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# Mitotic Phosphorylation of the Lamin B Receptor by a Serine/Arginine Kinase and p34$^{cdc2}$*

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**The lamin B receptor (LBR) is an integral protein of the inner nuclear membrane that is modified at interphase by a nuclear envelope-bound protein kinase. This enzyme (RS kinase) specifically phosphorylates arginine-serine dipeptide motifs located at the $NH_2$-terminal domain of LBR and regulates its interactions with other nuclear envelope proteins. To compare the phosphorylation state of LBR during interphase and mitosis, we performed phosphopeptide mapping of *in vitro* and *in vivo* $^{32}$P-labeled LBR and analyzed a series of recombinant proteins and synthetic peptides. Our results show that LBR undergoes two types of mitotic phosphorylation mediated by the RS and the p34$^{cdc2}$ protein kinases, respectively. The RS kinase modifies similar sites at interphase and mitosis (*i.e.* Ser$^{76}$, Ser$^{78}$, Ser$^{80}$, Ser$^{82}$, Ser$^{84}$), whereas p34$^{cdc2}$ mainly phosphorylates Ser$^{71}$. These findings clarify the phosphorylation state of LBR during the cell cycle and provide new information for understanding the mechanisms responsible for nuclear envelope assembly and disassembly.**

The nuclear lamina is a filamentous meshwork underlying the inner nuclear membrane (1, 2). In most cells this structure is a heteropolymer of type A and B lamins (3) linked to the inner nuclear membrane through integral membrane proteins. These lamin-binding proteins include the lamin B receptor (LBR[1] or p58; Ref. 4) and the lamina-associated polypeptides (5).

LBR possesses a long, hydrophilic $NH_2$-terminal domain protruding into the nucleoplasm, eight hydrophobic segments that are predicted to span the membrane, and a hydrophilic COOH-terminal domain (6, 7). The $NH_2$-terminal domain of LBR contains distinct sites for protein kinase A and p34$^{cdc2}$ kinase phosphorylation (8, 9) as well as a stretch rich in arginine-serine (RS) motifs (10). The RS motifs are specifically modified by a protein kinase that co-isolates with LBR and is part of a multimeric complex (8, 10). This LBR complex also includes the nuclear lamins and three polypeptides with molecular masses of 18 (p18), 150 (p150), and 34 (p34/p32) kDa, respectively (for pertinent information see Refs. 8, 10, and 12). The latter protein has been shown to interact with the splicing factor 2 (SF2) as well as with the HIV-1 proteins Rev and Tat (13–15). Phosphorylation of LBR by the RS kinase completely abolishes binding of p34/p32, suggesting that this enzyme regulates interactions among the components of the LBR complex (11).

At the onset of mitosis, the structure of the nuclear envelope is dramatically altered. The nuclear lamina depolymerizes as a result of hyperphosphorylation of the nuclear lamins at specific sites involved in lamin-lamin (16), lamin-chromatin (17), and lamin-membrane (5) interactions. Following depolymerization, the bulk of type A lamins disperse in the cytoplasm, whereas type B lamins remain bound to remnants of the nuclear envelope. At the same time, the nuclear envelope membranes break down into vesicular structures (1). Apart from lamin hyperphosphorylation, Courvalin *et al.* (9) also reported that LBR is phosphorylated on serine and threonine residues during mitosis.

As the events responsible for nuclear membrane breakdown are not completely understood and in light of the fact that LBR is phosphorylated by the RS kinase during interphase, we found it important to examine the specific modifications of LBR during mitosis. Results presented below reveal that during mitosis LBR is phosphorylated by both RS and p34$^{cdc2}$ protein kinases.

## EXPERIMENTAL PROCEDURES

*Materials*—Phosphocellulose and Affi-Gel 10 were purchased from Whatman Biosystems Ltd., United Kingdom, and Bio-Rad, respectively. Peptides $R_0$ ($^{70}$SSPSRRSRSRSRSRSPGRPAKG$^{91}$), $R_1$ ($^{61}$KQRKSQSSSSSPSRRSRSRS$^{80}$), $R_2$ ($^{78}$SRSRSR*S*PGRPAKG$^{91}$), and $R_4$ ($^{182}$KIFEAIK*T*PEKPSSKT$^{197}$) were made at the Protein Sequencing and Peptide Synthesis Facility of the European Molecular Biology Laboratory, Heidelberg, Germany. $R_0$ peptide was coupled to Affi-Gel 10 as described previously (11). Recombinant p34$^{cdc2}$/cyclin B was purchased from New England Biolabs Ltd., United Kingdom. Histone $H_1$ was obtained from Boehringer Mannheim GMbH, Germany. [$\gamma$-$^{32}$P]ATP (6000 Ci/mmol) as well as [$^{32}$P]phosphate (10 mCi/ml) were purchased from ICN Pharmaceuticals Ltd., United Kingdom. The anti-LBR antibody a$R_1$, raised against the peptide $R_1$, was prepared and affinity-purified as described previously (8). An anti-cyclin B antibody was kindly provided by Ingrid Hoffmann (Germany Cancer Research Center, Heidelberg, Germany). All other chemicals were purchased from Sigma.

*Construction of Plasmids and Expression of Fusion Proteins*—The pGEX-2T bacterial expression vector (Pharmacia Biotech Inc.) was used to construct plasmids that encode the wild type NH2 terminus (wtNt) and three mutated forms (wtNtA$^{71}$, wtNtA$^{84}$, and wtNtA$^{188}$) of the NH2-terminal domain of chicken LBR (6) fused with glutathione *S*-transferase (GST). To generate the cDNA coding for wtNt (amino acids

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This article is dedicated to the memory of Prof. Nikolaos Alexandrou.

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[1] The abbreviations used are: LBR, lamin B receptor; wtNt, wild type NH2 terminus; GST, glutathione *S*-transferase; SF, splicing factor; PAGE, polyacrylamide gel electrophoresis; SRPK1, SR protein kinase 1.


