Abstract. The temperature-sensitive cell cycle mutation nimA5 causes nuclei of Aspergillus nidulans to be blocked in late G2 at restrictive temperature. Under these conditions the spindle pole body divides but does not separate and the mitotic index drops to zero. If nimA5 is blocked for more than one doubling time and then shifted from restrictive to permissive temperature, nuclei immediately enter mitosis, the mitotic spindle forms, and the chromosomes condense (Oakley, B. R., and N. R. Morris, 1983, J. Cell Biol., 96:1155–8). We have cloned the wild-type nimA gene by DNA-mediated complementation of the nimA5 mutant phenotype and have characterized nimA mRNA expression by Northern blot analysis. The transcript is 3.6 kb in length and is under tight nuclear cycle regulation. In synchronously dividing cells, the levels of nimA mRNA become elevated as cells enter mitosis and drop sharply as cells progress through mitosis. Cells blocked in S-phase with hydroxyurea have very low levels of nimA mRNA. Cells blocked in mitosis, either by the antimitotic agent benomyl or by the cell cycle mutation bimE7, maintain elevated levels of the nimA transcript. These data demonstrate not only that nimA is required for entry into mitosis, but because the transcript is normally expressed cyclically and is under tight cell cycle control, they suggest that nimA may play a regulatory role in the initiation of mitosis.

Mitosis constitutes a major transition point of the nuclear division cycle and during normal growth it is regulated such that it occurs once per cell cycle at the appropriate time. The cell must therefore monitor its constituents and trigger the mitotic process after DNA replication has been completed and when all the mitotic machinery is available. Present evidence suggests that the trigger that promotes the G2-M transition may be a cytoplasmic factor. For instance, when a somatic interphase nucleus is introduced into the cytoplasm of a mitotic cell by cell fusion, it will prematurely undergo the G2-M transition (Rao and Johnson, 1970; Matsui et al., 1972). Similarly, interphase nuclei injected into the cytoplasm of Xenopus oocytes initiate mitosis when the oocyte undergoes meiotic maturation (Gordon, 1968; Ziegler and Masui, 1973). It is possible to detect the activity responsible for these effects by injecting cytoplasm into immature oocytes, which are naturally blocked in early meiotic prophase, and by monitoring the oocyte nucleus for initiation of meiosis. Such initiation can be induced by injection of cytoplasm from maturing oocytes (Masui and Markert, 1971; Smith and Ecker, 1971) or from mitotic cells, including a cdc mutant of Saccharomyces cerevisiae when it is blocked in mitosis (Kishimoto et al., 1982; Sunkara et al., 1979; Nelkin et al., 1980; Weintraub et al., 1982).

The cytoplasmic activity responsible for these phenomena has been called maturation-promoting factor or M-phase-promoting factor (MPF). The level of MPF is correlated with mitosis in frog eggs (Gerhart et al., 1984). It has also been demonstrated that addition and removal of partially purified MPF can drive the mitotic cycle in frog eggs and that stabilization of MPF activity by a second agent, called cyto-static factor, causes a mitotic arrest (Newport and Kirschner, 1984). In vitro analyses have also shown that a partially purified extract containing MPF is capable of inducing the early events of mitosis in isolated somatic nuclei (Miake-Lye and Kirschner, 1985; Lohka and Maller, 1985). These data clearly indicate that there are gene products in mitotic cytoplasm which are responsible for the G2-M transition and which are not active or are not present before the transition.

In recent years it has become possible to isolate euakaryotic genes by transformation with DNA gene banks to complement recessive mutations. This approach has allowed the isolation of numerous genes from Saccharomyces cerevisiae (e.g., Nasmyth and Reed, 1980) and Schizosaccharomyces pombe (e.g., Beach et al., 1982) that function during various stages of the cell cycle. The ability to transform Aspergillus nidulans (Balance et al., 1983; Tilburn et al., 1983; Yelton et al., 1985; Yelton et al., 1985; Johnson et al., 1985) allows

1. Abbreviations used in this paper: MPF, maturation-promoting factor; POK, 3-phosphoglycerate kinase; YG, 0.5% yeast extract, 2% glucose, trace elements.
a similar approach to be applied to this organism, as we have previously isolated recessive temperature-sensitive mutations (Morris, 1976a) that define specific cell cycle genes. In Aspergillus, unlike S. cerevisiae but like those of higher eukaryotes, the chromosomes become visibly condensed during mitosis and can be viewed in a condensed state at mitosis under the light microscope (Robinow and Caten, 1966). This feature has allowed us to identify mutations specifically affecting the mitotic process. In particular we have isolated and characterized a temperature-sensitive mutation in A. nidulans (nimA5) that blocks the G2-M transition at restrictive temperature (Morris, 1976a). The terminal phenotype of nimA5 as revealed by light and electron microscopy is characterized by a total lack of mitotic spindles and by the presence of a duplicated spindle-pole body. However, if this mutant is returned to permissive temperature, full mitotic spindles and condensed chromosomes synchronously appear in 80% of the cells within 7.5 min (0.075 fraction of cell cycle) of the shift (Oakley and Morris, 1983). The product of the nimA gene must therefore function very near the onset of mitosis, and its normal cellular function may be to participate in the control of the initiation of mitosis.

In this paper we report the molecular cloning of nimA by complementation of nimA mutant phenotype and show that the transcript levels of this gene peak during the G2-M period. This pattern of expression should ensure that high levels of the nimA gene product accumulate at mitosis, and indicate that nimA could be related to cytoplasmic mitotic factors that can be detected only during mitosis.

