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Cloning and Characterization of Two Human Skeletal Muscle α-Actinin Genes Located on Chromosomes 1 and 11*

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Conserved sequences of dystrophin, β-spectrin, and α-actinin were used to plan a set of degenerate oligonucleotide primers with which we amplified a portion of a human α-actinin gene transcript. Using this short clone as a probe, we isolated and characterized full-length cDNA clones for two human α-actinin genes (ACTN2 and ACTN3). These genes encode proteins that are structurally similar to known α-actinins with ~80% amino acid identity to each other and to the previously characterized human nonmuscle gene. ACTN2 is the human homolog of a previously characterized chicken gene while ACTN3 represents a novel gene product. Northern blot analysis demonstrated that ACTN2 is expressed in both skeletal and cardiac muscle, but ACTN3 expression is limited to skeletal muscle. As with other muscle-specific isoforms, the EF-hand domains in ACTN2 and ACTN3 are predicted to be incapable of binding calcium, suggesting that actin binding is not calcium sensitive. ACTN2 was mapped to human chromosome 1q42-q43 and ACTN3 to 11q13-q14 by somatic cell hybrid panels and fluorescent *in situ* hybridization. These results demonstrate that some of the isoform diversity of α-actinins is the result of transcription from different genetic loci.

The spectrin gene superfamily encodes a diverse group of cytoskeletal proteins including the α- and β-spectrins, α-actinins, dystrophin, and a recently identified protein called DRP (reviewed in Refs. 1–4). Family members are characterized by a central rod domain composed of 4 (α-actinin) to 24 (dystrophin) repeated units (5). Other common features include an EF-hand calcium-binding domain that may be functional, as in nonmuscle α-actinins and the α-spectrins, or degenerate as in the muscle α-actinins and dystrophin (6–9). There is also a highly conserved, amino-terminal actin-binding domain in β-spectrin, α-actinin, and dystrophin (10, 11), and a carboxyl-terminal domain of unknown function that is apparently unique to dystrophin and DRP (9, 12).

Located on the inner surface of the plasma membrane, the spectrins and dystrophin are thought to play a role in maintaining cellular integrity and flexibility as components of the membrane cytoskeleton (2, 13). On the other hand, the α-actinins have somewhat more diverse cellular functions reflected by their different subcellular localizations (reviewed in Ref. 3). A major function of the antiparallel α-actinin dimers is their actin filament cross-linking activity, and in nonmuscle cells, "cytoskeletal" α-actinin is found along microfilament bundles where it may mediate membrane attachment at adherens-type junctions in a dynamic manner, regulated by calcium binding at the EF-hands (14–17). In contrast, the calcium-insensitive muscle forms all function to anchor actin filaments in a constitutive manner. In skeletal and cardiac muscle they are a major component of the Z discs, while in smooth muscle, α-actinin is found at dense bodies and dense plaques that have a similar anchoring function (15, 18, 19).

Biochemical studies have identified a number of different α-actinin isoforms in various tissues and species, but without molecular characterization of their genes it has been difficult to determine their relationships to each other (15, 18, 20–24). Isoelectric focusing has resolved up to eight different variants in chicken gizzard and pectoralis muscle (15) but these same eight variants were seen in both muscle types, albeit in different ratios. Presently, it is not clear whether these isoforms result from posttranslational modifications, alternative mRNA splicing, transcription from multiple genes, or some combination of the above. On the other hand, amino acid analysis and peptide mapping of rabbit skeletal muscle α-actinins has revealed three isoforms, two in fast-twitch fibers and another in slow-twitch fibers (22). Schachat *et al.* (23) have identified two rabbit fast-twitch fiber forms although their correspondence with the forms of Kobayashi (22) is unclear, and cyanogen bromide peptide mapping suggests that one of these is the same as the slow muscle α-actinin. Finally, an antibody raised against a dystrophin fusion peptide has been shown to cross-react with α-actinin only in fast-twitch glycolytic skeletal muscle myofibers of the mouse (25).

Invertebrate α-actinin genes have been characterized in *Dictyostelium discoideum*, *Caenorhabditis elegans*, and *Drosophila melanogaster* (6, 26, 27). In vertebrates, two chicken genes, one encoding a skeletal muscle isoform and one encoding the smooth and nonmuscle isoforms, have been identified (7, 8, 28). Alternative splicing of an exon containing EF-hands of differing calcium sensitivity is responsible for the differences between smooth muscle and nonmuscle forms, however, generation of skeletal muscle isoform diversity is as yet unexplained. In humans, only one gene has been identified, that of the nonmuscle cytoskeletal isoform (ACTN1) which maps to chromosome 14q22–24 (29, 30).

Here we report the cloning and characterization of cDNAs from two genes for human skeletal muscle α-actinins. These genes were identified following polymerase chain reaction

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(PCR)[1] with oligonucleotide primers designed to amplify conserved sequences within the actin-binding domains of β-spectrin, dystrophin, and α-actinin. We are utilizing this approach to identify uncharacterized members of the spectrin superfamily on the assumption that such genes may play a role in human disease. One of the α-actinin genes appears to be a homolog of the previously sequenced chicken skeletal muscle-specific gene while the second represents a previously unidentified gene product.

