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Type B lamins remain associated with the integral nuclear envelope protein p58 during mitosis: implications for nuclear reassembly

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p58 (also referred to as the lamin B receptor) is an integral membrane protein of the nuclear envelope known to form a multimeric complex with the lamins and other nuclear proteins during interphase. To examine the fate of this complex during mitosis, we have investigated the partitioning and the molecular interactions of p58 in dividing chicken hepatoma (DU249) cells. Using confocal microscopy and double immunolabelling, we show here that lamins B1 and B2 co-localize with p58 during all phases of mitosis and co-assemble around reforming nuclei. A close juxtaposition of p58/lamin B-containing vesicles and chromosomes is already detectable in metaphase; however, p58 and lamin reassembly proceeds slowly and is completed in late telophase–G_1. Flotation of mitotic membranes in sucrose density gradients and analysis of mitotic vesicles by immunoelectron microscopy confirms that p58 and most of the type B lamins reside in the same compartment. Co-immunoprecipitation of both proteins by affinity-purified anti-p58 antibodies shows that they are physically associated in the context of a mitotic p58 'sub-complex'. This sub-assembly does not include the type A lamins which are fully solubilized during mitosis. Our data provide direct, *in vivo* and *in vitro* evidence that the majority of type B lamins remain connected to nuclear membrane 'receptors' during mitosis. The implications of these findings in nuclear envelope reassembly are discussed below.

Key words: lamin B/mitosis/nuclear membrane/p58

Introduction

The nuclear lamins are the building blocks of the nuclear lamina, a karyoskeletal structure interposed between the inner nuclear membrane and the chromatin network. The lamins are members of the intermediate filament protein family and can be divided into type A and type B subunits (for a recent review see Nigg, 1992).

Unlike the rest of the lamin proteins, lamin A is synthesized as a larger precursor (Gerace *et al.*, 1984; Lehner *et al.*, 1986a). The lamin A precursor and the type B lamins contain a characteristic COOH-terminal CaaX motif (cysteine/aliphatic/aliphatic/any amino acid), the target of post-translational isoprenylation (Wolda and Glomset, 1988; Krohne *et al.*, 1989). Interestingly, the COOH-terminal segment of the lamin A precursor which harbors the CaaX box and the isoprenyl tail is removed by proteolytic processing (Weber *et al.*, 1989). Thus, the mature lamin A molecule does not possess a lipid tail. However, the isoprenoid tail remains intact in the type B lamins while these proteins undergo trimming at the last few COOH-terminal amino acids and carboxymethylation of the (terminal) cysteine residue (Kitten and Nigg, 1991).

Initially, the ability of the lamins to associate with the inner nuclear membrane has been attributed to interactions between their isoprenoid tails and the phospholipids of the inner nuclear membrane (Krohne *et al.*, 1987; Nigg, 1992). Nevertheless, more recent data suggest that the lamins may also interact with integral membrane proteins of the nuclear envelope. In avian cells, one such protein is the polypeptide p58 (also referred to as the lamin B receptor), a major intrinsic component of the nuclear envelope which binds to lamin B *in vitro* with an affinity of ~200 nM (Worman *et al.*, 1988a, 1991; Bailer *et al.*, 1991). Apart from p58, there are also several other proteins (termed lamina-associated polypeptides, or LAPs), recently identified in rat liver nuclei, which bind to the lamins *in vitro* in a phosphorylation-dependent manner (Senior and Gerace, 1988; Foisner and Gerace, 1993). Whereas p58-related proteins have been identified in a wide variety of organisms (Worman *et al.*, 1988a; Georgatos *et al.*, 1989; Bailer *et al.*, 1991; Shimanuki *et al.*, 1992; Chaudhary and Courvalin, 1993), it is not yet known whether the LAPs occur in non-mammalian cells. Thus, for the time being, p58 represents the only well-characterized lamin-binding protein of the nuclear envelope which seems to be as widespread as the type B lamins.

p58 is phosphorylated at multiple sites during interphase and mitosis (Appelbaum *et al.*, 1990; Bailer *et al.*, 1991; Courvalin *et al.*, 1992). While the role of the mitotic phosphorylation has not been studied in detail, there is evidence from *in vitro* experiments that interphasic phosphorylation of p58 facilitates lamin B binding (Appelbaum *et al.*, 1990). Futhermore, p58 has been recently immunoisolated in the form of a native complex which comprises the nuclear lamins A and B, a kinase which specifically modifies p58 *in vivo*, a 34 kDa protein, an 18 kDa protein and a 150 kDa protein (Simos and Georgatos, 1992). The p58 kinase-mediated phosphorylation may affect the interactions of p58 with lamin as well as non-lamin proteins during interphase.

Recent work by Chaudhary and Courvalin (1993) has suggested that lamin B and p58 may dissociate during mitosis. To investigate this problem further, we decided to examine the fate of the p58 complex during cell division using more refined morphological and biochemical methods. Our observations show that, whereas lamin A dissociates from the mitotic membranes, a large fraction of B type lamins remain associated with p58 during mitosis. The implications of these data for nuclear envelope dynamics and nuclear lamina reassembly are discussed below.

