

Distinct nuclear assembly pathways for lamins A and C lead to their increase during quiescence in Swiss 3T3 cells

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SUMMARY

The expression of A-type lamins coincides with cell differentiation and as A-type lamins specifically interact with chromatin, a role in the regulation of differential gene expression has been suggested for A-type lamins. Using the mouse Swiss 3T3 cell line as a model, the change in two A-type lamins, lamins A and C, during cellular quiescence has been investigated. This well established model system mimics the first stages of differentiation when cells exit the cell cycle. In fact, quiescence in Swiss 3T3 cells was accompanied by a significant increase (2.6-fold) in lamin A protein levels and a smaller but reproducible increase (1.4-fold) in lamin C. These effects were fully reversible upon restimulation of the cells with serum. No effect upon lamin B levels was observed. Conversely, levels of A-type lamin mRNA decreased markedly as a result of quiescence suggesting transcriptional mechanisms are involved in the change in levels of lamins A and C. No difference in the incorporation of microinjected human lamin A into nuclei of quiescent or proliferating cells was observed. These data suggest A-type lamin binding sites were not limiting and indicated little difference between A-type lamin assembly mechanisms in quiescent and proliferating cells. The data did demonstrate lamin A and lamin C incorporation into

the nuclear lamina proceeded by different pathways when microinjected in Swiss 3T3 cells. The incorporation of recombinant lamin C into the nuclear lamina was delayed compared to lamin A and proceeded via intranuclear foci. Such foci were not seen with microinjected lamin A. Instead, recombinant lamin A was rapidly (<20 minutes) incorporated into the nuclear lamina. Comicroinjection of lamin A with lamin C did not prevent foci formation but assisted in the rapid clearing ($t_{1/2}$ =30 minutes) of these structures and the incorporation of both lamins A and C into the lamina. These data suggest that the incorporation of lamin C into the lamina is facilitated by lamin A. They demonstrate a distinct difference in the nuclear assembly pathways of lamins A and C and show for the first time a functional distinction for these two splice variants of the A-type lamin gene. From the differences in assembly pathways and changes in protein levels accompanying quiescence in 3T3 cells, we suggest distinct roles for lamin A and lamin C in proliferating and quiescent states of the cell cycle.

Key words: Nucleus, Intermediate filament, Lamin, Cell cycle, Quiescence

INTRODUCTION

The nuclear lamins are members of the intermediate filament protein family. Two groups have been recognised on the basis of sequence homologies, the A-type lamins and the B-type lamins. The lamins were first described as integral components of the nuclear lamina which underlie the inner nuclear membrane (Dwyer and Blobel, 1976; Gerace et al., 1978). Three major proteins were identified and termed lamins A, B and C. Subsequent studies have revealed that lamins A and C are splice variants of the same gene (Lin and Worman, 1993). In the case of somatic mammalian B-type lamins, two major proteins have been identified (Lehner et al., 1986), namely lamin B1 (Peter et al., 1989) and lamin B2 (Vorburger et al., 1989). These B-type lamins are the products of separate genes (Höger et al., 1988, 1990; Zewe et al., 1991).

A- and B-type lamins are distinguished by a number of criteria. These include their post-translational processing, their solubility properties during mitosis and their pattern of

expression. A-type lamins but not B-type lamins bind specifically to chromosome surfaces (Glass et al., 1993) and to specific DNA sequences (Baricheva et al., 1996). On the other hand, B-type lamins are always membrane bound (Gerace and Blobel, 1980; Stick et al., 1988). This is in part due to the isoprenylation of the CaaX-box in B-type lamins (Kitten and Nigg, 1991) and although lamin A also possesses such a signal, subsequent proteolytic processing removes this modification (Gerace et al., 1984; Weber et al., 1989; Hennekes and Nigg, 1994). Lamin C, another A-type lamin, does not possess a CaaX-box raising questions about the role of the isoprenylation and subsequent proteolytic steps in the association of A-type lamins with the inner nuclear membrane. Subsequent analyses have demonstrated that these modifications are not absolutely required for assembly into the peripheral nuclear lamina (Sasseville and Raymond, 1995), which means that other mechanisms must contribute to the lamin association with the inner nuclear membrane.

Such additional mechanisms include specific membrane

receptors for lamins and the specific assembly characteristics of lamins. In the case of B-type lamins, specific receptors are important for facilitating membrane association (Ye and Worman, 1994) and integral membrane proteins which specifically interact with A-type lamins have also been identified (Foisner and Gerace, 1993) although their importance has not been determined. Another important factor in the association of A-type lamins is the preference for heterotypic interactions between A-type and B-type lamins (Krohne et al., 1987; Georgatos et al., 1988; Ye and Worman, 1995) and therefore by implication B-type lamins must always be present to assist in the incorporation of A-type lamins into the lamina. As the A-type lamins are differentially expressed during development (Benavente et al., 1985; Lebel et al., 1987; Lehner et al., 1987; Stewart and Burke, 1987) and the B-type lamins are present in almost all nuclei (for an apparent exception see Broers et al., 1993) then lamin coassembly is also a potentially important factor in nuclear lamina association of A-type lamins.

During development, the appearance of A-type lamins is coincident with the emergence of differentiated phenotypes (Lehner et al., 1987; Stewart and Burke, 1987; Lourim and Lin, 1989; Röber et al., 1989). In cell lines which have the capacity for differentiation, A-type lamin expression is also induced under appropriate conditions (Lebel et al., 1987; Stewart and Burke, 1987; Paulin Levasseur et al., 1989; Guilly et al., 1990; Hass et al., 1990; Horton et al., 1992). The observed preferential binding of A-type rather than B-type lamins to chromatin (Höger et al., 1991; Glass et al., 1993; Baricheva et al., 1996) as well as the ability of transfected A-type lamins to induce differential gene expression (Lourim and Lin, 1992) and the observed rearrangement of lamin A accompanying differentiation in HL60 cells (Collard et al., 1992) also suggest A-type lamins are involved in differential gene expression.

The precise nature of this involvement has been the subject of some debate. For instance, the overexpression of one of the A-type lamins, lamin A, in myoblast cells caused a transient expression of muscle specific genes (Lourim and Lin, 1992) but in P19 embryonic carcinoma cells, the overexpression of heterologous lamin A did not induce differentiation (Peter and Nigg, 1991). These data appear to question a direct role for A-type lamins in differential gene expression. A-type lamins could assist differentiation by facilitating growth arrest (Lebel et al., 1987; Nigg, 1989; Hass et al., 1990; Lourim and Lin, 1992). Our recent data (Coates et al., 1996) support this view. Two markers of cellular quiescence, the BU31 antigen (Rowlands et al., 1994) and statin (Wang, 1985) were shown to be synonymous with lamins A/C. So A-type lamins are linked to the switch from proliferation to quiescence but again the critical unanswered question is whether A-type lamins are directly or indirectly involved.

To help resolve such key questions two key issues have to be settled. Firstly, are A-type lamin protein levels required to change during differentiation? Current results are inconclusive as some studies demonstrate a change in lamin A and C protein levels is required (Lebel et al., 1987; Lourim and Lin, 1989) whereas others suggest that a rearrangement in endogenous A-type lamin assemblies is sufficient (Collard et al., 1990, 1992). The Swiss 3T3 model provides a system to unequivocally demonstrate whether lamin A/C levels increase or remain unchanged during quiescence.

