

A Mutation in *Aspergillus nidulans* that Blocks the Transition from Interphase to Prophase

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ABSTRACT In order to develop a method for obtaining mitotic synchrony in *Aspergillus nidulans*, we have characterized previously isolated heat-sensitive *nim* mutations that block the nuclear division cycle in interphase at restrictive temperature. After 3.5 h at restrictive temperature the mitotic index of a strain carrying one of these mutations, *nimA5*, was 0, but when this strain was subsequently shifted from restrictive to permissive temperature the mitotic index increased rapidly, reaching a maximum of 78% after 7.5 min. When this strain was examined electron-microscopically, mitotic spindles were absent at restrictive temperature, but were abundant in material fixed 5 min after shifting from restrictive to permissive temperature. From these data we conclude that at restrictive temperature *nimA5* blocks the nuclear division cycle at a point immediately preceding the initiation of chromosomal condensation and mitotic microtubule assembly, and upon shifting to permissive temperature these processes begin almost immediately. Thus, *nimA5* allows one to exercise a precise control over the initiation of microtubule assembly and chromosomal condensation in vivo through a simple temperature shift and, consequently, *nimA5* should be a powerful tool for studying these processes. Electron-microscopic examination of spindles of material synchronized in this manner reveals that spindle formation, although very rapid, is gradual in the sense that spindle microtubule numbers increase as spindle formation proceeds.

The molecular nature of the signals that stimulate the onset of mitosis is unknown, as are the chemical events that cause mitotic microtubules to assemble and chromosomes to condense. Here we report the characterization of a heat-sensitive mutation of *Aspergillus nidulans* that should be a powerful tool for studying these processes. This mutation, *nimA5*, blocks the transition from interphase to mitosis at restrictive temperature. When *nimA5* that has been blocked at restrictive temperature is shifted to permissive temperature, spindle formation and chromosomal condensation initiate rapidly and are complete within 7.5 min. Thus *nimA5* allows one to exercise a precise control over the initiation of mitosis in vivo through a simple temperature shift.

MATERIALS AND METHODS

The strains used in this study have been described previously (3). YAG (0.5% yeast extract, 2% glucose, 2% agar) was used as solid medium and YG (0.5% yeast extract, 2% glucose) as liquid medium. Aceto-orcein staining was carried out following the method of Robinow and Caten (9) and mithramycin staining was performed as previously described (5). Mithramycin was generously donated by Pfizer Inc., New York, NY.

Since *A. nidulans* is coenocytic and all nuclei within a hyphal segment enter mitosis together, the mitotic index was defined as the percentage of hyphal tip segments in which the nuclei have condensed chromosomes rather than simply

as the percentage of nuclei with condensed chromosomes (3). Preparation for electron microscopy has been described previously (5, 6).

RESULTS

Our initial goal was to develop a method for obtaining mitotic synchrony in *A. nidulans*. We chose a strategy that made use of previously isolated heat-sensitive (hs⁻) *nim* mutations (3). These mutations block the nuclear division cycle in interphase at restrictive temperature (3) and, thus, are defective for genes whose products are necessary for the completion of interphase. If the product of a hs- *nim* gene normally functioned at a single point in interphase, nuclei would be unable to proceed past that point at restrictive temperature and would thus accumulate at that blockage point. If the mutant were subsequently shifted to permissive temperature and the product of the defective gene became functional simultaneously in all nuclei, the nuclei should resume the nuclear division cycle at the same point and eventually enter mitosis in synchrony. We tested strains carrying each of the 26 *nim* mutations to determine whether we could obtain synchrony in this fashion, and we obtained some degree of synchrony with several mutants. Here, we report results obtained with one mutation, *nimA5*.

