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Remodelling of the Nuclear Periphery During Muscle Cell Differentiation In Vitro

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Abstract We have examined the composition and ultrastructure of the nuclear periphery during in vitro myogenesis of the rat myoblast cell line, L6E9. Immunofluorescence labelling and immunoblotting showed that lamins A/C and B were all present in undifferentiated cells, but that they increased significantly before extensive cell fusion had occurred, with lamins A/C increasing proportionately more. Electron microscopic observations were consistent with these results, showing an increase in the prominence of the lamina during differentiation. On the other hand, immunofluorescence labelling suggested that the P1 antigen began to disappear from the nuclear periphery as the cells were fusing, after the increase in lamin quantity, and was no longer detectable in multinucleated cells. Unexpectedly, however, P1 was readily detected in isolated nuclei, whether prepared from myoblast or differentiated cultures, as well as in both myoblast and myotube nuclear matrices. It appears probable, therefore, that the fading of P1 labelling is due to masking of the epitope by a soluble factor recruited to the nuclear periphery as cells differentiate. These data, together with evidence that the genome is substantially rearranged during L6E9 myogenesis [Chaly and Munro, 1996], suggest that L6E9 cells are a useful model system in which to study the interrelationship of nuclear envelope organization, chromatin spatial order, and nuclear function.

Key words: lamina, lamins, antigen P1, L6E9, nuclear matrix, immunofluorescence, immunoblotting, electron microscopy

In interphase nuclei, chromatin is folded into loop domains that are anchored at sites throughout the nucleus to a nuclear scaffold, or matrix [Filipski et al., 1990]. By analogy with DNA loops in lampbrush chromosomes, interphase chromatin loop are thought to organize DNA into functional units that can coil or uncoil as appropriate for transcription and replication [Bonifer et al., 1991; Brasch, 1990; DeJong et al., 1990; Filipski et al., 1990; vanDriel et al., 1991]. The lamina, a fibrous proteinaceous layer lining the inner membrane of the nuclear envelope and a component of nuclear matrices, is believed to provide anchoring sites for the loops at the nuclear periphery [Gerace and Burke, 1988; Gerace and Foisner, 1994; Luderus et al., 1992, 1994; Spector, 1993].

Lamins are the most abundant and best-characterized lamina proteins [Gerace and Burke, 1988; Gerace and Foisner, 1994]. They are members of the intermediate filament protein family, and comprise two subtypes. A B-type lamin is found in all vertebrate cells, but A-type lamins, represented by lamins A and C in mammals, are present primarily in differentiated cells [Gerace and Foisner, 1994]. Although the function of lamins is not fully understood, many lines of evidence indicate that they participate in the interaction of chromatin and the inner nuclear membrane. Both the A- and B-type lamins are chromatin-binding proteins [Glass and Gerace, 1990; Hoger et al., 1991; Luderus et al., 1992, 1994], and lamins A, B_1, and B_2 have been shown to interact specifically with matrix-attachment region (MAR) DNA [Luderus et al., 1992, 1994]. Microscopy has also shown that concentrations of lamin B are highly correlated with the distribution of condensed chromatin masses, coating the envelope-associated surface of the masses [Belmont et al., 1993]. Furthermore, there appears to be growing consensus that lamins play a role in directing post-mitotic reassembly of the nuclear envelope on the surface of chromosomes [Gerace and Foisner, 1994; Lourim and Krohne, 1994].

As noted above, expression of A-type lamins is developmentally regulated in many cell types. For instance, A-type lamins are absent or reduced in early mouse [Stewart and Burke, 1987]

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and chicken [Lehner et al., 1987] embryos, in undifferentiated embryocarcinoma cells [Lebel et al., 1987; Stewart and Burke, 1987], in embryonic myocytes [Lourim and Lin, 1989] and in promyelocytic leukemia cells [Paulin-Levasseur et al., 1989]. The A-type lamins become expressed later in mouse and chicken embryogenesis, and during embryocarcinoma, promyelocyte and muscle differentiation [Lebel et al., 1987; Lehner et al., 1987; Lourim and Lin, 1989; Paulin-Levasseur et al., 1989; Prather et al., 1991; Stewart and Burke, 1987]. In addition, a number of myeloid and lymphoid cell lines lacking or reduced in lamins A/C have also been identified [Guilly et al., 1987, 1990; Paulin-Levasseur et al., 1988].

Since the three-dimensional organization of chromatin is considered to exert global effects on gene expression [Blobel, 1986; Manuelidis, 1990], it has been proposed that the lamina, through its role in organizing chromatin, may also play a role in regulating chromatin functions [Lourim and Lin, 1989; Moir and Goldman, 1993]. Specifically, it has been suggested that changes in lamin composition during development could effect changes in chromatin disposition at the nuclear periphery and thus affect chromatin function (and cell development) indirectly [Lourim and Lin, 1989]. Relatively little attention has been paid, however, to the role of non-lamin proteins of the lamina in modulating the properties of the nuclear periphery. Several such proteins have been described, including the P1 antigen [Chaly et al., 1984, 1989], perichromin [McKeon et al., 1984], and statin [Wang, 1985a]. Like lamins, these antigens are localized on the nucleoplasmic face of the nuclear envelope, in the region of the lamina. The function of these proteins has not been established, but statin expression, at least, is also developmentally regulated, rising during senescence and differentiation [Connolly et al., 1988; Fedoroff et al., 1990; Wang, 1985a, 1985b]. Conceivably, such non-lamin proteins might also be involved in the association of chromatin with the nuclear envelope, possibly by mediating chromatin interaction with lamins.

Muscle differentiation in vitro appears to be a good model system for further studies of the relationship between the lamina and gene expression. Myoblasts become committed to differentiate during G1, withdraw from the proliferative cell cycle and fuse into multinucleated myotubes. Concurrently, an array of muscle-specific genes begins to be expressed, while other genes are repressed [Nadal-Ginard, 1978; Nguyen et al., 1983; and references therein]. If the function of chromosomes is related to their spatial organization [Blobel 1986; Manuelidis 1990], then changes in the spatial distribution of chromosomes could reasonably be expected to occur during myogenesis. In an earlier study we have reported such a change in genome architecture in the L6E9 system, evidenced by repositioning of centromeres to the nuclear periphery in myotubes and a parallel aggregation and accumulation of condensed chromatin at the nuclear periphery [Chaly and Munro, 1996]. Further, if chromosome organization is mediated by the nuclear periphery as proposed, then reorganization of components of the nuclear lamina could also be reasonably expected. Indeed, changes in lamin composition and statin expression during muscle differentiation have been reported in rat and chicken [Connolly et al., 1988; Lehner et al., 1987; Lourim and Lin, 1989; Wedrychowski et al., 1989].

