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Gene Switching in Myogenesis: Differential Expression of the Chicken Actin Multigene Family†

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ABSTRACT: We described the construction of an α-actin complementary deoxyribonucleic acid (cDNA) clone, pAC269 [Schwartz, R. J., Haron, J. A., Rothblum, K. N., & Dugaiczyk, A. (1980) *Biochemistry 19*, 5883], that was used as a hybridization probe in the current investigation to examine the induction of actin messenger ribonucleic acid (mRNA) during myogenesis. A T_m difference of 10–13 °C between skeletal muscle α-actin and nonmuscle β- and γ-actin mRNAs and pAC269 allowed us to establish the highly stringent hybridization conditions necessary to measure separately the content of α-actin mRNA and β- and γ-actin mRNA during muscle development in culture. We observed low levels of α-actin mRNA (~130 molecules/cell) in replicating prefusion myoblasts. The vast majority of actin mRNA (2000 molecules/cell) present at this stage was accounted for by β- and γ-actin mRNA. Beginning at myoblast fusion, α-actin mRNA accumulated and within 30 h reached a level 270-fold greater than that observed in the undifferentiated state. At 95 h in culture when myotube formation was completed, α-actin content was at its peak (36 000 molecules/nucleus). Conversely, β- and γ-actin mRNA content began to decline at the beginning of fusion, and by the end of myotube formation β- and γ-actin mRNAs were undetectable by our techniques. A rapid depression of α-actin mRNA levels was observed after 95 h in the absence of cell death. At 6 days after the initiation of myotube formation, the content of α-actin mRNA was reduced by 80% in comparison to peak values and remained at that level. The switching of actin mRNA species was inhibited in myoblasts treated with bdU. The accumulation of α-actin mRNA and the disappearance of β- and γ-actin mRNA were observed following the reversal of the bdU block and coincident with the onset of myoblast fusion. We found that the expression of actin genes within the actin multigene family is switched in myogenesis through a strict developmental pattern.

Muscle development in culture has provided a cell differentiation system to study the regulation of contractile protein synthesis. Myogenesis follows a succession of morphological stages which includes the proliferation of myoblasts, the fusion of mononucleated cells, and the appearance of functional myofibrils (Dienstman & Holtzer, 1975). The fusion of myoblasts into multinucleated myotubes results in the coordinate appearance of a number of new muscle-specific contractile proteins (Buckingham, 1978; Devlin & Emerson, 1979; Strohman et al., 1977). The biochemical properties of one of these proteins, actin, has been studied extensively. Actin was once thought to be a single highly conserved protein but has recently been shown to represent at least six different polypeptides (Vandekerckhove & Weber, 1978) which are coded by a middle repetitive gene family in eukaryotic cells (Fryberg et al., 1980; Schwartz & Rothblum, 1980; Tobin et al., 1980). Of all the actins, α-actin appears to be selectively induced during myogenesis and is retained as a major constituent of the contractile apparatus in skeletal muscle (Gordon et al., 1977). Other types of actins including β and γ isoforms appear to be ubiquitous cytoskeletal proteins found in nonmuscle tissues including prefusion replicating myoblasts (Whalen et al., 1976; Garrels & Gibson, 1976).

Several studies on the appearance of mRNAs[1] in cultured muscle cells have suggested that myogenesis is regulated by transcriptional control (Strohman et al., 1977; Paterson & Bishop, 1977; Benoff & Nadal-Ginard, 1980). However, the mechanism(s) for the selective induction of α-actin mRNA within the actin multigene family during muscle development has not been previously elucidated. Overall actin mRNA content was detected in muscle cells by in vitro translation assays and shown to increase following myoblast fusion (Paterson et al., 1974). Other studies showed the qualitative appearance of α-actin during myogenesis but did not directly quantitate the induction of α-actin mRNA or the contribution of nonmuscle actin mRNA species to total actin mRNA content (Whalen et al., 1976; Hunter & Garrels, 1977). We have recently described the construction of a nearly full length α-actin cDNA recombinant DNA clone (95% full length), which can be used as a hybridization probe (Schwartz et al., 1980). However, due to a preexisting population of nonmuscle β- and γ-actin mRNA in prefusion myoblasts and their homology to the α-actin cDNA, it has been difficult to separately analyze the regulation of the α-actin gene during myogenesis. Therefore, we developed highly stringent hybridization conditions to specifically quantitate the content of α-actin mRNA.

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[1] Abbreviations used: mRNA, messenger ribonucleic acid; DNA, deoxyribonucleic acid; cDNA, complementary DNA; hnDNA, heterogeneous nuclear DNA; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; poly(A), poly(adenylic acid); Pipes, 1,4-piperazinediethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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In addition, we found that the expression of actin genes within the actin multigene family is switched during myogenesis in culture.

Materials and Methods

Materials. Fertilized White Leghorn chicken eggs were obtained from Rich Glo, Inc. (Houston, TX), and were incubated at 38 °C. Thigh muscles from 12-day-old embryos were used as the source of tissue for all primary chick muscle cultures. Restriction endonucleases were purchased from Bethesda Research Laboratories. Nonlabeled deoxynucleotide triphosphates were purchased from P-L Biochemicals. S_1 nuclease was purchased from Miles Laboratories. DNA polymerase I was purchased from Boehringer Mannheim. [^{3}H]dCTP (25 Ci/mmol) and [^{32}P]dATP (300 Ci/mmol) were purchased from New England Nuclear.

Bacterial Strain. A full-length actin cDNA clone, pAC269, constructed and described by Schwartz et al. (1980), was grown in M9 glucose minimal media in host *Escherichia coli* K12 strain RR_1. Recombinant plasmid DNA was purified by the method of Katz et al. (1977). All procedures using the chimaeric plasmid in its host were performed under approved P1 physical containment conditions.

