

# Regulation of the mRNA Levels of *nimA*, a Gene Required for the G2-M Transition in *Aspergillus nidulans*

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**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.

**M**ITOSIS 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 (Gurdon, 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).<sup>1</sup> 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 cytostatic 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 eukaryotic 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., 1984; Yelton et al., 1985; Johnson et al., 1985) allows

1. *Abbreviations used in this paper:* MPF, maturation-promoting factor; PGK, 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 *nimA5* 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*; *pyrG89*; *cnxE16*; *choA1*), SO7 (*nimA5*; *wA2*), SO5 (*nimA5*; *yA2*; *pyrG89*), 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 *pyrG89* 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  $-190^{\circ}\text{C}$ , transferred to and substituted in anhydrous acetone containing 2% osmium tetroxide and 0.1% uranyl acetate at  $-85^{\circ}\text{C}$ . After substitution for 48 h, samples were passively rewarmed to room temperature, infiltrated, and polymerized in quetol 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,  $10^8$ – $10^9$  conidia were inoculated into 50 ml of YG containing 10 mM uridine. Growth was at  $32^{\circ}\text{C}$  for 5.5 h with shaking or until germ tube emergence was just visible. The germinated conidia were harvested by centrifugation and resuspended in 40 ml of lytic mix containing 0.4 M ammonium sulphate, 50 mM  $\text{K}^+$  citrate, pH 6.0, 0.5% yeast extract, 0.5% sucrose, 10 mM  $\text{MgSO}_4$ , and 10 mg/ml Novozyme 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 5.0% in 10 mM  $\text{Tris-Cl}^-$ , pH 8.0. After centrifugation the precipitated protein was freeze dried and stored at  $4^{\circ}\text{C}$ .) Digestion of cell wall was allowed to proceed for 2 h at  $32^{\circ}\text{C}$  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  $\text{K}^+$  citrate, pH 6.0, at  $4^{\circ}\text{C}$ . The protoplasts were

resuspended in 1 ml of 0.6 M KCl, 50 mM  $\text{CaCl}_2$ , 10 mM  $\text{K}^+$  2-(*N*-morpholino)ethane sulfonic acid, pH 6.0, and were stored at  $4^{\circ}\text{C}$ .

For transformation, 100  $\mu\text{l}$  of the protoplasts were added to 4  $\mu\text{g}$  of DNA in 2  $\mu\text{l}$  of  $\text{H}_2\text{O}$ , followed by 50  $\mu\text{l}$  of 25% polyethylene glycol (PEG) 6000 containing 50 mM  $\text{CaCl}_2$ , 0.6 M KCl, and 10 mM  $\text{Tris-Cl}^-$ , 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  $\mu\text{l}$ ) were plated in 3 ml 0.5% yeast extract, 0.4 M ammonium sulphate, 1% sucrose, trace elements, 1% agar, at  $47^{\circ}\text{C}$  onto plates containing the same but solidified with 2% agar. For some experiments 10 mM uridine was incorporated into the regeneration media and protoplasts were allowed to regenerate for 18 h at  $32^{\circ}\text{C}$  before shifting to  $42^{\circ}\text{C}$ .

### 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  $10^6/\text{ml}$  and allowed to grow at  $37^{\circ}\text{C}$  for 18 h with vigorous shaking. The mycelia were harvested by filtration onto Miracloth (Calbiochem-Behring Corp., La Jolla, CA), rinsed completely with deionized water and dried by blotting between absorbent paper towelling. The dried mycelia were ground to a fine powder using a mortar and pestle cooled in liquid  $\text{N}_2$ . 2 g of this material was added to 10 ml of extraction buffer at  $65^{\circ}\text{C}$  containing 50 mM  $\text{Tris/Cl}^-$ , 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  $37^{\circ}\text{C}$  and incubated with 100  $\mu\text{g}/\text{ml}$  RNase A for a further 30 min. Proteinase K was then added to a concentration of 100  $\mu\text{g}/\text{ml}$  and the incubation continued for 3 h more. 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  $\mu\text{l}$  TE.

