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Control of Embryonic Development: Isolation and Purification of Chick Heart Myosin Light Chain mRNA and Quantitation with a cDNA Probe†

Hans-Henning Arnold and M. A. Q. Siddiqui*

ABSTRACT: Myosin light chain mRNAs were isolated from the myosin light chain synthesizing polysomes of the 16-day-old chick embryonic heart tissue by immunoadsorption of total polysomes to myosin light chain specific antibodies. The mRNA, purified by successive sucrose gradient centrifugations and oligo(dT)-cellulose chromatography, was assessed for purity by translation in mRNA-dependent rabbit reticulocyte lysate, electrophoretic separation on denaturing gels, and hybridization assays. Proteins synthesized in response to the RNA in a cell-free system were two myosin subunits of 24 000 and 18 000 molecular weight, identical with those of authentic myosin light chain subunits obtained from the homologous chick heart tissue. The RNA resolved on a denaturing polyacrylamide gel into two RNA bands of approximately 1090 and 980 nucleosides in chain length, the putative mRNAs for the myosin light chain subunits LCM_1 and LCM_2. Additional evidence for purity comes from hybridization kinetics of the mRNA with the complementary DNA (cDNA) synthesized with avian myeloblastosis virus reverse transcriptase. The RNA sequence complexity based on the hybridization assay was in excellent agreement with the combined molecular sizes of the two mRNAs. The observed $R_0t_{1/2}$ values for cDNA hybridization with the purified mRNAs, poly(A)-containing RNA, and total polysomal RNA indicated that the LCM mRNAs comprise 2.0 and 0.02% of poly(A) RNA and polysomal RNA, respectively. The availability of pure myosin light chain mRNAs and the cDNA probes should facilitate the analysis of the mechanism(s) underlying embryonic heart induction and differentiation during chick development.

The phenomena of morphogenesis and cell differentiation during early embryonic development are poorly understood at the molecular level. Heart muscle differentiation, which is an early event in chick embryonic development (Romanoff, 1960), provides a model system to investigate the mechanism(s) controlling the transition of presumptive heart cells to well defined, highly differentiated myocytes. The transition is characterized by several biochemical parameters, including the synthesis of muscle specific proteins, light and heavy chain myosin and actin, the major constituents of muscle proteins. Myosin consists of a large molecular weight (200 000) heavy chain myosin polypeptide (HCM)[1] and two to three small polypeptides, the light chain myosin subunits (LCM) (Taylor, 1972). The molecular weights of LCM subunits range between 15 000 and 27 000. While actin is synthesized in appreciable amounts in various cell types, the occurrence of specific differences in both composition and content of LCM subunits (Lowey & Risby, 1971; Sarkar et al., 1971; Yaffe & Dym, 1972; Sarkar, 1972; Low et al., 1971; Sreter et al., 1975) makes the latter a suitable marker for monitoring changes during muscle development. Also, a particular advantage of the chick embryo heart muscle system is the availability of a fate map of embryonic cells which facilitates the identification and isolation of the presumptive heart-forming cells from the very early stages of developing embryo (DeHaan et al., 1970; Rosenquist, 1970). These cells, when grown in culture, differentiate predominantly into well-defined heart muscle tissue.

According to the current concepts, changes in early morphogenetic pattern and cell differentiation, which are caused by differential expression of gene activity, are controlled in

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[1] Abbreviations used: HCM and LCM, heavy and light chain myosin, respectively; AMV, avian myeloblastosis viral; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-N^1-2-ethanesulfonic acid.

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some way by elements (information molecules) regionalized in the cytoplasm of the fertilized egg (Gurdon & Woodland, 1968; Davidson & Britten, 1971, 1973; Raff, 1977). Both RNA and/or protein translated from it at the appropriate developmental stages are implicated to play such a regulatory role (Davidson & Britten, 1971; Spirin, 1966; Brothers, 1976; Dickson & Robertson, 1976; Diener et al., 1972; Pilch et al., 1975; Colby et al., 1971). We have recently reported that a low molecular weight RNA (7S CEH-RNA) isolated from the differentiated 16-day-old heart tissue appears to play a key role in control of heart muscle differentiation, since it can cause heart-like differentiation in undifferentiated early chick embryonic cells in culture (Siddiqui et al., 1977; Deshpande & Siddiqui, 1977, 1978; Deshpande et al., 1977; Arnold et al., 1978a,b).

In order to relate the process of embryonic heart induction and differentiation to changes in specific gene activity in the RNA-induced system both in vitro and in situ, we undertook to monitor the synthesis and quantitate the levels of mRNAs for the muscle specific marker proteins during the early chick development. Specifically, our ultimate goal is to determine when the mRNAs for LCM subunits appear during the period precardiogenic cells are induced to become cardiac specific myoblasts and then differentiate into well-defined myocytes. To this end, we describe here the isolation and purification of myosin light chain mRNAs of the embryonic chick heart tissue by immunoadsorption of LCM-synthesizing polysomes, the synthesis of DNA complementary to the mRNA, and its characterization as a potential probe to measure the mRNA levels.

Materials and Methods

Materials. Sixteen-day-old chick embryos were obtained from Spring Lake Farms, NJ. Oligo(dT)-cellulose (T3) was purchased from Collaborative Research, Inc., ϕX 174 DNA was from Miles Laboratory, and micrococcal nuclease and sodium heparin were from Sigma. Radio-labeled and non-labeled deoxynucleoside triphosphates and amino acids were obtained from Amersham/Searle. Avian myeloblastosis viral (AMV) reverse transcriptase was kindly supplied by Dr. J. W. Beard of Life Sciences, Inc., St. Petersburg, FL. S_1 nuclease was prepared according to Vogt (1973). S_1 nuclease and ϕX 174 were also gifts of Dr. Miller of the Roche Institute of Molecular Biology. Hearts were removed from the chick embryos, freed of excess blood by quick washing in ice-cold Ringer's solution, and immediately frozen and stored at −70 °C until used. Prolonged storage was avoided when the tissues were used for isolation of RNA.

