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Regulation of Muscle Differentiation: Cloning of Sequences from α-Actin Messenger Ribonucleic Acid†

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ABSTRACT: α-Actin is found exclusively in skeletal muscle tissue and appears to be induced during myogenesis. The mechanism underlying the induction of muscle gene transcription can be studied with a deoxyribonucleic acid (DNA) probe complementary to the α-actin messenger ribonucleic acid (mRNA) sequence. Such a probe was produced by cloning complementary DNA (cDNA) transcribed from a breast muscle RNA preparation enriched for actin mRNA. Double-stranded DNA was inserted into the *Pst*I restriction site of plasmid pBR322, and the resulting hybrid DNA molecules were used to transform *Escherichia coli* RR1. Bacterial colonies were preliminarily screened by hybridization to two different [^{32}P]cDNA preparations, i.e., one of which contained sequences of α-actin mRNA and its major contaminant, while the other contained only the major contaminant sequence. The presence of an actin-specific gene insert was documented in plasmid pAC269 with translation assays. Total muscle mRNA was hybridized to pAC269 DNA-cellulose, and the hybridized message was then eluted and translated in a mRNA-dependent reticulocyte lysate. The mRNA which hybridized to pAC269 directed the translation of a protein of 42 000 M_r which was subsequently identified as actin by electrophoretic mobility (one and two dimensions), deoxyribonuclease I (DNase I) affinity, and cyanylation peptide mapping. Restriction endonuclease and heteroduplex mapping of pAC269 detected a 1.4-kilobase insert which is ~95% of the previously measured length of the actin mRNA. When pAC269 was used as a hybridization probe, it was found that the muscle-specific α-actin sequence had only a 70% homology with the nonmuscle β- and γ-actin sequences. Also a difference of 13 °C was observed in thermal melts between muscle and nonmuscle actin mRNAs hybridized to pAC269. These differences in homology and thermal melting will allow the specific quantitation of actin mRNA content during myogenesis and should also aid in the identification of the α-actin natural gene.

The actins are a family of contractile proteins found in all eukaryotic cells (Garrels & Gibson, 1976; Storti et al., 1976; Goldman et al., 1976). Actin was once thought to be a single highly conserved protein, but multiple forms of actin were later identified (Gruenstein et al., 1975; Elzinga & Lu, 1976). α-Actin is found only in skeletal muscle where it is a major constituent of the contractile apparatus. Five other forms of actin, one from cardiac tissue, two from smooth muscle, and two from nonmuscle cells (β and γ), have been identified (Vandekerchove & Weber, 1978; Elzinger & Lu, 1976).

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Amino acid sequence differences between the various actins cause an isoelectric focusing heterogeneity and prove that the actins are all products of different genes (Elzinger & Lu, 1976; Vandekerchove & Weber, 1978). This conclusion is supported by recent experiments which show that actin polypeptides are coded by a middle repetitive gene family in lower eukaryotes (Kindle & Firtel, 1978), insects (Tobin et al., 1980), and vertebrates (Schwartz & Rothblum, 1980).

Currently, changes in morphogenesis and cell differentiation are thought to be caused by selective gene expression. In muscle development, the increased accumulation of one gene product and the loss of the other polymorphic forms appear to be major regulatory events. For example, α-actin is induced during myogenesis (Whalen et al., 1976; Garrels & Gibson, 1976), while β- and γ-actins are repressed. This induction of α-actin is paralleled by an increase in total actin mRNA (Paterson et al., 1974), but we have shown that α-actin mRNA[1] comprises 8% of the total mRNA in differentiated muscle (Schwartz & Rothblum, 1980).

We believe that this apparent regulation at the transcriptional level can best be studied with a hybridization probe to the α-actin mRNA. Here we describe our use of a partially purified α-actin mRNA population to construct and select a bacterial clone carrying the α-actin structural sequence on a plasmid. This plasmid DNA can now be used as a pure α-actin hybridization probe to study actin induction during myogenesis and to search among genomic DNA sequences for the α-actin natural gene.

Materials and Methods

Materials. Three-week-old White Leghorn chicks were purchased from Animal Specialties, Houston, TX. Breast muscles were removed, rinsed in cold phosphate-buffered saline, 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. Liquified phenol was purchased from Fisher Scientific Co. and distilled immediately before use. Oligo(dT)–cellulose (6–18-mer average) was purchased from Collaborative Research. Restriction endonucleases and calf thymus terminal deoxynucleotidyl transferase were purchased from Bethesda Research Laboratories. Nonlabeled deoxynucleotides were purchased from P-L Biochemicals. S_1 nuclease, poly(dT), and poly(rA) were purchased from Miles Laboratories. [^{3}H]dCTP (25 Ci/mmol) was purchased from New England Nuclear, and [^{35}S]-methionine (755 Ci/mmol) and [α-^{32}P]dCTP (400 Ci/mmol) were purchased from Amersham Corp. Avian myeloblastosis virus (AMV) reverse transcriptase was supplied by Dr. J. W. Beard, Life Sciences, Inc., St. Petersberg, FL.

Bacterial Strains and Growth Conditions. Escherichia coli K12 strain RR1 containing plasmid pBR322 (Amp^r, Tc^r) was provided by Dr. H. W. Boyer (University of California, San Francisco). For isolation of pBR322 (Bolivar et al., 1977), RR1 was grown in M9-glucose minimal medium. *E. coli* RR1 was the recipient strain for plasmid transformation experiments.

[1] Abbreviations used: mRNA, messenger ribonucleic acid; DNA, deoxyribonucleic acid; dCTP, deoxycytidine 5′-triphosphate; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; cDNA, complementary DNA; DTT, dithiothreitol; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; dTTP, deoxythymidine 5′-triphosphate; dATP, deoxyadenosine 5′-triphosphate; dGTP, deoxyguanosine 5′-triphosphate; tRNA, transfer RNA; SSC, standard saline citrate; Pipes, 1,4-piperazinediethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Cl_3AcOH, trichloroacetic acid.

