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Temporal Resolution and Sequential Expression of Muscle-Specific Genes Revealed by *in Situ* Hybridization

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The expression of muscle-specific mRNAs was analyzed directly within individual cells by *in situ* hybridization to chicken skeletal myoblasts undergoing differentiation *in vitro*. The probes detected mRNAs for sarcomeric myosin heavy chain (MHC) or the skeletal, cardiac, and β isoforms of actin. Precise information as to the expression of these genes in individual cells was obtained and correlated directly with analyses of cell morphology and interactions, cell cycle stage, and immunofluorescence detection of the corresponding proteins. Results demonstrate that mRNAs for the two major muscle-specific proteins, myosin and actin, are not synchronously activated at the time of cell fusion. The mRNA for α-cardiac actin (CAct), known to be the predominant embryonic actin isoform in muscle, is expressed prior to cell fusion and prior to the expression of any isoform of muscle MHC mRNA. MHC mRNA accumulates rapidly immediately after fusion, whereas skeletal actin mRNA is expressed only in larger myofibers. Single cells expressing CAct mRNA have a characteristic short bipolar morphology, are in terminal G_1, and do not contain detectable levels of the corresponding protein. In a pattern of expression reciprocal to that of CAct mRNA, β-actin mRNA diminishes to low or undetectable levels in myofibers and in cells of the morphotype which expresses CAct mRNA. Finally, the intracellular distribution of mRNAs for different actin isoforms was compared using nonisotopic detection of isoform-specific oligonucleotide probes. This work illustrates a generally valuable approach to the analysis of cell differentiation and gene expression which directly integrates molecular, morphological, biochemical, and cell cycle information on individual cells.

INTRODUCTION

The differentiation of skeletal muscle is characterized by the fusion of mononucleated myoblasts into multinucleated myofibers which express a variety of muscle-specific proteins (Coleman and Coleman, 1968; Paterson and Strohman, 1972; Devlin and Emerson, 1978). During the differentiation process, myoblasts first undergo a proliferative phase after which they withdraw from the cell cycle during G_1, align, and fuse together into syncytial myofibers. The biochemistry of this process has been extensively studied, and it has been shown that muscle-specific proteins are coordinately expressed at the time of cell fusion (Devlin and Emerson, 1978). While myoblast fusion has been considered a potential "trigger" for the activation of muscle-specific genes, it is now clear that cell fusion and activation of muscle-specific genes are not obligatorily connected *in vitro*; myoblasts inhibited from fusion by a variety of techniques can still terminally differentiate and produce muscle-specific proteins (Emerson and Beckner, 1975; Chi *et al.*, 1975; Konieczny *et al.*, 1982). However, under normal conditions both *in vitro* and *in vivo*, terminal differentiation generally occurs concomitant with cell fusion (Coleman and Coleman, 1968).

To elucidate factors which may be involved in controlling the terminal differentiation of skeletal myoblasts, it is important to define the sequence of gene expression events involved in myogenesis. The time of expression of muscle-specific mRNAs has been studied using conventional hybridization procedures which involve extraction of RNA from heterogeneous cell populations derived from tissues or cultures (Devlin and Emerson, 1979; Paterson *et al.*, 1974; Saidapet *et al.*, 1984). The general conclusion from such studies is that different muscle-specific genes are activated at approximately the same time and that activation of these genes is coincident with cell fusion. The presence of muscle-specific mRNAs in a culture has been found, in most cases, to closely parallel synthesis of the corresponding protein (Devlin and Emerson, 1978, 1979). Therefore the control of skeletal muscle differentiation is thought to be primarily, if not entirely, at the transcriptional level. However, analysis of mixed cell populations allows only an approximation of the timing of this process. Finer resolution of the sequence of events is possible through the use of *in situ* hybridization, which allows a more precise descriptive analysis of specific mRNA expression in individual cells of particular morphotypes.

A primary objective of earlier methodological work for optimization of *in situ* hybridization to mRNA of cultured chicken skeletal muscle (Lawrence and Singer, 1985) was to describe the molecular events of myogen-

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esis at the single cell level in their full morphological context. In the work presented here, we have investigated the two major classes of differentiation specific genes expressed during skeletal myogenesis: myosin and actin. We have focused on the sarcomeric myosin heavy chain and the sarcomeric α-actins. In addition, we have investigated the repression of the β-actin isoform present in nonmuscle cells. With regard to α-actin, it was of particular interest to distinguish between the expression of two distinct isoforms, α-cardiac actin (CAct) and α-skeletal actin. The former has been shown to be the major form present during the early differentiation of embryonic skeletal muscle *in vitro* and *in vivo* (Paterson and Eldridge, 1984; Chang *et al.*, 1984; Gunning *et al.*, 1983). Filter hybridizations have shown that these two actin isoforms are expressed sequentially with the cardiac isoform mRNA present at significant levels in cultures at early stages of fusion, whereas skeletal actin mRNA levels accumulate appreciably only after fusion is nearly complete (Hayward and Schwartz, 1986). In the work presented here we have investigated the kinetics of expression of MHC mRNA relative to cardiac, skeletal and nonmuscle actin mRNAs during the differentiation *in vitro* of chicken embryonic myoblasts. In addition we have used immunofluorescence for MHC and cardiac actin to correlate the production of these proteins with the expression of their corresponding mRNAs. Finally, previous observations as to the intracellular localization of actin mRNA (Lawrence and Singer, 1986a) have been extended to a comparison of mRNA distributions for different isoforms.

MATERIALS AND METHODS

Cell Culture and Fixation

Skeletal myoblasts were isolated from the pectoral muscles of 12-day chicken embryos. Muscle tissue was minced and centrifuged in Hank's balanced salt solution (HBSS) without Ca and Mg. The tissue was then incubated at room temperature in 1% trypsin (Worthington) in HBSS for 10 min and cells were dissociated further by a brief vortexing. Prior to final plating, the cell suspension was preplated for 30 min on nongelatinized plastic to allow fibroblasts to attach preferentially, thus enriching the cell suspension for myoblasts. Cells were plated at a density of 2×10^6/100-mm plate into plates containing either tissue culture plastic coverslips (Lux) or glass coverslips which had been previously autoclaved in 0.5% gelatin. Culture medium consisted of Dulbecco's modified essential medium (DMEM), 10% fetal calf serum, 2% chicken serum or 2% embryo extract, and either penicillin–streptomycin or gentamycin as antibiotics. Medium was replaced after 2 days of culture and cultures were incubated at 37°C in a humidified chamber with 5% CO_2.

