Sequential Expression of Chicken Actin Genes during Myogenesis

Lawrence J. Hayward* and Robert J. Schwartz*‡

*Program in Neuroscience, and ‡Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030

Abstract. Embryonic muscle development permits the study of contractile protein gene regulation during cellular differentiation. To distinguish the appearance of particular actin mRNAs during chicken myogenesis, we have constructed DNA probes from the transcribed 3′ noncoding region of the single-copy α-skeletal, α-cardiac, and β-cytoplasmic actin genes. Hybridization experiments showed that at day 10 in ovo (stage 36), embryonic hindlimbs contain low levels of actin mRNA, predominantly consisting of the α-cardiac and β-actin isotypes. However, by day 17 in ovo (stage 43), the amount of α-skeletal actin mRNA/μg total RNA increased more than 30-fold and represented ~90% of the assayed actin mRNA. Concomitantly, α-cardiac and β-actin mRNAs decreased by 30% and 70%, respectively, from the levels observed at day 10. In primary myoblast cultures, β-actin mRNA increased sharply during the proliferative phase before fusion and steadily declined thereafter. α-Cardiac actin mRNA increased to levels 15-fold greater than α-skeletal actin mRNA in prefusion myoblasts (36 h), and remained at elevated levels. In contrast, the α-skeletal actin mRNA remained low until fusion had begun (48 h), increased 25-fold over the prefusion level by the completion of fusion, and then decreased at later times in culture. Thus, the sequential accumulation of sarcomeric α-actin mRNAs in culture mimics some of the events observed in embryonic limb development. However, maintenance of high levels of α-cardiac actin mRNA as well as the transient accumulation of appreciable α-skeletal actin mRNA suggests that myoblast cultures lack one or more essential components for phenotypic maturation.

ACTINS are essential components for many forms of cellular organization and mobility. Actin proteins have been well characterized in warm-blooded vertebrates, where at least seven different actins comprise a multigene family that is expressed in both a temporal- and tissue-specific manner (2, 9, 10, 16, 18, 33, 37, 45). In particular, the switching of actin gene expression has been studied during in vitro myogenesis. Our laboratory (37) as well as others (1, 28, 34) have shown that the nonmuscle β- and γ-cytoplasmic actin mRNAs are the predominant actin mRNA species in cultured replicating myoblasts. During subsequent myoblast fusion and formation of multinucleated myotubes, the nonmuscle actin mRNAs are reduced in content. Concomitantly, the sarcomeric α-actin mRNAs are induced and attain relatively high levels within myotubes cultured in the absence of neurons.

As members of a multigene family, the sarcomeric α-actin isoforms are encoded by the two closely related α-skeletal and α-cardiac genes (10). Comparison of the α-skeletal and α-cardiac amino acid sequences reveals only four amino acid substitutions within 375 residues, among the highest conservation found in vertebrate actins (46). Vandekerckhove and Weber (47) have shown that primitive chordates were the first eukaryotes to express a striated muscle–specific form. During the evolution of primitive amphibia or stem reptiles, the striated muscle type branched into the α-skeletal and α-cardiac actin isoforms presumably through a gene duplication event (47). Indeed, both of these genes are identically organized within their coding regions, which are interrupted by five introns at corresponding locations (10, 13, 16). Even in the highly divergent noncoding regions, one region of 12 nucleotides has been conserved in the α-skeletal and α-cardiac actin genes of chickens, rats, and humans (10, 24). Comparison of the promoter regions between the two different sarcomeric α-actin genes also shows striking similarities (10). First, the chicken α-skeletal and α-cardiac actin genes contain highly G+C rich domains that encompass the transcriptional promoter regions. Second, several short homologous sequences of 9–12 nucleotides, which comprise direct and indirect repeats in the α-cardiac actin gene, appear as a single stretch of 18 nucleotides in the promoter region of the α-skeletal actin gene. These homologous sequences may provide additional evidence for a common origin of the sarcomeric α-actin genes.

It is possible that some of these homologous sequences might serve to control co-expression of the sarcomeric α-actin genes during myogenesis. During the formation of somites in *Xenopus laevis* embryos, for example, both of the sarcomeric α-actin mRNA species appear to increase in content in a coordinate manner (29). Since primary cultures composed of dissociated embryonic myoblasts mimic many events of early myogenesis in vivo (20), one might also expect to find coordinated expression of both the α-skeletal and α-cardiac actin mRNAs in myoblast cultures. However, the sarcomeric α-actin genes might also be influenced by different regulatory

signals at appropriate stages during development. Such control of actin gene expression would be expected to occur at a stage between middle embryonic life and hatching or birth, when the α-skeletal and α-cardiac actin products assume a tissue-specific distribution comparable to that found in adult muscle tissues (15, 28, 36, 46).

To distinguish the appearance of individual actin mRNA species, we have constructed hybridization probes from the transcribed 3′ noncoding regions of the chicken α-skeletal, α-cardiac, and β-cytoplasmic actin genes. These probes were used to quantitate the steady-state levels of actin mRNAs during stages of chicken muscle development in the intact embryo and in a primary culture system. Our studies reveal that in both embryonic muscle and in cultured myoblasts the sarcomeric α-actin mRNAs accumulate in a sequential rather than a coordinate manner.

Materials and Methods

Plasmid DNA

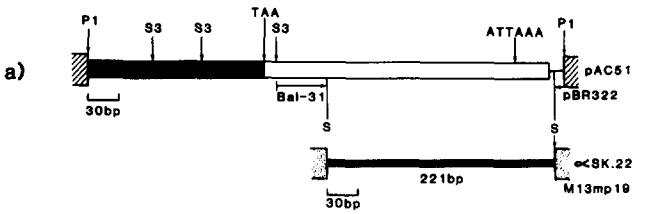
Three chicken actin plasmids were used to construct 3′ noncoding probes. A short α-skeletal actin cDNA clone, pAC51, was characterized by Schwartz et al. (36). A recombinant cDNA clone containing the β-cytoplasmic actin coding sequence, pA1, was a generous gift from D. Cleveland (11). The entire α-cardiac actin gene was subcloned into the *Eco*RI site of pBR322 (pAC 7.5; reference 9) and sequenced (10). Transformed *Escherichia coli* K12 strain RR1 were grown in M9 glucose minimal media followed by selective amplification of the plasmid in chloramphenicol. Recombinant plasmid DNA was purified by the method of Katz et al. (19). M13 vectors were transformed into *E. coli* K12 JM 101 and DNA phage stocks were grown as described (27). All procedures with recombinant DNA were done under approved P1 physical containment conditions.

