S-Phase phosphorylation of lamin B2

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Abstract   Lamin B2 modification in synchronously dividing populations of human diploid fibroblasts was determined by 2-dimensional gel electrophoresis and [^{32}P]orthophosphate labelling. In quiescent (G_0) and G_1 cultures of HDF, lamin B_2 migrated as 2 spots on 2-dimensional gels. In contrast, in S-phase populations of HDF lamin B_2 migrated as a single basic species. The level of lamin B_2 phosphorylation was determined after immunoisolation from [^{32}P]orthophosphate labelled cells. The results of these experiments indicated a 2–3-fold increase in the steady state level of lamin B_2 phosphorylation in S-phase HDF compared with G_0 HDF. Consistent with this evidence, tryptic peptide maps revealed the presence of a phosphopeptide in S-phase lamin B_2 which was absent from G_0 lamin B_2. Since all of the phosphate incorporated into S-phase and G_0 lamin B_2 was recovered in serine residues we conclude that the S-phase specific phosphopeptide did not represent either of the cdc2 sites associated with entry nuclear lamina breakdown.

Key words: Human fibroblast; Cell cycle; Lamin phosphorylation

1. Introduction

The eukaryotic cell nucleus is a well-ordered structure which provides a permissive environment for DNA replication [1,2]. Several elements of the nucleus are thought to be important for DNA replication, including the nuclear envelope [3,4], the nucleoskeleton [5,6] and replication factories [7]. Although little is known about the nucleoskeleton and replication factories, significant information has recently emerged concerning the nuclear envelope. The nuclear envelope comprises a double unit membrane which is periodically interrupted by nuclear pores [8]. Underlying the nuclear membranes is a filamentous substructure termed the nuclear lamina. The nuclear lamina is assembled from type V intermediate filament proteins or lamins [9,10]. The lamins are presently classified into two sub-families termed A-type and B-type. This classification is based on amino acid sequence homologies, post-translational modification and behaviour at mitosis [11]. Of these sub-families, B-type lamins appear to be required as functional components of all cells [12,13] whereas A-type lamins are thought to be components of differentiated cells [14].

The nuclear lamina appears to perform several functions. A small amount of lamin is required for nuclear envelope re-assembly at telophase [15]. Moreover, the lamina provides structural support for the nuclear envelope during interphase [16,17]. It has also been proposed that the lamina is involved in the organisation of chromatin looped domains [11]. Con-

sistent with this view is the observation that lamina assembly in sperm pronuclei is essential for the initiation of DNA replication [18,19]. This observation has led us to propose that the lamina is the key component of the nuclear envelope which is required for DNA replication [20].

Despite this evidence it is not clear how lamins influence DNA replication. In indirect immunofluorescence studies the lamina appears to be situated beneath the nuclear envelope while replication factories are distributed throughout the nucleoplasm [21,22]. Thus most sites of DNA replication are not in contact with the lamina. However, recent studies have shown that, during S-phase, B-type lamins re-distribute from the nuclear envelope to active sites of DNA replication [23] indicating a direct role for lamins in DNA synthesis.

As with other components of the nuclear envelope, the nuclear lamina is broken down and re-assembled during mitosis [8]. Lamina disassembly and re-assembly at mitosis is regulated by phosphorylation/dephosphorylation [24,25,26]. For complete disassembly of the lamina at mitosis, cdc2 consensus sites adjacent to the amino-terminal and carboxyl-terminal ends of the central rod domains must be phosphorylated [27,28,29]. However, interphase phosphorylation sites have also been identified. In particular PKC sites are phosphorylated in lamins following mitogenic stimulation of quiescent mammalian cells in culture [30]. One function of PKC phosphorylation is to regulate lamin uptake into the nucleus [31]. Therefore it seems likely that PKC phosphorylation may influence lamin dynamics during interphase. Since B-type lamins re-distribute from the nuclear envelope to replication factories during S-phase we wished to determine whether specific phosphorylation events were correlated with this process. Using 2-dimensional gel electrophoresis and tryptic peptide mapping we demonstrate that human lamin B_2 becomes phosphorylated at a unique site as human diploid fibroblasts enter S-phase, after re-stimulation from a quiescent state.