Cultures were rinsed twice in HBSS and fixed for 15 min at room temperature in 4% paraformaldehyde (Fisher) in phosphate-buffered saline (PBS) containing 5 m*M* $MgCl_2$. Coverslips containing cells were then stored in 70% ETOH at 4°C until use.

Hybridization

Derivation and details of the hybridization protocol have been published previously (Lawrence and Singer, 1985; Singer, *et al.*, 1986). Cells were removed from 70% ETOH and rehydrated in PBS plus 5 m*M* $MgCl_2$ for 10 min, followed by 10 min in 0.1 *M* glycine, 0.2 *M* Tris–HCl, pH 7.4. Just prior to hybridization, coverslips were warmed to 65°C for 10 min in 50% formamide, 2× SSC. Each coverslip was then placed cell side down on parafilm on a 20-μl drop of hybridization solution consisting of 10 ng probe DNA, 10 μg sonicated salmon sperm DNA (Sigma), and 40 μg *Escherichia coli* tRNA (Boehringer) in 50% formamide, 2× SSC (0.3 *M* sodium citrate buffer), 1% BSA, 10 m*M* vanadyl sulfate, 100–300 m*M* DDT, and 10% dextran sulfate (Sigma). Hybridization was for 3 to 4 hr at 37°C. Coverslips were rinsed three times for 30 min each in 2× SSC, 50% formamide at 37°C, 1× SSC, 50% formamide at 37°C, and 1× SSC on a shaker at room temperature. Hybridization of the oligonucleotide probe was as above except that hybridization was for 1 hr and 2 ng of probe was applied per sample, as previously described (Taneja and Singer, 1987).

Cells to be processed for autoradiography were dehydrated through 70, 95, and 100% ethanol and air-dried. Autoradiography was performed as previously described (Lawrence and Singer, 1986a), using undiluted Kodak NTB-2 photographic emulsion. Exposure time for ^{35}S was from 3 to 6 days and for ^{3}H was 5 to 8 weeks at 4°C. Slides were developed in D19 followed by Kodak fixer and stained either with the DNA fluorochrome, DAPI (diaminophenylindole), or toluidine blue. For the detection of biotinated probes, an enzymatic method was employed using alkaline phosphatase linked to avidin. Details of this method have been previously described (Singer *et al.*, 1986).

Muscle cultures were fixed on Days 1 through 4 after plating and hybridized *in situ* with ^{35}S- or ^{3}H-labeled probes in order to evaluate the timing of expression of certain differentiation-specific mRNAs. The distribution of autoradiographic label was evaluated in parallel samples at progressive stages of differentiation in randomly chosen fields under 40× objective magnification. Single cells were evaluated for grain density, multinucleation, morphology, and, in some cases, immunofluorescence and cell cycle stage (see below).

Probes

The α-cardiac actin probe was provided by Bruce Paterson and consisted of a cDNA clone in pBR322 complementary to 600 nucleotides of the 3′ end of cardiac actin mRNA, 300 nucleotides of which were complementary to the coding region (Eldridge *et al.*, 1985; Chang *et al.*, 1984). Similarly the cDNA clone for α-skeletal actin is specific for this isoform since it encodes 200 nucleotides of the 3′ nontranslated region of this mRNA (Fornwald *et al.*, 1982). The myosin heavy chain cDNA clone contains 5.9 kilobases of sequences complementary to the mRNA (Molina *et al.*, 1987). The clone includes coding sequences that are highly conserved among sarcomeric isoforms; therefore, this clone cross-hybridizes with different sarcomeric MHC isoforms but not the cytoplasmic forms (Gulick *et al.*, 1987). The oligonucleotide probe was made to a 51-nucleotide region of cardiac actin mRNA (residues 1630–1680 in the coding region; Taneja and Singer, 1987) which has 36 and 73% homology to β- and α-smooth muscle actin, respectively. Under the stringent conditions of hybridization, these homologies are insufficient to maintain stable hybrids. The oligonucleotide probes for β-actin were made to nucleotides 3061–3111 and 1442–1495 in the coding region (Kost *et al.*, 1983). Double-stranded probes were labeled by standard nick-translation to a specific activity of $2\text{–}5 \times 10^8$ cpm/μg for ^{35}S or $1\text{–}3 \times 10^7$ cpm/μg for ^3H-labeled probes. Oligonucleotide probes were end-labeled using terminal transferase with sulfonated dATP to a specific activity of 10^9 cpm/μg. Oligonucleotides to be detected nonisotopically were labeled with biotin-11-dUTP (BRL) using terminal transferase (Boehringer) and detected with steptavidin conjugated to alkaline phosphatase (Dako; Taneja *et al.*, in preparation).

Immunofluorescence

Slides were reacted with antibodies after hybridization but prior to coating with autoradiographic emulsion (see Singer *et al.*, 1987, for details of double labeling). Prior to applying antibodies, cells were treated with 0.5% Triton in PBS for 30 min. The myosin heavy chain antibody, XM1B, is a mouse monoclonal antibody specific for muscle myosin (Konieczny *et al.*, 1982) and was provided by John Coleman. This antibody was applied undiluted in culture medium for 30 min and was detected by rhodamine anti-mouse IgG (Zymed). The α-actin antibody has been described previously (Bulinski *et al.*, 1983). This is a rabbit polyclonal antibody made against a synthetic peptide which recognizes both the cardiac and skeletal α-actin isoforms. It was used at a 1:10 dilution and detected with rhodamine anti-rabbit IgG (Zymed).

RESULTS

Using probes to three actin mRNAs (β, α-cardiac, and α-skeletal) and to sarcomeric myosin heavy chain mRNA, *in situ* hybridization allowed detailed resolution of the timing of expression of each of these sequences. By combining this molecular technique with morphological, biochemical, and cell cycle analyses of each individual cell, it was possible to demonstrate a unique pattern of expression to each of these four genes and to correlate precisely their temporal expression with other events in myogenesis.

The major part of our work concerned analysis of genes activated during muscle differentiation: Sarcomeric MHC and sarcomeric α-actin (cardiac and skeletal). The results of mRNA analysis for two representative experiments are presented quantitatively in Fig. 1 and pictorially in Figs. 2 and 3. Figure 1 shows results of *in situ* hybridization of the three markers for muscle gene expression in mononucleated cells (A) and multinucleated myofibers (B).