A vector was constructed by ligating the *pyr4* gene of *Neurospora crassa* from AIPGM1 (May et al., 1985) as a 2.2-kb Eco RI fragment into the Eco RI site of pBR322 to yield the plasmid pGM3 (6.7 kb). *A. nidulans* DNA fragments were generated by limited digestion of 60  $\mu\text{g}$  of DNA with 50 U of Taq I for 15, 30, 45, and 60 min at  $65^{\circ}\text{C}$ . The pooled DNA was subjected to 0.7% agarose gel electrophoresis and a subpopulation of fragments between 6 and 10 kb were cut from the gel and collected by electroelution into dialysis bags. After phenolization and ethanol precipitation these Taq I 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* JM83 (Vieira and Messing, 1982) to ampicillin resistance.  $1.8 \times 10^4$  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  $7.0 \pm 2$  kb. The primary library therefore contained  $>4.0$  genomic equivalents, assuming a genome size of  $2.3 \times 10^4$  kb (Timberlake, 1978) for *A. nidulans*. The primary library was harvested from the transformation plates into Luria broth containing 20% glycerol and stored at  $-70^{\circ}\text{C}$ . For amplification, the primary library cells were grown for 6 h at  $37^{\circ}\text{C}$  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  $\mu\text{g}$  of DNA isolated from SO6TR5 was digested with 20 U of Bgl II in a volume of 200  $\mu\text{l}$  at  $37^{\circ}\text{C}$ . 40- $\mu\text{l}$  samples were removed at 0, 10, 20, 30, and 40 min from the digestion and added to 40  $\mu\text{l}$  of phenol to terminate the reaction. The digestion products were extracted with chloroform and brought to 500  $\mu\text{l}$  with ligation buffer (Maniatis et al., 1982) and incubated for 14 h at  $14^{\circ}\text{C}$  in the presence of 8 U of T4 DNA ligase to circularize linear fragments. Each ligation was precipitated with ethanol and resuspended in 50  $\mu\text{l}$  of TE (10 mM  $\text{Tris/Cl}^-$ , pH 8.0, 1 mM EDTA, pH 8.0). 25- $\mu\text{l}$  aliquots of each ligation were used to transform 200  $\mu\text{l}$  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)<sup>+</sup> mRNA

RNA was isolated from cells that were frozen with liquid N<sub>2</sub> 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/isoamyl 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/isoamyl alcohol (50:48:2) and twice with chloroform/isoamyl 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)<sup>+</sup> RNA was isolated by chromatography through 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. Quantitation of RNA was by absorbance at 260 nm.

## 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 400 ml YG containing 0.2% Tween 80 and 20 mM hydroxyurea to a final concentration of  $2.0 \times 10^7$ /ml. The conidia were allowed to germinate for 5 h at 37°C at 200 rpm in a 2-liter flask before being washed by centrifugation with 800 ml YG. Growth was continued in 400 ml YG for various periods of time. Samples were taken at regular intervals. Samples for the isolation of RNA were cooled rapidly by the addition of ice before a brief centrifugation in order to concentrate the cells, which were harvested by filtration through Miracloth and promptly frozen on dry ice. These samples were subsequently lyophilized and processed to isolate RNA. Parallel samples were fixed in glutaraldehyde and stained with 4,6-diamidino-2-phenylindole to determine the number of nuclei per germling as described previously (Bergen and Morris, 1983).

## Restrictive Temperature Blocks and Release

Conidia were inoculated into 200 ml YG to a final concentration of  $2 \times$

$10^7$ /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.

