

Cellular Localization of Muscle and Nonmuscle Actin mRNAs in Chicken Primary Myogenic Cultures: The Induction of α -Skeletal Actin mRNA Is Regulated Independently of α -Cardiac Actin Gene Expression

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Abstract. Specific DNA fragments complementary to the 3' untranslated regions of the β -, α -cardiac, and α -skeletal actin mRNAs were used as in situ hybridization probes to examine differential expression and distribution of these mRNAs in primary myogenic cultures. We demonstrated that prefusion bipolar-shaped cells derived from day 3 dissociated embryonic somites were equivalent to myoblasts derived from embryonic day 11–12 pectoral tissue with respect to the expression of the α -cardiac actin gene. Fibroblasts present in primary muscle cultures were not labeled by the α -cardiac actin gene probe. Since virtually all of the bipolar cells express α -cardiac actin mRNA before fusion, we suggest that the bipolar phenotype may distinguish a committed myogenic cell type. In contrast, α -skeletal actin mRNA accumulates only in mul-

tinucleated myotubes and appears to be regulated independently from the α -cardiac actin gene. Accumulation of α -skeletal but not α -cardiac actin mRNA can be blocked by growth in Ca^{2+} -deficient medium which arrests myoblast fusion. Thus, the sequential appearance of α -cardiac and then α -skeletal actin mRNA may result from factors that arise during terminal differentiation. Finally, the β -actin mRNA was located in both fibroblasts and myoblasts but diminished in content during myoblast fusion and was absent from differentiated myotubes. It appears that in primary myogenic cultures, an asynchronous stage-dependent induction of two different α -striated actin mRNA species occurs concomitant with the deinduction of the nonmuscle β -actin gene.

MYOGENESIS in culture parallels embryonic muscle development to the extent that myoblasts proliferate, withdraw from the cell cycle, fuse to form myotubes, and elaborate functional myofibrillar sarcomeres (reviewed in reference 30). Biochemical differentiation, as evidenced by the induction of muscle-specific gene products and the repression of subsets of nonmuscle genes, normally occurs after myoblast terminal commitment and fusion. Myoblasts grown in Ca^{2+} -deficient medium have demonstrated that biochemical differentiation does not require irreversible withdrawal from the cell cycle or the formation of multinucleated myotubes (12, 23, 25, 31, 33). Myoblast fusion may also be inhibited by certain *N*-carbobenzyloxy dipeptide amides that compete with the protein substrate of a soluble cytoplasmic metalloendoprotease (MEPr)¹ (9, 10). Recent evidence suggests that MEPrs may be required for biochemical differentiation (3, 12) as well as fusion.

We examined the switch in expression of the chicken actin

multigene family during the terminal differentiation of skeletal myoblasts (29). We recently showed that primary cultures of chicken myoblasts express specific actin mRNAs in a sequential pattern leading to terminal differentiation (17). Nonmuscle β -actin mRNA was detected predominantly in proliferating myogenic cultures and declines in content as myogenesis continues. Within 24 h after the plating of dissociated embryonic myoblasts, α -cardiac actin mRNA is induced and later is maintained as the major postfusion actin mRNA. In contrast, α -skeletal actin mRNA, the predominant skeletal muscle actin mRNA in vivo, is induced only after fusion is under way (17).

We reasoned that the temporal pattern of induction and deinduction of actin genes could be explained by the spatial distribution of actin mRNAs among cells at different stages of myogenesis. Therefore, several questions regarding myogenic regulation of the actin multigene family remained unanswered by previous studies. Specifically: (a) Does the low, but significant, level of β -actin mRNA in late muscle cultures result from low constitutive expression in myotubes or from abundant β -actin mRNA in the relatively few residual

1. *Abbreviations used in this paper:* LCM, low Ca^{2+} -containing medium; MEPr, metalloendoprotease.

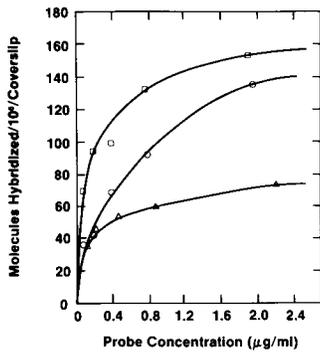


Figure 1. Effect of probe concentration upon in situ hybridization. ^{32}P -labeled single-stranded RNA probes to α -skeletal (\circ), α -cardiac (\square), and β -actin (\triangle) mRNA were hybridized at various concentrations to 76-h myogenic cultures for 4 h at 45°C . A background control probe consisting of the α -skeletal probe sequence in reverse orientation (i.e., non-complementary to mRNA) was also incubated on separate coverslips under the same conditions. Nonspecific background radioactivity was subtracted from hybridization signal at each concentration and the resulting signal was converted to molecules of probe hybridized (See Materials and Methods). Background radioactivity increased linearly with a slope of 9.6×10^6 molecules per $\mu\text{g}/\text{ml}$ probe. Each point represents the mean from four determinations.

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proliferating and nonmuscle cells? (b) Is the muscle-specific α -cardiac actin mRNA expressed synchronously by all myoblasts in early cultures or is the α -cardiac actin gene selectively expressed within a developmental subset of myoblasts? (c) Is the expression of the α -skeletal actin gene fusion dependent? These questions were answered by determining the cellular localization of actin mRNA transcripts by in situ hybridization in primary myogenic cultures.

Materials and Methods

Plasmid DNA

M13 clones containing nucleotide sequences from the 3' untranslated region of the chicken α -skeletal, α -cardiac, and β -actin genes were described previously (17). For synthesis of single-stranded RNA probes, the M13 probe sequences were inserted into plasmids pTZ18R and pTZ19R (Pharmacia Fine Chemicals, Piscataway, NJ), which contain the T7 RNA polymerase promoter and fl origin of replication. Plasmids were purified according to Katz et al. (18).

Synthesis of Radiolabeled Hybridization Probes

Radioactive single-stranded DNA probes were prepared by the procedure of Bergsma et al. (4). The calculated specific activity for ^{32}P -labeled DNA probes was $\sim 1.5 \times 10^9$ cpm/ μg , while that for ^{125}I -labeled probes was 1×10^8 cpm/ μg . Single-stranded RNA probes were generated using T7 RNA polymerase (Pharmacia Fine Chemicals) according to protocols obtained from the manufacturer. The specific activity of the ^{32}P -labeled RNA probes was 1.5×10^8 cpm/ μg .

