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Structure and developmental expression of the chick α-actin gene

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ABSTRACT

Recombinant DNA clones containing chick α-actin mRNA sequence have been isolated and used as probes to analyze the structure and developmental expression of the chick α-actin gene. The full length, 2000 nucleotide α-actin mRNA is detected in poly(A) RNA at early and late stages of in vivo leg muscle development. As expected, the α-actin mRNA is present at very low levels at early myogenic stages but is a high abundance species in terminally differentiated muscle. However, most of the α-actin mRNA from fused leg muscle is shorter than 2000 nucleotides, and occurs in relatively discrete size classes. An α-actin-like mRNA can be detected in poly(A) RNA from early embryonic brain, indicating that transcription of the α-actin gene may not be strictly muscle-specific at all stages of development.

We have identified at least 3, very short (< 100 base pairs) intervening sequences in the α-actin gene which was isolated from a chick genomic library. The structure of the chick α-actin gene differs, therefore, from the structures of actin genes from yeast and Drosophila, both of which contain a single, relatively long, intervening sequence.

INTRODUCTION

During embryonic cell differentiation, concerted programs of gene expression are involved in the elaboration of the various cellular phenotypes. In developing chick leg muscle, at least two diverse gene sets appear to be regulated according to different programs. One, consisting of genes coding for muscle-specific proteins, is transcribed at low levels during the early stages of myogenesis, but at increasingly higher levels during the terminal, fusion phase of myogenesis. Included in this set are the genes for contractile proteins (actin, myosin, etc.) and those for muscle-specific enzymes (creatine kinase, lactate dehydrogenase, etc.). A second, less well understood class of genes are regulated in "reverse-phase", compared to the muscle-specific genes, because they are transcribed at the early stages of muscle differentiation but not at the later, terminal stages. In order to study how the embryonic muscle cell regulates these two gene sets we have begun to analyze representatives from each set using recombinant DNA methods. Elsewhere (1) we have reported the molecular cloning of representatives of

reverse-phase regulated mRNA sequences from embryonic muscle. Here, we report the analysis of the structure and developmental expression of the gene for α-actin, a major muscle-specific contractile protein.

α-actin is one member of a group of actin isoproteins which are involved in cell shape and motility (2,3). In mammals, and probably other vertebrates, at least 6 different actin proteins have been identified, indicating that there are 6 different actin genes (4). The two cytoplasmic actins, β- and γ-actin, are virtually ubiquitous and are major components of the cytoskeletal system. The remaining 4 actins are muscle actins involved with muscle contractility. Each of these is specific for a different muscle type; skeletal muscle actin (α-actin), cardiac muscle actin, and a major and minor form of smooth muscle actin.

Despite this genic diversity, the different actins share extensive homology in their amino acid sequences. This extensive homology exists not only between the different actins of the same species, but also between actins from organisms of widely separated phyla (4,5). The conservation of the actin coding sequence is so strong that the DNA sequences encoding actins have been found to cross hybridize between vertebrates and nonvertebrates (6). Thus, the actin gene is not only very old, in evolutionary terms, but it is also one of the most highly conserved eucaryotic genes known.

These unusual properties of the actin genes make them interesting from several points of view. These include the nature of the constraints upon amino acid sequence and nucleotide sequence during the course of evolution and the mechanisms by which isogenes arise during phylogeny and diverge in function while strongly conserving amino acid coding regions. Finally, the mechanisms by which different tissues recognize the various isogenes and independently regulate their expression during the course of ontogeny can be explored.

METHODS AND MATERIALS

Preparation of Nucleic Acids

RNA, cDNA and chimeric plasmids were prepared and characterized as described elsewhere (1). A library of Charon 4A phage containing chick genomic DNA fragments was the generous gift of Richard Axel. Details of the construction and characterization of this library have been published (7). Screening for the actin genes was performed essentially as described by Benton and Davis (8) using ^{32}P labeled cDNA clones for actin (pα-actin 2, see Figure 1). Purification of phage DNA was performed as described elsewhere (9).

Restriction Nuclease Digestions and Blot Hybridizations

Restriction nucleases were purchased from New England Biolabs and were used according to the instructions of the supplier. RNA was blotted from agarose gels onto diazobenzyloxymethyl-paper as described previously (1), DNA was similarly blotted onto nitrocellulose paper (S&S products) by the method of Southern (10). Labeling of DNA probes, conditions of hybridization and washing of blots was conducted as described (1).

