Distinct changes in intranuclear lamin A/C organization during myoblast differentiation

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SUMMARY

Intranuclear lamin foci or speckles have been observed in various cell types. In order to explore the possibility of changes in internal lamin organization during muscle differentiation, we have examined the appearance of A-type lamin speckles that associate with RNA splicing factor speckles in C2C12 myoblasts and myotubes. Lamin speckles were observed in dividing myoblasts but disappeared early during the course of differentiation in postmitotic myocytes, and were absent in myotubes and muscle fibers. However, no changes were seen in the typical peripheral organization of lamins A/C or B1 or in RNA splicing factor speckles. Lamin speckles were also absent in quiescent myoblasts but reappeared as cells were reactivated to enter the cell cycle. These changes were not observed in other quiescent cell types. Immunoblot analysis indicated that the abundance and migration of lamins A and C was not altered in differentiated myoblasts. When myotube or quiescent myoblast nuclei were extracted with nucleases and detergent, a uniformly stained internal lamina was revealed, indicating that lamins A/C were antigenically masked in these cells, probably owing to structural reorganization of the lamina during differentiation or quiescence. Our results suggest that muscle cell differentiation is accompanied by regulated rearrangements in the organization of the A-type lamins.

Key words: Nuclear lamina, Lamin A, Muscle differentiation, Myoblasts

INTRODUCTION

The lamins form a network of filaments underlying the inner nuclear membrane termed the nuclear lamina. The lamina is considered to be an important determinant of interphase nuclear architecture as it plays an essential role in maintaining the integrity of the nuclear envelope and provides anchoring sites for chromatin (Moir et al., 1995; Gant and Wilson, 1997; Stuurman et al., 1998). In addition to the well-characterized peripheral location of lamins, there is considerable evidence for the intranuclear distribution of lamins (Goldman et al., 1992; Bridger et al., 1993; Hozak et al., 1995; Pugh et al., 1997), in particular their association with DNA replication centers in S-phase cells (Moir et al., 1994) and with RNA splicing factor speckles in interphase cells (Jagatheesan et al., 1999). Two major types of lamins are present in most mammalian cells: the B-type lamins (B1 and B2), which are found in nearly all somatic cells, and the A-type lamins (A and C), which are expressed primarily in differentiated cells, in a stage-specific manner depending upon the cell lineage (Stuurman et al., 1998). Lamins A, C and germ cell-specific lamin C2 are alternatively spliced products of the lamin A gene, *LMNA*, whereas lamins B1 and B2 are coded by separate genes.

Genetic studies have shown that the majority of mutations in human *LMNA* lead to autosomal dominant Emery-Dreifuss muscular dystrophy (EDMD) affecting skeletal and cardiac muscle (Bonne et al., 1999), and different mutations cause dilated cardiomyopathy (Fatkin et al., 1999), limb girdle muscular dystrophy (Muchir et al., 2000) or partial lipodystrophy (Shackleton et al., 2000; Cao and Hegele, 2000). Valuable insights into lamin A function have been obtained by the knock-out of mouse *LMNA* (Sullivan et al., 1999). Mice that lack *LMNA* develop severe muscle wasting, similar to human EDMD, by 3-4 weeks and die 8 weeks after birth. The reason for the selective effect on muscle cells is not known, though it has been suggested that the forces generated during muscle contraction might exacerbate physical damage to muscle cell nuclei (Sullivan et al., 1999), or that lamins might influence gene expression in progenitor cells (Wilson, 2000). A further possibility that could be examined is whether muscle differentiation is accompanied by specific changes in nuclear architecture that involve the lamins.

Differentiation of myoblasts into myotubes is coordinated by two families of transcription factors: the MyoD family, which includes the muscle-specific transcription factors MyoD, Myf5, myogenin and MRF4 (Lassar et al., 1994; Rudnicki and Jaenisch, 1995); and the MEF2 family of transcription factors (Black and Olson, 1998). Muscle differentiation follows a highly ordered, temporally distinct sequence of events. Myoblasts are first committed to the differentiation pathway in a step marked by the expression of the transcription factor myogenin, which is followed by expression of cell cycle regulators such as the inhibitor p21 and irreversible, asynchronous cell cycle withdrawal (Andrés and Walsh, 1996). The cells then differentiate phenotypically, express contractile genes and finally fuse into multinucleated myotubes. Specific changes have been reported to occur in nuclear organization during the process of myogenesis such as in the distribution of proteosomes on the nuclear matrix (De Conto et al., 2000), in

the localization of the E2F family of proteins (Gill and Hamel, 2000), as well as the disappearance of the nuclear mitotic apparatus protein NuMA (Merdes and Cleveland, 1998). However, no changes have been observed in the typical peripheral localization of the lamins, except for a lowering of lamin B1 levels in skeletal muscle (Manilal et al., 1999). The distribution of emerin, an inner nuclear membrane protein that binds to lamins, is also not altered during myogenesis (Manilal et al., 1996).

In this study we have explored the possibility of changes in the intranuclear lamin network during myoblast differentiation, by examining lamin A localization in the C2C12 cell line, a well-characterized murine skeletal muscle cell line (Yaffe and Saxel, 1977; Blau et al., 1983), using a monoclonal antibody to lamin A (LA-2H10), which labels intranuclear lamin speckles that colocalize with RNA splicing factor speckles or foci (Jagatheesan et al., 1999). We have earlier documented certain unusual features in the immunoreactivity of mAb LA-2H10 (Jagatheesan et al., 1999). This antibody specifically detects lamins A and C in immunoblots of cellular fractions and uniformly labels dispersed lamins in mitotic cells. However, in interphase cells, LA-2H10 exclusively stains intranuclear speckles without labeling the peripheral lamina, even in nuclei that have been treated with detergent, salt-extracted and nuclease-treated to reveal the nucleoskeletal framework. We have attributed these properties to subtle differences in the associations between lamin protofilaments at the periphery and at intranuclear sites, or to an unknown post-translational modification in peripheral lamin A, which leads to differential accessibility of the epitope region spanning amino acids 171-246. As this segment is present in both lamins A and C, mAb LA-2H10 does not distinguish between these proteins. Our findings from the present work indicate that lamin A/C speckles disappear when myoblasts differentiate into myotubes or are induced to become quiescent; this is due to antigenic masking, which probably results from a reorganization of the internal lamina. These changes are not seen with non-muscle quiescent cells such as serum-starved mouse C3H10T1/2 fibroblasts, suggesting that A-type lamin rearrangements are specific to muscle cell differentiation.

MATERIALS AND METHODS

Cell culture

C2C12 mouse skeletal myoblasts (obtained from H. Blau, Stanford University, CA) were maintained at subconfluent densities in DMEM supplemented with 20% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2. Myogenic differentiation was induced by changing subconfluent (60-70%) cells to DMEM containing 2% horse serum (differentiation medium). In order to arrest the growth of myoblasts, we used the method of Milasincic et al. (Milasincic et al., 1996) with some modifications (J. D., S. Ramkumar, C. Sachidanandan, unpublished). Briefly, subconfluent cultures were trypsinized and suspended at a final density of 10^5 cells per ml in DMEM containing 1.5% Methocel[TM] A4M (Sigma), supplemented with 20% FBS. Suspended cells were recovered for replating by dilution of methylcellulose-containing medium with four volumes of sterile phosphate-buffered saline (PBS) followed by centrifugation at 2000 *g* for 10 minutes, at room temperature. Cells were replated in DMEM containing 20% FBS. C3H10T1/2 mouse fibroblasts were grown in DMEM containing 10% FBS and arrested by incubation in 0.5% FBS for 72 hours.