Conidiospores of *nimA5* and a wild-type control were inoc-

ulated onto dialysis tubing on solid medium and incubated for 12 h at permissive temperature (32°C). At this point the plates were transferred to restrictive temperature (42°C) for 3.5 h (~1.4 generation times at this temperature) after which the dialysis tubing carrying the hyphae was removed and rapidly submerged in gently shaking liquid medium equilibrated at 32°C. Samples were fixed at intervals after the shift, stained with aceto-orcein, and mitotic indices were determined. The results of this experiment are shown in Fig. 1. The mitotic index of *nimA5* at 32°C on YAG is normally 4–5%, like that of the parental strain FGSC 154. After 3.5 h at restrictive temperature the mitotic index of *nimA5* was 0, but 5 min after the shift to permissive temperature the mitotic index was >65% and after 7.5 min the mitotic index was 78%. The mitotic index for the wild-type control remained between 2.5% and 6% throughout the experiment. The rapidity with which nuclei enter mitosis after the downshift suggests that *nimA5* blocks the nuclear division cycle at a point immediately preceding the onset of mitosis and that upon shifting to permissive temperature the block is released and nuclei enter mitosis rapidly and in synchrony. However, we recognized a possible alternative explanation. Since the mitotic indices in this experiment reflect only the state of chromosomal condensation but not microtubule assembly, *nimA5* might simply be blocking chromosomal condensation at restrictive temperature, but not other events of mitosis such as spindle formation.

Confirmation that *nimA5* affects spindle formation as well as chromosomal condensation was provided by electron-microscopic examination of a strain carrying *nimA5*. First, we examined hyphae that were fixed after blockage at 42°C for 2 h before fixation. (Upon prolonged maintenance at restrictive temperature, nuclei in strains carrying *nimA5* become large, raising the possibility that they may be polyploid. We chose to examine hyphae that had been blocked at restrictive tempera-

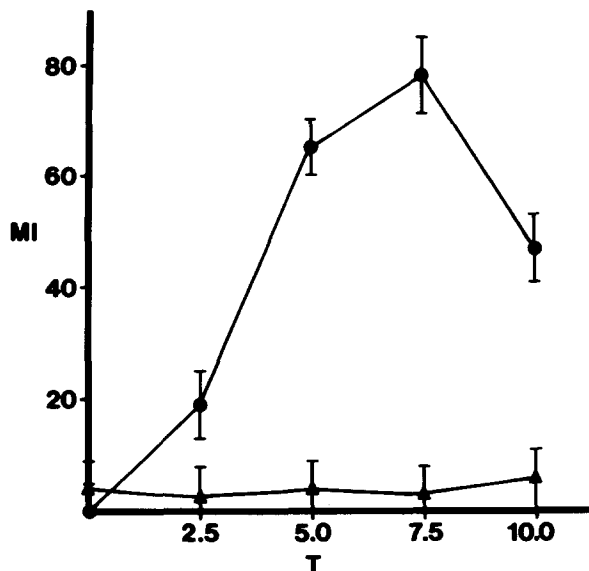


FIGURE 1 Mitotic synchronization in a strain carrying *nimA5*. Mitotic indices (*MI*) are plotted with respect to time (*T*) (in minutes) for a strain carrying *nimA5* (●) and for a wild-type strain (▲) (values are bracketed by standard errors). Both strains were grown for 12 h at 32°C, shifted to 42°C for 3.5 h and then (at *T* = 0) shifted back to 32°C. At the time of the shift the mitotic index of the strain carrying *nimA5* was 0 but it increased rapidly to 78% after 7.5 min. In contrast, the mitotic index of the wild-type strain remained at ~5% throughout the experiment.

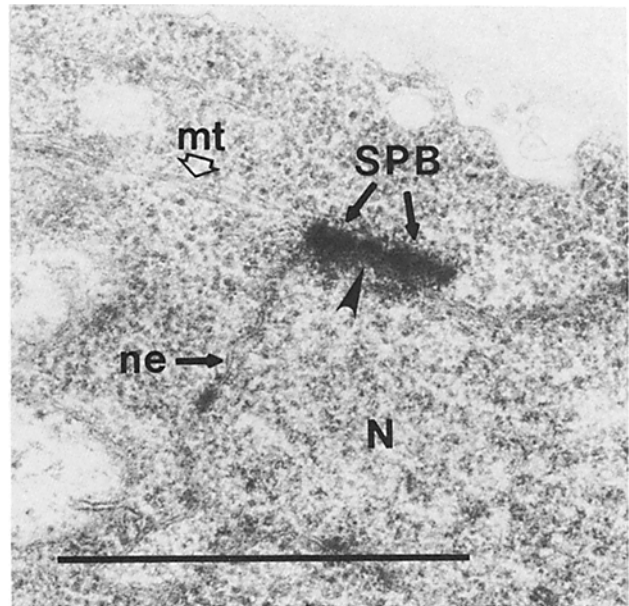


FIGURE 2 A strain carrying *nimA5* blocked in interphase at restrictive temperature. Two spindle-pole-bodies (*SPB*) are addressed to the nuclear envelope (*ne*). The *SPB* are joined by a small bridge (arrowhead). Microtubules (*mt*) are present in the cytoplasm but are absent from the nucleoplasm (*N*). Bar, 1 μ m. \times 54,500.