In this study we have examined the organization of the nuclear periphery as a function of differentiation, focussing on the relationship between the P1 antigen and lamins during myogenesis of the rat L6E9 myoblast cell line.

P1 is a highly conserved antigen detected in mammalian, invertebrate and echinoderm cells, as well as in cells of higher and lower plants [Chaly et al., 1984, 1986, 1988; Schatten et al., 1985]. Immunofluorescence and immunoelectron microscopy localizes P1 at the nuclear periphery during interphase in a distribution similar to but not identical with that of the lamins [Chaly et al., 1984, 1989]. The P1 labelling is thicker and more uneven, including irregularly shaped aggregates that project into the nucleoplasm. Furthermore, the behaviour of P1 and of the lamins is distinct during mitosis. While the lamins are released into the cytoplasm during prophase [Gerace and Foisner, 1994], P1 remains associated with the chromosome mass, outlining the surface of each chromosome [Chaly et al., 1984, 1986].

Lamins and P1 also behave differently during nuclear matrix preparation. We have shown with mouse 3T3 fibroblasts that the P1 antigen is displaced from the nuclear periphery when chromatin is solubilized by high salt (1–2M NaCl), becoming artefactually associated with the residual internal network of the nuclear matrix; in contrast, the distribution of the lamins is unaf-

fected by this treatment [Chaly et al., 1985]. On the basis of their behaviour during the mitotic cell cycle and during nuclear matrix preparation, we have proposed that the P1 antigen and the lamins may be acting in concert to mediate attachment of chromatin to the nuclear periphery during interphase, with P1 continuing to perform a "girdling" role around the chromosomes throughout mitosis [Chaly et al., 1985].

In this report, we show that the lamina, the lamins, and the P1 antigen all exhibit differentiation-related changes during L6E9 myogenesis.

MATERIALS AND METHODS

Cell Culture

Rat L6E9 myoblasts (gift from M. McBurney, University of Ottawa) were cultured and differentiated as previously described [Chaly and Munro, 1996]. Briefly, cultures were maintained in medium supplemented with 10% fetal bovine serum and antibiotics. For immunofluorescence labelling or electron microscopy, cells were seeded onto glass or plastic coverslips, respectively, and had formed a confluent monolayer one day later (day 1). To induce differentiation, cells were transferred to medium supplemented with 2% Hybrimax CPSR4 (Sigma), and cells were cultured for a further period of up to 6 days (i.e., to day 7).

Nuclear Matrix Preparation

Nuclear matrices were prepared by transferring cells on coverslips through solutions dispensed in weigh boats as previously described [Chaly et al., 1985]. Briefly, cells were treated sequentially with Triton X-100, 10 μg/ml deoxyribonuclease I, hypotonic buffer, 1 M NaCl, 2 M NaCl, and 50 μg/ml each of deoxyribonuclease I and ribonuclease A. All solutions contained phenylmethyl sulfonyl fluoride (1 mM), soybean trypsin inhibitor (10 μg/ml), and sodium tetrathionate (0.5 mM).

Indirect Immunofluorescence Labelling

Fixation, permeabilization, and primary antibodies. For staining of muscle-specific myosin, samples were fixed in 95% methanol at −20°C, air dried, and incubated with mouse monoclonal antibody MF20 (hybridomas provided by M. McBurney) [Bader et al., 1982] for 30 min.

For staining of the nuclear antigen P1 and lamins, samples were fixed in 3% paraformaldehyde for 5 min, permeabilized in 0.2% Triton X-100 for 20 min, and incubated with primary antibody for 45 min.

Antibody P1 (ascites fluid, 1:50–1:250) is a mouse monoclonal IgM antibody and has been previously described [Chaly et al., 1984]. Polyclonal guinea pig anti-lamin A/C (gpA) (1:75) and a human autoimmune anti-lamin B (2486) (1:75) were gifts from Y. Raymond (Institut du Cancer de Montreal). Polyclonal guinea pig anti-lamin A/B/C (1:200) was a gift from G. Krohne (German Tumour Research Centre, Heidelberg, Germany) [Benavente and Krohne, 1986].

Nuclear matrix preparations were labelled after fixation in paraformaldehyde, as previously described [Chaly et al., 1985].

Secondary antibodies. The secondary antibodies were one of the following, as appropriate: fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM (μ-chain specific) (1:150) (Cappell); FITC-conjugated goat anti-mouse IgG (H + L) (1:500) (Cappell); tetramethyl rhodamine isothiocyanate-conjugated goat anti-guinea pig IgG (H + L) (1:50) (Cappell); FITC-conjugated goat anti-human IgG (H + L) (1:150) (Cappell).

Samples were counterstained with 1.5 μg/ml Hoechst 33258 and mounted in 50% glycerol/phosphate buffered saline containing p-phenylene diamine to retard fluorescence bleaching. Preparations were examined with a Zeiss Photomicroscope III equipped for epifluorescent illumination, using a X 100/1.3 n.a. Neofluar phase or a X 63/1.25 n.a. Plan-Neofluar objective, and were photographed on Ilford XP1-400 film.

Electron Microscopy

For electron microscopy, cells growing on plastic coverslips were fixed in 2% paraformaldehyde/2% glutaraldehyde in 0.05 M sodium phosphate buffer, pH 7.4, for 2–4 h at room temperature. They were washed overnight in the buffer, post-fixed on ice in buffered 1% osmium tetroxide, and dehydrated on ice with a graded ethanol series and propylene oxide. Samples were infiltrated in Epon/Araldite, mounted on silicone-coated microscope slides and polymerized at 65°C, as previously described [Chaly, 1988].

Small portions of the monolayers were cut out, glued onto pre-formed blocks of resin, and sectioned parallel to the monolayer surface. Sections were double-stained with uranyl acetate

and lead hydroxide, and examined in a Philips EM420.

Isolation of Myoblast and Myotube Nuclei

Nuclei were isolated from day 1, 3, 5, and 7 cells by a modification of the method of Lourim (personal communication) [Lourim and Lin, 1989], as follows: After removal of the medium, cells were washed once with PBS containing 5 mM $MgCl_2$ and 1 mM EGTA, once with 50 mM Tris, and once with 15 mM Tris. Cells were then scraped into a pre-chilled Dounce homogenizer and left on ice for 10 min. A one-tenth volume of lysis buffer (1% Triton X-100, 100 mM EDTA, 2 μg/μl leupeptin) was then added and mixed in with one stroke of the Dounce. After 5 min on ice, the sample was homogenized with 10–15 strokes of the Dounce, until cell breakage was essentially complete as determined by phase contrast microscopy. Samples were then transferred to microfuge tubes and spun at 3000 rpm in a Beckman microfuge for 10 min. The pellet was resuspended in 5% glycerol in TTEL buffer (10 mM Tris, 0.1% Triton X-100, 10 mM EDTA, 2 μg/μl leupeptin), layered over a cushion of 10% glycerol in TTEL, and spun at 14,000 rpm in a Beckman microfuge for 10 min. The pellet containing the nuclei was then respun in TTEL without glycerol for 10 min. All buffers were at pH 7.4 and contained 1 mM phenylmethyl sulfonyl fluoride.