Antibodies

Antibodies to recombinant rat lamins used in this study and characterized in detail previously are mAb LA-2H10, which recognizes intranuclear lamin A speckles, mAb LA-2B3, which stains the nuclear periphery, and LB-P, which is a rabbit polyclonal antibody to lamin B1 (Jagatheesan et al., 1999). A mouse monoclonal antibody against SC-35 (Fu and Maniatis, 1990) was provided by Dr J Gall (Carnegie Institution of Washington, Baltimore, MD); and a rabbit polyclonal antibody to U5-116 kDa (Fabrizio et al., 1997) and a mouse monoclonal antibody to the trimethylguanosine cap of snRNAs (Bochnig et al., 1987) were obtained from Dr R Lührmann (University of Marburg, Germany). Mouse monoclonal antibodies to myosin heavy chain (A41025) were obtained from H. Blau, to BrdU from Sigma and to p21 (Pharmingen, USA) from G. K. Pavlath (Emory University, GA). A rabbit polyclonal antibody to myogenin was obtained from Santa Cruz Biotech, USA.

Immunofluorescence microscopy

Undifferentiated or differentiated C2C12 cells were washed with PBS and then routinely fixed by treatment with methanol at −20°C for 10 minutes, or with 3.7% formaldehyde for 15 minutes followed by 0.5% (v/v) Triton X-100 for 6 minutes at room temperature, as indicated. After washing with PBS, 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. Samples were mounted in Vectashield (Vector Laboratories, USA) containing 1 µg/ml 4,6-diamidino-2-phenylindole (DAPI). For double labeling experiments with LA-2H10 and rabbit polyclonal antibodies to U5-116 kDa or myogenin, cells were fixed in formaldehyde, blocked and 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, myosin or p21 (IgG subtypes), cells were fixed in formaldehyde, blocked and incubated with LA-2H10, followed by biotinylated anti-mouse antibody and avidin-Cy3. After this step, cells were incubated with the other primary antibody, followed by FITC-conjugated second antibody specific for IgG subtype. Incubations were for 1 hour each and were carried out sequentially with intervening washes with PBS, as this gave optimal labeling. There was no cross-reactivity of the secondary antibodies in control experiments in which either primary antibody was omitted. For staining of muscle sections, unfixed frozen sections (10 µm) of adult mouse skeletal muscle (tibialis anterior) were blocked with 10% horse serum, and incubated with mAb LA-2H10 or LA-2B3 for 1 hour, followed by biotinylated anti-mouse antibody and avidin-Cy3 for 1 hour each. Antibody conjugates were from Jackson Laboratories 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 fitted with a 60×, 1.4 NA objective lens, with excitation at 515, 488 and 351-364 nm (Argon-ion laser). All samples were also routinely viewed under phase contrast (representative images are shown in Fig. 2). Image analysis, including crossover subtraction and estimation of colocalized speckles, was carried out using DASY master program V4.19 (Meridian Instruments, USA.) and images were assembled using Adobe Photoshop 5.0.

DNA synthesis assay

DNA synthesis was measured by a standard assay as follows. C2C12 cells were plated on coverslips and incubated with BrdU at a final concentration of 100 µM for 15 minutes (pulse label) or 15 µM for 2-24 hours (cumulative label). For dual labeling studies, cells were rinsed with PBS, fixed with 3.5% formaldehyde for 5 minutes, permeabilized with 0.2% Triton X-100 for 15 minutes and blocked in 10% horse serum and 0.2% Triton X-100 for 1 hour. Samples were then labeled with mAb LA-2H10 for 1 hour followed by biotinylated

anti-mouse antibody and avidin-Cy3 for 1 hour each. After rinsing with PBS, cells were post-fixed with 3.5% formaldehyde for 5 minutes, DNA was denatured with 2 M HCl containing 0.5% Triton X-100 and 0.5% Tween-20 for 30 minutes, followed by neutralization with 1 mg/ml sodium borohydride. The samples were incubated with FITC-conjugated anti-BrdU antibody (Boehringer Mannheim, Germany) for 1 hour, rinsed and mounted in Vectashield containing DAPI and viewed by confocal microscopy. For quantitative analysis of BrdU incorporation, samples were stained with a peroxidase conjugate and DAB substrate according to the manufacturer's instructions (Vector Laboratories).

Nuclear extractions

Cells were extracted by the protocol described by De Conto et al. (De Conto et al., 2000) with minor modifications. Samples of undifferentiated myoblasts, differentiated myotubes (72 hours) or quiescent myoblasts plated on coverslips were rinsed twice with TM buffer (50 mM Tris-HCl, pH 7.5, 3 mM MgCl$_2$) and then incubated for 10 minutes on ice in TM buffer containing 0.4% Triton X-100, 0.5 mM CuCl$_2$ and 0.2 mM PMSF. Cells were rinsed and incubated with DNase I (20 units/ml) and RNase A (20 µg/ml) for 20 minutes at 37°C in TM buffer. The samples were then treated with 2 M NaCl for 5 minutes on ice, washed with TM buffer, fixed with formaldehyde and stained as described above.

Immunoblot analysis

Undifferentiated and differentiated C2C12 cells (24-72 hours) were harvested, lysed in Laemmli's sample buffer, boiled and electrophoresed on SDS-10% polyacrylamide gels. Gels were electroblotted onto nitrocellulose membrane filters and blocked overnight in 3% bovine serum albumin, in Tris-buffered saline. Filters were incubated with primary antibody for 2 hours, followed by peroxidase conjugated-secondary antibody in Tris-buffered saline containing 0.05% Tween-20 for 1 hour. Bound antibody was visualized using a chemiluminescence kit (Amersham Pharmacia, USA).

RESULTS

We initially examined the pattern of staining by mAb LA-2H10 in undifferentiated C2C12 mouse skeletal muscle myoblasts in dual labeling immunofluorescence assays with antibodies to the RNA splicing factors SC-35 and U5-116 kDa. Both SC-35, which is an essential pre-mRNA splicing factor belonging to the SR-rich family of non-snRNP proteins (Fu and Maniatis, 1990), and U5-116 kDa, a specific component of U5 snRNPs (Fabrizio et al., 1997), are localized in interchromatin granules and fibrils in the nucleus and display a speckled pattern of labeling in fluorescence assays. mAb LA-2H10 labeled 20-50 large intranuclear speckles or foci in all cells of the population and these were colocalized to >90% with RNA splicing speckles stained with antibodies to SC-35 or U5-116 kDa, as shown in Fig. 1. This is consistent with our earlier studies with other cell lines such as HeLa and F-111 rat fibroblasts (Jagatheesan et al., 1999).

