

Expression of Nuclear Lamin A and Muscle-specific Proteins in Differentiating Muscle Cells In Ovo and In Vitro

David Lourim and Jim Jung-Ching Lin

Department of Biology, University of Iowa, Iowa City, Iowa 52242

Abstract. Primary cultures and tissue samples of chicken embryonic muscle were immunologically probed for the expression of muscle-specific proteins, such as myosin heavy chain and the tropomyosins, as well as for the nuclear lamina protein, lamin A. As determined by quantitative immunoblotting, the expression of lamin A and the muscle-specific proteins were at low levels or absent in predifferentiation myoblasts both in vitro and in ovo. During differentiation, an increase of lamin A expression preceded the induction to high levels of expression of muscle-specific proteins. Immunofluorescence staining of chicken em-

bryonic muscle cells in culture also indicates an accumulation of lamin A before the induction of muscle-specific proteins expression. Furthermore, the accumulation of lamin A reached a plateau before the muscle-specific proteins during muscle development. In two dimensional NEPHGE gel analysis of immunoprecipitated lamin A, no detectable change in the ratio of the acidic/basic isoelectric variants of lamin A was observed during myogenesis. A potential role for lamin A in the mechanisms which underlie the differential and coordinate expression of muscle-specific genes is proposed.

THE transition of embryonic myoblasts to multinucleated myotubes is marked by the withdrawal of myoblasts from the cell cycle, and is accompanied by considerable changes in the expression of muscle-specific contractile proteins (Nadal-Ginard, 1978; Coleman and Coleman, 1968). The transcriptional activations of a number of muscle-specific genes are developmentally and coordinately regulated (Paterson and Bishop, 1977; Devlin and Emerson, 1978, 1979; Affara et al., 1980; Endo and Nadal-Ginard, 1987), suggesting the existence of regulatory mechanisms common to the expression of many muscle-specific genes. At the present time, the molecular basis of these mechanisms are not fully understood. Several lines of evidence have indicated that proteins capable of inducing muscle-specific gene expression are present in replicating myoblasts (Blau et al., 1983; Seiler-Tuynes et al., 1984; Minty et al., 1986; Tapscott et al., 1988), suggesting that the expression of muscle-specific genes may be controlled by additional levels of regulation other than *trans*-acting factors. Consequently, a chromosome-mediated repression mechanism has been postulated in the coordinate regulation of muscle-specific gene expression (Minty et al., 1986; Muscat et al., 1988).

It has been proposed that the structural organization of chromatin contributes to the state of activity of eukaryotic genes. The nuclear lamins form a fibrillar meshwork at the nucleoplasmic surface of the inner nuclear membrane (Aebi et al., 1986), and it has been proposed that this peripheral structure may organize one level of chromatin by serving as attachment sites for the chromatin fibers (Lebkowski and Laemmli, 1982; Gerace et al., 1984). The nuclear lamins

are found in a wide variety of organisms (Krohne and Benavente, 1986). Three major lamins have been identified in mammals and designated as lamins A, B, and C (Gerace and Blobel, 1980). B type lamins (i.e., lamin B) are implicated in anchoring the lamina to the nuclear envelope (Burke and Gerace, 1986; Worman et al., 1988), whereas A type lamins (i.e., lamins A and C) are suggested to directly or indirectly bind chromatin, and by assembling with B type lamins (Georgatos et al., 1988), associate chromatin with the nuclear envelope (Lebkowski and Laemmli, 1982; Gerace et al., 1984; Burke and Gerace, 1986; Benavente and Krohne, 1986).

It has been demonstrated that muscle-specific gene expression is accompanied by changes in chromatin structure, as detected by the differential immunoreactivity to anti-Z-DNA antibodies (Briane et al., 1987), and DNase 1 sensitivity (Affara et al., 1980; Carmon et al., 1982). To determine if changes in the nuclear lamina are associated with the differential gene expression observed during myogenesis, we have biochemically and immunologically characterized the temporal sequence of expression of lamin A and the muscle-specific isoforms of tropomyosin (TM) and myosin heavy chain (MHC), both in ovo and in vitro. We have determined that in undifferentiated myoblasts lamin A expression was at low levels, whereas in differentiated myotubes lamin A was expressed at relatively high levels. The increase in lamin A

1. *Abbreviations used in this paper:* CEF, chick embryo fibroblast; CEM, chicken embryonic muscle; MHC, myosin heavy chain; TM, tropomyosin.

expression preceded the increase in expression of muscle-specific proteins. However, the ratio of acidic to basic isoelectric variants of lamin A did not change as chicken embryonic muscle (CEM) differentiation proceeded. These results are consistent with the suggestion that the differential expression of lamin A during the process of myogenesis may be associated with the events which regulate muscle-specific gene expression.

Materials and Methods

Cell Culture

CEM cells were isolated from leg and thigh muscle of 10-d-old embryos by a modification (Lin et al., 1985) of the procedure of Konigsberg (1979). Cells were maintained in DME containing 15% horse serum, 2% chick embryo extract, and incubated at 37°C in a humidified chamber with 5% CO₂ and 95% air. For immunofluorescence experiments, cells were grown on 12-mm round, collagen-coated glass coverslips. Chick embryo fibroblasts (CEF) were prepared as described (Lin et al., 1984), and maintained in DME containing 10% FCS.

Monoclonal Antibodies

Anti-TM monoclonal antibodies (CH1 and CH291) were prepared and characterized as previously described (Lin et al., 1985). The antibody CH1 recognizes both the α - and β -TM isoforms, whereas the antibody CH291 reacts with only the α -TM isoform in chicken skeletal muscle. Anti-lamin A antibody (C23) was prepared and characterized as described (Lin et al., 1988). Anti-MHC antibody (MF20) and anti-desmin antibody (D3) were the kind gift of D. Fischman (Cornell Medical College, New York) (Bader et al., 1982; Danto and Fischman, 1984). The rabbit anti- α actin antiserum was the generous gift of J. Bulinski (University of California, Los Angeles; also see Bulinski et al., 1983).

Immunofluorescence Microscopy

The purified monoclonal antibodies, C23 and CH291, were conjugated to lissamine rhodamine sulfonyl chloride (Rho) and FITC by the method of Sanger (1975). For double-label direct immunofluorescence, a mixture of Rho and FITC conjugated antibodies were applied to the fixed and permeabilized cells on coverslips. The stained cells were observed and photographed using a Zeiss epifluorescence photomicroscope III with either a Zeiss 16 \times or 63 \times phase lens as previously described (Blöse, 1979). Identical staining results were observed when antibodies were conjugated to either fluorochrome.

Gel Electrophoresis and Quantitative Immunoblotting

Total cellular proteins from CEM cultures and leg tissues were analyzed at various times after cell plating or days of incubation, respectively. For gel and immunoblot analysis, the samples were adjusted to equal DNA concentrations, rather than using equal protein concentrations, which would dilute out nuclear proteins. Samples whose DNA content had been equilibrated were applied to 12.5% SDS-PAGE gels for protein separation. Densitometer analysis of the Coomassie blue staining of core histone content was used only for confirmation of approximately equal DNA content per sample.

Cell cultures, after washing three times with 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, were solubilized by the addition of sample buffer containing 0.1 M DTT, 2% SDS, 80 mM Tris, pH 6.8, and 15% glycerol. The samples were then placed into a boiling water bath for 3–5 min, passed 5–10 times through a 27-gauge syringe needle, and returned to the water bath for an additional 2–3 min before centrifugation and storage of the supernatant at –20°C.

