Expression of wild-type and nuclear localization-deficient human lamin A in chick myogenic cells

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Summary

Previous analysis of chick embryonic muscle (CEM) differentiation in vivo and in ovo demonstrated that lamin A accumulation to steady-state levels preceded the accumulation of muscle-specific proteins. These observations have suggested the appearance of A-type lamins may be important for differentiation. To test this hypothesis, we have temporally and quantitatively altered the expression of A-type lamins in CEM cells by transient transfection of wild-type (wt; pHLA) or nuclear localization-deficient (NLd; pHLA-del) human lamin A expression plasmids. Transfected CEM cells synthesized the wt and NLd human lamin As to high levels, both of which were resistant to high-salt extraction. The wt human lamin A localized to the nucleus, whereas the NLd protein showed cytoplasmic staining patterns, as well as time-dependent nuclear localization. The presence of endogenous chicken lamins A and B2 in NLd human lamin A cytoplasmic structures suggested the interspecies lamin copolymerization. Thus, this approach may provide a possible method for analysis of lamin-lamin or lamin-lamina component interactions in vivo. With regard to muscle differentiation, CEM cells transfected with either pHLA or pHLA-del demonstrated moderate and transient increased levels of the muscle-specific myosin heavy chain and creatine kinase activity. These increases appeared temporally and quantitatively to reflect the transient accumulation of the human lamin As. In contrast, β-tubulin and skeletal tropomyosin protein accumulations appeared unaffected. On the basis of these results, we suggest that nuclear lamina content and structure may play a limited, permissive and indirect role in the temporally regulated expression of the myogenic phenotype.

Key words: nuclear lamina, myogenesis, differentiation.

Introduction

The nuclear lamina is a proteinaceous network of fibers tightly associated with the inner membrane of the nuclear envelope and interposed between the inner membrane and chromatin (Fawcett, 1966; Aebi et al., 1986; Paddy et al., 1990). The major protein components of the nuclear lamina, the nuclear lamins, are members of the intermediate filament gene superfamily (McKeon et al., 1986; reviewed by Franke, 1987; Stewart, 1990), and are observed in a variety of organisms (reviewed by Krohne and Benavente, 1986; Nigg, 1989). Lamin isoforms are generally categorized as either A-types or B-types. A-type lamins have near neutral isoelectric points (pI values), and are cytoplasmically dispersed and soluble in mitotic cells, whereas B-type lamins have a slightly acidic pI, and remain associated with the vesicle remnants of mitotic cell nuclear membranes (Gerace and Blobel, 1980; Lehner et al., 1986a,b; Krohne et al., 1987; Kaufmann, 1989; Hoger et al., 1990). Within the interphase nuclear lamina, the molecular level at which the A-type lamins associate with the B-type lamins is unknown.

Developmentally regulated expression of lamin isoforms was first reported in Xenopus (Krohne et al., 1981; Stick and Schwarz, 1983; Krohne et al., 1984; Benavente et al., 1985; Stick and Hausen, 1985; reviewed by Krohne and Benavente, 1986), and subsequently, during in ovo development of Drosophila (Smith et al., 1987; Gruenbaum et al., 1988; Smith and Fisher, 1989), avian (Lehner et al., 1987; Lourim and Lin, 1989), and murine species (Stewart and Burke, 1987; Lebel et al., 1987; Houlston et al., 1988; Worman et al., 1988; Rober et al., 1989). In a cell type-specific manner during chick embryonic development, the relative amounts of lamin B2 remained fairly constant, lamin B1 decreased, and lamin A increased (Lehner et al., 1987). Furthermore, a very early event in human promyelocytic leukemia cell differentiation is a change in the localization of lamin A within the nucleus (Collard et al., 1992). In relation to muscle differentiation, the increase in lamin A expression temporally preceded the high-level expression...
of muscle-specific parameters (Lourim and Lin, 1989, 1990). Moreover, using myogenic inhibitory agents we have been unable temporally to dissociate myogenic differentiation from lamin A accumulation (Lourim and Lin, 1990, 1991). Taken together, these results may indicate a function for A-type lamin content or organization in processes which accompany or regulate cell differentiation.

In the present study we have transiently transfected chick myoblasts with either a wild-type (wt; pHLA), or a nuclear localization-deficient (NL4; pHLA-del Ser 407) human lamin A expression plasmid (Loewinger and McKeon, 1988). We have presented the hypothesis that in undifferentiated myoblasts, the premature expression of human wt lamin A would result in the precocious expression of lamin A function. In contrast, the cytoplasmic accumulation of the human NL4 lamin A would serve as a sink for the endogenous lamin A, thereby depleting adjacent nuclei of newly synthesized lamin A peptides and, consequently, lamin A function or activity. We report the wt and NL4 human lamin A proteins accumulated to high levels in transfected CEM cells, both of which were resistant to high-salt extraction during subcellular fractionation. A majority of the wt human lamin A localized to the nucleus and was biochemically similar to the lamin A of HeLa cells. The NL4 human lamin A was predominately cytoplasmic at early times post-transfection (pt), but increased in nuclear localization with time. Interestingly, the cytoplasmic human NL4 lamin A structures also contained the endogenous chicken lamins A and B2. These results demonstrate that the human wt and NL4 lamin As are able to interact with the endogenous chicken lamins. Surprisingly, forced-expression of either wt or NL4 human lamin As in CEM resulted in a significant increase in the accumulation of a subset of muscle-specific proteins. We discuss mechanisms consistent with this data by which alteration of the nuclear lamina content or organization could be involved in the temporal regulation of aspects of myogenic differentiation.