Analysis of MHC mRNA in individual cells showed a pattern of expression expected of a gene activated concurrent with fusion. One day (20 hr) after plating, when approximately 20% of the nuclei in the culture had been incorporated into multinucleated myotubes, all myotubes were heavily labeled for MHC mRNA, even the smallest (and most recently differentiated) binucleated myofibers (Figs. 1B and 2A–2C). In contrast, almost all mononucleated cells were clearly negative (Fig. 1A). The abundance of label over the smallest binucleate myofibers indicated that MHC mRNA expression was striking in its sudden onset. It was occasionally observed that a negative "myoblast" may have progressed to an advanced stage of fusion in that it appeared, using phase-contrast optics, to have conjoined a heavily labeled myofiber through a thick cytoplasmic bridge, but had not yet expressed MHC mRNA (Fig. 2C). While a small fraction (6%) of single cells labeled heavily for MHC mRNA, these invariably had the highly elongated, bipolar morphology characteristic of terminally differentiated "myocytes" which have been shown to express the same muscle-specific properties as small myofibers (see Coleman and Coleman, 1968; Konieczny *et al.*, 1982; see Fig. 2D and Discussion). As the percentage of nuclei in myofibers increased to 60% on Day 4, all multinucleated cells labeled heavily for MHC mRNA. Mononucleated cells were negative except for the progressively less frequent presumptive "myocyte," which constituted less than 1% of the population by Day 3.

Unlike the expression of MHC mRNA, cardiac actin mRNA was detected in a substantial number of mononucleated cells during the period of active cell fusion (Figs. 1A, 2E, and 2F). In Day 1 cultures an average 27%

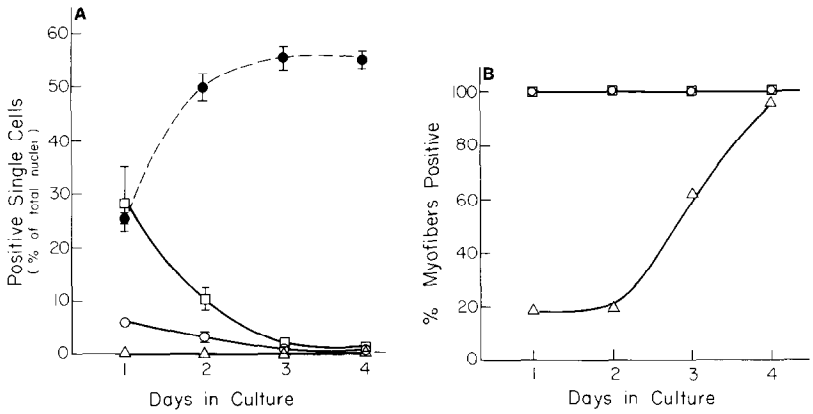
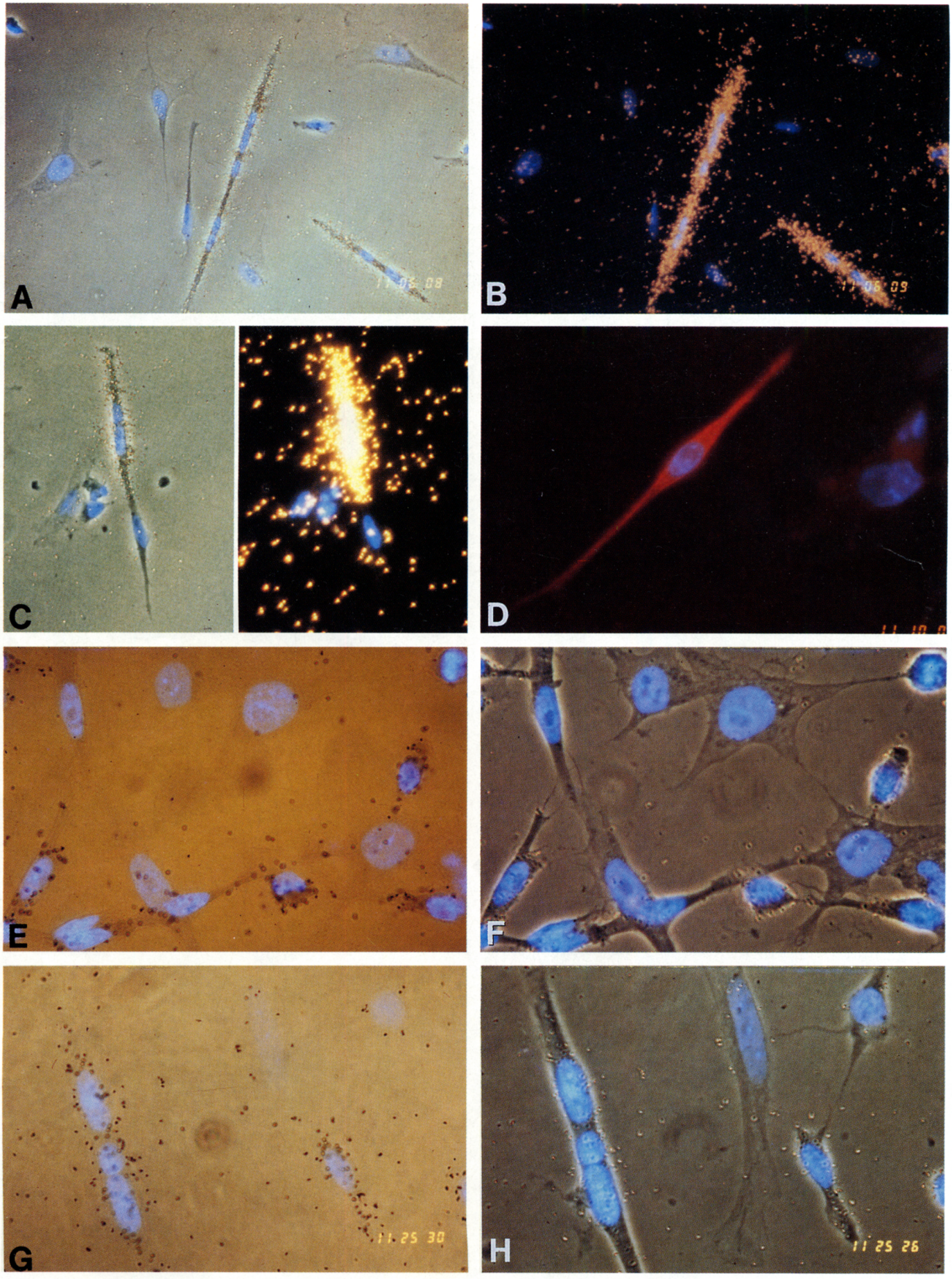


