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Premature chromatin condensation upon accumulation of NIMA

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The NIMA protein kinase of *Aspergillus nidulans* is required for the G_2/M transition of the cell cycle. Mutants lacking NIMA arrest without morphological characteristics of mitosis, but they do contain an activated $p37^{nimX}$ kinase (the *Aspergillus* homologue of $p34^{cdc2}$). To gain a better understanding of NIMA function we have investigated the effects of expressing various NIMA constructs in *Aspergillus*, fission yeast and human cells. Our experiments have shown that the instability of the NIMA protein requires sequences in the non-catalytic C-terminus of the protein. Removal of this domain results in a stable protein that, once accumulated, promotes a lethal premature condensation of chromatin without any other aspects of mitosis. Similar effects were also observed in fission yeast and human cells accumulating *Aspergillus* NIMA. This phenotype is independent of cell cycle progression and does not require $p34^{cdc2}$ kinase activity. As gain of NIMA function by accumulation results in premature chromatin condensation, and loss of NIMA function results in an inability to enter mitosis, we propose that NIMA functions in G_2 to promote the condensation of chromatin normally associated with entry into mitosis.
Key words: apoptosis/*Aspergillus*/chromatin condensation/fission yeast/NIMA

Introduction

Crucial to the regulation of the transition from the G_2 phase of the cell cycle into mitosis is the activity of a protein kinase known as maturation or M phase promoting factor (MPF). MPF consists of a catalytic subunit which is encoded by homologues of the fission yeast $cdc2^+$ gene ($p34^{cdc2}$), and a regulatory subunit known as cyclin B. $p34^{cdc2}$ activity requires its association with cyclin B and is regulated by alterations in the phosphorylation of key tyrosine and threonine residues. Studies in organisms spanning a wide evolutionary distance have found that the final step in the activation of MPF, namely the dephosphorylation of the Tyr15 residue of $p34^{cdc2}$ by homologues of the fission yeast *cdc25*-encoded protein-tyrosine phosphatase, is required and is usually rate limiting for entry into mitosis (Nurse, 1990).

In the filamentous fungus *Aspergillus nidulans* the NIMA protein kinase is required in addition to MPF for the G_2/M transition (Osmani *et al.*, 1991a,b). Mutants lacking NIMA activity arrest in the G_2 phase of the cell cycle with no morphological features of mitosis, such as chromatin condensation or cytoskeletal reorganization (Morris, 1976; Oakley and Morris, 1983). However, such strains do show one feature that is thought to be characteristic of the mitotic state: they contain activated MPF, the catalytic subunit of which in this organism is known as $p37^{nimX}$ (Osmani *et al.*, 1991b, 1994). This phenotype suggests that NIMA is required to enter mitosis at a point subsequent to $p37^{nimX}$ activation.

NIMA is a 79 kDa protein kinase with an N-terminal catalytic domain and a C-terminal domain of hitherto unknown function (Osmani *et al.*, 1988, 1991a). The kinase activity of NIMA is directed against serine and threonine residues and can be measured *in vitro* using β-casein as a substrate (Lu *et al.*, 1993a). Studies using peptide substrates have suggested that FRXS/T may be the preferred site of NIMA phosphorylation (Lu *et al.*, 1994), but to date no *in vivo* substrates have been identified. In *Aspergillus*, NIMA protein levels are extremely low but the activity can be assayed in immunoprecipitates. NIMA activity is low throughout most of interphase, but rises sharply appearing as a peak coincident with the G_2/M transition, and is then lost upon exit from mitosis. Much of the cyclic appearance of NIMA can be accounted for by a transient stability of an otherwise unstable protein. However, the phosphorylation of NIMA and regulation of gene expression may, in addition, be important for the regulation of NIMA activity (Osmani *et al.*, 1987, 1988, 1991a,b; Lu *et al.*, 1993b). The C-terminal non-catalytic domain of NIMA contains several PEST sequences which may contribute to the instability of the NIMA protein (Rogers *et al.*, 1986). Gain-of-function phenotypes have been seen in *Aspergillus* when the *nimA* cDNA has been expressed to high levels using a heterologous promoter in constructs present in multiple copies. It has been reported that under these conditions the accumulated NIMA promotes premature and somewhat aberrant mitotic events (Osmani *et al.*, 1988). Similar high levels of expression of a catalytically inactive mutant or of the non-catalytic domain of NIMA alone result in G_2 arrest (Lu and Means, 1994), presumably as a result of interference with wild-type NIMA function.

Functional homologues of NIMA from other organisms have yet to be described, but given the conservation of other mitotic controls across wide evolutionary distances, it is likely that they exist. There have been several reports describing proteins from various species that show significantly more sequence similarity to NIMA than to other protein-serine/threonine kinases; however, this is restricted only to the catalytic domain (Letwin *et al.*,

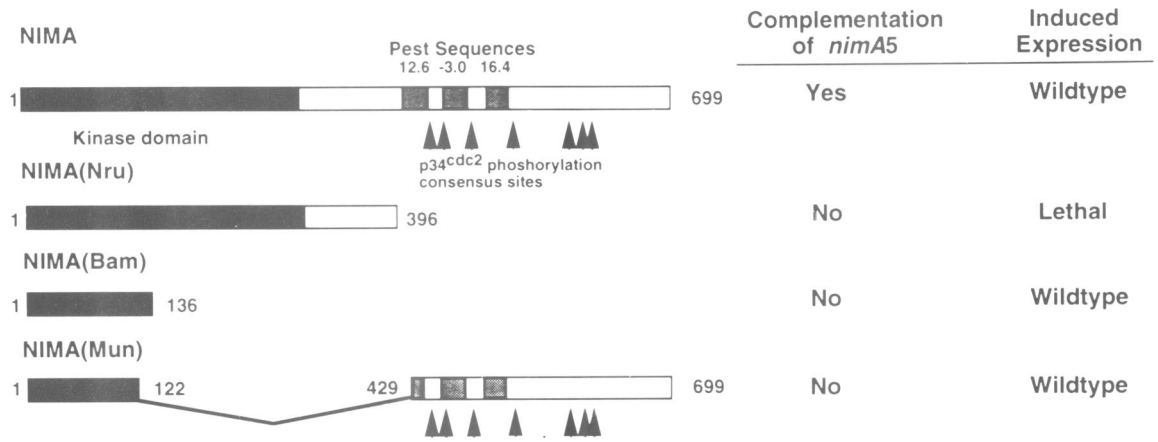

Fig. 1. Structure–function analysis of NIMA. Full-length NIMA is shown with the positions of the catalytic (kinase) domain (solid bar) and PEST sequences (shaded bar) with PEST scores calculated by the method of Rogers *et al.* (1986). The positions of $p34^{cdc2}$ phosphorylation consensus sites (Moreno and Nurse, 1990) are shown by arrows. The details of the constructions used are in Materials and methods. Complementation of *nimA*5 was assayed by transforming plasmids into MO79 and scoring their ability to grow on ethanol or glycerol media and their inability to grow on glucose medium at 42°C. Phenotype of induced expression was assessed using constructs transformed into MO27 on ethanol medium at 37°C.

1992; Schultz and Nigg, 1993). Functional studies may yet prove one or more of these to be true NIMA homologues.

We have studied the effect of expressing *nimA* and mutated derivatives in fission yeast as part of an effort to identify a functional NIMA homologue in this organism. One approach has involved expressing the *Aspergillus nimA* cDNA in *Schizosaccharomyces pombe* to look for strains that require such expression for viability. Another approach used was to identify dominant gain-of-function alleles of *nimA* in *Aspergillus*, by altering the level of either *nimA* gene expression or protein stability, with a view to using them in genetic suppression studies in fission yeast. These experiments have not identified a fission yeast NIMA gene but they have been useful in gaining insight into NIMA function. They have also led us to extend these NIMA expression studies into human cells. In all three experimental systems, the accumulation of NIMA resulted in chromatin condensation without other measurable aspects of mitosis. This phenotype was independent of the point in the cell cycle and did not require $p34^{cdc2}/p37^{nimX}$ kinase activity. The phenotypes of both gain- and loss-of-function *nimA* alleles suggest that NIMA may function in the regulation of chromatin condensation at the onset of mitosis, and that in the absence of NIMA function a checkpoint monitoring the onset of chromatin condensation prevents entry into mitosis, even though MPF has been activated.

Results

Instability of NIMA requires the C-terminal non-catalytic domain

Previous studies in *Aspergillus* have shown that high levels of *nimA* expression derived from the ethanol-inducible *alcA* promoter can prematurely promote the irreversible onset of mitotic events (Osmani *et al.*, 1988). We constructed plasmids containing both full-length and truncated *nimA* cDNAs linked to the *alcA* promoter, which differed from those reported previously in that they lacked several small open reading frames (ORF) 5′ to the initiation codon to maximize expression of NIMA and derivatives from *alcA*. These constructs were transformed into both a wild-type strain and one carrying the temperature-sensitive *nimA*5 mutation (Materials and methods). Only full-length *nimA* was capable of complementation of the *nimA*5 mutation in *trans*. Strains expressing only the catalytic domain of NIMA [NIMA(Nru)] showed an irreversible arrest, even at relatively low levels of expression on glycerol medium (Figure 1). No transformants were obtained with full-length *nimA*, which showed similar lethality to that reported previously (Osmani *et al.*, 1988). We do not know whether this is due to copy number effects or, given the lethality of C-terminally truncated NIMA, chromosomal rearrangements which are common in strains carrying multiple copies of tandemly integrated constructs. For these reasons, all further experiments were performed with strains carrying a single integrated copy of *alcA*::*nimA* at the same chromosomal locus (*argB* on chromosome 2).