Preparation of Myosin Light Chain and Affinity Support. Total myosin was prepared from the heart tissue according to procedure described by Wikman-Coffelt et al. (1973). The LCM subunits were dissociated from the heavy chain at pH 11.5 followed by a precipitation of the heavy chain with 0.8 M potassium citrate (Gaetjens et al., 1968). The purity of light chain myosin subunits (LCM_1 and LCM_2) was routinely examined on 10% or 12% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (see below). The proteins were also examined by two-dimensional separation on acrylamide gels according to O'Farell (1975). The couplings of LCM and of anti-myosin IgG fraction to CNBr-activated Sepharose 4B were done by incubating the mixture at pH 9.0 for 2 h at room temperature in 0.1 M $NaHCO_3$ containing 0.5 M NaCl (Parikh et al., 1974). The material was washed with 1.0 M NaCl in 0.1 M sodium acetate, pH 4.0, to free the column of unbound proteins followed by a second wash with 1.0 M NaCl in 0.1 M sodium borate buffer, pH 8.0. This washing procedure was repeated three times.

Preparation of Antibodies. Rabbits were immunized with electrophoretically pure total cardiac myosin prepared as described above. Four milligrams of the protein mixed with complete Freund's adjuvant was injected intramuscularly four times, at intervals of 1 week each. Two weeks later the rabbits were bled and the serum was precipitated with 40% ammonium sulfate. The immunoglobulin (IgG) fraction thus obtained was enriched in the LCM specific antibody by adsorption to the Sepharose 4B–LCM affinity column prepared above. The column was then washed with 0.05 M potassium phosphate buffer, pH 7.3, containing 0.3 M KCl and 2% Triton X-100. The anti-LCM IgG was eluted with 4.5 M $MgCl_2$. RNase activity was then removed by passing the antibody through a column containing CM-cellulose over DEAE-cellulose (Schimke et al., 1974). Goat (anti-rabbit) IgG antiserum, which was a generous gift of Dr. Hans Hager, Hoffmann-La Roche Inc., was partially purified by ammonium sulfate precipitation (40%) followed by removal of RNase activity as above. An insoluble matrix of goat (anti-rabbit) IgG antiserum was made by cross-linking the antibody with glutaraldehyde (Innis & Miller, 1977). The matrix was washed and equilibrated with the polysome buffer (see below).

Isolation of Myosin Light Chain Synthesizing Polysomes. Polysomes from the heart tissue were prepared as described by Palmiter (1974). In a typical experiment 100 g of frozen heart tissues was first homogenized briefly in a Waring blender at low speed and then in a loose-fitting Dounce homogenizer in 7 × the volume of polysome buffer (0.025 M triethanolamine, pH 6.4, 0.15 M NaCl, 5 mM $MgCl_2$, 0.2 M sucrose, and 1 mg/mL sodium heparin). After an initial homogenization (five strokes) sodium deoxycholate and Triton X-100 were added to a final concentration of 1% and 2%, respectively, and the mixture was further homogenized, first with the loose-fitting pestle followed by a tight-fitting pestle for three additional strokes each. Polysomes were then precipitated from the 25000*g* supernatant of the homogenate by adding $MgCl_2$ to a final concentration of 0.1 M and stirring the solution for 1 h at 0 °C. Polysomes were collected by sedimentation through a cushion of 1 M sucrose in polysome buffer and the pellet was suspended and dialyzed in the same buffer for overnight. One hundred grams of heart tissue yielded between 4000 and 5000 A_{260} units of polysomes.

The LCM-synthesizing polysomes were then isolated by the double-antibody precipitation method described by Schimke et al. (1974). Polysomes were incubated in polysome buffer at a concentration of 20 A_{260}/mL with the anti-LCM IgG (3 mg per 1000 A_{260} polysomes) for 1 h at 4 °C. The insolubilized goat (anti-rabbit) IgG matrix was then added (300 mg per 1000 A_{260} units polysomes) and the incubation was continued for an additional 3 h. Polysomes adsorbed to the matrix were recovered by centrifugation at 5000*g* and washed three times with polysome buffer containing 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, and 0.5 M sucrose followed by an additional wash with the polysome buffer alone. Polysomes were freed of the matrix by incubation with 4 × the volume of 0.05 M EDTA for 15 min. The incubation was repeated once to strip the matrix totally free of polysomes.

Preparation of Myosin Light Chain mRNA. RNA from the LCM-synthesizing polysomes was dissociated free of polysomal protein in 1% $NaDodSO_4$ and recovered by precipitation with 2 M LiCl in 0.01 M EDTA, pH 7.4. The pellet was washed three times with ice-cold 2 M LiCl, dissolved in H_2O, and reprecipitated in ethanol containing 2% sodium acetate, pH 4.5. The RNA was then fractionated by cen-

trifugation on 15–35.9% isokinetic sucrose gradient in 0.02 M Tris-Cl, pH 7.4, 5 mM EDTA, and 0.5% $NaDodSO_4$. The centrifugation was done at 20 °C for 14 h and 40000 rpm in a Beckman SW 41 rotor. The RNA samples were heated to 68 °C and rapidly cooled prior to centrifugation. Fractions of 0.6 mL each were collected and scanned in an ISCO fractionator Model 640.

RNA from the total nonimmunoadsorbed polysomes was extracted twice with a phenol–chloroform mixture (1:1) and the RNA in the aqueous phase was recovered by ethanol precipitation as above.

The poly(A)-containing RNA was isolated from the immunoadsorbed polysomal RNA fractions of sucrose gradient or from the total polysomal RNA by passing through oligo(dT)-cellulose (T-3) essentially as described by Aviv & Leder (1972). RNA dissolved in 0.01 M Tris-Cl, pH 7.4, 1 mM EDTA, and 1% $NaDodSO_4$ was first heated to 68 °C and rapidly cooled. The RNA solution was brought to 0.4 M NaCl and applied to the column. Elution was done in two steps, first with 0.1 M NaCl in Tris–EDTA buffer above followed by an elution in the same buffer without NaCl. The bound RNA was always recycled through oligo(dT)-cellulose under the same conditions to free the ribosomal RNA contamination.