Muscle Cell Culture. Primary cultures of trypsinized 12-day-old chick embryo muscle were prepared by the method of Moss et al. (1979). The initial plating density was 10^7 cells/100-mm plastic dish, and after three serial preplatings cells were finally plated at 3×10^6 cells/100-mm gelatin-coated dish. The technique of serial preplating selectively removes fibroblasts and allows for myoblasts to compose ~90% of the cellular population (Yaffe, 1968). Culture medium consisted of 10% horse serum, 1% penicillin–streptomycin, 0.25% fungizone, and 2.5% chick embryo extract in Dulbecco's modified Eagle's medium (Gibco). Cultures were fed on the second day after plating and on every other day thereafter. At 50 h postplating, 10^{-5} M cytosine arabinoside was added to the cultures for a period of 2 days in order to selectively kill remaining proliferating cells including fibroblasts. We found that myoblasts replicate for the first 30–40 h of culture. Following withdrawal from the cell cycle, myoblasts proceed to align for fusion. This occurred on a large scale around 45 h in culture. Myoblast fusion and the formation of myotubes was complete for the most part by 100 h in culture. After 5 days in culture, myofibrillar elements were organized into functional sarcomeres, which were readily observed under phase microscopy.

In other experiments, the thymidine analogue 5′-bromodeoxyuridine (bdU; 3×10^{-5} M) was added following the final plating. This low concentration of bdU results in the enhancement of cell number while myogenic cells fail to undergo fusion. Suppression of myogenesis is reversible by changing to normal medium supplemented with thymidine (10^{-5} M). Myotubes appear ~4 days later than myotubes in normal primary culture. Under these conditions, cytosine arabinoside is not added to cultures, since there is no overgrowth of myogenic cells by fibroblasts (Bischoff & Holtzer, 1970).

Isolation of RNA. Frozen cultures were scraped with a rubber policeman in the presence of buffer containing 0.1 M NaCl, 0.05 M Tris-HCl, pH 8.0, 1 mM Na_2EDTA, and 0.5% $NaDodSO_4$. The cell extracts were extracted with buffer-saturated phenol and chloroform. The aqueous phase was digested with proteinase K (40 μg/mL) and then extracted again with phenol and chloroform. The aqueous layer from the final extraction was brought to 0.3 M NaCl, and nucleic acids were precipitated by the addition of 2 volumes of absolute ethanol at −20 °C. DNA content was determined by a sensitive fluorometric assay (Kissane & Robins, 1958). DNA was then selectively removed from RNA by ethanol–cellulose chromatography as described by Moss & Schwartz (1981). Poly(A)-containing RNA from 3-week-old chick breast muscle was isolated as described by Schwartz & Rothblum (1980).

Hybridization Assay. The *Hha*I restriction digest fragments of pAC269, which contains 1400 base pairs of the actin cDNA, were isolated by preparative agarose gel electrophoresis (Schwartz et al., 1980). These fragments were nick-translated with [^{3}H]dCTP (25 Ci/mmol) as described by Maniatis et al. (1975) to a specific activity of 10^7 cpm/μg. Complementary single-stranded actin DNA (1 μg) was made by hybridization to poly(A)-containing muscle RNA (200 μg) in buffer containing 80% formamide, 7.2 mM Na_2EDTA, 0.4 M NaCl, and 10 mM Pipes (pH 6.4) at 48 °C for 45 min. The preparation was digested with 3000 units of S_1 nuclease in a final concentration of 3% formamide, 0.5 M NaCl, 2.5 mM $ZnCl_2$, and 0.2 mM NaOAc (pH 4.5). Hybridization and S_1 nuclease digestion were repeated to reduce self-annealing of actin cDNA to a level of 5–10%. [^{3}H]Actin cDNA was treated with alkali and sized to contain DNA fragments in the range of 100–400 nucleotides.

Hybridizations were performed in a final volume of 50 μL containing 0.6 M NaCl, 0.01 M Hepes (pH 7.0), and 0.002 M Na_2EDTA. Each hybridization reaction contained 3000 cpm of [^{3}H]actin cDNA (0.3 ng) and varying amounts of purified RNA. Following heat denaturation for 5 min at 100 °C, incubations were performed for 52 h at 68 °C, and the reaction was terminated by freezing at −80 °C. The extent of hybridization was determined by S_1 nuclease digestion for 2 h at 37 °C in a buffer containing 0.2 M NaOAc (pH 4.5), 0.475 M NaCl, 2.5 mM $ZnCl_2$, and 1600 units of S_1 nuclease followed by Cl_3CCOOH precipitation. The data from the hybridization experiments were expressed as the percent hybridization vs. the ratio of input RNA to [^{3}H]actin cDNA.

In thermal stability experiments [^{3}H]actin cDNA was hybridized to excess RNA at a ratio of 1:2000 as described above. Reactions were diluted to 0.2 M Na^+ and then allowed to equilibrate for 5 min at each temperature. The samples were S_1 nuclease digested and processed as described.

Actin mRNA concentration was determined by comparing the kinetics of hybridization of each RNA sample with that of poly(A)-containing RNA. The data were plotted as 1/(fraction of single-stranded plasmid cDNA) vs. the concentration of RNA × time (C_rt) according to Bishop (1972). The data should approximate a straight line as long as the reaction is <60% complete (Bishop, 1972; Tokarz et al., 1979). The slope is proportional to the concentration of actin mRNA. By comparison of the slope of an unknown sample to that of a poly(A) RNA standard, the absolute concentration of actin mRNA in the unknown sample can be determined. All slopes were determined by a linear regression program. Eight percent of 3-week-old chick breast muscle poly(A) RNA was assumed to contain actin mRNA (Schwartz & Rothblum, 1980).