Isolation of mRNA. Total nucleic acids were isolated from frozen muscle by successive $NaDodSO_4$–phenol extractions as previously described (Schwartz & Rothblum, 1980). Briefly, DNA was selectively removed from RNA by a series of 3 M NaOAc (pH 6.0) washes (Palmiter, 1974). The poly(A)-containing RNA was obtained from total RNA by two passages through an oligo(dT)–cellulose column as described by Aviv & Leder (1972). Heat-denatured RNA was then fractionated by centrifugation on 5–20% linear sucrose gradients in 0.02 M NaOAc (pH 5.0) and 0.001 M Na_2EDTA. The RNA fraction sedimenting between 15 and 18 S, which contained α-actin mRNA activity when translated in rabbit reticulocyte lysate, was recovered by ethanol precipitation. Fractionation by size of the poly(A)-containing RNA was also accomplished by chromatography on Sepharose 4B in 0.1 NaOAc and 0.001 M Na_2EDTA (pH 5.0) at 4 °C (Woo et al., 1974). Selected RNA samples were further purified by electrophoresis on 3.3% polyacrylamide containing the disulfide cross-linker *N′,N′*-cystamine bis(acrylamide) (Hanson, 1976). Gels were stained with methylene blue, and selected RNA bands were sliced out of the gel and dissolved in β-mercaptoethanol as previously described (Schwartz & Rothblum, 1980). RNA was removed from acrylamide polymers by passage over a cellulose column in the presence of buffered ethanol (Franklin, 1966). Aliquots of fractions isolated from gels and sucrose gradients were analyzed by translational activity and then used for cDNA synthesis.

Translation Assay. RNA was translated in a mRNA-dependent rabbit reticulocyte lysate after treatment with micrococcal nuclease according to Pelham & Jackson (1976) as described (Schwartz & Rothblum, 1980).

Binding of Actin to DNase–Agarose. Muscle G-actin has the unusual property of inhibiting DNase activity by binding rapidly to form a 1:1 stable complex with the enzyme (Mannherz et al., 1975). This highly specific interaction was used to identify actin synthesized in vitro (Schwartz & Rothblum, 1980).

Two-Dimensional Gel Electrophoresis. Actin synthesized in vitro was isoelectrically focused on a 5% polyacrylamide gel for 9000 V h. The pH gradient was established by a 2% (w/v) solution of ampholytes (LKB) of which 80% was pH 4–6 and 20% was pH 3–10 (O'Farrell, 1975). Proteins were electrophoresed in the second dimension in $NaDodSO_4$–10% polyacrylamide slab gels (Laemmli, 1970).

Cyanylation Cleavage of Actin. The cleavage reaction of cysteinyl residues was carried out under conditions similar to those of Jacobson et al. (1973). One hundred micrograms of chicken skeletal actin (Spudich & Watt, 1971) and actin synthesized in vitro was denatured in 6 M guanidine hydrochloride containing 0.5 mM DTT, 1 mM EDTA, and 20 mM Tris-HCl buffer (pH 8.0). This was then treated with 10 mM dithiodinitrocyanobenzoic acid (TNB-CN, Eastman) at 25 °C for 30 min. The mixture was exhaustively dialyzed against 50% acetic acid and then evaporated to dryness under a stream of nitrogen gas. The actin powder was then dissolved in 8 M urea containing 1 M borate buffer (pH 9.2), and the solution was incubated at 37 °C for 12 h. The reaction was terminated by the addition of excess dithiothreitol. The actin fragments were dialyzed against 1% $NaDodSO_4$ containing 50 mM Tris-HCl (pH 7.5) in dialysis tubing with a 3000 M_r limit.

Enzymatic Synthesis of Double-Stranded cDNA. The synthesis of the first strand of cDNA followed the conditions of Monahan et al. (1976). The reaction mixture contained 50 mM Tris-HCl (pH 8.3), 20 mM dithiothreitol, 6 mM $MgCl_2$, 1 mM each of dTTP, dATP, and dGTP, 0.5 mM

[^{3}H]CTP (2.75×10^4 cpm/μmol), 12.5 μg/mL oligo(dT)$_{18-20}$, 40 μg/mL actinomycin D, 125 μg of mRNA, and 200 units/mL AMV reverse transcriptase. The reaction was carried out at 46 °C for 20 min. The size of the cDNA was monitored by alkaline agarose gel electrophoresis (McDonnell et al., 1977).

The second strand was synthesized with AMV reverse transcriptase by utilizing the self-priming activity of the single-stranded cDNA according to Monahan et al. (1976). Two hundred microcuries of [^{32}P]dATP (400 Ci/mmol), dried by lyophilization, was resuspended in 100 μL of buffer containing 50 mM Tris-HCl (pH 8.3), 20 mM dithiothreitol, 8 mM $MgCl_2$, 400 μM each of dGTP, dCTP, and dTTP, 100 μM dATP, and ~6 μg of cDNA. AMV reverse transcriptase was added to a final concentration of 200 units/mL and incubated for 4 h at 46 °C. The double-stranded cDNA was digested for 30 min at 37 °C with 100 units of S_1 nuclease in 0.2 M sodium acetate (pH 4.5), 0.4 M NaCl, and 2.5 mM $ZnCl_2$ for 30 min at 37 °C. The DNA was deproteinized by phenol extractions and recovered by ethanol precipitation.

Construction of Hybrid DNA Molecules. Poly(dC), ~15 nucleotides in length, was added to the 3′ termini of S_1-treated DNA by using terminal deoxynucleotidyl transferase in the presence of $CoCl_2$ (Roychoudhury et al., 1976). pBR322 was cleaved by the restriction endonuclease *Pst*I and tailed with dGTP to a length of 15 dG/terminus. This was mixed with an equal molar amount of tailed cDNA in 0.2 M NaCl, 10 mM Tris (pH 7.6), 1 mM EDTA, and a final DNA concentration of 8 μg/mL. This mixture was heated to 60 °C and allowed to cool to room temperature over a period of 4 h.