Cell-Free Protein Synthesis in Rabbit Reticulocyte Lysate. Analysis of cell-free protein synthesis was done according to Pelham & Jackson (1976) using rabbit reticulocyte lysate (Hunt & Jackson, 1974) as described earlier (Arnold et al., 1978a). The reaction mixture, 50 μL, contained 25 μL of nuclease-treated lysate, 0.02 M Hepes, pH 7.4, 1 mM ATP, 0.2 mM GTP, 1 mM $MgCl_2$, 0.01 M creatine phosphate, 0.08 M KCl, 20 μM hemin, 2.5 μg of creatine phosphokinase, 10–20 μCi of [^{35}S]methionine, 50 μM each of amino acids mixture (except methionine), and 0.1–2.0 μg of poly(A) RNA. Incubation was done at 30 °C for 60 min, and 5-μL aliquots were taken for CCl_3COOH-insoluble cpm measurement or for analysis on $NaDodSO_4$–polyacrylamide gel (see below).

Immunoprecipitation. For immunopreciptation of the translation products, an aliquot (5–30 μL) was incubated overnight at 4 °C with Sepharose-bound anti-LCM IgG in 50 mM Tris-HCl, pH 7.4, 0.4 M KCl containing 2% Triton X-100. The mixture was centrifuged in an Eppendorf microfuge at full speed for 5 min and washed five times in the incubation buffer. The final pellet was then boiled for 3 min in 60 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 1% $NaDodSO_4$, and 8 M urea and centrifuged as before, and aliquots from the supernatant were subjected to gel analysis and radioactivity measurements. The IgG fraction from nonimmunized rabbits was used as control. Highly purified [^{125}I]myosin was also used in parallel as standard for the recovery measurements.

Gel Electrophoresis. All electrophoretic separations were performed on slab gels using a Bio-Rad electrophoresis apparatus. Proteins were examined on 10% or 12% polyacrylamide gel (15 × 100 × 140 mm) containing $NaDodSO_4$ as described by Laemmli (1970) with a 3% polyacrylamide stacking gel. Electrophoresis was done at 15 mA for the first hour and then continued at 30 mA until the marker dye reached the bottom. Gels were fixed in 10% Cl_3CCOOH and developed for fluorography (Bonner & Laskey, 1974).

Proteins were also analyzed by two-dimensional gel separation using isoelectric focusing at pH 3–10 in the first dimension and 12% polyacrylamide–$NaDodSO_4$ gel electrophoresis in the second dimension. The conditions were identical with those described by O'Farell (1975), except that the equilibration time for first dimensional gel was reduced to 10 min. Proteins were located either by staining or by fluorography as before.

Electrophoresis of RNA was done both on 2% agarose–urea gel according to Rosen et al. (1975) and on 4.25% polyacrylamide gel (6 × 100 × 140 mm) in 98% freshly deionized formamide as described earlier (Deshpande et al., 1977). Gels either were examined under the UV light after treatment with ethidium bromide (10 μg/mL) in 0.05 M Tris-Cl, pH 8.0, 0.1 M NaCl, 2 mM EDTA for 15 min or were stained with 0.005% Stains-All in 50% formamide and then scanned with a Gilford scanning spectrophotometer.

Preparation of cDNA. Labeled complementary DNA (cDNA) to LCM mRNA was prepared according to procedure described by Friedman & Rosbash (1977) with a few modifications. Reaction was done in a total volume of 50 μL containing 5 μg/mL oligo$(dT)_{12-18}$, 800 μM each of deoxynucleoside triphosphate except [^{3}H]deoxycytidine triphosphate which was 500 μM, 20 μg/mL of mRNA, and 20 units of AMV reverse transcriptase per μg of RNA. Incubation was done at 37 °C for 60 min and terminated by addition of 0.5 mL of 0.01 M EDTA and chilling on ice. The enzyme and oligo(dT) were in saturating amounts. Forty micrograms of *E. coli* tRNA was added as carrier, and the mixture was extracted with phenol–chloroform (1:1). The cDNA was freed of unreacted deoxynucleoside triphosphate by passing the mixture through an Ultragel pkA 44 (LKB) column. cDNA was then examined on an alkaline isokinetic (10–30.9%) sucrose gradient prepared in 0.1 N NaOH, 0.9 M NaCl, and 5 mM EDTA. Centrifugation was done at 4 °C for 21 h at 40000 rpm in a Beckman SW 41 rotor. φX 174 DNA was used as marker.

Hybridization Assay. Hybridization of cDNA with excess RNA was done in siliconized and heat sterilized glass vials (Regis Inc., IL). The reaction mixture, 100–200 μL, contained 0.25 M potassium phosphate buffer, pH 7.0, 1 mM EDTA, 0.2% $NaDodSO_4$, 10000–20000 cpm of [^{3}H]cDNA, and 40 μg/mL of carrier RNA. The mixture was overlayered with paraffin oil to prevent evaporation and the reaction was carried out at 65 °C for times required to drive the hybridization to R_0t values indicated. Aliquots of 10–15 μL were removed and diluted to 0.5 mL with S_1 nuclease digestion buffer (30 mM sodium acetate, pH 4.5, 280 mM NaCl, 2 mM $ZnSO_4$) containing 2 A_{260}/mL carrier RNA. Nuclease digestion was done at 37 °C for 30 min. These conditions allowed digestion of more than 90% single-stranded DNA, whereas the double-stranded DNA remained virtually undigested. CCl_3COOH-insoluble radioactivity was assayed on Millipore membrane filters before and after nuclease digestion. The data are expressed as percent of cDNA that remained resistant to S_1 treatment vs. the log of the product of RNA concentration (moles of nucleotide per liter) and time (seconds).