FIG. 1. Quantitative summary of muscle-specific mRNA expression *in vitro*. Cells were hybridized *in situ* with ^{35}S-labeled probes to (○) myosin heavy chain mRNA, (□) cardiac actin mRNA, or (△) skeletal actin mRNA. (A) Expression of mRNAs in mononucleated cells, presented as a percentage of total nuclei in the culture. Cells were scored as positive for an mRNA if the density of grains over the cell was a minimum of fivefold greater than the average grain density over unlabeled cells in the same culture or over RNase-treated samples or samples hybridized with pBR322 probe under identical circumstances. For comparison, the (●--●) broken curve indicates the kinetics of fusion in the same cultures (expressed as the percentage of total nuclei in myofibers). (B) Expression of mRNAs in myofibers, presented as the percentage of myofiber nuclei in labeled myofibers. Symbols are the same as in A.

of nuclei were in mononucleated cells labeled for cardiac actin mRNA, which decreased progressively to a low of 1% by Day 4. Since cardiac actin is a sarcomeric isoform present only in muscle tissues (Paterson and Eldridge, 1984), it is reasonable to suggest that single cells expressing this mRNA are in the early stages of terminal differentiation just prior to fusion into multinucleated myofibers. The kinetics of cell fusion, as indicated by the percentage of multinucleated cells during progressive days of culture (Fig. 1A), closely mirrors the decrease of CAct-positive single cells. This inverse correlation between the number of CAct-positive single cells and the number of cells undergoing fusion further supports the suggestion that cells expressing CAct mRNA are in the process of differentiating into myotubes. As observed for MHC mRNA, even the smallest myofibers were always labeled for CAct mRNA (Fig. 1B). Hence, expression of CAct and MHC mRNAs differed primarily in the single cell population, with CAct mRNA being expressed in a large number of morphologically distinct single cells (see below) as well as in myotubes (Figs. 3A and 3B).

Skeletal actin mRNA displayed a third pattern of expression during terminal differentiation. Single cells were always negative for this mRNA (Fig. 1A). On Days 1 and 2 of culture most myofibers were negative as well. In general small myofibers (two to eight nuclei) did not exhibit label for skeletal actin mRNA. Larger myofibers (greater than 10 nuclei) were weakly to moderately positive, as judged by significantly increased grain densities relative to mononucleated cells. Increases in grain density were not observed over RNase-treated myofibers in the same experiment. Half of the myofibers were expressing skeletal actin mRNA by Day 3 (see Figs. 1B, 3C, and 3D), and by Day 4 almost all myotubes were unambiguously labeled with grain den-

FIG. 2. A-C and E-H show autoradiographic detection of *in situ* hybridization with ^{35}S-labeled probes to muscle-specific mRNAs. (A, B) Myosin heavy chain mRNA detection in very early (1 day) myofibers. Note that even small binucleate myofibers label heavily, while mononucleated cells are negative (275×). (A) Phase photograph with a double exposure for DAPI nuclear fluorescence. (B) Dark-field image to show distribution of silver grains, with DAPI nuclear fluorescence. (C) MHC mRNA detection in fusing cells. Unlabeled myoblasts which apparently have fused or are in the process of fusing with a labeled myofiber are observed. Left, Phase/DAPI; right, dark-field/DAPI (275×). (D) Rhodamine immunofluorescence for myosin heavy chain protein, with DAPI nuclear fluorescence (blue). Photograph shows a mononucleated cell expressing MHC protein. This cell shows the characteristics of a myocyte: a highly attenuated bipolar morphology, a condensed nucleus, and expression of muscle-specific proteins confined to myofibers (700×). (E, F) Expression of cardiac actin mRNA in mononucleated cells of a 1-day culture, detected using a recombinant clone for the 3′ end of cardiac actin mRNA. Note the condensed nature of the nuclei in positive cells. Upper right corner shows a rounded mitotic cell which does not label (700×). (E) Transmitted light to show label, with DAPI nuclear fluorescence. (F) Phase/DAPI for cell morphology. Note preservation of morphological detail and fine cellular contacts throughout the hybridization procedure. (G, H) Cardiac actin mRNA detected using an oligonucleotide probe in a 1-day culture. Field shows a small labeled myofiber, one labeled cell, and an unlabeled cell. Note the similar grain densities over the labeled single cell and the myofiber, as well as the similarity in nuclear morphology. (G) Transmitted light/DAPI to show label. (H) Phase/DAPI to show morphology. Note that the labeled single cell has approximately the same density of grains as the small myofiber (700×).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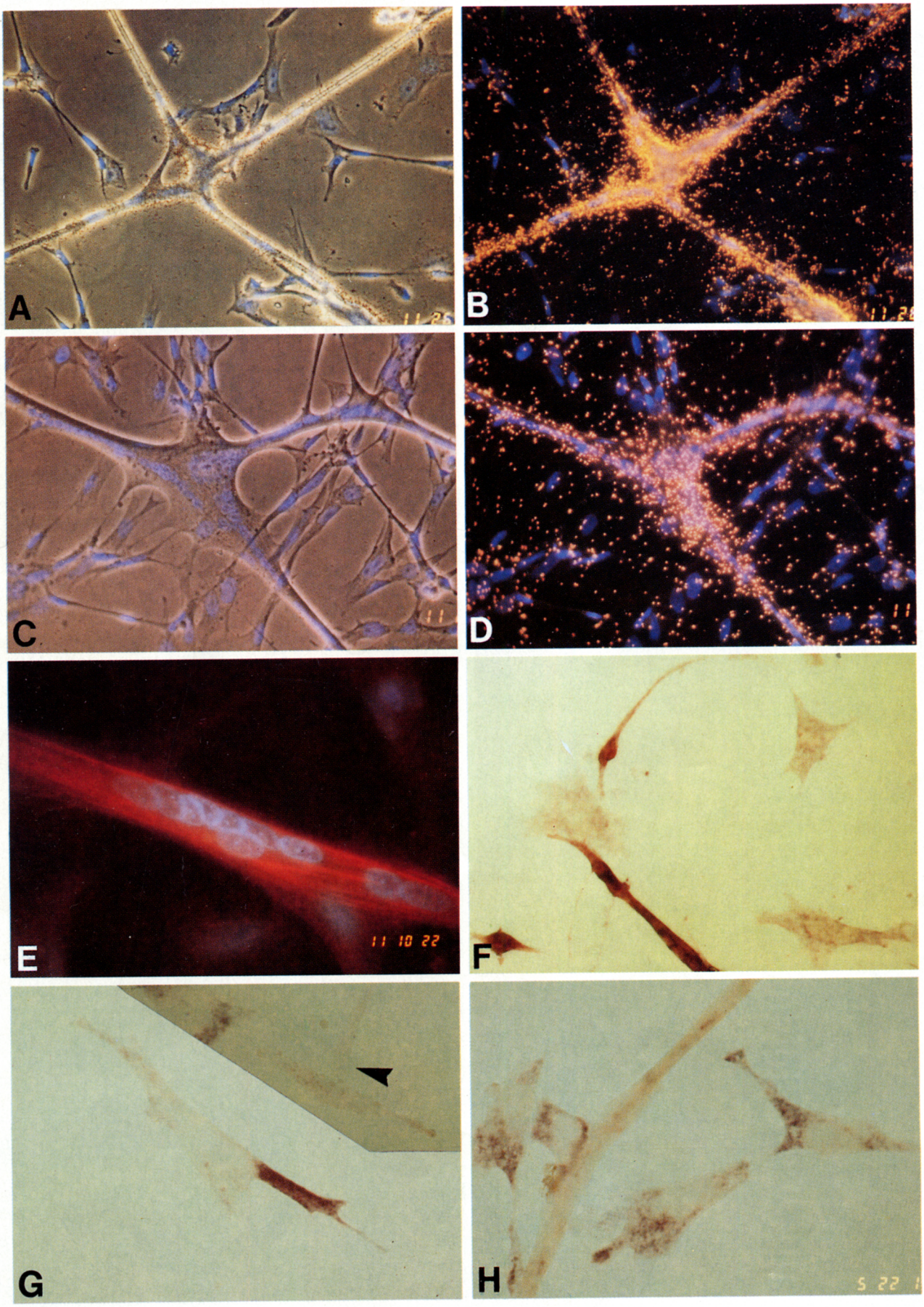
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sities similar to parallel samples hybridized for cardiac actin mRNA. These results are consistent with filter hybridization studies (Hayward and Schwartz, 1986) showing that SKAct mRNA is not present in significant levels until Days 3 and 4 of culture, but augment these results at a single cell level by indicating the point of myofiber development when this mRNA is expressed (some of these positive myofibers are present very early in culture).