2. Experimental

2.1. Cell culture

Human dermal fibroblasts (HDF), strain 2DD, passages 4–8, were prepared from a juvenile foreskin and have been described previously [32]. Cells were seeded onto 90 mm plastic tissue culture dishes at a density of 3×10^3 cm^{-2}, and were grown in Dulbecco's modification of Eagle's medium (DMEM, Gibco) supplemented with 10% (v/v) newborn calf serum (NCS) and antibiotics (10 units·ml^{-1} penicillin and 50 μg·ml^{-1} streptomycin). 76 h after seeding cells were made quiescent by washing twice in serum-free DMEM then re-feeding with DMEM supplemented with 0.5% (v/v) NCS and antibiotics for 7 days. Synchronised populations of cells were obtained by re-feeding quiescent cultures with DMEM supplemented with 10% (v/v) NCS and antibiotics and cells were harvested at appropriate times following re-stimulation. Re-stimulated cells were arrested in S-phase by addition of 1 mM hydroxyurea (HU) to the culture medium 8 h after serum re-stimulation and then used in the appropriate analysis at 24 h.

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2.2. Indirect immunofluorescence

Cells grown on coverslips were washed with phosphate buffered saline (PBS) and fixed by treatment with methanol/acetone (1:1, v/v) for 4 min at 4°C. Cells were then incubated with Ki-67 antibodies (diluted 1:30, Dako) at room temperature for 1 h in an humidified atmosphere. After washing with PBS the cells were incubated with rhodamine-conjugated rabbit anti-mouse Ig (diluted 1:30, Dako) at room temperature for 1 h in an humidified atmosphere. The cells were then washed with PBS and mounted onto glass microscope slides using 30% glycerol (v/v) in 10 mM Tris-HCl (pH 8.0) containing 12% (v/v) Mowiol (Hoechst), 1 μg·ml^{-1} 4,6-diamidino-2-phenylindole (DAPI), and 2.5% 1,4-diazobicyclo-[2.2.2]-octane (DABCO; Sigma). Slides were viewed with a Zeiss Axioskop using a 100× oil immersion objective. The proportion of stained cells was determined by counting 1000 total or 200 positive nuclei in randomly selected fields.

2.3. Detection of DNA synthesis

Cells on coverslips were incubated with 25 μM 5-bromodeoxyuridine (BrdUrd) in the presence of 2 μM fluorodeoxyuridine to inhibit thymidilate synthetase [33], for the indicated times. Cells were washed with PBS, then fixed with methanol/acetone (1:1, v/v) at 4°C for 4 min and washed with PBS. Cells were then treated with 1.5 N HCl for 30 min at room temperature to denature DNA, then incubated with MAS 250b (anti-BrdUrd antibodies diluted 1:10, Sera Labs) and incubated for 1 h at room temperature in an humidified atmosphere. After washing with PBS, cells were incubated with an FITC-conjugated rabbit anti-rat antibody (1:30, Dako) for 1 h as before. After washing, coverslips were mounted onto glass slides, and scored as described above.

2.4. Two-dimensional Western blotting

Cells on tissue culture dishes were rinsed three times with Tris-buffered saline (TBS; 25 mM Tris-HCl, 0.8% (w/v) NaCl, 0.02% (w/v) KCl, pH 7.5) and once with 10 mM Tris-HCl then scraped directly into sample buffer (final concentration = 4.5M Urea, 1% (v/v) Triton X-100, 2.5% (v/v) β-mercaptoethanol, 0.8% (v/v) 5–7 ampholines, 0.2% 3–10 ampholines). Isoelectric focusing was carried out according to standard protocols [34] using a BioRad mini 2D electrophoresis kit. Proteins were transferred to nitrocellulose membranes using a BioRad Mini-Trans Blot apparatus for 1 h. Membranes were probed with LN43.2 antibodies (anti-lamin B2 diluted 1:1000, a kind gift from Prof. Birgit Lane, University of Dundee). Bound antibodies were visualised using alkaline phosphatase-conjugated goat anti-mouse and developed in 25 mM Tris-HCl, pH 9.4, containing 250 μg·ml^{-1} 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and 500 μg·ml^{-1} p-nitroblue tetrazolium chloride (NBT).

