Regulation of Muscle Differentiation: Isolation and Purification of Chick Actin Messenger Ribonucleic Acid and Quantitation with Complementary Deoxyribonucleic Acid Probes†

Robert J. Schwartz* and Katrina Rothblum

ABSTRACT: Actin mRNA was isolated from total nucleic acid extracts of 3-week-old chick muscle. mRNA purified by standard nucleic acid isolation procedures was assessed for purity by translation product analysis, electrophoretic migration on denaturing gels, and hybridization assays. Proteins synthesized in response to enriched actin mRNA yielded a product (M_r 42 000) identified by actomyosin formation, F-actin polymerization, and DNase binding. Enriched actin mRNA contained a predominant RNA species at 1575 nucleotides but was contaminated (~50%) with a single lower molecular weight mRNA (band II, 1390 nucleotides). Full-length complementary DNA transcripts of the individual mRNA were characterized for reassociation kinetics with actin and band II mRNA. Band II cDNA was hybridized to its pure mRNA with an $R_0t_{1/2}$ of 3×10^{-3} which was in agreement with the mRNA sequence complexity of 1390 nucleotides. Hybridization of actin-enriched cDNA in RNA excess showed that the cDNA preparation consisted of band II mRNA and a RNA moiety that contained a complexity of 1800 nucleotides, sufficient to represent a single molecule of α-actin mRNA. In fact, this value is in agreement with a single polymorphic form of α-actin which was resolved by two-dimensional gel electrophoresis. The pure band II mRNA was exploited to hybridize and remove band II DNA sequences by hydroxylapatite chromatography which resulted in a highly purified actin cDNA. The equivalent $R_0t_{1/2}$ values for hybridization of actin cDNA with chicken muscle indicated that actin mRNA composed 8% of the poly(A)-enriched RNA and 0.09% of the total cellular RNA. Actin cDNA was utilized to determine the gene dosage in chicken genomic DNA and represents a middle repetitive gene with 10 to 11 copies per genome. The availability of pure actin cDNA probes should facilitate the analysis of the regulation of muscle differentiation during chicken myogenesis.

Differentiation of embryonic skeletal muscle in culture has been described as a succession of developmental phases named prefusion, the proliferation of presumptive muscle cells, fusion, the formation of multinucleated myotubes from mononucleated muscle cells, and postfusion, the differentiation of functional fibers (Hermann et al., 1970). The end points of pre- and postfusion provide well-defined morphological markers of cell differentiation which are further complemented by changes in the levels of contractile proteins and muscle-specific enzymes (Buckingham, 1978). Perhaps the most complex of these morphological markers is the appearance and specific orientation of thick and thin filaments to form sarcomeres. Most molecular studies have concentrated on the biosynthesis of the predominant myofibrillar proteins such as heavy-chain myosin (Strohman et al., 1977; John et al., 1977), actin (Paterson et al., 1974; Hunter & Garrels, 1977), and tropomyosin (Carmon et al., 1978). Of these proteins, actin was once thought to be a single, highly conserved protein in all cell types, although recently multiple forms have been found (Gruenstein & Rich, 1973; Elzinga et al., 1976). In dividing myoblasts, actin was found to exist in three forms possessing similar biochemical properties and molecular weights (42 000) but different isoelectric points and amino acid sequences (Vanderkerchkove & Weber, 1978). Two of the forms (β and γ) are found in prefusion dividing myoblasts and in nonmuscle cells (Whalen et al., 1976). The third form (α), the most acidic, is the only one found in fetal muscle tissue and becomes the predominant species in a culture of fused muscle cells (Whalen et al., 1976); Garrels & Gibson, 1976).

According to current concepts, changes in morphogenesis and cell differentiation may be caused by selective gene expression. Overall actin mRNA activity was detected in muscle cells by in vitro translation assays and shown to increase following myoblast fusion (Paterson et al., 1974). However, the mechanism(s) for increased mRNA accumulation and the selective induction of α-actin mRNA in muscle development has not been elucidated. In order to relate the process of skeletal muscle differentiation to changes in specific gene activity, we undertook to monitor the synthesis and quantitate the levels to mRNAs for the muscle-specific marker proteins during myogenesis. To this end, we describe here the isolation and purification of actin mRNA of chick breast muscle, the synthesis of DNA complementary to the mRNA, and its characterization as a potential probe to measure the actin mRNA levels.

Materials and Methods

Materials. Three-week-old Rhode Island chicks were obtained from Animal Specialties. Oligo(dT)–cellulose (T3) was purchased from Collaborative Research, Inc. SV_{40} DNA and *Hin*dIII restriction endonuclease were from Bethesda Research, Inc. Nonlabeled deoxynucleoside triphosphates and micrococcal nuclease were purchased from P-L Biochemicals. Avian myeloblastosis viral (AMV) reverse transcriptase was kindly supplied by Dr. J. W. Beard of Life Sciences, Inc., St. Petersburg, FL. ^{3}H-Labeled ovalbumin cDNA and chicken liver DNA were a gift of Dr. Ming Tsai. Chick superior pectoral muscles were removed, immediately frozen in liquid nitrogen, and stored at −80 °C until used. Storage greater than 1 week was avoided when tissues were used for isolation of RNA.

Preparation of Total RNA. Extraction of RNA from frozen breast muscles from 3-week-old Rhode Island Red chickens was performed according to the procedure of Rosen

† From the Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030. *Received June 11, 1979.* This work was supported by U.S. Public Health Service Grant NS 15050, by National Science Foundation Grant PCM 77-27336, and by a grant from the Muscular Dystrophy Association of America. R.J.S. is a recipient of U.S. Public Health Service Research Career Development Award.

0006-2960/80/0419-2506$01.00/0

et al. (1975). Frozen tissue (100 g) stored at −80 °C was broken into small pieces and homogenized at room temperature in a small Waring blender in 5 volumes (v/w) of sodium dodecyl sulfate ($NaDodSO_4$), 0.025 M Na_2EDTA, and 0.075 M NaCl (pH 8.0) containing 5 volumes of buffer-saturated phenol (pH 8.0). The emulsion was chilled for 30 min in ice and centrifuged in 250-mL polypropylene bottles at 6500*g* for 30 min. The aqueous upper phase and the protein interphase were removed and reextracted with an equal volume of buffer-saturated phenol (pH 8.0) for 30 min at room temperature. Following centrifugation, the aqueous phase was removed and made 0.2 M in NaCl and total nucleic acids were precipitated with the addition of 2.5 volumes of 95% ethanol at −20 °C for 18 h. Total nucleic acid was pelleted by centrifugation at 10000*g* for 1 h and resuspended in autoclaved deionized distilled water. DNA was selectively removed from RNA by a series of 3 M NaOAc (pH 6.0) washes (Palmiter, 1974). A single preparation routinely yielded between 60 and 80 mg of total RNA.

Purification of Poly(A)-Containing mRNA. Affinity chromatography using oligo(dT)–cellulose (6–18 oligomers, Collaborative Research, Inc.) was performed at room temperature by an adaptation of the method of Aviv & Leder (1972). The total RNA extract was dissolved in 1 mM Na_2EDTA, pH 7.0, and heat denatured at 65 °C for 5 min, followed by rapid cooling to 4 °C. This prevents mRNA–rRNA aggregation and improves both the resolution and recovery of the affinity chromatography procedure. Then KCl and Tris buffer were added to the total RNA extract (1 mg/mL) at room temperature to yield a final concentration of 0.5 M KCl and 0.01 M Tris-HCl, pH 7.5. After thorough washing, poly(A)-containing mRNA was eluted with 0.01 M Tris-HCl, pH 7.5. In addition, the bound fraction was melted again and reapplied to the dT column to further remove rRNA contamination. A yield of ~500–700 μg of poly(A)-enriched RNA was isolated from 70 mg of phenol-extracted RNA.

