Colocalization of intranuclear lamin foci with RNA splicing factors

G. Jagatheesan[1], S. Thanumalayan[1], Bh. Muralikrishna[1], Nandini Rangaraj[1], Anjali A. Karande[2] and Veena K. Parnaik[1,*]

[1]Centre for Cellular and Molecular Biology, Hyderabad 500 007, India
[2]Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India
*Author for correspondence (e-mail: veenap@ccmb.ap.nic.in)

SUMMARY

The lamins form a fibrous network underlying the inner nuclear membrane termed the nuclear lamina. In order to gain insights into the role of lamins in nuclear organization, we have characterized a monoclonal antibody (LA-2H10) raised against recombinant rat lamin A that labels nuclei in a speckled pattern in all cells of unsynchronized populations of HeLa and rat F-111 fibroblast cells, unlike the typical nuclear periphery staining by another monoclonal antibody to lamin A, LA-2B3. In immunolocalization studies the lamin A speckles or foci were found to colocalize with the RNA splicing factors SC-35 and U5-116 kD, but not with p80 coilin found in coiled bodies. Lamin B1 was also associated with these foci. These foci dispersed when cells entered mitosis and reformed during anaphase. The differential reactivity of LA-2H10 and LA-2B3 was retained after nuclei were extracted with detergents, nucleases and salt to disrupt interactions of lamins with chromatin and other nuclear proteins. Using deletion fragments of recombinant lamin A, the epitope recognized by LA-2H10 was located between amino acids 171 and 246. Our findings are consistent with a structural role for lamins in supporting nuclear compartments containing proteins involved in RNA splicing.

Key words: Nuclear lamina, Nuclear speckle, Spliceosome

INTRODUCTION

The nuclear lamins are the major components of a network of filaments underlying the inner nuclear membrane termed the nuclear lamina. The lamins play an important role in nuclear envelope disassembly and reassembly during mitosis, and have been proposed to be involved in the organization of the interphase nucleus (reviewed by Nigg, 1992; Hutchinson et al., 1994; Moir et al., 1995; Gant and Wilson, 1997). Two major classes of lamins are found in most mammalian cells: the B-type lamins (B1 and B2) that are expressed in all somatic cells, and the A-type lamins (A and C) that are present only in differentiated cells. Lamins A and C are alternatively spliced products of the same gene whereas B1 and B2 are coded by separate genes. Germ cells contain additional variants of the A and B-type lamins. The lamins belong to the intermediate filament family of proteins. Like other proteins in the family, the lamins have a central α-helical rod domain flanked by an amino-terminal head domain and a carboxy-terminal tail domain. The rod domains of two lamin monomers associate to form a two-stranded coiled-coil α-helix, which is the basic subunit involved in formation of the higher order structure of the lamin filament (Aebi et al., 1986; Stuurman et al., 1998). The association of lamins with chromatin (Glass et al., 1993) and integral membrane proteins (Gerace and Foisner, 1994) has been well-documented.

In addition to the well-characterized peripheral location of the lamins, internal lamin structures have been reported in G$_1$

cells (Bridger et al., 1993), in association with DNA replication centres in S phase cells (Moir et al., 1994), as well as during lamina assembly (Pugh et al., 1997). Hozak et al. (1995) have observed an internal nucleoskeleton of lamin filaments in HeLa cells at all stages of the cell cycle. Based on earlier morphological data (Hozak et al., 1993, 1994) the authors have proposed that this nucleoskeleton contains sites involved in transcription, RNA processing and, in S-phase cells, DNA replication. There is now considerable evidence for the colocalization of lamins with the DNA replication machinery (Moir et al., 1994; Luderus et al., 1992), and a structural role for lamins in DNA replication has been demonstrated (Ellis et al., 1997; Spann et al., 1997). Although interactions between splicing factors and certain nuclear matrix antigens have been described (Blencowe et al., 1994), lamin association with sites involved in RNA splicing or transcription has not been reported.

Several nuclear factors involved in events such as RNA splicing and transcription have been observed to localize to distinct, dynamic subnuclear compartments (reviewed by Lamond and Earnshaw, 1998; Spector, 1993). Antibodies that stain components of the pre-mRNA splicing machinery including the serine/arginine-rich (SR) family of proteins (such as SC-35) produce a speckled pattern of labeling of interchromatin granules and diffuse staining of perichromatin fibrils (Spector et al., 1991; Carmo-Fonseca et al., 1991). Another class of discrete structures that contain a human autoantigen termed p80 coilin as well as certain components

of snRNPs are the coiled bodies, and these vary from one to five or more per nucleus (Andrade et al., 1991; Bohmann et al., 1995). Major sites of mRNA transcription occur in thousands of foci throughout the nucleoplasm (Iborra et al., 1996). Other nuclear substructures whose functions are less well-defined are, for instance, the PML nuclear bodies (Asoli and Maul, 1991) and the PIKA (polymorphic interphase karyosomal association) compartment (Saunders et al., 1991). In a recent report, Fricker et al. (1997) have described deep tubular invaginations of the envelope within nuclei that are lined with nuclear pores and lamins.

In this study, we show that the lamins are associated with RNA splicing factors in nuclear speckles. We have raised monoclonal antibodies (mAbs) to recombinant rat lamin A and characterized two antibodies that recognize different aspects of the lamina. mAb LA-2B3 typically stained the peripheral lamina whereas mAb LA-2H10 revealed large internal lamin foci in all cells of unsynchronized populations of HeLa cells and rat F-111 fibroblasts. Both antibodies reacted exclusively with lamins A and C on one- and two-dimensional immunoblots. In localization studies, the lamin foci recognized by mAb LA-2H10 colocalized with marker proteins of the RNA splicing machinery but not with coiled bodies. Lamin B1 was also associated with these foci. These foci or speckles dispersed when cells entered mitosis and reformed during anaphase. The epitope recognized by mAb LA-2H10 was narrowed down to a segment of lamin A protein spanning amino acids 171 to 246. The differential immunoreactivity of lamin antibodies was retained in nuclease and salt extracted nuclei. We propose that the lamins are a structural component of nuclear speckles or domains containing proteins involved in RNA splicing.

MATERIALS AND METHODS

Generation of lamin antibodies

The rat lamin A cDNA described earlier (spanning amino acid residues 26-610; Hamid et al., 1996) was expressed from a pET-3 vector in the BL21(DE3)plys strain of *Escherichia coli* after induction with IPTG (Rosenberg et al., 1987). After confirmation of the amino-terminal sequence, recombinant lamin A protein was purified by electroelution from SDS-PAGE gels and used to immunize BALB/c mice by a protocol described previously (Visweswariah et al., 1987). The initial injection contained 100 µg of purified lamin A in complete Freund's adjuvant given subcutaneously and was followed at 3-week intervals by three boosters of 50 µg of protein in incomplete Freund's adjuvant. Subsequently 300 µg of protein in 0.01 M sodium phosphate, pH 7.5, 0.15 M NaCl (PBS) was injected intraperitoneally and three days later the animals were killed. Spleen cells from the immunized mice were fused with SP2/0 mouse myeloma cells and hybridomas were selected. Culture supernatants from fusion wells were screened initially by ELISA using purified lamin A as the antigen and subsequently with rat liver nuclear envelopes (isolated as described in a later section). Positive clones were subcloned by limiting dilution in 96-well microtiter plates. Isotyping of the antibodies was carried out using a commercial kit (Sigma Chemical Co., USA). Hybridoma supernatants were either used directly in immunoblots or concentrated tenfold by ultrafiltration before use in immunofluorescence assays. Recombinant lamin B1 was expressed from a rat lamin B1 cDNA (a gift from Dr M. R. S. Rao, Indian Institute of Science, Bangalore, India) cloned into the pET-11 vector, as described above. Polyclonal antibodies to recombinant lamin B1 were raised in rabbits by standard methods.