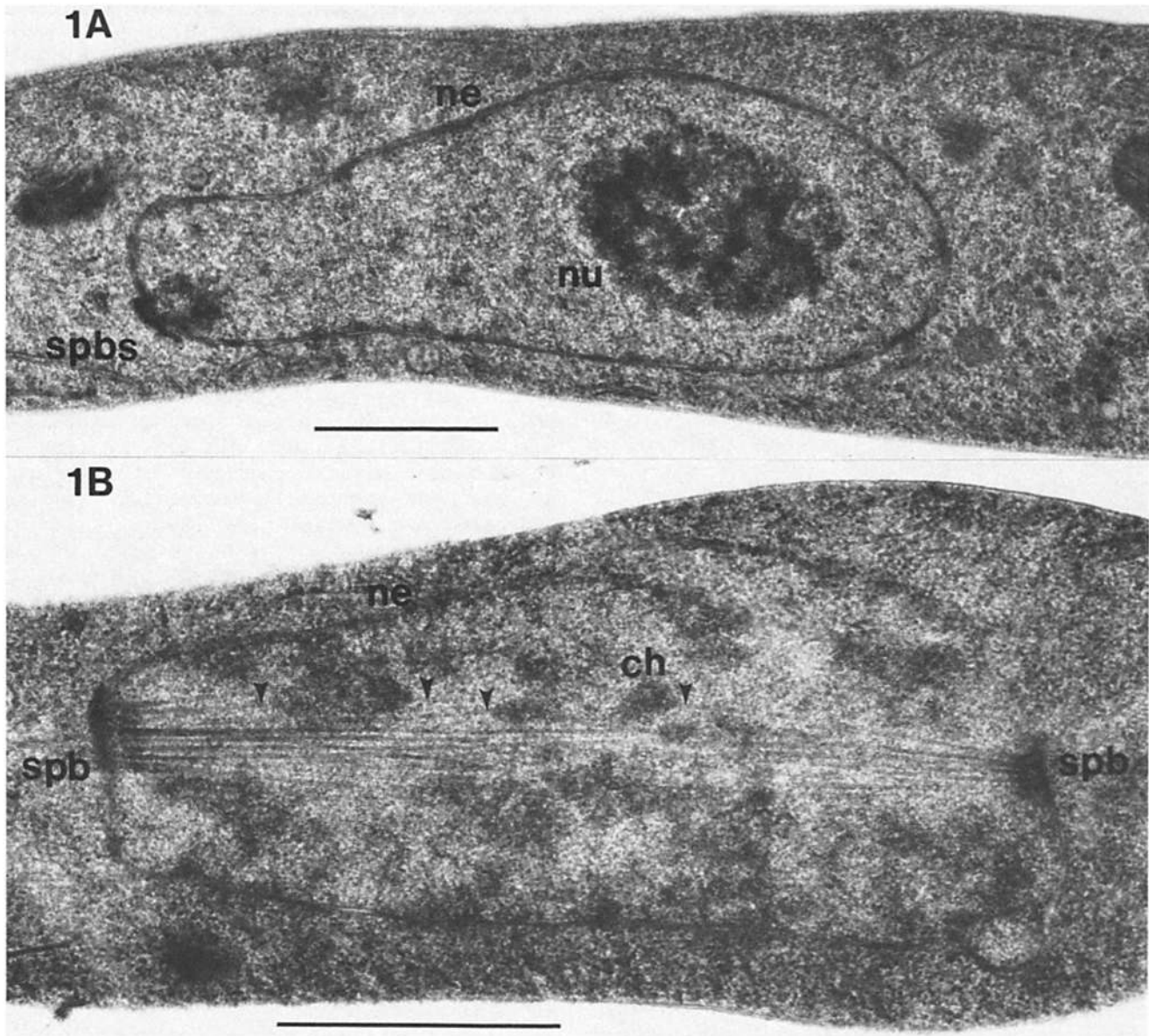
## Results

### 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 (SAOLIB1, 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 SAOLIB1 DNA was used to transform  $6.5 \times 10^8$  protoplasts isolated from a strain carrying *nimA5* and *pyrG89* (SA06).  $2.2 \times 10^4$  *pyr4*<sup>+</sup> transformants were obtained, seven of which exhibited growth at restrictive temperature (42°C), and were designated SO6TR1–SO6TR7. After streaking to single colony all temperature-insensitive transformants showed wild-type growth characteristics at both permissive (32°C) and restrictive temperature except SO6TR3, 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 (SO6TR2, TR4, and TR5) showed