The second key question concerns the relative importance of

lamin A and lamin C as these products of the A-type lamin gene differ by only 98 residues. Evidence that lamins A and C could have distinct roles comes from apparent differences in the assembly properties of lamins A and C in cells. For instance, although newly synthesised lamins A and C can be incorporated directly into the nuclear lamina (Gerace et al., 1984), the relative rate of incorporation was 12 times faster for lamin A ($t_{1/2}=5$ minutes) than for lamin C ($t_{1/2}=60$ minutes; Gerace et al., 1984). In the case of P19 embryonic carcinoma cells, in the absence of lamin A, lamin C was apparently only incorporated into the nuclear lamina after the cells have undergone mitosis (Horton et al., 1992) which was not the case for lamin A. Once incorporated though, both lamins A and C remain associated with the nuclear lamina fraction after extraction with detergents and high salt containing buffers (Horton et al., 1992). These studies therefore suggest that lamin A and lamin C are not equivalent in their assembly characteristics and this needs clarifying.

In this study we have addressed these two questions using the well established Swiss 3T3 cell model (Greenberg and Ziff, 1984). We show that A-type lamin protein levels are significantly increased (2.6-fold). A reproducible but smaller increase in lamin C levels was also observed (1.4-fold). The increase in protein levels was the inverse to changes in mRNA levels which decreased substantially in quiescent cells. The nuclear lamina of proliferating and quiescent cells showed no difference in the incorporation of microinjected lamins A and C suggesting that the number of A-type lamin binding sites at the inner nuclear membrane was not limiting. These studies revealed that lamins A and C follow separate but dependent assembly pathways and suggest that the rapid incorporation of lamin C depends upon lamin A levels. Collectively these data suggest a functional distinction between lamin A and lamin C.

MATERIALS AND METHODS

Cell culture

Swiss 3T3 murine fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) foetal calf serum (FCS) and supplemented with glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 µg/ml). For proliferating cell cultures, cells were grown to 30–40% confluency. For quiescent cultures, cells were first grown as above and then maintained in DMEM containing 0.1% FCS (v/v) for 7 days (Pardee et al., 1978). To restimulate quiescent cells, 10% (v/v) FCS was added. This induces the cells to reenter the cell cycle synchronously, undergoing DNA synthesis 14–20 hours later (Todaro et al., 1965). The cells were harvested after 24 hours and 48 hours. For immunocytochemical studies, cells were plated onto 13 mm diameter glass coverslips. In some instances, cells were incubated with 25 µM bromodeoxyuridine (BrdU; Sigma) for 30 minutes to monitor the proliferative activity of the cells.

Antibodies

The mouse monoclonal anti-lamin A (133A2; Hozak et al., 1995) and anti-lamin A/C (131C3) antibodies were used in these studies as well as mouse monoclonal antibodies to rat lamin B₂ (a gift from Dr L. Gerace, The Scripps Research Institute, La Jolla, USA) and the mouse monoclonal antibodies BU31 (Rowlands et al., 1994) which recognizes lamins A/C (Coates et al., 1996). Polyclonal mouse anti-human lamin A/C antibodies (Pc170) were generated against the N-terminal 171 amino acids of human lamins A/C which had been expressed as a glutathione S-transferase (GST) fusion protein. Serum was preadsorbed with glutathione S-transferase bound to glutathione-Sepharose beads prior to use. The rabbit anti-vimentin polyclonal antibodies (Moscinski and Evans, 1987) were

a gift from Dr R. Evans (Department of Pathology, University of Colorado Health Sciences Centre, Colorado, USA).

To monitor the proliferative activity of the cells, BrdU incorporation was detected using mouse monoclonal anti-BrdU antibodies (Sigma UK). Cells were fixed with methanol/acetone (1:1) and treated with 1.5 M HCl for 30 minutes prior to incubation with the anti-BrdU antibody.

Secondary anti-mouse and anti-rabbit antibodies were purchased from Dako, UK.

Isolation of nuclei from growing and quiescent Swiss 3T3 fibroblasts

Proliferating or quiescent cell cultures were washed twice in ice-cold phosphate-buffered saline and once with nuclear isolation buffer (NIB: 10 mM Tris-HCl, 10 mM NaCl, 3 mM MgCl₂, 0.5% (v/v) Triton X-100, pH 7.6; Hutchison, 1994) at 4°C. The cells were incubated in NIB for 5 minutes at 4°C, collected by scraping and burst using a Dounce homogenizer with a loose fitting pestle. Nuclei were counted using a haemocytometer and recovered by centrifugation at 3,000 rpm in an M4 swing-out rotor in a Jouan 4-22 centrifuge for 10 minutes through a 5 ml sucrose cushion (30% w/v in NIB). The nuclear pellet was dissolved in Laemmli sample buffer (Laemmli, 1970).

Preparation of cytoskeletal extract from proliferating and quiescent cells

Proliferating or quiescent Swiss 3T3 fibroblasts were collected and washed as described above and then were resuspended in cytoskeletal extraction buffer (10 mM Tris-HCl, 140 mM NaCl, 1.5 M KCl, 5 mM EDTA and 0.5% (w/v) Triton X-100, pH 7.6) and homogenized using a Dounce homogenizer. The homogenate was centrifuged at 3,500 rpm for 40 minutes at 4°C, the pellet washed once in PBS and dissolved in Laemmli sample buffer (Laemmli, 1970).

RNA isolation and northern blotting

RNA was isolated as described (Chomczynski and Sacchi, 1987). Total RNA from equal numbers of proliferating and quiescent cells were electrophoresed on 1.2% (w/v) agarose (SeaKem) gels containing 0.2 M formaldehyde. Staining with ethidium bromide was used to check the integrity of the 18S and 28S rRNA bands. Separated RNA species were transferred to Hybond-C membranes (Amersham, UK) from the agarose gel using a standard northern blotting protocol (Sambrook et al., 1989). After transfer the filter was baked at 80°C for 2 hours. The random hexanucleotide primer method in kit form (BCL, UK) was used to label the probes. Unincorporated [α^{32} P]dCTP was separated from labelled DNA by centrifuging the reaction mixture through a Sephadex G-50 (Sigma, UK) spin column preequilibrated with Tris-EDTA-NaCl (TEN) buffer (Sambrook et al., 1989). The specific activity of the probe was determined using a scintillation counter and the probe stored at -20°C until required. After hybridisation and washing steps the blot was exposed to Kodak X-OMAT film at -80°C.

The probe used for lamins A and C was a 450 bp fragment encompassing residues 171-319 (Fisher et al., 1986; McKeon et al., 1986). The complete coding region of human vimentin (Ferrari et al., 1986; Ralton et al., 1994) and human PCNA (Kill et al., 1991) were used as controls.

Immunofluorescence labelling

Cells were routinely fixed in methanol/acetone (1:1, v/v) for 4 minutes at -20°C and then were washed twice in phosphate buffered saline (PBS) and blocked for 30 minutes with 10% (v/v) goat serum (Sigma, UK) in PBS. Cells were incubated in primary antibodies. Antibodies 131C3, 133A2 and Pc170 were used at 1 in 2,000, 1 in 1,500 and 1 in 30 dilution, respectively. Vimentin and lamin B₂ antibodies were used at 1 in 500 dilution. All primary antibodies were incubated at room temperature for 1 hour. Bound primary antibody was detected by indirect immunofluorescence using a goat anti-mouse IgG FITC-conjugated antibody (1 in 100 dilution; Dako, UK). Coverslips were mounted onto glass slides and viewed using a Bio-Rad MRC 600

confocal laser scanning microscope. Digitally stored images were printed using a Sony video printer.