Other antibodies

A mouse monoclonal antibody against SC-35 (Fu and Maniatis, 1990) was kindly provided by Dr J. Gall (Carnegie Institution of Washington, Baltimore, USA); rabbit polyclonal antibodies to U5-116 kD (Fabrizio et al., 1997) and a mouse monoclonal antibody to the trimethylguanosine (m3G) cap of snRNAs (Bochnig et al., 1987) were gifts from Dr R. Luhrmann (University of Marburg, Germany); a rabbit polyclonal antibody to p80 coilin (Bohmann et al., 1995) was a gift from Dr A. Lamond (University of Dundee, Scotland), and a mouse monoclonal antibody that recognizes hnRNP K (Risau et al., 1983) was generously supplied by Dr H. Saumweber (Humboldt University, Germany) and used at the recommended dilutions.

Immunofluorescence microscopy

HeLa cells and F-111 rat fibroblasts were grown on coverslips to about 70% confluency in Petri dishes containing Dulbecco's minimum essential medium supplemented with 10% fetal calf serum. Cells were washed with PBS and then fixed by three different procedures. (1) Treatment with 3.7% formaldehyde for 15 minutes followed by 0.5% (v/v) Triton X-100 for 6 minutes at room temperature. This procedure of fixation was routinely used, except as indicated in Figs 2 and 3. (2) Treatment with methanol at −20°C for 10-15 minutes. (3) Treatment with methanol:acetone (2:1, v/v) at 4°C for 15 minutes. After extensive washing with PBS in each case, cells were incubated with 0.5% gelatin in PBS for 1 hour followed by incubation with first antibody for 1 hour and then FITC-conjugated second antibody for 1 hour at room temperature. Cells were washed with PBS after each step. Samples were mounted in PBS containing 90% glycerol, 10% *p*-phenylenediamine as anti-fade reagent, and 1 µg/ml 4,6-diamidino-2-phenylindole (DAPI). For double labeling experiments with LA-2H10 and rabbit polyclonal antibodies to U5-116 kD or p80 coilin, cells were fixed in formaldehyde as described in method (1) above. After blocking, cells were incubated with LA-2H10 followed by biotinylated anti-mouse antibody and avidin-Cy3, and then with the other primary antibody and FITC-conjugated second antibody at the recommended dilutions. For double labeling studies with mouse mAbs LA-2H10 (IgM subtype) and SC-35 (IgG subtype), cells were fixed in formaldehyde, blocked and incubated with SC-35, followed by FITC-conjugated second antibody specific for IgG subtype. After this step, cells were incubated with LA-2H10, followed by AMCA-conjugated second antibody specific for IgM subtype. Incubations were for 1 hour each and were carried out sequentially (with washes in PBS after each step) as this gave optimal labeling. Antibody conjugates were from Jackson Laboratories, USA and Vector Laboratories, USA.

Confocal laser-scanning immunofluorescence microscopy (CLSM) was carried out on a Meridian Ultima scan head attached to an Olympus IMT-2 inverted microscope with excitation at 515, 488 and 351-364 nm (Argon-ion laser). In double labeling experiments, the percentage cross-over for each dye was calculated from singly labeled specimens and corrected for. For quantitative analysis of colocalisation, the data from individual sections (0.5 µm thickness) was queried by test lines through the speckles and fluorescence intensities for both dyes were viewed graphically. The number of overlapping and non-overlapping peaks was estimated for one hundred speckles for each sample and the percentage of colocalised peaks was calculated. Images were assembled using Adobe Photoshop 3.0.

Extraction of nuclei

HeLa cell nuclei were extracted by a modification of the protocol of Nickerson et al. (1992), as described by Dyer et al. (1997). HeLa cells grown on coverslips were washed three times in ice-cold cytoskeleton buffer containing 10 mM Pipes pH 6.8, 10 mM KCl, 300 mM sucrose, 3 mM MgCl$_2$, 1 mM EDTA, 0.05 mM phenylmethylsulphonyl fluoride (PMSF) and 10 µg/ml aprotinin. The cells were then incubated in cytoskeleton buffer containing 0.5% (v/v) Triton X-100

for 10 minutes at 4°C and rinsed three times in ice-cold RSB buffer (42.5 mM Tris-HCl, pH 8.3, 8.5 mM NaCl, 2.6 mM MgCl$_2$, 0.05 mM PMSF and 10 μg/ml aprotinin). This was followed by incubation of the cells in RSB buffer containing 1% (v/v) Tween-20 and 0.5% (v/v) sodium deoxycholate for 10 minutes at 4°C. The cells were then rinsed twice in ice-cold digestion buffer (10 mM Pipes, pH 8.3, 50 mM NaCl, 300 mM sucrose, 3 mM MgCl$_2$, 1 mM EGTA, 0.05 mM PMSF and 10 μg/ml aprotinin) and incubated in 100 units/ml of DNase I in digestion buffer for 30 minutes at 30°C. To remove digested material, 1 M (NH$_4$)$_2$SO$_4$ was added slowly to the cells in digestion buffer to a final concentration of 0.25 M and incubated for 5 minutes at 4°C followed by two washes in ice-cold digestion buffer. One of these samples was further extracted with 2 M NaCl for 5 minutes at 4°C, and another was digested with RNase I for 30 minutes at 30°C. After washing in ice-cold digestion buffer, samples were fixed and processed for immunofluorescence microscopy.

Construction of lamin A deletions

Restriction fragments encompassing different regions of the lamin A cDNA were subcloned into the pGEX (Smith and Johnson, 1988) or pET (Rosenberg et al., 1987) series of expression vectors. Induction of protein expression was carried out as described above. The pGEX clones were expressed as fusions with the 27 kDa glutathione-S-transferase protein at the amino terminus, whereas the pET clones coded for polypeptides containing eleven amino acids of the T7 phage gene 10 protein at the amino terminus. Induced proteins were examined for their reactivity with lamin A antibodies by immunoblot analysis.

Immunoblot analysis

Rat liver nuclei were purified by sucrose density centrifugation. Nuclear envelopes were obtained by nuclease treatment and salt extraction of purified rat liver nuclei by the procedure of Kaufman et al. (1983) and characterized as described (Pandey and Parnaik, 1989). These preparations of nuclear envelopes retained the total lamin content of nuclei, and gave excellent separation of A and B-type lamins in isoelectric focussing (IEF) gels. Samples of nuclear envelopes, purified nuclei, soluble nuclear proteins released after the above extractions, recombinant lamin B1 or recombinant lamin A and its deletion fragments were separated by SDS-PAGE or by two-dimensional IEF-SDS-PAGE by the method of O'Farrell (1975), and then electroblotted onto nitrocellulose membrane filters. Immunodetection was carried out as described earlier (Pandey et al., 1994) using undiluted monoclonal antibody supernatants or rabbit polyclonal antibodies at a dilution of 1:100, and alkalkine phosphatase-conjugated secondary antibodies.

