Characterization of A 54-kD Protein of the Inner Nuclear Membrane: Evidence for Cell Cycle–dependent Interaction with the Nuclear Lamina

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Abstract. Using a mAb (R-7), we have characterized a 54-kD protein of the chicken nuclear envelope. Based on its biochemical properties and subnuclear distribution p54 is likely to be an integral membrane component specific to the inner nuclear membrane. Fractionation experiments indicate that p54 interacts, directly or indirectly, with the nuclear lamina, and analysis of p54 in cultured cells suggests that this interaction is controlled by cell cycle-dependent post-translational modification, most likely phosphorylation. Modification of p54 results in a slightly reduced electrophoretic mobility, and it converts the protein from a detergent-resistant to a detergent-extractable form. Detergent solubilization of p54 can be induced in vivo by treating isolated nuclei or nuclear envelopes with highly purified cdc2 kinase, one of the most prominent kinases active in mitotic cells. These results suggest that mitotic phosphorylation of p54 might contribute to control nuclear envelope dynamics during mitosis in vivo.

THE nuclear envelope is a double membrane system, fenestrated by nuclear pore complexes (for reviews see Franke et al., 1981; Gerace and Burke, 1988; Kessel, 1988; Nigg, 1989; Burke, 1990). It controls all exchange of macromolecules between the nucleus and the cytoplasm. The outer nuclear membrane is continuous with the ER; it is studded with ribosomes and active in protein synthesis. The inner nuclear membrane is lined at its nucleoplasmic surface by the nuclear lamina, a karyoskeletal structure composed of intermediate filament-type proteins, the lamins (Aebi et al., 1986; Franke, 1987). The structure, biogenesis and cell cycle dynamics of the nuclear lamina have been elucidated in considerable detail, and substantial progress has been made also toward a molecular analysis of the nuclear pore complex (for reviews see Newport and Forbes, 1987; Gerace and Burke, 1988; Nigg, 1988, 1989; Burke, 1990). In contrast, much remains to be learned about the composition and properties of the nuclear membranes per se. In particular, one would like to know how the nuclear lamina is associated with the nuclear envelope, and how the reversible disassembly and reassembly of the nuclear membranes is controlled during mitosis.

Recently, a number of nuclear envelope proteins have been identified which do not form part of either the pores or the lamina (Senior and Gerace, 1988; Worman et al., 1988, 1990; Padan et al., 1990). A mAb was shown to recognize three proteins of 75, 68, and 55 kD in rat liver nuclear envelopes, but only the 55-kD protein in a variety of tissue culture cells (Senior and Gerace, 1988). These proteins were shown to be integral components specific to the inner nuclear membrane, and biochemical evidence strongly suggested that they might interact, directly or indirectly, with the nuclear lamina (see also Powell and Burke, 1990). Independently, a 58-kD protein (p58) was characterized in avian nuclear envelopes (Worman et al., 1988). Based on a specific interaction with lamin B (but not lamin A) in a filter-binding assay, p58 was proposed to function as a lamin B–specific nuclear envelope receptor. More recently, p58 was shown to be a substrate for cAMP-dependent protein kinase, and phosphorylation was reported to strengthen in vitro binding to lamin B (Appelbaum et al., 1990). A cDNA was cloned based on partial sequence information obtained from purified p58 (Worman et al., 1990); this cDNA predicts a 73-kD protein with seven to eight potential transmembrane domains. (The discrepancy between the predicted and the observed molecular weights is attributed to aberrant migration of p58 on SDS gels.) Whether or not the avian p58 is structurally related to the mammalian 55-kD protein described by Senior and Gerace (1988) is not yet established. Finally, a 53-kD inner nuclear membrane protein, called otefin, has been characterized in *Drosophila* (Harel et al., 1989). According to its primary structure otefin contains at most one potential membrane anchoring domain (Padan et al., 1990), and is clearly distinct from p58.

The mAb R-7 described here was identified in the course of screening hybridoma cell lines for production of antibodies against novel nuclear envelope antigens. It specifically

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recognizes a 54-kD protein (p54) of the chicken nuclear envelope. We show that p54 represents an integral component of the inner nuclear membrane, and interacts, directly or indirectly, with the nuclear lamina. Finally, we present evidence suggesting that the interaction between p54 and the nuclear lamina is under control of cell cycle–dependent phosphorylation, possibly involving cdc2 kinase.

Materials and Methods

Antibodies and Cell Culture

Production and characterization of mAbs was carried out as described in detail by Lehner et al. (1986*a*). The mAb R-7 was obtained from a BALB/c mouse immunized intraperitoneally with a pore complex-lamina preparation prepared from livers of 18-d-old chicken embryos according to Dwyer and Blobel (1976), as modified by Lehner et al. (1986*b*). As determined by Ouchterlony double diffusion and a dot blot assay (Beyer, 1984), the mAb R-7 is of the IgG_1 class. The mAbs L3-4B4 (anti-lamin A) and E-3 (anti-lamin B_2) as well as the rabbit serum recognizing both chicken nuclear lamins A and B_2 have been characterized previously (Lehner et al., 1986*b*; Stick et al., 1988). Rabbit antibodies specific for proteins of the rough ER (Louvard et al., 1982) were kindly provided by B. Dobberstein (EMBL Heidelberg, Germany).

PAI myeloma cells and hybridoma clones were grown according to Lehner et al. (1986*a*), and the chicken erythroblast cell line HD3 (Graf et al., 1978) was cultured as described by Beug et al. (1982). Fibroblasts were prepared from 8–10-d-old chicken embryos and cultured as described (Borer et al., 1989). The conditions for culture and cell cycle synchronization of chicken DU249 hepatoma cells have also been described previously (Nakagawa et al., 1989).

Preparation of Nuclei and Subnuclear Fractionation

Nuclei from 3–18-d-old chick embryos were prepared as described previously (Lehner et al., 1987). Subfractionation of embryonic (18 d) chicken liver nuclei was carried out according to Dwyer and Blobel (1976) and Snow et al. (1987), with the following minor modifications: Nuclei (50-mg protein) were washed with TKM (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2.5 mM KCl, 1 mM PMSF, 1% Trasylol (Bayer, Leverkusen, Germany), 3 mM β-mercaptoethanol) and resuspended on a vortex by dropwise addition of 5 ml of ice cold 0.1 mM $MgCl_2$. Then, 25 μl each of DNase I and RNase A stock solutions (1 mg/ml in 10 mM Tris-HCl, pH 7.5, 0.1 mM $MgCl_2$, 10% sucrose) were added, followed by rapid addition of 20 ml digestion buffer 1 (DB1; 10 mM Tris-HCl, pH 8.5, 0.1 mM $MgCl_2$, 10% sucrose, 1 mM PMSF, 1% Trasylol, 3 mM β-mercaptoethanol)[1]. After digestion for 15 min at room temperature, the nuclear residues were pelleted (20,000 *g* for 10 min) and resuspended in 5 ml of digestion buffer 2 (DB2; 10 mM Tris-HCl, pH 7.5, 0.1 mM $MgCl_2$, 10% sucrose, 1 mM PMSF, 1% Trasylol, 3 mM β-mercaptoethanol). Additional 25-μl aliquots of DNase I and RNase A stock solutions were added and digestion was repeated for 15 min at room temperature. The crude nuclear envelopes were then pelleted (20,000 *g* for 10 min) and resuspended in DB2 containing 0.5 M NaCl. After a 10-min incubation on ice, the salt-washed nuclear envelopes were pelleted (10 min at 20,000 *g*) and resuspended in either DB2 containing 2% Triton X-100, DB2 containing 2% Triton X-100 and 0.5 M NaCl or HS/Triton X-100 (20 mM Mes-KOH, pH 6.0, 300 mM KCl, 2 mM EDTA, 2% Triton X-100, 10% sucrose, 1 mM PMSF, 1% Trasylol, 3 mM β-mercaptoethanol; Snow et al., 1987). In all cases, nuclear envelopes were extracted for 30 min on ice and centrifuged for 10 min at 20,000 *g*. To distinguish between integral and peripheral membrane proteins, salt-washed nuclear envelopes were incubated for 5 min on ice in either 4 M guanidine-HCl or 0.1 M NaOH. They were loaded over a 30% sucrose cushion and centrifuged for 45 min in a rotor (SW-40; Beckman Instruments, Inc., Palo Alto, CA) (30,000 rpm at 4°C). Alternatively, they were extracted for 5 min at room temperature with 8 M urea and sedimented (17,000 *g* for 10 min).