MATERIALS AND METHODS

PCR and Library Construction—Oligonucleotide primers were synthesized on an Applied Biosystems 380B DNA synthesizer (Foster City, CA) and purified on polyacrylamide gels as described (31) or on Oligonucleotide Purification Cartridges (ABI, Foster City, CA). The forward degenerate primer contained an *Sfi*I site and was 5′-GGG GGC CAA GTC GGC CTC AAR ACN TTY ACN RMN TGG-3′ where R = G + A, N = A + C + G + T, Y = T + C, and M = C + A. The positive control primer was nondegenerate and had the corresponding dystrophin sequence (9). The degenerate reverse primer included a *Not*I site and had the sequence 5′-GGG GGC CGC CTA NAR NTC NGG NCK RTG NBD RTG-3′ where K = T + G, B = C + G + T and D = T + A + G. As above, the nondegenerate control primer was based on the dystrophin sequence. Human fetal skeletal muscle RNA was isolated and reverse transcribed using oligo(dT) primers as described (32). Forty ng of single-stranded cDNA was amplified in 50-μl PCR reactions using Gene Amp reagents (Perkin-Elmer Cetus) for 25 cycles as follows: 94 °C, 30 s of denaturation; 50 °C, 10 min of annealing; and 65 °C, 4 min of elongation. The initial denaturing step was 7 min and the final elongation step was for 10 min at 65 °C. Products were phenol extracted and ethanol precipitated before digestion with *Not*I and *Sfi*I. After another phenol extraction/ethanol precipitation, products were cloned into λgt11 Sfi-Not (Promega, Madison, WI), packaged, and plated using standard protocols (33).

Library Screening—The human fetal skeletal muscle cDNA library in λgt10 has been described previously (34). Libraries were screened at high stringency (34) (0.1 × SSC, 0.1% sodium dodecyl sulfate, 55 °C wash), and clones were isolated using standard protocols (33). Positive clones were subcloned into pBSII SK+ (Stratagene Inc., La Jolla, CA) for further analysis and sequencing.

Northern Blot Analysis—Northern blots of human and mouse RNA were performed as described (35). Briefly, total cellular RNA was isolated from indicated tissues using 5.0 M guanidinium isothiocyanate, and 10 μg was separated on 1% formaldehyde gels and transferred to Pall Biodyne A membranes. Hybridization was done in 50% formamide and 5 × SSC at 45 °C and washes were with 1 × SSC and 0.1% sodium dodecyl sulfate at 55 °C for 1 h (33).

Chromosome Mapping—Use of a human-hamster somatic cell hybrid panel has been previously described (36). For *in situ* hybridization, chromosomes were prepared from phytohemagglutinin-stimulated peripheral blood lymphocytes, synchronized by a methotrexate block with bromodeoxyuridine release, and harvested by standard procedures. Cell suspensions were dropped onto precleaned microscope slides, stored at −20 °C, and baked at 60 °C for 1 h prior to hybridization. Purified plasmid DNAs were nick-translated with biotin-16-dUTP (Boehringer Mannheim GmbH, Germany), hybridized to chromosomal DNA, and detected by avidin-fluorescein (Vector Laboratories, Burlingame, CA) as previously described (37). Chromosomes were counterstained with DAPI (4,6-diamino-2-phenyl-indole) (2 μg/ml phosphate-buffered saline). Slides were viewed on a Zeiss axiophot microscope with a Zeiss plan neofluar oil immersion objective (×100, 1.3 numerical aperture). Fluorescein was viewed with a double band pass filter with excitation wavelengths centered around 490 and 560 nm and emission wavelengths at 530 and 650 nm (Omega, Brattleboro, VT). Twenty metaphase cells with hybridization on both chromatids of at least one chromosome/cell were examined for each probe. Representative chromosomes were photographed on Kodak Ektar 1000 film.

DNA Sequencing—DNA sequence analysis was performed on double-stranded template using the Sequenase version 2.0 kit (U. S. Biochemical, Cleveland, OH). In addition to T3 and T7, specialized primers were synthesized and purified as above. All sequences were determined on both strands, and overlapping sequences from parental clones were obtained for all restriction sites used in subcloning. The sequence of clone p18D1 was determined by Lofstrand Laboratories Inc. (Gaithersburg, MD). Sequence data was compiled and analyzed using the Wisconsin Genetics Computer Group version 6.2 package of sequence analysis programs (38). Some database homology searches were performed at the National Center for Biotechnology Information using the BLAST network service (39).

RESULTS

Degenerate PCR and Cloning of α-Actinin cDNA Fragments—Degenerate oligonucleotide primers for PCR were designed based on two small segments of high homology in the amino-terminal domains of dystrophin, β-spectrin, and α-actinin (11) (see "Materials and Methods"). The first (corresponding to amino acids 19–24 of dystrophin) has the sequence KTFT(K/A)W and has been implicated as an actin-binding site (40). The second corresponds to dystrophin amino acids 170–176 and has the sequence H(S/K/R)HRP(D/E)L. Using cDNA synthesized from human fetal skeletal muscle as a template, control nondegenerate primers corresponding to the dystrophin sequence properly amplified a 522-base pair product. The degenerate primers amplified a band slightly smaller in size superimposed on a background smear. These PCR products were digested with *Not*I and *Sal*I and cloned into λgt11 yielding approximately 5000 recombinant phage. When this library was screened at high stringency with a dystrophin cDNA probe, a single plaque hybridized, and sequence analysis confirmed that this contained known dystrophin sequence (data not shown). Another eight random recombinant phage were isolated, and the inserts were subcloned into pBSII SK+. Sequencing revealed that one (designated pAd7) contained a fragment of α-actinin identified by virtue of having 86% DNA sequence homology with the known chicken skeletal muscle α-actinin (8). The other clones apparently resulted from nonspecific priming events and consisted of several common muscle-specific transcripts (two myosin, one actin, one fibronectin, and one calcineurin B) as well as two clones with no homology to any known sequences in the GenBank (release 70.0) and EMBL (release 26.0) databases. When pAd7 was used as a probe to screen the PCR-amplified product library in λgt11, approximately one-fifth of the plaques hybridized intensely and another one-fifth hybridized with a somewhat weaker signal suggesting that the predominant PCR product consisted of sequences from two or more α-actinin genes.

Isolation of Full-length α-Actinin cDNA Clones—To obtain full-length coding sequences for the human α-actinin PCR product, we used pAd7 as a probe to screen a human fetal skeletal muscle cDNA library. Nineteen hybridizing phage were plaque purified and 11 inserts (ranging in size from 2.7–3.6 kilobases (kb)) were subcloned into pBSII SK+ for further analysis. Restriction enzyme analysis revealed that these clones fell into two classes with different restriction maps. The first class (hereafter designated as ACTN2) had eight clones and appeared to be heterogenous at both ends by restriction mapping. However, there was no evidence for alternative splicing of internal sequences as the restriction maps in areas that overlapped were all similar. The second class (designated ACTN3) had three clones with no apparent differences detectable by restriction mapping. Subsequent sequence analysis revealed that the PCR product pAd7 corresponds to the ACTN2 transcript (data not shown).