Materials and Methods

Aspergillus Strains and Growth

The Aspergillus strains used were R153 (wA3; pyroA4), SO6 (nimA5; wA2; yA2; chaA1; pyrG9B; cexA16; choA1), SO7 (nimA5; wA2), SO5 (nimA5; yA2; pyrG9B), and FGSC4 (wild type). YAG (0.5% yeast extract, 2% glucose, trace elements, 2% agar) was used as solid medium and was supplemented with 10 mM uridine for growth of pyrG9B strains (May et al., 1985). YG (YAG without agar) was used for growth in liquid culture.

Electron Microscopy

Samples for electron microscopy were quick frozen in liquid propane at -190°C, transferred to and substituted in anhydrous acetone containing 2% osmium tetroxide and 0.1% uranyl acetate at -85°C. After substitution for 48 h, samples were passively rewarmed to room temperature, infiltrated, and polymerized in Araldite resin. Serial gold thin sections were poststained with uranyl acetate and lead citrate and examined in a Hitachi H 600 electron microscope.

Aspergillus nidulans Transformation

For the preparation of protoplasts, 10⁶-10⁷ conidia were inoculated into 50 ml of YG containing 10 mM uridine. Growth was at 32°C for 5.5 h with shaking until germ tube emergence was visible. The germinated conidia were harvested by centrifugation and resuspended in 40 ml of lytic mix containing 0.4 M ammonium sulphate, 50 mM K⁺ citrate, pH 6.0, 0.5% yeast extract, 0.5% sucrose, 10 mM MgSO₄, and 10 mg/ml Novo-zyme 234 (lot No. 1030; Novo Industries, Bagsvaerd, Denmark). (Because some Novozyme preparations lysed protoplasts, all our Novozyme was subjected to an 80% ammonium sulphate precipitation at a concentration of 50% in 10 mM Tris-C1, pH 8.0. After centrifugation the precipitated protein was removed and the supernatant solution at 4°C.) Digestion of cell wall was allowed to proceed for 2 h at 32°C with gentle shaking after which the lytic mix was removed by two washes in 50 ml of 0.4 M ammonium sulphate, 1% sucrose, and 50 mM K⁺ citrate, pH 6.0, at 4°C. The protoplasts were resuspended in 1 ml of 0.6 M KCl, 50 mM CaCl₂, 10 mM K⁺ 2-(N-morpholino)ethane sulfonic acid, pH 6.0, and were stored at 4°C.

For transformation, 100 µl of the protoplasts were added to 4 µg of DNA in 2 µl of H₂O, followed by 50 µl of 25% polyethylene glycol (PEG) 6000 containing 50 mM CaCl₂, 0.6 M KCl, and 10 mM Tris-C1, pH 7.5. After incubation on ice for 15 min a further 1 ml aliquot of the PEG solution was added and the cells were allowed to incubate at room temperature for 15 min. Aliquots of this mixture (up to 500 µl) were plated in 3 ml 0.5% yeast extract, 0.4 M ammonium sulphate, 1% sucrose, trace elements, 1% agar, at 47°C onto plates containing the same but solidified with 2% agar. For some experiments 3 mM uridine was incorporated into the regeneration media and protoplasts were allowed to regenerate for 18 h at 32°C before shifting to 42°C.

General Techniques

Genetic techniques for A. nidulans have been described (e.g., Pontecorvo et al., 1953; Clutterbuck, 1974; Cove, 1977). Standard molecular cloning techniques were performed as described by Maniatis et al. (1982).

Isolation of DNA and Construction of Gene Bank from A. nidulans

Conidia of FGSC4 were inoculated into 500 ml YG to a final concentration of 10⁷/ml and allowed to grow at 37°C for 18 h with vigorous shaking. The mycelia were harvested by filtration onto Miracloth (Calbiochem-Behring Corp., Costa Mesa, CA), rinsed completely with distilled water, and then eluted by boiling between absorbent paper towelling. The dried mycelia were ground to a fine powder using a mortar and pestle cooled in liquid N₂. 2 g of this material was added to 10 ml of extraction buffer at 65°C containing 50 mM Tris-C1, pH 8.0, 0.1 M EDTA. 0.1% vol of 20% sarkosyl was added and allowed to incubate for 30 min. The mixture was then cooled to 37°C and incubated with 100 µg/ml RNase A for a further 30 min. Proteins were then added to a concentration of 100 µg/ml and the incubation continued for 3 h. Debris was removed by centrifugation for 10 min at 10,000 g and protein removed by successive phenol and chloroform extractions. The DNA was precipitated with an equal volume of 2.5 M NaCl/20% PEG 6000 and resuspended in TE, followed by an isopropanol precipitation, washed with 70% alcohol and dissolved in a final volume of 500 µl TE.

A vector was constructed by ligating the pyrG gene of Neurospora crassa from AlpGMI (May et al., 1985) as a 2.2-kb Eco RI fragment into the Eco RI site of pBR322 to yield the plasmid pGM3 (67 kb). A. nidulans DNA fragments were generated by limited digestion of 60 µg of DNA with 50 U of Taq I for 15, 30, 45, and 60 min at 65°C. The pooled DNA was subjected to agarose gel electrophoresis and a subpopulation of fragments between 6 and 10 kb were cut from the gel and collected by electrophoresis into dialysis bags. After phenolization and ethanol precipitation these Taq 1 fragments were ligated with pGM3 DNA that had been treated with Cla I and calf intestinal alkaline phosphatase and were used to transform Escherichia coli JM38 (Veira and Messing, 1982) to ampicillin resistance. 1.8 x 10⁸ ampicillin-resistant colonies were obtained, 80% of which were sensitive to tetracycline. Plasmid DNA was isolated from nine of these colonies, all of which contained insert DNA at the Cla I site with an average size of 70 ± 2 kb. The primary library therefore contained >4.0 genomic equivalents, assuming a genome size of 2.3 x 10⁹ kb (Timmerlake, 1978) for A. nidulans. The primary library was harvested from the transformation plates into Luria broth containing 20% glycerol and stored at -70°C. For amplification, the primary library cells were grown for 6 h at 37°C before isolating plasmid library DNA using the alkaline lysis procedure followed by banding in cesium chloride (Maniatis et al., 1982).

