p34cdc2 Acts As a Lamin Kinase in Fission Yeast

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Abstract. The nuclear lamina is an intermediate filament network that underlies the nuclear membrane in higher eukaryotic cells. During mitosis in higher eukaryotes, nuclear lamins are phosphorylated by a mitosis-specific kinase and this induces disassembly of the lamina structure. Recently, p34cdc2 protein kinase purified from starfish has been shown to induce phosphorylation of lamin proteins and disassembly of the nuclear lamina when incubated with isolated chick nuclei suggesting that p34cdc2 is likely to be the mitotic lamin kinase (Peter, M., J. Nakagawa, M. Dorée, J. C. Labbe, and E. A. Nigg. 1990b. Cell. 45:145–153).

To confirm and extend these studies using genetic techniques, we have investigated the role of p34cdc2 in lamin phosphorylation in the fission yeast. As fission yeast lamins have not been identified, we have introduced a cDNA encoding the chicken lamin B2 protein into fission yeast. We report here that the chicken lamin B2 protein expressed in fission yeast is assembled into a structure that associates with the nucleus during interphase and becomes dispersed throughout the cytoplasm when cells enter mitosis. Mitotic reorganization correlates with phosphorylation of the chicken lamin B2 protein by a mitosis-specific yeast lamin kinase with similarities to the mitotic lamin kinase of higher eukaryotes.

We show that a lamin kinase activity can be detected in cell-free yeast extracts and in p34cdc2 immunoprecipitates prepared from yeast cells arrested in mitosis. The fission yeast lamin kinase activity is temperature sensitive in extracts and immunoprecipitates prepared from strains bearing temperature-sensitive mutations in the cdc2 gene. These results in conjunction with the previously reported biochemical studies strongly suggest that disassembly of the nuclear lamina at mitosis in higher eukaryotic cells is a consequence of direct phosphorylation of nuclear lamins by p34cdc2.

The fission yeast, Schizosaccharomyces pombe, has been a useful experimental system for the study of the eukaryotic cell cycle because of numerous mutants deficient in cell cycle control that have been identified (for review see reference 22). A central controlling element is the protein kinase p34cdc2, activation of which is required for initiation of mitosis. Recent work has shown that cdc2 has been highly conserved throughout evolution and is required for initiation of mitosis in all eukaryotic cells (for review see reference 36). Entry into mitosis is characterized by profound changes in cellular organization (for review see reference 25), and it is important to determine how cdc2 activation causes these mitotic events.

It is likely that protein phosphorylation plays an important role in promoting mitotic changes in structure as entry into mitosis is accompanied by a general increase in protein phosphorylation (16, 21, 23, 28). One prominent mitotic substrate is the nuclear lamina, a structure composed of polymers of intermediate filament type proteins called lamins. During interphase the nuclear lamins are found in an insoluble matrix that underlies the inner nuclear membrane. During mitosis the nuclear lamina depolymerizes and lamins are either found free in the cytoplasm (A-type lamins) or as-
metaphase chick cells (40). We conclude that fission yeast contain a mitosis-specific lamin kinase similar to the lamin kinase of higher eukaryotes. The yeast lamin kinase activity can be detected in vitro in crude extracts and p34<sup>cd2</sup> immunoprecipitates prepared from yeast arrested in mitosis. The lamin kinase activity is temperature sensitive in extracts and p34<sup>cd2</sup> immunoprecipitates prepared from a temperature-sensitive cdc2 mutant. Thus, we proposed that the p34<sup>cd2</sup> kinase directly phosphorylates the chicken lamin B<sub>2</sub> expressed in yeast. As p34<sup>cd2</sup> controls entry into mitosis in all eukaryotic cells, it seems likely that such a mechanism determines lamina disassembly in higher eukaryotes as well, as has been proposed (40). Recent work has shown that p34<sup>cd2</sup> purified from starfish can directly phosphorylate lamins and induce disassembly of the nuclear lamina in isolated nuclei (40). Our results confirm and extend these findings, showing that mutation of cdc2 has a direct effect on lamin kinase activity.

We discuss the possibility that the nuclear structure we observe in fission yeast expressing the chicken lamin B<sub>2</sub> protein could form as a result of interactions between the chicken lamin B<sub>2</sub> protein and components of an endogenous yeast karyoskeleton. The mitotic reorganization of this structure indicates that the yeast nucleus may undergo a process analogous to mitotic disassembly of the nuclear lamina. This was unexpected, as the yeast nuclear membrane does not breakdown during mitosis. If yeast have a karyoskeleton, this type of reorganization may be required to accommodate the rapid changes in nuclear structure necessary for nuclear division. This would be consistent with a growing body of evidence that nuclear structures are fundamentally similar in higher and lower eukaryotes.

**Materials and Methods**

**Plasmids and Yeast Strains**

Media and standard yeast techniques were as described by Mitchison (27). The strain referred to as wild-type is 972h- (17). The plasmid pEVP-LamB2 (Fig. 1 A) was constructed using an Eco RI fragment containing a 2-kb cDNA encoding the chicken lamin B<sub>2</sub> protein (48). The ends of this fragment were filled in and subcloned into a filled-in Bam HI site in the plasmid pUC119 (42). This plasmid was introduced by transformation into leu<sup>−</sup>-strain (17) and isieucine prototrophs were selected. To isolate a strain in which the plasmid had integrated stably, leu<sup>+</sup> transformants were grown to saturation in yeast extract media, conditions that are nonselective for the leucine marker on the plasmid. This culture was plated on minimal plates lacking leucine, indicating that the leucine marker was stably inherited. DNA was prepared from this strain, and Southen blotted using the chicken lamin B<sub>2</sub> cDNA and a fragment of the adh promoter as probes. These experiments confirmed that the plasmid pEVP-LamB2 had integrated at the adh locus to give the structure shown in Fig. 1 B. This strain was designated LamB2.