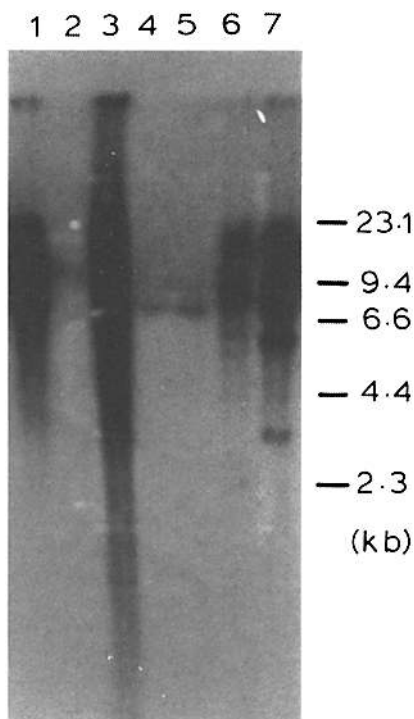
**Figure 1.** A mitotic transition induced by *nimA5*. A strain carrying *nimA5* was held at restrictive temperature (42°C) for a period of 2 h (A) then returned to permissive temperature (32°C) for 7.5 min (B). *ne*, nuclear envelope; *nu*, nucleolus; *spb*, spindle pole body; *ch*, condensed chromosome. The arrowhead indicates putative kinetochore microtubules. Bars, 1  $\mu$ m.

a single copy of the pBR322 sequence (based on band number and intensity) in their genomes and the rest showed varying numbers and integration sites. SO6TR5 was selected to re-isolate *nimA*.

To obtain evidence that SO6TR5 had been transformed by the wild-type allele of *nimA*, this strain was allowed to go through a self cross. Progeny were isolated which were auxotrophic but were temperature insensitive (i.e., *pyr4*<sup>-</sup>; *nimA*<sup>+</sup>). In this manner a two-step gene replacement was achieved (Miller et al., 1985). If SO6TR5 had been complemented by homologous integration at the *nimA* locus these progeny would be cell cycle wild type and yield no temperature-sensitive segregants when crossed to a wild-type strain. This was found to be the case. Of 104 segregants tested from crosses of a wild-type strain to *nimA* gene-replaced strains (isolated from SO6TR5) all were temperature insensitive.

#### *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  $\mu$ g 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-



**Figure 2.** Southern blot analysis of transformants of SO6 capable of growth at restrictive temperature. DNA (5  $\mu$ g) 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.

mids, 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.

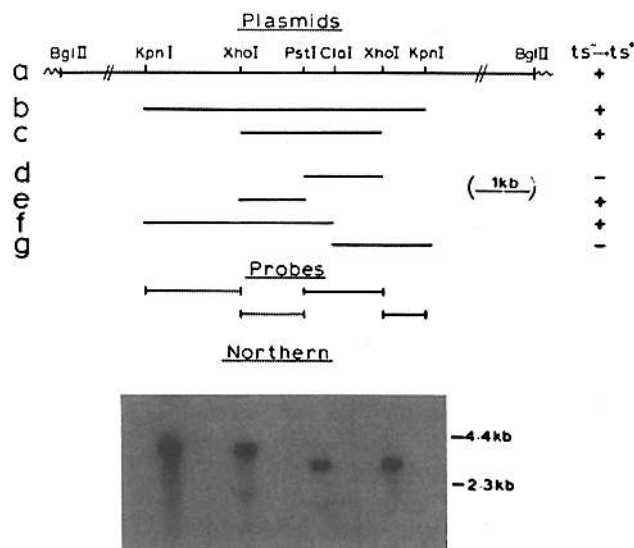
To isolate a specific probe for *nimA* we subcloned fragments of pSO6TR5.26. A series of plasmids were constructed in pUC18, pSAO1-pSAO6, which spanned various portions of the insert of pSO6TR5.26 as shown in Fig. 3. These plasmids were tested for their ability to complement a *nimA5* strain (SO6) and such activity was found to reside in a 5.4-kb Kpn I fragment (Fig. 3; pSAO1). Of the remaining subclones only pSAO3 and pSAO6 did not retain this activity; both lie to the right of the central Pst I site of pSO6TR5.26. When a series of restriction fragments spanning the entire insert of pSAO1 was used to probe *poly(A)*<sup>+</sup> RNA isolated from wild-type *A. nidulans*, two transcripts, one 3.6 and the other 2.8 kb in length, were seen to hybridize (Fig. 3). The data shown in Fig. 3 locate the *nimA* gene in part on the 3.5-kb insert of pSAO5 and demonstrate the mature transcript of this