2.5. In situ isotopic labelling of cells in culture

Cultures were rinsed twice with phosphate-free DMEM containing 0.5% (v/v) dialysed NCS (for quiescent cultures) or phosphate-free DMEM containing 10% (v/v) dialysed NCS (for re-stimulated cultures). Each 90 mm dish containing approximately 10^6 cells was then incubated with 2.5 ml phosphate-free DMEM (containing either 0.5% or 10% (v/v) dialysed NCS as appropriate) containing 1 mCi·ml^{-1} ^{32}P$_i$ for 4 h at 37°C in an humidified CO$_2$ incubator.

2.6. Immunoisolation of lamin B2

Cells in culture were solubilised according to Ward and Kirschner [35]. Dynabeads (M280, Dynal) are superparamagnetic polystyrene beads linked to sheep anti-mouse IgG. Dynabeads have been used previously to immunodeplete lamin B3 from Xenopus egg extracts [19]. LN43.2 antibodies were bound to Dynabeads and incubated with cell extracts overnight at 4°C. After extensive washing in TBS, lamin B2 bound to LN43.2-coated Dynabeads was solubilised in SDS-sample buffer and subjected to SDS-PAGE through 8% gels. For 1-dimensional Western blotting, proteins were transferred to nitrocellulose membranes and probed using LN43.2 antibodies or with L6 8A7 antibodies (anti A-type lamins diluted 1:500; a kind gift from Dr. Reimer Stick, University of Goettingen) as described for 2-dimensional Western blotting.

2.7. Two-dimensional tryptic phosphopeptide mapping

^{32}P-Labelled lamin B2 immunoisolated from solubilised cell extracts was subjected to SDS-PAGE through 8% gels. Gels were stained with Coomassie blue, photographed and dried down under vacuum. To

detect labelled phosphoprotein bands, the dried gels were autoradiographed using pre-flashed X-ray film. Immunoisolated ^{32}P-labelled lamin B bands were excised from the dried gels and prepared for trypsinisation. Trypsinisation of samples was achieved by four successive incubations with 10 μl of a 1 mg·ml^{-1} solution of trypsin at 37°C for 30 min each. Dried peptides were re-suspended in 5 μl of pH 1.9 buffer, spotted onto TLC plates and subjected to electrophoresis towards the anode in pH 1.9 buffer for 90 min at 500 V. Following electrophoresis, the peptides were subjected to ascending chromatography using n-butanol/pyridine/acetic acid/H$_2$O (75:50:15:60) as solvent. ^{32}P-Labelled peptides were visualised by autoradiography against pre-flashed film.

For phospho-amino acid analysis, ^{32}P-labelled samples of immunoisolated lamin B2 were precipitated with TCA then dissolved in 6 N HCl and heated at 110°C for 1 h. Following lyophilisation, the sample was re-suspended in pH 1.9 buffer containing 15 parts buffer to 1 part each of non-radioactive phosphoamino acids (1 mg·ml^{-1} each of phosphoserine, phosphothreonine and phosphotyrosine) and spotted onto TLC plates. The samples were subjected to electrophoresis in the first dimension for 1 h at 500 V in pH 1.9 buffer, then in the second dimension for 1 h at 500 V in pH 3.5 buffer (acetic acid/pyridine/H$_2$O; 50:5:945). Unlabelled phosphoamino acids were visualised using 0.2% (w/v) ninhydrin in acetone and labelled phosphoamino acids were visualised by autoradiography against pre-flashed film.