Preparative Sizing Technique. (1) Sucrose gradients were performed by using a Beckman SW40 rotor for 9 h at 39000 rpm at 4 °C. Approxmately 300 μg of poly(A)-containing RNA was dissolved in 0.002 M Na_2EDTA, pH 7.0, and heated to 65 °C for 5 min, quick cooled to 4 °C, and immediately layered on a linear 5–20% (w/v) sucrose gradient in 0.02 M NaOAc (pH 5.0) and 0.001 M Na_2EDTA. Fractions were collected, and absorbance was measured at 260 nm.

(2) Fractionation by size of poly(A)RNA was also accomplished by chromatography on Sepharose 4B in 0.1 M NaOAc and 0.001 M Na_2EDTA (pH 5.0) at 4 °C (Woo et al., 1974). Approximately 2.5 mg of total poly(A)RNA was applied to a 1 × 40 cm column and eluted at a flow rate of 10 mL/h.

Preparative Electrophoresis of RNA. Selected RNA samples were purified by electrophoresis on 3.3% polyacrylamide gels containing a disulfide cross-linker, *N′,N′*-cystaminebis-(acrylamide) (Pierce), which was developed by Hanson (1976). The poly(A) muscle RNA enriched for actin mRNA was heat denatured, quick cooled, and then loaded in buffer containing 20% sucrose, 0.01 M Tris, pH 7.9, 0.001 M Na_2EDTA, and 70% formamide onto 10 × 0.5 cm disulfide cross-linked polyacrylamide cyclindrical gels. The electrophoresis buffer was composed of 0.04 M Tris, pH 7.9, 0.005 M NaOAc, and 0.001 M Na_2EDTA, and electrophoresis was carried out for 10 h at 4 mA/gel at 4 °C. Gels were stained with 0.02% methylene blue in 0.4 M NaOAc(pH 4.5) for 1 h and destained in autoclaved distilled water for 1 to 2 h at room temperature. Selected bands were sliced out of the gel and dissolved in the presence of 1.3 M β-mercaptoethanol and 0.005M Na_2EDTA at 37 °C for 1 h. The dissolved gels were then made 0.1% $NaDodSO_4$, 0.1 M NaCl, and 0.05 M Tris-HCl, pH 7.5, and extracted for an additional 2 h. The RNA was then removed from acrylamide polymers by passage over a cellulose column (1 × 2 cm) in the presence of 30% ethanol (Franklin, 1966). Under these conditions RNA was selectively bound to the cellulose matrix, while acrylamide polymers were removed by extensive washing. RNA was eluted with 15% ethanol in a volume of 1 to 2 mL and then precipitated by the addition of 2 volumes of ethanol. This procedure allows for a 5–10% yield of intact RNA from gel slices and is an improvement over Hanson's (1976) original protocol because it eliminates the passage over hydroxylapatite which causes degradation of RNA.

Cell-Free Protein Synthesis in Rabbit Reticulocyte Lysate. A simple method has been described to convert the standard rabbit reticulocyte cell-free extract into an mRNA-dependent protein synthesis system (Pelham & Jackson, 1976). The nuclease-treated lysate (30 μL) was combined with the following components: 10 mM Tris-HCl, pH 7.4, 1 mM ATP, 0.2 mM GTP, 100 mM KCl, 25 μM hemin, 0.01 M creatine phosphate, 2.5 μg of creatine phosphokinase, 10–20 μCi of [^{35}S]methionine (300–800 Ci/mmol) (Amersham), 10 μM of 19 amino acids minus methionine, and 0.1–2.0 μg of poly(A) RNA in a total volume of 60 μL. Incubation was done at 32 °C for 2 h, 5-μL aliquots were taken for trichloroacetic acid (Cl_3AcOH) insoluble counts per minute measurement, and the remainder was used for product analysis.

Identification of Actin. (1) Actomyosin was prepared according to Paterson et al. (1974). Cell-free reaction products containing the actin polypeptides were adjusted to contain 0.4 M KCl and 100 μg of chicken breast myosin. The lysate was clarified at 10000*g* for 10 min, and the actomyosin was precipitated by the addition of 10 volumes of cold water. The precipitate was washed 3 times in cold phosphate-buffered saline and then resuspended in buffer containing 1% $NaDodSO_4$, 1% β-mercaptoethanol, 8 M urea, and 50 mM Tris-HCl, pH 7.0, for electrophoresis on polyacrylamide gel.

(2) The protocol of Bag & Sarkar (1975) was used to identify actin as an in vitro translated product by a single cycle of reversible salt-dependent transformation of globular (G) to fibrillar (F) actin.

(3) Muscle G-actin has the unique property of inhibiting DNase activity by binding rapidly to form a 1:1 stable complex between the two proteins (Mannherz et al., 1975). This highly specific interaction was used to identify in vitro synthesized actin. The translated products were treated with 0.5% Triton X-100, 0.75 M guanidine hydrochloride, 0.5 M NaOAc, 1 mM ATP, 1 mM $CaCl_2$, and 0.01 M Tris, pH 7.5, to depolymerize all synthesized actins (Bilkstad et al., 1978). This preparation was then reacted with 100 mg of DNase I–Sepharose, prepared according to Lindberg & Eriksson (1971), for 1 h. The agarose beads were washed consecutively with buffer A, which contained 0.01 M Tris-HCl, pH 7.5, 0.75 M NaCl, and 1 mM $CaCl_2$, and buffer B, which contained 0.75 M guanidine hydrochloride, 0.5 M NaOAc, 1 mM $CaCl_2$, and 0.01 M Tris-HCl, pH 7.5, and eluted with buffer containing 3 M guanidine hydrochloride, 0.5 M NaOAc, and 0.01 M Tris-HCl, pH 7.5. The released actin was dialyzed against 0.01 M Tris-HCl, pH 7.5, and concentrated by lyophilization.

Polyacrylamide Gel Electrophoresis. All electrophoretic separations were performed on slab gels with the Bio-Rad electrophoresis apparatus. Proteins were examined on 10% polyacrylamide gel (1.5 × 100 × 250 mm) containing $NaDodSO_4$ as described by Laemmli (1970) with a 3% poly-

acrylamide stacking gel. Electrophoresis was done at 40 mA for 5 to 6 h. Gels were fixed and stained in 0.1% Coomassie blue and 50% Cl_3AcOH and destained in 7% acetic acid and 30% methanol. Gels were infiltrated with PPO (Bonner & Laskey, 1974) and fluorographed with Kodak royal blue X-O-Mat film.

Two-Dimensional Gel Electrophoresis. In vitro synthesized [^{35}S]actin was isoelectric focused with chick skeletal actin isolated by the procedure of Spudich & Watt (1971). Isoelectric focusing in the first dimension was performed on a 5% polyacrylamide gel that contained a pH gradient estabished by 2% (w/v) ampholines of which 80% was pH 4–6 and 20% was pH 3–10 at 9000 V h according to the protocol of O'-Farrell (1975). These conditions allow for the resolution of α-, β-, and γ-actins. The second dimension was electrophoresed on $NaDodSO_4$–10% polyacrylamide slab gels (Laemmli, 1970).