Purification and Analysis of Actin mRNA

Actin mRNA was purified from embryonic day 18 chick leg muscle total poly(A) RNA using cloned actin cDNAs affixed to nitrocellulose filters (Millipore, 13mm HAWP). Plasmid DNA was linearized by digestion with Eco RI and 10 ug adsorbed to nitrocellulose filters as described by Kafatos et al. (11). Immediately prior to hybridization, filters were incubated in hybridization buffer (5x SSC (1x SSC=150 mM NaCl, 15 mM Na citrate), 20 mM Tricine NaOH (pH 8), 0.2% sodium dodecyl sulfate (SDS), 50% deionized formamide) for 2 hr at 42°C, which was then removed and replaced with fresh hybridization buffer (800 ul) containing 100 ug muscle poly(A) RNA. The 42°C incubation was continued for 16 hr with shaking. Non-hybridized RNA was removed using the following washing schedule: (i) three 5 min washes at room temperature with 15 ml each of 2x SSC, 0.1% SDS: (ii) two 30 min washes with 5 ml each of hybridization buffer at 42°C; (iii) three further washes as in (i); (iv) three 5 min washes with 15 ml each of 0.1x SSC at 52°C; (v) one 1 min rinse in ice cold distilled water. Specifically hybridized mRNA was then eluted by boiling for 3 min in 360 ul distilled water. After adjusting the eluate to 25 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 ug/ml tRNA (Sigma), the mRNA was concentrated by ethanol precipitation and dissolved in 10 ul distilled water. Five ul of the purified mRNA was then translated in a reticulocyte lysate translation system which had been treated with micrococcal nuclease to reduce endogenous mRNA (12). The reticulocyte lysate was purchased from New England Nuclear and used according to their protocols. In vitro synthesized polypeptides were labeled by addition of ^{35}S-methionine to the translation mixtures.

Translation products were analyzed either by electrophoresis on SDS polyacrylamide (10%) gels (13) or by 2-dimensional gel electrophoresis (14). Limited proteolysis of authentic chick muscle actin (Sigma) and the labeled in vitro translation products was performed as described by Cleveland et al (15).

Electron Microscopy

R-loop mapping was conducted essentially as described elsewhere (16,17).

Briefly, DNA from plasmid pGα-actin A1 (see Figure 4) was linearized by digestion with Eco RI, extracted with chloroform and concentrated by ethanol precipitation. One ug of day 18 embryo leg muscle poly(A) RNA was mixed with 0.5 ug linearized plasmid DNA, lyophilized to dryness and redissolved in 10 ul R-loop buffer (70% deionized formamide, 100 mM Tricine-NaOH (pH 8), 500 mM NaCl, 10 mM EDTA). After incubating overnight at 52°C, the hybridization aliquots were diluted 20 fold into ice cold R-loop buffer, and cytochrome C added to a final concentration of 100 ug/ml. Specimens were spread onto a water hypophase, the DNA adsorbed onto parlodian coated grids, stained with uranyl acetate and shadowed with platinum-paladium and carbon.

RD-loop mapping was performed as described elsewhere (18). Briefly, one ug day 18 embryonic leg muscle poly(A) RNA was mixed with 0.2 ug denatured, linearized pGα-actin A1 DNA and incubated at room temperature for 30 min in 80% formamide, 200 mM NaCl, 80 mM Tris-HCl (pH 8). At the end of the incubation the RD-loops were prepared for electron microscopy as described above. Electron microscopy was conducted on either a Phillips 300 or JEOL 100B microscope.

RESULTS

Preparation and Identification of cDNA clones containing α-actin mRNA Sequence

Clones containing α-actin mRNA sequence were selected from cDNA libraries constructed using total poly(A) RNA from embryonic and adult leg muscle. Muscle-specific cDNA clones were identified by differential colony hybridization to cDNAs synthesized using muscle and brain poly(A) RNA.

Figure 1 shows a restriction map of two such muscle-specific cDNA clones, pα-actin 1 and pα-actin 2, which were subsequently found to contain α-actin mRNA sequences (see below). The size of the cDNA inserts and the position of the restriction sites were determined by restriction endonuclease digestion followed by gel electrophoresis. On the basis of cross-hybridization pα-actin 1 was shown to partially overlap the left-hand (3') portion of pα-actin 2 (data not shown).

To determine the polypeptide coded for by these two overlapping clones, their complementary mRNA was isolated by affinity hybridization. Plasmid DNA from each clone was linearized, denatured and affixed to nitrocellulose filters by the method of Kafatos et al. (11). Total poly(A) RNA from fused embryonic leg muscle was hybridized to the filter-bound plasmid DNA and the specifically hybridized mRNA was isolated by thermal elution and translated in a reticulocyte lysate cell-free translation system in the presence of ^{35}S-methionine. Figure 2A shows a fluorogram of the translation products after electrophoresis

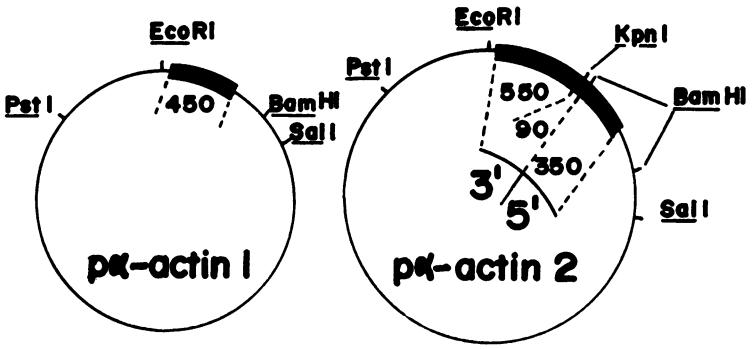

Figure 1. Restriction maps of α-actin cDNA clones. The cDNA inserts (heavy lines) reside in the Hind III site of pBR322 (19). The Hind III site was destroyed in preparation of the vector, therefore to isolate inserts, the plasmids were digested with Bam HI and Eco RI and the inserts isolated from agarose gels (20). The small numbers between the dashed lines indicate the approximate size, in base pairs, of the indicated segment. The segment pα-actin 2(3') is thereby isolated essentially free of pBR322 sequence while both the pα-actin 1 and pα-actin 2(5') inserts carry approximately 375 base pairs of pBR322 sequence with them.