Transformations, and all subsequent procedures using the chimeric plasmid in its host, were performed under approved P-3 physical containment conditions by using an approved vector and host. Transformation of *E. coli* K12 strain RR1 was under the same conditions as those used for X1776 (McReynolds et al., 1977) with minor modifications. Recombinant plasmid DNA was purified by the method of Katz et al. (1977).

Restriction Endonuclease Cleavage of Plasmid DNA. Reaction conditions for restriction endonuclease cleavage of plasmid DNA varied with the particular enzyme and were essentially as suggested by the supplier. Enzymes were added at 1 unit/μg of DNA substrate and incubated 1 h at 37 °C except for *Pst*I which was incubated at 30 °C. Digestion products of plasmid DNA were separated by agarose slab gel electrophoresis in buffer containing 50 mM Tris (pH 8.4), 20 mM NaOAc, 18 mM NaCl, and 2 mM EDTA at 70 V. Detection of fragments which contained inserted DNA was accomplished by transfer to nitrocellulose (Southern, 1975) and hybridization to [^{32}P]cDNA synthesized from enriched actin mRNA.

Isolation of Actin mRNA with Cloned DNA-Cellulose. Plasmid DNA from actin clone 269 (1 mg) was digested with *Hha*I and covalently linked to finely divided cellulose (40 mg) that had been treated according to the protocol of Noyes & Stark (1975). The DNA-cellulose was then treated with 2% glycine in Denhardt's buffer (Denhardt, 1966) at 4 °C for 24 h in order to hydrolyze any remaining diazo groups and block the nonspecific sites on the cellulose. Hybridizations were performed by placing 200 μL of packed cellulose into an Eppendorf centrifuge tube (1.5-mL capacity) with 50% formamide, 0.05 M Tris (pH 7.5), 1 mM EDTA, 0.4 M NaCl, 0.5% $NaDodSO_4$, 200 μg of poly(A)-containing RNA, 50 μg poly(A), and 50 μg of *E. coli* tRNA in a final volume of 600 μL. The tubes were heated to 80 °C for 2 min and then the contents allowed to hybridize at 37 °C in a shaking water bath for 18 h. The DNA-cellulose was washed with 600 μL of hybridization buffer, 2 times at 37 °C, 2 times at 55 °C, and then 2 times with 1 mL of ice-cold 2 × SSC. The DNA-cellulose was resuspended in 200 μL of chelexed formamide, heated to 80 °C for 2 min, and centrifuged. The supernatant was removed and made to contain 50% formamide, 0.5 M NaCl, and 2.5 volumes of ethanol. Precipitated RNA was translated in the reticulocyte lysate system.

Saturation Hybridization. Plasmid pAC269, digested with *Hha*I, was nick translated with [^{3}H]dCTP (25 Ci/mmol) as described by Maniatis et al. (1975). Complementary single-stranded pAC269 DNA (10 000 cpm/ng) was made by hybridization to poly(A)-containing muscle RNA at a ratio of 2 μg of DNA to 400 μg of poly(A)-containing RNA in buffer containing 80% formamide, 7.2 mM Na_2EDTA, 0.4 M NaCl, and 10 mM Pipes (pH 6.4) at 48 °C for 45 min. The preparation was digested with 3000 units of S_1 nuclease in a final concentration of 3% formamide, 0.5 M NaCl, 2.5 mM $ZnCl_2$, and 0.2 mM NaOAc (pH 4.5). Hybridization and S_1 nuclease digestion was repeated to reduce self-annealing of complementary pAC269 DNA to a level of 5–10%. pAC269 [^{3}H]-DNA was treated with alkali and sized to contain DNA fragments in the range of 100–400 nucleotides.

Saturation hybridization was performed in a final volume of 50 μL containing 0.6 M NaCl, 0.01 M Hepes (pH 7.0), and 0.002 M Na_2EDTA. Each hybridization reaction contained 3000 cpm of complementary pAC269 [^{3}H]DNA (0.3 ng) and varying amounts of purified RNA. Following heat denaturation for 5 min at 100 °C, incubations were performed for 52 h at 68 °C, and the reaction was terminated by freezing at −80 °C. The extent of hybridization was determined by S_1 nuclease digestion for 2 h at 37 °C in a buffer containing 0.2 M NaOAc (pH 4.5), 0.475 M NaCl, 2.5 mM $ZnCl_2$, and 1600 units of S_1 nuclease, followed by Cl_3AcOH precipitation. The data from the hybridization experiments were expressed as the percent of hybridization vs. the ratio of input RNA to pAC269 DNA.

In thermal stability experiments pAC269 [^{3}H]DNA was hybridized to excess RNA at a ratio of 1:2000 as described above. Reactions were diluted to 0.2 M Na^+ and then allowed to equilibrate for 5 min at each temperature assayed. The samples were then processed and S_1 nuclease digested as described.