Melting of cDNA:RNA Hybrid. Hybridization of [^{3}H]-cDNA with excess RNA was performed as above to the desired hybridization extent. Temperature was then raised at intervals of 5 °C each and aliquots were removed, diluted immediately with ice-cold S_1 nuclease digestion buffer, and assayed as above. All values were normalized by subtracting the counts that remained S_1 nuclease resistant at 100 °C.

All laboratory glassware were heat sterilized. Buffers and other liquid media were either autoclaved or treated with diethyl pyrocarbonate (0.2%) to protect against nucleases.

Results

Embryonic chick heart contains two LCM subunits, LCM_1 and LCM_2, which migrate with mobilities of 18000 and 24000 daltons respectively on 12% $NaDodSO_4$–polyacrylamide gel

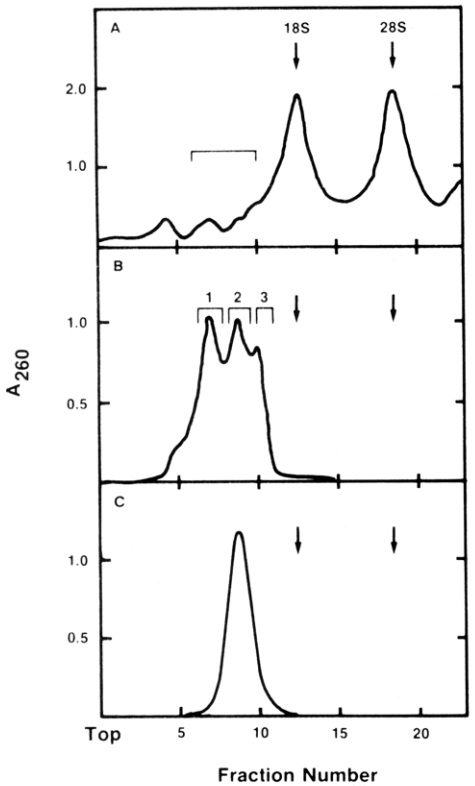

FIGURE 1: Sedimentation profiles of RNA from LCM-synthesizing polysomes on isokinetic sucrose gradient. (A) Twenty A_{260} units of immunoprecipitated LCM-polysomal RNA was sedimented through a 15–35.9% isokinetic sucrose gradient as described in Materials and Methods. The gradient was fractionated into 0.6-mL aliquots and scanned by an ISCO density gradient fractionator Model 640. The RNA sedimenting between 9 and 14S, as indicated, was pooled from several gradients, concentrated, and run again on a separate gradient under identical conditions (B). The material from peaks 1, 2, and 3 was recovered separately and subjected to translational assays using the rabbit reticulocyte lysate (see Figure 2). Peak 2 RNA which displayed the template activity for LCM_1 and LCM_2 exclusively was centrifuged again on sucrose gradient as above after two successive passages through oligo(dT)-cellulose as described in Materials and Methods (C). The arrows indicate the positions of 18S and 28S rRNA.

(see below, Figures 2 and 3). No other low molecular weight myosin peptides were found in detectable amounts in the 16-day-old chick embryonic heart. For the purpose of these studies, the LCM_1 and LCM_2 were purified together for the preparation of antibodies and the mRNAs isolated from the immunoadsorbed polysomes contained two distinct RNA species, the putative mRNA for LCM_1 and LCM_2. No attempt was made to purify the individual mRNA for the respective proteins separately, since the methodology employed for amplification of cDNA by molecular recombination via A/T tailing and cloning (Higuchi et al., 1976), currently in progress in our laboratory, would yield plasmids containing individually the cDNA for LCM_1 or the cDNA for LCM_2 mRNA.

Isolation and Purification of LCM mRNA. The procedure for isolation of LCM mRNA used in this study involved a modification of the double-antibody precipitation method utilizing an insoluble glutaraldehyde cross-linked matrix of goat (anti-rabbit) IgG, as described by Innis & Miller (1977). This minimized the amount of second antibody and, therefore, the contamination of nonspecifically bound polysomes. The specificity of this method for isolation of LCM-synthesizing polysomes was readily demonstratable by the translation of RNA using nuclease-treated rabbit reticulocyte lysate. The RNA isolated from the immunoadsorbed polysomes was

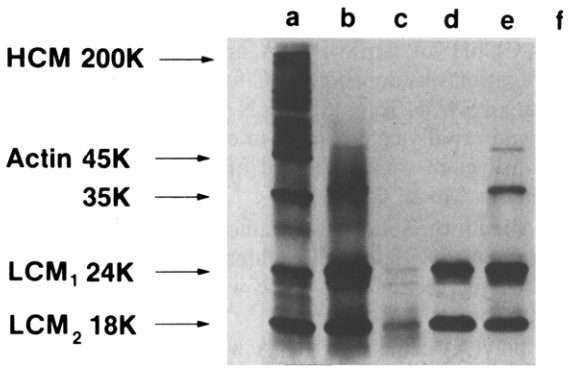

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis of translation products of chick heart mRNA. mRNA-dependent translation was performed in micrococcal nuclease treated rabbit reticulocyte lysate as described in Materials and Methods. Five-microliter aliquots were taken at the end of incubation and applied directly to a 12% $NaDodSO_4$–polyacrylamide gel. Nonlabeled marker proteins were run simultaneously on the same gel. The positions of marker proteins were ascertained by staining the gel in Coomassie Blue. The gel was then processed for fluorography, exposed to X-ray film developed after 48 h. (a) Total polysomal RNA; (b) immunoabsorbed polysomal RNA; c, d, and e are peak 1, 2, and 3 RNA, respectively (Figure 1B); (f) endogenous nuclease-treated lysate (without RNA); HCM, heavy chain myosin; LCM_1 and LCM_2, light chain myosin subunits 1 and 2, respectively.