**Materials and methods**

**Cell culture and DNA transfections**

Chick embryo myogenic cells (CEM) were prepared from leg muscles of day 10 embryos as described previously (Lourim and Lin, 1989). For each transfection experiment duplicate CEM cultures were utilized. Chick embryo fibroblast (CEF) and human HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal calf serum (FCS).

For DNA transfection, cells were refed at 12 hours post-plating with CEM conditioned complete medium (DMEM, 15% horse serum, 2% chick embryo extract) to which 5% FCS and 5% tryptophane phosphate had been added. The cells were transfected with various plasmids by calcium phosphate coprecipitation three hours following refeeding (Loewinger and McKeon, 1988). Briefly, identical amounts of control, pHLA, and pHLA-del plasmids were dissolved in sterile water before addition of CaCl2 to a concentration of 250 mM in a total volume of 125 µl. The amount of plasmid used for independent experiments ranged from one to five µg of plasmid DNA. The DNA/Ca2+ mixture was then added dropwise to 125 µl of 2×HEPES buffered saline (2×=280 mM NaCl, 49 mM HEPES, 1.48 mM Na2HPO4, pH 7.05), while gently flicking the HEPES solution. The mixture stood for 10-20 minutes at room temperature before being added dropwise directly into the culture medium. After 5 to 7 hours, the cells were washed and refed with fresh complete medium. The time of refeeding after transfection was designated as t=0 post-transfection (pt).

**Plasmids**

The human lamin A plasmids, pHLA and pHLA-del Ser 407, which utilize a SV40 promoter for expression, were a kind gift from Dr. Frank McKeon (Harvard Medical School; Loewinger and McKeon, 1988). A control plasmid, pECD, was constructed by excising the human lamin A cDNA from pHLA at the XbaI and EcoRI sites, filling in the overhangs with Klenow fragment, then performing blunt-ended ligation. To control for nonspecific promoter effects, a second set of eukaryotic expression plasmids, pCHLA and pCHLA-del 407, were constructed by inserting the human lamin A cDNA into the BglII/XbaI sites of pCB6, an expression vector utilizing a CMV immediate early gene promoter, generously provided by Dr. Mark Stinski (University of Iowa). CEM cells transfected with either set of expression plasmids produced human lamin A peptides which displayed identical biochemical characteristics and, relative to the endogenous lamin A, were expressed at high levels. Plasmids for transfection experiments were purified using Qiagen columns as recommended by the manufacturer (Qiagen Inc., Chatsworth, CA).

**Antibodies**

The anti-skeletal muscle tropomyosin antibodies, CH1 and CL2, and the anti-skeletal muscle myosin heavy chain antibody, MF20, have been described previously (Lourim and Lin, 1989). The broad species-specific anti-A-type lamin antibody C23 was prepared and characterized as described (Lin et al., 1988). The human-specific anti-A-type lamin monoclonal antibody 1E4 was a generous gift from Dr. Frank McKeon (Harvard Medical School; Loewinger and McKeon, 1988). The monoclonal anti-chicken lamin B2 antibody L3-5D10 was a generous gift from Dr. Reimer Stick (University of Munich, Germany; Lehner et al., 1987). The monoclonal anti-β-tubulin antibody DM1B was a generous gift from Dr. Stephen Blose (Cold Spring Harbor Laboratory; Blose et al., 1984). The chicken-specific anti-A-type lamin antibodies O1, P2 and P5 were a generous gifts from Dr. Erich Nigg (Swiss Institute for Experimental Cancer Research; Lausanne, Switzerland).

**Fluorescence microscopy**

For direct immunofluorescence, purified monoclonal antibodies were conjugated to FITC by the method of Sanger (1975). For double-labeling using monoclonal antibodies of the same IgG subclass (1E4, L3-5D10 and C23), coverslips were incubated with unconjugated primary antibodies followed by goat anti-mouse (GAM) secondary antibodies conjugated with tetramethyl rhodamine isothiocyanate (Rho). Unreacted antibody binding sites on the bound GAM secondary antibodies were blocked with an excess of normal mouse serum before incubating the coverslips with FITC-conjugated primary antibody. For double-labeling using monoclonal antibodies of different subclasses, IgG class primary antibodies were visualized by GAM-FITC γ-chain-specific antibody (Sigma) and the IgM class primary antibodies (CL2) were visualized by GAM-Rho μ-chain-specific antibody (Cappel).