Plasmid Recovery from A. nidulans Transformants

A total of 10 µg of DNA isolated from SOS6T5 was digested with 20 U of Bgl II in a volume of 200 µl at 37°C. 40-µl samples were removed at 0, 10, 20, 30, and 40 min from the digestion and added to 40 µl of phenol to terminate the reaction. The digestion products were extracted with chloroform and brought to 500 µl with ligation buffer (Maniatis et al., 1982) and incubated for 14 h at 4°C in the presence of 8 U of T4 DNA ligase to circularize linear fragments. Each ligation was precipitated with ethanol and resuspended in 50 µl of 10 mM Tris-C1, pH 8.0, 0.1 M EDTA, 5 µg/ml RNase A, at 4°C. 25-µl aliquots of each ligation were used to transform 200 µl of JM83 to ampicillin resistance and plasmids were isolated from these transformants using the alkali lysis mini-prep procedure (Maniatis et al., 1982).
Isolation of Poly(A)+ mRNA

RNA was isolated from cells that were frozen with liquid N2 and ground to a powder. The broken cell mass was transferred to a tube containing extraction buffer (50 mM Tris-HCl, pH 8.0, 0.3 M NaCl, 5 mM EGTA, 2% SDS) and one-half volume of phenol preheated to 65°C and vortexed for 1 min. After cooling to room temperature, one-half volume of chloroform/isooamyl alcohol (24:1) was added and the tube was vortexed again and centrifuged at 12,000 g for 10 min. The aqueous phase was transferred to a fresh tube and the organic layer was reextracted with one-half volume of extraction buffer and combined with the first aqueous phase. The combined aqueous material was extracted twice with phenol/chloroform/isooamyl alcohol (50:48:2) and twice with chloroform/isooamyl alcohol (24:1). Nucleic acids were precipitated by the addition of 2 vol of ethanol and storage at −20°C overnight, sedimented at 12,000 g for 10 min, dried, dissolved in water, and stored frozen. Poly(A)+ RNA was isolated by chromatography using oligo dT cellulose as described in Maniatis et al., 1982.

Small-scale RNA Isolation

Between 0.1 and 0.2 g of mycelia were harvested by filtration and frozen on dry ice in 1.5 ml eppendorf tubes before lyophilization (Raeder and Broda, 1985). The dried mycelia were ground to a fine powder in the tube using a Kontes pellet pestle and extracted as described above but using 1/10 vol and carrying out the initial extraction at room temperature, not at 65°C. RNA was isolated from cells that were frozen with liquid N2 and ground to a powder. The broken cell mass was transferred to a tube containing extraction buffer (50 mM Tris-HCl, pH 8.0, 0.3 M NaCl, 5 mM EGTA, 2% SDS) and one-half volume of phenol preheated to 65°C and vortexed for 1 min. After cooling to room temperature, one-half volume of chloroform/isooamyl alcohol (24:1) was added and the tube was vortexed again and centrifuged at 12,000 g for 10 min. The aqueous phase was transferred to a fresh tube and the organic layer was reextracted with one-half volume of extraction buffer and combined with the first aqueous phase. The combined aqueous material was extracted twice with phenol/chloroform/isooamyl alcohol (50:48:2) and twice with chloroform/isooamyl alcohol (24:1). Nucleic acids were precipitated by the addition of 2 vol of ethanol and storage at −20°C overnight, sedimented at 12,000 g for 10 min, dried, dissolved in water, and stored frozen. Poly(A)+ RNA was isolated by chromatography using oligo dT cellulose as described in Maniatis et al., 1982.

Northern Blots

RNA was denatured with methylmercury and separated by electrophoresis through 1% agarose as described by Bailey and Davidson, 1976. RNA was transferred to a Gene Screen membrane (New England Nuclear, Boston, MA) according to the manufacturer’s instructions. Hybridization and washing were as described for Southern blots but included 10% dextran in the hybridization media with 5 ng/ml nick-translated probe and used 3× SSC at 65°C in the final wash. Filters were exposed on Kodak XAR-5 film with Dupont Cronex Lightning-Plus intensification screens at −80°C.

Southern Blots

DNA was digested, electrophoresed, and transferred to nitrocellulose as described previously (May et al., 1985). Hybridizations and nick translations were performed as described previously (May et al., 1985).

Plasmid Construction and Propagation

Plasmids were constructed in pUC18 and propagated in JM83 as described previously (May et al., 1985). pSA01 was constructed from the 5.4-kb Kpn I fragment of pSO6TR5.26 (see Results) ligated into the Kpn I site of pUC18. pSA02 contains the 2.6-kb Xho I fragment of pSA01 ligated into the Sal I site of pUC18. pSA03 contains the 1.4-kb Pst I-Xho I fragment of pSA02 isolated by cutting pSA02 with Pst I and by self-ligating. pSA04 contains the 1.2-kb Pst I fragment of pSA02 ligated into the Pst I site of pUC18. pSA06 contains the 1.8-kb Cla I-Kpn I fragment of pSA01 ligated into Kpn I/Acc I cut pUC18. pSA05 contains the 3.5-kb Kpn I-Cla I fragment of pSA01 ligated into Kpn I/Acc I cut pUC18.