Skeletal muscle tissue samples were collected from the legs of embryos at the times indicated. After homogenizing in 10 mM Tris, pH 7.4, 25 mM KCl, 25 mM NaCl, 1.5 mM MgCl₂, 0.5 mM PMSF, 1.0 mM DTT, and 50 mg/ml Aprotinin, an equal volume of sample buffer was added, and the sample was rehomogenized. Tissue samples were then processed as for culture samples.

The DNA content of each sample was determined by a modification of the method of Burton (1968). Briefly, aliquots of culture and tissue samples

in sample buffer were diluted 1:3 in 5 mM NaOH, and adjusted to 0.1 M NaCl. Two volumes of cold 100% ethanol were added, and the mixture incubated at 4°C overnight. The precipitated DNA was washed two times with 70% ethanol and air dried. To the dried pellets, equal volumes of 5 mM NaOH and 1 N HClO₄ were added, and the solution heated to 70°C for 15 min. The solution was then processed for colorimetric analysis as described by Burton (1968).

SDS-PAGE was carried out according to Laemmli (1970) with a low concentration of bisacrylamide (12.5% acrylamide and 0.104% bisacrylamide). Isoelectric focusing gel electrophoresis was performed according to the method of O'Farrell (1975), including four to six ampholines (4%), and 3.5–10.0 ampholines (1%) in the first dimension gel mixture. NEPHGE gels were based on a modification of the method of O'Farrell et al. (1977), as described by Lehner et al. (1986).

Protein immunoblotting was performed according to Towbin et al. (1979), as modified by Lin et al. (1985). The resulting autoradiographs were quantified with a Hoefer CS300 densitometer. The relative values quantified for each time point were determined under conditions of linearity as established for each protein independently by the scanning of serially diluted sample autoradiographs. The use of different densitometer sensitivity settings allowed the relative values determined for protein accumulation to be presented on the same log scale, and do not reflect the absolute amounts of the proteins. It should be noted that the absolute values quantified for lamin A accumulation were approximately one log unit less than those for MHC. For autoradiography and fluorography of gels, a modified method of Bonner and Laskey (1974) was performed as described previously by Lin et al. (1984).

Radioactive Labeling and Immunoprecipitation

CEF and CEM culture cells were grown on 100- or 150-mm collagen-coated culture dishes as described, and labeled for 12 h with 100 μ Ci of [³⁵S]methionine (1,110 Ci/mmol) per dish in methionine-free DME containing 2.5% FCS. For immunoprecipitation experiments, the solubilized samples were diluted to a final concentration of 0.3% SDS using immunoprecipitation buffer A (1% Triton X-100, 10 mM Tris, pH 7.5, 0.1% EGTA, 150 mM NaCl). To reduce nonspecific binding, samples were preabsorbed with protein A-Sepharose beads. To immunoprecipitate lamin A, 2–5 μ l of the monoclonal antibody C23 ascites fluid was added to the preabsorbed samples, mixed, and incubated for 1 h at 4°C. Protein A-Sepharose beads were added and further incubated for 30 min. The beads containing the antibody-antigen complex were then collected by centrifugation and washed three times with buffer A plus 0.15% SDS. Proteins were eluted from the beads by the addition of sample buffer and heating in a boiling water bath for 3 min. After autoradiography, the spots identified as the lamin A basic and acidic isoelectric variants were excised from the gel, eluted with 2% SDS for 24 h, and the radioactivities of the fractions were determined using a liquid scintillation counter.

Results

Lamin A Accumulates before the Muscle-specific Proteins during Skeletal Muscle Differentiation In Vitro and In Ovo

The induction to a high level of expression of the muscle specific proteins, α -TM, MHC, as well as lamin A in CEM cultures, was analyzed at various times (12, 24, 36, 48, and 60 h, and 3, 4, 5, 6, 7, and 8 d) after plating by quantitative immunoblotting. The Coomassie blue-stained protein patterns of these samples are shown in Fig. 1 A. Immunoblot autoradiographs of these samples using monoclonal antibodies, MF20, C23, and CH291 directed against MHC, lamin A, and α -TM are shown in Fig. 1, B, C, and D, respectively.

As can be seen in Fig. 1 B, muscle-specific MHC was present in substantial amounts in refusion myoblasts at 12 h after plating (Fig. 1 B, lane 1). However, a decrease in MHC content was consistently observed in the cells cultured between 24 and 48 h. The initial decrease in MHC content may be due to a down regulation of certain MHC isoforms in culture, similar to that observed in quail myogenic cultures