Synthesis and Characterization of Complementary DNA. Tritiated complementary DNA to actin-enriched mRNA was prepared according to the procedure described by Efstratiadis et al. (1975) and Monahan et al. (1976), with a few modifications. The reaction was done in a total volume of 20 μL containing 2.5 μg/mL oligo(dT)$_{12-13}$, 50 mM Tris-HCl, pH 8.3, 20 mM dithiothreitol, 6 mM $MgCl_2$, 400 μM each of the deoxynucleoside triphosphates except [^{3}H]deoxycytidine triphosphate (ICN; 25 Ci/mmol), which was 100 μM, 50 μg/mL mRNA, and 5 units of AMV reverse transcriptase per μg of RNA. The reaction was carried out at 46 °C for 15 min, and the cDNA was isolated free of nucleotides and RNA as previously described (Harris et al., 1973). Under a 40% scintillation counting efficiency, the [^{3}H]cDNA had a specific radioactivity of 17.8×10^6 cpm/μg. The size of the cDNA was determined by electrophoresis on alkaline 4% polyacrylamide gels prepared according to McDonnell et al. (1977). DNA fragments of SV_{40} DNA digested with *Hin*dIII restriction endonuclease served as molecular weight standards.

DNA and RNA Excess Hybridization. DNA excess hybridizations were carried out in a final volume of 50 μL in tapered reaction vials (Regis Chemical Co.). A constant ratio of unlabeled DNA to [^{3}H]cDNA was maintained in each vial. Each reaction vial contained approximately 2000 cpm of [^{3}H]cDNA and 200 μg of sheared chicken liver DNA. Hybridization was performed in 0.6 mM NaCl, 0.01 M Hepes, pH 7.0, and 0.002 M Na_2EDTA at 62 °C. The extent of hybridization was determined by S_1 nuclease resistance.

RNA excess 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 2000 cpm of [^{3}H]cDNA and varying amounts of purified RNA. Following heat denaturation for 5 min at 100 °C, incubations were performed for 1–16 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 (Miles) followed by Cl_3AcOH precipitation. Between 5 and 10% of the [^{3}H]cDNA incubated in the absence of added RNA was resistant to S_1 nuclease treatment. The data from the hybridization experiments were expressed as the percent hybridization vs. the equivalent C_0t for DNA excess hybridization and the equivalent R_0t for RNA excess hybridization.

Results

Total nucleic acid (650 mg) was isolated from 500 g of frozen breast muscle by an $NaDodSO_4$–phenol extraction procedure. DNA plus low molecular weight RNA was selectively removed from the total nucleic acid preparation by 3 M sodium acetate washes. The total RNA prepartion (400 mg) was separated into a poly(A)-enriched fraction by chromatography on oligo(dT)–cellulose. Total RNA was heated to 65 °C in 1 mM EDTA, pH 7.0, for 5 min and then quick cooled in a dry ice–ethanol bath prior to two passages over oligo(dT)–cellulose. The removal of DNA is a critical step in eliminating aggregation of RNA–DNA complexes before oligo(dT)–cellulose chromatography. Total cellular RNA and poly(A)-enriched muscle RNA were electrophoresed on 3% agarose gel in the presence of 0.025 M sodium citrate, pH 3.5, and 5 M urea (Figure 1). After oligo(dT)–cellulose chromatography, essentially all the rRNA and 4S and 5S RNA were removed, while large selective increases in two RNA species (band I, 5.5×10^5 apparent M_r; band II, 4.5×10^5 apparent M_r) were observed in the poly(A)-enriched RNA.

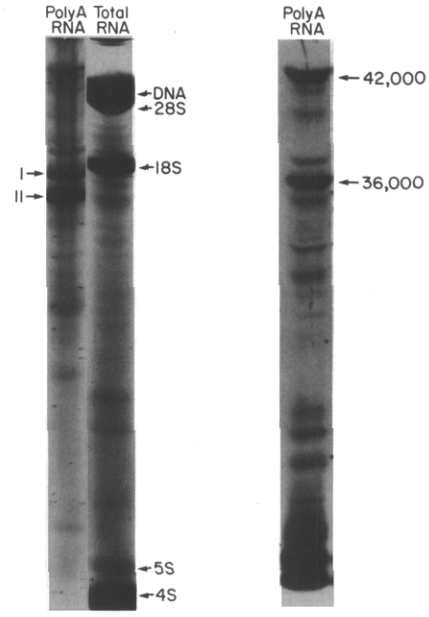

FIGURE 1: Electrophoretic analysis of total muscle poly(A)-enriched RNA and in vitro translation products. RNA samples (left), including total RNA before sodium acetate washes and poly(A)-enriched RNA after oligo(dT)–cellulose chromatography, were electrophoresed on 3% agarose gels according to the protocol of Rosen et al. (1975). The poly(A)-enriched RNA preparation was translated in a mRNA-dependent reticulocyte lysate (right). [^{35}S]Methionine-incorporated products were electrophoresed on $NaDodSO_4$–10% polyacrylamide gel and processed for autoradiography as described under Materials and Methods.

The incorporation of [^{35}S]methionine in a nuclease-treated reticulocyte lysate to which total poly(A)-enriched muscle RNA had been added was at least 10-fold greater than in extracts without added RNA. Incorporation was linear for 90 min and was maximal at an RNA concentration of 20–40 μg/mL. The mRNA-dependent translation products were electrophoresed on $NaDodSO_4$–10% polyacrylamide gels, and labeled polypeptides were visualized by fluorography on X-ray film (Figure 1). Polypeptides of M_r 42 000 and 36 000 comigrated with authentic chick breast actin and α-tropomyosin standards which individually accounted for ~10% of the total incorporated methionine into labeled polypeptides (Figure 1). Translation products which comigrated with actin were further identified by biochemical assays.

Identification of Translated Actin. Actomyosin formation, the globular to fibrillar actin transformation, and the binding to DNase are three inherent properties of actin. The property of actin and myosin to form an actomyosin complex was first used to identify the presumptive synthesized actin product.

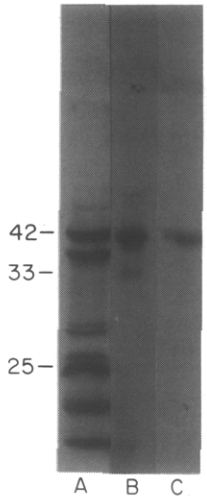

FIGURE 2: Translation product analysis of actin-containing mRNA. Total muscle poly(A)-enriched RNA (1 μg) was translated in a mRNA-dependent reticulocyte lysate (60 μL), and actin products were analyzed by (A) coprecipitation with chicken breast myosin, (B) F-actin coprecipitation, and (C) binding to DNase–Sepharose as described under Materials and Methods and Results.

The actomyosin complex was formed by the addition of purified chicken skeletal muscle myosin to the lysate in the presence of 0.4 M NaCl and was precipitated by the reduction of salt to 0.04 M NaCl. The actomyosin complex was pelleted by centrifugation and electrophoresed on $NaDodSO_4$–10% polyacrylamide gel. In Figure 2A, actin composed 15% of the radioactivity in the actomyosin pellet, while additional autoradiographic bands were associated with polypeptides which appeared to comigrate with tropomyosin (M_r 36000) and the troponins and myosin light chains.

The reversible polymerization of G-actin to F-actin is a characteristic property of all actins. Because of its large size, F-actin can be separated from contaminating soluble protein by centrifugation. The in vitro synthesized products directed by muscle poly(A)-enriched RNA were subjected to a single cycle of low salt–high salt actin polymerization in the presence of low concentrations of adult muscle actin carrier. The F-actin pellet was collected by centrifugation at 100000*g* for 2 h and subjected to electrophoresis in $NaDodSO_4$–polyacrylamide gel (Figure 2B). After one cycle of polymerization, the F-actin pellet contained the muscle actin stained with Coomassie blue and 80% of the radioactivity.