Cloning into M13 Vectors

Nucleic acid sequences and restriction endonuclease maps of actin structural genes and cDNA clones were used to develop several cloning strategies for 3′ untranslated regions. A short α-skeletal actin cDNA clone called pAC51 (44), which overlaps the last 100 nucleotides of the coding region, was digested with *Sau*3A and *Pst*I to yield a 272-bp fragment. This small fragment was isolated by electrophoresis in 2% agarose gels and electroelution (22). The recovered DNA fragment was blunt-end ligated into the *Sma*I site of M13 vectors as previously described (10, 27). This α-skeletal actin DNA fragment contained a stretch of oligo(dG) from the original cDNA tail that hybridized with a non-actin RNA species of ~3,500 nucleotides found in mature breast tissue (data not shown). We eliminated all but five residues of the oligo(dG) tail by partial digestion of the *Sau*3A/*Pst*I fragment with Bal-31. The shortened insert in M13mp19 extends from the 61st nucleotide in the 3′ untranslated region and contains 221 nucleotides (Fig. 1 *a*). Construction of the α-cardiac actin probe αCA.16 involved isolation of the *Ava*II/*Bgl*II fragment of pAC7.5 (10). This fragment was partially digested with *Dde*I to yield a 164-bp insert that was ligated into the *Sma*I site of M13mp19 (Fig. 1 *b*). The 3′ end of the β-cytoplasmic actin sequence in pAC1 was removed by *Pvu*II/*Pst*I digestion, and then was treated with exonuclease III followed by S_1 nuclease to remove the poly(A) region derived from the cDNA. The 213 nucleotide fragment was ligated into the *Sma*I site of M13mp10 (Fig. 1 *c*). The construction of M13 clones was confirmed by dideoxy sequencing according to Sanger et al. (35). Probe sequences were compared to the published sequences for the chicken α-skeletal (13), α-cardiac (10), and β-cytoplasmic (21) actin 3′ untranslated regions. We obtained recombinant DNA clones that contained inserted DNA in two orientations. The (+) sequence DNA may be used as a template to synthesize labeled probe complementary to each mRNA species, while the (−) sequence DNA may be bound directly to nitrocellulose filters for quantitation of labeled RNA in future studies.

Preparation of ^{32}P-labeled DNA Probes

Uniformly labeled single-stranded DNA probes were synthesized from M13 templates by primer extension with 40 μCi each of [α-^{32}P] dCTP [α-^{32}P] TTP (3,000 Ci/mmol) according to Bergsma et al. (2). Cleavage at a single restriction site within the downstream cloning cassette generated probes of defined length

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        10          20          30          40          50          60
CTGCTGAGGA  CCTGCACCAG  CCATGCAACT  TTCTATTTTG  TAACAATTTC  TGGTTACTGT
        70          80          90         100         110         120
TGCTGCAAAG  CTCCATGTGA  CACAGTGTAT  GTAAAGTGTA  CATAAATTAA  TTTATTTTAC
       130         140         150         160         170         180
CTCGTTTTGT  TTGTTTTTAA  AACCAATGCC  CTGTGGAAGG  AAACATAAAA  CTTCAAGAAG
       190         200         210         220
CATTAAATCA  TCAGTCATTC  TGTCACACCC  CTAAAACCCC  C
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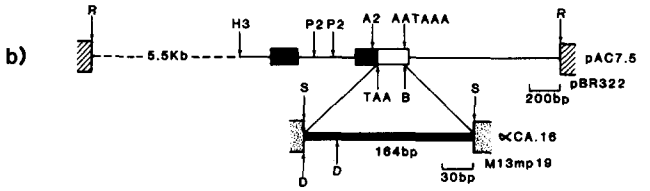


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        10          20          30          40          50          60
TAAGCACTTT  TCCCTACATT  TACTTCCCAC  TCAGGATGAC  GACAGTATGC  TTCTTGGAGT
        70          80          90         100         110         120
CTTCTGGCAG  CCCTTCCTGA  ACTCCTCCGT  ACTTGTACAG  TTTGTTTACA  CACTTGTGCA
       130         140         150         160
ATTTATTTGT  GCTTCTAATA  TTTATTGCTT  TATAAATAAA  GATC
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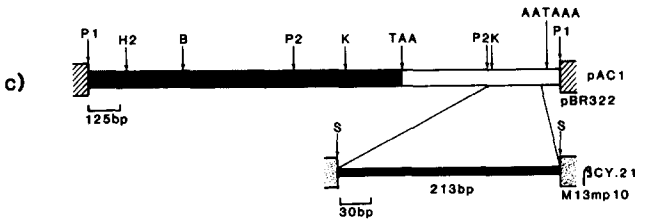


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        10          20          30          40          50          60
AGATGATAAT  TGCTTGTCTG  TAAATTATGT  AACCCAACAA  GTGTCTTTTT  GTATCTTCCG
        70          80          90         100         110         120
CCTTAAAAAC  AAAACACACT  TGATCCTTTT  TGGTTTGTCA  AGCAAGCGGG  CTGTGTTCCC
       130         140         150         160         170         180
CAGTGATAGA  TGTGAATGAA  GGCTTTACAG  TCCCCCACAG  TCTAGGAGTA  AAGTGCCAGT
       190         200         210
ATGTGGGGGA  GGGAGGGGCT  ACCTGTACAC  TGA
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Figure 1. Restriction maps and nucleic acid sequences of the transcribed 3′ noncoding regions of the (*a*) α-skeletal, (*b*) α-cardiac, and (*c*) β actin genes that were subcloned into M13 vectors. These clones were named αSK.22, αCA.16, and βCY.21, respectively. Amino acid coding regions of plasmid clones are shaded black, whereas 3′ untranslated regions are represented by open boxes. Restriction endonuclease abbreviations: A2, *Ava*II; B, *Bgl*II; D, *Dde*I; H2, *Hin*cII; H3, *Hin*dIII; K, *Kpn*I; P1, *Pst*I; P2, *Pvu*II; R, *Eco*RI; S, *Sma*I; S3, *Sau*3A. Sequence differences between this and previous studies (10, 13) were found at nucleotide position 72 (T insertion) of αSK.22 and positions 5 (C insertion), 40 (C instead of T), and 146 (T insertion) of αCA.16.

(~200 nucleotides long) that could be easily isolated from the linearized template (7.7 kb) and the remaining extension products by electrophoresis on a denaturing 5% polyacrylamide gel. Specific activity of the electroeluted probes was ~1.5 × 10^9 cpm/μg.