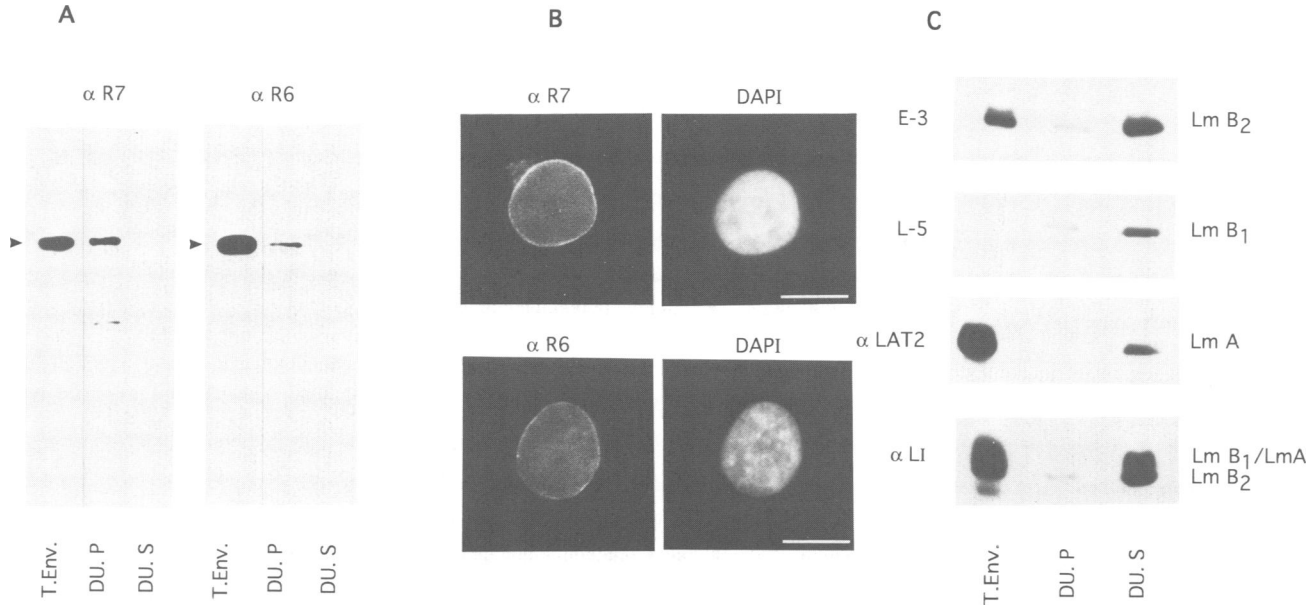

Fig. 1. Anti-p58 and anti-lamin antibodies used in this study. (**A**) Chicken hepatoma (DU249) cells were extracted with 8 M urea and ultracentrifuged as specified in Materials and methods. The membrane-containing pellet (DU. P) and the urea-solubilized material (DU. S) were analyzed in parallel with a sample of turkey erythrocyte nuclear envelopes (T.Env.) by SDS–PAGE (10% acrylamide gels) and immunoblotted using affinity-purified αR7 or αR6 antibodies. The arrowhead marks the position of p58. (**B**) Indirect immunofluorescence microscopy with affinity-purified αR7 and αR6 antibodies. Unsynchronized DU249 cells growing on coverslips were fixed with formaldehyde and permeabilized with Triton X-100, as explained in Materials and methods. Samples were then incubated with the anti-peptide antibodies, followed by FITC-conjugated goat anti-rabbit IgG or DAPI as indicated. Bars represent 10 μm. (**C**) The urea-insoluble residue (DU. P) and the urea extract (DU. S) of DU249 cells were analyzed in parallel with isolated turkey erythrocyte nuclear envelopes (T.Env.) by SDS–PAGE (7.5% acrylamide gels). Replica blots of these gels were probed with the monoclonal antibodies E-3 and L-5 and the anti-peptide antibodies αLI and αLAT2, as indicated. The relevant parts of the gels are shown. The characterization of these antibodies, except αLAT2, has been described previously (Lehner *et al.*, 1986a; Djabali *et al.*, 1991; Simos and Georgatos, 1992). Note that the autoradiogram shown in panel αLAT2 was exposed for 48 h, whereas all other autoradiograms were exposed for 16 h.

Results

Production and characterization of antibodies

To prepare reagents suitable for detecting p58 in cultured cells, we used as immunogens the peptides R6 and R7, two synthetic derivatives representing different parts of the NH_2-terminal domain of chicken liver p58 (for details see Materials and methods). The rabbit anti-peptide antibodies (αR7 and αR6) were affinity-purified and tested by immunoblotting and indirect immunofluorescence microscopy. Both antibodies reacted specifically with turkey erythrocyte p58 (Figure 1A, T.Env. lanes) and stained the nuclear periphery of interphasic chicken hepatoma (DU249) cells (Figure 1B). However, neither of the two antibodies reacted with the mammalian form of p58 (J.Meier and S.D.Georgatos, unpublished observations). Consistent with previous data, when whole DU249 cells were fractionated into a urea-insoluble, membrane-containing pellet and a urea-soluble supernatant, αR6 and αR7 detected a 58 kDa protein in the former but not in the latter fraction (Figure 1A, DU. P and DU. S lanes). The same 58 kDa band, and a minor degradation product, were also labelled by a polyclonal guinea pig antibody developed against intact turkey erythrocyte p58 (not shown). Because the αR7 serum had a higher titer, we used primarily this antibody in most of the subsequent analysis. The αR6 antibody was used for confirmatory work and gave similar results in all assays tested.

To monitor the lamin proteins of DU249 cells, we used several antibodies. As shown in Figure 1C, the anti-peptide antibody αLAT2 recognized exclusively lamin A, the monoclonal antibodies E-3 and L-5 (a generous gift of E.A.Nigg, ISREC, Epalinges, Switzerland) reacted with lamins B2 and B1, respectively, whereas the anti-peptide antibody αLI [for characterization see Djabali *et al.* (1991) and Simos and Georgatos (1992)] recognized all lamin isotypes.

In situ studies with intact mitotic cells

To examine the fate of p58 during mitosis we performed indirect immunofluorescence microscopy. Unsynchronized cultures of DU249 cells were briefly permeabilized with digitonin, formaldehyde-fixed and doubly labelled with αR7 and either E-3 or L-5. This permeabilization/fixation method allowed rapid access of the fixative to internal cellular structures [see also Belmont *et al.* (1993)]. Specimens were screened to identify mitotic cells fixed at different stages of mitosis and visualized by laser scanning confocal microscopy. In late prophase/early prometaphase, p58 started dissociating from chromatin, while in late prometaphase the protein was dispersed in a variety of round and tubular structures (probably membrane vesicles) (Figure 2a and f). In metaphase, the distribution of p58 did not change much, except that there was a noticeable accumulation of p58-containing particles near the metaphase plate (Figure 2b and g, arrowheads). In anaphasic cells, the majority of the p58 vesicles were still dispersed in the cytoplasm but a few of them clearly associated with the reforming nuclei (Figure 2c and h). p58 reassembly around the daughter nuclei was complete by late telophase–early G_1 (Figure 2d, i, e and j).