The above results indicate that cardiac actin mRNA is expressed prior to cell fusion and prior to the expression of both MHC mRNA and skeletal actin mRNA. These results were obtained in several experiments using a probe for the 3′ end of cardiac actin mRNA (Eldridge *et al.*, 1985). This CAct probe appeared to show little or no cross-hybridization with β-actin mRNA, as evidenced by the specific labeling of only myofibers in Days 3 and 4 cultures, and not single cells. However, to confirm that the high fraction of single cells positive for CAct mRNA on Days 1 and 2 were not due to cross-hybridization to β-actin mRNA, hybridizations were performed using an oligonucleotide probe representing a 51-nucleotide sequence of the cardiac actin mRNA (see Experimental Procedures). We have shown that this probe does not hybridize to isoforms other than cardiac actin under the conditions employed (Taneja and Singer, 1987). As illustrated in Figs. 2G and 2H, hybridization with this synthetic CAct probe, produced equivalent results, with 30% of mononucleated cells labeling on Day 1, compared to 27% with the untranslated region probe.

At the other extreme, we were interested in hybridizing to all the muscle isoforms of MHC mRNA; for this mRNA we used a 6-kb, full-coding sequence MHC probe to verify the maximal number of single cells expressing any isoform of sarcomeric MHC mRNA (Figs. 2A–2C). This probe has been shown to cross-hybridize with sarcomeric MHC mRNAs, including the cardiac MHC isoform but not cytoplasmic isoforms (Molina *et al.*, 1987). Even with this general probe, MHC mRNA was detected in only a maximum of 7% of mononucleated cells at Day 1 of culture. These results confirm the presence specifically of the α-cardiac isoform of actin mRNA in a population of mononucleated cells in which mRNA for sarcomeric isoforms of MHC were undetectable. It should be emphasized that the cardiac actin was detected with a 51-nucleotide oligomer and that sarcomeric MHC was undetectable even with a 9-kb probe (presumably 180 times more sensitive).

Morphological Observations on Mononucleated Cells Expressing Cardiac Actin or MHC mRNA

In cell populations undergoing active cell fusion, categorizing cells as simply "mononucleated" or "multinucleated" does not fully represent the more precise morphological information afforded by *in situ* hybridization. Above results suggest that CAct mRNA-positive single cells eventually become incorporated into myofibers. These cells may be in a very early stage of cell fusion prior to myotube formation. Therefore CAct mRNA-positive mononucleated cells from the experiment in Fig. 1 were further classified into two general categories: (1) cells clearly *not* in the process of fusing with another cell, and (2) cells which may be in the process of fusing with another cell. Time-lapse microscopy, as well as electron microscopy, has shown that cell fusion is typically preceded by close alignment of bipolar myoblasts or the extension of a process from one myoblast to another myoblast or myofiber (Lipton and Konigsberg, 1972; Powell, 1973; Huang *et al.*, 1978). Myoblasts engaged in this intimate contact, but for which membranes did not appear to have fused as judged by high magnification phase-contrast optics or immunofluorescence (see below), were scored as single cells that "may be fusing." However, if the cytoplasm of a cell appeared to be contiguous with another cell or myofiber, even if only through a cell process, then this was considered to be part of a myofiber and not a mononucleated cell. Of the CAct mRNA-positive single cells, slightly more than half were not engaged in cell fusion by the criteria stated above, whereas some of the other 49% may have been just beginning to undergo fusion.