Tissue Distribution of α-Actinin mRNA—To determine the tissue distribution of ACTN2 and ACTN3 expression, we probed Northern blots with representative clones for each gene (p18D1 and p7M1). These probes each recognized a

[1] The abbreviations used are: PCR, polymerase chain reaction; kb, kilobase(s); NRM, Nemaline rod myopathy.

predominant band of ~3.5 and ~3.4 kb, respectively, in mouse skeletal muscle RNA. Probe p18D1 (ACTN2) also hybridized with a similarly sized transcript in mouse cardiac muscle, but no transcripts were detected in small intestine (smooth muscle), kidney, liver, or brain (Fig. 1). In contrast, ACTN3 mRNA was only detected in skeletal muscle. ACTN3 transcripts in human skeletal muscle were also a single size of ~3.5 kb, however, p18D1 hybridized to three major ACTN2 transcripts (~3.3, 3.8, and 5.6 kb) and a more rare one of ~4.7 kb. A probe containing nucleotides 3902–4181 of the ACTN2 transcript recognized the two largest ~5.6- and ~4.7-kb transcripts while a nucleotide 2938–3907 probe hybridized to these and the ~3.8-kb message. Thus, these size differences reflect differences in the length of 3′-untranslated sequences.

Mapping of the ACTN2 and ACTN3 Genes to Human Chromosomes—A primary interest of ours is to assess whether α-actinin genes play a role in the etiology of any human genetic diseases. To facilitate this approach, we mapped both genes to human chromosomes. Hybridization of an ACTN2 cDNA to Southern blots containing DNAs from human/hamster somatic cell hybrids revealed that ACTN2 segregated with human chromosome 1 (Table I). The discordant fractions for the other autosomes and the sex chromosomes ranged from 0.18 to 0.80. The ACTN3 cDNA probe 7M1 segregated with human chromosome 11 with discordant fractions for the other chromosomes of 0.11–0.67 (Table II). One of the hybrids with a positive hybridization signal for ACTN3 contained a deleted chromosome 11 containing 11pter-q23, indicating that ACTN3 is located within this interval. To independently confirm these assignments, and to sublocalize the genes further, p18D1 (ACTN2) and p7M1 (ACTN3) were used as probes for fluorescent *in situ* hybridization to human lymphocyte chromosomes. ACTN2 mapped to 1q42-q43 in 20/20 metaphase spreads that had a fluorescein hybridization signal over at least one chromatid (Fig. 2, *A* and *B*), while ACTN3 mapped to 11q13-q14 by the same criteria (Fig. 2, *C* and *D*).

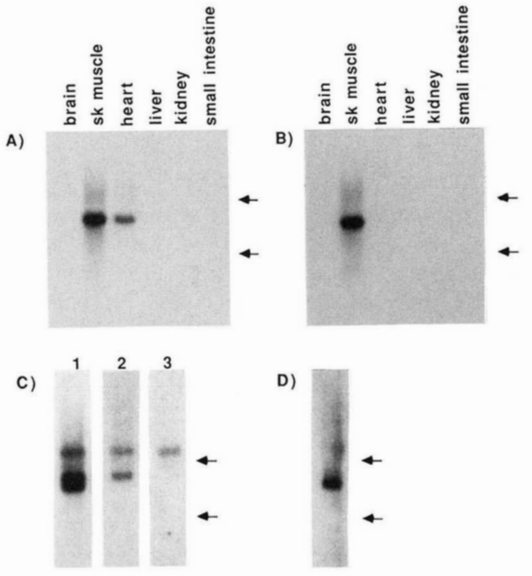

FIG. 1. **Northern blot analysis of ACTN2 and ACTN3 transcripts.** *Arrows* at *right* indicate locations of ribosomal RNA bands. *A* and *B* are the same filter containing adult mouse mRNA from indicated tissues probed with p18D1 (cDNA clone of ACTN2 containing nucleotides 1–3431) and p7M1 (cDNA clone of ACTN3 containing nucleotides 1–2894), respectively. *C*, human fetal skeletal muscle RNA probed with p18D1 (*lane 1*). After the signal decayed, this was reprobed with subclones of ACTN2 cDNA clones containing nucleotides 2938–3907 (*lane 2*) and 3902–4181 (*lane 3*). *D*, human fetal skeletal muscle RNA probed with p7M1.

TABLE I

Segregation of ACTN2 (p7D1) with human chromosomes in DNAs from somatic cell hybrids

p7D1 is a cDNA clone of ACTN2 that contains nucleotides 124–3256 as defined in Fig. 3.

Chromosome[a]	Hybridization pattern[b]				Discordant fraction[c]
	+/+	−/−	+/−	−/+	
1	2	9	0	0	0.00
2	0	5	0	3	0.38
3	2	5	0	3	0.30
4	1	5	1	3	0.30
5	1	6	1	3	0.36
6	1	4	1	4	0.50
7	1	5	1	4	0.45
8	1	3	1	6	0.64
9	0	7	2	2	0.36
10	1	4	0	4	0.44
11	2	7	0	2	0.18
12	2	5	0	3	0.30
13	1	5	1	4	0.45
14	2	3	0	5	0.50
15	2	7	0	2	0.18
16	1	4	1	5	0.55
17	0	5	2	4	0.55
18	1	4	1	5	0.55
19 and 19der[d]	1	1	0	8	0.80
20	2	2	0	7	0.64
21	2	4	0	5	0.45
22	1	6	1	3	0.36
X	1	7	1	1	0.20
Y	0	9	2	0	0.18

[a] Human chromosome complements of the hybrids were determined by isozyme and cytogenetic analysis (55) as well as by hybridization with DNA probes from each autosome and the *X* chromosome.