Primary Myoblast Cultures

Myoblasts were obtained from 11–12-d chicken embryonic pectoral muscle and were cultured as described previously (17) except that 50 $\mu\text{g}/\text{ml}$ gentamicin (Sigma Chemical Co., St. Louis, MO) was substituted for penicillin/streptomycin. Cells were grown on 22-mm-diam Thermanox plastic coverslips (Miles Scientific Div., Naperville, IL) coated with rat tail collagen in 8-well multiplates (Miles Scientific Div.) containing 1.5 ml of media per well in preparation for in situ hybridization. Some cultures were incubated with a low Ca^{2+} -containing medium (LCM; 110–150 μM Ca^{2+}) consisting of 93% suspension culture MEM (Gibco, Grand Island, NY) supplemented with 5% horse serum, 2% chick embryo extract, and gentamicin. Myoblasts to be grown in LCM were rinsed twice with Hank's CMF at 24 h after plating and then maintained in LCM until confluency (66–72 h after plating).

RNA Dot Blots and Hybridization

Total nucleic acid and total RNA were isolated from muscle cultures as described (17). RNA was spotted onto Biotrans nylon membranes (ICN Radiochemicals, Div. ICN Biomedicals Inc., Irvine, CA) and hybridized according to Bergsma et al. (4). The DNA content of total nucleic acid preparations was determined by a fluorescence assay using the DNA-binding dye bis-benzimidazole (Hoechst 33258) and a Hoefer mini-fluorometer (Hoefer Scientific Instruments, San Francisco, CA).

In Situ Hybridization

In situ hybridizations of cultured cells were carried out according to the protocols of Lawrence and Singer (20, 21) with minor modifications. For detection and quantitation of ^{32}P -labeled probes, coverslips were cut in half and placed in scintillation vials containing PBS to measure Cerenkov radiation at an efficiency of $\sim 30\%$. Nuclei were Fielgen stained and the number of mononuclear cells and nuclei within myotubes was determined. For autoradiographic detection of ^{125}I -labeled probes, coverslips were mounted on microscope slides and dipped in NTB-2 nuclear track emulsion (Eastman Kodak Co., Rochester, NY) at 45°C , stored at 4°C for 7–9 d. Slides were developed and stained with hematoxylin and eosin. Photographs were taken using Kodak Tri-X film and a $50\times$ oil immersion objective.

Results

Optimization of Conditions for Hybridization In Situ

The effect of probe concentration on hybridization signal was assessed with single-stranded ^{32}P -labeled RNA and DNA probes hybridized to muscle cells grown on plastic coverslips. The ^{32}P assay (20) proved an efficient means of simulating conditions that would later be used for hybridization of probes suitable for autoradiography. Hybridizations with single-stranded RNA probes were carried out for 2–4 h at 45°C , while single-stranded DNA probes were hybridized for 10–14 h at 37°C . Fig. 1 indicates that hybridization of single-stranded RNA probes to α -skeletal, α -cardiac, and β -actin mRNAs of 76-h myogenic cultures appears to saturate at probe concentrations exceeding 2 $\mu\text{g}/\text{ml}$. The signal-to-background ratio at half-maximal hybridization (0.1–0.3 $\mu\text{g}/\text{ml}$) was routinely 20–60:1 for RNA probes under these hybridization and washing conditions. Similar hybridizations of ^{32}P -labeled DNA probes yielded signal-to-background ratios of 50:1 at probe concentrations up to 0.4 $\mu\text{g}/\text{ml}$, the maximum tested.

Cellular Localization of Specific Actin mRNAs during Muscle Development

^{125}I -labeled single-stranded DNA probes were hybridized in situ to primary skeletal muscle cultures to determine the distribution of actin mRNAs among the heterogeneous cell types present. Autoradiographs in Fig. 2 summarize the distribution of actin mRNAs at three time points after plating: 18 h (prefusion), 38 h (start of fusion), and 76 h (postfusion).

Early primary cultures 18 h after plating (Fig. 2, A, D, G, and J) consisted mainly of proliferating myogenic and non-myogenic cells. All cells contained β -actin mRNA (Fig. 2 A), as expected for proliferating cells, since the nonmuscle actins form the microfilaments necessary for mitotic cytokinesis. A subset of 17% of cells had begun to express α -cardiac actin mRNA by 18 h at substantial levels, while the majority of cells remained unlabeled (Fig. 2 D). Binding of the α -skeletal actin probe (Fig. 2 G) was comparable to the level of nonspecific binding of a reverse-orientation control probe (Fig. 2 J).