Absence of lamin A/C speckles in myotubes

Myogenesis can be induced in culture by depriving dividing myoblasts of growth factors by lowering serum concentrations to 2%. Myoblasts form multinucleated myotubes in an ordered series of events over a period of 72 hours in culture. Undifferentiated C2C12 myoblasts and myotubes after 72 hours of serum deprivation were labeled with antibodies to
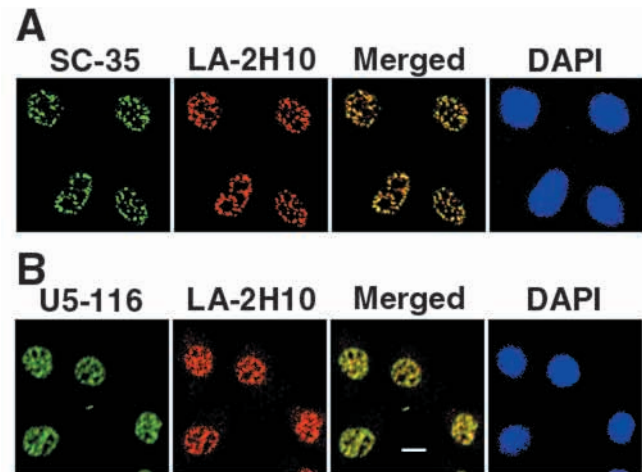

Fig. 1. Colocalization of lamin A with RNA splicing factors. Methanol-fixed C2C12 myoblasts were labeled with mAb LA-2H10 and antibodies to (A) SC-35 or (B) U5-116 kDa. Confocal overlays of the doubly stained cells are shown in the merged panel, where the yellow color highlights structures stained by both antibodies in a single optical section of 0.5 µm. Scale bar: 10 µm.

lamins, RNA splicing factors and myosin as illustrated in Fig. 2A,B. Intranuclear speckles labeled with LA-2H10 were observed in undifferentiated cells but were absent in myotubes. Lack of staining was apparent in both formaldehyde-fixed (data not shown) and methanol-fixed samples. However, the typical nuclear rim staining of lamins A/C by mAb LA-2B3, which recognizes only peripheral lamins, and lamin B1 by LB-P was unchanged in differentiated cells. RNA splicing factor speckles stained for SC-35 and U5-116 kDa were also observed with both cell types. Thus, the splicing speckles were not disrupted but they were not colocalized with lamin A/C in myotube nuclei. The contractile apparatus protein myosin was expressed significantly only in myotubes as expected. The distinct labeling patterns obtained with mAbs to A-type lamins in cultured myotubes were also observed with sections of adult mouse skeletal muscle. As shown in Fig. 2C, myofiber nuclei were not stained with LA-2H10, though the peripheral lamina was stained with LA-2B3 in most nuclei.

### Absence of lamin A/C speckles is due to their reorganization

In order to check the abundance and migration of lamin A and C proteins in differentiated and undifferentiated cells, we have analyzed the immunoreactivity of LA-2H10 towards C2C12 cell lysates sampled over a period of 0, 1, 2 and 3 days in differentiation medium. As shown in Fig. 3, LA-2H10 recognized lamins A and C in undifferentiated myoblasts, and there was no change in the reactivity of LA-2H10 during the course of differentiation in terms of the levels detected or sizes of proteins. This was seen even in the 3 day sample, where the cells had almost exclusively formed multinucleated myotubes and LA-2H10-positive mononucleated cells represented less than 6% of the population in immunofluorescence assays. Similarly, myoblasts clearly expressed peripheral lamin A and lamin B1 and there was no change in the amounts of lamins detected by LA-2B3 or LB-P, whereas myosin levels increased during the course of differentiation as expected.
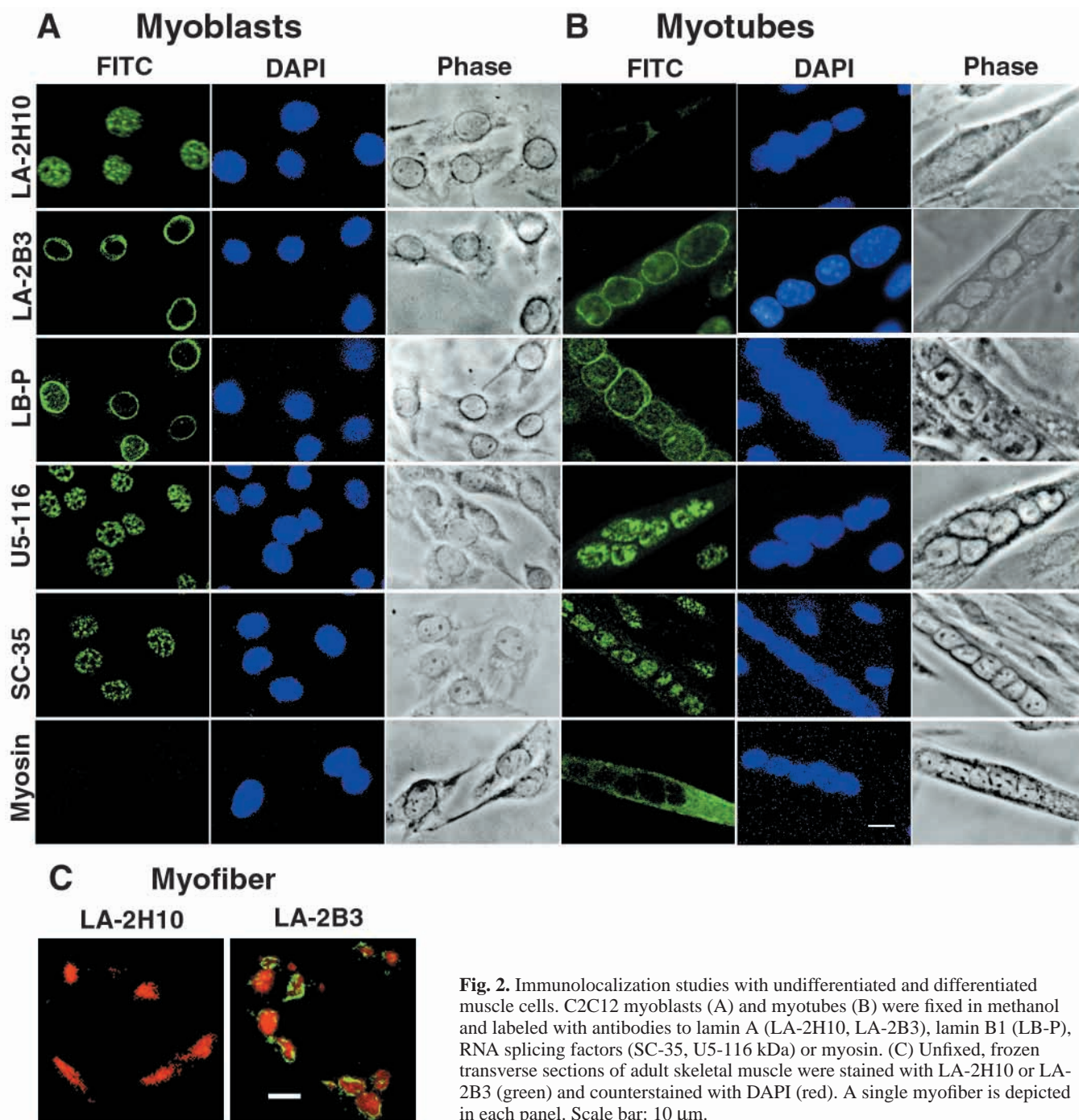
Fig. 2. Immunolocalization studies with undifferentiated and differentiated muscle cells. C2C12 myoblasts (A) and myotubes (B) were fixed in methanol and labeled with antibodies to lamin A (LA-2H10, LA-2B3), lamin B1 (LB-P), RNA splicing factors (SC-35, U5-116 kDa) or myosin. (C) Unfixed, frozen transverse sections of adult skeletal muscle were stained with LA-2H10 or LA-2B3 (green) and counterstained with DAPI (red). A single myofiber is depicted in each panel. Scale bar: 10 μm.