A small aliquot of each sample was removed to determine the concentration of DNA in each sample with a Burton assay, as described by Lourim and Lin [1989], and samples were stored at −70°C.

Gel Electrophoresis and Immunoblotting

Samples were suspended in sample buffer, boiled, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to standard protocols [Bio-Rad manual] in 12% gels. Sample concentration was adjusted so that an equal amount of DNA (10 μg) was loaded in each lane.

Immunoblotting was performed according to standard protocols [Bio-Rad Manual]. For immunodetection, the nitrocellulose was soaked in blocking buffer (phosphate-buffered saline/3% powdered skim milk) for 2–4 h, and incubated with human anti-lamin B or guinea pig anti-lamin A/C in blocking buffer overnight. Blots were then washed in blocking buffer and incubated in secondary antibody (biotinylated goat anti-human Ig (1:250) (Vector Labs) or goat anti-guinea pig Ig (1:250) (Cappell)) for 1 h, washed again, and incubated with avidin D-horseradish peroxidase (1:100) (Vector Labs) for 20 min. The reaction was then developed with 5-chloro-1-naphthol and hydrogen peroxide. All steps were carried out at room temperature.

RESULTS

Time Course of Differentiation

On day 1, i.e., 24 h after plating, L6E9 cells formed a confluent monolayer of fusiform myoblasts containing a single ovoid nucleus. Replacing the medium on day 1 with differentiation medium induced the cells to begin fusing by day 3, with maximum fusion achieved by about day 7. The cells first became greatly elongated and oriented in parallel arrays, and then fused to form bi- and tri-nucleated myotube cells. Nuclear profiles in myoblasts were ovoid, but became increasingly elongated after cell fusion. As fusion continued, the nuclei flattened and became arranged in irregular clusters or circular aggregates.

Results from immunofluorescence labelling with antibody MF20 (Fig. 1), directed against muscle-specific myosin [Bader et al., 1982], were consistent with these observations. Samples prepared on days 1, 3, 5, and 7 of culture clearly showed that differentiation was not synchronous (Fig. 2). Using labelling intensity and the number of nuclei per cell as criteria, 500 cells in each sample were classified into five categories, from 1 (undifferentiated) to 5 (highly differentiated) (Fig. 2). Brightness categories were subjectively established within each experiment, with the dullest cells in day 1 and day 3 samples characterized as "faint," the brightest cells in day 7 samples categorized as "very bright," and the other categories interpolated between these extremes.

On day 1, myoblasts were virtually unlabelled by MF20 ($< 0.1\%$) and were all mononucleated (category 1). By day 3, 4.3% of the cells contained detectable levels of muscle-specific myosin and 1–2 nuclei (category 2), while another 2.5% contained 3–4 nuclei and were brightly labelled by the antibody. The proportion of labelled cells, the labelling intensity, and the number of nuclei per cell continued to increase with time in culture. By day 7, less than 1.5% of the cells were in category 1 (mononucleated, unlabelled) with 90% containing at least 3–4 nuclei

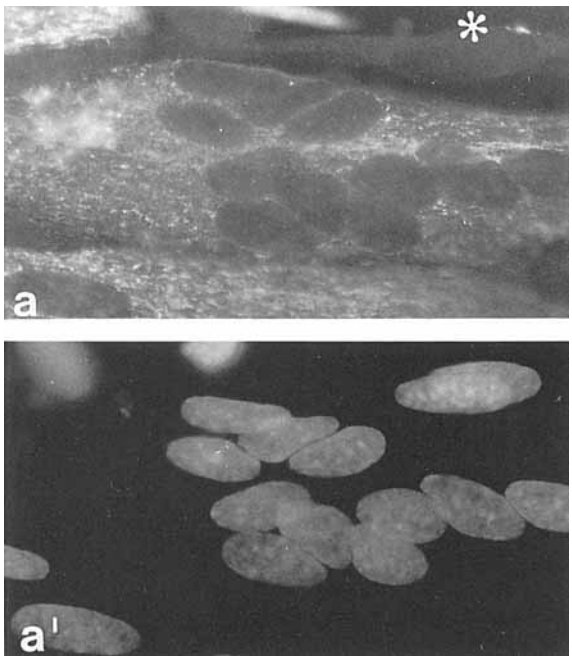

Fig. 1. Day 7 culture labelled with anti-myosin (a) and counterstained with Hoechst (a'). Most cells are labelled, including a large multinucleated myotube in the centre. One cell (*), however, has not differentiated and is unstained. ×1800.

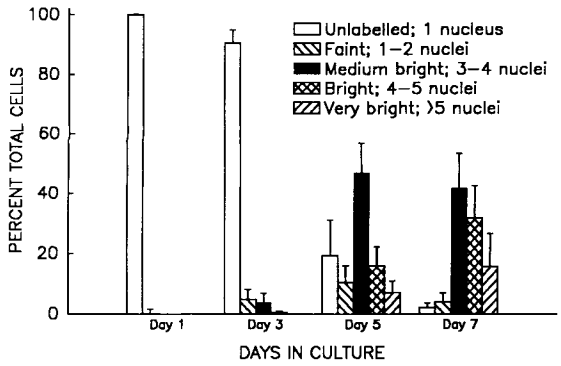

Fig. 2. Histogram showing the change in anti-myosin labelling and in the number of nuclei per cell during differentiation. Whereas all cells were mononucleated and essentially none contained myosin on day 1, few unlabelled mononucleated cells remained on day 7. Over 90% exhibited at least 3–4 nuclei and were stained at least moderately brightly on day 7. The data are averaged from five experiments, with 500 cells counted in each sample of each experiment. Error bars represent the standard deviation.

and exhibiting medium bright to very bright labelling for myosin (categories 3, 4, and 5).

Immunolabelling of Intact Cells

Lamins. Anti-lamin B, -lamin A/C, and -lamin A/B/C produced essentially indistinguishable peripheral nuclear staining, but cytoplasmic and nucleoplasmic background labelling was much higher with the anti-lamin B and -lamin A/C, obscuring detail. Only micrographs of samples labelled with anti-lamin A/B/C are shown.