Preparation of Microsomal Fractions

Microsomal fractions were prepared from livers of 18-d-old chicken embryos, using a differential centrifugation procedure similar to the one described by Schenkman and Cinti (1978). Briefly, the livers of five chicken embryos were washed once in TM (5 mM Tris-HCl, pH 7.5; 1 mM $MgCl_2$, 1 mM PMSF, 1% Trasylol, 3 mM β-mercaptoethanol) and once in TM containing 0.25 M sucrose. After addition of an equal volume of the latter buffer, a homogenate was prepared in a Potter-Elvehjehm homogenizer and filtered three times through three layers of a nylon filter (110 mesh). Nuclei were pelleted by centrifugation in a rotor (HB4; Sorvall Instruments, Newton, CT) for 10 min at 600 *g*. The supernatant was removed and centrifuged a second time for 10 min at 10,000 *g* to yield a postmitochondrial supernatant. Microsomes were then pelleted by centrifugation in a Beckman SW27 rotor for 90 min at 105,000 *g* and then washed once in TM (25,000 *g* for 15 min). Proteins were precipitated by addition of 0.5 vol of 50% TCA and incubation for 60–90 min on ice, washed with 10% TCA, followed by acetone, and processed for SDS-PAGE.

Cell Fractionation

Subcellular fractionation of interphase and mitotic DU249 cells was done as described by Stick et al. (1988). To obtain the particulate fractions of mitotic cells used in the dephosphorylation experiments, nocodazole-treated DU249 cells were collected by mechanical shake off and washed in ice-cold PBS (138 mM NaCl, 4 mM KCl, 1.5 mM KH_2PO_4, 6 mM Na_2HPO_4, pH 7.1). Cells were resuspended in TKM and incubated for 10 min. Subsequently, they were broken using a Dounce homogenizer and the particulate fraction was sedimented (17,000 *g* for 10 min at 4°C). The pellet was washed once in TKM and resuspended in dephosphorylation buffer (50 mM Tris-HCl, pH 8.0, 1 mM $ZnCl_2$, 1 mM $MgCl_2$, 1 mM PMSF, 1% Trasylol). Aliquots were then incubated in the presence or absence of 50 U of bacterial alkaline phosphatase (500 U/1.6 ml; Sigma Chemical Co., St. Louis, MO). After 1 h at room temperature the particulate fractions were sedimented, washed once with TKM to remove excess enzyme, and processed for SDS-PAGE.

In Vitro Solubilization of p54 by Incubation with cdc2 Kinase

Nuclei were prepared from 7-d-old chicken embryos, as described above, and in vitro disassembly assays were performed essentially as described previously (Peter et al., 1990*a*). In brief, $\sim 5 \times 10^7$ nuclei/ml were resuspended in kinase assay buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 6 mM EGTA, 1 mM ATP, 1 mM PMSF, 1% Trasylol). Reactions were carried out at 30°C in final volumes of 50 μl; they were started by addition of cdc2 kinase (1 μl; 300 pMol/μl/min). The kinase used in these experiments was obtained from a 70% ammonium sulfate precipitate after the P11-phosphocellulose step of the previously described procedure (Labbé et al., 1989). After a 90-min incubation, nuclei were treated for 10 min on ice with 0.5% Triton X-100 and centrifuged (15,000 *g*) for 10 min at 4°C. Pellets and supernatants were taken up in gel sample buffer and analyzed by SDS-PAGE. The distribution of p54 was then analyzed by immunoblotting.

Gel Electrophoresis and Immunoblotting

For gel electrophoresis, proteins were solubilized by incubation in gel sample buffer for 20 min at room temperature. This procedure was chosen because of a pronounced tendency of p54 to aggregate during boiling in gel sample buffer. Proteins were then resolved on discontinuous SDS–polyacrylamide gels according to Laemmli (1970). Immunoblotting was carried out as described previously (Lehner et al., 1987), except that 5% milk powder was used instead of 3% BSA for preparation of the blocking buffer. Primary antibodies were hybridoma supernatants (undiluted), ascites fluids (diluted 1:1,000 in blocking buffer), or rabbit serum (diluted 1:250 in blocking buffer). Peroxidase-coupled secondary reagents (Medac and Nordic) were diluted 1:1,000 in blocking buffer. In some experiments, mouse IgG were detected using ^{125}I-labeled sheep secondary antibodies (Amersham Corp., Arlington Heights, IL), diluted to 0.1 μCi/ml in blocking buffer.

Indirect Immunofluorescence and Immunoelectron Microscopy

Immunofluorescent staining of cultured cells was carried out as described previously (Lehner et al., 1987). For staining of cryosections, small pieces of tissues from 18-d-old chicken embryos were fixed for 2 h in PBS containing 3% formaldehyde and 2% sucrose and washed three times for 5 min in PBS. After overnight perfusion with 2.3 M sucrose in PBS, the tissue blocks were frozen in liquid nitrogen and sectioned (0.8 μm) using an

1. *Abbreviations used in this paper*: DBl–2, digestion buffer 1–2; GuHCl, guanidine hydrochloride.

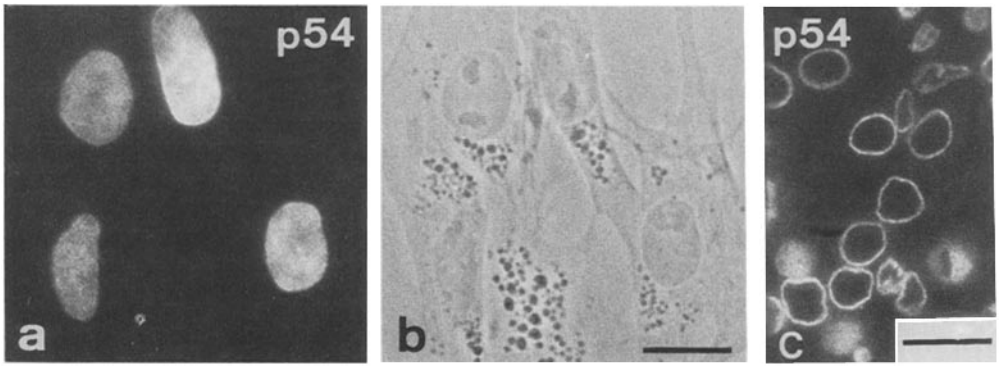

Figure 1. Immunofluorescence localization of chicken p54. (*a* and *b*) Chick embryo fibroblasts were grown on coverslips, fixed with formaldehyde, and permeabilized with Triton X-100. They were then incubated with R-7 (anti-p54) hybridoma supernatant (undiluted), followed by rhodamine-conjugated goat anti-mouse IgG. (*c*) Cryosection of an embryonic liver (prepared as described in Materials and Methods) was labeled with R-7 antibody. Note exclusive staining of the nuclear periphery. Bars: (*b*) 20 μm; (*c*) 10 μm.

ultramicrotome (Reichert-Jung, Vienna). Sections were treated for 5 min at room temperature with 0.5% Triton X-100 in PBS, washed three times with PBS, and then incubated with antibodies. Hybridoma supernatants were used undiluted, whereas ascites fluids were diluted 1:1,000 and the rabbit serum against chicken lamins A and B_2 was diluted 1:300. Secondary antibodies were rhodamine-conjugated goat anti-mouse IgG or fluorescein-conjugated goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA). diluted 1:300 in PBS. Fluorescence microscopy was performed with a microscope (model 18; Zeiss, Oberkochen, Germany) equipped with a Planapo ×63 oil immersion objective and filters (Zeiss) for rhodamine and fluorescein excitation.

Immunoelectron microscopic experiments on cryosections were done exactly as described (Griffiths et al., 1983, 1984). For these studies, ascites fluids were diluted 1:200 in the case of L3-4B4 and 1:150 in the case of R-7.