Results

As shown in Figure 1, slot C, total muscle poly(A)-containing RNA electrophoresed on denaturing gels is enriched in two major RNA species, designated band I (5.2×10^5 M_r) and band II (4.6×10^5 M_r). Unfortunately, with sucrose gradient fractionation, Sepharose 4B chromatography, and preparative polyacrylamide gels, we were unable to completely purify the actin mRNA-containing band I (Figure 1, slot A) away from its lower molecular weight contaminant (Figure 1, slot B; Schwartz & Rothblum, 1980). This was shown by both DNase-agarose binding (Table I) and by autoradiography on $NaDodSO_4$-polyacrylamide gels (Figure 1, slots D–F) after using the various RNA preparations to direct polypeptide synthesis in a reticulocyte lysate. Such analysis revealed increased binding of the band I translation products to DNase-agarose with each RNA purification step. The maximal purity obtained, however, was 51%. When purified band I directed translation products were analyzed on a $NaDodSO_4$-polyacrylamide gel (Figure 1, slot E), 50% of the incorporated [^{35}S]methionine migrated at 42 000 M_r (actin), while most of the remaining radioactivity migrated at 36 000

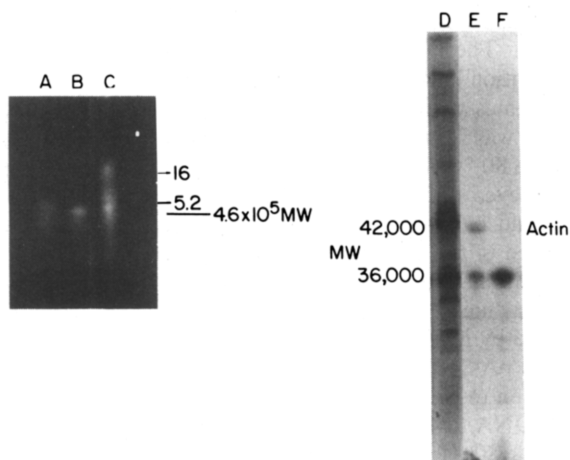

FIGURE 1: Electrophoretic analysis of actin-enriched RNA and in vitro translation products. RNA samples were electrophoresed on denaturing 2% agarose gels (Bailey & Davidson, 1976). Slot A is band I (actin mRNA containing RNA), slot B is band II RNA, and slot C is total poly(A)-containing RNA. These were translated in reticulocyte lysate, and the products were run on $NaDodSO_4$–10% polyacrylamide gels and autoradiographed on X-ray film. Slot E shows products from the translation of band I, slot F from band II, and slot D from total poly(A)-containing RNA.

Table I: Purification of Actin mRNA Measured by Translational Activity

source of poly(A)-containing RNA	total [^{35}S]-methionine[a] incorpn (cpm/μg of RNA)	radioact bound[b] to DNase-agarose (cpm/μg of RNA)	% incorpd into actin
muscle	218 000	22 200	10
sucrose gradient	350 000	67 400	19
Sepharose 4B	400 000	185 600	46
preparative polyacrylamide gels	360 000	184 000	51
hybridized to pAC269 DNA-cellulose	470 000	422 000	90

[a] Total incorporation of acid-precipitable [^{35}S]methionine in a 60-μL translation assay. [b] Radioactivity eluted from 100 μL of packed DNase-agarose beads with 3 M guanidine hydrochloride as described under Materials and Methods and by Schwartz & Rothblum (1980).

M_r. Treatment of the purified band II directed products in a similar fashion resulted in 95% of the incorporated label migrating with the unidentified 36 000 M_r polypeptide (Figure 1, slot F).

Amplification of Chimeric Plasmids. When conventional methods of RNA purification failed to completely purify the actin mRNA, we decided to complete the purification by cloning actin cDNA in *E. coli*. To this end, double-stranded DNA was synthesized from chicken breast muscle poly(A)-containing RNA which was enriched for actin by using only the 15–18S fraction from a sucrose gradient (Table I; Schwartz & Rothblum, 1980). In two different experiments, 15% and 20% of the input mRNA were reverse transcribed, as calculated from the incorporation of [^{3}H]dCTP into the first strand. After copying the second strand, the resultant hairpin loop was cleaved with S_1 nuclease as described under Materials and Methods. The double-stranded cDNA was examined by autoradiography on 5% polyacrylamide gels and found to migrate predominantly as two bands between 1100 and 1400 base pairs. Small amounts of double-stranded cDNA of heterogenous size (from 1100 to 300 base pairs) were also observed. Approximately 15 dC residues were then added to

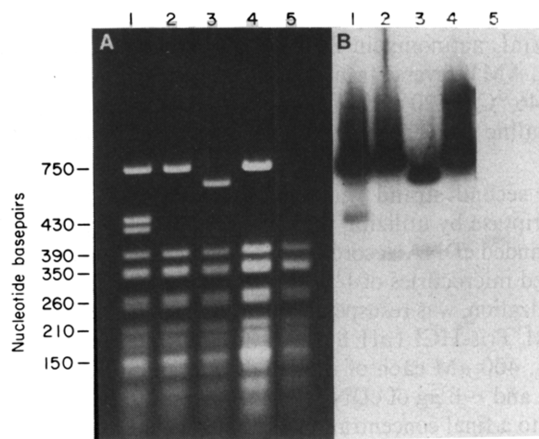

FIGURE 2: Sequence homology of actin recombinant DNA plasmids determined by Southern blot restriction mapping. In panel A, *Hha*I digests of plasmid DNA were analyzed on a 2% agarose slab gel. Electrophoresis was performed as described under Materials and Methods, and the gel was stained in ethidium bromide (5 μg/mL) and visualized under ultraviolet light. The samples shown are the *Hha*I digests of pAC269 (slot 1), pAC51 (slot 2), pAC50 (slot 3), pAC56 (slot 4), and of pBR322 (slot 5). Panel B is an autoradiogram of [^{32}P]cDNA (7 × 10^6 cpm in 8 mL) hybridized to Southern blots of *Hha*I fragments and viewed in the same order as panel A. *Hha*I-digested pBR322 and *Hae*III-digested SV40 DNA (not shown) were used as DNA molecular weight standards.

the 3′ termini of double-stranded cDNA by using terminal deoxynucleotidyl transferase. The bacterial plasmid pBR322 was cleaved with *Pst*I at the single site (Bolivar et al., 1977) and then "tailed" with 15 dG residues. Equimolar amounts of double-stranded DNA and the plasmid DNA were then used to transform *E. coli* RR1. Four hundred clones that were Amp^s and Tc^r were obtained from 1.2 μg of plasmid DNA and 0.3 μg of the double-stranded DNA.