gene to be 3.6 kb in length. The 3.5-kb Kpn I-Cla I insert of pSAO5 was used in the rest of this study as a specific probe to monitor the levels of the *nimA* 3.6-kb transcript during various stages of the nuclear division cycle of *A. nidulans*.

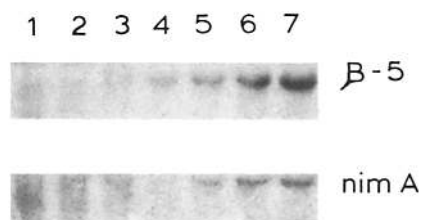
### Nuclear Cycle Regulation of *nimA* Transcript Levels

*Aspergillus* forms dormant uninuclear spores blocked in G1 (conidia) which, when exposed to nutrients, germinate and grow into mature mycelia. The germination and outgrowth of conidia are partially synchronous (Bainbridge, 1971) with the first nuclear doubling occurring at  $\sim$ 5 h. We followed the levels of the transcripts of the *benA*  $\beta$ -tubulin gene (May et al., 1985) and the *nimA* gene during the process of conidial germination in a mitotically wild-type strain (R153, *wA2*, *pyrA4*). No transcript for *nimA* or tubulin was detected in RNA isolated from dormant ungerminated conidia. By 3 h of germination, a clear band of tubulin mRNA was detected (Fig. 4). The *nimA* transcript, however, was not apparent until 4 h of growth, indicating a later requirement for this gene product than for *benA*  $\beta$ -tubulin. The appearance of *nimA* transcript coincides with the time at which the first nuclear division occurs (Fig. 4).

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 3.** Subcloning of pSO6TR5.26. A restriction map of the insert of pSO6TR.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)*<sup>+</sup> RNA is shown in the bottom panel.



**Figure 4.** Appearance of *nimA* and *benA*  $\beta$ -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  $\mu$ g) was analyzed on a Northern blot using radiolabeled p $\beta$ -5 ( $\beta$ -tubulin) (May et al., 1985) and the 3.5-kb Kpn I-Cla I insert of pSAO5 (*nimA*) DNA as hybridization probes.