purified by centrifugation on successive isokinetic sucrose gradients. Figure 1A shows the sedimentation pattern of total polysomal RNA. The major portion of RNA sedimented as 18S and 28S rRNA, as expected, and was relatively free of LCM synthesizing activity but the RNA sedimenting between 9 and 14S region contained LCM mRNA like activity. The slow-moving RNA peak near the top of gradient appeared to be non-poly(A) RNA, presumably 4S and 5S RNA or a degradation product of rRNA. The 9–14S RNA when pooled (as indicated in Figure 1A) and run again on sucrose gradients under identical conditions was resolved into three prominent peaks (Figure 1B). Of the three fractions (peaks 1, 2, and 3), the RNA in peak 2 directed the translation of polypeptides that were indistinguishable from the authentic LCM_1 and LCM_2 of the chick heart muscle when examined by electrophoresis on 10% $NaDodSO_4$–polyacrylamide gel (Figure 2). The purity of peak 2 mRNA was further documented by a two-dimensional separation of the translation products using isoelectric focusing in the first dimension and 12% $NaDodSO_4$–polyacrylamide gel electrophoresis in the second dimension (Figure 3). The extent of contaminant RNAs, if any, appeared to be negligible. Furthermore, more than 90% of CCl_3COOH-insoluble radioactivity was immunoprecipitable when assayed with antibodies against LCM_1 and LCM_2. The specificity of the immunoprecipitation assay is demonstrated by polyacrylamide gel electrophoretic analysis of immunoprecipitated LCM and the translation products of LCM mRNA (Figure 4). The RNA from peak 1 appeared to be deficient in translation of LCM_1 relative to LCM_2 (Figure 2, column c), whereas the translational products of peak 3 RNA contained additional polypeptides (column e). The RNA from peak 2 was recovered from several gradients and further purified by two passages through an oligo(dT)-cellulose column. When examined on sucrose gradient following the chromatography, the peak 2 RNA sedimented as a symmetric peak (Figure 1C) of about 13S size, but on denaturing gels, consisting of 4.25% polyacrylamide in 98% formamide or on agarose–urea, it resolved into two major RNA bands (inset in Figure 5). The RNA bands were identified by their migration relative to purified marker RNA run on the same gel. The electrophoretic mobilities of these two RNA species

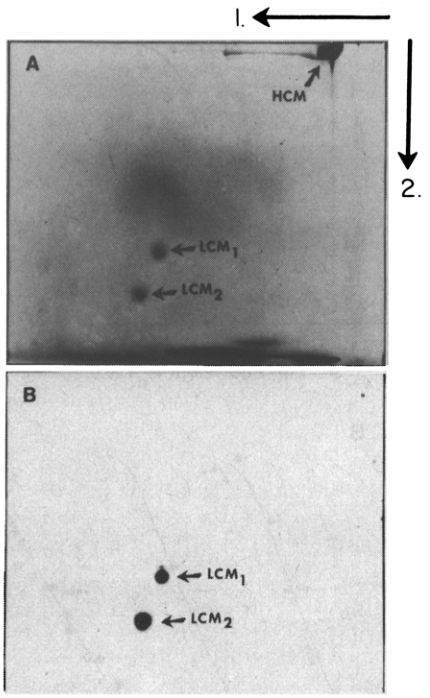

FIGURE 3: Two-dimensional separation of labeled translation products and purified myosin subunits. A 5-μL aliquot ($\simeq$40 000 cpm) of the rabbit reticulocyte lysate translation mixture with the purified LCM mRNA and purified myosin preparation was subjected to the two-dimensional separation by isoelectrofocusing in the first dimension and polyacrylamide gel electrophoresis in the second dimension as described in Materials and Methods. The labeled products were identified by fluorography (Bonner & Laskey, 1974) and the marker LCM subunits by staining with Coomassie Blue. (A) Marker purified myosin subunits; (B) [^{35}S]methionine-labeled translation products of LCM mRNA. Migration on the first dimension was from right to left and on the second dimension from top to bottom. HCM, heavy-chain myosin; LCM_1 and LCM_2, light-chain myosin subunits 1 and 2.

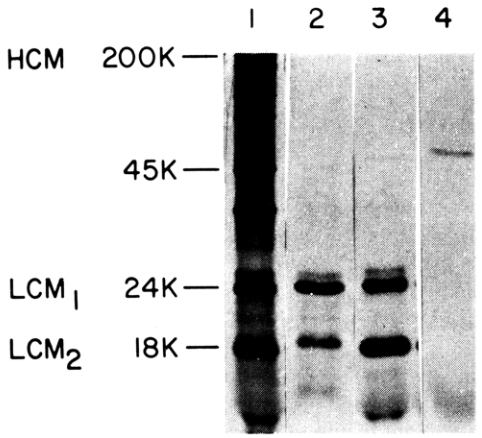

FIGURE 4: Analysis of translation products on $NaDodSO_4$–polyacrylamide gels with and without immunoprecipitation. An aliquot (35 μL) of the rabbit reticulocyte lysate assay using total chick heart polysomal RNA as messenger was immunoprecipitated with the LCM specific antibodies as described in Materials and Methods and analyzed on the $NaDodSO_4$–polyacrylamide gel along with the products of purified LCM mRNA and the marker proteins. (1) translation products of total polysomal RNA; (2) same as 1 after immunoprecipitation with anti-LCM antibodies; (3) translation products of purified LCM–mRNA; (4) blank (–RNA).

corresponded to 360 000 and 320 000 daltons (Figure 5). These values account for approximately 1090 and 980 nucleosides per chain. The molecular weights of the translational products of peak 2 RNA, as shown in Figure 1, were 24 000 and 18 000, identical with those of LCM subunits of the 16-day-old chick