Chromatin was visualized with the fluorochrome DAPI (4′, 6′-diamidino-2-phenylindole; 0.5 µg/ml; Sigma), specific for A+T-rich DNA regions, or 7-AAD (7-amino-actinomycin D, 10 µM; Molecular Probes, Inc.), specific for G+C-rich DNA regions. 7-AAD was viewed with a standard Rho fluorescence filter set (excitation filter BP540 and barrier filter LP590), whereas DAPI was viewed with excitation filter G365 and barrier filter LP420. To view antibody-conjugated fluorochromes along with DAPI, either
a ×40 objective (Neofluar, PH 2), or a ×100 objective (Neofluar, PH 3) was used. To view fluorochromes conjugated to antibodies along with 7-AAD, a ×63 (Planapo PH 3) oil objective was used. Cells were photographed using a Zeiss epifluorescence photomicroscope III as described previously (Blose, 1979).

**Gel electrophoresis and immunoblotting**

Total cellular proteins from CEM cultures were analyzed at various times after transfection. For gel and immunoblot analysis, samples were prepared as described previously (Lourim and Lin, 1989). SDS-PAGE, IEF gel electrophoresis, protein immunoblotting, autoradiography and fluorography were performed as described (Lourim and Lin, 1989). Nonequilibrium pH gel electrophoresis (NEPHGE) was based on a modification of the method of O’Farrell et al. (1977), as described by Lehner et al. (1986b) and included pH 3.5-10 Ampholines (4%) and pH 6-8 Ampholines (1%) in the first dimension gel mixture.

**Subcellular fractionation**

CEM cells for subcellular fractionation were harvested 48 hours post-transfection. All procedures were carried out at 4°C. Briefly, CEM monolayers were washed twice with phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8.0 mM Na2HPO4, pH 7.3) containing 5 mM MgCl2 and 0.2 mM EGTA, and once with a low-salt buffer (50 mM Tris, 1 mM PMSF, 1 mM MgCl2, 10 µg/ml leupeptin, pH 7.5). Cells were then washed with hypotonic lysis buffer (HLB: 10 mM Tris-HCl, 1 mM PMSF, 1 mM MgCl2 and 10 µg/ml leupeptin, pH 7.5), scrape harvested into a chilled Dounce homogenizer, 1 ml of cold HLB was added, and the mixture incubated on ice for 15 minutes. Cell lysis was monitored by phase-contrast microscopy. The cells were disrupted by 10-15 strokes with a B-pestle before adding 1/10 volume of 50% glycerol in 10xNIM (1xNIM: 10 mM Tris-HCl, 83 mM KCl, 17 mM NaCl, 1 mM MgCl2, 1 mM PMSF, 1 mM DTT, 10 µg/ml leupeptin, pH 7.5), and mixed by 5-10 strokes with the B-pestle. The cell homogenates were spread over 0.5 ml of 10% glycerol in NIM, and centrifuged for 10 minutes at 4,000 revs/min in an Eppendorf microfuge. The supernatants (S-1) were removed and the pellets washed once with 10% glycerol in NIM, then twice in NIM alone. The pellets were resuspended in 400 µl of NIM containing 0.1% Triton X-100, 0.6 M KCl, and incubated on ice for 10 minutes before centrifugation at 14,000 revs/min for 10 minutes. The KCl extract supernatants (S-2) were removed, and the pellets washed once with NIM containing 0.1% Triton X-100, then once with NIM. The S-1 and S-2 supernatants were combined with their respective washes, PMSF and SDS were added to 1 mM and 0.1%, respectively, and the supernatants dialyzed overnight against two to three changes of 0.1% SDS, 0.1 mM PMSF, 5 mM Tris-HCl (pH 7.5). Equal percentages of the fractions were analyzed by SDS-PAGE and immunoblotting.

**Muscle creatine kinase enzymatic assay**

Skeletal muscle creatine kinase (MCK) activity was assayed essentially as described by Dawson and Eppenberger (1970), using coupling reactions and spectrophotometrically monitoring the change in absorbance at 340 nm for the reduction of NADP. All procedures were carried out at 4°C. Briefly, CEM cells were washed three times with PBS-Mg2+-EGTA, two times with sonication buffer (PBS containing 1 mM EDTA and 0.1 mM 2-mercaptoethanol), scrape harvested into 1.7 ml centrifuge tubes, and the sample volume was adjusted to approximately 100 µl/35 mm dish with sonication buffer. Cells were lysed by sonication for 30 seconds at a setting of 5 with a 50% duty cycle using a Vibra Cell Sonicator (Sonic and Materials, Inc.). Cell homogenates were then centrifuged (Eppendorf Microfuge) for 10 minutes, and the supernatant stored at ~20°C. Protein concentration was measured as described by Lowry et al. (1951). Data were plotted, and statistical significance was evaluated with Student’s t-tests.