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1–205), 30 cycles of the polymerase chain reaction were performed as described (11). Full-length LBR cloned to the *Eco*RI site of Bluescript $SK^-$ was used as a template. The LBR-$SK^-$ clone was a generous gift of G. Blobel (Rockefeller University, New York) and H. J. Worman (Columbia University, New York). The sense primer contained nucleotides +1 to +21 of LBR preceded by a *Bam*HI site. CAGTA was added 5′ to the *Bam*HI site. The antisense primer was complementary to nucleotides +598 to +615 of LBR. A complementary stop codon was added 5′ to this sequence, preceded by an *Eco*RI site. GC was added 5′ to the *Eco*RI site. The polymerase chain reaction product was purified using the QIAEX gel extraction kit (QIAGEN Inc., Chatsworth, CA). Purified DNA was digested with *Eco*RI and *Bam*HI, repurified, and ligated into the *Bam*HI/*Eco*RI site of pGEX-2T. *Escherichia coli* strains XL-1 Blue were transformed by standard methods.

An oligonucleotide-directed *in vitro* mutagenesis system (Altered Sites®II *In vitro* Mutagenesis system, Promega, Corp., Madison, WI) was used to mutate the sites that are potentially phosphorylated by $p34^{cdc2}$/cyclin B protein kinase. Using the oligonucleotides 5′-TCTGGAAGGAGCACTTGAGGA-3′, 5′-GACCAGGAGCTCTGGATCG-3′, and 5′-TTTCTCCGGAGCTTTTATTGC-3′, $Ser^{71}$, $Ser^{84}$, and $Thr^{188}$ were mutated to Ala. The mutated cDNAs ($wtNtA^{71}$, $wtNtA^{84}$, and $wtNtA^{188}$) were sequenced and subcloned into the pGEX-2T expression vector as described previously for wtNt. GST fusion proteins were produced in bacteria and purified as described (11). A fusion protein missing the RS motifs (deletion of residues 75–84; construct termed GST-ΔRSNt) as well as a protein containing the five arginine-serine repeats of LBR fused to GST (residues 75–84; construct termed GST-RS) was generated as described previously (11).

*Purification of LBR Kinase*—LBR kinase was isolated from turkey erythrocyte nuclear envelopes as described previously (11). Briefly, the 1 M NaCl extract of nuclear envelopes (following dilution to 0.3 M and clarification by centrifugation) was applied to a phosphocellulose column previously equilibrated with 20 mM Tris-HCl (pH 7.5), 0.3 M NaCl, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. The bound proteins were eluted by a linear (0.3–1 M) NaCl gradient. Kinase-containing fractions were pooled, the salt concentration was adjusted to 0.35 M, and the material was further chromatographed through an Affi-Gel 10 column containing the $R_0$ peptide. The column was subsequently washed with 0.9 M NaCl, and elution of the kinase activity was accomplished by a linear (0.9–2.2 M) NaCl gradient. The active fractions were pooled, concentrated with an Amicon device, and used in subsequent experiments.

*Cell Culture and Synchronization; in Vivo and in Vitro Phosphorylation*—Chicken hepatoma cells, DU249, were grown according to Meier and Georgatos (18). To obtain mitotic cells, the cultures were synchronized by a double block of 2 mM thymidine (16 h) and 20 ng/ml nocodazole (4 h) as described elsewhere (19). Mitotically arrested cells were detached by mechanical agitation. For *in vivo* $^{32}P$ labeling, the cells were incubated in suspension for 3 h with 1 mCi/ml $^{32}P_i$ in phosphate-free medium containing nocodazole, washed with cold phosphate-buffered saline (155 mM NaCl, 20 mM sodium phosphate, pH 7.4), and collected by centrifugation. To obtain interphase cells, cultures were grown under similar conditions as above except that thymidine and nocodazole were not included in the growth medium. After the end of incubation with $^{32}P_i$, the dishes were vigorously washed two times with cold phosphate-buffered saline to remove mitotic cells. Adherent interphase cells were collected with a rubber policeman. Both mitotic and interphase cells were lysed in 1 ml of 50 mM Tris-HCl (pH 7.4), 0.2% SDS, 1% Triton X-100, 100 mM NaCl, 50 mM NaF, 0.1 mM sodium orthovanadate, 2 mM EDTA, and a mixture of protease inhibitors (8). The lysates were clarified by centrifugation at 12,000 × *g* for 10 min, and then immunoprecipitation of LBR was carried out as described previously (8).

In order to obtain mitotic cell extracts, cells were harvested by centrifugation at mitosis, washed once in ice-cold KHM buffer (78 mM KCl, 50 mM Hepes-KOH (pH 7.0), 4 mM $MgCl_2$, 8.37 mM $CaCl_2$, 10 mM EGTA, 1 mM dithiothreitol, 20 μM cytochalasin B, and 1 mM phenylmethylsulfonyl fluoride), resuspended at 0 °C in KHM buffer, and Dounce-homogenized. Membrane-free cytosol was prepared by ultracentrifuging the samples at 400,000 × *g* for 1 h at 4 °C. Immunodepletion of mitotic extracts with an anti-cyclin B antibody was performed essentially as described by Hoffmann *et al*. (20). Briefly, 25 μl of protein A-Sepharose were incubated with antiserum for 2 h at 4 °C and washed three times with phosphate-buffered saline containing 1% Triton X-100. Extracts were incubated twice with the Immunobeads for 2 h at 4 °C on a rotator and recovered after centrifugation for 30 s in a microcentrifuge. The immunoprecipitates were washed three times with 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1% Triton X-100 and subsequently used as a source of $p34^{cdc2}$/cyclin B protein kinase.

For $p34^{cdc2}$/cyclin B phosphorylation, 1 μl of the enzyme preparation (activity, 2000 units/ml; 1 unit is the amount of $p34^{cdc2}$/cyclin B required to catalyze the transfer of 1 pmol of phosphate to histone $H_1$ in 1 min at 30 °C) was incubated with 6 μg of GST-wtNt or with 1.5 μg of electroeluted LBR in a buffer composed of 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EGTA, and 50 μM [γ-$^{32}$P]ATP (6000 Ci/mmol) in a reaction volume of 25 μl.

For RS kinase phosphorylation, 6 μl of the enzyme preparation (activity, 10 units/ml; 1 unit is the amount of enzyme required to catalyze the transfer of 0.1 nmol of phosphate to 1.5 μg of electroeluted LBR in 30 min at 30 °C) was incubated with 6 μg of GST-wtNt in a buffer composed of 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 200 mM NaCl, 1 mM dithiothreitol, and 50 μM [γ-$^{32}$P]ATP (6000 Ci/mmol) in a reaction volume of 25 μl.

Samples were incubated for 30 min at 30 °C, and the reaction was stopped by adding the appropriate volume of 5 × Laemmli buffer (21) and heating at 95 °C for 3 min. Electroeluted LBR was obtained from urea-insoluble nuclear envelopes as described previously (11).

