 mutation was investigated in more detail, Osmani et al. (1991a) showed that although such cells could enter mitosis, the mitotic events which did occur were very perturbed. In particular, the nuclear envelope and spindle were abnormal, but other aspects such as chromatin condensation and spindle pole activation were normal. This suggests that (1) bimE+ acts as an inhibitor of mitotic events and (2) that nimA+ function is required for at least some mitotic functions. A model of how bimE and various nim mutations might interact to regulate entry into mitosis is shown in Fig. 6.

Involvement of calcium in the G2/M transition

Calcium has been widely implicated in cell cycle transi-
tions in plants and animals, but few cell cycle mutants have been shown to have defects in their calcium metabolism. The gene for calmodulin, one of the major internal cellular receptors for calcium, has been cloned and sequenced (Rasmussen et al. 1990). Using site-specific gene replacement, the calmodulin gene has been placed under the control of the inducible aclA promoter (Lu et al. 1992). These strains become ethanol-dependant, growing normally when grown on inducing media. If expression from the aclA promoter is repressed, most (>80%) cells arrest in G2. This block is reversible by returning cells to inducing media. Another interesting feature of aclA-calmodulin strains is that over-expression of calmodulin allows growth on very low calcium media.

Structural and motor proteins required for cell cycle progression

Coordination of the complex series of events which occur during mitosis may involve another set of checkpoints. For instance, defects in the spindle may lead to prolonged mitosis. The cell can apparently sense that mitosis is incomplete and therefore does not return to interphase. Mutation screens based on this logic have recently identified genes (in the budding yeast) which are required to monitor microtubule function (Hoyt et al. 1991; Li and Murray, 1991). These mutants, supersensitive to anti-microtubule drugs, fail to arrest if progress through mitosis is delayed. Ascertaining that mitosis has been correctly and completely executed is therefore a critical checkpoint in the cell cycle. Under normal circumstances anti-microtubule drugs block the cell cycle at M, probably because a crucial checkpoint which monitors the completion of mitotic events has not been satisfied.

For similar reasons, mutations in the structural genes for the α- and β-tubulins also lead to mitotic blocks. Aspergillus tubulins, like most other eukaryotes, are encoded by a small multigene family of two α- and two β- tubulin genes. The benA gene encodes two of the three β- tubulin polypeptides (Sheir-ness et al. 1978). A temperature-sensitive mutation, benA33, has hyperstable microtubules and blocks in mitosis with a persistent spindle (Oakley and Morris, 1981). Microtubule turnover is therefore necessary for normal completion of mitotic events.
\(\alpha\)-tubulin, gene, \(\text{tubA}\), was identified as a suppressor of \(\text{benA}\) mutations, which conferred resistance to the antimicrotubule fungicide, benomyl (Morris et al. 1979). Molecular disruption of the \(\text{tubA}\) gene leads to a mitotic block in vegetative cells (Doshi et al. 1991), while disruption of the other \(\alpha\)-tubulin gene, \(\text{tubB}\), leads to a block in meiosis (Kirk and Morris, 1991). Although there may be subtle differences in function between these tubulins, the likely reason for their different functions is differential expression: \(\text{tubA}\) is the major vegetative tubulin while \(\text{tubB}\) is highly expressed during sexual development.

**Gamma-tubulin**

Biochemical analysis of microtubules, from a variety of sources, established long ago that the basic backbone consisted of equimolar amounts of \(\alpha\) and \(\beta\)-tubulin molecules. However, suppressor analysis of mutations in the \(\text{benA}\) gene led to the discovery of a completely new member of the tubulin gene superfamily, \(\gamma\)-tubulin (Oakley, 1992). \(\gamma\)-Tubulin is encoded by the \(\text{mipA}\) gene which was identified as an allele-specific suppressor of the \(\text{benA33}\) mutation (Weil et al. 1986). The primary amino acid sequence of \(\gamma\)-tubulin is clearly related to both \(\alpha\)- and \(\beta\)-tubulin but is sufficiently distinct from either to define a completely new class (Oakley and Oakley, 1989). \(\gamma\)-Tubulin is located at the spindle poles, where it appears to be necessary for normal microtubule assembly during both interphase and mitosis (Oakley et al. 1990). Its location, the phenotype of cells lacking it and the genetic evidence that \(\text{mipA}\) interacts with \(\beta\) and not \(\alpha\)-tubulin have led to the suggestion that \(\gamma\)-tubulin determines both the location and polarity of microtubule initiation (Oakley, 1992). Another fascinating aspect of \(\gamma\)-tubulin is that cells lacking this protein appear to lose the ability to monitor successful completion of mitosis: the mitotic index (as judged by chromatin condensation) of a \(\gamma\)-tubulin disruptant is similar to wild type. Chromatin appears to condense and decondense at about normal rates, yet there is no spindle formation and therefore no chromatin separation. The single nucleus in such cells becomes large and probably polyploid, suggesting that the \(\Delta\text{mipA}\) cell ascertains (correctly) that chromatin has condensed and decondensed, and therefore that the next round of DNA replication may proceed, but ascertains (incorrectly) that DNA segregation has occurred. Alternatively, Oakley (1992) has suggested that \(\gamma\)-tubulin may be involved in monitoring the M-G1 transition.