The reactivity of LA-2H10 towards myotube cell lysates in immunoblots but not in immunofluorescence assays suggested that the lamin speckles were organized in the myotube nuclei in a manner that masked their reactivity towards the antibody. The extraction of cell monolayers with detergent, followed by DNase I and salt has been shown to disrupt interactions between lamins and chromatin or other nuclear proteins, and yield a filamentous network consisting of lamins and tightly bound nuclear proteins, also termed the nuclear matrix; this procedure results in unmasking of epitopes on lamins and nuclear matrix-associated proteins, and effectively rules out covalent modification as a mechanism for antigenic disappearance (Nickerson et al., 1992; Dyer et al., 1997; De Conto et al., 2000). This treatment does not disrupt intranuclear lamin A/C speckles in fibroblasts and HeLa cells (Jagatheesan et al., 1999). In the present study, C2C12 myoblasts and myotubes were treated with detergent and nucleases, followed by salt extraction, as described by De Conto et al. (De Conto et al., 2000). This method was preferred for these cells as the method of Nickerson et al. (Nickerson et al., 1992) led to considerable lysis of myoblasts and myotubes. As illustrated in Fig. 4A, extracted myotubes labeled with LA-2H10 displayed nearly uniform nuclear staining, unlike the large speckles seen in myoblasts. The speckled pattern of lamin A/C
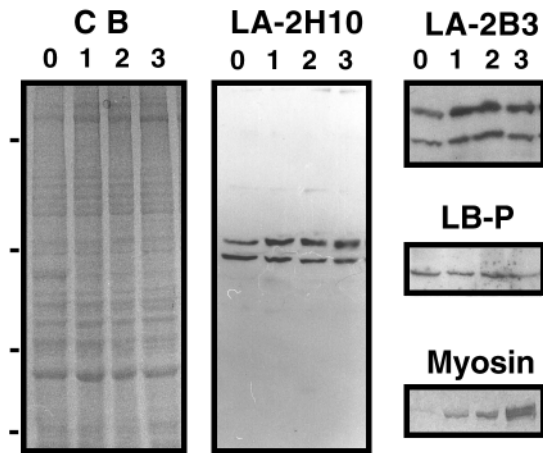
Fig. 3. Immunoblot analysis of C2C12 cell lysates. Cell lysates were prepared from C2C12 cells kept in differentiation medium for 0-3 days and samples (50 µg protein) were resolved by 10% SDS-PAGE, transferred to nitrocellulose filters and immunoblotted with antibodies to lamins and myosin. A representative gel stained with Coomassie Blue is also shown (CB). Molecular mass markers indicated on the left (from top to bottom) are: phosphorylase b, 94 kDa; albumin 67 kDa; ovalbumin, 43 kDa; and carbonic anhydrase, 30 kDa.

staining was retained in extracted myoblasts, indicating that the treatment had not significantly altered the intranuclear lamin network in these cells. The speckled pattern of SC-35 was also retained after extraction (Fig. 4C). The efficiency of the nuclease digestion was confirmed by staining with DAPI for DNA for each sample, and control experiments with an antibody to trimethylguanosine-capped RNA confirmed that RNA had been completely digested (Fig. 4D). The typical peripheral lamina staining by LA-2B3 was not altered in myoblasts or myotubes that had been extracted (Fig. 4B). Hence, though lamins A/C are not visible as speckles in myotubes, they are still present within myotube nuclei and become accessible to the antibody only after chromatin is removed. Based on the above results we can conclude that lamins A/C are present but are antigenically masked in differentiated myotubes, and that antigenic masking is most likely due to structural reorganization, though it is possible that new binding proteins could also block antibody access to the epitope.

Disappearance of lamin A/C speckles is an early event during differentiation

In order to determine the stage of differentiation at which lamin A/C speckles disappeared, differentiated cell cultures were stained with LA-2H10 and myogenic markers. C2C12 myoblasts were switched to differentiation medium and initially examined at 24, 48 and 72 hours by labeling with LA-2H10 and myogenin, an early marker for commitment to the myogenic pathway. Undifferentiated myoblasts contained only 2-3% cells that were positive for myogenin. After 24 hours of serum deprivation, approximately 30% of cells expressed myogenin, as shown in Fig. 5A. Multinucleated myotubes were rarely observed in these cultures at this time point. Most of the nuclei that were positive for myogenin were depleted of intranuclear lamin speckles. By 48 hours, several smaller myotubes could be observed. These were myogenin-positive

but were devoid of speckles. Larger myotubes that were formed by 72 hours exhibited a similar pattern of labeling. The 48- and 72-hour samples also contained a few mononucleated cells that were negative for both lamin speckles and myogenin, and were likely to be quiescent (Yoshida et al., 1998). When differentiating myoblasts were stained with an antibody to myosin (Fig. 5B), positive mononucleated cells and multinucleated myotubes were observed only after 48 or 72 hours in low serum medium, which was expected because myosin is a late marker of differentiation. Cells that expressed myosin did not display intranuclear lamin speckles. These data indicate that the disappearance of lamin A/C speckles is an early event during commitment of myoblasts to the myogenic pathway.

Although myogenin is an early marker for cells that are committed to differentiate, it is not a marker of the postmitotic state, as subsequent expression of the cell cycle inhibitor p21 has been shown to correlate with establishment of the postmitotic state (Andrés and Walsh, 1996). In order to determine whether disappearance of lamin A/C speckles occurred in postmitotic cells or at an earlier stage, myoblasts were induced to differentiate from 6-24 hours and stained to detect the presence of myogenin, p21 and lamin speckles. As shown in Fig. 5C,D, myogenin expression was evident 6 hours after growth factor withdrawal, and increased thereafter. A considerable percentage of myogenin-positive cells also displayed lamin speckles initially, but this number decreased as differentiation proceeded. The percentage of cells positive for p21 was low in the initial stages of differentiation and increased by 18 hours. A significant finding was that lamin speckles were not observed in cells that expressed p21 at any time point. A quantitative analysis of the data with a larger cell population is shown in Fig. 5E,F. Whereas lamin speckles were observed in at least 50% of myogenin-positive cells, p21-expressing cells never showed speckles. Thus, the disappearance of lamin A/C speckles occurs after induction of myogenin expression in differentiating myoblasts, when cells have started withdrawing from the cell cycle.