*Phosphopeptide Mapping and Phosphoamino Acid Analysis*—Proteolytic peptide mapping was performed essentially as described by Luo *et al.* (22) and Simos and Georgatos (8). Briefly, immunoprecipitates of *in vivo* phosphorylated LBR or *in vitro* phosphorylated GST-wtNt were run on SDS-PAGE and then transferred to a nitrocellulose sheet. The radioactive LBR/GST-wild type $NH_2$ terminal bands were excised, soaked in 0.5% polyvinylpyrrolidone 360 in 100 mM acetic acid for 1 h at 37 °C and washed extensively with water. The protein was digested by trypsin in 50 mM $NH_4HCO_3$ at 37 °C overnight. The released peptides were dried, resuspended in water, and loaded on a cellulose TLC plate (Eastman Kodak Co.). Electrophoresis (in the first dimension) was run at pH 8.9 (1% ammonium carbonate) for 1 h at 500 V; ascending chromatography (in the second dimension) was performed using as a solvent a mixture of 1-butanol/pyridine/glacial acetic acid/water at a ratio of 75:50:15:60.

For phosphoamino acid analysis, the tryptic digest was treated with 5.7 M HCl at 110 °C for 90 min, dried, and subjected to electrophoresis on cellulose TLC plates at pH 3.5 (pyridine/glacial acetic acid/water, 1:7:992) for 45 min at 1500 V.

*Other Methods*—SDS-PAGE was performed according to Laemmli (21) using 12% gels. Dried gels were exposed to Kodak x-ray film with intensifying screens. Protein concentration was determined by the method of Bradford (23).

## RESULTS

To determine whether LBR is a substrate for $p34^{cdc2}$ protein kinase, electroeluted LBR as well as salt-washed nuclear envelopes preheated at 60 °C for 10 min (to inactivate the endogenous RS kinase) were used as substrates for *in vitro* phosphorylation assays. Fig. 1 shows that $p34^{cdc2}$ modifies both the envelope-associated and the purified LBR protein. Under these conditions $p34^{cdc2}$ also phosphorylated lamin A, lamin B, and residual histones $H_1$ and $H_5$ left behind after salt extraction of the nuclear envelopes.

Inspection of the amino acid sequence of chicken LBR revealed the presence of three potential $p34^{cdc2}$ phosphorylation sites ($Ser^{71}$, $Ser^{84}$, and $Thr^{188}$; see Fig. 2*A*) conforming to the consensus Ser/Thr-Pro-*X*-*X* (24). To assess the ability of $p34^{cdc2}$ to modify these sites, we synthesized three peptides ($R_1$, $R_2$, and $R_4$) modeled after the published sequence, each one containing one potential phosphorylation site (Fig. 2*A*). As shown in Fig. 2*B*, the phosphorylation of purified LBR was inhibited by peptides $R_1$ and $R_2$, which acted as substrates for the kinase. In contrast, peptide $R_4$ was poorly phosphorylated by $p34^{cdc2}$ and did not significantly inhibit the phosphorylation of purified LBR. To express these results quantitatively, the same type of *in vitro* phosphorylation assays was performed using a range of peptide concentrations. Data presented in Table I document that $R_1$ was the strongest inhibitor of $p34^{cdc2}$-mediated phosphorylation of LBR and the best substrate for $p34^{cdc2}$, whereas $R_4$ was the weakest inhibitor and the poorest substrate for the kinase.

In agreement with these observations, only phosphoserine could be detected when *in vitro* phosphorylated LBR was ana-

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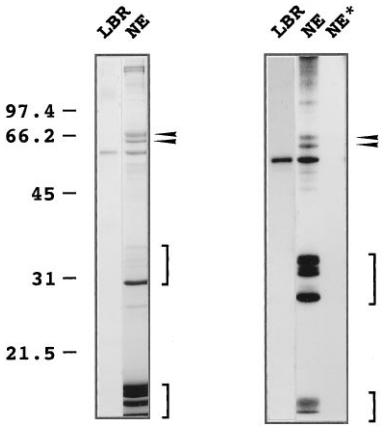


FIG. 1. ***In vitro* phosphorylation of nuclear envelope proteins and purified LBR by p34$^{cdc2}$ protein kinase.** 1.5 $\mu$g of electroeluted LBR or 15 $\mu$g of salt-washed nuclear envelopes (*NE*) preheated at 60 °C for 10 min were incubated with [$\gamma$-$^{32}$P]ATP and p34$^{cdc2}$ protein kinase. Samples were analyzed by SDS-PAGE and stained with Coomassie Blue (*left panel*) or autoradiographed (*right panel*). *NE** shows the phosphorylation of nuclear envelope proteins preheated at 60 °C for 10 min in the absence of p34$^{cdc2}$ protein kinase. *Arrowheads* indicate the positions of lamins A and B. *Brackets* indicate the positions of histone $H_1$ isoforms, histone $H_5$, and core histones $H_3$, $H_{2B}$, $H_{2A}$, and $H_4$ (from *top* to *bottom*). *Bars* on the left indicate molecular masses (in kDa). Note the phosphorylation of lamins A and B and of histones $H_1$ and $H_5$ by p34$^{cdc2}$ protein kinase.