Two affinity purified anti-NIMA antisera were produced in rabbits using amino acids 343–699 of NIMA as the immunogen. On Western blots of whole cell extracts, neither serum could detect full-length NIMA expressed from either its endogenous promoter or the stronger *alcA* promoter (Figure 2A). Other workers have found that NIMA is only detectable on Western blots when first immunoprecipitated from a large amount (>2 mg) of total protein (Osmani *et al.*, 1991a; S.Osmani, personal communication). This is also true in strains overproducing NIMA, suggesting that not only is the protein of low abundance but that it is also rapidly turned over. In contrast, NIMA(Nru) was easily detected on Western blots with either of our affinity purified antisera (Figure 2A). The C-terminal deletion of NIMA(Nru) removes three potential PEST sequences (Figure 1; Rogers *et al.*, 1986), which is consistent with the increased stability of the protein. Thus, from these experiments we conclude that the instability of NIMA requires the presence of its non-catalytic C-terminus, and that as the accumulation of NIMA caused by removal of the instability sequences results in lethality, this instability is an important mechanism in regulating NIMA for correct cell cycle progression.

As noted in Figure 1, the C-terminus of NIMA is also rich in $p34^{cdc2}$ consensus phosphorylation sites. The C-

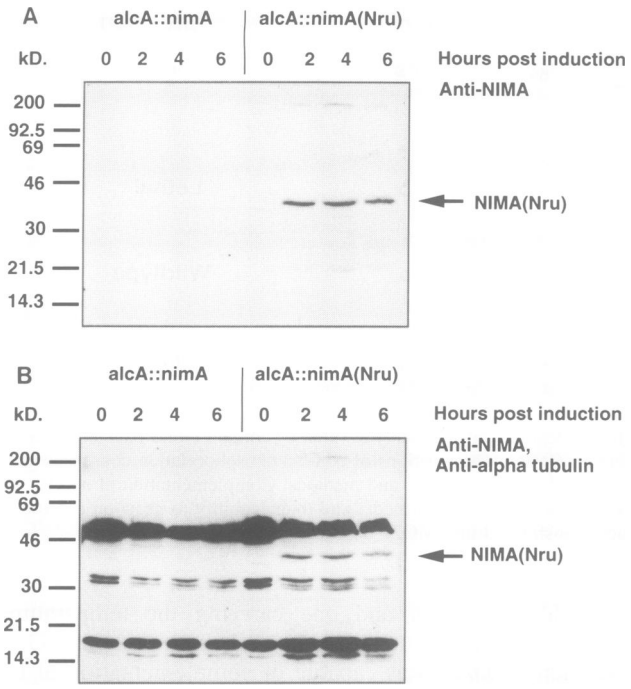

Fig. 2. C-terminally truncated NIMA(Nru) shows increased stability. (**A**) Anti-NIMA Western blot analysis of *A.nidulans* strain MO27 transformed with a single copy of *alcA::nimA* (pMO137) or *alcA::nimA*(Nru) (pMO152) integrated at *argB*. Strains were grown in glucose medium at 32°C for 14 h, harvested and washed extensively with carbon-free medium and then transferred into ethanol medium for the times indicated (hours post-induction). Extracts were made and Western blotting was carried out as described in Materials and methods, with a 1:100 dilution of affinity purified anti-NIMA antibodies. Each track contains ~100 μg of total soluble protein. (**B**) After detection of the NIMA signal in (A), the same filter was reprobed with anti-α-tubulin antibodies, using a 1:1000 dilution of the mouse monoclonal antibody B-5-1-2 (Sigma) as a loading control. The lower molecular weight bands in (B) may be proteolytic cleavage products of α-tubulin or cross-reacting proteins.

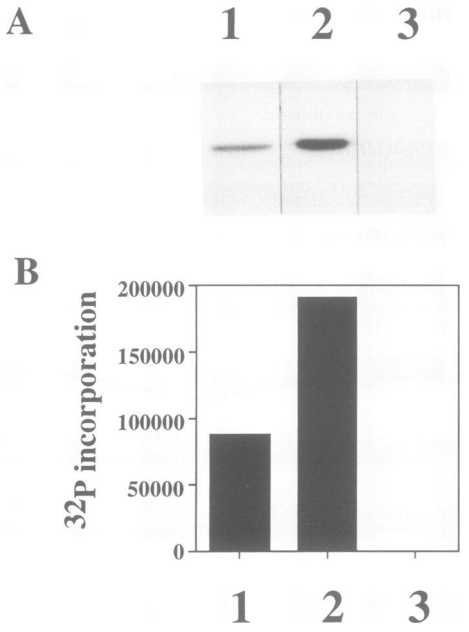

Fig. 3. The C-terminus of NIMA is phosphorylated by $p34^{cdc2}$ *in vitro*. A histidine-tagged protein containing amino acids 343–699 of NIMA was purified from bacteria (Materials and methods) and used as a substrate in $p34^{cdc2}$ kinase assays. (**A**) Autoradiograph of an SDS–polyacrylamide gel of reaction products from the kinase assays. 10 ng of NIMA protein were incubated for 20 min at 30°C with 200 μM [γ-^{32}P]ATP in HB15 buffer (Moreno *et al.*, 1991) containing immunoprecipitates prepared from 200 μg of fission yeast-soluble protein isolated with: lane 1, anti-$p56^{cdc13}$ antibodies (SP4; Moreno *et al.*, 1989); lane 2, anti-$p34^{cdc2}$ antibodies (C2; Simanis and Nurse, 1986); and lane 3, protein A beads only. Sample buffer was added to 1×, and products were separated on a 10% SDS–PAGE prior to autoradiography. (**B**) The ^{32}P incorporated into NIMA in (A) was quantified using a Molecular Dynamics PhosphorImager and is expressed as relative units.

terminus of NIMA (amino acids 343–699), which was produced for immunization of rabbits to produce anti-NIMA antisera, can be phosphorylated *in vitro* by $p34^{cdc2}$ immunoprecipitated from fission yeast (Figure 3). However, we do not have any evidence to suggest that phosphorylation of this region of NIMA would have any significance in terms of protein stability, nor do we know whether NIMA is phosphorylated by $p37^{nimX}$ *in vivo*.

Accumulation of NIMA in Aspergillus promotes chromatin condensation but not mitosis

We next looked in detail at the terminal phenotype caused by the accumulation of stable NIMA. When expression from the *alcA* promoter was induced by growth in ethanol medium, cells with hypercondensed nuclei accumulated coincidently with the appearance on Western blots of the stable NIMA(Nru) protein. Over the time-course of the experiment (6 h) all cells (>99%) exhibited this phenotype. These cells resembled those which have been described previously as the mitotic arrest phenotype associated with high-level expression of full-length NIMA. However, two observations revealed that although containing condensed nuclei, these cells were not in mitosis. First, immunofluorescent anti-tubulin staining showed that as the cells arrest, no mitotic spindles were formed and interphase microtubules were lost. However, on the rare occasions when microtubules were still present in cells with condensed chromatin (presumably at the onset of or early in the arrest), only remnants of interphase microtubule arrays were seen (Figure 4). Thus we found no evidence of mitotic spindle formation in these cells. In a normal asynchronous population of *Aspergillus* cells, interphase microtubule arrays are seen in ~96% of cells, indicating that under these conditions of NIMA accumulation cells were prematurely condensing chromatin during interphase without the formation of a normal mitotic spindle. Control cells, expressing full-length NIMA from the *alcA* promoter (single copy of pMO137 at *argB*) grown under inducing conditions (ethanol medium), were indistinguishable from wild-type cells in mitotic index and microtubule morphology (not shown). Secondly, $p37^{nimX}$ kinase activity was assayed from $p13^{suc1}$ precipitates of soluble protein. Over the time-course of the experiments, $p37^{nimX}$ activity did not increase significantly, and averaged 5- to 10-fold lower than that seen in controls of wild-type mitoses showing a similar percentage of cells with condensed nuclei (Figure 5). As microtubular spindle formation and $p37^{nimX}$ kinase activation do not occur under these conditions, the accumulation of stable NIMA promotes chromatin condensation in interphase cells but does not