**Results**

**Expression and localization of wt and NLΔ human lamin A in CEM cells**

Transfection of the plasmids pHLA and pHLA-del Ser 407 into CEM cells resulted in the expression and accumulation of human wt and NLΔ lamin A proteins, respectively. As is demonstrated by Western blotting of cell homogenates in Fig. 1, the antigens recognized by the broad species-specific antibody C23 (Fig. 1B) included lamin A and C of HeLa extracts (lane 1), lamin A of CEM cells (lanes 2, 3, 4), the human wt lamin A expressed in pHLA (lane 3), and the human NLΔ lamin A expressed in pHLA-del (lane 4) transfected CEM cells. The human lamin A proteins expressed in CEM cells were of the expected molecular masses, which for the wild-type lamin A (lane 3) was of the same mobility as authentic lamin A of human HeLa.

![Fig. 1. Western blotting of CEM cells transfected with pHLA and pHLA-del shows the high level expression of human lamin As. Total homogenates of HeLa cells (lanes 1), control CEM cells (lanes 2), pHLA transfected CEM (lanes 3), or pHLA-del transfected CEM (lanes 4), were prepared and analyzed by Western blotting. Transfected CEM homogenates were harvested at 40 hours pt. After gel electrophoresis, proteins were either stained with Coomassie Blue (A), or processed for immunoblotting with the antibodies: C23 for A-type lamins (B); 1E4 for human A-type lamins (C); or L3-5D10, for chicken lamin B2 (D). Bound primary antibodies were visualized with 125I-labeled goat anti-mouse IgG antibodies. std, the migration positions of molecular mass standards. k, kilodaltons.](image-url)
cells (lane 1). The human-specific anti-A-type lamin antibody 1E4 (Fig. 1C), recognized the HeLa A-type lamins (lane 1), and the human wt and NL\textsuperscript{d} lamin A proteins expressed in transfected CEM cells (lanes 3, 4). The monoclonal antibody L3-5D10 (Fig. 1D) did not react with human lamin proteins (lane 1), and did react with chicken lamin B\textsubscript{2} (lane 2). NEPHGE analysis indicated that the wt human lamin A was efficiently processed from a precursor form, and isoelectrically modified like the lamin A of HeLa cells (data not shown). In NEPHGE analysis of the NL\textsuperscript{d} human lamin A, four to five phosphorylated NL\textsuperscript{d} variants were observed, along with a single nonphosphorylated iso-variant (data not shown).

The cellular localizations of the human lamin As expressed in CEM cells were analyzed by immunofluorescence microscopy (Fig. 2). Consistent with the Western blotting data, the chicken A-type lamin and the human lamin As were recognized by the monoclonal antibody C23 (Fig. 2 C,F,I), whereas the antibody 1E4 recognized the human wt lamin A of pHLA transfected CEM cells (Fig. 2E), the human NL\textsuperscript{d} lamin A of pHLA-del transfected CEM (Fig. 2H), and failed to recognize nuclear lamins in pECD (control) transfected (Fig. 2B) and nontransfected CEM cells. In pHLA transfected CEM cells (Fig. 2, D-F), the wt human lamin A appeared predominately in nuclei, although cytoplasmic wt human lamin A fibers were occasionally observed (see inset Fig. 2 D-F). pHLA-del transfected CEM cells (Fig 2, G-I) showed diffuse, fibrillar, and phase-dense vesicle-like cytoplasmic staining for the human NL\textsuperscript{d} lamin A peptide, as well as nuclear staining.

Subcellular fractionation
To determine biochemically the subcellular localization of the human wt and NL\textsuperscript{d} lamin A expressed in CEM cells,
we fractionated 65 hour pt CEM cells (Fig. 3). Western blotting for A-type lamins showed that the endogenous mature chicken lamin A of control CEM cells (Fig. 3B, Control) was not observed in the cytoplasmic supernatant (lane 2), or in the 0.6 M KCl-extracted S2 fraction (lane 3), and remained nearly quantitative in the salt-resistant, nuclei-enriched pellet (lane 4). Under these extraction conditions, the majority of the skeletal-muscle tropomyosins (Fig. 3C) were found in the cytoplasmic supernatant (lane 2), and the remainder were found in the 0.6 M KCl extract (lane 3).

In pHLA transfected CEM cells (Fig. 3, pHLA), both the mature and the putative human lamin A precursor were observed quantitatively in the salt-resistant, nuclei-enriched pellet (lane 4). Although not quantified, the unprocessed human lamin A peptide accounted for a minor, though detectable, amount of the total human lamin A expressed in CEM cells. In pHLA-del transfected CEM cell, the majority of the human NL\textsuperscript{d} lamin A were found in the salt-resistant pellet (Fig. 3, pHLA-del lane 4). However, detectable amounts of the human NL\textsuperscript{d} lamin peptide were observed in the cytoplasmic supernatant (lane 2), and in the 0.6 M KCl extract (lane 3). A peptide migrating above the mature chicken lamin A was observed in the cytoplasmic fraction of pHLA-del transfected CEM (Fig. 3, pHLA-del lane 2, c\text{LaA}o), which was not observed in the pellet fraction (lane 4). A small amount of this peptide was observed in the control and wt transfected CEM cell cytoplasmic fractions (lanes 2). On the basis of pulse-chase experiments, this peptide appears to represent the chicken lamin A precursor (c\text{LaA}o) (data not shown; Lehner et al., 1986a). Chicken lamin B\textsubscript{2} was not observed in detectable quantities in fractions other than the salt-resistant pellet in Western blotting experiments (data not shown).