on a 10% polyacrylamide gel. Lane "a" shows the products obtained in the absence of exogenous message, where the band marked "E" is a protein product of an endogenous reticulocyte mRNA. Lanes "b" and "c" show the translation products obtained using mRNA selected by pα-actin 2 and pα-actin 1 DNA, respectively. The single band marked "A" co-migrates with both purified chick muscle actin and chick muscle creatine kinase. Therefore, in order to distinguish between these two abundant muscle proteins, we subjected the translation products to 2-dimensional electrophoresis where both co-migrated with α-actin (Figure 2D). Finally, when the pα-actin 1 specified polypeptide and authentic α-actin were challenged with increasing amounts of protease from S. aureus by the procedure of Cleveland et al. (15), identical fragment patterns were obtained upon electrophoresis (Figure 2, B and C). These results confirm that the cDNA clones pα-actin 1 and pα-actin 2 contain sequence derived from actin mRNA. That these actin clones specifically contain α-actin as opposed to cytoplasmic actin sequence is established by RNA-blot hybridization (see below).

In order to determine the orientation of the α-actin mRNA contained in pα-actin 1 and pα-actin 2, total poly(A) RNA was cleaved and separated into fractions containing the 3' and 5' regions by the method of Ouellette et al (21). The insert of pα-actin 1 hybridized exclusively to the 3' fraction, while the 350 base pair insert of pα-actin 2 (designated 5' in Figure 1) hybridized both to the 3' and 5' fractions (data not shown). Thus, pα-actin 1 must be derived from a region of α-actin mRNA which is 3' to that in pα-actin 2. The extent

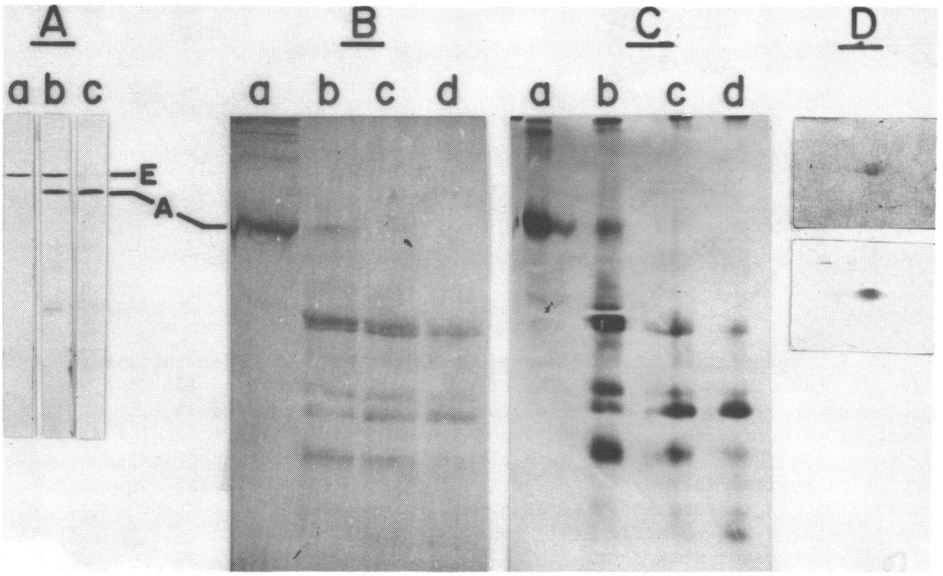

Figure 2. Identification of α-actin as the polypeptide programmed by the mRNA selected by hybridization with pα-actin 1 and pα-actin 2.
mRNA complementary to the plasmids shown in Figure 1 was isolated and translated as described in Methods and Materials.
Panel A. Products of in vitro translation displayed on a 10% polyacrylamide gel. Lane a-products obtained in the absence of exogenous message. Lane b-products obtained using mRNA selected by pα-actin 2. Lane c-products obtained from mRNA selected by pα-actin 1. the Band marked "A" co-migrates with authentic α-actin.
Panel B. α-actin was digested with Staphlococcus aureus protease as described by Cleveland et al. (15). The concentrations of protease used were: lane a-0 ug/ml; lane b-10 ug/ml; lane c-33 ug/ml; lane d-100 ug/ml.
Panel C. Translation product programmed by pα-actin 1 selected mRNA was co-digested in the same gel slots with the authentic α-actin in panel B.
Panel D. 2-D gel electrophoresis of authentic α-actin (upper panel) and the translation product programmed by pα-actin 1 (lower panel). The isoelectric focussing direction was left (basic) to right (acidic) and electrophoresis with SDS from top to bottom. Only the relevant portions of the gel are shown.

All gels were treated with NEN Enhance prior to fluorography.

of overlap between these two clones has not yet been unequivocally determined and therefore the sum of the α-actin mRNA sequence represented in pα-actin 1 and 2 is a minimum of approximately 1000 and a maximum of 1400 nucleotides out of a total of 2000 nucleotides (see below).