Results

Identification of p54, A Protein Component of the Inner Nuclear Membrane

The mAb R-7 was isolated while screening hybridoma supernatants for antibodies that recognize nuclear envelope proteins. As shown below, this mAb reacts with a single protein of 54 kD (hereafter called p54) in a variety of chicken tissues and cell types. By indirect immunofluorescence microscopy, the R-7 antibody specifically stained the nucleus of chick embryo fibroblasts (Fig. 1 *a*). Staining was homogeneous and virtually indistinguishable from that produced by anti-lamin antibodies (not shown), except that the intensity of R-7 staining was generally weaker and varied considerably among different cells (Fig. 1 *a*). By appropriate focusing of the microscope, a pronounced rim staining of the nuclear periphery could be seen (not shown), indicating that the R-7 antigen might be located preferentially, or exclusively, at the nuclear envelope. This notion was confirmed by immunofluorescence microscopy on cryosections prepared from embryonic liver tissue. As shown in Fig. 1 *c*, labeling by R-7 antibody was restricted to the nuclear envelope and completely absent from either the nucleoplasm or the cytoplasm.

To confirm the above immunofluorescence data, the subcellular distribution of p54 was determined by biochemical analysis. Liver tissue of 18-d-old chicken embryos was fractionated to enrich for proteins of nuclei, nuclear envelopes, and microsomal membranes, respectively (Fig. 2 *A*). By immunoblotting, the R7 antibody recognized a single 54-kD protein which was present in nuclei (Fig. 2 *B*, lane *2*) and nuclear envelopes (Fig. 2 *B*, lane *3*) but not in microsomal fractions (Fig. 2 *B*, lane *1*). An identical fractionation pattern was seen when probing parallel samples with antibodies against lamin B_2 (Fig. 2 *B*, lanes *4–6*). Conversely, a rabbit serum raised against the ER of dog pancreas (Louvard et al., 1982) reacted with two proteins of 58 and 91 kD that were present in microsomal membranes (Fig. 2 *B*, lane *7*) but virtually undetectable in whole nuclei (Fig. 2 *B*, lane *8*). Since the outer nuclear membrane is continuous with the ER, the absence of p54 in the microsomal fraction strongly suggests that this protein is specific to the inner nuclear membrane.

The above interpretation is supported and extended by the results of immunoelectron microscopy (Fig. 3). By indirect immunogold labeling of whole cell cryosections (Griffiths et al., 1983, 1984), p54 was found to be specifically located in close proximity to the inner nuclear membrane (Fig. 3, *A* and *B*). Labeling was fairly uniform and no specific associations with nuclear pores could be detected (Fig. 3 *B*). The distribution of lamin A, stained for control, was similar, although gold particles were more numerous and appeared to be scattered somewhat further toward the nuclear interior (Fig. 3 *C*).

Evidence for Interaction of p54 with the Nuclear Lamina

To obtain more detailed information on the mode of membrane association of p54, its properties were examined by biochemical fractionation of nuclei and extraction of nuclear envelopes by various procedures. In a first series of experiments (Fig. 4), chromatin was released from nuclei by nuclease treatment; the resulting crude nuclear envelopes were washed and extracted further with non-ionic detergent (Triton X-100). Aliquots of the various fractions were then analyzed by SDS-PAGE and stained with Coomassie blue (Fig. 4 *A*) or subjected to immunoblotting using mAbs R-7 (anti-p54; Fig. 4 *B*) or E-3 (anti-lamin B_2; Fig. 4 *C*). As expected, p54 was recovered quantitatively in the crude envelope fraction when chromatin and ribonucleoprotein particles were released from nuclei by digestion with nucleases (Fig. 4 *B*, lane *2'*). The R-7 antigen also remained associated with nuclear envelopes after washing with buffer containing 0.5 M

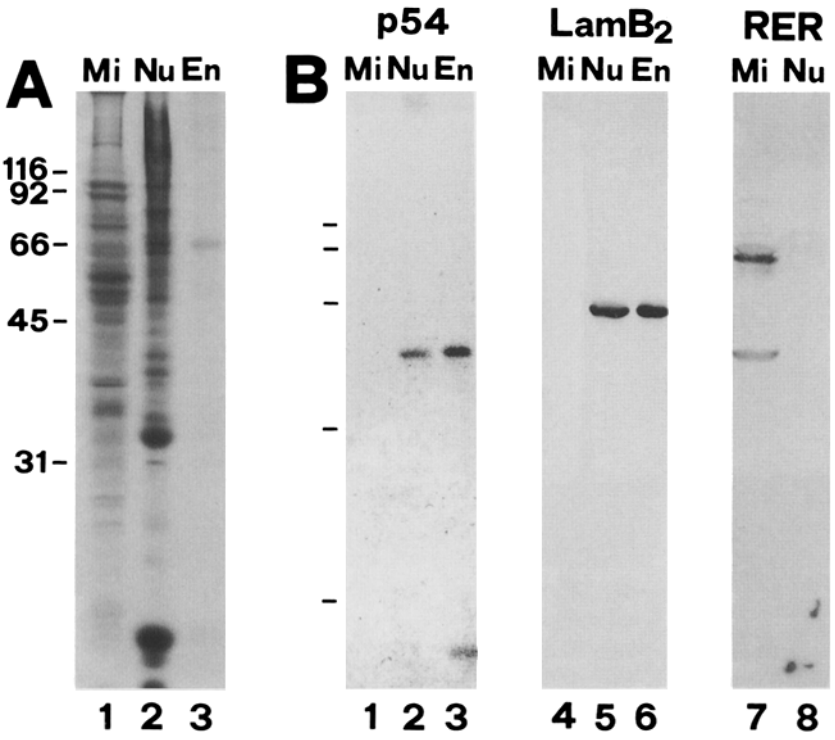

Figure 2. Biochemical analysis of the subcellular distribution of p54. Liver tissue prepared from 18-d chicken embryos was fractionated into microsomal (*Mi*), nuclear (*Nu*), and nuclear envelope (*En*) preparations (see Materials and Methods). Proteins were separated by SDS-PAGE (*A*, 12%; *B*, 10% polyacrylamide) and either stained with Coomassie blue (*A*) or transferred to nitrocellulose (*B*). Filters were probed with the mAbs R-7 (anti-p54, lanes *1–3*), E-3 (anti-lamin B_2, lanes *4–6*), or a rabbit serum specific for proteins of the ER (anti-RER, lanes *7* and *8*). Molecular masses of marker proteins are indicated (from top to bottom: β-galactosidase, 116 kD; phosphorylase B, 92 kD; BSA, 66 kD; ovalbumin, 45 kD; carbonic anhydrase, 31 kD).

NaCl (Fig. 4 *B*, lane *3'*) and it largely resisted extraction with 2% Triton X-100 (Fig. 4 *B*, lanes *4'* and *5'*). Under all of the above conditions, lamin B_2 fractionated in parallel with p54 (Fig. 4 *C*).

In contrast, separation of p54 from lamin proteins could be induced by extracting nuclear envelopes with 4 M guanidine hydrochloride (GuHCl), 8 M urea, or 0.1 N NaOH (Fig. 5). Whereas the lamins were solubilized by all of these reagents (Fig. 5 *B*), consistent with their peripheral association with the inner nuclear membrane, p54 was recovered almost quantitatively from the particulate fractions (Fig. 5 *A*). While these criteria indicate that p54 is an integral membrane protein, its resistance to extraction by Triton X-100 (Fig. 4 *B*) suggests that p54 interacts not only with the lipid bilayer but also with a component of the nuclear envelope that resists extraction by non-ionic detergent. An obvious candidate for this component is the nuclear lamina. To test this possibility, nuclear envelopes were extracted with various combinations of non-ionic detergent and high salt (Fig. 5 *C*). Whereas DB2 (see Materials and Methods) supplemented with 2% Triton X-100 and 0.5 M NaCl solubilized both lamin B_2 and p54 (Fig. 5 *C*; NaCl/T), the use of the high salt/Triton X-100 buffer described by Snow et al. (1987) led to solubilization of p54 but not lamin B_2 (Fig. 5 *C*; KCl/T). Thus, whereas two different combinations of Triton X-100 with high salt resulted in extraction of p54, only one of these procedures allowed extraction of lamin B_2. According to these operational criteria, lamin–lamin interactions would appear to be stronger than those between p54 and lamin proteins, a notion supported also by the results shown below (see Fig. 10).