Synchronous Culture of A. nidulans

Conidia of R153 were inoculated into 200 ml YG to a final concentration of 2 × 10⁸/ml and incubated with shaking at 32°C, 200 rpm for 7.5 h. At this time YG at 70°C was added to bring the temperature rapidly to 42°C. Incubation was continued at 42°C for 3 h before addition of YG at 4°C to bring the temperature rapidly to 32°C for release experiments. Incubation was continued at 32°C for a half hour more. Samples were taken and processed as described for synchronous culture samples.

Results

The nimA5 Mutation Causes a Very Late and Readily Reversible Block in G2

Mitosis in A. nidulans is completed in 5 min (0.05 fraction cell cycle) after a well-defined G2 period of 20 min (Bergen and Morris, 1983). Nuclei in G2 are characterized by a lack of condensed chromatin coupled with the presence of a duplicated spindle pole body that resides on the nuclear membrane. Progression into mitosis is defined by condensation of chromatin and the formation of an intranuclear spindle formed between the separated spindle pole bodies. Therefore, a very sharp morphological transition occurs in nuclei during progression from G2 into M. Strains of A. nidulans carrying the temperature-sensitive mutation nimA5 become blocked in late G2 when grown at the restrictive temperature, arresting nuclei in the mitotic cycle with uncondensed chromatin but with a duplicated spindle pole body (Fig. 1 A). If blocked cells are returned to permissive temperature they synchronously enter mitosis. The duplicated spindle pole body separates, an intranuclear spindle composed of nonkinetochore and kinetochore microtubules is formed between the spindle pole bodies, and the chromosomes become condensed within 5–7 min of the shift (Fig. 1 B; Oakley and Morris, 1983). The micrographs of Fig. 1 clearly show that the decondensed chromatin in nimA5-blocked nuclei goes through a process of condensation after downshift to permissive temperature. This improved preservation of chromosome structure, compared with our earlier data, is due to the use of freeze substitution to fix samples in the present study.

DNA-mediated Complementation of the Mutant nimA5 Phenotype

A gene bank was constructed (SAILIB1, Materials and Methods) in an integrative plasmid vector, pGM3, that contains the pyr4 gene of N. crassa (which encodes orotidine 5'-phosphate decarboxylase) ligated into the Eco RI site of pBR322. The neurospora pyr4 gene complements uridine auxotrophy caused by the pyrG89 mutation of A. nidulans. A total of 25 μg of SAILIB1 DNA was used to transform 6.5 × 10⁸ protoplasts isolated from a strain carrying nimA5 and pyrG89 (SA06). 2.2 × 10⁴ pyr4+ transformants were obtained, seven of which exhibited growth at restrictive temperature (42°C), and were designated S06TR1-S06TR7. After streaking to single colony all temperature-insensitive transformants showed wild-type growth characteristics at both permissive (32°C) and restrictive temperature except S06TR3, which conidiated poorly. These strains were assumed to be heterozygous for nimA and also to possibly contain extra copies of other genes that may have cotransformed with nimA. DNA was isolated from each temperature-insensitive transformant and probed for pBR322 sequences (Southern, 1975) using nick-translated plasmid DNA (Fig. 2). Three of these strains (S06TR2, TR4, and TR5) showed Osmani et al. Mito tic Transition in Aspergillus
Figure 1. A mitotic transition induced by nimA5. A strain carrying nimA5 was held at restrictive temperature (42°C) for a period of 2 h (A) then returned to permissive temperature (32°C) for 7.5 min (B). ne, nuclear envelope; nu, nucleolus; spb, spindle pole body; ch, condensed chromosome. The arrowhead indicates putative kinetochore microtubules. Bars, 1 μm.

Isolation and Subcloning of nimA from SO6TR5
To re-isolate the wild-type copy of nimA transformed into SO6TR5, total DNA from this strain was subjected to partial digestion with Bgl II for various periods of time (see Materials and Methods). This was ligated with T4 DNA ligase at a low DNA concentration to promote intramolecular ligation and used to transform E. coli cells to ampicillin resistance. To ensure a high yield of partial digestion products, conditions were selected such that at early time points of the digestion few E. coli transformants were obtained. From a total of 10 μg of DNA treated in this manner 144 ampicillin-resistant colonies were obtained, and plasmids from 14 were analyzed by Eco RI digestion and agarose gel electrophoresis. All produced a diagnostic 2.3-kb Eco RI fragment corresponding to a fragment containing the pyr4 gene present in pGM3. As ampicillin resistance was used to select these plas...
Southern blot analysis of transformants of SO6 capable of growth at restrictive temperature. DNA (5 μg) isolated from strains SO6TR1-SO6TR7 (lanes 1-7) was digested to completion with Bgl II and subjected to electrophoresis through 0.7% agarose and transferred to nitrocellulose. The filters were hybridized with nick-translated pBR322 DNA. The position and size in kilobases of lambda Hind III markers is indicated on the right.

We conclude that they contain the original vector sequence. In addition, by subtracting the vector sequence these plasmids were found to contain *Aspergillus* genomic DNA of the following sizes: 1.5, 8.4, and 13.6 kb, designated as pSO6TR5.23, pSO6TR5.26, and pSO6TR5.31, respectively. These plasmids were used to transform the nimA5 strain SO6 to determine if they contained sequences capable of complementing nimA5 and pyrG89. Each of the re-isolated sequences contained a functional pyr4 gene, as predicted by the restriction enzyme analysis, and was able to alleviate uridine auxotrophy after transformation. Moreover, transformation with either pSO6TR5.26 or pSO6TR5.31 also allowed growth at the restrictive temperature, although transformation with pSO6TR5.23 did not.