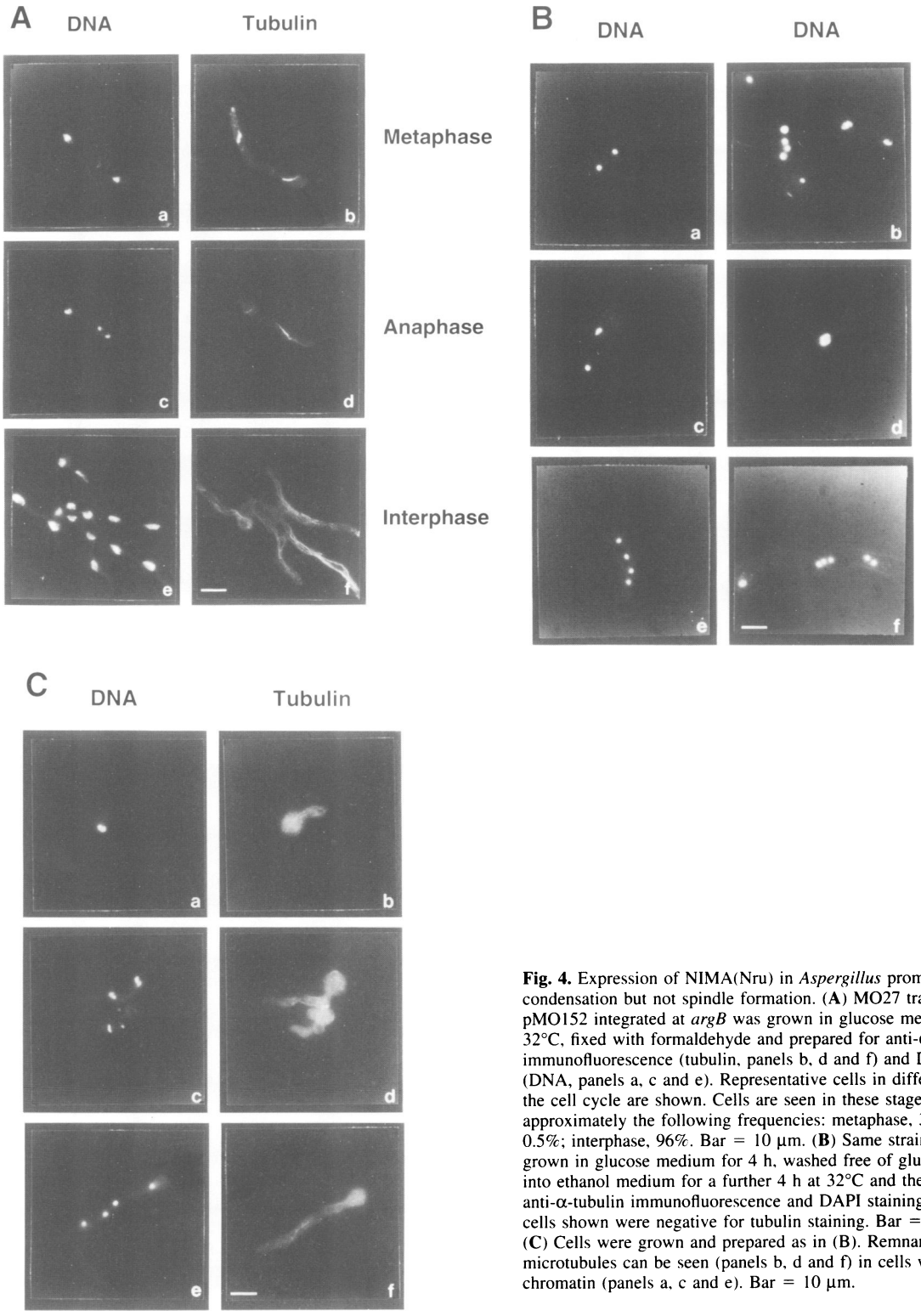

Fig. 4. Expression of NIMA(Nru) in *Aspergillus* promotes chromatin condensation but not spindle formation. (**A**) MO27 transformed with pMO152 integrated at *argB* was grown in glucose medium for 8 h at 32°C, fixed with formaldehyde and prepared for anti-α-tubulin immunofluorescence (tubulin, panels b, d and f) and DAPI staining (DNA, panels a, c and e). Representative cells in different stages of the cell cycle are shown. Cells are seen in these stages with approximately the following frequencies: metaphase, 3.5%; anaphase, 0.5%; interphase, 96%. Bar = 10 μm. (**B**) Same strain as in (A) was grown in glucose medium for 4 h, washed free of glucose, transferred into ethanol medium for a further 4 h at 32°C and then prepared for anti-α-tubulin immunofluorescence and DAPI staining. DAPI-stained cells shown were negative for tubulin staining. Bar = 10 μm. (**C**) Cells were grown and prepared as in (B). Remnants of interphase microtubules can be seen (panels b, d and f) in cells with condensed chromatin (panels a, c and e). Bar = 10 μm.

advance *Aspergillus* cells prematurely into a complete mitotic state.

Expression of NIMA in S.pombe

Both the full-length *nimA* cDNA and NIMA(Nru) were expressed in *S.pombe* from various promoters. Full-length *nimA* had no effect on cell viability when expressed from the weak CaMV-tet promoter, but was lethal when expressed from a single integrated copy of the strong *nmt1* promoter when the expression was derepressed by the depletion of thiamine. When using even the weakest available expression systems for fission yeast (CaMV-tet in the presence of the tet-repressor protein expressed from the *adh1* promoter; Forsburg, 1993), no transformants could be obtained in several independent experiments with constructs containing the stable C-terminally truncated NIMA(Nru). Although this expression system is repressible, there is still a residual background constitutive

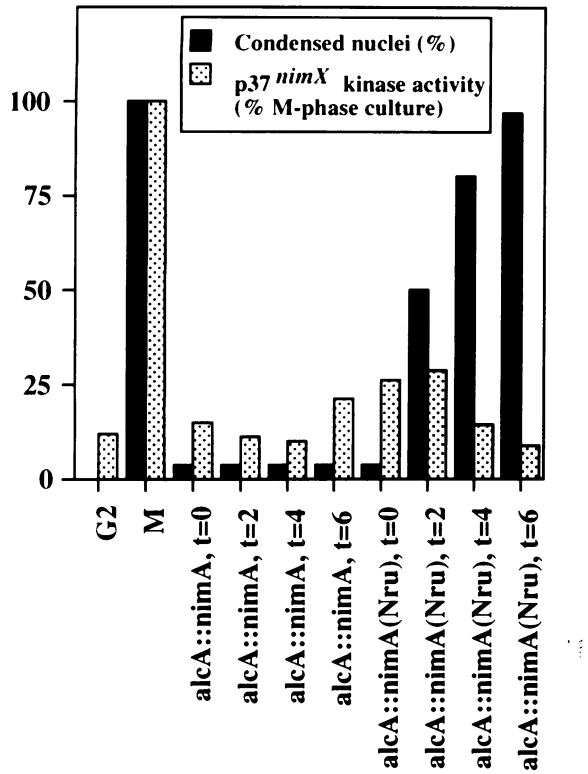

Fig. 5. NIMA(Nru)-induced chromatin condensation is not associated with mitotic levels of p37^{nimX} kinase activity. Liquid mycelial cultures were grown as described in the legend to Figure 2. Time post-induction (*t*) is shown in hours. The percentage of cells with condensed nuclei was determined by DAPI staining. p37^{nimX} kinase activity was determined with a peptide substrate (Materials and methods) and is expressed as a percentage of an M-phase culture. G_2 and M-phase samples were prepared as follows. A strain carrying a *nimT*23 mutation (O'Connell *et al.*, 1992; MO73: *pabaA*1; *nimT*23) was grown in glucose medium for 14 h at 32°C, and transferred to 42°C for 3 h. At this stage the culture was split in two, and half was processed for protein extraction (G_2 sample); the other half was returned to 32°C in the presence of 5 μg/ml benomyl for 45 min (M sample) prior to harvesting and protein extraction.

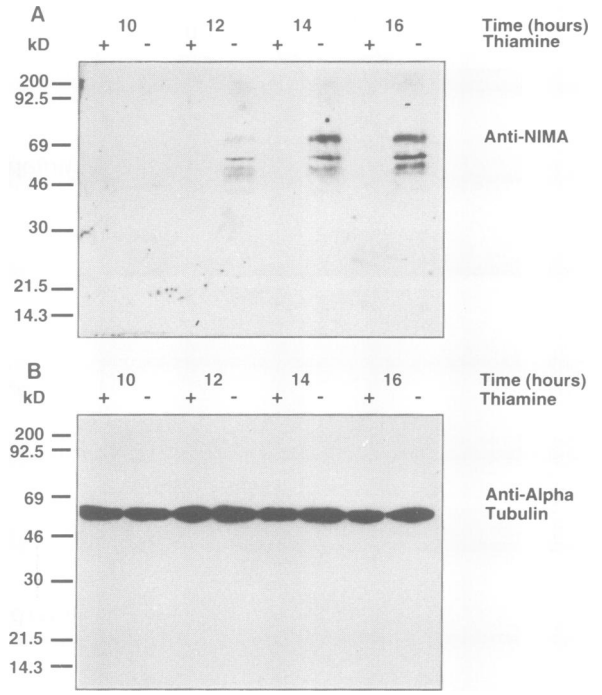

Fig. 6. Western blot analysis of NIMA expression in fission yeast. *S.pombe* cells carrying an integrated copy of pMO159 (*nmt1::nimA*) were grown in minimal medium at 32°C in the presence or absence of 5 μM thiamine for the times indicated. Soluble protein was extracted, separated by SDS–PAGE (100 μg/track) and blotted to nitrocellulose. (**A**) Western blot probed with a 1:100 dilution of affinity purified anti-NIMA. (**B**) Same filter as in (A) reprobed after NIMA detection, with a 1:1000 dilution of the mouse monoclonal anti-α-tubulin antibody B-5-1-2 (Sigma) as a loading control.

activity from this promoter which we assume provides sufficient NIMA(Nru) for lethality. Similarly, we could not obtain transformants with NIMA(Nru) expressed from the *nmt1* promoter under repressing conditions. From this we conclude that fission yeast, like *Aspergillus*, is extremely sensitive to the lethal effects of NIMA accumulation, which can be promoted either by very high-level expression or by removal of the non-catalytic C-terminal domain.