To select colonies which contained actin recombinant DNA, we first replica plated the Tc^r clones 2 times and transferred them directly to nitrocellulose filters. These clones were then hybridized in situ (Grunstein & Hogness, 1975) with [^{32}P]-cDNA synthesized from band I RNA (50% actin, 50% contaminant) and band II RNA (95% contaminant). Comparison of the autoradiograms from these two hybridizations allowed us to select 25 of the 400 Tc^r clones as possibly containing actin sequences.

Determination of Lengths of Inserted DNA in Recombinant Plasmids. Plasmid DNA from four of the positive clones was prepared from 1-L cultures. Plasmid pAC269, pAC51, pAC50, and pAC56 and parental pBR322 DNA were digested with *Hha*I, and the resulting fragments were separated by electrophoresis on 2% agarose gels (Figure 2). When compared to *Hha*I digestion of parental pBR322 (slot 5), digestion of pAC269 revealed three extra fragments of 750, 480, and 430 base pairs in length (slot 1). The other pAC plasmids each contained a single additional fragment of 750 base pairs or less (slots 2–4). In pAC269, the clone with the largest insert, the lengths of the three fragments add up to ~1660 base pairs. A total of 337 of these base pairs are plasmid DNA containing the *Pst*I insertion site as computed from the available pBR322 DNA sequence data (Sutcliffe, 1978). Therefore, the net DNA insert length is ~1330 base pairs.

The DNA fragments were then transferred to nitrocellulose by the Southern procedure (Southern, 1975). It was found that the largest fragments in all four pAC clones contained actin DNA sequences by hybridization to [^{32}P]cDNA made from band I RNA. This cDNA was made from mRNA excised from a polyacrylamide gel, and there was a tendency to make short cDNA due to either nicking of RNA or to some

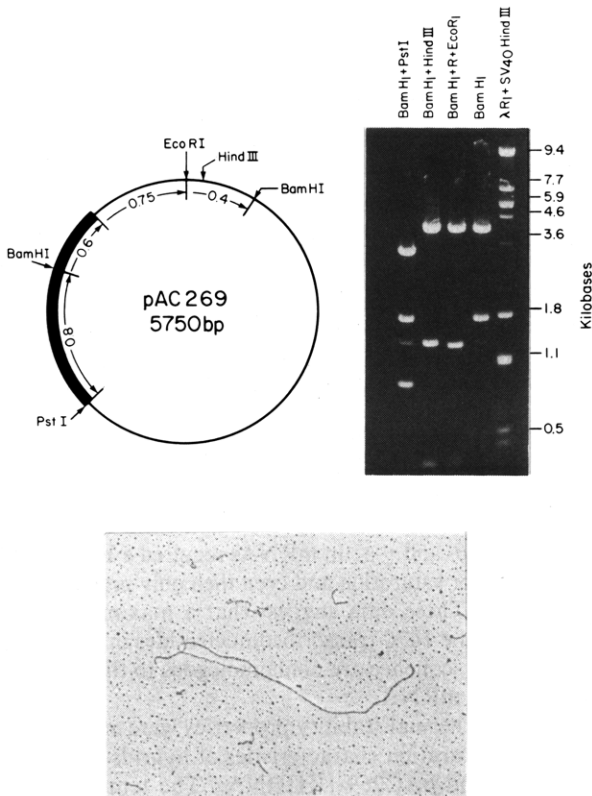

FIGURE 3: Partial restricition map of pAC269. The map (left) shows the inserted actin DNA fragment and its orientation (in heavy black) in the pBR322 plasmid DNA. The cleavage sites of *Bam*HI, *Pst*I, *Hin*dIII, and *Eco*RI in pAC269 (right) are indicated. The R loop of pAC269 and actin mRNA was prepared by Dr. Myles Mace, Jr., under the conditions of Chow et al. (1977). The orientation of the R loop is described under Results.

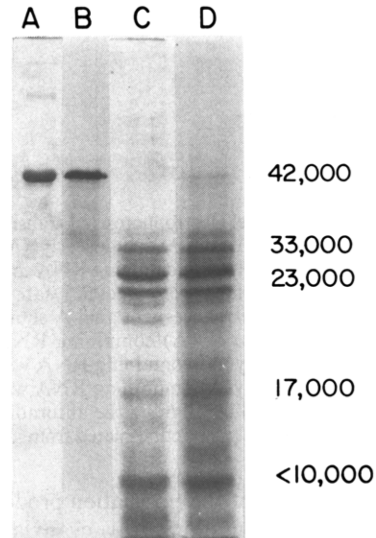

FIGURE 4: Electrophoresis of translation products of RNA selected by hybridization to pAC269 DNA. Total poly(A)-containing muscle RNA was hybridized to pAC269 DNA–cellulose as described under Materials and Methods. Hybridized RNA was eluted, translated, and then electrophoresed on a NaDodSO$_4$–12% polyacrylamide slab gel. In slot A is authentic chick muscle actin stained with Coomassie blue. In slot B is the autoradiograph of the translation product. In slot C is stained cyanylation fingerprint of chick muscle actin. In slot D is the autoradiograph of the cyanylation fingerprint of the translation product.

impurities which remained with the RNA. However, the fact that most of the cDNA molecules were shorter than most of the plasmid inserts and the fact that all of the cDNAs began their synthesis on the poly(A) tail of a mRNA molecule allowed us to determine the 3′–5′ orientation of the actin DNA insert in pAC269. The largest *Hha*I fragment (750 base pairs) shows the most hybridization (Figure 2B) and is complementary to the 3′ end of the mRNA. The 480 base pair fragment is intermediate, and the 430 base pair fragment with the least amount of cDNA hybridized is complementary to the 5′ end of the mRNA.