To isolate a specific probe for nimA we subcloned fragments of pSO6TR5.26. A series of plasmids were constructed in pUC18, pSAO1-pSAO6, which spanned various portions of the insert of pSO6TR5.26 as shown in Fig. 3. These plasmids were tested for their ability to complement a nimA5 strain SO6 and such activity was found to reside in a 5.4-kb Kpn I fragment (Fig. 3; pSAO1). Each of the re-isolated sequences contained a functional pyr4 gene, as predicted by the restriction enzyme analysis, and was able to alleviate uridine auxotrophy after transformation. Moreover, transformation with either pSO6TR5.26 or pSO6TR5.31 also allowed growth at the restrictive temperature, although transformation with pSO6TR5.23 did not.

We next compared the mRNA levels of nimA in RNA isolated from cells undergoing synchronous nuclear division. To provide an internal control, the mRNA levels of *nimA* were standardized to those of a constitutive gene, *A. nidulans* 3-phosphoglycerate kinase (PGK) (Clements and Roberts, 1985). We have also included a positive control, *A. nidulans* histone H2A, (May, G. S., and N. R. Morris, unpublished observation), a gene known to be regulated at the level of mRNA in other organisms. To obtain synchronization of mitosis, conidia of R153 were germinated in the presence of 20

**Figure 2.** Southern blot analysis of transformants of SO6 capable of growth at restrictive temperature. DNA (5 μg) isolated from strains SO6TR1-SO6TR7 (lanes 1-7) was digested to completion with Bgl II and subjected to electrophoresis through 0.7% agarose and transferred to nitrocellulose. The filters were hybridized with nick-translated pBR322 DNA. The position and size in kilobases of lambda Hind III markers is indicated on the right.

**Figure 3.** Subcloning of pSO6TR5.26. A restriction map of the insert of pSO6TR5.26 (a) is shown above subcloned plasmids pSAO1 (b); pSAO2 (c); pSAO3 (d); pSAO4 (e); pSAO5 (f); and pSAO6 (g), all of which were constructed in pUC18. The ability of these plasmids to complement the temperature sensitivity of a nimA5 strain is indicated to the right. Restriction fragments of plasmid b (pSAO1) were isolated to use as nick-translated probes as indicated. The hybridization pattern of these probes to poly(A)+ RNA is shown in the bottom panel.
Figure 4. Appearance of nimA and benA β-tubulin transcripts during germination of conidia. Conidia of R153 were germinated for 6 h. RNA was extracted from cells removed from the culture at time zero (lane 1); 1 h (lane 2); 2 h (lane 3); 3 h (lane 4); 4 h (lane 5); 5 h (lane 6); 6 h (lane 7). The RNA (5 μg) was analyzed on a Northern blot using radiolabeled pβ-5 (β-tubulin) (May et al., 1985) and the 3.5-kb Kpn I-Cla I insert of pSAO5 (nimA) DNA as hybridization probes.

mM hydroxyurea for 5 h to block cells at the beginning of S phase (Bergen and Morris, 1983). This treatment completely blocks nuclear division (Fig. 5). The cells were subsequently washed free of hydroxyurea and allowed to progress through the nuclear division cycle. For 30 min no nuclear division was apparent, but between 30 and 70 min a synchronous nuclear doubling was observed (Fig. 5).

RNA isolated from these synchronously dividing cells was analyzed on RNA blots by hybridization with radiolabeled nimA, H2A, and PGK probes. The level of RNA hybridizing to the PGK probe remains fairly constant but quantitation of the levels of nimA and H2A mRNA by densitometry revealed that there were marked fluctuations in the levels of these transcripts through the nuclear division cycle (Fig. 5). The level of the H2A transcript followed the pattern predicted by the results of Hereford and Osley (1981) who demonstrated that the level of H2A mRNA increased during the S phase of S. cerevisiae. In Aspergillus the levels of H2A and mRNA peaked at 10 and 60 min after release from the hydroxyurea block (Fig. 5). In contrast, the level of nimA mRNA remained low during those periods of the nuclear division cycle when H2A message levels were high, i.e., S phase, but showed a marked accumulation during the period of nuclear division, i.e., M phase. The level of nimA mRNA 40 min after release from the hydroxyurea block increased 16-fold compared with the level found in cells blocked in S by hydroxyurea treatment (Fig. 5). As the frequency of nuclear division decreased, the level of nimA mRNA also decreased coincident with the second increase in H2A mRNA levels (Fig. 5).

We have extended these studies to analyze the transcript levels of nimA during two consecutive nuclear division cycles to eliminate the possibility that the first nuclear division during germination is atypical. If hydroxyurea-released cells are allowed to progress through two nuclear divisions, then the level of the nimA transcript goes through two oscillations with peaks of accumulation occurring during the two periods of nuclear doubling (data not shown). These data clearly demonstrate that the transcript levels of nimA and H2A both oscillate with H2A transcripts peaking in S and nimA at the G2-M boundary.

Relationships between nimA mRNA Levels and Mitosis

As nimA transcript levels appear to be elevated during mitosis and to be lowered soon after mitosis, we have tried to elucidate the relationship between these two events. It is known from previous work that at restrictive temperature the nuclei of strains carrying nimA5 become blocked in late G2 and upon return to permissive temperature synchronously enter mitosis (Oakley and Morris, 1983). This system allows precise control over the initiation of mitosis. We have followed the level of nimA mRNA during a nimA5 block/release-
induced, synchronous mitosis (Fig. 6). After 3 h at restrictive temperature, an asynchronous culture of a strain carrying nimA5 had a chromosome mitotic index of <1% (Fig. 6). During this incubation H2A mRNA levels fell to 15% of the steady-state level detected at 32°C and the level of the nimA transcript increased threefold (Fig. 6). Upon return to permissive temperature the chromosome mitotic index of these cells jumped to >70% (Fig. 6). Coincident with this synchronous mitosis was a precipitous drop in the level of nimA5 transcript back to the level present before the restrictive temperature block. The level of H2A transcript did not vary significantly during the initial 10-min period at 32°C but it did begin to increase during the next 20 min of growth as the number of cells in mitosis began to decline, indicating entry of these cells into S phase on a time scale consistent with normal cellular growth.