2.8. Quantification of gels, immunoblots and autoradiographs

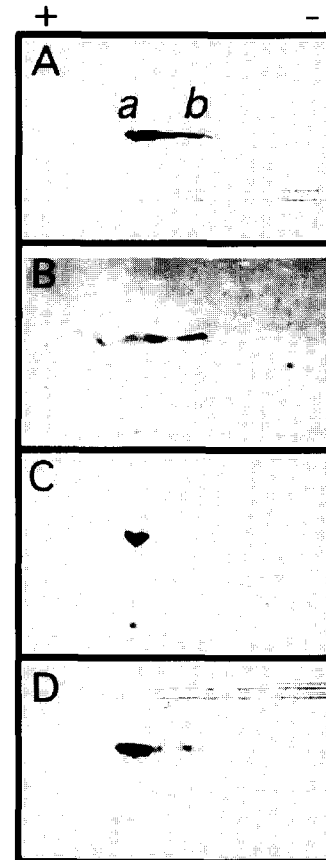Digitised images of gels, blots or autoradiographs were obtained

Fig. 1. 2-Dimensional Western blot analysis of lamin B. Quiescent HDF (A); quiescent HDF re-stimulated with serum for 18 h (B) or 24 h (C); quiescent HDF re-stimulated with serum for 24 h in the presence of hydroxyurea (D). Proteins were solubilised in 2D sample buffer and separated by IEF in the first dimension and by SDS-PAGE in the second dimension. Proteins were transferred to nitrocellulose membranes and probed with LN43.2 (anti-lamin B), then alkaline phosphatase-conjugated rabbit anti-mouse antibodies. Secondary antibodies were visualised with BCIP and NBT. 'a' and 'b' indicate acidic and basic species, respectively.
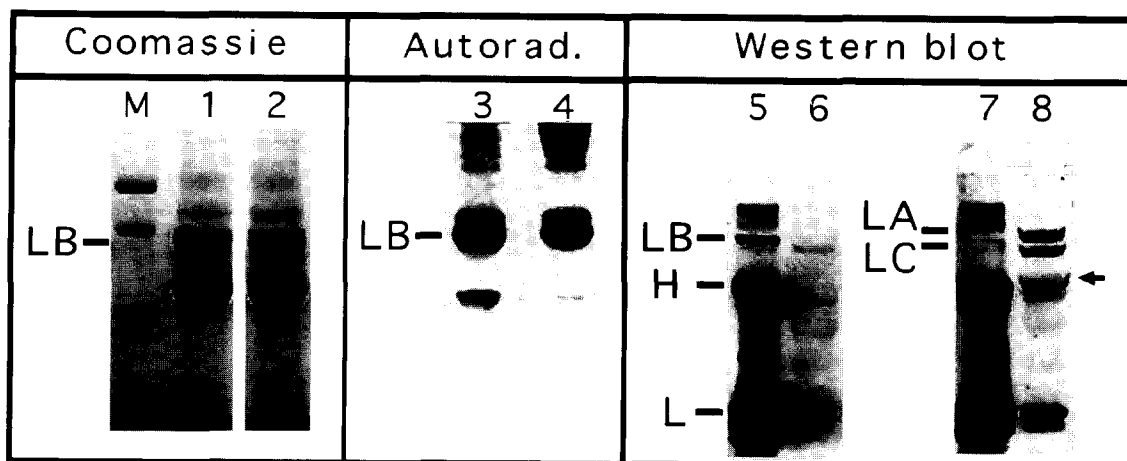
Fig. 2. Analysis of lamin B immunoisolated from solubilised samples of HDF. Cultures of quiescent HDF (tracks 1 and 3) or S-phase HDF (tracks 2 and 4) were incubated in the presence of [^{32}P]phosphate for 4 h then prepared for immunoisolation using LN43.2-linked Dynabeads. Dynabead fractions were separated by electrophoresis through 8% gels, stained with Coomassie blue, photographed and then dried under vacuum. Dried gels were subjected to autoradiography against pre-flashed X-ray film. LB indicates the position of lamin B. The markers shown (lane m) are 106, 80, 49.5 and 32.5 kDa. For 1-dimensional Western blotting, cultures of HDF were solubilised and incubated with Dynabeads linked to anti-lamin B antibodies. The Dynabead fraction, which included LN43.2 antibodies and adsorbed proteins was solubilised in SDS sample buffer. The supernatant remaining following Dynabead recovery was precipitated with methanol/acetone and the resultant pellet solubilised in SDS sample buffer. Dynabead fractions and precipitated supernatants were separated by SDS-PAGE through 8% gels, transferred to nitrocellulose membranes and probed with LN43.2 (anti-lamin B, tracks 5 and 6) or L6 8A7 (anti-lamins A/C, tracks 7 and 8). Tracks 5 and 7 = Dynabead fractions; tracks 6 and 8 = supernatant fractions. LA, LB, and LC indicate the position of lamin A, B and C, respectively. H and L indicate the positions of IgG heavy and light chains, respectively. L6 8A7 cross-reacts with a protein of M_r 58–60 (arrow).