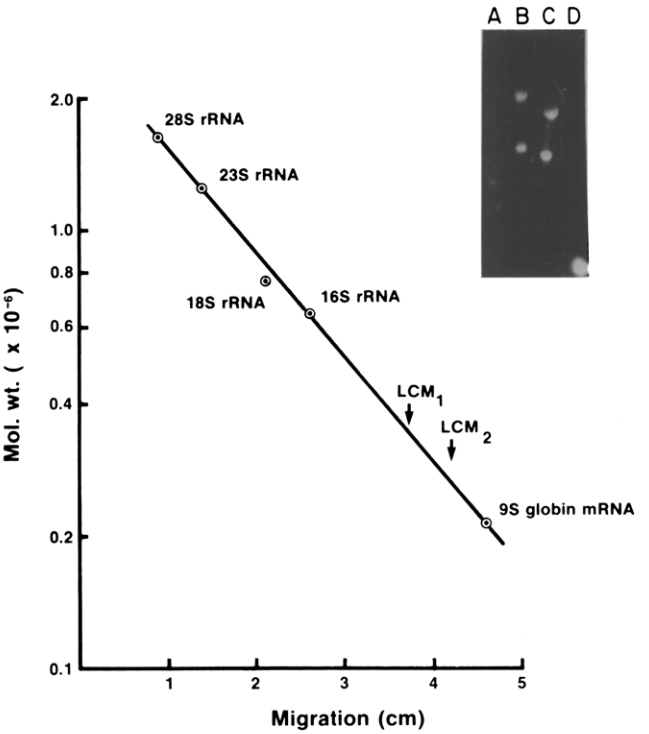

FIGURE 5: Molecular weight estimation of LCM mRNA by polyacrylamide–formamide gel containing 98% formamide along with the marker RNAs of known molecular weights. The RNAs were located on gel under UV light after ethidium bromide treatment or by staining as described in Materials and Methods. The inset shows the separation of RNAs on an agarose–urea gel as detailed in Materials and Methods. (A) LCM mRNA; (B) 18S and 28S rRNA; (C) 16S and 23S rRNA; (D) 4S RNA.

embryonic heart. This would suggest that the mRNAs for LCM_1 and LCM_2 contain about 370 and 430 nucleosides more than expected to code for these proteins, respectively.

When total polysomal RNA from the 16-day-old chick embryonic heart was translated and the products were examined on $NaDodSO_4$–acrylamide gels as before, the majority of the labeled polypeptides migrated with mobilities identical with those of muscle specific proteins (Figure 2, column a). Among those identified with the aid of nonlabeled markers were the HCM, actin, tropomysin, and two LCM subunits with molecular weights of 200 000, 45 000, 35 000, 24 000, and 18 000, respectively. The LCM subunits represented about 15% of the total radioactivity (see Discussion). Column b in Figure 2 shows the pattern of the material translated from the poly(A) RNA obtained after immunoadsorption of polysomes with antibodies against LCM subunits. Although the products appear to contain some unknown polypeptides, the LCM subunits constituted a major part ($\sim$70%) of the total products. The RNA from peak 2 (column d), on the other hand, was translatable into polypeptides LCM_1 and LCM_2 exclusively, which accounted for 96% of the total radioactivity. This quantitation was based both on immunoprecipitation assay and on scanning of the X-ray films after a brief exposure to gels.

Synthesis of [^{3}H]DNA Complementary to LCM mRNA. [^{3}H]cDNA was synthesized using AMV reverse transcriptase and the purified LCM mRNA templates. Under the conditions chosen (see Materials and Methods), 0.4 μg of cDNA was made per μg of template RNA. The specific activity of the product was 1×10^7 cpm per μg. The cDNA had an average size of 1050 nucleosides per chain when examined by centrifugation on an alkaline sucrose gradient using ϕX 174 DNA as a marker (Figure 6A). A high proportion of the

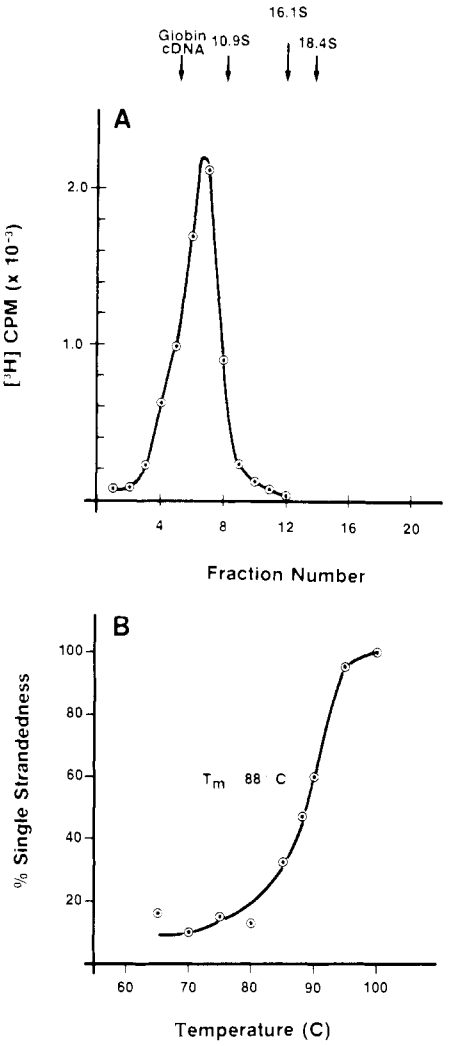

FIGURE 6: (A) Alkaline sucrose density gradient centrifugation on LCM cDNA. [^{3}H]cDNA was prepared using AMV reverse transcriptase and purified LCM mRNA template as described in Materials and Methods. The DNA was centrifuged on a 10–30.9% alkaline sucrose isokinetic gradient for 21 h at 40000 rpm in a Beckman SW 41 rotor. The sample was preheated at 80 °C for 30 min. Aliquots of 5 μL diluted with H_2O and neutralized with CCl_3COOH were assayed for radioactivity. ϕX 174 DNA which sediments at 18.4S, 16.9S, and 10.9S under these conditions was used as marker. The migration of β-globin cDNA run on a parallel gradient is shown by the arrow. (B) Melting profile of [^{3}H]cDNA:mRNA hybrid. [^{3}H]cDNA was hybridized to mRNA to R_0t 1 × 10^{-2} at 65 °C as described in Materials and Methods. Temperature was then raised at intervals of 5 °C each at which time aliquots were removed, diluted immediately in ice-cold S_1 nuclease digestion buffer, and assayed for single-stranded cDNA. All values were normalized for the residual enzyme resistant radioactivity (6%) at 100 °C.