Developmental Expression of the α-actin Gene

When α-actin sequence probes were hybridized to RNA from muscle and non-muscle tissue blotted onto DBM paper, a distinctive hybridization pattern was observed (Figure 3). Panel A shows the hybridization obtained using labeled

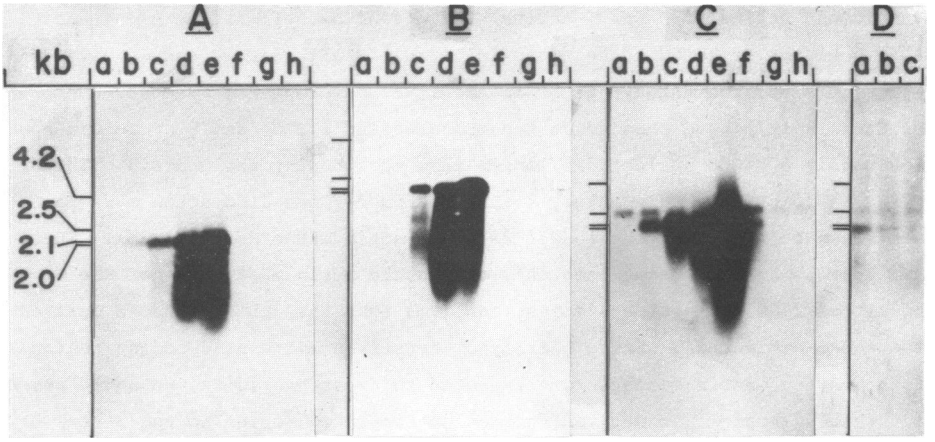

Figure 3. Hybridization of α-actin mRNA cloned segments to muscle and brain total cell poly(A) RNA.
Cloned cDNA inserts were isolated, labeled with ^{32}P by nick translation, and hybridized to RNA blots. In panels A and D, 15 ug total poly(A) RNA was loaded in each lane. In panels B and C, 5 ug total poly(A) RNA was loaded per lane. The probes used were; panel A and D-pα-actin 1; panel B and C-the 3' and 5' portions of pα-actin 2, respectively. For panels A, B and C the lane assignments are; a-stage 24 limb bud; b-embryonic day 10 (ED 10) leg muscle; c-ED 14 leg muscle; d-ED 18 leg muscle; e-adult leg muscle; f-ED 11 brain; g-ED 18 brain; h-adult brain. In panel D the lane assignments are; a-ED 11 brain; b-ED 18 brain; c-adult brain. The RNA sizes are given in kilobases with 4.2 and 2.1 kb being the size of marker chick ribosomal RNA (22).
Description of the developmental stages of chick leg muscle have been published (1, 23-26). Briefly, they are as follows; stage 24 limbs-mesodermal core of cells pluripotent for muscle and other phenotypes; ED 10 leg muscle-predominantly mononucleate myoblasts that do not accumulate muscle-specific markers; ED 14 leg muscle-a mixture of mononucleate myoblasts and fused, multinucleate myotubes which are undergoing terminal differentiation; ED 18 leg muscle-predominately composed of fused, multinucleate myotubes and myofibers.

insert from pα-actin 1. Panels B and C show the hybridization of the 3' and 5' portions of pα-actin 2, respectively (see Figure 1). All three probes hybridize to a poly(A) RNA species approximately 2000 nucleotides in length, which is present in poly(A) RNA from muscle tissue but is absent in poly(A) RNA from either brain or from limb buds prior to the appearance of muscle. Moreover, this 2000 nucleotide poly(A) RNA species accumulates during muscle development, as expected for a muscle-specific mRNA.

In addition to recognizing the 2000 nucleotide species, the inserts from pα-actin 2 also hybridize to a 2500 nucleotide species present in all RNA preparations. This second band is detected using either the 3' or 5' halves of pα-actin 2, although in Figure 3 it is best seen for the 5' half (panel C). This second band of hybridization is probably due to the mRNAs coding for the

cytoplasmic actins (β- and γ-actins) for 3 reasons. First, β- and γ-actin mRNAs would be expected to be found in virtually all tissues and we have detected this species throughout brain and liver development. Second, Hunter and Garrels (27) have shown that the mRNAs coding for β- and γ-actin are approximately 500-600 nucleotides longer than that coding for α-actin. Third, in the calf, cDNAs complementary to the coding regions of α-actin mRNA have been shown to recognize the longer β- and γ-actin mRNAs as well (28). The fact that pα-actin 1 recognizes only the muscle actin mRNA, and not the ubiquitous 2500 nucleotide species, confirms that this cDNA clone is derived from the α-actin mRNA. Since the highly conserved amino acid coding regions of the various actin messages are expected to cross hybridize, we would predict that pα-actin 1 is derived from an untranslated region at the 3' end of α-actin mRNA.

At early stages of brain development (embryonic day 11) both the 2500 nucleotide and 2000 nucleotide actin mRNA bands can be detected (Figure 3, panel C, lane f). To determine if the 2000 nucleotide band was α-actin mRNA, we hybridized blots of brain poly(A) RNA with the pα-actin 1 probe which is specific for the α-actin mRNA. Figure 3, panel D shows that in overexposed radiographs of such experiments, hybridization to a 2000 nucleotide α-actin-like mRNA can be seen in day 11 embryo brain RNA (lane a) but not in RNA from later stage brains (lanes b and c). This indicates, therefore, that the α-actin gene may be transcribed at low levels in early stages of brain development.