CEM CULTURE

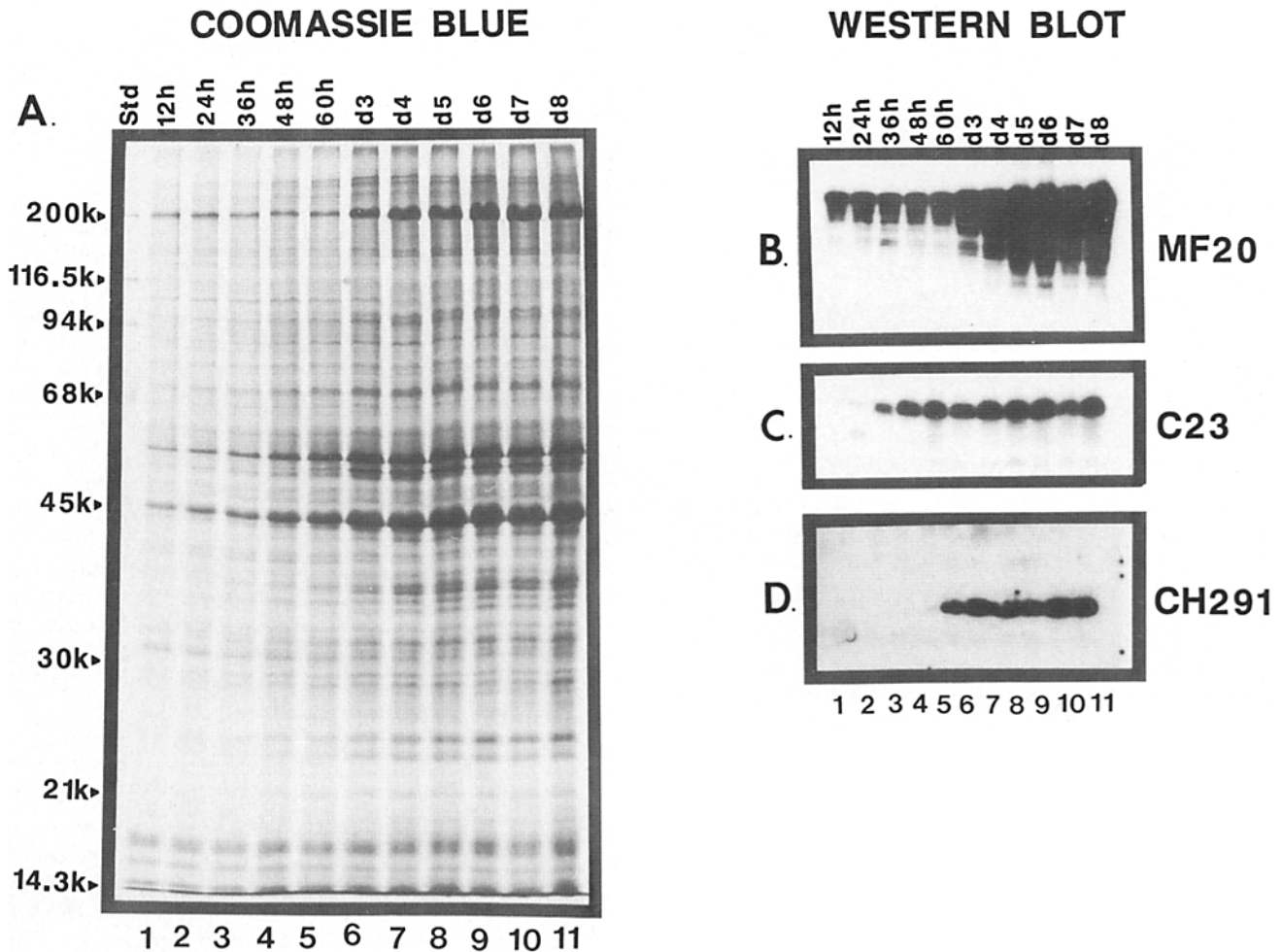


Figure 1. Expression of lamin A, myosin heavy chain, and α -TM in cultured CEM cells. After normalization for DNA content of CEM culture samples at different stages of differentiation, samples were prepared and analyzed by Western blotting. After gel electrophoresis, proteins were either stained with Coomassie blue (A) or processed for immunoblotting with the antibodies: MF20 for muscle-specific myosin heavy chain (B), C23 for lamin A (C), or CH291 for muscle specific α -TM (D). Primary antibodies were visualized with 125 I-labeled goat anti-mouse IgG antibodies. Samples were taken at (lane 1) 12 h, (lane 2) 24 h, (lane 3) 36 h, (lane 4) 48 h, (lane 5) 60 h, (lane 6) 3 d, (lane 7) 4 d, (lane 8) 5 d, (lane 9) 6 d, (lane 10) 7 d, and (lane 11) 8 d after plating. Binding of these monoclonal antibodies has been previously characterized, and in skeletal muscle, found to be confined to the areas shown. Lamin A autoradiograph exposure times were typically three to four times longer than for either TM or MHC. *Std*, molecular mass markers in kilodaltons.

when fed growth-promoting medium (Devlin et al., 1982). Nevertheless, after ~ 60 h in culture an increase in the accumulation of MHC was apparent (Fig. 1 B, lane 6). At that time, the accumulation of α -TM also became evident (Fig. 1 D, lane 6). Furthermore, the coordinate accumulation of α -actin, myosin light chains 1 and 2, and desmin was also observed after ~ 60 h in culture (data not shown). However, the appearance of lamin A can be detected at 12 and 24 h after plating and is readily apparent after 36 h in culture (Fig. 1 C, lanes 1-3). Lamin A accumulates rapidly, such that between 48 and 60 h in culture, it has approached a maximal value (Fig. 1 C, lanes 4-11).

To quantify the relative levels of muscle-specific proteins and lamin A accumulation, densitometric scans of immuno-

blots from three to five separate experiments were performed. Fig. 2 illustrates the plots of the relative values of MHC, lamin A, and α -TM accumulation as a function of time in culture. Lamin A content increased rapidly from 12 h to ~ 60 h after plating, at which time lamin A approached a steady state value. However, both MHC and α -TM started to accumulate significantly after ~ 3 d after plating, and reached their plateau after lamin A had reached a maximal level.

To determine whether the *in vitro* temporal relationship for the induction for lamin A and muscle-specific proteins is also observed *in ovo*, leg muscle samples were collected at specific times of embryogenesis, normalized for DNA content, and processed for immunoblotting with monoclonal

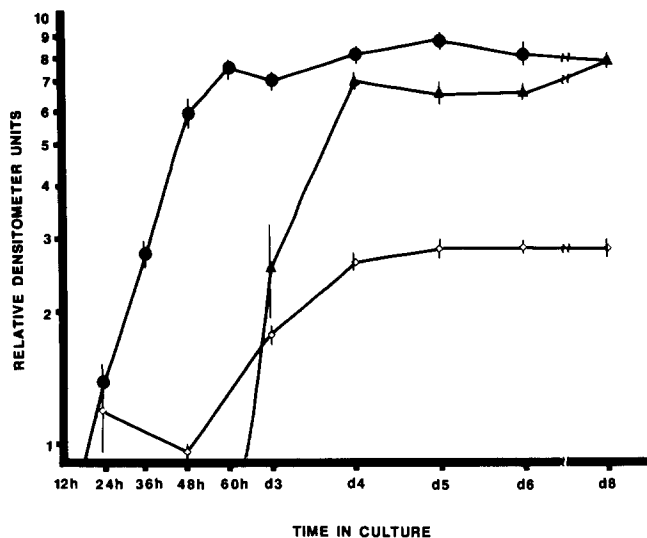


Figure 2. Relative expression of lamin A, myosin heavy chain, and TM in cultured CEM. Immunoblot autoradiographs of samples sets (as in Fig. 1) and serial dilutions of day 8 samples were scanned at predetermined sensitivity settings with a densitometer. The use of different sensitivity settings allowed the plotting of all relative values measured to be displayed on the same log cycle and consequently do not reflect absolute amounts for comparison purposes. Densitometer measurements of serially diluted immunoblotted CEM samples show a linear relationship when plotted on a log scale (not shown). Experimental samples yielded values that fell within the range of linearity as determined from the scanning of serially diluted CEM sample autoradiographs. The areas under the scanning line corresponding to the full size protein were quantified and used for the plotting of the curves. Vertical bars indicate the standard error of the means as calculated from four separate experiments. (◇) Myosin heavy chain; (●) lamin A; (▲) TM.

antibodies against MHC, lamin A, or both the α - and β -TM isoforms. The Coomassie blue-stained protein patterns of the tissue samples are shown in Fig. 3 A. As can be observed in the Western blots of the skeletal muscle tissue samples (Fig. 3, B, C, and D), negligible amounts of MHC had accumulated in the day 7 sample (Fig. 3 B, lane 1). By day 10, a detectable level of MHC was observed, with accumulation remaining at a low level until about day 14. After which, the MHC content increased rapidly (Fig. 3 B, lanes 2–6) and approached the steady-state value at approximately the time of hatching (Fig. 3 B, lanes 7 and 8). These trends were readily observed in Fig. 4, which plots the relative values of antigen accumulation as quantified by densitometer scans of immunoblot autoradiographs. TM expression was detected on day 12, with the α -TM isoform appearing first (*lower band*) (Fig. 3 D, lane 3). TM accumulation proceeded rapidly once initiated, approaching a maximal level on days 18–20 (Fig. 3 D, lane 6). Lamin A was expressed in minor amounts relative to the muscle specific proteins, and its presence was undetectable, or at extremely low levels on day 7 (Fig. 3 C, lane 1). The accumulation of lamin A continued throughout myogenesis (Fig. 3 C, lanes 2–7), and appeared to reach a plateau before the time of hatching (Fig. 3 C, lane 7).

It should be noted that the antibody CH291 used for the culture samples recognizes only the α -TM isoform, whereas

the antibody CH1 used for the tissue samples recognizes both the α - and β -TM isoforms. Therefore, the apparent variability of the relative ratios of lamin A/TM accumulation between the *in vitro* and *in ovo* blots can be accounted for from the use of different primary antibodies in each instance. Furthermore, the ^{125}I -labeled secondary antibodies may exhibit different affinities for these two antibodies. However, the ratio of lamin A/MHC appears to be comparable between the tissue and culture samples.