Restriction endonuclease cleavage analysis using several other enzymes confirmed the size of the inserted actin DNA in clone pAC269 (Figure 3). DNA was initially digested with *Bam*HI and divided into four aliquots, three of which were digested with a second endonuclease. The complete restriction map of pBR322 as presented by Sutcliffe (1978) was used to verify our results and to determine the exact size of the fragments. The digestion with *Bam*HI and *Pst*I released three fragments (Figure 3). The largest 3.2-kilobase fragment contained parental plasmid DNA. The second fragment of 1.75 kilobases contained a 0.6-kilobase portion of actin DNA and a 1.15-kilobase fragment of pBR322 because of an unreconstituted *Pst*I site in the inserted actin DNA. The 0.8-kilobase fragment was caused by a single recovered *Pst*I site and a single internal *Bam*HI site centrally located in the actin insert. Double digestion of *Bam*HI and *Hin*dIII showed that *Hin*dIII did not cut the actin insert but released fragments which were 1.45 and 0.3 kilobases. *Eco*RI cuts close to the *Hin*dIII site within pBR322 DNA and produces two small fragments of 1.35 and 0.35 kilobases plus a large fragment of 4 kilobases. *Bam*HI alone causes a release of a 1.75-kilobase fragment which contains ~0.6 kilobase of actin DNA sequences. The partial restriction endonuclease cleavage map of pAC269 shows that 1400 base pairs of actin DNA were inserted into the recombinant plasmid and provides sufficient orientation to determine the insert size by the technique of R looping.

Electron microscopy was used to analyze R loops formed between *Eco*RI linearized pAC269 DNA and muscle poly(A)-containing RNA. Almost all of the DNA molecules observed possessed an R-loop structure when hybridization was performed in mRNA excess according to Chow et al. (1977). Examination of heteroduplexes reveals a single protruding RNA tail of heterogeneous length, which is located toward the short arm of the asymmetrically split DNA (bottom of Figure 3) and represents the 3′ poly(A) sequence of the mRNA. This observation is consistent with the orientation determined by Southern analysis (Figure 2). The overall length of the actin insert determined by R looping is 1400 bases, essentially identical with 1330 base pairs determined by endonuclease mapping. An additional 3450 and 800 bases of plasmid DNA flank the DNA insert. Since no RNA strands were observed at the 5′ termini of the actin DNA, we assume that the actin DNA insert in pAC269 is close to full length in size.

Positive Confirmation of the Actin Insert by Hybridization to DNA–Cellulose. To confirm the identity of pAC269 as actin cDNA, we covalently linked the cloned plasmid DNA to diazobenzyloxymethylcellulose and used it to isolate actin mRNA by affinity chromatography (Noyes & Stark, 1975). Poly(A)-containing RNA from chick breast muscle was hybridized to an excess of pAC269–cellulose, and the bound RNA (5–10% of the input) was subsequently eluted. This RNA was translated and the protein products were electrophoresed on a NaDodSO$_4$–polyacrylamide gel. The single translation product was found to have the same electrophoretic mobility as actin purified from chick skeletal muscle (Figure

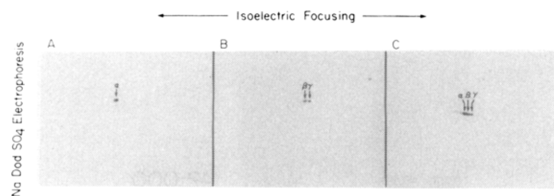

FIGURE 5: Two-dimensional gel electrophoresis of translation products of RNA hybridized to pAC269 DNA–cellulose. RNA preparations selected by hybridization of poly(A)-containing RNA to pAC269 were translated in a mRNA-dependent reticulocyte lysate and analyzed by two-dimensional gel electrophoresis. Panel A shows translation products produced when the poly(A)-containing RNA source was muscle, panel B when the poly(A)-containing RNA was from brain, and panel C when muscle poly(A)-containing RNA was mixed with brain. α-Actin was identified by aligning the autoradiographic spot with the stained nonradioactive pure actin isolated from skeletal muscle.

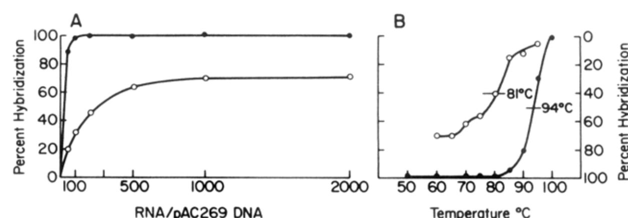

FIGURE 6: Homology of pAC269 DNA sequences with β- and γ-actin mRNAs. In panel A, RNA excess hybridizations were performed with total muscle RNA (●) and total embryonic brain RNA (○) by using single-stranded complementary pAC269 [^{3}H]DNA as described under Materials and Methods. In panel B pAC269 [^{3}H]DNA–RNA hybrids were incubated for 5 min at each temperature and then S_1 nuclease digested. The line through each melt curve is the T_m point.

4). Further comparison of the translation product and pure actin was made by fragmentation with a cyanylation reagent. α-Actin has five cysteinyl residues at the 10th, 217th, 256th, 284th, and 373rd amino acid positions, and cleavage of actin with TNB-CN gave rise to many fragments which were observed on $NaDodSO_4$–12% polyacrylamide slab gel (Figure 4). When an autoradiograph of the cyanylated translated product was compared with a stained TNB-CN digest of pure actin, the two gels were found to have exactly the same number of fragments (17) in the same relative positions and the same relative densities (Figure 4; slots C and D).

Translation products of the affinity-chromatographed mRNA were also tested for ability to bind to DNase I–agarose. Actin is a naturally occurring inhibitor of DNase, and the inhibition of DNase I activity has been used in the isolation of actin (Lazarides & Lindberg, 1974). When the translation products were incubated with DNase I–agarose, 90% of the radioactive proteins were bound (Table I). The labeled translation product was eluted from DNase I–agarose and found to comigrate with the 42 000 M_r actin band (Schwartz & Rothblum, 1980).

