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α-Cardiac Actin Is the Major Sarcomeric Isoform Expressed in Embryonic Avian Skeletal Muscle

Abstract. *A primer extension assay that is diagnostic for the messenger RNA's (mRNA's) transcribed from the β-cytoplasmic, α-cardiac, and α-skeletal actin genes of the chicken was used to measure the mRNA levels for these actin isoforms. Measurements were made in chicken breast muscle during myogenesis in vivo and in vitro. α-Cardiac actin mRNA accounts for more than 90 percent of the sarcomeric actin transcripts expressed in avian embryonic breast muscle. Five weeks after hatching, α-skeletal actin mRNA is the only detectable sarcomeric actin transcript.*

Analysis of the various actin isoforms poses a problem since there is little divergence in their protein sequences (*1*). The pioneering work of Vandekerckhove and Weber (*1, 2*) has shown that there are at least six actin isoforms in adult mammalian tissues: two sarcomeric forms (α-cardiac and α-skeletal muscle actin), two smooth muscle forms (α- and γ-smooth muscle actin), and two cytoplasmic forms (β- and γ-cytoplasmic nonmuscle actin). The conservation in amino acid sequence is striking in that the α-skeletal muscle isoform differs in 4 of 375 amino acids from α-cardiac actin, 6 of 375 amino acids from the γ-smooth isoform, 8 of 375 amino acids from the α-smooth isoform, and 24 or 25 of 375 amino acids from the β- and γ-cytoplasmic actins. In order to avoid possible problems due to homology between the various isoforms, a primer extension assay was used in place of RNA dot blots or Northern blots (*3*) to determine the level of expression of the different actin genes during myogenesis. This assumes that each actin gene initiates transcription at a point that yields a primer-extended fragment of unique length for each gene.

The sequence of the 5′ coding exon for each of the actin genes used in this study is given in Fig. 1. Both the sarcomeric actins encode the amino terminal methionine-cysteine dipeptide which is not present in the β-cytoplasmic actin gene. However, the cysteine residue is absent from the processed sarcomeric actin (*1, 2*). The function of this cysteine residue is not clear, but similar results have been reported for other vertebrate sarcomeric actin genes (*4–7*). The encoded amino acid sequence for the amino terminus of each actin gene agrees exactly with the diagnostic sequence characteristic for each isoform (*1, 2*). The primer sequences used in this study are underlined in Fig. 1.

The lengths of the extended primers (Fig. 2A) indicate that the β-cytoplasmic, α-cardiac, and α-skeletal actin messenger RNA's (mRNA's) have 5′ noncoding leader sequences of approximately 90, 60, and 75 base pairs (bp), respectively. Our results agree with the reported leader length for α-skeletal actin mRNA (*4*).

The β-actin isoform represents more than 90 percent of the total actin transcripts measured in dividing myoblasts 36 hours after plating (lane a in Fig. 2A and Table 1). The low level of expression of the α-cardiac isoform in 36-hour cultures (lane b in Fig. 2A) represents the small percentage of myoblasts (5 to 10 percent) that have undergone fusion (*8, 9*) and are expressing the differentiation-specific cardiac isoform. No α-skeletal actin transcripts are detectable in the prefusion cultures with this exposure time. After most of the cells have fused (approximately 70 to 80 percent), there is a marked reduction in the expression of β-actin mRNA (lane d in Fig. 2A and Table 1) and a concomitant increase in the expression of the α-cardiac (lane e in Fig. 2A and Table 1) and α-skeletal actin transcripts (lane 1 in Fig. 2B and Table 1). However, in the latter case, α-skeletal actin represents less than 5 percent of the sarcomeric actin transcripts expressed in differentiated cultures.