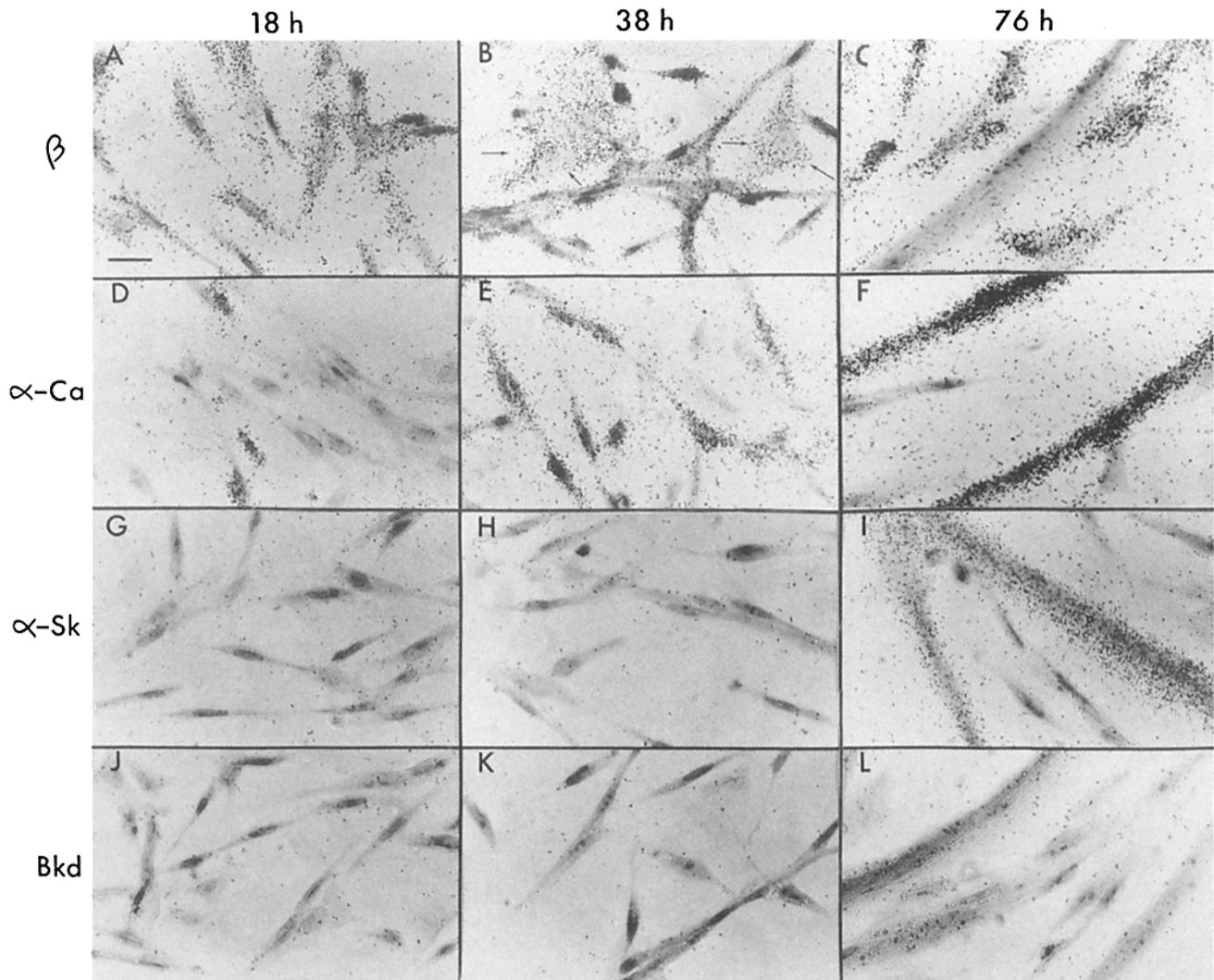


Figure 2. Content and localization of actin mRNAs during myogenesis in culture. ^{125}I -labeled DNA probes complementary to β -actin (A–C), α -cardiac (D–F), and α -skeletal (G–I) mRNA sequences, and a reverse-orientation α -cardiac probe (J–L) were hybridized to primary myogenic cultures fixed at 18 (A, D, G, and J), 38 (B, E, H, and K), or 76 (C, F, I, and L) h after plating. The probe-specific activity was 1×10^8 cpm/ μg , probe concentration was 0.1 $\mu\text{g}/\text{ml}$, and exposure was for 9 d. Cells were stained with hematoxylin and eosin. Arrows in B point to fibroblasts labeled with the β -actin mRNA probe. Bar, 20 μm .

By 38 h, as myoblasts began to fuse (Fig. 2, B, E, H, and K), β -actin mRNA had become preferentially restricted to nonfused cells. Fibroblasts at this stage were recognized as pale-staining, pleiomorphic cells (arrows in Fig. 2 B), while myoblasts were spindle-shaped, bipolar-appearing cells with dark-staining, elongated nuclei. Grains representing hybridization to β -actin mRNA were localized over the cytoplasm and at the cell periphery but were minimal over cell nuclei. In contrast, α -cardiac actin mRNA (Fig. 2, D and E) was distributed throughout myoblast cytoplasm and over nuclei. At this time, 58% of the mononucleated myoblasts expressed α -cardiac actin mRNA, while fibroblasts did not (Fig. 2 E). This analysis excludes $\sim 20\%$ of cells, which were fusing at this time in more densely populated clusters and labeled positive for α -cardiac actin mRNA. Expression of α -skeletal actin mRNA was not detected above background in myoblasts before fusion (Fig. 2, H and K).

By 76 h (Fig. 2, C, F, I, and L), β -actin mRNA was found exclusively in nonfused myoblasts and fibroblasts interspersed between newly formed myotubes (Fig. 2 C). α -Cardiac actin mRNA attained very high levels in myotubes but was scarce in surrounding cells (Fig. 2 F). At the completion of fusion, α -skeletal actin mRNA was detected in myotubes but was not present above background in neighboring unfused cells (Fig. 2, I and L).

Calculation of the Number of Probe Molecules Hybridized Per Differentiated Cell and the Efficiency of In Situ Hybridization

We quantitated the number of probe molecules hybridized per differentiated cell. Table I indicates the total RNA content per coverslip based on the DNA content of total nucleic acid preparations and the number of nuclei per coverslip.

Table I. Determination of Nucleic Acid Content during Myogenesis in Culture

Time in culture	DNA*	RNA‡	Nuclei/ coverslip§	RNA
<i>h</i>	$\mu\text{g}/\mu\text{g}$ of total nucleic acid	$\text{pg}/\text{nucleus}$	$/10^6$	$\mu\text{g}/\text{coverslip}$
18	0.294	6.2	0.033	0.20
38	0.291	6.3	0.072	0.46
76	0.203	10.2	0.271	2.77

* Determined by fluorometric assay of total nucleic acid preparations.

‡ This number = $(1/[\mu\text{g DNA}/\mu\text{g total nucleic acid}] - 1) \times (2.6 \times 10^{-12} \text{ g of DNA per chick nucleus})$.

§ Cell nuclei were counted from coverslips stained by the Fuelgen technique.

|| Calculated from $(\text{pg RNA}/\text{nucleus}) \times (\text{nuclei}/\text{coverslip per } 10^6)$.

The mass of total RNA per coverslip increases ~14-fold between 18 and 76 h after plating. Table II incorporates data describing the relative amounts of specific actin mRNAs per μg total RNA with the saturation values for in situ hybridization at 76 h (from Fig. 1). A conversion factor was obtained from these independent measurements that could be used to estimate the maximal in situ hybridization signal at saturation for cells fixed at the 18- and 38-h time points.

The number of probe molecules hybridized (i.e., the number of accessible mRNA molecules) per differentiated cell was calculated by determining the number of nuclei per coverslip and the fraction of cells labeled by the three different actin mRNA probes. β -Actin mRNA was expressed in unfused myoblasts and fibroblasts such that 220–250 molecules were accessible, on average, per cell (Table II). Fibroblasts became distinguishable by 38 h and contained the majority of grains (Fig. 2 B). By 76 h, the amount of β -actin gene probe hybridized increased to >1,000 copies per fibroblast. α -Cardiac actin mRNA averaged ~300 molecules hybridized per differentiated myoblast but increased to 800 molecules hybridized per nucleus in myotubes. On the other

hand, levels of α -skeletal actin mRNA were not significant until 76 h when >700 copies of probe hybridized per myotube nucleus. In situ hybridization efficiency was estimated at ~10% of the α -striated actin mRNA molecules (15,000 copies) previously detected per 75-h myotube nucleus (30).