Immunofluorescence Microscopy of CEM Cells during Differentiation

To analyze the temporal appearance of lamin A and α -TM within individual CEM cells, double-labeled direct immunofluorescence was performed. Cells were scored and classified into one of four categories based on the relative staining intensity with both the anti-lamin A and anti-tropomyosin antibodies. As can be seen in Table I (and Fig. 5, A–C), at 24 h in culture a majority (82.6%) of the cells showed weak nuclear staining with the anti-lamin A antibody C23, and negative tropomyosin staining with the antibody CH291. Approximately 4.6% of the cells showed a strong nuclear staining, and yet were negative for TM staining. In addition, among the population (11.2%) with a strongly positive C23 and CH291 staining, most of these cells were multinucleated. This latter category may represent the cells which are in a more advanced stage of differentiation, as suggested by a fusion index of 27.6% at 24 h in culture. As differentiation progressed with time in culture, the population of cells with strong nuclear staining increased, whereas the percentage of cells with weak nuclear staining concurrently decreased to 44.4% by 72 h after plating (Table I and Fig. 5, G–I). Furthermore, the class of cells with strong lamin A/negative TM staining also increased substantially. These results are in good agreement with the immunoblotting data shown in Figs. 1 and 2, and demonstrate that within individual CEM cells, the expression and accumulation of nuclear lamin A preceded the induction to a high level of expression of TM. It should be noted that this method of counting cells places an equal weight to contaminating mononucleated CEF cells as to syncytial myotubes, and consequently, the percentages calculated by this method of analysis actually undervalues the contributions of multinucleated cells to these ratios, especially at the later times in culture.

Fig. 5 shows phase contrast and immunofluorescent micrographs of cells at various stages of early CEM differentiation with 24 (A–C), 48 (D–F), and 72 (G–I) h cultures. As differentiation proceeded, as judged by cell fusion and/or TM staining, the size of nuclei increased (compare Fig. 5, A, D, and G). The increase in nuclear size roughly paralleled the increase in the intensity of C23 staining. This suggests that the increase in lamin A content typically accompanies the enlargement of the nucleus. However, occasional large nuclei can be observed with diffuse staining for lamin A.

At 48 h in culture, the fusion index had risen to 60.7% (Table I). Of the strongly positive lamin A staining cells, most were positive for TM staining. However, a considerable percentage of the strongly positive lamin A cells remained negative for TM expression (Fig. 5, D–F, *arrowhead*; see Table I). By 72 h in culture the number and the intensity of positive lamin A (Fig. 5 H) and TM (Fig. 5 I) staining cells had in-

CEM TISSUE

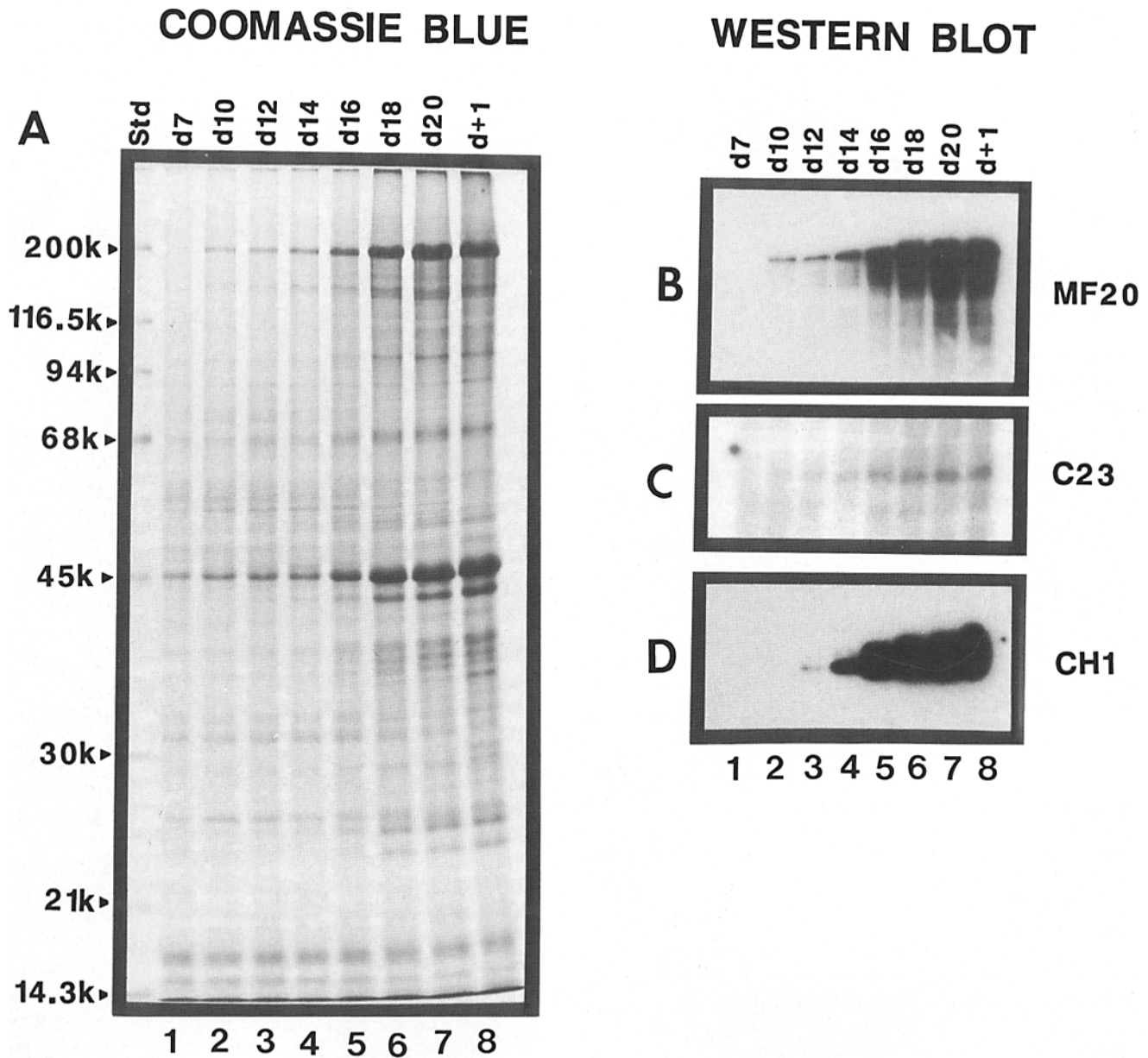


Figure 3. Expression of lamin A, myosin heavy chain, and TM in embryonic leg muscle at different stages of development. Samples were generated from embryos or hatched chicks on the following days after the initiation of incubation, days 7 (lane 1), 10 (lane 2), 12 (lane 3), 14 (lane 4), 16 (lane 5), 18 (lane 6), 20 (lane 7), and one day after hatch (*d + 1*, lane 8). The DNA concentration of these samples was determined and used to normalize the amounts of samples loaded into the gels. After electrophoresis, proteins were either stained with Coomassie blue (*A*), or processed for immunoblot analysis with MF20 against MHC (*B*), C23 against lamin A (*C*), or CH1 against both α - and β -TM (*D*). Note the use of an anti-TM antibody CH1 that recognizes both the α - and β -TM isoforms gives the appearance of a greater induction of TM expression when compared to culture samples that were probed with an anti-TM antibody CH291 which recognizes only the α -TM isoform.

creased (see Table I), with a vast majority of nuclei within syncytial cells (86%). TM staining had increased in intensity, and was associated with longitudinally arrayed fibers, although a striated pattern was not yet apparent (Fig. 5 I).