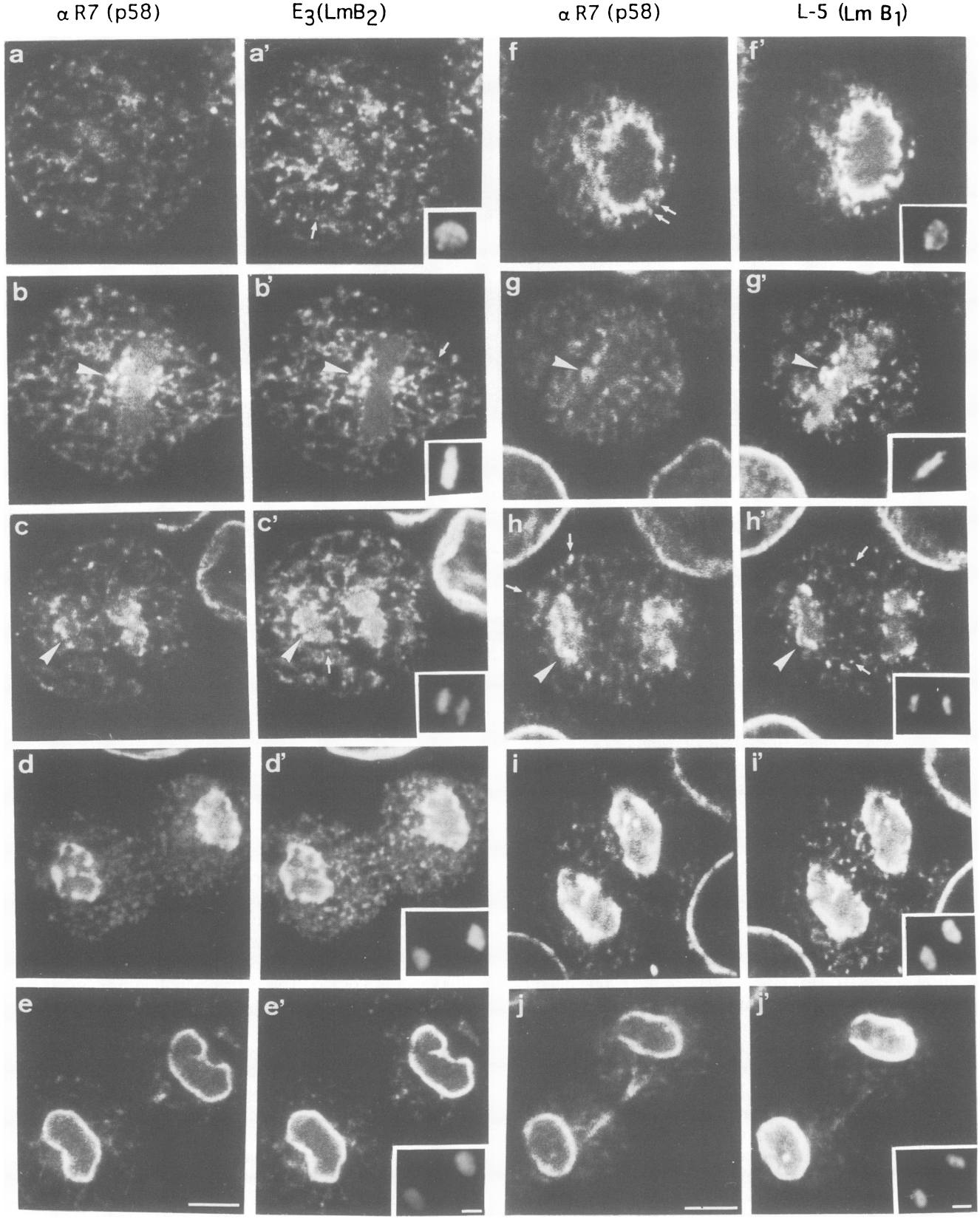

Fig. 2. Distribution of p58 and lamin B during mitosis as revealed by indirect immunofluorescence and confocal microscopy. Non-synchronized DU249 cells were permeabilized with digitonin and fixed with formaldehyde as specified in Materials and methods. The specimens were triply stained with affinity-purified αR7/FITC goat anti-rabbit IgG, DAPI and either E-3/Texas Red goat anti-mouse IgG, or L-5/Texas Red goat anti-mouse IgG, as indicated. Shown here are confocal sections corresponding to prometaphase (a and a′, late prometaphase; f and f′, late prophase/early prometaphase); metaphase (b, b′, g and g′); anaphase (c c′, h and h′); telophase (d, d′, i and i′); and early G_1 (e, e′, j and j′). Insets show the DAPI staining of the corresponding cells. Arrowheads show p58 and lamin-containing particles associated with chromosomes. Arrows show structures which are singly labelled with the anti-p58 or the anti-lamin antibodies. Bars correspond to 5 μm.

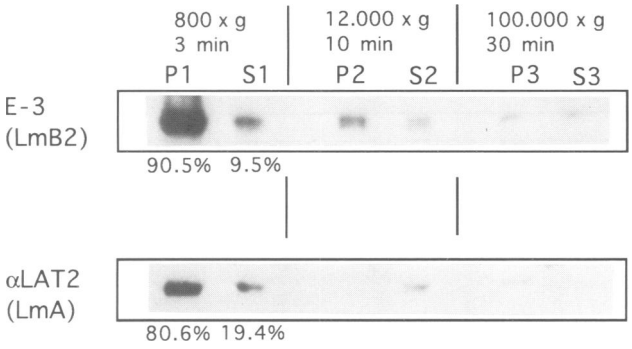

Fig. 3. Probing the release of nuclear lamins from mitotic cells treated with digitonin. Mitotic DU249 cells obtained by synchronization with thymidine and nocodazole were treated with digitonin exactly as in indirect immunofluorescence assays. After this treatment, the cell suspension was centrifuged at 800 *g* for 3 min, yielding a pellet (P1) and a supernatant (S1). The S1 fraction was then centrifuged at 12 000 *g* for 10 min, yielding a pellet (P2) and a supernatant (S2) fraction. Finally, the S2 fraction was spun at 100 000 *g* for 30 min, yielding a pellet (P3) and a supernantant (S3) fraction. Equivalent portions (~10%) of each fraction were resolved by SDS–PAGE and immunoblotted with anti-lamin A and anti-lamin B antibodies, as indicated. The autoradiograms were scanned to calculate the relative intensity of each band; the percentages are given underneath the first two lanes. Note that digitonin releases ~10% of lamin B2 and ~20% of lamin A. Whereas most of the released lamin B2 is associated with vesicles pelletable at 12 000 *g*, the bulk of the released lamin A is soluble and does not pellet by moderate or high speed centrifugation. The relevant parts of the gels are shown.

Table I. Distribution of lamin B2- and p58-positive particles in 11 optical sections from five anaphasic DU249 cells as determined by double immunofluorescence and confocal microscopy

Location	Marker	Total number of particles counted	Number of particles showing no match (no colocalization)
Cytoplasm	p58	613	12
	LmB2	636	20
On or near chromosomes	p58	226	13
	LmB2	234	12

Parallel monitoring of lamin B1 and lamin B2 yielded very similar immunostaining patterns (Figure 2, compare a′–e′ with f′–j′ respectively) and, despite the differences in fluorescence intensity, each lamin B particle in one channel could be matched with a p58 particle in the other channel. Thus, p58 and type B lamins appeared to co-localize during all phases of mitosis. Comparable results were obtained when synchronized DU249 cells were examined instead of non-synchronized cells (J.Meier and S.D.Georgatos, unpublished observations). The apparent co-localization of p58 and type B lamins was not due to 'cross-talk' between Texas Red and FITC (the two fluorochromes used in these assays), as a minority of brightly stained vesicles appeared only in one channel (Figure 2, arrows).

To rule out the possibility that a large fraction of lamin B had leaked out of the mitotic cells before fixation, we incubated synchronized DU249 cells (95% mitotic as judged by DAPI staining) with digitonin under exactly the same conditions used in indirect immunofluorescence assays and examined the cell residue and the supernatant by SDS–PAGE and immunoblotting. Data depicted in Figure 3 show that <10% of membrane-bound lamin B was released by this treatment. Parallel monitoring of the lamin A material released by digitonin showed that only ~20% of lamin A was removed, although this protein is largely soluble during mitosis (see below). These data indicate that the loss of lamin material during the brief digitonin treatment is minimal.

Furthermore, to examine potential variations among different mitotic cells and to exclude peculiarities due to the focusing procedure, we performed morphometric analysis. This involved the exact mapping of >800 anti-lamin- and anti-p58-decorated particles in magnified photographs corresponding to 11 confocal sections obtained from five different anaphasic cells similar to the ones shown in Figure 2. The results of this analysis, presented in Table I, clearly show that the majority of type B lamins co-localize with p58. The same data also demonstrate that only one-third of the p58- and lamin B-containing vesicles are associated with chromosomes during anaphase, whereas two-thirds of these vesicles are still in an unassembled state.

Subcellular fractionation and immunoisolation of mitotic membranes

To assess the partitioning of p58 and lamin B during mitosis, we examined subcellular fractions of nocodazole-arrested (prometaphasic) DU249 cells. Homogenates of such cells (95% mitotic as judged by DAPI staining) were first separated into a membrane-rich pellet (P1) and a supernatant (S1) fraction by centrifugation at 13 000 *g*. Western blotting of these fractions showed that most of the type B lamins and p58 partitioned with the P1 fraction, whereas lamin A partitioned almost entirely with the S1 fraction (Figure 4, lanes S1 and P1 and Table II). Ultracentrifugation of the S1 fraction (200 000 *g* for 1 h) and immunoblotting showed that >70% of the lamin B material in this fraction is pelletable in the absence of Triton X-100 and non-pelletable in the presence of detergent (data not shown). Thus, the lamin B in the low-speed supernatant does not represent 'soluble' protein but rather protein attached to small membrane vesicles which do not sediment at 13 000 *g* (for relevant information see Lourim and Krohne, 1993).