FIG. 3. (A, B) Detection of cardiac actin mRNA (cloned probe) in muscle fibers within a 3-day culture (275×). (A) Phase/DAPI. (B) Dark-field/DAPI to highlight autoradiographic label. (C, D) Detection of skeletal actin mRNA in muscle fibers within a 3-day culture. Large myofibers are clearly labeled, but often to a lesser extent than samples treated identically for detection of cardiac actin mRNA with probes of comparable specific activity (A, B) (275×). (E) Rhodamine immunofluorescence for α protein in a 2-day culture, with DAPI nuclear fluorescence. Intense staining of myofibrils is observed in myotubes. Mononucleated cells with fluorescent staining above background levels are not observed (700×). (F) Alkaline phosphatase detection of cardiac actin mRNA in a 2-day culture after hybridization with one biotinated oligonucleotide probe. Fibroblasts show low levels of background staining, whereas the myofiber and two single cells stain darkly. Note that the cell in the lower left shows characteristic short bipolar morphology. Label is distributed throughout both positive single cells with the central regions staining darker. The background level of staining in the fibroblast cells resulted from the long time (4 hr) of color development for alkaline phosphatase (275×). (G) Done as in F, except two oligonucleotide probes used were for β-actin mRNA which is heavily localized in lamellipodia. Inset shows a short bipolar cell and part of a small myotube from the same sample (arrow) which did not stain for β-actin mRNA. Color development was for 1 hr (440×). (H) Same as in G, detection of β-actin mRNA. Detection of single cells is evident and the myotube in the field contains much less β-actin mRNA than cardiac actin mRNA, compared to the myotube in F. Same development time as in G (440×).

We conclude that cardiac actin mRNA is found in a substantial fraction of mononucleated cells for which fusion is not imminent, demonstrating that this mRNA is expressed prior to the earliest stages of myofiber formation.

Further morphological observations were possible using this approach. Many single cells expressing cardiac actin mRNA were morphologically distinct from the smaller fraction of single cells which express MHC mRNA. The latter were generally highly elongated bipolar myoblasts, whereas the cardiac actin mRNA-positive cells were most typically short (less than 20 μm) bipolar "spindle-shaped" cells (see Figs. 2E, 2F, and 3F). This difference was most pronounced on Days 1 and 2 of culture. For instance 65% of single cells expressing cardiac actin mRNA on Day 1 were characterized as short bipolar cells. In contrast, for MHC mRNA 18 cells out of 20 showed the extremely attenuated, thin morphology typical of single cells expressing this mRNA and protein (see Fig. 2D). Short bipolar myoblasts positive for cardiac actin mRNA were scored as possibly fusing with some frequency (38/90), since clusters of single cells beginning alignment often expressed this mRNA. Essentially none of the cells expressing cardiac actin mRNA exhibited flattened fibroblastic morphology, whereas all these cells expressed β-actin (see below).

Immunofluorescence Analysis of α-Actin and Myosin Proteins

Indirect immunofluorescence was performed to determine whether single cells expressing cardiac actin mRNA or MHC mRNA also expressed the corresponding protein. Cell cultures were stained with antibodies made against a synthetic peptide of α-actin which reacts to both the skeletal and cardiac isoforms (Bulinsky *et al.*, 1983) or a monoclonal antibody to myosin (Konieczny *et al.*, 1982). Analysis of individual cells evaluated simultaneously for cardiac actin mRNA or MHC mRNA and their cognate proteins demonstrated that all of the single cells expressing myosin mRNA also contained myosin protein (Fig. 2D). In contrast to this result, cells expressing cardiac actin mRNA did *not* express detectable levels of cardiac actin protein. Because the hybridization process and autoradiography weaken the immunofluorescence staining slightly, the experiment was repeated where one immunofluorescence sample was analyzed directly and the other was hybridized with a probe for the corresponding mRNA and processed for autoradiography. Once again, there was a very close correlation between expression of MHC as judged by immunofluorescence and by *in situ* hybridization to mRNA. The immunofluorescence staining (Table 1) shows that 5% of nuclei on Day 1 were in single cells positive for protein, very close to the 6% which had been found in parallel samples analyzed for mRNA expression *in situ* (Fig. 1A). The previous result on mononucleated cells positive for cardiac α-actin mRNA (Fig. 1A) was also repeated using parallel samples; these cells did not express detectable immunofluorescence for α-actin protein. Almost all single cells showed only a low level of background fluorescence. Most small myofibers exhibited moderately bright staining and large myofibers stained very brightly (Fig. 3E). Hence, significantly fewer cells were scored positive for α-actin protein as compared to cardiac actin mRNA (Table 1 versus Fig. 1A). These results indicate that many single cells containing significant quantities of cardiac actin mRNA do not express detectable levels of the corresponding protein (see Discussion).

Cell Cycle Analysis

It is well documented that as skeletal myoblasts undergo terminal differentiation they withdraw from the cell cycle during the G_1 phase, resulting in postmitotic myofiber nuclei which express a variety of muscle-specific products (see Holtzer *et al.*, 1975; Hill *et al.*, 1986). If single cells expressing cardiac actin mRNA are indeed in an early stage of terminal differentiation, it might be expected that they would be noncycling, G_1 stage cells. This was also suggested by the somewhat condensed appearance of their nuclei. To determine their cell-cycle stage, the DNA content of individual cells was measured by microfluorimetry of nuclei stained with the DNA fluorochrome, DAPI. As previously described, DAPI fluorescence is directly proportional to DNA content and provides a reliable tool for determining cell cycle stage in a given cell population (Coleman *et al.*, 1981).

After *in situ* hybridization and autoradiography, cultures were stained with DAPI and individual cells were

TABLE 1
IMMUNOFLUORESCENCE ANALYSIS OF α ACTIN AND MHC PROTEIN EXPRESSION

	Day 1		Day 2		Day 3	
	Actin	MHC	Actin	MHC	Actin	MHC
+ Single Cells	1-3[a]	5	0-1	2	0	0
− Single Cells	66	69	43	40	32	34
+ Myofiber	21	25	47	57	68	66
− Myofiber	14	0	9	0	0	0

Note. Results are presented as percentages of total nuclei. At least 500 nuclei were scored on each day for each antibody.

[a] A range is presented to indicate the upper limit obtained when cells with immunofluorescence marginally above background are scored as positives.

simultaneously evaluated for mRNA expression and DNA content. Results are presented in Fig. 4. Since myofiber nuclei have G_1 DNA content, measurement of individual myofiber nuclei provides an internal standard for the fluorescence intensity of G_1 cells. As expected, data from myofiber nuclei fall into a relatively homogeneous population ranging from 10–14 arbitrary fluorescence units. In contrast, measurement of DNA content for single cells not expressing cardiac actin mRNA (cycling myoblasts and fibroblasts) results in a much broader range of fluorescence intensities (11–28), reflecting the S phase and G_2 content of cells in this population. Analysis of single cells expressing cardiac actin mRNA on the same slide reveals that the DNA content profile of this cell population, ranging from 10–14 fluorescence units, is identical to that of the myofiber nuclei rather than that of the cycling cells. These results indicate that cardiac actin-positive single cells have G_1 DNA content and may have withdrawn from the cell cycle, further substantiating the view that these represent a distinct subset of cells early in the terminal differentiation process.