To obtain chicken lamin B<sub>2</sub> expressing strains that could be arrested in mitosis, the strains nda3<sup>−</sup> LamB2 and cdc13<sup>−</sup> LamB2 were constructed. LamB2 was crossed to cdc13<sup>−</sup> leu<sup>−</sup>-32 (32, 37) or nda3<sup>−</sup> KM311 leu<sup>−</sup>-32 (47). As the leucine marker in LamB2 is closely linked to the chicken lamin B<sub>2</sub> coding sequences (Fig. 1 B), all leu<sup>−</sup> progeny of this cross must contain the chicken lamin B<sub>2</sub> gene. Thus, the progeny of the above crosses were screened for colonies that were leu<sup>+</sup> and either temperature sensitive (in the case of cdc13<sup>−</sup>) or cold sensitive (in the case of nda3<sup>−</sup>). The presence of the chicken lamin B<sub>2</sub> protein was confirmed by immunofluorescence and Western blotting (data not shown).

Figure 1. Expression of chicken nuclear lamin B2 in fission yeast. (A) Plasmid pEVP-LamB2 constructed to express chick nuclear lamin B2 in fission yeast. See Materials and Methods for complete description. (B) Organization of pEVP-LamB2 sequences after stable integration of the plasmid at the fission yeast adh locus. (C) Western blot using anti-chicken lamin B2 antibodies. Lane 1, whole cell extract from chick DU249 cells; lane 2, extract from yeast cells transformed with pEVP11; lane 3, extract from yeast cells transformed with pEVP-LamB2.

**Preparation of Protein Extracts, Immunoprecipitation, and Western Blotting**

Approximately 2 × 10<sup>7</sup> logarithmically growing yeast cells (O.D. <sub>395</sub> of 0.2–0.4) were pelleted by low-speed centrifugation, washed once with cold STOP buffer (0.9% NaCl, 5 mM Na<sub>2</sub>P<sub>2</sub>O<sub>4</sub>, 10 mM EDTA, 1 mM Na<sub>2</sub>HPO<sub>4</sub>), transferred to a 3-ml Falcon tube and then resuspended in 20 ml of HE buffer (50 mM Hepes, pH 7.9, 5 mM EDTA, 5 mM EGTA, 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 50 mM β-glycerophosphate, 1 mM DTT, 1 μg/ml leupeptin and pepstatin, 1 mM PMSF). 1 g of glass beads was added to the suspension, and the mixture was vortexed vigorously for 1 min. For Western blotting experiments, 1 ml of SDS-PAGE sample buffer was added and the sample was incubated at 80°C for 5 min. The buffer was collected and spun in an Eppendorf centrifuge (Brinkman Instruments Inc., Westbury, NY) for 10 min to pellet cel-
and the cells were incubated at 80°C for 5 min. The beads were washed with 1 ml RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1% Triton X-100) and then spun for 10 min in an Eppendorf microfuge to pellet debris. To analyze the extractability of the chicken lamin B2 protein, cells were lysed with glass beads as described above in 20 μl of HE buffer, and then incubated in either 1 ml of LS buffer (20 mM Tris, pH 8.0, 5 mM MgCl₂, 2.5 mM KC1, 1 mM PMSF) or 1 ml of HS buffer (LS + 1 M NaCl, 1% Triton X-100) for 20 min at room temperature on a rotary shaker. At the end of the incubation, the liquid was removed and spun for 15 min in an Eppendorf microfuge. SDS, DTT, and glycerol were added to the supernatant to a final concentration of 2%, 1 mM and 10%, respectively. These samples were designated "supernatant fractions." A volume of either LS or HS supplemented with SDS, DTT, and glycerol equal to the volume of the relevant supernatant was added to each pellet. These samples were designated "pellet fractions." All the samples were then incubated at 80°C for 5 min. The fractions containing HS were desalted by passing them through a Sephadex G-50 spin column equilibrated with LS + SDS, DTT, and glycerol. Just before electrophoresis bromophenol blue was added to each sample to a final concentration of 0.1%.

Western blots were done using Immobilon (Amersham International, Amersham, UK) according to the manufacturer's instructions. Blots were probed with cell culture supernatant from the monoclonal mouse cell line L4, which produces monoclonal antibody against chicken lamin B2 (20) and developed using alkaline phosphate-conjugated secondary antibody directed against mouse IgG (Sigma Chemical Co., Poole, England). Chicken lamin B2 immunoprecipitations were done using 1 μl of ascites fluid from the E-3 mouse anti–chicken lamin B2 monoclonal line (20) for every 1 ml of yeast extract prepared as described above. To precipitate the antigen–antibody complex the extract was incubated first with rabbit anti–mouse IgG (Sigma Chemical Co.) for 30 min and then adsorbed to protein A-Sepharose beads for 45 min. In vivo labeling with 32P-inorganic phosphate was done as described in Gould and Nurse (8). Phosphoamino acid analysis was done as described in Cooper et al. (3). Tryptic peptide–mapping experiments were performed as described in Peter et al. (40). 32P-labeled laminas from interphase and metaphase DU249 chicken cells were isolated as described in Peter et al. (40). Tissue culture and whole cell extract preparation of DU249 cells were carried out as described in Nakagawa et al. (31).