SDS-PAGE and immunoblotting

Protein samples were electrophoresed on 7.5-17.5% (w/v) polyacrylamide gels or 5-15% (w/v) minigels as described previously (Ralton et al., 1994). Samples were electrophoretically transferred to nitrocellulose membranes using a Bio-Rad transblot (Kyhse-Andersen, 1984). Nitrocellulose membranes were blocked overnight at 4°C with 3% (w/v) Marvel. Bound primary antibody (133A2 and 131C3 at 1 in 5,000 dilution) was detected using a goat anti-mouse peroxidase-conjugated antibody (Dako, UK) or a pig anti-rabbit peroxidase-conjugated antibody used at 1 in 1,000 dilution (Dako). Blots were developed using diaminobenzidine and hydrogen peroxide in Tris buffered saline (TBS). The relative proportions of lamins A and C were determined from immunoblots using the BioImager system and Wholeband analysis software (Bio Image, UK).

Expression of recombinant proteins

Three fragments of human lamin C were expressed as described (Dyer et al., 1997) in *E. coli* as fusions with glutathione S-transferase (GST) using the pGEX expression system (Smith and Johnson, 1988). The three fragments were as follows: GST-lamin C 1-171, GST-lamin C 171-319 and GST-lamin C 319-572. The fragments were purified first by affinity chromatography using glutathione-agarose (Sigma, UK) and then by ion exchange chromatography using a 1 cm × 10 cm Fractogel EMD DEAE-650S column (Merck-BDH, UK) linked to a Merck-Hitachi Biochromatography system. The proteins were eluted using a 0-1 M NaCl linear gradient in a buffer of 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 25 mM β -mercaptoethanol. Bacterially expressed full length human lamin B was a gift from Dr M. Stewart (MRC, Cambridge, UK). Recombinant human lamins A and C for microinjection were produced and purified as described (Moir et al., 1990) using the Merck-Hitachi Biochromatography system.

Fluorescence labelling of human lamins A and C

Lamins A and C were fluorescently labelled with both tetramethylrhodamine 5-iodoacetamide (5-TMR1A; Pierce) and 5-iodoacetamidofluorescein (5-IAF; Pierce), respectively, as described (Schmidt et al., 1994). Briefly, 400 μ l of lamins A and C were dialysed into a buffer containing 8 M urea, 20 mM Na₂HPO₄, pH 7.0, 1 μ M leupeptin. A stock solution of the appropriate label (4 mg/ml in dimethylsulphoxide) was added in aliquots of 2 μ l until a sixfold molar excess had been achieved. The labelling reaction was performed in the dark at room temperature for 12-16 hours. To remove non-bound dye the protein was precipitated as described (Wessel and Flügge, 1984). The mixture was mixed thoroughly and centrifuged at 3,500 rpm for 5 minutes. The supernatant was removed, 3 volumes of methanol added and the denatured protein recovered by centrifugation at 3,500 rpm for 5 minutes. The pellet was dried briefly and then dissolved to a final protein concentration of approximately 3.0 mg/ml in a buffer containing 8 M urea, 20 mM Na₂HPO₄, pH 8.0, and stored at -80°C. The labelling efficiency was determined as described (Simon and Taylor, 1986).

Microinjection of fluorescently tagged lamins

For microinjection studies, 20 μ l of fluorescently labelled lamins A and C were dialysed against lamin injection buffer (20 mM Na₂HPO₄, 300 mM KCl, pH 8.2; Schmidt et al., 1994) for 1 hour at room temperature. Following dialysis the concentrations of the labelled proteins were determined using a Bradford assay and adjusted to 0.5 mg/ml for microinjection. Cells were grown on 13 mm diameter coverslips (BDH) for microinjection which was performed using Eppendorf femtotips and an Eppendorf microinjector 5242 (Eppendorf). Microinjected cells were incubated at 37°C for various times before being fixed in methanol/acetone (1:1, v/v) for 5 minutes at -20°C and analysed by immunofluorescence microscopy. In those experiments where both

lamins A and C were microinjected, then, for example, 5-TMRIA lamin A would be coinjected along with 5-IAF lamin C so that lamin A could be followed in the rhodamine channel and lamin C in fluorescein channel in the one cell at the same time. The incorporation characteristics of lamins A and C were independent of the fluorescent tag used.

RESULTS

Characterization of the antibodies used in this study

Previous studies had shown that epitome masking could prevent the detection of lamin A in cells (Collard et al., 1990; Collard and Raymond, 1992). To ensure that any changes seen by immunofluorescence in the levels of lamins A/C in quiescent cells were proportional to changes in protein levels, we used a panel of antibodies which recognized three different domains in lamins A and C. A polyclonal mouse serum was raised against a GST-fusion protein containing the N-terminal 171 amino acids of lamins A/C (Fig. 1A). This serum reacts specifically with lamins A and C but not lamin B (Fig. 1B, lanes 5-7). Three GST-fusion proteins spanning the entire length of lamin C (Fig. 1A) were used to characterize monoclonal antibody 131C3. This antibody recognizes an epitope located within residues 319-566 of lamins A/C (Fig. 1B, lane 14). Monoclonal antibody 133A2 has been characterized previously and recognizes an epitope within residues 598-611 of human lamin A (Hozak et al., 1995).

Antibodies to lamins A/C preferentially stain quiescent nuclei

Antibodies specific for lamins A/C were used to stain Swiss 3T3 fibroblasts which were either actively proliferating as

shown by BrdU staining (75% positive cells: data not shown) or grown to confluency and then maintained in DMEM containing 0.1% (v/v) FCS for 7 days (<0.1% of cells had incorporated BrdU; data not shown) to induce quiescence.

Monoclonal antibody 131C3 stains the majority of proliferating nuclei weakly (Fig. 2A,B). A few nuclei display intranuclear lamin staining (Goldman et al., 1992; Bridger et al., 1993) which is frequently due to extensions of the nuclear membrane rather than foci in Swiss 3T3 cells (Schmidt et al., 1994). After cells have been maintained in 0.1% (v/v) FCS for 7 days, all the cells displayed strong nuclear rim staining (Fig. 2C-F). This was irrespective of the location of the epitope since all three monoclonal antibodies 131C3 (Fig. 2C,D), 133A2 (Fig. 2E,F) and BU31 as well as polyclonal Pc170 antibodies (data not shown) gave similar staining patterns.

These results suggest a significant increase should be observed in A-type lamin protein levels in quiescent Swiss 3T3 cells as has been observed in other differentiating culture models (Lebel et al., 1987; Stewart and Burke, 1987; Lourim and Lin, 1989; Paulin Levasseur et al., 1989). Therefore we have quantitated the extent of the increase in lamins A and C with respect to nuclear number.