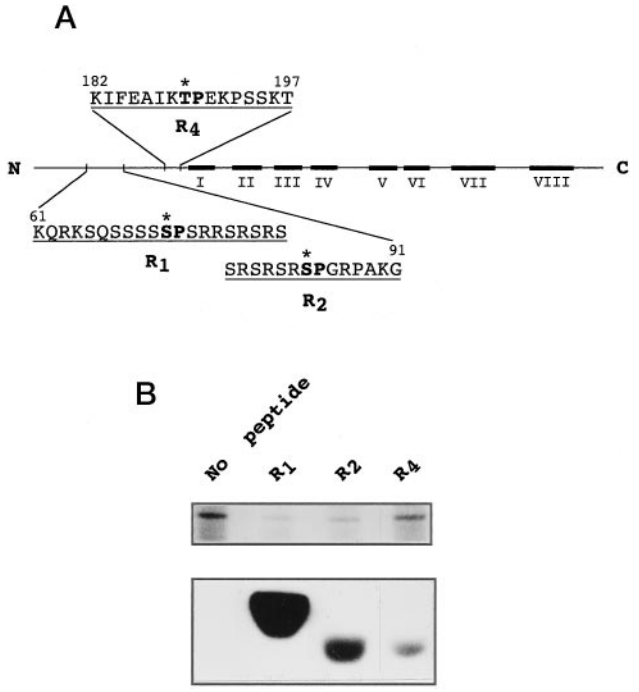


FIG. 2. **Phosphorylation of purified LBR by p34$^{cdc2}$ protein kinase in the presence of various synthetic peptides.** *A*, amino acid sequences of the peptides used. Consensus motifs are shown in *bold*, and the *asterisk* denotes predicted phosphorylation sites. The relative position of the peptides in the LBR molecule is schematically indicated. *Black boxes* along the LBR sequence, numbered with Roman numerals, represent potential transmembrane domains. *B*, 1.5 $\mu$g of electroeluted LBR were incubated with p34$^{cdc2}$ protein kinase in the presence of 0.5 mM of each peptide as described under "Experimental Procedures." Samples were subsequently analyzed by SDS-PAGE on 10–20% gradient gels and autoradiographed. The *upper panel* shows the phosphorylation of LBR and the *lower panel*, the phosphorylation of the added peptides. Only the relevant parts of the autoradiogram are shown.

TABLE I

*Concentration of synthetic peptides required for 50% inhibition of the LBR phosphorylation ($IC_{50}$) by exogenously added p34$^{cdc2}$ protein kinase and relative phosphorylation rates (RPR) of the respective peptides by p34$^{cdc2}$*

| Peptide | $IC_{50}$[a] | RPR[b] |
|---|---|---|
| | *mM* | |
| $^{61}$KQRKSQSSSSSPSRRSRSRS$^{80}$ ($R_1$) | 0.14 | 100 |
| $^{78}$SRSRSRSPGRPAKG$^{91}$ ($R_2$) | 0.40 | 60 |
| $^{182}$KIFEAIKTPEKPSSKT$^{197}$ ($R_4$) | 0.60 | 11 |

[a] 1.5 $\mu$g of electroeluted LBR were incubated with p34$^{cdc2}$ protein kinase and [$\gamma$-$^{32}$P]ATP as described under "Experimental Procedures," in the presence of different concentrations of $R_1$, $R_2$, and $R_4$. Phosphorylation of LBR was monitored with autoradiography.

[b] Peptides $R_1$, $R_2$, and $R_4$ were incubated at a concentration of 100 $\mu$M with p34$^{cdc2}$ protein kinase and [$\gamma$-$^{32}$P]ATP. Phosphorylation of the peptides was monitored with autoradiography.

lyzed by phosphoamino acid analysis (Fig. 3*A*). Pursuing this point further, we expressed in *E. coli* a fusion protein consisting of GST and the $NH_2$-terminal domain of LBR (residues 1–205; construct termed GST-wtNt) as well as various derivatives of this fusion protein in which Ser$^{71}$, Ser$^{84}$, and Thr$^{188}$ were changed to Ala (constructs termed GST-wtNtA$^{71}$, GST-wtNtA$^{84}$, and GST-wtNtA$^{188}$, respectively). The wild type and the three mutated proteins were used as substrates in *in vitro* phosphorylation assays. p34$^{cdc2}$ could efficiently phosphorylate GST-wtNt, GST-wtNTA$^{84}$, and GST-wtNtA$^{188}$, whereas the phosphorylation of GST-wtNtA$^{71}$ was significantly impaired (Fig. 3*D*). This was not due to a global misfolding of the polypeptide chain induced by the replacement of Ser$^{71}$ because both GST-wtNt and GST-wtNTA$^{71}$ were efficiently modified by the RS kinase (Fig. 5*A*).

To confirm these results we performed two-dimensional proteolytic peptide mapping. Fig. 4 shows that p34$^{cdc2}$-phosphorylated GST-wtNt yielded one major phosphopeptide (peptide designated *b*), one phosphopeptide of moderate intensity (peptide designated *c*), and two minor phosphopeptides (peptides designated *a* and *d*). Phosphopeptide mapping of *in vitro* phosphorylated GST-wtNtA$^{71}$, GST-wtNtA$^{84}$, and GST-wtNtA$^{188}$ by p34$^{cdc2}$ revealed that the major phosphopeptide (b) corresponds to phosphorylation of Ser$^{71}$, peptides a and c correspond to phosphorylation of Ser$^{84}$, and peptide d corresponds to phosphorylation of Thr$^{188}$. From the sum of all these observations it can be concluded that Ser$^{71}$ of avian LBR is the major site phosphorylated by p34$^{cdc2}$ protein kinase under *in vitro* conditions, whereas Ser$^{84}$ is weakly modified by the enzyme. It is noteworthy that in some of our experiments we have been unable to detect the spot corresponding to Thr$^{188}$. The very low extent of Thr phosphorylation could explain our inability to detect it when we performed phosphoamino acid analysis of *in vitro* phosphorylated LBR by p34$^{cdc2}$ (see Fig. 3*A*).

That LBR can be directly phosphorylated by p34$^{cdc2}$ protein kinase implies that the lamin B receptor protein has the potential of being an *in vivo* substrate for mitotic kinases. To explore this idea, we performed experiments using mitotic cell extracts. Fig. 5*C* shows that membrane-free cytosol prepared from nocodazole-arrested chicken hepatoma (DU249) cells contained both p34$^{cdc2}$ and RS protein kinase activities. This can be deduced from the fact that mitotic extracts phosphorylated GST-ΔRSNt (which lacks the RS region of LBR and is not a substrate for the RS kinase), GST-wtNtA$^{71}$ (which is not phosphorylated by p34$^{cdc2}$), and GST-RS (a fusion protein consisting of GST and five RS repeats but missing the putative p34$^{cdc2}$ site of LBR). The same results were obtained with mitotic extracts from HeLa cells (Fig. 5*D*). To confirm that p34$^{cdc2}$/cylin B is truly the kinase responsible for mitotic LBR phosphorylation, we immunodepleted HeLa cell extracts with an anti-cyclin B polyclonal antibody. Fig. 5*E* shows that extracts pretreated with the anti-cyclin B antibody had lost their ability to