When the P1 fraction was resuspended in buffer and subfractionated in sucrose density gradients, we observed that 34–38% of the original amount of type B lamins and 41% of p58 floated together in the 0.9 M sucrose phase (Table II and Figure 4, panels αR7, L-5, E-3 and αLI). This fraction contained nuclear membrane vesicles and endoplasmic reticulum (ER) membranes, as could be shown by immunoblotting with specific anti-ER antibodies (J.Meier and S.D.Georgatos, unpublished observations). In contrast to lamin B, traces of lamin A (which contaminated the P1 fraction) remained entirely in the loading zone and did not float to the 0.9 M sucrose phase (Figure 4, panel αLAT-2). When interphasic preparations of DU249 cells were analyzed by sedimentation through the same sucrose gradients, all of the p58 and type B lamins were found in the loading zone and the pellet fraction [not shown; for relevant information see Maison *et al.* (1993)]. These data are consistent with previous observations showing that type A lamins are fully dissociated from the fragments of the nuclear envelope, whereas type B lamins remain membrane-bound during mitosis (Gerace and Blobel, 1980). The results also indicate

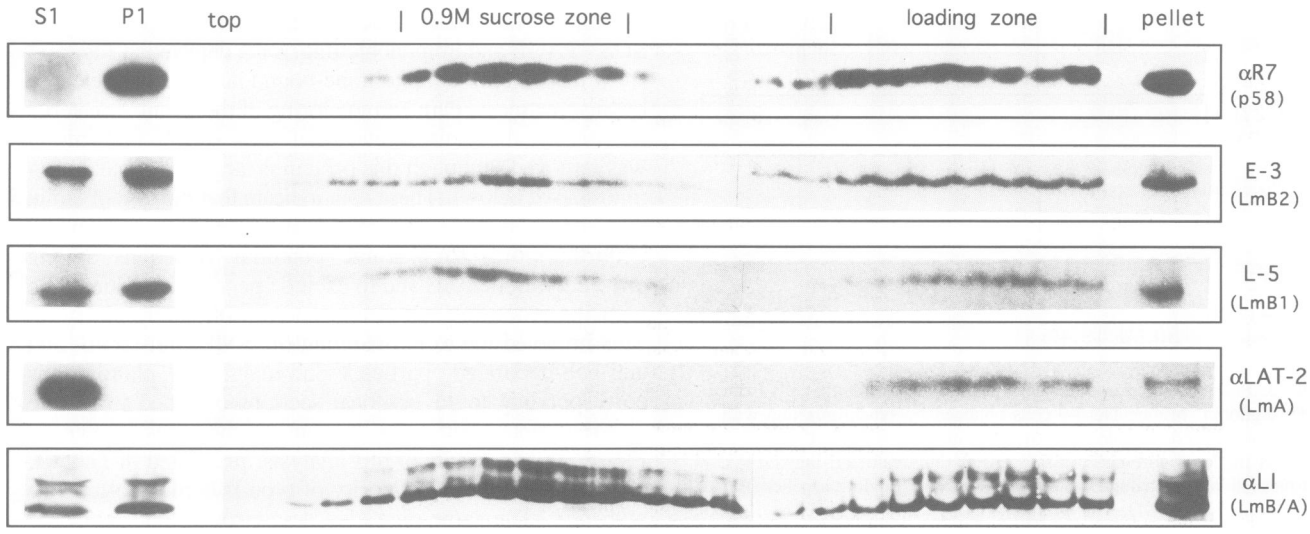

Fig. 4. Subcellular fractionation of mitotic DU249 cells by flotation in sucrose gradients. Nocodazole-arrested DU249 cells were homogenized and centrifuged at 13 000 *g*, yielding a cytosolic supernatant (S1) and a membrane-containing pellet (P1). The P1 fraction was resuspended in homogenization buffer, loaded at the bottom of sucrose step gradients, and centrifuged as described in Materials and methods. The collected fractions (numbers 1–23) and the pellet were analyzed by SDS–PAGE in 7.5% acrylamide gels and Western blotting using affinity-purified αR7, E-3, L-5 and affinity-purified αLI and αLAT2. The fractions corresponding to the loading zone, the 0.9 M sucrose phase, the top and the bottom of the gradient (pellet) are indicated. Note that some p58 and the type B lamins float up to the 0.9 M sucrose phase, whereas residual lamin A present in the P1 fraction remains in the loading zone. The autoradiogram shown in panel αLAT-2 was exposed for 48 h, whereas all other autoradiograms were exposed for 16 h.

that p58 and type B lamins reside either in the same mitotic vesicles, or in membranes which co-fractionate in sucrose gradients. For further biochemical experiments, we used primarily the fraction of mitotic membranes floating at the 0.9 M sucrose zone and not the material found in the loading zone and the pellet of the gradient, because the latter two fractions might have been contaminated with residual cytosolic proteins and unbroken cells.

To determine whether lamin B and p58 reside in the same mitotic membranes, we performed whole mount immunoelectron microscopy. To identify p58-carrying membranes, we used affinity-purified αR7. Pilot experments (J.Meier and S.D.Georgatos, unpublished) confirmed that the NH_2-terminal epitopes of p58 recognized by this antibody are indeed exposed to the mitotic cytoplasm and are accessible to exogenous proteases, as could be predicted from the tentative topology of p58 (Worman *et al.*, 1990; Smith and Blobel, 1993; Soullam and Worman, 1993). To decorate the type B lamins, we employed the polyclonal anti-lamin antibody αLI because the monoclonal antibodies did not work well in immunoelectron microscopy assays. The fact that αLI reacted with both type A and type B lamins did not present a problem because, as demonstrated above, the mitotic membranes collected at the 0.9 M sucrose phase were free of lamin A protein.

Double staining revealed that the floated mitotic vesicles contained both p58 and lamin B (Figure 5A). Morphometric analysis showed that >60% of the p58-positive vesicles also carried lamin B (Table III). In contrast to this, only 4% of the p58-free membranes were stained with αLI (Table III). Similar data were obtained when we immunostained unfractionated membranes taken from fresh mitotic homogenates (Figure 5B). Thus, the co-localization of p58 and lamin B does not appear to be restricted in the membrane fraction that floats at the 0.9 M sucrose phase. The diameters of the p58- and lamin B-containing vesicles ranged from 220 to 400 nm. In general, these results are consistent with the confocal microscopy data, except that the percentage of doubly stained vesicles appears to be lower *in vitro* than *in vivo*. This may be due to a number of factors, including *in vitro* proteolysis (see Discussion).

Table II. Relative distribution of lamin B and p58 in various sucellular fractions of mitotic DU249 cells, as estimated by immunoblotting

Fraction	p58	LmB2	LmB1
S1	10%	24%	22%
P1	90%	76%	78%
Pellet after flotation (subfraction of P1)	16%	12%	8%
Loading zone of flotation gradient (subfraction of P1)	33%	30%	32%
0.9 M sucrose zone of flotation gradient (subfraction of P1)	41%	34%	38%

To approach the same problem by a biochemical method, we immunoisolated lamin B-carrying mitotic membranes. For these purposes, floated mitotic membranes (Figure 4) were incubated with magnetic beads carrying affinity-purified αLI antibodies. Examination of the immunoisolates by thin-sectioning and electron microscopy showed that the beads coated with anti-lamin antibodies carried distinct membraneous structures on their surfaces (range of diameters = 200–300 nm), whereas the beads coated with normal rabbit IgG were free of membranous material (Figure 6B). The immunoisolated vesicles also contained fuzzy fibrillar material that may correspond to vimentin filaments [for relevant information see Maison *et al.* (1993)]. Western blotting analysis of the αLI immunoisolates showed that the bead-bound membranes contained both p58 and type B