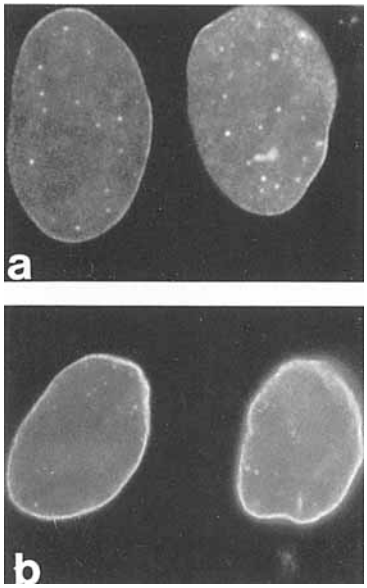

Fig. 3. Immunofluorescent labelling of nuclei (a) and nuclear matrices (b) in day 1 cultures by anti-lamin A/B/C. ×1150.

In day 1 cells, anti-lamin staining was peripheral with small speckles of intense labelling (Fig. 3a). As reported by others [Schmidt et al., 1994], the speckles are interpreted as representing cross-section views of small invaginations of the nuclear envelope.

Upon formation of parallel arrays of elongated nuclei, the labelling appeared to undergo a subtle but consistently observed qualitative change. Beginning on day 3, the staining at the rim of the nuclei progressively became more crisply delineated (Fig. 4a). Whether this change was due to alterations in lamin organization, or whether it was simply an optical consequence of nuclear flattening is not clear.

Antigen P1. The rim of most (94.7%) nuclei in undifferentiated L6E9 cells was brightly labelled by antibody P1 (Figs. 5a–a', 6a–a', 7), as has been previously reported for many cell types [Chaly et al., 1984, 1985, 1986, 1988; Schatten et al., 1985].

Labelling intensity declined during differentiation, and nuclei in large myotubes were essentially unstained in day 7 samples (Figs. 5–6). To establish the kinetics of this change, the labelling intensity of nuclei in differentiating samples was evaluated by comparison with the labelling

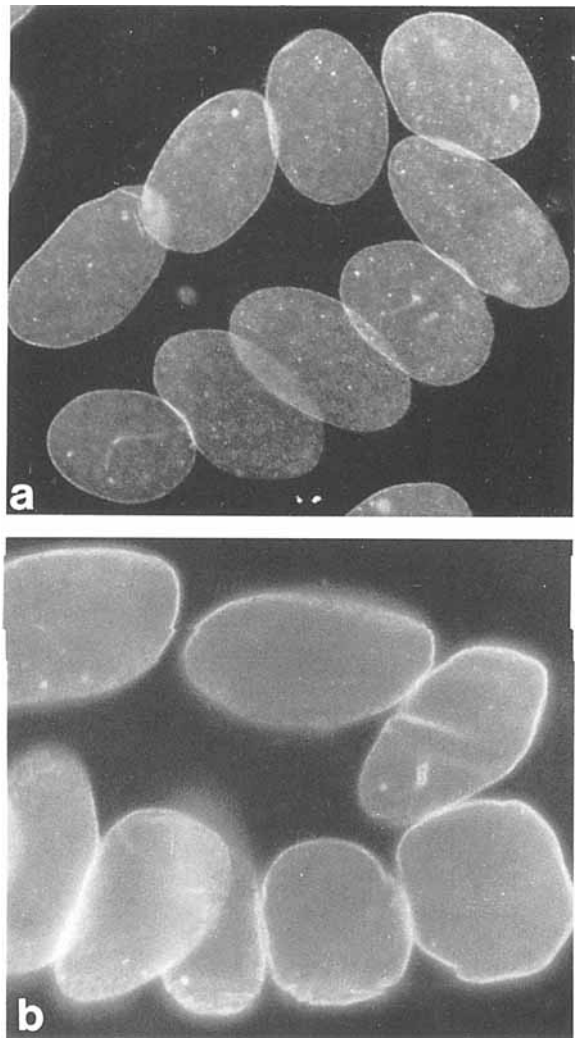

Fig. 4. Immunofluorescent labelling of nuclei (a) and nuclear matrices (b) in Day 7 myotubes by anti-lamin A/B/C. ×1250.

intensity of the majority of nuclei in day 1 samples from the same experiment. Nuclei that were significantly duller than these were classified as medium in brightness, or fainter (Fig. 7).

The proportion of brightly labelled cells had decreased to 83% by day 3 (Figs. 5b–b′, 7). Within a multinucleated cell, all nuclei in these and more differentiated samples stained with similar intensity. The number of brightly stained cells continued to decrease (Figs 5c–c′, 7), and by day 7, only 13% were brightly labelled, with 19% exhibiting medium fluorescence intensity, and 68% in which the nuclei were barely distinguishable from the cytoplasm, i.e., were essentially unlabelled (Figs. 5d–d′, 6b–b′, 7).

There was some variability in staining intensity at all time points, so that even on day 7 some mononucleated cells were labelled (Fig. 7). This is consistent with the asynchronous pattern of differentiation observed in experiments with anti-myosin (Figs. 1 and 2).

Immunolabelling of Nuclear Matrices

We had previously shown that P1 was redistributed to the internal network of nuclear matrices by salt extraction, whereas the lamins were unaffected [Chaly et al., 1985]. A possible explanation for the change in P1 labelling of L6E9 was biochemical modification of the P1 antigen during myogenesis. This raised the prospect that its interaction with the nuclear periphery might be different in myoblasts than in non-myogenic cells. One way of testing this possibility was to examine the behaviour of lamins and P1 during nuclear matrix preparation.

Nuclear matrices were prepared from day 1 and day 7 samples as previously described [Chaly, 1988; Chaly et al., 1985]. By phase contrast microscopy, day 1 and day 7 L6E9 matrices showed an internal reticulum and residual nucleoli within a residual nuclear envelope-pore complex-lamina, and the amount of DNA retained in the matrices was small as judged from the very faint staining with Hoechst 33258 (data not shown). We have reported similar results for mouse 3T3 nuclear matrices [Chaly, 1988; Chaly et al., 1985].

As expected, nuclear matrix preparation did not affect the labelling pattern with anti-lamin A/B/C in either the day 1 (Fig. 3b) or the day 7 (Fig. 4b) samples.

In Day 1 samples, the P1 antigen behaved as previously reported for mouse 3T3 fibroblasts and bovine lymphocytes [Chaly et al., 1985; Setterfield et al., 1985]. That is, it was relocalized (Fig. 6c), forming a meshwork of patches and strands coincident with the internal network visualized by phase microscopy (not shown). Surprisingly, however, whereas P1 had been largely undetectable in day 7 cells (Figs. 5d, 6b), day 7 matrices were labelled almost as brightly as the day 1 (Fig. 6d). The staining again coincided with the phase dense internal reticulum.