RESULTS

Specificity of monoclonal antibodies

Monoclonal antibodies to recombinant lamin A were produced as described in Materials and Methods. Two antibodies, LA-2B3 and LA-2H10 (both IgM isotypes), that showed strong and specific reactivity to samples of rat liver nuclear envelopes in an ELISA assay were chosen for further analysis. The reactivity of these antibodies as well as polyclonal antibodies to lamin B1 were checked by immunoblotting of rat liver nuclear envelopes, purified nuclei, soluble nucleoplasmic proteins and purified recombinant lamins A and B1. As shown in Fig. 1C and D, both monoclonal antibodies to lamin A showed very similar reactivity, and recognized proteins migrating at the position of lamin A (70 kDa) and lamin C (62 kDa) in nuclear envelopes and nuclei, and there was no significant cross-reactivity with other proteins in these fractions, or with soluble nucleoplasmic proteins. The

polyclonal antibody to lamin B1 recognized a single protein migrating at the position of lamin B1 (65 kDa), as illustrated in Fig. 1B. All three antibodies recognized their respective recombinant lamins. In order to confirm the specific reactivity of these antibodies to the A-type and B-type lamins, immunoblotting was carried out with samples of nuclear envelopes separated by two-dimensional IEF-SDS-PAGE. By this procedure, the A-type lamins which migrate as a series of spots of pI 6.9-7.1 due to differential phosphorylation (Ottaviano and Gerace, 1985) are well resolved and separated from the B-type lamins which migrate at pI 5.6-5.8 (Fig. 1E). The two antibodies to lamin A recognized only the A-type lamins (all separable isoforms) and did not cross-react with the B-type lamins, and the antibody to lamin B1 reacted exclusively with lamin B1.

Lamina structures revealed by immunofluorescence

The distribution of the A-type lamins in proliferating populations of HeLa and F-111 cells was analyzed by indirect immunofluorescence using CLSM. With LA-2B3, cells displayed a typical staining at the nuclear periphery (Fig. 2E,F). However, with LA-2H10, a distinct pattern of large speckles or foci within the nucleus was observed with no obvious staining at the nuclear periphery as illustrated in Fig. 2A,B. This pattern was observed in all the cells examined in these unsynchronized cell populations. When optical sectioning was carried out at 0.5 μm intervals, the lamin structures stained with LA-2H10 could be seen throughout the nucleus but excluding the nucleolar region (data not shown). HeLa cells were also stained with both LA-2B3 and LA-2H10 simultaneously in order to ascertain whether labeling with one antibody might interfere with binding to the other. In this sample, the nuclei in every cell exhibited large foci as well as peripheral lamina staining, which was a combination of the reactivity of each antibody (Fig. 2K). It may be noted that the intranuclear staining by mAb LA-2H10 was revealed after 1 hour of incubation with the antibody and did not require a long period of incubation.

With a view to ruling out the possibility that the staining patterns observed were due to an artefact of the fixation procedure, we also tested each antibody with two other fixation procedures, as illustrated in Fig. 2C,D,G,H. Intranuclear lamin foci were revealed with LA-2H10 with methanol and methanol/acetone fixation also, whereas LA-2B3 gave a peripheral nuclear fluorescence under these conditions. Since the distinctive patterns of internal and peripheral lamina distribution were obtained with two different cell lines and three procedures of fixation, we concluded that this differential immunolocalization was not an artefact resulting from a particular method of fixation or an unusual structure seen only in a particular cell type. Intranuclear lamin A foci were also observed in COS-1 monkey kidney epithelial cells, J774 mouse macrophage-like cells, and C3H mouse fibroblasts (data not shown). In a later section the mapping of the mAb LA-2H10 epitope to residues 171-246 of the lamin A protein is described. When HeLa cells were labeled with LA-2H10 antibody that had been preincubated with the 171-246 peptide, staining was reduced considerably, whereas preincubation of LA-2H10 with a control peptide (291-355) did not affect labeling (Fig. 2I,J). This confirmed the specificity of mAb LA-2H10.
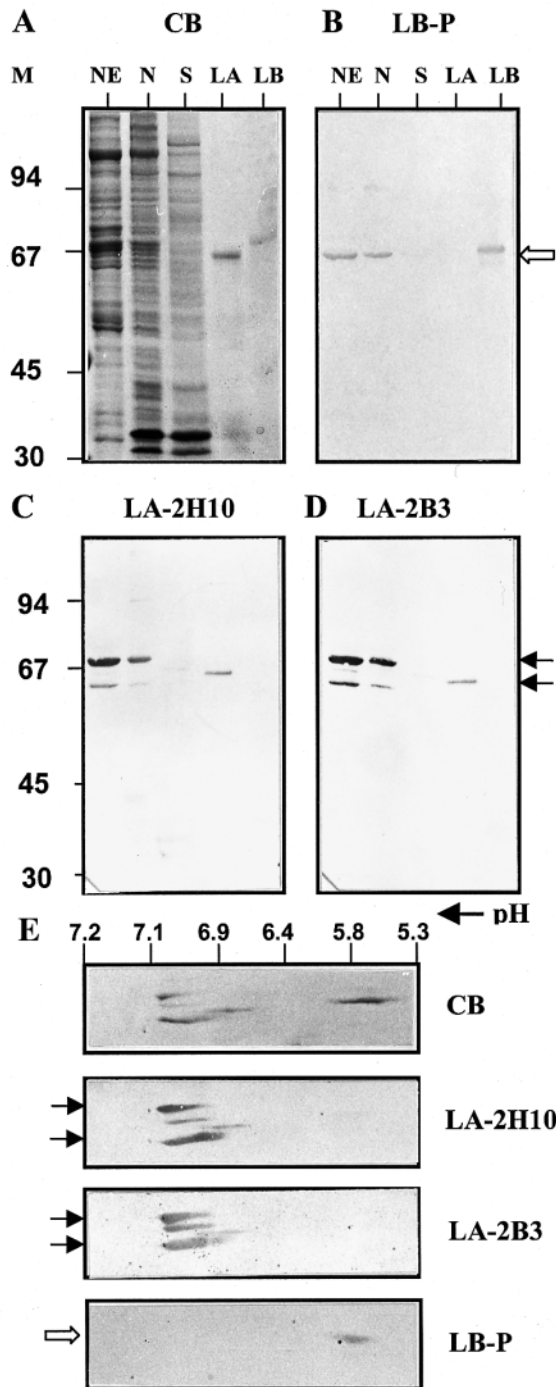
Fig. 1. Specificity of lamin antibodies. (A) Rat liver nuclear envelopes (NE, from 1.5×10^6 nuclei), purified nuclei (N, about 1.5×10^6 nuclei), soluble nuclear proteins (S, from 1.5×10^6 nuclei), recombinant lamin A (LA) and recombinant lamin B1 (LB) were resolved by 8% SDS-PAGE and stained with Coomassie Blue (CB). Identical gels were transferred to nitrocellulose filters and blotted with LB-P (B), LA-2H10 (C) and LA-2B3 (D). (E) A two-dimensional IEF-10% SDS-PAGE separation of nuclear envelopes (from 3×10^6 nuclei) stained with Coomassie Blue, and immunoblots with LA-2H10, LA-2B3 and LB-P. The pH gradient of the IEF run is indicated. Positions of rat A-type lamins are marked by filled arrows and lamin B1 by an open arrow. Molecular mass markers (M) are: phosphorylase b, 94 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; and carbonic anhydrase, 30 kDa.