α -Cardiac Actin mRNA Accumulates in Postmitotic Myoblasts

Previously, Hayward and Schwartz (17) and Bains et al. (2) have shown high level expression of α -cardiac actin in prefusion-replicating myoblasts. As shown here (Fig. 2 and Table II), α -cardiac actin mRNA appeared asynchronously in myogenic cultures until virtually all prefusion myoblasts express α -cardiac actin mRNA before fusion. However, these studies did not demonstrate that any cell synthesizing α -cardiac actin mRNA was replicative or postmitotic. To determine whether myoblasts that express α -cardiac actin mRNA have withdrawn from the cell cycle, prefusion myogenic cultures were pulsed for 30 min with [methyl, 1', 2'-³H]thymidine and processed for hybridization with [¹²⁵I]-

Table II. Quantitation of In Situ Hybridization to Actin mRNAs

Actin mRNA probe	Time in culture	Dot blot binding*	Probe hybridized	Labeled cells	Probe hybridized**
	<i>h</i>	$\text{cpm}/\mu\text{g total RNA}$	$\text{molecules}/10^6 \text{ coverslip}$	%	$\text{molecules}/\text{differentiated cell}$
β	18	399	7.0§	≥99.0	217
β	38	455	17.9§	≥99.0	251
β	76	319	76.0‡	27.8	1,010
α -Ca	18	137	2.3§	16.8	425
α -Ca	38	288	10.9§	66.8	228
α -Ca	76	695	160.0‡	72.2	818
α -Sk	18	7	0.2§	≤1.0	ND
α -Sk	38	10	0.5§	≤1.0†	ND
α -Sk	76	501	142.0‡	72.2	726

* Determined by hybridization of actin gene probes to dot blots of total RNA.

‡ Saturation value obtained from Fig. 1. This value was used to calculate a conversion factor between the signal obtained from RNA dot blots and that obtained by in situ hybridization: $(\text{dot blot cpm}/10^6 \text{ molecules hybridized}) = \{(\text{dot blot cpm}/\mu\text{g RNA}) \times (\mu\text{g RNA}/\text{coverslip}) \div (10^6 \text{ molecules hybridized}/\text{coverslip})\}$ for the 76-h myotube culture. For this particular hybridization, the conversion values (units as listed) were 11.6, 12.0, and 9.8 for the β -, α -cardiac, and α -skeletal actin probes, respectively. Thus, the efficiency of hybridization for all three probes at saturation appears to be equivalent.

§ Calculated from $\{(\text{dot blot cpm}/\mu\text{g total RNA}) \times (\mu\text{g total RNA}/\text{coverslip}) \div (\text{dot blot cpm}/10^6 \text{ molecules hybridized})\}$.

|| Determined from cultures hybridized with [¹²⁵I]-labeled actin gene probes and extent of fusion. Values represent percent of total nuclei correlated with labeling. See text for details.

† Some α -skeletal mRNA labeling was detected in groups of three or more precociously fused myogenic cells at this time point and was excluded from the analysis.

** $\{(\text{molecules hybridized}/\text{coverslip}) \div (\text{nuclei}/\text{coverslip}) \div (\text{fraction of cells labeled})\}$.