Control experiments using a wild-type strain showed no significant decrease in chromosome mitotic index when subjected to growth at 42°C or upon shifting to 32°C, nor did the level of nimA mRNA drop precipitously when returned to 32°C (data not shown).

If progression through mitosis were important in order to lower the level of nimA mRNA, one might predict that a block in mitosis would prevent this response. We have tested this using two different methods to block mitosis, i.e., by using the antimitotic, antimicrotubule drug benomyl and the temperature-sensitive mutation bimE7. The effect of benomyl on an asynchronous culture of A. nidulans was to cause an elevation of the chromosome mitotic index in a mitotic cycle–dependent manner (Fig. 7). Coincident with this elevation in mitotic index was a parallel elevation in the level of nimA mRNA levels over the first 75 min of the incubation (Fig. 7). Over the same period of time H2A mRNA levels fell to a new lower steady state level. These data strongly suggest that, in response to the addition of benomyl, cells are being blocked in mitosis and are expressing an elevated level of nimA mRNA due to the absence of mitotic progression. However, on continued incubation in the presence of benomyl, nimA mRNA levels did begin to fall. At later time points the chromosome mitotic index also fell rapidly and a sharp increase in H2A mRNA levels was apparent.

We also investigated the consequence of a mitotic block on nimA transcript levels by using bimE7, a temperature-sensitive mutation of A. nidulans that arrests the nuclear division cycle in mitosis at the restrictive temperature (Morris, 1976a, b). The mutation caused by bimE7 led to a time-dependent increase of the chromosome mitotic index of an asynchronous culture when it was shifted to restrictive temperature, after an initial decrease due to a transient tempera-
tured to increase the steady-state level of clear division cycle in part by completion of DNA synthesis permissive temperature, the chromosome mitotic index of nimA mRNA appears at about the time of the first nuclear division. If germination is blocked at S by hydroxyurea, the level of nimA mRNA is very low, but this low level increases 16-fold if hydroxyurea is removed to allow a synchronous nuclear division cycle. This increase in nimA mRNA level is not sustained, leading to a peak of accumulation of the transcript during the period of nuclear division, followed by a decrease after completion of nuclear division.

To more clearly define the points in the nuclear division cycle at which nimA mRNA levels are controlled and to confirm the synchronous culture data, we unexpectedly observed that when a block was imposed in mitosis either with the temperature-sensitive bimE7 mutation or with benomyl. These experiments show that mitotic blocks cause a marked elevation in nimA mRNA levels. The addition of benomyl to an asynchronous culture causes cells to become blocked due to depolymerization of microtubules as they attempt to proceed through mitosis. This leads to a buildup of nuclei with condensed chromatin and a concurrent fivefold elevation in the level of nimA mRNA, such that the curves depicting the kinetics of both responses are coincident. Similar data were obtained when bimE7 was used to impose a mitotic block on an asynchronous culture, leading, in this case, to a five- to sixfold increase in the level of nimA mRNA.

Incubation at restrictive temperature of an asynchronous culture of an A. nidulans strain carrying the nimA5 mutation leads to a G2-M arrest and a threefold increase in the level of nimA mRNA. When nimA5-blocked cells are returned to permissive temperature, the nimA5 gene product becomes reactivated, possibly due to its renaturation. The reactivation of the nimA gene product causes a synchronous mitosis to occur and the elevated level of nimA mRNA is rapidly reduced.

In order to explain these phenomena, we developed the following working hypothesis. The appearance of nimA mRNA is linked to mitosis and is regulated during the nuclear division cycle in part by completion of DNA synthesis such that during the G2-M transition a control system(s) operates to increase the steady-state level of nimA mRNA.

Discussion
The molecular cloning of the G2-M-specific gene, nimA, from A. nidulans by mutant rescue of nimA5 has allowed us to identify the transcript of this gene and to monitor its level at various stages of the nuclear division cycle. We have shown that nimA mRNA is absent from dormant assexual spores (conidia) of A. nidulans. Upon germination of conidia nimA mRNA appears at about the time of the first nuclear division. If germination is blocked at S by hydroxyurea, the level of nimA mRNA very low, but this low level increases 16-fold if hydroxyurea is removed to allow a synchronous nuclear division cycle. This increase in nimA mRNA level is not sustained, leading to a peak of accumulation of the transcript during the period of nuclear division, followed by a decrease after completion of nuclear division.

To more clearly define the points in the nuclear division cycle at which nimA transcript levels are controlled and to confirm the synchronous culture data, we monitored the nimA transcript levels when a block was imposed in mitosis either with the temperature-sensitive bimE7 mutation or with benomyl. These experiments show that mitotic blocks cause a marked elevation in nimA mRNA levels. The addition of benomyl to an asynchronous culture causes cells to become blocked due to depolymerization of microtubules as they attempt to proceed through mitosis. This leads to a buildup of nuclei with condensed chromatin and a concurrent fivefold elevation in the level of nimA mRNA, such that the curves depicting the kinetics of both responses are coincident. Similar data were obtained when bimE7 was used to impose a mitotic block on an asynchronous culture, leading, in this case, to a five- to sixfold increase in the level of nimA mRNA.

Incubation at restrictive temperature of an asynchronous culture of an A. nidulans strain carrying the nimA5 mutation leads to a G2-M arrest and a threefold increase in the level of nimA mRNA. When nimA5-blocked cells are returned to permissive temperature, the nimA5 gene product becomes reactivated, possibly due to its renaturation. The reactivation of the nimA gene product causes a synchronous mitosis to occur and the elevated level of nimA mRNA is rapidly reduced.