Colocalization studies with nuclear marker proteins

Our observation that mAb LA-2H10 stained the lamina in a speckled manner prompted us to compare this pattern of localization with that of known proteins that label well-defined nuclear substructures. We have carried out colocalization studies with antibodies to SC-35, a constituent of SR-rich snRNPs (Spector et al., 1991) and U5-116 kD, a conserved component of U5 snRNPs (Fabrizio et al., 1997), both of which are found predominantly in nuclear speckles and partly dispersed in the nucleoplasm. We have also employed antibodies to p80 coilin which is a constituent of coiled bodies. Our results demonstrate that lamin A speckles colocalize with those of SC-35 (checked with both HeLa and F-111 cells) as well as U5-116 kD but not with coilin, as seen in the confocal overlays of single sections scanned in the same optical plane in Fig. 3A-L, and confirmed by quantitative analysis. Analysis of the double labeling data with LA-2H10 and antibodies to SC-35 and U5-116 kD confirmed the colocalization of >90% of the lamin speckles with SC-35 or U5-116 kD against a non-overlapping background of diffuse nucleoplasmic labeling of SC-35 or U5-116 kD. Furthermore, no overlap was obtained in the analysis with mAb LA-2H10 and antibody to p80 coilin. The distribution of speckles throughout the nucleus excluding the nucleolar regions is consistent with that reported earlier for splicing factors.

Since the peripheral lamina in differentiated cells is composed of both A-type and B-type lamins, we compared the intranuclear pattern of distribution of lamin A with that of lamin B1 (Fig. 3M-O). The polyclonal antibody to recombinant rat lamin B1 (LB-P) stained the nuclear rim and intranuclear speckles in HeLa cells. In localization studies with LA-2H10 and LB-P, the majority of speckles (67%) were found to colocalize. However, LA-2H10 labeled a number of additional, non-overlapping speckles (28%) that did not colocalize. It is possible that these speckles may contain another B-type lamin such as B2. Only 5% of the nuclear speckles stained by LB-P were not labeled by LA-2H10, which is close to the background level for this analysis. (The prominent labelling of intranuclear foci by LB-P polyclonal antibody in HeLa cells was also seen with COS-1 cells, but was less pronounced in fibroblasts such as F-111 and C3H). When HeLa cells were labeled with LA-2H10 and WGA-FITC, which binds to nuclear pore complexes (Finlay et al., 1987) and stains the nuclear rim, no colocalization was observed (data not shown).

Immunolocalization in mitotic cells

The A-type lamins have been observed in earlier studies to be dispersed in the cytoplasm from the onset of mitosis until anaphase, with a fraction of the lamins subsequently becoming associated with decondensing chromosomes in telophase (Gerace et al., 1978). Nuclear speckles containing splicing factors have also been shown to disperse during mitosis, with the speckled pattern reforming at anaphase and localizing around chromosomes by telophase (Spector et al., 1991). When HeLa cells were labeled with LA-2H10 and cells at different stages of mitosis were examined (Fig. 4), the lamins were found to be mostly dispersed in the cytoplasm until metaphase, with speckles becoming prominent by anaphase. (A few speckles were occasionally seen in a metaphase cell.) Subsequently, the lamins were reconcentrated around the

chromosomes in early telophase, with a fraction of lamins remaining in the cytoplasm. The localization of lamin A during mitosis as detected by LA-2H10 was similar to the distribution of A-type lamins observed in earlier studies, except for the presence of speckles in anaphase. When mitotic HeLa cells were labeled with LA-2H10 as well as antibody to U5-116 kD, both proteins were found to be dispersed upto metaphase, with prominent speckles seen by telophase (Fig. 5). Lamin A and U5-116 kD were found to colocalize in 56% of the speckles in the telophase cell, whereas 38% of the speckles contained only lamin A. This is in contrast to the interphase nucleus where >90% of the speckles were colocalised.

Immunolocalization in extracted nuclei

The treatment of detergent-solubilized monolayers of cultured cells with DNase I followed by salt extraction has been shown to result in the removal of >95% of the chromatin and yield a filamentous network consisting of the lamins and other nuclear proteins, also termed the nuclear matrix (Nickerson et al., 1992). Interactions between lamins and chromatin or other nuclear proteins are generally disrupted in such preparations, and masked epitopes should, in principle, be revealed if masking is due to such interactions (Dyer et al., 1997). When extracted HeLa cell nuclei were labeled with LA-2H10, the staining pattern was almost identical to that seen prior to extraction (Fig. 6A-C). It may be noted that the peripheral lamina was not labeled with LA-2H10 even after extraction. In extracted nuclei stained with LA-2B3, the nuclear rim was prominently labeled as in untreated cells, and intranuclear foci were not revealed (Fig. 6F-H). Extracted nuclei labeled with the polyclonal antibody to lamin B1 exhibited both peripheral and intranuclear staining (Fig. 6U-W). The speckled pattern of localization of SC-35 and U5-116 kD was retained after extraction, although the diffuse

nucleoplasmic labeling was eliminated (Fig. 6K-M, P-R). Samples were monitored for efficiency of extraction by counterstaining with DAPI, which was reduced to undetectable levels in all extracted cells (Fig. 6a). Upon further treatment of extracted nuclei with 2 M NaCl or RNase A, the speckled pattern of SC-35 and U5-116 kD was mostly retained (Fig. 6N,O,S,T), which is consistent with earlier reports on the nuclease insensitivity of speckles containing SC-35 and certain snRNP proteins (Spector et al., 1991; Carmo-Fonseca et al., 1991). The peripheral and speckled labeling of lamins was also not altered by 2 M NaCl or RNase A (Fig. 6D,E,I,J) which suggests that these lamina structures are distinct from RNase-sensitive nuclear matrix filaments (Nickerson et al., 1992). The efficacy of RNase digestion was confirmed by the absence of staining with anti-m3G antibody (Fig. 6d) which labels capped snRNAs (Bochnig et al., 1987; Carmo-Fonseca et al., 1991); but this labeling was not affected by DNase I digestion (Fig. 6c) or subsequent treatment with 2 M NaCl (data not shown). The efficiency of 2 M NaCl treatment was checked by analyzing the labeling of hnRNP K by mAb Q18 (Risau et al., 1983). Upon DNase I digestion, brightly stained foci were still visible though the diffuse staining was lost (Fig. 6f). Further treatment with 2 M NaCl reduced labeling to a considerable extent (Fig. 6g), thus confirming that 2 M NaCl was effective in disrupting large assemblies of proteins.
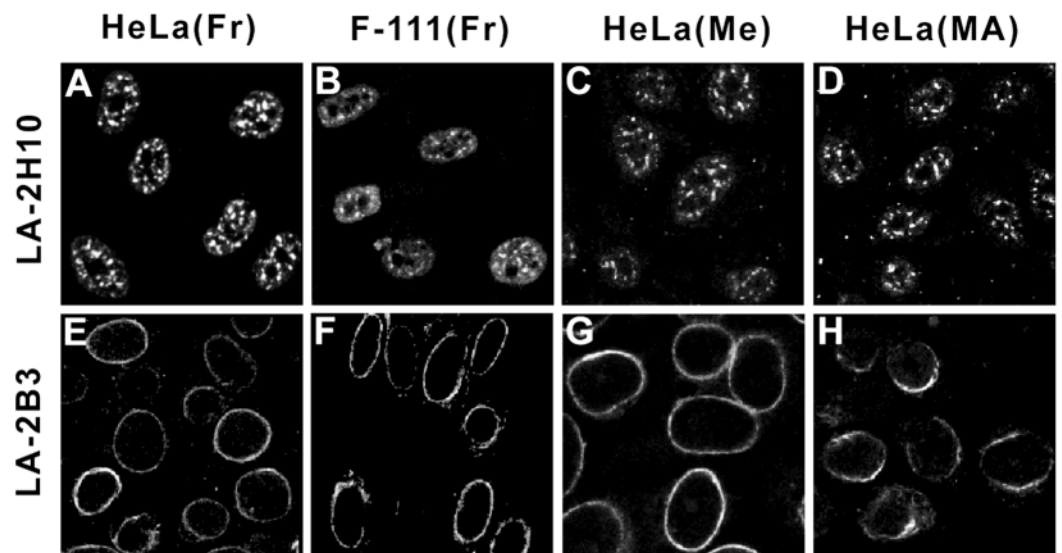