As can be seen in Fig. 5 H, the lamin A staining pattern was not uniformly strong for every nuclei in syncytial cells, with occasional small nuclei staining more intensely than the larger nuclei. Furthermore, mononucleated bipolar cells were

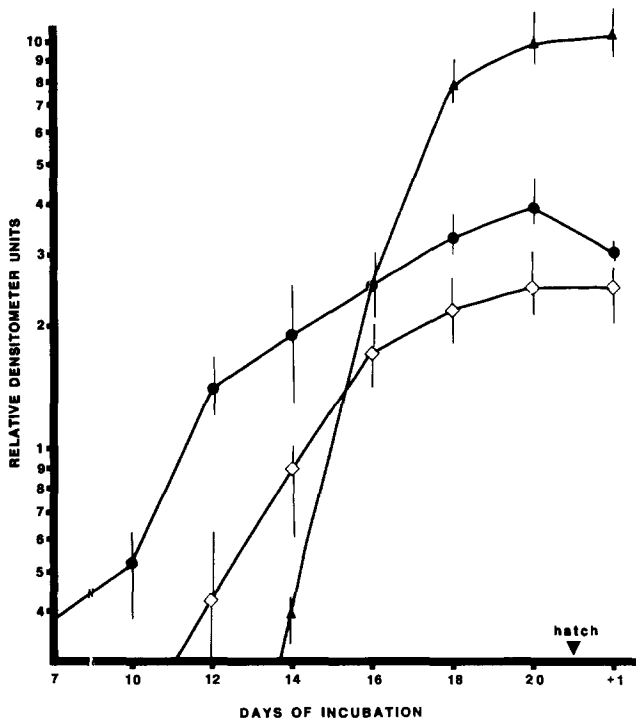


Figure 4. Relative expression of lamin A, muscle specific myosin heavy chain, and TM in embryonic leg skeletal muscle. Densitometer scans yield relative values of expression of lamin A, TM, and MHC. Relative values determined as for Fig. 2. (◇) Myosin heavy chain; (●) lamin A; (▲) TM.

observed that displayed a weak nuclear staining pattern, which were also negative for TM staining (Fig. 5, G, H, and I, arrowhead).

Two-Dimensional Gel Analysis of Lamin A Isoelectric Variants Observed during Myogenesis In Vitro

To ascertain if changes in lamin A isoelectric variants are observed during myogenesis, total homogenates and the C23 immunoprecipitates of [³⁵S]methionine-labeled differentiating CEM cultures were analyzed by two-dimensional isoelectric focusing and NEPHGE gels, respectively. The predominant isoelectric variant of lamin A at all stages of differentiating muscle cultures was the more basally migrating species (Fig. 6, B and D). Based on quantitative measurement of the immunoprecipitated lamin A, the ratio of the acidic to basic variants in CEM cells did not change significantly during differentiation (data not shown). At 36 h in culture, the TM isoforms were predominately composed of nonmuscle isoforms, with only minor amounts of the muscle-specific isoforms synthesized (Fig. 6 A). There is also little accumulation of the muscle-specific myosin light chains 1 and 2, troponin C, and desmin. The lamin A from the 36-h sample migrates predominately as a single spot, with quantitatively minor isoelectric variants migrating to more acidic positions (Fig. 6 B). By 132 h in culture an increase in the synthesis of the muscle-specific protein isoforms of TM, myosin light chains 1 and 2, desmin, and α -actin was evident (Fig. 6 C). However, despite the increase in the total amount of lamin A, the ratio of acidic to basic variants did not change (compare Fig. 6 B with 6 D). Within differentiated myotubes,

Table I. Fusion Index and Staining Intensity of CEM Cells at 24, 48, and 72 h in Culture with Anti-Lamin A (C23) and Anti- α -TM (CH291) Antibodies

	Time in Culture (in hours)		
	24	48	72
Fusion index*	27.6%	60.7%	86.0%
C23/CH291‡			
+/-	82.6%	64.0%	44.4%
+ +/ -	4.6%	13.4%	16.1%
+ +/ +	11.2%	20.8%	38.0%
+ / +	1.5%	1.8%	1.4%
n§	605	283	279

* Fusion index was calculated as the percentage of the total number of nuclei that are found within syncytial cells.

‡ The staining intensity of C23 (lamin A) was arbitrarily defined as strong (+ +) or weak (+); and CH291 (tropomyosin) staining as negative (-), or positive (+).

§ n, the number of cells scored.

the major variant of lamin A was the most basally migrating form. In contrast, lamin A from CEF cells had a much higher ratio of acidic to basic variants (data not shown). These results suggest that phosphorylation of lamin A is not a component of the mechanisms for the differential gene expression of differentiating muscle cells.

Discussion

In the present study, we have demonstrated that in nondifferentiated myoblast cultures and embryonic muscle tissues, lamin A was present in low quantities, and that during differentiation, lamin A accumulated rapidly. Based on quantitative immunoblotting, the increase in lamin A expression temporally preceded the induction to a high level of expression of muscle-specific protein isoforms. The temporal relationship of the expression and accumulation of lamin A before the induction to a high level of expression of muscle-specific genes is further demonstrated on a cellular level by results obtained from the immunofluorescence staining of differentiating CEM cells.

It is interesting to note that the intensity of lamin A staining can differ between nuclei within individual myotubes. This may reflect the presence of nuclei at different stages of the differentiation pathway. For example, in Fig. 5 H, a bipolar cell that is weakly positive for lamin A staining appears to be in the process of fusing with a myotube, which contains nuclei strongly staining for lamin A. As a consequence of this fusion, a heterogenous staining intensity will be observed between nuclei within the syncytial myotube. Alternatively, a heterogeneity in nuclear morphology and activity for acetylcholine receptor synthesis has also been observed within the same muscle fibers (Merlie and Sanes, 1985; Englander and Rubin, 1987; Pavlath et al., 1989). Thus, the differential expression and accumulation of lamin A in the myonuclei of muscle fibers may have further biological significance.

It has previously been reported that mouse early embryo and teratocarcinoma stem cells contain only a single major lamin polypeptide, resembling lamin B (Stewart and Burke,