[b] Number of hybrids with indicated pattern of hybridization signal and chromosome. Column designations are as follows: +/+ = hybridization signal and chromosome both present; −/− = hybridization signal and chromosome both absent; +/− = hybridization present but chromosome absent; −/+ = hybridization absent but chromosome present.

[c] Hybrids with a rearranged chromosome or in which the chromosome was present in fewer than 15% of cells were excluded for calculation of discordant fractions.

[d] This includes nine hybrids containing the der 19 translocation chromosome 19pter-q13::Xq24-qter and one hybrid containing 19qter-p13::Xq13-qter.

Sequence Analysis of Two α-Actinin cDNAs—Representative clones from each class (p18D1 = ACTN2 and p7M1 = ACTN3) were sequenced in their entirety as were the 5′ and 3′ ends of all the clones and the region of a clone, R31–2 (nucleotides 3431–4181 in Fig. 3), that extended beyond the 3′ end of clone 18D1 (Figs. 3 and 4).

The sequence of ACTN2 contains a putative initiator methionine fitting the Kozak consensus (41) at nucleotide 174 and an in-frame stop codon at position 2856 predicting a polypeptide of 894 amino acids or approximately 104 kDa. The ACTN2 clones represent three different forms of the same transcript, apparently utilizing alternate polyadenylation signals in the 3′-untranslated region. Two clones had poly(A) tracts at position 3269, three ended at 3431, and one extended to an *Eco*RI site at 4181. Presumably, the actual 3′ end of this transcript was lost in the cloning process which involved digestion of methylated cDNAs with *Eco*RI. Since then, we have characterized another independently derived clone (pR31–2) that ends at the same *Eco*RI site indicating that this extra 3′ material is not the result of a cloning artifact. There was some heterogeneity, presumably due to incomplete reverse transcription, at the 5′ ends of these clones. However, three of them began within 30 base pairs of p18D1 (+5, +13,

TABLE II

Segregation of ACTN3 (p7M1) with human chromosomes in DNAs from somatic cell hybrids

p7M1 is a cDNA clone of ACTN3 that contains nucleotides 1–2894 as defined in Fig. 4.

Chromosome[a]	Hybridization pattern[b]				Discordant fraction[c]
	+/+	−/−	+/−	−/+	
1	1	6	3	0	0.30
2	0	3	3	2	0.56
3	1	3	1	3	0.50
4	0	3	4	2	0.67
5	2	4	2	2	0.40
6	2	4	1	2	0.33
7	1	3	3	3	0.60
8	1	2	2	4	0.67
9	0	4	4	2	0.60
10	1	2	3	3	0.67
11 and 11q^{-}[d]	4	5	0	0	0.00
12	2	4	1	2	0.33
13	3	5	0	1	0.11
14	2	2	2	3	0.56
15	1	5	2	1	0.33
16	2	2	2	4	0.60
17	1	4	3	2	0.50
18	1	3	3	3	0.60
19 and 19der[e]	3	1	1	5	0.60
20	3	1	1	5	0.60
21	2	3	2	3	0.50
22	2	4	2	2	0.40
X	0	4	2	1	0.43
Y	0	6	4	0	0.40

[a] Human chromosome complements of the hybrids were determined by isozyme and cytogenetic analysis (55) as well as by hybridization with DNA probes from each autosome and the *X* chromosome.

[b] Number of hybrids with indicated pattern of hybridization signal and chromosome. Column designations are as follows: +/+ = hybridization signal and chromosome both present; −/− = hybridization signal and chromosome both absent; +/− = hybridization present but chromosome absent; −/+ = hybridization absent but chromosome present.

[c] Hybrids with a rearranged chromosome or in which the chromosome was present in fewer than 15% of cells were excluded for calculation of discordant fractions.

[d] One hybrid with a positive hybridization signal contained chromosome 11pter-q23 indicating that 7M1 maps proximal to 11q23.

[e] This includes nine hybrids containing the der 19 translocation chromosome 19pter-q13::Xq24-qter.

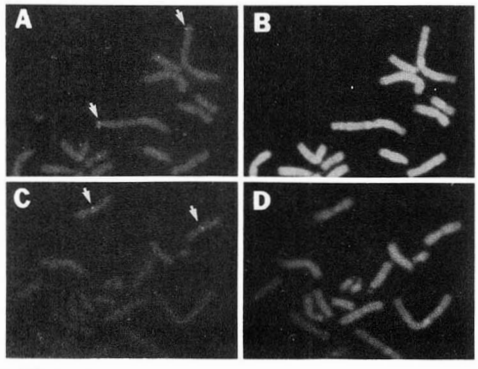

FIG. 2. **Fluorescent *in situ* hybridization of α-actinin cDNA probes to human metaphase chromosomes.** Fluorescein- (*A*) and DAPI- (*B*) labeled images of p18D1 (ACTN2) showing hybridization (*arrows*) to both chromatids at 1q42-q43; and fluorescein- (*C*) and DAPI- (*D*) labeled images of p7M1 (ACTN3) showing hybridization (*arrows*) to both chromatids at 11q13-q14.

and +30) suggesting that the actual 5′ end is at or near this point.

All three ACTN3 clones began and ended at the same places. They encoded a 103-kDa polypeptide of 901 residues as defined by an initiator methionine and in-frame stop codon at positions 19 and 2722, respectively (Fig. 4).