Hybridization of pAC269 to Electrophoresed RNA Covalently Linked to Diazobenzyloxymethyl (DBM) Paper. RNA samples were separated by electrophoresis on slab gels containing 2% agarose and 5 mM methylmercury hydroxide according to Bailey & Davidson (1976). The fractionated RNA was transferred to diazobenzyloxymethyl (DBM) paper according to Alwine et al. (1977). Following transfer the DBM paper was hybridized to nick-translated ^{32}P-labeled pAC269 DNA, (sp act. 1×10^8 cpm/μg).

Results

We showed that α-actin cDNA cross-hybridizes to the

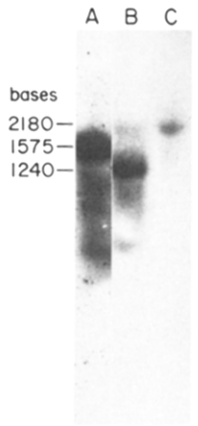

FIGURE 1: Detection of actin mRNA species with pAC269 DNA. Poly(A)-containing RNA samples from chicken breast muscle (5 μg, slot A), gizzard (20 μg, slot B), and embryonic brain (20 μg, slot C) were separated by electrophoresis under denaturing conditions (Bailey & Davidson, 1976) on 2% agarose gels and transferred to DBM paper (see Materials and Methods). Nick-translated ^{32}P-labeled pAC269 *Hha*I fragments containing the actin DNA insert were hybridized to DBM-cellulose-bound RNA and autoradiographed on X-ray film.

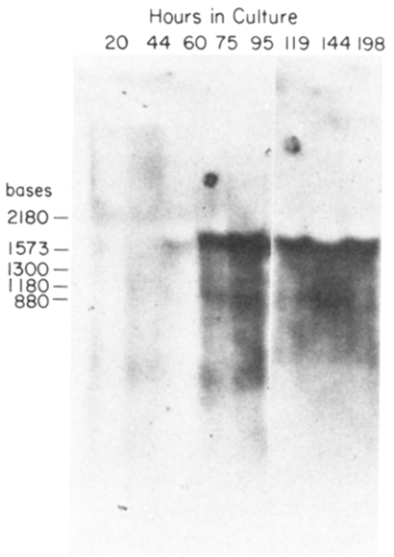

FIGURE 2: Detection of actin mRNA species during myogenesis. RNA isolated from myogenic stages (40 μg/slot) was electrophoresed on denaturing 2% agarose gel and transferred to DBM paper. Nick-translated ^{32}P-labeled pAC269 *Hha*I fragments containing the actin DNA insert were hybridized to DBM-cellulose-bound RNA and autoradiographed on X-ray film.

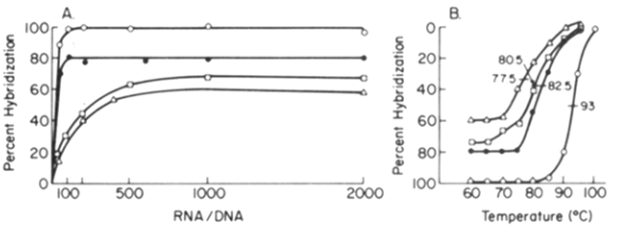

FIGURE 3: Discrimination between actin mRNA species by saturation hybridization and melting of hybrids formed with α-actin cDNA. In panel A, saturation hybridization was performed with total RNA isolated from chick breast muscle (○), rabbit muscle (●), chicken embryonic brain (□), and chicken gizzard (△) by using [^{3}H]actin cDNA (0.3 ng of DNA, 3000 cpm) under conditions described under Materials and Methods for 48 h at 68 °C. In panel B, [^{3}H]actin cDNA–RNA hybrids were diluted to 0.2 M Na^+, incubated for 5 min at each temperature, and then digested with S_1 nuclease.

middle repetitive actin gene family in the chicken genome (Schwartz & Rothblum, 1980). The capability of skeletal muscle α-actin cDNA to hybridize with nonmuscle actin mRNA species is shown in Figure 1. Unfractionated RNA from chick muscle (slot A), gizzard (slot B), and brain (slot C) was electrophoresed on denaturing agarose gel, linked to DBM paper, and hybridized to ^{32}P-labeled pAC269 DNA. The autoradiograph reveals that actin DNA hybridizes to actin mRNA species which differ in molecular weights. Skeletal muscle which expresses only α-actin contained a major autoradiographic band sized at 1575 nucleotides (slot A). Actins which are found in brain tissue as well as the cytoplasm of most nonmuscle cells (β and γ isoforms) are coded for by mRNAs which comigrate at 2200 nucleotides. Gizzard contributed two actin mRNA species, one which coded for a reduced amount of β-actin sized at 2200 nucleotides and the predominant smooth muscle actin mRNA which was sized at 1240 nucleotides. These autoradiographs reveal, by virtue of the different molecular weights of actin mRNAs, that muscle α-actin is the predominant actin mRNA (≥95%) expressed in mature skeletal muscle. These results also illustrate the capacity for differential expression of actin mRNA species coded by the actin multigene family.

These differences in mRNA chain length can be used as an analytical tool to demonstrate the differential accumulation of one actin gene product vs. the other polymorphic forms during myogenesis in culture (Figure 2). In dividing myoblasts a faint autoradiographic band was sized at 2200 nucleotides which corresponds to β- and γ-actin mRNA (see Figures 1 and 2; 20-h time point). With increasing time in culture, this autoradiographic band was reduced in intensity and then finally disappeared at 95 h, a late fusion time point.