The specific binding of actin to DNase I was utilized to develop a reagent for quantitating actin mRNA translational activity. In order to determine binding specificity, we incubated radioactive polypeptides synthesized in vitro in the presence of either chicken oviduct, reticulocyte, and/or muscle poly(A)-enriched RNA with DNase–Sepharose and an excess of either actin (200 μg) or bovine serum albumin (200 μg). Pretreatment of translated products with 0.75 M guanidine was utilized to depolymerize actin and enabeled the dissociated [^{35}S]actin to bind to DNase–Sepharose beads. The affinity resin was washed consecutively with buffers A and B to remove unincorporated label and soluble proteins. Actin was eluted from DNase–Sepharose beads with 3 M guanidine hydrochloride. In Table I, ~1.8–2.2% of the radioactive protein synthesized by either oviduct or reticulocyte mRNA was bound to the resin, while 10% of the labeled protein synthesized in the presence of muscle mRNA was bound to the DNase matrix. The actin polypeptides were specifically bound since at least 78% of the radioactive material was competed with an excess of cold actin (200 μg), while BSA was ineffectual.

Table I: Specificity of [^{35}S]Actin Binding to DNase–Sepharose

source of poly(A) RNA	in vitro synthesized protein[a] (cpm)	standard elution[b] (cpm)	actin competition[c] (cpm)	BSA competition[d] (cpm)
oviduct	240 500	5 400	4500	3 290
reticulocyte	193 900	3 500	3690	2 570
muscle	218 000	22 200	5050	21 550

[a] Total incorporation of acid-precipitable [^{35}S]methionine in a 60-μL translation assay. [b] Radioactivity eluted from 100 μL of packed DNase–Sepharose beads with 3 M guanidine as described under Materials and Methods. [c] Actin (200 μg) was incubated with DNase–Sepharose and translation lysate and then eluted with 3 M guanidine hydrochloride. [d] Bovine serum albumin (200 μg) was incubated with DNase–Sepharose and translation lysate and then eluted with 3 M guanidine hydrochloride.

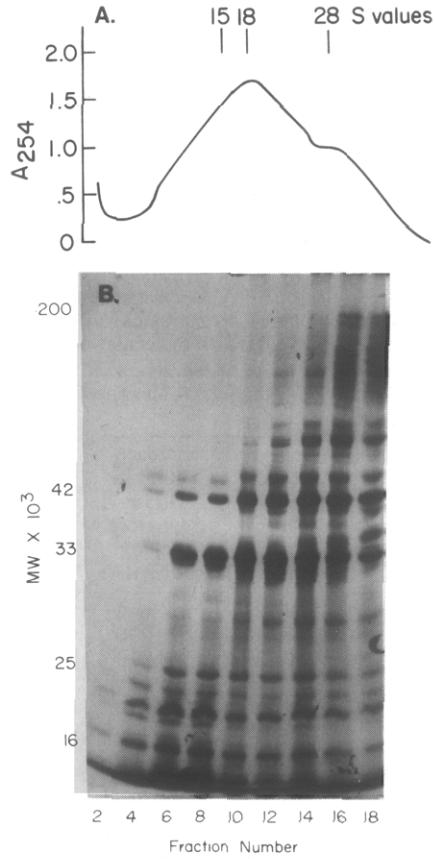

FIGURE 3: Translation products of poly(A)-enriched RNA fractionated on sucrose gradients. Panel A shows the absorbance profile of 300 μg of poly(A)-enriched RNA centrifuged for 9 h on a 12.5-mL 5–20% sucrose gradient at 39000 rpm. In panel B, RNA fractions were collected by ethanol precipitation and then translated in the reticulocyte lysate. The radioactive products were electrophoresed on a $NaDodSO_4$–10% polyacrylamide slab gel and autoradiographed.

Actin eluted from DNase–Sepharose beads with 3 M guanidine hydrochloride was electrophoresed on $NaDodSO_4$–polyacrylamide gels as shown in Figure 2C. Essentially all the radioactivity (95%) comigrated with the actin band (M_r 42000). The high degree of specificity of binding to DNase–Sepharose will serve in quantitating actin mRNA purification.

Actin mRNA Isolation and Purification. Since actin mRNA comprised a large percentage of the total mRNA activity in the breast muscle, it should be possible to purify the individual mRNAs by a combination of sizing and chromatographic procedures. After heat denaturation to prevent mRNA aggregation, the oligo(dT)-purified RNA was ana-

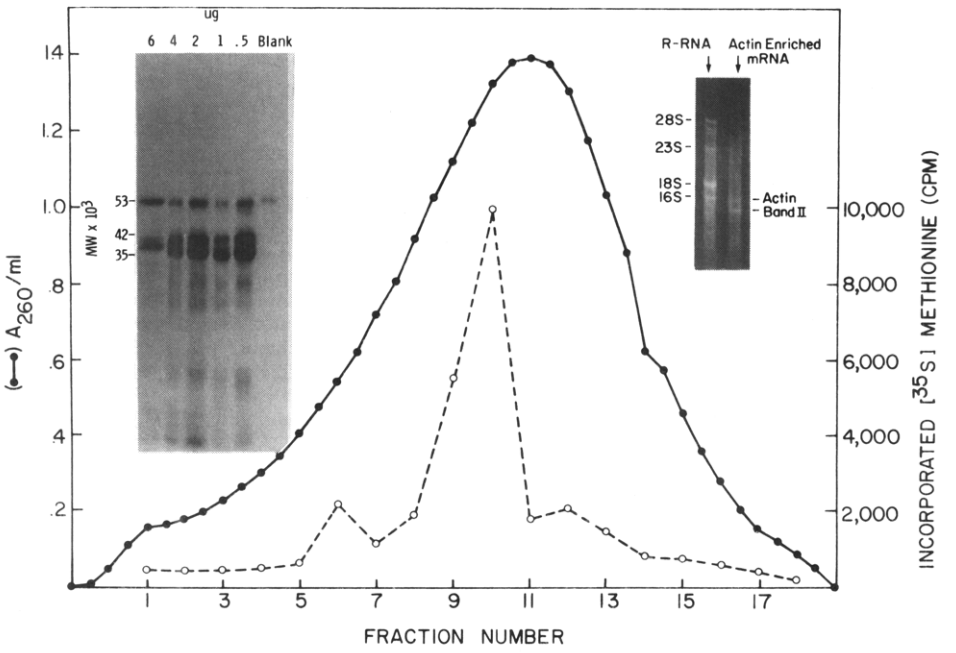

FIGURE 4: Separation of sucrose gradient fractionated RNA on Sepharose 4B. Sucrose gradient fractions 6–10 were collected (2.5 mg) and applied to a 1 × 50 cm Sepharose 4B column in 0.01 M sodium acetate and 0.011 M Na_2EDTA, pH 5.0. Fractions (1 mL) were collected and ethanol precipitated, and then every other fraction was assayed for translational activity for actin by binding to DNase–Sepharose (○). The left insert is the autoradiograph of the actin-enriched mRNA-directed translation products of fraction 10. The right insert is the electrophoretic analysis of actin-enriched mRNA on 2% agarose gel containing 5 mM methylmercury hydroxide stained with ethidium bromide (Bailey & Davidson, 1976). Ribosomal RNA of chicken 28 S (M_r 1.65 × 10^6) and 18 S (M_r 0.71 × 10^6) and *Escherichia coli* 23 S (M_r 1.07 × 10^6) and 16 S (M_r 0.53 × 10^6) served as molecular weight standards.

Table II: Purification of Actin mRNA Measured by Translational Activity

stage of purifn	total [^{35}S]Met incorpn (cpm/ μg of RNA)	radioact. bound to DNase–Sepharose (cpm/ μg of RNA)	% incorpd into actin	x-fold purifn
total RNA	3 520	642	18	1
poly(A) RNA	218 140	22 230	10	35
sucrose gradient	350 430	67 410	19	105
Sepharose 4B	395 000	185 650	47	289
preparative polyacrylamide gels	360 780	184 130	51	210

lyzed by centrifugation on 5–20% sucrose gradients (Figure 3). A broad peak containing actin mRNA was detected from 12 to 28 S with the maximum mRNA activity occurring between 15 and 18 S. These activities correspond to a large peak of absorbance at 260 nm. An additional shoulder at 26–28 S corresponded to mRNA coding for assorted large proteins such as myosin heavy chain. The gradient peak from fraction 9 through 13 was collected and represented about a 100-fold increase in actin translational activity over the starting total RNA extract (Table II).