Analysis of the cells arrested by high levels of NIMA expression revealed that fission yeast responds to the accumulation of NIMA in a way similar to that described above for *Aspergillus* cells accumulating NIMA(Nru). Coincident with the ability to detect NIMA on Western blots (Figure 6), was the cessation of cell division and the appearance of cells with single hypercondensed nuclei (Figures 7A and B, and 8A). The degree of chromosome condensation was significantly greater than that seen in wild-type cells entering mitosis (Figure 8). The three fission yeast chromosomes were sometimes visible as three distinct lobes of DAPI staining material (Figure 8). This has also been seen previously in cells arrested in mitosis for an extended period (e.g. the *nda*$^{-}$ mutants; Toda *et al.*, 1983) where presumably signals for chromosome condensation are sustained and lead to hypercondensation. As with *Aspergillus*, the condensation of nuclei was associated with a loss of cytoplasmic microtubules, and no mitotic spindles were formed as the nuclei condensed (Figure 7C). p34^{cdc2} kinase activity was only slightly elevated when compared with the +thiamine control (Figure 7D). It is notable that although NIMA could be detected on Western blots, it did not accumulate to very high levels; this may have been due to proteolysis of NIMA (Figure 6). Derepression of the *nmt1* promoter requires 12–14 h growth at 32°C (Maundrell, 1990); the condensed chromatin phenotype appeared quite soon after initial derepression. This indicated that relatively low levels of NIMA are capable of inducing chromatin condensation. Unlike many mitotic mutants of fission yeast, these cells did not display a 'cut' phenotype in which a septum forms through the nucleus. From these experiments we conclude that the accumulation of *Aspergillus* NIMA in fission yeast causes premature chromatin condensation without other aspects of mitosis.

Accumulation of NIMA induces chromatin condensation from any point in the cell cycle

We next addressed the question of whether NIMA accumulation could induce chromatin condensation from any point in the cell cycle. Although we had established that

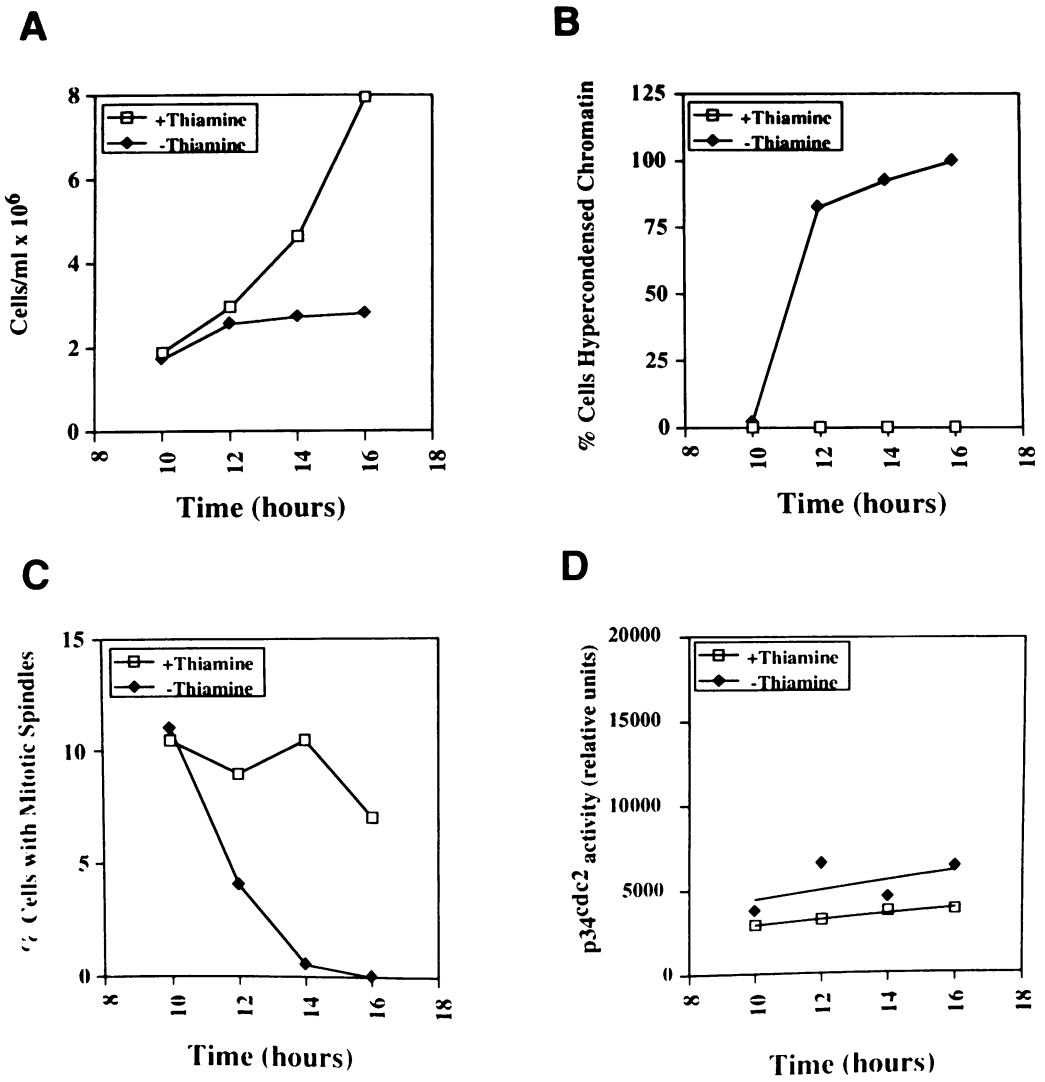

Fig. 7. Accumulation of NIMA promotes chromatin condensation in fission yeast. Cultures were grown as described in the legend to Figure 6. In each panel, the culture grown in the presence of 5 μM thiamine is represented by the open squares and the culture grown in the absence of thiamine is represented by closed diamonds. Time refers to hours of growth at 32°C. (**A**) Culture cell numbers were determined using a Coulter Counter Model ZM. Data are the average of three readings. (**B**) The percentage of cells with condensed chromatin was determined by DAPI staining of ethanol-fixed cells. For each point n = 200. (**C**) The percentage of cells with mitotic spindles was determined as described in Materials and methods. Data presented are for mixed aldehyde-fixed cells (n = 200). Very similar results were obtained with methanol-fixed cells (not shown). (**D**) $p34^{cdc2}$ kinase in SP4 (Moreno *et al.*, 1989) immunoprecipitates activity quantitated with a peptide substrate and presented in relative units. Values are the average of two samples.

NIMA accumulation could promote chromatin condensation in interphase cells, we did not know, for example, whether this could occur in cells that had yet to commit to mitosis by passage of START. There is a good collection of *Aspergillus* mutant strains defective in G_2 progression, but there are no well-characterized mutants that arrest cells in the G_1 phase of the cell cycle. Further, monitoring the cell cycle arrest point after inducing the expression of NIMA(Nru) was not technically feasible due to the problems with flow cytometric analysis of DNA content in this organism (Osmani *et al.*, 1994). We therefore chose to pursue these experiments in fission yeast.

Temperature-sensitive loss-of-function mutations in cell cycle control genes and the DNA synthesis inhibitor hydroxyurea were used to arrest cells in the cell cycle. Once the cells were arrested, derepression of the *nmt1* promoter led to the accumulation of lethal levels of NIMA; the effects on chromatin condensation were monitored by DAPI staining (Figure 8). NIMA was shown to induce high levels of chromatin condensation in >95% of cells from pre-start G_1 (*cdc10-V50*; Marks *et al.*, 1992), early S phase (hydroxyurea) and G_2 (*cdc25-22*, Nurse *et al.*, 1976). These data show that accumulation of NIMA can induce chromatin condensation from any point in the fission yeast cell cycle. This is in keeping with the finding that the arrest point in *Aspergillus* is associated with the presence of interphase microtubule arrays. Further, the NIMA-induced chromatin condensation did not require $p34^{cdc2}$ activity, as the effects of NIMA were also epistatic to the *cdc2-33* mutation (Nurse *et al.*, 1976). This is consistent with the finding that in both *Aspergillus* and fission yeast the arrest phenotype is not associated with elevated levels of $p34^{cdc2}/p37^{nimX}$ activity in wild-type cells.