A second autoradiographic band of 1600 bases which corresponds to α-actin mRNA appears in midfusion muscle cells at 60 h in culture. The intensity of this autoradiographic band was observed to increase to a maximal level by the 95th hour in culture. The intensity of the α-actin mRNA band declined at later developmental time points. Interestingly, a series of lower molecular RNA species (1330, 1180, and 880 bases) homologous to actin DNA also followed the pattern of induction and deinduction of α-actin mRNA. These minor actin mRNA bands were observed in autographs of poly(A) RNA isolated from adult chicken muscle (Figure 1). Currently, it is not known whether these low molecular weight RNA species are due to a precise degradation process of actin mRNA, a mistake in nuclear processing of actin hnRNA, or perhaps allelic actin genes expressed at a level 2–5% of that of the α-actin gene. Nevertheless, these autoradiographs (Figure 6) show qualitatively a reduction in β- and γ-actin mRNA species and the induction of α-actin mRNA following the onset of myoblast fusion.

Homology and Sequence Divergence of Actin mRNA. In order to quantitate the induction of α-actin mRNA and the repression of β- and γ-actin mRNAs during the process of skeletal muscle differentiation, it was necessary to develop a stringent hybridization assay for α-actin mRNA, which would help to eliminate cross-hybridization to other members of the actin gene family. Previous studies have shown that a ΔT_m of at least 5 °C is necessary to discriminate between related nucleic acid sequences in globin (Benz et al., 1977), vitellogenin (Wahli et al., 1979), and chorion (Sim et al., 1979) multigene families. Therefore, comparisons of the homology and sequence divergence of nonmuscle actin mRNAs to skeletal muscle α-actin mRNA were made by saturation hybridization to single-stranded [^{3}H]actin cDNA and thermal melting of hybrids.

Panel A of Figure 3 shows the complete hybridization of α-actin cDNA with unfractionated muscle RNA with an RNA

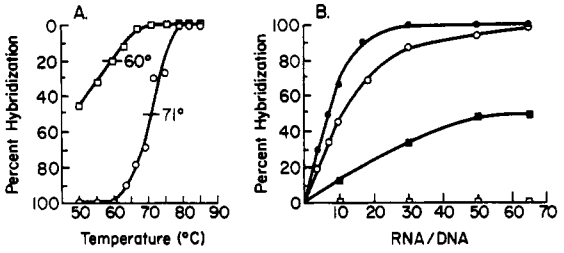

FIGURE 4: Development of stringent hybridization conditions for α-actin mRNA. In panel A are the melting profiles of hybrids formed between muscle poly(A) RNA from muscle (○) and brain (□) with actin cDNA in 50% formamide and 0.5 M NaCl at 50 °C for 48 h. In panel B, hybridization with [^{3}H]actin cDNA was performed in the above medium either at 50 °C (filled symbols) or at 65 °C (open symbols) for 48 h.

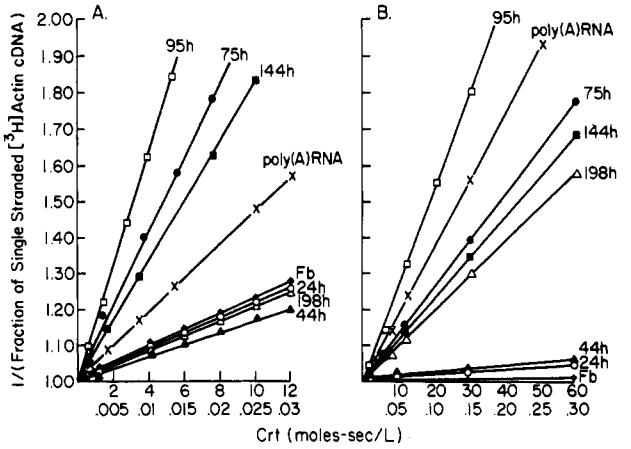

FIGURE 5: Hybridization of actin cDNA to RNA isolated from myogenic stages. [^{3}H]Actin cDNA was hybridized to total RNA samples (higher C_rt values) isolated from the following in vitro muscle culture stages: 24 h (○), 44 h (▲), 75 h (●), 95 h (□), 144 h (■), 198 h (△), and passaged fibroblasts (♦). Poly(A) RNA from 3-week-old chick breast muscle served as a standard (lower C_rt values). In panel A, hybridizations were performed under nonstringent aqueous conditions at 68 °C as described under Materials and Methods. In panel B, hybridizations were performed under highly stringent conditions of 50% formamide and 0.5 M NaCl at 65 °C.

to DNA ratio of 100:1. Similarily, RNA isolated from rabbit muscle hybridized with a low RNA input but saturated only 80% of the chicken actin probe. Brain RNA which contains β- and γ-actin mRNA required a ratio of 500:1 to reach a maximal 70% protection of the actin DNA from S_1 nuclease digestion. The same level of protection was obtained with gizzard RNA. The protected nucleotide sequence of ~1030 nucleotides is almost sufficient to code for the entire actin polypeptide. Sequence differences in the noncoding region of the actin may be responsible for the 30% nonhomology between α-actin mRNA and the other actin mRNA species (Cleveland et al., 1980). We have mapped the α-actin cDNA (pAC269) with ^{32}P-end-labeled actin mRNAs and confirm that the bulk of nonhomologies between actin mRNA species reside primarily at the 3′ noncoding regions (J. A. Haron and R. J. Schwartz, unpublished experiments). Substantial differences in the untranslated 3′ region have been detected between β- and γ-actin mRNA (Cleveland et al., 1980).