DNA copies were of the average length of 1050 nucleosides, although some batches of AMV reverse transcriptase yielded cDNA of substantially smaller size. The S_1 nuclease digestion of cDNA indicated that between 8 and 12% of the DNA contained double-stranded structure. The fidelity of the DNA transcript was ascertained by examining the thermal melting properties of the cDNA:mRNA duplex hybridized with an excess of mRNA to a R_0t of 1.0 × 10^{-2}. As shown in Figure 6B, melting of the hybrid occurred with a sharp transition. The T_m was 88 °C, indicating that an accurate base pairing was present in the cDNA:mRNA complex.

Hybridization of [^{3}H]cDNA with Excess LCM mRNA. For further characterization of the cDNA and for quantitation of the mRNA and its complexity, the kinetics of reassociation of cDNA with LCM mRNA were studied. Figure 7A shows

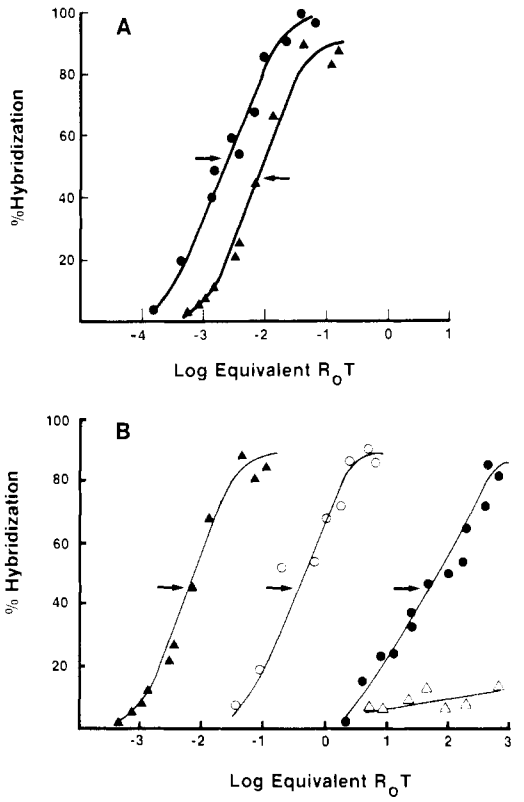

FIGURE 7: (A) Kinetics of hybridization of purified mRNA with the [^{3}H]cDNA. Purified LCM mRNA and β-globin mRNA were hybridized with their respective cDNA and the hybrids were assayed as described in Materials and Methods. The percent of the cDNA resistant to nuclease digestion in controls lacking the mRNA (6% for LCM cDNA and 5% for β-globin cDNA) was subtracted from all values. Arrows indicate the $R_0t_{1/2}$ values. (●) β-Globin cDNA:mRNA; (▲) LCM cDNA:mRNA. (B) Hybridization kinetics of [^{3}H]cDNA with purified LCM mRNA, poly(A) RNA, and total polysomal RNA of the chick heart. RNA from total polysomes, poly(A) RNA, and the purified LCM mRNA were prepared as described in Materials and Methods. Rat liver RNA was a gift of Drs. Innis and Miller. Hybridization was carried to the indicated R_0t values by varying the RNA concentration and incubation times of the respective reactions. Arrows indicate the $R_0t_{1/2}$ values. (▲) LCM mRNA; (○) poly(A) RNA; (●) total polysomal RNA; (△) rat liver RNA.

the kinetics of cDNA:mRNA hybridization performed with excess RNA. The reaction took place within the range of 100 R_0t values displaying a pseudo-first-order reaction rate. At completion, 90% of [^{3}H]cDNA was hybridized to LCM mRNA. The $R_0t_{1/2}$ observed was 6.3 × 10^{-3}. Hybridization kinetics of β-globin cDNA and purified β-globin mRNA was followed in a parallel experiment for comparison (Figure 7A). β-Globin cDNA was prepared under identical conditions using a purified rabbit β-globin mRNA preparation (a generous gift of Drs. Innis and Miller). The size distribution analysis on an alkaline sucrose gradient indicated that globin cDNA was also of full length (680 nucleosides). The $R_0t_{1/2}$ observed for globin cDNA:mRNA hybridization was 2 × 10^{-3}, in agreement with the previously observed values (Innis & Miller, 1977). 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 unrepeated sequences of nucleic acid, one can calculate the sequence complexity of LCM mRNA by comparing its reaction kinetics with that of globin mRNA. Using 700 nucleosides as the chain length of β-globin mRNA (Innis & Miller, 1977), we obtained a complexity of 2225 nucleosides for LCM mRNA. This value is in excellent agreement with the

Table I: Analysis of LCM mRNA Activity in Different RNA Fractions

RNA	yield (mg)[b]	LCM mRNA act. (cpm/μg)[c]	sp LCM mRNA act. (%) immuno-precipitation	sp LCM mRNA act. (%) gel scanning	content of LCM mRNA (%)[d]	purification factor[d]
total polysomal RNA	236	3090	15	nd	0.017	1
immunoabsorbed polysomal RNA	3	2560	nd	nd	nd	nd
poly(A) RNA[a]		186000	14.8	15.5	1.85	
LCM mRNA after suc. grad. & oligo(dT)-cellulose chromatog.	0.018	237000	96	94	93	5472

[a] Isolation of poly(A) RNA on oligo(dT)-cellulose and its translation were not used as routine purification steps for LCM mRNA preparation. [b] Yield was based per 100 g of frozen 16-day-old chick heart tissues. [c] Activity was determined by rabbit reticulocyte lysate translation assay (see Materials and Methods). [d] LCM mRNA content and its purification were calculated based on hybridization data with LCM cDNA. nd, not determined.

combined sizes of the two LCM mRNA (1090 nucleosides for LCM_1 and 980 for LCM_2) based on the electrophoretic separation on denaturing gel (see Figure 5). It appears, therefore, that the LCM mRNA preparation consists of two molecular species of high purity, and the sequences of both are present in the cDNA transcripts. Table I summarizes the extent of purification of LCM mRNA and its content in the RNA populations.