Further purification of sucrose gradient fractions 6–10 was accomplished by chromatography on Sepharose 4B on 0.1 M sodium acetate (pH 5.0) containing 1 mM Na_2EDTA, pH 5.0, at 4 °C. Under these conditions any 28S rRNA contaminant remains selectively bound to the Sepharose 4B beads, while the remaining RNA was fractionated by size (Figure 4). Actin mRNA activity was measured by translation product analysis of all eluted fractions by DNase–Sepharose binding assays. The Sepharose 4B elution profile shows actin eluted in one major peak fraction (fraction no. 9–10 in Figure 4). Examination of actin-enriched mRNA-directed translation products on $NaDodSO_4$–polyacrylamide slab gels (Figure 4, left insert) revealed that about half of the incorporated [^{35}S]methionine resided in a polypeptide which comigrated with actin while the remainder resided with a lower molecular weight polypeptide (M_r 36 000). Translation of these RNA species showed a complete loss of mRNA coding for protein of a molecular weight greater than 42 000, while high doses of input RNA failed to stimulate a significant translation of protein with a molecular weight less than 36 000. Thus, different size classes of mRNA could be separated by chromatography on Sepharose 4B, while a selective enrichment of two- to threefold for actin mRNA in comparison to sucrose gradient fractionation (Table II) could be obtained by pooling the appropriate Sepharose 4B fractions. The distribution of RNA components in the actin-enriched mRNA fraction was analyzed by electrophoresis of RNA on denaturating 2% agarose gels which contained 5 mM methylmercury hydroxide. Two distinct ethidium bromide stained bands with apparent molecular weights of 5.2 × 10^5 (1575 nucleotides) and 4.6 × 10^5 (1390 nucleotides) appear to correspond to the major translation products (Figure 4, right insert).

In order to separate these two RNA species, it was necessary to use a preparative type electrophoretic procedure. We elected to use electrophoresis on cylindrical polyacrylamide gels which were composed of disulfide bis(acrylamide) cross-links (Hanson, 1976) and contained 3.3% polyacrylamide. The RNA was heated to 65 °C for 30 min in 1 mM EDTA and overlaid in 70% formamide and 10% sucrose onto 10-cm long gels. After electrophoresis, RNA bands were stained with 0.02% methylene blue in 0.4 M NaOAc, pH 4.5, and gels were destained in autoclaved distilled water for 1 to 2 h at room temperature. Under these conditions the RNA is neither fixed nor modified within the gel. The stained RNA bands were excised from the gel and completely dissolved in concentrated β-mercaptoethanol and 5 mM EDTA. The dissolved gels were treated with 0.1% $NaDodSO_4$, 0.1 M NaCl, 1 mM EDTA, and 50 mM Tris-HCl, pH 7.5, for 2 h at 37 °C. The RNA was then removed from acrylamide polymers by passage over

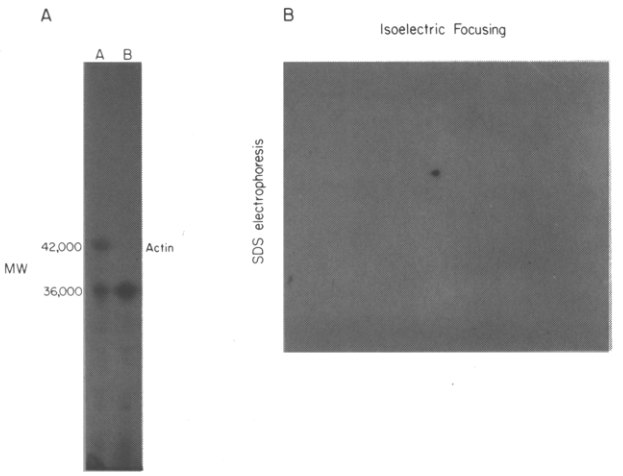

FIGURE 5: Electrophoretic analysis of purified actin-enriched mRNA-directed translation products. In panel A, the translation of actin-enriched mRNA (slot A) and band II mRNA (slot B) was from preparative gel electrophoresis. In each assay, ~0.5 μg of RNA was translated in the mRNA-dependent reticulocyte assay. In panel B, actin-enriched mRNA translated product was displayed by two-dimensional gel electrophoresis as described under Materials and Methods.

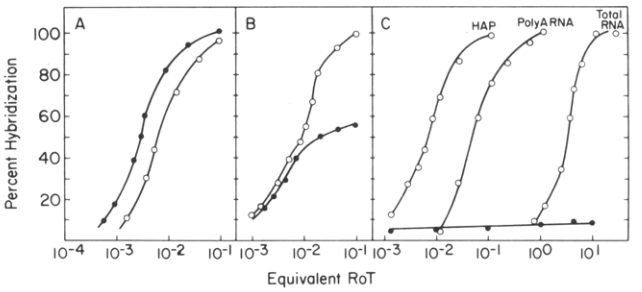

FIGURE 6: Specificity of hybridization of purified actin and band II mRNA with their [^{3}H]cDNAs. RNA excess hybridizations were performed with purified band II mRNA (●) and actin-enriched mRNA (○) by using band II [^{3}H]cDNA in panel A, actin-enriched [^{3}H]cDNA in panel B, and hydroxylapatite-purified actin [^{3}H]cDNA in panel C. Hybrids were assayed as described under Materials and Methods. In panel C, actin-enriched [^{3}H]cDNA (50 ng) was hybridized to band II mRNA (5 μg) to a R_0t of 10^{-1}. The reaction mixture was incubated with 1 mL of hydroxylapatite (Clarkson) at 60 °C in 0.01 M $NaPO_4$ (pH 6.7). Single-stranded actin [^{3}H]cDNA was eluted at 0.12 M $NaPO_4$, applied to a column (1 × 50 cm) containing Sephadex G-50 with a pad of Chelex 100 (Bio-Rad), and eluted in buffer containing 0.05 M Tris, pH 7.5, 0.1 M NaCl, and 1 mM Na_2EDTA. The actin [^{3}H]cDNA was coprecipitated with carrier *E. coli* tRNA (10 μg/mL) and 2 volumes of ethanol and 0.5 M NaCl. The actin [^{3}H]cDNA was 95% sensitive to S_1 nuclease.

a cellulose column (1 × 2 cm) in the presence of 30% ethanol (Franklin, 1966). RNA is selectively bound to the cellulose, while the majority of the acrylamide is removed by extensive washing. RNA was eluted with 15% ethanol in a volume of 1–3 mL and precipitated by the addition of 2 volumes of ethanol. Frequently, a small amount of residual acrylamide coelutes with RNA and must be removed by an additional phenol–chloroform extraction. Nevertheless, this procedure has allowed us to partially separate the two RNA species.

The isolated RNA species were translated in the mRNA-dependent reticulocyte lysate. Analysis of the translated products of band I RNA in Figure 5A, slot A, showed that ~50% of the incorporated [^{35}S]methionine resided with actin, while the remainder was found to migrate with a polypeptide of M_r 36 000. The translated products of band II RNA revealed that 95% of the total incorporated label migrated with a polypeptide of M_r 36 000 (Figure 5A, slot B). Further identification of the translated products as well as the homogeneity was examined directly by two-dimensional gel electrophoresis (Figure 5B). Acidic conditions (pH 3–6) were necessary to ensure resolution of actin within the isoelectric focusing gel. Inspection of autoradiographs of two-dimensional slab gels, for band I directed products, resolved a single spot with a molecular weight of 42 000 which focused within a pH range of 5.4–5.5 (Figure 5B). This autoradiographic spot comigrated with skeletal actin standard and corresponds to the α form of actin. From these findings actin mRNA can be unequivocally assigned to band I (M_r 5.2 × 10^5; Figures 1 and 5).