Since its discovery, \(\gamma\)-tubulin has been identified in several other eukaryotes, including mammals, insects and yeast (see Oakley, 1992, for review), but its potential role in the M-G1 transition in other organisms remains to be clarified.

**Motors and mitosis**

The structure of the spindle undergoes extensive changes during its brief existence. For instance, components such as the spindle pole bodies need to be brought into alignment at opposite ends of the nucleus. Although microtubules are required for this, other proteins are necessary to provide the motive force. A likely candidate motor protein is the BIMC protein. A temperature-sensitive mutant, \(\text{binC3}\), fails to separate its spindle pole bodies. Microtubule elongation is not impaired and a unipolar spindle is made. Spindle microtubules run from the spindle pole bodies but do not overlap and consequently a normal metaphase spindle does not form. The mitotic block in \(\text{binC3}\) is transient and the cells exit mitosis after some time. Cloning and sequencing of \(\text{binC}\) revealed that this gene could encode a protein with an amino-terminal domain similar to that of kinesin, a motor protein involved in microtubule-dependent transport (Enos and Morris, 1990). Similar proteins have now been found in fission yeast (\(\text{cut7}\); Hagan and Yanagida, 1990). These proteins are probably components of the spindle apparatus, since antibodies raised against the \(\text{cut7}\) gene product decorate the mitotic spindle in fission yeast (Hagan and Yanagida, 1992). There may be a family of kinesin-like molecules in *Aspergillus* - a new member, \(\text{klp}\), has been isolated using PCR by O’Connel and Morris (see Reference quoted by Morris and Enos, 1992). \(\text{klp}\) has a carboxy-terminal motor domain and resembles the \(\text{KAR3}\) gene of budding yeast. \(\text{klp}\) function in *Aspergillus* is not known but the \(\text{KAR3}\) gene in yeast is needed for nuclear fusion (Meluh and Rose, 1990).

**Other structural proteins and mitosis**

\(\text{bim}\) encodes a member of the tetrameric peptide repeat family of proteins and may be a component of the nuclear scaffold. Mutation or molecular disruption of the \(\text{bim}\) gene cause a block in metaphase (O’Donnell et al. 1991) showing that this gene product is required for nuclear separation. The reason for the mitotic block is unclear: the spindle appears normal and chromatin condenses. \(\text{BIMA}\) may be a structural nuclear protein required for progression through M. \(\text{bim}\) is similar to the \(\text{nu}\) gene in fission yeast (Hirano et al. 1988) mutations in which cause a similar mitotic phenotype.

**Protein phosphatase is required for completion of anaphase**

During mitosis many proteins become phosphorylated, but at anaphase these phosphoproteins are dephosphorylated. A temperature-sensitive mutation in the \(\text{binG}\) gene, which encodes a type1 protein phosphatase (PP1), leads to the formation of large nuclei which have failed to complete anaphase correctly (Doonan and Morris, 1989). This mutation is pleiotropic, also affecting cell polarity and cell wall formation (Borgia, 1992). Anti-tubulin staining reveals that spindles fail to elongate normally and often break in the middle before nuclear separation is complete. The first indication that dephosphorylation might be defective was provided by comparing MPM-2-staining of wild-type and \(\text{binG}\) cells. Wild-type cells have very low levels of MPM-2-reactive proteins except at mitosis when the spindle poles react. The \(\text{binG}\) cells, even at permissive temperature, have increased levels of MPM-2 reactive proteins in the spindle pole bodies and at restrictive temperature the nucleolus also reacts (Doonan and Morris, 1989). The \(\text{binG}\) encodes a protein over 80% identical to mammalian PP1. Protein phosphatase assays, using phosphorylase A as an *in vitro* substrate, confirmed that the \(\text{binG}\) mutation caused a dramatic reduction in PP1 activity from cell extracts (Doonan et al. 1991). A mammalian PP1 gene complements the *Aspergillus* mutation. A rabbit PP1 cDNA was cloned into an inducible expression vector containing the \(\text{alcA}\) pro-
moter and this was transformed into the bimG11 mutant. Expression of the mammalian cDNA from this promoter fully complements all aspects of the bimG11 mutation including the reduction in PPase activity, the cell wall defect and the failure to separate daughter chromosomes (Doonan et al. 1991). A related protein phosphatase from mammalian cells, type 2A, which is nearly 50% identical to PP1 could not complement the bimG11 mutation. PP1 is also required for normal anaphase separation of chromatin in both fission yeast (Ohkura et al. 1989) and in Drosophila (Axon et al. 1990).