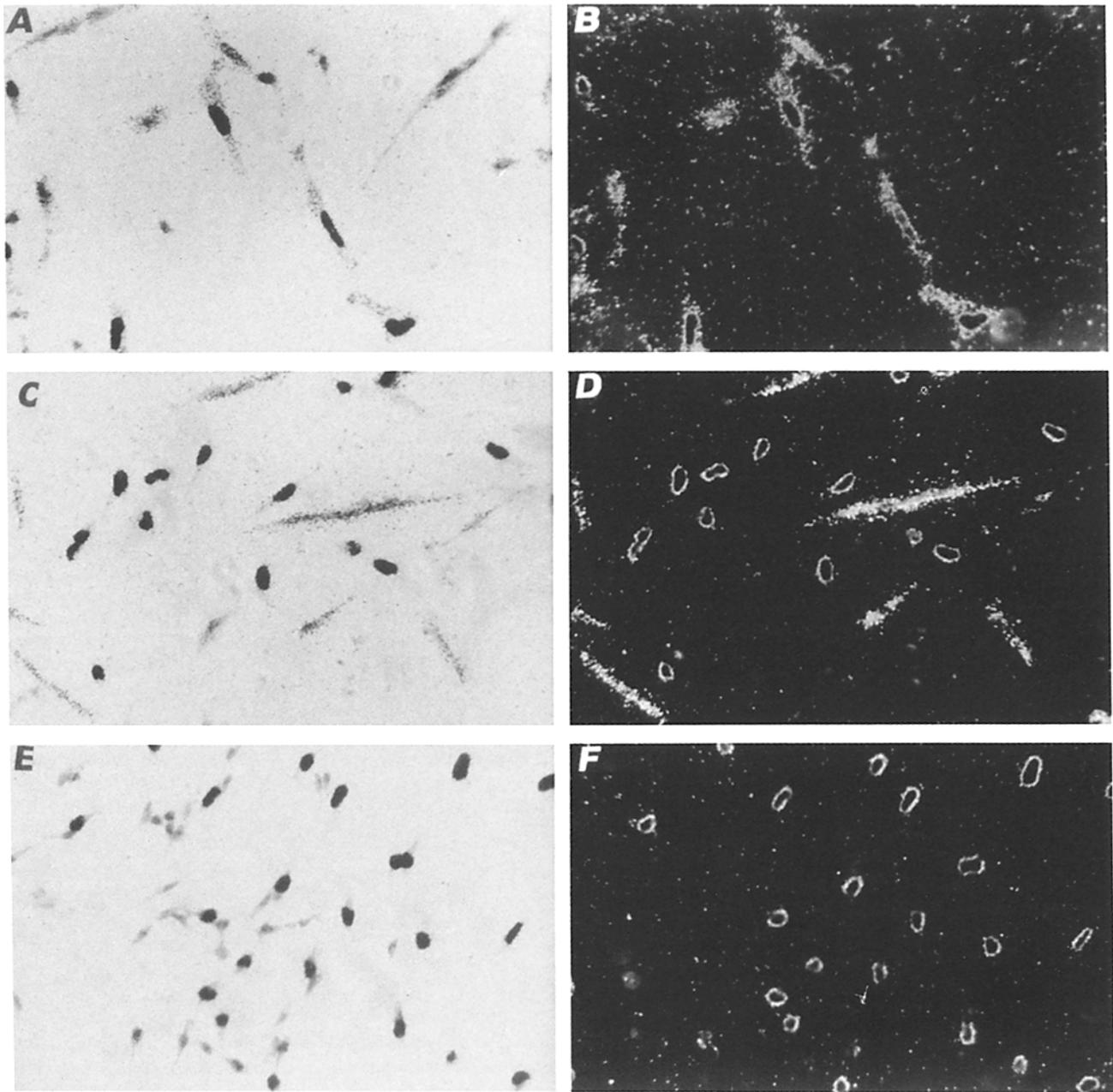


Figure 3. Labeling of myogenic culture with [³H]thymidine and actin mRNA probes. Primary muscle cultures (30 h after plating) exposed to 4 μCi/ml [³H]thymidine for 30 min before fixation (*A-F*). Cells were hybridized in situ with ¹²⁵I-labeled 3' noncoding probes to β-actin (*A* and *B*) and α-cardiac actin (*C* and *D*). Probe concentration was 0.1 μg/ml and exposure was for 7 d. Dark field illumination is shown in *B*, *D*, and *F*.

labeled probes. Autoradiographs in Fig. 3 show the dual labeling of myogenic cultures. Replicating cells were identified by incorporated [³H]thymidine localized as clustered grains over nuclei with no apparent grains over the cellular cytoplasm. Under darkfield illumination (Fig. 3, *E* and *F*) labeled nuclei were displayed as halo-like images with dark epicenters. β-Actin mRNA revealed by hybridization to ¹²⁵I-labeled probes was clearly expressed in most cells regardless of the proliferative state (Fig. 3, *A* and *B*). Fig. 3, *C* and *D*, indicates that those cells that were still dividing (and whose nuclei thus incorporated [³H]thymidine) did not appear to

express α-cardiac actin mRNA in their cytoplasm. In contrast, cells that did express α-cardiac actin mRNA exhibited a highly elongated morphology. Thus, cells that express α-cardiac actin mRNA appear to have withdrawn from the cell cycle and entered the postmitotic state.

Fusion Blockade in Calcium-deficient Medium

To determine if α-skeletal actin mRNA expression, which was temporally correlated with fusion, was also fusion dependent, a nontoxic method was sought to prevent fusion. A

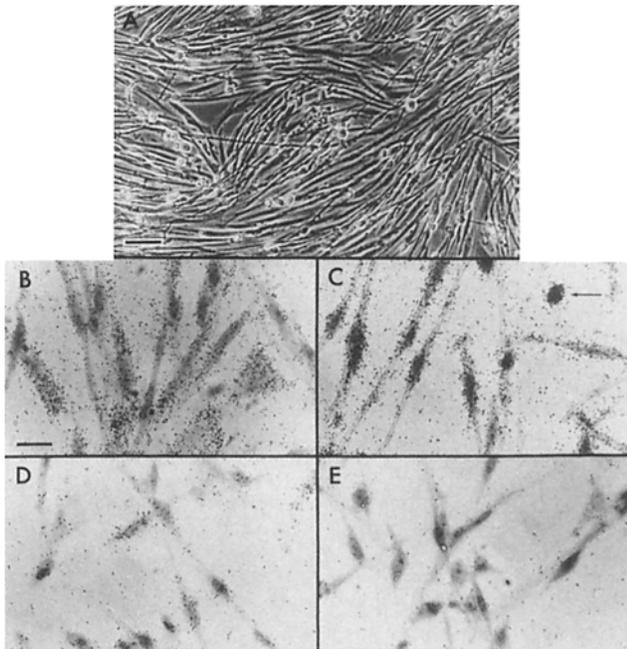


Figure 4. In situ hybridization of actin gene probes to myoblasts grown in LCM. (A) Phase-contrast micrograph of myoblasts maintained in LCM starting at 24 h and continuing to 72 h. Myoblasts were hybridized to β -cardiac (B), α -cardiac (C), α -skeletal (D), and reverse α -cardiac (E) actin mRNA probes as in Fig. 2. The arrow in C points to a rounded cell expressing α -cardiac actin mRNA. Bars: (A) 100 μ m; (B) 20 μ m.

procedure was adopted in which myoblasts were seeded in normal plating medium containing 1.8 mM Ca^{2+} and fed at 24 h with an LCM (110–150 μM Ca^{2+}). Primary myoblast cultures maintained in LCM from 24 to 72 h after plating grew to confluency but did not fuse, as shown in Fig. 4 A. These myoblasts exhibited a strikingly elongated morphology and tended to align longitudinally with each other. Upon feeding with medium containing 1.8 mM Ca^{2+} , these myoblasts rapidly fused to form myotubes within 6–12 h. The results of in situ hybridization of the ^{125}I -labeled actin mRNA probes are summarized in Fig. 4, B–E. Both β - and α -cardiac actin mRNAs were expressed in myoblasts that had not yet fused (Fig. 4, B and C). α -Skeletal actin mRNA was expressed at only very low levels, slightly above the background hybridization of the control probe (Fig. 4, D and E).

The levels of actin mRNA expression were quantitated on a total RNA basis by hybridization with specific 3' untranslated region actin probes to RNA dot blots. For this experiment, cells were maintained in LCM until 72 h after plating and then fed with either fresh LCM or normal medium. RNA was extracted at the time of the medium change and at 6, 12, and 24 h later (Fig. 5). α -Skeletal actin mRNA was expressed at low levels (<1% of the level in 3-wk breast muscle) in the cultures maintained in LCM but increased threefold by 12 h in cultures allowed to fuse in normal medium. In contrast, α -cardiac and β -actin mRNAs were expressed initially at high levels and exhibited a modest increase by 12 h of 60 and 30%, respectively, over expression in cells maintained in LCM. By 24 h, the relative increase in expression