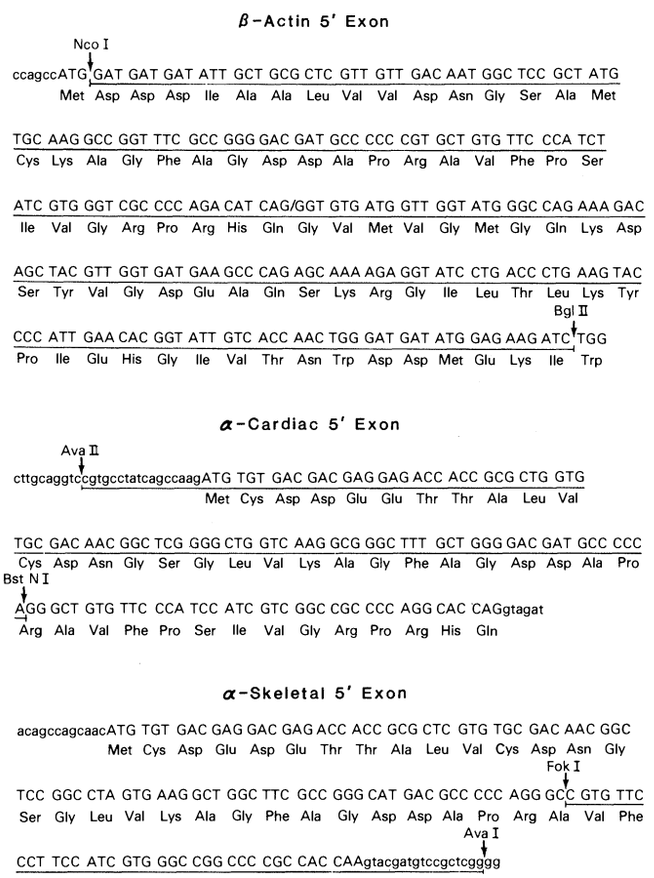

Fig. 1. The nucleotide sequence of the 5′ coding exon for the β-actin, α-cardiac actin, and α-skeletal actin genes in the chicken. The primer fragments are underlined in the sequence, the 3′ end of the primer being on the 5′ side of the depicted sequence. The lengths of the primers are as follows: β-actin, 252 bp; α-cardiac actin, 105 bp; and α-skeletal actin, 54 bp. The β-actin primer was prepared from the full size β-actin cDNA clone, pA-1, constructed by Cleveland *et al.* (*12*); the genomic sequence data obtained in our laboratory were used to select the restriction enzyme sites. The sarcomeric actin primers were cut from the appropriate subclones of the complete genomic fragments prepared as described earlier (*11*). Lower-case letters indicate flanking or intron sequence positions. The slash in the β-actin sequence is the position of the first intron within the coding portion of the genomic sequence. The recognition site for Fok I is 13 bp to the 5′ side of the cut site. The sequence data reported here for the α-skeletal actin agree with those reported previously (*4*).

Table 1. Relative expression of the actin isoform mRNA's in different chicken muscle tissues. Relative actin isoform transcript levels were measured using 10 μg of total RNA. Specific bands were scanned with a soft laser and the area under each peak was determined by weight. Data were taken from Fig. 2. Numbers represent arbitrary units of peak area. N.D., not detectable.

Isoform	36-hour myoblasts (unfused cultures)	92-hour myofibrils (fused cultures)	Cardiac muscle from 1-day-old chicks	15-day embryonic breast muscle	Breast muscle from 5-week-old chicks	15-day embryonic brain
β-actin	38.0	10.9	11.2	12.7	1.0	45.2
α-Cardiac	1.5	12.6	19.2	14.10	N.D.	N.D.
α-Skeletal	N.D.	0.8	3.0	1.0	62.1	N.D.

Cardiac muscle from 1-day-old chicks gives a picture very similar to the differentiated muscle tissue culture cells. Low levels of β-actin mRNA are being transcribed, but the major actin transcript represents the α-cardiac actin isoform (lane h in Fig. 2A and Table 1). Although low levels of the α-skeletal actin mRNA are observed, they represent less than 5 percent of the α-cardiac mRNA level (lane i in Fig. 2A and Table 1).

Fifteen-day embryonic breast muscle gives the same pattern as seen in the differentiated cultures (lanes d to f versus lanes j to l in Fig. 2A), with α-cardiac actin mRNA representing the major sarcomeric isoform transcript expressed in developing breast muscle. Low levels of α-skeletal actin mRNA are also seen (lane 3 in Fig. 2B and Table 1).

In the young adult chicken, 5 weeks after hatching, the mRNA transcript pattern in breast muscle is that expected for the adult skeletal muscle tissue (lanes m to o in Fig. 2A and Table 1). The α-skeletal actin mRNA is the only detectable sarcomeric species (lanes 5 and 6 in Fig. 2B). Extremely low levels of the β-actin transcript are still expressed in the young adult muscle (lane m in Fig. 2A). This level of expression represents less than 1 percent of the actin transcripts measured in this assay (Table 1).