Developmental Expression of p54

The above results strongly suggested that p54 might interact, directly or indirectly, with the nuclear lamina. With the hope of determining which lamin isoform(s) might play a role in anchoring p54, we compared the expression of p54 with that of individual lamin proteins in various cell types and tissues. In particular, we took advantage of the earlier observation that A- and B-type lamins show differential expression during avian and mammalian development (Lehner et al., 1987; Stewart and Burke, 1987; Röber et al., 1989, 1990). Nuclei were prepared from various parts of 3 to 18-d-old chicken embryos, and equal amounts of nuclear proteins were resolved by SDS-PAGE. Coomassie blue staining of histones was used to control for equal loading of gel lanes (not shown). Duplicate gels were then subjected to immunoblotting analyses, using antibodies specific for p54 (Fig. 6 *A*), lamin B_2 (Fig. 6 *B*), and lamin A (Fig. 6 *C*), respectively. In these experiments, lamin B_2 can be considered as a marker also for the second B-type lamin (B_1); as shown previously, expression of lamin B_1 largely parallels that of lamin B_2 (Lehner et al., 1987). In most samples and throughout the developmental period investigated, expression of p54 was similar to that of lamin B_2 and levels of both proteins were relatively constant (compare Fig. 6, *A* and *B*). However, we note that amounts of p54 were significantly lower in brain than in liver tissues (Fig. 6 *A*, compare lanes *6, 8,* and *10* to lanes *7, 9,* and *11*). In this respect, expression of p54 parallels the expression of lamin A which also appears in brain much later than in liver (Fig. 6 *C*). In contrast to p54, however, lamin A is not expressed in early embryos (Fig. 6 *C*; see also Lehner et al., 1987).

By immunoblotting (Fig. 7) and immunofluorescent staining of cryosections (not shown) we also analyzed the expression of p54 in nuclei isolated from 18-d embryonic brain, liver, heart, skeletal muscle, and erythrocytes. As shown by Coomassie blue staining similar amounts of total protein were analyzed for each tissue (Fig. 7 *A*). These experiments

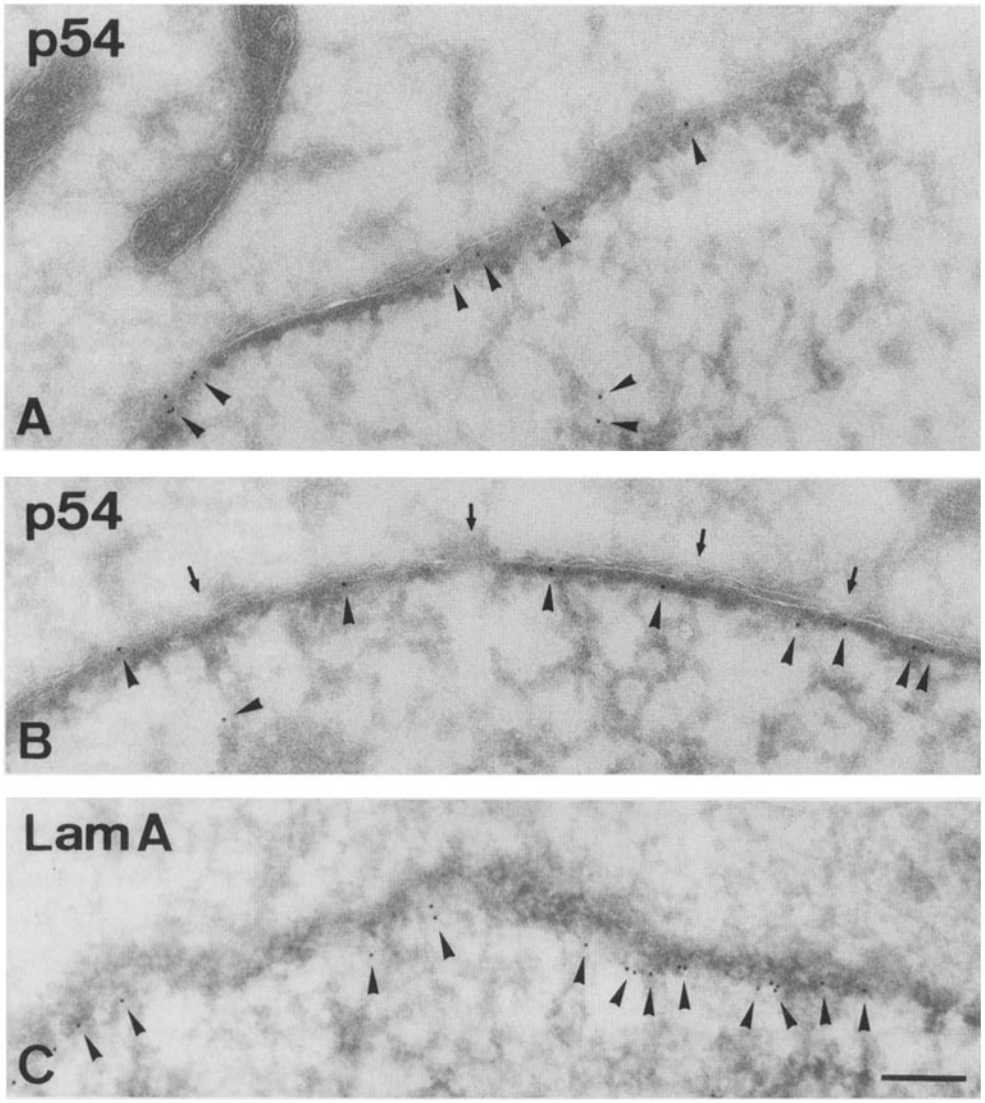

Figure 3. Immunoelectron microscopic localization of p54. Ultrathin cryosections were prepared of chicken erythroblasts (line HD3 AGts34 AEV (Graf et al., 1978)) and incubated with the mAbs R-7 (anti-p54) (*A* and *B*) or L3-4B4 (anti-lamin A) (*C*), followed by rabbit anti-mouse IgG antibodies and gold-conjugated (9 nm) protein A. For better visualization, each gold particle is marked by an arrowhead. The arrows in *B* point to nuclear pores. Bar, 200 nm.

show that expression of p54 (Fig. 7 *B*) did not parallel expression of lamin B_2 (Fig. 7 *C*). For instance, erythrocytes expressed large amounts of p54, as well as lamin A (Lehner et al., 1987), but only low amounts of lamin B_2 (Fig. 7, *B* and *C*, lane *1'*). Conversely, heart and skeletal muscle nuclei contained comparatively large amounts of lamin B_2 but very little p54 (Fig. 7, *B* and *C*, lanes *4'* and *5'*). Based on the results shown in Figs. 6 and 7 we conclude that expression of p54 is not correlated with the expression of any particular lamin isoform. Instead, the data suggest that p54 might interact with both A- and B-type lamins.

Distribution of p54 during Mitosis

In view of the dramatic changes in the structure of the nuclear envelope that are characteristic of mitosis in higher eukaryotes, it was of interest to compare the distribution of p54 and lamins A and B_2 at various stages of fibroblast division. To this end, mitotic cells were examined by double immunofluorescence microscopy, using antibodies for p54 and lamins A/B_2 (Fig. 8). During mitotic prophase, staining of the nuclear periphery by either antibody became progressively less pronounced, concomitant with fragmentation of the nuclear envelope (not shown). During metaphase, both p54 and lamins were distributed similarly throughout the cells (Fig. 8, *a*, *b*, and *c*), but in telophase p54 reassembled at the nuclear periphery before the reassociation of the lamins with the nuclear envelope was complete (Fig. 8, *d*, *e*, and *f*).