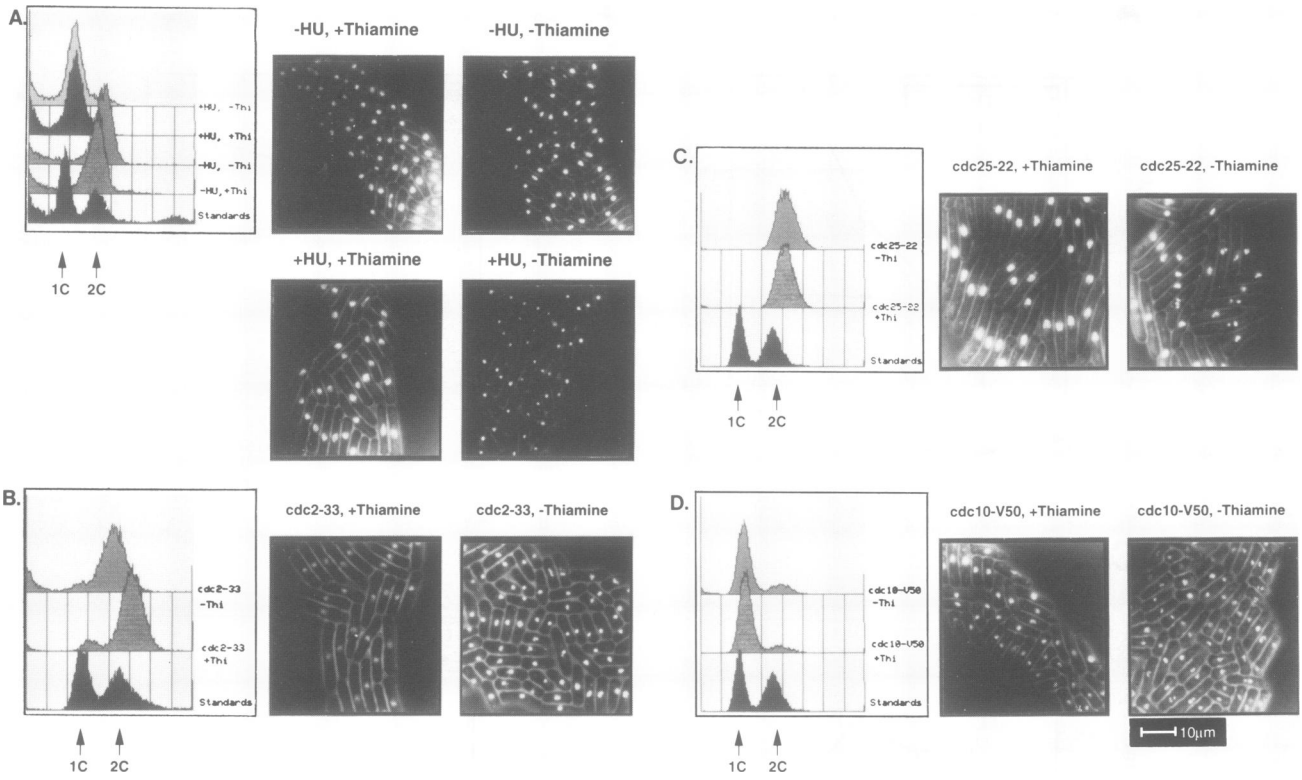

Fig. 8. Accumulation of NIMA promotes chromatin condensation from multiple points in the fission yeast cell cycle. All strains carry a single integrated copy of pMO159. Cells shown were grown in minimal medium with or without 5 μM thiamine as indicated. For wild-type cells (−HU), cells were grown at 32°C for 16 h. For hydroxyurea-treated cells (+HU), cultures were grown in minimal medium without hydroxyurea for 10 h at 32°C, after which hydroxyurea was added to 11 mM. A second aliquot of hydroxyurea was added after a further 3.5 h. Samples shown have been grown for a total of 16 h. For strains containing ts *cdc* alleles, cultures were grown at 25°C for 14 h, and shifted to 36°C for a further 4 h. Pilot experiments indicated this to be an appropriate time frame to ensure that the *cdc* phenotype manifested prior to the NIMA-dependent chromatin condensation. Panels depict the DNA content measured on PI-stained cells by flow cytometric analysis (FACScan, Becton-Dickinson; Sazer and Sherwood, 1990) and DAPI-stained cells. The genetic backgrounds of strains are described in Materials and methods. (**A**) Wild-type background in the presence and absence of hydroxyurea (HU). (**B**) *cdc2-33*. (**C**) *cdc25-22*. (**D**) *cdc10-V50*. In each case condensed chromatin is seen in >95% of the cells in cultures without thiamine, although some cells do not appear as condensed because they lay in a different focal plane.

Expression of NIMA in HeLa cells induces chromatin condensation and apoptosis

As we observed similar effects of NIMA accumulation in *Aspergillus* and fission yeast, we wished to test whether these effects of NIMA accumulation were confined only to ascomycete fungi or could also be seen in mammalian cells. To this end we expressed NIMA and NIMA(Nru) from the CMV promoter in transiently transfected human cervical carcinoma (HeLa) cells. Included in these constructs was the lymphocyte CD2 cell surface marker which was expressed from the SV40 promoter. This allowed transfectants to be identified soon after transfection using a monoclonal antibody against CD2 for immunofluorescent staining. This antibody was also utilized to separate transfected cells from untransfected cells through the use of immunomagnetic beads. As a negative control, the human CDC2 cDNA, which has been shown previously to have little effect on cell viability or cell cycle progression (Heald *et al.*, 1993; C.Norbury, unpublished results), was expressed from identical constructs.

The expression of both forms of NIMA had the same effects on HeLa cells, although in keeping with the findings in *Aspergillus*, NIMA(Nru) affected a greater proportion of transfected cells [37% for NIMA(Nru), 24% for NIMA; Figure 10C]. Cells were observed with a rounded cell morphology and altered nuclear morphology (Figure 9). The chromatin was either condensed into a single mass without individual chromosomes being distinguishable (abnormally condensed), or resembled that of apoptotic cells with chromatin fragmented into several discrete bodies (late apoptotic) (Figure 9a–c). The chromatin condensation pattern did not resemble that of cells entering mitosis, and immunofluorescent staining of transfected cells showed no reactivity with the MPM-2 anti-mitotic phosphoprotein monoclonal antibody (Figure 9; Davis *et al.*, 1983). The cells with condensed chromatin also lacked discernible nuclear lamin-A staining, indicating that nuclear lamins had been degraded rather than depolymerized; tubulin staining revealed either interphase microtubule arrays which were collapsed around the nuclear mass or a complete absence of microtubule staining (Figure 9). Thus, although these cells displayed chromatin condensation, albeit abnormal, they did not have any other morphological signs of mitosis. Further, flow cytometric analysis showed that the DNA content of the transfected cells was identical to that of controls, indicating that they were not arresting at any particular point in the cell cycle (Figure 10A). Analysis of $p34^{cdc2}$ kinase activity indicated that, as in *Aspergillus* and fission yeast, the chromatin condensation associated with accumulation of NIMA in HeLa cells did not require mitotic levels of MPF activity (Figure 10B). This is in keeping with most cells arresting