The potential of cDNA for LCM mRNA as a probe for quantitation of LCM mRNA sequences present in various RNA preparations was investigated by hybridization with the total polysomal RNA and the poly(A) RNA of chick heart. As shown in Figure 7B, hybridization of cDNA with the three RNA preparations occurred to the same extent over a range of 100 R_0t values. The $R_0t_{1/2}$ observed were 0.35 and 39 for poly(A) RNA and polysomal RNA, respectively. These values would suggest that LCM mRNA comprises about 2.0% of poly(A) RNA and 0.02% of polysomal RNA. The hybridization of RNA obtained from the rat liver tissue with LCM cDNA was insignificant. These experiments demonstrate the specificity of cDNA as a probe and the stringency of hybridization assays.

Discussion

Specific immunadsorption of LCM-synthesizing polysomes to antibodies enriched for LCM was employed for the isolation of LCM mRNA. The mRNAs were assessed for purity by translation in an mRNA-dependent rabbit reticulocyte lysate, electrophoretic separation on denaturation gels, and hybridization assays. The reticulocyte lysates were rendered mRNA dependent by eliminating the endogenous mRNA activity by micrococcal nuclease treatment (Pelham & Jackson, 1976). This facilitated a direct evaluation of cell-free products on polyacrylamide gels without the necessity of immunoprecipitating the products first. Portions of lysates were also subjected to immunoprecipitation assays when required for quantitation. The polypeptides synthesized in response to the purified RNA of peak 2 (Figure 1) migrated identically with the marker LCM subunits which were isolated from the homologous heart tissues and purified to homogeneity. The two-dimensional separation of the labeled polypeptides clearly demonstrates that the mRNAs were free of other contaminant mRNAs, although electrophoresis on one dimension, on occasions, showed the presence of minor components (see Figures 2 and 4). Thus, from the electrophoretic separation and immunoprecipitation data, we concluded that proteins synthesized in response to peak 2 RNA were two complete LCM subunits of 24000 and 18000 molecular weight. The ratio (1.2:1.0) of label in the subunits, LCM_1 and LCM_2, was consistently reproduced and reflected the ratio of the respective mRNA in the peak 2 RNA fraction. The radioactivity in the subunits together, however, constituted 15% of the total products of polysomal RNA, suggesting that translatable LCM mRNA comprises 15% of the total polysomal mRNA population. This evaluation, however, is misleading, since the relative amounts of the products of the translation in rabbit reticulocyte lysate do not necessarily reflect the concentrations of the respective mRNAs. The translational efficiency of one class of mRNA varies significantly from that of others in the same mixed population of mRNAs. We have already observed (experiments to be reported elsewhere) that ionic concentrations and incubation conditions which were optimum for LCM mRNA translation resulted in a poor translation of HCM mRNA. Hybridization assay would yield a more accurate estimate of LCM mRNA content in the total mRNA of the chick heart (see Table I).

The extent of mRNA purification was also evident by the sedimentation profile of peak 2 RNA on sucrose gradient and its migration on denaturing gels. Additional evidence for purity comes from the kinetics of hybridization with the cDNA. If the LCM mRNA preparation contains heterogenous RNA as a contaminant, it would affect the R_0t values 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 of cDNA to both LCM mRNA and total poly(A) RNA occurred within a range of 100 R_0t units and to the same extent, as would be expected if the cDNA were hybridizing to the same RNA species. Furthermore, an independent determination of the molecular sizes was made based on the comparative hybridization data between the cDNAs of globin and LCM and their respective mRNAs. The close length sizes of the two cDNA (globin and LCM) would minimize the possible effects on hybridization rates. The maximum complexity thus estimated by hybridization for LCM mRNA (2225 nucleosides) and the minimum estimate of complexity based on physical separation of two mRNA on formamide gels (2070 nucleosides) were in excellent agreement and suggest that the level of contamination, if any, was less than 7%. Since the calculated value of sequence complexity is the same as the sizes of two molecules of mRNA, it would mean that the mRNA in peak 2 RNA fraction consists of two single RNA molecules, the putative mRNA for LCM_1 and LCM_2. Indeed, LCM_2 was the product of translation of a slower moving LCM mRNA fraction obtained from a separate sucrose gradient, whereas the RNA fraction next higher in size produced LCM_1 predominantly (unpublished results).

Thus, the specific cDNA probe synthesized in this study will afford a reliable detection and quantitation of LCM mRNA sequences and their complexity in developing presumptive heart cells. Recent evidence on sequency complexity analysis (Peterson & Bishop, 1977) of primary cell culture of the

12-day-old embryonic breast muscle suggested that mRNA sequences, in exceptionally high concentration, accumulate in cells developing toward myofibrillar formation. The sequences contain mRNA templates for the synthesis of muscle specific proteins. Buckingham et al. (1974, 1976), using similar cell culture derived from the fetal calf muscle, reported that the putative mRNA for HCM undergoes a stabilization prior to the terminal differentiation suggesting the existence of a control mechanism operating posttranscriptionally. The HCM and LCM subunits are also known to be synthesized coordinately (Devlin & Emerson, 1978) and on monocistronic messengers (Low et al., 1971; Sarkar & Cooke, 1970). Similar information with respect to cardiac muscle differentiation is not available, but, more importantly, the information on molecular events in situ in developing cells prior to myoblast formation is totally lacking. The purification of muscle specific mRNA and the availability of the respective cDNA probes will thus facilitate our analysis of the mechanism underlying the process of embryonic induction and cell differentiation during heart muscle formation.

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