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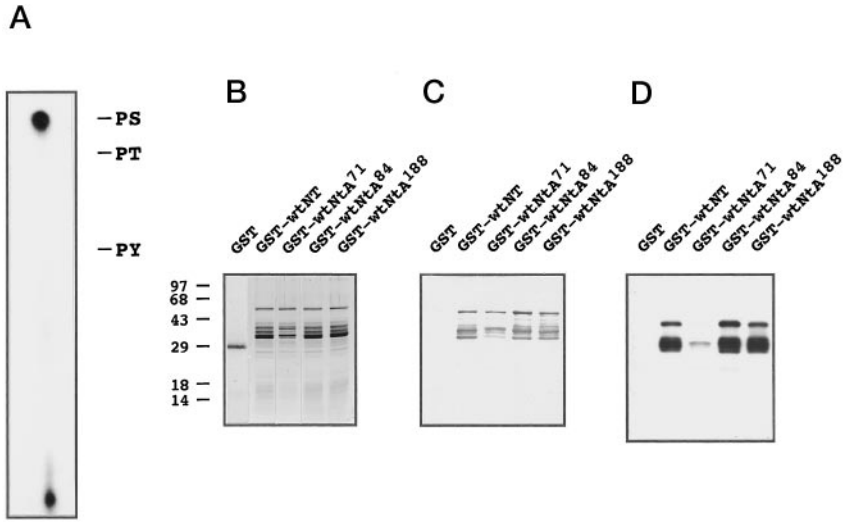


FIG. 3. **Identification of the site on LBR that is phosphorylated *in vitro* by p34$^{cdc2}$ protein kinase.** *A*, phosphoamino acid analysis following *in vitro* phosphorylation of electroeluted LBR by p34$^{cdc2}$ protein kinase. $^{32}$P-Labeled LBR was analyzed by SDS-PAGE, blotted onto nitrocellulose filters, and subsequently excised and subjected to hydrolysis and phosphoamino acid analysis as described under "Experimental Procedures." Migration of phosphoserine (*PS*), phosphothreonine (*PT*), and phosphotyrosine (*PY*) standards is shown at left. *B*, SDS-PAGE analysis and Coomassie Blue staining of GST, GST fusion protein containing the $NH_2$-terminal domain of LBR (GST-wtNt, amino acids 1–205), and similar fusion proteins except that Ser$^{71}$, Ser$^{84}$, and Thr$^{188}$ were mutated to Ala (constructs termed GST-wtNtA$^{71}$, GST-wtNtA$^{84}$, and GST-wtNtA$^{188}$, respectively). The full-length fusion protein migrates with an apparent molecular mass of approximately 51 kDa. The *lower bands* represent degradation products (see also Ye and Worman (7) and Nikolakaki *et al.* (11)). *C*, immunoblotting of bacterially expressed proteins using an affinity-purified anti-LBR antibody raised against peptide $R_1$ ($aR_1$). The blots were stained using an alkaline phosphatase-conjugated goat anti-rabbit antibody. Note that in addition to full-length fusion protein, $aR_1$ also reacts with degradation products. *D, in vitro* phosphorylation of bacterially expressed proteins by p34$^{cdc2}$ protein kinase. The samples were analyzed by SDS-PAGE and autoradiographed. Molecular mass standards are shown at *left* (in kDa).

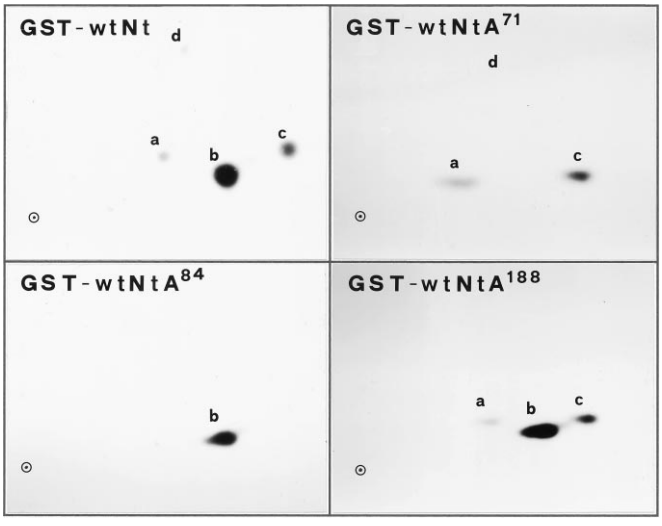


FIG. 4. **Tryptic phosphopeptide analysis of GST-wtNt and alanine mutants of GST-wtNt (for nomenclature, see text and legend to Fig. 3).** *In vitro* phosphorylated fusion proteins, by p34$^{cdc2}$ protein kinase, were transferred to nitrocellulose and digested with trypsin. The eluted phosphopeptides were separated by electrophoresis at pH 8.9 (horizontal direction; cathode to the right) and by ascending chromatography. Origins of sample application are marked by ⊙.

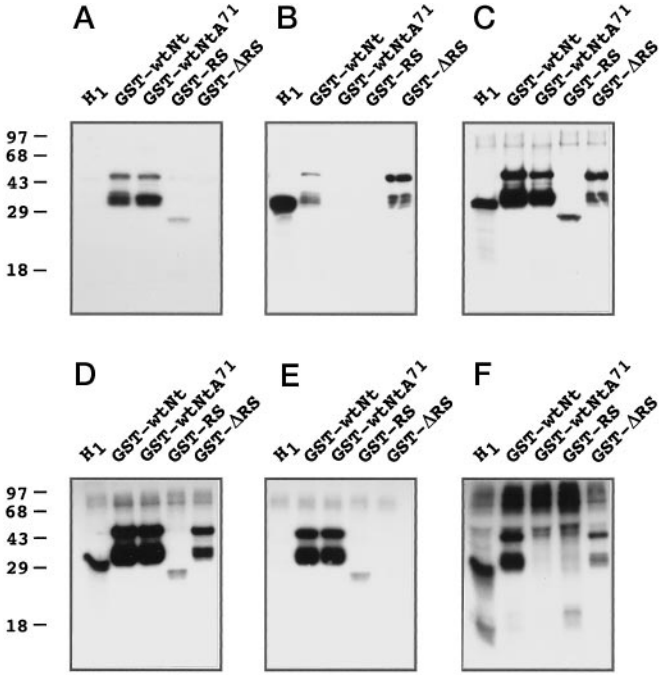


FIG. 5. **Mitotic cell extracts contain both p34$^{cdc2}$ and RS protein kinase activities.** Autoradiograms of *in vitro*-phosphorylated histone $H_1$, GST-wtNt, GST-wtNtA$^{71}$, GST-RS, and GST-ΔRSNt (for nomenclature, see text and legend to Fig. 3) by partially purified RS kinase (*A*), p34$^{cdc2}$ protein kinase (*B*), cell extracts made from DU249 cells arrested in mitosis (*C*), mitotic cell extracts made from HeLa cells (*D*), mitotic cell extracts from HeLa cells pretreated with an anti-cyclin B antibody (*E*), and immunoprecipitates of mitotic HeLa cell extracts with the anti-cyclin B antibody (*F*). Additional bands represent phosphorylated proteins associated with protein A-Sepharose/anti-cyclin B beads. Molecular mass standards are shown at left (in kDa).

phosphorylate histone $H_1$ and GST-ΔRSNt and contained only the RS kinase activity, whereas a typical p34$^{cdc2}$ pattern was obtained with the immunoprecipitated activity (Fig. 5*F*).