Lamin A/C speckles reappear after reactivation of quiescent myoblasts

The disappearance of lamin A/C speckles in postmitotic myocytes prompted us to examine the dynamics of the speckles in quiescent cells and cells reactivated to enter the cell cycle. As cell cycle arrest in serum-deprived myoblasts is generally irreversible, we have induced reversible growth arrest by culturing myoblasts in methylcellulose-containing media by a modification of the method of Milasincic et al. (Milasincic et al., 1996) as described in Materials and Methods. Importantly, quiescent cells in suspension are viable and can be reactivated by replating on a solid substratum in the presence of serum. C2C12 cells were held in suspension culture for 48 hours and subsequently reactivated for 24 hours. Growth arrest and reactivation were confirmed by analysis of DNA synthesis. Incorporation of BrdU was 31% for proliferating myoblasts, 1.4% for quiescent myoblasts and 3% for myotubes (data not shown). Dividing, quiescent and reactivated myoblasts were stained with antibodies to lamins and RNA splicing factors. As shown in Fig. 6, lamin speckles were prominent in dividing cells but absent in quiescent cells and reappeared in reactivated cells. The typical peripheral localization of lamins A/C and

lamin B1, and the speckled staining observed for SC-35 were not altered in quiescent cells. We also checked for the presence of lamin A/C speckles in quiescent mouse C3H10T1/2 fibroblasts as an example of a non-muscle cell type. No significant changes were observed when C3H10T1/2 cells were serum-starved for 72 hours, indicating that lamin A/C speckles were not reorganized in quiescent, non-muscle cells. For C3H10T1/2 cells, the incorporation of BrdU was 35% for proliferating cells, 5% for serum-starved cells and 40% for cells restimulated with 10% FBS for 24 hours (data not shown).

In order to determine the time course of reappearance of lamin speckles, quiescent C2C12 cells were replated in the presence of serum and cumulative DNA synthesis was monitored by continuous labeling with BrdU. Speckles started reappearing between 6-18 hours after replating and were observed in 76% of the cells by 18 hours (Fig. 7A). At this time 37% of cells had undergone DNA replication and most cells that stained positive for BrdU also displayed speckles. By 24 hours, the majority of the cells displayed lamin A/C speckles and had also replicated their DNA. A quantitative analysis of the data is shown in Fig. 7B. Thus the reappearance of lamin speckles correlated with the ability of the cells to enter the cell cycle, suggesting that the speckles might be reformed before entry into S phase. Although quiescent cells did not display speckles, extraction of the cells with detergent and nucleases revealed a uniformly stained lamin network (Fig. 7C), suggesting that lamin A/C speckles are antigenically masked in quiescent cells as in myotubes. Comparison of extracted quiescent and myotube nuclei indicated that the distribution of internal lamins in both cell types was similar, with most cells displaying a uniform pattern of staining.

DISCUSSION

Lamina reorganization and intranuclear lamins A/C

In this study we have presented evidence for a marked change in intranuclear lamina organization during skeletal myoblast differentiation in culture. Undifferentiated C2C12 myoblasts are labeled by mAb LA-2H10 to lamin A/C in a pattern of intranuclear lamin speckles that colocalize with RNA splicing factors, as observed earlier with several other mammalian cell lines. As myoblasts are induced to differentiate in low serum medium, these lamin speckles disappear at an early stage, when cells have started withdrawing from the cell cycle. The lamin speckles are absent in reversibly arrested myoblasts and reappear as cells re-enter
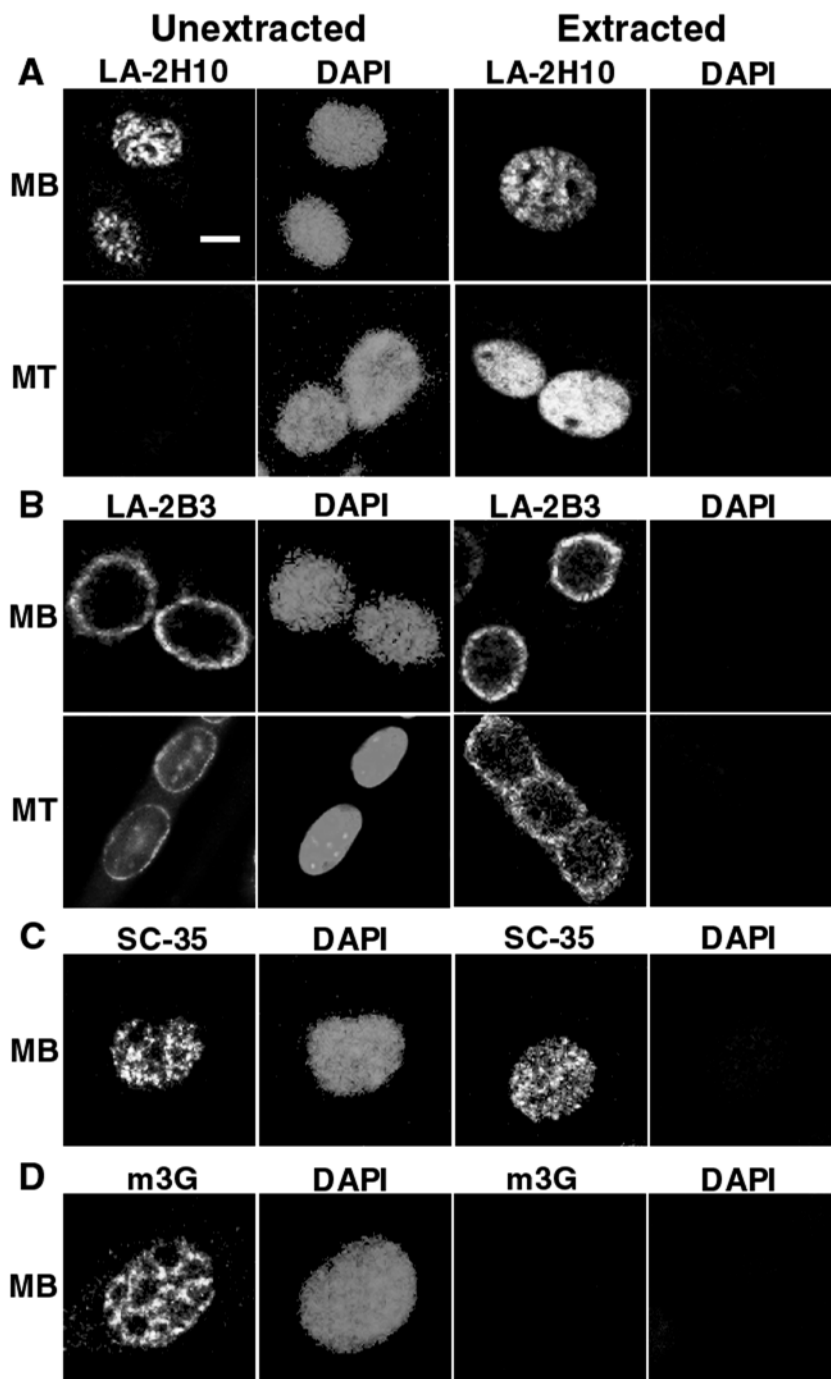

Fig. 4. Localization studies in extracted cells. C2C12 myoblasts (MB) and myotubes (MT) were extracted with detergents, digested with nucleases and treated with 2M NaCl, followed by formaldehyde fixation and labeling with (A) mAb LA-2H10, (B) mAb LA-2B3, (C) mAb SC-35 and (D) m3G antibody to capped RNA, and with DAPI. Unextracted cells are shown as controls. Images of optical sections of 1 μm are displayed. Scale bar: 10 μm.