Sequence divergence between nonmuscle actin mRNAs and skeletal muscle actin mRNA was examined by using the denaturation characteristics of hybrids formed between RNA and [^{3}H]actin cDNA. The fidelity of the α-actin DNA sequence was ascertained by examining the thermal melting of α-actin cDNA–muscle mRNA duplexes. The observed T_m was 93 °C and occurred with a sharp transition indicating that accurate base pairing was present in the hybrids (Figure 3B). Even though there are no amino acid substitutions between rabbit and chicken actin proteins (Vandekerckhove & Weber, 1978), a reduced T_m of 83 °C was observed in melts of cross-species hybrids, which suggests a greater divergence at the nucleic acid level. Thermal properties of brain β- and γ-actin mRNA–actin DNA hybrids showed a broad multiphasic melt curve in which denaturation began as early as 70 °C. An overall T_m of 81 °C was measured for β- and γ-actin mRNA–α-actin DNA hybrids which is consistent with the amino acid substitutions found between these proteins (Vandekerckhove & Weber, 1978). Thermal melts of smooth muscle actin mRNAs–α-actin DNA duplexes also showed a reduced T_m of 78 °C. Because of a T_m difference of ≥10 °C between skeletal muscle and nonmuscle isoforms of actin mRNA, it was possible to develop stringent hybridization conditions to separately quantitate α-actin mRNA.

Stringent Hybridization Conditions. We found that hybridization of RNA to [^{3}H]actin cDNA at 78 °C could discriminate between α-actin and the other actin mRNA species. Since prolonged incubation at this temperature resulted in extensive breakdown of RNA, we did not exploit this specificity achieved under aqueous hybridization conditions. Instead we utilized formamide to reduce the high thermal requirement for very stringent hybridization as illustrated in Figure 4. In this experiment muscle and brain poly(A)-containing RNAs were first allowed to anneal with [^{3}H]actin cDNA in a buffer containing 50% formamide and 0.5 M NaCl at 50 °C and were then melted in the same buffer. Heterologous hybrids formed with β- and γ-actin mRNA melted with a T_m of 60 °C, while self-hybrids formed with α-actin mRNA melted with a T_m of 71 °C (panel A). The ΔT_m of 11 °C between muscle and nonmuscle actin mRNAs is consistent with thermal denaturation under standard aqueous conditions. A test of high stringency was performed with actin mRNA hybridized with α-actin cDNA at either 50 or 65 °C in the presence of 50% formamide (panel B). Hybridization at 50 °C allowed α-actin mRNA to saturate the actin DNA probe while β- and γ-actin mRNA protected 45% of the actin cDNA. The very stringent conditions of hybridization at 65 °C allowed for the complete saturation of α-actin cDNA, albeit at a rate 4–5 times slower than in aqueous conditions. More importantly, the stringent hybridization conditions eliminated annealing with β- and γ-actin mRNA. The use of highly stringent hybridization conditions made it possible to quantitate total actin mRNA content (α-, β-, and γ-actin mRNA) as well as α-actin mRNA in cultured muscle cells.

Switching of Actin mRNAs. To study the mechanism(s) responsible for the expression of the actin genes in chick myoblasts, RNA isolated from cells at various stages of myogenic development was hybridized with α-actin cDNA. The concentration of total actin mRNA was determined by the kinetics of hybridization of each RNA sample with that of a standard source of actin mRNA. In this case, the standard was poly(A)-containing RNA isolated from 3-week-old chick breast muscle of which 8% is actin mRNA (Schwartz & Rothblum, 1980). Hybridization assays to determine total actin mRNA content were performed under permissible aqueous conditions as described under Materials and Methods. The contribution of α-actin to total actin mRNA levels was examined by performing hybridizations to α-actin cDNA under stringent conditions. Figure 5 is a graphic representation of hybridization data used to determine the accumulation of actin mRNA in cultured muscle cells. Table I provides the slopes of each curve and the fractional contribution of the actin mRNA species to cellular RNA. The

Table I: Hybridization Quantitation of Actin mRNA Sequences during Myogenesis

source of RNA	DNA[a] (μg)	slope[b,c] (L mol^{-1} s^{-1})	slope[b,d] (L mol^{-1} s^{-1})	actin mRNA[c] (% of total RNA)	α-actin mRNA[d] (% of total RNA)	β- and γ-actin mRNA (% of total RNA)
std[e]		15.1	3.8	8	8	
myoblast cultures						
24 h	1.7	0.025	0.0005	0.013	0.0010	0.012
44 h	3.7	0.019	0.0005	0.010	0.0010	0.009
75 h	3.8	0.095	0.0209	0.050	0.0435	0.0065
95 h	4.1	0.114	0.0411	0.060	0.0855	ND[f]
119 h	4.1	0.050	0.0132	0.027	0.0275	ND
144 h	3.9	0.051	0.0132	0.027	0.0275	ND
198 h	3.6	0.024	0.0115	0.013	0.0240	ND
fibroblast		0.024	0.0001	0.013	0.0002	0.013

[a] DNA content was determined on total nucleic acid preparations of myogenic cultures per 100-mm dishes (see Materials and Methods). [b] Slopes were determined by linear regression for each of the RNA samples (see Results and Materials and Methods): % actin mRNA = [(slope of sample)/(slope of std)](% actin mRNA in std). [c] RNA samples hybridized under nonstringent conditions (see Materials and Methods). [d] RNA samples hybridized under stringent conditions (see Results). [e] The standard RNA solution is breast muscle poly(A) RNA which contains 8% actin mRNA (Schwartz & Rothblum, 1980). [f] ND is the abbreviation for not detectable.