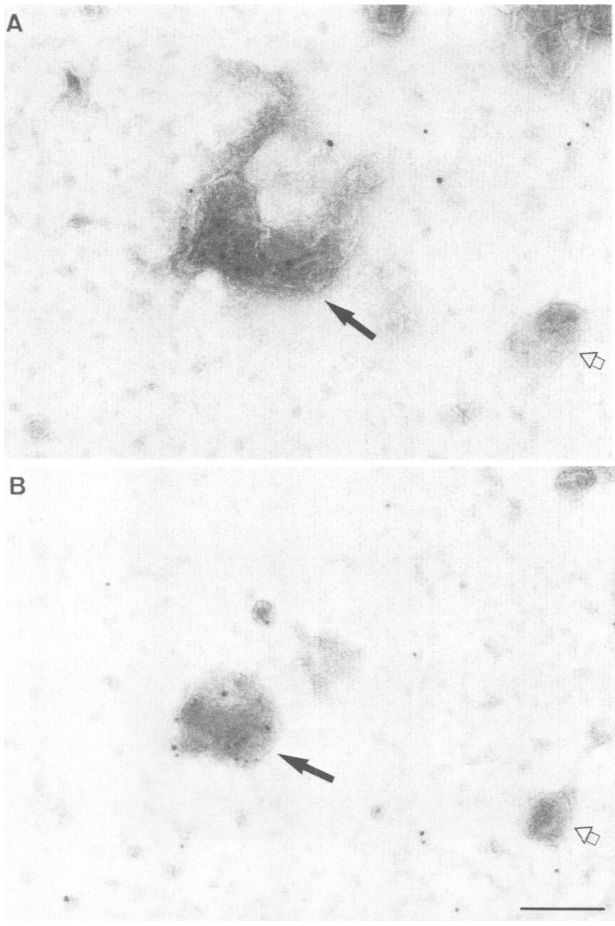

Fig. 5. Double-immunostaining of mitotic membranes. Representative electron micrographs of specimens taken from floated mitotic membranes (peak at 0.9 M sucrose, panel **A**), or from a fresh mitotic cell homogenate (panel **B**) and decorated with affinity-purified αR7/protein A−6 nm gold and with affinity-purified αLI/protein A−14 nm gold. Closed arrows show doubly stained vesicles, whereas open arrows show non-decorated vesicles. Bar represents 250 nm.

Table III. (**A**) Double labelling of mitotic vesicles, obtained from the 0.9 M sucrose fraction of the flotation gradients, with anti-p58 (αR7) and anti-lamin (αLI) antibodies as revealed by immunoelectron microscopy

Assay	No. of vesicles examined	No. αR7(+)	No. αLI(+)	No. αR7(+)/ αLI(+)
1	265	29	11	43
2	153	11	6	15
3	106	5	5	17
4	126	1	0	6
Total	650	46	22	81

Table III. (**B**) Distribution of lamin B among p58-containing and p58-free mitotic vesicles

	p58-containing vesicles (%)	p58-free vesicles (%)
Lamin B present	63.2 (58−85)	4.2 (0−6)
Lamin B absent	36.2 (15−42)	95.8 (94−100)

lamins (Figure 6A). Although one should not judge from immunoblotting data generated with two different antibodies, the αLI immunoisolates seem to contain more lamin B than p58. This may be explained by the fact that DU249 cells and floated mitotic vesicles generally contain more lamin B than p58 (Table IV), and by the fact that a significant amount of lamin B dissociates from p58 under *in vitro* conditions (see above).

Co-immunoprecipitation of p58 and lamin B from detergent extracts of mitotic membranes

To examine whether p58 and type B lamins are physically associated during mitosis or whether they merely co-localize on the same membranes, we performed immunoprecipitation experiments. Crude membranes of mitotic DU249 cells were first fractionated by flotation in sucrose gradients and the mitotic vesicles migrating in the 0.9 M sucrose zone collected and extracted with Triton X-100. The clarified extract, which contained the bulk of p58 and lamin B associated with the membranes, was then used for immunoprecipitation with the affinity-purified αR7 antibody. SDS−PAGE and silver staining of the immunoprecipitate revealed that five polypeptides migrating at 76, 74, 70, 68 and 45 kDa were co-isolated with p58 (Figure 7A). Western blotting showed that the 68 kDa band corresponded to lamins B1 and B2 which co-migrate in 10% polyacrylamide gels (Figure 7B). None of these proteins could be detected in material mock-precipitated with normal rabbit IgG and protein A−Sepharose (Figure 7B). The lack of specific antibodies did not allow us to examine whether the other components of the interphase 'p58 complex' [e.g. the p58 kinase and the other p58-associated proteins; see Simos and Georgatos (1992)] were also present in the mitotic 'p58 sub-complex' which lacks lamin A. Addition of [^{32}P]ATP to the immunoprecipitates did result in phosphorylation of p58; however, since other proteins in this preparation were also phosphorylated, we could not distinguish whether the phosphorylation of p58 was due to the p58 kinase or to another mitotic kinase (J.Meier, G.Simos and S.D.Georgatos, unpublished observations). Further experiments are needed to determine the identity of the non-lamin partners of p58 during mitosis.

To assess the relative amount of lamin B and p58 present in the mitotic 'p58 sub-complex', we performed quantitative immunoblotting (for technical details see Materials and methods) and measured the concentrations of lamin B and p58 in the Triton X-100 extract of the mitotic vesicles and in the αR7 immunoprecipitates. As shown in Table IV, the amount of lamin B co-isolated with p58 was very close to the quantity expected to be bound to p58 if one assumes a 1:1 stoichiometry and a K_d of 200 nM [for relevant information see Worman *et al.* (1988a)]. However, since the polyoxyethylene-type detergent Triton X-100 is known to affect interactions of membrane proteins (Morein and Simons, 1985), we also performed the same experiment using CHAPS, a zwitterionic detergent thought to preserve integral membrane protein complexes (e.g. Bennett *et al.*, 1992). Under these conditions, the amount of lamin B co-immunoprecipitated with p58 was substantially higher, although the percentage of the immunoprecipitated p58 in the presence of the two detergents was not much different (Table IV). This provides an indication that lamin B may bind to p58 as a small oligomer which is not preserved in