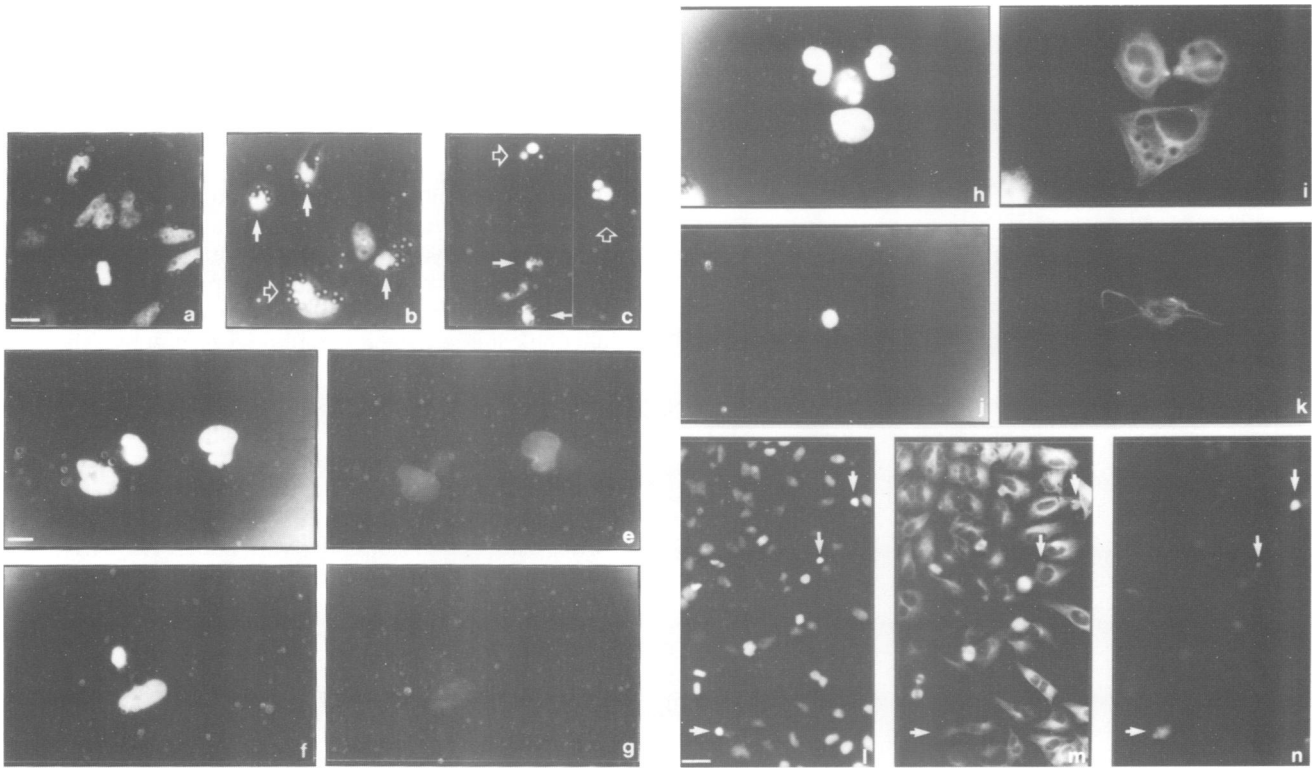

Fig. 9. Effects of NIMA and NIMA(Nru) expression on HeLa cells. Cells were transfected and prepared for immunofluorescence as described in Materials and methods. (**a**–**k**) Transfected cells selected with magnetic beads, which can be seen as the small spherical objects in the Hoescht 33258-stained panels. (**l**–**n**) are not bead-selected. (a–c) depict bead-selected cells stained with Hoescht 33258. Cells are expressing: (a) human CDC2; (b) NIMA; and (c) NIMA(Nru). Cells scored as abnormally condensed are indicated by solid arrows, and those scored as late apoptotic are indicated by open arrows. Bar (in a) = 20 μm. (d–k) depict bead-selected cells expressing NIMA(Nru). Transfected cells not showing condensed chromatin are included in (d–i) as an internal control. (d, f, h and j) Hoescht 33258-stained cells. (e) Anti-lamin A staining (central cell has condensed chromatin but lacks lamin A staining). (g) MPM-2 staining (upper cell has condensed chromatin but lacks MPM-2 staining). (i and k) Anti-α-tubulin staining. In (i), the cell with condensed chromatin (central) lacks microtubules. In (k), the cell is displaying condensed chromatin and has microtubules collapsed around the nucleus. Bar (in d) = 10 μm. (l–n) depict a single field of unselected NIMA(Nru)-transfected cells stained with: (l) Hoescht 33258; (m) anti-α-tubulin; and (n) anti-CD2. Arrows in each panel identify $CD2^+$ (transfected) cells, all which have condensed chromatin and lack microtubules. Bar (in l) = 40 μm.

with a G_1/S DNA content (Figure 10A) which is prior to $p34^{cdc2}$ activation. We therefore conclude that the accumulation of *Aspergillus* NIMA in human cells can also induce premature chromatin condensation in the absence of mitosis.

Discussion

The nature of the *Aspergillus nimA*$^-$ loss-of-function phenotype has for some time proved difficult to reconcile with conventional models for regulation of the G_2/M transition. Experiments in most systems have indicated that the activation of MPF by the dephosphorylation of tyrosine 15 on $p34^{cdc2}$ is the final step required for mitotic entry; yet *nimA*$^-$ mutants arrest in G_2 even though this step is completed and cells have activated MPF (Osmani *et al.*, 1991b). Without loss-of-function mutations in NIMA homologues of other species, it has been impossible to test whether an additional level of control involving NIMA is also present subsequent to MPF activation outside of *Aspergillus*. In addition, we have had little clue as to the function of NIMA late in G_2 that is required for entry into mitosis. The experiments described here provide some insight into these problems.

We have shown that in *Aspergillus* removal of the C-terminal non-catalytic domain of NIMA results in greatly increased stability of the protein. This truncation removes three potential PEST sequences, two of which have extremely high PEST scores (Rogers *et al.*, 1986) and so are likely to be involved in the instability of NIMA. We have also noted that this region of NIMA contains several consensus sites for phosphorylation by homologues of $p34^{cdc2}$, and have found that the C-terminus of NIMA (amino acids 343–699) can be efficiently phosphorylated *in vitro* by $p34^{cdc2}$. It is possible that NIMA may be up-regulated by $p37^{nimX}$ phosphorylation of residues in the non-catalytic domain. However, this is not required for NIMA to be active when it is overexpressed, and so cannot alone account for the accumulation of NIMA protein and activity as cells enter mitosis. Other factors, such as an increase in *nimA* mRNA (Osmani *et al.*, 1987), may also contribute to the accumulation of NIMA as cells enter mitosis.

Regulated expression in *Aspergillus* of the stable form of NIMA [NIMA(Nru)] resulted in lethality with cells containing condensed chromatin. These experiments show that regulation of NIMA stability must be an important mechanism in maintaining normal cell cycle progression in *Aspergillus*. It has been reported previously that the accumulation of full-length NIMA in *Aspergillus* results in a premature and irreversible mitosis (Osmani *et al.*, 1988). In these experiments, mitosis was assayed by the

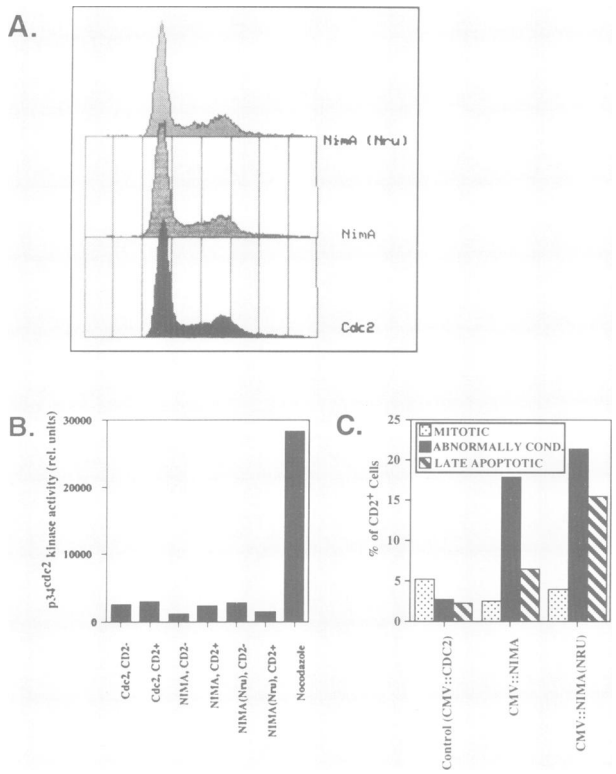

Fig. 10. Accumulation of NIMA causes premature chromatin condensation in HeLa cells. Transfected cells were collected using anti-CD2 antibodies and magnetic beads. (**A**) FACS analysis of DNA content in $CD2^+$ cells. Normal FACS profiles are seen in the control (Cdc2) and cells expressing either full-length NIMA (NimA) or C-terminally truncated NIMA [NimA(Nru)]. (**B**) Relative $p34^{cdc2}$ kinase activity was assayed using anti-$p34^{cdc2}$ immunoprecipitates made from 40 μg of protein from whole-cell extracts. Samples were taken from both $CD2^+$ (transfected) and $CD2^-$ (untransfected) cells from the same culture plate. Nocodazole-arrested cells were used as a control for cells arrested in mitosis. (**C**) Quantification of the phenotypes of bead-selected $CD2^+$ cells with condensed chromatin.

appearance of condensed nuclei and mitotic spindles. The presence of spindles was transient and was followed by the loss of microtubular staining. In contrast to this we did not observe any spindle formation in cells arrested by the expression of NIMA(Nru); rather, we observed that while most cells lacked any specific tubulin staining, those cells that did stain for microtubules contained the remnants of an interphase array. Further, we did not observe mitotic levels of the $p37^{nimX}$ kinase, a marker of mitosis not measured in the study reported previously. Although the C-terminal truncation may result in an inability to interact with other elements of mitotic control, we were unable to generate any transformants in which expression of full-length NIMA from the *alcA* promoter resulted in a promoter-dependent lethality. This may have been due to copy number effects and/or gene rearrangements in the high copy number transformants which were described previously (Osmani *et al.*, 1988).

The effects of expression of the stable NIMA(Nru) could not be investigated in fission yeast as it was lethal in this organism even at a low level of expression. Fission yeast cells accumulating NIMA arrested with hypercondensed chromatin without entering mitosis. This was assayed by spindle formation and $p34^{cdc2}$ activation. Further, we showed that this arrest phenotype is not only independent of the cell cycle stage, showing that chromatin condensation was truly premature, but also that it did not require $p34^{cdc2}$ activity. Therefore, in *Aspergillus* and fission yeast NIMA accumulation has very similar phenotypic effects, promoting premature chromatin condensation without any other aspects of mitosis.

Aspergillus and fission yeast are both ascomycete fungi and show sequence conservation in several cell cycle control genes involved in the G_2/M transition (O'Connell *et al.*, 1992; Osmani *et al.*, 1994). Despite this phylogenetic relatedness and a considerable effort making use of both function- and sequence homology-based approaches, we have not yet identified a fission yeast NIMA homologue; it is possible that no such homologue exists in this organism. However, as the NIMA gain-of-function phenotype is essentially identical in both organisms, then targets for NIMA action, which we presume are relevant substrates for the protein kinase activity of NIMA, must be conserved.

We also expressed both NIMA and NIMA(Nru) in transiently transfected human (HeLa) cells. As with both fungal systems, HeLa cells accumulating NIMA exhibited premature chromatin condensation without entering a true mitotic state. By analogy to the fungal systems, this is likely to require an active kinase. These experiments indicate that human cells may also have targets for NIMA action. Several protein kinases that are structurally related to NIMA have been identified in human cells and, like *nimA* in *Aspergillus*, one of these is maximally expressed in cells at G_2/M (Schultz and Nigg, 1993; Schultz *et al.*, 1994). Although these are good candidates for a human NIMA, there are no functional studies to confirm this. In our experiments, considerable levels of apoptosis were seen particularly in cells accumulating NIMA(Nru), and thus it is possible that the apoptosis is a direct consequence of NIMA accumulation. Disruptions to normal cell cycle controls have been shown to induce cell death by apoptosis in some instances (Kung *et al.*, 1990); the premature chromatin condensation induced by NIMA may result in a cell sensing that it is entering mitosis prematurely. However, it is also possible that the apoptotic cell death is a secondary effect of the chromatin condensation, which would be expected to inhibit transcription and so metabolically arrest the cells.