Pursuing this point further, interphase DU249 cells and cells arrested at prometaphase were labeled metabolically with [$^{32}$P]orthophosphate, and the *in vivo* phosphorylated LBR was immunoprecipitated by $aR_1$ antibodies (see "Experimental Procedures"). The level of phosphorylation was similar in interphase and mitotic cells (data not shown; see also Ref. 9). The 58-kDa bands corresponding to immunoprecipitated LBR were excised and processed for phosphoamino acid analysis and two-dimensional tryptic phosphopeptide mapping. Only phosphoserine could be detected, irrespective of whether phosphorylation occurred during the interphase or the prometaphase (data

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not shown; for a typical TLC profile see Fig. 3*A*). The maps of mitotically phosphorylated LBR and LBR modified at interphase were qualitatively similar (some residues phosphorylated at interphase were phosphorylated to a lower extent at mitosis, *i.e.* phosphopeptides 1, 3, 8, and 9) except for one spot that was present in the former but absent in the latter (Fig. 6, compare *panels C* and *D*). This spot represented the major phosphopeptide (phosphopeptide *b*) corresponding to $Ser^{71}$ as shown by mixing equal counts/min of the tryptic digests, *in vitro* phosphorylated GST-wtNt by $p34^{cdc2}$, and LBR modified *in vivo* at interphase (Fig. 6, compare *panels B* and *C* with *panel E*). This mix reproduced the phosphopeptide pattern of LBR that had been modified by mitotic kinases *in vivo*. The same mitotic pattern was also obtained by mixing equal counts of *in vitro* phosphorylated GST-wtNt by $p34^{cdc2}$ and LBR modified *in vivo* at mitosis (data not shown). To confirm that $Ser^{71}$ is the additional site phosphorylated at mitosis by $p34^{cdc2}$, we performed the following experiment. Mitotic extracts (prepared from DU249 cells as described under "Experimental Procedures") were used to phosphorylate either GST-wtNt or GST-$wtNTA^{71}$. The phosphorylated proteins were then analyzed by two-dimensional tryptic phosphopeptide mapping. The phosphopeptide map of *in vitro* phosphorylated GST-wtNt was identical to the map derived from mitotic LBR *in vivo* (data not shown), whereas the map of *in vitro* phosphorylated GST-$wtNtA^{71}$ was similar to the map derived from interphase LBR phosphorylated *in vivo*; that is, phosphopeptide b was conspicuously missing (compare *panels F* and *C*). From the sum of these observations two major conclusions can be drawn. First, the RS and the $p34^{cdc2}$ protein kinases are both responsible for the mitotic phosphorylation of LBR and second, $Ser^{71}$ is the major site phosphorylated *in vivo* by $p34^{cdc2}$.

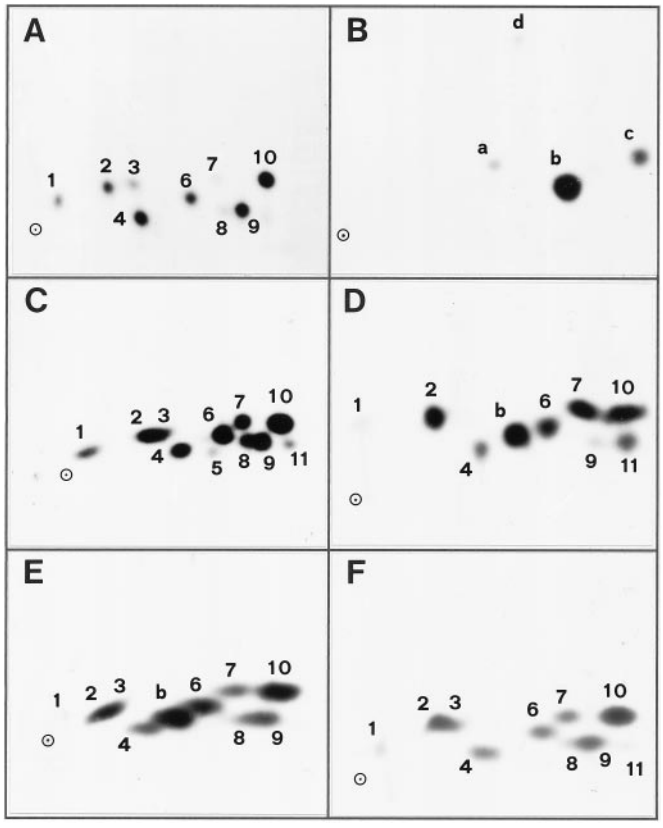


FIG. 6. **Tryptic phosphopeptide analysis of LBR following phosphorylation during interphase and mitosis *in vivo* or by RS and $p34^{cdc2}$ protein kinases *in vitro*.** *A*, GST-wtNt was phosphorylated *in vitro* by partially purified RS kinase. *B*, GST-wtNt was phosphorylated *in vitro* by $p34^{cdc2}$ protein kinase. *B* is the same as the *top right panel* in Fig. 4. *C*, LBR was immunoprecipitated from $^{32}P$-labeled DU249 cells arrested at interphase. *D*, LBR was immunoprecipitated from $^{32}P$-labeled DU249 cells arrested at mitosis. *E*, equal Cerenkov counts of digests of *in vitro* phosphorylated GST-wtNt by $p34^{cdc2}$ protein kinase (*B*) and *in vivo* phosphorylated LBR during interphase (*C*). *F*, GST-$wtNtA^{71}$ was phosphorylated *in vitro* by mitotic extracts prepared from DU249 cells (as described under "Experimental Procedures"). Analysis of phosphopeptides was carried out as described in Fig. 4. Anode is on the left and cathode on the right. Origins of sample application are marked by ⊙.