Evidence for Mitotic Phosphorylation of p54

Although R-7 antibodies reacted with a single 54-kD protein in all tissues analyzed (see above), we frequently observed a doublet of closely spaced bands (at 54 and 55 kD) when carrying out immunoblotting experiments on lysates of cultured cells (see, for example, Fig. 9 *A*, lane *1*). One possible explanation for the appearance of the slower migrating band was modification of p54 by a posttranslational mechanism, particularly phosphorylation. To test the idea that such a hypothetical modification might be related to cell cycle progression, we compared the electrophoretic mobility of the R-7 antigen in interphase and mitotic (i.e., M phase arrested)

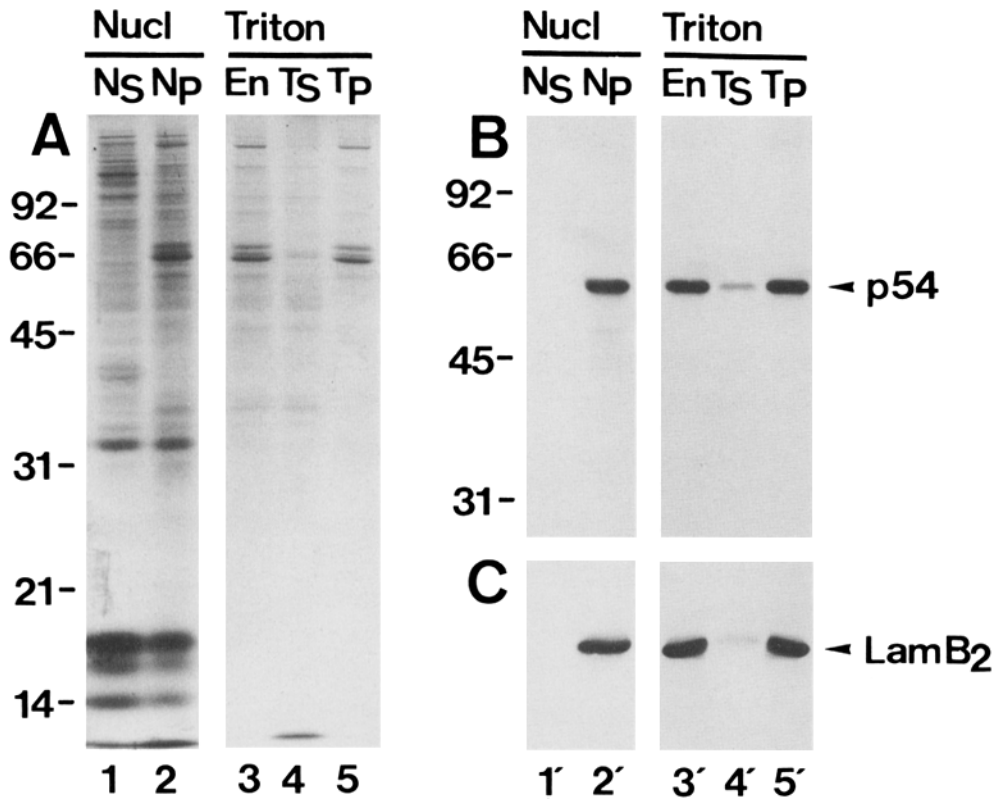

Figure 4. Nuclear envelope association of p54. Nuclei were prepared from livers of 18-d old chicken embryos. After treatment with DNase I and RNase A, crude nuclear envelopes (N_P; lanes *2* and *2'*) were separated by centrifugation from the bulk of nuclease-sensitive components (N_S, lanes *1* and *1'*). Envelopes were further washed with 0.5 M NaCl (*En*, lanes 3 and 3') and extracted with 2% Triton X-100 to separate detergent-soluble (T_S, lanes 4 and 4') and insoluble (T_P, lanes *5* and *5'*) fractions. Proteins were separated by SDS-PAGE (*A*, 12%; *B* and *C*, 8% polyacrylamide) and either stained with Coomassie blue (*A*) or transferred to nitrocellulose and probed by immunoblotting (*B* and *C*). Filters were analyzed for the distribution of p54 (using mAb R-7; *B*) and lamin B_2 (using mAb E-3; *C*). Molecular masses of marker proteins are indicated (from top to bottom: phosphorylase B, 92 kD; BSA, 66 kD; ovalbumin, 45 kD, carbonic anhydrase, 31 kD; trypsin inhibitor, 21 kD; lysozyme, 14 kD).

cells. As shown in Fig. 9 *A*, a doublet of closely spaced immunoreactive bands was seen in interphase cells, but only one band, comigrating with the upper band of the interphase doublet, was seen in M phase–arrested cells (lanes *1* and *2*).

To investigate the biochemical properties of p54 during mitosis in more detail, interphase (I) and M phase–arrested (M) DU249 cells were fractionated in the presence or absence of non-ionic detergent (Fig. 9 *B*). When hypotonic homogenization was carried out in the absence of Triton X-100, both forms of the R-7 antigen present in interphase cells sedimented with the particulate fraction (Fig. 9 *B*, compare lanes *2* and *3*). Remarkably, however, the two forms were separable upon addition of Triton X-100 in that the 54 kD form still sedimented (Fig. 9 *B*, lane *5*) but the 55-kD variant was completely solubilized (Fig. 9 *B*, lane *4*). In homogenates of M phase–arrested cells only one band (corresponding to the upper band of the interphase doublet) could be detected (Fig. 9 *B*, lane *6*; see also Fig. 9 *A*, lane *2*). After

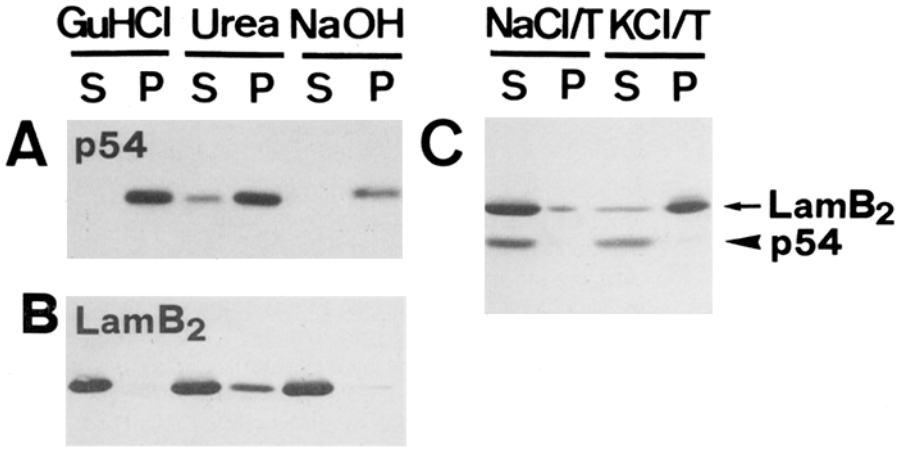

Figure 5. Solubility properties identify p54 as an integral membrane protein. Salt-washed nuclear envelopes were prepared from nuclei of 18-d chicken livers as described in the legend to Fig. 4. These were then extracted (*A* and *B*) with 4 M guanidine-HCl (*GuHCl*), 8 M urea, or 0.1 M NaOH, and centrifuged to yield supernatants (*S*), containing peripheral membrane proteins, and pellets (*P*), containing integral membrane proteins. Salt-washed envelopes were treated also (*C*) with either 0.5 M NaCl/2% Triton X-100 in DB2 (see Materials and Methods) (*NaCl/T*) or HS/Triton X-100 buffer (Snow et al., 1987) (*KCl/T*), and fractionated by centrifugation in soluble (*S*) and particulate components (*P*). Proteins were separated by 8% SDS-PAGE and analyzed by immunoblotting, using mAbs R-7 (anti-p54, *A*) and E-3 (anti-lamin B_2, *B*) or both antibodies together (*C*).

