Differential Patterns of Transcript Accumulation during Human Myogenesis

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We evaluated the extent to which muscle-specific genes display identical patterns of mRNA accumulation during human myogenesis. Cloned satellite cells isolated from adult human skeletal muscle were expanded in culture, and RNA was isolated from low- and high-confluence cells and from fusing cultures over a 15-day time course. The accumulation of over 20 different transcripts was compared in these samples with that in fetal and adult human skeletal muscle. The expression of carbonic anhydrase 3, myoglobin, HSP83, and mRNAs encoding eight unknown proteins were examined in human myogenic cultures. In general, the expression of most of the mRNAs was induced after fusion to form myotubes. However, several exceptions, including carbonic anhydrase and myoglobin, showed no detectable expression in early myotubes. Comparison of all transcripts demonstrated little, if any, identity of mRNA accumulation patterns. Similar variability was also seen for mRNAs which were also expressed in nonmuscle cells. Accumulation of mRNAs encoding α-skeletal, α-cardiac, β- and γ-actin, total myosin heavy chain, and α- and β-tubulin also displayed discordant regulation, which has important implications for sarcomere assembly. Cardiac actin was the only muscle-specific transcript that was detected in low-confluence cells and was the major α-actin mRNA at all times in fusing cultures. Skeletal actin was transiently induced in fusing cultures and then reduced by an order of magnitude. Total myosin heavy-chain mRNA accumulation lagged behind that of α-actin. Whereas β- and γ-actin displayed a sharp decrease after initiation of fusion and thereafter did not change, α- and β-tubulin were transiently induced to a high level during the time course in culture. We conclude that each gene may have its own unique determinants of transcript accumulation and that the phenotype of a muscle may not be determined so much by which genes are active or silent but rather by the extent to which their transcript levels are modulated. Finally, we observed that patterns of transcript accumulation established within the myotube cultures were consistent with the hypothesis that myoblasts isolated from adult tissue recapitulate a myogenic developmental program. However, we also detected a transient appearance of adult skeletal muscle-specific transcripts in high-confluence myoblast cultures. This indicates that the initial differentiation of these myoblasts may reflect a more complex process than simple recapitulation of development.

Studies with a variety of organisms have demonstrated that the differentiation of myoblasts in culture to form myotubes is accompanied by a profound change in gene expression. Using mRNA-cDNA hybridization kinetics analysis, Paterson and Bishop (39) and Affara et al. (1) demonstrated that the abundant mRNA sequence class present in myotubes is almost totally absent from myoblasts and that the converse is largely true of the abundant mRNAs of proliferating myoblasts. This change in mRNA accumulation was paralleled by a change in the chromatin structure, as assayed by DNase I sensitivity, of genes encoding the abundant myotube mRNAs (1). Such data support the idea that the reciprocal switch within the high-abundance class of myoblast and myotube mRNAs is mediated at the level of transcription.

The induction of muscle-specific gene expression during in vitro myogenesis appears to be highly synchronous. Devlin and Emerson (11) observed that as myoblasts begin to differentiate, the synthesis of contractile muscle proteins is initiated coordinately, increases in parallel, and reaches maximal levels at the same time. Measurement of translatable mRNAs in these cultures demonstrated that this coordinate induction of protein synthesis was regulated by mRNA levels (12). Subsequent studies with cDNA probes specific for muscle mRNAs supported this observation (6, 15, 37, 46, 47), with one possible exception (6, 55). The finding of both temporal and quantitative coordination of contractile protein gene expression raised the exciting possibility that these genes may be regulated by a common mechanism (12). Extension of protein synthesis analysis to noncontractile proteins has further suggested that gene regulation during myogenesis may be the result of coordinate regulation of several large groups of genes, or "gene sets" (24). Devlin and Emerson (11) followed the kinetics of induction of protein synthesis during quail myogenesis for a large number of proteins visualised by two-dimensional gel electrophoresis. They found that the kinetic profiles could be collected into five groups. Similarly, Garfinkel et al. (16) detected four developmentally related groups of abundant muscle mRNAs by their expression at different stages of myogenesis. These observations are consistent with the Hastings and Emerson (24) model of muscle gene expression based on coordinate regulation of muscle gene sets. The apparent existence of gene sets raises the possibility that a

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small number of regulatory molecules may be capable of coordinating the regulation of transcript accumulation during myogenesis. With the recent development of an assay for gene sequences which compete for the same regulatory agents (45), it has become possible to test such models and to determine whether muscle gene regulation is controlled by a small number of activating agents. We recently isolated a number of cDNAs which correspond to abundant adult human skeletal muscle mRNAs (17). These cDNAs have presented us with the opportunity of evaluating the regulation of mRNA levels during human myogenesis.

In this report, we used our muscle cDNA clones to follow the accumulation of the corresponding mRNAs in primary myogenic cultures generated from cloned adult human myoblasts. We found that the individual patterns of transcript accumulation could be grouped into broad categories as described by Devlin and Emerson (11). However, detailed analysis indicates that no two mRNAs are identically expressed. This suggests that each gene may have its own unique determinants of transcript accumulation. We also compared expression within myogenic cultures with that observed in fetal and adult human skeletal muscle. Patterns of transcript accumulation established within the myobute cultures are consistent with the hypothesis that myoblasts isolated from adult tissue recapitulate a myogenic development program. However, we also detected a transient appearance of adult skeletal muscle-specific transcripts in high-confluence myoblast cultures. This indicates that the initial differentiation of these myoblasts may reflect a more complex process than simple recapitulation of development.

MATERIALS AND METHODS

Materials. All enzymes used were obtained from New England BioLabs, Inc., Beverly, Mass. All radionucleotides were obtained from Amersham Corp., Arlington Heights, Ill. Nitrocellulose paper (BA 85) was a product of Schleicher & Schuell, Keene, N.H.

Preparation of plasmid DNA and DNA fragments. Plasmid DNA was prepared from chloramphenicol-amplified cultures as described previously (48). All plasmids have been previously described (17, 18, 41) with the exception of the full-length human myoglobin cDNA, pMbO (50), which was kindly provided by S. Boxer and R. Varadarajan. Enzyme digestions and purifications of DNA fragments were carried out by standard procedures (28).

DNA sequence analysis. DNA fragments were isolated from the myosin heavy-chain (MHC) and tubulin cDNA clones, subcloned into the M13 vectors mp10 and mp11 (32), and sequenced by the dideoxy method of Sanger et al. (44). Some DNA fragments were sequenced by the method of Maxam and Gilbert (30).

Cell culture. Human skeletal muscle cells were isolated from the vastus lateralis muscle of a 63-year-old adult male (XXVIII) and from the left quadriceps of a young male (32 months old) diagnosed with Duchenne muscular dystrophy (DXII) by the procedure described by Blau and Webster (3). Muscle cells derived from three individual clones were pooled and maintained in mitogen-rich growth medium containing Ham F-10 nutrient mixture supplemented with 15% fetal calf serum and 0.5% chicken embryo extract. After reaching confluence, the cells were exposed for 18 h to mitogen-poor starvation Dulbecco modified Eagle medium (with 1 μM insulin and 2.5 μM dexamethasone) as described previously (I. D. Kaplan and H. M. Blau, Exp. Cell Res., in press). Afterwards, the cultures were maintained in fusion medium (with 5% horse serum, 0.5% chicken embryo extract, 1 μM insulin, and 2.5 μM dexamethasone). Medium was replaced every day.