In order to explain these phenomena, we developed the following working hypothesis. The appearance of nimA mRNA is linked to mitosis and is regulated during the nuclear division cycle in part by completion of DNA synthesis such that during the G2-M transition a control system(s) operates to increase the steady-state level of nimA mRNA.
the elevation in H2A mRNA occurs, there could be a requirement to reduce the level of the nimA gene product in order to complete mitosis and initiate DNA replication during normal growth.

Previous data from this laboratory have demonstrated that nimA is required for mitosis and that this gene normally functions very near to the onset of mitosis. If the nimA gene product is inactivated by the temperature-sensitive mutation nimA5, nuclei are unable to enter mitosis. What then is the function of the nimA gene product? Perhaps nimA is a structural component of the mitotic machinery or is associated with condensation of chromatin. However, it is difficult to reconcile either of these two possibilities with the fact that no mitotic events occur during a nimA5 block. The spindle pole body remains unseparated, the spindle fails to form, and chromatin remains uncondensed (Fig. 1A). It is possible to completely inactivate spindle function using microtubule poisons such as colchicine or benomyl while allowing chromosome condensation to occur, e.g., Fig. 7. For this reason we do not believe that nimA is a structural component of the mitotic spindle because nimA5 stops both formation of the mitotic spindle and chromosome condensation. Conversely, if nimA function is required to condense chromatin, why does a spindle not begin to form during a nimA5 block? Clearly nimA5 effects both the process of spindle formation and chromosome condensation. Equally clear is the fact that the block imposed by nimA5 is very late in G2 at a period during which the nucleus is able to maintain itself in a mitotically inactive stage for a considerable period of time while nevertheless retaining the ability to progress directly into mitosis (Fig. 1 and Oakley and Morris, 1983). It is with these considerations in mind that we suggest nimA may serve to trigger the mitotic process. We have shown that there is a tight positive correlation between the mitotic state and elevated levels of nimA mRNA and presumably the nimA gene product. The nimA gene product is therefore required in a defined amount or in an elevated amount in order for nuclei to enter and complete mitosis. Regulation would thus occur in the following manner. During interphase the level of nimA gene product is held at a low level due to reduced expression of the gene. During the G2 period of the cycle increased expression of nimA mRNA occurs, leading to an increase in the level of the nimA gene product. When the level of the nimA gene product reaches a critical level mitosis is initiated.

There is a strong parallel between the behavior of nimA and the behavior of MPF, which varies in a cell-cycle dependent manner similar to the fluctuations we have observed for nimA mRNA. It is possible that nimA is the MPF of A. nidulans. Particularly pertinent to this suggestion is the fact that colchicine is often used to block higher eukaryotic cells in mitosis in order to obtain elevated MPF activity. Analogous to this situation the elevating e effect a benomy block has on the expression of nimA mRNA in Aspergillus (Fig. 7). Similarly, the detection of MPF in a mitotically blocked mutant yeast strain and the increased expression of nimA in bim E7–blocked cells of A. nidulans have obvious similarities.

nimA is unique in that it is the only nuclear division gene known to function at either G2 or M that is controlled at the level of its mRNA. Having cloned the nimA gene we are now in a position to directly test the hypothesis that elevated nimA mRNA causes cells to enter mitosis (and possibly to sustain mitosis) and to use the techniques of molecular biology to gain insights into the structure and biochemical function of the nimA gene product.