the cell cycle. However, no significant changes are observed in the typical peripheral localization of A-type lamins or lamin B1, or in the speckled appearance of RNA splicing factors in differentiated or quiescent cells. The retention of immunoreactivity of mAb-2H10 on western blots of differentiated myoblasts and the uniform nuclear staining of
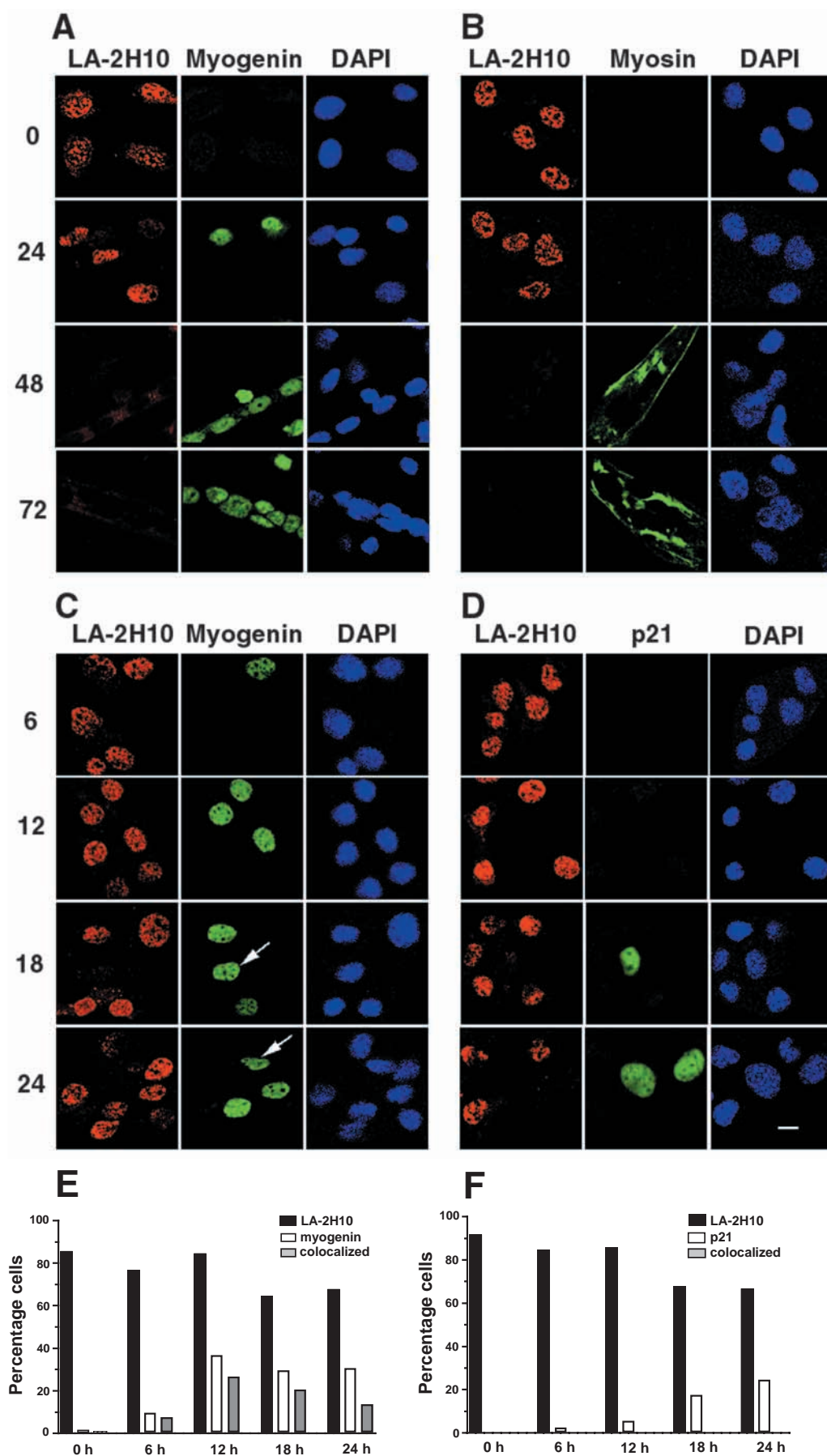
Fig. 5. Localization of lamin A speckles and markers for myoblast differentiation. (A,B) C2C12 cells were kept in differentiation medium for 0-72 hours, fixed with formaldehyde and labeled with mAb LA-2H10 and myogenin antibody or LA-2H10 and myosin antibody. (C,D) C2C12 cells were maintained in differentiation medium for 6-24 hours, fixed with formaldehyde and labeled with LA-2H10 and myogenin antibody, or LA-2H10 and p21 antibody. (E,F) Quantitative analysis of data in C,D, with n=150 cells at each time point. Arrows in C indicate cells expressing myogenin but not lamin A speckles. Scale bar: 10 μm.
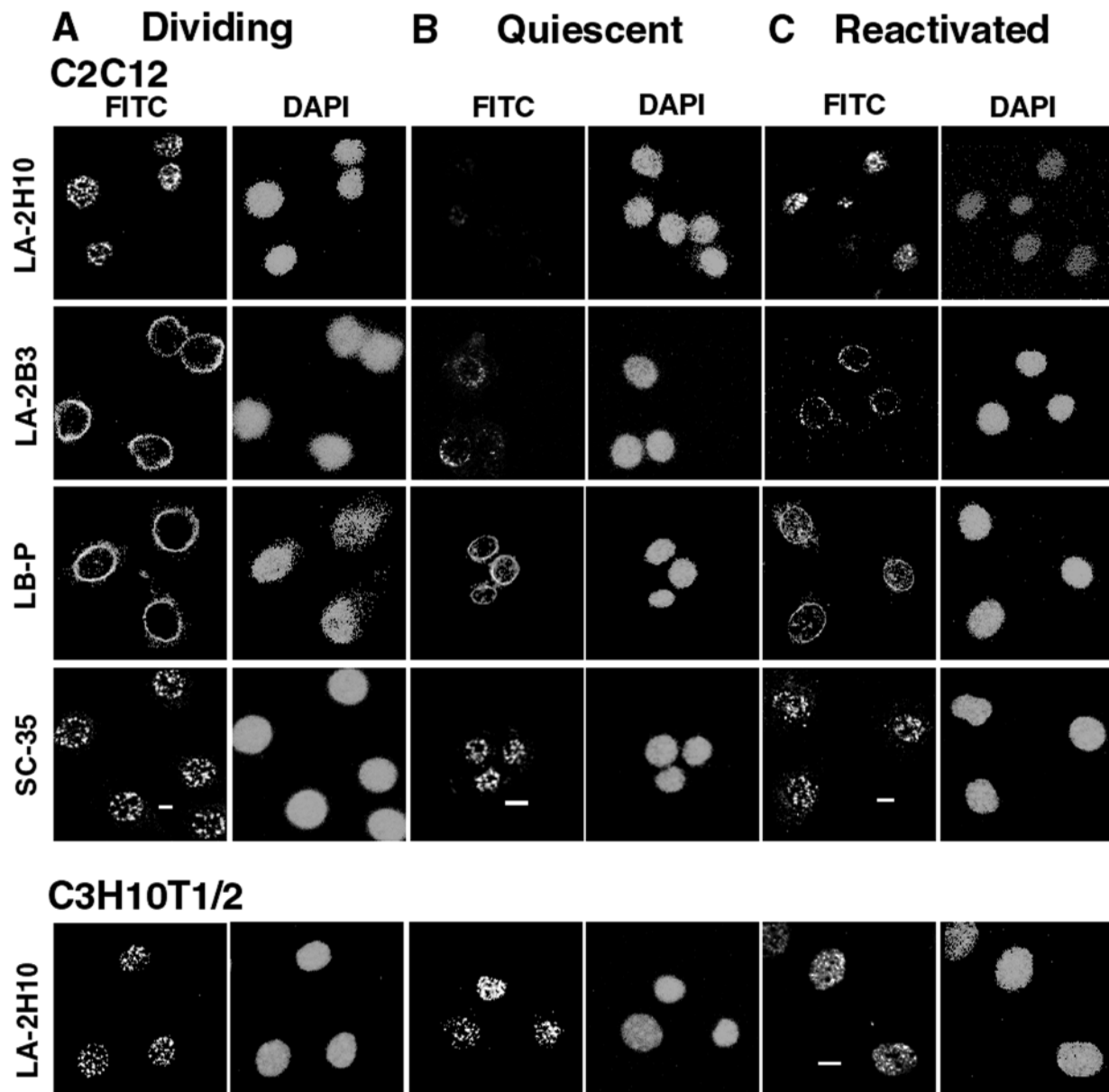
Fig. 6. Localization studies in quiescent cells. (A-C) C2C12 myoblasts that were normally dividing, induced to become quiescent or subsequently reactivated for 24 hours, were fixed and labeled with the indicated antibodies. Similarly, dividing, quiescent and reactivated non-muscle cells (mouse C3H10T1/2 fibroblasts, bottom) were fixed and labeled with mAb LA-2H10. Scale bar: 10 μm.