## DISCUSSION

In this study we demonstrated that LBR undergoes mitotic phosphorylation and that the RS kinase is the main protein kinase responsible for this modification. Comparison of tryptic phosphopeptide maps of *in vivo* $^{32}P$-labeled LBR immunoprecipitated from chicken cells indicates that the enzyme modifies similar sites at interphase and mitosis. Some serine residues of the RS motif phosphorylated at interphase are phosphorylated to a lower extent at mitosis. Furthermore, we demonstrated that LBR is also a substrate for $p34^{cdc2}$ protein kinase during mitosis. Using recombinant proteins produced in bacteria, phosphoamino acid analysis and two-dimensional phosphopeptide mapping of *in vitro* and *in vivo* $^{32}P$-labeled LBR, we have been able to demonstrate that $Ser^{71}$ is the major site phosphorylated by $p34^{cdc2}$ at mitosis. Courvalin *et al.* (9) reported that $Thr^{188}$ is likely to be phosphorylated by this enzyme during mitosis. According to our results the extent of Thr phosphorylation is very low and most probably $Thr^{188}$ represents a minor site modified by $p34^{cdc2}$. In line with our observations is the fact that the phosphoamino acid analysis presented by Courvalin *et al.* (9) demonstrated that mitotic LBR contained mainly phosphoserine, whereas phosphothreonine was hardly detectable.

Previous reports have shown that the RS protein kinase is strongly associated with LBR, participating in a subassembly of nuclear envelope proteins termed "the LBR complex" (8, 11). The enzyme phosphorylates LBR in a constitutive fashion during interphase (8, 11) and belongs to a novel class of protein kinases that specifically modify arginine-serine (RS) dipeptide motifs. Other members of this novel class of enzymes include a kinase associated with small nuclear ribonucleoprotein particles, which phosphorylates the U1 small nuclear ribonucleoprotein 70-kDa protein and ASF/SF2 (25), and a cell cycle-regulated serine kinase (SRPK1, SR Protein Kinase 1) that can

TABLE II
*Existence of a $p34^{cdc2}$ phosphorylation motif in RS-containing proteins*

Predicted $p34^{cdc2}$ phosphorylation sites are shown in bold and RS motifs are underlined. Numbers in parentheses correspond to the appropriate references.

| | |
|---|---|
| ·S**SP**SRRSRSRSRSRSPG | Chicken LBR (6) |
| ·R**SP**RRRRRSRSRSRSRSRSRSRY | Human splicing factor SC35 (42) |
| ·R**SP**SYGRSRSRSRSRSRSRSRSNS | Human splicing factor SF2 (13) |
| ·P**SP**SRRSRSRSRSRSKS | Mussel sperm-specific protein PHI-2B (43) |
| ·P**SP**TRRSSKSRSKSRSRSRSAS | Mussel sperm-specific protein PHI-1 (43) |
| ·K**SP**SRSRSRSRSKSNA | Yeast cytoskeleton assembly control protein SLA1 (44) |
| ·R**SP**NRGRGGSSGGPTTRSQSRSLSRSRSRSRSRSRG | Human papillomavirus E2 protein (45) |
| ·R**TP**CSFADQLLSTFIANNYLCYFYRRRRSRSRSRSRSRSRSPH | Nuclear polyhedrosis virus polyhedral envelope protein (46) |

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phosphorylate splicing factors of the SR family (26, 27). The SRPK1 activity was initially identified in nuclear extracts (26), and later the same group (27) also purified and characterized SRPK1 from cytosolic extracts. Thus, the cellular distribution of SRPK1 remains to be clarified. Interestingly, its fission yeast homologue, Dsk1, was found to be cytoplasmic in interphase cells, migrating to the nucleus before mitosis (28). Recently, Colwill *et al.* (29) reported that mammalian Clk/Sty, which is the prototype for a family of dual specificity kinases (termed LAMMER kinases), also interacts with members of the SR family of splicing factors and phosphorylates ASF/SF2.

The function of these enzymes appears to be the regulation of protein-protein interactions through phosphorylation of RS domains. In fact, LBR kinase regulates interactions among the components of the LBR complex (11), whereas SRPK1 and Clk/Sty regulate the intranuclear distribution of SR splicing factors (26, 29, 30). SRPK1 may also be responsible for the redistribution of splicing factors as cells enter mitosis (26).

LBR is also a substrate for $p34^{cdc2}$ protein kinase, a key mitotic kinase. Several substrates for this enzyme have been identified to date (reviewed in Ref. 31). However, the detailed mechanisms by which $p34^{cdc2}$ induces the profound structural changes characteristic of mitotic cells remain quite obscure. Although the physiological significance of LBR phosphorylation by $p34^{cdc2}$ remains to be examined by other approaches, it is interesting to note here that, at least *in vitro*, the $p34^{cdc2}$-modified LBR and the nonmodified protein do not significantly differ in their lamin B binding properties.[2] This is in line with the fact that lamin B, although disassembled by $p34^{cdc2}$ phosphorylation (32–34), remains associated with membrane vesicles during mitosis (1, 18).

On the other hand, LBR, together with the integral membrane protein Lap2, is the most obvious candidate to mediate the association of interphase nuclear membranes to chromatin (5, 19, 35, 36). The idea that the LBR complex mediates chromatin anchorage during interphase is further reinforced by the fact that lamin A has been found to interact with components of interphase chromatin (17, 37–40).

During mitosis, the nuclear envelope breaks down and the nuclear lamina is depolymerized (1, 32–34). Already, at prophase, binding of the membranous structures to chromosomes is weakened. At the end of mitosis, the first step in nuclear envelope reformation appears to be the binding of mitotic vesicles to the surfaces of chromosomes, followed by fusion of these vesicles and assembly of an envelope structure around chromatin (reviewed in Ref. 41). Taking into account that LBR is phosphorylated by the RS kinase and by $p34^{cdc2}$ protein kinase and that the major phosphorylation target of $p34^{cdc2}$ is $Ser^{71}$ (which is located near the RS repeats), one might consider that there is some cross-talk between these two phosphorylation events. An intriguing possibility would be that phosphorylation of LBR, mediated by $p34^{cdc2}$ protein kinase, together with RS phosphorylation function as a switch preventing premature membrane assembly around chromosomes. This idea is consistent with the previously reported observation that phosphorylation of Lap2 by mitotic cytosol inhibits its binding to chromosomes (5). Along these lines, it is also noteworthy that at least part of $p34^{cdc2}$ and RS protein kinase activities is associated with chromosomes.[3]

Finally, we need to note the existence of a $p34^{cdc2}$ phosphorylation site among other proteins containing an RS motif and, even more interestingly, that this site is located a few amino acids upstream of the RS repeats, as in the case of LBR (Table II). Some of these proteins have been shown to undergo RS-specific phosphorylation such as the splicing factors SC35 and SF2 (11, 26), whereas the phosphorylation status of the others remains to be examined in future studies.