In contrast to the solubility of the chicken lamin A precursor, the insoluble nature of the mature chicken lamin A, the mature wt lamin A and its precursor, and the majority of human NL\textsuperscript{d} lamin A may indicate polymerization in transfected CEM cells.

**Cytoplasmic lamin structures contain endogenous lamin A and B\textsubscript{2}**

Both pHLA (only occasionally) and pHLA-del transfected cells contained cytoplasmic human lamin A structures. To determine if the endogenous chicken lamin A and B\textsubscript{2} were associated with these cytoplasmic structures, we utilized chicken-specific antibodies against lamin B\textsubscript{2} (L3-5D10), and against lamin A (P5) in an immunofluorescence analysis (Fig. 4). The cytoplasmic human lamin A fibers occasionally found in the pHLA transfected cells (Fig. 4B) also contained chicken lamin B\textsubscript{2} (Fig. 4C). More clearly, the cytoplasmic phase-dense bodies of pHLA-del transfected CEM cells (Fig. 4D-I) contained both chicken lamin B\textsubscript{2} (Fig. 4F) and chicken lamin A (Fig. 4I) in addition to the
human NL<sup>d</sup> lamin A (Fig. 4E, H). Cytoplasmic lamin vesicle-like structures often displayed a rim-like lamin staining pattern (arrowheads in Fig. 4D-I), and did not contain the cytoskeletal intermediate filament protein desmin, or skeletal muscle tropomyosin isoforms in detectable quantities (data not shown). Furthermore, the nuclear staining for chicken lamin A and B<sub>2</sub> appeared relatively weak in cells positive for cytoplasmic human lamin As (Fig. 4C, F, I). The decrease in the nuclear staining for chicken lamin As and B<sub>2</sub> was likely due to the cytoplasmic human NL<sup>d</sup> lamin As acting as a cytoplasmic sink for the endogenous lamin. However, we cannot rule out the possibility that the epitopes of the anti-chicken lamin antibodies were masked in the nucleus by interaction with the exogenous human wt and NL<sup>d</sup> lamin A.

Time-dependent nuclear localization of the NL<sup>d</sup> peptide, and distribution of DNA in CEM cells expressing human lamin As

To follow the time course of the appearance in the nucleus of the NL<sup>d</sup> lamin A, and to ascertain the distribution of DNA in cells transfected with the human lamin A expression plasmids, we utilized the DNA-dependent fluorochromes DAPI or 7-AAD (7-AAD staining shown in Fig. 5). In the human wt lamin A expressing CEM, the majority of the lamin A staining appeared associated with the nuclear periphery, and the DNA often appeared indistinguishable from nontransfected CEM cells (Fig. 5, A-H). At later times pt, the syncytial CEM cells generally showed a gradient of wt human lamin A localization, with a decrease in lamin A staining with increasing distance from a central
Ectopic expression of lamin A in CEM cells

strongly staining nucleus, or small group of nuclei (Fig. 5, E-H). In a minority of wt lamin A-expressing cells, the DNA revealed by 7-AAD staining appeared condensed, and in proximity to the nuclear envelope (for example, see Fig. 5E,F). These results suggest that the exogenous human wt lamin A was capable of altering, either directly or indirectly, the distribution of chromatin in CEM nuclei.

The cytoplasmic vesicle-like structures of pHLA-del transfected CEM did not contain DNA, as shown by the lack of staining by the DNA dye 7-AAD (Fig. 5, I-P), or DAPI (data not shown). Comparison of cells stained for the human NLd peptide at 24 hours (Fig. 5, I-L), to 104 hours pt cells (Fig. 5, M-P), revealed that with increasing time following transfection, the frequency and size of the vesicle-like structures had decreased, and the relative amount of human NLd lamin A with nuclear localization had increased. Furthermore, often the entire complement of nuclei within syncytial CEM cells stained for the NLd peptide (Fig. 5, M-P). Whether the NLd lamin with nuclear localization, as observed at later time points, was derived from a redistribution of the NLd lamin of the cytoplasmic NLd structures or from newly synthesized lamins, is unknown.

Time-dependent accumulations of human lamin As and cytoskeletal proteins in transfected CEM

To determine the time-dependent accumulation of the human wt and NLd lamin As during CEM differentiation, we performed Western blot analysis on CEM homogenates (Fig. 6) prepared at 24 hours (lanes 1-3), 72 hours (lanes 4-6), and 104 hours (lanes 7-9) pt. As assayed by the broad-species-specific antibody C23 (Fig. 6B), and the human-specific antibody 1E4 (Fig. 6C), the wild-type human lamin A accumulated to high levels by 24 hours pt (lanes 2 in Fig 6B and C), which, however, steadily (and reproducibly) decreased with time, such that relative to 24 hour pt CEM cells, progressively smaller amounts were present by 72 hours (lanes 5 in Fig. 6B and C), and 104 hours (lanes 8