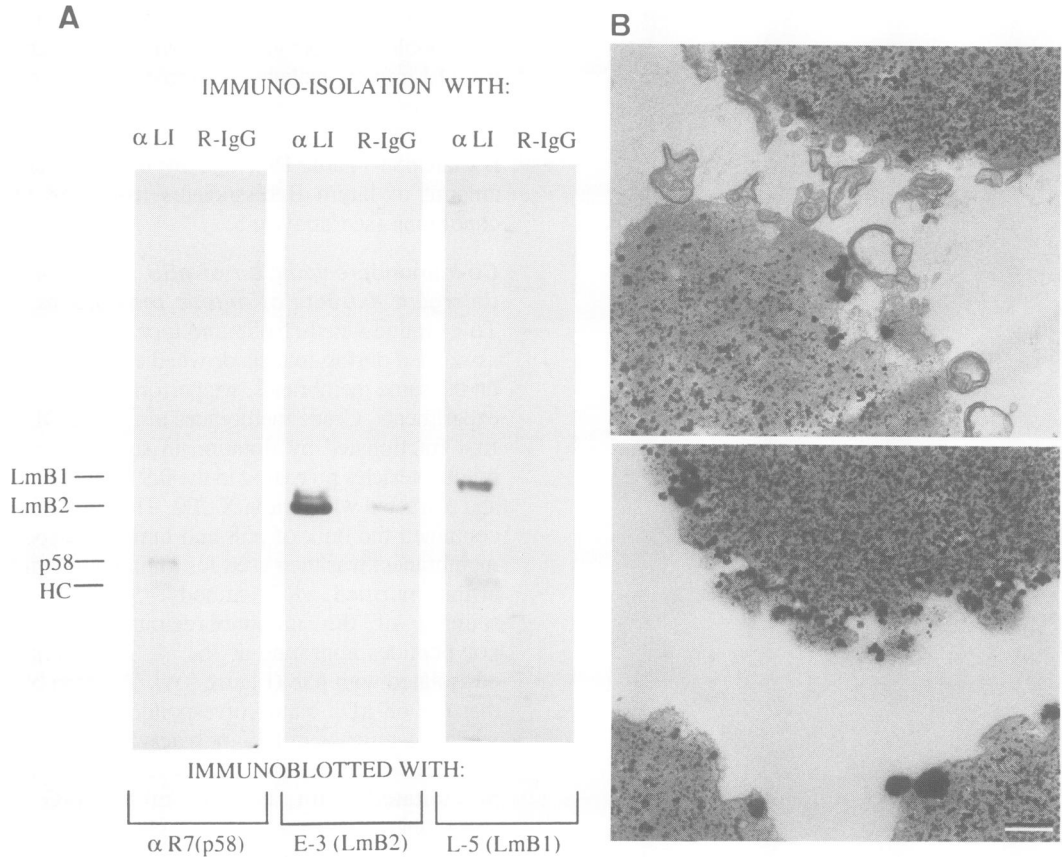

Fig. 6. Immunoisolation of p58- and lamin B-carrying mitotic vesicles. Floated mitotic material was collected from the 0.9 M sucrose phase of a sucrose step gradient (see Figure 5) and diluted in PBS. This material was incubated with magnetic beads carrying either affinity-purified αLI antibodies (αLI), or rabbit IgG (R-IgG). Immunocomplexes were harvested with the help of a magnet and the samples were analyzed by SDS–PAGE and Western blotting (**A**) or by electron microscopy (**B**). In (A), the immunoisolates were probed with affinity-purified αR7 (anti-p58), E-3 or L-5, as specified. The positions of lamins B1 (LmB1) and B2 (LmB2), p58 (p58), and the heavy chains of IgG (HC) are indicated. The electron micrographs shown in (B) represent thin sections of material bound to αLI-coated beads (top panel) or to normal rabbit IgG-coated beads (lower panel). Bars correspond to 300 nm.

Table IV. Relative amounts of p58 and lamin B in the αR7 immunoprecipitates and the non-precipitated material, as determined by quantitative immunoblotting

Fraction[a]	p58	Lamin B	Amount of lamin B expected to be in a complexed form
Triton X-100 extract of vesicles (nM)	3.8	22	
αR7 immunoprecipitate of Triton X-100 extract (ng)	270 (31%)	35	25.2
CHAPS extract (nM)	1.2	16	
αR7 immunoprecipitate of CHAPS extract (ng)	65 (24%)	38	4.89

[a]Vesicles found in the 0.9 M sucrose fraction were extracted with either Triton X-100 or CHAPS, and the extracts were immunoprecipitated with αR7.

The molar ratio of lamin B to p58 in homogenates of whole DU 249 cells has been estimated to 2.6. The amount of lamin B expected to be in a complexed form with p58 was calculated by the mass action law, using a K_d of 0.2 μM (see Worman *et al.*, 1988a) and an assay volume of 3 ml. The quantity of lamin B in the cell homogenates, the immunoprecipitates and the non-precipitated fractions were determined by quantitative immunoblotting as specified in Materials and methods.

the presence of Triton X-100. Although the quantity of lamin B co-immunoprecipitated with the p58 antigen can only be underestimated because of the repeated washings of the immunoisolates and the steric hindrance imposed by the binding of p58 to the anti-p58 antibodies, these experiments clearly show that the majority of type B lamins are physically associated with p58 in the context of a mitotic 'p58 sub-complex'.

Discussion

A multiplicity of lamin–membrane interactions during the cell cycle

Recent studies have advanced the idea that the nuclear lamins associate during interphase with several integral membrane proteins of the nuclear envelope. Avian cells contain at least one such protein, p58 (Worman *et al.*, 1988a; Bailer *et al.*,

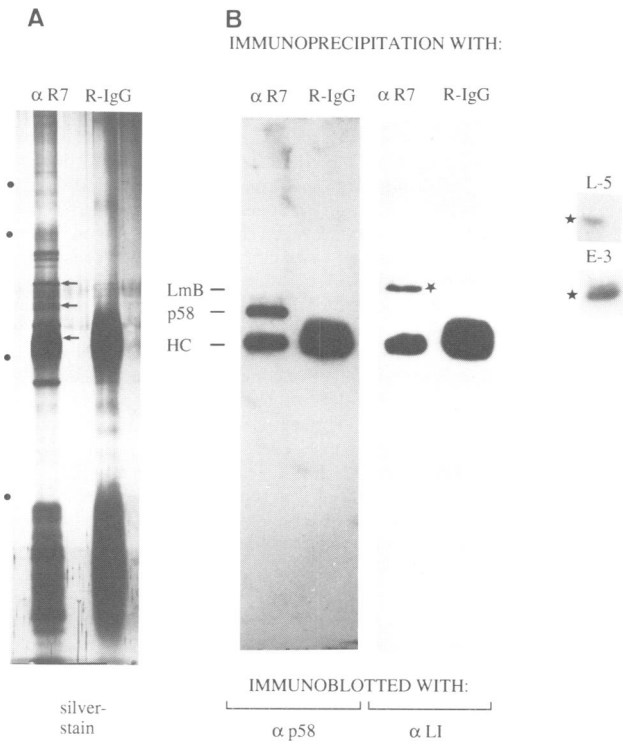

Fig. 7. p58 and type B lamins are associated during mitosis. Mitotic membranes isolated by flotation were extracted with 1% Triton X-100 and centrifuged at 12 000 *g*. The resulting supernatant was incubated either with affinity-purified αR7 (αR7), or with control antibodies (rabbit anti-mouse IgG, R-IgG). After this incubation, protein A–Sepharose was added and the immunocomplexes were harvested by centrifugation. Samples were analyzed by SDS–PAGE and either stained with silver (panel **A**), or tested by Western blotting (panel **B**), as indicated. Note in (B) that lamins B1 and B2 comigrate in the gel system used and yield a single band upon immunoblotting with the αLI antibody. This band comprises both of the type B lamins, as indicated by immunoblotting with E-3 and L-5 (asterisks). Dots mark molecular weight standards of 106, 80, 49.5 and 32.5 kDa and arrows indicate (from top to bottom) the positions of lamin B, p58 and IgG heavy chains.

1991), whereas mammalian cells contain at least three distinct types of lamin-binding proteins: p58, the LAPs 1A, 1B and 1C and the LAP 2 protein (Powell and Burke, 1990; Chaudhary and Courvalin, 1993; Foisner *et al.*, 1993). The multiplicity of lamin-binding proteins suggests that the coupling of the nuclear lamina to the nuclear membrane could be accomplished by several different molecular mechanisms. For instance, p58 and LAP 2 may recognize unique features of the lamin B (including the isoprenyl tail), behaving essentially as 'farnesyl/lamin B receptors', as first proposed by Nigg (1992). On the other hand, the LAP 1 class of proteins may recognize common features of type A and B lamins. Irrespective of these interactions, lamin B, which is constitutively expressed in most eukaryotic cells, could operate as a docking site for lamin A (Georgatos *et al.*, 1988; Krohne *et al.*, 1991). This redundancy may offer structural and functional flexibility to the different nuclear laminae, since eukaryotic cells are known to express variable amounts and different types of lamin proteins during development and differentiation (Lehner *et al.*, 1987; Stewart and Burke, 1987; Worman *et al.*, 1988b; Röber *et al.*, 1989).