**Lamin Kinase Assays in Crude Mitotic Extracts**

Native yeast extracts for lamin kinase assays were prepared from strains containing nda2-KM311 mutation (14) which had been arrested in mitosis by incubation for 5 h at 20°C. High levels of p34cdc2 histone kinase activity can be detected in native extracts prepared from cells arrested in this manner and 50–60% of the cells have condensed chromosomes (29). To obtain a mitotic extract with a temperature-sensitive p34cdc2 histone H1 kinase (29), the strain nda2-KM311 cdc2-L7 was generated by crossing cdc2-L7 (37) and nda2-KM311 and selecting recombinants that were both cold and temperature sensitive. The double mutant was viable at temperatures between 29°C and 33°C. Extracts for kinase assays were prepared as described in Moreno et al. (29). Briefly, 2 × 10⁶ cells were washed in STOP buffer (see above), transferred to a Falcon tube (Falcon Labware, Oxnard, CA), resuspended in 20 μl of HB buffer (60 mM β-glycerophosphate, 15 mM p-nitrophenylphosphate, 25 mM MOPS, pH 7.2, 15 mM MgCl₂, 15 mM EGTA, 1 mM DTT, 0.1 mM sodium orthovanadate, 1 mM PMSF, 20 μg/ml leupeptin, pepstatin, and aprotinin), and lysed by vortexing with 2.5-μg acid–washed glass beads. The beads were washed with 1 ml HB buffer and the extract was centrifuged for 15 min in an Eppendorf microfuge (Brinkman Instruments, Inc.) to pellet debris.

Lamin substrate for in vitro phosphorylation was prepared using a bacterial strain containing the chicken lamin B2 cDNA subclone in the expression plasmid pR3038 (41). This plasmid and preparation of chicken lamin B2 protein will be described in more detail elsewhere (Heitlinger, E. M., Peter, M. Haener, A. Lustig, U. Aebi, and E. A. Nigg, manuscript submitted for publication). Briefly, chicken lamin B2 expression was induced by infection with bacteriophage λ CE6 as described in Rosenberg et al. (41). 2 h after induction the cells were harvested by centrifugation, resuspended in RIPA buffer, and lysed by sonication. The lysate was centrifuged for 10 min and chicken lamin B2 was immunoprecipitated from the supernatant as described above. The protein A-Sepharose beads from the immunoprecipitation were washed twice with RIPA + 20 mM NaF + 50 mM β-glycerophosphate, twice in HB buffer and then resuspended in 40 μl HB buffer and 5 μl yeast extract. As a control, immunoprecipitations using extracts from uninduced bacteria were treated in the same manner (data not shown). The beads and extract were preincubated for 10 min at either 25°C or 35.5°C. The kinase reaction was started by the addition of unlabeled ATP to a final concentration of 10 μM and 15–30 μCi of γ-32P-ATP (Amersham International). The reaction was incubated for 15 min and then stopped with 1 ml of RIPA buffer. The protein A-Sepharose beads were washed twice with RIPA buffer, once with PBS and then boiled in SDS-PAGE sample buffer before gel electrophoresis. In each experiment, histone H1 kinase activity was measured in parallel as described in Moreno et al. (31). Where indicated, bacterially synthesized p34cdc2 prepared as described (2, 29) was added to a final concentration of 0.5 μM.

**Lamin Kinase Assays in p34cdc2 Immunoprecipitates**

p34cdc2 was immunoprecipitated from crude yeast extracts prepared as described above using the previously described antiserum AB4711 (8) as described in Gould et al. (9). 1-ml extracts from 3 × 10⁶ cells were incubated with 5 μl of serum for 30 min, followed by incubation with protein A-Sepharose for 1 h. The protein A-Sepharose was then washed extensively with HB buffer (see above). For kinase assays as much residual HB buffer as possible was removed and the immunoprecipitates were preincubated at the desired temperature for 15 min. The reaction was started by the addition of 10 μl of lamin buffer containing 0.2 μg/ml solubilized bacterially produced chicken lamin B2, 150 mM NaCl, 1 mM DTT, 1 mM EGTA, 15 mM MgCl₂, 50 μM ATP and 10 micromolars γ-32P-ATP (Amersham International) and incubated for 15 min. Soluble chicken lamin B2 was prepared from bacterial extracts using a method described in detail elsewhere (Heit-
Expression of Chicken Lamin B2 in Fission Yeast

To express the chicken lamin B2 protein in fission yeast the plasmid pEVP-LamB2 (shown schematically in Fig. 1 A) was constructed (see Materials and Methods). The plasmid contains a cDNA encoding the chicken lamin B2 protein (48) under the control of the S. pombe adh promoter, sequences required for replication in bacteria and yeast and the S. cerevisiae LEU2 gene as a selectable marker (42). This plasmid was introduced into a leul-32 strain, and colonies capable of growing on minimal media were selected. Protein extracts from this strain were probed by immunoblotting with a monoclonal antibody against the chicken lamin B2 protein (20) and compared to protein isolated from cultured chick DU249 cells. As shown in Fig. 1 C, a protein the size of the chicken lamin B2 protein (lane I) can be detected in yeast transformed with the plasmid pEVP-LamB2 (lane 3). No such protein is detected in a strain transformed with a control plasmid (lane 2). Previously it has been observed that lamin B2 protein isolated from chicken cells migrates more rapidly than the primary translation product of the cDNA (48, 49). This is likely to be the result of isoprenylation, a posttranslational modification that appears to be required to target laminas to the nuclear envelope (Kitten and Nigg, unpublished data; 15, 18, 49). As the mobility of the chicken lamin B2 protein isolated from yeast is identical to that of the modified chicken protein, it seems likely that fission yeast can also modify laminas in this fashion.

To obtain a strain that expressed the chicken lamin B2 protein uniformly, the original transformants were screened for variants in which the plasmid had integrated stably into the yeast chromosomes (see Materials and Methods). Southern blot analysis of a stable variant showed that the plasmid had integrated at the adh locus to give the structure shown in Fig. 1 B (data not shown). As indicated, the chromosome in this strain has two tandem copies of the adh promoter, one of which remains linked to the S. pombe adh gene and a second copy that drives expression of the chicken lamin B2 cDNA. We will refer to this strain as LamB2.