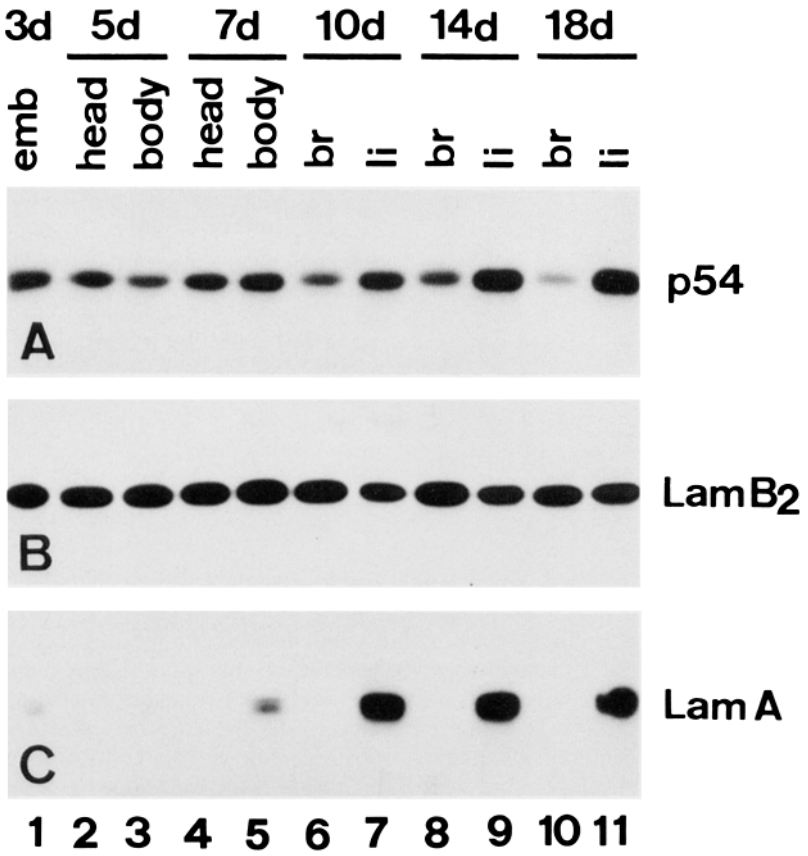

Figure 6. Developmental expression of p54. Nuclei were isolated from various tissues at different developmental stages of chicken embryogenesis. Nuclear proteins were resolved by SDS-PAGE and either stained with Coomassie blue to assess protein loading (not shown) or transferred to nitrocellulose and probed by immunoblotting. The following mAbs were used: *A*, R-7 (anti-p54); *B*, E-3 (anti-lamin B_2); *C*, L3-4B4 (anti-lamin A). Nuclei were prepared from 3-d (*3d*) embryos (lanes *1*); 5-d embryos, head (lanes *2*); 5-d embryos, body (lanes *3*); 7-d embryos, head (lanes *4*); 7-d embryos, body (lanes *5*); 10-d embryos, brain (lanes *6*); 10-d embryos, liver (lanes *7*); 14-d embryos, brain (lanes *8*); 14-d embryos, liver (lanes *9*); 18-d embryos, brain (lanes *10*); 18-d embryos, liver (lanes *11*). Only the relevant parts of the immunoblots are shown.

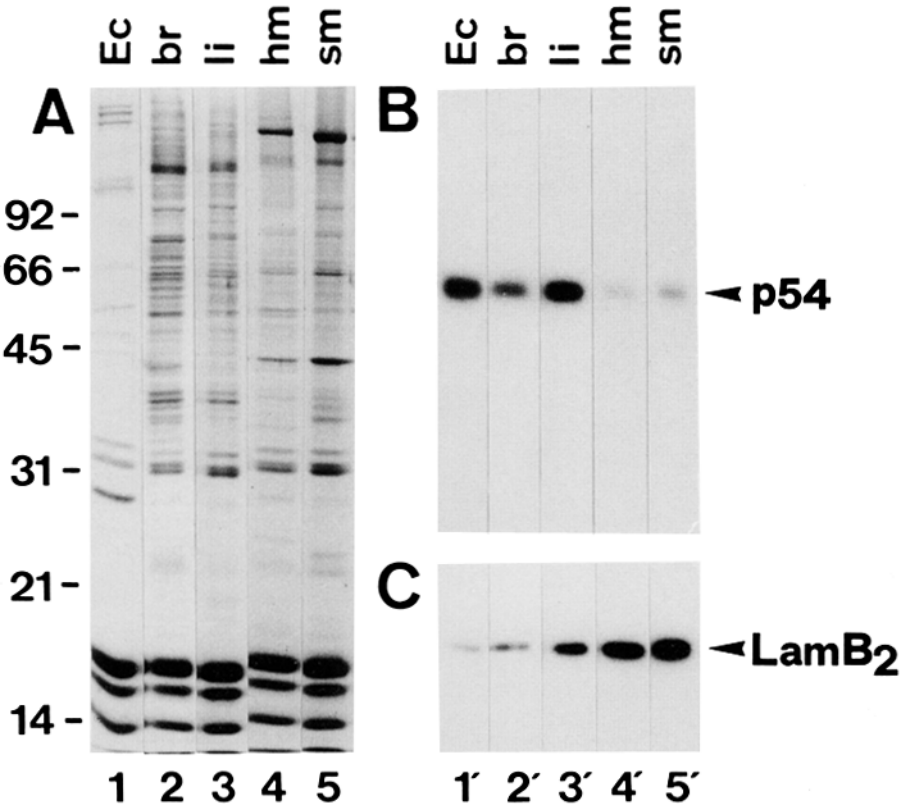

Figure 7. Comparison of the expression of p54 and lamin B_2 in selected chicken embryonic tissues. Nuclei were prepared from tissues of 18-d old chicken embryos. Proteins of isolated nuclei and whole (lysed) erythrocytes were resolved by SDS-PAGE (*A*, 12%; *B* and *C*, 8% polyacrylamide) and either stained by Coomassie blue (*A*) or transferred to nitrocellulose (*B* and *C*). Filters were probed with mAbs R-7 (*B*, anti-p54) or E-3 (*C*, anti-lamin B_2). Shown are erythrocytes (lanes *1* and *1′*) and nuclei isolated from brain (lanes *2* and *2′*), liver (lanes *3* and *3′*), heart (lanes *4* and *4′*), and skeletal muscle (lanes *5* and *5′*). Marker proteins are described in the legend to Fig. 4.

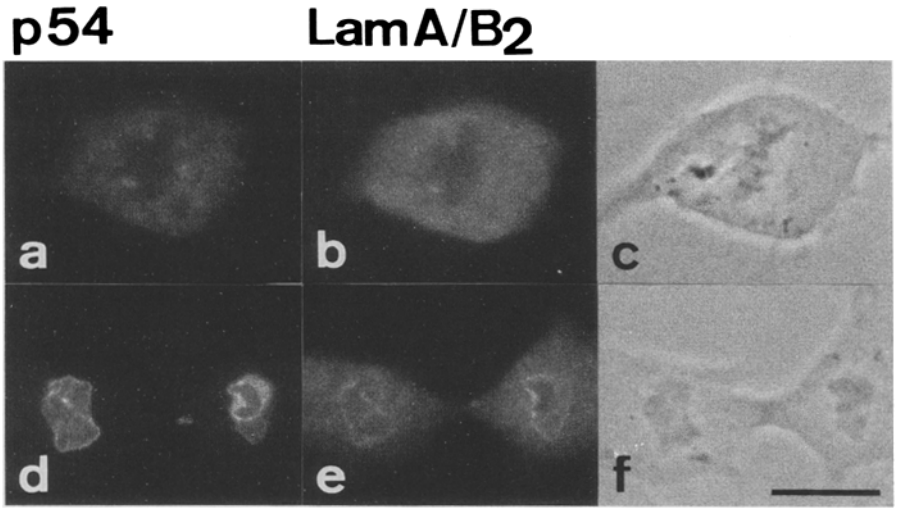

Figure 8. Mitotic distribution of p54. Chicken embryo fibroblasts were grown on coverslips, fixed with formaldehyde, and permeabilized with Triton X-100. Double immunofluorescent staining was done using mAb R-7 (anti-p54, *a* and *d*) and a rabbit serum recognizing both lamins A and B_2 (Lehner et al., 1986*b*; *b* and *e*). Secondary reagents were rhodamine-conjugated anti-mouse IgG antibodies and fluorescein-conjugated anti-rabbit IgG antibodies. Coverslips were searched for spontaneously occurring mitotic cells. *a–c*, metaphase cell; *d–f*, telophase cell. *c* and *f* represent the phase contrast micrographs corresponding to *a/b* and *d/e*, respectively. Bar, 10 μm.

homogenization in the absence of detergent this protein was found in the particulate fraction (Fig. 9 *B*, lanes *7* and *8*), but it could readily be solubilized by addition of detergent (Fig. 9 *B*, lanes *9* and *10*). As shown previously (Stick et al., 1988), and confirmed in the present study (not shown), lamin B_2 displayed a mitotic fractionation behavior indistinguishable from that of the R-7 antigen.