Protein levels of lamins A and C are increased in quiescent nuclei

Immunoblotting analysis using the antibody 131C3 demonstrated an increase in the level of lamin A when cell extracts were compared for proliferating and quiescent cells (Fig. 3). Whole cell extracts revealed an increase in A-type lamin levels whereas vimentin protein levels dropped due to quiescence (data not shown). To give more accurate quantitative compar-

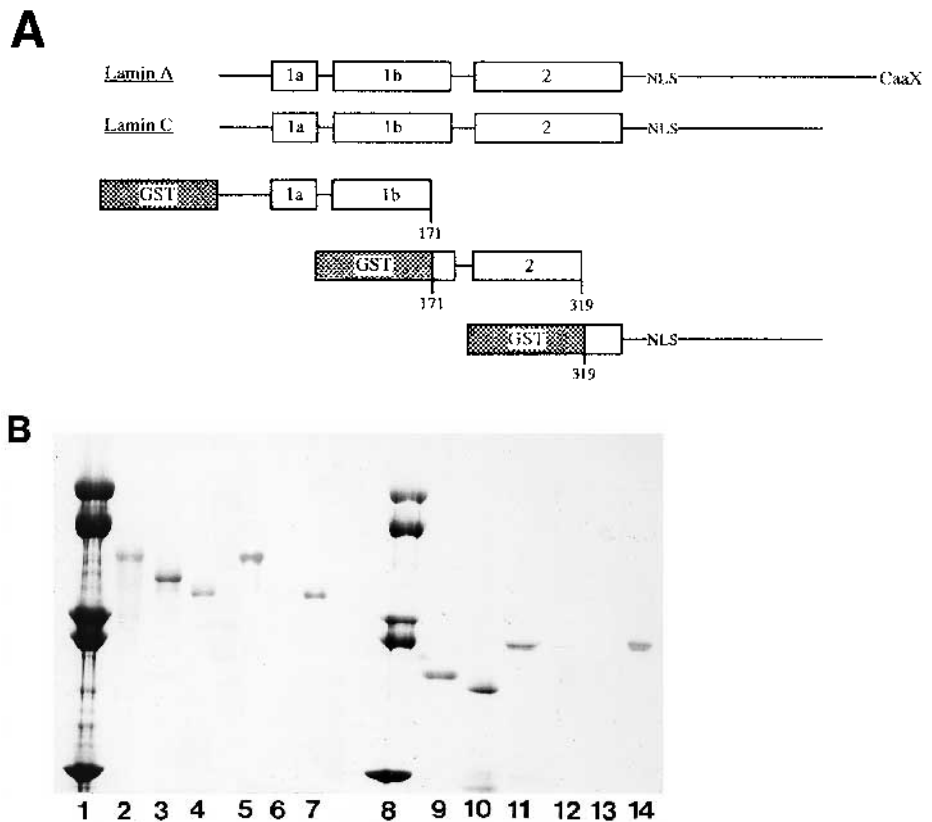


Fig. 1. Characterisation of the antibody tools used in this study. (A) Schematic representation of human lamins A and C. Lamin C, which is identical to lamin A apart from 6 amino acids at the C terminus, and the three fragments of human lamin C expressed in bacteria as fusions with GST. Rod domains, nuclear localisation signals (NLS) and CaaX box are all indicated. (B) Bacterially expressed, purified human lamins A, B and C were subjected to SDS-PAGE and either stained with Coomassie Blue (lanes 2, 3 and 4, respectively) or blotted with mouse polyclonal antibodies Pc170 (lanes 5, 6 and 7). Pc170 reacts with human lamins A and C only (lanes 5 and 7). Three fragments of human lamin C comprising residues 1-171, 171-319 and 319-572 were subjected to SDS-PAGE and either stained with Coomassie Blue (lanes 9, 10 and 11, respectively) or blotted with monoclonal antibody 131C3. 131C3 reacts specifically with an epitope located within residues 319-572 (lane 14). Molecular mass markers used are 116, 97, 58, 53 and 29 kDa, respectively (lanes 1 and 8).

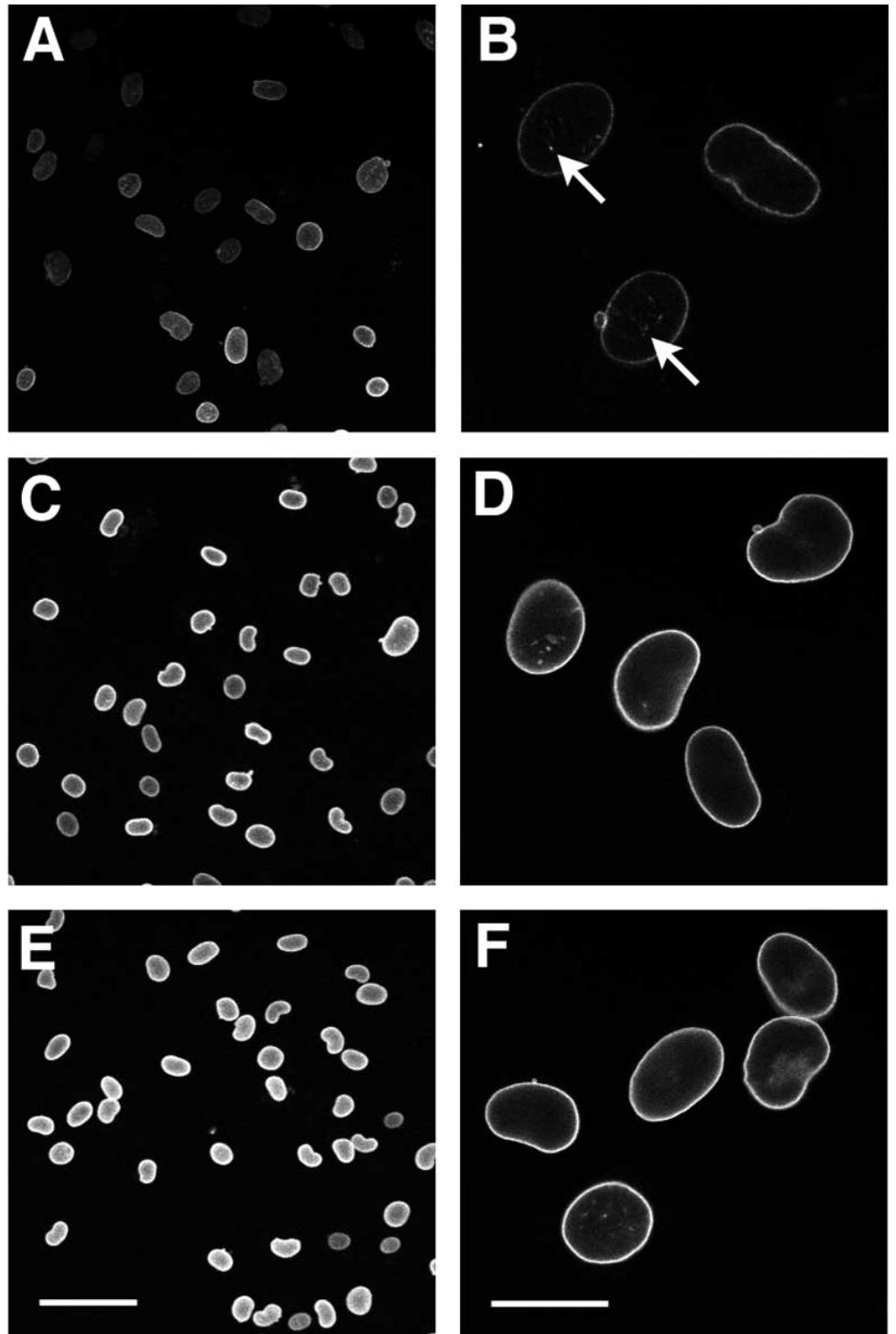


Fig. 2. Confocal images of proliferating and quiescent Sw3T3 fibroblasts probed with monoclonal antibodies 131C3 and 133A2. Cells were maintained in DMEM containing 10% (v/v) FCS and grown to 40-50% confluency or grown to confluency and maintained in DMEM containing 0.1% (v/v) FCS for 7 days, fixed in methanol/acetone (1:1; v/v) and indirectly immunofluorescently labelled with 131C3 (A-D) or 133A2 (E,F). (A and B) Confocal images ($\times 16$ and $\times 63$, respectively) of proliferating nuclei. There is variation in the intensity of nuclear envelope staining with a majority of cells displaying a weak nuclear rim. Arrows denote intranuclear staining with lamin A/C antibodies (Goldman et al., 1992; Bridger et al., 1993; Schmidt et al., 1994). (C and D) Confocal images ($\times 16$ and $\times 63$, respectively) of quiescent nuclei. There is a marked increase in the intensity of nuclear envelope staining with all the nuclei displaying a strong nuclear rim. (E and F) Immunolabelling of proliferating and quiescent Sw3T3 fibroblasts with 133A2. Images (E) $\times 16$ and (F) $\times 63$ of quiescent nuclei show strong nuclear envelope staining with this antibody. In proliferating cells, these antibodies gave similar staining patterns as seen with the monoclonal antibody 131C3 (data not shown). Bars: 100 μm (A,C,E); 25 μm (B,D,F).