Unexpectedly, the major contaminant of actin mRNA translation products, which appeared to comigrate with α-tropomyosin in one-dimensional polyacrylamide gel electrophoresis, failed to enter isoelectric focusing gels from the basic end. In fact, the M_r 36 000 polypeptide encoded by band II mRNA is not tropomyosin for the following reasons: (1) the mRNA coding for tropomyosin was found to be smaller in size (~14 S) than band II RNA; (2) the M_r 36 000 polypeptide failed to coprecipitate with affinity-purified precipitating tropomyosin antibody; (3) the M_r 36 000 protein contains proline while tropomyosin is void of proline; (4) cyanylation of the ^{35}S-labeled M_r 36 000 polypeptide revealed vastly different fingerprints; (5) the M_r 36 000 polypeptide is basic with an isoelectric point of ≤8, contrary to that of tropomyosin which is 4.5. Translation analysis has yet to determine whether the major actin contaminant, the M_r 36 000 product, is a single polypeptide or its proper identity. However, this product is encoded by band II mRNA (M_r 4.6 × 10^5), a high abundant poly(A)-containing RNA which accounts for 8% of the mRNA content in breast muscle.

Hybridization of [^{3}H]cDNA with Excess Actin and Band II mRNA. Several reports have shown that full-length complementary DNA transcripts of individual mRNAs can be synthesized by using AMV RNA-directed DNA polymerase and adjusting both the deoxynucleotide triphosphate substrate concentration and the time and temperature of incubation (Efstratiadis et al., 1975; Monahan et al., 1976). RNA coding for actin and M_r 36 000 protein was used as templates for AMV reverse transcriptase. Under the conditions chosen (see Materials and Methods), 0.45 μg of cDNA was made per μg of complete RNA with a specific activity of 1.7 × 10^7 cmp/μg. Copy DNA made to actin-enriched mRNA was sized to 1400–1600 nucleotides. The band II cDNA had an average size of 1400 nucleotides per chain when examined by electrophoresis on alkaline polyacrylamide gel. A high proportion of the DNA copies are full-length, although some batches of AMV reverse transcriptase yielded cDNA of substantially smaller size. The complete removal of acrylamide from the preparative electrophoresis purification step was also a critical factor in attaining full-length cDNA. The S_1 nuclease digestion of cDNA indicated that between 5 and 10% of the DNA contained double-stranded structure.

For further characterization of cDNA and for quantitation of mRNA and its complexity, the kinetics of reassociation of cDNA with actin or band II mRNA was studied. Figure 6A shows the kinetics of band II cDNA–mRNA hybridization performed with excess mRNA. The reaction took place within the range of 100 R_0t units, displaying a pseudo-first-order reaction rate. At completion, 98% of the ^{3}H-labeled band II cDNA was hybridized to its pure mRNA with an equivalent $R_0t_{1/2}$ of 3 × 10^{-3}. Band II cDNA was utilized to determine the extent of contamination of the actin-enriched mRNA with band II mRNA. The rate of the cross-hybridization reaction (band II cDNA vs. actin mRNA) was almost twice as slow

as that of the homologous reaction, i.e., $R_0t_{1/2} = 6 \times 10^{-3}$, but both reactions went to greater than 95% completion (Figure 6A). Therefore, actin mRNA appears to contain a considerable band II mRNA contaminant, which is consistent with the analysis of protein translation products (Figure 6A).

Similar hybridization experiments were also performed with actin-enriched cDNA with excess actin mRNA (Figure 6B). Analysis of the data indicated that a hybridization curve containing two different RNA classes provided the best fit for the data. The $R_0t_{1/2}$ value for the first abundant class was 5×10^{-3}, while the $R_0t_{1/2}$ of the other highly abundant component was 1×10^{2}. When actin-enriched cDNA was reacted with band II RNA, the $R_0t_{1/2}$ was 7×10^{-3} and 56% of the cDNA was found to be complementary. These data suggest that the initial abundance class for actin cDNA which hybridized to mRNA was due to the high level of band II mRNA contamination. If a correction is made for the fact that each component in the hybridization of actin cDNA–mRNA represents a fraction of the total RNA population, then the $R_0t_{1/2}$ pure for the second component is 3.9×10^{-3}. Since the $R_0t_{1/2}$ is directly proportional to the RNA sequence complexity (Wetmur & Davidson, 1968), defined as the total number of nucleoside residues present in an unrepeated sequence of nucleic acid, one can calculate the sequence complexity of actin mRNA by comparing its reaction kinetics with that of band II mRNA. By use of 1390 nucleotides as the chain length of band II mRNA (Figure 4), a complexity of 1800 nucleotides was obtained for actin mRNA. This value is in agreement with a singled polymorphic form of actin which was resolved by isoelectric focusing. It appears that the actin-enriched mRNA preparation consists of two molecular species; one is actin and the other appears to be band II RNA.

As a further means of purification of actin-specific sequences in the cDNA preparation, we exploited the highly purified band II RNA to remove contaminating band II cDNA sequences. ^{3}H-Labeled actin-enriched cDNA (50 ng) was incubated with a 100-fold mass excess of band II mRNA (5 μg) and hybridized to a R_0t of 10^{-1}, which is sufficiently high to ensure completion of the reaction. Under these conditions cDNA complementary to actin mRNA will remain single stranded. Subsequent to the hybridization reaction, single- and double-stranded material was separated by chromatography on hydroxylapatite according to the protocol of Alt et al. (1978). Approximately 55% of the radioactive material was recovered in the double-stranded fraction, while the remainder was detected as single-stranded DNA. On the assumption that the relative abundance of sequences in the cDNA preparation is representative of the mRNA population from which it was derived, this recovery roughly corresponds to that expected for the actin-specific sequence. The maximum hybridization of this cDNA function was 100% to actin-enriched mRNA at a $R_0t_{1/2}$ of 7×10^{-3} and it did not significantly (~5–8%) hybridize to band II mRNA sequences (Figure 6C). This result suggests that the purified actin cDNA preparation consists mainly of sequences complementary to similar species of mRNA.

The potential of ^{3}H-labeled actin cDNA as a probe for quantitation of actin mRNA sequences was investigated by hybridization with RNA from different stages of purification. As shown in Figure 6C, the $R_0t_{1/2}$ observed for the starting RNA preparation was 3.5. This kinetic value suggests that actin mRNA comprises only 0.09% of the total RNA obtained from chick breast muscle. These data also show an overall 1400-fold purification of the actin mRNA by the steps already described.

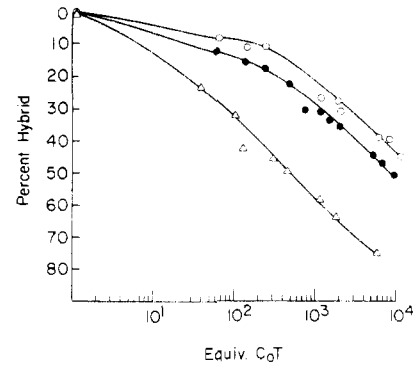

FIGURE 7: Determination of actin gene dosage in chicken DNA. DNA excess hybridizations were performed as described by using 200 μg of sheared chicken liver DNA (400-nucleotide number-average molecular weight) and 1.13×10^{-4} μg of [^{3}H]cDNA (sp act. 17.8×10^6 cpm/μg) made to ovalbumin mRNA (○), band II mRNA (●), and actin mRNA (△). The extent of hybridization was measured by S_1 nuclease digestion as described. A background of 8% was subtracted from all data points.