Gel Electrophoresis and Immunoblotting

Coomassie stained gels and immunoblots of day 1, 3, 5, and 7 nuclear proteins are shown in Figure 8. On the gels (Fig. 8a), a high molecular weight band (> 200 kD), prominent in day 5 and 7 samples, was weaker at day 3 and absent in day 1 nuclei. This band is most likely muscle myosin. Also, the region 35–80 kD appeared to

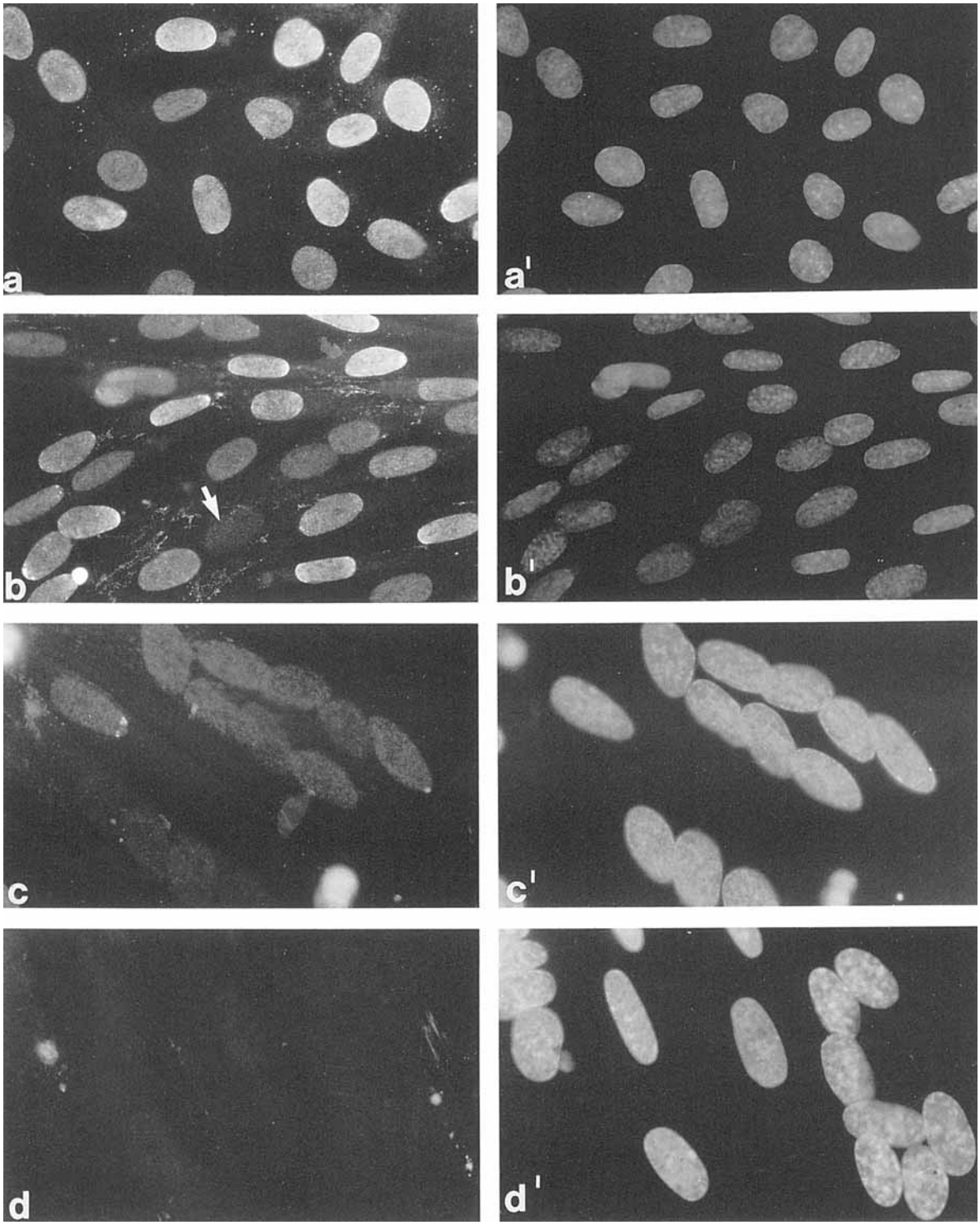

Fig. 5. Immunofluorescent labelling of day 1 (**a–a'**), day 3 (**b–b'**), day 5 (**c–c'**), and day 7 (**d–d'**) cultures by antibody P1 (a, b, c, d), counterstained with Hoechst (a', b', c', d'). Whereas all nuclei in day 1 samples were moderately or brightly stained (a–a'), some nuclei in day 3 samples (b–b') were essentially unlabelled (*arrow*) and fewer appeared bright. Staining intensity decreased to barely detectable levels as differentiation proceeded (c–d'). ×460.