isons of changes in lamin levels, nuclei were first purified and comparisons made on the basis of equal numbers of nuclei for the different cell states (Fig. 3). When equal numbers of nuclei were loaded per gel lane, then a small but measurable increase in the level of lamin C (Fig. 3, cf lane 2 with lane 1) as well as a significant increase in lamin A levels were observed (Fig. 3, cf track 2 with lane 1) in serum starved Swiss 3T3 cells. Lamin B levels remained apparently unaffected (Fig. 3, lanes 5, 6). Using these results and four other different preparations

of nuclei from both proliferating and quiescent cells, it was found that the mean increase in lamins A levels was 2.6-fold (± 0.4 $n=5$) whilst lamin C levels increased 1.4-fold (± 0.6 $n=5$) in quiescent cell nuclei compared to proliferating cell nuclei.

Serum restimulation of quiescent cultures results in a rapid decrease in the levels of lamins A and C

In Swiss 3T3 cells, the readdition of serum has been shown to stimulate the cells to reenter the cell cycle and proliferate

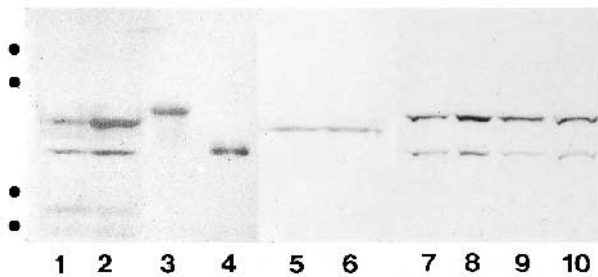


Fig. 3. Lamin A and lamin C protein levels are increased as a result of quiescence and this effect is reversible upon reentry of the cells into the cell cycle. To compare directly levels of lamins A and C in proliferating and quiescent cells, equivalent numbers of nuclei were analyzed by SDS-PAGE and immunoblotting. Nuclei were extracted directly with Laemmli sample buffer after purification. The monoclonal antibody 131C3 was used to detect lamins A and C. The levels of lamins A/C (lanes 1, 2 and 7-10) and lamin B₂ (lanes 5, 6) were compared in isolated nuclei from proliferating (lanes 1, 5 and 7, respectively) and quiescent cells (lanes 2, 6 and 8) and the reversibility of the lamin A/C increase investigated by readdition of serum (lanes 9 and 10). In quiescent cells (lane 2) the levels of lamin A and lamin C were increased 2.6 and 1.4-fold, respectively, as determined by quantification of the immunoreactive bands compared to levels in nuclei isolated from proliferating cells (lane 1). Levels of lamin B₂, detected using the antibody RL24 did not appear to change in isolated nuclei of proliferating (lane 5) and quiescent (lane 6) cells. These effects upon lamins A and C are reversible upon reentry of the cells into the cell cycle with the addition of 10% FCS serum (lanes 7-10). Proliferating cells (lane 7) were serum starved for 7 days (lane 8) before the readdition of serum. After 24 hours (track 9) and 48 hours (lane 10), lamin A levels are reduced and are comparable to those found in proliferating cells (lane 7). Recombinant human lamin A (lane 3) and lamin C (lane 4) were included for reference. Notice that the recombinant lamin A has a slower relative mobility than the Swiss 3T3 lamin A because of missing post-translational modifications (Gerace et al., 1984; Weber et al., 1989; Hennekes and Nigg, 1994). The relative mobility of marker proteins β -galactosidase (116 kDa), phosphorylase B (94 kDa), pyruvate kinase (58 kDa) and glutamate dehydrogenase (53 kDa) are indicated (\bullet).

(Todaro et al., 1965). Vimentin levels increase (Rittling et al., 1985). If the changes observed in lamins A and C are linked to the quiescent state then protein levels would be expected to return to those observed in proliferating cells.

Equivalent numbers of nuclei from proliferating, quiescent and restimulated cells were analyzed by SDS-PAGE and immunoblotting (Fig. 3, lanes 7-10). The readdition of 10% (v/v) FCS to the quiescent cultures (Fig. 3, lane 8) caused the reduction of lamins A and C levels (Fig. 3, lanes 9, 10) to those found in proliferating nuclei (Fig. 3, lane 7) within 24 hours (Fig. 3, lanes 9). No further change occurred over the subsequent 24 hours (Fig. 3, lanes 10).

With these changes in protein levels, a change in the immunofluorescence signal would also be expected after restimulation of quiescent cells with 10% (v/v) serum. This was indeed the case, with most nuclei becoming only weakly positive for lamin A (Fig. 4B) from having been almost uniformly stained with the anti-lamin A antibodies (Fig. 4A). Readdition of serum therefore reversed the increase in lamin A signal.

A noticeable change in nuclear morphology occurred after restimulation with serum. The quiescent nuclei appeared more compact, sometimes 'bean-shaped', whereas the nuclei of re-