There have been reports that chick embryonic brain expresses the α-skeletal actin mRNA (*10*). However, only β-cytoplasmic actin mRNA could be detected by primer extension with embryonic brain RNA (lanes p to r in Fig. 2A and Table 1).

Our results establish that the mRNA for both sarcomeric actin isoforms is transcribed in cultured 12-day embryonic breast muscle and in 15-day embryonic breast muscle; however, the predominant sarcomeric actin mRNA transcript represents the α-cardiac isoform. Actin isoform expression in embryonic mouse muscle suggests a different picture. Using RNA dot-blot and Northern blot analysis with a putative α-cardiac 3′ complementary DNA (cDNA) clone and an α-skeletal actin 3′ cDNA clone, Minty *et al.* (*3*) found that 17- to 20-day-old fetal mouse skeletal muscle expresses 60 to 70 percent α-skeletal actin and 30 to 40 percent fetal actin mRNA (presumed by these authors to be the cardiac isoform). In cultures of embryonic mouse muscle the two sarcomeric actins are expressed to equivalent extents. Although our results are in general agreement with those of Minty *et al.* (*3*), the method of detection may account for the numerical differences. Low levels of expression and

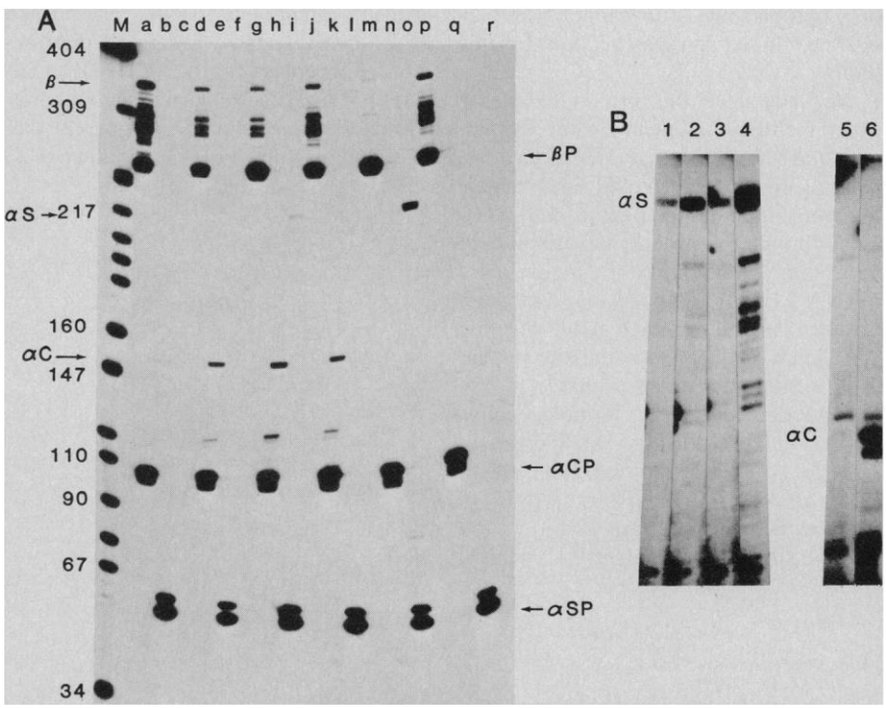

Fig. 2. Primer extension analysis of the actin isoform mRNA's during development in vitro and in vivo. End-labeled (*14*) double-stranded primer (approximately 5000 count/min) (Fig. 1) was mixed with 10 μg of total RNA (*13*), precipitated with ethanol, and suspended in 10 to 20 μl of 80 percent formamide, 40 m*M* PIPES (*p*H 6.4), 1 m*M* EDTA, and 400 m*M* NaCl. The samples were heated to 68°C for 10 minutes and then annealed for 12 to 16 hours at 50°C. The hybrids were diluted fivefold with stop buffer to give a final salt concentration of 300 m*M* sodium acetate (*p*H 5.2), 10 m*M* $MgCl_2$, and 10 μg of calf liver transfer RNA per reaction. The samples were washed with ethanol, dried, and suspended in 19 μl of reverse transcriptase buffer containing 50 m*M* tris (*p*H 8.3), 50 m*M* KCl, 6 m*M* $MgCl_2$, and 2 m*M* dithiothreitol. The reaction was started with the addition of reverse transcriptase, 12 to 15 units per microliter, and incubated at 41°C for 1 hour. The DNA was ethanol precipitated after a brief treatment with 0.025 m*M* EDTA and 0.2*N* NaOH for 15 minutes at 42°C to cleave the RNA in the reaction. Samples were loaded on a buffer gradient gel containing 6 percent polyacrylamide and 7*M* urea (*15*). (A) The positions of the primer-extended fragments specific for α-cardiac actin (α*C*) (approximately 149 base pairs), α-skeletal actin (α*S*) (approximately 238 bp), and β-cytoplasmic actin (β) (approximately 347 bp) are shown; *P* designates the primer location. The additional bands between the primer and the end point of the extended primer are thought to be reverse transcriptase pause points as described by Ghosh *et al.