ture for 2 h because this period, which is slightly shorter than one generation time at 42°C, is long enough to allow the great majority of nuclei to reach their blockage point and short enough to avoid polyploidy.) We examined serial sections of 20 nuclei and found that each nucleus contained two spindle-pole-bodies (*SPB*) joined by a bridge (Fig. 2). Spindle microtubules were absent in all cases although cytoplasmic microtubules were present (Fig. 2) and in many cases cytoplasmic microtubules were seen to terminate at *SPB*. Examination of serial sections of nuclei from wild-type *A. nidulans* growing logarithmically showed that about one-half of the nuclei had double *SPB*. Therefore, in wild-type *Aspergillus* the spindle-pole-body must replicate approximately halfway through interphase. Since spindle formation does not begin in *A. nidulans* until 5–10 min before the completion of nuclear division (7, and Oakley and Morris, unpublished observations), the morphology of *nimA5* nuclei at restrictive temperature suggests that *nimA5* arrests the nuclear division cycle in the second half of interphase.

To determine whether spindle formation occurred after shifting to permissive temperature, we examined material that was blocked at 42°C for 2 h, then shifted to 32°C for 5 min before fixation. 25 of 50 nuclei examined contained mitotic spindles. Thus mitotic microtubule assembly is initiated rapidly after the shift from restrictive to permissive temperature. The chromosomal mitotic index obtained by aceto-orcein staining of the same material was 54%. (The mitotic index was slightly lower than for the previous experiment because the blockage period was slightly shorter than one generation and a small percentage of the nuclei probably had not reached the blockage point at the time of the shift.) Thus the increase in the chromosomal mitotic index after the shift to permissive temperature was accompanied by a similar increase in the percentage of nuclei containing mitotic spindles. From these data we conclude that *nimA5* does not simply block chromosomal condensation at restrictive temperature. Rather, *nimA5* blocks the nuclear di-

vision cycle in late interphase at restrictive temperature and, upon shifting to permissive temperature, nuclei rapidly enter mitosis.

While a detailed analysis of mitotic ultrastructure in *Aspergillus* is beyond the scope of this study, several facts about spindle formation are apparent from the spindles we have examined. First, the spindles in material fixed 5 min after the shift from restrictive to permissive temperature were not in early stages of spindle formation (Fig. 3 shows a typical spindle). Most (~80%) had reached a stage analogous to medial nuclear division as defined by Byers and Goetsch (1). Thus spindle formation is complete in many nuclei within 5 min of the shift to permissive temperature. Second, although spindle formation is rapid, it is also gradual in the sense that spindle microtubules increase in number as spindle formation proceeds. In early stages of spindle formation in which the two SPB are close together (Fig. 4), comparatively few microtubules are present. (For example, serial sections revealed that the spindle shown in Fig. 4 contains no more than 15 microtubules). As the SPB move farther apart the number of microtubules increases until, at medial nuclear division, 35–50 microtubules are present (6, 7, and unpublished observations). Third, SPB separation and the initiation of spindle microtubule assembly must occur at approximately the same time because in the 50 nuclei examined we found no separated SPB without spindle microtubules nor joined SPB with spindle microtubules. In addition, serial sections of early spindles reveal that microtubules from the two SPB intermingle in the nucleoplasm and in the cytoplasm outside the nuclear envelope. This con-

figuration is consistent with a role for spindle and/or cytoplasmic microtubules in SPB separation.

The preceding experiments were carried out on hyphae growing on dialysis tubing on agar plates. A limitation of this procedure is that it would be difficult to obtain large quantities of mitotic hyphae for biochemical analysis. To overcome this limitation we repeated the synchronization procedure in liquid culture. *NimA5* hyphae were grown in Erlenmeyer flasks at permissive temperature and shifted to restrictive temperature for 4 h before cool medium was added to obtain a rapid shift to permissive temperature. Samples were fixed 7.5 min after the shift and stained with the specific DNA stain mithramycin. Hyphae treated in this manner gave a mitotic index of 90%. Thus one can obtain mitotic synchrony in liquid culture with *nimA5*.