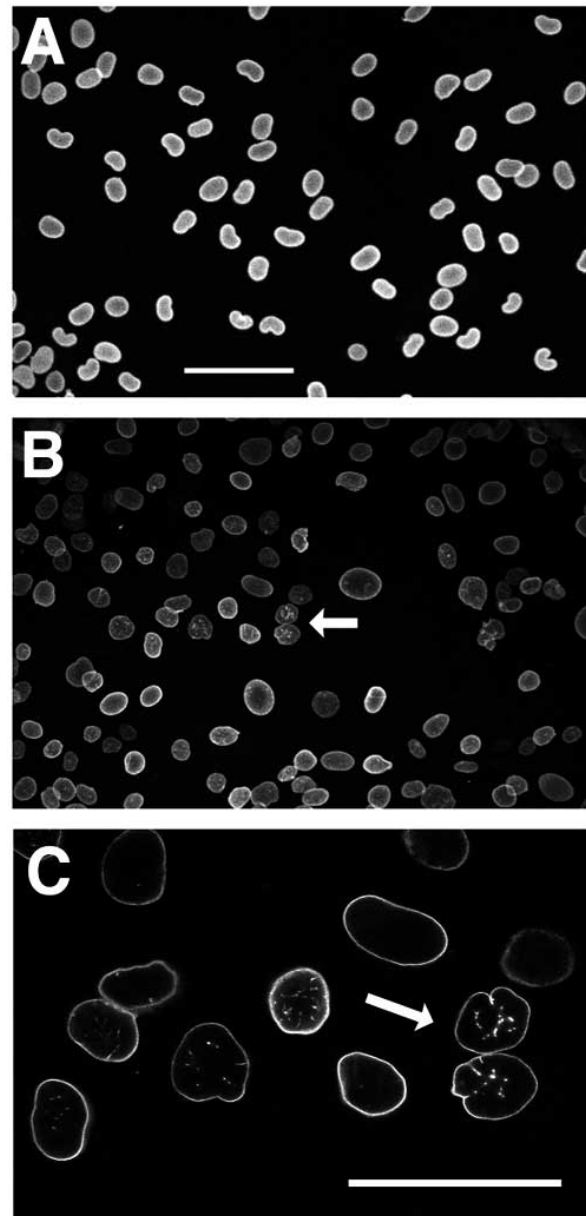


Fig. 4. The increase in immunofluorescence signal for lamins A and C is reversible in quiescent Swiss 3T3 cells by restimulation with serum. Serum starved Swiss 3T3 cells (A) were restimulated with 10% (v/v) serum (B,C). Before stimulation (A) the field of cells is uniformly stained with the anti-lamin A antibodies 133A2. After 24 hours exposure to 10% fetal calf serum (B,C) the immunofluorescence signal for lamin A has reduced significantly. The arrow in B points to a group of cells displaying prominent intranuclear staining. An enlargement of this area (C) clearly demonstrates that the intranuclear staining is due the highly folded nuclear membrane in serum restimulated cells. The structures are more frequent in restimulated cells (B) compared to proliferating cells (Fig. 2A). Bars, 100 μ m.

stimulated cultures were more diverse in size, often possessing highly folded nuclear membrane (Schmidt et al., 1994; Fig. 4C). These intranuclear projections appeared more frequent in the nuclei of restimulated cultures than in proliferating cultures (cf. Fig. 4B with Fig. 2B).

Levels in lamin A and C transcripts are substantially reduced in quiescent 3T3 cells

To investigate the possible mechanisms which control the increase in lamin A and C levels, northern blot analyses were undertaken (Fig. 5). Lamin A and C mRNA levels were substantially reduced in quiescent cells (Fig. 5A) and interestingly, lamin C mRNA was barely detectable in the quiescent cell samples. We also monitored changes in PCNA (Fig. 5B) and vimentin mRNA levels (Fig. 5C) and as previously reported these transcripts were also reduced in quiescent 3T3 cells (Rittling et al., 1985). It is important to note that whereas the change in vimentin protein levels correlated with a decrease in transcript, the change in lamin A and C protein levels was the inverse of the change in transcript levels.

Quiescent and proliferating cells efficiently incorporate fluorescently tagged lamins A and C

To investigate the contribution of post-translational mechanisms in regulating the incorporation of A-type lamins into the lamina of quiescent and proliferating cell nuclei, we used microinjection to introduce fluorescently tagged lamin A and lamin C into cells (Fig. 6). We observed that lamin A was rapidly incorporated into the nuclear lamina, even by 20 minutes (Fig. 6; lamin A) but that lamin C incorporation into the lamina was significantly delayed (Fig. 6; lamin C). Very importantly, no difference between proliferating (Fig. 6) and quiescent cells (data not shown) were seen in the incorporation of the tagged lamins. Even 24 hour post-microinjection (Fig. 6) appreciable signals for both lamins A and C were still detectable. Where mitosis occurred after microinjection, transmission of the tagged lamins to both daughter cells also occurred.

This approach did reveal another surprising observation. Lamin A was apparently incorporated by a separate pathway compared to lamin C (Fig. 6). Specifically, lamin A was found in the nuclear periphery by 20 minutes post-microinjection (Fig. 6) whereas lamin C showed no such direct incorporation into the lamina but formed intranuclear foci in the nucleoplasm at this time point. Lamin C foci were still present at the 180 minute time point but there appeared to be a steady incorporation into the nuclear lamina. The identity of the intranuclear foci was confirmed by 3-dimensional reconstruction of optical sectioned nuclei to distinguish these structures from intra-nuclear projections of nuclear membranes (Schmidt et al., 1994). The signals for both lamin A and C appeared quite stable as no significant reduction was observed after 24 hours. From these data, it appears that protein availability per se determines lamin A/C levels in the lamina as the microinjected protein incorporated equally efficiently in both quiescent and proliferating cells. This suggests that the post-translational mechanisms controlling lamin A/C incorporation appear the same in the two cell states.

Lamin A assists in the incorporation of lamin C into the nuclear lamina

To assess the relative importance of lamin A and lamin C in the incorporation process, equimolar amounts of the two lamins were microinjected into both quiescent (data not shown) and proliferating cells (Fig. 7). In the presence of equimolar lamin A, lamin C was efficiently and rapidly incorporated into the lamina. After 20 minutes, tagged human lamin C was now detected in the nuclear lamina. Foci of lamin A and C were formed but were cleared much more efficiently from the nucle-

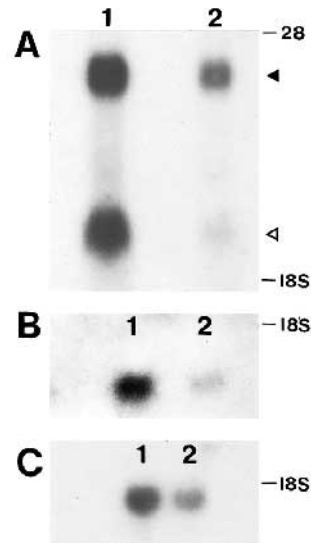


Fig. 5. Levels of lamin A and C RNA are significantly reduced in quiescent cells. Cells were detached from their growth surface by trypsinisation and counted prior to extraction so that a direct comparison of the levels of mRNA species corresponding to equivalent numbers of proliferating and quiescent cells could be made because in quiescence all RNA levels drop (Pardee, 1989). From 540,000 proliferating and quiescent cells, 30 μ g and 21 μ g total RNA was isolated, respectively, and subjected to electrophoresis. 18S and 28S RNAs were detected by ethidium bromide staining which confirmed that excessive degradation had not occurred before blotting onto nitrocellulose membranes and probed with several cDNA probes. A 450 bp fragment was excised from a vector containing the complete cDNA for human lamin C (pLCII Hulam C) and 32 P-labelled. Using this probe lamin A (A, filled arrowhead) and lamin C (A, open arrowhead) mRNAs were detected in both proliferating cells (A, lane 1) and quiescent cells (A, lane 2). Interestingly the levels of lamin A and C transcripts were significantly lowered in quiescent cells even taking into account the difference in the total amounts of RNA loaded for the two samples. Two controls were employed to compare the decrease in RNA levels in proliferating and quiescent cells; a proliferating-cell nuclear antigen (PCNA) probe (B) and a vimentin probe (C). Both PCNA and vimentin mRNAs are known to be down-regulated in quiescent cells (Rittling et al., 1985). The complete cDNAs for human PCNA (pCMV HuPCNA; kindly donated by Dr C. Midgely, Dundee University). An abundance of PCNA mRNA was detected in the proliferating cells following hybridisation (B, lane 1). As expected PCNA displayed a barely detectable level of transcription in quiescent cells (B, lane 2). The vimentin probe hybridised to a transcript present in both proliferating and quiescent cells (C). As previously demonstrated (Rittling et al., 1985) the level of hybridisation to the vimentin transcript was significantly lower in quiescent cells (C, lane 2) compared to proliferating cells (C, lane 1).

oplasmic compartment and were not detected 1 hour post-microinjection. From these data, lamin A appears to facilitate the incorporation of lamin C into the lamina.