* (*16*). *M* indicates the Hpa II–cut pBR322 markers. Sources of RNA: (lanes a to c) 36-hour prefusion myoblast cultures; (lanes d to f) 92-hour fused myofibril cultures; (lanes g to i) cardiac tissue from 1-day-old chicks; (lanes j to l) 15-day chick embryonic breast muscle; (lanes m to o) breast muscle from 5-week-old chicks; and (lanes p to r) 15-day chick embryonic brain tissue. Exposure, 24 hours. (B) Longer exposure of the film shown in (A) to demonstrate the detection of the α-skeletal actin mRNA transcripts in embryonic muscle, in vitro and in vivo: (lane 1) 92-hour fused myofibril cultures; (lane 2) cardiac muscle from 1-day-old chicks; (lane 3) 15-day embryonic breast muscle; (lane 4) breast muscle from 5-week-old (adult) chicks; (lane 5) breast muscle from 5-week-old chicks; and (lane 6) cardiac muscle from 1-day-old chicks. α-Cardiac actin mRNA transcripts are not seen in breast muscle from 5-week-old (adult) chicks. Exposure, 6 days.

unambiguous identification of each isoform may not be determined as precisely by hybridization methods as by the primer extension assay. Alternatively, this discrepancy may reflect differences in the avian and murine muscle tissues analyzed. Chicken breast muscle is a pure fast muscle (*8*), whereas the fetal mouse tissue is a mixture of slow and fast muscle types (*3*).

In contrast to Ordahl *et al.* (*10*), we find no evidence for the expression of the muscle-specific sarcomeric actin isoforms in embryonic chick brain. In the absence of sequence data on the α-actin probe used by these authors, the presence of coding regions homologous to β- or γ-cytoplasmic actin cannot be rigorously excluded and may account for this finding.

We have used this primer extension assay technique to measure the expression and regulation of the chicken actin gene isoforms transfected into mouse myogenic and nonmyogenic cells (*11*). Even though the primers described here have some homology to the mouse actin mRNA's, the length of the extended primer is unique for each gene; thus one can simultaneously measure the regulation and expression of both the mouse and chicken actin genes in mouse cells.

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1,25-Dihydroxyvitamin D_3: A Novel Immunoregulatory Hormone

Abstract. *The hormonal form of vitamin D_3, 1,25-dihydroxyvitamin D_3 [1,25(OH)$_2$D$_3$], at picomolar concentrations, inhibited the growth-promoting lymphokine interleukin-2, which is produced by human T lymphocytes activated in vitro by the mitogen phytohemagglutinin. Other metabolites of vitamin D_3 were less effective than 1,25(OH)$_2$D$_3$ in suppressing interleukin-2; their order of potency corresponded to their respective affinity for the 1,25(OH)$_2$D$_3$ receptor, suggesting that the effect on interleukin-2 was mediated by this specific receptor. The proliferation of mitogen-activated lymphocytes was also inhibited by 1,25(OH)$_2$D$_3$. This effect of the hormone became more pronounced at later stages of the culture. These findings demonstrate that 1,25(OH)$_2$D$_3$ is an immunoregulatory hormone.*