DISCUSSION

Although mutations that block the nuclear division cycle in interphase have been identified in several organisms recently reviewed by Nurse (4) and Pringle (8), *nimA5* appears to be unique among characterized mutations in that it blocks the nuclear replication cycle immediately before the onset of mitosis and produces high mitotic indices when blocked hyphae are shifted to permissive temperature. *NimA5* and the *Saccharomyces cerevisiae* mutation *cdc4* are similar in that they each block the nuclear division cycle after SPB duplication and before SPB separation. These mutations differ, however, in some important ways. First, *cdc4* is not rapidly reversible, SPB

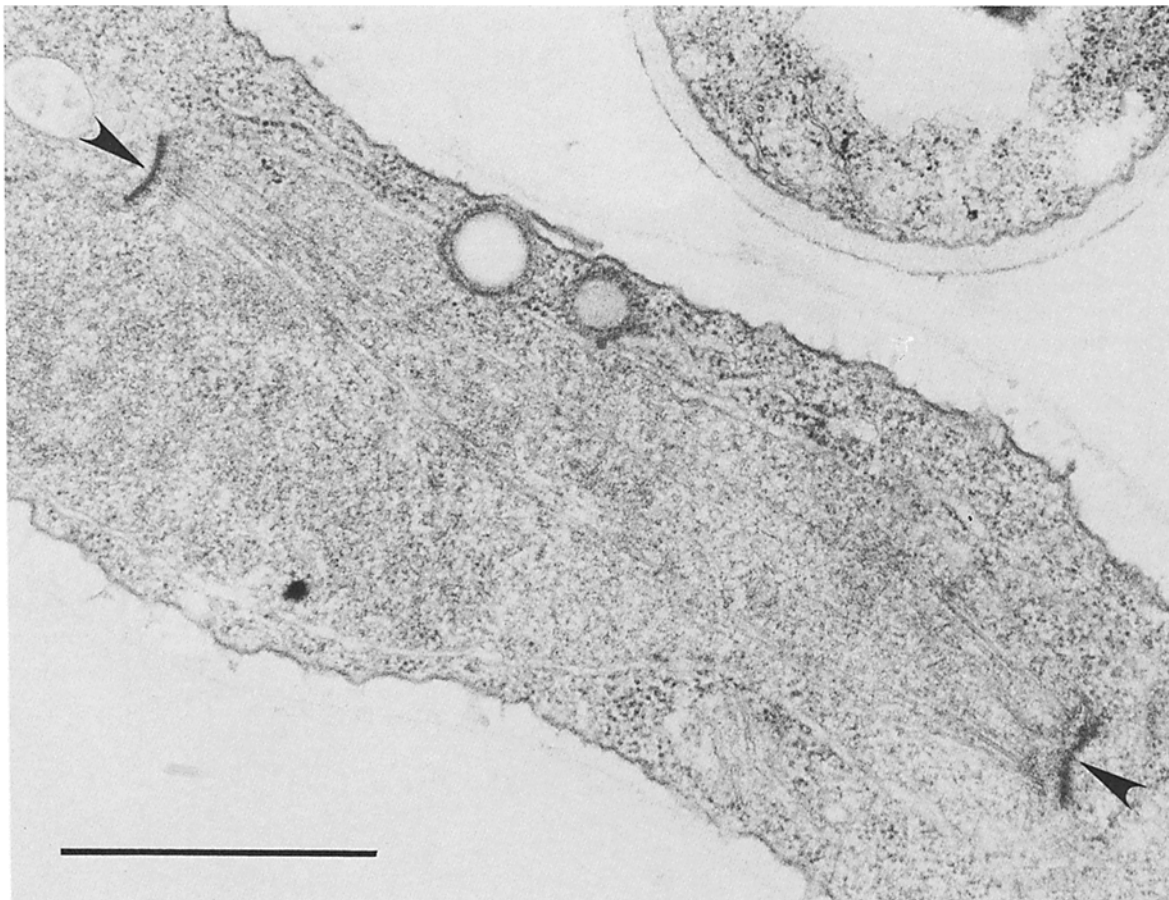


FIGURE 3 A medial nuclear division spindle. Microtubules are abundant in the nucleoplasm between the spindle-pole-bodies (arrowheads). Bar, 1 μm . $\times 43,000$.