Primary Myoblast Cultures

White Leghorn eggs, from Texas A&M Poultry Science, College Station, Texas, were incubated at 38°C and staged according to Hamburger and Hamilton (17). Primary myoblast cultures were prepared from day 11 (stage 37) embryonic hindlimbs, dissected free of skin and connective tissue, and mechanically

dissociated in calcium-, magnesium-free Hank's balanced salt solution by a modification of the method of Fischbach (12). The resulting cell suspension was filtered through two layers of sterile cheesecloth and two layers of lens paper to remove aggregates, and single cells were preplated (37°C, 5% CO_2) for 20 min on 100-mm plastic dishes. Myoblast-enriched populations were harvested and then plated at a density of 2–2.5 × 10^6 cells/dish on 100-mm dishes (Falcon Labware, Oxnard, CA) coated with rat tail collagen. Cells were maintained for 24 h in 84% Eagle's minimum essential medium that contained Earle's salts and L-glutamine (Gibco, Grand Island, NY), 10% horse serum (Gibco), 5% chick embryo extract, 100 U/ml penicillin, and 100 μg/ml streptomycin. Thereafter, the medium contained 2% chick embryo extract (other components unchanged) and was replaced every 48 h. In some experiments, 10 μM cytosine arabinoside (Sigma Chemical Co., St. Louis, MO) was included in the medium at 72 h to eliminate replicating cells and was maintained until 120 h.

Isolation of Total RNA

Thigh and breast muscle tissue was dissected from embryos at days 10 through 17, frozen in liquid N_2, and stored at −70°C. Samples of mature breast, heart, gizzard, and brain were taken from 3–4 wk hatchling chickens for use as standards. RNA was isolated from frozen tissue as previously described (36). Total RNA was isolated from pooled muscle cell cultures according to Moss and Schwartz (30).

Nucleic Acid Electrophoresis, Transfer, and Hybridization

RNA samples were denatured in 1 M deionized glyoxal by the method of McMaster and Carmichael (25). Denatured RNA was electrophoresed on 1% agarose slab gels (150 V for 3–6 h) in a recirculating 20 mM sodium phosphate, pH 7.0, buffer. RNA was then transferred to nitrocellulose filters according to Thomas (41). Filters were baked at 80°C for 2 h. Glyoxal was removed from the bound RNA by placing the filter in boiling 20 mM Tris (pH 8.0) for 5 min (42) and then allowing it to air dry. An RNA dot hybridization method modified from White and Bancroft (49) was used for quantitating the relative levels of the actin mRNA species. Samples of total RNA were treated in 25 μl 3.7% formaldehyde in 6× standard saline citrate (SSC) at 60°C for 20 min. The samples were diluted to 200 μl/spot with 15× SSC and spotted onto a moist nitrocellulose filter under a low vacuum (10 μl/s) in a Minifold apparatus (Bio-Rad Laboratories, Richmond, CA). Wells were rinsed with 200 μl of 20× SSC (3.0 M NaCl, 0.3 M sodium citrate, pH 7.0), and the filters were baked at 80°C for 2 h. RNA filters and dot blots were prehybridized for 4 h at 50°C in plastic seal-and-save bags that contained 15 ml of 4× SSC, 20 mM sodium phosphate (pH 7.0), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 50 μg/ml calf thymus DNA. This buffer was removed and replaced with a hybridization buffer that consisted of 50% formamide, 5× SSC, 20 mM sodium phosphate (pH 7.0), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.1% diethylpyrocarbonate, 25 μg/ml *E. coli* tRNA (Sigma Chemical Co.), and 2.5 × 10^5 cpm/ml of a ^{32}P single-stranded DNA probe and incubated at 42°C for 12–18 h. The blots were then washed four times (30 min each wash) with agitation in 400 ml 2× SSC, 0.5% SDS at 42–45°C. Blots were air dried and exposed to Kodak XAR-5 film overnight at −20°C. Individual filter spots on dot blots were then quantitated by liquid scintillation counting. Background binding of probe to nitrocellulose (20–30 cpm) was subtracted from all samples. Nonspecific binding to equivalent amounts of *E. coli* tRNA controls was below 10 cpm/μg tRNA under these conditions.

Nuclear DNA was isolated from adult chicken liver tissue as previously described (9). Restriction endonuclease digestion products of genomic DNA (15 μg per lane) were separated by 0.8% agarose slab gel electrophoresis in TEA buffer (40 mM Tris, 20 mM EDTA, 20 mM sodium acetate, 20 mM NaCl, pH 7.5) at 40 volts for 16 h. DNA bands were partially depurinated with acid and then transferred to nitrocellulose by the method of Southern (39). Filters were hybridized with ^{32}P-labeled DNA probes according to the conditions described in Chang et al. (9).

Results

Development of Specific 3′ Noncoding Actin mRNA Probes

To obtain probes specific for a particular actin mRNA type, it was necessary to subclone DNA regions of low homology from a given actin gene. Sequences downstream from the translational stop codon (3′ untranslated regions) have diverged for each known actin gene and are of sufficient length (160–600 nucleotides) to serve as hybridization probes. DNA fragments within this transcribed 3′ noncoding portion of the α-skeletal, α-cardiac, and α-actin mRNAs were subcloned by blunt-ended ligation into the *Sma*I-site of the bacteriophage vector M13 (Fig. 1). No significant sequence homology is shared between the subcloned portions of the three actin mRNA species (Fig. 1).