Actin Gene Dosage in Chicken DNA. Since actins appear to be products of multiallelic genes, it was not apparent whether these proteins are coded by unique or moderately repetitive sequences in the chick genome. Therefore, utilizing the rate of hybridization of [^{3}H]cDNA to its complementary strands present in a large excess of sheared chicken liver DNA (400-nucleotide mean average) at a ratio of 1 to 1.8×10^6, it was possible to assess the number of copies of these sequences in the genome. Full-length ovalbumin cDNA (1890 nucleotides) was used as an analytical standard for a unique gene sequence. The equivalent $C_0t_{1/2}$ of 1.8×10^3 for ovalbumin (Figure 7) was similar to previously published values (Sullivan et al. 1973; Monahan et al., 1976). ^{3}H-Labeled actin cDNA (≤90% pure) hybridized to 83% completion and had a $C_0t_{1/2}$ of 1.5×10^2 when reacted with chicken DNA (Figure 7). There are 11-fold more actin genes than ovalbumin. Since α-actin cDNA cross-hybridizes to an extent of 70% to nonmuscle β- and γ-actin mRNA isolated from either gizzard or embryonic brain tissue (data not shown), it is not clear to what degree each actin gene sequence is repeated in the chicken genome. However, the gene dosage experiment suggests that actin is a moderately repetitive gene family in higher eucaryotes. Interesting, the extent and rate of hybridization of [^{3}H]cDNA indicated that highly abundant band II mRNA was transcribed from nonrepetitive DNA sequences at 50% completion and a $C_0t_{1/2}$ of 7.5×10^2 (Figure 7). These data suggest that there are twice as many band II genes as those of ovalbumin.

Discussion

Identification of the product of cell-free translation of chick muscle actin mRNAs and preparation of DNA probes complementary to this mRNA will be required to permit further investigation of the possible regulation of actin biosynthesis in muscle development. Actin is synthesized in appreciable quantities during myogenesis and comprises ~10% of the myofibrillar proteins in muscle. Such a large abundancy of this component has allowed for both the purification of proteins and the detection of mRNA activity in total cell RNA extracts (Figure 1). Actin was identified by actomyosin formation, fibrillar actin polymerization, and quantitative binding to DNase–Sepharose (Figure 2, Table I). Approximately 10% of the translated products of the poly(A)-enriched RNA was detected as actin, in agreement with the cellular content in skeletal muscle. Furthermore, the translation product of actin

mRNA represented authentic actin because it demonstrated a pattern on two-dimensional gels identical with the α-actin standard and consistent with the reported limited heterogeneity of the mRNA (Garrels & Gibson, 1976).

Substantial purification of actin mRNA was accomplished by a combination of precise sizing techniques with the selective purification of poly(A)-enriched RNA by affinity chromatography. In our initial studies, actin mRNA was found to contain considerable ribosomal contamination and to migrate greater than 18 S on sucrose gradients. Attempts to disaggregate higher molecular weight complexes by heating to 70 °C or treatment with formamide proved unsuccessful. However, the removal of DNA from the total nucleic acid preparation by a series of sodium acetate washes was essential for the removal of ribosomal RNA contaminants following oligo(dT)–cellulose chromatography. This resulted in the selective enrichment of two major poly(A)-containing RNA species (bands I and II in Figure 1) in which band I appeared to code for actin and accounted for a purification of 135-fold over the total nucleic acid preparation by translational activity (Table II). Fractionation of the poly(A)-enriched RNA on sucrose gradients allowed for an additional threefold purification in which mRNA larger than 18 S in size was removed from actin mRNA which migrated between 15 and 18 S. Sepharose 4B chromatography was capable of fractionating mRNAs based on their respective sizes (Woo et al., 1974) and allowed for the removal of most RNA smaller than 15 S in size. At this step in the purification scheme, almost 95% of the total mRNA activity was present as actin and a major M_r 36000 protein contaminant (Figure 4). However, the apparent molecular weight of actin mRNA (5.2×10^5) and band II mRNA (4.6×10^5) was too close to enable separation by column chromatography. This problem was solved by electrophoresis on disulfide cross-linked polyacrylamide gel which permitted the complete separation and purification of band II mRNA away from the actin-enriched mRNA. The extent of mRNA purification was evident from the homogeneity of the eluted mRNA species and the translated products.

From the size determination on denaturing methylmercury hydroxide–agarose gels, it was possible to calculate the approximate number of noncoding nucleotides in the actin mRNAs. The mRNA which codes for α-actin is 1575 nucleotides in length, and, allowing for a poly(A) stretch of $\sim$100 nucleotides at the 3′ end, there are 1475 transcribed nucleotides. The size of the highly purified actin mRNA from chicken breast muscle agrees with a size estimate of α-actin mRNA from the L6 rat myoblast cell line (Hunter & Garrels, 1977). We know that 1122 nucleotides are required to code for α-actin from the amino acid sequence (Elzinga et al., 1973). The noncoding region of mRNAs at the 5′ region probably is $\sim$50 nucleotides in length (Proudfoot & Longley, 1976). The noncoding region at the 3′ and 5′ regions of the actin mRNA, therefore, contains 350 nucleotides. Interestingly, the nonmuscle β- and γ-actin mRNAs contain a much larger noncoding region of $\sim$1000 nucleotides (Hunter & Garrels, 1977). A few other cellular mRNAs are known to have long noncoding regions. Ovalbumin mRNA has been accurately sized and was found to contain $\sim$600 noncoding nucleotides (Woo et al., 1974), while lens α A_2 crystalline mRNA has more than 700 noncoding nucleotides (Bloemendal, 1977). The significance of a large untranslated region in the actin mRNAs is currently not known.

Additional evidence for purity comes from the kinetics of hybridization with the cDNA. If the mRNA preparations contain heterogeneous RNA as a contaminant, it would affect the R_0t value of the reaction. If the contaminants are poly(A)-containing RNA and are transcribed into cDNA, the reassociation will occur resulting in a gradual increase in the extent of hybridization over a wide range of R_0t values affecting the shape of the curve (Bishop et al., 1974). The hybridization reaction of band II cDNA to band II mRNA (Figure 6) occurred within a range of 100 R_0t units and displayed a pseudo-first-order reaction rate. At completion, 98% of the ^{3}H-labeled band II cDNA was hybridized to band II mRNA with an apparent observed $R_0t_{1/2}$ of 3×10^{-3}. This value is intermediate between the $R_0t_{1/2}$ of 4×10^{-3} for pure ovalbumin mRNA which contains 1890 nucleotides (Monahan et al., 1976) and the $R_0t_{1/2}$ of 1×10^{-3} for globin mRNA which contains 700 nucleotides (Ross et al., 1974) and is, therefore, consistent with a single species of RNA with the complexity of 1390 nucleotides. This mRNA represents $\sim$8% of the poly(A) mRNA sequences in chick breast muscle. Its translation product has been characterized and shown not to resemble any of the structural skeletal proteins including tropomyosin. The identification of this highly abundant RNA is under investigation.