Cellular Localization of Chicken Lamin B2 Expressed in Fission Yeast

To investigate the cellular localization of the chicken protein in fission yeast, LamB2 cells were fixed and processed for immunofluorescence (10), and probed with a monoclonal antibody against chick lamin B2 (20). To investigate the cellular localization of the chicken lamin B2 protein biochemically, cellular fractionation experiments were carried out. LamB2 cells were lysed and then extracted either with a low ionic strength buffer (Fig. 3, lanes 4 and 5), or a buffer containing 1% Triton X-100 and 1 M NaCl (Fig. 3, lanes 2 and 3). In addition, one sample was boiled in SDS-PAGE sample buffer to analyze total protein (Fig. 3, lane I). After a 20–30-min incubation, the extract was separated by centrifugation into soluble and insoluble fractions, each of which was boiled in SDS-PAGE sample buffer, and then fractionated by gel-electrophoresis. The gel was then electro-blotted and probed with a monoclonal antibody to the chicken lamin B2 protein. The bulk of the chicken lamin B2 protein remained in the insoluble fraction (Fig. 3, lane 2, high salt/detergent pellet; lane 4, low salt pellet) regardless of the extraction procedure. Only a small fraction of the chicken lamin B2 protein was solubilized as a result of either extraction procedure (Fig. 3, lane 3, high salt/detergent supernatant; lane 5, low salt supernatant). Thus, the chicken lamin B2 protein expressed in fission yeast appears to form a structure with solubility properties similar to those of the nuclear lamina of higher eukaryotes (6).

In higher eukaryotes the nuclear lamina disassembles as cells enter mitosis. To investigate whether the structure we observe in fission yeast undergoes a mitotic rearrangement we performed immunofluorescence studies on LamB2 cells arrested in mitosis. To arrest cells in mitosis we used the cold-sensitive mutant nda3-KM311 which blocks in mitosis because of a defect in the beta-tubulin protein (14). This strain was crossed with LamB2 (see Materials and Methods) to generate the strains nda3-LamB2. This strain was arrested in mitosis and the cellular location of the chicken lamin B2 protein was examined by indirect immunofluorescence as described above.

Figure 4. Redistribution of chicken lamin B2 during mitosis in fission yeast. Anti-chicken lamin B2 immunofluorescence and DAPI staining of nda3-LamB2 cells arrested in mitosis by incubation at 20°C for 5 h. (A and C) Anti-chicken lamin B2 immunofluorescence; (B) cells shown in A stained with DAPI; (D) cells shown in C stained with DAPI. (A and B) Show an average field of cells. Arrow a indicates a cell in which chicken lamin B2 remains associated with the nucleus; arrow b indicates a cell in which the chicken lamin B2 becomes widely distributed throughout the cytoplasm. C and D show a good example of two cells in which the chicken lamin B2 protein is distributed throughout the cytoplasm.

1. Abbreviation used in this paper: DAPI, diaminophenylindine.
In vivo phosphorylation of chicken lamin B2 expressed in fission yeast. (A) Chicken lamin B2 was immunoprecipitated from 32P-labeled yeast cells and analyzed using SDS-PAGE and autoradiography. Lane 1, wild-type cells. Lane 2, LamB2 cells. A phosphoprotein of the correct molecular weight is detected only in LamB2 (arrow). (B) Phosphorylated amino acids present in chicken lamin B2 protein expressed in fission yeast. 32P-labeled chicken lamin B2 was immunoprecipitated and fractionated by SDS-PAGE as described above. The chicken lamin B2 band was excised from the gel and subjected to acid hydrolysis. The resulting phosphoamino acids were separated by two dimensional electrophoresis. The positions of unlabeled standards as detected by ninhydrin staining are indicated (s, serine; t, threonine; y, tyrosine).

The results are shown in Fig. 4. Fig. 4 A and C show indirect immunofluorescence using anti-chicken lamin B2 antibodies; Fig. 4 B and D show the same cells stained with the DNA-binding dye DAPI. As can be seen in the majority of the cells the ringlike structure associated with the nucleus characteristic of interphase cells (Fig. 2) has disappeared and is replaced by much more diffuse, punctate staining. In many of the cells (the cell indicated as a in Fig. 2 A is an average example), some staining is still roughly associated with the nucleus although in about 20% of the cells (for example, cell b in Fig. 2 A) no clear nuclear structure can be observed. Fig. 2 C and D show two cells of the latter type; here it can be seen that the anti–chicken lamin B2 staining is punctate and dispersed throughout the cytoplasm.

In higher eukaryotes, disassembly of the nuclear lamina is also accompanied by an increase in extractability of the lamin protein. To see if an analogous change in extractability occurs in fission yeast we extracted LamB2 cells arrested in mitosis as described above. However, in contrast to the situation in higher eukaryotes, the bulk of the chicken lamin B2 protein remained insoluble even when the nuclear structure was dispersed as judged by immunofluorescence (data not shown). It is possible that we failed to observe solubilization of the lamins because complete disassembly of the nuclear structure only occurred in some of the cells. Alternatively, the cytological changes we observe may be due to a partial depolymerization of large lamina polymers, and filamentous fragments may remain entangled with cellular components. Indeed the cytoplasmic chicken lamin B2 we observed in mitotically arrested fission yeast is found in discrete blobs rather than being finely dispersed as might be expected if the lamina had been completely solubilized (Fig. 4 C). Incomplete solubilization may be due to substoichiometric phosphorylation of the chicken lamin B2 protein on some sites necessary for mitotic disassembly (see below).