using a video camera linked to a SunnSparc workstation running the Millipore Bioimager software. Quantification of the relative intensities of bands and spots was performed using the software supplied, expressed as the integrated optical density (I.O.D.).

3. Results

Following serum re-stimulation from a quiescent (G_0) state cultures of human diploid fibroblasts enter S-phase as a synchronous population [32]. Progression from G_0 to S-phase can be monitored by indirect immunofluorescence using both anti-BrdUrd antibodies (after labelling cultures with BrdUrd) and Ki67 antibodies [36]. Typically cells were in G_1-phase 18 h after serum re-stimulation. By 24 h post-serum re-stimulation, however, 63% of cells had entered S-phase (as judged by incorporation of BrdUrd into DNA and the presence of Ki67 in nucleoli — see Table 1). In order to compare lamin B_2 modification in G_0, G_1 and S-phase cells, whole cell extracts were separated on 2-dimensional gels and Western Blotted with the lamin B_2 specific monoclonal antibody LN43.2. In G_0 (Fig. 1A) and G_1 cultures (Fig. 1B), lamin B_2 migrated as two spots (a and b). By contrast, in S-phase cultures lamin B_2 migrated as a single spot (Fig. 1C) which had the same relative mobility as spot A. To confirm that the disappearance of spot B occurred contemporaneously with entry into S-phase, cultures of HDF were accumulated in S-phase by incubation with 1 mM HU following serum re-stimulation. We have previously demonstrated that following this treatment HDF become arrested in an early S-phase state [37]. Whole cell extract were again prepared from HU arrested cultures, separated on 2-dimensional gels and Western blotted with mAb LN43.2. In these experiments, lamin B_2 again migrated as a single spot which had the same relative mobility as spot A (Fig. 1D).

Since spot A is a more basic species, the simplest explanation for the disappearance of spot B is the accumulation of one or more phosphorylated residues in lamin B_2 as HDF enter S-phase. To test this hypothesis, G_0 or S-phase cultures of HDF were first labelled with [^{32}P]orthophosphate then solubilised. Lamin B_2 was then immunoisolated from the ^{32}P-labelled cell extracts using mAb LN43.2 linked to paramagnetic Dynabeads. To confirm that lamin B_2 was isolated from the extracts samples were separated on 1-dimensional SDS-PAGE and either stained with Coomassie Brilliant Blue (Fig. 2, lanes 1 and 2) or Western blotted and probed with either mAb LN43.2
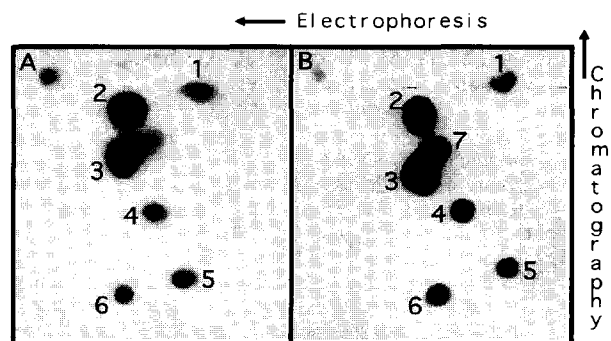