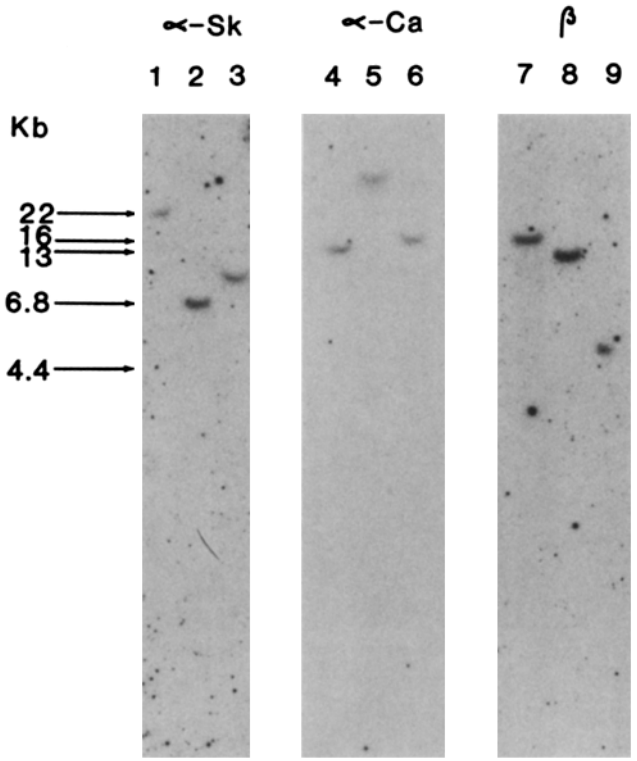

Figure 2. Restriction endonuclease-digested chicken genomic DNA hybridized with actin 3′ noncoding gene fragments. Adult liver DNA was digested with restriction enzymes *Eco*RI (lanes *1*, *4*, and *7*), *Bam*HI (lanes *2*, *5*, and *8*), and *Bgl*I (lanes *3*, *6*, and *9*). The DNA was separated on a 0.8% agarose/1X TEA gel run for 16 h at 40 V. ^{32}P-labeled single-stranded probes (2 × 10^6 cpm/ml) were hybridized to each blot at 68°C for 24 h. Exposure period for the probes αSK.22 (lanes *1*, *2*, and *3*) and βCY.21 (lanes *7*, *8*, and *9*) was 5 d while time for αCA.16 (lanes *4*, *5*, and *6*) was 3 d.

The specificity of the 3′ noncoding actin gene probes was demonstrated by hybridization with chicken liver nuclear DNA fragments obtained by digestion with restriction enzymes *Eco*RI, *Bam*HI, and *Bgl*I. Southern blot analysis shown in Fig. 2 allowed us to determine the number of coding loci for each of these actin gene probes in the chicken genome. Autoradiographs confirmed that each probe hybridized only with DNA from its parent actin gene and verified our earlier study which showed that each of these three actin types is represented by a single copy per haploid chicken genome (9).

α-Skeletal Actin mRNA Levels Increase Greater Than 30-fold between Days 10 and 17 of Myogenesis In Ovo

Changes in the steady-state content of the three actin mRNA species during chicken embryonic thigh development were examined by RNA blotting techniques. First, RNA Northern blots in Fig. 3 display relative actin mRNA levels in total RNA isolated from embryonic hindlimbs at various stages of development. α-Cardiac and β-actin mRNAs are the predominant actin transcripts in embryonic day 10 or day 11 thigh

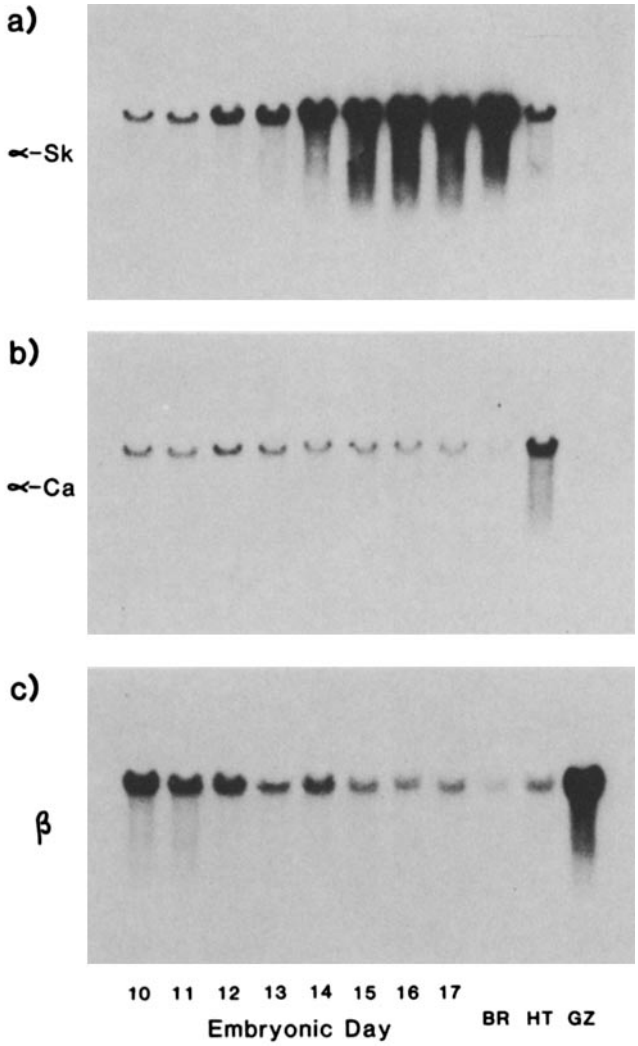

Figure 3. RNA blot analysis of actin mRNAs in staged embryonic hindlimbs. Total RNA was isolated from chicken thighs from embryonic days 10–17 (10 μg/lane) and separated by electrophoresis on a 1.0% agarose/20 mM sodium phosphate gel. Blots were hybridized with labeled single-stranded probes from (*a*) αSK.22, (*b*) αCA.16, and (*c*) βCY.21 templates. RNA samples from 3-wk tissues were chosen as standards: breast (*BR*), heart (*HT*), and gizzard (*GZ*). Exposure period for each blot was (*a*) 24 h, (*b*) 6 h, and (*c*) 24 h.

muscle (the α-cardiac blot was exposed to film for a shorter time than the others). At this developmental stage the hindlimb is composed of mesenchymal tissue that includes a population of replicating and postreplicative myoblasts (18). By day 12 embryonic hindlimbs contain a large population of postmitotic and fusing myoblasts.

The major accumulation of the α-skeletal actin mRNA occurred between the 13th and 16th embryonic days. During this period of development the thigh is composed of myotubes that are polyinnervated by motor neurons and responsive to electrical stimulation (4). At day 14 of thigh muscle development, α-skeletal actin mRNA became the major actin mRNA species, and by day 17 the levels of both α-cardiac and β-cytoplasmic actin mRNAs were reduced to less than 10% of the total actin mRNA detected.