A Cell Cycle–Regulated Lamin Kinase in Fission Yeast

In higher eukaryotes nuclear lamins are phosphorylated. Furthermore, at mitosis, the overall level of nuclear lamin phosphorylation increases, and phosphorylation on mitosis-specific residues is detected (6, 38, 40, 50). Phosphorylation on two of these sites has been shown to be required for mitotic disassembly of the nuclear lamina (13). To determine if dispersal of the structure we observed in fission yeast strains expressing chicken lamin B2 could be due to similar events, the phosphorylation state of the chicken protein was...
a 67-kD phosphoprotein in but not in wild-type cells (Fig. 5 A, lane 1). To determine the presence of inorganic 32P-phosphate under the same conditions. Cells were lysed in RIPA buffer as described in Gould and Nurse (8) and the chicken lamin B2 protein was immunoprecipitated from the indicated in vivo labeled samples, eluted from gels, and digested with trypsin. The resulting phosphopeptides were separated by electrophoresis and chromatography as described in reference 40. (A) cdc13-LamB2 cells arrested in mitosis after incubation at 35.5°C for 4 h; (B) chick DU-249 cells arrested in mitosis as described in reference 40; (C) asynchronous LamB2 cells cultured at 35.5°C as in (A); (D) interphase chick DU-249 cells. Mitosis-specific peptides detected in both yeast and chick cells are designated M1 and M2. An interphase phosphopeptide detected in both yeast and chick cells is designated 1.

We next investigated whether chicken lamin B2 phosphorylation increases during mitosis. Mitotically arrested cells were generated using the strains cdc13-LamB2, which arrests in mitosis at 36°C because of a defect in cyclin B (11), and nda3-LamB2 (see above). Both strains were arrested in mitosis by incubation at the restrictive temperature in the presence of inorganic 32P-phosphate. As controls, asynchronous cultures of LamB2 cells were incubated under the same conditions. Cells were lysed and 32P-labeled chicken lamin B2 protein was analyzed as described above for Fig. 5. The results are shown in Fig. 6. Cells arrested in mitosis using either the cdc13 mutation (Fig. 6, lane 2) or the nda3 mutation (Fig. 6, lane 4) contain higher levels of phosphorylated chicken lamin B2 than asynchronous control cultures incubated under the same conditions (Fig. 6, lane 1; LamB2 cells incubation as for cdc13 LamB2; Fig. 6, lane 3, LamB2 cells incubation as for nda3-LamB2 cells). Densitometric scanning of the autoradiogram reveals that the levels of phosphorylated chicken lamin B2 increased 4.2-fold in the cdc13-LamB2 and 3.8-fold in nda3-LamB2 relative to asynchronous cultures grown under the same conditions. Western-blotting experiments showed that the total amount of chicken lamin B2 protein was the same in mitotic and asynchronous cultures. Fig. 6, lanes 5 and 6 show the results of a typical experiment. Roughly equal quantities of chicken lamin B2 can be detected in LamB2 (Fig. 6, lane 5) and mitotically arrested nda3-LamB2 (Fig. 6, lane 6).

At present we have no information on the stoichiometry of phosphorylation of the chicken lamin B2 protein at mitosis. However we note that a band of slightly lower electrophoretic mobility increases in intensity in the mitotically arrested cells (indicated by small arrow). The decrease in electrophoretic mobility could be because of phosphorylation of a particular mitotic site on a fraction (roughly 10–20%) of the chicken lamin B2 protein. Substoichiometric phosphorylation of this site may explain why the interphasic structure is not completely dispersed, as judged by immunofluorescence, and why there is no increase in chicken lamin B2 solubility in cells arrested in mitosis (see above). We note that in these strains, chicken lamin B2 protein is expressed at comparatively high levels as it is under the control of the strong adh promoter. Such high levels of chicken lamin B2 may result in formation of structures in which not all of the chicken protein is accessible to the mitotic kinase.

To compare the lamin kinase in fission yeast to the lamin kinase of higher eukaryotes, we analyzed the sites of lamin phosphorylation in fission yeast and compared them to the sites of phosphorylation in cultured chicken DU249 cells. 32P-labeled chicken lamin B2 protein was prepared from mitotically arrested and asynchronous cultures as described above. The phosphorylated chicken lamin B2 protein was eluted from an SDS gel, digested with trypsin and the resulting peptides were separated in two dimensions by

Figure 7. Tryptic phosphopeptide analysis of chicken lamin B2 from interphase and mitotic chicken cells and interphase and mitotic fission yeast. 32P phosphorylated chicken lamin B2 was immunoprecipitated from the indicated in vivo labeled samples, eluted from gels, and digested with trypsin. The resulting phosphopeptides were separated by electrophoresis and chromatography as described in reference 40. (A) cdc13-LamB2 cells arrested in mitosis after incubation at 35.5°C for 4 h; (B) chick DU-249 cells arrested in mitosis as described in reference 40; (C) asynchronous LamB2 cells cultured at 35.5°C as in (A); (D) interphase chick DU-249 cells. Mitosis-specific peptides detected in both yeast and chick cells are designated M1 and M2. An interphase phosphopeptide detected in both yeast and chick cells is designated 1.