Homology of pAC269 DNA to β- and γ-Actin mRNA. α-, β-, and γ-actins are products of different genes and are separable from each other by two-dimensional gel electrophoresis. We have shown that actin cDNA cross hybridizes to the repeated family of actin DNA sequences in the chicken genome. We therefore assumed that the α-actin clone pAC269 would be sufficiently homologous to the β- and γ-actin mRNAs to allow the latter sequences to be isolated by affinity chromatography. The labeled translation products of affinity-purified α-actin mRNA were examined by two-dimensional gel electrophoresis and autoradiography and found to yield a single spot with a molecular weight of 42 000 which focused within a pH range of 5.4–5.5 (Figure 5A). This autoradiographic spot comigrated with purified skeletal actin and was thus identified as α-actin.

Poly(A)-containing RNA was isolated from 14-day embryonic chick brain and hybridized to pAC269 DNA–cellulose. The bound RNA was eluted, translated, and two dimensionally electrophoresed as above and found to yield two spots corresponding to β- and γ-actin (Figure 5B). When affinity purified α-actin mRNA was mixed with β and γ mRNA and translated, three radioactive spots were found on the autoradiograph of the two-dimensional gel which corresponded to α-, β-, and γ-actins (Figure 5C).

The homology between the β- and γ-actin mRNA mixture and α-actin mRNA was preliminarily investigated by saturation hybridization of total cellular RNA to complementary single-stranded pAC269 [^{3}H]DNA. Figure 6 (panel A) shows the saturation hybridization of the pAC269 DNA probe as measured by protection of acid precipitable radioactivity from S_1 nuclease digestion. Saturation was observed at an RNA to DNA ratio of 100:1. Embryonic chick brain RNA which contained both β- and γ-actin mRNAs required a RNA/DNA ratio of 500:1 for saturation and even then only protected 70% of the probe. These data suggest that a maximum of 440 nucleotides of the 1470 transcribed nucleotides in the α-actin structural gene sequence are nonhomologous to the combined β- and γ-actin mRNA.

Finally, the thermal stability properties were examined by using the denaturation characteristics of hybrids formed between RNA and pAC269 [^{3}H]DNA. The temperature at which 50% of the hybridized sequences have melted (T_m) is a useful parameter in relating the degree of complementarity between hybridized nucleic acid species. The fidelity of cloned pAC269 DNA was ascertained by examining the thermal melting of the α-actin DNA–mRNA duplex. The T_m was 94 °C and occurred with a sharp transition indicating that accurate base pairing was present (Figure 6B). Thermal properties of brain β- and γ-actin mRNA–pAC269 DNA hybrids revealed a broad multiphasic melt curve in which hybrids began to denature as early as 70 °C. An overall T_m of 81 °C was measured for the nonmuscle actin mRNA–muscle actin DNA hybrids.

Discussion

α-Actin is undetectable in prefusion myoblasts but comprises ~8% of the total protein synthesized in fully differentiated muscle (Paterson et al., 1974; Schwartz & Rothblum, 1980). Since α-actin is specifically induced in muscle, a hybridization probe to α-actin mRNA will serve in the analysis of the early gene expression in muscle development. However, when we attempted to purify the α-actin mRNA with several conventional techniques, a maximum of 50% purity was obtained (Table I; Schwartz & Rothblum, 1980). Therefore, we decided to utilize molecular cloning to isolate the α-actin structural sequence from the preparation of actin-enriched mRNA. Since we did not have a pure probe to select actin clones, we utilized the relative purities of band I RNA (50% actin sequence, 50% contaminant) and band II RNA (95% contaminant) to screen a large number of clones. Actin-containing clones were then identified as those which hybridized to cDNA made from band I RNA, but not with that made from band II. The plasmid DNA from each "actin clone" was then digested with *Hha*I and analyzed by Southern mapping with band I cDNA to determine the size of its actin sequence insert (Figure 2).

The fidelity of the largest inserted sequence (in clone pAC269) was then documented by linking the plasmid DNA to cellulose and using this to purify actin mRNA from total

muscle mRNA by affinity chromatography. The bound RNA was then eluted and translated, and the translation product was positively identified as actin on the high-resolution two-dimensional gel system of O'Farrell (1975). The cyanylation fingerprint of this translation product was found to be identical with the fingerprint of purified skeletal muscle actin (Figure 4). The translation products were also effectively bound by DNase I–agarose which was an indication that the actin synthesized in vitro was functional. We take these results to be unequivocal evidence that pAC269 DNA is complementary to actin mRNA.

The size of chicken α-actin mRNA determined under denaturing conditions is 1575 bases (Schwartz & Rothblum, 1980) and is comparable to the size estimated from the L6 rat myoblast line (Hunter & Garrels, 1977). Our Southern mapping and heteroduplex data indicate that the actin insert in pAC269 is 1400 $\pm$ 50 base pairs in length. Subtracting a poly(A) stretch of 100 nucleotides from the mRNA size of 1575 base pairs, pAC269 is calculated to contain 95% of the α-actin mRNA sequence. Skeletal muscle actin contains 374 amino acids (Elzinga & Lu, 1976). Thus, the coding region accounts for only 1122 bases of the message, and the 350 remaining bases must exist in noncoding regions. Interestingly, β- and γ-actin mRNA contain much larger noncoding regions of $\sim$1000 nucleotides (Hunter & Garrels, 1977). The significance of such extraordinarily large untranslated regions in the β- and γ-actin mRNAs is currently not known. However, sequence differences in the noncoding regions of the actins might be responsible for the 30% overall nonhomology between α-actin mRNA and the mRNAs of the other actin species.