Fig. 2. Immunolocalization of lamins by confocal microscopy. HeLa or F-111 cells were fixed with formaldehyde (Fr), methanol (Me) or methanol:acetone (MA) and stained with mAb LA-2H10 (A,B,C,D) or LA-2B3 (E,F,G,H). In K, formaldehyde-fixed HeLa cells were labeled with a combination of LA-2H10 and LA-2B3. In I and J, formaldehyde-fixed HeLa cells were labeled with mAb LA-2H10 that had been preincubated for 1 hour at ambient temperature with a 100-fold molar excess of peptide 171-246 or peptide 291-355, which correspond to deletion fragments 8 and 7 described in Fig. 7. Bar, 10 μm.
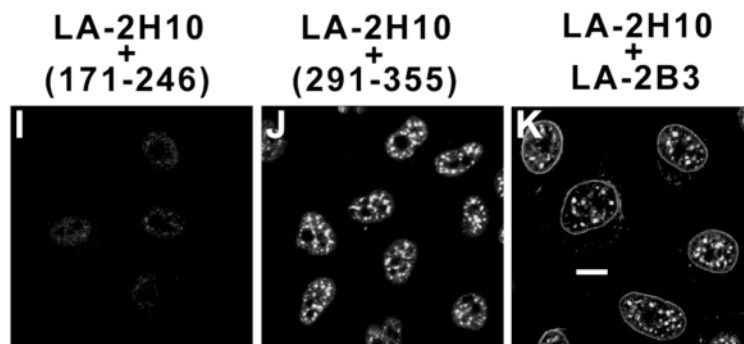
Fig. 3. Colocalization of lamins with nuclear proteins. Formaldehyde-fixed HeLa cells (and F-111 cells in SC-35-F) were doubly labeled with mAb LA-2H10 and antibodies to SC-35 (A-F), U5-116 kD (G-I), or coilin (J-L). For staining with LB-P and LA-2H10 (M-O) HeLa cells were fixed with methanol. Confocal overlays of the doubly stained cells are shown in the merged panel, where the yellow colour highlights structures stained by both antibodies. Arrows in N and O indicate speckles stained by LA-2H10 but not by LB-P. Bar, 10 μm.
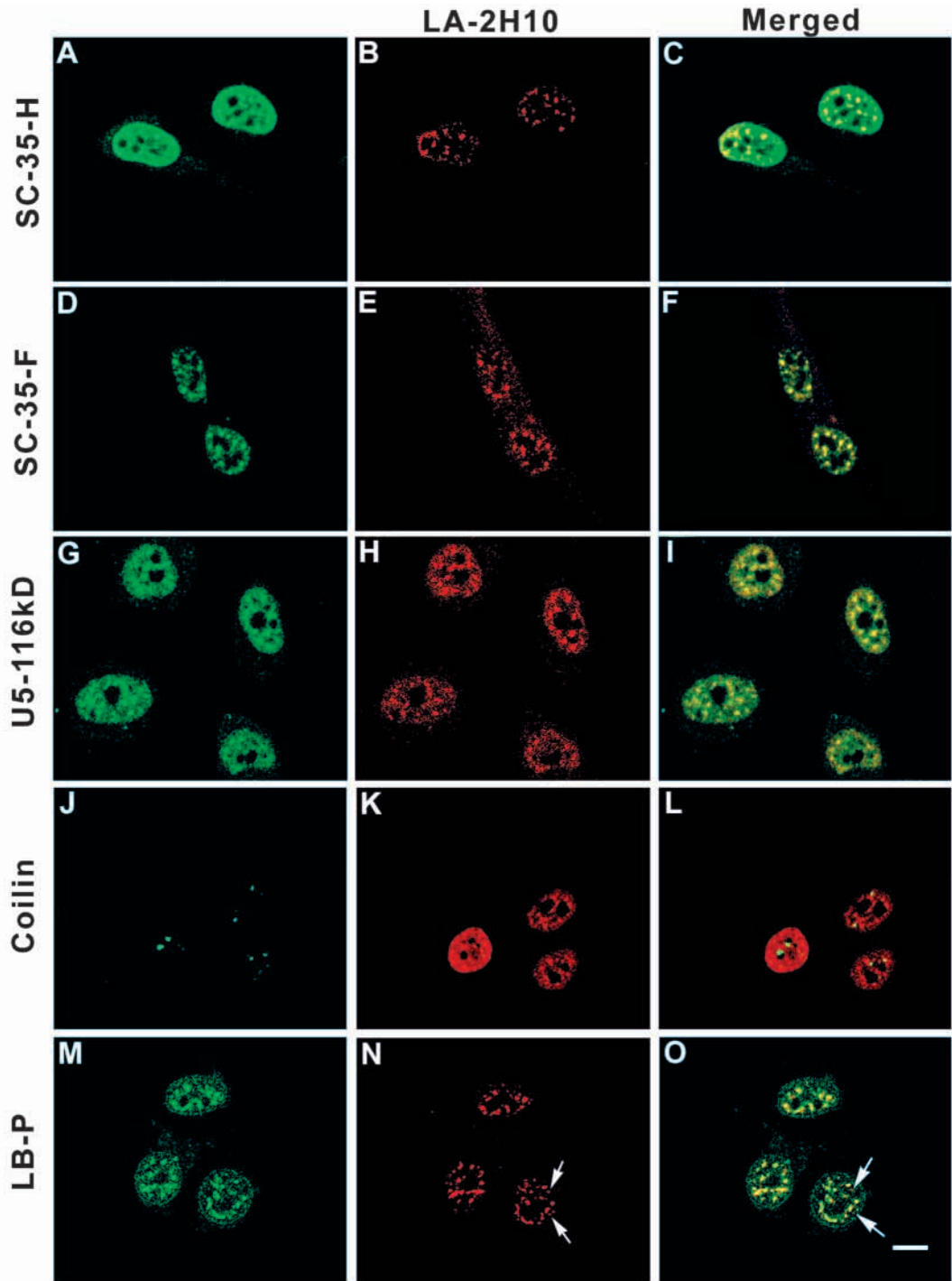