RNA dot blots were used to quantitate the actin mRNA levels in the embryonic hindlimbs. Total cellular RNA was denatured in formaldehyde, diluted over a range of 0.1–1.6 μg, and applied to nitrocellulose filters under conditions in which >95% of the applied RNA was bound and available for hybridization. Each filter also contained standards of total RNA isolated from 3–4 wk chicken breast muscle, heart, gizzard, and brain tissue. Blots were then hybridized with the specific single-stranded 3′ noncoding fragments and autoradiographed. The amount of ^{32}P DNA hybridized per dot was counted, background radioactivity was subtracted, and representative linear plots were drawn (Fig. 4, *a–c*). These results

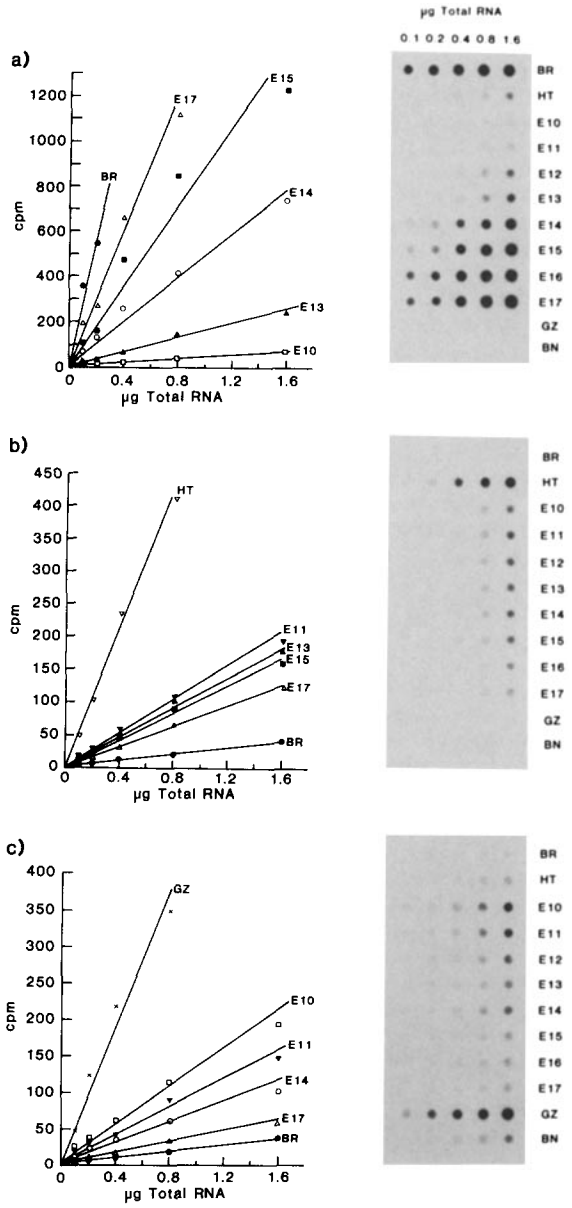

Figure 4. Dot blot quantitation of embryonic thigh muscle mRNA hybridized with 3′ noncoding actin DNA probes. Successive twofold dilutions of total RNA were spotted onto nitrocellulose filters in triplicate and hybridized with ^{32}P-labeled single-stranded (*a*) αSK.22, (*b*) αCA.16, and (*c*) βCY.21 probes. Exposure period for each blot was (*a*) 6 h, (*b*) 6 h, and (*c*) 12 h. The amount of radioactivity associated with hybridization to each spot was measured by liquid scintillation, and representative linear plots are shown. Standards included total RNA from 3–4 wk breast (*BR*), heart (*HT*), gizzard (*GZ*), and brain (*BN*) tissues. □, *E10*; ▼, *E11*; ▲, *E13*; ○, *E14*; ■, *E15*; △, *E17*; ●, *BR*; ▽, *HT*; ×, *GZ*.

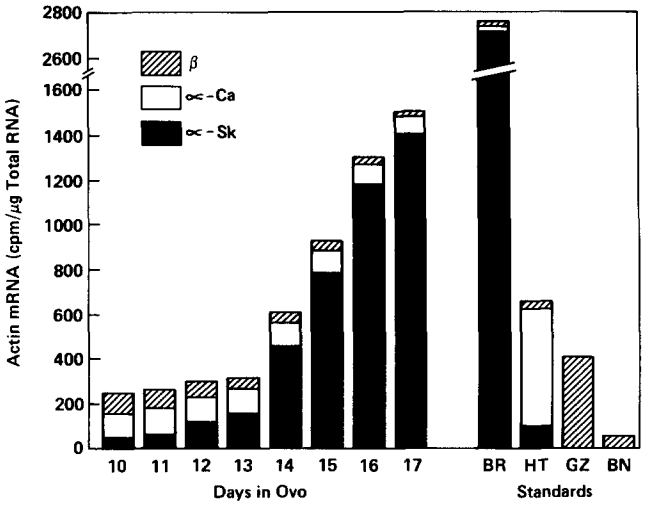

Figure 5. Summary of actin mRNA levels during embryonic thigh muscle development. Linear slope values (cpm/μg total RNA) as calculated from dot blot data from Fig. 4 are plotted for days 10–17 embryonic and for 3–4-wk tissue standards. The contribution of each actin mRNA species is indicated by the height of each shaded block. Abbreviations follow those of Fig. 4.

were summarized by plotting the slopes of these lines (cpm/μg total RNA) versus days of development (Fig. 5). The α-skeletal actin mRNA in embryonic chick thigh muscle increased more than 30-fold between 10 and 17 days in ovo. By contrast, during the same period, the concentration of α-cardiac actin mRNA in embryonic hindlimbs steadily decreased such that it was reduced by 30% at day 17, and, similarly, β-cytoplasmic actin mRNA was 70% lower than its level at day 10. Actin gene expression in pectoralis major muscle tissue followed a similar pattern to that seen in the thigh tissue, except the major increase in α-skeletal actin mRNA was delayed by ~24 h relative to that observed for hindlimb tissue (data not shown).

Since these DNA hybridization probes were synthesized at the same time and under the same conditions using labeled thymidine and deoxycytidine triphosphate nucleotides, their specific activities should differ only according to base composition, which varied from 51 to 57% T+C. By normalizing the radioactive signal to account for base composition and chain length, which varied within 15–20%, it was possible to assess the relative content of each of the three actin mRNA species.

The expression of α-skeletal actin mRNA appears to be restricted to adult striated muscle tissues. We could not detect α-skeletal actin RNA in brain tissue, which contains a vascular smooth muscular component, nor in gizzard tissue, a visceral smooth muscle. Interestingly, α-skeletal actin mRNA made up 10–15% of the actin mRNA assayed in 3–4 wk whole heart tissue (Fig. 5). Gunning et al. (15) detected α-skeletal actin mRNA in human heart tissue, but at higher levels (~50%). It is not known if the α-skeletal actin mRNA levels change with further age of the heart nor is it known if both sarcomeric α-actins are expressed in the same muscle fibers.