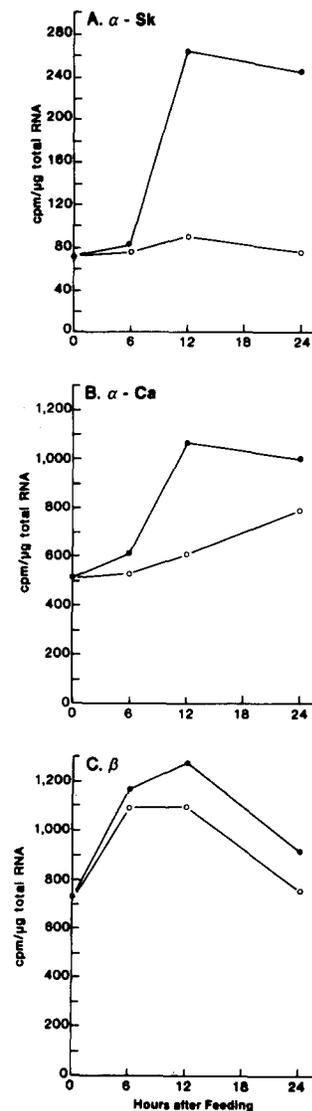


Figure 5. Expression of actin mRNAs in Ca^{2+} -deficient and Ca^{2+} -restored muscle cultures. α -Skeletal (A), α -cardiac (B), and β -actin (C) mRNA probes were hybridized to dot blots of total RNA from myoblasts maintained in LCM until 72 h after plating and then fed with either fresh LCM (○) or normal media (●). RNA was isolated at 0, 6, 12, and 24 h after the medium change. The expression of α -skeletal, α -cardiac, and β -actin mRNAs per μg total RNA in 3–4-wk breast muscle, heart, and gizzard RNA standards was 8,100, 1,150 and 1,360 cpm, respectively.

had declined to 20 and 10%, respectively. To rule out differential processing of nascent RNA as a result of growth in LCM, Northern blots demonstrated that the actin mRNAs isolated from these cultures were the same size as those from muscle cultures grown in 1.8 mM Ca^{2+} and that each actin mRNA was represented by a single band (data not shown). Thus, the α -skeletal actin gene is regulated distinctly from the α -cardiac and β -actin genes and appears to be fusion dependent.

Effect of Metalloendoprotease Inhibitors on Fusion and Actin Gene Expression

Couch and Strittmatter (9) demonstrated that MEPr inhibitors can block the Ca^{2+} -dependent fusion of rat primary myoblasts. These synthetic substrates can competitively inhibit MEPr's and block fusion in a reversible manner when added to the culture medium in the millimolar range. When the MEPr inhibitor Cbz-Ser-Leu-amide was added to unfused chicken myoblast cultures at 1.5 mM and examined 24 h later, fusion was prevented and accumulation of α -skeletal and α -cardiac mRNA was abolished (Fig. 6, A, C, and E).

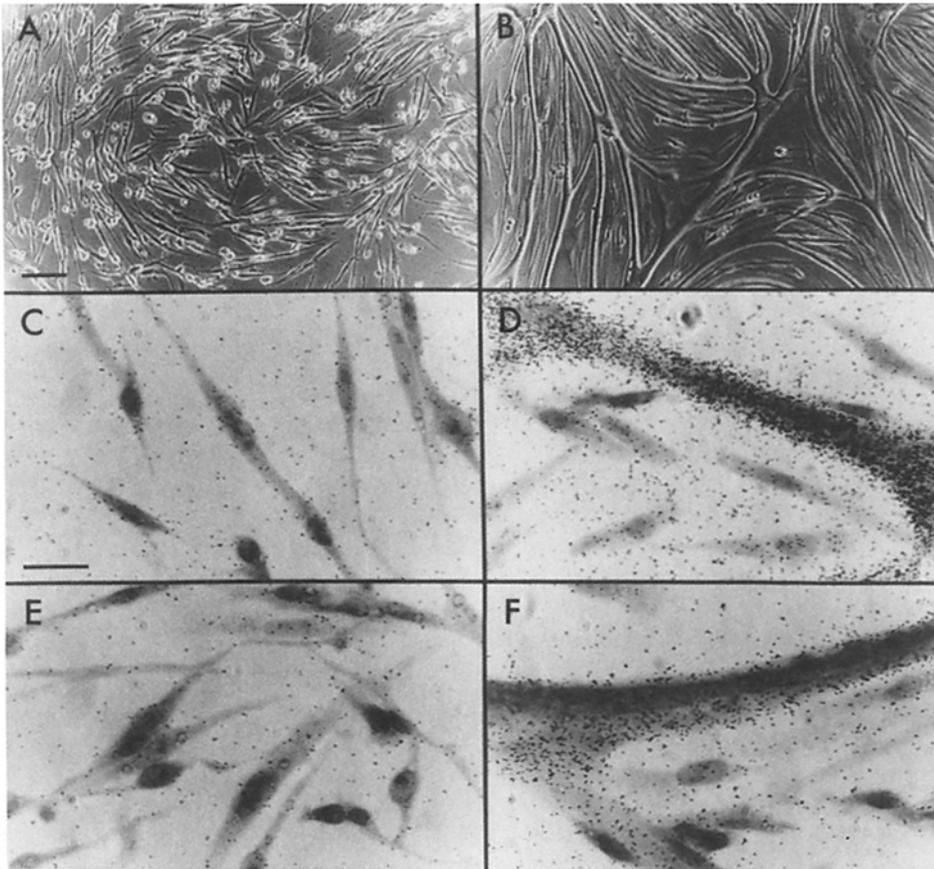


Figure 6. Effect of dipeptide metalloendoprotease inhibitors on fusion and α -striated actin gene expression. Myoblasts grown in LCM until 52 h were treated for 24 h in HCM with 1.5 mM Cbz-Ser-Leu-amide (A, C, and E) or 1.5 mM Cbz-Gly-Gly-amide (B, D, and F). Phase micrographs in A and B indicate culture morphology after 24-h treatment. ^{125}I -labeled actin gene probes to α -cardiac actin (C and D) and α -skeletal actin (E and F) were hybridized in situ as in Fig. 2. The final concentration of dipeptide solvent (dimethylformamide) was 0.75%. Bars: (A) 100 μm ; (B) 20 μm .

In contrast, cultures treated with 1.5 mM of the control dipeptide, Cbz-Gly-Gly-amide, formed normal myotubes and expressed both actin mRNAs (Fig. 6, B, D, and F).

The concentration dependence of the dipeptide amides on actin gene expression was examined in myoblasts maintained in LCM and then fed with medium containing 1.8 mM Ca^{2+} plus various concentrations of inhibitor. Fig. 7 summarizes the dot blot data for samples of total RNA from treated cultures that were hybridized with actin 3' untranslated region probes. Accumulation of α -skeletal and α -cardiac but not β -actin mRNAs was inhibited after a 24-h exposure to the dipeptide Cbz-Ser-Leu-amide (Fig. 7, open symbols). The extent of inhibition was dosage dependent with α -skeletal actin mRNA accumulation being twice as sensitive to inhibitor (50% inhibition at 0.28 mM dipeptide) as was that of α -cardiac actin mRNA (50% inhibition at 0.57 mM dipeptide). None of the actin mRNA levels were affected significantly by treatment with the control dipeptide Cbz-Gly-Gly-amide (Fig. 7, closed symbols). This experiment indicates that an inhibitor which prevents fusion in the presence of Ca^{2+} has similar inhibitory effects upon biochemical differentiation.