![Fig. 5. Distribution of DNA in pHLA and pHLA-del transfected CEM cells. CEM cells transfected with pHLA (A-H) or pHLA-del 407 (I-P), were stained at 24 hours pt (A-D, I-L), or at 104 hours pt (E-H, M-P). Cells were double stained for human lamin A peptides with the antibody 1E4 (A, C, E, G, I, K, M, O) and the DNA dye, 7-AAD (B, D, F, H, J, L, N, P). A decreasing gradient of nuclear wt human lamin A staining was observed in some syncytial cells (see the strongly staining central nuclei indicated by the arrow in E, F). In 24 hour pHLA-del post-transfected CEM cells, the cytoplasmic human lamin A vesicle-like structures (open arrows in I-L) did not colocalize with the 7-AAD staining structures (filled arrows in I-L). Bar, 10 µm.](image-url)
in Fig. 6B and C). The accumulation of NL<sup>d</sup> peptide increased from 24 hours (lanes 3 in Fig. 6B and C) to 72 hours (lanes 6 in Fig. 6B and C). But as with the human wt lamin A, the NL<sup>d</sup> peptide had decreased in abundance by 104 hours pt (lanes 9 in Fig. 6B and C). The endogenous chicken lamin A of control transfected CEM, relative to histone content, was in low abundance at 24 hours pt (approximately 48 hours post-plating) (Fig. 6B, lane 1), and increased in amount by 72 hours (Fig 6B, lane 4), when it appeared to have reached a steady-state level (compare Fig 6B; lane 4 to lane 7). These results are consistent with our previous observation that lamin A accumulation increases during CEM differentiation (Lourim and Lin, 1989). In both wt and NL<sup>d</sup> transfected CEM cells the endogenous lamin A accumulated to levels similar to control CEM, suggesting that exogenous lamin As did not affect the expression of the endogenous lamin A.

To determine the effects the exogenous human wt and NL<sup>d</sup> lamin As on the synthesis and accumulation of muscle-specific and nonmuscle-specific cytoskeletal proteins, the CEM homogenates were Western blotted with antibodies against various cytoskeletal proteins. Based on the Coomassie Blue stained gel (Fig. 6A), no differences were observed in the accumulations of the bulk of proteins from control and transfected CEM samples from the same time points. Immunoblotting for β-tubulin (Fig. 6D) and skeletal muscle tropomyosins (Fig. 6F,G) indicated that their accumulations appeared unaltered by the presence of exogenous human lamin A in 24, 72 and 104 hour pt cells. In contrast, the skeletal muscle isoform of myosin heavy chain (MHC) (Fig. 6E) had accumulated to higher amounts at 24 hours pt in both pHLA and pHLA-del transfected CEM cells when compared with control transfected CEM cells. Differences in MHC accumulations between control (lane 4), and pHLA transfected (lane 5) cells were negligible by 72 hours pt. However, MHC accumulation in the 72 hour pHLA-del transfected cells (lane 6) remained higher than control cells. At 104 hours pt (Fig. 6E, lanes 7-9), no differences were apparent in the amounts of MHC that had accumulated in control, pHLA or pHLA-del transfected CEM cells. Comparison of the accumulation patterns of human wt and NL<sup>d</sup> lamin A with MHC indicate that the increase in muscle-specific MHC roughly parallel the accumulation of the human wt and NL<sup>d</sup> lamin A proteins.

**Muscle creatine kinase activity in CEM expressing human wt and NL<sup>d</sup> lamin As**

Utilizing an enzymatic assay for muscle creatine kinase (MCK) activity, significant differences in MCK levels were also observed between control, pHLA and pHLA-del transfected CEM cell homogenates at 79 hour pt (Fig. 7, 79 hours). In seven independent experiments, the MCK activity measured from CEM cells transfected with pHLA and pHLA-del was consistently higher than that measured from control transfected CEM cells. The mean MCK activity of pHLA transfected cells (1.3 × 10<sup>3</sup> ± 0.69 × 10<sup>3</sup> ΔA<sub>340</sub>/µg per min) was significantly (P<0.05) higher than control transfected CEM cells (0.67 × 10<sup>3</sup> ± 0.25 × 10<sup>3</sup> ΔA<sub>340</sub>/µg per min). The mean MCK activity measured from pHLA-del transfected CEM cells (1.09 × 10<sup>3</sup> ± 0.58 × 10<sup>3</sup> ΔA<sub>340</sub>/µg per min), although higher than control CEM
with control, pHLa or pHLa-del expression plasmids. CEM cells generated at 79 hours and 120 hours pt. The values of the 79 hours independent experimental culture sets. The values of the 120 hour pt samples are averages of 2 independent experimental culture sets. The values of the 120 hour pt samples are averages of 7 independent experimental culture sets.

values, was not significantly different in statistical analysis $(P>0.1)$.