Apparently, the associations of the nuclear lamins with the inner membrane are modulated during nuclear envelope breakdown. For instance, type A lamins completely dissociate from nuclear envelope fragments and disperse as lower oligomers in the mitotic cytoplasm (Gerace and Blobel, 1980). This may be due to hyperphosphorylation-induced depolymerization and weakening of the heterotypic interactions between type A and type B lamins (Georgatos *et al.*, 1988). In contrast, type B lamins remain bound to membrane vesicles throughout mitosis (Gerace and Blobel, 1980) in an aggregation state that has not yet been precisely determined. The origin of the mitotic membranes to which type B lamins bind is presently debated. Previous data have suggested that the lamin B2 fully dissociates from nuclear envelope-derived vesicles and re-distributes in ER membranes (Stick *et al.*, 1986). However, more recent studies indicate that the lamin B-carrying membranes are distinct from the bulk of the ER (Maison *et al.*, 1993). Implicit in the second scenario is the idea that the type B lamins may not dissociate from their inner nuclear membrane 'receptors' during mitosis, a possibility that we have directly addressed in this study.

Undoubtedly, some lamin B detaches from the nuclear membrane upon sample processing *in vitro* and this by itself suggests that the mitotic p58 'sub-complex', which lacks lamin A, is less tight than its interphase counterpart. A factor contributing to *in vitro* dissociation is probably proteolysis: we have noticed that upon prolonged handling of mitotic cell homogenates, a significant amount of p58 is degraded into a 50 kDa product (J.Meier and S.D.Georgatos, unpublished observations). Despite this limitation, it is clear that under *in vivo* conditions most of the p58 and lamins B1 and B2 co-localize in the same mitotic membranes, associate in a specific fashion, and co-assemble around the reforming nuclei at the end of mitosis. These data are at variance with the conclusions reached in a recently published study involving mitotic HeLa cells (Chaudhary and Courvalin, 1993), although a partial co-localization of lamin B and p58 has also been observed in this case. Since avian and mammalian cells contain a different lamin and 'lamin receptor' complement, it is conceivable that the different results may reflect species-specific differences in the reassembly of the nuclear envelope. However, because the studies on HeLa cells did not involve double labelling with anti-lamin B and anti-p58 antibodies, or biochemical analysis by immunoprecipitation, a direct comparison between the two sets of results is not possible. To resolve potential differences due to technique, we explored various methanol fixation protocols, as reported by Chaudhary and Courvalin (1993). Under these conditions, the immunostaining pattern of p58 was similar to the one reported here. However, when we decorated methanol-fixed mitotic DU249 cells with the anti-lamin B antibodies, we observed a very weak and diffuse staining, almost indistinguishable from 'background' fluorescence. Although this diffuse pattern can be interpreted as evidence for a dissociation of lamin B from p58, we find it more reasonable to suggest that mitotic chicken lamin B may not be adequately fixed by methanol.

Implications for nuclear reassembly

Different mechanisms have been postulated to explain the reformation of a nuclear envelope around chromatin at the end of mitosis. One set of observations have suggested that nuclear membrane reassembly does not involve the lamins

because mitotic vesicles assemble around chromatin after immunodepleting mitotic *Xenopus* egg extracts of lamin LIII, previously thought to be the only lamin form present in this system (Newport *et al.*, 1990; Meier *et al.*, 1991). This 'lamin-independent' pathway is based on technically sound observations and is supported by microinjection studies (Krohne and Benavente, 1986). However, it is unlikely that it reflects the *in vivo* situation, as the egg extracts also contain another type B lamin, which is not recognized well and, therefore, not affected by the anti-LIII antibody immunodepletion (Lourim and Krohne, 1993). In contrast to these studies, other experiments employing mammalian, amphibian, and insect cell-free extracts have indicated that the presence of type B lamins is essential for the targeting of nuclear envelope vesicles to the chromosomes (Burke and Gerace, 1986; Dabauvalle *et al.*, 1991; Ulitzur *et al.*, 1992). Given that a significant fraction of type B lamins remain associated with p58 throughout mitosis, our data seem more consistent with the second alternative. In our view, the membrane and p58-associated lamin B may be utilized for the initial targeting of nuclear envelope vesicles to the surfaces of chromosomes [for relevant data on lamin B–chromatin interactions see Höger *et al.* (1991)]. In addition, p58 itself may bind directly to chromatin through specific DNA-binding motifs located in its NH_2-terminal, nucleoplasmic domain (Worman *et al.*, 1990) and further stabilize this interaction. Thus, 'lamin-independent' binding of mitotic vesicles to chromosomes, which could occur *in vitro*, might be less efficient and slower than the binding of normal, lamin-containing membranes to chromatin. As far as we could tell, there is hardly any quantitative information comparing the kinetics and chromosome binding capacities and affinities of lamin-free and lamin-containing vesicles.

It is interesting that a relatively 'early' association of lamin B and p58 with the chromosomes has been detected during metaphase (this report). A similar observation has been made by Höger *et al.* (1991) who observed the existence of type A lamins on metaphase chromosomes in *Xenopus* somatic cells and by Kouklis *et al.* (1993) who detected small quantities of lamins on the surfaces of chromosomes isolated from 3T3 cells. In our hands, prometaphasic chromosomes isolated from various different cell types often carry membrane vesicles on their surfaces, readily detectable by thin-sectioning and transmission electron microscopy (A.Pyrpasopoulou, C.Maison and S.D.Georgatos, unpublished observations). In view of these data, a possibility worth investigating is that some fragments of the inner nuclear membrane carrying p58 and lamin B remain bound to chromatin throughout mitosis. Such chromatin-bound vesicles may serve as 'seeds' which initiate the targeting of other lamin and p58-containing vesicles back to the chromosomes at the end of cell division. The presumed 'homotypic' interactions between chromatin-associated and free nuclear envelope vesicles would not be without precedent. For example, fragments of the Golgi apparatus and the ER generated during mitosis are known to reassociate with each other at the end of cell division reforming the original organelles (Warren, 1993).

Materials and methods

Cell culture and subcellular fractionation

Chicken hepatoma cells [DU249 cells (Langlois *et al.*, 1974)] were kindly provided by R. Zeller (EMBL). They were grown in DMEM, supplemented with 10% fetal calf serum (Biolabs), 2% chicken serum (Sigma), 2 mM glutamine and antibiotics (100 IU/ml penicillin; 100 mg/ml streptomycin). The cells were synchronized by a combined thymidine (2 mM, for 12 h) and nocodazole (20 ng/ml, for 8 h) treatment (Nakagawa *et al.*, 1989). Mitotic cells were collected by shake-off. This method yielded $\sim 5 \times 10^6$ cells/15 cm dish with a mitotic index of 95%, as judged by DAPI staining. Cells were homogenized at 4°C in KHM buffer (78 mM KCl, 50 mM HEPES-KOH, pH 7.0, 4 mM $MgCl_2$, 10 mM EGTA, 8.37 mM $CaCl_2$, 1 mM DTT and 20 mM cytochalasin B), as described by Burke and Gerace (1986). In some experiments, the whole mitotic homogenate was used without further treatment, whereas in other cases the homogenate was centrifuged at 13 000 *g* for 30 min at 4°C. The resulting pellet (P1 fraction) was resuspended in ice-cold KHM buffer for further analysis. The same procedure was used to fractionate interphasic cells. For flotation experiments, the procedure described in Wilson and Newport (1988) was followed. Whole mitotic homogenates, or the resuspended P1 fraction of mitotic or interphasic cells were supplemented with 2.3 M sucrose in KHM buffer to a final concentration of 1.3 M sucrose. The samples were loaded at the bottom of a SW50.1 Beckman centrifuge tube, overlaid with 1.1, 0.9 and 0.7 M sucrose in KHM buffer and spun for 2 h at 200 000 *g* at 4°C. After centrifugation, fractions of ~220 μl were collected using an AutoDensi Flow fractionator and analyzed by SDS–PAGE and/or immunoblotting. Urea extraction of DU 249 cells was carried out according to Georgatos and Blobel (1987).