investigated. LamB2 and wild-type cells were grown in the presence of inorganic 32P-phosphate under the same conditions. Cells were lysed in RIPA buffer as described in Gould and Nurse (8) and the chicken lamin B2 protein was immunoprecipitated using a monoclonal antibody directed against chicken lamin B2 (20), and analyzed by SDS-PAGE and autoradiography. As shown in Fig. 5 A, this procedure detects a 67-kD phosphoprotein in LamB2 cells (Fig. 5 A, lane 2) but not in wild-type cells (Fig. 5 A, lane 1). To determine which amino acids were phosphorylated, the 32P-labeled chicken lamin B2 protein was eluted from the gel and then hydrolyzed in 6 N HCl. The resulting phosphoamino acids were then separated by two-dimensional electrophoresis (3). As shown in Fig. 5 B the chicken lamin B2 protein expressed in fission yeast was phosphorylated mainly on serine, although some phosphothreonine was also detected. No phosphotyrosine was observed. A similar pattern of amino acid phosphorylation has been reported for lamin B1 in metaphase chicken cells (40).
electrophoresis and thin-layer chromatography. $^{32}$P-labeled 
lamin B$_2$ protein prepared from either mitotically arrested 
or asynchronous cultures of chick DU249 cells were ana-
alyzed in parallel. The results of this analysis are shown in 
Fig. 7. In mitotically arrested fission yeast cells (Fig. 7 A) 
a peptide which is designated M1 is phosphorylated. A sec-
ond peptide designated M2 is also phosphorylated to a lesser 
extent. These same two spots are also detected in $^{32}$P-
labeled chicken lamin B$_2$ from mitotically arrested chicken 
cells (Fig. 7 B). Mixing experiments were done to confirm 
that the fission yeast and chicken phosphopeptides comigrate 
(data not shown). The serine phosphorylated in the M1 pep-
tide has been identified as Ser-16 (40) and mutation of the 
equivalent serine in the human lamin A protein is able to 
block mitotic disassembly of the nuclear lamina (13). In 
avynchronous fission yeast cells phosphorylation of the M1 
and M2 peptides decreases substantially and a third phos-
phopeptide, designated I, is detected (Fig. 7 C). This resem-
bles the changes in lamin phosphorylation observed in asy-
chronously growing cultured chicken cells (Fig. 7 D). Here 
also the levels of M1 and M2 peptide phosphorylation de-
crease, while phosphorylation of peptide I increases. Other 
peptides labeled to a lesser extent were also seen to be in 
common between yeast and chicken (compare Fig. 7, A and 
B, Fig. 7, C and D). These experiments show that the mitotic 
increase in phosphorylation of the chicken lamin B$_2$ protein 
expressed in fission yeast is due to qualitative and quantita-
tive changes in lamin kinase activity. Moreover, in both mi-
totic and interphase fission yeast cells, the sites of phos-
phorylation are a subset of those phosphorylated in chicken 
cells suggesting that the fission yeast kinases are related to 
the chick kinases.

**Phosphorylation of Chicken Lamin B$_2$ by p34<sup>cdc2</sup> 
Kinase In Vitro**

The above results demonstrate that fission yeast have a lamin 
kinase, indicating that this activity is evolutionarily con-
served. Recent studies have shown that the cell cycle control 
protein p34<sup>cdc2</sup>, a protein serine-threonine kinase that is 
activated at mitosis, is found in all eukaryotic cells (for review 
see reference 36) and biochemical studies have shown that 
p34<sup>cdc2</sup> purified from starfish can phosphorylate nuclear la-
mains and induce lamina disassembly when incubated with 
isolated chick nuclei (40). It therefore seemed possible that 
the mitotic lamin kinase activity we detect in fission yeast 
could be p34<sup>cdc2</sup>. To investigate this possibility, we took ad-
vantage of an in vitro assay for fission yeast p34<sup>cdc2</sup> protein 
kinase (29). In this assay p34<sup>cdc2</sup> protein kinase can be 
detected in crude yeast extracts by virtue of its ability to phos-
phorylate exogenous H$_1$ histone. The activity can be shown 
to be because of p34<sup>cdc2</sup> and not to other kinases because it 
is temperature sensitive in extracts prepared from <i>cdc2</i>$^+$ 
strains. Moreover, the temperature sensitivity of the activity 
in mutant extracts can be rescued by the addition of purified 
p13<sup>mut</sup> (29). p13<sup>mut</sup> is the product of the <i>sccl</i> gene and 
genetic and biochemical evidence suggests that it interacts 
physically with p34<sup>cdc2</sup> (2, 12).

To investigate the role of p34<sup>cdc2</sup> in chicken lamin B$_2$ 
phosphorylation we used the above assay system except that 
bacterially produced chicken lamin B$_2$ was added to the ex-
tracts instead of histone H$_1$. Extracts were prepared from mi-
totically arrested fission yeast cells which either had a wild-
type <i>cdc2</i> gene or a mutation known to result in temperature 
sensitivity of the <i>cdc2</i> kinase (see Materials and Methods). 
Substrate was prepared from extracts of <i>Escherichia coli</i> 
strains expressing the chicken lamin B$_2$ proteins. <i>E. coli</i> 
extracts were incubated first with the monoclonal antibody 
against chicken lamin B$_2$ and then with protein A con-
jugated to Sepharose beads (see Materials and Methods). 
The beads were then washed and incubated with extracts pre-
pared from various yeast strains in the presence of $^{32}$P-
ATP for 15-30 min at either 25 or 36°C. At the end of the 
incubation period, the beads were washed, boiled in SDS-
PAGE sample buffer, and then the $^{32}$P-labeled proteins were 
analyzed by gel electrophoresis and autoradiography (Fig. 8 
A). Histone H$_1$ kinase assays of the same extracts were car-
ried out in parallel (Fig. 8 B).

When extracts from yeast carrying a wild-type <i>cdc2</i> gene 
were used, phosphorylation of a 67-kd protein was detected 
(Fig. 8 A, lanes 1 and 2). This phosphoprotein was absent 
when the assay was done using extracts from bacteria that 
didn't express chicken lamin B$_2$ (data not shown). The level 
of kinase activity was roughly the same whether the assay 
was carried out at 25°C (Fig. 8 A, lane 1) or 36°C (Fig. 8 A, 
lane 2). However in strains bearing a temperature-sensitive 
mutation in the <i>cdc2</i> gene, lamin kinase activity at 36°C 
(Fig. 8 A, lane 4) was significantly lower than lamin kinase 
activity at 25°C (Fig. 8 B, lane 3). However if this extract 
was supplemented with bacterially produced p13<sup>mut</sup>, 
kinase activity at 36°C was rescued (Fig. 8 A, lane 5). As has previ-
ously been shown (29), histone H$_1$ kinase activity in these 
extracts showed a similar pattern of temperature sensitivity 
and rescue by p13<sup>mut</sup> (Fig. 8 B, lanes 3–5). Tryptic peptide 
mapping of the in vitro phosphorylated chicken lamin B$_2$ 
protein revealed that both the M1 and M2 sites were phos-
phorylated (data not shown).