Relative to the values of 79 hour control cells, the MCK activities measured at 120 hours pt from control, pHLa and pHLa-del transfected CEM cells had increased (Fig. 7, 120 hours). However, significant differences were no longer apparent in the MCK activities between control, pHLa and pHLa-del transfected CEM cells. In addition, we noted that the MCK activity of the pHLa and pHLa-del transfected cells at 120 hours pt had not significantly increased relative to the values measured at 79 hours for pHLa and pHLa-del transfected CEM. Like the pattern of muscle-specific MHC protein accumulation, the pattern of increased MCK activity temporally paralleled the accumulation patterns of the human wt and NL\textsuperscript{d} lamin A proteins.

**Discussion**

In this study we have demonstrated that CEM cells expressed wt or NL\textsuperscript{d} human lamin A proteins when transiently transfected with the human lamin A expression plasmids, pHLa and pHLa-del, respectively. The human lamin A accumulated to high levels relative to the endogenous nuclear lamin proteins, and decreased in amounts with increased time post-transfection. In subcellular fractionation experiments, the vast majority of both the wt and NL\textsuperscript{d} lamin proteins were resistant to high salt extraction. The wt human lamin A protein appeared to be efficiently processed, transported into the nucleus, and assembled into the CEM nuclear lamina. At early times post-transfection the NL\textsuperscript{d} lamin A localized predominately to the cytoplasm, whereas at later times post-transfection the majority of the protein localized to the nucleus. The endogenous chicken lamins colocalized with the wt and NL\textsuperscript{d} lamins whether located in the nucleus or the cytoplasm. This suggests the formation of inter-species oligomers, and a mechanism for the nuclear localization of the human NL\textsuperscript{d} lamin A. With regard to muscle differentiation, CEM cells transfected with either pHLa or pHLa-del demonstrated moderate and transient increased levels of the muscle-specific myosin heavy chain protein and creatine kinase activity, which temporally and quantitatively reflected the transient accumulation of the human lamin As. In contrast, the expressions of β-tubulin and the skeletal muscle tropomyosins appeared refractory to the presence of the exogenous lamin As. These results suggest that the changing composition of the nuclear lamina, in particular the increase in A-type lamin content, may function as a permissive component in the mechanisms which temporally regulate limited aspects of the expression of the myogenic phenotype.

**Interactions and assembly of human wt and NL\textsuperscript{d} lamin A with the endogenous chicken lamins**

We have noted a number of interesting features of A-type lamin biochemistry in these experiments. Previous analysis of the lamin nuclear localization signal (NLS) had suggested that nuclear localization of NL\textsuperscript{d} human lamin A (expressed from the same construct used in this study) was due to heterodimer formation with endogenous lamin peptides (Leowinger and McKeon, 1988). In our experiments we have made several observations consistent with the oligomerization of the endogenous lamins with the exogenous human lamins and, therefore, a mechanism for NL\textsuperscript{d} lamin A nuclear transport. First, the demonstration in vivo that the endogenous lamins A and B\textsubscript{2} localized to the cytoplasmic human NL\textsuperscript{d} lamin A structures; second, in subcellularly fractionated pHLa-del transfected cells, the increase in the relative abundance in the soluble fractions of the chicken lamin A precursor with the NL\textsuperscript{d} lamin A peptide; and third, the time-dependent nature of NL\textsuperscript{d} peptide nuclear localization, which temporally paralleled the muscle differentiation-dependent expression pattern of the endogenous A-type lamins.

From these experiments, the question arises of whether the interspecies polymers responsible for NL\textsuperscript{d} nuclear transport were in the form of A-type homo-oligomers or A-B hetero-oligomers. We have performed immunoprecipitation experiments using species- and isoform-specific antibodies and observed that the endogenous chicken lamin A, not B-type lamins, were coimmunoprecipitated with human NL\textsuperscript{d} lamin A (Lourim and Lin, unpublished). This suggests that nuclear transport of the human NL\textsuperscript{d} lamin A may be due to oligomerization with A-type chicken lamins. Exclusive formation of A-type lamin homopolymers would be consistent with recent reports of the unique assembly properties of lamin A, lamin C and B-type lamins in embryonal carcinoma cells (Horton et al., 1992) and nuclear envelope localizations in predifferentiation human promyelocytic leukemia cells (Collard et al., 1992). Using the methodologies outlined in these experiments in conjunction with immunological and TEM methods, the lamin isoforms responsible for nuclear localization of the NL\textsuperscript{d} lamin A and,
consequently, the higher-order structure of the interphase lamina may be further defined. In addition, immunological analysis of the NL\textsuperscript{d} lamin cytoplasmic structures may lead to identification of lamina-dependent nuclear envelope protein interactions.