Expression and Intracellular Localization of β- and cardiac actin mRNAs

The objective of the above experiments was to analyze the expression of genes activated during terminal differentiation, hence expression of "constitutive" β-actin was not a focus of these studies. However, having detected cardiac actin mRNA in a subset of mononucleated cells, it was then of interest to compare the intracellular distribution of this mRNA with that of β-actin mRNA, which previous work had shown to concentrate at the cell periphery, in lamellipodia, and in cell protrusions (Lawrence and Singer, 1986a). In the above experiments, using ^{35}S-labeled probes and subsequent autoradiography, CAct mRNA did not appear to exhibit a similar localization pattern in expressing single cells; however, it was necessary to evaluate this question using higher resolution techniques. We approached this problem in two ways, using cardiac or β-actin mRNA-specific probes which were tritium-labeled and detected with autoradiography or biotinated specific oligonucleotide probes detected by avidin-alkaline phosphatase. Both approaches allowed us to compare the intracellular distribution of the respective mRNAs and at the same time revealed a consistent reciprocal relationship of the expression of these two mRNAs in different cell types.

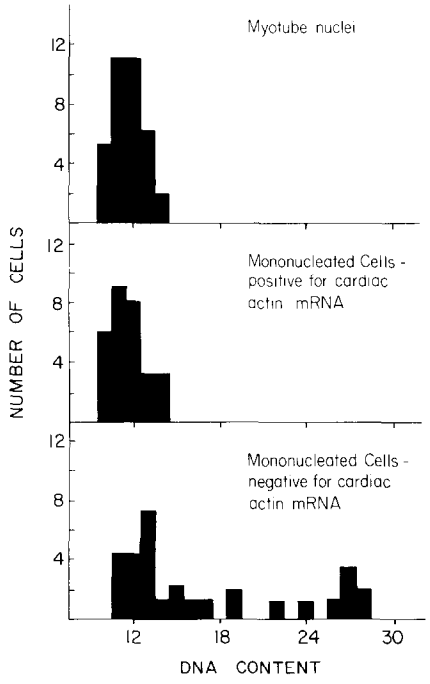

FIG. 4. Fluorimetric measurements of DNA content. After hybridization and autoradiography, cultures were stained with the DNA fluorochrome, DAPI, and the relative DNA content of individual nuclei was determined using microfluorimetry (see Coleman *et al.*, 1981). DNA content is expressed in arbitrary units.

Hybridization of biotinated oligonucleotides to the cardiac and β-actin isoforms, detected with avidin-alkaline phosphatase, is shown in representative photomicrographs presented in Figs. 3F–3H. This nonisotopic analysis confirmed the presence of CAct mRNA in single cells; approximately 17% were positive on Day 2 of culture, in accord with previous autoradiographic results (Fig. 1A). In addition these results revealed a significant relationship in the expression of β- and cardiac actin isoforms. Whereas the CAct probe stained myofibers and some bipolar mononucleated cells heavily (Fig. 3F), the β-actin probe stained fibroblastic cells most heavily, with comparatively little stain in myofibers (Figs. 3G and 3H). Single cells with the characteristic myocyte or short spindle morphology (presumptive CAct-positive cells) frequently showed little label with the β isoform (Fig. 3G, inset). These results indicate that the expression of β-actin mRNA most likely decreases, prior to and after fusion, in a pattern reciprocal to that of CAct mRNA.

With regard to intracellular localization, the nonisotopic hybridization with the β-actin oligonucleotide confirmed our previous finding of preferential localization of this mRNA at the cell periphery and in lamellipodia (Fig. 3G). As suggested from our autoradiographic results (Figs. 2E and 2F), the alkaline phosphatase detection of CAct mRNA showed that this mRNA was distributed throughout the cell (Fig. 3F) sometimes with higher concentrations near the nucleus, a pattern quite distinct from that of β-actin mRNA. This suggested that mRNAs for the cardiac and β-actin isoforms either were differentially localized within a

given cell type or their respective distribution was characteristic of the different cell types in which they were expressed. Our analyses showed that fibroblastic or myoblastic cells with well-defined lamellipodia showed pronounced localization of β-actin mRNA but did not express CAct mRNA. The small fraction of single cells which expressed CAct mRNA on Day 2 or 3 of culture had a more rounded, spindle-shaped morphology. While cells of this morphotype contain substantially less β-actin than CAct mRNA, the intracellular distribution of these mRNAs is not significantly different; both mRNAs appear to be present throughout the cell with peak concentrations near nuclei. Finally, with regard to intracellular distribution of mRNAs in myofibers, our analyses showed that myofibers consistently labeled throughout for both cardiac actin mRNA and MHC mRNA; however, it was noted for both mRNAs that regions containing many nuclei were often more heavily labeled than regions devoid of nuclei.

DISCUSSION

Using an approach which allowed molecular analysis of individual cells undergoing skeletal myogenesis *in vitro*, we have characterized the expression of muscle-specific genes to an extent not feasible by conventional filter hybridization techniques. Microscopic analysis of individual cells allowed precise conclusions as to the presence of certain nucleic acid sequences, coincident with several other cytologic features including morphology, cell interactions, cell cycle, and immunofluorescence for specific proteins. The direct integration of molecular, morphological, biochemical, and cell-cycle analysis on individual cells provides a powerful and comprehensive approach generally applicable to the analysis of cell differentiation. Application of this *in situ* hybridization approach to populations of cells cultured from embryonic pectoral muscle revealed a defined sequence of expression for several muscle-specific markers. As outlined in Fig. 5, our results indicate that the following sequence of events occurs during the terminal differentiation of skeletal myoblasts *in vitro:* a proliferative phase involving expression of β-actin genes, withdrawal from the cell cycle, activation of cardiac actin gene expression, repression of β-actin genes, myoblast fusion, activation of MHC gene expression, myofiber growth, and maturation, and activation of skeletal actin gene expression. While the expression of cardiac and skeletal actin mRNAs was sufficiently separated in time to have been discernible from conventional hybridization studies (Paterson and Eldridge, 1984; Hayward and Schwartz, 1986), the relationship of cardiac actin expression to cell fusion, to MHC mRNA expression, or to withdrawal from the cell cycle could be unequivocally resolved by *in situ* hybridization. An abstract reporting the basic finding that cardiac actin mRNA expression precedes both fusion and MHC mRNA expression was published previously (Lawrence and Singer, 1986b). After initial completion of this manuscript, a paper by Hayward *et al.* (1988) showed expression of cardiac actin mRNA in nonreplicative single cells and suppression of β-actin mRNA in myofibers, utilizing *in situ* hybridization. On specific points of overlap concerning actin mRNA expression, our work and that of Hayward and Schwartz, done independently, are in agreement. While other studies have shown that some single cells (i.e., myocytes) are capable of expressing the battery of muscle markers, especially under conditions where fusion is prevented, work presented here shows quantitatively that in the normal course of differentiation *in vitro* the cardiac actin gene is expressed in single cells prior to fusion and prior to the expression of other muscle-specific markers such as MHC. The demonstration that cardiac actin mRNA is expressed in the absence of muscle MHC mRNA indicates that these postmitotic cells are in a discrete category in an early phase of myogenic differentiation.