Based on these results we conclude that the mitotic lamin 
kinase we detect in fission yeast is either p34<sup>cdc2</sup> itself or a 
kinase that is very directly dependent on p34<sup>cdc2</sup> for activity. 
To investigate this possibility further we immunoprecipitated 
p34<sup>cdc2</sup> from mutant and wild-type extracts and then assayed 
the immunoprecipitates for lamin-kinase activity. As shown 
in Fig. 8 C, high levels of lamin kinase activity could be de-
tected in immunoprecipitates from wild-type cells (Fig. 8 C, 
lane 4). This activity was reduced over sevenfold in im-
munoprecipitates of extracts containing a temperature sensi-
tive <i>cdc2</i> protein (Fig. 8 C, lane 3). Both immunoprecipiti-
tes contained equal amounts of p34<sup>cdc2</sup> (data not shown). 
Lamin-specific kinase activity was not detected in either ex-
tract when immunoprecipitates were made using preimmune 
sera (Fig. 8 C, lanes 1 and 2). From these experiments we 
conclude that p34<sup>cdc2</sup> is likely to be directly responsible 
for mitotic phosphorylation of the chicken lamin B$_2$ protein.

**Discussion**

**The Role of p34<sup>cdc2</sup> in Mitotic Events**

The protein kinase p34<sup>cdc2</sup> controls entry into mitosis in all 
eukaryotic cells. Entry into mitosis correlates with the ap-
pearance of many new phosphoproteins (16, 21, 23). By anal-
ogy with control of other cellular processes by protein phos-
phorylation, p34<sup>cdc2</sup> could initiate mitosis by activating a
p34°d¢2 is directly responsible for phosphorylation of chicken lamin B2 in vitro. (A and B) Mitotic extracts from strains with wild-type or temperature sensitive cdc2 proteins were assayed for lamin kinase activity (A) or H1 histone kinase activity (B). For lamin kinase assays bacterially produced chicken lamin B2 was immunoprecipitated using protein A-Sepharose beads, and the beads were incubated with yeast extracts at the indicated temperatures in the presence or absence of bacterially produced pl 3°w¢1. At the end of the incubation the beads were washed, boiled in SDS-PAGE sample buffer and phosphorylation of chicken lamin B2 was analyzed by SDS-PAGE and autoradiography. H1 histone kinase assays were performed as described in reference 29. The arrow in panel A indicates the position of phosphorylated chicken lamin B2. The arrowhead in B indicates the position of phosphorylated Histone H1. Lane 1, wild-type extract assayed at 25°C; lane 2, wild-type extract assayed at 35.5°C; lane 3, cdc2° extract assayed at 25°C; lane 4, cdc2° extract assayed at 35.5°C; lane 5, cdc2° extract assayed at 36°C in the presence of 0.5 µM pl 3°w¢1. (C) Lamin kinase assays of immunoprecipitates using preimmune and anti-p34°d¢2 antiserum. Lane 1, preimmune control, cdc2° extract; lane 2, preimmune control, wild-type extract; lane 3, p34°d¢2 immunoprecipitate from a cdc2° extract; lane 4, p34°d¢2 immunoprecipitate from a wild-type extract. Phosphorylated chicken lamin B2 is indicated by an arrowhead. All assays were performed at 36°C.

cascade of secondary kinases which would in turn phosphorylate mitotic substrates. Alternatively, p34°d¢2 could initiate mitosis by directly phosphorylating key mitotic substrates.

We have investigated the role of p34°d¢2 in mitotic protein phosphorylation by expressing a well-characterized mitotic substrate, chicken nuclear lamin B2, in fission yeast. We have used the resulting strains to show that fission yeast contain a mitosis-specific lamin kinase that phosphorylates chicken lamin B2 proteins on the same sites as a lamin kinase detected in mitotic chick cells. Moreover, phosphorylation by the fission yeast kinase correlates with a striking change in the intracellular distribution of the chicken lamin B2 at mitosis. We conclude that fission yeast contain a mitosis-specific lamin kinase that resembles the lamin kinase of higher eukaryotes.

The fission yeast lamin kinase can be detected in vitro in crude extracts prepared from mitotically arrested cells. We find that the kinase activity is temperature sensitive in extracts prepared from yeast strains having mutations that render the p34°d¢2 protein kinase inactive at 36°C in vitro (29). Moreover, the temperature sensitivity is rescued by addition of pl 3°w¢1, the protein product of sucl, a gene that suppresses the temperature sensitivity of cdc2° mutants in vivo (12) and of the kinase activity in vitro (29) in an allele-specific manner. These results indicate that p34°d¢2 is likely to be the fission yeast lamin kinase. It is formally possible that we are detecting a distinct kinase which is being dynamically maintained in an active state by p34°d¢2 in the extracts. However, this seems unlikely for a number of reasons. Firstly, the extract is prepared from mitotically arrested cells. Thus, a downstream kinase component of a p34°d¢2-dependent cascade should already be active, and indeed lamin kinase activity can be detected when the extracts are
assayed at 25°C. The extract is prepared using a buffer that contains phosphatase inhibitors which should prevent inactivation of the kinase by dephosphorylation. Secondly, as the extract is substantially more dilute than the cytoplasm of intact cells, the protein concentrations are probably not high enough to reconstitute a p34\textsuperscript{cdc2}-dependent kinase cascade. Finally, a lamin kinase activity that is temperature sensitive in cdc2 mutants can be specifically immunoprecipitated using anti-p34\textsuperscript{cdc2} antibodies.