detergent and nuclease-treated myotube indicate that the speckles are antigenically masked in these cells. It may be noted that we could clearly detect the expression of lamins A/C in undifferentiated C2C12 myoblasts by immunoblot analysis and the abundance of the lamins did not change upon differentiation, unlike an earlier report of the absence or low levels of lamin A expression in chicken myoblasts and increased expression in differentiated cells (Lourim and Lin, 1989). This discrepancy may be attributed to differences in the sensitivity of the experimental technique as in the earlier work samples with very low protein concentration were analyzed at early stages of differentiation. However, we cannot rule out the possibility of differences in lamin expression profiles between avian and mammalian cells.

Our observation that lamin speckles are antigenically masked in myotube and quiescent myoblast nuclei, and yield a predominantly uniform staining with LA-2H10 after detergent extraction and nuclease digestion, has important implications because it suggests a physical rearrangement of the lamina during muscle differentiation. Antigen masking could conceivably also occur by binding of new proteins, but this is unlikely to lead to a distinct change in the staining pattern from large speckles to a uniform one. Epitope masking of lamin antibodies due to interactions of lamins with chromatin (Collard et al., 1990) or phosphorylation of lamins (Dyer et al., 1997) is well known. The reorganization of lamin speckles to a more uniformly stained internal structure raises the possibility that such an internal structure might be part of
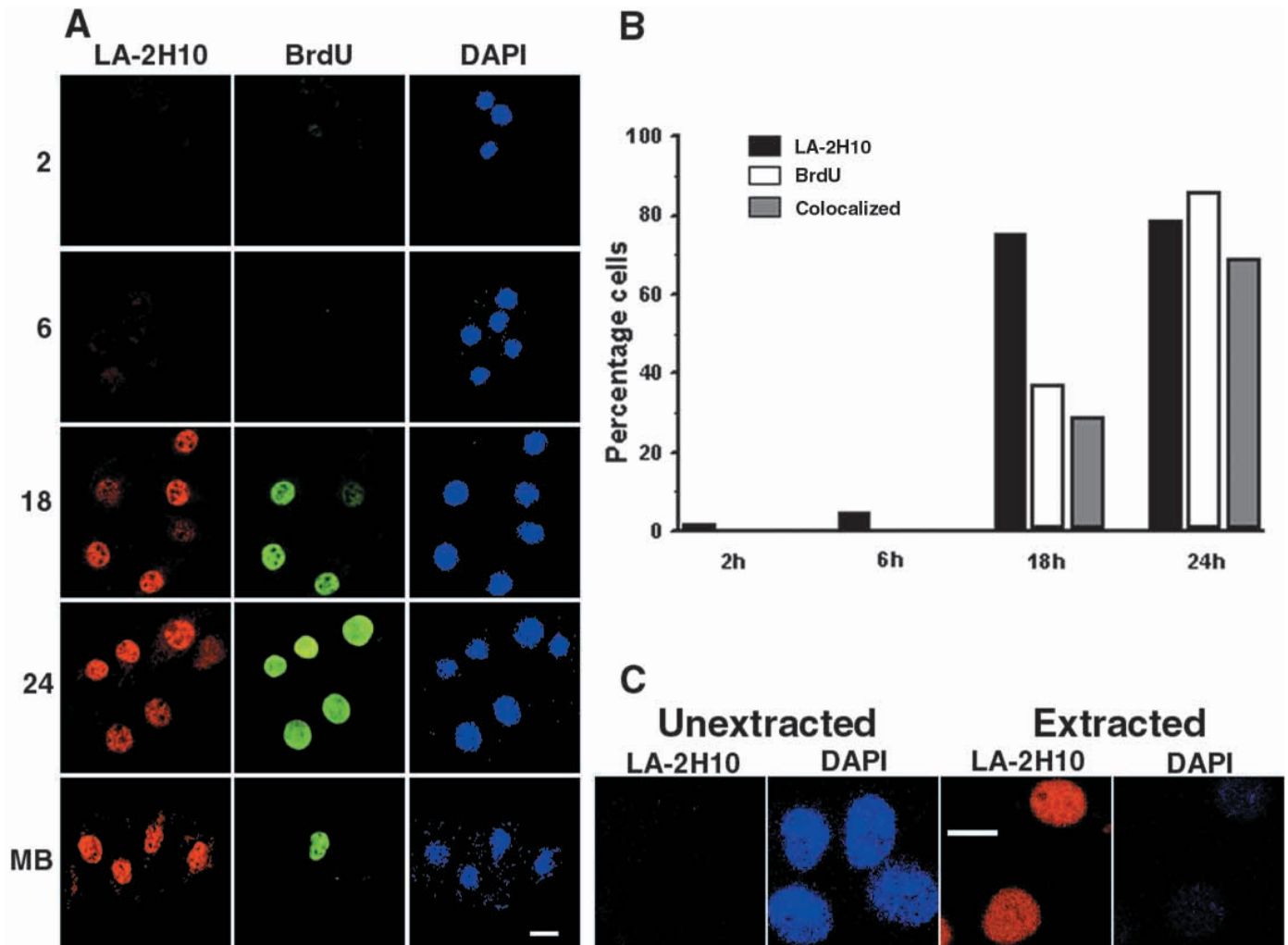
Fig. 7. Reactivation of quiescent C2C12 cells. (A) Incorporation of BrdU and staining with LA-2H10 were assayed with quiescent C2C12 cells reactivated for 2-24 hours. (B) Quantitative analysis of data in A with *n*=120 cells at each time point. (C) Quiescent C2C12 cells were extracted with detergent and nucleases as described in Fig. 4 and stained with mAb LA-2H10 and DAPI. Scale bars: 10 μm.

a stable network that can contribute to formation of specific lamin compartments such as speckles when required, as for example when quiescent myoblasts are reactivated.