Size Heterogeneity of α-actin mRNA

The α-actin mRNA is heterogeneous with respect to size, and the degree of heterogeneity increases in terminally differentiated muscle. As illustrated in panels A, B and C of Figure 3, a smear of hybridizing RNA below the 2000 nucleotide band, primarily in day 18 embryo and adult leg muscle RNA (lanes d and e) is routinely observed. In contrast, almost no size heterogeneity is seen in poly(A) RNA from day 10 embryo leg muscle (lane b) and relatively little is seen in day 14 embryo leg muscle RNA (lane c).

Several considerations suggest that this heterogeneity reflects the *in vivo* status of the α-actin mRNA in leg muscle and not an artifact. First, in a large number of experiments, similar heterogeneity is seen for the α-actin mRNA from terminally differentiating leg muscle and not for other tissues; second, probes for other mRNAs hybridized to these same blots do not show evidence of degradation of those mRNAs in the embryonic day 18 or adult leg muscle lanes; third, at low exposure levels, discrete hybridization bands are noted, indicating that the lower molecular weight forms of α-actin mRNA occur

as relatively discrete size classes. This latter point is clearly illustrated in Panel B, lane c of Figure 3 where two bands of hybridization are seen at approximately 1400 and 1100 nucleotides.

Isolation and Characterization of a Cloned Genomic DNA Segment Containing the Chick α-actin Gene.

In order to study the structure of the α-actin gene a chick genomic library was screened by the procedure of Benton and Davis (8). Since ultimately it is of interest to compare the structures of all the different actin genes, we performed the initial screening with p α-actin 2 which recognizes non-muscle actin sequences as well as the α-actin sequence. Twelve independent clones were isolated using this probe, many of which were subsequently found to be derived from overlapping segments of genomic DNA. Of these 12, three hybridized with the muscle-specific actin cDNA clone pα-actin 1. A restriction endonuclease map for one of these clones (λGα-actin A) revealed no recognition site for Eco RI within several thousand bases around the α-actin gene (Figure 4). Southern blot hybridization of restriction nuclease digests of this genomic DNA segment with pα-actin 1 and 2 as probes indicated that the actin coding region was contained within an internal 6.2 kilobase Hind III fragment (Figure 4B). This fragment was isolated and subcloned into the Hind III site of pBR-322 (subclone designation pGα-actin A1) to permit more detailed analysis of the α-actin gene.

The location and orientation of the α-actin gene within the pGα-actin A1 subclone was determined by Southern blot hybridization using the 3 α-actin cDNA insert segments shown in Figure 1. In Figure 5A, a blot of pGα-actin A1 was hybridized with pα-actin 1 (lane b), pα-actin 2(3') (lane c), and pα-actin 2(5') (lane d). Both pα-actin 1 and pα-actin 2(3') can be seen to hybridize to the 2.7 kb fragment which lies to the left of the Bam HI site as drawn in Figure 4B, while pα-actin 2(5') hybridizes to the right hand 3.5 kb fragment. In panel B a similar experiment is shown in which pGα-actin A1 DNA was digested with Hind III and Xho I. As expected, pα-actin 1 hybridizes to the left hand 2.5 kb fragment (lane b) while pα-actin 2(5') hybridizes to the right hand 3.7 kb fragment. On the other hand, pα-actin 2(3') hybridizes both to the 2.5 and 3.7 kb fragments indicating that the Xho I site lies within the region which encodes the α-actin mRNA segment corresponding to pα-actin 2(3'). Since there is no Xho I site in pα-actin 2(3') we conclude that this site in pGα-actin A1 lies within an intervening sequence which is spliced out of the mature α-actin mRNA.

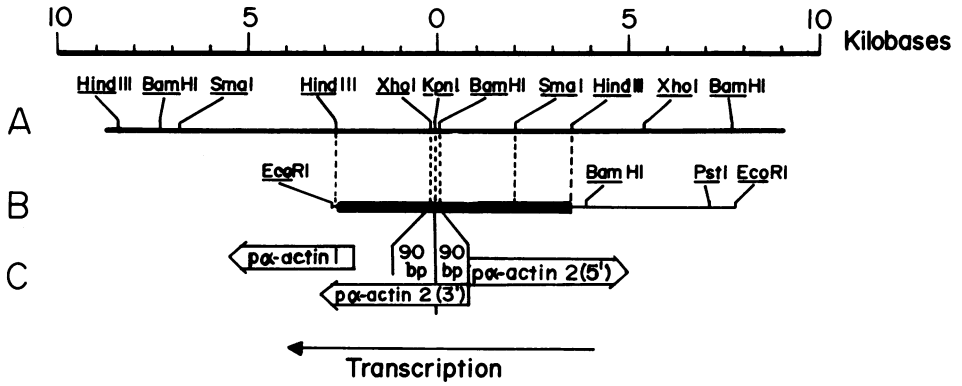

Figure 4. Restriction endonuclease map of chick genomic DNA segment containing the α-actin gene.
A-Position of various 6-base recognition restriction sites in λGα-actin A.
B-Structure of the subclone, pGα-actin A1, containing the internal 6.2kb fragment which contains the α-actin gene. Thick line, 6.2 kb insert; thin line, pBR322 DNA vehicle. Plasmid is shown linearized by Eco RI digestion.
C-Summary of results of Southern blot hybridization experiment from Figure 5. The regions homologous to the different cDNA clone segments are shown relative to the Xho I, Kpn I and Bam HI sites. This drawing is not in the same scale as shown at the top of the figure.