α-Skeletal and α-Cardiac Actin Gene Expression during Myogenesis in Culture Is Not Coordinate

We next examined the expression of the actin gene family during myogenesis in embryonic chicken primary myoblast

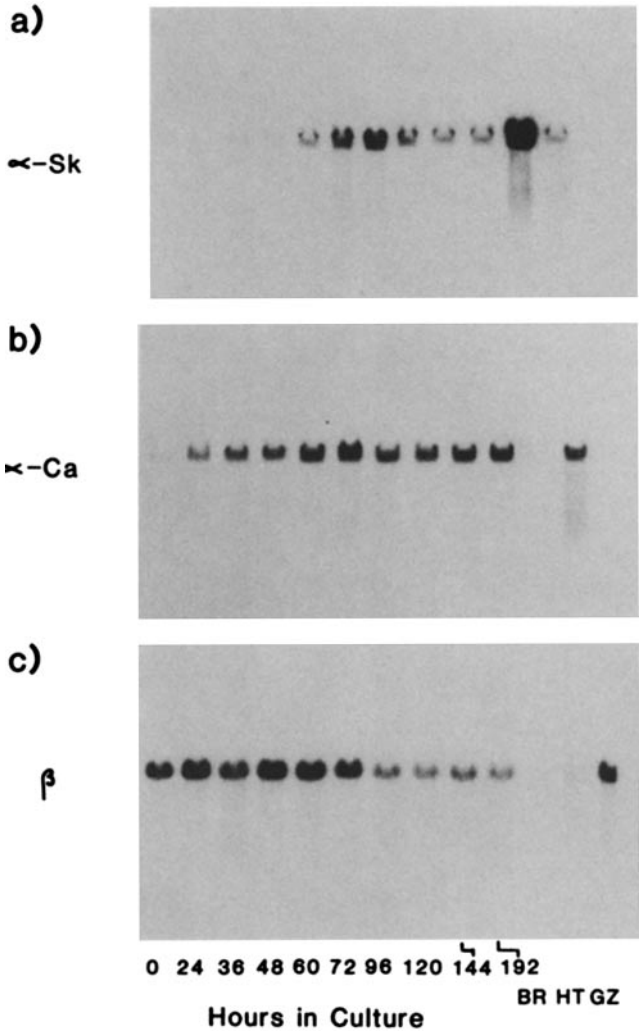

Figure 6. RNA blot analysis of actin mRNA species in cultured chicken muscle. Total RNA was isolated from the myoblast cell suspension after dissociation (0 h), and at various times after plating. RNA (10 μg/lane) was separated by electrophoresis as described in Fig. 3, and nitrocellulose blots were hybridized with (*a*) αSK.22, (*b*) αCA.16, and (*c*) βCY.21 probes as described in Materials and Methods. Exposure period for the three blots was 12 h.

cultures. Total RNA was isolated from cultures at prefusion (0–36 h), fusion (48–72 h), and postfusion (>72 h) stages. The time course of actin mRNA accumulation in vitro was assessed by RNA Northern blots (Fig. 6) and dot blots hybridized with ^{32}P-labeled 3′ noncoding actin probes. Fig. 7 summarizes the time course of actin gene expression, as quantitated by dot blots, for the average of two representative cultures.

In freshly dissociated myoblasts (0 h) the levels of α-skeletal and α-cardiac actin mRNAs were only 15% and 40%, respectively, of the mRNA levels found in the day 11 hindlimb source (Fig. 7). If the bulk of the α-actin mRNAs were present in myotubes, then the loss of multinucleated fibers would account for the decrease in sarcomeric actin mRNAs. However, the majority (85–90%) of myogenic cells in day 11 embryonic hindlimbs are mononucleated (18). Furthermore, Nguyen et al. (31) showed that a rapid preferential destabilization of muscle-specific mRNAs occurs when postmitotic myoblasts reenter the cell cycle. A cessation of gene transcrip-

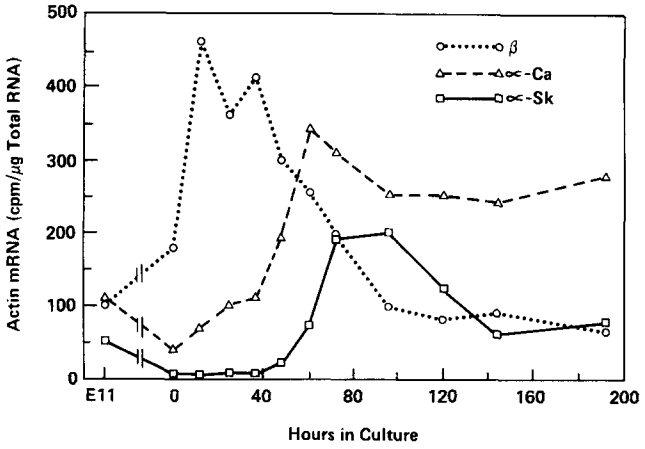

Figure 7. Hybridization of actin probes to dot blots of total RNA from primary muscle cultures. The relative levels of each actin mRNA/μg total RNA are plotted against time in culture; a sample of the embryonic day 11 thigh tissue source (E11) is included for comparison. A mature breast muscle RNA standard included on the blot probed with αSK.22 yielded a signal of 2,590 cpm/μg total RNA, which was similar to the signal for the corresponding standard in Fig. 5. Probes used were αSK.22, αCA.16, and βCY.21.

tion may also be coupled to mRNA turnover. Thus, it is possible that the immediate loss of α-actin mRNA and the increase in cytoplasmic β-actin mRNA at the time of plating may result from mitogenic effects of cell dissociation on actin gene activity (14).

β-Actin mRNA doubled in content during the proliferative phase before fusion and by 24 h accounted for 75% of the total actin mRNA detected. The β-actin mRNA level had peaked by 36 h in culture and steadily decreased with the cessation of nuclear DNA replication and the onset of myoblast fusion. The α-skeletal actin mRNA was barely detectable in prefusion myoblast cultures, amounting to ~1–3% of the assayed actin mRNA. In contrast, the α-cardiac actin mRNA content was 15-fold greater than that of the α-skeletal actin mRNA by 36 h in culture and represented 20–25% of the total assayable actin mRNA. Therefore, it appears that α-cardiac actin mRNA is induced when myoblasts are primarily mononucleated (>90%) and continues to accumulate to its highest level during fusion.

The α-skeletal actin mRNA accumulated appreciably only after fusion was nearly complete (60–72 h in culture). By 96 h the α-skeletal message had increased ~25-fold over the initial level in proliferating myoblasts. The sequential appearance of α-cardiac and α-skeletal actin mRNA followed the same temporal pattern in each of four culture sets.