Mapping of epitope region recognized by mAb LA-2H10

Various deletion mutants of lamin A were constructed and expressed in *E. coli*, either in the pGEX vector series (fragments 2, 3 and 7, see Fig. 7) or the pET vector series (fragments 1, 4, 5, 6 and 8). The bacterially expressed lamin A fragments were tested for their reactivity against mAb LA-2H10 and LA-2B3 by immunoblot analysis. As illustrated in Fig. 7, the smallest fragment recognized by LA-2H10 was a segment spanning amino acids 171 to 246. This segment falls in the carboxy-terminal half of coil 1B in the rod domain. As mAb LA-2B3 reacted with segment 171-291 but not with segment 171-246, its epitope is likely to lie between amino acids 246 to 291.

DISCUSSION

Colocalization of lamins with RNA splicing factors

In this study, we have presented evidence for the colocalization of a subset of lamin structures with components of the RNA splicing machinery. The antibody mAb LA-2H10 raised against recombinant rat lamin A was found to label the nuclei of HeLa and F-111 cells in a pattern of 20-50 speckles in all cells of unsynchronized populations using different methods of fixation. Since the speckled pattern was reminiscent of that obtained with antibodies directed against proteins involved in pre-mRNA splicing (Spector, 1993), double labeling experiments were carried out with antibodies to marker proteins of nuclear speckles and other substructures. Intranuclear lamin speckles colocalized with SC-35 and U5-116 kD but not with p80 coilin. SC-35 is an essential pre-mRNA splicing factor belonging to the SR-rich family of non-snRNP proteins that is localized in a speckled pattern of interchromatin granules and diffuse perichromatin fibrils (Spector et al., 1991; Fu and Maniatis, 1990). U5-116 kD is a specific component of U5 snRNPs that is essential for splicing

(Fabrizio et al., 1997) and is also localized in speckles and fibrils in the nucleus. Recent studies suggest that the splicing of nascent transcripts occurs near sites of transcription in the perichromatin fibrils, and that splicing factors are recruited from interchromatin granules (speckles) to sites of active transcription. The interchromatin granules may also be involved in the pre-assembly of spliceosomes (Misteli and Spector, 1998). Another nuclear substructure called the coiled body is comprised of proteins such as fibrillarin, p80 coilin, NOPP140 as well as certain snRNPs (Andrade et al., 1991; Bohmann et al., 1995). The functions of coiled bodies are not

well understood at present (Lamond and Earnshaw, 1998). We have observed that the speckled pattern of localization of SC-35, U5-116 kD and lamins was resistant to extraction of nuclei with detergent, nucleases and salt, although these treatments eliminated the diffuse staining of SC-35 and U5-116 kD. Based
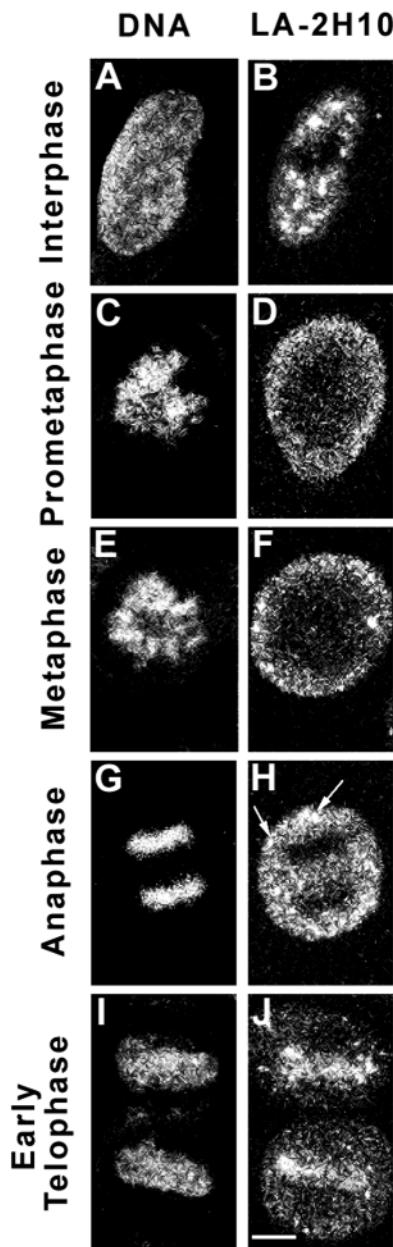

Fig. 4. Immunolocalization with mAb LA-2H10 in mitotic HeLa cells. HeLa cells were labeled with mAb LA-2H10 and DAPI to stain DNA, and scanned for mitotic cells. Cells in different phases of mitosis are shown. Speckles in an anaphase cell in H are marked by arrows. Bar, 10 µm.
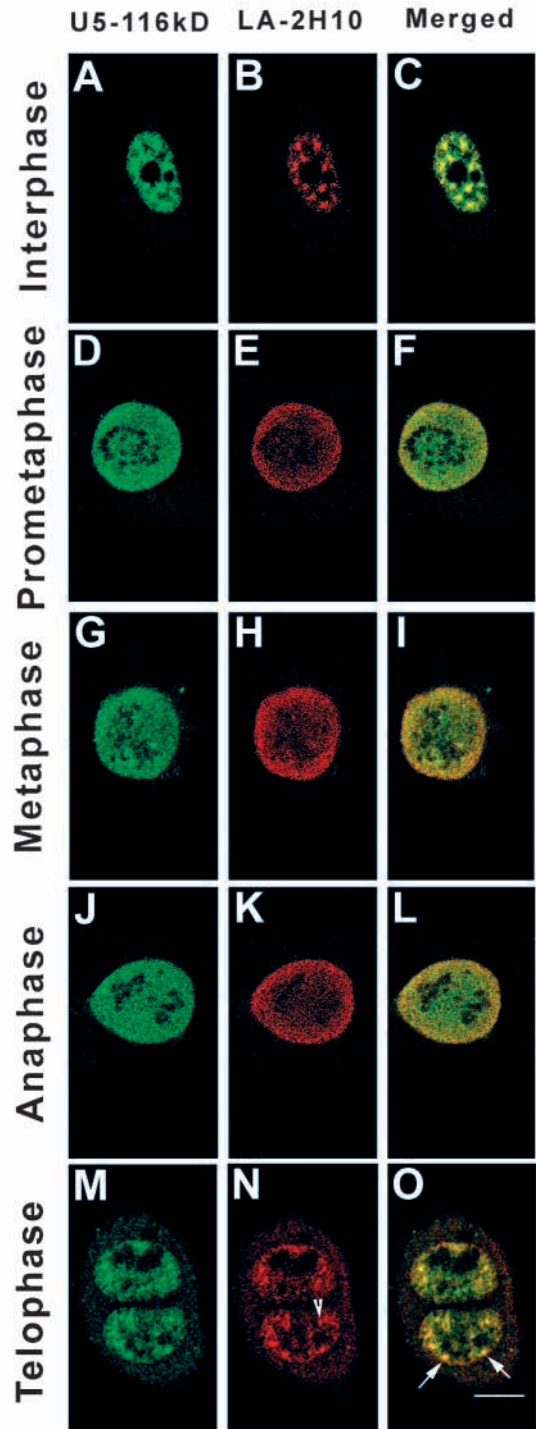