RNA isolation. Total cellular RNA was isolated from myoblasts, myotubes, and 24-week-old human fetal skeletal muscle by a modification of the guanidine hydrochloride procedure (7). Total RNA from adult human skeletal muscle (19) was isolated by the phenol-chloroform method described by Palmiter (38).

Gel electrophoresis of RNA and hybridization. Samples (2 μg) of total RNA were electrophoresed on 1% agarose gels containing 2.2 M formaldehyde and transferred to nitrocellulose exactly as described by Maniatis et al. (28). Nick-translated DNA probes (42), 10^6 dpm/μg, were hybridized to RNA blot transfers at 10^6 dpm/ml in a solution containing 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50 mM NaH2PO4 (pH 7.0), 5× Denhardt solution (13), and 10% (wt/vol) dextran sulfate at 60°C for 12 to 18 h. The blots were then washed twice quickly in 1× SSC at room temperature, three times for 30 min each in 1× SSC-0.1% sodium dodecyl sulfate (SDS) at 65°C, and finally for 30 min in 0.5× SSC-0.1% SDS at 65°C. This washing regimen was followed for all blots with the following exceptions: for MHC and skeletal and cardiac actin, washing was performed at 50°C, and for α- and β-tubulin it was done at 60°C. The filters were exposed to Kodak XAR film for 1 to 10 days. Following autoradiography, the filters were stained with methylene blue to visualize 28S and 18S rRNAs (28) and to verify that equivalent amounts of RNA were transferred.

Densitometry. Autoradiograms were scanned with an E-C densitometer (E-C Apparatus Corporation, St. Petersburg, Fla.) attached to an Omniscire chart recorder (Houston Instruments, Austin, Tex.). Areas under the peaks were measured by cutting out the peaks and weighing them. The linear range of the machine was calibrated by scanning an autoradiogram containing serial dilutions of adult human skeletal muscle RNA electrophoresed, blot transferred, and hybridized to the skeletal α-actin probe. All measurements were made within the established linear range.

Characterization of an MHC cDNA. We previously isolated an MHC cDNA from our adult human skeletal muscle cDNA library (17). This clone, originally named C13a and renamed pHMsMHC-1, was subjected to restriction mapping and partial DNA sequence analysis. Recently, Saez and Leinwand (43) isolated and sequenced an MHC cDNA, pSMHCA, which was obtained by screening the same cDNA library. Comparison of the restriction map and nucleotide sequence of pSMHCA with that of pHMsMHC-1 reveals that they are colinear transcripts from the same gene and appear to start within 6 base pairs (bp) of each other at the 5′ end.

To generate an MHC DNA probe which would hybridize similarly to all sarcomeric MHC mRNAs, we isolated the 1,190-bp HindIII fragment from pHMsMHC-1 (bp 1450 to 2640), which covers the amino-terminal half of the light meromyosin segment encoded by the clone (43). We hybridized this HindIII fragment to nitrocellulose blots of human genomic DNA that had been digested with various restriction endonucleases and fractionated on an agarose gel. At the low stringency employed (0.5× SSC, 50°C), each digest revealed the presence of more than 15 strongly hybridizing genomic DNA fragments (data not shown). We conclude that this probe recognizes a large multigene family encoding sarcomeric MHC sequences. We also used this probe at the same low stringency to hybridize to nitrocellulose blots of gel-fractionated human skeletal muscle and heart RNA and...
to rabbit soleus, psosas, and ventricular RNA. Strong hybridization was seen to all these muscle RNAs (unpublished observation), which contain mRNAs for different MHC isotypes. Thus, we assume that for the purposes of this study, this probe, when used under low-stringency hybridization conditions, can be used to measure total sarcomeric MHC mRNA levels.

Isolation and characterization of α- and β-tubulin cDNAs. A human fibroblast cDNA library (36) was screened with chicken α- and β-tubulin cDNA probes (8) exactly as described previously for β- and γ-actin (21). The nucleotide sequence of the longest α-tubulin clone is identical to that of a human α-tubulin cDNA, ba1, characterized by Cowan et al. (9). To measure total α-tubulin mRNA levels during myogenesis, we used the 1,770-bp BamHI DNA fragment as a hybridization probe.

Comparison of the nucleotide sequences of two cloned β-tubulin cDNAs pHFBT-1 and pHFBT-2 with the data of Lee et al. (27) demonstrated that these two cDNAs were the precursors of the human α-tubulin gene identified by M40 and that the 5' end of pHFBT-2 probably corresponds to a full-length copy of the corresponding mRNA. To measure total β-tubulin mRNA levels during myogenesis, we used a 1,050-bp BamHI DNA fragment isolated from pHFBT-1 (bp 610 to 1660).

Subcloning human β-tubulin 3' UTR. The 3' untranslated region (UTR) of pHFBT-1 was isolated as an HaeIII-BamHI DNA fragment and cloned into the EcoRI (made blunt with T4 DNA polymerase)-BamHI sites of pBR322 to generate the clone pHFBT-1-3 UT. The ca. 340-bp insert was excised by digestion with EcoRI plus BamHI and contained the last 5' bp of coding region, the 180-bp 3' UTR, ca. 125 bp of polydeoxyadenylic acid tail, and 30 bp of simian virus 40 primer. This 3' UTR probe only hybridized to the 2.8- and 1.7-kilobase (kb) mRNAs generated by the human β-tubulin M40 gene (27).

RESULTS

Sequence analysis of muscle cDNAs. We recently reported the isolation of a group of cDNAs corresponding to abundant mRNAs expressed in adult human skeletal muscle (17). To identify the proteins encoded by these cDNAs, many of which are full-length copies of the mRNAs (17), we subcloned DNA fragments from the 5' half of these cDNAs into M13 vectors and determined their nucleotide sequences. Over 20 cDNAs were analyzed, and in all cases an open reading frame was identified in the mRNA strand. Both the nucleotide sequence and the derived amino acid sequence were compared with those present in the Genbank data base. In only three cases were we able to identify the corresponding encoded protein. The first of these, H16a, was identified as encoding carbonic anhydrase III (CAIII), and the complete sequence has been published (51). The second, H22h, encodes the slow isofrom of troponin T, and the complete sequence of this cDNA will be published elsewhere (Gahlmann, Trout, Wade, Gunsing, and Kedes, J. Biol. Chem., in press). The third of these, C15b, was found to encode a protein virtually identical to the Drosophila melanogaster heat shock protein HSP83. Of the 56 codons of C15b determined, 51 of the encoded amino acids were identical to those in HSP83 (data not shown). This is the first isolation of a vertebrate HSP83 gene sequence which corresponds to a full-length copy of the mRNA (17).