The predicted sequences of these two proteins clearly indicate that they are two different human α-actinins as they both have strong sequence homology to all of the other known α-actinin sequences (Table III and Fig. 5) (proteins are designated in Table III according to species (*e.g.* Ch = chicken, Hu = Human) and primary site of expression (*e.g.* Nm = nonmuscle, Sk = skeletal muscle)). Both HuActSk1 (ACTN2) and HuActSk2 (ACTN3) sequences are collinear with each other and with each of the other human and chicken genes with the exception that neither has the extra five amino acids found between the two EF-hands of the nonmuscle forms (Fig. 5) and that the amino termini differ in length (see below). Among the vertebrate genes (*e.g.* chicken and human) there is generally about 80% amino acid sequence identity and 90% sequence similarity between different genes. HuActSk1 and HuActSk2 are as dissimilar from each other as they are from the human nonmuscle and chicken smooth muscle forms characterized by Youssoufian *et al.* (30) and Baron *et al.* (7, 28). However, HuActSk1 (ACTN2) appears to be the evolutionary homolog of the chicken skeletal muscle form characterized by Arimura (8) as these two proteins have the highest degree of homology (Table III and Fig. 5) and their amino-terminal sequences are very similar (Fig. 6). Comparison of HuActSk2 (ACTN3) with the chicken skeletal muscle form gave a pattern of amino acid differences much like that for the comparison between HuActSk1 and HuActSk2 (not shown).

Pairwise comparisons between the known vertebrate genes show that amino acid changes have occurred throughout the molecule (Fig. 5). The most conserved region is the amino-terminal actin-binding domain, but there are other short stretches (up to ~20 amino acids) of complete conservation in repeats 1 and 2. The region of greatest variability among all the known sequences is in repeats 3 and 4 from approximately residue 570 to 700 (numbering based on the final length of the sequences used to generate figure 5 after the addition of gaps). Although the middle of this region (amino acids 641–660) is conserved between the two human skeletal muscle forms, there are two differences in this region between the chicken and human skeletal muscle homologs (*ChActSk* and *HuActSk1* in Fig. 5).

In contrast to the rest of the actin-binding domain, the amino-terminal ends of all the α-actinins are the most divergent as different initiator methionines are apparently used (Fig. 6). However, it is difficult to ascribe any functional significance to this observation, especially as subsequent modification and acetylation may alter the final protein products (42). At the carboxyl terminus, the last eight amino acids have been conserved across all five vertebrate genes suggesting some selective pressure for these sequences (Fig. 5).

To assess whether HuActSk1 and HuActSk2 have potentially functional calcium-binding EF-hands, we compared the sequences of these domains with the EF-hand consensus of Kretsinger (43) and with the other known α-actinins (Fig. 7). Both human skeletal muscle α-actinins have only 11/16 matches in the first EF-hand with either an arginine or lysine at the *Y* position suggesting that these peptides would probably not be able to coordinate calcium binding properly (7, 8, 43). In contrast, the second EF-hands have 13 and 14/16 matches, respectively, similar to the chicken smooth and skeletal muscle isoforms. Neither has the five amino acid spacer that is found between the two EF-hands in the chicken and human nonmuscle forms (7, 8, 29, 30), however, the absence of this spacer is apparently not absolutely correlated