Several laboratories have shown that changes in T_m are related to the number of nucleotide differences (sequence divergence) between nonidentical hybridizing sequences (Laird et al., 1969; Ullman & McCarthy, 1974; Leder et al., 1973). The reduction in T_m has been variably estimated to be 1.6–3.4 °C for each 1% sequence divergence (Benz et al., 1977). Seventy percent of the α-actin DNA sequence which hybridized to brain β- and γ-actin mRNA melted with a ΔT_m of 13 °C. Thus, there is a minimum of 3.8% and a maximum of 8.1% sequence divergence between skeletal muscle and cytoplasmic actin mRNA species, presumably within the coding region of the mRNAs. Unfortunately, it is not yet possible to separate β- and γ-actin mRNAs, and that is why this data is reported as an average of the two nonmuscle actin species.

Myogenesis is a process of cell differentiation which involves changes in contractile proteins, muscle-specific enzymes, and the mRNA populations of embryonic cells (Buckingham, 1978). The availability of a hybridization probe to α-actin mRNA will allow us to study the mechanism underlying the induction of muscle gene transcription. Also, the difference in thermal melting of pAC269 DNA hybrids with α-actin mRNA and β- and γ-actin mRNAs allows stringent hybridization conditions to be used for the specific quantitation of α-actin mRNA (R. Schwartz and K. Rothblum, unpublished experiments). The nonhomology between the actin mRNAs can also be used to develop DNA fragments which can serve as α-actin-specific hybridization probes. Finally, the actin sequence nonhomology should also make it possible to identify and isolate the α-actin natural gene from the middle repetitive actin gene family (Tobin et al., 1980; Schwartz & Rothblum, 1980) and to subsequently determine the sequence organization of this gene.

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Deoxyribonucleic Acid Sugar Damage in the Action of Neocarzinostatin[†]

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ABSTRACT: Neocarzinostatin (NCS), an antitumor protein antibiotic which causes base release and both true and alkali-labile, single-strand breaks almost exclusively at thymidylic acid and, to a lesser extent, at deoxyadenylic acid residues in deoxyribonucleic acid (DNA) in the presence of mercaptans and oxygen in vitro, produces gaps which are not the result of simple splitting of the phosphodiester bond. We show that sugar damage plays an important role in the NCS-induced DNA strand scission reaction. The release of ^{3}H-labeled compounds, mainly as formic acid and H_2O from [5′-^{3}H]thymidine-labeled λDNA, accounts for 50–80% of the thymine released without or with postincubation alkaline treatment, respectively. The release of these labeled sugar degradation, products correlates well with the increase in both thymine release and 5′-phosphate ends due to postincubation alkaline treatments. In addition, a malonaldehyde-like substance (characterized by its reaction with thiobarbituric acid and its chromatographic properties), containing tritium label derived from the 1′,2′ carbons of the deoxyribose moiety of thymidylate in DNA, is produced in a bound form concomitantly with the strand breaks and thymine release; its production is dependent on the presence of mercaptans and oxygen, is stimulated by 2-propanol, and is inhibited by α-tocopherol. Labeled malonaldehyde-like material and H_2O are released from DNA only after alkaline treatment and account for ~40% of the corresponding thymine released. The correlation between thymine release and strand breaks is examined to elucidate the nature of the gaps produced by NCS. The number of thymines released by various postincubation treatments is consistent with the number of 5′-phosphate ends generated at the DNA gaps as determined by the combined use of alkaline phosphatase and polynucleotide kinase; after alkaline treatment (0.3 N NaOH, 37 °C, 30 min) both values are in good agreement with the number of single-strand breaks, as estimated by alkaline sucrose gradients. Taken together, these data indicate that a significant fraction of the DNA damage caused by NCS requires alkaline treatment for removal of both the DNA base and a sugar fragment from the 5′-phosphate end of the DNA gap.

There is a substantial body of evidence [reviewed in Goldberg et al. (1980)] that cellular deoxyribonucleic acid (DNA) is the primary target in the action of the antitumor protein antibiotic neocarzinostatin (NCS).[1] In addition to placing single-strand breaks in DNA in vivo, NCS causes single-strand breaks in DNA in vitro in a reaction that is markedly enhanced by mercaptans (Beerman & Goldberg, 1974; Beerman et al., 1977). The breaks in the DNA are not simple phosphodiester nicks (Kappen & Goldberg, 1977; Poon et al., 1977) but consist of gaps that bear 3′- and 5′-phosphoryl termini (Kappen & Goldberg, 1978a). Cleavage of the DNA occurs almost exclusively at thymidylic acid and, to a lesser extent, at deoxyadenylic acid residues (Hatayama et al., 1978; D'Andrea & Hazeltine, 1978) and is associated with the release of the corresponding bases (Poon et al., 1977; Ishida & Takahashi, 1976). Evidence suggesting a free-radical mechanism in the DNA cleavage event has been presented (Kappen & Goldberg, 1978b; Sim & Lown, 1978).

While the primary sequence of the NCS protein has been known for some time (Meienhofer et al., 1972), we have only recently discovered a nonprotein chromophore to be associated with the protein (Napier et al., 1979) and have found that it possesses the cytotoxic and in vitro DNA strand scission activities of the parent compound (Kappen et al., 1980). The apoprotein stabilizes the chromophore and controls its release (Kappen et al., 1980). Fluorescence, dichroism, and other studies indicate that the chromophore binds tightly and specifically to the target DNA, by an intercalative mechanism (Povirk & Goldberg, 1980; L. F. Povirk, N. Dattagupta, B. C. Warf, and I. H. Goldberg, unpublished data). A partial structure of the chromophore (M_r 661) has recently been

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[1] Abbreviations used: NCS, neocarzinostatin; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; TBA, 2-thiobarbituric acid; DNase, deoxyribonuclease; ATP, adenosine 5′-triphosphate.

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