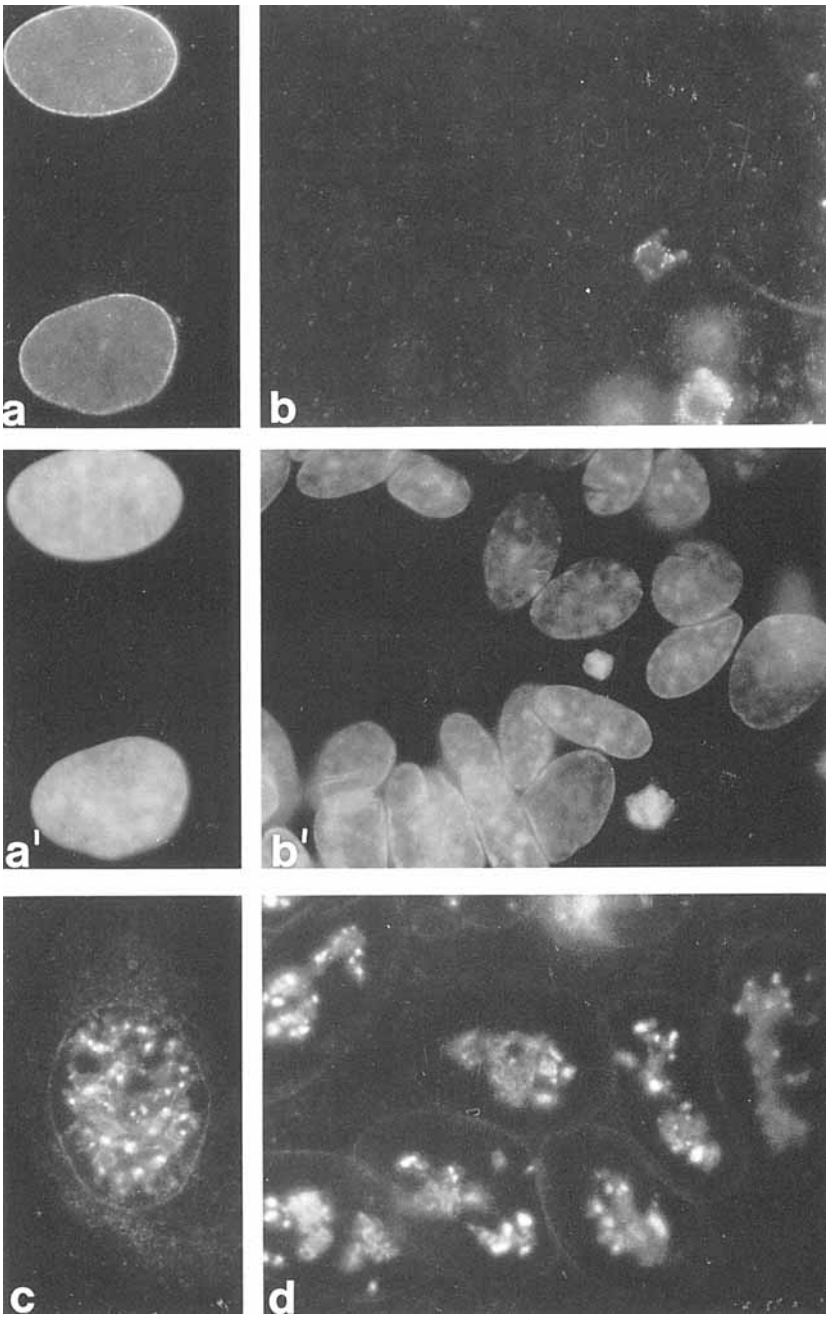

Fig. 6. Higher magnification view of nuclei (**a–a'**, **b–b'**) and nuclear matrices (**c, d**) in day 1 (a–a', c) and day 7 (b–b', d) cultures, labelled with antibody P1 (a, b, c, d) and counterstained with Hoechst (a', b'). a–a', ×1800; b–b', ×900; c, d, ×1700.

contain fewer bands in day 1 and, to some extent, day 3 nuclei.

Immunoblotting with anti-lamin antibodies showed that all three lamins were present in day 1 and day 7 samples, but that they increased significantly during differentiation, with lamins A/C increasing proportionately more (Figs. 8b–c). To determine the timing of these changes, samples from days 3 and 5 were also tested (Figs. 8b–c). It appears that the increase in lamins occurred between days 1 and 3, since the lamin bands are similar in intensity in day 3, 5, and 7 immunoblots.

We had previously reported that the P1 antibody detected bands at molecular weights of 27–30 kD in mouse splenocyte nuclear matrices

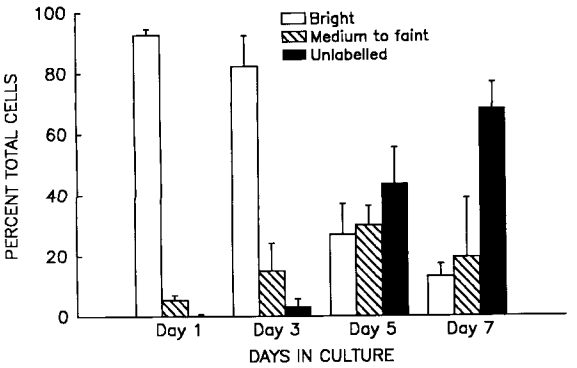

Fig. 7. Histogram showing the change in P1 labelling during differentiation. Results are presented as the proportion of cells with a particular brightness of nuclear staining in a culture, since all nuclei in a multinucleated cell exhibited similar staining intensity. The data are averaged from three experiments, with 500 cells counted in each sample of each experiment. Error bars represent the standard deviation.

[Chaly et al., 1984]. For other antibodies, we have found that heparin treatment largely suppresses non-specific binding of antibodies, e.g., of secondary antibodies, without affecting specific reactions significantly. In carrying out the present study, we found that the 27–30 kDa bands detected by P1 were eliminated by heparin blocking (data not shown), and conclude that they represent non-specific binding of P1. However, no other bands were revealed.

It was formally possible that P1 had been solubilized during nuclear preparation. Therefore, to verify that the samples being used for immunoblotting did contain the antigen, isolated nuclei were formaldehyde-fixed immediately after the last centrifugation and processed for immunofluorescence microscopy with antibody P1, without further permeabilization. In day 1 samples, all nuclei (Figs. 9a–a′) were brightly stained at the periphery, with the labelled band somewhat broader and less crisply delineated than in unfractionated cells (cf. Figs. 6a–a′). In day 7 samples, myotube nuclei were clearly distinguishable by their elongated shape from those of less differentiated cells (Fig. 9b′). Unexpectedly, in these samples as well, all nuclei were also labelled by antibody P1 (Figs. 9b–b′). Similarly, all nuclei in samples isolated from cultures at days 3 and 5 were stained by the P1 antibody (data not shown).

Therefore the P1 antigen was clearly present in all the nuclear samples used for immunoblotting. However, despite considerable further effort, we have been unsuccessful so far in obtaining consistent specific binding of the antibody in immunoblots. The P1 antibody is of the IgM class, and difficulties in obtaining meaningful immunoblots with such antibodies are not uncommon.

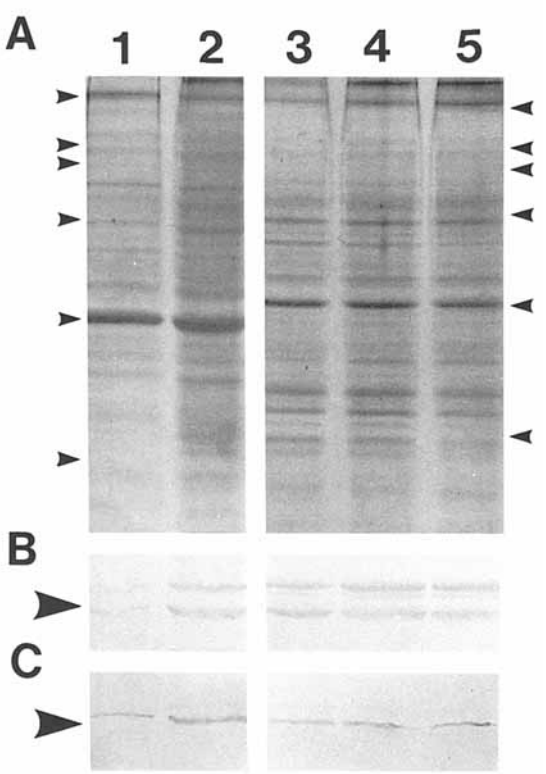

Fig. 8. Coomassie-stained gels **(A)**, immunoblots with anti-lamin A/C **(B)**, and -lamin B **(C)** of day 1 *(lane 1)*, day 3 *(lane 3)*, day 5 *(lane 4)* and day 7 *(lanes 2 and 5)* nuclear samples. *Arrowheads* in **A** indicate, from the top, the position of the 200, 116, 97, 66, 45, and 31 kDa molecular weight markers. Arrowheads in **B** and **C** indicate the position of the 66 kDa molecular weight marker.