Premature condensation of chromatin from interphase, and defects in decondensation of chromatin after mitosis, have been observed in fission yeast and mammalian cells carrying mutations in *pim1*/RCC1 (reviewed by Dasso, 1993; Sazer and Nurse, 1994). The premature chromatin condensation seen in mammalian cells lacking RCC1 is not, however, analogous to that described here. Cells lacking RCC1 show other morphological aspects of mitosis when they exhibit premature chromatin condensation. Further, premature chromatin condensation in these cells requires activated MPF. It is, however, possible that premature chromatin condensation in RCC1-deficient cells requires NIMA activity, but this has not yet been tested as a *pim1*/RCC1 homologue has not been identified in *Aspergillus*.

The gain-of-function phenotypes which we have described here suggest a model for wild-type NIMA function in *Aspergillus* (Figure 11). Artificial accumulation of NIMA prematurely promotes chromatin condensation, so it is therefore likely that NIMA normally accumulates at

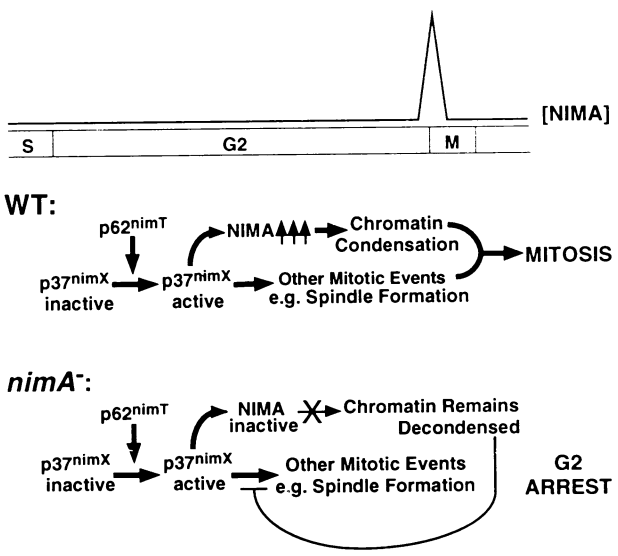

Fig. 11. Model for NIMA function in *Aspergillus*. NIMA is unstable and present in low levels throughout interphase, but accumulates to peak at the G_2/M transition. In G_2, $p37^{nimX}$ (homologue of cdc2; Osmani *et al.*, 1994) is activated by $p62^{nimT}$ (homologue of cdc25; O'Connell *et al.*, 1992). Once active, $p37^{nimX}$ may be involved in the stabilization and accumulation of NIMA (see text), which then promotes condensation of chromatin. Other mitotic events such as spindle formation are not affected by NIMA accumulation. In *nimA* mutants, $p37^{nimX}$ is still activated by $p62^{nimT}$, but as NIMA activity is low, chromatin does not condense. The inability to condense chromatin blocks further progression into mitosis and the cells arrest in G_2.

the onset of mitosis to promote the chromatin condensation usually associated with mitosis. This function for NIMA would explain why *nimA*$^-$ mutants arrest in G_2, as they would be unable to condense their chromatin in preparation for mitosis. A feedback mechanism may prevent further entry into mitosis despite $p37^{nimX}$ dephosphorylation on tyrosine, because in the absence of such a mechanism there would be a lethal entry into mitosis with disorganized chromatin. However, as MPF is activated in these cells, what could the target of this checkpoint control be to prevent entry into mitosis? One possibility could be regulation of the subcellular localization of the activated MPF. That is, in *nimA*$^-$ cells the activated MPF may be confined to the cytoplasmic compartment; so although active as a kinase in assays *in vitro*, it would be unable to gain access to and phosphorylate nuclear substrates and therefore be unable to promote mitosis. An alteration in the subcellular location of activated MPF has been reported for cells overproducing the WEE1 protein kinase (Heald *et al.*, 1993).

With this model we would still propose that $p37^{nimX}$ activity is required for the other aspects of mitosis, but that the NIMA kinase is only involved in the organization of the chromatin. If $p37^{nimX}$ is required to promote chromatin condensation, then this must be via NIMA, possibly by promoting NIMA stability. A requirement for active MPF to condense chromatin is therefore bypassed by the artificial accumulation of NIMA; under these conditions there is an uncoupling of the control of chromatin condensation from other mitotic events, such as mitotic spindle formation, which require active MPF. Our experiments suggest that NIMA substrates exist in other organisms and thus that NIMA kinase may be present as well. If so, then our model for the role of NIMA in chromatin condensation during mitosis may be more generally applicable to other eukaryotes.

Materials and methods

Aspergillus methods

All *A.nidulans* strains are derivatives of a single isolate, FGSC4. Methods for the propagation and manipulation of *Aspergillus* were as described previously (O'Connell *et al.*, 1992, 1993). For this study, plasmids were introduced into either MO27 (*wA2*; *argB2*; *nicA5*) or MO79 (*wA2*; *argB2*; *nimA5*; *nicA5*). Transformants were selected by their ability to grow in the absence of arginine. Complementation of *nimA5* was assayed by the ability to form colonies at 42°C on either glycerol or ethanol medium. Indirect immunofluorescent staining of microtubules was performed on formaldehyde-fixed cells grown on coverslips as described (Mirabito and Morris, 1993), using a 1:100 dilution of the anti-α-tubulin mouse monoclonal antibody B-5-1-2 (Sigma) and a 1:100 dilution of Cy-3-conjugated sheep $F(ab')_2$ anti-mouse (Sigma). DAPI staining was either as described previously (O'Connell *et al.*, 1993) on glutaraldehyde-fixed cells, or on heat-fixed cells mounted in 50% glycerol, 1 mg/ml p-phenylenediamine and 1 μg/ml DAPI.

Fission yeast methods

S.pombe strains were propagated and manipulated as described (Moreno *et al.*, 1991). Indirect immunofluorescent staining of microtubules was performed on cells fixed with either methanol or formaldehyde and glutaraldehyde, as described (Moreno *et al.*, 1991), using a 1:5 dilution of the mouse anti-α-tubulin monoclonal antibody TAT-1 (a gift from Dr K.Gull, University of Manchester, UK) and a 1:50 dilution of Cy-3-conjugated sheep anti-mouse $F(ab')_2$ (Sigma). For DAPI staining of nuclei, cells were washed with water, heat-fixed to a microscope slide and mounted in 50% glycerol, 1 mg/ml p-phenylenediamine and 1 μg/ml DAPI. NIMA expression studies from the *nmt1* promoter were carried out in derivatives of *leu1*-32, *ura4*-D18, *ade6*-704, *h*$^-$. CaMV-tet expression studies were carried out in the same strain with or without a *sup3*−5 selected copy of the tet-repressor expressed from the *adh1* promoter (Forsburg, 1993).

Plasmid constructions

Standard procedures were used for all DNA manipulations (Sambrook *et al.*, 1989). Unless otherwise indicated, enzymes were obtained from New England BioLabs and plasmids were propagated in *Escherichia coli* strain XL-1 Blue (Stratagene).

The *nimA* cDNA used for expression in *Aspergillus*, HeLa cells and *S.pombe* from the CaMV-tet promoter spanned from 29 nucleotides 5′ to the initiation codon. These constructs were based on a *nimA* cDNA that was constructed as follows. Nucleotides 421–639 of the published *nimA* sequence were amplified by PCR using Vent DNA polymerase (New England BioLabs) and the following oligonucleotides: 5′ end, GGGGATCCGTCGACGCTCGATCCCTGC; and 3′ end, TGTCGGAGAGAGCTCAGG. The resulting product was digested with *Sal*I and *Eco*RI and cloned into pBluescript KS$^+$ (Stratagene) to make pMO134. The remaining portion of the *nimA* cDNA was added to this construct as an *Eco*RI fragment from p3.3B (Osmani *et al.*, 1987), which was kindly supplied by Dr S.Osmani (Geisinger Clinic, PA) to make pMO135.

For expression in *Aspergillus*, the insert of pMO135 was cloned into the *argB* marked *alcA* expression vector pKK12 (Kirk and Morris, 1993) as a *Kpn*I−*Xma*I fragment to make pMO137. Internal deletions in *nimA* were constructed by restriction digestion and religation to make the following plasmids: pMO139 [NIMA(Bam), *Bam*HI digestion]; pMO152 [NIMA(Nru), *Nru*I/*Sma*I double digestion]; and pMO158 [NIMA(Mun), *Mun*I digestion].