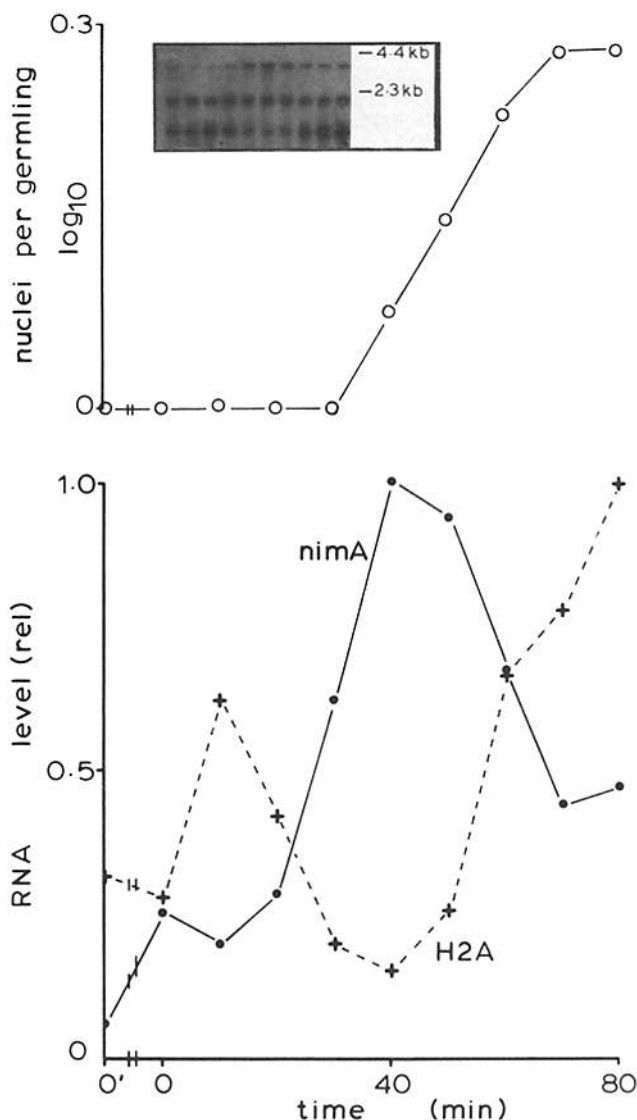
mM hydroxyurea for 5 h to block cells at the beginning of S phase (Bergen and Morris, 1983). This treatment completely blocks nuclear division (Fig. 5). The cells were subsequently washed free of hydroxyurea and allowed to progress through the nuclear division cycle. For 30 min no nuclear division was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

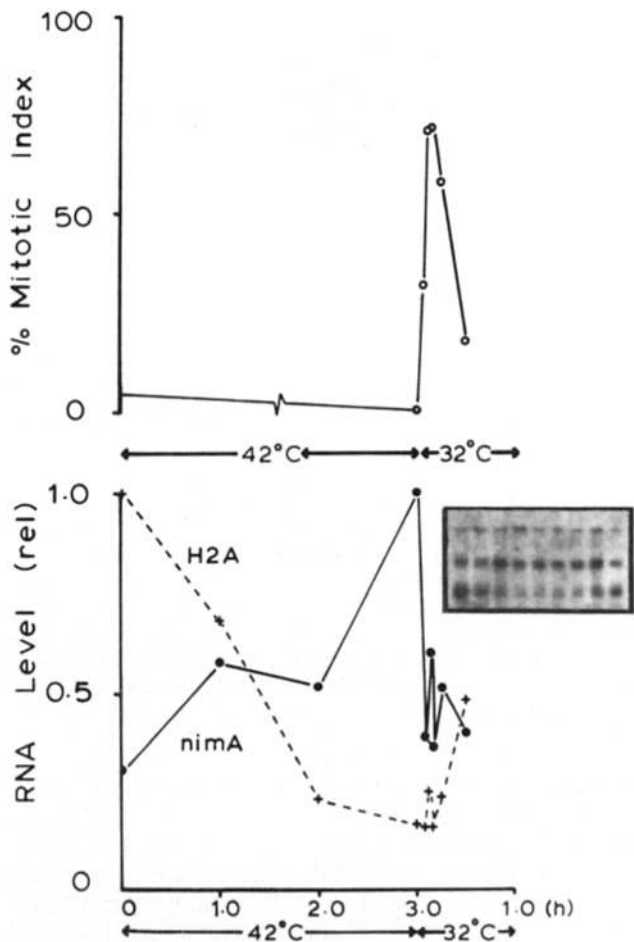
#### Relationships between *nimA* mRNA Levels and Mitosis

As *nimA* transcript levels appear to be elevated during mitosis and to be lowered soon after mitosis, we have tried to elu-