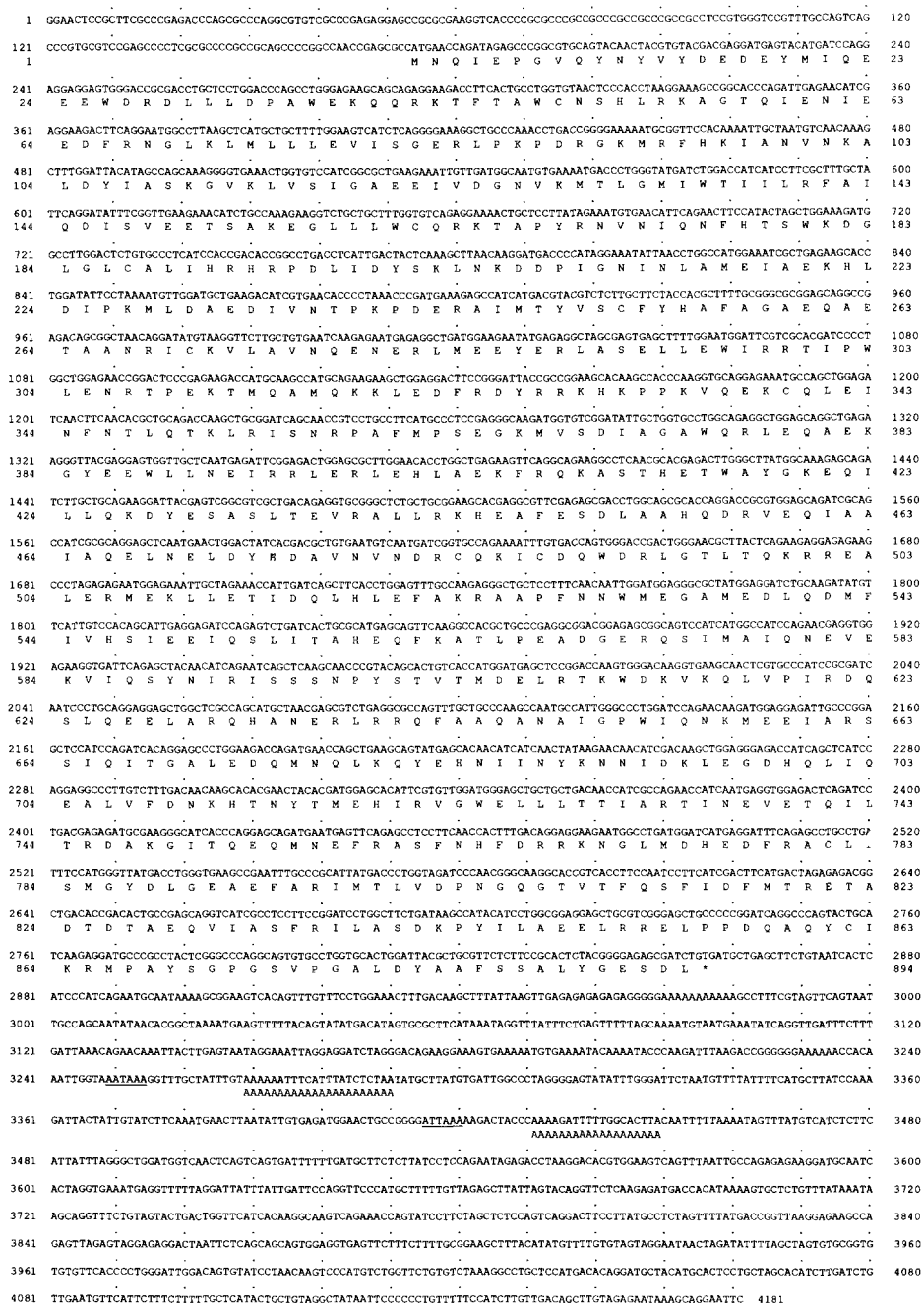

FIG. 3. **cDNA and predicted amino acid sequence of the ACTN2 gene transcript.** * = the termination codon. Two alternate poly(A) tracts are shown below the sequence line and their respective polyadenylation signals are *underlined*. The sequence ends at a presumed natural *Eco*RI site at 4176 in the 3′-untranslated region. Sequences distal to this were not cloned.

with muscle-specific isoforms as demonstrated by the *Drosophila* skeletal muscle protein (*DrActSk* in Fig. 7) (27).

DISCUSSION

Using PCR with degenerate primers based on known sequences for members of the spectrin gene superfamily, we amplified a fragment of human α-actinin cDNA and used this to isolate cDNAs from two human skeletal muscle-specific genes. Our PCR strategy utilized primers with a high degree of degeneracy (8192-fold and 8.4×10^6-fold) designed to hybridize with all the possible sequences coding for the conserved portions of the amino-terminal domains of dystrophin, β-spectrin, and α-actinin. Because of the low effective concentration of any one primer pair, we used a long annealing step to increase the effective CoT. We were probably also aided by the fact that perfect homology is not necessary for PCR primer binding and activity (44). It is not surprising that the most abundant specific PCR product in our library (~2/5 of all clones by hybridization) was from a relatively abundant transcript, α-actinin. In contrast, we only identified a single dystrophin clone reflecting the fact that, although enriched in the PCR library, this transcript is much more rare. The relatively high rate of nonspecifically primed PCR products (seven out of eight random clones that were sequenced) is probably a consequence of the high degree of primer degeneracy.

The α-actinins were first identified by Ebashi and Ebashi in 1965 (45). They have since been well described biochemically, but a molecular genetic characterization of α-actinin genes is necessary to understand the basis for their diversity of isoform structure and function. Previous studies in chickens, rabbits, and mice have characterized two or three different forms of skeletal muscle α-actinin on the basis of amino acid composition, pI, proteolytic digestion, and antigenic cross-reactivity (18, 20–24), but to date only one skeletal

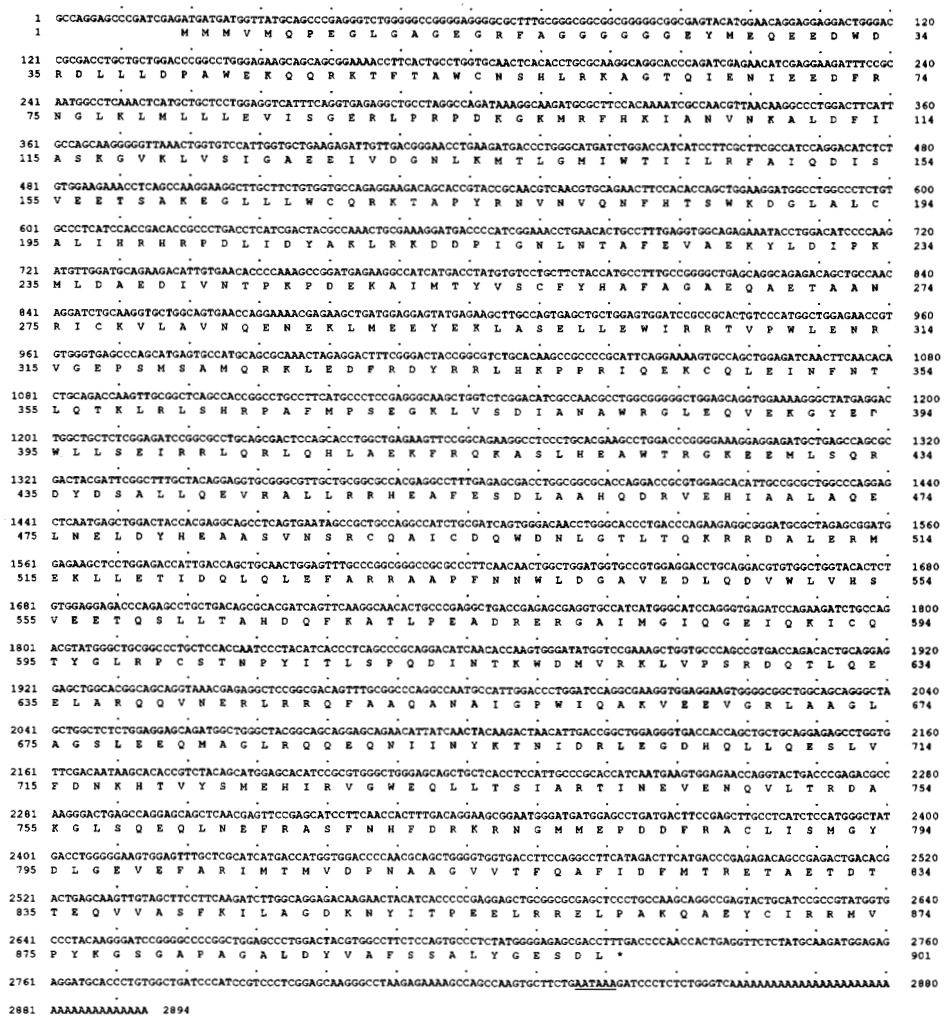

FIG. 4. **cDNA and predicted amino acid sequence of the ACTN3 gene transcript.** * = the termination codon and a polyadenylation signal is *underlined.*

TABLE III

Amino acid sequence comparisons among known α-actinins

Predicted amino acid sequences of the known α-actinin proteins were compared using the program Gap (38). Shown are % similarity(% identity) for indicated pairwise comparisons. HuActNm = the human nonmuscle ACTN1 (30), HuActSk1 = human skeletal muscle ACTN2, HuActSk2 = human skeletal muscle ACTN3, ChActSk = chicken skeletal muscle α-actinin (8), ChActSm = chicken smooth muscle isoform (7), DrActSk = *Drosophila* skeletal muscle α-actinin (27), DctAct = *D. discoideum* α-actinin (6). ChActNm (not shown) = chicken nonmuscle α-actinin (8) which is identical to ChActSm except for an exon encoding part of the first EF-hand (7, 8).