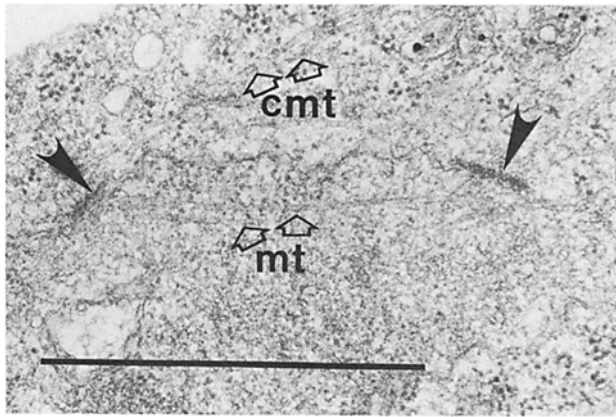


FIGURE 4 An early nuclear division spindle. Microtubules (*mt*) are present in the nucleus but are relatively sparse. The spindle-pole bodies (arrowheads), shown in glancing section, have separated a short distance. Cytoplasmic microtubules (*cmt* and unlabeled) are also present. Bar, 1 μm . $\times 51,000$.

separation occurring 7–8 h or more after shifting from restrictive to permissive temperatures (Dr. Breck Byers, personal communication). Second, spindle microtubules are present for much of the cell cycle in *S. cerevisiae* (2) and are present in blocked *cdc4* (1), consequently even if *cdc4* were rapidly reversible, it would not permit one to examine the control of microtubule assembly. Third, since chromosomal condensation is not observed in *S. cerevisiae*, *cdc4* can not be used to study the initiation of chromosome condensation.

NimA5 allows one to regulate the initiation of mitosis experimentally in a precise and simple fashion and, consequently, should be useful in ascertaining the cellular changes associated with spindle formation and chromosomal condensation. For example, *nimA5* should allow one to determine whether SPB from mitotic and nonmitotic cells differ in their abilities to nucleate microtubule assembly, whether posttranslational modification of microtubule proteins occurs during spindle formation, etc. While these experiments can, in principle, be performed in any organism in which the cell cycle can be synchronized, *nimA5* is particularly useful for such experiments because large amounts of *Aspergillus* mycelium containing either mitotic or nonmitotic nuclei can be obtained easily. *NimA5* should also be useful for testing the effects of metabolic inhibitors on spindle formation and chromosomal condensation. With *nimA5* one can block at a point immediately preceding the onset of mitosis, add inhibitors of protein synthesis, DNA synthesis, etc., allow inhibitors ample time to take effect and then shift to permissive temperature. If an inhibitor blocks the entrance of *nimA5* nuclei into mitosis upon shifting to permissive temperature, the inhibitor must affect a process that is essential to the initiation of mitosis and occurs within the 7.5 min preceding the onset of mitosis. It is important to note, however, that failure of an inhibitor to block mitotic initiation would be less meaningful. Several independent pathways of

cell cycle events are likely to exist (8) and some events necessary for, and proximal to, mitotic initiation may not be in the “*nimA5*” pathway. Since events that are not in the “*nimA5*” pathway are likely to be completed under restrictive conditions, subsequent addition of inhibitors of these events would not block mitotic initiation. Thus, for example, failure of a protein synthesis inhibitor to block mitosis would not necessarily mean that protein synthesis is not required during the 7.5 min before mitotic initiation, but only that there was no requirement for protein synthesis in the “*nimA5*” pathway subsequent to the *nimA5* block.

Finally, identifying and characterizing the *nimA5* gene product should reveal a great deal about the initiation of mitosis. Since *nimA5* blocks the nuclear replication cycle in interphase at restrictive temperature, a functional *nimA5* gene product must be necessary for the progression into mitosis. Since the majority of nuclei enter mitosis within 5 min after shifting from restrictive to permissive temperature with maximal mitotic indices obtained with 7.5 min, the *nimA5* gene product must function within 7.5 min of the onset of mitosis. The figure of 7.5 min is conservative. During this period, hyphae must equilibrate to permissive temperature, the *nimA5* gene product must become functional, and chromosomal condensation must occur. Thus the interval between *nimA5* becoming functional and nuclear commitment to mitosis must be even less. Because the product of *nimA5* functions so near to the onset of mitosis its normal cellular function may be to participate in the control of the initiation of mitosis. However, it is also possible that the *nimA5* gene product does not normally function in the regulation of mitosis but becomes the rate-limiting factor when it is dysfunctional. In either case the product of *nimA5* is necessary for mitotic initiation and, consequently, its characterization should help to elucidate this process.

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