Electron Microscopic Mapping of the α-Actin Gene

In order to gain a more comprehensive picture of the organization of the α-actin gene we used electron microscopy to visualize hybrids between it and α-actin mRNA. pGα-actin A1 DNA was linearized by Eco RI digestion (Figure 4B) and then incubated with day 18 embryo leg muscle poly(A) RNA under R-loop hybridization conditions (16,17). Figure 6 (A and B) shows two representative R-loop structures. Each contains an RNA-DNA hybrid which formed several short points of contact with the displaced single-stranded DNA. Measurements of 20 representative R-loop structures (Figure 7) revealed an average RNA-DNA hybrid length of approximately 1650 nucleotides. The points of contact between the RNA-DNA hybrid and the displaced single-strand DNA were found to be non-random and the position of these are plotted in Figure 7. These points of contact indicate the presence of short intervening sequences in the α-actin gene.

To more clearly establish the identity and position of these short intervening sequences we formed RD-loops (18) between α-actin mRNA and the denatured α-actin gene. In this type of experiment the presence of intervening sequences would be seen as regions of DNA not in base pair register with RNA. In Figure 6 (C and D) two representative RD-loop structures are shown, where

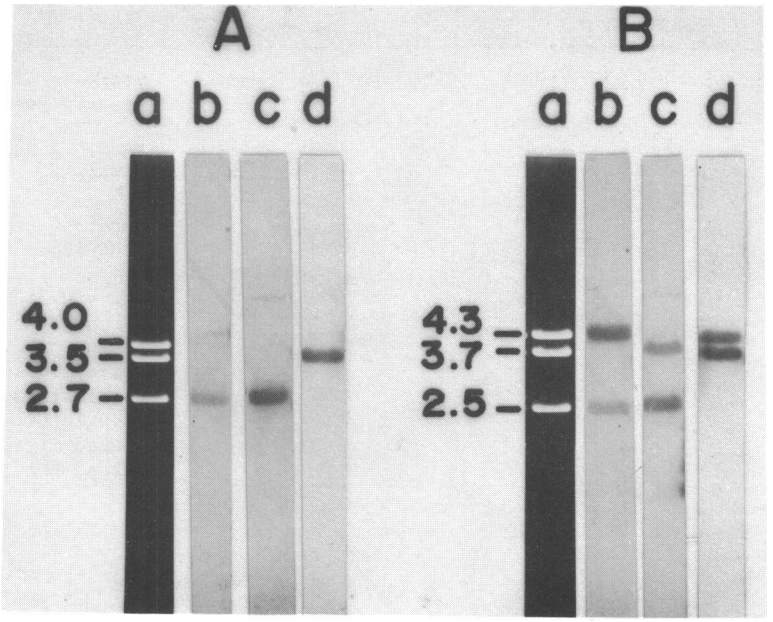

Figure 5. Southern blot hybridization of pGα-actin A1 to determine the position of the regions homologous to pα-actin 1 and 2.
pGα-actin A1 DNA was digested with restriction nucleases, electrophoresed, blotted and hybridized with α-actin cDNA probes labeled with ^{32}P by nick translation.
Panel A-pGα-actin A1 DNA digested with Bam HI and Hind III.
Panel B-pGα-actin A1 DNA digested with Xho I and Hind III.
Lane assignments for both panels are; lane a-ethidium bromide stain of fragments; lanes b, c and d-the blotted DNA is hybridized to ^{32}P pα-actin 1, pα-actin 2(3') and pα-actin 2(5'), respectively.

the thicker RNA-DNA hybrid is distinguishable from the thinner single-strand DNA regions, and the short intervening sequences are seen as small "knobs" at various points along the RNA-DNA hybrid. The position of the intervening sequences (Figure 7) corresponds well with those determined by R-loop mapping.

The results obtained from the electron microscopic mapping of the α-actin gene suggest that we have not visualized the entire gene because the RNA-DNA hybrid lengths are shorter than expected for the length of the intact message. Assuming a poly(A) tail of 200 nucleotides, the full length mRNA should contain at least 1800 nucleotides of transcribed sequence. This result is not surprising since the data in Figures 3 and 4 demonstrate that the majority of α-actin mRNA purified from fused embryonic muscle by oligo(dT) cellulose chromatography is shorter than full length message. Because these messages are selected by their 3' poly(A) tracts, we assume that the region