	HuActSk1	HuActSk2	HuActNm	ChActSk	ChActSm	DrActSk	DctAct
HuActSk1	100	90(80)	89(80)	97(95)	89(81)	82(69)	60(38)
HuActSk2		100	89(78)	89(79)	89(78)	83(68)	59(37)
HuActNm			100	89(80)	98(96)	83(69)	60(38)
ChActSk				100	90(81)	82(68)	60(38)
ChActSm					100	83(69)	60(39)
DrActSk						100	59(38)
DctAct							100

muscle-specific gene has been identified in chickens (8). Here, we demonstrate that there are at least two different genes expressed in human skeletal muscle with as many differences between themselves as between each of them and the human nonmuscle gene, ACTN1 (30). Expression of the ACTN2 gene product is specific to striated muscle, as mRNA was detected in both skeletal and cardiac muscle. In contrast, ACTN3 transcription is limited to skeletal muscle. Both gene products are predicted to have calcium-insensitive EF-hands, supporting the idea that they encode myofibrillar α-actinins.

Amino acid sequence comparisons (Table III and Fig. 5) demonstrate that the chicken (ChActNm) and human (HuActNm) nonmuscle isoforms are probably encoded by homologous genes, as are the chicken (ChActSk) and human (HuActSk1) skeletal muscle α-actinins. The high degree of conservation among these respective proteins implies that the gene duplication events that gave rise to the different isoforms occurred before the divergence of the two species and that the homologous genes have been conserved due to functional constraints that are distinctive for each isoform. It is interesting that the *Drosophila* skeletal muscle gene is equally dissimilar from all the vertebrate genes. Furthermore, it is the only known muscle-specific gene with an EF-hand that is predicted to be calcium-sensitive (27). It may be that the sequenced *Drosophila* clones are nonmyofibrillar isoforms of a gene that is expressed in both muscle and nonmuscle cells.

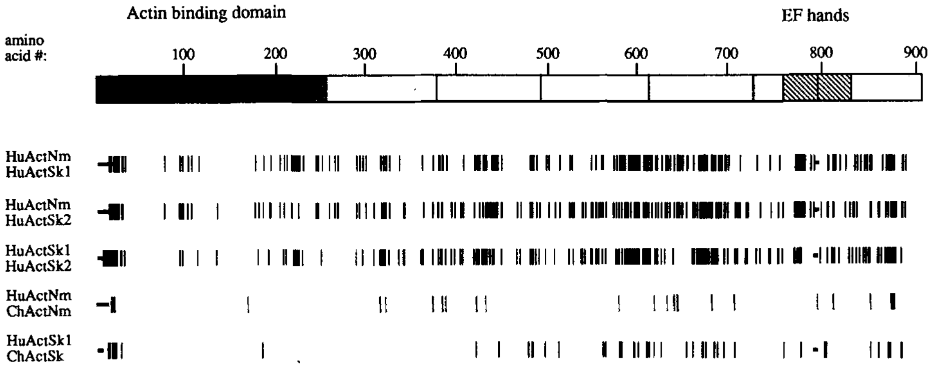

FIG. 5. **Pairwise sequence comparisons of the known human α-actinin proteins and their chicken homologs.** Above is a schematic of α-actinin indicating locations of the amino-terminal actin-binding domain (*gray*), the four central repeats (*open*) and the *shaded* carboxyl-terminal domain containing two EF-hand structures (*diagonal hatches*). Aligned below are results of pairwise comparisons between the proteins indicated at the *left* (names as in Table III). The amino acid sequences were iteratively aligned to each other using the program Gap (38) to optimize the alignments. The program added gaps only at the amino termini and between the EF-hands. These "gapped sequences" (indicated at the *left*) were then compared in pairwise fashion using the program Gapshow to draw a vertical line at each position of nonidentity. *Horizontal lines* indicate locations of added spaces in one or both of the compared sequences.

```
HuActNm                        MDHYDS QQTNDYMQPE EDWDRDLLLD PAWEKQQRKT FTAW
HuActSk1             MNQ IEPGVQYNYV YDEDEYMIQE EEWDRDLLLD PAWEKQQRKT FTAW
HuActSk2  MMMVMQPEGL GAGEGRFAGG GGGGEYMEQE EDWDRDLLLD PAWEKQQRKT FTAW
ChActNm                       MDHHYDP QQTNDYMQPE EDWDRDLLLD PAWEKQQRKT FTAW
ChActSk           MNSMNQ IETNMQYTYN YEEDEYMTQE EEWDRDLLLD PAWEKQQRKT FTAW
DrActSk                     MMMENGLSM EYGDGYMEQE EEWEREGLLD PAWEKQQKKT FTAW
DctAct                                 MSEEPTP VSGNDKQLLN KAWEITQKKT FTAW
VertCon                                   EYM QE EDWDRDLLLD PAWEKQQRKT FTAW
```