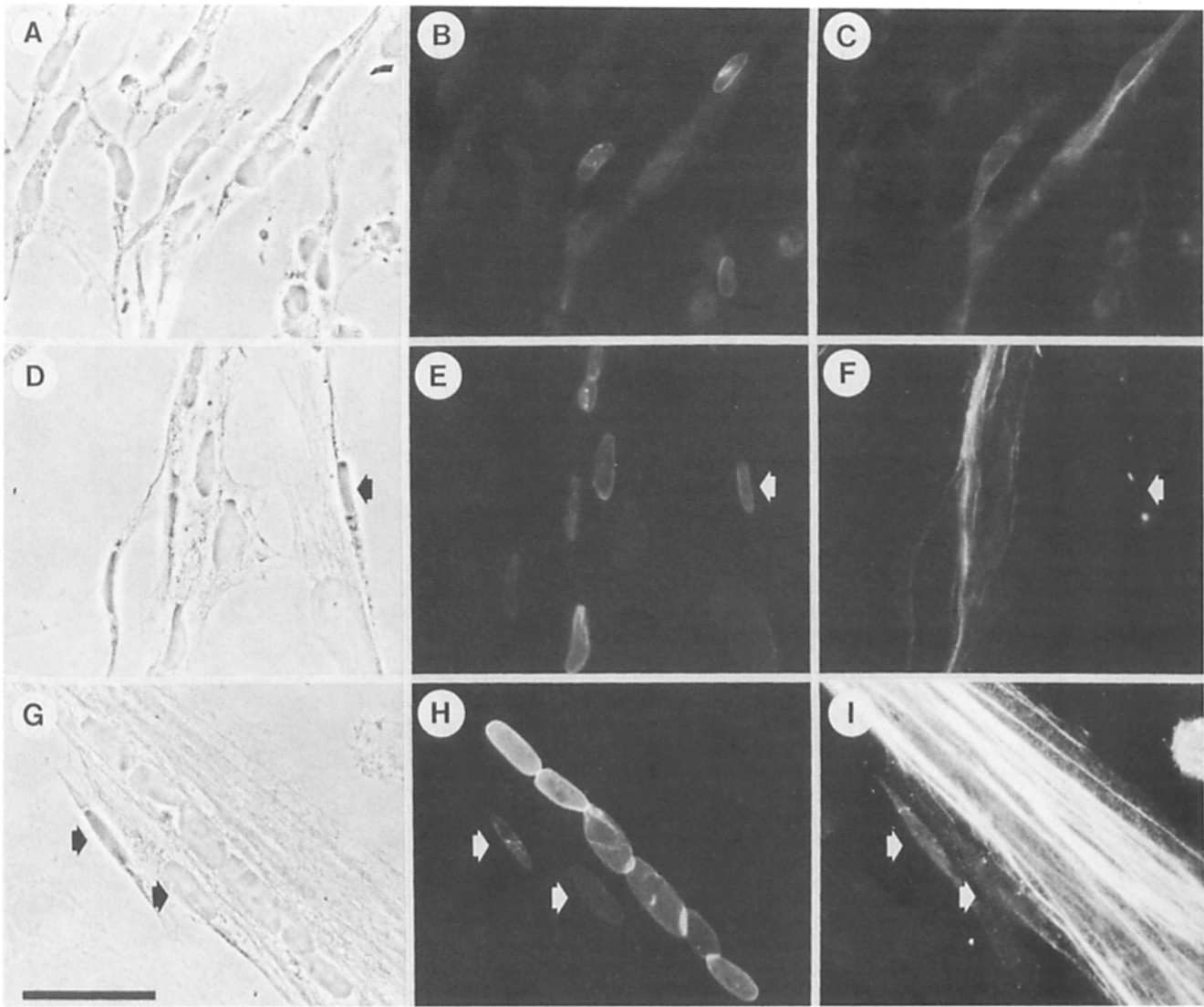


Figure 5. Double-label direct immunofluorescence of cultured CEM cells with the monoclonal antibodies C23 (lamin A) and CH291 (α -TM). Cells were fixed and stained with rho-C23 (B, E, and H) and FITC-CH291 (C, F, and I). Cells in the same field were viewed with either phase contrast (A, D, and G), or fluorescence optics (B, C, E, F, H, and I) at 24 (A, B, and C), 48 (D, E, and F), or 72 (G, H, and I) h after plating. Bar, 20 μ m.

1987), and that the induced differentiation of mouse carcinoma cells results in the expression of lamins A and C (Lebel et al., 1987). The differential expression of nuclear lamina proteins during chicken development has also been reported. Of the three nuclear lamina proteins described in chicken (A, B1, and B2), none have been detected in pachytene oocytes, spermatocytes, and spermatids (Stick and Schwarz, 1982, 1983). The B type lamins appear to be the predominate form in early chicken embryos, with little or no lamin A observed (Lehner et al., 1987). During embryonic development, lamin A becomes increasingly prominent, in a developmental and tissue-specific manner (Lehner et al., 1987). These findings are consistent with our results, in which we have extended to demonstrate the expression and accumulation of nuclear lamin A before the differentiation-dependent expression of muscle-specific proteins at high levels.

The differentiation of myogenic cells involves the induc-

tion to a high level of expression of a large number of muscle-specific genes. How these genes are coordinately induced remains unclear. The induction of muscle-specific genes in nuclei of a variety of nonmuscle cells by mouse muscle heterokaryons (Hardeman et al., 1986), and the identification of at least two separate genes capable of inducing muscle-specific gene expression in nonmuscle cells (Davis et al., 1987; Pinney et al., 1988), suggest that diffusible *trans*-acting factors may regulate muscle-specific gene expression. However, despite the presence of muscle-specific *trans*-acting factors in replicating myoblasts, most muscle-specific genes are not expressed at high levels (Hardeman et al., 1986; Minty et al., 1986; Tapscott et al., 1988). This suggests that muscle-specific genes may be repressed by mechanisms which limit the interaction of *trans*-acting factors with muscle-gene regulatory regions. These observations have suggested (at least) two levels in the transcriptional regula-