Fig. 3. Two-dimensional tryptic maps of quiescent and S-phase-associated lamin B labelled with [^{32}P]phosphate. Lamin B was immunoisolated from cultures of quiescent (a) and S-phase (b) HDF that had been labelled with [^{32}P]phosphate, and were separated by electrophoresis through 8% SDS-PAGE gels. Excised lamin B bands were then subjected to trypsin digestion, and tryptic peptides were separated on cellulose thin-layer plates by electrophoresis in the first dimension and by chromatography in the second dimension. [^{32}P]Phosphate-containing peptides were visualised by autoradiography against pre-flashed X-ray film. Numbers indicate major lamin B phosphorylated peptides.
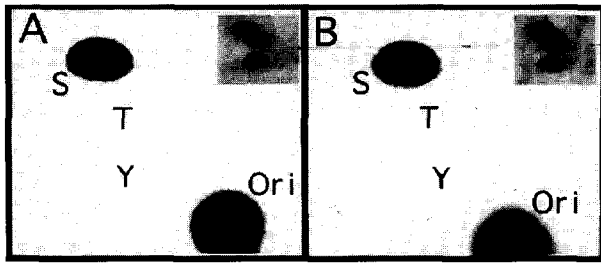
Fig. 4. Phosphorylated amino acids present in lamin B derived from quiescent and S-phase HDF. Lamin B was immunoisolated from cultures of quiescent (A) and S-phase (B) HDF that had been labelled with [^{32}P]phosphate, and were separated by electrophoresis through 8% SDS-PAGE gels. Excised lamin B was then subjected to hydrolysis in 6 N HCl and separated on cellulose thin-layer plates by electrophoresis in pH 1.9 buffer in the first dimension and by electrophoresis in pH 3.5 buffer in the second dimension. [^{32}P]Phosphoamino acids were visualised by autoradiography against pre-flashed X-ray film. Migration positions of nonradioactive phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y) added to each sample and stained with ninhydrin after electrophoresis (insets).

(Fig. 2, lanes 5 and 6) or the anti-A-type lamin antibody mAb L6 8A7 (Fig. 2, lanes 7 and 8). Coomassie stained gels indicated that lamin B_2 was the major polypeptide isolated from both G_0 and S-phase cultures in these experiments. This result was confirmed by Western blotting experiments which revealed that while lamin B_2 was recovered efficiently from cell extracts (Fig. 2, lanes 5 and 6) A-type lamins were not recovered and remained in the extract (Fig. 2, lanes 7 and 8). To determine the extent of ^{32}P-incorporation into lamin B_2, Coomassie stained gels were dried under vacuum and subjected to autoradiography (Fig. 2, lanes 3 and 4). Autoradiograms revealed extensive incorporation of ^{32}P into lamin B_2 immunoisolated from both G_0 and S-phase HDF. To determine the extent of incorporation in each culture the bands were excised from each gel and analyzed by Cherenkov counting. The results of three separate experiments consistently revealed a 2–3-fold increase in the steady state level of lamin B_2 phosphorylation in S-phase HDF compared to G_0-phase HDF (Table 2).