FIG. 6. **Lineup of the amino termini of the known α-actinin proteins.** Names are as in Table III. *VertCon* = vertebrate consensus identified by the program Lineup (38) with invariant amino acids in *boldface*.

```
                             EF1                                           EF2
           n  nn  nX  Y Z  -X  -Zn  nn  n                      n  nn  n X Y Z  -X  -Zn  nn  n
           EL  LL  LD  D DG ID   EL  LL  L                      EL  LL  L D D DG ID   EL  LL  L
HuActNm    EFRASFNHFD RDHSGTLGPE EFKACLISLG YDIGNDPQGE AEFARIMSIV DPNRLGVVTF QAFIDFMSRE
HuActSk1   EFRASFNHFD RRKNGLMDHE DFRACLISMG YDL.....GE AEFARIMTLV DPNGQGTVTF QSFIDFMTRE
HuActSk2   EFRASFNHFD RKRNGMMEPD DFRACLISMG YDL.....GE VEFARIMTMV DPNAAGVVTF QAFIDFMTRE
ChActNm    EFRASFNHFD RDHSGTLGPE EFKACLISLG YDIGNDAQGE AEFARIMSIV DPNRMGVVTF QAFIDFMSRE
ChActSm    EFRASFNHFD RKKTGMMDCE DFRACLISMG YNM.....GE AEFARIMSIV DPNRMGVVTF QAFIDFMSRE
ChActSk    DFRASFNHFD RRKNGLMDHD DFRACLISMG YDL.....GE AEFARMMSLV DPNGQGTVTF QSFIDFMTRE
DrActSk    EFRSSFNHFD KNRTGRLSPE EFKSCLVSLG YSIGKERQGD LDFQRILAVV DPNNTGYVHF DAFLDFMTRE
DctAct     EFKACFSHFD KDNDNKLNRL EFSSCLKSIG DEL.....TE EQLNQVISKI DTDGNGTISF EEFIDYMVSS
```

FIG. 7. **Lineup of EF-hand amino acid sequences of the known α-actinin proteins with spaces added where appropriate to optimize the alignments.** Names are as in Table III. Above are indicated the positions of hydrophobic residues on the inner face of helices E and F (*n*) and positions of the oxygen-containing residues at the vertices of the calcium-chelating octahedron (*X*, *Y*, *Z*, *−X*, *−Z*) as defined by Kretsinger (43). Just below are shown the 16 positions of which 12 must match for functional calcium binding (defined in (43)). Mismatched residues in the sequences below are *underlined*.

Alternatively, these facts may simply reflect the great evolutionary distance between the species and/or the *Drosophila* gene may represent a distinct α-actinin isoform whose vertebrate homolog has yet to be identified.

About half of the sequence differences between different α-actinin genes result in conservative amino acid substitutions. However, the pattern of nonconservative changes is not strikingly different from that of all changes shown in Fig. 5 (data not shown). Therefore, conserved regions illustrated in Fig. 5 should accurately reflect the effects of functional constraints on the amino acid sequence. The amino-terminal actin-binding domain is the most conserved, consistent with the fact that this region is thought to bind actin (40, 46) which is also highly conserved (47). In contrast, as noted previously (7, 27), the central repeats, three and four in particular, are much more divergent between the isoforms. We have found that this is also true for the comparison of the chicken and human skeletal muscle homologs (Fig. 5), suggesting that these domains serve a structural function that is not dependent on strict sequence conservation. Despite this generalization, there are several short regions of conservation (*e.g.* amino acids 566–572 and 650–660 on the gapped consensus sequence used to generate Fig. 5) that may serve specific functional purposes for α-actinins in general.

Our analysis of multiple cDNA clones, together with the Northern blot studies, did not provide any evidence for alternative splicing of protein coding sequences for either ACTN2 or ACTN3. However, we cannot rule out the possibility of less abundant spliceoforms of similar size, particularly in cardiac muscle which was not examined in humans. The multiple sized ACTN2 transcripts detected in human skeletal muscle are accounted for by differences in the usage of polyadenylation signals and are not predicted to alter the encoded protein product. Furthermore, the homologous mouse locus encodes only a single sized transcript, suggesting that these differences are not likely to be functional. An analogous situation has been reported for the nidogen gene which encodes two different sized transcripts in the mouse and only one in humans (48). In this case, the difference is accounted for by the absence of one polyadenylation signal in the human gene.

With the exception of two clusters of myosin heavy chain genes (49, 50), most human structural protein isoform genes that have been mapped are scattered with respect to one another. The three known α-actinin genes fit this general rule as they are also all on different chromosomes. However, the localization of ACTN2 on chromosome 1 may be noteworthy since the erythroid α-spectrin gene has been mapped to 1q22-q25 (51) and Youssoufian *et al.* have previously shown that ACTN1 and erythroid β-spectrin are in close proximity at 14q22–24 (30). However, the *in situ* mapping places ACTN2 at the distal end of 1q. If, by analogy to the ACTN1/β-spectrin synteny, ACTN2 and α-spectrin were once located near each other, the location of ACTN2 at 1q42-q43 may reflect an ancient paracentric inversion of 1q that has been postulated by de Grouchy *et al.* (52).

Several mutations of an *X*-linked *Drosophila* α-actinin gene have recently been identified (27). Hemizygous null mutants for this gene survive embryogenesis but die in the second day of larval growth. In contrast, alleles with missense mutations result in flight muscle defects caused by disruption of Z discs and myofibrillar attachments. No disease-causing mutations

of α-actinin have been demonstrated in humans, but there is one inherited muscle disease, nemaline rod myopathy (NRM), that is characterized by abnormalities of α-actinin expression. Skeletal muscle from patients with NRM contains disordered Z lines and rod-shaped bodies that are composed predominantly of α-actinin (53). Recent linkage studies in one large Australian family with NRM have established linkage to markers at 1q21-q23 (54), thus ruling out ACTN1, ACTN2, and ACTN3 as candidate genes for the disease in this family. However, it is still possible that an as yet unidentified α-actinin gene may cause this disease and/or that other families with NRM may have ACTN2 or ACTN3 mutations.

An important next step will be to characterize the fiber type and developmental patterns of ACTN2 and ACTN3 expression and to investigate possible differences in isoform function. Isoform-specific DNA probes and antisera will be used in this approach and will also be useful for characterizing the origin of α-actinin in nemaline rods in muscle from patients with NRM (53).

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