Although one can easily assay for PP1 activity in Aspergillus extracts, there is no apparent change in the level of PP1 activity through the cell cycle (Doonan, unpublished). While this could be an artifact of the assay procedure, it suggests that either the level of protein phosphorylation is regulated by modulation of kinase activity against a constant background of PPase activity or that PPase activity might be controlled by altering access to the substrate. The latter idea has some support from a recent paper on the cell cycle-specific entry of PP1 into the mammalian nucleus during G2 (Fernandez et al. 1992). Evidence that other gene products may influence PP1 activity comes from genetic analysis of bimG11 suppressors. The bimG11 allele is highly susceptible to extragenic suppression by mutation of other genes. Cold-sensitive alleles of at least three of these bimG11 suppressors (sug) have a defect in anaphase, similar to the temperature-sensitive anaphase defect in bimG11 (Doonan and Scofield, unpublished data). Cold-sensitive mutations in sugB lead to a more severe defect in anaphase than the original temperature-sensitive bimG11 mutation. Anaphase bridges are particularly obvious when these mutants are grown under cold conditions. The mechanism of suppression may provide insight into the function and/or regulation of this phosphatase.

Mutations which affect the fidelity of chromosome separation: another way of isolating cell cycle mutations

By screening for mutations which lower the fidelity with which chromosome segregation occurs, Upshall and Mortimore (1984) have identified novel genes required for cell cycle progression. Hfa (high frequency of aneuploidy) strains when grown at semi-permissive temperature give rise to a high percentage of asexual progeny with abnormal numbers of chromosomes. This implies that these strains are defective in chromosome segregation. We have found that a mutation in one of these genes, HfaL, produces a temperature-sensitive defect in anaphase, reminiscent of bimG11 and its suppressors (Doonan, unpublished).

The reasons for mis-segregation in the other strains is less clear, but at restrictive temperature two of these have another intriguing phenotype. The HfaB and HfaF mutants were shown to be temperature-sensitive for entry into mitosis, blocking in G2 (Bergen et al. 1984). Unlike many of the nim and bim mutants, the HfaB and HfaF mutants appear to undergo successive rounds of DNA replication despite failure to enter M when grown at restrictive temperature (Bergen et al. 1984; Upshall and Mortimore, 1984). This implies that HfaB and HfaF normally function to inhibit DNA replication if mitosis has not occurred. Genetic mapping studies suggest that the HfaB mutant is very closely linked to sodN1A, so that there is some doubt as to whether these mutations represent complementing alleles of the same gene (Upshall and Mortimer, 1984). The latter mutation causes a temperature-sensitive block in S-phase (Bergen et al. 1984). The molecular nature of the genes involved in karyotype stability remains to be determined but should provide some interesting insights into cell cycle checkpoints.

Building an organism: spatial and temporal aspects of cell division in a multicellular organism

As more is understood about the molecules immediately responsible for the control of nuclear division, it is clear that similar sets of proteins regulate mitosis in most if not all eukaryotes. It is also clear that the interactions between the regulatory molecules are complex and that as understanding increases, the level of complexity is likely to increase. This is particularly so in the case of multicellular organisms. Aspergillus is an excellent organism in which to investigate the relationship between cell division and morphogenesis. At least two distinct developmental pathways (sexual or asexual) are possible and each involves several recognisable cell types. During asexual development foot cells are formed within the vegetative hyphae. These foot cells give rise to aerial hyphae which grow away from the substratum. At a given height the tip of such hyphae swell to form a vesicle and subsequently metulae (primary sterigmata) are divided off on the surface of the vesicle. Phialides (secondary sterigmata) are formed on the metulae and by repeated division generate asexual conidia (summarised in Fig. 7). The sexual pathway involves the formation of other, quite distinct cell types, including Hulle cells, fertile hyphae and sexual ascospores. In both cases, these cells appear in a well-defined sequential order and the molecular control of many aspects of morphogenesis are understood (reviewed by Timberlake, 1990). The different cell types vary in a number of cell cycle parameters: some cells are multinucleate (the hyphae); others binucleate (fertile hyphae) or uninucleate (conidia). The potential for cell division is either unlimited (as in the case of hyphal tip cells) or strictly limited (in the case of primary sterigmata which divide once).