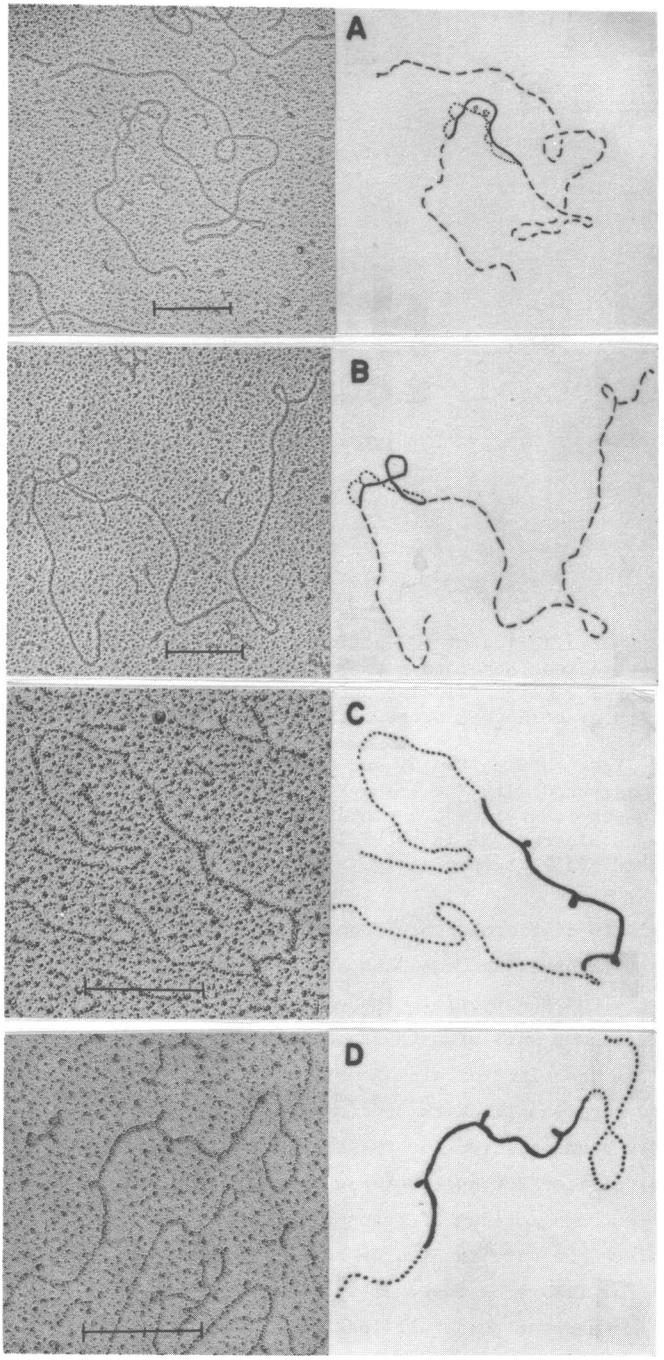

Figure 6.

R-loop and RD-loop analysis of the α-actin gene.

Panels A and B:
R-loops

Panels C and D:
RD-loops

Bar = 0.25 um

In the tracings; solid lines=RNA-DNA hybrids; dotted lines =single-strand DNA: dashed lines-double-strand DNA

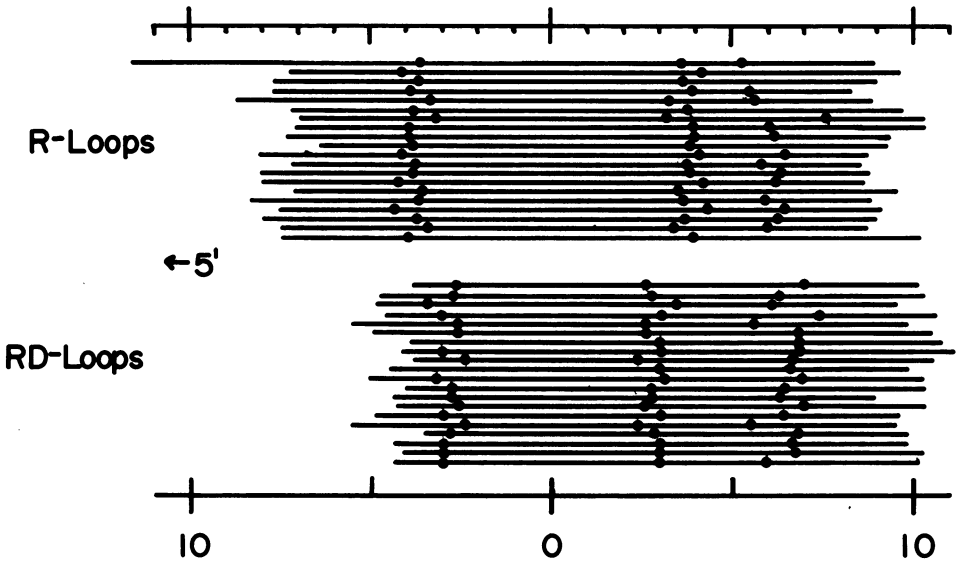

Figure 7. Lengths of R-Loops and RD-Loops and Intervening Sequence Positions. R-loops and RD-loops were measured for total length and the position of intervening sequences noted. Dots show the position of single-strand DNA contacts with RNA-DNA hybrids in the R-loops or the position of knobs in RD-loop structures. The axis for comparison of intervening sequence positions was arbitrarily placed in the center of the largest RNA-DNA hybrid segment. The scale is in kilobases.

of the α-actin gene we are failing to visualize by microscopy is its extreme 5' end.