As the fission yeast kinase resembles the kinase of higher eukaryotes, we propose that p34\textsuperscript{cdc2} is also responsible for nuclear lamin phosphorylation in higher eukaryotes. This confirms the recent finding that highly purified p34\textsuperscript{cdc2} prepared from starfish can directly phosphorylate chicken nuclear lamin and is sufficient to induce nuclear lamina disassembly when incubated with chick nuclei in vitro (40). The genetic approach we have used complements and extends this earlier study because we have shown that mutations that alter the cdc2 protein directly affect lamin kinase activity. This excludes the possibility that the previously obtained results were due to an unrelated kinase in the chick nuclei or a contaminant in the starfish p34\textsuperscript{cdc2} kinase preparation. Conversely, our present results do not rigorously exclude the possibility that a distinct kinase that is part of a p34\textsuperscript{cdc2} complex is responsible for lamin phosphorylation. However, the previous studies with purified starfish p34\textsuperscript{cdc2} suggest that this explanation is unlikely as lamin kinase activity was detected in a purified complex shown to consist of only two major components, p34\textsuperscript{cdc2} and cyclin B (19).

Purified starfish p34\textsuperscript{cdc2} also appears to directly phosphorylate the nucleolar proteins nucleolin and NO38 (Ib,39). Thus, it is plausible that p34\textsuperscript{cdc2} induces at least some mitotic events by directly phosphorylating key structural proteins rather than by triggering a cascade of secondary kinase reactions (for review and discussion see references 28 and 40).

A Nuclear Lamina-like Structure in Fission Yeast?

Immunofluorescence of fission yeast cells expressing chicken lamin B\textsubscript{I} revealed that the protein is found in a ringlike structure associated with the fission yeast nucleus. Fractionation studies show that this structure is resistant to extraction with detergent and high salts. Thus chicken lamin B\textsubscript{I} protein expressed in yeast appears to be in a structure with some similarities to the nuclear lamina of higher eukaryotes. We suggest that the chicken protein may be interacting with endogenous yeast proteins and becoming incorporated into a yeast structure analogous to the nuclear lamina of higher eukaryotes. In support of this possibility, lamin modifying activities similar to those found in higher eukaryotes were also detected in fission yeast. These include a kinase that phosphorylates chicken lamin B\textsubscript{I} during interphase (Fig. 7) and an activity, most likely a farnesyltransferase, that increases the electrophoretic mobility of chicken lamin B\textsubscript{I}, (Fig. 1), as well as the mitotic lamin kinase p34\textsuperscript{cdc2}. As yet neither nuclear lamins or intermediate filament proteins have been identified in fission yeast. However, budding yeast have proteins immunologically related to nuclear lamins, and these proteins can interact with vertebrate nuclear lamina components in vitro (5). If similar interactions can occur in vivo, the structure we observe in LamB2 may be the fission yeast nuclear lamina.

Alternatively, the chicken lamin B\textsubscript{I} protein by itself may be sufficient for assembly of a nuclear lamina. However, we do not detect any novel structures in the LamB2 strain by light microscopy, and the strain is able to grow and divide at normal rates. One might expect a nonphysiological insoluble structure to have a more noticeable effect on cell morphology or viability. We also note that we have no direct evidence that the chicken lamin B\textsubscript{I} protein is intranuclear; the immunofluorescence data are also consistent with it being found in a cytoplasmic structure that forms around the nucleus during fixation. Excluding this possibility will require detailed studies of the LamB2 strain using ultrastructural techniques. Clear proof that yeast contain lamins ultimately requires identification of fission yeast genes that could encode intermediate filament or nuclear lamin proteins.

The existence of a nuclear lamina-like structure in fission yeast would be compatible with a number of studies showing that lower eukaryotes contain structures resembling the karyoskeleton of higher eukaryotes. As already noted, the budding yeast, S. cerevisiae, contains proteins immunologically related to nuclear lamins (5), and to nuclear pore components (4, 33). In addition, a structure that resembles the higher eukaryotic nuclear matrix and may interact with chromatin in an analogous manner has been identified (I.a). Also, the budding yeast protein REP2, which plays a role in segregation of the 2-\textmu m circle, has some similarity to intermediate filament proteins and is found associated with an insoluble nuclear fraction (51).

The nuclear-associated structure we detect in the LamB2 strain is reorganized during mitosis when chicken lamin B\textsubscript{I} protein is phosphorylated by p34\textsuperscript{cdc2}, becoming dispersed throughout the cytoplasm. This is surprising as fission yeast mitosis takes place in the absence of nuclear membrane breakdown. Dispersal could be explained if the chicken protein is extranuclear (see above) and is held in place by components of the cytoskeleton that reorganize at mitosis. Investigating this possibility will require ultrastructural studies of the mitotic and interphase structures that we have observed. However, it is worth considering the possibility that a correctly located lamina-like structure may be able to disassemble in the absence of nuclear envelope breakdown. Pachytene meiotic cells have been shown to lack a nuclear lamina although the nuclear membrane is still intact (43) and lamina disassembly in the absence of nuclear envelope breakdown has also been observed in vitro in nuclei added to cell-free extracts that support mitosis (34, 40). Possibly the nuclear membrane can be disrupted sufficiently to allow lamina disassembly without completely breaking down. Partially disassembled "fenestrated" nuclear membranes are a feature of mitosis in some fungi (52) and have also been observed in mitotic cells of the thymus (30). Perhaps the mitotic nuclear membrane of fission yeast contains "gaps" which are too small or infrequent to have been detected in previous ultrastructural studies (24, 45) but which still permit disassembly of the yeast karyoskeleton. Disassembly of a karyoskeleton may be necessary to accommodate the rapid distortion of nuclear shape that takes place as the nucleus extends along the length of the cell and then divides in two (24, 45, 46). The contrasting modes of mitosis in yeast and animal cells could be points along a continuum of degrees of nuclear envelope breakdown, rather than mechanistically distinct pro-
cesses. It may be possible to use fission yeast strains expressing vertebrate lamins to explore this possibility further.

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Note Added in Proof: Similarity between nuclear lamins and another S. cerevisiae protein, the product of the SIR4 gene, has recently been reported (Diffley, J. F. X., and B. Stillman. 1990. Nature [Lond.]. 342:24).

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