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References
Bailey, J. M., and N. Davidson. 1976. Methylmercury as a reversible denaturing agent for agarose gel electrophoresis. Anal. Biochem. 70:75–85.
Bainbridge, B. W. 1971. Macromolecular composition and nuclear division during spore germination in Aspergillus nidulans. J. Gen. Microbiol. 66: 319–325.
Balance, D. J., F. P. Buxton, and G. Turner. 1983. Transformation of Aspergillus nidulans by the ortidine-5-phosphate decarbosylase gene of Neurospora crassa. Biochem. Biophys. Res. Commun. 112:284–289.
Beach, D., D. Durkacz, and P. Nurse. 1982. Functional homology of cell cycle control genes in budding and fission yeast. Nature (Lond.). 300:706–709.
Bergen, L. G., and N. R. Morris. 1983. Kinetics of the nuclear division cycle of Aspergillus nidulans. J. Bacteriol. 156:155–160.
Clements, J. M., and C. F. Roberts. 1983. Molecular cloning of the 3-phosphoglycerate kinase (PGK) gene from Aspergillus nidulans. Clutterbuck, A. J. 1974. Aspergillus nidulans. In Handbook of Genetics. Vol. I. C. King, editor. Plenum Publishing Corp., New York. 447–510.
Clutterbuck, A. J. 1974. Molecular cloning of the 3-phosphoglycerate kinase (PGK) gene from Aspergillus nidulans. Clutterbuck, A. J. 1974. Aspergillus nidulans. In Handbook of Genetics. Vol. I. C. King, editor. Plenum Publishing Corp., New York. 447–510.
Cove, D. J. 1977. The genetics of Aspergillus nidulans. In The Genetics and Physiology of Aspergillus nidulans. J. E. Smith and J. A. Pateman, editors. Academic Press Inc., Ltd., London.
Gambino, J., L. G. Bergen, and N. R. Morris. 1984. Effects of mitotic and tubulin mutations on microtubule architecture in actively growing protoplasts of Aspergillus nidulans. J. Cell Biol. 99:830–838.
Gerhart, J., M. Wu, and M. Kirschner. 1984. Cell cycle dynamics of an M-phase-specific cytoplasmic factor in Xenopus laevis oocytes and eggs. J. Cell Biol. 98:1247–1255.
Gardon, J. B. (1968). Changes in somatic cell nuclei inserted into growing and maturing amphibian oocytes. J. Embryol. Exp. Morphol. 20:401–414.
Hereford, L. M., M. A. Osley, J. R. Ludwik II, and C. S. McLaurthin. 1981. Cell-cycle regulation of yeast hisone mRNA. Cell. 24:367–375.
Johnson, I. L., S. G. Hughes, and A. J. Clutterbuck. 1985. Cloning an Aspergillus nidulans developmental gene by transformation. EMBO (Eur. Mol. Biol. Organ.) J. 4:1307–1311.
Kishimoto, T., T. Kuriyama, H. Kondo, and K. Kantani. 1982. Generality of the action of various maturation-promoting factors. Exp. Cell Res. 137:121–126.
Lohka, M. J., and J. L. Millar. 1985. Induction of nuclear envelope breakdown, chromosome condensation and spindle formation in cell-free extracts. J. Cell Biol. 101:518–523.
Maniatis, T., E. F. Fritsh, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1–545.
Masui, Y., and C. L. Markert. 1971. Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. J. Exp. Zool. 177:129–146.
Matsui, S.-I., H. Yoshida, H. Weinfeld, and A. A. Sandberg. 1972. Induction of prophase in interphase nuclei by fusion with metaphase cells. J. Cell Biol. 54:120–132.
May, G. S., J. Gambino, J. A. Weatherbee, and N. R. Morris. 1985. Identification and functional analysis of beta-tubulin genes by site specific integrative transformation of Aspergillus nidulans. J. Cell Biol. 100:712–719.
Miske-Lye, R., and M. W. Kirschner. 1985. Induction of early mitotic events in a cell-free system. Cell. 41:165–175.
Miller, B. L., K. Y. Miller, and W. B. Timberlake. 1985. Direct and indirect gene replacement in Aspergillus nidulans. Mol. Cell. Biol. 5:1709–1721.
Morris, N. R. 1976a. Mitotic mutants of Aspergillus nidulans. Genet. Res. 26: 237–254.
Morris, N. R. 1976b. A temperature-sensitive mutant of Aspergillus nidulans reversibly blocked in nuclear division. Exp. Cell Res. 98:204–210.
Nasmuth, K. A., and S. L. Reed. 1980. Isolation of genes by complementation in yeast: molecular cloning of a cell-cycle gene. Proc. Natl. Acad. Sci. USA. 77:2119–2123.
Nelkin, B. L., Nichols, and B. Vogelestein. 1980. Protein factor(s) from mitotic CHO cells induce mitotic maturation in Xenopus laevis oocytes. FEB (Fed. Eur. Biochem.) Lett. 109:233–238.
Newport, J. W., and M. W. Kirschner. 1984. Regulation of the cell cycle during early Xenopus development. Cell. 37:731–742.

Oakley, B. R., and N. R. Morris. 1983. A mutation in Aspergillus nidulans that blocks the transition from interphase to prophase. J. Cell Biol. 96:1155–1158.

Pontecorvo, G., J. A. Roper, C. M. Hemmons, K. D. MacDonald, and A. W. J. Buthon. 1953. The genetics of Aspergillus nidulans. Adv. Genet. 5:141–238.

Raeder, U., and P. Broda. 1985. Rapid preparation of DNA from filamentous fungi. Lett. Appl. Microbiol. 1:17–20.

Rao, P. N., and R. T. Johnson. 1970. Mammalian cell fusion: studies on the regulation of DNA synthesis and mitosis. Nature (Lond.). 225:159–164.

Robinow, C. F., and C. E. Caten. 1966. Mitosis in Aspergillus nidulans. J. Cell Sci. 5:403–437.

Smith, L. D., and R. E. Ecker. 1971. The interaction of steroids with Rana pipiens oocytes in the induction of maturation. Dev. Biol. 25:233–247.

Southern, E. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503–517.

Sunkara, P. S., D. A. Wright, and P. N. Rao. 1979. Mitotic factors from mammalian cells: a preliminary characterization. J. Supramol. Struct. 11:189–195.

Tilburn, J., C. Searzzocchio, G. G. Taylor, J. H. Zabicky-Zissman, R. A. Lockington, and W. R. Davies. 1983. Transformation by integration of Aspergillus nidulans. Gene (Amst.). 26:205–221.

Timberlake, W. E. 1978. Low repetitive DNA content in Aspergillus nidulans. Science (Wash. DC). 202:773–775.

Vieira, J., and J. Messing. 1982. The pUC plasmids, an M13 mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primer. Gene (Amst.). 19:259–268.

Weintraub, H., M. Buscaglia, M. Ferrer, S. Weiller, A. Boulet, F. Fabre, and E. E. Baulieu. 1982. Mise en evidence d'une activite "MPF" chez Saccharomyces cerevisiae. C. R. Acad. Sci. Ser. III Sci. Vie. 295:787–790.

Yelton, M. M., J. E. Hamer, and W. E. Timberlake. 1984. Transformation of Aspergillus nidulans by using a trpC plasmid. Proc. Natl. Acad. Sci. USA. 81:1470–1474.

Yelton, M. M., W. E. Timberlake, and C. A. M. J. Van Den Hondel. 1985. A cosmid for selecting genes by complementation in Aspergillus nidulans: selection of the developmentally regulated yA locus. Proc. Natl. Acad. Sci. USA. 82:834–838.

Ziegler, D. H., and Y. Masui. 1973. Control of chromosome behavior in amphibian oocytes. 1. The activity of maturing oocytes inducting chromosome condensation in transplanted brain nuclei. Dev. Biol. 35:283–292.