Actin Gene Expression in Cultures of Early Embryonic Muscle

Finally, we sought to determine if the asynchronous appearance of α -striated actin mRNAs was part of a developmental program established in early embryonic myogenic cells. This question was addressed by dissecting somites and limb buds from 3–4-d embryos and growing dissociated cells in cul-

ture. Short myotubes formed from dissociated limb buds that expressed α -skeletal and α -cardiac mRNA after 2 d in culture (data not shown). Some cultures were maintained in LCM after 18 h so that elongated myoblasts would be clearly distinguished from the abundant nonmuscle cell types present. Fig. 8 shows the hybridization of actin gene probes to somite-derived cells after 60 h in culture. β -actin mRNA was present in all cells, while α -cardiac actin mRNA was expressed only in myoblasts (Fig. 8, A and B). Binding of the

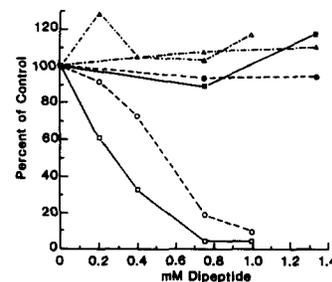


Figure 7. Concentration dependence of dipeptide MEPr inhibitors on specific actin mRNA content. Myoblasts were grown in LCM until 66 h, at which time baseline actin mRNA levels were determined. Remaining cultures were treated for 24 h in high Ca^{2+} -containing medium with 0, 0.4, 0.75, or 1.0 mM Cbz-Ser-Leu-amide (open symbols) or 0.75 or 1.33 mM Cbz-Gly-Gly-amide (closed symbols) with the final concentration of dimethylformamide solvent at 0.75% in all cases. Total RNA was isolated and hybridized with probes for α -skeletal (\square), α -cardiac (\circ), β -actin (\triangle) actin mRNAs. Binding of probes to dot blots (cpm/ μg total RNA) was normalized to the solvent control (100% activity [cpm/ μg total RNA] = 92, 568 and 294 for α -skeletal, α -cardiac, and β -actin probes, respectively).

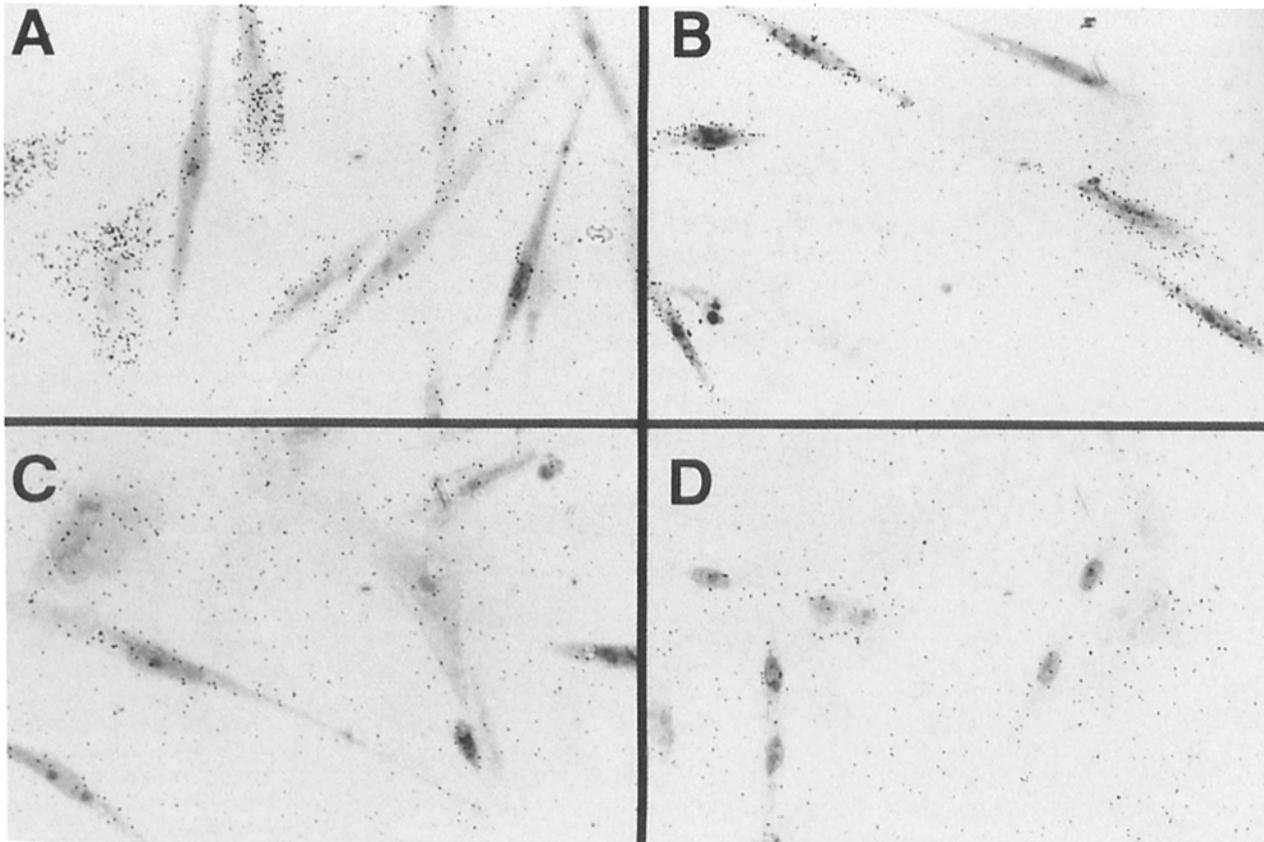


Figure 8. Expression of specific actin genes in 3-d embryonic somite cultures. Cells were dissociated from 3-d chick embryo somites and plated following the same procedure used for myoblast cultures (Materials and Methods). At 18 h, cultures were fed with LCM and spindle-shaped myoblasts became distinguishable from nonmyogenic cells. Cells were fixed at 60 h and hybridized with probes as in Fig. 2. *D* represents hybridization to the control reverse-orientation α -cardiac actin probe.