The above results strongly suggested a correlation between the sensitivity of a modified form of the R-7 antigen to detergent extraction and the reduced mobility of this form in SDS-gels. To test the possibility that the slower migrating form of the R-7 antigen might be generated by phosphorylation, membrane fractions were prepared from nocodazole-arrested DU249 cells and incubated in the presence or absence of bacterial alkaline phosphatase (Fig. 9 *C*). Compared to envelopes that were kept on ice (Fig. 9 *C*, lane *1*) or in-

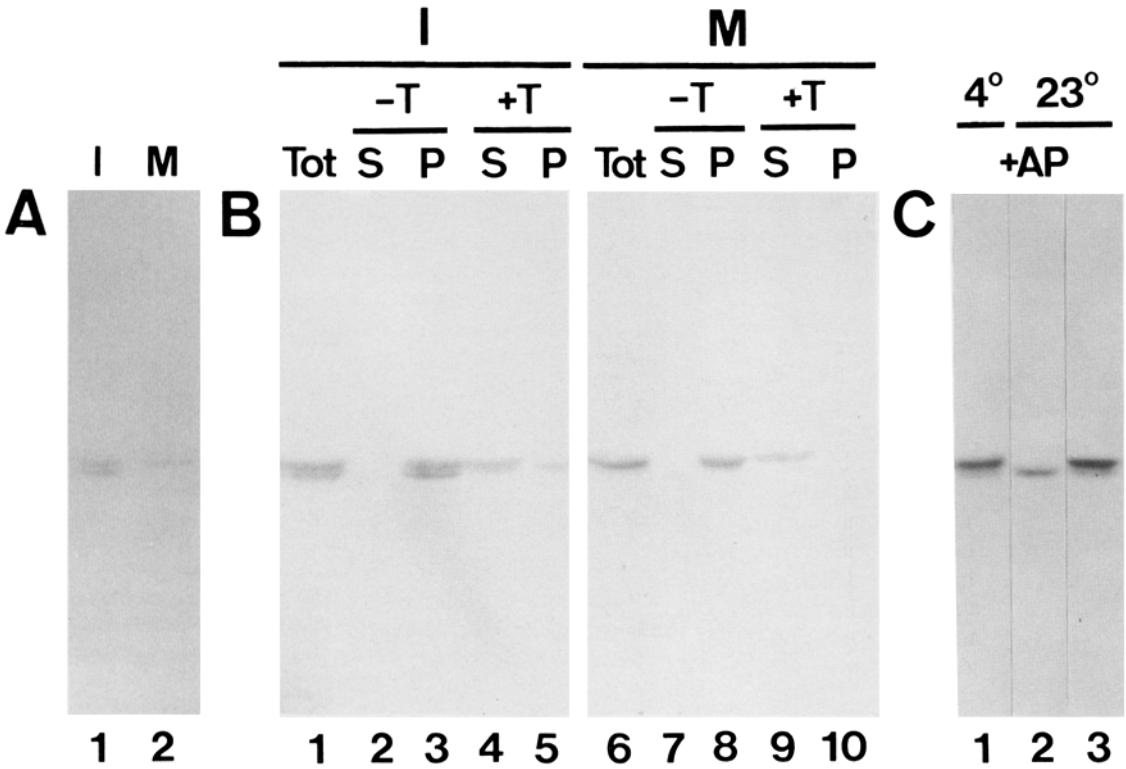

Figure 9. Biochemical analysis of p54 distribution in interphase and metaphase cells. (*A*) DU249 chicken hepatoma cells were harvested in interphase (*I*, lane *1*) or nocodazole-induced metaphase (*M*, lane *2*) and the electrophoretic mobility of p54 was assessed by immunoblotting. (*B*) Interphase (*I*) and metaphase-arrested (*M*) cells were fractionated into soluble (*S*) and particulate fractions (*P*) in the presence (*+T*) or absence (*−T*) of Triton X-100, and the extractability of p54 was determined by immunoblotting. (*C*) Particulate fractions were prepared from nocodazole-arrested DU249 cells (lane *1*). Aliquots were then incubated for 1 h at either 4°C (lane *1*) or room temperature (lanes *2* and *3*) in the presence (*+AP*, lane *2*) or absence (lanes *1* and *3*) of bacterial alkaline phosphatase (500 U/1.6 ml, Sigma Chemical Co.). Proteins were separated by 8% SDS-PAGE and the distribution of p54 was determined by immunoblotting.

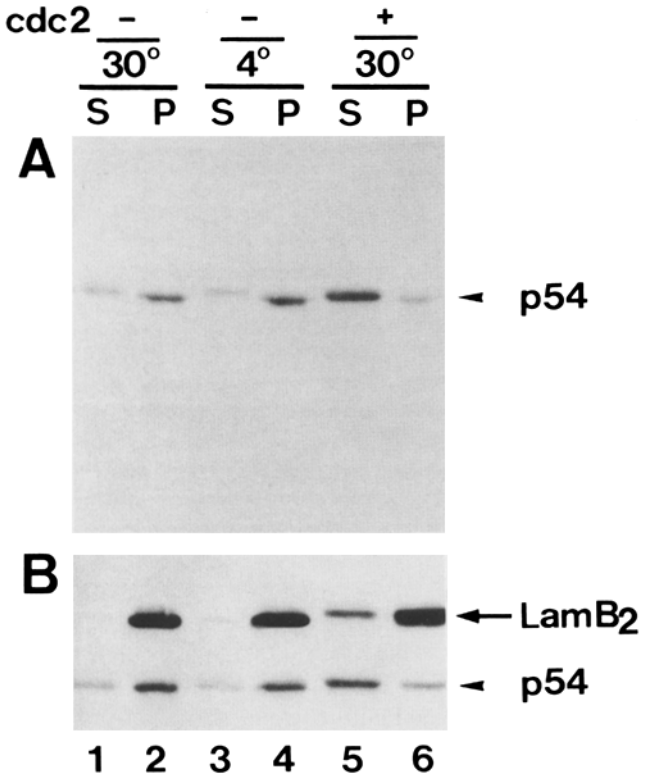

Figure 10. Solubilization of p54 by cdc2 kinase. Embryonic nuclei (*A*) and nuclear envelopes (*B*) were prepared as described in Materials and Methods. Aliquots were then incubated at either 4°C (lanes *3* and *4*) or 30°C (lanes *1* and *2* and *5* and *6*) for 90 min with cdc2 kinase (lanes *5* and *6*) or assay buffer without kinase (lanes *1–4*). To assess the detergent solubility of p54, samples were treated for 10 min on ice with 0.5% Triton X-100 and subjected to centrifugation for 10 min at 12,000 *g*. The distribution of p54 in supernatants (*S*) and pellets (*P*) was analyzed by immunoblotting. The filter shown in *B* was probed also for the partitioning of lamin B_2. The positions of R-7 antigen and lamin B_2 are indicated; only the relevant parts of the immunoblots are shown.

cubated at room temperature in the absence of phosphatase (Fig. 9 *C*, lane *3*), incubation with phosphatase converted the slower migrating form of the R-7 antigen to the faster migrating form (Fig. 9 *C*, lane *2*). In parallel experiments, the mobility of lamin B_2, a protein known to be highly phosphorylated during mitosis, was similarly enhanced by incubation with alkaline phosphatase (not shown; see Nakagawa et al., 1989; Peter et al., 1990*a*). Given that both the R-7 antigen and lamin B_2 showed a similar change in mobility in response to alkaline phosphatase, while no evidence was obtained for proteolytic degradation, these experiments strongly suggest that the conversion of the 54-kD form of the R-7 antigen to the 55-kD form is caused by phosphorylation.

Detergent Solubilization of p54 by cdc2 Kinase

If the above interpretation is correct, the results shown in Fig. 9 *B* indicate that the association of p54 with a detergent-resistant structure at the nuclear envelope is regulated by phosphorylation. Moreover, the predominance of the 55-kD form of the R-7 antigen in M phase–arrested cells (Fig. 9, *A* and *B*) suggests a role for a cell cycle–regulated protein kinase in phosphorylating p54. Prominent among the protein kinases that are activated at the onset of mitosis is the cdc2 kinase (Nurse, 1990). This kinase is required for entry of cells into mitosis, and several abundant nuclear proteins are candidate physiological substrates of this enzyme (Langan et al., 1989; Peter et al., 1990*a*,*b*; Belenguer et al., 1990; Meijer et al., 1991; for reviews see Pines and Hunter, 1990; Moreno and Nurse, 1990; Nigg, 1991). To test whether p54 might represent a potential substrate for cdc2 kinase, interphase nuclei as well as nuclear envelopes were isolated from 18-d-old chicken embryos and then incubated with a cdc2 kinase preparation from starfish oocytes (Labbé et al., 1989). Then, the detergent extractability of the R-7 antigen was assessed by immunoblotting. As shown in Fig. 10, cdc2 kinase treatment caused conversion of the bulk of the R-7 antigen to a detergent-extractable state (Fig. 10, *A* and *B*, compare lanes *5* and *6* with lanes *1–4*). Lamin B_2 could also be released in response to phosphorylation by cdc2 kinase (see Peter et al., 1990*a*). However, under appropriate conditions, the R-7 antigen could be solubilized preferentially over lamin B_2 (Fig. 10 *B*, lanes *5* and *6*). This observation suggests that lamin–lamin interactions are stronger than those responsible for anchoring of p54, and that the release of p54 cannot be attributed solely to phosphorylation of lamins and subsequent release of p54–lamin complexes. Finally, we note that the detergent-extractable R-7 antigen generally migrated with a slightly reduced electrophoretic mobility (e.g., Fig. 10, *A* and *B*; see also Fig. 9), suggesting that extractability of the R-7 antigen correlates with phosphorylation of p54 itself. This correlation was observed irrespective of whether phosphorylation had occurred before isolation of nuclei (Fig. 10, *A* and *B*, lanes *1* and *3*) or was induced by cdc2 kinase in vitro (Fig. 10, *A* and *B*, lanes *5* and *6*).