DISCUSSION

Elevated lamin A levels accompany quiescence in Swiss 3T3 cells

This study demonstrates unequivocally that cellular quiescence

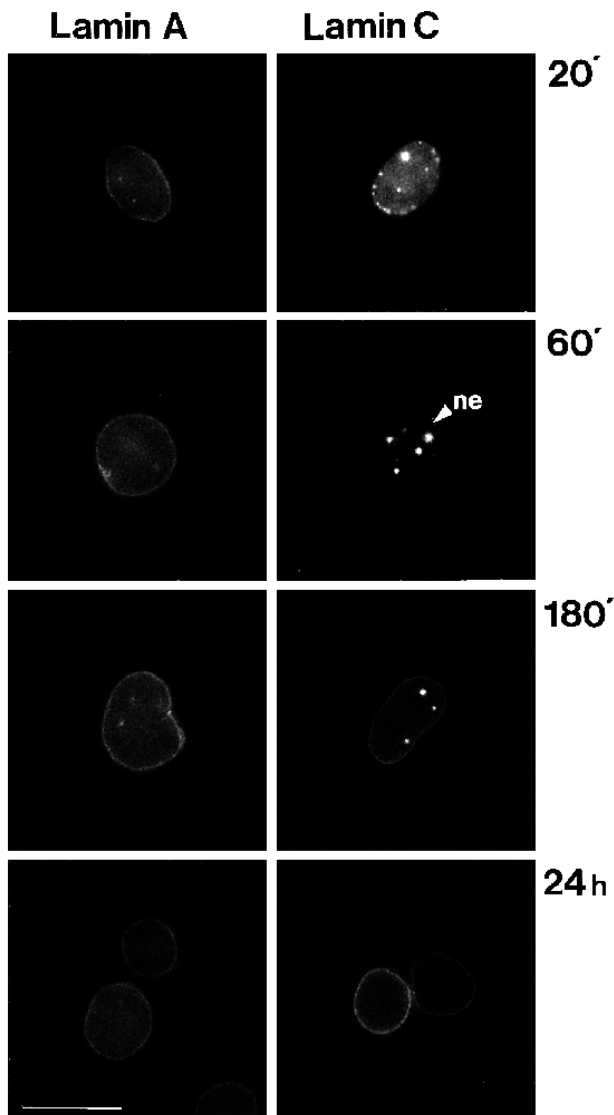


Fig. 6. Lamin A incorporation into the nuclear lamina is different from that of lamin C. Proliferating 3T3 cells were microinjected with fluorescently tagged human lamin A (3.2 mole label/mole lamin A) and lamin C (0.5 mole label/mole lamin C). Cells were fixed and processed for immunofluorescence microscopy after 20 minutes, 60 minutes 180 minutes and 24 hours. In the case of lamin A, incorporation into the nuclear lamina occurred after 20 minutes. This result was independent of the labelling efficiency of the lamin A as similar results were obtained with a sample labelled at 0.3 mole label/mole lamin A. The nuclei in 3T3 cells are often folded (Schmidt et al., 1994) and by immunofluorescence these can appear as spots like those seen after 20 minutes in the lamin A sample. Lamin C formed distinct intranucleoplasmic foci as confirmed by optical sectioning using the confocal microscope. Only after 180 minutes postinjection was the microinjected lamin C detected at the nuclear periphery although foci were still detectable. This demonstrates a difference in the pathway of incorporation for the two lamins. These observations were independent of the proliferation status of the cells as similar results were obtained in quiescent cells (data not shown).

in Swiss 3T3 cells is accompanied by a significant increase in protein levels of lamin A and a smaller but detectable increase in lamin C. This is the first study to quantitate changes in the

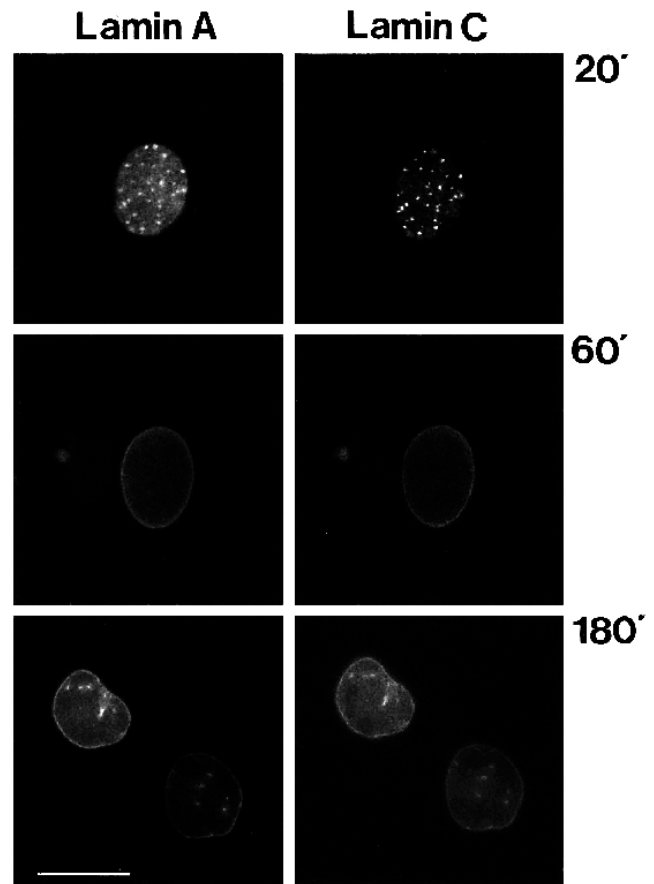


Fig. 7. Lamin A facilitates the incorporation of lamin C into the nuclear lamina. To investigate whether lamin A or lamin C had a more dominant role in the lamina incorporation process, proliferating cells were microinjected with equimolar amounts of fluorescently tagged lamin A (5-TMRIA) and lamin C (5-IAF). After 20 minutes, lamin A was not detected in the nuclear rim. Similarly lamin C colocalised with lamin A as foci and as unincorporated signal in the nuclear compartment. After 1 hour (60 minutes), both lamin A and C were almost exclusively found at the nuclear rim. These data indicate that lamin A is able to facilitate the incorporation of lamin C.

expression of lamins A and C accompanying quiescence. We have also demonstrated a down-regulation of lamin A and C protein levels upon reentry of Swiss 3T3 cells into the cell cycle which is accompanied by distinct changes in nuclear membrane morphology. These data establish a definite link between regulation of the cell cycle and relative levels of A-type lamins in the cell.

Earlier studies had shown that changes in A-type lamin protein levels accompanied cell differentiation (Lebel et al., 1987; Stewart and Burke, 1987; Lourim and Lin, 1989; Paulin Lévassieur et al., 1989) but subsequent investigations have shown that A-type lamins do not control differentiation. For instance, the overexpression of heterologous lamin A in P19 embryonic carcinoma cells (Peter and Nigg, 1991) did not induce differentiation. Also, not all terminally differentiated cells express lamins A/C such as pancreatic islet cells and Purkinje cells of the cerebellum (Cance et al., 1992). However, in chick embryonic muscle primary cells, the overexpression of lamin A did induce the transient expression of muscle

specific proteins (Lourim and Lin, 1992). The results presented here offer an explanation for the involvement of A-type lamins, particularly lamin A and to a lesser extent lamin C, in differentiation by helping to 'stabilize' cells (Nigg, 1989) which have exited from the cell cycle and entered a quiescent state prior to embarking on the differentiation process.