DISCUSSION

We have isolated and analyzed recombinant DNA clones containing the chick α-actin mRNA sequence and chick genomic DNA segments containing the α-actin gene. It is anticipated that the cloned actin genes will be useful for the study of the mode of differential expression of the actin gene family during the course of embryonic development.

The actin gene family consists of a number of genes which encode proteins whose amino acid coding sequence is strongly conserved within the same species, as well as between organisms as widely separated in evolution as yeast and mammals (4,5,6). In the chick, a portion of the α-actin cDNA clone pα-actin 2 hybridized to at least two mRNA bands, one approximately 2000 nucleotides in muscle RNA lanes, and a second at approximately 2500 nucleotides found in RNA from every tissue tested. A similar hybridization pattern for rat actin cDNA has recently been reported by Katcoff et al (28). In

contrast, a 3' specific region of α-actin mRNA, encoded in pα-actin 1, is clearly specific for α-actin mRNA (Figure 3). Similar results have been reported by Cleveland et al. (6), who recently showed that the β- and γ-actin mRNAs in chick contain regions near their 5' ends which are specific for each of those messages and do not cross hybridize. It is possible that α-actin mRNA contains a specific 5' region as well but we have not determined this as yet because we do not have probes for this region. It remains to be determined whether or not these distal regions are translated. The possible functions of untranslated, message-specific regions between mRNAs which code for highly conserved proteins is an intriguing question, especially if they are involved in the recognition and therefore the regulation of the different actin isogenes and their transcripts.

There is considerable heterogeneity in the size of the α-actin mRNA from terminally differentiated muscle (Figure 3A,B,C; lanes d and e). This α-actin mRNA heterogeneity seems to be specific for fused embryonic myotubes and adult muscle fibers because it is seen to a much lesser extent in muscle at early stages of development (Figure 3A,B,C; lanes b and c). Moreover, it is not seen for other messages analyzed on the same RNA blots. The expected minimum length of the amino acid coding region for actin is approximately 1100 nucleotides, or just over 50% of the total length of the mRNA, and implies that there must be 700-800 nucleotides of 5' and 3' untranslated RNA. It is possible, therefore, that the α-actin mRNA size reduction could result from loss of untranslated regions. It is also possible that the shorter α-actin mRNAs derive from multiple α-actin genes. We do not favor this latter hypothesis because the α-actin specific probe, pα-actin 1, which hybridizes to the shorter α-actin mRNAs (Figure 3A) only recognizes a single restriction fragment in chicken genomic DNA (data not shown).

Finally, the α-actin mRNA may not be exclusively muscle-specific. Using α-actin mRNA-specific cDNA clone, pα-actin 1 (Figure 3A) we can detect hybridization to a similar sized mRNA present at early stages of brain development (Figure 3D). This finding was somewhat unexpected because α-actin is generally considered to be a muscle-specific protein. We cannot, as yet, rule out the possibility that this hybridization is due to a similar sized mRNA in brain which shares partial homology with the 3' muscle-specific region of the α-actin mRNA. The transient transcription of histo-specific genes in inappropriate tissue types at very early stages of development is consistent with an irreversible gene repression model for gene regulation of embryonic development (29). On the other hand, α-actin may simply be a normal low

level constituent of many non-muscle cells because Rubenstein and Spudich (30) have reported that the α-actin polypeptide is a normal constituent of some non-muscle cells such as fibroblasts.

Our preliminary analysis of the organization of the chick α-actin gene (Figures 4-7) shows that it is remarkable in one major respect; i.e., that the 3 intervening sequences identified so far all appear to be very short (<100 base pairs). There may be more than 3 intervening sequences which are too short to visualize by R-loop or RD-loop mapping. In addition, because we probably have not visualized the extreme 5' end of the gene, either by hybridization analysis or by electron microscopy, there may be additional intervening sequences in this region. Our failure to obtain cDNA clones for the 5' end of the α-actin mRNA or to visualize it by electron microscopy, is due to the fact that only a small minority of the actin mRNA isolated by oligo(dT) cellulose chromatography are full length (see Figure 3).

A relatively small number of actin genes have been mapped to date and these differ considerably from the one described in this report. Both the Drosophila and the yeast actin genes contain a single intervening sequence (1.65 and 0.3 kb, respectively) (31,5). Interestingly, in both these cases, the single intervening sequence lies close to the 5' end of the gene. Dictyostelium contains multiple actin genes, none of which has yet been shown to contain an intervening sequence (32,33). Thus, while the coding sequences are highly conserved, the organization of the coding segments in the genome is very different. This is in marked contrast to the α- and β-globin genes, whose coding sequences have diverged at the same time as the organization of the 3 coding blocks in each gene has remained constant (7,34,35). Although it is possible that the chick α-actin gene does not share a common ancestor with the actin genes of yeast and Drosophila it is also clear that intervening sequences can be deleted during the course of evolution. Deletion of intervening sequences has been observed in a rat pre-proinsulin gene (36) and in an α-globin-like gene in the mouse (37). It will be interesting to compare the structure of the chick α-actin gene to its isogenes in the chick, and to actin genes from related vertebrate phyla to determine the pattern of phylogenetic change in the coding and intervening sequence regions. Studies towards this end are currently underway.

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