Dynamic behavior of lamin A/C speckles during cell cycle

The changes that occur in the peripheral lamina organization during nuclear envelope assembly and disassembly in mitotic cells have been well documented (Gant and Wilson, 1997; Moir et al., 2000b). There is now accumulating evidence for the dynamic behavior of the intranuclear lamin network during the cell cycle. Lamin foci have been shown to associate with DNA replication centers in S-phase nuclei (Moir et al., 1994). Studies with mutant lamins suggest that normal lamina assembly is required to establish DNA replication centers (Ellis et al., 1997) and that lamins are essential for the elongation phase of DNA synthesis (Spann et al., 1997) and are involved in the organization of the replication factor complex and PCNA (Moir et al., 2000a). Intranuclear lamin foci have also been observed at internal sites in nuclei of G1 phase cells in association with heterochromatin (Bridger et al., 1993).

We have earlier demonstrated that mAb LA-2H10 to lamin A labels nuclei of interphase cells in a pattern of 20-50 large intranuclear speckles that colocalize with RNA splicing proteins (Jagatheesan et al., 1999). Although RNA splicing factors are localized in both interchromatin granules and perichromatin fibrils (Fakan and Puvion, 1980; Spector, 1993; Lamond and Earnshaw, 1998), the splicing of nascent transcripts is proposed to occur near sites of transcription in perichromatin fibrils (Fakan, 1994), with splicing factors being recruited from interchromatin granules, which may also be involved in the preassembly of spliceosomes (Misteli and Spector, 1998). Based on our earlier observations, we have proposed that lamin speckles perform an important structural role in the organization of RNA splicing factor speckles, especially in their reassembly towards the end of mitosis (Jagatheesan et al., 1999). Our present data demonstrate the absence of lamin A speckles in quiescent myoblasts and their early reassembly in cells reactivated to enter the cell cycle, suggesting that lamin A speckles might also play a significant role during exit from quiescence. However, their exact function has yet to be elucidated. It appears that considerable flexibility

is possible in the formation of the intranuclear lamin network in different cell lineages, as reorganization of lamin A speckles is observed in quiescent myoblasts and myotubes, but not in quiescent C3H10T1/2 mouse fibroblasts or non-dividing cell types such as adult hepatocytes (data not shown). A more extensive screening of other cell lineages is under way.

Nuclear reorganization during myoblast differentiation

There are indications that adaptation of the nucleus of the myoblast cell to the postmitotic muscle fiber cell may require changes in nuclear organization, in particular in elements of the nucleoskeleton. For example, the nuclear mitotic apparatus protein (NuMA), which is a component of the intranuclear matrix in interphase nuclei and translocates to the spindle pores during mitosis (Lydersen and Pettijohn, 1980) is observed to be degraded during myotube formation, being completely lost after 48-96 hours of culture (Merdes and Cleveland, 1998), but can be detected in most other tissues. A further example is that prosomes, which are the core of 26S proteasomes and are associated with the nuclear matrix, undergo changes in their distribution pattern upon fusion of myoblasts to myotubes, with one subclass losing immunoreactivity in myotubes due to epitope masking by chromatin, as revealed by nuclear extraction procedures (De Conto et al., 2000). In addition to changes in nuclear structure, the location of transcription factors such as the E2F family of factors shifts from the nucleus to the cytoplasm during myoblast differentiation, in order to maintain nuclei in a quiescent state in terminally differentiated myotubes (Gill and Hamel, 2000). There is presently no evidence for reorganization of nuclear membrane proteins such as emerin during myoblast differentiation or of changes in the typical peripheral location of the lamins, except for a lowering of lamin B1 levels in adult muscle (Manilal et al., 1996; Manilal et al., 1999) (B. M., J. D., N. R. and V. K. P., unpublished), which might be a late event as we have not observed any changes in lamin B1 expression during myoblast differentiation. Our finding that lamin speckles are reorganized in postmitotic, differentiating myoblasts as well as in reversibly arrested myoblasts suggests that these rearrangements may be regulated by common events that occur during cell cycle withdrawal in myoblasts. These changes in lamina structure and the reported degradation of NuMA in myotubes raise the possibility of considerable reorganization of the internal nucleoskeleton upon muscle differentiation.

Implications of lamina reorganization during muscle differentiation

Mutations in the lamin A gene cause progressive muscle wasting and weakness in the human autosomal dominant disease EDMD (Bonne et al., 1999), and in the lamin A knockout mouse (Sullivan et al., 1999). Furthermore, mutations in emerin, an inner nuclear membrane protein that associates with lamin A (Fairley et al., 1999) also result in EDMD of the X-linked form (Bione et al., 1994; Nagano et al., 1996; Manilal et al., 1996). It is not clear why lamin A or emerin mutations should predominantly affect muscle tissue when emerin is ubiquitously expressed and lamin A is present in almost all differentiated cells. It has been proposed that in the absence of lamin A or emerin, nuclear envelope integrity is compromised and muscle nuclei may be unable to withstand

the mechanical stress to which the muscle fiber is subjected (Sullivan et al., 1999). A role for emerin in regulating gene expression in heart and muscle has also been suggested (Östlund et al., 1999). Other inner membrane proteins that interact with lamins and have the potential to be involved are the lamin B receptor or LBR (Worman et al., 1988) and the lamina-associated polypeptides (Gerace and Foisner, 1994), as well as the nucleoskeletal protein LAP2α (Dechat et al., 2000). Recently, a model has been proposed for the association of the A-type lamins with the lamina and the inner nuclear membrane through interactions with lamin B, emerin and LAP1C, which might explain why mutations in either emerin or lamin A lead to EDMD (Hutchison et al., 2001). It has also been suggested that lamin A might affect gene expression in progenitor mesenchyme stem cells by influencing the spatial organization of chromatin (Wilson, 2000). Manilal et al. (Manilal et al., 1999) have suggested that since cardiac and skeletal muscle nuclei have lower levels of lamin B1, they may be particularly sensitive to the loss of either emerin or lamin A.

Our findings provide evidence for changes in internal lamin A/C organization that occur primarily during muscle differentiation. Lamin speckles are reorganized in postmitotic, terminally differentiated myotubes, but are present in other differentiated cell types. These rearrangements in the internal lamina might be part of the overall process of nuclear architectural changes during muscle differentiation. If this is hindered by mutant lamins or absence of lamins, it could lead to muscle-specific disease symptoms. In this context, an important conclusion obtained from the recent in-depth analysis of gene expression profiles in Duchenne and limb-girdle muscular dystrophies is that dystrophic muscle fibers are in a state of incomplete differentiation (Chen et al., 2000). It is now evident that, in addition to a role for the peripheral lamina in maintaining nuclear integrity, the possibility of tissue-specific variations in the internal lamina network needs to be investigated in detail in order to understand the complex disease phenotypes that result from different mutations in the *LMNA* gene.

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