Electron Microscopy

To determine whether the changes described above in lamins and in the P1 antigen during myogenesis were reflected in structural reorganization of the nuclear envelope-lamina region, cell samples were examined by electron microscopy.

As shown in Figure 10, a distinct difference is visible between the samples. In day 1 myoblasts, the lamina was faintly stained and often difficult to recognize (Fig. 10a), resembling that in many tissue culture cells, such as mouse 3T3 fibroblasts [Chaly, 1988; Chaly et al., 1989]. In day 7 samples, on the other hand, the lamina was considerably more prominent. It was stained more intensely and appeared thicker (Fig. 10b).

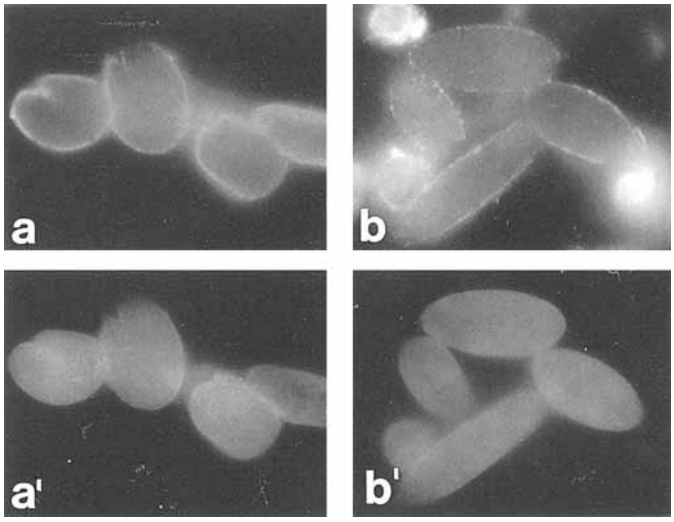

Fig. 9. Labelling of isolated day 1 (**a–a'**) and day 7 (**b–b'**) nuclei by antibody P1 (a, b), counterstained with Hoechst (a', b'). In contrast to intact samples (cf. Figs. 5, 6), all nuclei in both samples are labelled by the antibody. ×1000.

DISCUSSION

In this study, we have shown that reorganization of the nuclear periphery during myogenesis involves a change in lamin composition, but also includes thickening of the lamina at the electron microscope level, masking of the P1 antigen, and recruitment of a differentiation-specific factor.

Immunoblotting showed an overall increase in the quantity of lamins A, B, and C during differentiation, as well as a marked relative increase in the amount of the A-type lamins. Further, the lamina was more prominent in myotubes at the electron microscope level. These results are generally consistent with reports on myogenesis in chicken. Studies of lamin expression in whole embryos [Lehner et al., 1987], as well as in primary cultures and tissue samples of embryonic chicken muscle [Lourim and Lin, 1989] have shown that the avian lamin A is absent or present only at low levels in early embryos and predifferentiation myoblasts, but that it increases prominently in the first 10 days of embryogenesis [Lehner et al., 1987] and during myogenesis in vitro and in vivo [Lourim and Lin, 1989]. The avian B-type lamins B1 and B2, on the other hand, are present in substantial amounts even in early embryos [Lehner et al., 1987].

Another study has characterized the lamins in the rat myoblast line, L8E63, which appears to differentiate with kinetics similar to those of L6E9 [Wedrychowski et al., 1989]. It found that the three lamins were present at similar levels in myoblasts and myotubes, with an additional lamin C' detected in myotubes. However, the earliest sampling of the L8E63 myoblasts was after 3 days in culture, at which time the L6E9 cells used in this study had already undergone differentiation-related stimulation of lamin A/C expression.

The timing of the change in lamins A/C in L6E9 is also consistent with data in the study of Lourim and Lin [1989]. They reported that the expression of lamin A preceded that of muscle genes such as the myosin heavy and light chains, tropomyosin, troponin C, and desmin. They also reported that dimethyl sulfoxide, an inhibitor of early muscle cell differentiation and fusion, reversibly prevented the appearance of lamin A, and that, during recovery from the drug, lamin A expression again preceded that of muscle-specific genes. We have similarly shown that lamins A/C were already present at high levels in L6E9 cells on day 3. At this time, the cell population was still comprised almost exclusively of mononucleated myoblasts and was expressing relatively little myosin heavy chain, as estimated by immunofluorescence labelling and gel electrophoresis.

Whether the L6E9 system is directly analogous to tissues and primary cultures is not clear, however. In undifferentiated chicken muscle precursors, the A-type lamin was essentially undetectable [Lourim and Lin, 1989]. Both mamma-

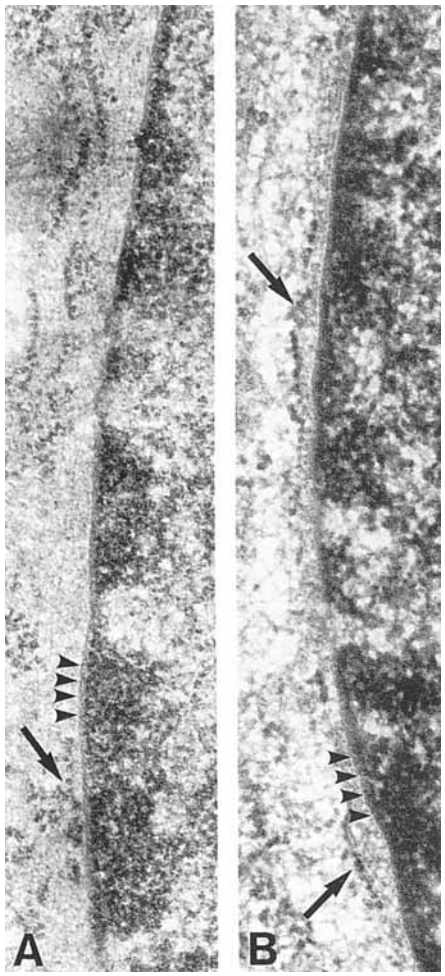

Fig. 10. Electron micrographs of the nuclear periphery of a myoblast nucleus in a day 1 sample **(A)** and of one nucleus in a multinucleated myotube in a day 7 sample **(B)**. The lamina is overall more prominent in the myotube **(B)** than in the myoblast **(A)** (*arrowheads*), staining more darkly and appearing thicker. As well, ribosomes (*arrows*) are more numerous on the cytoplasmic face of the myotube nuclear envelope, and the envelope lumen in these cells is distended and contains fibrillar material (at arrows in B). ×50,000.

lian A-type lamins were, however, clearly present at significant levels in L6E9 myoblasts, since the nuclei were stained by anti-lamin A/C, and lamins A/C were visible in immunoblots. This suggests that the L6E9 myoblasts may be at a more advanced stage of myogenic commitment than the muscle precursors investigated in other studies [Lourim and Lin, 1989].