α -skeletal actin mRNA probe could not be detected above background levels (Fig. 8, *C* and *D*). Thus, myoblasts derived from embryonic muscle as early as day 3 accumulate actin mRNAs in parallel to that of cultures derived from day 11 embryonic myoblasts.

Discussion

We localized actin mRNAs at the cellular level during the developmental transition from proliferating myoblasts to postmitotic, multinucleated myotubes. Within hours after plating, dissociated myoblasts began to accumulate high levels of α -cardiac actin mRNA. In contrast, α -skeletal actin mRNA was observed to accumulate only in multinucleated myotubes and not in isolated myoblasts or fusion-arrested myoblasts. Uniform labeling of myotubes with either α -cardiac or α -skeletal actin probes implies coexpression of these genes within the same multinucleated fibers. Northern blots of total cellular RNA in earlier studies could not discern whether β -actin mRNA was expressed in fused myotubes or in nonfused myoblasts and contaminating fibroblasts. Our analysis shows that the β -actin transcripts are present in both myoblasts and fibroblasts but become reduced in content in fusing myoblasts and appear to be absent in well-differentiated myotubes. Thus, in the same myogenic cells, stage-dependent induction of the two α -striated actin mRNA spe-

cies occurs concomitantly with deinduction of the β -actin gene.

The temporal correlation between α -skeletal actin mRNA expression and the onset of fusion encouraged us to examine the extent to which α -skeletal actin mRNA expression is fusion dependent. Myoblasts maintained in LCM accumulated only low levels of α -skeletal actin mRNA but expressed both α -cardiac and β -actin mRNAs at high levels. When 1.8 mM Ca^{2+} was added back to these cultures to allow fusion, the level of α -skeletal actin mRNA increased within 12 h to a level 50% of its maximum observed *in vitro*. Thus, growth in LCM appeared to separate the appearance of α -skeletal actin transcripts from the induction of α -cardiac actin mRNA. However, this phenomenon could result from the inhibition of a Ca^{2+} -dependent process that is distinct from fusion (11). To determine if this were the case, experiments were conducted using MEPr inhibitors to block fusion in the presence of Ca^{2+} .

The MEPr inhibitor, Cbz-Ser-Leu-NH₂, prevented fusion and also inhibited accumulation of both α -skeletal and α -cardiac but not β -actin mRNAs. Baldwin and Kayalar (3) showed that Cbz-Ser-Leu-NH₂ prevented induction of muscle-specific creatine kinase activity in L6 myoblasts. They postulated that an MEPr may function in initiation of myoblast terminal differentiation. Such a proposal leaves open the question of where such a protease might act. One

possibility is that it acts at the myoblast surface in a direct step necessary for membrane fusion. In this case, triggering of muscle-specific gene expression would be secondary to the fusion process. This seems unlikely, since myotubes treated with MEPr inhibitors failed to maintain their accumulated α -skeletal and α -cardiac actin mRNAs (data not shown). Perhaps more likely, an MEPr might be necessary for myoblast entry into a differentiative pathway that includes fusion as a later step. The idea that fusion may be secondary to such a differentiative pathway is supported by experiments with other fusion inhibitors such as transforming growth factor β , which block biochemical differentiation even in non-fusing cells (12, 21). Purification and analysis of the muscle MEPr and its substrate are essential for a clearer understanding of its role in muscle differentiation.

This study has also contributed to the identification of a committed myogenic phenotype. Konigsberg (19) recognized morphological differences between myoblasts and fibroblasts and showed that virtually all bipolar cells in low-density cultures yielded purely myogenic clones, while flattened unipolar cells gave rise to fibroblastic clones. Bipolar-shaped cells in pre-fusion cultures were noted in a previous report to contain actin filaments oriented along their longitudinal axis (26). In contrast, fibroblastic cells contain multidirectional arrays of actin filaments which span the interior of the cell in broad, parallel sheets. Although we cannot directly identify the pluripotent precursor cell (1, 28) that initiates the myogenic pathway, we have demonstrated that labeling with the α -cardiac actin probe is restricted to the bipolar cell type. Also, bipolar cells derived from dissociated embryonic 3-d somites appeared to have already established a pattern of α -cardiac actin mRNA expression equivalent to that of later myoblast lineages present in embryonic 11–12-d pectoral tissue with respect to the expression of the α -cardiac actin gene.

Autoradiographs of embryonic 11–12-d pectoral myoblasts revealed that α -cardiac actin mRNA labeling increased asynchronously in pre-fusion myoblasts. By the time of fusion, almost all the bipolar cells had accumulated α -cardiac mRNA (Table II). This asynchrony reflects a cell cycle-dependent sensitivity to myoblast terminal differentiation in which myoblasts stop proliferating and begin to express muscle-specific genes at a particular point in the cell cycle. For example, Clegg et al. (8) determined that deprivation of fibroblast growth factor during the G_1 phase of the cell cycle initiates terminal differentiation of MM14 mouse myoblasts. Since virtually all mononucleated bipolar cells in culture eventually express α -cardiac actin mRNA before fusion, we suggest that the bipolar cell represents the phenotype of a committed but not necessarily terminally differentiated myogenic cell type.

The recent observations of Blau et al. and Hardeman et al. (5, 16) provide evidence that α -striated actin genes are regulated by a diffusible cytoplasmic transacting factor(s). The transfection of actin genes into myogenic and nonmyogenic cells has provided strong supporting evidence for transacting factors which stimulate the transcription of the α -striated actin genes (14, 24, 27). We observed expression of the α -cardiac and α -skeletal actin genes in a distinct, sequential manner during muscle development. These α -striated actin isoform genes share common evolutionary origins (32), genomic organization (6, 7, 13, 14), and regulatory sequences

within the promoter regions (6, 7) yet their expression can be differentially manipulated in myogenic cultures. Recent nuclear transcription runoff experiments have indicated that both α -cardiac and α -skeletal actin genes are regulated primarily at the transcriptional level for tissue- and stage-specific expression (French, B., L. J. Hayward, and R. J. Schwartz, manuscript in preparation). Thus, certain muscle-specific gene inductive factors that influence the transcription of the α -skeletal actin gene may be synthesized as part of a postfusion-dependent differentiative pathway. The sequential accumulation of α -cardiac followed by α -skeletal actin mRNA during myogenesis in culture indicates that differential regulation of these genes is sensitive to additional signals or factors that arise during myogenesis.

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