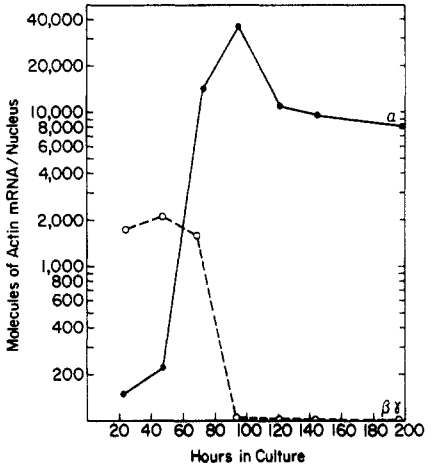

FIGURE 6: Switching of actin mRNA species during myogenesis. The content of α-actin mRNA (●) and β- and γ-actin mRNA (○) was derived from data in Table I and the RNA content of myoblast cultures expressed per diploid nucleus. α-Actin mRNA content was determined by utilizing a molecular weight of 5.2×10^5. Content of β- and γ-actin mRNAs was determined by utilizing a molecular weight of 7.2×10^5 for both RNA species and a factor to normalize for the 70% protection of the actin cDNA probe (see Figure 3A).

content of β- and γ-actin mRNA was estimated by subtraction of α-actin mRNA values from total actin mRNA (Table I). We expressed actin mRNA content on a per diploid nucleus basis, because of the formation of multinucleated myotubes from single nucleated myoblasts (Figure 6).

Under the culture conditions employed, myogenic cells attached themselves to the collagen-coated dishes and underwent replication through the first 30–40 h in culture. Actin mRNA content was estimated at 2000 molecules/cell and was predominantly of β- and γ-actin mRNA. The content of muscle-specific actin mRNA was estimated at a minimum level of 130 molecules/cell (Figure 6). In comparison, the content of α-actin mRNA in passaged fibroblasts was not detected above the hybridization background. At the beginning of fusion there was no increase in the number of actin mRNA molecules per cell. Midway through fusion (75 h in culture) α-actin mRNA content increased to 14 600 molecules/cell nucleus, and at the completion of myotube formation the α-actin mRNA concentration reached a peak level of 36 000 molecules/cell nucleus. The rapid accumulation of α-actin mRNA represented a 75-fold increase (with respect to total RNA) and a 270-fold increase in concentration per cell nucleus. Unexpectedly, the amount of α-actin mRNA declined during the next 100 h in culture. During this time,

Table II: Effect of bdU on Actin mRNA Content during Myogenesis

source of RNA	α-actin mRNA molecules/ diploid nucleus	β- and γ-actin mRNA molecules/ diploid nucleus
25 h bdU	150	4200
72 h bdU	200	4000
48 h postreversal	550	1600
96 h postreversal	40 000	⩾50

in which sarcomeres are functional, there was no loss of myotubes as determined by measurement of cellular DNA content per culture dish (Table I). Importantly, there was no indication of cell death in older myotube cultures. A maintenance level of 8000 molecules of α-actin/nucleus was reached following the completion of muscle cell differentiation. The reduction of nonmuscle β- and γ-actin mRNA content observed by autoradiography of electrophoresed RNA (Figure 2) was quantitated by hybridization assays (Table I, Figure 6). In dividing myoblasts at least 94% of the actin mRNA is β- and γ-actin. At the onset of myoblast fusion β- and γ-actin mRNAs were reduced to 30% of the total actin mRNA, whereas 70% was the α iso form. When fusion was completed, all the actin mRNAs were accounted for by α-actin mRNA. These data support a specific developmental program in myogenesis in which the expression of the actin genes is switched.

bdU Inhibits the Induction of α-Actin mRNA. DNA synthesis and cell replication appear to have essential roles in the differentiation of muscle (Okazaki & Holtzer, 1966). Inhibition of myogenesis by the thymidine analogue bdU has received considerable attention due to its apparent selective effect on the expression of differentiated functions (Stockdale et al., 1964; Coleman et al., 1970; Bischoff & Holtzer, 1970). Shortly after plating, bdU (3×10^{-5} M) was administered to myoblast cultures. The low concentration of bdU resulted in the failure of myogenic cells to fuse and initiate synthesis of contractile protein filaments. The content of α-, β-, and γ-actin mRNAs was assayed in bdU-treated cultures (Table II) and for direct comparisons was examined by autoradiography following hybridization of [^{32}P]actin cDNA to electrophoresed RNA linked to DBM paper (Figure 7). The content of β- and γ-actin mRNA was maintained (4000 molecules/cell) during the first 40–72 h in bdU, while α-actin mRNA remained at low detectable levels (150 molecules/cell; Table II). Suppression of myogenesis was reversed by feeding the cultures

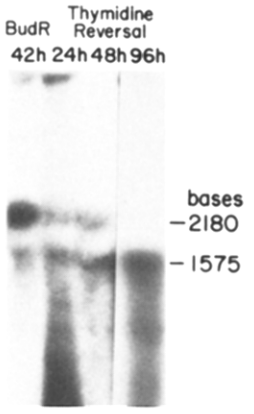

FIGURE 7: Detection of actin mRNA species in bdU-treated myoblasts. Nick-translated ^{32}P-labeled pAC269 was hybridized with RNA isolated from bdU-treated and thymidine-reversed cultured muscle cells (40 μg/slot), which were electrophoresed on denaturing 2% agarose gel and transferred to DBM paper. Hybridization was performed in the presence of 10% dextran sulfate. Autoradiographs of the first three time points were exposed to X-ray film for 40 h. The last time point (95 h of thymidine reversal) was exposed to X-ray film for 5 h.

normal medium supplemented with thymidine (10^{-5} M). Cell proliferation continued for at least an additional 48 h in which the α-actin mRNA level remained virtually unchanged (Table II; Figure 7). Myotubes appeared in the thymidine-reversed cultures ~4 days later than myotubes in normal primary culture. At this time, the α-actin mRNA concentration was found to be 40 000 molecules/nucleus, while β- and γ-actin mRNAs were reduced to undetectable levels. These results are consistent with observations made in other developmental systems that the incorporation of bdU into DNA prevents the expression of cell differentiation products (Ingram et al., 1974; Wilt & Anderson, 1972). In this case, continued myoblast proliferation inhibits the induction of the α-actin and the repression of β- and γ-actin gene expression.