The failure to identify any other clones by this method led us to enter all possible amino acid sequences derived from the six possible reading frames of each cDNA clone into the data base. We were again completely unsuccessful in identifying any more of the clones. Thus, we conclude that the majority of the cDNAs we isolated do not correspond to previously sequenced abundant muscle mRNAs or proteins.

Transcript accumulation in human myotubes. Previous workers have discerned groups of coregulated transcripts based on both translatable mRNA and direct mRNA measurements in avian and mammalian myogenic cultures (6, 12, 16, 37, 47). Since we have previously cloned a group of abundant human skeletal muscle mRNAs, we sought to identify coregulated subgroups by comparing the accumulation of these transcripts in myoblasts, myotubes, and adult skeletal muscle. Our muscle cDNAs have already been categorized into three broad subgroups: those expressed (i) primarily in skeletal muscle, (ii) primarily in both heart and skeletal muscle, and (iii) in both sarcomeric muscle and nonmuscle fibroblasts (17). It was therefore of particular interest to evaluate whether members of each subdivision would display common patterns of transcript accumulation during myogenesis.

Three clonal myoblasts isolated from a 63-year-old man were expanded in culture. When the cells reached 30 to 40% confluence, RNA was prepared from one set of culture dishes. At this time, less than 1% of the nuclei on the dishes were present within myotubes. A second set of cultures were allowed to reach 80% confluence, transferred to differentiation medium, and harvested for RNA after 4 days, when approximately 60 to 70% of the cell nuclei were in myotubes. Samples (2 μg) of total RNA from the myoblasts, myotubes, and adult human skeletal muscle were electrophoresed on a 1% agarose–2.2 M formaldehyde gel, blot transferred to nitrocellulose, and hybridized with the various cDNA probes.

Figure 1 shows a set of autoradiograms resulting from the hybridization of these cDNA probes to the myoblast, myotube, and adult muscle RNAs. We have previously demonstrated that clones P2a, H22h, C11e, and CAIII are highly expressed in adult skeletal muscle but barely, if at all, in heart or fibroblasts (17). These four cDNAs fell into three broad categories of transcript accumulation (Fig. 1). The two P2a transcripts were induced to almost adult muscle levels in the 4-day myotubes, whereas the H22h and C11e transcripts were induced to levels well below that in the adult and CAIII transcripts were undetectable in the myotubes. The transcripts corresponding to the four cDNAs expressed in both skeletal muscle and heart (H14d, C9c, P1e, and C23a) appeared to show more coordinate induction than the skeletal muscle-specific transcripts. H14d was induced in myotubes to a level slightly higher than in the adult, whereas
The data we present suggest significant maturation-related changes. During myogenesis, the accumulation of transcripts which had accumulated to only low levels in 4-day-old myotubes relative to their level in adult muscle would continue to rise as myotube cultures were allowed to mature. To this end, we created a more detailed time course of RNA expression patterns from the same myoblast clones used to generate the data shown in Fig. 1.

RNA was harvested from human myoblasts that were maintained at very low density, about 5% confluence; this is referred to as the low-confluence (LC) myoblast sample. Close inspection of over half the dishes at this stage failed to reveal the presence of any myotubes in these cultures. After growth for 8 days, the cells had reached 80% confluence, and a second RNA sample was taken, referred to as the high-confluence (HC) myoblast sample. At this time, approximately 5% of the nuclei on the plates were within multinucleated myotubes. The cells were transferred to starvation medium, and within 24 h the fraction of nuclei in myotubes increased to 40%. The cells were thereafter maintained in fusion medium for 14 days. During this time a progressive increase of cell fusion into myotubes was observed.

The fusion index (Fig. 2) indicated an apparent biphasicity. First, a rapid increase from 5 to 40% fusion occurred between days 0 and 1. Second, a slower rise occurred from days 2 to 15, during which the percent fusion doubled. During this second phase, myoblasts appeared to proliferate, and a fraction of these fused into myotubes. Examination of the cultures revealed little evidence of myotubes lifting off the plates with increasing time, and thus most of the myotubes present after the overnight starvation were still present on day 15. The use of dexamethasone and insulin accounts for the long-term persistence of myotubes in these cultures (Kaplan and Blau, unpublished observations).

The accumulation of mRNAs detected by our cDNA probes was measured in total RNA samples isolated from the primary cells in culture and from 24-week-old fetal and pentageneric adult human skeletal muscle. Autoradiograms of these RNA samples, size fractionated and hybridized to the appropriate DNA probes, are shown in the following figures. Densitometric quantitation of the individual transcript levels is presented graphically above the corresponding autoradiograms. The graphs are plotted on a log scale so that severalfold differences between time points are clearly evident whatever the absolute level of expression, unlike a linear scale, which emphasizes differences which amount to only a fractional change at high levels of expression and diminishes differences which are manyfold at low levels of expression. On the basis of similar patterns of transcript accumulation, we subdivided the results obtained with these cDNAs into three groups: (i) rapid inducers, (ii) late inducers, and (iii) inducers which are not muscle specific.

Rapid inducers. Autoradiograms and graphs of transcript accumulation for the transcripts detected by the cDNAs P2a, H14d, and P1e are shown in Fig. 3. All three cDNAs detected transcripts which were induced to substantial levels in the day 1 myotubes. However, the kinetics of transcript accumulation during the cell culture time course and the levels of fetal and adult skeletal muscle indicated independent regulation of each transcript.

P2a detected two transcripts of 1.8 and 2.2 kb which were already present at about 20% of the maximal level in HC cells and coordinately and progressively increased to the...
70% maximum level by day 15 (Fig. 3). In contrast, the H14d and P1e transcripts displayed more complex induction kinetics very different from those of the P2a transcripts and also from each other. In particular, the H14d transcript underwent a sixfold increase from HC to day 1, was then reduced by half to day 2, and thereafter progressively increased to a day 15 maximum level of expression that was more than twice that of adult muscle (Fig. 3). The P1e transcript underwent a fivefold increase between HC and day 1, similar to H14d, but then was reduced by half to a low point at day 5 and finally increased to a value at day 15 that was less than one-third the level in adult muscle (Fig. 3).

Thus, these three cDNA clones detected rapidly induced transcripts, but the kinetics of accumulation were substantially different within the cells in culture, and this difference was even more apparent when levels of expression were compared with those in adult skeletal muscle. In particular, it should be noted that transcripts could be induced in culture to levels significantly above (H14d), similar to (P2a), or significantly below (P1e) the level observed in adult skeletal muscle. It is of further interest that when the day 15 and adult skeletal muscle values were significantly different, the level in fetal muscle was intermediate between the two values.

In addition to the 2.2-kb mRNA detected by the P1e cDNA, this probe also detected the transient appearance of two additional transcripts (arrows, Fig. 3). These two transcripts were readily seen in the HC RNA sample, at a reduced level at day 1 and a higher level in adult skeletal muscle, but were not seen in any other samples (Fig. 3) even after prolonged autoradiography (not shown). A similar transient appearance of transcripts was seen with the other two rapidly induced transcript probes, C9c and C23a.