The effect of exogenous lamin As on muscle protein accumulation

Myogenic differentiation is suggested to be regulated by the activity of a group of muscle-specific trans-acting factors, the MyoD family of proteins (for reviews see: Olson, 1990; Weintraub et al., 1991). As MyoD family proteins have been observed in replicating, undifferentiation myogenic cells, they are suggested to be negatively trans-regulated (Benezra et al., 1990; Thayer and Weintraub, 1990). However, the expression of muscle-specific reporter constructs in undifferentiated myoblasts (Tsika et al., 1990), or extrachromosomal muscle-specific genes (Seiler-Tuyns et al., 1984; Minty et al., 1986), has suggested that muscle-specific trans-acting factors are functional in myoblasts. Moreover, the lack of expression from the corresponding endogenous genes has implied that muscle-specific gene expression may involve a chromatin-mediated component in a two-level regulatory mechanism (Minty et al., 1986). In this regard, it is interesting that a number of investigators have demonstrated that lamins bind to DNA, poly nucleosomes or chromatin in vitro (Lebkowski and Laemmli, 1982; Yuan et al., 1991; Hakes and Berezney, 1991; Glass and Gerace, 1990; Burke, 1990; Hoger et al., 1991). Possible in vitro functions of lamin proteins have been implied by an inhibition of chromatin decondensation following microinjection of lamin antibodies into mitotic cells (Benavente and Krohne, 1986). In addition, in vitro analysis has suggested that lamin peptides may be indirectly involved in a wide array of nuclear events (Burke and Gerace, 1986; Newport, 1987; Sheehan et al., 1988; Blow and Sleeman, 1990; Meier et al., 1991), including replication (Newport et al., 1990; Meier et al., 1991).

In designing these experiments, we made the assumption that nuclear localization of A-type lamins would be required if the differential expression of lamin A exerted its effects on gene expression levels via regulating chromatin topology. Consequently, the ability of both the nuclear human wt and cytoplasmic NL\textsuperscript{d} lamin A to increase the accumulation of a subset of muscle-specific proteins to approximately equal degrees presents a paradox. Possibly, the quantity of the NL\textsuperscript{d} lamin A transported into the nucleus compensated for the amount of endogenous A-type lamins cytoplasmically trapped. However, this explanation does not account for the increase in muscle-specific protein accumulation at early times post-transfection, the time when the NL\textsuperscript{d} peptide was predominately cytoplasmic, and the nuclei were apparently depleted of the endogenous lamins.

A preferred interpretation is as follows. Predifferentiated myogenic cells have been demonstrated to possess the molecular machinery (e.g. trans-acting factors) necessary for the expression muscle-specific genes. However, muscle-specific gene expression in these cells is presumed to be suppressed by the presence of an inhibitory activity (Mueller and Wold, 1989; Benezra et al., 1990; Peterson et al., 1990; Thayer and Weintraub, 1990; Lee et al., 1991). We suggest that as myogenic cells undergo differentiation, the increase in the nuclear lamina lamin A content may result in an inactivation of the putative inhibitory activity. The release of myoblasts from this inhibition then permits high level expression from muscle-specific genes. According to this hypothesis, the forced expression and precocious nuclear accumulation of wt human lamin A would sequester or inactivate the presumed inhibitory activity, resulting in the premature expression of muscle-specific genes. The cytoplasmic NL\textsuperscript{d} human lamin A, in contrast, produced the observed effect by depleting nuclei of the inhibitory activity, possibly via the endogenous lamins in the cytoplasm. Thus, the human NL\textsuperscript{d} lamin A cytoplasmic structures would serve as a cytoplasmic sink for the inhibitory activity, as well as for the endogenous lamins. This implies the inhibitory activity is associated with the nuclear lamina, lamina-associated protein complexes (for examples see, McKeon et al., 1984; Powell and Burke, 1990; Seydel and Gerace, 1991; Bailar et al., 1991; reviewed by Gerace and Burke, 1988), or possibly, B-type lamins themselves.

As myogenic trans-acting factors appear present and functional in predifferentiation myoblasts when lamin A content is low, the increase in A-type lamin protein accumulation may be necessary, but by itself is not sufficient, for the differentiation-dependent high level muscle-specific gene transcription or post-transcriptional processing events. Therefore, like the apparent indirect role of the nuclear lamins in DNA replication (Newport et al., 1990; Meier et al., 1991; Mills et al., 1989; reviewed by Jackson, 1990), A-type lamins would not be directly responsible for transcriptional or post-transcriptional regulation, but would rather serve as a permissive component in a hierarchy of regulatory mechanisms. If a multiple-level regulatory model of gene expression is applied to the differentiation of embryonal carcinoma cells, the lack of effect on differentiation of p19 cells by ectopic expression of lamin A (Peter and Nigg, 1991) can be attributed to defects in the expression of necessary trans-acting factor(s).

The studies reported here have indicated that the expression patterns of a subset of muscle-specific proteins were quantitatively and temporally affected by the ectopic expression of exogenous lamin A. The lack of more pronounced effects produced by the exogenous A-type lamins may be attributed to the differential expression of the endogenous A-type lamin during differentiation of the primary myogenic cells. Consistent with this interpretation, it was at early times post-transfection, when the accumulation of the endogenous lamins was low, that the effects of the exogenous lamins were most observable. In the future, a more definitive answer to the question of the function of the increase in A-type lamins during differentiation may be derived from experiments which focus on the disruption of A-type lamin expression, possibly using lamin A antisense constructs, or “gene disruption” techniques.

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Ectopic expression of lamin A in CEM cells

References


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