Hybridization experiments performed on actin-enriched cDNA in RNA excess showed that the cDNA preparation consisted essentially of two molecular species. Band II mRNA was a major contaminant which composed $\sim$50% of the cDNA, while the remaining sequences were shared with a moiety that contained a complexity of 1800 nucleotides, sufficient to represent a single actin mRNA species. Indeed, a single isoelectric focusing form of actin was found to be the translation product of the actin-enriched mRNA. These results were expected since actin isolated from chick breast muscle contains only the single α-actin form. Nevertheless, in order to use the actin cDNA as a hybridization probe, it was necessary to remove the major contaminating DNA sequences. This was facilited by reacting the actin-enriched cDNA preparations with band II mRNA and the subsequent removal of the single-stranded actin cDNA from double-stranded band II cDNA–RNA hybrids by hydroxylapatite chromatography. This procedure was successfully utilized by Hastie & Bishop (1976) to isolate cDNA to different mRNA abundancy classes and by Alt et al. (1978), who used it to purify dihydrofolate reductase cDNA. The resultant actin cDNA hybridizes back to actin-enriched mRNA at a $R_0t_{1/2}$ of 7×10^{-3} ($R_0t_{1/2}$ pure of 3.9×10^{-3}) and did not cross-react with band II RNA sequences. The ability of the actin cDNA to selectively inhibit translation of actin mRNA following hybridization supports its assignment as actin cDNA (R. Schwartz, J. Haron, and A. Dugaiczyk, unpublished experiments).

Actin cDNA was used to quantitate actin mRNA content during mRNA purification. Hybridization analysis demonstrated that actin mRNA composed only 0.09% of the starting RNA and indicated an overall 1400-fold purification by the steps described. The cDNA hybridized to muscle poly(A)-rich mRNA with an $R_0t_{1/2}$ of 5×10^{-2} and represented 7.6% of total muscle RNA sequences. The actin mRNA was calculated to be present at $\sim$8800 copies/muscle cell diploid nucleus by assuming a content of $\sim 10^{-7}$ μg of poly(A) RNA mRNA per diploid nucleus.

In muscle development in culture, the bulk of the mRNA is transcribed from single-copy DNA sequence while $\sim$20–30% of the highly abundant poly(A) mRNA sequences are transcribed from moderately repetitive sequences. Actin was identified as one of the seven prominent sequences in the highly abundant class of myogenic mRNA (Paterson & Bishop, 1977). Since protein isolation studies have indicated

the potential of multiallelism, initial experiments were designed to evaluate the number of actin gene sequences in total chicken genomic DNA. Utilizing ovalbumin cDNA as a unique gene standard, we compared the rate and extent of hybridization of band II cDNA and actin cDNA (Figure 7). We determined that there are twice as many band II genes as ovalbumin genes in the chicken genome, while actin mRNA complementary sequences are repeated in the chick genome approximately 10–11-fold. Recently, Kindle & Firtel (1978) have shown that actin sequences are repeated 15–20-fold in the slime mold genome. It appears that actin polypeptides may be coded for by a middle repetitive gene family both in lower eucaryotes and vertebrates. Since there is more than sufficient genomic DNA to code for the six muscle and nonmuscle actin polypeptides which are currently known, it will be important to determine whether the orientation of the actin genes in the chick genome is in a classic repeat spacer arrangement analogous to the sea urchin histone gene (Kedes, 1976) or perhaps in a more random fashion.

It appears that formation of highly conserved proteins such as actin falls into two classes, muscle and nonmuscle, which are composed of separate gene elements. The increased accumulation of one gene product vs. the other polymorphic forms appears to be a major problem in gene regulation in myoblasts and other cell types. In order to understand how a choice in gene expression is conducted, it will be necessary to know the genomic organization of these contractile protein genes. As an initial approach to understanding the molecular events controlling the expression of muscle-specific genes, we have begun cloning of the actin structural genes.

Acknowledgments

We thank Earl Scharff, III, for excellent technical assistance.

References

Alt, F., Kellems, R. E., Bertino, J. R., & Schimke, R. T. (1978) *J. Biol. Chem. 253*, 1357.

Aviv, H., & Leder, P. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1408.

Bag, J., & Sarkar, S. (1975) *Biochemistry 14*, 3800.

Bailey, J. M., & Davidson, N. (1976) *Anal. Biochem. 70*, 75.

Bilkstad, I., Markey, F., Carlsson, L., Perrson, T., & Lindberg, U. (1978) *Cell 15*, 935.

Bishop, J. O., Morton, J. G., Rosbash, M., & Richardson, M. (1974) *Nature (London) 250*, 199.

Bloemendal, H. (1977) *Science 197*, 127.

Bonner, W. M., & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83.

Buckingham, M. E. (1978) *Int. Rev. Biochem. 15*, 209.

Carmon, Y., Newman, S., & Yaffe, D. (1978) *Cell 14*, 393.

Efstratiadis, A., Maniatis, T., Kahatos, F. C., Jeffrey, A., & Vournakis, J. W. (1975) *Cell 4*, 367.

Elzinga, M., Collins, J. H., Kuehl, W. M., & Adelstein, R. S. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2687.

Elzinga, M., Maron, B. J., & Adelstein, R. S. (1976) *Science 191*, 94.

Franklin, R. M. (1966) *Proc. Natl. Acad. Sci. U.S.A. 55*, 1304.

Garrels, J. I., & Gibson, W. (1976) *Cell 9*, 793.

Gruenstein, E., & Rich, A. (1973) *Biochem. Biophys. Res. Commun. 64*, 472.

Hanson, J. N. (1976) *Anal. Biochem. 76*, 37.

Harris, S. E., Means, A. R., Mitchell, W. M., & O'Malley, B. W. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3776.

Hastie, N. D., & Bishop, J. O. (1976) *Cell 9*, 761.

Hermann, H., Heywood, S. M., & Marchok, A. C. (1970) *Curr. Top. Dev. Biol. 5*, 181.

Hunter, T., & Garrels, J. I. (1977) *Cell 12*, 767.

John, H. A., Patrunou-Georgoulas, M. & Jones, K. W. (1977) *Cell 12*, 501.

Kedes, L. H. (1976) *Cell 8*, 321.

Kindle, K. L., & Firtel, R. A. (1978) *Cell 15*, 763.

Laemmli, V. K. (1970) *Nature (London) 227*, 680.

Lindberg, U., & Eriksson, S. (1971) *Eur. J. Biochem. 18*, 474.

Mannherz, H. G., Barrington-Leigh, J., Leberman, R., & Pfrang, H. (1975) *FEBS Lett. 60*, 34.

McDonnell, M. W., Simon, M. N., & Studier, F. W. (1977) *J. Mol. Biol. 110*, 119.

Monahan, J. M., Harris, S. E., Woo, S. L. C., Robberson, D. L., & O'Malley, B. W. (1976) *Biochemistry 15*, 223.

O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007.

Palmiter, R. D. (1974) *Biochemistry 13*, 3606.

Paterson, B. M., & Bishop, J. O. (1977) *Cell 12*, 751.

Paterson, B. M., Roberts, B. E., & Yaffe, D. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4467.

Pelham, H. R. B., & Jackson, R. J. (1976) *Eur. J. Biochem. 67*, 247.

Proudfoot, N. J., & Longley, J. I. (1976) *Cell 9*, 733.

Rosen, J. M., Woo, S. L. C., & Comstock, J. P. (1975) *Biochemistry 14*, 2895.

Ross, J., Gielen, J., Packman, S., Ikawa, Y., & Leder, P. (1974) *J. Mol. Biol. 87*, 697.

Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866.

Strohman, R. C., Moss, P. S., Micou-Eastwood, J., Spector, D., Przybyla, A., & Paterson, B. (1977) *Cell 10*, 265.

Sullivan, D., Palacios, R., Stavenezer, J., Taylor, J. M., Faras, A. J., Kiely, M. L., Summers, N. M., Bishop, J. M., & Schimke, R. T. (1973) *J. Biol. Chem. 248*, 7530.

Vanderkerchkove, J., & Weber, K. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1106.

Wetmur, J. G., & Davidson, N. (1968) *J. Mol. Biol. 31*, 349.

Whalen, R. G., Butler-Brown, G. S., & Gros, F. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2018.

Woo, S. L. C., Harris, S. E., Rosen, J. M., Chan, L., Sperry, P. L., Means, A. R., & O'Malley, B. W. (1974) *Prep. Biochem. 4*, 555.