The cDNA probe C9c detected two transcripts of 9.0 and 9.5 kb which were coordinately induced to close to maximal levels by day 1 (Fig. 4). The level of the 9.0-kb transcript was reduced to one-half of the day 1 level by day 2 and thereafter increased to its maximum level by day 10. In contrast, the 9.5-kb transcript was reduced to less than one-fifth of the day 1 level by day 2 and remained essentially undetectable thereafter. Unlike the minor P1e transcripts, the 9.5-kb C9c transcripts was present at a high level in fetal muscle but not in the adult (Fig. 4).

The C23a probe also detected the transitory appearance of a transcript (Fig. 4). However, the kinetics of its induction were quite different from those of the 9.5-kb C9c transcript. The 1.5-kb C23a transcript reached a maximum level in the HC sample, was reduced by day 1, and was not detectable in any other sample but that of adult muscle, which contained an amount equivalent to that observed at HC (Fig. 4). The 1.2-kb C23a transcript was first detectable at day 1 and displayed an accumulation profile thereafter which was similar, but not identical, to that seen with the C9c 9.0-kb transcript (Fig. 4).

Finally, it should be noted that whereas the 30 to 40% confluence myoblast sample previously analyzed (Fig. 1) contained detectable amounts of the P2a, H14d, P1e, and C23a transcripts, none of these transcripts were detectable in the very low confluence myoblast sample (LC) shown in Fig. 3 and 4. This is consistent with the possibility that a small number of postmitotic cells within intermediate-confluence myoblast cultures account for the low levels of muscle-specific transcripts (Fig. 1).

Late inducers. Autoradiograms and graphs of transcript accumulation for the transcripts detected by the cDNA probes C11e, CAII, and myoglobin are shown in Fig. 5. Unlike the transcripts detected by the rapid-inducer cDNAs, we could not detect any C11e, CAII, or myoglobin mRNA in the early myotube samples. Furthermore, the accumulation kinetics for these three late inducers were profoundly different for each.

The 3.6-kb C11e transcript was first detected at day 5 and increased to reach 60% of adult levels by day 15 (Fig. 5). This demonstrates that a timing mechanism must be operating in these cells in culture which can result in staggering the time of accumulation of these transcripts compared with that of the rapid inducers. Furthermore, the progressive increase from day 5 to day 15 suggests that C11e transcript levels may indicate myotube maturation.

The 1.7-kb CAII mRNA had not been detected previously in the 4-day-old myotube sample (Fig. 1) and in this case was only just detectable in the day 10 and 15 samples after prolonged autoradiography (Fig. 5). To accurately quantitate the level of CAII expression, we performed an RNA dilution experiment and determined that CAII was expressed at 0.03 and 0.3% of adult levels in the day 10 and
15 myotubes, respectively, and appeared to indicate late maturation (data not shown).

The slower accumulation profile was seen with myoglobin (Fig. 5). We could not detect the 1.2-kb myoglobin mRNA in the myotubes, and it was only barely detectable in fetal muscle. To accurately measure the level of myoglobin mRNA in fetal muscle, we electrophoresed a serial dilution of adult muscle RNA together with 10 μg of day 15 and fetal muscle RNA. The level of myoglobin mRNA in 10 μg of fetal muscle RNA was intermediate between the amounts of 0.1 and 1.0 μg of adult RNA and identical to the result obtained by Weller et al. (54) for human fetal myoglobin mRNA levels. We estimated from RNA dilution experiments that if myoglobin mRNA is generated in the myotubes, it is accumulated to a level below 0.3% of adult levels.

In addition to the mature myoglobin 1.2-kb RNA, we also detected two transiently induced transcripts of 1.4 and 2.3 kb. These two transcripts were detected in the day 1 myotubes and in adult skeletal muscle but in none of the other samples shown in Fig. 5. Furthermore, we could detect these two transcripts in the absence of mature mRNA at day 1 (Fig. 5) and also the mature mRNA but not the 1.4- and 2.3-kb transcripts in fetal muscle (data not shown). Thus, while the failure to detect myoglobin mRNA in the cells in culture would suggest that the myoglobin gene is repressed in early myogenesis, the detection of the 1.4- and 2.3-kb transcripts suggests at least a transient activity of this locus.

To confirm that the 1.4- and 2.3-kb transcripts were derived from the myoglobin gene locus, we prepared cDNA probes containing exclusively 5' and 3' UTRs from the full-length myoglobin cDNA. These probes, together with a probe containing most of the coding region and 3' UTR,
were hybridized to the HC, day 1, and adult RNAs (Fig. 6). The *HindIII-NcoI* probe containing only the 5' UTR hybridized very strongly to the 1.4- and 2.3-kb transcripts in all three RNA samples. Similarly, the *Smal-BglII 3' UTR* probe recognized both transcripts, although it was evident that the 5' UTR probe yielded stronger 1.4- and 2.3-kb signals relative to the 1.2-kb mRNA than did the 3' UTR probe (Fig. 6). This trend was even more evident with the *Nco-BglII* coding region plus 3' UTR probe, which in this particular hybridization barely detected the 1.4- and 2.3-kb transcripts (Fig. 6).

We therefore conclude that the 1.4- and 2.3-kb transcripts are products of the myoglobin locus and that these transcripts contain the myoglobin 5' UTR and part of the 3' UTR but probably little if any coding region. Because of the differential hybridization of the different myoglobin probes to these transcripts, it is not likely that the 1.4- and 2.3-kb transcripts derive simply from readthrough of the first polyadenylation signal to generate a longer 3' UTR. Nor is it likely that they are intron-containing transcripts, because each of the human myoglobin introns is too large to be contained in these transcripts (53). Rather, it would appear that the 1.4- and 2.3-kb transcripts represent non-myoglobin-coding transcripts which can be generated from the myoglobin locus independent of the production of mature mRNA.

**Non-muscle-specific transcripts.** Autoradiograms and graphs of the accumulation of transcripts detected by the non-muscle-specific cDNAs are shown in Fig. 7. In parallel with our observations concerning the muscle-specific mRNAs, we again found that accumulation of non-muscle-specific transcripts appeared to be unique to each transcript. The HSP83 mRNA detected by the C15b cDNA increased fivefold from LC to HC and was maintained at a high level throughout the time of culture. Fetal muscle and adult muscle showed slightly reduced levels of HSP83 mRNA (Fig. 7), but compared with all other cDNA probes described here (unpublished), was the closest to a truly constitutively expressed mRNA. The C8f mRNA showed a smaller increase than HSP83 between LC and HC and was reduced to a much greater extent in fetal and adult muscle. However, between the HC and day 15 time points, the HSP83 and C8f transcript levels remained largely unchanged. In contrast, the H6a cDNA probe detected a 10-kb transcript which displayed accumulation kinetics resembling a bell-shaped curve (Fig. 7). The H6a transcript showed a progressive increase in level from LC to a peak value in day 10 myotubes and thereafter a progressive decrease back to a low level in the adult. Finally, it should be noted that the level of each of these transcripts in fetal muscle was intermediate between the corresponding levels in day 15 myotubes and adult muscle.