Discussion

With the acid of a novel mAb, R-7, we have characterized a 54-kD protein (p54) of the chicken nuclear envelope. As indicated by its biochemical properties and the results of immunocytochemistry, p54 is an integral membrane protein located predominantly at the inner nuclear membrane. Subcellular fractionation experiments strongly suggest that p54 interacts, directly or indirectly, with the nuclear lamina. This interaction appears to be regulated during the cell cycle, most probably via phosphorylation of p54. As suggested by in vitro experiments, cdc2 kinase may play a role in phosphorylating p54 during mitosis.

p54: A Lamin Receptor?

p54 resisted extraction of nuclear envelopes with 0.1 M NaOH and 4M guanidine-HCl, and, by these criteria, has to be considered as an integral membrane protein. However, p54 could not easily be extracted from nuclear envelopes by non-ionic detergent, indicating that it is not merely embedded within the lipid bilayer, but also interacts with detergent-insoluble components of the nuclear envelope. An association of p54 with nuclear pore complexes is unlikely, because immunolabeling with R-7 antibodies, at both the light and the electron microscopic level, revealed a homogeneous distribution of p54 at the nuclear envelope. Thus, p54 is most probably linked to the nuclear lamina. Consistent with this view, p54 could not be detected in microsomal membranes, indicating that it is enriched at the inner nuclear membrane.

In chicken, the lamina consists of at least three proteins, lamins A, B_1, and B_2 (Lehner et al., 1986*b*; Peter et al., 1989; Vorburger et al., 1989*a*). These lamins show pronounced variations in their expression during chicken embryonic development; the two B-type lamins are expressed in virtually all cell types, but lamin A is undetectable in early embryos and appears at specific times during development of different tissues and organs (Lehner et al., 1987; see also Röber et al., 1989, 1990). When the expression of p54 was compared to the expression of individual lamins, no correlation could be observed between levels of p54 and either A- or B-type lamins. Thus, while our data do suggest a link between p54 and the lamina, they do not provide evidence for a specific interaction of p54 with any particular member of the lamin family.

Does Phosphorylation Control the Lamina Association of p54?

In cultured cells, we observed a variant of p54 displaying a slightly reduced electrophoretic mobility. As indicated by its sensitivity to alkaline phosphatase, this variant almost certainly arises from phosphorylation. Consistent with this view, the slower migrating form of the R-7 antigen was not routinely observed in isolated nuclei and subnuclear fractions, presumably because of phosphatase action during sample preparation. Because the R-7 antigen does not recognize its antigen in immunoprecipitation experiments, we could not use in vivo ^{32}P-labeling experiments to directly demonstrate phosphate incorporation into p54, and evidence for phosphorylation of p54 is therefore indirect. Despite this reservation, we shall assume in the subsequent discussion that the slower migrating variant of the R-7 antigen does represent a phosphorylated protein.

Most interestingly, phosphorylation of p54 converted the protein from a detergent resistant to a detergent extractable state, and the phosphorylated form of p54 predominated in nocodazole-arrested M phase cells, although it could be detected also in interphase cells (Fig. 9). Taken together, these results indicate that phosphorylation of p54 is under cell cycle control, and contributes to regulate the association of p54 with the nuclear lamina. In vitro experiments showed that cdc2 kinase was able to confer sensitivity to detergent-extraction to p54, and, concomitantly, converted p54 to a slower migrating form (Fig. 10). This result raises the intriguing possibility that p54 might be a substrate for cdc2 kinase in vivo. Because of the impossibility to carry out immunoprecipitation experiments with R-7 antibody, no direct proof for phosphorylation of p54 by cdc2 kinase could be obtained. However, by incubating nuclear envelopes with cdc2 kinase in the presence of radiolabeled ATP, phosphate incorporation into a protein comigrating with p54 could be demonstrated (Peter, M., S. M. Bailer, and E. A. Nigg, unpublished results).

Also, we recently found that chicken p54 is immunologically related to turkey p58, the protein that was proposed to function as a lamin B receptor (Worman et al., 1988, 1990). After immunoprecipitation with a specific human autoimmune serum (Courvalin et al., 1990), p58 in fact comigrated precisely with the R-7 antigen, and it was readily recognized by R-7 antibody in immunoblotting experiments (Bailer, S. M., and E. A. Nigg, unpublished results). Although the extent of the structural relationship between the R-7 antigen and the purported lamin B receptor remains to be established, it is intriguing in the context of our phosphorylation results that p58 contains at least two motifs (SPXR) (Worman et al., 1990) which qualify as potential phosphoacceptor sites for cdc2 kinase (Langan et al., 1989; Shenoy et al., 1989; Peter et al., 1990*b*).

One might argue that the observed effects of cdc2 kinase on p54 extractability might reflect a release of lamin-p54 complexes, because of phosphorylation of lamins and subsequent disassembly of the lamina network (Peter et al., 1990*a*). However, we consider this explanation to be unlikely for the following reasons: first, incubation of nuclear envelopes with cdc2 kinase resulted in a change in electrophoretic mobility of p54, consistent with phosphorylation of p54 itself. Second, under appropriate conditions p54 could be solubilized more efficiently than lamin B_2, indicating that mitotic phosphorylation weakened not only interactions between neighboring lamin proteins but also between lamins and p54. Third, considerable amounts of phosphorylated p54 could be seen also in interphase cells; these modified forms of p54 were extractable by detergent during a stage of the cell cycle when the bulk of the nuclear lamins occurs in a polymeric detergent-resistant state. These observations argue in favor of a regulatory role of phosphorylation in controlling the interaction between p54 and the lamina.

At present we do not know what kinases are responsible for the presence of comparatively large amounts of phosphorylated p54 during interphase (Fig. 9, lane *I*). One possibility would be that mitotically added phosphate groups display a very low turnover on p54. Alternatively, different kinases may act on p54 during different stages of the cell cycle. In this context, it is interesting that turkey p58, the purported lamin B receptor, was recently reported to be a substrate for cAMP-dependent protein kinase (Appelbaum et al., 1990). Given the immunological relationship between chicken p54 and turkey p58, our results are in agreement with the suggestion that phosphorylation might control interactions between integral membrane proteins and the nuclear lamina. However, whereas phosphorylation of p54 by cdc2 kinase is shown here to weaken these interactions, Appelbaum and colleagues reported that phosphorylation of p58 by cAMP-dependent protein kinase strengthened binding of lamin B to the receptor. If p54 were identical with p58, this result would be difficult to reconcile with our present observation that the interphase phosphorylated p54 displays increased sensitivity to detergent extraction, and hence a reduced interaction with the nuclear lamina.

What Mediates Membrane Association of B-type Lamins during Mitosis?

Our evidence suggests that p54 interacts (directly or indirectly) with both A- and B-type lamins in interphase nuclei, and that phosphorylation weakens these interactions during mitosis. This might contribute to explain why A-type lamins are released in a soluble state during mitotic nuclear envelope breakdown. On the other hand, the question arises what mediates the persistent membrane attachment characteristic of B-type lamins (Gerace and Blobel, 1980; Stick et al., 1988). As proposed previously (Vorburger et al., 1989*b*; Kitten and Nigg, 1991), membrane association of B-type lamins may involve hydrophobic modification (isoprenylation and carboxyl methylation) of the COOH-terminal CaaX box

(Chelsky et al., 1987; Wolda and Glomset, 1988; Beck et al., 1988). If mitotic membrane association of B-type lamin dimers (or tetramers) were mediated primarily via lipophilic modification (rather than via interactions with integral membrane proteins), rapid lateral diffusion within communicating lipid bilayers would be facilitated. This might explain the observed extensive redistribution of lamin B_2 between remnants of the nuclear envelope and elements of the endoplasmic reticulum (Stick et al., 1988).

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