Discussion

Gene dosage experiments suggest that the actin gene family consists of 7–10 genes in the chicken genome (Cleveland et al., 1980; Schwartz & Rothblum, 1980). Differences in the molecular weight of actin mRNA as shown in Figure 1 allowed us to observe a limited expression of the actin multigene family. Skeletal muscle expresses the α-actin mRNA (1570 nucleotides), to the exclusion of other actin mRNA species (Schwartz & Rothblum, 1980). In a similar fashion, brain tissue expresses the ubiquitous cytoplasmic β- and γ-actin mRNA types which are both 600 nucleotides longer than α-actin mRNA (Hunter & Garrels, 1977; Cleveland et al., 1980). As for smooth muscle actins, the predominant actin mRNA (Saborio et al., 1979) is 300 nucleotides shorter than α-actin mRNA (Figure 1, panel B). These differences in mRNA chain length can be used as an analytical tool to demonstrate the qualitative accumulation of one actin gene product vs. the other polymorphic forms within the actin multigene family. In the case for myogenic cells, the accumulation of α-actin mRNA and the diminution of β- and γ-actin mRNA were observed by autoradiography following hybridization of [^{32}P]actin cDNA to electrophoresed RNA linked to DBM paper (Figures 2 and 7).

Previously, it was not possible to quantitate by hybridization techniques the content of specific actin mRNA species during muscle differentiation because of homologies which exist within the protein coding regions (Schwartz et al., 1980; Katcoff et al., 1980). However, differences at the nucleic acid level have also provided sufficient sequence divergence to discriminate between closely related genes and by the use of stringent hybridization conditions to separately quantitate similar mRNA species (Benz et al., 1977; Sim et al., 1979). In this study hybrid melting experiments were performed to determine the sequence relationship between α-actin cDNA and nonmuscle actin mRNA. In general, ΔT_m values parallel the difference in amino acid sequences observed in the corresponding polypeptide chains (as observed of globin chain; Benz et al., 1977). Several investigators have shown that ΔT_m is related to the number of nucleotide differences (sequence divergence) between nonidentical hybridizing sequences (Laird et al., 1969; Ullman & McCarthy, 1973). The reduction in T_m has been variously estimated as 1.6–3.4 °C for each 1% sequence divergence (Leder et al., 1974; Benz et al., 1977). Seventy percent of the α-actin DNA sequence which hybridized to brain β- and γ-actin mRNA melted with a ΔT_m of 10–13 °C. Thus, there is a minimum of 3.8% and a maximum of 8.1% sequence divergence between skeletal muscle and cytoplasmic actin mRNA species, which is consistent with ~25 amino acid substitutions between these proteins (Vandekerckhove & Weber, 1978). It is also possible that the actin mRNAs may differ in positions leading to synonymous codons with little change in polypeptide structures. Such divergence of coding sequence with little or no change in amino acid sequence is known to occur in sea urchins, in which different species show widely different sequences in histone genes coding for identical proteins (Schaffner et al., 1978). These changes in codon usage might contribute to the sequence divergence between avian and mammalian skeletal actin mRNAs (Figure 3).

The ΔT_m of 10–13 °C between α-actin mRNA and β- and γ-actin mRNA allowed us to establish a highly stringent hybridization condition to differentially measure the content of total actin mRNA and α-actin mRNA. In passaged chick fibroblasts there was no detectable α-actin mRNA within a C_rt of 60. However, in order to detect extremely low levels of muscle-specific actin mRNA in fibroblasts or other nonmuscle tissues, it would have been necessary to extend hybridization rates to C_rt's of 10^3–10^4. Such experiments have revealed the presence of globin and ovalbumin RNA sequences at less than 1 molecule/cell in nonerythroid and nonestrogen target tissues (Ono & Cutler, 1978; Tsai et al., 1979). In comparison, during myogenesis we observed significant low levels of α-actin mRNA (~150 molecules/cell) in replicating prefusion myoblasts. It is not evident whether α-actin mRNA is constitutively expressed in all replicating myoblasts or is the result of a few precocious cells found in primary muscle cultures. It would be necessary to perform in situ hybridization in these cells with an α-actin mRNA specific probe, such as pAC23, an α-actin cDNA clone to the 3′ noncoding terminus, to resolve this problem. Nevertheless, the vast majority of actin mRNA (~2000 molecules/cell) present at this stage was accounted for by β- and γ-actin mRNA (Table I, Figure 6). α-Actin was dramatically and transiently induced during the early stages of myogenesis. Beginning at myoblast fusion, α-actin mRNA accumulated and within 30 h reached a level 270-fold greater than that observed in the undifferentiated state. At 95 h in culture, when myotube formation is completed, α-actin mRNA content is at its peak (36 000 molecules/nucleus). Conversely, β- and γ-actin mRNA content began to decline at the beginning of fusion and by the end of myotube formation was undetectable by our techniques ($\leq$25 molecules/nucleus). Although myotubes did not die or dedifferentiate following the 95-h time point, a rapid depression

of α-actin mRNA levels was observed. At 6 days after the initiation of myotube formation, the level of α-actin mRNA was reduced by 80% in comparison to peak values and remained at that level.

The reduction of α-actin mRNA content which follows its accumulation appears to be a general phenomenon in the regulation of cell differentiation products. Benoff & Nadal-Ginard (1980) utilized a pure myosin heavy-chain cDNA probe and showed that myosin mRNA is induced prior to fusion and then deinduced during terminal myogenesis in vitro. In agreement, Leibovitch et al. (1979) showed a decrease of the abundant class muscle-specific mRNA during terminal muscle differentiation. In the case of erythroid differentiation, the induction and deinduction of the globin genes in Friend's erythroleukemia cells seems to be due to activation of specific transcription. A transient stabilization of the de novo synthesized globin mRNA during the early stages of induction was followed by destabilization (Lowenhaupt & Lingrel, 1978). Although a number of investigators have shown muscle-specific mRNA to be increased during myogenesis in culture (Paterson & Bishop, 1977; Devlin & Emerson, 1979; John et al., 1977), few groups have observed the depression of mRNA species after morphological differentiation. This is because of the failure of many investigators to examine cultures once the point of maximal differentiation has been obtained.