**Reproducibility of expression in different cultures.** The absolute levels of transcript accumulation which we measured in these cultures may be quite typical of differentiating human cultures. This derives from the observation that we obtained identical mRNA accumulation patterns for a number of these mRNAs in myogenic clones derived from a second individual, a 32-month-old boy with Duchenne muscular dystrophy. It has been reported that myoblasts isolated from patients suffering from Duchenne muscular dystrophy show altered morphology and limited growth potential compared with those isolated from normal individuals (4). We expanded approximately 10 clonal myoblasts in parallel with the culture described above for the normal myoblasts isolated from a 63-year-old man. RNA was similarly isolated from 30 to 40% confluent myoblasts and from 4-day myotubes, electrophoresed on an agarose gel, and analyzed as described above. We found virtually identical levels of transcript accumulation in the normal and dystrophic myotubes (not shown). The precise levels of transcript accumulation in these RNA samples were quantitated by dot blot analysis. Table 1 shows the levels of transcript accumu-

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**FIG. 6.** Autoradiogram of hybridization of different regions of the myoglobin cDNA to human myogenic RNA samples. A restriction map of the human myoglobin cDNA is shown, with the coding region indicated as a solid box, the UTRs as hatched boxes, and the abutting vector regions as open boxes; numbers indicate base pairs. Three DNA fragments, *HindIII-NcoI*, containing the 5' UTR, *Nco-BglII*, containing coding region plus 3' UTR, and *Smal-BglII*, containing the 3' UTR, were hybridized to 2-μg samples of the indicated RNAs. See Fig. 3 legend for details.

**FIG. 7.** Autoradiograms and graphs of C15b, C8f, and H6a transcripts in human myogenic RNA samples. Analysis was performed exactly as described in the legend to Fig. 3.
TABLE. 1. Transcript accumulation in human myogenic culturesa

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<th>Transcript</th>
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<td>Myoblast</td>
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<td>H6a</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>C8f</td>
<td>16</td>
<td>8</td>
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a Myoblasts isolated from an adult and from a patient with Duchenne muscular dystrophy were cultured as described in the text. RNA was isolated from 30 to 40% confluent myoblasts and myotubes which had been exposed to differentiation medium for 4 days. The level of transcript accumulation was quantitated by using both RNA gel blot analysis as shown in Fig. 1 and dot blots as described in the text. All values were calculated as a fraction of the amount of transcript present in adult human skeletal muscle.

lation measured by using each of the cDNA probes in both normal and dystrophic myoblasts and myotubes compared with the levels in adult human skeletal muscle. We found that expression of the different mRNAs in the dystrophic samples was virtually identical to that in the normal samples. This suggests to us that the levels of transcript accumulation we are measuring may be intrinsic to the differentiation of clonal human myoblasts in cell culture and are not altered in myoblasts with the Duchenne muscular dystrophy genotype.

The degree of differential regulation of various mRNAs observed in these experiments was quite surprising considering the results of previous studies (6, 12, 37, 47). However, it is important to note that virtually all previous works have studied the regulation of structural protein mRNA levels. Thus, we felt it was absolutely necessary to evaluate the expression of a group of structural proteins in order to relate our observations to those of other research groups in the field. Since we have cloned cDNA probes specific for the sarcomeric actins and MHC and for the nonmuscle actins and tubulins, we measured the expression of these mRNAs in the same series of RNA samples.

Sarcomeric actin and MHC expression. Autoradiograms and graphs of transcript accumulation for the mRNAs encoding cardiac and skeletal actin and total sarcomeric MHC are shown in Fig. 8 and 9. Again, it is clear that differential regulation of transcript levels best characterizes the results obtained.

Cardiac actin was induced very rapidly in these cultures. Cardiac actin mRNA levels were close to maximal by day 1 and remained largely unchanged throughout the culture time course (Fig. 9). The level in fetal muscle was only partially reduced from this maximum but was substantially reduced in the adult sample. We further found, in contrast to all other muscle-specific mRNAs examined, that the LC myoblasts expressed significant levels of cardiac actin, equal to those expressed in adult muscle (Fig. 8 and 9). It is also notable that when cardiac actin was most highly expressed (days 1 to 15, Fig. 8), three to four high-molecular-weight transcripts were seen in addition to the mature RNA. This pattern of transcript accumulation was also seen, albeit at a much higher level relative to the mRNA, in mouse L cells transfected with the human cardiac actin gene (20). We therefore hybridized a separate panel of these RNAs with the human cardiac actin intron 4 probe (20). Hybridization was seen to all the high-molecular-weight transcripts (data not shown). We conclude that substantial quantities of immature intron-containing cardiac actin transcripts accumulate in these cells. That the levels of these immature transcripts parallel the expression of the mRNA also suggests that the regulation of expression of this gene is largely transcriptional.

In contrast to the expression of cardiac actin, the expression of skeletal actin was more complex. The level of skeletal actin mRNA transiently reached a peak at day 1, rapidly dropped to a low point at day 5, and only increased slightly by day 15 (Fig. 8 and 9). Fetal skeletal muscle expressed skeletal actin at approximately the same level as adult muscle. At much longer autoradiographic exposures than that shown in Fig. 8, there was no detectable skeletal actin transcript present in the LC myoblasts (not shown). Thus, it is apparent that cardiac actin is the only sarcomeric actin mRNA present at significant levels in LC myoblasts.

Since the level of cardiac actin mRNA in the adult skeletal muscle sample is known to be 5% of the level of skeletal actin mRNA (19), we have expressed the levels of both these mRNAs relative to that of skeletal actin mRNA in adult muscle. At all time points in cell culture, cardiac actin was the predominant sarcomeric actin mRNA and was expressed at levels equal to that of skeletal actin mRNA in adult skeletal muscle (per microgram of total RNA) (Fig. 9A). In LC myoblasts, cardiac actin mRNA levels exceeded that of skeletal actin by over 40-fold and were predominant to a similar extent in day 15, 10, and 15 myotubes. However, in day 1 myotubes, skeletal actin reached about one-third the level of cardiac actin before undergoing a reduction of almost one order of magnitude. The high level of cardiac actin expression was also found in 24-week-old human fetal
skeletal muscle, in which it accounted for 32% of total sarcomeric actin mRNA. This is remarkably similar to the value of 30 to 40% cardiac actin in 17- to 20-day-old mouse fetal skeletal muscle (33).

The fusion index (Fig. 2) provides a convenient biological clock against which to compare the time course of mRNA accumulation. Even though muscle cell fusion can be experimentally uncoupled from gene expression (14), it is still possible that these two sets of events are coordinated in muscle cell differentiation. Comparison of the actin mRNA accumulation curves (Fig. 9A) with the fusion index (Fig. 2) revealed that cardiac actin transcript accumulation preceded fusion to form myotubes. In the HC myoblast culture containing only 5% myotubes, cardiac actin mRNA was already at 38% of maximum, and by day 1, when 40% of the nuclei are in myotubes, the mRNA level was 84% of maximum. This indicates that as the myoblasts fuse into myotubes, they already express high levels of cardiac actin mRNA.