The accumulation of sarcomeric actin mRNAs in myoblast cultures was examined more critically by simultaneously hybridizing two DNA probes of equivalent specific activities to replicate RNA blots (Fig. 8). In this experiment, the β-actin probe served as an internal hybridization standard, since the β-actin mRNA (2.0-kb RNA) could be easily discriminated on RNA Northern blots from the smaller RNA species hybridized to the α-skeletal actin (1.7-kb RNA) and the α-cardiac actin (1.6-kb RNA) probes. RNA from several stages in culture was transferred equally to the nitrocellulose filters, as verified by the intensity of the β-actin autoradiographic bands shown in Fig. 8. At 36 h in culture, α-cardiac actin mRNA was almost fully induced, but α-skeletal actin mRNA

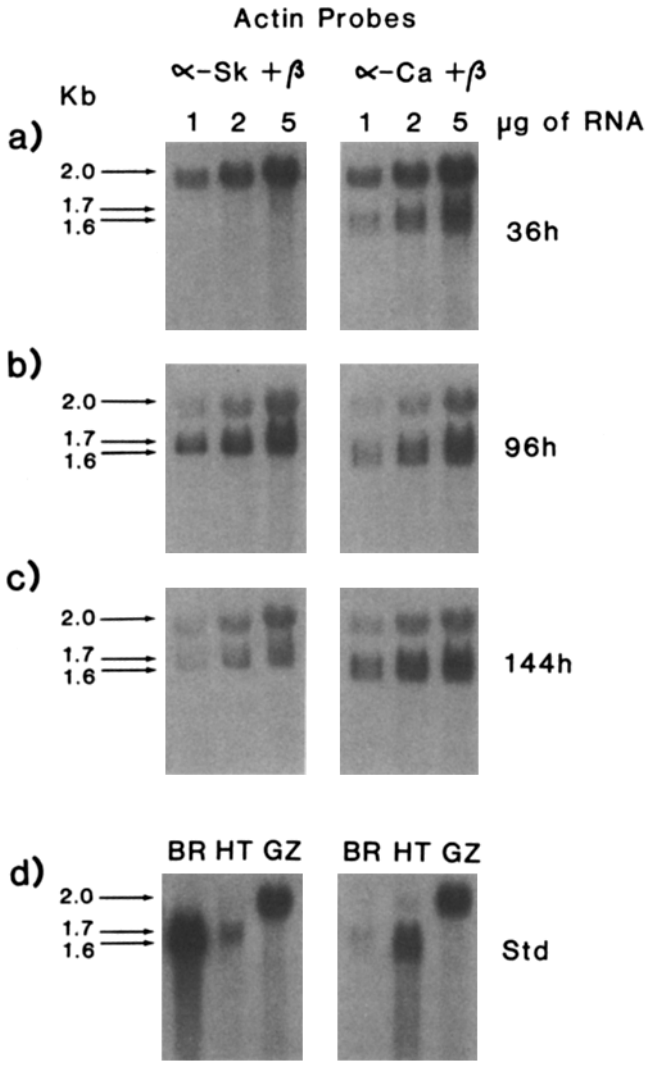

Figure 8. Relative amounts of actin mRNA species in muscle culture visualized by size separation. RNA blots hybridized with βCY.21 plus either αSK.22 or αCA.16 probes depict the amounts of sarcomeric α-actin mRNAs with respect to the amount of β-actin mRNA. Each pair of blots (*a–c*) was obtained from a single 1.0% agarose gel that was loaded with duplicate RNA samples (in the amounts indicated) from muscle cultures at (*a*) 36 h, (*b*) 96 h, or (*c*) 144 h after plating. Standards from 3–4-wk tissues (*d*) were loaded in duplicate at 10 μg/lane. Gels were run for 6 h at 150 V. Arrows indicate approximate length of each mRNA species. Exposure period was for 12 h.

was not yet detectable. After the completion of myoblast fusion (96 h), similar amounts of the sarcomeric α-actin mRNAs were seen. Under the standard growth conditions used, primary muscle cultures after 4 d attain α-skeletal actin mRNA levels (on an mRNA/total RNA basis) equivalent to those found in embryonic muscle on days 13–14 in ovo. However, by 144 h in vitro, the level of α-skeletal actin mRNA declined to less than one-third its value at 96 h and remained low at later time points. In contrast, the α-cardiac actin mRNA level remained at ~80% of its peak amount (60% of that found in 3–4 wk heart tissue) through at least 192 h in culture.

Clearly, three major differences in actin gene expression were observed between myogenesis in culture and in the intact embryo. First, α-cardiac actin mRNA appeared at high levels

in cultured myoblasts before the detection of significant levels of α-skeletal actin mRNA. Second, the α-cardiac actin mRNA did not decline in late myogenic cultures as was seen in late stages of embryonic thigh muscle. Third, the peak content of α-skeletal actin mRNA was approximately one-seventh of that observed in day 17 embryonic leg muscle on an mRNA per microgram total RNA basis. Thus, primary myoblast cultures do not accumulate sarcomeric actin mRNAs characteristic of development in ovo.

Discussion

The high degree of nucleic acid sequence homology within the protein coding regions of isotypic genes renders probes derived from these regions relatively nonspecific. DNA probes constructed from actin coding sequences, for instance, cross-hybridize with other actin mRNA species (31, 37). Levels of some isotypic mRNAs within the actin multigene family can be distinguished using thermally stringent hybridization assays that rely on sequence mismatches between the probe and particular mRNA species (37). Such conditions have allowed us to demonstrate a switch in the expression of the more divergent sarcomeric and nonmuscle actin genes during myogenesis in culture (37), as well as to detect and isolate different actin genes (9). However, thermally stringent hybridization assays are limited in application and may not adequately discriminate between the highly conserved α-skeletal and α-cardiac actin gene products. The transcribed noncoding portion of several chicken actin genes provides unique gene probes that make possible the quantitation of actin mRNA levels on a total RNA basis during skeletal muscle development.

We showed that expression of the α-skeletal, α-cardiac, and β-cytoplasmic actin genes in primary myoblast cultures differed significantly from that in the intact embryo. During myogenesis in ovo, accumulation of α-skeletal actin mRNA increases ~30-fold between days 10 and 17, while α-cardiac and β-actin mRNAs decrease during the same period. At 3 wk after hatching, the latter actin mRNA types comprise <2% of the assayed actin mRNA in breast muscle. Since even the most careful dissection cannot remove connective tissue that envelops groups of muscle fibers, the low levels of β-cytoplasmic actin mRNA may represent expression products of residual nonmuscle cells. It is also possible that these actin mRNA species are expressed at higher levels in muscle satellite cells, which would presumably exhibit an early embryonic phenotype (23). Alternatively, these genes may not be fully repressed in mature myofibers.