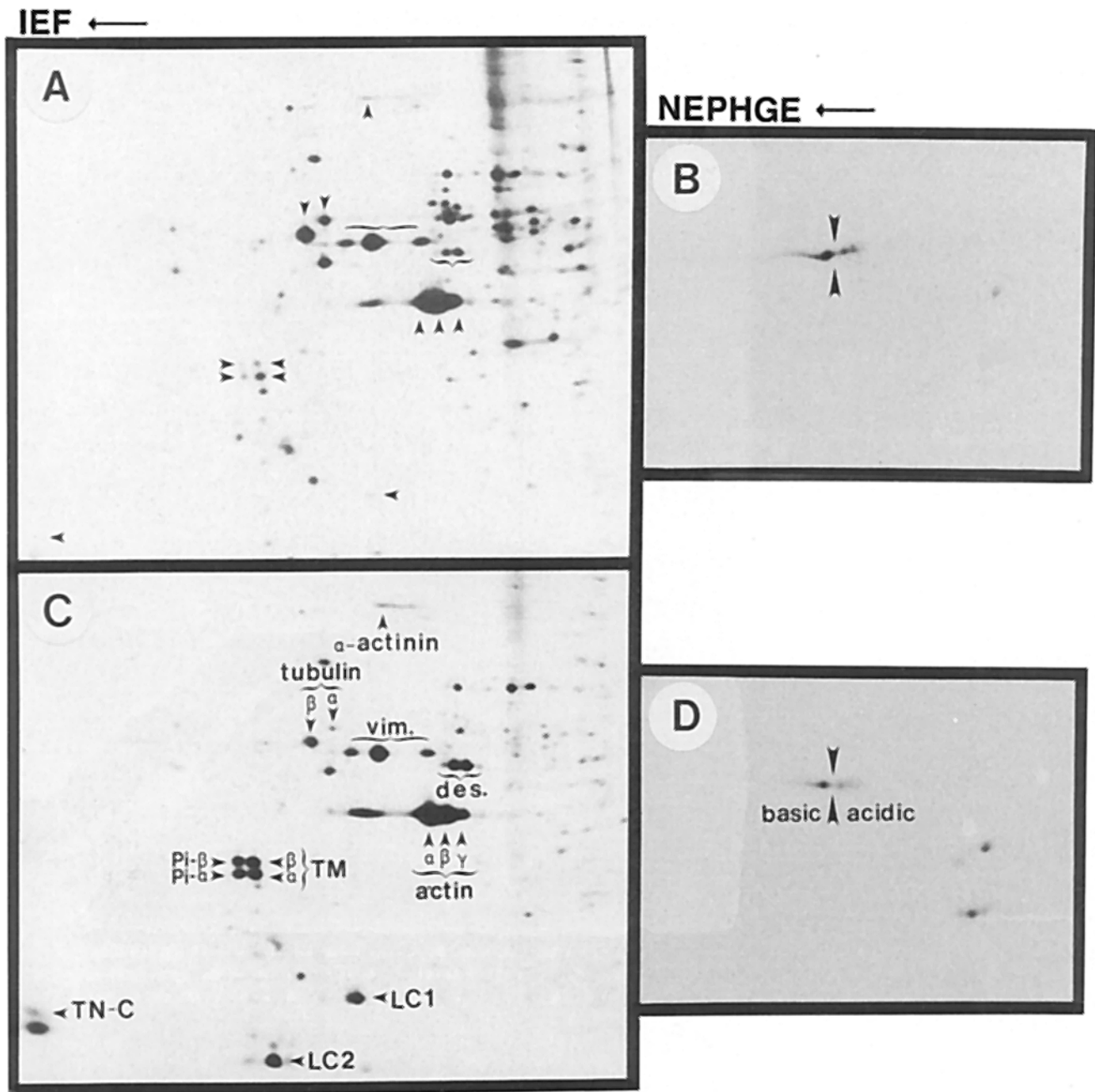


Figure 6. Lamin A from myoblasts and myotubes. Two-dimensional gel analysis of total homogenates and anti-lamin A immunoprecipitates of cultured CEM at different stages of differentiation. Cells were labeled with [³⁵S]methionine for 12 h at 1 (*A* and *B*) or 5 (*C* and *D*) d after plating. Total homogenates and anti-lamin A antibody immunoprecipitates were prepared as described in Materials and Methods. 200,000 cpm of TCA precipitable counts were loaded on isoelectric focusing gels for total homogenates. An equal number of counts (7,200 cpm) were loaded into NEPHGE gels for C23 immunoprecipitated samples. NEPHGE gels were aligned based on the migration of vimentin and lamins B1 and B2. In *C*, myosin light chains 1 and 2, α -TM, phosphorylated α -TM, β -TM and phosphorylated β -TM, and troponin C are designated by *LC1*, *LC2*, *TM α* , *α -Pi*, *TM β* , *β -Pi*, and *TN-C*, respectively. Desmin and vimentin are indicated by *des.* and *vim.*, respectively. The corresponding positions of these proteins are indicated by arrowheads in *A*. The arrowheads in *B* and *D* indicate the point of separation of immunoprecipitated lamin A for the determination of the ratio of acidic/basic lamin A isoelectric variants.

tion of muscle-specific gene expression: first, a chromatin-mediated activation of the muscle-specific genes; and second, a *trans*-acting factor modulation of transcriptional activity (Minty et al., 1986; Gunning et al., 1987).

In this study we have demonstrated an increase in nuclear lamin A during myoblast differentiation. This increase in lamin A temporally precedes the expression of a number of

muscle-specific genes. While these results do not elucidate the molecular mechanisms of muscle-specific gene induction, it suggests that the nuclear lamina may serve as a permissive component in the regulatory mechanisms of muscle differentiation. We can envision that by regulating the lamina content and density, chromatin topology and/or degree of condensation may be altered. These effects may be mediated

by chromatin binding directly to lamina proteins (Lebkowski and Laemmli, 1982), or indirectly, with chromatin-lamina associations mediated by peripheral chromatin proteins such as perichromin (McKeon et al., 1984). As a consequence of these suggested changes in chromatin topology, a permissive environment for the interaction of *trans*-acting regulatory factors to muscle-specific genes may then be present. Furthermore, the increase in lamin A expression may be the result of an induction by *trans*-acting factors which are present during myogenic differentiation, providing an integrated system for the coordinate expression of the differentiated phenotype. Regardless of the molecular role that lamin A plays in the induction observed during myogenic differentiation, due to the nearly ubiquitous presence of lamin A, it is doubtful that lamin A, or any isoelectric variants induces muscle-specific gene expression specifically. It is considerably more likely that the peripheral lamina acts in a more global manner, for instance, by altering loop domain organization, or by sequestering heterochromatin. Furthermore, we consider it highly likely that the increase in lamin A expression during myogenesis may be reflective of an increase in other nuclear structural proteins.

To further examine the role of lamin A expression in muscle differentiation, we have observed that in preliminary experiments with DMSO treatment of differentiating muscle cells, the expression of lamin A is inhibited concurrently with muscle-specific proteins. Furthermore, during the recovery of CEM cells from DMSO treatment, the accumulation of lamin A precedes the induction to a high level of expression of the muscle-specific genes in a temporal manner similar to the results we have reported in this study. These results support the suggestion that the appearance of lamin A before the induction of muscle-specific proteins may be an event of importance in the mechanisms which regulate the myogenic differentiation program.