We wished to determine whether the increase in steady state phosphorylation observed as lamin B_2 entered S-phase resulted from an overall increase in phosphorylation at all residues or from the appearance of novel S-phase phosphorylation sites. Immunoisolated ^{32}P-labelled lamin B_2 was separated on 1-dimensional gels, excised and subjected to trypsin digestion. Tryptic peptides were separated on cellulose thin layer plates by electrophoresis in the first dimension and chromatography in the second dimension. ^{32}P containing peptides were visualised by autoradiography against pre-flashed X-ray film. Following trypsin digestion, six ^{32}P-labelled peptides were reproducibly released from lamin B_2 that was immunoisolated from G_0 cells (Fig. 3A). Each of these peptides was also released from lamin B_2 which was immunoisolated from S-phase cells. The intensity of each spot was consistently higher in autoradiographs of S-phase forms of lamin B_2. In addition, a seventh peptide was also released from S-phase forms of lamin B_2 that was not detected in G_0 forms of lamin B_2 (Fig. 3B). This peptide contained 10% of the total phosphate incorporated in S-phase lamin B_2. To determine the distribution of ^{32}P between amino acid residues phosphoamino acid analysis was performed. Analysis of both S-phase and G_0 forms of lamin B_2 revealed

that all ^{32}P was incorporated into serine residues (Fig. 4A and B). Since previous studies have indicated that phosphorylation of lamin B_2 at mitosis results in the accumulation of phosphothreonine residues [27] we conclude that peptide seven does not correspond to either of the cdc2 sites implicated in lamina disassembly.

4. Discussion

Using 2-dimensional gel electrophoresis and tryptic phosphopeptide analysis we have identified an S-phase specific phosphorylation of human lamin B_2. B-Type lamins have previously been reported to undergo extensive modification by phosphorylation as cells progress through the cell cycle [24,26]. These phosphorylation changes include modification of cdc2 sites, which control lamina assembly/disassembly at mitosis [27,28], and modification of a single PCK site to control lamin uptake into the nucleus [31]. As far as we are aware this is the first report of an S-phase specific modification of a B-type lamin. Other papers have reported on changes in B-type lamin phosphorylation accompanying mitogen stimulation of cells grown in culture [30,38] but neither paper identifies the cell cycle phase in which these changes occur.

Experiments using cell-free extracts of *Xenopus* eggs have highlighted the importance of the lamina in the initiation of DNA replication [18,19,20]. The recent discovery that B-type lamins redistribute from the nuclear envelope to replication factories, in S-phase fibroblasts [23] has served to emphasise this role. It seems likely that a re-distribution of B-type lamins from the nuclear envelope to replication factories would require

Table 1
Percentages of proliferating cells in different HDF cultures

Assay	Time post-restimulation (h)		
	Q	18	24
Ki-67	0.31 ± 0.23	3.47 ± 0.46	62.53 ± 4.75
BrdUrd	1.09 ± 0.05	6.37 ± 1.72	63.23 ± 7.57

Percentages of cells staining for KI-67 and the percentages of cells incorporating BrdUrd following exposure to BrdUrd for 24 h (quiescent culture) or continuous exposure to BrdUrd in cultures restimulated with serum for 18 or 24 h. Values indicate the mean ± standard error of the mean (S.E.M.; $n = 3$).

Table 2
Phosphorylation levels of lamin B derived from quiescent and S-phase cultures of HDF

Culture condition	Relative level of lamin B phosphorylation (cpm O.D.$^{-1}$)
Quiescent	850 ± 337
S-phase	2219 ± 240

Lamin B was immunoisolated from quiescent and S-phase cultures of HDF which had been incubated in the presence of ^{32}P$_i$ for 4 h, and separated by SDS-PAGE through 8% gels. After Coomassie staining and vacuum drying of the gels, lamin B bands were excised and incorporation of ^{32}P determined by Cherenkov counting. Relative protein concentrations of lamin B were determined by densitometric analysis of photographs of the Coomassie-stained gels. Relative specific ^{32}P-incorporation into lamin B was determined by dividing the counts per minute (cpm) of the gel chips by the integrated optical density (I.O.D.) of the same band on the Coomassie-stained gel prior to excision. Average values are shown ± S.E.M., $n = 3$. Analysis of variance shows a significant difference between the two samples of $P = 0.008$.

modification by phosphorylation. That S-phase phosphorylation of lamin B_2 occurs lends weight to this hypothesis.

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