In culture, β-cytoplasmic actin mRNA levels increased sharply during the proliferative phase before fusion. As myoblasts withdrew from the cell cycle and replicating fibroblasts were killed with cytosine arabinoside, the levels of β-actin mRNA steadily declined. In contrast, α-cardiac mRNA increased to a level approximately 15 times over that of the α-skeletal actin mRNA in prefusion mononucleated myoblasts. The α-skeletal actin mRNA levels remained low through the onset of myoblast fusion (48 h), but rose rapidly by 60 h in culture. It is possible that postreplicative mononucleated myoblasts primarily express α-cardiac actin mRNA, while newly formed myotubes contain both α-cardiac and α-skeletal actin mRNA. In situ hybridization studies in progress should clarify the cellular distribution of actin transcripts during myogenesis in vitro.

During later times in culture the expression of the two α-actin mRNAs did not appear tightly linked. At the completion of myoblast fusion, both sarcomeric α-actin mRNAs had reached maximal levels and were expressed in similar amounts. After 96 h in culture the α-skeletal actin mRNA species was reduced to 40% of its peak content and remained at that level through at least 192 h, while the α-cardiac actin mRNA was retained at three times the α-skeletal actin mRNA level in differentiated myotubes.

The detection of α-cardiac actin mRNA in chicken primary skeletal myoblasts was not unexpected since previous studies have shown the co-expression of sarcomeric α-actin genes in amphibian embryos (29) and mouse myoblast cultures (28). Recent studies in mammalian myogenic cells have also shown a switch in the expression of nonmuscle and sarcomeric actin genes (1, 28). Bains et al. (1) used 3′ noncoding regions of human actin cDNA clones to quantitate actin mRNA levels in C2C12 myoblasts, which are derived from regenerating adult mouse skeletal muscle (50). α-Cardiac actin mRNA was found to be the major α-actin gene product during the first 72 h of differentiation in culture, reaching a peak during the period of most active fusion (24–36 h). As seen in the present study, the induction of high levels of α-cardiac actin mRNA preceded the slower appearance of α-skeletal actin mRNA. However, C2C12 cell expression of α-cardiac actin mRNA fell to <10% of its peak value by 96 h and remained low for at least 7 d in culture. The rapid decline in α-cardiac actin mRNA is in contrast to our results described here and to the recent study of Blau et al. (3), in which α-cardiac actin mRNA accumulated steadily over a 6-d period in human primary myotube cultures. Thus, C2C12 cell differentiation parallels some aspects of primary myoblast differentiation but may, because of the cell line's adult tissue origin, reflect a later stage of commitment in which the α-cardiac gene is repressed after fusion. Alternatively, cell line establishment or other culture variables may account for the observed differences between primary cultured myoblasts and myogenic cell lines in sarcomeric α-actin gene expression.

Our results do not agree with a report by Paterson and Eldridge (34), in which chicken primary myoblast cultures were quantitated by a primer extension assay. In that study, the α-cardiac actin mRNA level was found to be 16-fold greater than that of α-skeletal in 92-h myotube cultures and 14-fold greater in 15-d embryonic skeletal muscle tissue. It is possible that the hybridization conditions for the primer extension study may have favored detection of α-cardiac actin mRNA (8), since the hybridizable length of the α-cardiac actin DNA primer (105 bases) was 2.8 times that of the α-skeletal actin mRNA probe (37 bases). A 3.8-fold difference in the required reverse-transcribed extension length, where α-cardiac is the shorter extension product, may additionally have precluded complete extension from the α-skeletal transcripts, thus complicating the analysis of such data.

Many contractile proteins and metabolic enzymes that are synthesized in embryonic skeletal muscle differ from neonatal and adult isoforms (7). The switching of isotypic mRNA expression may result from selective transcription or repression of members of a multigene family (26) or from differences in RNA processing (5) or stability. Such control mechanisms

are hypothesized to underlie developmental changes in myoblast populations in vertebrate embryos (38). It was initially thought that gene amplification might also play a role in control of contractile gene expression (51). However, we have been unable to reproducibly demonstrate amplification of actin genes during chicken myogenesis.

Several protein isoforms characteristic of mature cardiac muscle appear during early skeletal myogenesis and decline at later stages. Cardiac forms of actin (28), the troponins (43), myosin light chains (40, 48), C-protein (32), and lactate dehydrogenase (6) are present in embryonic skeletal muscle but are reduced significantly in neonatal and adult muscles. We observed that cultured differentiated myotubes failed to decrease the level of α-cardiac actin mRNA, in contrast to its reduction in late-stage embryonic hindlimb muscle. A transient rise in α-skeletal actin mRNA, in which the maximal level attained in culture was only one-seventh the level observed in day 17 embryonic thigh muscle, suggests that myoblast cultures lack one or more essential components for phenotypic maturation.

Toyota and Shimada (43, 44) have recently demonstrated by immunofluorescence techniques that co-culture of embryonic chicken skeletal muscle with spinal cord motor neurons inhibits the expression of cardiac troponins. Nerve extracts as well as adrenergic innervation provide a comparable effect. This lends support to the hypothesis that diffusible factors present in nerves influence, directly or indirectly, the development of chicken muscle fibers. That such effects result from release of specific mediators of muscle maturation has not been documented.

We have shown that accumulation of specific actin mRNAs during muscle development follows a sequential pattern in which low levels of α-cardiac and β-actin mRNAs are replaced by high levels of the α-skeletal isotype. Whether such changes in mRNA levels are mediated by transcriptional mechanisms or by altered mRNA stability or both has not yet been resolved. Muscle cells in culture express α-skeletal actin mRNA only transiently and to a modest extent but attain high levels of α-cardiac actin mRNA. To characterize in detail the neuronal influences on muscle actin gene expression, we have begun co-culture of chicken nerve and muscle. Parallel experiments using electrical stimulation of myotubes in vitro and pharmacological blockade of endogenous activity will focus on the role of contractile activity in actin gene switching.

We thank Drs. Derk Bergsma, Sherry Bursztajn, and Kun Sang Chang for valuable discussions. Dr. Henry Epstein provided critical comments concerning the manuscript.

This work was supported by U.S. Public Health Service grant NS-15050 and a grant from the Muscular Dystrophy Association of America to R. J. Schwartz. L. J. Hayward is a fellow of the Medical Scientist Training Program and was partially supported by NS-07182.

Received for publication 13 September 1985, and in revised form 26 December 1985.

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