It seems clear that lamins are imported into nuclei by signal-mediated transport as soluble proteins [Goldman et al., 1992; Nigg 1992; Sasseville and Raymond, 1995]. As well, immunolabelling studies with antibodies to the immature form of lamin A [Sasseville and Raymond, 1992] and after microinjection with biotinylated lamin A [Goldman et al., 1992] indicate that lamins are integrated into the lamina throughout interphase. Development-related changes in lamin composition at the nuclear periphery can, therefore, occur even in cells which are no longer dividing. It will be interesting to examine more closely the temporal relationship between the withdrawal of myoblasts from the proliferative cycle and changes in lamin expression, and to investigate the pathway of incorporation of the newly synthesized lamins into the myotube lamina.

Unlike the lamins, the P1 antigen seemed to disappear during differentiation when fixed and detergent-permeabilized cells were processed for immunofluorescence. This is not likely due to long-term culture, since HeLa cells maintained in culture for similar lengths of time retain normal levels of P1 labelling [Chaly, unpublished observations]. Nor is it due directly to withdrawal from the cell cycle [Nadal-Ginard, 1978], since P1 is brightly stained in resting, G_0, lymphocytes [Chaly et al., 1988]. We conclude, therefore, that the disappearance of P1 staining of nuclei in intact cells during L6E9 myogenesis in vitro is differentiation-specific. That P1 labelling is bright throughout early embryogenesis in mice [Schatten et al., 1985] suggests that the reduction observed in the L6E9 system may be specific to muscle differentiation.

The timing of the disappearance of P1 labelling indicates that it may be related to myogenic events occurring after withdrawal from the cell cycle, as L6E9 cells are undergoing fusion. Although cell counts showed that some nuclei were unlabelled or faintly labelled on day 3, a large reduction in P1 labelling was not observed until day 5 of differentiation. By day 3, there was little fusion or myosin heavy chain expression but both fusion and anti-myosin staining intensity increased greatly between days 3 and 5. In contrast, the increase in expression of lamins A/C had already reached near-maximum levels by day 3. The loss of P1 labelling would therefore follow L6E9 lamin restructuring, which, in chicken, apparently takes place at about the time of commitment [Lourim and Lin, 1989].

Fading of P1 labelling during myogenesis appears to occur as the result of progressive masking of the epitope. In general, P1 has been readily detected by immunofluorescence in all cell types examined so far [Chaly et al., 1984, 1986, 1988,

1989; Schatten et al., 1985], with the exception of mouse sperm [Schatten et al., 1985]. This includes cells that reportedly acquire lamins A/C at the nuclear periphery only during development, such as the unstimulated and lectin-stimulated lymphocyte [Chaly et al., 1988; Guilly et al., 1987] and the mouse embryo [Schatten et al., 1985; Stewart and Burke, 1987], as well as cell types that constitutively express both A- and B-type lamins, such as HeLa, Chinese Hamster Ovary and mouse 3T3 cells [Chaly et al., 1986]. It would appear, therefore, that the lamins are unlikely to be directly implicated in the fading of P1 labelling.

The fading does not appear to be due to large scale degradation of the antigen during myogenesis, nor to a differentiation-related modification in the antigen epitope, since P1 was visualized in isolated myotube nuclei and in myotube nuclear matrices. The apparent disappearance of the antigen in myotube cultures fixed before extraction would seem, therefore, to be due to progressively reduced accessibility of the epitope. Furthermore, the observation that P1 was revealed in isolated myotube nuclei suggests that a factor recruited to the nuclear periphery during development is responsible for concealing the P1 epitope in differentiated cells, and that this factor is extracted during nuclear isolation. Again, since lamins are highly insoluble and are not extracted by these protocols, they are unlikely to be the proteins responsible.

These results indicate that the change observed in the ultrastructure of the myotube nuclear lamina reflects not only the increase in lamin quantity, but also an increase in other non-lamin lamina proteins, such as the unidentified soluble factor above. At least one other non-lamin peripheral nuclear protein, statin, also exhibits increased expression during myogenesis [Connolly et al., 1988]. Statin is a 57 kD protein localized at the nuclear periphery in the region of the lamina [Wang, 1985a, 1985b]. In general, statin is absent in proliferating cell populations and becomes reversibly expressed as cells senesce or withdraw from the cell cycle [e.g., Fedoroff et al., 1990; Wang, 1985a, 1985b]. Immunolabelling of primary cultures of myogenic cells has shown that statin is absent from newborn rat and chicken myocytes and appears as the myocytes begin to fuse in vitro [Connolly et al., 1988]. It appears, therefore, that at least three types of proteins of the nuclear lamina region—the lamins, statin and the P1 antigen—may be undergoing differentiation-related modifications during myogenesis.

The significance of these changes to the myogenic process is still largely a matter of speculation. On the one hand, it is possible that the changes are not specific to muscle differentiation, but rather reflect lamina modifications associated with its generalized role in nuclear envelope structure. On the other hand, as noted earlier, it has been proposed that the lamina may play a role in regulating gene expression by modulating the global organization of chromatin [Lourim and Lin, 1989], and we have demonstrated macroscopic changes in the genome architecture of differentiating L6E9 cells [Chaly and Munro, 1996]. Direct evidence as to whether changes in lamina composition might affect gene expression by mediating genome reorganization is contradictory, however. Overexpression of lamin A in differentiating myoblasts affected expression of some muscle-specific genes, suggesting at least a limited role for lamina composition in expression of the muscle phenotype [Lourim and Lin, 1992]. On the other hand, ectopic expression of lamin A did not induce differentiation of P19 embryocarcinoma cells [Peter and Nigg, 1991]. However, the data show that, during L6E9 differentiation at least, modifications to the lamina involve more than just the lamins, and raise the possibility that altering lamin composition alone may be insufficient. Coordinate changes in both lamin and non-lamin proteins may be necessary to reorganize chromatin and, consequently, remodel gene expression for differentiation.

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