A prediction from such a proposed role for A-type lamins is that the absence of A-type lamins should be more conducive to rapid cell growth. The data in this study demonstrate that a decrease in A-type lamin levels correlates with proliferation in Swiss 3T3 cells, an observation seen also immunohistochemically in other cells including cancerous cells (Rowlands et al., 1994; Coates et al., 1996). Our results demonstrate lower levels of A-type lamins in proliferating cultures (Figs 2 and 3) and restimulated quiescent cultures which have re-entered the cell cycle also reduce their A-type lamin content (Figs 3 and 4). A correlation between significantly reduced levels or the absence of A-type lamins with the proliferative tendency of some tumours has been noted previously (Cance et al., 1992) so that transformation does not necessarily abolish A-type lamin function. Indeed, levels of A-type lamins were significantly reduced in small cell carcinomas (SCLC) compared to other lung non-SCLC lines (Broers et al., 1993). Still more interesting is the observation that expression of the *v-rasH* oncogene in SCLC lines not only changed the cellular phenotype to that of a non-SCLC but also increased A-type lamin expression by at least 10-fold (Kaufmann et al., 1991). These data help establish a link between the level of A-type lamin expression and reduced cell proliferation and now focus future investigations on the mechanisms whereby this influence is exerted.

Lamin A and lamin C are incorporated into the nuclear lamina by different pathways

Previous studies investigating the assembly pathway and kinetics of incorporation of lamin A and lamin C into the nuclear lamina had noted that lamin C always lagged behind lamin A (Gerace et al., 1984; Horton et al., 1992). In this study we have directly addressed this point using a microinjection strategy and found that lamin C was first incorporated into foci before entering the lamina whereas lamin A was incorporated directly into the lamina (Fig. 6). This result reconciles an apparent discrepancy in the literature concerning the presence (Goldman et al., 1992) or absence (Schmidt et al., 1994; Schmidt and Krohne, 1995) of lamin A in foci after microinjection into 3T3 cells. As biotinylated lamin A formed foci, we suggest that the biotinylation procedure, which modified lysine residues, altered the bioactivity of recombinant lamin A so that this biotinylated protein behaved more like lamin C than native lamin A. In agreement with this suggestion, others have shown that the labelling procedure can affect paracrystal formation for lamin A (Schmidt et al., 1994). The data here demonstrate a clear difference between lamin A and lamin C for the kinetics of incorporation.

These data also demonstrate that lamin A and lamin C differ in their preferred pathways of incorporation into the nuclear lamina. Nevertheless, these pathways do overlap as the microinjection of lamin A with lamin C caused lamin A to default to the lamin C pathway with the formation of the intranuclear foci (Fig. 7). This suggests that lamin A and C form hetero-oligomeric complexes in this situation with kinetics of incorporation into the lamina intermediate between that of the individual lamins. Comparison of the two experi-

ments in Figs 6 and 7 suggest that when lamin A is in excess (Fig. 6), then incorporation is independent of lamin C and does not result in foci formation. The functional importance of these different pathways is that they provide an additional mechanism to control A-type lamin assembly.

A question which follows from our observations concerns the functional distinction of lamin A and lamin C. They both arise by differential splicing from the lamin A gene (Lin and Worman, 1993) and lamin A only differs from lamin C by 98 C-terminal amino acid residues (Fisher et al., 1986). Lamin A and C form mixed oligomers (Krohne et al., 1987; Moir et al., 1991; Ye and Worman, 1995) and so it is perhaps difficult to reconcile this apparent difference in the assembly pathway for the two variants. A major difference between the two variants is the absence of the CaaX box from lamin C. This motif can significantly alter the pathway of incorporation into the lamina and removal of this motif induces the formation of intranuclear aggregates (Schmidt and Krohne, 1995) as seen for lamin C. This difference as well as the preferred interaction of lamin A with B-type lamins rather than lamin C (Georgatos et al., 1988) are consistent with our observation that lamin A and lamin C differ in their assembly pathway in the nuclei of Swiss 3T3 cells.

The recent description of another lamin A gene variant, lamin A Δ 10 (Machiels et al., 1996), adds further weight to this argument. This variant differs from lamin A through the lack of exon 10 which contains the polyhistidine and polyacidic residue domain. In GLC-A1 cells where the ratio of lamin A to C is reduced 8-fold and lamin A Δ 10 is the most abundant A-type lamin, the assembly pathway for lamin A Δ 10 has been described (Machiels et al., 1995). Lamin A Δ 10 does not efficiently incorporate into the lamina and was also observed as intranuclear aggregates (Machiels et al., 1995). These can be extracted with Triton-X-100 (Machiels et al., 1995) and therefore differ fundamentally from the lamin A/C intranuclear foci. These collective studies raise an important question concerning the functional significance of the three A-type lamin variants.

Functional roles for A-type lamins

Our studies have implicated lamin A and to a lesser extent lamin C in regulating the cell cycle in the establishment of quiescence. A role in proliferation is also implied as reduced levels of A-type lamins facilitate rapid cell proliferation. The question is whether A-type lamins play a passive or active role in these fundamental cellular processes. Previous studies have demonstrated that lamins A and C (residues 296-355; Ozaki et al., 1994) interact with negative growth regulators such as p110^{Rb} (Shan et al., 1992; Ozaki et al., 1994). A-type lamin expression is also modulated by oncogene expression with concomitant changes in cell morphology (Kaufmann et al., 1991). These data suggest that lamin influence is mediated through their interaction with known modulators of the cell cycle. For instance, the binding of A-type lamins to the p110^{Rb} binding site would be expected to alter its interaction with other cell cycle regulators (Ozaki et al., 1994). By such mechanisms, A-type lamins could facilitate and stabilize specific events in the cell cycle.

Our assembly data have demonstrated that A-type lamins can also occupy an intranuclear compartment as well as being found in the lamina. This is apparently dependent upon lamin C levels. So depending upon the rate of synthesis, the A-type lamin composition of this compartment can be varied but the

question is whether this is of functional significance. Some have suggested that these intranuclear foci indicate a specific functional compartment (Hozak et al., 1995). The close association with heterochromatin (Bridger et al., 1993) and the binding of chromatin by A-type lamins (Glass et al., 1993; Taniura et al., 1995) are all highly suggestive. Our studies indicate that the intranuclear foci also perform an assembly-related role and the indication from other studies (Machiels et al., 1995; Sasseville and Raymond, 1995) is that intranuclear foci result from inefficient A-type lamin assembly. The more important conclusion for A-type lamin function is that levels of lamin A determine the efficient assembly of both lamin C (this study) and lamin A Δ 10 (Machiels et al., 1995).

Another important observation to arise from these results is the lack of any demonstrable difference between the incorporation kinetics of exogenous lamin A and C into the lamina of either proliferating or quiescent nuclei. This indicates that lamin A incorporation sites can not be limiting in the two cell cycle states concurring with the view that it is an increase in lamin A protein levels which is the principal cause of the change in the lamina characteristics of quiescent 3T3 cells. In other cell types, other mechanisms can operate such as the local rearrangement of A-type lamins (Collard et al., 1992) and interactions with other nuclear components (Collard et al., 1990; Dyer et al., 1997). The present studies do not exclude other mechanisms but establish the importance of changes in lamin A levels to the establishment of the quiescent state in Swiss 3T3 cells.

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