Fig. 5. Localization with mAb LA-2H10 and antibody to U5-116 kD in mitotic HeLa cells. Cells in different phases of mitosis are shown. Colocalized speckles in a telophase cell in O are marked by arrows. A speckle that is labeled only by LA-2H10 in N is marked by an arrowhead. Bar, 10 µm.
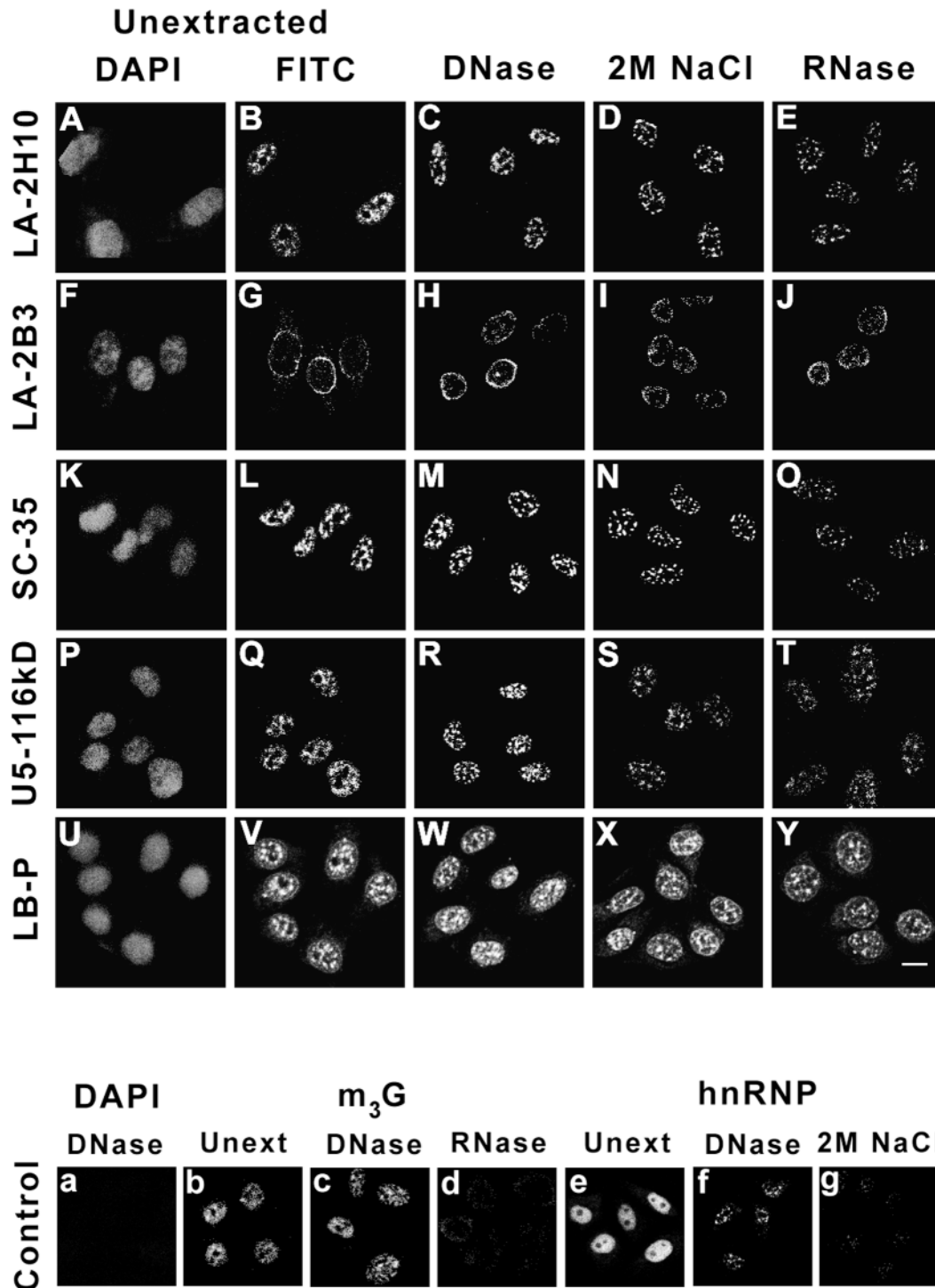
Fig. 6. Localization studies in extracted nuclei. (A-Y) HeLa cells were extracted with detergents and treated with DNase I, followed by 2 M NaCl extraction or RNase A digestion, fixed and labeled with the indicated antibodies. Unextracted cells were labeled with DAPI and indicated antibodies (FITC). (a-g) The control panel displays a typical DNase I-treated sample stained with DAPI (a); labeling of unextracted cells and cells after DNase or RNase digestion with anti-m3G antibody (b,c,d); and labeling of unextracted cells and cells after DNase digestion and 2 M NaCl treatment with an antibody to hnRNP K (e,f,g). Bar, 10 µm.

on our findings, we propose a structural role for lamins in maintaining interchromatin granules. As lamins have not been detected in active splicing complexes from HeLa nuclear extracts by mass spectrometric methods (Neubauer et al., 1998), they are unlikely to play a functional role in the splicing

reaction. However, we cannot rule out a role for lamins in events related to splicing occurring in the interchromatin granules such as spliceosome pre-assembly that are presently not well understood.

Our colocalization data indicate that the B-type lamins are

also associated with lamin A intranuclear foci, since the majority of nuclear speckles labeled by LA-2H10 are also stained by LB-P antibody to lamin B1. Hence, this internal lamin framework is likely to maintain its functions in undifferentiated cells which express only B-type lamins.
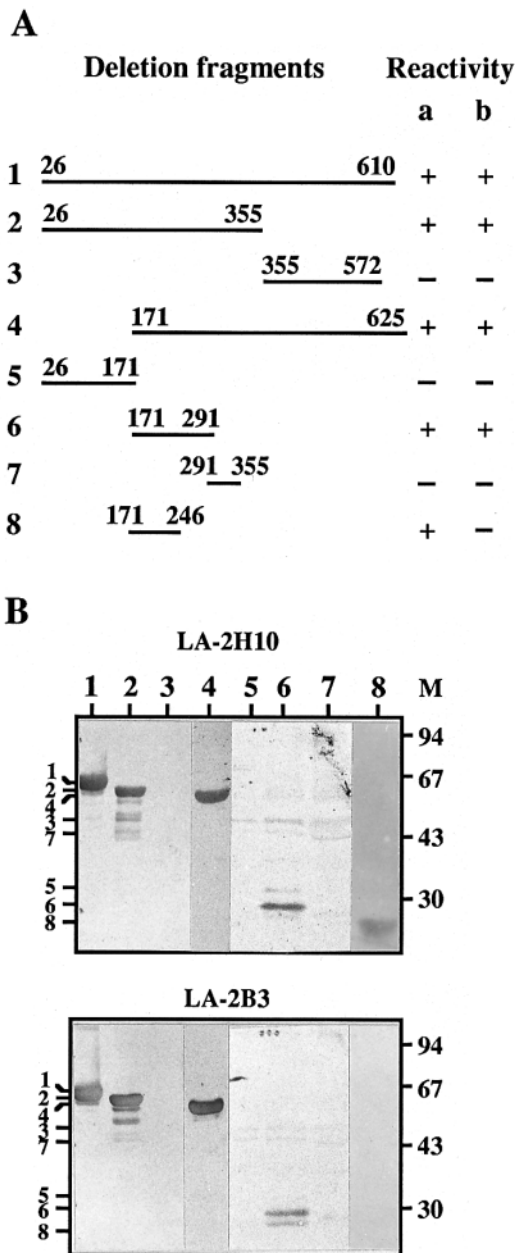