The sarcomeric MHC transcripts accumulated with kinetics that closely paralleled the fusion index at the start of the time course. On days 0 and 1, MHC mRNA levels were 7 and 42% of maximum, respectively (Fig. 9B), and the fusion index was 5 and 40%, respectively (Fig. 2). The level of MHC mRNA increased only slightly through the remainder of the time course, and fetal and adult muscle accumulated slightly higher levels of the mRNA than the myotubes (Fig. 9B). Comparison of the MHC mRNA level with that of total actin revealed that the accumulation of MHC mRNA lagged at least 1 day behind that of sarcomeric actin (Fig. 9B). However, between days 1 and 15, the accumulation curves for actin and MHC mRNAs were qualitatively identical. Finally, longer-exposure autoradiograms than that shown in Fig. 8 failed to reveal any detectable MHC mRNA in the LC myoblasts.

The expression of cardiac actin in LC myoblasts is particularly surprising, since no expression of any other muscle-specific transcript was detected in these cells. It is normally assumed that the expression of muscle-specific transcripts only occurs in postmitotic cells (24). This raises the question of whether the expression of cardiac actin in LC myoblasts is due to the presence of a small population of postmitotic “triggered” cells. The HC sample contained a large fraction of postmitotic myoblasts, which expressed cardiac and skeletal actin mRNAs in the ratio of 4:1 (Fig. 9A). However, the LC cells expressed a cardiac-to-skeletal actin mRNA ratio of greater than 40:1 (Fig. 9A). An identical result was obtained by comparing cardiac actin and MHC mRNA levels in LC and HC samples (Fig. 9A and B). This indicates that, at most, 10% of cardiac actin mRNA levels in LC myoblasts could be explained by the presence of postmitotic cells of the type seen at HC. We therefore conclude that either cardiac actin mRNA is accumulated at significant levels in the majority of these proliferating myoblasts or that postmitotic cells differ between HC and LC in their accumulation of these mRNAs. The second possibility would necessarily mean that accumulation of mRNAs encoding skeletal actin and MHC but not cardiac actin in postmitotic cells is dependent on cell density and/or cell contact.

**Nonmuscle actin and tubulin expression.** Autoradiograms and graphs of β- and γ-actin and α- and β-tubulin mRNA accumulation are shown in Fig. 10 and 11. We again found that although there were striking similarities between β- and γ-actin and between α- and β-tubulin, the accumulation pattern of each mRNA was nevertheless unique.

The time course of β- and γ-actin mRNA accumulation

FIG. 9. Levels of sarcomeric actin and MHC mRNAs during human myogenesis. The autoradiograms shown in Fig. 10 were quantitated by densitometry. (A) Levels of cardiac (○) and skeletal (△) actin mRNAs are shown as percentages of the level of skeletal actin in adult human skeletal muscle. (B) Levels of cardiac plus skeletal actin (○) and MHC (△) are expressed as percentages of the maximal level observed for each. The values for cardiac plus skeletal actin were obtained by adding the values shown in panel A. See Fig. 3 legend for details.
During in vitro myogenesis was practically identical. There was a two- to threefold increase in the level of both mRNAs as the cells progressed from LC to HC. After 18 h in starvation medium, this value dropped by 40% to 50% and was maintained as this level for the remainder of the time course (Fig. 11A). Thus, the only significant decrease in nonmuscle actin mRNAs occurred at the time of greatest increase in sarcomeric actin mRNA levels, between days 0 and 1. Whereas the extent of the decrease in β- and γ-actin mRNA levels, 40% to 50%, correlates closely with the increase in myotube formation from 5 to 40%, the failure to observe any further reduction in mRNA levels with increasing cell fusion into myotubes was surprising. However, it should also be noted that the second phase of cell fusion, days 1 to 15, was not accompanied by any further increase in sarcomeric actin mRNA levels. It is therefore apparent that virtually all changes in actin mRNA levels are complete by day 2 and that subsequent cell fusion does not influence them.

Previous studies of primary cultures have also reported only small reductions in nonmuscle actins during in vitro myogenesis and have suggested that this is due to the continued presence of nonmuscle cells (11, 25, 47). With clonally derived myoblasts, it is clear that myogenic cells are continuing to synthesize β- and γ-actin. However, it is not clear whether the nonmuscle actin mRNAs are located exclusively in the proliferating myoblasts or are also present within myotubes. Resolution of this point will require in situ hybridization studies.

Analysis of fetal and adult skeletal muscle revealed the existence of discordant regulation of β- and γ-actin during late muscle development. If we take the ratio of β- to γ-actin mRNA as 1 in the myotube and myoblast samples, then the corresponding ratios in fetal and adult muscle were 1.6 and >11, respectively (Fig. 11A). The result suggests that the

FIG. 10. Autoradiograms of β- and γ-actin and α- and β-tubulin mRNAs in human myogenic RNA samples. Analysis was performed exactly as described in the legend to Fig. 3. The subclones pHFB-3'UT and pHG-3'UT (41) were used to visualize the β- and γ-actin transcripts. The 1,770-bp BamHI fragment of pHFBT-1 and the 1,050-bp BamHI fragment of pHFBT-1 were used to visualize the α- and β-tubulin transcripts, respectively. Following hybridization, the filters were washed with 0.5 SSC-0.1% SDS at 60°C.

FIG. 11. Levels of β- and γ-actin and α- and β-tubulin mRNAs during human myogenesis. The autoradiograms shown in were quantitated by densitometry. (A) Levels of β (○) and γ (●) actin mRNAs are shown as percentages of the maximal level observed for each. (B) Levels of α (○) and total (●) tubulin transcripts are shown as a percentage of the maximal level observed for each. (C) Levels of the 1.8-kb (●) and 2.7-kb (○) β-tubulin transcripts are shown as a percentage of the level of the 18S (1.8-kb) β-tubulin transcript in the day 10 sample. See Fig. 3 legend for details.
ratio of β- to γ-actin in the muscle changes during maturation. However, it is not known whether this reflects a change in the myofibers or in nonmuscle cells and/or myoblast satellite cells associated with myofibers. Finally, the γ-actin-specific probe detected two other transcripts, one larger and one smaller than the mature mRNA (Fig. 10). These additional transcripts were only detected in the HC, day 1, and adult RNA samples. The β-actin-specific probe also detected two transcripts which were larger than the mRNA (Fig. 10). One was more prominent in the adult and the other was more prominent in the fetal sample. On longer-exposure autoradiograms than that shown in Fig. 10, we also clearly detected these two transcripts in the day 1 RNA sample. At present, the composition of these extra acting transcripts is unknown, but their regulation did parallel that seen with a number of the other cDNA probes (Fig. 4 and 5).