Immunochemical procedures

For immunization, the synthetic peptides R7 [corresponding to residues 135–156 of chicken liver p58 as described in Worman *et al.* (1990)] and the peptide R6 (corresponding to residues 119–139 of chicken liver p58), were coupled to keyhole limpet hemocyanin (Sigma) through an additional NH_2-terminal cysteine, using *N*-succinimidyl 3-(2-pyridyldithio)propionate (Pierce Chemical, Rockford, USA). The coupled peptides were used for immunizing rabbits as described in Simos and Georgatos (1992). Anti-R7 (αR7) and anti-R6 (αR6) antisera were screened by immunoblotting and immunofluorescence. αR7 and αR6 IgG were prepared by affinity chromatography over an R7 or R6–Affigel 10/15 column (10 mg of R7/ml of resin), followed by elution with 200 mM glycine-HCl pH 2.3, 500 mM NaCl and dialysis against PBS. The anti-peptide antibody αLI, recognizing all type A and type B lamins (Djabali *et al.*, 1991; Simos and Georgatos, 1992), was modelled after residues 1–32 of human lamin A (Fisher *et al.*, 1986). The monoclonal anti-chicken lamin B2 antibody E-3 and the monoclonal anti-chicken lamin B1 antibody L-5 (Lehner *et al.*, 1986b) were a kind gift of E.A.Nigg (ISREC, Switzerland). The anti-peptide antibody αLAT2, exclusively recognizing lamin A, was raised against two peptides representing residues 562–583 and 571–593 in the tail domain of chicken lamin A [for sequences see Peter *et al.* (1989)]. The peptides were coupled to keyhole limpet hemocyanin (Sigma) using 3.3% glutaraldehyde (Sigma). The coupled peptides were used for immunizing rabbits as described in Simos and Georgatos (1992). Anti-LAT2 antisera were screened by immunoblotting and immunofluorescence. αLAT2 IgG was prepared by affinity chromatography over a peptide–Affigel 15 column (10 mg of LAT peptides per 2 ml of resin). Immunoblotting assays were performed as described previously (Georgatos and Blobel, 1987). For detection of immunoreactive proteins, ^{125}I-labelled protein A or ^{125}I goat anti-mouse (both Amersham) was used. For conventional indirect immunofluorescence microscopy, DU249 cells were grown on glass coverslips or allowed to adhere to Alcian Blue-coated coverslips. The cells were washed with PBS, fixed with 3.5% formaldehyde in PBS for 10 min at room temperature and permeabilized with 0.5% Triton X-100. For confocal microscopy, DU249 cells were grown on glass coverslips. The cells were washed with PBS and then permeabilized with 20 μg/ml digitonin in PBS, for 3 min at room temperature. After permeabilization, cells were fixed in 3.5% formaldehyde in PBS for 10 min. After quenching with 50 mM NH_4Cl and blocking in IF buffer (20 mM HEPES, pH 7.9, 250 mM KCl, 1% BSA, 0.1% cold fish gelatin and 0.02% NaN_3), cells were incubated with anti-p58 antibodies (αR7, diluted 1:50 in IF buffer containing 0.05% Triton X-100) and either with anti-chicken lamin B2 antibodies (E-3, diluted 1:1000 in IF buffer containing 0.05% Triton X-100) or with anti-chicken lamin B1 antibodies (L-5, diluted 1:1500 in IF buffer containing 0.05% Triton X-100). As secondary antibodies, FITC-coupled goat anti-rabbit antibodies or Texas Red-conjugated goat anti-mouse antibodies (both from Cappel, Organon Teknika, West Chester, USA) were used. Samples were examined in a modular confocal microscope, developed and constructed at EMBL. Double labelling experiments were performed at an excitation wavelength of 514 nm using an argon-ion laser.

For immunoprecipitation, mitotic membranes were solubilized with 1% Triton buffer (1% Triton X-100, 50 mM Tris–HCl pH 7.4, 150 mM NaCl) containing protease inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin, 5 μg/ml

aprotinin and 1 mM PMSF) at 4°C for 20 min and then centrifuged for 30 min at 4°C at 13 000 *g*. The supernatant was incubated with either αR7 or αLI (both at 10 μg/ml) for 1 h at 4°C. After this incubation, protein A–Sepharose was added (10 μl/ml) and incubation continued for another 45 min. Immunocomplexes were harvested and washed twice with 1% Triton buffer and once with 0.1% Triton buffer. Immunoprecipitation with CHAPS was performed in a similar way, except that the media contained 30 mM CHAPS instead of Triton X-100.

Quantitative immunoblotting was done as follows: different amounts of turkey erythrocyte nuclear envelopes were electrophoresed in 10% polyacrylamide gels. The amount of p58 and lamin B was measured by staining the gel with Coomassie blue, extracting the dye with pyridine, and measuring absorbance at 605 nm (Fenner *et al.*, 1975). Replica gels were transferred to nitrocellulose filters and probed with anti-p58 and anti-lamin antibodies/^{125}I-labelled protein A. The amount of ^{125}I-labelled protein A bound (via the antibody) to each protein was measured by excising the corresponding bands with the aid of an autoradiogram and γ-counting. From these experiments standard curves were generated, correlating the amount of p58 and lamin B (in μg) with the amount of radioactivity in the blots after antibody/protein A decoration. To calculate the amount of p58 and lamin B in detergent extracts and immunoprecipitates, a fraction of each preparation was analyzed by Western blotting and the ^{125}I-labelled protein A radioactivity measured as above. The values obtained were fitted to the standard curves and the mass of each antigen was deduced.

Immunoisolation of mitotic vesicles was carried out as follows. Affinity-purified αLI (10 μg), affinity-purified αR7 (10 μg) or normal rabbit IgG (10 μg) were mixed with 50 μl of magnetic beads coated with sheep anti-rabbit IgG (Dynabeads, Dynal, Norway). After overnight incubation, the beads were washed with PBS and mixed either with mitotic homogenates or with floated mitotic membranes fractionated in sucrose gradients. After incubation for 2 h, immune complexes were harvested from the liquid phase with the help of a magnet and washed three times with PBS and either processed for Western blotting, or fixed with 1% glutaraldehyde in 0.2 M cacodylate buffer pH 7.4, Epon-embedded, thin-sectioned and visualized by EM.

Immunoelectron microscopy

For single labelling immunoelectron microscopy, samples adsorbed to EM grids were first blocked for 15 min with 0.5% (w/v) cold fish gelatin in KHM buffer and then incubated for 30 min with the appropriate antibody solution (1/10 in gelatin/KHM for αLI; 1/2 in gelatin/KHM for αR7). After three washes with gelatin/KHM, samples were incubated with protein A–gold (diluted 1/100 to 1/250) for another 20 min. The samples were washed (three times in KHM buffer for 5 min and once in distilled water for 2 s) and stained with 2% uranyl acetate. For double immunodecoration, the samples were incubated first with one antibody and then with protein A–9 nm gold, blocked with 0.1% buffered glutaraldehyde, washed with 20 mM glycine/KHM and incubated with the second antibody and protein A–14 nm gold. After appropriate washings, the samples were stained with 2% uranyl acetate. The specimens were visualized in a Philips 301 or a Philips 400 microscope operated at 80 kV.

Other methods

Polyacrylamide gel electrophoresis was done as described by Laemmli (1970). Protein concentration was measured by a Bio-Rad kit (Bio-Rad, Richmond, CA).

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