Fig. 7. Mapping of epitope region recognised by mAbs LA-2H10 and LA-2B3. (A) Deletion fragments of lamin A used for the analysis, encoded by the indicated amino acid residues, and a summary of their reactivities with mAbs LA-2H10 and LA-2B3 (columns a and b, respectively). (B) Immunoblots of deletion fragments 1-8 with mAbs LA-2H10 and LA-2B3. Positions of the bacterially expressed deletion fragments in a Coomassie Blue-stained gel are shown on the left of each blot. Molecular mass markers (M) shown on the right of each blot are: phosphorylase b, 94 kDa; albumin 67 kDa; ovalbumin, 43 kDa; and carbonic anhydrase, 30 kDa.

Dynamic behaviour of lamin speckles

It is becoming increasingly evident that lamins can occur at sites within the nucleus. Hozak et al. (1995) have demonstrated that lamin A is part of an internal skeleton that ramifies throughout the nucleus from the nucleolus to the periphery, and is partly associated with dense structures, which the authors propose to be sites of DNA replication, transcription and RNA processing. The association of lamins with DNA replication foci in S-phase has been reported (Moir et al., 1994) and there is now considerable evidence for a structural requirement for a lamin nucleoskeleton for DNA replication to occur (Zhang et al., 1996; Ellis et al., 1997; Spann et al., 1997). Hutchison and coworkers have proposed that lamina assembly is a prerequisite for the correct assembly of a nuclear matrix (or nucleoskeleton) which in turn supports replication centres, but that lamins are not directly involved in DNA replication events (Zhang et al., 1996; Ellis et al., 1997). Intranuclear lamin foci are transiently seen during lamina assembly (Pugh et al., 1997), and have also been observed at internal sites in nuclei of G1 phase cells in association with heterochromatin (Bridger et al., 1993). These intranuclear lamin foci are distinct from nuclear-envelope derived channels and do not colocalize with membranes (Bridger et al., 1993) or nuclear pore complexes (Moir et al., 1994). The above studies collectively point towards a significant role for intranuclear lamins in nuclear organisation. Our results on the colocalization of a class of internal lamin speckles with RNA splicing factors lend further support to this view. As these speckles are seen in cells at all stages of the cell cycle and are only 20-50 in number per nucleus, it is highly unlikely that they correspond to the S or G_1-phase specific foci. We have observed that at the onset of mitosis, when transcription and splicing are downregulated, lamin A and U5-116 kD are dispersed in the cytoplasm. Speckles containing both these proteins are reformed by anaphase, though formation of lamin A speckles appears to precede colocalisation with U5-116 kD. Our findings are consistent with the view that the lamin network is in dynamic equilibrium (Stuurman et al., 1998), and new lamin substructures can be formed in a stage-specific manner depending on the functional requirements of the cell.

Epitope masking by antibodies

The differential immunoreactivity of different lamin structures has been reported in previous studies, and is primarily due to epitope masking, especially in the tail domain (Collard et al., 1990) and at phosphorylated sites in the lamin protein (Dyer et al., 1997). Masking of the epitope recognized by mAb LA-2H10 (which lies between residues 171 and 246) is unlikely to be due to phosphorylation since this region does not contain sites of phosphorylation by cdc2 kinase, protein kinase C or other lamin kinases (Heald and McKeon, 1990; Eggert et al., 1993). Sites of lamin glycosylation (Ferraro et al., 1989), methylation (Chelsky et al., 1987) or isoprenylation (Wolda and Glomset, 1988) also do not fall in this segment. In addition, a database search has indicated that this sequence does not have significant homology with nuclear proteins other than the lamins. On the other hand, the segment 171-319 has been reported to be involved in at least three major kinds of interactions: lamin-chromatin (Glass et al., 1993), lamin-lamin (Moir et al., 1991) and lamin binding to the retinoblastoma protein (Ozaki et al., 1994; but this occurs outside the 171-246

epitope segment of LA-2H10). Thus it is likely that such interactions may result in epitope masking of different kinds, as seen for example with mAb L68A7 which labels the nuclear periphery in quiescent cells but not in dividing cells (Dyer et al., 1997), and with the two antibodies we have described here. However, the differential reactivity of LA-2H10 and LA-2B3 to different lamin structures is unlikely to be due to lamin interactions with chromatin or other extractable nuclear proteins since in immunolocalization studies with nuclease-treated, salt-extracted nuclei, the labeling patterns of LA-2H10 and LA-2B3 were not altered. We have also not found any proteins co-precipitating with lamins in immunoprecipitation experiments with these antibodies (data not shown). Hence the differential reactivity is either due to an unidentified posttranslational modification, or interaction with a tightly-bound protein, or due to subtle differences in the associations between lamin filaments at the periphery and at intranuclear sites.

Complexity of the lamin network

There is now considerable evidence for a more elaborate network of filaments within the nucleus, although the exact role and topography of lamins within this framework remains to be fully understood. Hozak et al. (1995) have reported the presence of both peripheral and internal lamin filaments in detergent-extracted, encapsulated HeLa cells. Nuclease-treated, salt-extracted nuclei also reveal a peripheral meshwork connected through a network of core or matrix filaments to more dense intranuclear structures (Nickerson et al., 1992). Sperm pronuclei assembled in *Xenopus* egg extracts display differences in labeling with lamin antibodies between peripheral and internal core filaments (Zhang et al., 1996). Differences in RNase sensitivity between matrix filaments and the lamin speckles we have described point towards different intermolecular interactions in the two structures. In addition, in vitro studies on lamin assembly demonstrate that lamin dimers can associate laterally to form at least three different kinds of antiparallel filaments (Stuurman et al., 1998). These reports highlight the complexity of the lamina network and also point to the inherent difficulties in observing and analyzing it due to inaccessibility to antibodies as well as epitope masking. The antibodies we have described here should provide a useful handle for further studies on the intranuclear lamin network.

It is becoming increasingly evident that the regulation of biological processes in the crowded environment of the nucleus is likely to be facilitated by nuclear compartmentalization of essential factors. Our data on the colocalization of lamins with RNA splicing factors in nuclear speckles is consistent with a structural role for lamins in supporting such nuclear compartments. Hence it would be of relevance to understand how distinct compartments are assembled and utilized in various nuclear events, and the role of lamins in this process.

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