In contrast to the nonmuscle actin mRNAs, the 1.8-kb transcripts encoding α- and β-tubulin progressively increased from LC myoblasts to a peak value at days 5 to 10 and then showed a significant decrease by day 15 (Fig. 10 and 11B). This suggests that α- and β-tubulin mRNA accumulation continued to increase as fusion proceeded. The decrease seen in tubulin mRNA levels by day 15 correlated with progressive decreases seen in fetal and adult skeletal muscle. Thus, we concluded that the levels of the 1.8-kb α- and β-tubulin mRNAs show tightly coordinated expression during the cell culture time course and that this involves both initial induction and subsequent repression of mRNA levels.

The regulation of α- and β-tubulin mRNA levels during late muscle development was somewhat discordant. If we take the relative expression of β- to α-tubulin mRNA as 1 at day 5, this ratio changed progressively from 3.0 in fetal muscle to >8 in adult muscle, and it is apparent that this trend was already present in the day 10 and 15 myotube samples (Fig. 11B). This demonstrates that differential regulation of these two tubulin mRNA types occurred during muscle differentiation. Furthermore, the finding that β-tubulin mRNA levels per microgram of total RNA in adult skeletal muscle were equal to that in LC myoblasts and one-quarter of the maximal levels seen in this time course indicates that β-tubulin mRNA is probably expressed at quite high levels within adult muscle fibers.

The β-tubulin cDNA probe also detected a 2.7-kb transcript which displayed a much more complex accumulation pattern than that seen for the 1.8-kb transcript (Fig. 10). It is apparent that these two transcripts were independently regulated in HC myoblasts and the myotube and fetal muscle samples (Fig. 11C). In fact, the 2.7-kb β-tubulin transcript displayed the most complex regulation of all the transcripts we examined.

Finally, the β-tubulin cDNA probe detected two other transcripts of 2.8 and 1.7 kb, which were most noticeable in the adult sample (arrows, Fig. 10). When we performed more stringent hybridizations with our β-tubulin coding region cDNA probe, we observed preferential hybridization to these 1.7- and 2.8-kb transcripts and in particular also observed the 2.8-kb transcript in the HC and day 1 samples (data not shown). Since the sizes of the 1.7- and 2.8-kb transcripts correlate closely with the size of the two β-tubulin cDNAs we have cloned (data not shown), we generated a gene-specific 3' UTR probe from the shorter of our two cDNAs to specifically visualize the transcripts from the corresponding gene. Figure 12 shows an autoradiogram resulting from the hybridization of this 3' UTR probe to the various RNA samples. The 1.7- and 2.8-kb mRNAs were only detected in the HC, day 1, and adult RNA samples. Note that the HC and day 1 but not the adult also showed a 1.8-kb transcript in the longer-exposure panel shown in Fig. 12. This may reflect heterogeneity at the 5' end of the transcript, because the promoter of the corresponding gene does not contain a TATA box (27) and as such may use multiple initiation sites for transcription. Furthermore, it appears that the 1.7- and 2.8-kb transcripts may be differentially regulated, since the 2.8-kb transcript was the more abundant of the two in the HC sample, whereas the converse was true for the adult muscle. Thus, we conclude that the human M40 β-tubulin gene is transiently expressed during early myogenesis in culture and is also expressed in adult muscle.

DISCUSSION

This paper presents a detailed analysis of transcript accumulation in differentiating human myoblasts. Among the unique features of this study are the use of primary myoblasts under conditions in which the potential effects of nonmuscle cells are completely eliminated; the use of clonal myoblasts obtained from an adult; and the ability to compare the accumulation of a large number of different transcripts encoding both structural and nonstructural proteins. Thus, these results provide information on (i) the ability of adult myoblasts to recapitulate a developmental program, (ii) the mechanisms controlling and coordinating transcript accumulation, and (iii) the relationship between structural protein gene expression and sarcomere assembly.

Do adult myoblasts recapitulate early development? Myoblasts from adult human skeletal muscle, when induced to form myotubes, express sarcomeric actin mRNAs in a manner totally dissimilar from the composition of the adult myofibers with which they were originally associated. Whereas adult skeletal muscle accumulates skeletal and cardiac actin mRNA in a ratio of 20:1, the myotubes created by fusion of adult myoblasts expressed a ratio of 1:20. This high ratio of cardiac to skeletal actin seen in human myotubes does not appear to be affected by culture conditions. We recently found that when human myoblasts are grown under very different culture conditions which alter the kinetics of cardiac and skeletal actin transcript accumulation, the final relative expression of the two is identical to that described here (23). Furthermore, many of the other transcripts (e.g., P1e, CAIII, myoglobin, and H6a) are expressed in myotubes at levels very different from that in adult muscle, and the fetal muscle samples usually express an intermediate level. Thus, it appears that in general the patterns of transcript accumulation in the myotubes are
characteristic of a precursor relationship to expression in fetal and adult muscle.

A notable finding of our study is the similarity of transcript accumulation in adult human myoblasts and chicken embryo myoblasts. Hayward and Schwartz (25) followed the expression of skeletal and cardiac actin in cultures in day 11 chicken embryonic hind limb myoblasts induced to form myotubes. Their mRNA accumulation curves are virtually identical, both qualitatively and quantitatively, to those described here. The chicken myoblasts were isolated from a hind limb when the major population of myogenic cells in that tissue were mononucleated and involved in primary myofiber formation in ovo. The similarity of sarcomeric actin expression in an embryonic and an adult vertebrate system suggests that the adult human myoblasts are recapitu-
lating an early developmental myogenic "program" and that the detailed pattern of regulation of these genes during early myogenesis is highly conserved in evolution. Furthermore, in ovo studies in the chicken support the predominance of cardiac over skeletal actin in early development, although the magnitude of this is subject to debate (25, 40).

Compelling evidence that cardiac actin is the predominant α-actin in early human myogenesis is also provided by the recent heterokaryon studies of Hardeman et al. (23). They observed that human fibroblasts, when allowed to form heterokaryons with mouse C2C12 myotubes, accumulate cardiac and skeletal actin in the ratio of 20:1, reminiscent of the ratio in human primary culture, despite the finding that the C2C12 cells are accumulating these transcripts at a ratio of approximately 2:1. This suggests that the predominance of cardiac actin is intrinsic to early stages of human myogenesis and is independent of the mechanism which initiates the myogenic program (23).

Studies of myoblasts isolated from adult chicken skeletal muscle also indicate that they synthesize embryonic isoforms when they fuse in culture (29). Similarly, Whalen et al. (52) detected the synthesis of embryonic MHC and myosin light chains in primary cultures generated from adult rat myoblasts. The appearance of embryonic MHC also precedes that of neonatal and adult MHC in cultures derived from adult human muscle (S. Webster and H. Blau, unpublished observation).