The importance of 1,25-dihydroxyvitamin D_3 [1,25(OH)$_2$D$_3$] in mineral and skeletal metabolism is well established (*1*). However, the widespread distribution of receptors for 1,25(OH)$_2$D$_3$ in tissues not regarded to participate in mineral metabolism has made it apparent that 1,25(OH)$_2$D$_3$ plays a wider biologic role than was previously thought (*2*). We and others have found that 1,25(OH)$_2$D$_3$ receptors are present in normal human monocytes and malignant lymphocytes but are absent from resting T and B lymphocytes (*3*). Nevertheless, T and B lymphocytes obtained from normal human subjects express the 1,25(OH)$_2$D$_3$ receptor when the cells are activated in vitro by mitogenic lectins and Epstein-Barr virus, respectively (*3*). The mitogenic lectin phytohemagglutinin (PHA) stimulates T lymphocyte proliferation (*4*) and induces the production of various lymphokines, including interleukin-2 (IL-2), which is important for the growth of T cells (*5*). In the present investigation we report that 1,25(OH)$_2$D$_3$ suppresses IL-2 and inhibits the proliferation of PHA-stimulated lymphocytes.

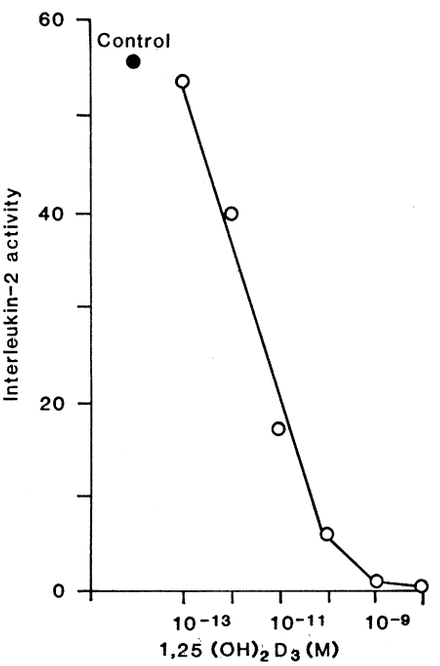

Fig. 1. Effect of 1,25(OH)$_2$D$_3$ on IL-2. Human peripheral blood mononuclear cells (1×10^6), purified by Ficoll-Hypaque gradient, were cultured with various concentrations of 1,25(OH)$_2$D$_3$ in 1 ml of medium RPMI 1640 supplemented with PHA (1 percent), fetal bovine serum (2 percent), 2-mercaptoethanol (5.6×10^{-5} *M*), indomethacin (1 μg/ml), Hepes buffer (10 m*M*), penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 m*M*), sodium pyruvate (1 m*M*), and ethyl alcohol (1 percent). Control cultures were prepared similarly, but in the absence of 1,25(OH)$_2$D$_3$. After 2 days of culture at 37°C in a humidified atmosphere of 5 percent CO_2, the media were assayed for IL-2 content with the CTLL-2 murine cell line as described (*5*). Interleukin-2 activity is expressed as the inverse of the medium dilution giving 50 percent of maximal control culture growth.

Peripheral mononuclear leukocytes were isolated by Ficoll-Hypaque gradients from blood samples obtained from normal adults. The cells were cultured for 2 days in medium containing PHA (1 percent) alone or in the presence of increasing concentrations of 1,25(OH)$_2$D$_3$ (10^{-13} *M* to 10^{-8} *M*). The IL-2 content of the media was determined by means of a bioassay that involves titration of the media on the proliferation of the murine cell line CTLL-2; proliferation of the CTLL-2 cells depends strictly on the presence of IL-2 (*5*). Using this assay method, we found that the media from activated lymphocytes grown in the presence of 1,25(OH)$_2$D$_3$ for 2 days showed reduced IL-2 activity. This effect was dependent on the concentration of 1,25(OH)$_2$D$_3$. Fifty percent reduction of IL-2 activity in the medium, in comparison with activity in media from control cultures grown without 1,25(OH)$_2$D$_3$, occurred at 2×10^{-12} *M* 1,25(OH)$_2$D$_3$ (Fig. 1). This effect was reproduced in four additional experiments with blood cells from different normal individuals. The concentration of 1,25(OH)$_2$D$_3$ causing 50 percent reduction in IL-2 activity in the four experiments was 4×10^{-12} *M*