For expression in *S.pombe* from the CaMV-tet promoter, *nimA* was subcloned from pMO135 as a *Kpn*I−*Nsi*I fragment into the *ura4*$^+$-based vector pSLF102 (Forsburg, 1993) to make pMO150, and as a *Sal*I−*Nsi*I fragment into the *LEU2*-based vector pSLF101 (Forsburg, 1993). To express NIMA(Nru) in *S.pombe*, pMO150 and pMO151 were each digested with *Nru*I and *Sph*I, end-filled with the Klenow fragment of DNA polymerase I (Boehringer-Mannheim) and religated to make pMO165 and pMO164, respectively. For the expression of *nimA* from the *nmt1* promoter, a construct pANDX was kindly provided by Dr S.MacNeill (University of Edinburgh) which has *nimA* from the initiation codon to the 3′ end of the published sequence as an *Nde*I fragment in pRIP1/s (Maundrell, 1993). To express NIMA(Nru) from this promoter,

pANDX (pMO159) was digested with *Nru*I and *Sma*I and religated to make pMO160.

For expression in HeLa cells, the human CDC2 cDNA (Lee and Nurse, 1987), the full-length NIMA ORF and the *Nru* truncation were each inserted immediately downstream from the human cytomegalovirus (hCMV) immediate early promoter region in the vector pCD2/CMV. This vector is derived from pKV461 (Sowden *et al.*, 1989; kindly provided by A.Kingsman), but contains in addition a cDNA coding for a truncated and biologically inactive rat CD2 cell surface antigen, driven by the SV40 early promoter. The CD2 expression cassette was excised from pERCD2–2 (He *et al.*, 1988; kindly provided by A.N.Barclay) as an *Nde*I–*Eco*RI fragment and inserted into the unique *Eco*RI site of pKV461 to generate pCD2/CMV. The transcriptional orientation of the CD2 gene in this vector is opposite to that of the hCMV promoter. Supercoiled plasmids were purified for transfection by two rounds of CsCl gradient centrifugation.

For the production of NIMA protein in *E.coli*, the *Xho*I–*Nsi*I fragment (corresponding to amino acids 343–699) of the *nimA* cDNA was subcloned into *Sal*I/*Pst*I-digested pQE9 (Qiagen), which contains the *lacZ* promoter and six N-terminal histidine codons in-frame with the NIMA sequence to make pMO145.

HeLa manipulations

HeLa S3 cells were maintained in Dulbecco's modified eagle's medium containing 10% fetal calf serum in a humidified 5% CO_2 incubator at 37°C. Derivatives of pCD2/CMV expressing human CDC2, NIMA or NIMA(Nru) were transfected into HeLa cells by the calcium phosphate coprecipitation technique (Graham and van der Eb, 1973), using 30 mg plasmid DNA in 1.5 ml precipitate per subconfluent 150 mm dish, or 4 mg DNA in 200 ml precipitate for cells grown on coverslips in 35 mm dishes. After incubation with the precipitate for 16 h, the cells were washed with phosphate-buffered saline (PBS) and re-fed with complete medium (time zero). After a further 24 h, cells growing on coverslips were fixed for immunofluorescence studies by immersion in freshly prepared 3% paraformaldehyde, 2% sucrose in PBS for 15 min at 25°C. After three brief washes in PBS, cells were permeabilized by 5 min incubation in 0.5% Triton X-100 in PBS. For anti-tubulin staining, cells were post-fixed for 5 min in 100% methanol at −20°C. After three further washes in PBS, cells were blocked with 10% fetal calf serum in PBS for 30 min at 37°C, then incubated with the appropriate primary antibody diluted in the same blocking solution. The primary antibodies used were rabbit anti-lamin A (kindly provided by B.Burke, Harvard Medical School, MA) diluted at 1:50, anti-mitotic phosphoprotein mouse monoclonal MPM-2 (kindly provided by P.N.Rao, University of Texas, TX) diluted at 1:500 and anti-α-tubulin monoclonal B-5-1-2 (described above) diluted at 1:200. The coverslips were again washed three times with PBS before incubation with Texas Red (Amersham) or Cy-3 (Sigma)-conjugated secondary antibodies, followed in some experiments by further washing with PBS and incubation with FITC-conjugated anti-rat CD2 (OX-34; SeroTec). After three final washes in PBS, the second of which contained Hoescht 33258 (Sigma) at 1 mg/ml, the coverslips were rinsed briefly in distilled water, allowed to dry in the dark at room temperature and mounted in 90% glycerol containing 1 mg/ml p-phenylenediamine and 10 mM Tris, pH 8. Fluorescence microscopy and photography were carried out using a Zeiss Axioskop.

For flow cytometric studies, cells were harvested 24 h after transfection by immersion in PBS containing 4 mM EDTA for 10 min at room temperature and vigorous pipetting. After combination with the culture medium (to collect non-adherent cells), the cell suspension was collected by centrifugation (2500 *g*, 3 min) and incubated for 30 min at 4°C in complete medium (1 ml per 150 mm dish of cells) containing FITC-conjugated mouse anti-rat CD2 monoclonal (OX34; SeroTec) at 5 mg/ml. After washing once in PBS, cells were fixed by the addition of 70% ethanol (2 ml/150 mm dish of cells) while being mixed on a vortex, and were then left at 4°C for at least 30 min. The fixed cells were washed once in PBS and then stained in PBS (1 ml/150 mm dish) containing propidium iodide (PI; 40 mg/ml) and RNase A (100 mg/ml) for 15 min at room temperature, before analysis by flow cytometry (FACScan, Becton Dickinson). A gate was set to collect PI fluorescence data from cells with FITC (CD2) staining at least 10-fold higher than the background level, determined using mock-transfected cells stained in parallel. Data were collected from 10 000 $CD2^+$ cells and analysed using Lysis II software.

For selection with immunomagnetic beads, cells were washed off culture dishes 8 h after transfection using PBS/EDTA as described above. Magnetic beads coated with rat anti-mouse IgG2a (Dynabeads M-450; Dynal) were secondarily coated with the anti-CD2 monoclonal (OX-34; SeroTec) according to the manufacturer's instructions, and were then used to fractionate the transfected cells into $CD2^+$ and $CD2^-$ subpopulations. The cells were then either grown for a further 24–48 h on coverslips and processed for immunofluorescence as described above, or lysed for Cdc2 immunoprecipitations [performed as described previously by Norbury *et al.* (1991)] and subsequent peptide kinase assays as described above.

Preparation of anti-NIMA antisera

Amino acids 343–699 of NIMA, with an N-terminal tag of six consecutive histidine residues, were produced in *E.coli* strain M15 harbouring pMO145 and purified under denaturing conditions by Ni-agarose chromatography as directed by the manufacturer (Qiagen). The purified protein was excised from an SDS–polyacrylamide gel; the slice was lyophilized, ground in a mortar and pestle to a fine powder and emulsified with Freund's adjuvant. Each of two rabbits were initially immunized with 250 μg of protein, and then 2 weeks' later were immunized with 100 μg every 2 weeks for a further 14 weeks. Sera were collected and affinity purified against his-tagged NIMA (amino acids 343–699) which had been blotted to nitrocellulose as described (Harlow and Lane, 1988). Bound antibodies were washed extensively with 10 mM Tris, pH 7.5, 500 mM NaCl, prior to elution. A dilution of 1:100 of affinity purified sera was used for Western blotting. Pre-immune sera were negative for reactivity with bacterially expressed NIMA, or NIMA(Nru) expressed in *Aspergillus*. Antibodies bound to Western blots were detected with horseradish peroxidase-conjugated sheep anti-rabbit antiserum (Amersham) diluted 1:1000 and ECL detection reagents (Amersham) using the recommended conditions of the manufacturer.

Protein extracts and kinase assays

Aspergillus mycelial liquid cultures were growth to low density, harvested by filtration through miracloth (Calbiochem), washed in cold stop buffer (Moreno *et al.*, 1991) and frozen in liquid nitrogen until extraction of protein. For protein extraction, frozen mycelia were ground under liquid nitrogen with glass beads in a mortar and pestle into a fine powder, which was then extracted with HB15 buffer (Moreno *et al.*, 1991) containing the protease inhibitors aprotinin, leupeptin and phenylmethylsulfonyl fluoride (PMSF) each at 1 μg/ml. The slurry was vortexed at room temperature 3×1 min, with 1 min incubations on ice between each vortexing. Mycelial fragments were removed by centrifugation at 2500 *g* for 10 min at 4°C. The extracts were cleared by 2×10 min centrifugations at 15 000 *g* at 4°C. For assays of $p37^{nimX}$-associated kinase, $p13^{suc1}$ beads [5 mg/ml $p13^{suc1}$ coupled to Affigel-15 (Bio-Rad)] were used to isolate $p37^{nimX}$, and activity was measured using a peptide substrate-based assay system under the conditions recommended by the manufacturer (Amersham).

Protein extracts of *S.pombe* were made as described (Moreno *et al.*, 1991) by vortexing with glass beads using HB15 as the extraction buffer containing the protease inhibitors aprotinin, leupeptin and PMSF each at 1 μg/ml. Extracts were cleared by 2×10 min centrifugations at 15 000 *g* at 4°C prior to immunoprecipitation. Assays of $p34^{cdc2}$ kinase activity were performed on immunoprecipitations of $p56^{cdc13}$-associated kinase isolated using SP4 affinity purified antisera (Moreno *et al.*, 1989) and a peptide substrate-based assay system under the conditions recommended by the manufacturer (Amersham).

HeLa soluble protein was extracted as described previously (Norbury *et al.*, 1991).

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