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References

- Aebi, U., J. Cohn, L. Buhle, and L. Gerace. 1986. The nuclear lamina is a meshwork of intermediate-type filaments. *Nature (Lond.)*. 323:560-564.
- Affara, N., B. Robert, M. Jacquet, M. Buckingham, and F. Gros. 1980. Changes in gene expression during myogenic differentiation. I. Regulation of messenger RNA sequences expressed during myotube formation. *J. Mol. Biol.* 140:441-458.
- Bader, D., T. Masaki, and D. Fischman. 1982. Immunological analysis of myosin heavy chain during avian myogenesis in vivo and in vitro. *J. Cell Biol.* 95:763-770.
- Benavente, R., and G. Krohne. 1986. Involvement of nuclear lamins in postmitotic reorganization of chromatin as demonstrated by microinjection of lamin antibodies. *J. Cell Biol.* 103:1847-1854.
- Blau, H., C. Chiu, and C. Webster. 1983. Cytoplasmic activation of human nuclear genes in stable heterokaryons. *Cell*. 32:1171-1180.
- Blose, S. 1979. Ten nanometer filaments and mitosis: maintenance of structural continuity in dividing endothelial cells. *Proc. Natl. Acad. Sci. USA*. 76:3372-3376.
- Bonner, W., and R. Laskey. 1974. A film detection method of tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46:83-88.
- Briane, D., D. Delain, H. Senechal, T. Taillandier, J. Wahrmann, and B. Herve. 1987. Differential immunoreactivity for Z-DNA in rat myoblast nuclei during their terminal differentiation. *Exp. Cell Res.* 170:453-468.
- Bulinski, J., S. Kumar, K. Titani, and S. Hauschka. 1983. Peptide antibody specific for the amino terminus of skeletal muscle α -actin. *Proc. Natl. Acad. Sci. USA*. 80:1506-1510.
- Burke, B., and L. Gerace. 1986. A cell free system to study reassembly of the nuclear envelope at the end of mitosis. *Cell*. 44:639-652.
- Burton, K. 1968. Determination of DNA concentration with diphenylamine. *Methods Enzymol.* 12:163-166.
- Carmon, Y., H. Czosnek, U. Nudel, M. Shani, and D. Yaffe. 1982. DNaseI sensitivity of genes expressed during myogenesis. *Nucleic Acids Res.* 10:3085.
- Coleman, J., and W. Coleman. 1968. Muscle differentiation and macromolecule synthesis. *J. Cell Physiol.* 72:19-34.
- Danto, S., and D. Fischman. 1984. Immunochemical analysis of intermediate filaments in embryonic heart cells with monoclonal antibodies to desmin. *J. Cell Biol.* 98:2179-2191.
- Davis, R., H. Weintraub, and A. Lassar. 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell*. 51:987-1000.
- Devlin, R., and C. Emerson. 1978. Coordinate regulation of contractile protein synthesis during myoblast differentiation. *Cell*. 13:599-611.
- Devlin, R., and C. Emerson. 1979. Coordinate accumulation of contractile protein mRNAs during myoblast differentiation. *Dev. Biol.* 69:202-216.
- Devlin, R., P. Merrifield, and I. Konigsberg. 1982. The activation of myosin synthesis and its reversal in synchronous skeletal-muscle myocytes in cell culture. In *Muscle Development: Molecular and Cellular Control*. M. Pearson and H. Epstein, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 355-366.
- Endo, T., and B. Nadal-Ginard. 1987. Three types of muscle-specific gene expression in fusion blocked rat skeletal muscle cells: Translational control in EGTA-treated cells. *Cell*. 49:515-526.
- Englander, L., and L. Rubin. 1987. Acetylcholine receptor clustering and nuclear movement in muscle fibers in culture. *J. Cell Biol.* 104:87-95.
- Gerace, L., and G. Blobel. 1980. The nuclear envelope lamina is reversibly depolymerized during mitosis. *Cell*. 19:277-287.
- Gerace, L., C. Comeau, and M. Benson. 1984. Organization and modulation of nuclear lamina structure. *J. Cell Sci.* 1(suppl.):137-160.
- Georgatos, S., C. Stourmaras, and G. Blobel. 1988. Heterotypic and homotypic associations between the nuclear lamins: site-specificity and control by phosphorylation. *Proc. Natl. Acad. Sci. USA*. 85:4325-4329.
- Gunning, P., E. Hardeman, R. Wade, P. Ponte, W. Bains, H. M. Blair, and L. Kedes. 1987. Differential patterns of transcript accumulation during human myogenesis. *Mol. Cell. Biol.* 7:4100-4114.
- Hardeman, E., C. Chiu, A. Minty, and H. Blau. 1986. The pattern of actin expression in human fibroblasts x mouse muscle heterokaryons suggests that human muscle regulatory factors are produced. *Cell*. 47:123-130.
- Konigsberg, I. 1979. Skeletal myoblasts in culture. *Methods Enzymol.* 58:511-527.
- Krohne, G., and R. Benavente. 1986. The nuclear lamins: a multigene family of proteins in evolution and differentiation. *Exp. Cell Res.* 162:1-10.
- Laemmli, U. 1970. Cleavage of structural proteins during assembly of the head of the bacteriophage T4. *Nature (Lond.)*. 227:680-685.
- Lebel, S., C. Lampron, A. Royal, and Y. Raymond. 1987. Lamins A and C appear during retinoic acid-induced differentiation of mouse embryonal cells. *J. Cell Biol.* 105:1099-1104.
- Lebkowski, J., and U. Laemmli. 1982. Non-histone proteins and long-range organization of HeLa interphase DNA. *J. Mol. Biol.* 156:325-344.
- Lehner, C., V. Kurer, H. Eppenberger, and E. Nigg. 1986. The nuclear lamin protein family of higher vertebrates. Identification of quantitatively minor lamin proteins by monoclonal antibodies. *J. Biol. Chem.* 261:13293-13301.
- Lehner, C., R. Stick, H. Eppenberger, and E. Nigg. 1987. Differential expression of nuclear lamin proteins during chicken development. *J. Cell Biol.* 105:577-587.
- Lin, J. J.-C., F. Matsumura, and F. Yamshiro-Matsumura. 1984. Tropomyosin-enriched and alpha-actinin-enriched microfilaments isolated from chicken embryo fibroblasts by monoclonal antibodies. *J. Cell Biol.* 98:116-127.
- Lin, J. J.-C., C.-S. Chou, and J. L.-C. Lin. 1985. Monoclonal antibodies against chicken tropomyosin isoforms: production, characterization and application. *Hybridoma*. 4:223-242.
- Lin, J. J.-C., J. L.-C. Lin, E. Davis-Nanthakumar, and D. Lourim. 1988. Monoclonal antibodies against caldesmon, a Ca^{++} /calmodulin- and actin-binding protein of smooth muscle and nonmuscle cells. *Hybridoma*. 7:273-288.
- McKeon, F., D. Tuffanelli, S. Kobayashi, and M. Kirschner. 1984. The redistribution of a conserved nuclear envelope protein during the cell cycle suggests a pathway for chromosome condensation. *Cell*. 36:83-92.
- Merlie, J., and J. Sanes. 1985. Concentration of acetylcholine receptor mRNA in synaptic regions of adult muscle fibres. *Nature (Lond.)*. 317:66-68.
- Minty, A., H. Blau, and L. Kedes. 1986. Two-level regulation of cardiac actin gene transcription: muscle-specific modulating factors can accumulate before gene activation. *Mol. Cell. Biol.* 6:2137-2148.
- Muscat, G., T. Gustafson, and L. Kedes. 1988. A common factor regulates

- skeletal and cardiac α -actin gene transcription in muscle. *Mol. Cell. Biol.* 8:4120-4133.
- Nadal-Ginard, B. 1978. Commitment, fusion and biochemical differentiation of a myogenic cell line in the absence of DNA synthesis. *Cell.* 15:855-864.
- O'Farrel, P. Z. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007-4021.
- O'Farrel, P. Z., H. Goodman, and P. H. O'Farrel. 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell.* 12:1133-1142.
- Paterson, B., and J. Bishop. 1977. Changes in the mRNA population of chick myoblasts during myogenesis in vitro. *Cell.* 12:751-765.
- Pavlat, G., K. Rich, S. Webster, and H. Blau. 1989. Localization of muscle gene products in nuclear domains. *Nature (Lond.)* 337:570-573.
- Pinney, D., S. Pearson-White, S. Konieczny, K. Latham, and C. Emerson. 1988. Myogenic lineage determination and differentiation: evidence for a regulatory gene pathway. *Cell.* 53:781-793.
- Sanger, J. 1975. Changing patterns of actin localization during cell division. *Proc. Natl. Acad. Sci. USA.* 72:1913-1916.
- Seiler-Tuyns, A., J. Eldridge, and B. Paterson. 1984. Expression and regulation of chicken actin genes introduced into mouse myogenic and nonmyogenic cells. *Proc. Natl. Acad. Sci. USA.* 81:2980-2984.
- Stewart, C., and B. Burke. 1987. Teratocarcinoma stem cells and early mouse embryos contain only a single lamin polypeptide closely resembling lamin B. *Cell.* 51:1099-1104.
- Stick, R., and H. Schwarz. 1982. The disappearance of the nuclear lamina during spermatogenesis: an electron microscopic and immunofluorescence study. *Cell Differ.* 11:235-243.
- Stick, R., and H. Schwarz. 1983. Disappearance and reformation of the nuclear lamina structure during specific stages of meiosis in oocytes. *Cell.* 33:949-958.
- Tapscott, S., R. Davis, M. Thayer, P.-F. Cheng, H. Weintraub, and A. Lassar. 1988. MyoD1: a nuclear phosphoprotein requiring a Myc homology region to convert fibroblast to myoblasts. *Science (Wash. DC)* 242:405-411.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
- Worman, H., J. Yuan, G. Blobel, and S. Georgatos. 1988. A lamin B receptor in the nuclear envelope. *Proc. Natl. Acad. Sci. USA.* 85:8531-8534.