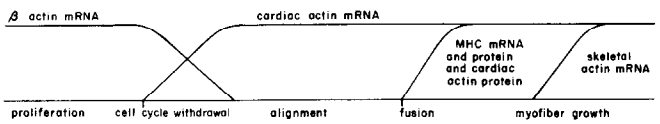

FIG. 5. Schematic representation of the suggested sequence of events during the terminal differentiation of skeletal myoblasts *in vitro.*

Single cell analysis allowed us to correlate directly the expression of α-cardiac actin mRNA with cell cycle stage, using DNA microfluorimetry. Mononucleated cells expressing cardiac actin mRNA consistently had a 2C DNA content, indicating that these were postmitotic cells in terminal G_1 (G_0). A less likely possibility is that the CAct mRNA is expressed only during G_1 and is degraded prior to entry into S. However, the conclusion that these cells are noncycling is further supported by the characteristic appearance of their highly condensed nuclei, identical to the characteristic morphology of postmitotic myofiber nuclei, and by the data showing that the expression of CAct mRNA in single cells parallels the period when postmitotic myoblasts are fusing into myofibers. Since cardiac actin mRNA was expressed by cells most likely in terminal G_1 (G_0), and myosin heavy chain mRNA was expressed after fusion of these same cells, the expression of these two genes is separated by as much as 12 hr or more (average time from mitosis to fusion; Konigsberg *et al.*, 1978).

Defining the sequence of gene expression during muscle differentiation is important for understanding the nature of the molecular controls regulating the differentiation process. Our results showing the sequential expression of muscle-specific genes would not support a

single "master gene" which directly activates a battery of muscle-specific genes simultaneously (Davis *et al.*, 1987). However, it is not inconsistent with a regulatory gene initiating a cascade of events which results in differential timing of particular genes; for instance, those involved in a cardiac muscle program versus a skeletal muscle program. In addition, our work suggests that, in single cells and perhaps young myotubes, cardiac actin sequences are expressed without a concomitant expression of protein, as judged by immunofluorescence. Although it is possible that differential sensitivities of detection by antibodies versus *in situ* hybridization and autoradiography may account for the detection of message versus protein, several indirect arguments make this a less likely explanation. First, the cardiac actin mRNA in single cells, in many cases, is abundant as evidenced by our ability to detect it with an oligonucleotide probe. As evidenced by labeling density, on a per nucleus basis, the relative abundance of cardiac actin mRNA in single cells was equivalent to that of young myotubes (Figs. 2G and 2H). Since the antibody detects α-actin staining in most of these myotubes, and since there is no appreciable skeletal actin mRNA present, the immunofluorescence must represent the presence of cardiac α-actin. The absence of detectable protein in the mononucleated, bipolar myoblasts, however, suggests that the cardiac actin mRNA may not be translated into protein until some time after these cells fuse. While conventional hybridization studies indicate that expression of most muscle genes is under transcriptional control, there has been some evidence for translational control in developing muscle cultures, for instance, with regard to myosin light chains, where cell free extracts translated considerable quantities of this protein, while none was evident *in vivo* or in tissue culture (Yablonka and Yaffe, 1977). Again, the greater temporal resolution afforded by single-cell molecular and biochemical analysis may reveal instances of translational control that would elude studies on RNA or proteins extracted from cell populations. It is possible, however, that the amount of translation was insufficient to result in a detectable accumulation of cardiac actin in the cells before fusion. Yet another explanation for lack of immunofluorescent detection could be that the amino terminus of the actin may be unavailable for the antibody until sarcomere assembly.

The *in situ* hybridization approach also makes it possible to analyze the intracellular distribution of specific mRNAs within expressing cells. It was of particular interest to determine if the mRNAs for two different actin isoforms were distributed in the same way within the cell. We have shown that β-actin mRNA is localized peripherally to the leading edge or lamellipodia of motile cells (Lawrence and Singer, 1986). The cardiac actin isoform mRNA is clearly localized more centrally, as shown nonisotopically using alkaline phosphatase to detect a hybridized oligonucleotide. In cells of the same morphology we have not observed a significantly different distribution of β-actin; therefore, it is possible that a subpopulation of cells exists in which all actin message is perinuclear. While differential distributions of actin mRNA in different cell types may be a consequence of cell morphology, it is also of interest to consider that differential localization of this actin mRNA (i.e., sites of synthesis) contributes to the production or maintenance of these different morphologies.

Previous work suggests some species specific differences in the expression of cardiac actin and skeletal actin genes. Minty *et al.* (1986) has suggested coexpression of these genes in mouse as has been reported in *Xenopus* (Mohun *et al.*, 1984). This report conclusively shows that cardiac actin is expressed in mononucleated chicken cells, well before the activation of the skeletal actin genes, which lag behind even that of myosin. Miller and Stockdale (1986) have shown that at least two lineages of myoblasts are involved in the development of chicken limb, one of which fuses early in development. Our examination of cultured embryonic muscle may include predominantly a subset of myoblasts which fuse late in their developmental program and which may, conceivably, be more likely to express muscle-specific genes as single cells. An *in vivo* analysis may clarify these different myogenic subsets further.

Two aspects not addressed by current methodology will be of importance for future work. The first is the transcriptional activation of specific genes as evidenced by nuclear RNA, and the second is the ability to detect the presence of the mRNAs for two actin isoforms in the same cell. Both of these approaches may contribute significantly more precise information concerning individual cells undergoing terminal differentiation.

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