However, the transcripts expressed in HC myoblasts suggest that the recapitulation hypothesis may not completely account for the behavior of these myoblasts. With a number of cDNA probes, we detected the transient appearance in HC myoblasts and day 1 myotubes of transcripts which also appeared in either fetal (C9c) or adult (P1e, C23a, myoglobin, γ-actin, and β-tubulin) skeletal muscle. These data suggest that the adult myoblasts initially synthesize transcripts characteristic of a late stage of development and then revert to an embryonic pattern of expression. It is unclear whether this is a unique property of adult myoblasts or is intrinsic to myoblasts independent of developmental stage. Resolution of this will require analysis of fetal myoblasts in culture.

Differential regulation of transcript accumulation. The most striking feature of the results presented here is the clearly discordant regulation of accumulation of the majority of transcripts encoding both structural and nonstructural proteins. This contrasts with the small amount of data on preexisting transcript accumulation in the literature. Devlin and Emerson (12) found precise coordinate induction of mRNAs encoding MHC, myosin light chains 1 and 2, troponin T, and troponin C in fusing cultures of primary quail myoblasts. A similar precision of mRNA accumulation has been reported for MHC, α-actin, troponin T, and myosin light-chain 2 in the rat L6E9 cell line (15). Coordinate regulation of α-actin and MHC mRNAs and of MHC and myosin light-chain 2 mRNAs have also been reported for the mouse T984 cell line (6) and rat primary cultures (47), respectively. However, it must also be noted that none of these studies compared levels of transcript accumulation in cell culture with those in fetal and adult muscle and that all these studies have concerned only components of the contractile apparatus.

Nevertheless, some evidence for differential accumulation of muscle-specific transcripts does exist in the literature. Both Devlin and Emerson (11, 12) and Shani et al. (47) found that the α-actin mRNA is induced with faster kinetics than MHC mRNA in primary cultures of quail and rat myoblasts. Hayward and Schwartz (25) also observed that cardiac actin mRNA accumulation precedes that of skeletal actin mRNA in chicken primary cultures. Our data are in complete accord with these observations and also indicate that it is cardiac rather than skeletal actin mRNA which accumulates prior to that encoding MHC. Finally, it has been shown mouse T984 cells accumulate myosin light-chain 3 mRNA with much slower kinetics than that of MHC mRNA (6). Thus, whereas the bulk of the mRNAs encoding contractile proteins may accumulate coordinately, cardiac α-actin is a consistent exception and so too may be MLC3. Our studies therefore suggest that the coordinate regulation which appears to exist for a number of the contractile-protein mRNAs may not extend to the range of other muscle-specific transcripts.

The differential regulation of transcript accumulation observed in our myogenic cultures provides unambiguous evidence that some form of biological clock must exist which is intrinsic to these myogenic cells. Transcript accumulation can start at different times with different kinetics for different transcripts. Cardiac actin was the only muscle-specific transcript observed in LC cultures. The majority of transcripts were first detected in the HC cells, and some transcripts were first detected later at day 5 (C11e), day 10 (CAI1), and in fetal muscle (myoglobin mRNA). Does this mean that activation of muscle-specific genes is occurring at different times for different genes? The recent data of Minty et al. (34) indicate that cardiac actin gene expression involves at least two dissociable stages: first, activation of the gene, and second, modulation of its transcriptional activity. Thus, it is possible that all muscle-specific genes are coordinately activated and that observed differences in transcript accumulation result from differential modulation of transcript accumulation from each gene. Indeed, it appears that the myoglobin locus may be active in early myogenic cultures and that not only output but also the type of mature transcript generated are subject to modulation.

The modulation of transcript accumulation must play an important role in determining the phenotype of developing muscle. For example, variations in the level of skeletal actin, CAI1, myoglobin, and H6a indicate that muscle cells have the ability to regulate by several orders of magnitude the levels of transcripts accumulated from a specific gene. Furthermore, modulation in transcript levels can be either abrupt (e.g., H14d and 2.7-kb β-tubulin) or graded (e.g., H6a, C11e, 1.8-kb α- and β-tubulin) and can occur at different times. These modulations are unlikely to be merely a product of progressively increasing myoblast fusion into myotubes. This follows from the observation that these
modulations were unrelated to the fusion index and that cardiac actin mRNA levels were essentially fixed from days 1 to 15 of the time course. In fact, the latter finding suggests that the cellular response has been synchronized by day 1, possibly as a result of the 18-h starvation step between days 0 and 1. Thus, the signals controlling transcript accumulation within the myotube must change with time to account for this temporal regulation.

The data we have assembled are most consistent with the possibility that most muscle genes have their own unique determinants of transcript accumulation and that the phenotype of a muscle may not be so much determined by which genes are active or silent but rather by the extent to which their transcript levels are modulated. Nadal-Ginard and co-workers have clearly demonstrated that variations in both transcript processing (5) and mRNA half-life (31) can profoundly influence both the kinetics and net accumulation of muscle mRNAs. It is also likely that modulation at the level of transcription will be of importance (34). The extent to which these different mechanisms contribute to the individualization regulation of muscle gene transcript accumulation is clearly a central issue in understanding the control of muscle development.

**Gene expression and sarcomere assembly.** The finding that sarcomeric actin mRNA levels are elevated before either the induction of sarcomeric MHC or the onset of cell fusion seems to be a general property of primary myogenic cultures. This observation has now been made for quail (11, 12), chicken (25), and rat (47) mixed cultures and for the pure human cultures described here. The concordance of our data with that of Hayward and Schwartz for the chicken (25) further suggests that this is due to the early induction of the cardiac isoform of actin. These observations correlate very well with the proposal of Dlugosz et al. (13) that initial expression of sarcomeric α-actin results in the creation of either mixed stress fibers containing α-, β-, and γ-actin or α-actin homopolymers aligned with nonmuscle actin filaments. It is proposed that with time, the α-actin filaments would predominate as the synthesis of β- and γ-actin decreases and that these filaments would serve as the template for alignment of myosin thick filaments and completion of sarcomere assembly. Thus, the early expression of cardiac actin in the human myogenic cultures may reflect the need to create stress fiber-like α-actin filaments prior to the synthesis of other components of the contractile apparatus.

The progressive increase in α- and β-tubulin mRNA levels during myotube formation suggests that increased levels of tubulin may be required at an early stage of myogenesis. α- and β-tubulin mRNA levels peaked at days 5 to 10 of our time course, and their regulation was unrelated to the fusion index. Similarly, Tassin et al. (49) observed that microtubules are more prevalent in day 8 human myotubes than within fibroblasts and myoblasts in the same culture. This may represent a functional correlation between sarcomere assembly and tubulin levels, since myosin fibrils are first evident in day 2 human myotubes and the detection of striated sarcomeres progressively increases from days 3 to 5 (S. Webster and H. Blau, unpublished). This correlation is supported by the observations of Antin et al. (2), who found that in the presence of taxol, myotubes assemble striated myofibrillar structures containing interdigitating myosin thick filaments and microtubules from which actin thin filaments are excluded. Thus, it is possible that elevated levels of microtubules may play a transitory role in the process of sarcomere assembly.

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**LITERATURE CITED**