**Figure 5.** Levels of *nimA* and H2A transcripts during a synchronous nuclear division. Conidia of R153 were synchronized at S with hydroxyurea (time 0') and subsequently washed free of hydroxyurea (time 0) as described in Materials and Methods. (Top) The number of nuclei per germling was determined at time 0' and time 0 and then at 10-min intervals for 80 min. (Bottom) RNA was extracted from cells at the times indicated and analyzed on a Northern blot (5  $\mu$ g RNA) using radiolabeled DNA probes for *nimA*, PGK, and H2A. The autoradiogram is shown at the top. (The first and second lanes correspond to samples 0 and 0', respectively.) The autoradiogram was quantitated by densitometry to obtain the relative levels of *nimA*, PGK, and H2A. The mRNA levels of *nimA* ( $\bullet$ ) and H2A ( $+$ ) were determined as a ratio to the PGK level to eliminate minor variations in loading and are expressed relative to the highest ratio detected. Transcript sizes are *nimA*, 3.6 kb; PGK, 1.7 kb; H2A, 1.1 kb.

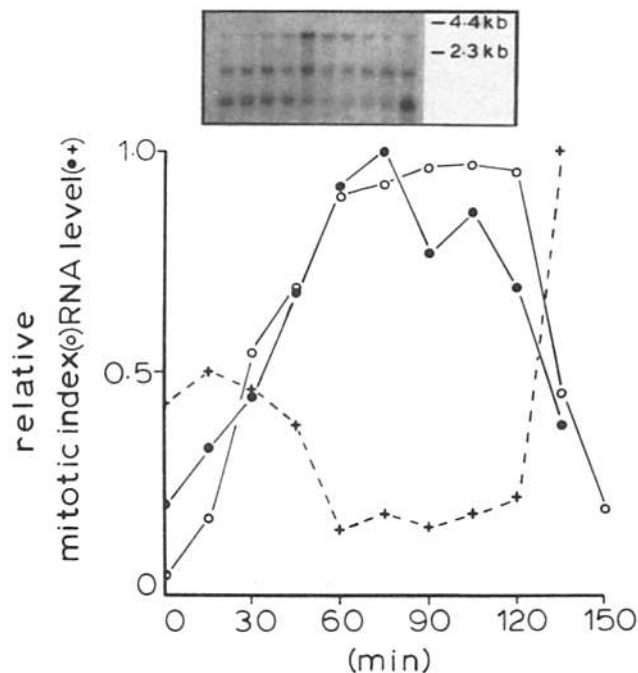
cidate the relationship between these two events. It is known from previous work that at restrictive temperature the nuclei of strains carrying *nimA5* become blocked in late G2 and upon return to permissive temperature synchronously enter mitosis (Oakley and Morris, 1983). This system allows precise control over the initiation of mitosis. We have followed the level of *nimA* mRNA during a *nimA5* block/release-



**Figure 6.** Levels of *nimA* and H2A transcripts during a *nimA5* block/release-induced synchronous mitosis. Conidia of a strain carrying *nimA5* were grown for 8 h at 32°C (time 0) followed by 3 h of growth at 42°C. The culture was then returned rapidly to 32°C. The chromosome mitotic index of the culture was determined (top) and samples extracted for RNA at the times indicated (bottom). RNA (5 µg) from these samples were analyzed on a Northern blot (insert above) and the relative levels of *nimA* (●) and H2A (+) mRNA determined as described in Fig. 4.

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



**Figure 7.** Levels of *nimA* and H2A during a mitotic block imposed by benomyl. Conidia of R153 were grown for 8 h (time 0) at which time benomyl was added to a final concentration of 2.4 µg/ml. Samples were taken at 10-min intervals to determine the chromosome mitotic index (○) of the culture and to isolate RNA. RNA (5 µg) was analyzed on a Northern blot (insert) and the relative level of *nimA* (●) and H2A (+) mRNA determined as described in Fig. 4.