The fusion of proliferating myoblasts can be stopped by growth in a low concentration of bdU (Okazaki & Holtzer, 1966). Inhibition occurs after one round of DNA synthesis and appears to result from the substitution of bdU for thymidine within single strands of the newly synthesized DNA (Bischoff & Holtzer, 1970; Coleman et al., 1970). Previous studies have shown that contractile proteins including myosin heavy chain were not synthesized under these conditions while, in general, protein synthesis and cell proliferation were not affected (Bischoff & Holtzer, 1970). In the present study, the levels of β- and γ-actin mRNA (4000 molecules/cell) were maintained in bdU-treated myoblasts, while switching of the actin genes was inhibited during cell replication. These findings are consistent with observations made in other developmental systems that the incorporation of bdU into DNA prevents further differentiation while permitting continued cellular proliferation. Importantly, these results clearly demonstrate that α-actin mRNA is not stored as an untranslatable mRNP in prefusion myoblasts. The induction of α-actin mRNA and the repression of β- and γ-actin mRNA were observed following the reversal of the bdU block and were coincident with the onset of myoblast fusion. Although the mechanism of bdU action is not clear, a report by Lapeyre & Bekhor (1976) utilizing chromatin reconstitution experiments has shown that only 50% of the nonhistone chromosomal proteins bind to unsubstituted control DNA. It is thought that bdU may partially interfere with the process of gene activation during embryogenesis at the level of binding of regulatory nonhistone chromosomal protein to DNA.

The accumulation of 36 000 molecules of α-actin mRNA/cell and the disappearance of β- and γ-actin mRNA in a period of 30 h strongly suggest activation and repression at the level of transcriptional regulation. However, regulation at posttranscriptional sites such as mRNA stability has been reported to increase during myogenesis (Singer & Kessler-Icekson, 1978) and may also provide an important role in controlling actin mRNA content. Pulse–chase measurements are the most effective techniques for analyzing molecules following synthesis. It is possible through their use to establish transcription rates, decay rates, and intermediates in decay. It should be feasible to use pulse-labeled nuclear RNA in similar experiments to determine whether the developmental pattern of actin gene expression is controlled at the level of transcription.

Added in Proof

Recent experiments in our laboratory (W. Zimmer, P. Hobus, and R. Schwartz, unpublished results) have demonstrated that the chicken α-actin structural gene is preferentially replicated ~10–100-fold during the induction of α-actin mRNA in both embryonic thigh muscles and myoblast cultures. The amplified actin DNA sequences disappear at older myogenic stages in which the deinduction of α-actin mRNA is normally seen. These results suggest that the differential expression of the α-actin gene during myogenesis in the chicken occurs through a gene amplification mechanism.

Acknowledgments

We thank Earl Scharff, III, for excellent technical assistance. We acknowledge Drs. Henry Epstein, Jeffrey Rosen, and Anthony Means and Edwin Stone for their thoughtful commentary in this study and in preparation of this manuscript.

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Polymerization of Clathrin Protomers into Basket Structures†

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ABSTRACT: The effects of pH, ionic strength, temperature, and protein concentration on the rate of clathrin (8 S) polymerization to form coat (or basket) structures (~300 S) have been measured by turbidity. The extent of polymerization has also been evaluated under the same experimental conditions by analytical centrifugation. The characteristic polygonal structure of the re-formed coat was confirmed by electron microscopy. The rate of polymerization is sensitive to all the variables investigated. The reaction is very slow at pH ~7 and becomes very rapid by pH ~6. The polymerization is readily reversed by increasing the pH slightly. The time dependence of the polymerization does not conform to either a first- or a second-order reaction but to a higher order. Increasing temperature increases the rate but decreases the extent of reaction. Increasing the salt concentration decreases the rate. The effects of several salts on the rate follow the Hofmeister ranking, with the exception of sulfate.

The coated pit regions of mammalian plasma membranes have been implicated as the site of receptor-mediated endocytosis for numerous proteins and hormones (Schlessinger et al., 1978; Maxfield et al., 1978; Goldstein et al., 1979; Brown & Goldstein, 1979). It has also been shown that the protein which forms the coat of these pits is the same as that found around coated vesicles inside cells (Pearse, 1975, 1976). These vesicles probably play a major role in intracellular transfer among organelles (Ockleford & Whyte, 1977; Rothman et al., 1980). Pearse (1975, 1976, 1978) and others (Ockleford & Whyte, 1977; Blitz et al., 1977; Woods et al., 1978; Woodward & Roth, 1978; Bloom et al., 1980) have isolated coated vesicles from various cell types and shown that one protein is the major constituent present in the coats of these vesicles. This protein, which was named clathrin by Pearse (1975), is capable of re-forming the coat structure observed in coated vesicles (Pearse, 1978; Schook et al., 1979; Keen et al., 1979; Woodward & Roth, 1979).

Clathrin has been isolated from coated vesicles of human and bovine brain by similar procedures and further characterized in this laboratory. A protomer with a molecular weight of 610000 and a sedimentation constant of 8.1 S has been isolated (Pretorius et al., 1981). It was shown to polymerize at pH 6.8 to yield two populations of clathrin baskets (coat structure) with average sedimentation rates of 150 S and 300 S and molecular weights of 25×10^6 and 100×10^6, respectively (Nandi et al., 1980).

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