Such diversity probably represents the imposition of additional controls on the basic cell cycle machinery. One could envisage that such controls might include spatial control of nuclear position and division, temporal control of nuclear division and the degree to which nuclear division is coupled to cytokinesis.

Nuclear positioning

Cytokinesis occurs close to the site of nuclear division in many organisms and therefore the position of the nucleus during cell division may have profound consequences for the subsequent development. Even in unicells such as yeast, the nucleus occupies defined positions at different stages in the cell cycle. Nuclear positioning takes on added importance for multicellular organisms: nuclei (and other organelles) are moved to achieve non-random distribution, often in response to environmental stimuli or morpho-
isolated which fail to form colonies at restrictive temperature due to a conditional inability to move nuclei along their hyphae (Morris, 1976). These mutations specifically inhibit only nuclear movement (Oakley and Reinhart, 1985). Nuclear movement seems to be independent of mitotic events in that nuclear division mutants (except mutations in tubulin genes) display normal nuclear migration (Osmani et al. 1990).

The interaction between the NUD proteins and the microtubule cytoskeleton is unclear: both immunofluorescence (Osmani et al. 1990) and EM studies (Meyer et al. 1988) show that all classes of microtubules in nudC3 and nudA2 are apparently normal. One of the nud genes, nudC, has been cloned and sequenced, encoding a product of 22 kDa, but has no homology to other known sequences (Osmani et al. 1990). In some organisms a considerable amount of nuclear movement occurs during telophase. For instance, in budding yeast telophase movement is responsible for moving the daughter nuclei through the neck into the bud. This aspect of nuclear movement appears normal in nud- backgrounds, suggesting that these genes are especially involved in interphase motion.

At least three other genes are needed for nuclear movement during morphogenesis - mutations in apsA and apsB prevent nuclear migration into the primary sterigmata (Clutterbuck, 1977) and the bcnA mutation causes abnormal nuclear movement into conidiospores, leading to binucleate and trinucleate spores (Pizzirani-Kleiner and Azevedo, 1986). The relationship between these and the nud genes is not clear - no complementation studies have been reported. Although not lethal, the aps mutations do slow colony growth, suggesting that they may have some role in vegetative growth.

Kinesin-like molecules (encoded by the KAR3 gene) are required for nuclear fusion in yeast and have been implicated in moving the nuclei just prior to fusion (Meluh and Rose, 1990). A homologue of KAR3 has been cloned from Aspergillus (O’Connell and Morris, unpublished; see References quoted by Enos and Morris, 1992). The function of this gene in Aspergillus has not been determined but such molecules are obvious candidates for generating microtubule-based motion.

Cytokinesis

Cytokinesis physically separates the daughter cells from one another. The manner in which this occurs can have far reaching consequences for further growth of those daughter cells. For instance, symmetrical division produces two physically equal daughters, but asymmetric division produces daughters with different amounts of cytoplasm and consequentially different growth potential. The degree of coupling between karyokinesis and cytokinesis also has profound effects on the types of cell produced. In Aspergillus this varies from a rather loose coupling in the hyphal stages where multinucleate cells are formed to strict coupling during spore formation where uninucleate cells are produced.

Cell and nuclear division are not tightly coupled in the hyphal stages: at least 32, maybe 64, nuclei can co-exist in a single cell and nuclear number in hyphal compartments
Conclusions

Many of the ground rules of cell division in *Aspergillus* are now at least partly understood: the protein kinases and phosphatases which are the immediate governors of cell cycle transitions have been identified and something of their functions are known. There are clear indications in yeast that the central regulatory molecule, the p34^cd2^ kinase, can be modified by information from diverse sources: nutritional status; DNA integrity; cell size. The next task is to understand what other layers of control might be involved in modification of these central cell cycle control systems during the development of multicellular differentiated organisms such as *Aspergillus*.

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References


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