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-

ture shock response (Fig. 8). In agreement with the benomyl effect this elevation in mitotic index also led to an increase in the mRNA level of *nimA* and a decrease in the mRNA level of H2A. Again there would appear to be a relationship between mitosis and the elevation of *nimA* transcript levels and a requirement for mitotic progression in order to lower these elevated levels quickly. Upon prolonged incubation the chromosome mitotic index of *bimE7*-blocked cells remained at 100% (Fig. 8), but the level of *nimA* mRNA decreased. It is known that, during prolonged incubation at restrictive temperature, the chromosome mitotic index of *bimE7* strains remains high, but the spindle mitotic index falls (Gambino et al., 1984).

## 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 asexual 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 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 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 build-up 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.

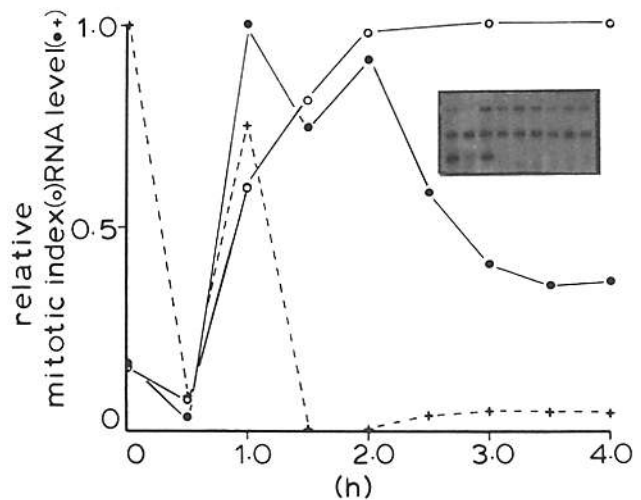


Figure 8. Levels of *nimA* and H2A during a mitotic block imposed by *bimE7*. Conidia of a strain carrying *bimE7* were grown for 8 h (32°C) at which time the culture was shifted to 42°C (time 0). Samples of the culture were taken at 30-min intervals to determine the chromosome mitotic index (○) of the culture and to isolate RNA. RNA (5 μg) was analyzed on a Northern blot (insert) and the relative levels of *nimA* (●) and H2A (+) mRNA determined as described in Fig. 4.

Normally the subsequent transition into and through mitosis activates a second control system(s) which decreases the elevated levels of *nimA* mRNA that are present during mitosis. If the execution point of this negative feedback control system(s) is not reached due to a mitotic block, then *nimA* mRNA remains at the high mitotic phase level. This model explains the negative effect a block in S by hydroxyurea has on *nimA* transcript levels. It also explains the elevating effect of a mitotic block caused by either *bimE7* or benomyl on *nimA* mRNA levels and the nuclear division cycle variations in the level of *nimA* mRNA observed in synchronously dividing cells.

We do not know if the variation in *nimA* transcript levels is due to changes in the rate of transcription from the gene or to changes in the rate of turnover of the mRNA. However, the fact that after release from a *nimA5* block the *nimA* transcript levels fall rapidly indicates that message stability is important in controlling *nimA* mRNA levels. The precise point during the mitotic process at which the *nimA* transcript becomes unstable is difficult to define, but present data suggest that completion of mitosis is not an absolute requirement for this response. Mitotic blocks imposed by either benomyl or *bimE7* both prevent completion of mitosis, but if either block is sustained *nimA* mRNA levels begin to fall. In the case of the block imposed by benomyl the decrease in *nimA* mRNA levels precedes the decline in chromosome mitotic index (Fig. 7). In addition, when both *nimA* mRNA levels and the chromosome mitotic index of cells blocked with benomyl have fallen, the transcript level of H2A rises rapidly, indicating initiation of DNA synthesis. This could mean that in the absence of spindle function certain nuclear division control systems still operate. Preliminary data indicate that during prolonged incubation in benomyl polyploid nuclei are generated in *A. nidulans* (our unpublished observation). Because, during an extended benomyl block, *nimA* mRNA levels fall before the decrease in chromosome mitotic index and before



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. 1 A). 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 is the elevating effect a benomyl 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 *bimE7*-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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