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REFERENCES

1. Gerace, L., and Blobel, G. (1980) *Cell* **19,** 277–287
2. Aebi, U., Cohn, J., Buhle, L., and Gerace, L. (1986) *Nature* **323,** 560–564
3. Gerace, L., Blum, A., and Blobel, G. (1978) *J. Cell Biol.* **79,** 546–566
4. Worman, H. J., Yuan, J., Blobel, G., and Georgatos, S. D. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 8531–8534
5. Foisner, R., and Gerace, L. (1993) *Cell* **73,** 1267–1279
6. Worman, H. J., Evans, C. D., and Blobel, G. (1990) *J. Cell Biol.* **111,** 1535–1542
7. Ye, Q., and Worman, H. J. (1994) *J. Biol. Chem.* **269,** 11306–11311
8. Simos, G., and Georgatos S. D. (1992) *EMBO J.* **11,** 4027–4036
9. Courvalin, J.-C., Segil, N., Blobel, G., and Worman, H. J. (1992) *J. Biol. Chem.* **267,** 19035–19038
10. Simos, G., and Georgatos S. D. (1994) *FEBS Lett.* **346,** 225–228
11. Nikolakaki, E., Simos, G., Georgatos, S. D., and Giannakouros, T. (1996) *J. Biol. Chem.* **271,** 8365–8372
12. Simos, G., Maison, C., and Georgatos, S. D. (1996) *J. Biol. Chem.* **271,** 12617–12625
13. Krainer, A. R., Mayeda, A., Kozak, D., and Binns, G. (1991) *Cell* **66,** 383–394
14. Luo, Y., Haifeng, Y., and Peterlin, B. M. (1994) *J. Virol.* **68,** 3850–3856
15. Fridell, R. A., Harding, L. S., Bogert, H. P., and Cullen, B. R. (1995) *Virology* **209,** 347–357
16. Peter, M., Heitlinger, E., Haner, M., Aebi, U., and Nigg, E. A. (1991) *EMBO J.* **10,** 1535–1544
17. Glass, J. R., and Gerace, L. (1990) *J. Cell Biol.* **111,** 1047–1057
18. Meier, J., and Georgatos, S. D. (1994) *EMBO J.* **13,** 1888–1898
19. Maison, C., Pyrpasopoulou, A., and Georgatos, S. D. (1995) *EMBO J.* **14,** 3311–3324
20. Hoffmann, I., Clarke, P. R., Marcote, M. J., Karsenti, E., and Draetta, G. (1993) *EMBO J.* **12,** 53–63
21. Laemmli, U. K. (1970) *Nature* **227,** 680–685
22. Luo, K., Hurley, T. R., and Sefton, B. M. (1991) *Methods Enzymol.* **201,** 149–152
23. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
24. Moreno, S., and Nurse, P. (1990) *Cell* **61,** 549–551
25. Woppmann, A., Will, C. L., Kornstadt, U., Zuo, P., Manley, J. L., and Luhrmann, R. (1993) *Nucleic Acids Res.* **21,** 2815–2822
26. Gui, J-F., Lane, W. S., and Fu, X-D. (1994) *Nature* **369,** 678–682
27. Gui, J-F., Tronchere, H., Chandler, S. D., and Fu, X-D. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 10824–10828
28. Takeuchi, M., and Yanagida, M. (1993) *Mol. Biol. Cell* **4,** 247–260
29. Colwill, K., Pawson, T., Andrews, B., Prasad, J., Manley, J. L., Bell, J. C., and Duncan, P. I. (1996) *EMBO J.* **15,** 265–275
30. Mermoud, J. E., Cohen, P. T. W., and Lamond, A. I. (1994) *EMBO J.* **13,** 5679–5688
31. Reed, S. I. (1992) *Annu. Rev. Cell Biol.* **8,** 529–561
32. Heald, R., and McKeon, F. (1990) *Cell* **61,** 579–589
33. Peter, M., Nakagawa, J., Doree, M., Labbe, J. C., and Nigg, E. A. (1990) *Cell* **61,** 591–602
34. Ward, G., and Kirschner, M. (1990) *Cell* **61,** 561–577
35. Ye, Q., and Worman, H. J. (1996) *J. Biol. Chem.* **271,** 14653–14656
36. Pyrpasopoulou, A., Meier, J., Maison, C., Simos, G., and Georgatos S. D. (1996) *EMBO J.*, **15,** 7108–7119
37. Burke, B. (1990) *Exp. Cell Res.* **186,** 169–176
38. Glass, C. A., Glass, J. R., Taniura, H., Hasel, K. W., Blevitt, J. M., and Gerace, L. (1993) *EMBO J.* **12,** 4413–4424
39. Hoger, T., Krohne, G., and Kleinschmidt, J. (1991) *Exp. Cell Res.* **197,** 280–289
40. Yuan, J., Simos, G., Blobel, G., and Georgatos, S. D. (1991) *J. Biol. Chem.* **266,** 9211–9215
41. Wiese, C., and Wilson, K. L. (1993) *Curr. Opin. Cell Biol.* **5,** 387–394
42. Fu, X.-D., and Maniatis, T. (1992) *Science* **256,** 535–538
43. Carlos, S., Jutglar, L., Borrell, I., Hunt, D. F., and Ausio, J. (1993) *J. Biol. Chem.* **268,** 185–194
44. Holtzman, D. A., Yang, S., and Drubin, D. G. (1993) *J. Cell Biol.* **122,** 635–644
45. Delius, H., and Hofman, B. (1994) *Curr. Top. Microbiol. Immunol.* **186**, 13–31
46. Oellig, G., Happ, B., Mueller, T., and Doerfler, W. (1987) *J. Virol.* **61,** 3048–3057

[2] G. Simos and S. D. Georgatos, unpublished observations.

[3] E. Nikolakaki, S. D. Georgatos, and T. Giannakouros, unpublished observations.

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